CHAPTER ONE

INTRODUCTION

and

LITERATURE REVIEW

Section 1

INTRODUCTION

Fig. 1.0 Central New South Wales cotton field, May, 2001

The gross annual value of Australian agricultural production reported in March 1998-99 was $29 billion (Aust. Bureau of Statistics, 2003). This contributed to the gross domestic product of $593,311 billion for that year. Of this, the cotton industry in Australia was worth $1.5b. It is important to realise that the raw materials for nearly all foods and other agricultural products, the clothing (including the primary minerals for synthetics), chemical and building industries, depend to some degree on the products taken from the soil. These raw materials support the employment of workers in primary, through tertiary industries, and all subsequent flow-on of the value-added product.

In the long term, agriculture must be sustainable. Australia is currently losing an estimated $2.4 billion in land degradation through salinity, acidity and sodicity annually (Cooperative Research Centre for Soil & Land Management [CRCSLM] 1999), and in 1999 around 20% of farms had experienced some form of land degradation, with 16% reporting productivity declines, and 10% removing some land from agricultural production (Kemp & Alexander, 2000). Sustainability of primary production depends on the maintenance of soil fertility, in turn requiring responsible management and the balancing of many factors. This includes replenishing the elements exported by the harvest, particularly in light of the age of Australian soils, thin topsoil and often unreliable rainfall.

One of the newer agricultural technologies has been the introduction of pest-resistant plants such as Ingard® cotton. An advantage of these plants was a

reduced need for pesticide sprays (at least 40-60%) in the first few years of commercial use (Pyke & Fitt, 1998). Since the commercial release of the genetically modified (GM) cotton which produces the *Bacillus thuringiensis* larvicidal protein, overall profitability of cotton production has increased because of the reduction in cost of insecticides used (currently estimated at $100 million each year in Australia). The *Bt* protein only targets one kind of agricultural pest (*Helicoverpa*), and other cotton plant herbivores such as mites, thrips, mirids and bugs still have to be controlled. The principal pest of cotton, however is *Helicoverpa*, and accounts for eighty per cent of the need to spray (Fitt, 2000).

Legislation dealing with genetically modified plants is administered by the Office of the Gene Technology Regulator (OGTR) in Australia under 'The Gene Technology Act (2000)', that determines guidelines for practical testing of any newly developed genetically modified organisms, either plant or animal, to determine whether they should be released. This testing is undertaken to estimate potential risks of new technologies such as the emergence of new weeds, pests and diseases. In the absence of precedent, Australia can be guided by overseas authorities.

The United States Environmental Protection Agency (EPA) *Bt* Plant-Pesticides Biopesticide Registration Action Document (accessed April 2000) states that 'the *Btk* expression levels validated by ELISA procedures measured 2.04 µg per gram (ppm) of fresh leaf tissue for field-grown plants'. The document also states that 'submitted data indicate that … an estimated 1.44 grams of *Bt* protein per acre (based on 60,000 plants per acre) would enter the soil as a result of post harvest incorporation of *Bt* cotton'. The EPA document further states that 'studies on the effects of invertebrate soil organisms were not required', reasoning that there would be an accumulation of organic detritus if soil organisms were significantly impacted. The observation that use of insecticides (such as the widespread use of chlorinated hydrocarbons in the 1950s and 1960s) had not resulted in the build-up of plant detritus in cotton fields, was taken as evidence that 'representative species such as Collembola and earthworms' were not adversely affected. Similarly, in the case of the delta-endotoxin, the submitted data also showed that production ceases at

senescence of the cotton plant, allowing time for protein degradation prior to harvest. The soil moisture was not noted in the report, however, and as pointed out by Nester et al. (2002, p. 7), the rate of microbial breakdown would depend on soil moisture. Degradation of insecticidal, as well as other proteins, requires water for hydrolysis and deamination, and in general, the drier the soil, the longer the insecticidal crystal protein will be stable.

Ream et al. (1993) reported that the *Bt* insecticidal protein was expressed most highly in the leaves of cotton plants, and yet results of protein tissue expression levels undertaken by 'antibody-based reagents' cited in the EPA Report (2000, page IIC17) showed expression of 2.04 ppm from cotton leaf tissue, 11.5 ppm from pollen, 1.62 ppm from seed. Expression of the protein in pollen was therefore five times that of the leaves. The EPA Report did not discuss either root tissue or tests on the soil surrounding the root.

A growing plant will continually produce the *Bt* protein, yet there is no mention was made in the EPA Report of the susceptibility of the soil-dwelling microbiota that contribute to mineralisation of plant organic material to this protein. Saxena et al. (1999) showed that *Bt* corn – the seed variety which gives protection from corn borer pests – releases the Cry1A(b) protein through its roots into the soil, precisely where most bacterial, fungal and micro- and mesofaunal activity takes place. Several insect-resistant plants have *Bt*-based toxicity for plant and animal parasitic nematodes (Edwards et al. 1988). Alteration in the ratios of higher orders of food-web predators may impact on other food-web communities. Additionally, the work of Saxena et al. (1999) was done on insect-resistant corn but so far no reference has been made to cotton, nor have studies been undertaken within the Australian soil system. The Report of the Food and Agricultural Organisation of the United Nations (FAO) on environmental effects of genetically modified crops (2003) recommended that environmental effects of *Bt* crops should be assessed on a case-by-case basis, including their potential impact on local soil microflora and biodiversity.

In Australia, the Cotton Research and Development Corporation (CRDC) Annual Operating Plan 2004 – 2005, acknowledged that 'soil biology is now

recognised by many cotton growers as an important component of maintaining a sustainable farming system', and that a benchmark needs to be established to monitor the potential positive or negative impacts of transgenic insect- and herbicide-tolerant cottons on soil.

The productivity of an asset such as soil requires some understanding of the interactions between its physical characteristics and complex biota which inhabit it. While the project described here was limited to the short-term impact of the soil microbiota during the life of the plant, each facet of knowledge gained from the interplay of soil chemistry, soil biology and soil physics collectively contributes to a better understanding of the relationship between a plant and the soil and its biota. It is also possible that insights into the effect of new technologies may provide an opportunity to harness benefits from natural (biological) processes.

The aims of this project were therefore to examine the rhizosphere of soils in which cotton was grown, in order to identify any impacts on the soil microflora and micro- and mesofauna, or potential soil community perturbation which may have arisen from the different proteins or other characteristics contributed by the genetically modified plants.

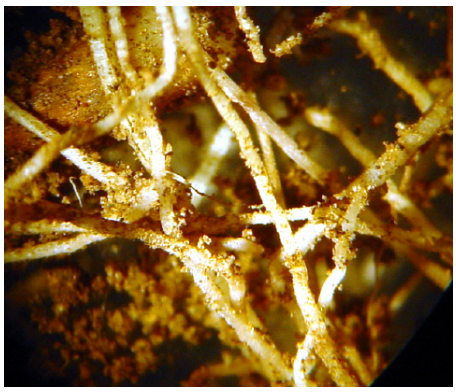


Fig. 2.0 The rhizosphere, where soil adheres to roots of a cotton plant.

Section 2

Literature Review

2.1 CONTEMPORARY AGRICULTURAL MANAGEMENT

A report co-sponsored by the United Nations Environmental Program, Environment Canada and UNESCO (1975) noted that human intervention in the environment, for example, by deforestation, strip mining and the construction of large dams and diversion of rivers, has become a force of geologic scale. Broad-acre large-scale farming systems which increase returns through economies of scale, are also a major contributor to the change in the landscape. Recently introduced technologies in farming include the use of genetically modified plants such as cotton and soybean. One such modification is the use of the *Bt* gene for expression of a protein toxic to insect pests. The cultivated area under genetically modified crop plants is increasing globally. The original *Bt* gene has been modified since its first transfer to tobacco plants. This modification, to enhance the expression of the protein in plant tissue, has now also been inserted into commercially valuable crops such as corn, cotton and potatoes.

The world area planted under GM-crops rose from 1.7 million hectares to 67.7 million hectares between 1996 and 2003, a 40-fold increase (ISAAA, 2004). This world trend is expected to continue and Australia is following. Llewellyn & Higgins (2002) anticipated that over half of the area planted would be to GM crops in 2002 and would move rapidly towards full adoption. In fact, in 2003 the principal crops for global GM area were soybean at 61%, maize at 23%, cotton at 11% and canola at 5%. Genetically modified plants were planted in a total area of 272 million hectares, and compared with their non-genetically modified plants, amounted to 25% of the area, up from 22% in 2002 (ISAAA, 2004). The trend is plotted in Figure 2.1.1.

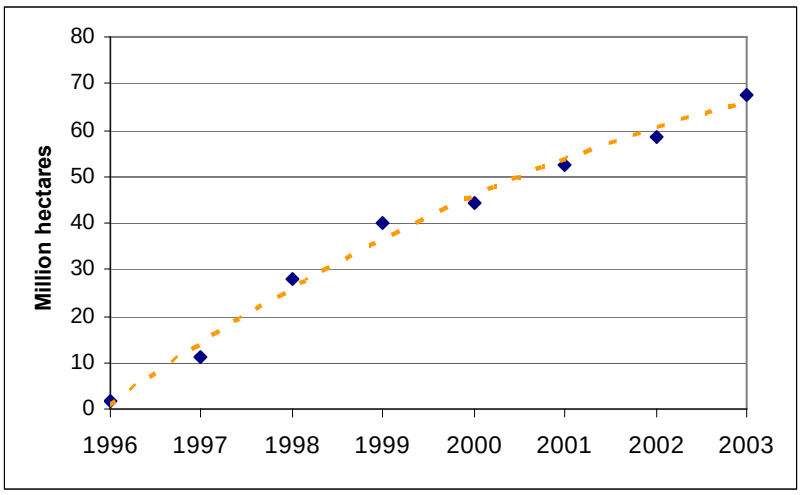


Figure 2.1.1 Total world area of GM crops for areas over 50,000 hectares of the four commercialised GM crops corn, canola, cotton and soy, (International Service for the Acquisition of Agri-biotech applications, 2004).

The potential impact of GM-crops on the soil biota needs to be understood to predict any long-term effects on plants and their soil microbiota. The issues on hazards to the soil environment are not as clear as, for example, the impact of a site development. In the soil environment no one factor is likely to represent the harm from an event, and the questions of what is acceptable by way of soil modification or degradation has not yet been fully answered. Human activities can have longer-term influences on soil microbiota than are sometimes appreciated. A crude oil spill is estimated to affect microbial communities for more than ten years; the effect of clear cutting of forests may take 300 years for remediation of the soil processes; pesticide application can take 4-16 weeks and the damage done by strip mining may last for 50-100 years (McKenzie, 1998).

2.2 AUSTRALIAN LEGISLATION ON SOIL CONSERVATION

While conservation of the Australian soil is mandatory for long-term agricultural sustainability, there is no definition of 'soil degradation' in the Soil Conservation and Landcare Act (1989). The legislation primarily covers fines after soil contamination, but does not address issues of prevention of degradation. The Act states that a landholder has a duty of care not to cause or risk causing degradation of land, but there is no specific legislation on the guidance of soil usage, apart from the clauses appearing in Part A, Schedule 1: Prescribed Activities of Environmental Significance, which relate to:

- Activities producing listed wastes including heavy metals, or infective substances being disposed of in the soil which are likely to pollute, or constitute an infective hazard, and
- Materials Handling and Transportation:
  Crushing, grinding or milling which is likely to become a dust or noise nuisance.

2.3 PHYSICO-CHEMICAL INFLUENCE OF SOIL MICROENVIRONMENTS

Field soil is a heterogeneous mixture of mineral particles and varying amounts of organic materials. The chemical flux of agricultural soils alters seasonally with high export of carbon through crop harvesting, and diurnally with CO_2 from respiration. Physical disruption to the matrix caused by tillage greatly alters the bacterial population (Kennedy, 1999; Simpfendorfer et al.1999). Further, Ferris (1982) found that plant parasitic nematodes comprised 21% of a nematode population in an undisturbed ecosystem compared to 35% in a disturbed environment. The application of inorganic fertilisers and methods of irrigation also contribute to variability in soil microbial populations. Cropping systems and soil nutrient availability can also affect soil mineral content and subsequent crops, and therefore, the microbial populations the soil can support. Some soil characteristics which impose restrictions on the microbiota are considered below.

2.3.1 Pore size

The water permeability within soil, also known as hydraulic conductivity, varies with the size of particles and the surrounding pores, and the nature of particle aggregation. Because they have more macropore space, sandy soils generally have higher saturated conductivity than finer-textured soils. Fine clay and silt can clog the small connecting channels between larger pores (Brady & Weil, 1999, pp. 192-193; 442).

Movement for nematodes is most rapid when the diameter of the soil particles are about one third of the length of the nematode (Nicholas, 1975). Penetration of microfauna and mesofauna in compact clays may not be possible due to the narrowness of the channels between the particles. Particularly with cracking clays, which open and close during the seasonal cycle, nematode populations encounter changing conditions (Cohn et al. 1996). Pore size can also limit the growth of fungal

hyphae (Otten et al. 1999), and waterlogging can lead to a decrease in microbial diversity by selection of facultative or obligate anaerobes under anoxic conditions. Soils with different physical characteristics are therefore required for studies comparing the effect of plants on the microbiota, and moisture content should be monitored to alleviate non-plant associated effects.

2.3.2 Trace elements

Different plants have different mineral requirements for growth, and a deficit in the availability of trace elements can affect the population dynamics of soil microbiota. Fluorescent pseudomonad bacteria cause an iron-dependent antagonism against a variety of microorganisms within the rhizosphere (Lynch, 1982; Simeoni et al. 1987; Lindsey & Jones, 1989, p. 191) because the pyoverdines or 'siderophores' they produce deplete available iron to target pathogens. This theory is supported by observations that disease suppression occurs only in soils with low iron availability and that addition of iron in a form assimilable by the pathogen reduces disease suppression (Simeoni et al. 1987). Potassium deficiency can also result in increased susceptibility to Alternaria leaf spot (Allen, 2000). High levels of carbon removal by the harvest of grain legume crops can also cause soil acidity problems, and manganese deficiency can lead to Take-all disease (French, 1995).

2.4 THE RHIZOSPHERE

The rhizosphere is the zone of interaction between the soil, plant roots and microbiota. This microenvironment is characterised by increased biomass of actinomycetes, fungi, nematodes, protozoa and other microfauna, and is strongly influenced by the interface of the plant root system and its exudates (Bowen & Rovira, 1999; Corbett et al. 1984; Marschner, 1997, p. 562). This live-plant influence has such a local effect on populations of microbes, protozoa and nematodes that no similar increases in population were measured in bulk soil >1.8mm away from the root, when compared with the microbial populations within a patch of leaf litter (Rønn et al. 1996). Microbial activity is also increased in the region of the plant root (Kroer et al. 1998). An example of this increased metabolic activity was shown to double when measured by the uptake of ^{3}H leucine CFU^{-1} h^{-1}. The benefits of the relationship between plant and soil microbes apply not only to the microbiota but also to the plant, for example, Kloepper et al. (1989) reported that wheat yield increased up to 30% with *Azotobacter* inoculation and up to 43% with

Bacillus inoculants. Non-specific rhizobacteria are also known to benefit plant growth, as with the plant-growth promoting rhizobacteria reported by Kloepper (1991), who indicated that different strains can increase crop yields, control root pathogens, increase resistance to foliar pathogens, promote legume nodulation and enhance seedling emergence.

The root surface is the zone of transfer of nutrients from the external environment, and interfaces with its own symbiotic microflora. This surface of exchange is sometimes known as the 'rhizoplane' and can therefore be likened to the intestinal membrane of an animal, where the gut lumen is coated with a protective layer of beneficial or neutral bacteria (Cato-Smith, 1996). This root/ microbe/soil boundary may therefore be loosely considered a biofilm, where plant-beneficial microflora interact symbiotically with the host plant, with the rhizosphere bacteria assisting plant growth through siderophores, antibiotics or HCN, or direct growth promotion through the production of plant growth factors (Bowen & Rovira, 1999). In this area there is competition for nutrients and protection of the root cells from pathogenic invasion and the interactive complexity is still largely not understood. Only occasionally will a root pathogen invade the epidermal cells and enter the cortex, and possibly the stele. This situation is analogous to animal gut infections, when the integrity of the epithelial cells are breached.

In *Bt* crops the plant tissues produce specific insecticidal crystal proteins in a soluble form (American Academy of Microbiology, 2002), and the concentration gradient of the *Bt* protein, as well as all other plant-produced proteins, would therefore be driven by diffusion depending on the moisture surrounding the root. The concentration is therefore anticipated to be lower in the surrounding soil than within the living plant cells, and would depend on the leakiness of the root cells. A scanning electron microscope image of a soil aggregate from which a plant root was extracted is shown in Figure 2.4.1. The centre of the picture illustrates the smooth soil surface adjacent to the root where it pushed past the grains of surrounding soil, causing a casing, where clay soil in particular, would closely surround the root surface. It also shows the mix of small stones and open channels in which the microbial rhizosphere populations interact. At the 1mm scale, there are regions of smoothly polished soil, impenetrable stones and aerobic channels through which water would flow, and be

trapped. Each soil aggregate has its own differing physical characteristics, and supports the microbiota suited for these conditions.

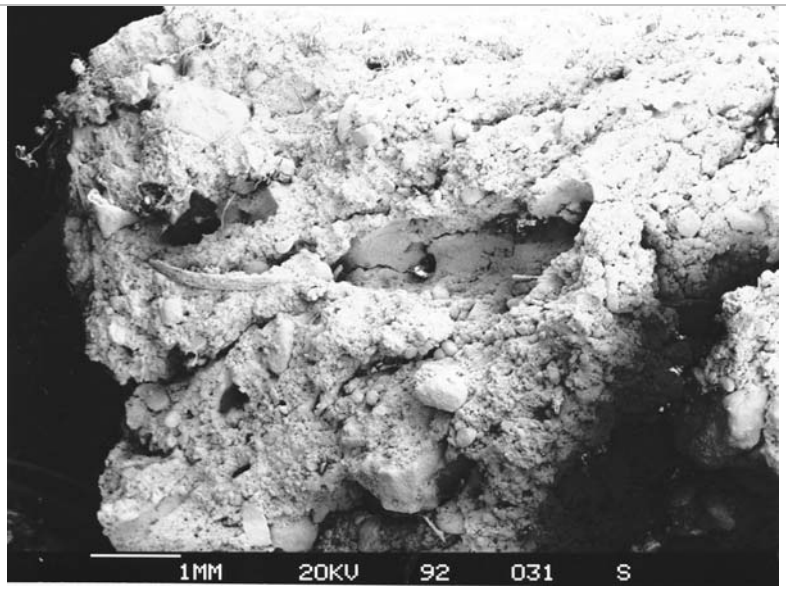


Figure 2.4.1 Scanning electron microscope image of a root-associated zone within a soil aggregate. Used with permission of CSIRO.

The apical growing regions of roots are sites of considerable material loss through abrasion, with possibly up to 20% of photosynthetic production being passed into the soil from cereal roots (Grant & Long, 1981). Selection of distinct physiological and taxonomic groups of microbiota occurs in this region, and an increased proportion of rhizosphere bacteria degrade cellulose, ferment sugars, solubilize phosphates, produce extra-cellular polysaccharides and synthesize growth factors. Taxonomically there is a high proportion of Gram-negative bacteria, particularly *Pseudomonas* species (Grant & Long, 1981). Since the rhizosphere is rich in exudates as an energy source, the microbial population can reach up to 1 x 10^9 cells cm^{-3}, 10-100 times larger than the population in the bulk soil. This rhizosphere-soil to bulk-soil ratio also varies with the type of plant and soil type, and adjacent soil microbial populations can in turn affect plant growth by their pathogenic or beneficial influences. Given that bacteria occur in higher numbers at root junctions, a multiple-branched root system would support higher numbers of bacteria than one with a simpler, less-branched system. A difference in the root system architecture of non-GM and GM plants could possibly influence the microbiota which surround the living root tissue.

A major issue for research in soil microbiology is the interactive nature of the ecosystem. No living entity exists in isolation, nor is it unaffected by its close

neighbours. As discussed above, soil physical and chemical characteristics can affect root growth, morphology and distribution of plant root systems (Marschner et al. 1997, p. 513). Soil structure is affected by changes in management, eg till or no-till, with resultant redistribution of soil pores (Churchman, 2002), and use of fertilisers. Because all of these factors affects the health of the plant, photosynthate material will vary, and this in turn will influence the living microbiota surrounding the root. Different plant species also influence the types of bacteria present in the rhizosphere through release of specific exudates (Lemanceau et al. 1995; Miller, et al. 1989; Marschner, 2001). It can be argued that the rhizosphere is a continually changing micro-environment and the rhizosphere microbial populations reflect the life that is possible under the continually changing conditions.

The adaptive nature of soil microbiota demonstrates a continuum of evolutionary forces which alter the composition of community members in favour of those able to survive the particular treatment and become the founding units of the new population. Food chains in soil are usually complex, involving many different species and unexpected results can arise through compensatory or modified competitive effects. Applications of pesticides often have unexpected results because the rate of decomposition of organic matter is depressed by populations of decomposers affected by the treatment. Most nematodes in soil feed on bacteria and fungi. Pesticides may remove some but not all species of the soil biota, thus permitting remaining species to multiply vigorously. *Trichodorus* spp. often reach higher population levels after soil fumigation than before treatment (Dropkin, 1989).

The doubling time for bacteria in rhizosphere soil has been estimated at 5 hours (pseudomonads have a doubling time of 5.2 hours in the rhizosphere, compared with 77 hours in the bulk soil [Bowen & Rovira, 1999]), and so within three days of hypothetical deletion of all but one of the bacteria, approximately 15 generations could have occurred. This represents a population increase from a single bacterium to 1×10^{13} bacteria, given adequate nutrient. This adaptability was demonstrated by Oger et al. (1997) when transgenic plants that produced opines altered the populations of rhizosphere bacteria. The levels of bacteria able to utilize mannopine were 80 times higher in the rhizospheres of the transgenic legume, *Lotus corniculatus*, than in the rhizospheres of non-engineered *L. corniculatus*. These population changes can therefore been seen as ephemeral.

Research by Buyer & Drinkwater (1997), comparing Biolog with PLFA for bacteria, showed a statistically significant replicate effect not observed in the substrate utilization assay, suggesting that structurally different microbial communities were functionally similar, and that the Biolog patterns therefore reflected the currently activated metabolic activities of the dominant microbes in the samples.

2.4.1 Effect of plant exudates on soil pH

The source of nitrogen alone can affect the root exudates and the surrounding root area (Marschner et al. 1982). With corn and sunflower plants, the pH of the area immediately adjacent to the roots was 4.5, lowered from 6.0 when NH_4^+ was used as the source of nitrogen, compared with no change in pH for plants which were grown with NO_3^- (Marschner et al. 1982). This difference in pH could therefore result in selection of more acid-tolerant microbial species within the area where an acidic type of fertiliser was applied.

Within limits, plants are able to respond to soil pH. Uptake of cations present as Ca^{2+}, Mg^{2+}, K^+ and NH_4^+ result in the release of H^+ ions into the soil, and the uptake of anions such as PO_4^{3-}, SO_4^{2-} and NO_3^- result in a net efflux of hydroxyl, bicarbonate or carboxylate anions as in the reaction $HCO_3^- \leftrightarrow OH^- + CO_2\uparrow$ (Kennedy, 1992). If the total cations exuded from the roots are greater than the total anions then net acidification will occur. Significant differences in pH measured by ash alkalinity can be found within the different plant tissues (Kennedy 1992; Robson, 1989), reflecting the different functions of leaves, roots etc. If the GM-plants cause an alteration in overall electronic balance with extrusion of H^+ ions through the permeable membranes, for example, by requirement of higher levels of nitrate for production of the extra protein, then the surrounding soil (and possibly the associated rhizosphere microbiota) may be acidified.

There are also indirect effects on the bacteria surrounding the root from the extrusion of organic anions from the root apices of aluminium-tolerant plants. Horst et al. (1982) found that the mucigel surrounding the root surface contained eight times more aluminium than the root tissue, suggesting that the mucilage acts as a semi-permeable membrane for the root. Being mucoidal, it may temporarily protect the root against desiccation under conditions of high transpiration (see also Kochian, 1995). Zhang et al. (2001) found evidence of malate-permeable channels and cation

channels activated by aluminium in the apical cells of wheat roots. These organic acids such as malate, citrate and oxalate not only act to chelate the metallic ions and reduce the acidity surrounding the plant roots, but offer a source of energy to those organisms able to utilise these common substances as a source of food. This rhizo-secretion has potential for genetic manipulation for root tolerance of harsh soil environments (lower pH and increased salt content) and for optimised plant nutrition through the trichoblasts. Alteration of the root secretory function could also possibly change the chemical compounds which signal the presence of the root to adjacent detrimental microorganisms.

2.4.2 Temporal effects on soil microbiota of changing plant root exudates

Plant root exudates vary in time, influencing changes in microbial populations at different stages of plant growth (Semenov et al. 1998). With cotton plants, for example, total carbohydrate concentration and concentrations of calcium, potassium and sodium were sharply lower in root exudates of 55-day-old cotton plants compared with 18- and 30-day old plants. For magnesium concentrations, the shift occurred between 18 and 30 days (Watkins, 1981). These differences in plant root exudates over time are reflected within microbial communities, as micro-habitats change according to the substrate type and availability of exudates along an expanding plant root system. This selectivity has been described as a travelling wave by Semenov et al. (1998), as the growing root passes the (stationary) micro-habitats.

2.4.3 Complexity of the soil biota

Current estimates of the number of species of soil organisms vary. An estimation by Patel (1999) quotes 10-20 million bacteria, 100,000 fungi, 50,000 algae and 30,000 protozoa per gram of fertile soil. Altieri (1999) estimated that a square metre of an organic temperate agricultural soil may contain 1000 species or organisms with population densities in the order of 10^6 m^{-2} for nematodes, 10^5 m^{-2} for micro arthropods and 10^4 m^{-2} for other invertebrate groups, and that one gram of soil may contain over a thousand fungal hyphae and up to a million or more individual bacterial colonies. The variability of microbial populations in each gram of soil was tabulated in Metting (1993, p. 13).

Table 2.4.3.1 Variability in numbers and biomass of soil microbiota per g soil (from an abridged version of Metting (1993, p. 13).

Organisms	Per g	Biomass (wet kg/ha)
Bacteria	10^8-10^9	300 -3000
Actinomycetes	10^7-10^8	300 -3000
Fungi	10^5-10^6	500 -5000
Microalgae	10^3-10^6	10 -1500
Protozoa	10^9-10^{10}	5 -200
Nematodes	10^1-10^2	1 -100

While estimates differ, each group influences others, as part of a complex association of primary feeders and higher trophic level feeders, and of varying predator/prey relationships. There may therefore be a disruption to soil microbiota in the food web if one section, unrelated to a target pest, is removed. This situation is similar to the eradication of one trophic level of soil microbiota (Whitford, 1982). In a litter-bag study, the pesticide chlordane was applied in three test-site desert soils in Arizona, Nevada and California. The pesticide killed virtually all the insects and mites. Without predatory mites to hold them in check, bacterivore nematodes multiplied rapidly and devoured a large portion of the bacterial colonies responsible for litter decomposition and nutrient cycling. The insecticide application reduced the rate of litter decomposition by nearly half due to the indirect effect of killing the predators of the nematodes. If the toxic *Bt* protein from the GM plants kills a subset of the predators within a soil ecosystem, this effect may translate to inhibition of the carbon recycling needed within agricultural soils for crop production in the next season.

2.4.4 The primary mineral recyclers

Ninety to ninety-five percent of all nutrient cycling in soil passes through bacteria to higher trophic levels, so microorganisms functioning at this primary level of mineralisation of organic matter are an important link in the recycling process. An estimated 10-30% of total organic matter in the soil consists of a labile fraction, which is recycled within weeks or months. Arguably, it is this pool of biomass which is the major reservoir of potentially available plant nutrients. The microbial component functioning as recyclers of this fraction consist of soil bacteria, fungi, protozoa and other micro- and macrofaunal soil populations (Wilson, 1987).

2.4.5 Interdependent and opportunistic population change

Genomic adaptation of microbial populations, such as the inclusion of multiple copies of rRNA which code for enzymes resulting in faster utilisation of available nutrients, can be inherited by bacteria carrying the trait, giving a selective advantage. Klappenbach et al. (2000) demonstrated multiple repeat sequences coding for a degradative enzyme for the substance 2-4-D among a microbial community which had been exposed to this substance. The substrate was degraded most rapidly by the bacteria which had larger numbers of repeat sequences than others.

Interrelationships between bacteria, plant and fungi are known to be beneficial to the members of this triple association (Azcón et al. 1991) when physiological and biochemical processes are successfully shared, and these associations can have effects that are not at first apparent. Galleguillos et al. (2000) found that a genetically modified strain of the bacterium *Sinorhizobium meliloti* and A-M fungi enhanced the positive effects achieved on the plant, improving the acquisition of nutrients. However, because of the specific interaction with A-M fungi, *Glomus intraradices* or *G. mosseae*, the length of the lateral roots colonised by these species was significantly affected by the presence of *S. meliloti*. This would impact on remaining soil bacterial populations because of changes in the availability of sites on the root for colonisation. Further, the triple association from the influence of bacteria which have been detected inside the spores of A-M fungi (Perotto & Bonfante, 1997), both of which occur within the living plant root tissue, is not known.

Other well known beneficial bacteria include nitrogen fixers such as *Rhizobium* and *Bradyrhizobium*, both Gram-negative motile rods which lead to the formation of root nodules. The plant-bacterial symbiosis in legumes, for example, is so interdependent that leghemoglobin, the important O_2 binding protein in the root nodule, is genetically coded for in part by both the plant and the bacterium. The globin (protein) portion of leghemoglobin is coded for by plant DNA while heme synthesis is coded for by bacterial genes (Brock & Madigan, 1991, p. 672).

The interactive nature of the soil ecosystem is also shown by the damage to plants caused by pathogens working in combination. This damage may be logarithmic, rather than additive. Watkins (1981) documented that plant-parasitic nematodes in association with pathogenic fungi cause more damage to cotton than the sum of

damage they cause separately. An important example of the compounding effect occurs with *Belonolaimus longicaudatus* (the sting nematode), a devastating parasite of cotton. When infestation of cotton occurs with this nematode and Fusarium-wilt fungus, almost total crop failure can result (Webster, 1972, p. 199). Another example of cumulative effect occurs with the infestation of cotton by *Rhizoctonia solani*, where 80% infection rate can occur in fungicide-treated seed, but the percentage of plants killed by the fungus is much lower. The plants infected have reduced yields and are more susceptible to other environmental stresses (Press & Kloepper, 1994). *Agrobacterium tumefaciens* is attracted to plant saccharides which it 'uses' to guide it towards plant wounds in the rhizosphere, and it will also migrate up a concentration gradient of vir-inducing phenolic wound exudates to colonise the wound site (Pickup & Saunders, 1996). Walker (1997) also found that infection by *Rhizoctonia* was increased in rootlings of grapevines in pot experiments when the nematode *Meloidogyne incognita* was present.

2.4.6 Regenerative potential of soil microorganisms

The potential for recovery of a target population after an environmental impact should be considered in a work of risk assessment. Microbial population dynamics are described by exponential growth until density-dependent factors (space, air) or carrying capacity (exhaustion of nutrients) has been reached. With a doubling time of 5 hours, for example, as with soil bacteria (Bowen & Rovira, 1999) and using the Malthusian growth model $P_t = P_0 e^{rt}$ (where P_t is the resulting bacterial population at time t, P_0 is some estimation of the initial population, *e* is the natural logarithm describing exponential growth and the exponents r and t are the rate and time factors), by rearrangement to

$$\frac{\left[\ln \frac{P_t}{P_0} \right]}{r} = t$$

would solve for the time the population needed to regenerate from the difference in numbers. Given, for example, 4×10^6 bacteria and 95% of this number (the point of difference in population number which is taken as a common consensual statistical difference, ie, 1×10^5 cells) it would take 7.7 minutes to regrow from the 95% of the population level back to the previous population of 4×10^6 colony forming units. For perspective, a major environmental impact on the soil bacteria which reduced the population numbers to 5% of the original, would take 7.49 hours to regenerate to the

full amount of 4×10^6, ie, less than a day, given unlimited nutrients, lack of predation and unrestricted and uncoordinated growth within non-density dependent conditions.

However, the complex interactivity of the living soil biota is such that a deficit in knowledge of the magnitude of the driving forces of the soil population ecology makes modelling difficult. The dynamics of unrestricted growth are modified by vertical impacts (predation) as well as horizontal influences (competition) and the nutrients in soil are not unlimited. None of these factors are known in the case of genetically modified cotton in a real soil system, and the results most closely describing a real soil system may therefore need to be taken from empirical observation under reasonably consistent conditions.

There have been several studies on the ability of soil microbiota to recover after the application of toxic chemicals:

1. The herbicides Logran, Hoegrass and Glean were added to soil in a study by Gupta and Neate (1998). The soil microbiota had largely recovered after 7 days.

2. Paraquat is a cell membrane disruptor and photosynthesis inhibitor that results in loss of chlorophyll and carotenoids, with a documented half-life of 1,000 days (http://ace.orst.edu/info/extoxnet/ tibs/ movement.htm, accessed 14/12/03). Metabolic activity of soil microbiota was inhibited temporarily by its application, but some bacteria were still able to utilise substrates in the presence of paraquat (http://www.btny.purdue.edu/ Pubs/WS/WS-24-W.pdf, accessed 11/08/04).

3. A study by Taiwo & Oso (1997) found that application of atrazine, pyrethrin and metobromuron and metolachor, especially when applied above the recommended dose rate, affected microbial populations, targeting particular groups. An initial rise of microbial populations was followed by significant reductions in species. *Mycobacterium*, *Agrobacterium*, *Nitrosomonas*, *Nitrobacter* and *Thiobacillus* spp. were completely eliminated in the treated soils, and *Fusarium*, *Mucor, Cephalosporium, Penicillium* and *Nocardia* spp., present in the control soils, were also eliminated in the chemically treated

soils. It was found also that some phenylamide herbicides affect the soil microbiota, inhibiting nitrification. While the pesticides in the study were toxic only to the pests they were designed to control, they were metabolised to yield products inhibitory to entirely dissimilar organisms.

4. The populations of soil microbiota may increase if a toxin, which affects a different target, can be utilised as a source of energy. Yassir et al. (1999) found accelerated degradation of atrazine where there was a history of repeated applications, compared to low degradation potential in soils where this herbicide was not applied previously.

5. The effect of antifungal compounds on soil microbiota was shown by the work of Glandorf et al. (2001) who found that inoculation of *Pseudomonas putida*, modified to constitutively produce the *phz* biosynthetic gene locus which produced the antifungal compound phenazine-1-carboxylic acid (PCA), only transiently changed the composition of the rhizosphere fungal microflora. The effects lasted for a maximum of 40 days. Glandorf et al. (2001) showed that none of the strains affected the metabolic activity of the soil microbial population (substrate-induced respiration), soil nitrification potential, cellulose decomposition, plant height, or plant yield in the wheat plant rhizospheres during the times of the trials where fungal suppression had shown a measurable effect.

The *Bt* 'toxin' is a protein of near neutral pH, with no recorded persistence in the soil environment. Saxena et al. (1999) reported that, in sterile Hoaglands solution, immunological and larvicidal assays became negative as soon as the solution in which the GM-plants were growing, was no longer sterile, due (presumably) to microbial breakdown.

The redundancy of function between individual members within an ecosystem, as described by Kennedy (1999) compensates for the effects of differing ratios of bacteria and fungi, unless a disturbance is extreme. This suggests that, within an agricultural soil environment, the *Bt* protein would be degraded even in the extremely unlikely event that all the bacteria were consumed by protozoa, or if the fungi were eaten by fungivorous nematodes.

2.4.7 Plasticity in microbial populations

The numbers of soil microbiota recover after application of a toxic substance, but the new community mix may not return to the same state as before the event (Wikström, et al. 2000). This shift in population structure by the soil microbiota to adapt to the new environmental conditions is known as plasticity. West-Eberhard, (1989) defined phenotypic plasticity as "the ability of a single genotype to produce more than one alternative form of morphology, physiological state, and/or behaviour in response to environmental conditions." Plasticity in soil microbial communities can occur through change in genotype, phenotype or significant alteration in population numbers.

The impact of pesticide use, tillage practices, crop rotation, water management and destruction of crop residues can also affect soil microbial populations and lead to selection pressure to promote the functional groups of microorganisms adapted to the new conditions (Marrone, 1993) and also decrease bacterial diversity (Christensen, 1989). However, even though organic compounds in soil will eventually be degraded, an associated change in the soil microbiota may not necessarily interact with the crop plants in a similar manner to those of the previous community mix. At Avon, SA, disease suppression to wheat pathogens increased from a low to high level over a period of 5-10 years following a change in management practices to full stubble retention, limited grazing and higher nutrient inputs to meet crop demand (Roget 1995). The level of disease suppressive activity in soils against fungal diseases is known to be a function of the population, activity and composition of the microbial community (Roget 1995). Cunningham, (1981) had also documented that the suppression of the serious pathogen *Gaeumannomyces graminis* var tritici occurred in America, and followed shorter crop rotations and reduced tillage of monocultures of wheat. It was found that the soil under these conditions became more suppressive to the pathogen, and the suppressive nature of the soil could then be transferred to conducive soils, rendering them suppressive. Suppressive soils can also occur in pasture as well as wheat monocultures, so is not associated entirely with the plant type (Wildermuth,1980). A change in environmental conditions can reverse the trend. A recent case in point occurred at Avon where mineral N had increased, particularly during the summer and early autumn period. As the amount of available N (i.e. nitrate N) in the topsoil increases during this non-crop period, the disease suppression occurring in the following crop season decreased (Roget, 1995).

A change in soil microbiota can also change through the build-up of numbers. It is now known that bacteria communicate by producing small, diffusible signal molecules called acyl homoserine lactones that allow them to assess their numbers by its concentration in the surrounding soil. This phenomenon is known as 'quorum sensing' and is currently the focus of important research on what triggers bacteria to become plant-pathogenic or plant-protective against pathogens, when microbial numbers reach critical levels (Bodman et al. 2003).

2.5 GENETICALLY MODIFIED PLANTS

2.5.1 Does the *Bt* protein get into the rhizosphere of cotton plants?

2.5.1.1 The arguments in favour

Ayers & Thornton (1968) showed that larger molecules reactive to ninhydrin such as amino acids (and proteins) were not detected in the rhizosphere and intact roots of wheat and peas, but were found with damaged roots after swirling with sand. Protein leakage from roots therefore occurs in the peripheral root region as the descending root is abraded by soil particles. Root-attacking microflora such as *Erwinia carotovora*, *Fusarium oxysporum* and plant parasitic nematodes would also expose proteins, including the *Bt* protein, to localised soil pockets outside the root. Tapp & Stotzky (1998) found that the *Bt* protein produced in corn plants was detected in soil surrounding the plant roots for up to 234 days.

Proteins that are targeted for export from the cell carry an endoplasmic reticulum (ER) secretion peptide sequence. However, no ER secretion peptide sequence has been found in registered crops (Kostichka & Warren 1996), and so these proteins are seen as cytoplasmic, and the excretion into the soil is expected to be incidental (passive), rather than actively exported from the cells. Protein leakage may still occur with mechanical damage resulting from rupture of the cell membrane.

2.5.1.2 The arguments against

The potential for export of the *Bt* protein from the plant root cells also comes from an indication of mechanical properties derived from S-S bonds. Proteins to be exported from eukaryotic cells, after undergoing spontaneous folding into their native conformations, are often covalently cross-linked by the formation of intrachain or interchain disulfide bridges between Cys residues (Lehninger et al. 1997, p. 927).

These disulphide linkages provide stability for proteins such as snake venoms, immunoglobulins, milk proteins and structural proteins, and contribute to the elasticity of hair and wool (Straub, 1967). Disulphide bridges can be predicted by conformation analysis, where Cys residues specifically align within 0.3µm of the tertiary protein structure. According to Swiss-Prot annotator (Auchincloss, 2004, pers. comm.), a check of all of the crystallised *Bt* insecticidal proteins revealed that the only strain that contained a disulphide bridge occurred with the *Bacillus thuringiensis* subsp. kurstaki Cry2Aa endotoxin. This had a single S-S bond contained within 635 amino acid residues, (Chen et al. 2003). The lack of disulphide bonds indicates that none of the *Bt* crystalline proteins are likely to be exported from the cells in which they were formed into the surrounding rhizosphere soil. In comparison, trypsin and chymotrypsin, the two digestive enzymes that cleave the active site of the *Bt* protein in the Lepidopteran gut, each have 6 disulphide bridges in 224 residues and 5 disulphide bridges in 242 residues (Schulz & Schirmer, 1979).

2.5.2 Susceptibility of the Soil Microbiota to the *Bt* Protein

Many researchers have published literature referring to the *Bt* protein as "the toxin". Paracelsus (1493-1541) the 'father' of modern toxicology, was quoted as having said "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy" (in Casarett & Doull, 1975).

In the case of the *Bt* protein, dose of the substance alone does not specify an index of toxicity. Specific gut receptors are required for its pore-forming and cellular lytic activity, as well as the number of the gut receptors in the target organism (Van Rei et al. 1989). Age of the target *Helicoverpa* larvae is also a factor. Sublethal concentrations of *B. thuringiensis* spore-crystal complexes have been documented to stunt the growth of susceptible Lepidoptera (Salama et al. 1981; Fitt et al. 1994). A dose of the *Bt* protein that was lethal for neonates, would severely retard, but not kill larger instars (Fitt et al. 1994). The type of host protease is also important for conversion of the pro-toxin to an active toxic substance (Griffitts et al. 2005).

The high specificity of the *Bt* protein for the gut receptors for *Helicoverpa* (shown in Table 2.5.2.1) illustrates that a slight change in the conformation brought about by an alteration in just a few bases could result in loss of function. This specificity has meant that the *Bt* protein is non-toxic for nearly all other living organisms tested,

including mammals, in doses far beyond that found in a normal diet. This means that it is a very specific insecticide which leaves other beneficial insects unaffected. Comparison of different dose loading of the Cry proteins for the targets is tabulated in Table 2.5.2.1.

Table 2.5.2.1 Specificity of the Cry1A(c) protein

Toxin	***M. sexta***		***H. virescens***	
	LC_{50}	CI_{90}	$LC_{50}^{(*)}$	CI_{90}
	Ng/ cm2 on artificial medium			
Bt 2 toxin	20	15-28	7	5-10
Bt3 toxin	20	15-29	157	43-374
Bt 73 toxin (Cry1A(c))	**9**	**6-12**	**2**	**1-4**

(*) CI_{95} represent confidence intervals at 95%, calculated from probit analysis. From Van Rei et al. 1989.

More recent research by Liao et al. (2002) suggests that other species within *Helicoverpa* differ in their susceptibilities to the Cry1A(c) protein, with *H. punctigera* being more sensitive than *H. armigera*.

If the *Bt* protein expressed by the cotton plant was exuded from the roots and into the rhizosphere, it could affect susceptible microbiota and change the ratio of components in the soil food web. It is also possible that unintentional changes to the plant may have occurred with the insertion of the exogenous genes, and these may influence the micro-ecology surrounding the root.

2.6 THE NEED FOR IMPROVEMENT IN PLANT PEST CONTROL

Conventional cotton is the most intensively sprayed crop in the world (Phipps & Park, 2002). Crop rotation is one alternative to application of agricultural chemicals but it is sometimes difficult to find viable alternative crops with comparable cash value. As well as the inconvenience and equipment and labour cost of mixing and spreading pesticides, the chemical sprays tend to be non-specific to crop pests, and thereby also eliminating many beneficial insects.

An alternative to non-selective insecticides was offered by the commercial product Dipel®, which is a dried preparation of *Bacillus thuringiensis*, the bacterium from which the gene has been cloned into transgenic plants. Dipel® has been registered as

a pesticide since 1961 (EPA Report, 2001). It has a high molecular potency compared to that of other pesticides and has been compared as being 300x higher in effect than synthetic pyrethroids and 80,000 times more effective than organophosphates (Feitelson et al. 1993). The commercial product of *B. thuringiensis* (the dried bacterium) is estimated to release into the environment a spore load of 10^{15} per hectare, at a rate of 3,000 tons per year (Vilas-Bôas et al. 2000). There are several variants of the product, all sold under the names of Dipel® eg. 2X, ES, HG, Forte etc., and while each specific formulation is confidential, the differences lie in the number of spores/crystals and the adjuvants used to maintain the active ingredient and attach it to plant tissue (National Registration Authority, Canberra, Aust., pers comm.).

The protection given by these pesticides can be short-lived, however, because surface-applied microbial *Bt* is degraded by UV light, dispersed by wind and washed from the plant leaves by rainfall, necessitating several applications over a growing season (NPTN Fact Sheets, 2000).

In response to these difficulties, various companies have developed different expression systems. Some of these include conjugal mating to transfer the large mobilizable plasmids on which the toxin genes reside to other *Bt* strains in order to broaden their host range. Mycogen Corporation (San Diego, CA) has encapsulated the crystal within stabilized cells of *Pseudomonas fluorescens,* by adding a chemical fixative to the final fermentation broth to rapidly kill and stabilize the cells by strengthening the cell wall and inactivating proteolytic enzymes (CellCap). Field trials indicate that products made through this encapsulation process persist longer than conventional *Bt* products, and since the organisms are dead and cannot spread from the site of application, the product also creates fewer environmental concerns. Crop Genetics International (Hanover, MD) introduced the toxin gene into an endophytic bacterium which colonizes the xylem of plants and provides a type of systemic immunity against susceptible insect pests (InCide) (Feitelson et al. 1992).

While there is a market for these applied pesticides, there are undoubted advantages in the use of plant-expressed proteins which target destructive pests, especially when the protection continues for the whole of the growing season, without the need for additional applications of pesticides.

2.6.1 How the *Bt* protein works

The toxicity of the *Bt* protein derives from both the oral dose and the specificity of target gut receptors. Given both conditions, the active site of the molecule, an amphipathic trans-membrane protein, is inserted into the mid-gut brush-border. The seven alpha-helices of domain 1 form a trans-membrane pore causing efflux of cellular potassium, which results in major ionic imbalance, causing the caterpillars to stop eating and die (Llewellyn et al.1992). A synergistic effect of the toxin can also occur, with secondary infection due to bacterial entry through the pore, in the event that the insect has received a sub-lethal dose.

2.6.2 Specificity of the *Bt* protein

The crystal protein of *Bacillus thuringiensis* subsp. kurstaki HD-73 (Cry1A(c)) was sequenced by Adang et al. (1985). It has three domains within the molecule, and the configuration of the B and C domains are thought to relate to the specificity of binding in the brush-border membrane in the gut of the insect (Van Rie et al. 1989; Chen et al, 1994). Variations within the protein domains at residues 335 to approximately 615 of the Cry1 proteins have been found to be specific to *H. virescens* (Yamamoto & Powell, 1993, pp. 3-41). A relatively large specificity-determining region which in this case encompasses virtually all of domains II and III is needed for identification of multiple receptors to Cry1A-type toxins in the midgut membranes of *Helicoverpa* (Van Rie et al. 1989).

The intracellular crystal proteins have been classified on the bases of their flagellar H-antigens and on their structure, encoding genes, and host range (Schnepf et al. 1998). The major groups and their target insects are listed in Table 2.6.2.1.

Table 2.6.2.1 ***Bacillus thuringiensis*** **protein crystal classification (Rukmini et al. 2000)**

Gene	Subclass	Host Specificity	Protoxin (kDa)	Toxin (kDa)
Cry1	IA(a)	Lepidopteran	130-160	ca. 60
	IA(b)	Lepidopteran/Dipteran	130-160	ca. 60
	IA(c)	Lepidopteran	130-160	ca. 60
	IB	Lepidopteran	130-160	ca. 60
	IC	Lepidopteran	130-160	ca. 60
	ID	Lepidopteran	130-160	ca. 60
	IE	Lepidopteran	130-160	Ca. 60
	IF	Lepidopteran	130-160	Ca.60
CryII	IIA	Lepidopteran/Dipteran	70-71	65
	IIB	Lepidopteran	70-71	65
	IIC	Lepidopteran	70-71	65
CryIII	IIIA	Coleopteran	73	55
	IIIB	Coleopteran	73	55
	IIIC	Coleopteran	73	55
	IIID	Coleopteran	73	55
CryIV	IVA	Dipteran	134	46-48
	IVB	Mosquitoes	138	46-48
	IVC	Black flies	58	not known
	IVD	Nematodes	72	30
	CytA		27	not known
CryV	V	Lepidopteran/Coleopteran	81.2	not known

2.6.3 Difference between the plant-produced *Bt* protein and the *Bacillus*

Most crystal proteins are protoxins that must be activated by proteolytic cleavage into toxic polypeptides. However, the plant-produced *Bt* protein only includes the active portion of the molecule, together with a modified plant promoter, and therefore does not need to be activated.

Within the *Bacillus*, there are separately produced cytolytic (Cyt) proteins which are also found to have toxic properties. These cyt-proteins are not receptor-related, but

bind to specific phospholipids and disrupt not only insect cells but mammalian cells (Yamamoto & Powell, 1993).

Given the diversity of spores and crystals and the synergistic effect between them, Dipel® is not an appropriate control for use in the comparison of toxicity with the plant-produced *Bt* protein.

The image below shows subcultured *Bacillus thuringiensis* at sporulation with the characteristic bi-pyramidal parasporal crystal. When the gene is transferred into a different vector such as *E. coli* the crystal is amorphous which suggests that post-translational modification of the protein crystal occurs within *Bacillus*.

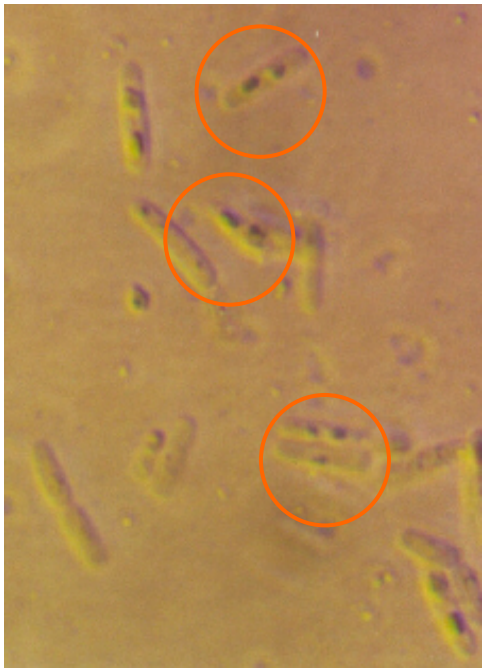


Figure 2.6.3.1 Phase-contrast image of *Bacillus thuringiensis* subs. Kurstaki from a culture obtained from the Insect Pathology Laboratory of the University of Adelaide.

Both the cry- and cyt-proteins are dependent on ingestion for their activity, and will not penetrate the intact exoskeleton of an insect.

Bacillus thuringiensis is moderately persistent in soil with an average half life of four months (NPTN Technical Fact Sheet, 2000) but tends to increase and sporulate within specialised niches of pH and nutrients such as in insect cadavers. Research by Donegan et al. (1995) on the effect of *Bt*k toxin on bacteria and fungi using biochemical tests of individual cultures, community substrate utilization and DNA fingerprinting, failed to show any influence on growth and species composition of

soil microorganisms apart from a transitory increase in numbers. This could have been influenced by factors other than *Bt* production. Previous work by Vilas-Bôas et al. (2000), showed that *Bacillus thuringiensis* vegetative cells did not multiply and that the spores did not germinate in the soil microcosm, even in sterilised soil, and spore germination has never been demonstrated in non-sterile soil.

2.6.4 Genetically modified plants: the advantages and disadvantages

The specificity of the *Bt* protein means that only target insects, and not other beneficial insects, are affected. If the sequence of the protein is altered slightly by a site mutation (such as the substitution of alanine for glutamic acid at position 92) the toxicity is almost completely abolished for Lepidoptera (Chen 1995). Other amino acid substitutions, while not resulting in death, may cause sub-lethal effects such as larval growth inhibition.

The *Bt* protein was shown by Dien et al. (2002) to denature within 15 minutes, at less than 80ºC, during a fermentation process to produce fuel ethanol. It does not contain prosthetic groups of metals, so it completely degrades. In soil, the Cry9C, Cry1A(c) and Cry3A proteins degraded with relatively short half lives of 4.5 days, and 2.2 to 46 days for the Cry1 and Cry3 proteins (Environmental Protection Agency Report Registration Action Document, 2000).

The cotton genome is estimated as 2.2×10^6 kb per haploid chromosome complement, with an estimated 40,000-60,000 genes (Liu, 1997). According to a personal communication received from Monsanto Pty Ltd (1 February 2001), transgenic technology only adds one to a few genes on the one vector, which would represent about 1/100,000th of the total genomic DNA. The *Bt* protein expressed by the plant only represents 0.2% of the total soluble plant protein produced, so it is insignificant in terms of metabolic energy expended by the plant.

Other advantages in terms of agricultural production are summarised from the EPA Report (2000):

- Savings from fuel, equipment and labour costs associated with the reduction in applications of chemicals
- Elimination of the potential for applicator and farm worker exposure associated with the use of more toxic compounds

- Reduced potential for human and environmental hazards from the elimination of drift into non-target areas
- Growers would be less dependent on weather for insecticide applications
- Adverse effects on target organisms should be reduced because the only organisms able to receive a dose are those feeding on the crop
- The plant is producing the protein continuously

The disadvantages are that the *Bt* protein expression varies during the growing season, and the decline begins during the flowering stage (Holt, 1998; Fitt, 2000; Olsen & Daly, 2000), necessitating application of insecticide at about two thirds of the way through the cotton growth cycle (Fitt 1998).

2.6.5 Perceived risks of GM-plants

Expression of Cry1(a) genes in cotton is influenced by one or more of the following: site of gene inserted, gene construct, background genotype, epistasis, somaclonal mutation and the physical environment (Sachs et al. 1998).

Plasmids, microinjection or gene guns are used to introduce DNA into plants, a largely random process, rather than recombination using gametes or compounds that induce mutagenesis. Viable plants are then screened for the expression of the traits. The genetic constructs inserted into the recipient genome may contain material that originated from unrelated organisms and the resulting plant propagules express traits that would not normally have been achieved with traditional genetic recombination through backcrossing. Because each (viable) insertion may be located at a different position along a chromosome, no consistency can be claimed across the sites of insertion for different modified plant types. The insertion of the gene is relatively random and may impact on the control of promoters or enhancers. Control of the novel regulatory elements may also be outside the normal homeostatic feedbacks of the plant.

There is therefore a possibility that effects on microbial and other soil populations will not be due to the transgenic products but will result from unintentional changes in plant characteristics due to the process of genetic engineering (Altieri, 2000; Godoy et al. 1998; Donegan et al. 1995).

With the insertion of various types of DNA to different genera of bacteria, there is now a possibility that through natural recombination, an unanticipated alteration in protein structural domains may result in some affinity to receptors of non-target rhizosphere soil-dwelling inhabitants. Some *Bt*-activity has been documented against mites, nematodes, platyhelminths and protozoa (Feitelson, 1993). Activity against free-living, plant-parasitic and myceliophagus nematodes was found by Ignoffo & Dropkin (1976), although it occurred with different *Bt*-type proteins, and not specifically with the Cry1A(c) protein inserted into the cotton plants. The effect of this activity on the soil microbiota is not known.

An example of an unexpected change in plant characteristics occurred with Round-up Ready® soybeans under conditions of severe drought. The GM-plants were less able to convert nitrogen from the air into usable forms than non-GM soybeans. Another case involved 'gene silencing', which turns off other unrelated genes. Insertion of the *Bt* gene into a strain of potato to make it resistant to the Colorado potato beetle, inactivated the gene for resistance to potato cyst nematode, and the trait was not detected until after approval of the variety (Agnet 2000).

While it is known that nematodes are susceptible to the CryV and CryVI proteins from some strains of *Bacillus* (Ignoffo & Dropkin, 1976), GM-cotton has had the Cry1A(c) gene inserted into the plant genome, and the protein from this particular DNA sequence has not been documented to affect nematodes.

Some of the currently debated risks of modified plants which may affect the soil microbiota are discussed below.

2.6.5.1 Horizontal gene transfer

It may be possible for the plant DNA encoding the *Bt* protein to be transferred to competent bacteria. This includes the transfer of functional DNA other than genes for the *Bt* protein, such as the possibility of the cauliflower mosaic virus promoter present in Roundup Ready® cotton recombining with the genomes of other organisms, leading to the production of new recombinant viruses, or re-activation of dormant viruses (Old & Primrose, 1994). However the transfer of plant-derived DNA to soil bacteria, and its expression, would depend on:

- The amount of non-degraded DNA from the plant material

- Recipient microorganisms or viruses able to incorporate the sequence into their genome.

Stotzky (2000) found that chromosomal and plasmid-DNA carrying the *Bt*-gene could transform competent *Bacillus* after adsorption to clays for up to 15 days (the longest time studied). Gallori et al. (1994) found that the level of transformation occurred between 1 in 10^{-5} and 10^{-8} cells, depending on the type of clay. These investigations, however, used purified linearised and plasmid DNA from donor *Bacillus* strains to transform auxotrophic receptor strains. Vilas-Bôas et al. (2000) used the 75 kb pHT73 plasmid and strain KT0pHT7 3-EmR as the donor and strains 407-1 and 407-0A as recipients to demonstrate conjugation of *B. thuringiensis* carrying the Cry1A(c) gene. None of the experiments used the plant-derived DNA, which is known to have been adapted for increased expression within plant cells by alteration of the A-T/G-C base ratio, so the particular plant-adapted protein may not have been expressed by the bacteria, even if it had been incorporated into the bacterial DNA complement. Vilas-Bôas et al. (2000) also noted that while the *Bt* spores persist in the soil for several years, spore germination and plasmid transfer between *B. thuringiensis* strains and correlated bacteria appears only to occur within non-laboratory environments during the active phase and within specific optimised environments such as within an insect.

The plant genes for *Bt* expression are chromosomal, and therefore less transferable than plasmid-borne genes, limiting the potential transfer to indigenous environmental microorganisms. Transformation of bacteria with plant transgenes has only been accomplished at low frequencies and under high selection pressure but where homology to existing DNA has not occurred, horizontal gene transfer has not been observed, even at extremely low frequencies of less than about 10^{-9} to 10^{-17} (Nielson et al. 1998).

As Clark & Paul (1970) pointed out, nucleic acids, (the constituents of DNA and RNA) are cyclic-N compounds connected to phosphate groups by ester linkages. These are readily degraded in soil, and do not appear to accumulate in soil organic matter. Dale et al. (2002) also noted that if a DNA fragment does not retain functional integrity, it is unlikely to have any environmental impact. In other words, even if plant-derived DNA is incorporated into a bacterial genome by transformation

– with its promoters, for synthesis into the protein – it will only survive if there is a selective advantage for the bacteria to carry the additional DNA. Nielsen et al. (1998) also advised that frequencies of DNA transfer should not be confounded with the likelihood of environmental implications, since the frequency of horizontal gene transfer is probably only marginally important compared with the selective force acting on the outcome. Stotzky (1993) noted that "even if the novel gene(s) is transferred to indigenous microorganisms, there should be little cause for concern unless the novel gene(s), either in the introduced GEM or in an indigenous recipient(s), results in some unexpected impacts on the environment".

2.6.5.2 The transfer of antibiotic resistance genes

The markers that lead to resistance to antibiotics in modified cotton plants are Streptomycin, Kanamycin and Spectinomycin (GMAC, 2001). They are linked to a bacterial promoter that does not function in the plants, so the antibiotic protein is not present in the plants. The American Academy of Microbiology (2002) stated that microbes are much more likely to obtain genes, such as those for antibiotic resistance, from other microbes in the environment, that the probability for such genes to be acquired from genetically engineered plant DNA is remote.

2.6.5.3 Accumulation of the *Bt* protein in the soil environment

It has been postulated that sub-lethal doses may build up to express toxic effects in non-target organisms (Stotzky, 2000; Saxena et al. 2002). The *Bt* protein could accumulate in the environment to concentrations that could constitute a hazard to non-target organisms, such as the soil microbiota, beneficial insects and microorganisms or enhance the selection and enrichment of toxin-resistant target insects. The accumulation and persistence of the Cry1A(b) protein in soil could also result in its leaching through soil to groundwater and in its horizontal movement to surface waters by rain, irrigation, snow melts, etc. as has been observed with heavy metals (Saxena et al. 2002).

This would presuppose that the protein did not degrade in its course through the soil. The product of the inserted genes, the *Bt* protein, is known to be biodegradable. After the reported 40 days growth of corn plants in 50 g of soil the plant roots would have nearly filled the container. The results of the leaching tests reported that the Quickstix test was positive for the *Bt* protein from the plant tubes, but that the

larvicidal effect of the soil (when added to the diet of *Manduca sexta*, a susceptible larva) caused only 12-20% mortality. Only 16 larvae were tested for each soil and the rate of mortality is known to be naturally high at the larval stage of development. After 12 hours of incubation in soil no *Bt* protein was detected from the leachates of any of the 50 g soil columns regardless of the concentration of purified protein. Given adequate moisture and a normal aerobic soil microbial complement expressing protease (normally ~1 x 10^{7-9} culturable cfu per gram soil), proteins will be hydrolysed to their constituent amino acids and degraded to their C, N and O components for recycling back to microbial or plant biomass – quite a different situation from the toxicity of heavy metals. For living organisms without the number of, or specific gut receptors for, the *Bt* 'toxin', the protein should perhaps be comparable with the non-toxic clay moiety itself, as it would have no effect. The postulation by Stotzky (2002) that such an ephemeral protein which cannot be detected after 24 hours even in its pure form, or from plant tissue in only 50 g soil, could constitute an environmental hazard, should be scientifically challenged.

2.7 MEASUREMENT OF *Bt* TOXICITY IN SOIL

The United States Environmental Protection Agency (EPA) *Bt* Plant-Pesticides Biopesticide Registration Action Document (2000, accessed 2003) states that the functional activity of the *Btk* HD-73 was estimated to have an LC_{50} of 0.28ppm against *Helicoverpa virescens*. (The HD-73 strain is the Cry1A(c) protein expressed in the single-gene Ingard® cotton plants, and is the strain against which the feeding larvae show the highest sensitivity.) This means that the *Helicoverpa* would need to ingest about 0.1 g soil if the protein was adsorbed as a 1:1 protein/soil compound. Any other soil microbiota susceptible to the protein would have to either ingest more soil than this, at a similar sensitivity, or demonstrate a higher susceptibility to the toxin than the target larvae.

2.7.1 Adsorption of the protein to clay soil

Smectite-clay minerals, by the nature of their negative molecular surface charge, are able to bond with organic matter, including proteins, generally around the positively charged polar N- entity. These protein-clay or organic-humic-clay complexes bind most strongly at the particular isoelectric point (IEP) of the organic compounds where their positive charge attracts the negative charge of the clay surface and would be influenced by the surrounding soil pH. The isoelectric point of the *Bt*-protein was

reported by Venkateswerlu and Stotzky (1992) as ranging between pH 4.4 and pH 5.5. At pH values above the IEP, the protein is negatively charged overall (Pagel-Wieder et al. 2004), and so would not adhere to neutral or alkaline soils as strongly, if ionic bonding alone were to be taken into account. An earlier work by Harter & Stotzky (1973) reported that binding of organic matter can also occur strongly, even when the pH of the clay suspensions is several units above the protein pI of 5.08 (Swiss-Prot/TrEMBL, available http://www.expasy.org/cgi-bin/pi_tool1?_Q4PNY8@noft@average).

Research by Churchman (2002) demonstrated that chemical bonding of proteins to clays is not due simply to hydrogen bonding, but is likely to include van der Waals interactions and also involve favourable entropy changes. The opposite electrostatic charges of the negative clay surface and positive regions of proteins creates a strong and instantaneous bond, even at high pH. Associated work by Palm et al. (1994) reported using the supernatant from fine pieces of leaf tissue which was incorporated into the soil just before centrifuging. Ipso facto, any *Bt* protein complexed to clay soils would be found in the soil pellet after centrifugation, if adsorption had occurred, and not in the supernatant. Palm (1994) reported the water content of the soil to be 45% of capacity. However the soil types used in the study were a fine sandy loam with a clay component of 10%, a coarse sandy loam containing 2.5% clay and a silt loam with 11.1% clay sized particles. The soil would have only been damp, and the 'fine pieces' of crushed frozen leaf tissue containing the Cry1A(c) protein may not have fixed to the clay. In this current investigation, washed and blotted roots of freshly harvested Coker 312 and 312i cotton plants, the same type as used by Palm (1994), were determined as having a density of $0.8_{(water)}$ by increased displacement. Any of Palm's plant tissue containing the protein and added just prior to centrifuging would therefore have been present in the supernatant, giving a false positive result.

It was suggested by Stotzky (2000) that ionic bonding is not the only factor in resistance to microbial breakdown of *Bt* protein. The physical structure of clay soils, with particle sizes of <2µm and the nature of their planar structure, means they can trap organic matter between the layers. More recent research by Saxena & Stotzky (2001) however, found that the [*Bt*] toxins only partially intercalated montmorillonite clay, a swelling 2:1 Si:Al clay mineral with a significantly higher cation-exchange capacity and specific surface area than kaolinite, a non-swelling 1:1,

Si:Al clay. These observations, however, are in contrast to the work of Estermann et al. (1959) who found that proteolytic enzymes can adsorb on a montmorillonite-protein complex, penetrate between the lattice layers, and hydrolyse protein.

2.7.2 Microbial breakdown of the *Bt* protein in soil

Stotzky (2000) reported the presence of the Cry1A(b) protein within rhizosphere soils, but did not quantify the protein against known standards. Fitt et al. (1994) investigated the declining levels of *Bt* expression once plants began to senesce by using *H. armigera* and *H. puntigera* insect toxicity assays, but did not cross-reference the plant tissue with an immunological assay. It is critical to quantify a substance in the environment in order to measure its toxicity and calculate its rate of breakdown within the rhizosphere.

Solubility facilitates catabolism of polypeptides and proteins, and the *Bt* protein has been described by Li et al. (1991) as amphipathic, as are most membrane-spanning proteins. The *Bt* protein is therefore unlikely to remain undegraded for long in the soil environment, given sufficient water for hydrolysis. The most likely soil animals to be affected by the *Bt* protein are those with a gut membrane that internalise their food, such as nematodes.

However, the localised domains of proteins having an opposite charge to negatively charged clay particles can chemically bond, and previous work by Saxena et al. (2002) reported that the *Bt* protein had been detected in rhizosphere soil surrounding the roots of *Bt*-corn (hybrid NK4640Bt plus 12 additional hybrids). The authors concluded "that the *Bt* toxin's breakdown is inhibited from microbial breakdown because it can adsorb to clay minerals, on the clay-size fraction of the soil on humic acids and on complexes of M-humic acids-Al hydroxypolymers. The binding of the toxins onto these surface-active particles reduced their availability to microbes, which is apparently responsible for their persistence in soil." This agrees with the observation by Wilson (1987) that colloid-bound enzymes may be stabilised and protected from biological attack, and may retain some activity towards substrates of relatively low molecular weight.

Associated work undertaken by Saxena et al. (1999) reported that in sterile Hoaglands solution, immunological and larvicidal assays became negative as soon as the solution in which the GM-plants were growing, was no longer sterile. This

suggests a rapid rate of microbial breakdown of the protein, which could also occur in clay soil, even if more slowly. Saxena et al. (2002) used the purified protein from Dipel® 2X at the rate of 3.2μg g^{-1} soil in 50 g soil, and detected in leachates 6 minutes after addition to the soil column (not long enough for bacterial degradation). Leachates were 'immunologically negative' after 12 and 24 hours in all soils (including the Montmorillonite clay soil which is known to strongly adsorb protein). In an agricultural field the probability of rapid breakdown of the estimated 1.44 grams per acre (based on 60,000 plants), is therefore high.

Regarding the presence of plant roots in rhizosphere soil, none of the procedures from published references relating to *Bt* protein in soils cited methods for guaranteeing the absence of roots within the test soils. Substances specific to root tissue in extremely small amounts within soil are difficult to isolate, and it is possible that some of the *Bt* protein detected within the rhizosphere soils from previous experiments could, in fact, have come from the presence of fine roots, and not from the surrounding rhizosphere soil.

Clay/organic material complexes have been reported to become resistant to bacterial hydrolysing enzymes (Pinck et al. 1954; Harter & Stotzky, 1973; Saxena & Stotzky, 2001) thereby slowing the degradation of organic matter, including proteins. Stotzky (2000) found that in the case of the Cry1A(b) endotoxin inserted into genetically modified corn plants, the *Bt* protein extruded from the roots and complexed onto clay surfaces remained detectable immunologically for up to 234 days. This indicates that the *Bt* protein was chemically stable. The findings of persistence of the protein in soil were similar to those of Donegan et al. (1995) who used an ELISA to detect the purified toxin after 56 days in two different soils. The active site of the protein showed that of the 150 amino acids only three histidine, two lysine and ten arginine residues were present to give a net positive charge, and four aspartate and four glutamate residues would have given a net negative charge. The active site is therefore largely uncharged, and would not preferentially attract the negatively-charged clay surface to the active site of the protein, so orientation of the protein onto a clay surface is likely to be random.

While Koskella & Stotzky (2002) hypothesised that [with *Bt* protein] "The toxins could accumulate to concentrations that may constitute a hazard to non-target

organisms ...” particularly on clay soils, it does not imply that the *Bt* protein would be selectively concentrated, as other soil organic matter with a similar pI and opposite charge would also be adsorbed onto the clay surfaces within the soil. This was confirmed by Pagel-Wieder, et al. (2004), who conducted experiments to determine the kinetics of the adsorption of organic-C compounds to smectite soil. The work confirmed that a first-order inverse relationship occurred for adsorption to 25% smectite clay with increasing organic-C concentration at neutral pH.

2.7.3 Effect of wetting/drying on adsorption of the *Bt* protein to soil surfaces

Surface adsorption of organic material affects the charge characteristics (as indicated by electrophoretic mobility) and hydrogen activity (pH) of the clays. Such changes also affect the suitability of the clay-protein complexes as a substrate for microbes, the biodegradability of the adsorbed protein, and the activity of adsorbed enzymes. Ranjard et al. (2000) found that drying of the montmorillonite-protein complex increased its stability toward microbial attack when rewetted, and so the degradation of proteins in association with clay soils may be multifactorial. One important factor in the breakdown of any protein is, of course, the amount of moisture available. The report of the American Academy of Microbiology (2002) stated that in general, the drier the soil, the longer an ICP will be stable, as there is less microbial degradation.

2.7.4 Other related work on rate of *Bt* protein degradation in soil

With the further commercialisation of GM-plants now under development, the world areas planted with these crops is anticipated to increase, but the effect on the rhizosphere microbial communities due to altered plant characteristics is not well understood. The complexity of the microbiota within the rhizosphere also includes interactions at a molecular level of enzymes and proteins and chemical bonds; and larger ecosystems, which involve an interplay of ecological factors within the broader environment. Some of the interactions which will be considered within the limits of this project are suggested in Figure 2.7.4.1.

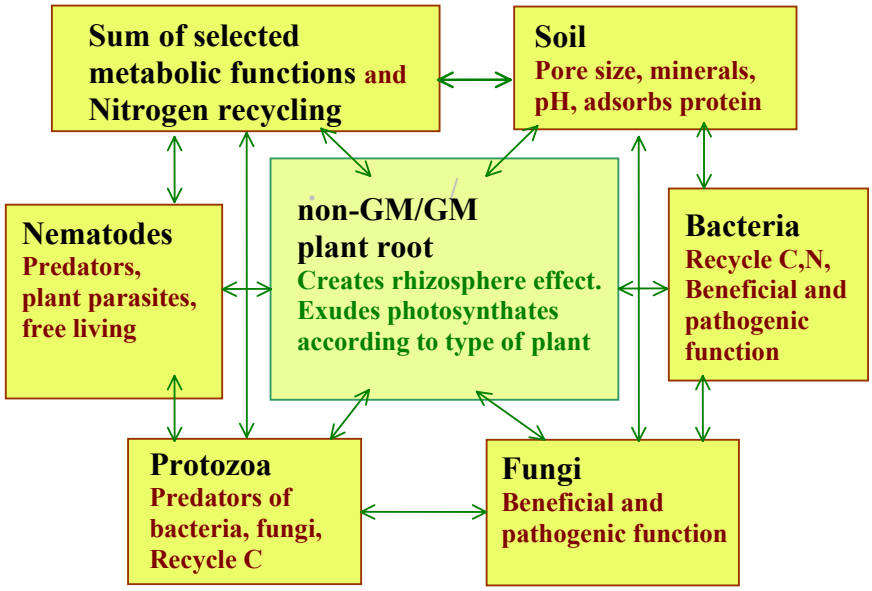


Figure 2.7.4.1 Possible interactions between soil microbial populations.

2.8 CONSIDERATION OF METHODS TO BE USED TO ANALYSE MICROBIAL POPULATIONS

2.8.1 Cultivable bacterial and fungal colony counts

Different methods of microbial detection in soil have particular advantages, as well as their own inherent limitations. Molecular methods using PCR can amplify a particular gene sequence, but bacteria are known to contain multiple and varying copies of gene complement (Klappenbach et al. 2000) and so a consistent ratio of colony count vs. function cannot be implied. Electron microscopy distinguishes bacteria by shape, but dead and live bacteria are not differentiated and the aggregate surface must be coated in carbon or gold, so sample preparation is time and resource intensive. This can be overcome by using light microscopy with viable stains such as acridine orange which stains DNA, but the opacity of the soil is a major barrier to accurate counting, and the method is time intensive. Specific subsets of bacteria can be detected by fluorescent antibody staining, but this is only useful in tracking a single microbial species within the microbial pool.

Two widely used methods available for estimating bacteria and fungi on a unit soil basis and still in use today include the soil plating, and the dilution method, both of which were described by Warcup in 1955. Another method of measurement of original soil populations is to separate the bacteria from the soil by dilution and

filtering at harvest, and to measure the sampled populations by haemocytometer. This result is not influenced by growth under unlimited and general nutrient, as occurs with plating. Although possibly giving a more accurate score of bacteria at the time of harvest, a count of the individual cells by haemocytometer does not provide information on colony morphology which is often valuable in differentiating ratios of major bacterial groups. A further disadvantage is the non-differentiation between viable and non-viable cells.

The plate method for comparing microflora has merit because the viable colonies grown on selective or general media give information about the function of the population with regard to nutrient acquisition. Colonies may also have a distinct appearance on a particular media, for example, the fluorescence of iron-chelating pseudomonad siderophores under ultraviolet light (Rosenthal, 1974) allows identification of pseudomonads able to grow on different carbon sources, and may give an indication of limiting elements between different soils. Another advantage of plating is found with the measurement of zone of clearance around antibiotic producers such as *Bacillus* and actinomycetes commonly found in soil, indicating the relative antagonism by the strength of antibiotics produced.

Brock and Madigan (1991, p. 612) illustrated the difference between 21% O_2 concentration (the partial pressure of air) and 0% in the core of a soil aggregate as 6 mm. Because of this, similar bacteria will not be distributed homogeneously through soil and the aerobic plating methods normally used may result in the loss of the anaerobes taken from the interior of the aggregate. This is particularly relevant with a denser clay soil such as Narrabri, NSW, where most cotton production occurs.

In this project which aims at the comparison of microbial growth at different trophic levels in the rhizosphere of GM and non-GM plants, identification of bacteria to species level is not required. Information on the potential degradation of protein and growth of identifiable saprophytes is appropriate, and this can be shown by using the soil plating method, even though amplification of bacterial growth through the selection of media and conditions of incubation is acknowledged.

2.8.2 Molecular methods for determining variability of bacteria

Molecular probes can detect the presence of a DNA or RNA sequence by selectively amplifying known segments, and the detection of mRNA for a particular gene can

demonstrate its active transcription. Tiedje & Zhou (1996) reported that the 16S rRNA gene is too conserved to be routinely useful for resolution at the species level which represent only a 0-3% difference in 16S rRNA sequence. Since then, variations in the 'collective genome' indicative of different microbial species have been detected by use of Denaturing Gradient Gel Electrophoresis (DGGE) which separates amplified sequences by base sequence or length. DGGE is reputedly able to resolve a difference in a single base within a sequence but it is limited by the resolution of number of bands on a gel, which currently is much less than the number of fatty acids detected by gas chromatography.

An alternative contemporary method for analysing the bacteria within rhizosphere soil is the amplification of 16S rRNA genes by PCR. This method reports the presence of bacteria within soil, as 16S RNA is specific to all bacteria. The relative intensity of colour development on the chromatographic band in the electrophoresis gel indicates the amount of RNA present after a set number of amplifications. The technique of PLFA analysis is able to indicate the presence of active bacteria (and fungi) because the fatty acid is only produced by metabolising microbial entities, and, ipso facto, the DNA had to be present. PLFA used in the rhizosphere soils was also able to indicate the change in signature for the total community, by the effect of a change in environment.

Terminally-labelled restriction fragment length polymorphism analysis (T-RFLP) allows measurement of the size polymorphism of terminal restriction fragments from a PCR amplified marker. The size polymorphism provides an estimate of the diversity of the marker in the community and, in some cases, may provide insight into the phylogenetic structure of the community (Holmes & Thies, 1999). Any marker with regions of conserved sequence (for example the nitrogen fixing gene (*Nif*)) can be used as a target.

The DNA re-association curves from the work of Torsvik et al. (1990) showed that the major part of DNA isolated from the bacterial fraction is extremely heterogeneous, with an estimated 4,000 completely different genomes of standard soil bacteria. Some of the DNA preparations had a rapidly reassociating fraction of only about 5% of the total DNA, and the major portion of the diversity was located in

that part of the community which could not be isolated and cultured by standard techniques. This includes the DNA within plasmids, phages and other viruses.

Other difficulties that arise when analysing mixed microbial communities in soil include:

- Inhibition of PCR amplification by co-extracted contaminants
- Differential amplification
- Formation of artefactual PCR products
- Contaminating DNA
- 16s rRNA sequence variations due to rrn operon heterogeneity,

which would unavoidably lead to a biased reflection of the microbial diversity (Wintzingerode et al. 1997).

This is potentially problematic as humic acids in the sampled soils are most likely to be included in the sampled soils used in the work, and humic acid substances strongly inhibit DNA modifying enzymes.

A record of the phylogenetic variability of the microbial community in itself does not show that the functioning capacity of the whole microbial complement was caused by the presence of one plant-produced protein. The microbial population is known to continually alter in response to substrate availability. The question of the redundancy of function in soil microbiota to degrade proteins and mineralise organic substances will not be addressed by a semi-quantitative measure of the variability of a gene sequence, but by observation of the living microbiota within the rhizosphere at the time of the growth of the plant.

2.8.3 The Biolog system

This method of prokaryotic identification relies on the ability of the bacteria in the sample to oxidise 95 different forms of organic carbon and is measured by the intensity of colour which develops by the breakdown of reducing sugars. It can therefore be used as an indicator of communal metabolic function. However, the system suffers from the following drawbacks:

1. The degree of substrate oxidation may be a function of inoculation density
2. Differing microbiota which use the same substrate may oxidise those substrates to different extents

3. The oxidation of the substrate may reflect the metabolic activities of only a certain proportion of the community (Ibekwe & Kennedy, 1998).

Wünsche et al. (1995) used two Gram-negative bacteria with completely different substrate utilization patterns and showed that the influence of inoculation density was largely removed by an extended incubation period. It does not negate the problem of failing to report the diversity of the community where the dominant utiliser of many compounds may in fact be one species. Knight et al. (1997) also found that when using GM Biolog substrate utilisation plates to compare three soil samples, bulked and thoroughly mixed, there was little or no reproducibility in terms of type or extent of substrate use. This difference from individual samples from a homogenous mix would mask differences across genuinely different soil samples.

The Biolog system is a semi-quantitative assay of carbon utilisation by bacterial populations, and is therefore a test of function: it does not enumerate the contribution made by the uncultivable and unidentifiable microorganisms of which there are an estimated 90% in the soil. Moreover, part of the research conducted by Knight et al. (1997) investigating the effect of heavy metals on soil microbiota compared three replicate, unamended soils. The three replicates were bulked and thoroughly mixed, had the same pH, and had been without the influence of plants for two years. The Biolog tests on these replicates showed variation of the dye formation after incubation of 60 hours, such that 'there was little or no reproducibility in terms of type or extent of substrate use.'

2.8.4 Estimation of Protozoan populations

Two commonly used methods for determining protozoan populations are direct microscope counting and most probable number techniques. It has been reported by Foissner (1999) that the widely used dilution culture techniques provide highly unreliable estimates of the number of active protozoa in natural soil samples, and that methods of direct counting are to be preferred. As Narrabri soil is known to contain a high (%) proportion of clay (Northcote, 1974), the opacity of the medium and the slow movement of some amoebae (Cowling, 1994) determine that the most probable number method is the more practicable in this work.

2.8.5 Phospholipid Fatty Acid Analysis

Whole soil PLFA analysis does not have the capability to identify microorganisms to species and strain level, but rather produces descriptions of microbial communities based on functional groupings of fatty acid profiles (Ibekwe & Kennedy, 1998). The method has the advantage of detecting all viable cells within the rhizosphere because phospholipids are known to rapidly degrade, and only the phospholipids from membranes from the living (or recently dead) organisms are present (Zelles et al. 1992). The membrane fatty acids of microbiota can be used to identify selected and cultivated populations by Fatty Acid Methyl Ester analysis by comparison with a database of known microorganisms. This method also gives information on the proportions of the different microbial groups, especially if there are known fatty acid signatures for them. PLFA also overcomes the bias of selective plating, where amplification of the cultivable bacteria occurs, which may not be representative of the community structure, and would only include those which were able to be selected and cultivated.

Fatty acids can vary according to time of incubation and growth media, and this variability can be used to advantage. If the conditions of culturing are equivalent between paired trials, PLFA is a sensitive method for detecting subtle changes within soil microbial populations.

Information on individual components of the microbial community have been described by several authors. Certain signature fatty acids have been found to be specific for groups of microbiota, eg the fatty acids unique to bacteria are the β-hydroxy, cyclopropane, and branched chain fatty acids, which are not common elsewhere (O'Leary & Wilkinson, 1988). Gram-negative bacteria contain a high proportion of even-numbered, straight chain and cyclopropyl fatty acids (Zelles et al. 1992) and also 16:1ω7, 18:1ω7 and 18:1ω9 fatty acids. The Gram-positive bacteria have been found to contain a substantial amount of odd-chain methyl-branched (eg iso- and anteiso-branched fatty acids (O'Leary & Wilkinson, 1988; Kaneda 1991). White et al. (1996) also noted that the terminally branched saturated fatty acids, i15:0, a15:0, i16:0, i17:0 and a17:0 are also often found in Gram-positive bacteria, but the branched fatty acids can also be indicative of eubacteria. O'Leary (1989, p. 456) reported that bacteria did not contain polyunsaturated fatty acids, and all *Bacillus* species contain major amounts of branched-chain plus small amounts of

saturated fatty acids, and *B. thuringiensis* also contains substantial amounts of unsaturated fatty acids. This bacterial unsaturation does not occur contiguously between the C-C bonds, but is connected by a saturated linkage in between the double bonds. Polyunsaturated phospholipids are found in micro-eukaryotes such as protozoa (White et al. 1996) and the plant phospholipids can also include epoxy-, hydroxy- or cyclopropene rings.

There are also other fatty acids which are common to many life forms such as the 14:0, 16:0 and 18:0 fatty acids (Cavigelli et al. 1995) and these are considered universal. According to Hitchcock (1975), plant fatty acids include the 12:0, 14:0, 16:0, 18:0, 18:1ω9; 18:2ω9,12; and 18:3ω3 and these fatty acids are not useful as taxonomic markers because they are ubiquitous within the rhizosphere. They can, however, be used semi-quantitatively as a measure of total biomass. One notable fatty acid signature specific to fungi is the 18:2ω6, or 9 sterol (ergosterol) (Olsson, 1999).

From the works of Wellburn et al. (1994), Rajendran et al. (1992) and Summit et al. (2000), Palmitic acid (16:0) appeared to be the most commonly occurring and predominant fatty acid, present in all samples. Ibekwe & Kennedy (1998) examined the difference in fatty acids between rhizosphere soils under field and greenhouse conditions for wheat, barley and peas, and also found a dominance of the 16:0 saturated fatty acid in all treatments. This fatty acid has been associated with plant roots (Wellburn et al. 1994); eubacteria (Cavigelli et al. 1995); nematodes (Chen et al. 2000); and fungi (Vestal & White, 1989). It is also known as a precursor of membrane lipids, fats and waxes (Lehninger et al. 1997, p. 653) and is considered a universal fatty acid.

The study of root-associated bacteria associated with field-grown canola and wheat undertaken by Germida et al. (1998), found that the rhizoplane communities grown at the same site differed significantly. The results were obtained, however, by culturing the bacterial colony forming units on trypticase soy broth and then randomly selecting the colonies in order to analyse the population diversity by fatty acid methyl ester analysis. TSA medium has been documented by Siminoff and Gottlieb (1951) to increase the growth of *Bacillus subtilis* over 20-fold at 16 days, with the addition of 2.5% tryptone. It can thus be argued that the enhancement of

growth from the medium did not necessarily reflect the true microbial population mix within the conditions of the soil.

The method proposed for use in this project extracts the fatty acids directly from the rhizosphere soil, avoiding the intermediate step of cultivation. The 'signature' described by the different proportions of phospholipids can be used to describe the overall community regardless of cultivability. The identification of specific phospholipid fatty acids also ensures that only the living entities are detected, rather than cellular storage components, detected by fatty acid methyl ester analysis. This gives the advantage of relating actively metabolising microbial populations within the rapidly changing soil microbial environment. By measuring a change in proportions of known biomarkers, PLFA can report a change in the overall soil population.

Total PLFA data has been used to estimate total numbers of cells with a conversion factor of 5.9×10^4 cells $pmol^{-1}$ of PLFA (Kieft et al. 1994). This presupposes that all cells contain the same amount of membrane phospholipids and that all cells are the same size. Prokaryotes vary roughly between 1 and 10µm in length, and eukaryotes including fungi, protozoa, nematodes and plant cells, between 10 and 100µm. Within a mixed microbial rhizosphere population, it can be argued that the differing mix of fatty acids is considered too generalised for numbers, but the total concentration of fatty acids from each sample may be a general indicator of the total of all active cellular activity, for comparison of different treatments.

Nevertheless, the recent paper by Saxena & Stotzky (2001), who used population numbering procedures found that there was no apparent toxic effect of *Bt* protein on earthworms, nematodes, protozoa, bacteria or fungi in soil, but a method of identifying a change of the type of members of the soil community by their signature fatty acids could yield very valuable information. Any significant deletion or variation in peak height between GM- and nonGM- plant rhizospheres could be traced to that genus of the affected member(s) and more specific tests performed on their susceptibility. This method was therefore chosen to assess differences in the living component of the whole soil populations of the rhizosphere, regardless of culurability.

2.8.6 ^{14}C radiolabelling of the *Bt* protein

^{14}C radiolabelling is useful for tracing the utilisation of the carbon moiety of the purified protein within the food web, but only through one or two trophic levels. Waste products (eg CO_2) are also labelled after breakdown of the protein and there may have been several cycles of predation and incorporation into metabolic products by detritivores before the probe is undertaken. It is already known that the protein can be broken down initially by the plant proteases for recycling within the cell and then by the ubiquitous protein-degrading bacteria within the soil. ^{14}C is therefore limited in its application and will not be used.

2.9 CHOICE OF PLANT AS A MODEL FOR THE CASE STUDY

Several works (Miller et al. 1989; Lemanceau et al. 1995; Marschner et al. 2001) have demonstrated that different plant species influence the types of bacteria present in the rhizosphere through the release of exudates specific for that plant type. When this project began, the only commercially available GM plants were cotton and carnations. The soil and its associated microbial consortia needs to be understood for optimum management to enable agricultural sustainability and productivity. Because of the scale on which cotton is grown in Australia, it was the plant of choice for a case study.

Bt cotton plant varieties are sold based on their particular agronomic traits. Within Australia, there are now eleven non-GM cotton strains and six GM cotton plant varieties available commercially which have been developed for resistance to some fungal diseases, for tolerance to soil salinity or drought and/or length of season etc (Aust. Cotton Seed Distributors, 2004). New strains are developed by backcrossing and do not relate directly to the parent line, so several of the same species of plant may show inter-species differences. Environmental effects on the plants were apparent from yield results, which varied by season and district for the same varieties.

The strain of cotton on which this research is based is known by the trade name Ingard®. It was released for commercial production on 14 September 2000 (OGTR, 2001). According to Monsanto Pty Ltd (pers. comm by email received 1 February 2001), the specific conventional variety and its GM-counterpart only currently adds one to a few genes on the one vector, a change of about 1/100,000 of the total plant

genomic DNA, or about 0.001%. Grown under similar conditions, the same variety of conventional cotton plant and the single-gene Ingard variety developed from it is therefore as close as is practically possible to a paired plant comparison for this work.

2.10 CONTAINMENT REQUIREMENT FOR GROWING GM-COTTON WITHIN THIS STUDY

The environmental conditions north and south of 22º S latitude in Australia are quite different. Based on these environmental conditions and the data that was available, the Australian Genetic Modification Advisory Committee (GMAC), (2000) allowed the commercial release of Ingard cotton south of this boundary. The Authority considered that the planting of GM cotton in this area would not pose risks to the environment or human health that could not be managed. North of this latitude there was no adequate data to support the same conclusions, and so field trials north of the boundary were subject to conditions designed to minimise any possible risk to the environment (OGTR Pers. Comm., by email received 2002).

Any GM-cotton strains not yet commercially released would constitute a Dealing of Notifiable Low Risk, and need to be grown in a secure glasshouse and not allowed to grow to flowering stage. Because all of the strains of GM-cotton used in this research are to be grown south of Latitude 22º and have been commercially released, the guidelines under Schedule 2, Part 1, 1.1(b) (i) of the Act relating to Dealings of Notifiable Low Risk do not apply.

SUMMARY OF CHAPTER 1

Chapter 1 contains a review of the relative literature and comparison of work by other researchers on soil microbiota, using genetically modified corn plants, cotton plant tissue in soil and the purified *Bt* protein in soils. There are unresolved questions relating to the quantification of the *Bt* protein in the rhizosphere soil of living cotton plants, using different soils. The work described in this thesis will address the specific issue of the effect on the microbiota from the rhizosphere of four strains of genetically modified cotton, grown in three Australian soils.

The research plan which follows, outlines the methods to be used.

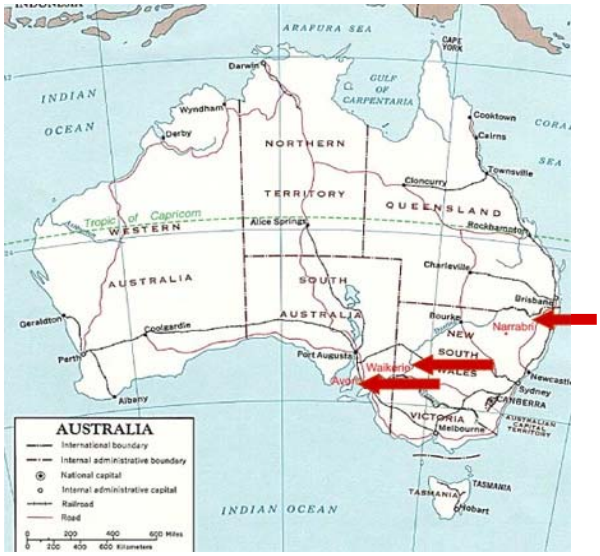
1. Analyse different soil samples with contrasting density and mineral content, to compare the effect on growth of cotton plants, including typical soil where most of the cotton is grown in Australia.
2. Choose suitable paired non-GM and GM cotton plants
3. Quantitatively measure the *Bt* protein within the cotton plant tissue and the surrounding soil
4. Measure the degradation rate of the *Bt* protein within the soil environment
5. Compare individual microbial populations for differences which could be attributed to the presence of the *Bt* protein from GM cotton
6. Compare the community metabolic dynamics for significant difference between the paired cotton plant trials
7. Determine, in terms of risk analysis framework, the environmental impact of the *Bt* protein on the soil microbiota within the rhizosphere, within the conditions of the trials

The experimental design will compare the microbial populations in the rhizosphere of four different parental cotton plants and their genetically paired GM counterparts, within three contrasting soil types, over different harvest ages.

The null hypothesis is that there is no significant impact on the functions of the rhizosphere microbiota of genetically modified and non-modified crop plants, under the conditions of the trials for paired GM-cotton strains and their non-GM isolines.

CHAPTER TWO

DEFINITION OF INITIAL PARAMETERS AND PROTEIN QUANTIFICATION



Section 3

Soil sampling and analysis

Fig. 3.0 The three soil sampling sites

3.1 INTRODUCTION

Because soils are rarely homogenous (Janik et al. 1998), either laterally or with depth, it is not possible to take a representative sample from even the same paddock. Because of soil variability, the publication '*SoilPak*' (McKenzie ,1998), written for Australian cotton farmers, had to be revised in light of the yield mapping now possible using the Global Positioning System. The irregularity in agricultural performance between the best and worst sections of some fields has been attributed to soil variation.

Plant root growth is affected by soil type, and the productivity of a crop in the next season may reflect the degree of compaction where soil is high in clay (Raper et al. 2000). Previous work by Palm et al. (1994) showed major differences in breakdown of the *Bt* protein within different soil types, and work by Marschner et al. (2001) found differences in the microbial communities within plant rhizospheres for different soil types. Montmorillonite clay adsorbs organic material of an opposite molecular charge, slowing the rate of protein degradation (Palm et al. 1994; Saxena & Stotzky, 2001). These findings, however, differed from those of Estermann et al. (1959).

Al and Fe are examples of elements present in soil in varying amounts, and occurring as a complex range of substances, with the proportions of soluble and insoluble forms strongly affected by the degree of acidity (Kennedy, 1992). The trivalent ions

of aluminium (Al^{3+}) and iron (Fe^{3+}) take part in hydrolytic reactions, a result of their high ratio of charge to ionic size (Kennedy, 1992). These hydrolytic reactions are involved in the enzymatic breakdown of proteins both within and external to living plant cells for component recycling, and in the degradation of plant tissue within the soil. Additionally, plants require Fe for their photosynthetic transport systems and chlorophyll formation and redox system, changing between Fe^{2+} and Fe^{3+}. Moreover, the abundance of Fe can influence the competitive advantage of chelating microorganisms such as *Pseudomonas* (see Literature Review). Production of root mucilage by the plant, which rhizosphere soil microbiota utilise as a C and N source, can be governed by Al toxicity, and mineral nutritional factors such as nitrogen and iron can also affect the populations of bacteria in the rhizosphere (Marschner, 1997).

Soil physical properties alone do not necessarily signify that the same bacterial communities will develop for a given set of conditions. An example of this difference in microbial communities was shown by Germida et al. (1998) where significantly different bacterial communities were found within crops grown at the same site. Because contrasting physico-chemical properties of soils have such an important influence on microbial populations (Literature Review, p. 7) it was necessary to incorporate soils of contrasting texture and composition into the plant trials described here, in order to investigate whether a significant difference exists between two paired plant types grown within the same soil, and between different soils. This study on the rhizosphere therefore required a measurement of the effects of both plant and soil type.

The soil analysis described in this section determined differences in the physical and chemical properties between recently cropped, unmodified and contrasting agricultural soils. The practical approach to dealing with the influence of the soil variability on the rhizosphere microbial populations was to accept that the sampled soil used, with its different mix of physical and chemical properties, would not be constant over soils of a similar type. The effect of the plant on the soil microbiota was compared from soil which had been mixed before being added to the pots, to establish a reasonably 'homogenised' mix for comparison of the same paired plant varieties.

3.2 MATERIALS AND METHODS

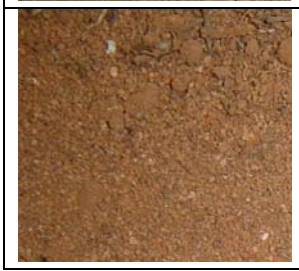
3.2.1 Regulatory compliance

An AQIS licence was obtained to transport soil from Narrabri in New South Wales to South Australia, and all soils from Narrabri soil pot trials were autoclaved after use.

3.2.2 Soil collection and storage

The three soils chosen for the trials included a sandy loam from Avon, South Australia, a sandy soil from Waikerie, South Australia, and a clay soil from Narrabri, New South Wales. The locations are marked in Figure 3.0 and the defined soil types and previous management are listed in Table 3.2.2.1 below. These soils were chosen because of the contrasting textural properties, and known neutrality of pH, similar to commonly cultivated agricultural soils.

Table 3.2.2.1 Definition of soils used

Images of sampled soils	Location	Soil classification[1]	Previous crop	Texture grade from % clay content [2]
	**Avon**: Sth Aust. 34º 14' 15"S, 138º 18' 40"E	Lithocalcic Calcarosol	Wheat	Fine sandy loam
	**Narrabri**: N.S.W. 30º 19'S, 149º 146'E	Black Vertosol	Cotton	Heavy clay
	**Waikerie**: Sth Aust. 34º 14' 16"S, 140º 02' 25"E	Arenic Rudosol	Pea	Red sand

[1] Isbell, 1996; [2] Northcote, 1974

All soils were sampled from 0 to 10 cm below the surface using a shovel. The soil moistures were determined gravimetrically by weighing approximately 100 g of

freshly collected soil, drying overnight at 84ºC and reweighing to obtain the dry weight equivalent.

The calculation for the proportional change in moisture content is

$$\Delta \text{ air-dry moisture} = \frac{(\text{Weight of moist soil + container}) - (\text{Weight of dry soil + container})}{(\text{Weight of dry soil + container}) - (\text{Weight of container})} \times 100\%$$

(Blakemore et al. 1987) [3.2.2.1]

Avon soil contained 2% moisture and was stored in a 7 cubic metre bulk container in a large storage shed. Waikerie soil, containing less than 3% moisture, was stored in sealed plastic bags. The Narrabri soils were transported by overnight freight from New South Wales in a sealed insulated container and contained 16% moisture on receipt. The pooled Narrabri soils were spread out on a sheet of black plastic for 24 hours to air dry, then replaced in woven mesh bags until potting.

3.2.3 Minimisation of soil heterogeneity within area samples

To ensure that any change in rhizosphere soil microbiota reflected the influence of the root exudates of the different cotton plant varieties and not from soil variability alone, all sampled soils from each area were passed through a 5mm sieve and then pooled prior to potting.

3.2.4 Laboratory analysis of soils

As the plant trials were conducted in non-draining plastic pots, determination of the field moisture capacity was required to avoid waterlogging and microbial shift due to anaerobic conditions. The detailed method and apparatus used to calculate field moisture capacity is shown as Appendix 3.1. Particle density and particle size distribution were also measured (Appendix 3.2 and 3.3) as these soil properties can affect hydraulic conductivity and the mobility of microfauna such as protozoa and nematodes, and the rate of enzymatic hydrolytic activity through water retention.

The pH was measured using a soil/water ratio of 1:5 (as per Rayment & Higginson, 1992, p. 17) to ensure that the soils were not highly acidic or basic, which could denature the *Bt* protein. The electrical conductivity of the three sampled soils was also measured to ensure that salinity would not inhibit the growth of cotton plants.

3.2.5 Determination of clay type in Narrabri soil by X-ray diffraction

Analysis of the mineral composition of the Narrabri soil used in the work was obtained from X-ray diffraction analyses to measure the proportion of smectite as per the method of Taylor (1991). The samples were ground in an agate mortar and pestle and passed through a 0.5mm sieve. A 1 g sub-sample was further ground for 10 minutes in a McCrone micronizing mill under ethanol. The resulting slurry was oven dried at 60°C then thoroughly remixed with an agate mortar and pestle before being lightly pressed into aluminium sample holders for X-ray diffraction analysis of the powdered sample.

X-ray diffraction patterns were recorded with a Philips PW1800 micro-processor-controlled diffractometer using Co Ka radiation, variable divergence slit, and graphite monochromator. The diffraction patterns were recorded in steps of 0.05°2θ with a 3.0 second counting time per step, and logged to data files for analysis.

Quantitative analysis was performed on the XRD data using the commercial package SIROQUANT from Sietronics Pty Ltd. The data was first background subtracted and calibrated for the variable divergence slit. Results were normalised to 100% and do not include amorphous or unidentified phases.

3.2.6 Determination of soil components by mid-infrared analysis

A BioRad analyser, FTS175, with 'Grams' software (Galactic NH) in the mid-infrared spectrum was used to predict the physical and chemical attributes of the sampled soils. This allowed comparison, and avoided bias from using only the one procedure. The analysis predicts elemental composition of soil, as well as structural properties such as particle size distribution and density. Using this method, 20 g soil samples were oven-dried overnight and pulverised to a fine powder by mortar and pestle, and then sealed pending submission for analysis. The amplitude of the peaks of wave numbers (cm^{-1}) were converted to digital values for each of the chemical and physical properties recorded, and the estimated values were compared with a dataset of soils previously analysed and correlated with the results of laboratory analyses which were done by standard 'wet chemistry'. This analysis resulted in rapid and simultaneous measurements of the soils. The investigated properties of the sampled soils are shown in Table 3.2.6.1.

Table 3.2.6.1 Soil characteristics measured by mid-infrared analysis

Carbon content organic carbon, charcoal content particulate organic carbon (mainly of plant origin) Carbonates	**Physical properties** % clay-sized particles % silt-sized particles % sand-sized particles Bulk density proportion quartz propn. kaolinite clay propn. smectite clay estimated. lime requirement plant-available water capacity	**Water holding capacity** at 0, 10, 30, 50 kPa, W100K, W500K; W5 bar and W15 bars which equals 1.5mPa, which is the consensual wilting point of plants[1]
pH and conductivity pH(water), pH($CaCl_2$), effective sodium % electrical conductivity effective cation exchange capacity		**Elemental analysis** Si, Al, Fe, Ca, Mg, exchangeable Ca exchangeable Mg total nitrogen

[1] (Waisel et al. 1996, p. 278)

3.2.7 Comparison of Aluminium and Iron content in soil

An acid soil digest was undertaken to resolve the variability of Al and Fe in Avon soil, and of Al in Waikerie soil that was predicted by the mid-infrared analysis. Soil samples were prepared by mixing with aqua regia (3:1 HCl and HNO_3). Each sample was then dried at 80ºC and dilute acid was added to known concentrations. Aerosols of the acidified soil samples were decomposed by inductively coupled plasma to disassociate the analyte compounds into individual atoms. The ionised elements were then identified by the individual wavelengths and the proportion of the total was calculated by the intensity of the reflected mid-infrared light beam. The compared results of the two methods are shown in Table 3.3.1.1.

3.3 RESULTS

3.3.1 Physical characteristics from mechanical analysis

Table 3.3.1.1 Soil texture, using the pipette method

	Size fractions (mm) [1]					
Sample	**Coarse sand 0.2-2**	**Fine sand 0.02-0.2**	**Silt & clay <0.02**	**Silt 0.002-0.02**	**Clay <0.002**	**Total**
Avon	34.6	40.9	24.8	8.9	15.9	100.3
Narrabri	7.7	14.2	78.8	19.3	59.5	100.7
Waikerie	62.1	28.0	9.8	2.5	7.3	99.9

[1] Weight as % of oven dry weight of soil sample

The particle density was determined by the pycnometer method (Appendix 3.2), and calculated from oven-dried soil as Avon 2.57, Narrabri 2.73 and Waikerie 2.66 g^{-cc}. For comparison, pure clay montmorillonite is listed as having a particle density between 2 and 3, and the value commonly accepted is about 2.8; and pure quartz is 2.65 g^{-cc} (CRC Handbook of Chemistry & Physics, 1968 (a)).

Field moisture capacity was measured using 10kPa suction on sintered glass from saturation. The results were: Avon 18.1%, Narrabri 34.5%, and Waikerie 8.2%. The field moisture capacity correlated well with the mechanical analyses of particle density, ie., the higher the clay and silt contents, the higher the water retention.

3.3.2 Determination of clay type in Narrabri soil by X-ray diffraction

The X-ray diffraction spectral scan of the Narrabri soil appears as Appendix 3.4. A summary of the analysis by the SIROQUANT software is tabled below.

Table 3.3.2.1 Mineral content of the Narrabri soil used in the pot trials

Mineral	% content (average of 3)
Quartz	32
Orthoclase	3
Albite	15
Smectite	45
Kaolin	2
Mica	2

The percentage of clay content differed from the pipette method by 14%, but showed that the soil was nevertheless very high in smectitic clay.

3.3.3 Determination of soil characteristics by mid-infrared spectral analysis and comparison with mechanical analysis

The tabulated mid-infrared predictions, with comparisons against mechanical analysis, are shown in Table 3.3.3.1 below. The number of significant figures shown are appropriate for the precision of each of the tests.

Table 3.3.3.1 Comparison of mid-infrared spectral analysis of soils and mechanical analysis

Results from mid-infrared analysis					Results of mechanical analysis		
MIR title		**Avon**	**Narra-bri**	**Waik-erie**	**Avon**	**Narra-bri**	**Waik-erie**
OC	total carbon in soil	1.5	1.2	1.3			
Char	total charcoal in soil	0.1	0.2	0.1			
POC	particulate organic carbon (litter)	1.0	0.2	0.3			
CO_3	Carbonate	9.4	1.7	3.8			
pHw	pH (H_2O)	8.7	7.7	9.0	8.5	7.7	8.5
pHc	pH ($CaCl_2$)	7.5	7.5	8.8			
ESP	exchangeable Na%	13.6	10.4	0.0			
EC	elect. conductivity	0.4	1.0*	0.2	0.2	0.2	0.1
ECEC	effective cation exchange capacity	15.9*	39.6*	17.5*			
Clay	clay sized particles	5.1*	49.4	25.3	15.9	59.5	7.3
Silt	silt sized particles	26	24.	12	8.9	19.3	2.5
Sand	sand sized particles	69	27	63*	75.5	21.9	90.1
BD	bulk density	1.3	1.1	1.4*	2.5	2.7	2.6
W.0kp	} water holding capac.	18*	42	27*			
W10kp	} water holding capac.	44*	46	42*	18.2	34.5	8.2
W30kp	} water holding capac.	41*	45	37*			
W50kp	} water holding capac.	30*	41	31*			
W100k	} water holding capac.	20	38	24			
W500k	} water holding capac.	0*	37	6*			
W5bar	} water holding capac.	0.5*	28	6*			
W15ba	(= 1.5 mPa): consen-sual plant wilting point	0*	25	3*			
Tot N	total nitrogen	0.07	0.08	0.09			
Qu	Proportion quartz	76*	36*	73*		32%	
Kaol	Proprtn kaloninite-clay	2*	19*	5*		2.3%	
Smect	Proprtn smectite-clay	12*	44*	18*		45%	
Lime Req	estim. lime require-ment for the soil	6.1*	3.7*	5.2*			

… Continued

Results from mid-infrared analysis					Results of mechanical analysis		
MIR title		**Avon**	**Narra-bri**	**Waik-erie**	**Avon**	**Narra-bri**	**Waik-erie**
Si-XRF	)	79*	64	75*			
Al-XRF	) elemental	6.3*	14.7	8.0*	9.5	17.55	
Fe-XRF	) analysis	4.4*	7.7	3.7	10.02	37.35	
Ca-XRF	)	4.7*	1.7	2.5*			
Mg-XRF	)	1.1*	1.1	0.7			
Ca-exch	exchangeable ca	9.3	20.5	11.7			
Mg-exch	exchangeable mg	1.4	7.0	1.4			
PAWC	plant available water capacity.	44*	22	39*			
	* Less reliable value due to spectral mis-match that results from variability in this characteristic from the calibration spectra against which other samples are compared						
	Mechanical (water) pH was determined at 20ºC by the method of Rayment & Higginson (1992).						
	Bulk density measured mechanically is without pores.						
	The proportions of quartz, kaolin and smectite have been extracted from the x-ray analysis. The spectra is shown as Appendix 3.4.						
	The Al and Fe component of Avon and Narrabri soils were analysed by acid digest and are shown as a percentage value, as an average of three samples.						

3.3.4 Comparison of Aluminium and Iron soil content

Acid soil digests were undertaken to resolve the proportion of the Al and Fe with greater precision than is shown in Table 3.3.3.1 and as described in paragraph 3.2.7.

From the soil digest, the Al content of each of three replicate samples showed that Narrabri soil had 1.8 times higher aluminium content and 3.7 times more Fe than Avon soil. The comparison of Al between the mid infrared spectral analysis and acid digest was very close, but Fe differed by a factor of 10, possibly because of differing levels of oxidation and peak overlap. Both methods confirmed that the Al and Fe content of Narrabri soil was significantly greater than that of the Avon soil.

3.4 DISCUSSION

The properties of the soils tested showed that each of the soils sampled contrasted sufficiently from each other to meet the requirements of varying soil influence within the paired cotton plant pot trials.

The X-ray analysis confirmed that the cationic smectites are present in the highest proportion in the Narrabri soil and would therefore bind strongly to protein and other organic matter of an opposite ionic charge. The use of highly smectitic soil allowed comparison of results from this project with the work of Palm et al. (1994).

The value in using more than one method for soil testing was seen in the comparison of the mid-infrared soil analysis when compared with traditional (wet) chemistry and X-ray analysis. The mechanical analysis was closer to the soil characteristics described by Northcote (1974) in the proportions of sand, silt and clay-sized particles for the Avon sandy loam, Narrabri heavy clay and Waikerie sandy soil. Similarly, the bulk density of the Narrabri soil was expected to be higher than the other soils because of the higher proportion of clay-sized particles. However, this was not reflected in the mid-infrared estimation at the time of the analysis. Soil characteristics according to the mid-infrared system were estimated from samples against which datasets of known soils were matched, and the sandy loams and sands were more represented at the time of the analysis than Narrabri soils.

From the results of both tests, it appeared that one of the strengths of the mid-infrared analysis was the estimation of the proportion of quartz and smectite clay as these were both similar for the individual tests. Estimates of soil pH_w were very close between the two methods, except perhaps for the Waikerie soil. However, the water holding capacity, measured at 10kPa, differed markedly, particularly within the Waikerie soil. Within this project, it was essential to avoid waterlogging and anaerobiosis at the base of the pots, and so the mechanical analysis was used. Because of the higher water holding capacity of Narrabri soil compared with the other two soils, the increased water within the pore-spaces required control via gravimetric watering to less than the field moisture capacity.

The soil pH showed neutral to slightly basic soils, possibly due to the calcium-carbonate content. The *Bt* protein would not be denatured within the relative neutrality of the three soils.

Fe and Al were present in both Avon and Narrabri soils, but on average were higher in the Narrabri than the Avon soil. As no soils had a pH_w less than 6, Fe toxicity would be unlikely to occur in the plants (Kapulnik, 1996). Total N was depleted in all soils at the beginning of the project but there was biologically available carbon in the form of leaf litter. This suggests that N was more limiting than C for incorporation into microbial biomass. The ratio of C:N will be addressed in later sections of this thesis.

Section 4

Growth of plants and harvesting of plant tissue and rhizosphere soil

Fig. 4.0 Cotton plants 189, 289i, C312 and C312i grown in Avon soil at 9 weeks.

4.1 INTRODUCTION

4.1.1 Inherent variability of plant root exudates

Organic compounds lost from roots include sugars, amino acids, organic acids, fatty acids, nucleotides, vitamins and enzymes and these vary with plant species. The structure of rhizosphere communities of field-grown annuals and perennials is also influenced by the plant species grown (Westover et al. 1997; Heuer et al. 2002; Dunfield & Germida 2001). Root exudates, even from a single plant species grown in sterile conditions, are complex and variable mixtures of chemicals (Bacic et al. 1986), so that virtually any process affecting plant growth or physiology could have an effect on the quality and quantity of the root exudates (Curl & Truelove, 1986). Watkins (1981) reported that 23 amino acids have been found in root exudates, but the quantity of amino acids exuded varies widely among plant species. Even within a monoculture of a field-grown cotton crop, carbohydrates in seed coats and root exudates differ significantly among cultivars (Watkins, 1981).

4.1.2 Possible change in physical attributes between the non-GM and GM cotton plants by random insertion of the *Bt* gene

If the signalling process determining the cell division or differentiation in the root apical meristem of a GM-plant is disrupted by an exogenous gene, then physical disruption of the developing root cells may alter the growth patterns or function (Assoc. Prof. Rob Reid, pers. comm.) and affect nutrient uptake such that the surrounding microbiota may also be influenced by the physically altered plant.

Response of plants to herbivory may also be different in non-GM and GM cotton plants. Insect damage in itself may alter root secretions by the release of phytoalexins which are produced on wounding. Takahashi et al. (2004) found that

the bacterial alarmone guanosine 5'-diphosphate 3'-diphosphate, which is involved in a key regulatory process governing bacterial gene expression, was secreted by plants under abiotic and biotic stress. If the expression of this chemical signal differed between the non-GM and GM plants under different levels of stress from herbivory, for example, the microbial community could possibly respond to the plant hormone excreted through the roots and trigger their own response mechanisms. In this work the plants were grown in a glasshouse, so this effect could not be confirmed but could be considered in future research.

4.1.3 Possible metabolic effect from the insertion of the exogenous protein-generating gene

Sullivan et al. (2003) described the highly specific plant system for the degradation of individual proteins that is necessary for the survival of plants. The mechanism of proteolysis is closely controlled by the plant and requires a tag of the protein with ubiquitin, ready for its destruction by the enzyme 26S proteasome, in preparation for degradation to release nitrogen and carbon in the form of amino acids. If cellular degradation of the constitutively expressed exogenous plant protein is not controlled, the nitrogen component may not be available for sequestration by the plant. Root exudates of a GM plant may subsequently vary from the non-GM plant, with the result that the ratio of bacteria and fungi in a microbial population may be altered because they have different growth optima depending on the C:N ratio.

4.1.4 Influence of plant growth under nutrient deficient conditions

Mineral nutrient supply can also affect root growth, morphology and distribution of root systems in the soil (Marschner, 1997, p. 513). The interaction of metabolic pathways in plants is complex, and deficiency in one element can affect several pathways simultaneously. Calcium, which is needed to preserve the integrity of the cell membrane, is an example. Calcium-deficient tissues have enhanced respiration and decrease the net rate of protein synthesis, and environmental cues result in depolarisation of the plasma membrane through activation of calcium channels (Marschner, 1997, pp. 292-3). Calcium is also required for cell wall stabilisation, and because of this, deficiency or altered protein synthesis pathways may lead to increased root leakiness of low molecular weight solutes (Marschner, 1997, pp. 292-3). This increased leakiness could lead to greater loss of the *Bt* protein from the roots of GM plants where Ca is deficient.

When plants are nutrient deficient the amounts of low molecular weight root exudates often increase and the composition of the exudates is altered (Marschner, 1997, p. 555). These and other conditions which control the plant root systems will probably influence the rhizosphere and its microbial inhabitants, as rhizosphere microorganisms can influence the acquisition of phosphorus, potassium and to some extent, also nitrogen, from soils mainly via their effects on root morphology and physiology (Marschner, 1997, p. 562). Because of this close communication between the plant root exudates and rhizosphere microbiota, any difference in plant mass, rate of growth, or type of exudates between the non-GM and GM plants, may cause a shift in the type of microbial populations within the surrounding rhizosphere.

It is therefore possible that GM plants may be relatively disadvantaged in comparison with the non-GM plants for N because of the extra N required to produce the *Bt* protein; and it is possible that other systemic proteins (including N-containing compounds secreted from the roots) may be limited by this deficit.

From a BLAST search, the elements required to produce the Cry1A(c) protein were calculated, and revealed that the C:N ratio required for its production was 3.3:1. No published work which refers to the effect of the additional implant of an endogenous protease for sequestration of N from the protein by the plant in the event of nutrient deficiency has been found. The nitrogen requirement for the additional protein may affect the composition of root exudates of the plants producing the exogenous protein, and the soil microbiota could respond to the altered root environment.

4.1.5 Control of incidental factors

Two major environmental factors that influences the growth of cotton plants are the combination of growth period and temperature. The Australian Cotton C.R.C. actually evaluates cotton growth in terms of degree-days (Bange & Milroy, 2000). When grown in a glasshouse with homogenised soil, controlled temperature, randomised rotation and gravimetric watering, environmental variability is greatly reduced. However, there may still be minor differences in carbohydrate production from photosynthesis (also available to the roots and exudates) which will vary according to seasonal inherent light energy and vigour of individual seeds. The microbial response may therefore differ between batches of plants, but variation would be expected to be minimal.

4.1.6 Influence of plant growth conditions in pot trials

Pot trials have been criticised for the artificial plant growth conditions that they provide thereby excluding the natural variability of an agricultural field (De Vries, 1980). The volume of the container and nutrient or water optimisation have a major influence on growth, and under glasshouse conditions there is often a lack of above-ground herbivory.

The microbial food web can also be altered by the regular watering of pot trials to onstant moisture. Clarholm (1981) found that two days after heavy rain on a podzol there was a ten-fold increase in bacterial biomass in the humus layer. There was a 20-fold increase in amoebae four days after the rain that coincided with a decrease in bacteria. This elimination of wetting/drying cycles could affect the soil microbial populations around the root zone. Treves et al. (2002) showed that different microbial populations can become dominant at different soil saturations. Care was taken here to ensure that pots were watered to less than field capacity. Altieri (1999) also reported that soil disturbance replaces stratified surface soil horizons with more homogenous physical characteristics and residue distribution patterns, and that the resulting loss of a stratified soil microhabitat caused a decrease in the density of species. It is also possible that in pots where root growth is restricted, they would fill the pore spaces and lead to anaerobic conditions that cause a shift in soil microbial populations, distinct from field conditions. This was avoided as much as possible by keeping the field moisture capacity below saturation.

Because the non-summer temperatures of South Australia lengthen the growing period of the cotton plants beyond a reasonable trial length for experimental analysis, the plants had to be grown in a glasshouse. There is thus a trade-off between results gained within what is considered a controlled and relatively artificial environment, and 'the real world', where rainfall, temperature and soil variability all impose their influence and the results may differ between pot trials and field conditions.

Taking these factors into account, the purpose of this section of this thesis is to describe the conditions under which the plants were grown, and to quantify the elemental plant tissue content of the non-GM and GM plants. This benchmark was used to determine whether any physical or elemental differences occurred in the

plants that may have been influenced by the genetic modification and its subsequent effect on the surrounding microbiota.

4.2 MATERIALS AND METHODS

4.2.1 Regulatory compliance

The regulatory conditions for all trials were met, as all of the cotton strains were released commercially at the time that the work commenced. Below latitude 22° S in Australia, "the growing and harvesting of the [Ingard] cotton, disposal of crop residues, and transport of seed is essentially unrestricted" (OGTR pers. comm., 2002). All plants were grown in a secure glasshouse and no GM plants were grown to flowering. All GM-plant tissue and soils were autoclaved after analysis. The growth of the pot trials at CSIRO Land and Water, Glen Osmond, South Australia was covered under the Australian Cotton Research Institute's licence to grow commercially-released GM-cotton under GMAC permit PR-55.

4.2.2 Choice of plant as a model for the case study

The trials were limited to four paired cotton plants and their parental non-GM equivalents, listed in Table 4.2.2.1. Further reference to the paired non GM/GM plants will use the notation summarised in this table, where the subscript 'i' refers to the (inserted protein of the) genetically modified paired plant. The seeds were obtained from the CSIRO Plant Industry, ACRI, at Narrabri, N.S.W.

Table 4.2.2.1 Cotton plant varieties used in all trials

Strain	Variety	Properties[1]
V15,V15i	Siokra	Okra leafed, old variety. Now replaced by V16.
V2, V2i	Sicala	Normal leaf. Now replaced with V3. *Fusarium* ranking 81; *Verticillium* ranking 100. This is the CSIRO standard against which all cotton strains are compared for Verticillium-wilt.
189, 289i	Sicot	*Fusarium* ranking 100; *Verticillium*- ranking 97. This is the cotton strain against which all Fusarium-wilt susceptibility is evaluated.
312, 312i	Coker	Susceptible to bacterial blight, Verticillium-wilt, Fusarium-wilt, *Alternaria* and black root rot[2]

(1) 2004 Disease Rankings as a comparison against a 'benchmark' cotton strain, where the value has been set to 100, as per the publication of the Cotton Seed Distributors Ltd.

(2) Constable, 2003, CSIRO Plant Industry, ACRI, Narrabri, Personal communication.

4.2.3 Plant growth conditions in pot trials

All pot trials were established using 5mm sieved soil. Three different soils were used and 1kg soil was weighed into each of the 1.05L pots.

The plants were kept in a secure glasshouse with the ambient temperature of 25°C ± 4°C. Six seeds were planted in each pot to a depth of 1 cm. The plants in the paired trials were watered every second day. For Avon soil, pots were brought up to 18% above mass of dry soil by weight as this was closest to the moisture capacity (refer Section 3). The Narrabri soils were kept at about $^2/_3$ the field capacity of 34%, to avoid possible anoxic conditions in the non-draining pots. The moisture capacity of 8% for Waikerie soil reflects its sandy nature (Section 3). In trials where Hoaglands nutrient solution was added, 10 ml was applied once weekly to each pot and watered in. For the plants grown under conditions of nutrient-deficiency, only water was added to the plants, ie, the only nutrients were gained from the soil. The chemical composition of the Hoaglands solution is given in Appendix 7.1.

All paired plants were kept in a water bath at 25ºC near the optimum temperature for cotton root growth (McMichael & Burke, 1996). Apart from differences in nutrient availability and differences in growth due to individual plant strain, overall plant growth is affected by the absorption of incident light and temperature. The pots were rotated during growth at each watering and moved to ensure that localised difference in temperature of the water bath did not occur, to avoid differential growth factors. A relative growth comparison by measurement of above-ground plant mass between the non-GM and GM plants under the conditions of each of the paired trials was therefore compared as a measurement of the sum total of all varying factors.

4.2.4 Comparison of plant growth under limited nutrients

Cotton grown commercially in Australia is monitored for nutrient requirement and N-fertiliser is applied as needed. Growth of cotton under nitrogen adequacy may not reveal differences that may otherwise be amplified under conditions of deficiency. V15 and V15i plant trials were therefore set up under the same conditions of growth but with some plants receiving only RO water from the time of planting until harvest at 9 weeks. The only nutrients available to the plant were therefore present in the soils at the start of the trials.

Potassium, nitrogen and magnesium are good indicators of nutritional status of the plant as these minerals are readily translocated to the young leaves (Marschner, 1997, p. 396). This information can be used to compare the ratio of mature to young leaves, and confirm nutrient deficiency.

4.2.5 Calculation of total root mass

The volume of the plant root reflects the amount of tissue required to supply the nutrient uptake requirement of the above-ground plant tissue. This root mass influence the surrounding soil microbiota both by habitable spaces and the associated root exudates.

An estimation of root volume is one of the measurements required to understand plant performance and to compare non-GM and GM plants as a microbial habitat. As noted by Pierret et al. (2000) the total root mass of plants is difficult to measure because of the inextricably linked association of the fine roots with soil particles. In addition, core washing techniques are extremely imprecise and labour-intensive. This has been found by other researchers specifically with cotton plants. Jordan (1983) reported that the total biomass of cotton plants produced by the root system comprises approximately 10 percent of total biomass produced by the plant during a growing season. However it is known to be virtually impossible to separate roots from soil without losing a significant amount of the root sample to be measured.

Pierret et al. (2000) developed a system of measuring root length density in intact samples using X-radiography and image analysis, although all techniques using spectral radiation are limited by the opacity of soil. Mid infrared analysis penetrates approximately 2.5μm and near infrared up to several millimetres (S. McClure, Electron microscopist, CSIRO, pers. comm). X-rays are inhibited by the scattering of the rays upon deflection of the multiple boundaries and CAT scans can detect boundaries, but the channels seen as root (which are less dense than soil) can be confused with pore spaces.

A general allometric model was developed by Enquist & Niklas (2002) that describes the mass of roots in relation to the above-ground leaf and stem tissue for all higher plants including gymnosperms and angiosperms. The model predicts that above-ground biomass (M_A) will scale in a nearly isometric manner with respect to M_R (ie, $M_L + M_S \alpha M_R^{3/4}$), where M_L is leaf mass, M_S is stem mass and M_R is root

mass) across and within clades and different habitats. Empirical studies undertaken by Enquist & Niklas (2002) confirmed that plant metabolic rate was proportionate to the ¾ power of M_T, (total mass), and so above-ground plant mass can be used to predict total plant mass. This estimation can determine whether the ratio of roots to above-ground mass differs such that there is a measurable effect on the plant where more or less root mass is required to support the above-ground plant.
The below-ground root mass can be calculated by the formula

$$M_R = \frac{M_A}{3.88}^{1/1.02}$$ (Enquist & Niklas, 2002)

where M_R is root mass, and M_A is above-ground plant tissue.

The relationship described above has been found to be nearly isometric for mature individuals no longer reliant for growth on the nutrients of the germplasm.

The pore space in the pots was calculated using Avon soil, categorised as a fine sandy loam from the particle size distribution tests (Northcote, 1974; Appendix 3.3). The particle density (totally devoid of pore spaces) was determined by pycnometer method (Appendix 3.2) as 2.5 g cc^{-1}. The volume of one kilogram of Avon soil at field moisture capacity (rounded) was calculated as 883 cubic centimetres. Therefore by subtraction of the density and calculation of the volume, the total pore space in the pot was calculated at 494 cubic centimetres: this was 56% of the total soil volume.

The density of freshly harvested cotton plant root was calculated by displacement (wetting) to be $0.8_{(water)}$, and using the Enquist & Niklas formula (above), the root volume could be calculated as mass/density. The maximum above-ground mass for all trials occurred with the C312i plants at 9.7 weeks in Avon soil, at 24.82 g. Using this maximum, the root volume of 7.84 cubic centimeters per pot was calculated. This would represent the upper limit of root mass before plant growth stopped.

Subtraction of the root mass from the available pore space showed that there was 487 cubic centimetres of non-particle space remaining in the pot. With an average transpiration of 30 g water each day from the watering records (= 30 cubic centimetres), this ensured that some pore space was available between the two-day watering. The Narrabri soils had a field moisture capacity of 34.5% (Section 3), but the pots were also kept at 18% of soil mass to ensure avoidance of waterlogging.

4.2.6 Harvesting of plant tissue and rhizosphere soil

Immediately after harvest, above-ground stems and leaves of the cotton plants were weighed. Fresh weight was used as the basis for the comparison of root mass. At least four replicates of each of the non-GM plants and four of the paired GM plant strains were grown for each of the trials.

Leaf sections were cut from freshly harvested adult plants for *Bt* protein analysis and chemical elemental composition, and where the plant growth development permitted, mature leaves from new growth were selected as per the method of leaf harvesting outlined by Reuter & Robinson (1997). The leaves were dried in paper bags in an oven at 60ºC overnight, pulverised in a mortar and pestle and stored in capped plastic vials. The leaves were not washed before analysis because some elements can be leached during washing (Fowles, 2004, pers. comm.).

In the pot trials, pockets of non-rhizosphere soil remained within the pot until about four weeks, and so rhizosphere soil was carefully separated from the roots with a narrow spatula, and sharp pointed knife. Only soil in close association with roots was used for analysis. Under the conditions of growth within the weighed 1kg pot soils, the plant roots had descended to the base of the pot within ten days. Plants grown for longer than five weeks had extensive root systems and the adjacent soils were so closely associated with roots that it was difficult to do a non-rhizosphere soil comparison. Extra pots of the respective sub-sampled soils were kept under similar conditions of nutrient and moisture, but without growing plants, to compare the plant rhizosphere microbiota with the non-plant associated microbial consortia.

Harvested rhizosphere soils were transferred to closed containers and stored at 8ºC overnight, pending the analyses to be undertaken the next day. Soils intended for longer-term storage were immediately placed into a freezer at -20ºC in sealed containers. Prior to microbial population or chemical analysis, moisture content of the fresh or frozen soils was determined by oven drying overnight. Weight of the soil was increased proportionately to give the same dry weight equivalent.

4.2.7 Elemental analysis of plant tissue

Plant enzymes and tissue require metals and other cofactors for correct functioning, and the paired plant types were compared for elemental content as differences in root

function may reflect uptake capacity of the roots. The Analytical Chemical Unit at the CSIRO Adelaide precinct analysed the desiccated, ground leaf tissue by the method of inductively coupled plasma calibrated against known standards, to measure Ca, Mg, P, K, S as percent content; and Al, B, Cu, Fe, Mn, Mo, Na and Zn as mg kg^{-1} content simultaneously. Between-run test consistency was checked by comparing previously analysed sub-samples with reanalysis in a later batch.

4.3 RESULTS

4.3.1 Plant growth conditions in pot trials

The extent of the restriction on plant growth within the size of the container was established in pilot trials. Table 4.3.1.1 below summarises the container volume to above-ground plant mass.

Table 4.3.1.1 Comparison of plant container volume to above-ground plant tissue

Volume of container	Number of replicate containers	Averaged above-ground plant mass (g)	Age at harvest (weeks)
50 ml Sarstedt tubes	15. (average 3 plants per container)	0.47 ± 0.12	10
400 ml non-draining pots with 30 g soil	14. (average 3 plants per container)	1.55 ± 0.20	9.3
1.05 litre non-draining pot with 1kg soil	7. (average 5 plants per container)	4.6 ± 1.88	9.7
The plants in each of the containers were supplied with Hoaglands nutrient solution			

Some plants grew more vigorously, even in very similar conditions, because of natural variability. The trend for plant growth is displayed in Figure 4.3.1.1.

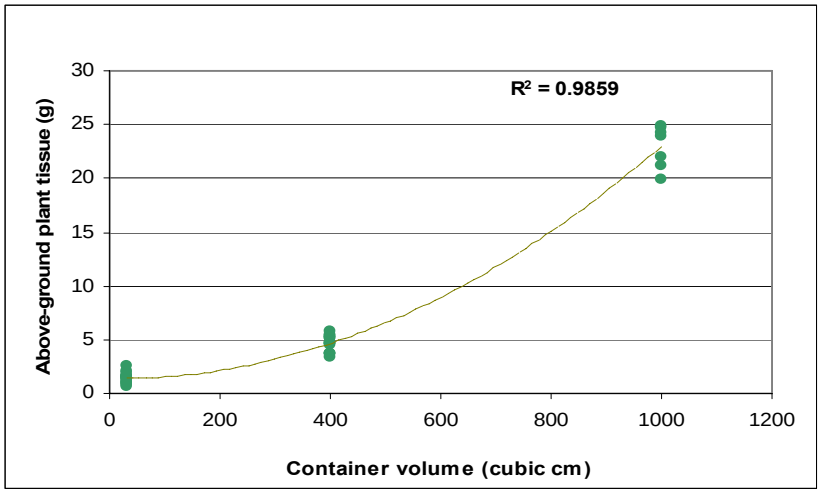


Figure 4.3.1.1 Correlation of above-ground plant mass with container size for cotton plants grown in 50 ml, 400 ml and 1.05 L containers.

To exclude the possible effect of plant growth from pot volume on the rhizosphere microbiota, all comparisons between non-GM and GM plant rhizospheres within this project have been taken from plants grown in 1.05 L pots into which 1kg soil was weighed, leaving space for watering. The pot positions were randomised within the water baths as described previously, but the light source was natural, and so day-length varied with the season between some trials.

Germination rate varied, but as six seeds were planted in each pot at the start of the trials, nearly all pots contained more than one plant. Not all individual plants were counted over all trials, but for 25 trials, 350 non-GM plants germinated, compared with 343 GM plants. Given the inconsistent germination and variability of individual plant vigor, a comparison of plant mass for individual plants cannot be made.

Of the trials where four replicate pots were harvested, the average above-ground wet plant mass was 267 g for non-GM, compared with 280 g for the GM plants, over all three soils. Thus, the totalled mass for all GM plants was slightly higher under the conditions of the paired trials. When the number of plants per pot were plotted as the abscissa and the plant mass as a covariate, the R^2 test of all mature plants older than 7 weeks showed 0.6% correlation for non-GM plants, and 0.005% for the paired GM plants (n=48 for each): neither relationship suggests that the number of plants in the pot resulted in a higher or lower mass. The competitive interaction between the plant roots in each pot is therefore not the major factor describing the growth of either plant type, but rather the restrictive volume of the pots.

Considering 32 trials individually, non-GM plants had greater mass in 12 trials, and 20 trials showed greater mass for the GM-plants. This variation is partly explained by germination rate of the individual seeds and individual plant vigour, from seeds which had not been pre-selected for uniformity of size. The maximum variation in total mass over all trials was 4.37 grams, and the generally higher mass for the GM plants was not consistent for soil type. A comparison by single factor ANOVA showed a non-significant difference for non-GM and GM-plant types for the three soils.

A time-series comparison of the matched plant types V15 and V15i for Avon and Narrabri soils is shown in Figures 4.3.1.2 and 4.3.1.3 respectively. The above-

ground plant mass was averaged over four replicates (separate pots) for each non-GM and GM plant type, for nine Avon soil trials and seven Narrabri soil trials. The figures show increasing mass with age, until about 8 weeks for all soils.

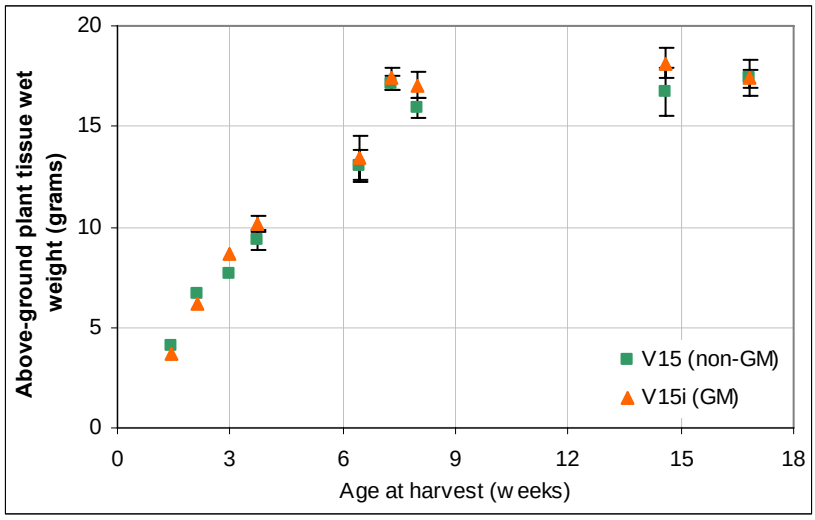


Figure 4.3.1.2 Above-ground plant mass for V15 and V15i plants grown in Avon soil.

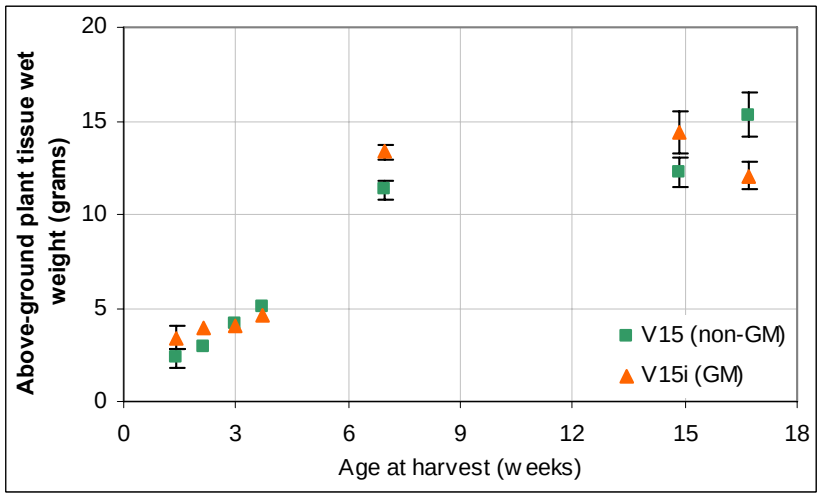


Figure 4.3.1.3 Above-ground plant mass for V15 and V15i plants grown in Narrabri soil.

The green square symbols represent the non-GM plants and the red filled triangles show the mass of the GM plants. The error bars show the standard error of the mean.

4.3.3 Establishment of root density

The soil inter-pore space was calculated from the C312/C312i plants with maximum above-ground plant mass at harvest. A subtraction of the root volume from the volume of soil at sowing (after compaction from watering) and known particle density from that soil (Section 3) confirmed that there was still some pore space in the soil at harvest, and as the soil was kept at less than the field moisture capacity,

the conditions were not anaerobic. Rhizosphere microbiota would not have been influenced by anoxic conditions in any of the pots. As the plant was receiving nutrients but had stopped growing, root density had developed to the point where the plant was pot-bound. The similar above-ground plant mass for all remaining paired plants at harvest suggested that the rhizosphere areas would therefore not have differed with respect to root density.

4.3.4 Effect of frequency of watering

The watering regime influenced plant growth particularly in the very early growth stages. Figure 4.3.3.1 shows the difference in growth of plants watered daily versus every second day. The plants were not harvested for measurement of mass at this early stage, but gravimetric watering records established that 30 g was lost with daily watering compared to approximately 40 g over two days by watering every alternate day.

Figure 4.3.4.1 Comparison of seedling growth at day 10. Plants to right of ruler received daily watering, and left of ruler were watered at two-day intervals. All grown in Avon soil.

The addition of 50 g plastic beads on the surface of the soils within the pots reduced evaporation from the soil surface, and were added two weeks post-germination. From the average loss of water of 8 replicate pots over three days without beads, followed by 8 replicates over three days with beads on the soil surface, the amount of water evaporation for the pots without beads amounted to 2% of soil mass. An average of 30 extra grams of water per day was used by the pots with plants, compared with pots containing soil only. The loss of moisture was comparable for both non-GM and GM plants.

4.3.5 Comparison of plant root architecture for non-GM/GM plants

The dicotyledonous tap-root systems and frequency of secondary roots were compared for relative thickness of primary root to length by visual observation at

harvest. Examination of the clustering of root hairs under a dissecting microscope was also undertaken at least once per four replicates for each plant type. The non-GM and GM plant root systems appeared indistinguishable.

4.3.6 Comparison of root exudate zone area for non-GM and GM plants

The area of the rhizosphere zone surrounding the roots of the cotton plants was illustrated and compared between the V15 and V15i paired plant types by growing seedlings in half-strength Hoaglands solution mixed with 2% agar, together with 0.06% bromocrescol purple. The liquid media was added to polycarbonate jars and autoclaved. Surface-sterilised seeds were aseptically placed onto the media after it solidified. The ammonium absorbed by the roots from $NH_4H_2PO_4$ in the nutrient solution resulted in the production of H^+ ions from the root exudates, turning the pH indicator yellow in the immediate area surrounding the roots. Comparison between the two plant types by visual inspection showed a similar volume of acid was exuded from the roots for both the non-GM and GM plants as shown in Figure 4.3.6.1.

Figure 4.3.6.1 Acidic exudates from cotton plant roots shown by Bromocrescol purple.

Left, non-GM plant Centre, non-plant control Right, GM plant.

Mauseth (1988, p. 272) reported that the mucigel exuded by the plant roots is the actual contact zone between the soil and the root surface and that roots grown axenically (under sterile conditions) have much less mucigel. This suggests that the root exudates would diffuse extensively in the soil surrounding the roots.

4.3.7 Plant tissue elemental analysis

The precision of the analysis of elemental content of the plant tissue composition was determined by comparing the results of the same pooled samples from the 189/289i leaf tissue grown in Avon soil, over two separate analytical runs.

The differences between the first and second analyses showed that the 189 and 289i plants differed markedly in the three elements Al, Fe and Na. These three elements had highest values and the highest variance between the two analyses. These differences occurred between the first and second analyses of the same non-GM plant, as well as between the two runs of its matched GM counterpart. The standard errors of the mean were calculated as the (high value – low value)/ square root of 2 and is shown as ± the average of the two values as shown in Table 4.3.7.1. The standard error of the mean (as with standard deviation) is dependent on the magnitude of the number, so the percentage variance of the mean was calculated, (σ^2/mean) to test true variance. In the cases of the three elements the % variances were Al at 36% and 17%; Fe at 37% and 15% and Na at 23% and 16%, all for non-GM and GM plants respectively. The disproportionate results and SEMs are shown in bold type.

Table 4.3.7.1 Variance for results for elemental content of dried cotton leaf tissue between two separate analyses

Element	Plant 189 (non-GM)	Plant 289i (GM)
Ca %	2.9 ± 0.16	2.86 ± 0.115
Mg %	0.55 ± 0.00	0.54 ± 0.02
P %	0.21 ± 0.01	0.28 ± 0.08
K %	1.64 ± 0.05	1.73 ± 0.32
S %	0.35 ± 0.00	0.34 ± 0.01
Al mg/kg	**349.36 ± 90.36**	**292.39 ± 36.39**
B mg/kg	76.86 ± 2.14	81.13 ± 0.13
Cu mg/kg	5.02 ± 0.22	3.85 ± 0.35
Fe mg/kg	**211.57 ± 55.57**	**154.79 ± 16.79**
Mn mg/kg	24.96 ± 0.04	24.66 ± 0.66
Mo mg/kg	1.33 ± 0.03	1.27 ± 0.02
Na mg/kg	**209.55 ± 34.55**	**193.06 ± 22.06**
Zn mg/kg	18.09 ± 0.91	17.85 ± 1.14

The plants were grown at the same time under the same conditions and randomly repositioned at watering. Plastic beads had been applied to the surface of all pots, and the watering was done by the gentle pouring of approximately 30ml of water onto the surface of the plastic beads, so no soil splash-up could occur. The variance was investigated by checking the values for Al against Reuter and Robinson (1997) which reported that this element was toxic at these levels, however the plants did not show signs of toxicity, ie, short and truncated roots.

The usual protocol of the Analytical Chemical Unit is to analyse one sample from every 10-15 in duplicate. Reagent blanks and control or standard samples are included in the digest and analysis process for quality control purposes (J. Cozens, ACU, pers. comm), so the standard error of the mean cannot be calculated for all samples.

Clay soil, by definition, has a high proportion of particles less than 2μm and particles of approximately 1μm can stay suspended in air over extended periods. Leaf tissue analysis only required between 0.2 and 0.3 g per analyte so a tiny particle of dust carrying AlOH or FeO_2 could have altered the composition of the leaf tissue and could explain the similarity between the paired trials affected by the high values, and the lack of these excessive elements in the separate trials grown under adequate or deficient nutrient conditions, at a different time. Moreover, if the plants contained levels of aluminium in the range recorded, they should have shown symptoms of toxicity (reduced root growth and short root length), and these symptoms were not observed.

A mid-infrared scan of the leaf tissue from the plant C312 showing the highest recorded Al content was compared with the leaf tissue of V15i showing the lowest amount of all the elemental tissue analyses. The two spectra were subtracted from each other, and the only differences between the two samples occurred at 3693 cm^{-1}, 3650 cm^{-1} and 3622 cm^{-1}. These were the peaks consistent with the distinct peaks of kaolinite clay minerals, and are strongly indicative of aerosol contamination. The remaining peaks of the scan for the paired leaf tissue samples were essentially equivalent within the influence of the paired trials. The subtracted scan is shown as Figure 4.3.7.1 below.

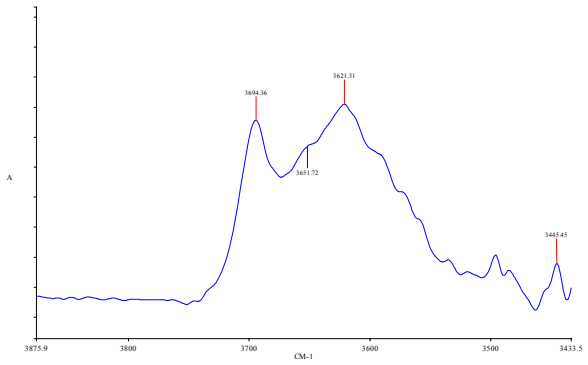


Figure 4.3.7.1 **Mid infrared high-low subtraction scan of compared plant tissues showing differences in position coincident with Kaolin (indicating a possible clay mineral content).**

It is difficult to give a precise interpretation of the elemental composition of the plant leaf tissue from mid-infrared analysis without many samples, and using chemometrics (L. Janik, pers. comm). However, from the information gained from the scan of the mid-infrared leaf tissue analysis which compared differences in peaks between the compared C312 and V15i plants (both different plant strains and both samples also differing in the genetic modification) the leaf tissue only formed distinct peaks at the frequency of silicon which formed the smectitic clay minerals, so the difference could be seen as non-plant associated Al. As this detection of contamination may be helpful for future work, the methodology and complete spectral scans are given in Appendix 4.2. The values of the elements Al, Fe and Na were therefore removed from further comparative results.

The results of the remaining elemental analysis of the leaf tissue for the non-GM and GM plants were then compared against the ranges of adequacy for cotton plant growth as reported by Reuter & Robinson (1997) and the Australian Cotton CRC (2004). (Both publications show similar values, and these can be compared in Tables 4.3.7.2 and 4.3.8.1, together with the results of the analysis. Both publications quoted the results as percent elemental content and so are absolute in terms of composition, regardless of method. The levels below the range considered adequate for normal growth are shown in green, and the values approximating toxicity are shown in red.

Table 4.3.7.2 Comparative elemental analysis of leaf tissue under nutrient adequacy

		Avon V2, V2i	Avon 189, 289i [1]	Avon C312, C312i	Narrabri C312, C312i	Adequate range (Reuter & Robinson)	Critical value (Aust. Cotton CRC)
Ca %	Non-GM	2.85	2.9	2.78	2.52	2.2 – 3.8	
	GM	3.02	2.86	2.86	3.07		
Mg %	Non-GM	0.56	0.55	0.66	0.61	0.3 – 0.9	
	GM	0.53	0.54	0.63	0.63		
P%	Non-GM	**0.58**	**0.21**	**0.59**	**0.23**	0.25 – 0.5	0.28 – 0.5
	GM	0.49	0.28	**0.68**	0.29		
K%	Non-GM	2.83	1.64	2.98	**1.27**	1.5 – 3.0	1.5 – 3.0
	GM	2.66	1.73	3.19	**1.42**		
S%	Non-GM	0.54	0.35	0.43	0.34	0.35	0.6 – 1.2
	GM	0.46	0.34	0.42	0.43		
B mg/kg	Non-GM	80.04	76.86	78.78	71.10	20 – 100	50 – 80
	GM	82.03	81.13	71.57	70.43		
Cu mg/kg	Non-GM	7.16	5.02	6.84	7.10	5 – 30	5 – 25
	GM	6.14	**3.85**	7.17	5.58		
Mn mg/kg	Non-GM	**17.01**	24.96	15.45	93.47	25 – 500	50 – 200
	GM	**15.47**	24.66	13.99	59.45		
Mo mg/kg	Non-GM	1.62	1.33	1.51	1.08	0.75	0.4 – 0.9
	GM	1.60	1.27	1.47	0.92		
Zn mg/kg	Non-GM	40.53	**18.09**	34.75	**17.08**	25 – 60	20 – 60
	GM	34.64	**17.85**	36.02	**15.55**		

(1) The arithmetic mean was used for the results of the 189/289i trial for the two independent analytical runs

The elemental composition of the paired non-GM and GM plants grown under the same conditions within the individual trials were more similar than the plant elemental tissue content of the paired non-GM and GM plants between trials. A comparison of plants grown under nutrient adequacy showed that there were no consistently higher or lower values for elements across the 3 non-GM plant types compared with their GM counterparts. Moreover, each of the paired plants showed similarity of adequacy or deficiency, with the exception of Cu and P. Copper was slightly lower for the GM plant 289i, and P was deficient in the non-GM plant. Both paired plants, however, approached the lowest point in the range for P.

The results of Chi-squared analysis of proportional change in the ratio of elements measured as percent of total are given in Table 4.3.7.3 below. The percentage probability of similarity is shown under the plant strain.

Table 4.3.7.3 **Chi square result of independence for proportional distribution of Ca, Mg, P, K and S compared between the non-GM and GM plants**

Avon V2/V2i	Avon 189/289i	Avon C312/C312i	Narrabri C312/C312i
45%	71%	33%	85%

(The value of 100% shows a perfect correlation of elemental ratio, and the value of 0 indicates a significantly changed relationship. Significance has been taken at $p \leq 0.05$)

None of the three paired non-GM and GM plants grown in Avon soil, nor the same paired plants grown in Narrabri soils, are significantly different at $p \leq 0.05$. A test of the non-GM plant types compared with each other to test variability between plant strains is shown in Table 4.3.7.4.

Table 4.3.7.4 **Chi square result of independence for proportional distribution of Ca, Mg, P, K and S compared between the non-GM parental plants**

Avon V2/189	Avon V2/C312	Avon 189/C312	Avon/Narrabri C312/C312
53%	40%	47%	52%

The proportion of elements within the leaf tissue between the non-GM and GM plants was therefore approximately in the range of difference between the non-GM parental plants.

4.3.8 Comparison of leaf tissue for plants grown under limited nutrients

At the 9 week harvest of a trial of the V15 and V15i plants grown without the addition of any nutrient solution, yellowing of the older lower leaves occurred, symptomatic of nitrogen (and possibly other) nutrient deficiency (Grundon, 1987). Visual observation of both non-GM and GM plants from all five replicates within Avon and Narrabri soils indicated similar nutrient deficiency. Leaf drop due to calcium and nitrogen deficiency was observed. Interveinal chlorosis was apparent.

Figure 4.3.8.1 **Plants grown under nutrient limitations showing chlorosis of older leaves indicative of nitrogen deficiency.**

The plant tissue for V15 and V15i plants grown under conditions of nutrient-deficiency was analysed and the results are shown in Table 4.3.8.1 below. The values lower than the range considered adequate are shown in green as per the previous table, and those considered above the range are shown in red.

Table 4.3.8.1 Comparative elemental analysis of ground leaf tissue For V15 (non-GM) and V15i (GM) nutrient-deficient plants.

		Avon V15, V15i, green leaf	Avon V15, V15i, yellow leaf		Narra-bri V15, V15i, green leaf	Narrabri V15, V15i, yellow leaf	Adequate range (Reuter & Robin-son)	Critical value (Aust. Cotton CRC)
Tot C %	Non-GM	40.02	37.57		39.47	38.47		
	GM	39.23	38.16		39.8	39.15		
Tot N%*	Non-GM	**1.96**	**1.08**		**1.18**	**0.76**		3.5 – 4.5
	GM	**1.95**	**1.46**		**1.12**	**0.85**		
Ca %	Non-GM	3.82	5.31		2.58	3.87	2.2 – 3.8	0.4 – 6.0
	GM	3.84	4.97		2.75	3.10		
Mg %	Non-GM	0.74	0.89		0.61	0.79	0.3 – 0.9	0.4 – 0.9
	GM	0.73	0.87		0.67	0.70		
P%	Non-GM	**0.57**	0.32		0.42	0.30	0.25 – 0.5	0.28 – 0.5
	GM	**0.52**	0.37		0.46	0.29		
K%	Non-GM	2.20	1.57		**0.99**	**0.56**	1.5 – 3.0	1.5 – 3.0
	GM	2.02	1.79		**1.04**	**0.70**		
S%	Non-GM	**0.27**	**0.27**		**0.13**	**0.17**	0.35	0.6 – 1.2
	GM	**0.26**	**0.28**		**0.13**	**0.11**		
B mg/kg	Non-GM	**141**	**241**		90	**256**	20 – 100	50 – 80
	GM	**125**	**228**		74	**125**		
Cu mg/kg	Non-GM	**4.5**	**3.9**		**2.9**	**2.5**	5 – 30	5 – 25
	GM	**3.9**	**3.6**		**2.7**	**2.3**		
Mn mg/kg	Non-GM	27	36		61	140	25 – 500	50 – 200
	GM	26	36		50	80		
Mo mg/kg	Non-GM	2.7	2.7		2.9	1.7	0.75	0.4 – 0.9
	GM	2.1	2.5		1.3	0.9		
Zn mg/kg	Non-GM	34	23		**20**	**9**	25 – 60	20 – 60
	GM	37	26		**17**	**9**		
* Also deficient according to Marschner (1997, p. 398) for a range of adequacy of 3.6-4.7.								

In all cases where the non-GM (V15) plant showed elemental deficiency or excess, the GM (V15i) plant also showed the same result, reflecting a similar plant response to the effect of the individual paired treatments.

The comparison between the older yellow and younger green leaves indicated the mobility of the different elements within the plant system, and was used to compare the function of transport between the plant tissue, across the two plant strains. The mobile elements C, N, P, K Cu and Zn are all mobile from the older to younger leaves, and this was reflected in the results. The less-mobile elements Ca, Mg, Fe,

Mn and B are also shown in higher levels in the older, yellow leaves, and the remaining element S, is inconsistent between the plants. The element Mo showed a plant-associated effect with the C312/C312i plants expressing higher Mo in the green leaves where the other two plants did not.

The only consistent difference between the non-GM and GM plants occurred with Nitrogen. It was consistently higher in the GM plants, within both Avon and Narrabri soils. As the carbon:nitrogen ratio of the plant-produced *Bt* protein was found to be 3.3:1 (see Literature Review), which indicates a very high nitrogen demand, the C:N ratio within the leaf tissue was calculated to investigate whether the GM-plants differed (Table 4.3.8.2).

Table 4.3.8.2 Carbon to Nitrogen ratio in plant leaf tissue for paired non-GM/GM plants grown under nutrient limiting conditions.

Soil	V15 (non-GM) green leaf	V15 (non-GM) yellow leaf	V15i (GM) green leaf	V15i (GM) yellow leaf
Avon	20:1	35:1	20:1	26:1
Narrabri	33:1	50:1	35:1	46:1

The higher C:N ratio can be seen in the yellow leaves, where nitrogen has been transported to the younger green leaves in both the non-GM and GM plants, for plants grown in both soils. The yellow leaves from the GM plants from both soils, however, had a lower C:N ratio indicating a higher N content in the yellow leaves of the non-GM plant.

4.4 DISCUSSION

The averaged GM- and non-GM above-ground plant tissue showed similar growth between the paired plants within the individual trials, with similar root architecture and elemental content of leaf tissue. Each comparison is detailed below.

4.4.1 Plastic response of plant growth by restrictive volume of the container

As shown in Figure 4.3.1.1, the correlation of 0.98 for the above-ground plant mass for volume of container for plants harvested within the same week clearly shows that the restrictive volume of the container was the major driver of the plant growth pattern.

The comparison of rate of growth for the paired V15 and V15i plants in Avon and Narrabri soils (as shown in Figures 4.3.1.2 and 4.3.1.3) demonstrated very minor differences when grown under similar conditions. It is noteworthy that the lowest correlation of all plants over time of growth occurred between four different GM plants within Narrabri soil, but as this amounted to a correlation of only 74% it can be seen that the major factor for the rate of growth is time, and not a difference in plant characteristics resulting from the genetic modification.

The same plant strain grown in Avon soil had an increased vigour over all times, and this included the plant tissue mass after cessation of growth at approximately eight weeks. Neither non-GM nor GM plants dominated in above-ground tissue mass at any harvesting time, so the effect of the plant modification was inconsequential with regard to plant vigour.

4.4.2 Plant physical structure of non-GM and GM plants

There was no difference (such as additional branching) in root structure of the paired cotton plants that would differentiate microbial habitat. This statement is supported by observations of 96 harvests of plants where there was no observable difference in root architecture of the tap-root and auxiliary roots of the C_3 plant root pattern between the non-GM and GM paired plants for all trials. Any microbial differences in rhizosphere soil for plant type therefore cannot be attributed to the physical characteristics of difference in root mass or rate of growth alone.

Some indication of cotton plant growth for the different varieties grown under field conditions can be gained from information from the Cotton Seed Distributors Variety Guide. However, the main concern for the producers is for the quality, strength and uniformity of the harvested product so the growth habits of the plants are irrelevant and are only indicated as short/compact, compact, medium or vigorous/tall. Stone (2003) compared the growth of cotton plants under field conditions in Redmill during the 2002 season of a height of 100-120 cm for conventional, Ingard and Bollgard II varieties, and for the averaged results of 80 commercial trials over two seasons. With comparable planting dates and soil types, the averaged heights were 150 cm (Addison, 2003). These independent field trials showed that the non-GM and GM cotton plants do not vary in growth pattern for similar seasonal influence, but do vary by seasons and location.

4.4.3 Rhizosphere zone area

The similar colour of the bromocrescol purple pH indicator (yellow below neutrality) for both the V15 and V15i plants in the sterile medium surrounding the roots indicated that the total cationic exchange was similar between the two plant strains. No difference in elemental deficiency would therefore have occurred through a pH effect. The similarity of area of the zone of diffusion also showed that the volume of root exudates did not vary greatly between the two plant types.

4.4.4 Plant tissue elemental content

The individual elements analysed are discussed below. Additional to the elements analysed under conditions of nutrient adequacy, the elements C and N were also investigated for the nutrient-deficient plants to test the C:N ratio.

Total C may not be an effective measure of difference in plant metabolism as it is a function of photosynthesis, and includes cellular structural material (cellulose, lignin) as well as sugars. It was also observed by Upchurch & Taylor (1990) that C is the only element used extensively by plants that is not taken up primarily through the root system. The difference in total C content between the green leaves and the yellow leaves of V15 and V15i plants grown in both Avon and Narrabri soils was less than 5%. Bioavailable carbon was also shown to be present in all three sampled soils at the beginning of the trials, (Section 4) so this element is unlikely to be a critical factor in growth under nutrient-limiting conditions.

The leaf tissue analysis of the yellow leaves from plants grown under nutrient limited conditions showed a consistent difference between the non-GM and GM plants for nitrogen, of major importance for plant cellular components including the production of the *Bt* protein. Independent research by Chen et al. (2004) also found that two *Bt* transgenic cotton cultivars Kumian No. 1 and Xingyang 822 were higher in N content than their parental lines but the study did not compare the nitrogen content with the *Bt* protein content. This will be discussed further in Section 5 which covers the measurement of the *Bt* protein in plant tissue.

Ca and **Zn**, two of the elements discussed in the introduction as important for the cell wall stability and leakiness of root exudates, are notably the two elements whose values fell within 5% difference for both the non-GM and GM plants.

Ca levels were within the range of adequacy for nutrient adequate plants within both soils, and the yellow leaves of the V15/V15i plants grown under nutrient deficiency in Avon soil showed an increase in level, but both non-GM and GM plants were similarly affected.

Mg was in the adequate range for both of the trials under adequate and deficient nutrients. The relative Mg content was the same for the non-GM and GM plants as occurred with Ca.

K only showed deficiency in the C312/C312i trial in Narrabri soil under nutrient adequacy: both the non-GM and GM plants were similarly affected. Under nutrient deficient conditions, K was shown to be deficient in both the V15 and V15i plants grown in Narrabri soil, but the same plant strains were not deficient in this element in Avon soil. As there was no consistent plant or soil effect with this element, and both non-GM and GM plants were similarly affected over the four trials under nutrient adequacy, and two trials using different soils under nutrient deficiency, the levels of K are seen as reflecting the conditions of the individual trials, rather than a differentiating effect between the non-GM and GM plants.

P content was the most variable of all the elements for the leaf content. It was deficient in two unrelated non-GM plant strains but adequate for the two related GM-strains, under adequate nutrient. The same strain (C312/C312i) was found to be above the normal range within Avon soil, but the plant type (GM/nonGM) was similar in content. The P content was slightly above the range for the V15 and V15i plants grown in Avon soil, but all others fell within the normal range for this element. The P content of the same pooled 289i sample is also notable in its variance of 46% between the first and second analytical run, and this cannot be explained.

The V15 and V15i plants grown without the addition of nutrient solution in both Avon and Narrabri soils and harvested at 9 weeks showed the phosphate component of the plant tissue was above the level of deficiency for the plants grown in both Avon and Narrabri soils, (Table 4.3.8.1). The analysis also showed that the green leaves of the plants grown in Avon soil had accumulated P at toxic levels according to Reuter & Robinson and the Aust. Cotton CRC guidelines. All plant roots were colonised by A-M fungi and this symbiotic association is known to amplify P in the

plant root system. The increased concentration of P in the roots of the plants grown under nutrient deficiency due to the amplification by the A-M fungi is not known, as all plants were grown in non-sterile soil and all were colonised. Another confounding factor with plant-available phosphate from soil is that there may be a large amount of phosphorus in dead bacterial cells surrounding the root (Nye & Tinker, 1977), and the phosphatase produced by the root could sequester P from this reserve.

Under adequate nutrient supply all plants showed the elemental content of **S** within the normal range, but under deficiency, all non-GM and GM plants were similarly affected, and all leaf tissue was deficient in both old and young tissue, for both soils.

The **Boron** leaf content was consistently higher in both the V15 and V15i plants grown under nutrient deficiency. Boron is not particularly mobile in plant systems (Grundon, 1987), and the elevated levels were shown in both the green and yellow leaves grown in Avon and Narrabri soils. The plants grown under nutrient limiting conditions did not show symptoms of boron toxicity (leaf cupping and chlorosis), so the effect did not cause visible symptoms. Marschner (1997) noted that under Zn deficiency or environmental stress conditions, plasma membranes lose their integrity which is directly related to the control of passive ion uptake. Here Zn was not deficient, so other environmental factors related to nutrient deficiency may have caused the passive uptake of boron. Research is currently being undertaken by the University of Adelaide on the role of membrane transport of boron in tolerance to abiotic stresses (Assoc. Prof. Rob. Reid, pers. comm.). Within current knowledge there appears to be only one boron transporter in *Arabidopsis thaliana*, so genetic manipulation for the regulation of the uptake of this element is potentially limited. If the uptake of boron occurred as a result of an imbalance of elemental uptake by the roots, then both non-GM and GM plants were affected similarly.

A higher leaf content of the elements **Al, Fe and Na** occurred in the older yellow leaves than the green leaves in both the V15 and V15i plants. The elements Al and Na accumulate in leaf tissue incidentally over time (Prof. Andrew Smith, pers. comm), so the concentration may be a function of the age of the leaves rather than an effect of the altered plant. From the mid-infrared soil analysis all of these elements were abundant so they would not have been limiting to the plant at the pH of the soil.

Cu has a wide range of adequacy between 5 and 30 mg kg^{-1}. The only plant that was shown to be deficient under adequate nutrient conditions was 289i, grown in Avon soil. Its paired 189 plant was not deficient, but was at the bottom of the adequate range. Every single plant, whether non-GM or GM, was found to be deficient under the conditions of nutrient deficiency, for both Avon and Narrabri soils.

The V2 and V2i plants grown in Avon soil showed a deficiency in **Mn** under nutrient adequacy, but the 189/289i plants grown in Avon soil, and C312/C312i plants grown both in Avon and Narrabri soils were within the adequate range. Under nutrient deficiency, all plants were within the range of adequacy, with the older non-GM plant leaves higher in this element, but still within the range of adequacy for the Narrabri soil. However, the two authorities cited (Reuter & Robinson and the Aust. Cotton CRC) differ with regard to the lowest critical value of this element for cotton.

The requirement for **Mo** is lower than for any of the other mineral nutrients, except nickel (Marschner, 1997, p. 369) so any difference in this element may be incidental.

Zn is an important element required for protein synthesis. Under nutrient deficiency, a decrease in protein and an increase in amino acid concentration occurs (Marschner, 1997, p. 369). Here, Zn was deficient according to the guidelines by Reuter & Robinson (1997) under the conditions of nutrient adequacy in the 189 and 289i plants grown in Avon soil, and the C312 and C312i plants grown in Narrabri soils. Neither the V2/V2i nor the C312/C312i plants grown in Avon soil were different in leaf content for this element. Under nutrient deficiency, Zn levels were low for the V15 and V15i plants grown in Avon soil, but only became deficient for these plants when grown in the Narrabri soil.

In summary, the variability of elemental tissue content can be related to individual strain (non-GM/GM) as well as to the environmental variables. This is indicative that as there was no consistent soil or plant strain effect, the non-GM and GM plants were similarly affected by the conditions of the individual trials, both under conditions of nutrient adequacy and deficiency. The difference in N leaf content in the GM plants however, may cause a difference in the soil microbiota around the root if a lower C:N ratio is found, which favours bacteria over fungus (Ferris & Matute, 2003). This possibility will be explored Section 7 describing Microflora.

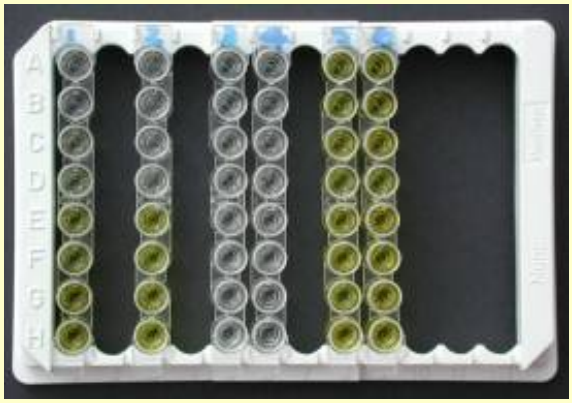


Fig. 5.0 An ELISA test showing colorimetric detection of the *Bt* protein from leaf tissue (lower left, and root tissue (two right lanes). Standards are shown in the left-most lane. Results of non-GM plants are negative (clear)

Section 5

Quantification of the Cry1A(c) protein

5.1 INTRODUCTION

The measure of toxicity within an environment requires quantification of the substance, the amount ingested or applied at which it becomes toxic, and its rate of breakdown. A genetically modified living plant is defined by a quantifiable difference of the production of particular inherited traits (OGTR, pers. comm. 2003). In the case of Ingard cotton, this is the *Bt* protein.

Variability of the *Bt* protein content in plants has been noted by several researchers. Adamczyk & Sumerford (2001(b)) compared thirteen varieties of transgenic Cry1A(c) cotton over two sites, and for two generations, and determined that the parental background had a stronger impact on the expression of Cry1A(c) than the environment. Fitt (1998) noted that *Bt* expression decreased as the plant aged, and Adamczyk et al. (2001(a)) showed that the expression of the protein was higher in the terminal leaves than squares, white flowers and bolls. None of the researchers compared the *Bt* expression in the roots of the plants and so no comparison has been made with the levels of protein in or around the roots which may affect the soil rhizosphere microbial communities.

Purified *Bt* protein was "immunologically negative" after 12 hours in the non-sterilised soils tested, including clay-amended soils, indicating probable microbial breakdown (Saxena et al. 2002). The Envirologix Quickstix® method used in the analysis (Envirologix Catalogue No. AS 003 LS) did not quantify the amount of the protein and an ELISA test is known to be more sensitive.

The United States Environmental Protection Agency (EPA) *Bt* Plant-Pesticides Biopesticides Registration Action Document (2001) estimated that 1.44 grams of *Bt* protein per acre (based on 60,000 plants per acre) would enter the soil as a result of post harvest incorporation of *Bt* cotton. However the document did not include *Bt* expression levels from root tissue using 'antibody-based reagents' tests.

It is also known that proteins of an opposite ionic charge adsorb to clay soils strongly, with the possibility of the active site being orientated towards the clay surface and away from the recognition site of the antigen. Work by Kostella & Stotzky (2002) used the methods of sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and Fourier-transform infrared (FT-IR) analyses and insect bioassays to confirm that the binding of the *Bacillus thuringiensis* subspp. kurstaki morrisoni toxins did not alter their structure, nor was the active site preferentially orientated with respect to the clay surface. Thus, in this work ELISA tests that could quantify the protein in soil were potentially useful for toxicity assays and detection of *Bt* protein in clay soils like that from Narrabri.

The *Bt* protein from corn was present in a mixture of 6% soil and approximately 94% montmorillonite which is a smectite clay (Saxena & Stotzky 2001). However moisture content was not mentioned in that work, and dryness is known to inhibit the rate of protein degradation in soil.

Communication from the manufacturer of the Envirologix ELISA kits advised that the kits had never been validated for use with soil (Envirologix personal communication received 20 September 2002), and that there could be cross-reactivity with other soil bacterial proteins and non-specific interference from humic acids. Therefore there was a need to test the kit by comparing its use with rhizosphere soil against known levels of *Bt* protein of the leaf and root tissue of the same plant.

The *Bt* protein from corn plants is non-detectable in a dry-mill fermentation process < 15 minutes after liquefaction at 80ºC, the first of the three steps (liquefaction, saccarification and fermentation) (Dien et al, 2002). In a comparison using a wet-mill process, only 28-40% of the protein was accounted for after 24 hours at 52ºC. Dien et al. (2002) proposed that possible causes of non-detection of the protein within the wet-milled process were denaturation due to the addition of sulphite, or

denaturation due to endogenous proteases or thermal deactivation (at 52ºC). However, in both wet and dry mill processes the point at which the protein had decreased or had become non-detectable was the addition of liquid. In an Australian summer in Narrabri there is more rainfall than in winter, so the *Bt* protein would be eliminated from the fields at soil surface temperatures.

Hausenbuiller (1973, p. 144) showed that, as a general principle, insulation from incoming heat radiation increases with depth of soils. Even with a surface temperature of 80ºC, the point at which the *Bt* protein denatures, the soil temperature would be less at a depth of a few centimetres, and at a depth of 60cm the diurnal heat exchange would be negligible. Shade from the leaf canopy of the plants would also shield the soil from heat, and so the rhizosphere soil (which continues to a depth greater than 1m below the surface) would provide a physical environment which protects the protein from thermal degradation. At the late harvest period irrigation is stopped, which also means that the protein would not be attacked by hydrolytic enzymes which require water for the breakdown of proteins.

Additionally, the presence of entomopathogen units [ELISA assay] do not necessarily signify infectivity, and toxicity must be related to bioassay results, a test of toxicity of the root tissue on the target organism (Hurst et al. 1997). Accordingly, there was also a need here to determine the toxicity of *Bt* protein on neonates of *Helicoverpa*.

The work in this section therefore addressed the points raised above, and aimed to quantify the plant-produced *Bt* protein in the leaf and root tissue and in the surrounding rhizosphere soil.

5.2 MATERIALS AND METHODS

5.2.1 Validation of the ELISA kit for the detection of Cry1A(c) in clay soil

An Envirologix Cry1Ab/Cry1Ac Plate Kit (AP 003, EnviroLogix Inc. Portland, USA) was used for all ELISA assays. The colorimetric process of the kit was not modified to increase the intensity of the result of *Bt* protein at or below the lowest calibrator of the kit because that was not the purpose for which the product was intended or warranted.

Leaf tissue of the same plant strains C312 and C312i, used by Palm et al. (1994) and grown for 9 weeks in Avon soil, was prepared as described in Appendix 5, pooled and homogenised by bead-beater. The homogenate was immediately put onto ice.

Three replicates were made of each 600µL tube of leaf homogenate, in a dilution series of 0.00 (negative control of buffer only) 200, 400, 600, 800 and 1000µL to determine the maximum amount of protein that could be measured colorimetrically from pooled leaf homogenate. In addition, triplicates were made of two additional repeated sets of dilutions using the same pooled leaf homogenate.

The Narrabri soil used in this study contained a minimum of 45% smectite clay (Section 2), taken from field soil from a known, non-GM site. The soil was ground finely by mortar and pestle, and weighed into 1.5 ml eppendorf tubes. The sampled soil contained approximately 1% moisture.

0.1 gram of the prepared soil was added to each of the triplicates of one of the sets of dilution series and 0.2 g soil was added to each of the remaining dilution series. The tubes with the soil, leaf homogenate and buffer were vigorously hand-shaken to disperse the soil in the mixture of leaf tissue and buffer. The same ELISA kit was used for the soil tests and for the plant leaf and root tissue.

The Cry1A(c) protein was estimated against standards for each of the four plant types within the pot trials according to manufacturer's instructions. The optical density was read immediately using a Biolog Microstation plate reader with λ1 set at 450nm with a reference λ2 at 650nm. The detailed method is described in Appendix 5.1.

Additionally, an ELISA test was made on the *Bacillus thuringiensis* culture which was obtained from the Insect Pathology Laboratory of the University of Adelaide. An illustration of the parasporal crystals of several *Bt* bacteria is shown in Figure 2.6.3.1 in the Literature Review.

5.2.2 Measurement of *Bt* protein from nitrogen-deficient plants

In an additional experiment, four replicate plants of the V15 and V15i cotton strain were grown in Avon soil, and another four replicates for each of V15 and V15i in Narrabri soil, to test the effect of *Bt* production in plant tissue under nitrogen-

deficient conditions. The nutrient deficient plants were watered adequately, but no nutrient solution was added to any of the pots.

The plants were grown to 14.8 weeks for Narrabri soil, and 16.8 weeks for Avon, harvested, and immediately frozen at -20ºC. The lower, older, leaves of all plants were yellow, with red pigmentation on the petioles indicative of nitrogen and possibly other deficiency (Grundon, 1987). The younger leaves were green, signifying normal growth.

5.2.3 Bioassay using live larvae

5.2.3.1 *Plutella zygostella*

The first assays used second instar *Plutella zygostella* donated by the Entomology Division of the University of Adelaide, Waite Campus. Twenty five larvae were transferred by fine paint-brush from a living Chinese lettuce plant to separate wells of two 5 x 5 tissue culture plates, one with ample freshly-harvested shredded leaves and the other with washed and blotted cotton roots. The insect larvae were kept at 25°C in indirect light on a laboratory bench.

5.2.3.2 *Helicoverpa armigera*

Trial 1: Plant tissue only

Second instar larvae were put into separate wells of two 5 x 5 tissue plates, as per the *Plutella* trial, with freshly harvested, washed and blotted leaf and root tissue, and kept at 25°C in indirect light on a laboratory bench. Plastic-wrap was used to seal gaps between the box and lid. Spring clips were applied to hold the lids on tightly.

Trial 2: Artificial diet

The *Helicoverpa* diet was prepared as per the Australian Cotton Research Centre (CRC) recipe (Appendix 5.2) and while still warm, approximately 200µL was delivered into 96-well microtitre plates, using cut pipette tips to avoid clogging. Freeze-dried leaves from cotton strains 189 and the equivalent GM 289i plants were ground in a mortar and pestle and distilled water was added to reconstitute the plant tissue to the moisture content at harvest, before aliquotting 100µl to each well. The same quantity of 5.3g ascorbic acid per litre of water was added to the media as a control.

On arrival from the CRC at Narrabri, paper disks with *Helicoverpa virescens* eggs were placed in a sealed plastic zip-lock bag with artificial diet in petri dishes. The plastic bags were kept at 25°C in the dark until hatching. On the day after hatching, the second instar larvae were transferred to the medium in the microtitre plates, using a fine soft paint brush. One larva per well was added to each of the 96 wells. Adhesive tape was applied to each column (8 wells) of the multi-well plate to keep the larvae in the wells, as the first instinct of the larvae is to disperse. Mylar film supplied by the CRC at Narrabri was placed over the base of the box and air-holes were punched through with a needle. The clear plastic lid into which air-holes were punched with a fine soldering iron, was placed over the top and four large spring clips were placed around each box to prevent escape. The larvae were kept in an incubation chamber with a 12-hour night-day cycle at 25°C until counting.

5.3 RESULTS

5.3.1 Detection of Cry1A(c) protein in highly smectitic clay soil by ELISA

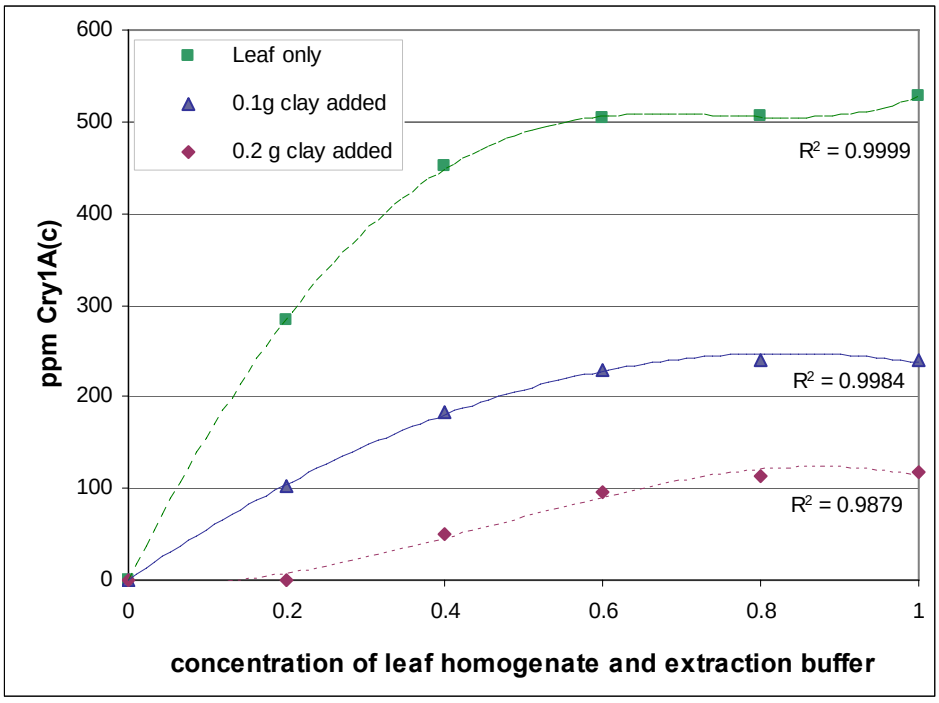


Figure 5.3.1.1 ELISA detection of the Cry1A(c) protein showing proportional increase of concentrations of leaf homogenate (green symbol), and increasing concentrations of pooled leaf homogenate with added clay soil (blue and red symbols) n = 3.

The Cry1A(c) in the pooled and homogenised leaf tissue was analysed using the ELISA kit up to 520ppm and thereafter the concentration of the protein was higher than the maximum level that could be reported (Figure 5.3.1.1). The simultaneously run negative controls (non-GM plant tissue) showed that no *Bt* protein was detected.

When the clay was added, the minimum level which was clearly above the negative control was detected at a dilution of 40% of the concentration of 527ppm, and included 0.2 g of added clay soil. That is, using the 0.02 g leaf tissue with clay added at 10 times the mass of the leaf tissue used in the analysis, 9.6% of the Cry1A(c) protein, or 50 ppm, was still detected.

5.3.2 Quantification of the protein in cotton plant root tissue by ELISA

Ream et al. (1993) reported that the *Bt* protein was most highly expressed in leaf tissue, but it was found in this study that for the V15i plants grown in all three soils the *Bt* protein in the leaf tissue of cotton plants was higher than in the root tissue in only 42% of all cases. It was reported by Palm et al. (1994) however, that the protein concentration varied 10-20 fold between plant varieties, and also varied within tissue types for the same plant.

Because the focus of this work was on the expression of the *Bt* protein in the soil, ELISA tests were mainly carried out on root tissue. However nine trials where both leaf and root tissue were analysed concurrently are shown in Table 5.3.2.1.

Table 5.3.2.1 Expression of Cry1A(c) protein in parts per million dry tissue equivalent

Soil, GM plant	***Age of plant at harvest (weeks)***	***Leaf tissue ppm***	***Root tissue ppm***
Avon, V15i	1.4	28.66 ± 3 n = 4	26.31 ± 5 n = 4
Avon V15i	14.8	181.52 ± 18 n = 2	116.99 ± 4 n = 2
Avon V15i	16.7	599.44 ± 30 n = 2	305.18 ± 18 n = 2
Avon V15i	16.9	124.53 ± 19 n = 4	172.99 ± 30 n = 4
Avon, C312i	9.0	527.30 ± 0 n = 1	302.17 ± 25 n = 4
Avon, C312i (dry, undisturbed)	41.0	253.40 ± 0 n = 1	358.71 ± 37 n = 4
Narrabri V15i	1.4	406.06 ± 0 n = 1	187.40 ± 54 n = 3
Narrabri, V2i	14.8	82.79 ± 0 n = 1	235.48 ± 92 n = 3
Narrabri, 289i	12.1	370.82 ± 38 n = 2	313.14 ± 18 n = 4

Comparative concentrations of the *Bt* protein from root tissue are shown in Figure 5.3.2.1.

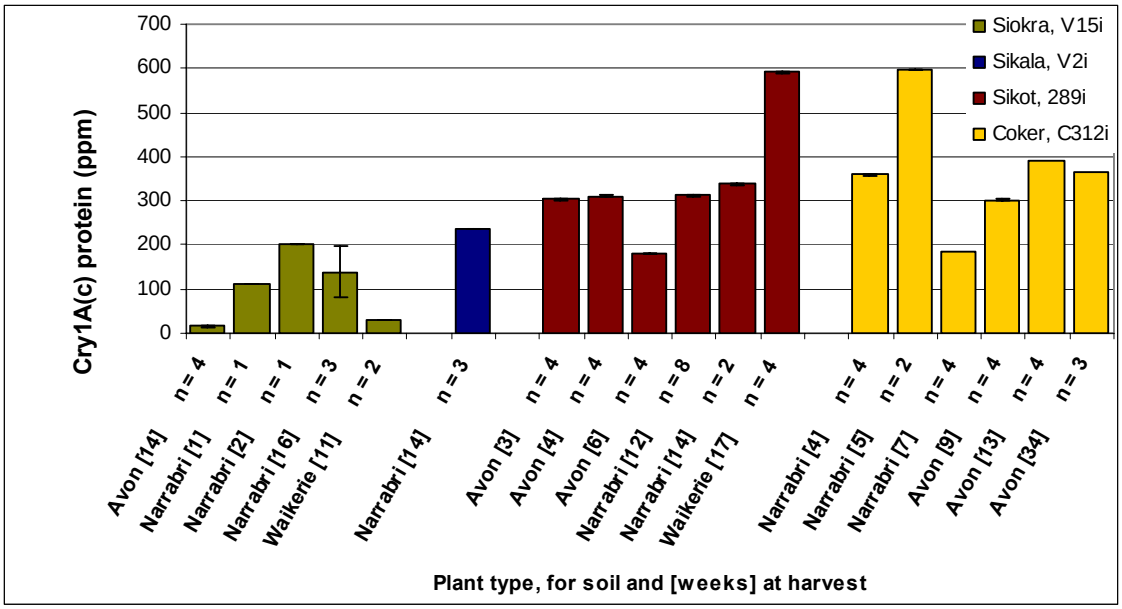


Figure 5.3.2.1 Comparison of *Bt* protein expression in cotton root tissue, for cotton plant cultivar and soil, using ELISA. The ages of the plants at harvest [weeks], are indicated within the square brackets.

None of the non-GM plants from any of the paired trials was positive for the protein.

Fresh leaf and root tissue was always used for the protein quantification and sub-samples of all plant tissue were dried in a herbage oven overnight for calculation of moisture content prior to ELISA. In light of the research on *Bt* corn by Dien et al. (2002), cotton root tissue from a herbage oven was included in an ELISA analysis to investigate the degradation of the *Bt* protein at 52ºC, under dry conditions. It was found that the concentration of the protein was slightly higher than the fresh cotton root tissue, so denaturation did not occur at this temperature from dry heat.

Regression analysis (r^2) showed that only 10% of the production of the *Bt* protein in leaf tissue was attributable to age of the V15i (GM) plants at harvest. This is compared to 0.7% in the roots (n=19 for each analysis), for all soils. A *Bt* protein concentration of 30% was explained by age at harvest for the 289i (GM) plant root over all soils. For the C312i plant 3.2% protein production in the root was explained by age of harvest, so the age at harvest accounted for less variability than individual plant and trial.

5.3.2.2 Measurement of *Bt* protein from nutrient-deficient plant tissue

A plant tissue analysis from the paired plant cultivars was undertaken by the CSIRO Chemical Analysis Section, which showed the C:N ratio of the leaf tissue as detailed in Table 5.3.2.2.1.

Table 5.3.2.2.1 Comparison of C:N % in the nitrogen-deficient plants

	C:N	***C:N***	***C:N***	***C:N***
	Non-GM Green leaf	***GM Green leaf***	***Non-GM Yellow leaf***	***GM Yellow leaf***
Avon V15/V15i, 16 weeks	20.3	20.1	34.4	26.1
Narrabri V15/V15i, 14 weeks	33.4	35.2	50.6	45.5

Compared with a different plant type below, not deficient

Avon C312/C312i, 9 weeks	22.6	24.5

As described in Section 4, the nitrogen content of leaf tissue was higher in the older (yellow) leaves of the GM plants than the non-GM plants for the plants grown under nutrient deficient conditions. This means that some of the nitrogen was not available to the GM plant, even though it was nutrient deficient. The leaves from the same plants were used to compare the amount of *Bt* protein. A comparative illustration of the amount of the *Bt* protein produced in the leaf is shown in Figure 5.3.2.2.1.

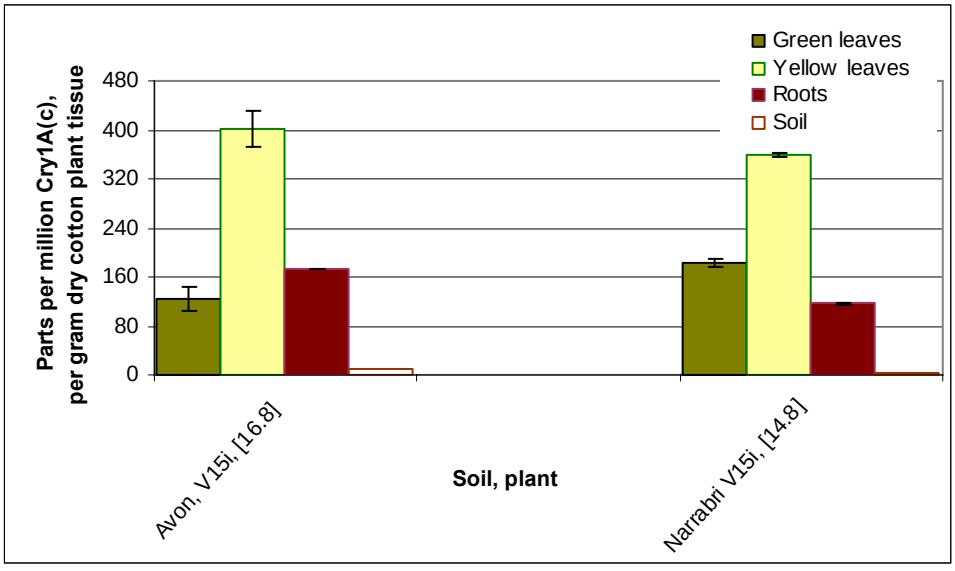


Figure 5.3.2.2.1 Cry1A(c) protein in leaves, roots and soil for Avon and Narrabri, showing increased *Bt* levels in nitrogen-deficient leaves.

(1) The small amount of *Bt* in the rhizosphere soil may have come from fine roots present.

The non-GM plants showed no detectable *Bt* protein for leaf, root and soil.

The higher expression of the protein in the older, yellowed leaves which were symptomatic of nitrogen deficiency, compared to the green leaves of the same plant suggests that the promoter for the Cry1A(c) gene is constitutive and the plant produces the protein regardless of nutritional status. The N, S, C and O of the Cry1A(c) protein would be unavailable to the plant for other metabolic pathways.

5.3.2.3 Measurement of *Bt* protein from the *Bacillus* (Dipel®) culture

The concentration of the Cry1A(c) protein the *Bacillus* culture (Dipel®) was measured at at 5,000ppm at a concentration of 1 in 500.

5.3.3 Quantification of the *Bt* Protein in Rhizosphere soil by ELISA

Of 50 ELISA tests for the Cry1A(c) protein on freshly harvested rhizosphere soil from amongst living root tissue:

- 42 results recorded zero ppm
- Five results were less than 1ppm
- One was 1ppm
- The remaining two results measured 2.05 and 10 ppm.

This indicates that either the protein is not exuded from the plant root or it is quickly degraded.

5.3.4 Bioassay using live larvae

Plutella zygostella

The larvae demonstrated feeding avoidance of the cotton leaf and root tissue after being transferred from the (softer) Chinese cabbage plants on which they hatched. Three days after transfer to the tissue plates the larvae had spun webs and ceased the active phase of their cycle. No conclusion can be formed from this result.

Helicoverpa armigera

The outcome of trial 1 with direct feeding on root tissue was similar to the *Plutella,* as the *Helicoverpa* did not eat the fresh root tissue and so the technique was changed to using insect artificial diet. The comparative results are shown in Table 5.3.4.1.

Table 5.3.4.1 Larval survival rate (% alive) from artificial diet and fresh cotton leaf tissue

Control (diet only)		Plant 189 (non-GM)		Plant 289i (GM)		Dipel® HG	
Day 3	**Day 13**	**Day 3**	**Day 13**	**Day 3**	**Day 13**	**Day 3**	**Day 13**
96.2 (n = 79)	93.6 (n = 47)	94.0 (n = 100)	81.1 (n = 74)	93.8 (n = 96)	77.2 (n = 57)	4.17 (n = 65)	nd.

* The decrease in numbers indicates that some larvae had escaped despite the efforts to keep them in the wells, or possibly burrowed down through the surface.

From the results the mortality rate of the larvae on the non-GM plant was 12.9%, compared with the control of 2.6%. While the 289i plant showed a higher mortality rate of 16.6% than the 189 plant, the Dipel® reduced the population to 4.17% in three days. This shows the high effectiveness of the bacterial product possibly with the synergistic effect of the spores and cytolysins in the bacterial cell that were not present in the plant-produced *Bt* protein. The ELISA test which was done on the leaves from which the assay was done, showed an average of 590ppm of Cry1A(c) protein (n=4) from the roots of the 289i plants used for the assays harvested at 17 weeks.

5.4 DISCUSSION

5.4.1 Quantification of the Protein in Plant Tissue by ELISA

The ELISA method used in this work consistently detected the presence of the Cry1A(c) protein in GM cotton plant leaf and root tissue and confirmed its absence in the tissue of non-modified plants.

The *Bt* protein content varied with the age of harvest for the individual trial, and between the leaf and root tissue within the same sampled plant. This is consistent with the work of Palm et al. (1994), Holt (1998) and Fitt (2000). The leaf tissue was not always found to be higher in the Cry1A(c) protein, even though it had been reported to be the case by Ream et al. (1993). Results in protein expression may differ between this work through different antibody-based tests conducted by independent laboratories and the moisture levels of plant tissue at harvest. Even so, the *Bt* protein measured from leaf and root tissue were all in excess of the 2.04 ppm protein reported in the Protectants Biopesticides Registration Action Document of the EPA (cited in the Literature Review), usually by several hundred fold.

5.4.2 Observations on the method of *Bt* protein detection in soil using polyclonal antibodies

The curves measuring the detection of the Cry1A(c) protein at the different concentrations are best described as a Type I isotherm for the identical monolayer adsorption, as described by Langmuir's (1918) equation, which is general for all adsorption spectra. This model describes a curve of decreasing gradient approaching a plateau where the concentration of the substance tested becomes saturated. The reason for this is that smectite carries a permanent negative ionic charge and any proteins or organic matter will be adsorbed at a rapid initial rate by the more available active clay sites, followed by a slower rate of adsorption as less active sites become available (Churchman, pers. comm.). This result was also confirmed by Pagel-Wieder et al. (2004) who found that with a higher organic carbon content, less protein is adsorbed onto soil clay fractions.

Similar results of adsorption isotherms of *Bt*-toxin/soil systems with a pronounced plateau were published by Venkateswerlu & Stotzky (1992), Tapp et al. (1994), and Crecchio and Stotzky (2001). This maximum concentration differed from the results of Pagel-Wieder (2004), who found that a plateau was not reached when *Bt* protein was adsorbed on a mixture of 25% smectite, 50% illite and 15% kaolinite with *Bt* proteins ranging from 0 to 80ng ml^{-1} under sterile conditions. The *Bt* protein used in the work of Pagel-Wieder however, was a purified and microbially-produced protein harvested from a genetically engineered *E. coli*. It therefore lacked other plant-produced proteins and organic substances derived from the homogenised plant leaves, which would also have been adsorbed onto the clay.

From the results of the ELISA test for Cry1A(c) protein with increasing clay content, the Narrabri soil was found to be unsaturated with organic material of the opposite ionic charge at the start of the experiment. It could accommodate proteins and organic matter by at least an additional 500µg g^{-1} within seconds of its addition. By extrapolation from the curve, the ELISA kit can be seen as having an upper limit of detection of approximately 520 ppm under the conditions of the assay used here.

Because the rhizosphere soil is intimately associated with plant roots, the process of cleaning and blotting the root in analysis work could possibly abrade the fine, delicate root hairs. In these trials extreme care was taken to separate the soil from the fine roots but there was no guarantee that fine root hairs may not have been

present. This problem of harvesting rhizosphere soil without roots has been documented by several authors. In investigating the presence of the Cry1A(b) protein in soil Saxena & Stotzky (2001) conceded that harvesting at 40 days by 'gently shaking the roots to dislodge adhering small clumps of soil' [from the 15 g soil in which they were grown] could have broken some fine roots 'and some toxin released from sloughed and damaged root cells' thus causing a positive result of finding the *Bt* protein in the soil. Similarly, Ayers and Thornton (1968) studied amino acid exudation by intact and damaged roots and concluded that a substantial portion of experimentally determined 'root exudate' may be released from abraded root hairs and tips. If this was the case, it could be argued that almost any manipulative technique may induce root damage and release organic materials and thus would be an artefact of the rhizosphere soil harvest.

Söderberg et al. (2004) argued that it was impossible to remove all roots from the bulk soil, and the lack of difference between bulk and rhizosphere soil may be partly attributed to the methods used. In this study the presence of fine root hairs may have caused a false positive ELISA result in the surrounding soil, and may particularly relate to the two results in 50 ELISA tests, where *Bt* protein measured 2.05 and 10 ppm (46 of the 50 tests measured either 0, or less than 1ppm).

The method of determining the amount of Cry protein external to the root is even problematic when plants are grown in soilless liquid (Hoaglands solution). Saxena et al. (1999) reported that the Cry protein was exuded from the roots of transgenic corn plants in laboratory studies. However the positive result may have occurred by lysing the cellular material after vortexing. Indeed, a representative of Envirologix company stated that the extraction buffer is intended, in combination with vortexing, to lyse plant cells.

5.4.3 Quantification of the Protein in Rhizosphere soil

Addition of clay soil inhibited the detection of the Cry1A(c) protein by the ELISA kit, as shown by lower readings where clay soil was added. However, even when so much clay was added to the leaf homogenate that pipetting was difficult, the protein could still be detected above the lowest calibrator of 0.5ppb. Under these conditions, the protein was still detected at 47% of the 527ppm of the pooled leaf tissue, when 1 g soil was added, and at 22% when 2 g soil was added. The kit was therefore

shown to detect the *Bt* protein in association with soils, including the highly absorptive smectite clay at 45% of its mineral content.

The fact that the protein was still detected by the antibodies while adsorbed onto the clay surface demonstrated that the tertiary conformation of the protein had not altered. It is possible that the active site of the protein could be cleaved away from the attached clay by digestive enzymes and the clay/protein moiety could therefore have retained some toxicity. This would give credence to Stotzky's (2002) insect assay where the (almost pure) clay and protein mixture resulted in some toxicity in *Helicoverpa* assays.

Because the polyclonal antibodies detected the *Bt* protein within the thick clay, this also suggests that soil proteases could attack the polypeptides of the *Bt* protein where it was exposed to an outer surface of the clay-protein moiety. The positive ELISA results from leaf tissue mixed with clay, as well as the negative results obtained from the non-GM plant tissue, verified that the protein was detectable even when mixed with soils, and that the humic acids and other organic material did not negate the value of the test for the protein.

In all but two ELISA tests, the measurement of the *Bt* protein in the rhizosphere soil was below the detectable limits of 1.5 $\mu g\ g^{-1}$ dry soil equivalent, even from the thickly root-bound pots. In the two results from Avon and Narrabri of 10ppm and 1 ppm respectively, it is possible that fine roots were present in the soil in these two isolated cases. This indicates that either the protein was not passively or actively exported from the root cells, or, like other proteinaceous substances, that it is degraded quickly when it enters the soil environment, or both. Prima facie, there would be no effect on the environment, if the 'toxic' substance was not present.

The original proposition by Saxena et al. (2002) that the *Bt* protein exuded from plant roots could persist in the soil environment is therefore not supported by the work described in this section.

5.4.4 Bioassay using live larvae

The method of measuring toxicity for the target organism *Helicoverpa* using the 96 well plates was appropriate for small larva and compared the effect of the *Bt* protein against the controls, with that of the live *Bacillus*. While the results of the

assay showed a toxic effect from the GM plant, the more effective method of control was the use of Dipel®. It is possible that the marginal difference in the effect of *Bt* plant tissue came from the method of application. The artificial diet must be applied to the wells while it is relatively liquid, and this occurs above 50ºC. Homogenised leaf tissue was applied to the surface of the artificial diet after it had cooled to avoid overheating and denaturing the protein. It is now known that *Helicoverpa* neonates often burrow vertically through surface layers of insect diet and into the lower layers. In this case the larvae could have avoided most of the toxin.

Dipel® differs substantially from the single Cry1A(c) toxin produced by Ingard® cotton in that it contains five crystal proteins and one spore in varying amounts, together with the conjugate used for adhering the product to the leaves etc. Dipel® SC produces the Cry1A(a), 1A(b), 1A(c) and 2A and 2B proteins, while Ingard cotton only contains the Cry1A(c) protein (Sumitomo Chemical, 2003). Thus there could have been a synergistic effect of the additional proteins. The effectiveness of the live *Bacillus* even at the dilution of 1 in 1,000,000 was shown by the mortality of the larva which resulted in only 4% surviving at the third day.

The results of *H. armigera* survival rate (Fitt et al. 1994) showed large differences for survival of the separate cohorts on transgenic leaves over the season. There were also increased rates of survival on transgenic leaves with the later (older) instars at commencement of the transgenic leaf diet. In one case, the cohort of five larvae showed 70% survival of neonates after five days on node 3 leaves from transgenic cotton plants (1992-93 season), and in another, all five second/third instar larvae survived. This was the stage of the *Helicoverpa* that was used in the assays described above. It would appear that not only the vigour of the larvae, but also the status of the plant (time of the season), and possibly other factors influence mortality.

The ELISA tests from the longer-term work of Head et al. (2002) also confirmed the absence of detectable Cry1A(c) protein in all of the soil samples, where shredded cotton plant tissue had been buried in field soils, suggesting that either little or no accumulation occurred over years or that there was rapid breakdown after each season (ie reduced to undetectable levels in three months). The toxicity studies also done by Head et al. (2002) using *Heliothis virescens*, (the previous name for Helicoverpa) had a mean survival rate of 97.7% for *Helicoverpa* for soil samples

collected from *Bt* cotton fields, and 98.0% for samples from outside the *Bt* cotton fields, indicating no toxic effect of the *Bt* protein on this highly susceptible insect.

However, the work of Head et al. (2002) focussed on the one target insect, and it is known that the microbiota within the soil ecosystem are root-feeders and form part of an extensive, complex, interactive food web. Plant roots are broken or abraded during growth, exposing the protein to micro-and mesofauna, any of which may be affected by differences in exudates from modified plants. More specific investigations on the soil inhabitants were therefore needed to determine if any effect could be found which altered the microbiota within the rhizosphere of the GM plants. These investigations will be addressed in the following sections of this thesis with specific tests for each of the selected soil microbiota. To address the possibility that there may be a difference in the rate of plant tissue degradation between the non-GM and GM plants by the soil detritivore, the second test for toxicity, that of persistence in the environment, was investigated using litterbag trials. This will be discussed in Section 6.

Section 6

Persistence of the *Bt* protein in the soil environment.

Fig. 6.0 Exhumation of litterbags in Avon soil

6.1 INTRODUCTION

The toxic effect of a substance within a particular environment must be quantified both in terms of the amount present, as well as its persistence. In the previous section it was confirmed that the *Bt* protein was produced in the leaf and root tissue for all of the GM plants tested. The plant-produced *Bt* protein has been sequenced and is known not to contain toxic intermediary substances on breakdown. However the effect of the *Bt* protein within agricultural soils containing leaf litter from genetically modified plants in various stages of decomposition is not known.

Proteins are decomposed readily by many bacteria and fungi (Schinner et al. 1995). The extracellular proteases can show a high degree of resistance when physically adsorbed onto soil colloids or covalently bound to soil organic matter, but are inhibited by desiccation. The *Bt* protein has been found to begin denaturing at about 70ºC (Dien et al. 2002), and while the surface temperatures of agricultural soils may approach this temperature, catabolism of proteins is countered by desiccation – a situation that would occur at maturation and senescence of the standing crop.

Decomposition of plant litter depends on soil temperature, soil aeration, soil available water and pH (Begon et al.1990, pp. 369-375), and solubility and chemical properties (Wilson, 1987, p. 124 and Henriksen & Breland, 1999, p. 1121). Begon et al. (1990, pp. 369-375) pointed out that 'the rate at which dead organic matter decomposes is strongly dependent on its content of available nitrogen, or on nitrogen (ammonium or nitrate) that is available from outside'. Henriksen & Breland (1999)

also noted that nitrogen availability affected carbon mineralisation, fungal and bacterial growth, and enzyme activities during decomposition of wheat straw in soil. It was shown in Section 4 that the GM leaf tissue contained more N than the non-GM plant. If the higher content of nitrogen in the leaves of GM plants differs from the non-GM plants, there could be a difference in the availability of N for the soil microbiota and therefore in litter breakdown.

Additionally, the rate of degradation of plant tissue depends on the different forms of organic C from plant tissue which are degraded at different rates according to the molecular structure causing chemical resistance to breakdown. Begon et al. (1990, p. 364) described the rate of plant tissue breakdown from the least resistant to the most resistant as: sugars < (less resistant than) starch < hemicelluloses, pectins and proteins < cellulose < lignins < suberins < cutins. With the availability of different forms of C, including the products of photosynthesis, different soil microbiota are able to degrade the different carbon compounds. If the plant tissues differ in structure, or the exudates differ in C content, the microbial populations may also differ in structure.

Microbial degradation can also depend on predation pressure of the higher trophic groups on the bacteria which are the primary agents of C mineralisation as per the example of Whitford et al. (1982), mentioned in the Literature Review. Foissner (1999) also reported that decomposition is delayed by 8-18 months if soil animals are excluded from the decomposer cycle. Therefore if *Bt* protein impacts in decomposing plant tissue affected any part of the detritivore food web (without compensatory effect), then the degradation process could be delayed.

This section addressed the issue of the persistence of the *Bt* protein at moderate temperatures and in dry conditions, such as could be found in an Australian agricultural field after harvest, and its rate of degradation after watering.

6.2 MATERIALS AND METHODS

6.2.1 Experiment 1: Litterbags in soil.

The trial was undertaken with C312 and C312i plant tissue harvested at 9 weeks. The cotton plants were grown in Avon soil where no GM crops had been planted. The freshly harvested and unmacerated plant tissue from each of the four GM-plant

replicates was inserted into 9 cm x 9 cm elongated hexagonal nylon mesh bags as per the method of Edwards & Heath, (1963), with a diameter of 1mm along the narrow side of the mesh opening. The mesh size was chosen to show whether initial fragmentation of leaves and roots by soil animals is a necessary prerequisite for extensive microbial decomposition of litter, as suggested by Whitford et al. (1982). The bags and GM plant tissue were buried in three 3-litre draining plastic pots, together with the paired non-GM plant tissue in separate bags. The bags containing the plant tissue were buried mid-depth in each of the pots with waterproof plastic tags, approximately 10 cm away from each other. The pots were watered weekly, and kept in a glasshouse at 25°C.

6.2.2 Test of chemical stability of the *Bt* protein from dry, undisturbed plants

Replicate C312 and C312i plants were grown under the same conditions as the plants described in 6.2.1, but watering was stopped at 9 weeks at the harvest of the first batch. The second batch of plants were kept in the glasshouse for 35 weeks (8½ months) without watering, to test for the persistence of the *Bi* protein in dead leaf, stem and root tissue. The tissue was tested by ELISA in the same way as for live plant tissue. A separate trial was additionally undertaken for the same paired plant types, but using Narrabri soil.

6.3 RESULTS

6.3.1 Experiment 1: Breakdown of plant tissue in Avon soil

The photographs following show the compared rate of breakdown of the plant tissue for the paired trials (Figs 6.3.1.1 through 6.3.1.6. The ELISA tests were negative for the Cry1A(c) protein in both GM and non-GM litter at the first litterbag exhumation at two weeks, and remained negative for the other tests until and including the last trial at 8 weeks when the fine roots had degraded, and only the thicker stalks remained. All images to the left are from non-GM plants and to the right from GM plant tissue.

Note: all illustrations have been archived as digital images to facilitate enlargement for future observation, if required. Each sample is shown within a 90mm petri-dish, for scale.

Figure 6.3.1.1 Avon bulk soil (5mm sieved) before planting

ELISA not done. Soil moisture 2%. Soil fine and powdery.
Some fine, dry plant material present in the soil from the previous harvest of wheat.

Figure 6.3.1.2 Cotton plant root tissue in Avon soil at nine weeks harvest

ELISA: non-GM plant 0 ppm **GM root tissue 302 ppm**

Soil moisture: 15%. Soil was damp but smelt aerobic. Geosmins present.

Figure 6.3.1.3 Cotton plant root tissue in Avon soil after 8½ months undisturbed and dry.

ELISA: non-GM plant 0.00 ppm **GM root tissue 364 ppm**

Soil moisture: 2%. Soil was powder-dry and the stems were hard and brittle.
Plant tissue types were clearly identifiable.

Figure 6.3.1.4 Cotton plant tissue in Avon soil from inside litterbags after wetting and exhumation at 2 weeks

ELISA: non-GM 0.00 ppm. GM plant 0.00 ppm

Soil moisture: 15%. Soil was damp and cohesive but not saturated. The fine, desiccated root tissue had swollen with the moisture, and the leaves and stems had become darker in colour. Tissue types partly broken down, but still identifiable.

Figure 6.3.1.5 Cotton plant tissue in Avon soil from inside litterbags after wetting and exhumation at 4 weeks

ELISA: non-GM 0.00 ppm GM plant 0.00 ppm

Soil moisture 15%. Soil damp but not saturated. Leaf tissue appeared slightly slimy. Larger leaves and stems still intact, but finer roots were harder to identify.

Figure 6.3.1.6 Cotton plant tissue in Avon soil from inside litterbags after wetting and exhumation at 6 weeks

ELISA: non-GM 0.00 ppm GM plant 0.00 ppm

Soil moisture 14%. Wet from previous 24 hour watering. Smelt musty, indicating active fungi. Less intact leaves and stems. No fine roots remained in either sample.

It can be clearly seen that the rate of breakdown of non-GM and GM cotton plant tissue is equivalent. Moreover, from the ELISA test, the *Bt* protein was not detected after moisture was added to the soil in which the dry plant tissue was buried.

6.3.2 Experiment 2: Additional trials using plants grown in Narrabri soils

Varieties C312 (non-GM) and C312i (GM) were also grown in Narrabri soil to 13 weeks and then harvested, and buried in known previous non-GM Narrabri soil. The first exhumation at 5 weeks showed the protein had decreased from an averaged 389ppm (n = 4) to 272ppm, and a clay mantle (pedotubule) had developed around the damp root. The soil surrounding the plant material was negative for the protein.

The second exhumation at 12 weeks showed that only a small remnant of plant tissue remained undegraded, with negative tests for the protein, in the leaves, roots and soil.

6.4 DISCUSSION

6.4.1 Litterbags.

This work demonstrated that the *Bt* protein was chemically stable when it was kept dry, but degraded at moderate temperatures when water was available. The proteolytic activity of soil microbiota is well documented, for example by the results of Ladd & Butler (1972), and the *Bt* protein was similarly degraded without effect to the soil microbiota, during litter degradation. The results are also in agreement with the work of Donegan and Seidler (1999) who compared the action of the microbiota in rhizosphere soil, using litterbags for measurement of the rate of degradation, Biolog community analysis, and rDNA fingerprint analysis of the soil populations. They reported that while the plant genomic DNA can be detected for several months, the effect of the plant genetic modification did not have a direct effect on the population of the soil microorganisms.

The coating of clay around the roots from the Narrabri soil appeared to have inhibited degradation of the *Bt* protein as some was detected at five weeks. The pot with Narrabri soil was only watered once after burial and the clay was dry at exhumation. By eight weeks the plant tissue had degraded in both soils to fine particulate plant detritus with low tensile strength, and the original tissue type was only just identifiable. ELISA measurements showed that the *Bt* protein in the plant litter in Avon soils had degraded in less than two weeks when the litterbags had been

kept moist. The result of the ELISA tests showed no *Bt* protein remained after this time for both soils, where moisture was present.

Similar results of rapid protein breakdown were found by other independent researchers. Saxena et al. (2002) used purified protein and found the soil to be 'immunologically negative' after 12 and 24 hours in all soils (including a similar smectite clay soil which was used in this research, and was known to adsorb protein). Palm et al. (1994) showed that when incubated in soil under laboratory conditions, Cry1A(c) protein contained in transgenic *Bt* cotton plant residues degraded in soil at a rate similar to or greater than pure Cry1A(c) proteins. Palm et al. (1994) also reported that from comparative studies of purified *Bt* protein recovered from γ-irradiated and unsterile 10% clay soil, the comparative loss of protein from non-sterile soil was attributed to microbial action, even when adsorbed onto clay surfaces. The ELISA tests from the work of Head et al. (2002) also confirmed the absence of detectable Cry1A(c) protein in all of the soil samples, where shredded cotton plant tissue had been buried in field soils, suggesting that either little or no accumulation over years and rapid breakdown after each season (ie reduced to undetectable levels in three months). Head et al. (2002) could not detect Cry1A(c) after 3-6 consecutive years of postharvest tillage of the transgenic cotton. Further, the Cry 1F protein, a similar delta-endotoxin, was ineffective against *Helicoverpa virescens* after incorporation into soil and its half-life was estimated to be less than one day (Herman et al. 2002).

6.4.2 Desiccation

The importance of water in the function of hydrolysing enzymes such as proteases, peptidases, esterases, glycosidases and phosphatases can be observed from the general formula $AB + H_2O \rightarrow AOH + BH$ (Laskin & Lechevalier, 1974). The microbial breakdown of proteins and other organic substances in soil is therefore dependent on soil moisture, and this in turn depends not only on the rainfall of the area, but also on the soil type and its water retention. Water availability for enzymatic hydrolysis may be impeded by a protective layer of clay which could mean that the cry1A(c) protein degradation occurs more slowly within the protective clay layer, particularly in the inter-cropping periods where neither irrigation nor rainfall occurs to degrade it. Within cotton-growing fields the post-harvest residue is not shredded to fine particles to facilitate degradation, but is simply ploughed in.

Desiccation of leaf tissue in a drying oven at 50ºC (Section 4) showed that the protein is not degraded. Another factor contributing to the rate of enzymic breakdown rate is temperature. This factor was not included here as all trials were run at the same temperature.

The breakdown of organic polymers to reduce decaying plant material to its elemental form is the summation of function of many interacting microbiota. Wilson (1987) noted that the rate of N mineralisation of legume material with a ratio of 11:1 proceeded more rapidly than wheat straw with a C:N ratio of 48:1. The preceding photographs show an equivalent rate of breakdown at the macroscopic level, even though the GM cotton plants contain a higher level of N than the non-GM plants. This equivalent rate of breakdown precludes any effect of toxicity to detritivores (Literature Review, p. 14) from the *Bt* protein or altered GM plant characteristics, on the predators of the bacteria that mineralise the plant tissue.

SUMMARY OF CHAPTER 2

The results of the soil analysis established that the sampled Narrabri soil contained a high content of smectite clay, and would be suitable to test the clay/protein interaction by antibody-based methods, as per the questions raised in the Literature Review. The Avon soil was shown to be a good contrast in physical properties, to compare plant growth and microbial consortia.

While the non-GM and GM plants appeared similar by observation, the plant tissue analysis showed that there were differences between the non-*Bt* producing plants and the *Bt*-protein producing plants in the two important elements Zn and N, both of which are required for protein synthesis. This difference could not be attributed to soil type. This implies that the root exudates could have differed between the two plant types, and this difference may lead to differentiation in the adjacent soil microbiota if the composition of the exudates differed sufficiently.

The measurement of the *Bt* protein within the plant tissue, roots and surrounding rhizosphere soils was required both to determine the level of protein expression and to investigate the difference in total plant nitrogen content with the measured protein. The ELISA method was found to be appropriate for the detection of the plant production of Cry1A(c) protein, even in 60% heavy clay soil. The plant leaf tissue analysis showed that there was a higher concentration of nitrogen in the modified plants, and the results suggested that the GM-plant may have been unable to sequester the nitrogen from the older senescing leaves at the same rate as the non-GM plants. The rate of degradation of the protein was found to be rapid, particularly in a moist soil environment.

The focus of this work, however, is not on the physiology of the GM-plants, but on the response of the soil microbiota to the plant root exudates, ie, whether there is a difference between the two rhizosphere soil environments that can be attributed to the genetically modified plants. This was addressed in the following section where tests described selected individual microbial populations which were harvested from the rhizosphere soils of the different plants from the paired GM/ non-GM trials.

CHAPTER THREE

MEASUREMENT OF INDIVIDUAL MICROBIAL POPULATIONS

Section 7
Microflora:
7.1 Bacteria

Fig. 7.1.0 Subcultured distinctive soil bacteria isolated from Narrabri soil, resistant to chloramphenicol and ampicillin, and fluorescing blue at 366nm.

7.1.1 INTRODUCTION

The first enzymatic step in protein degradation by bacteria occurs outside the cell. Proteins of high molecular weight are hydrolysed into polypeptides and amino acids and are then transported into the cells and deaminated there (Clarke, 1984, p. 11). Because the ingestion of proteins and amino acids occurs outside the bacterial cell, it can be argued that there may be no toxic effect from the plant-produced *Bt* protein, a gut toxin in insects, on bacteria, per se. As part of the rhizosphere food web, bacteria are prey to many microscopic soil-dwellers such as protozoa and nematodes. Whitford et al. (1982) found a difference in the microbial function of litter breakdown after pesticide application killed most of the insects and mites. This resulted in a decrease of bacteria due to predation by increased nematode populations, as the mites were predators of the nematodes. With the decrease in bacterial populations, recycling of C to the soil was inhibited. If predators of bacteria are susceptible to the *Bt* protein in the rhizosphere of cotton plants, the microbial flora may be affected, and the numbers of bacteria altered through the indirect pressure on predators.

The propensity of *Bacillus* species to catabolise protein in the soil was noted by Aslim et al. (2002), where 26 of 30 *Bacillus* strains isolated from soil recorded proteolytic activity. Extracellular proteolytic enzymes are also produced by many other heterotrophic bacteria including commonly occurring *Pseudomonas* (Hawker et al. 1960). Johansen & Binnerup, (2002) found extracellular proteolytic enzymes were

produced by pseudomonads wherever proteins occurred in the rhizosphere of growing barley plants. The plant-produced *Bt* protein in the rhizosphere of actively growing plant roots is likely to be susceptible to breakdown by these and by many other proteases secreted by resident soil microorganisms.

The composition of the microbiota surrounding plant roots differs with the type and age of the plants, as well as with temperature, irradiation, soil moisture content, soil and plant nutrient status and root injury or stress (Rovira, 1959). Soil type also has a significant influence on the soil microbiota (Sessitsch et al. 2002). Each bacterial community within a particular plant and soil ecosystem (and their predators) should therefore be considered as potentially different in its response to different root exudates within the rhizosphere.

Rouatt et al. (1960) classified 306 soil, rhizosphere and rhizoplane bacterial isolates into ten Genera. *Arthrobacter* predominated amongst the soil isolates and *Pseudomonas* amongst the rhizosphere isolates. The predominance of *Arthrobacter* in the rhizosphere was also found with other C3 plants (subterranean clover) by Sperber & Rovira (1959). More recent studies using PLFA and DGGE analysis (Söderberg et al. 2004) confirmed the relative abundance of Gram-negative bacteria in rhizosphere soil, and Gram-Positive bacteria in bulk soil. Kloepper et al. (1992) found that the number of identifiable species of *Pseudomonas* was greater than for other genera within the rhizosphere soil. The reason for this change in the rhizosphere bacteria is not known, but is important because of the potential of *Pseudomonas* as biological control agents against plant root diseases (Press & Kloepper, 1994).

Given the potentially different root exudates of non-GM and GM plants, information on the selectivity of microflora in the rhizosphere is needed to establish differences that may alter the balance and function of soil microflora.

The work described in this section compared the numbers and colony morphology of bacterial flora from three different rhizosphere soils of non-GM and GM cotton plants, to determine if there were differences in population numbers, or observable differences in colony morphology that would suggest functional disparity. Additionally, the recoverability of the microbial population after a major chemical impact was tested, to determine the plasticity, or characteristics of the surviving populations after the deletion of most of the bacteria present in the original soils.

7.1.2 MATERIALS AND METHODS

In comparing the methods of soil plate and dilution plate for culturing microflora, Montégut (1960) reported that compact soils with a high clay content have a constant risk of agglomeration, and the dilution method alone is likely to ensure complete uniformity. As Avon soil had a clay content of 16%, Waikerie contained 7% clay and Narrabri had 60% clay, the dilution method was chosen. The physical properties of the three soils were detailed in Section 3.

7.1.2.1 Media and antibiotics used

The three media listed in Table 7.1.2.1.1 were chosen to provide a source of protein or nitrogen and organic carbon nutrients which would normally be utilised by common aerobic soil bacteria. The components for each of the media are listed in Appendix 7.1.

Table 7.1.2.1.1 Media and antibiotics used for the estimation of bacterial populations

Media	Antibiotics used	Selective nature of medium
$^1/_{10}$ concentration tryptone soya (TSA) agar (Lawley et al. 1983)	No antibiotics	A protein-containing medium to cultivate mixed populations of aerobic bacteria able to utilise polypeptides
ISP4 Medium (1)	Cycloheximide, 75µg ml^{-1}, (antifungal)	A starch and inorganic-mineral medium which enriches bacteria such as actinomycetes that synthesize their own peptides from inorganic N
Medium B of King (1954) (2)	Ampicillin, (3) 50µg ml^{-1}; Chloramphenicol, 12.5µg ml^{-1}	Selective for Fe-chelating pseudomonads which are known to be versatile consumers of organic compounds

(1) A variation on International *Streptomyces* Project Medium 4, (Atlas, 1993, p. 461)
(2) Rosenthal, S.L. (1974)
(3) Antibiotics as per Simon & Ridge (1974)

7.1.2.2 Dilutions and plating technique

Two grams of moist, freshly harvested rhizosphere soil from each of the Avon, Narrabri and Waikerie were taken, as well as non-plant associated soils kept under the same conditions of added nutrient and moisture. These soils were added to 20 ml phosphate buffer and vortexed for two minutes in autoclaved McCartney bottles.

Serial dilutions were made to 10^{-3} and 10^{-5} concentrations of the mixture and 100µL was delivered onto all media plates using the spread-plate method, with an ethanol-flamed glass spreader. The inoculated plates were allowed to dry briefly in a laminar-flow cabinet.

Four replicates (from separate pots) of rhizosphere soil were plated for each of the paired plant strains, as well as two replicates of non-plant associated soil for comparison of the rhizosphere effect. Duplicate plates were cultured from each dilution, for each of the four sets of replicates for each treatment. The eight rhizosphere soils and two non-rhizosphere soils therefore incorporated forty plates for each of the three bacterial media, for each paired trial. All bacterial plates were inverted and incubated in darkness at 24ºC.

7.1.2.3 Method of scoring: plate counts

If the number of colonies exceeded 50 on any plate, or where confluence occurred, calculation was based on the lower concentration. Two colony counts were made, one on day 3, when the colonies were marked, and another on day 6 or 7 to detect slower growing bacteria.

7.1.2.4 Method of scoring: phenotypes

In addition to estimating colony numbers, the frequency of the most common bacterial forms appearing on the $^{1}/_{10}$ TSA medium were recorded by keywords. The occurrence of each categorised type of bacteria (see below) was noted if present, for each of the plates. From the presence or absence of the characterised colonies, binary logistical regression using SPSS® was used to estimate differences in the commonly-occurring bacterial populations within each trial for one of the dilutions, depending on the growth. The definitions of the characterised colonies appear in Table 7.1.2.4.1.

7.1.2.5 Potential for bacterial recoverability after a major environmental impact

A study was also undertaken to investigate the bacterial population from rhizosphere soil that had survived chloroform fumigation (discussed in Section 13). The method used freshly harvested Narrabri rhizosphere soil in which the 189 and 289i cotton plants were grown, as well as non-rhizosphere soil kept under the same conditions. Soil samples were prepared as per the established procedure of soil fumigation (Appendix 13.3). After ten days of fumigation, 2 g soil was taken from the jar and

diluted to 10^{-4} in autoclaved phosphate buffer and duplicates were plated aseptically onto the four media previously described. The plates were incubated under the same conditions as the previous trials and the colonies were counted on the sixth day.

Table 7.1.2.4.1 Descriptions of bacteria most commonly occurring on all media

Bacterial characteristics(1)	**Description and comments**
Filamentous, spore-forming,	**Putative identification:** Actinomycetes Discrete, usually white, chalky colonies, attached to the medium by substrate mycelium, which resist removal by scraping with a wire loop (Stotzky, 1993). Slow growing, usually only appearing after the third day. Where present, aerial mycelium of shorter length than fungi. Able to grow on TSA or ISP4 media, if not impeded by other faster-growing bacterial overgrowth.
Non-filamentous, non-spore-forming,	**Putative identification:** (*) *Arthrobacter*-like Raised white, yellow or cream mucoid. Members of the genus *Arthrobacter* are among the dominant bacteria in soil (Keddie, 1975) and may account for 50-60% of total bacterial counts. The non-motile rods are restricted in movement, so colonies tend to remain separate.
Non-filamentous, spore-forming,	**Putative identification:** (*) *Bacillus*-like Fast-growing, spreading clear or translucent colonies. Uneven margin. Often malodorous. The common soil genus of *Bacillus* is among the most common organisms to appear when streaked onto agar plates containing various nutrient media (Brock & Maddigan, 1991, p. 776).
Non-filamentous, Non-spore-forming, fluorescing at 366nm.	**Putative identification:** Siderophore-producing Pseudomonads

(1) Brock& Maddigan, 1991.

(*) The classification was distinguished by the characteristics of motility. It was not intended to imply that all of the bacterial colonies were able to be categorised into the two genera of *Bacillus* and *Arthrobacter*, but were representative of two most common soil bacteria, both of which are members of the fast-growing, cultivable, aerobic heterotrophs. Possibly *Pseudomonas aeruginosa* would be included with the category of '*Bacillus*-like' as this common soil bacteria typically has flat mucoid colonies with irregular edges which spread over the plate with time (Todar, 2004) and is well known to utilise a wide range of carbon sources.

The pseudomonads grown on Fe-deficient King's B medium were also observed under ultraviolet light at 366 nm to categorise different types of siderophore (Meyer et al.

2002). The nature of the difference in the acyl chain of the pyoverdine which results in a difference in colour does not affect Fe transport: non-fluorescing pseudomonads such as *P. corrugata*, *P. graminis* and others are capable of Fe-uptake without producing fluorescent pigments (Meyer et al. 2002).

7.1.3 RESULTS

7.1.3.1 General bacterial growth on $^{1}/_{10}$-strength Tryptone Soya Agar

7.1.3.1.1 Total number of colony forming units across all soils

Figure 7.1.3.1.1.1 shows the comparison of colony forming bacterial units on $^{1}/_{10}$-strength Tryptone Soya Agar without antibiotics. From Tukey's post hoc analysis, the homogenous subsets are shown by characters signifying differences between the trials, taking into account the variability of the replicates ($p = 0.05$). A combination of characters above the bars, for example abc, shows the span of the results across more than one category, attributable to variance from the mean by replicates of the trial.

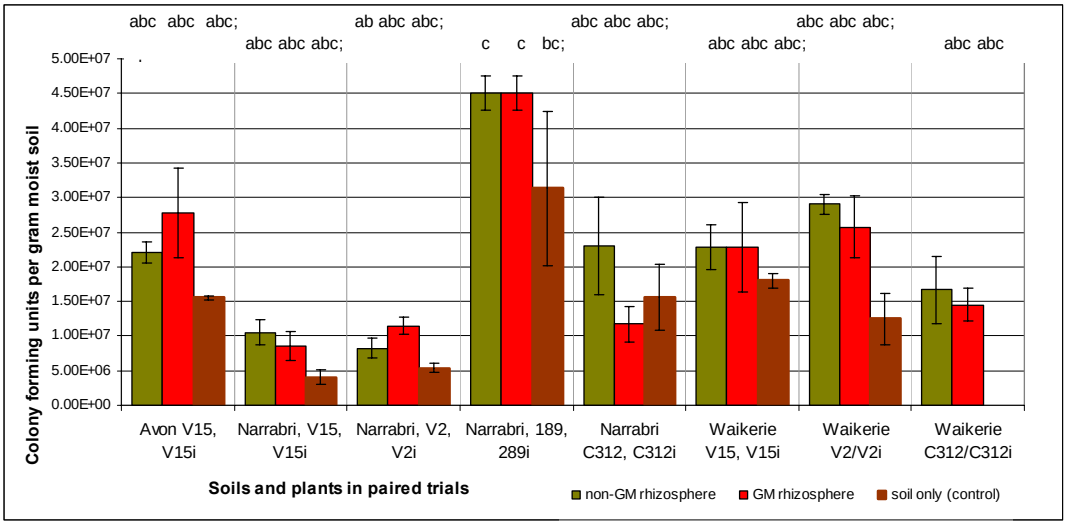


Figure 7.1.3.1.1.1 Bacterial colony-forming units for paired soil and plant trials, from $^{1}/_{10}$-strength TSA medium.

Except for the C312/C312i Narrabri trial, an overall rhizosphere effect can be seen for all trials, where colony numbers were less for the control (non-plant) soils. The exception to this occurred with the Narrabri C312i bacterial population because of overgrowth of clear, spreading bacteria on two of the replicates, and with Narrabri V2/V2i plate counts, where the numbers were similar.

Tukey post hoc tests were done on the number of colony forming units after ANOVA analysis were calculated (after $\log_{10}+1$ transformation). They showed that except for

the 189/289i plant trial grown in Narrabri soil, each of the paired trials resulted in a grouping within the same homogeneous subset for the compared plants, indicated by the letters above the bars. The relatively high colony count for rhizosphere bacteria from the 189 and 289i rhizosphere soils may have occurred because of addition of nutrient solution close to harvesting and were sufficiently different in numbers to be grouped as a separate subset. However, both the non-GM and GM rhizosphere soil bacterial colony numbers were similar.

A comparison of bacterial colony numbers on the (log-nontransformed) data for the V15 and V15i plant strains comparing different soil treatments showed more similarity between the Avon and Waikerie rhizosphere soils than the Narrabri soil. The arithmetic mean of the bacterial numbers in Avon rhizosphere soils showed a greater colony number for the GM plants, whereas the Narrabri rhizosphere soil bacteria showed a slight decrease, and the Waikerie GM rhizosphere soil bacterial count was approximately equal for the same plant strain.

A comparison of the colony numbers for the plant strain V2 and V2i showed a higher colony count for the GM plant rhizosphere soils in Narrabri soil, but a decrease for the same plants in the Waikerie soils. The individual trials therefore showed variation in colony numbers for plant type as well as for soil type, indicating the effect of varying microbial populations within the different rhizosphere environments, rather than a consistent effect across all trials.

The number of colony forming units ranged between 4.6×10^6 for a control soil in the Narrabri V15/V15i trial, to 4.5×10^7 for the 289i plant trial within the Narrabri rhizosphere soil. The bacterial populations from rhizosphere soil from the same paired plant cultivars, the C312 and C312i plants grown in Waikerie soil, showed a non-significant difference between the number of bacterial colonies, as did Avon and Narrabri (except C312/C312i).

When the total number of bacteria which grew on $^1/_{10}$-strength TSA was compared against age at harvest, a negative correlation (Pearson's R) of -0.128 resulted. This showed a weak trend for population decrease over time, under the conditions of the pot trials. When the Narrabri 189 and 289i trial was eliminated, the correlation was -0.256 which meant that the positive growth which resulted in increased numbers through nutrient addition outweighed the effect of age-related decrease.

7.1.3.1.2 Bacterial morphology comparison across all soils

Of the 82 plates where *Bacillus*-like growth was noted, 41 plates were from the non-GM rhizosphere trials, and 30 from the GM rhizosphere trials. The t-test analysis showed that neither the spreading *Bacillus*-like colonies nor the discrete *Arthrobacter*-like colonies were significantly different between the two paired plant types (at $p \leq 0.05$), although the comparison of *Bacillus*-like colonies was close to the point of chosen significance at $p = 0.08$.

At the 1×10^{-5} concentration alone, *Bacillus* occurred on 62% of the 78 duplicated $^{1}/_{10}$-strength TSA plates for all three soils. This common soil bacterium occurred on at least one of the replicates of all trials. The prediction of similarity using binary logistic regression for occurrence of *Bacillus* being dependent on the paired plant types, and taking all soils into account, resulted in a significance of 0.22 for the 64 cases of rhizosphere soils.

Of 30 records of colonies having the raised white, yellow or cream discrete colonies (categorised as *Arthrobacter*-like) at the 10^{-5} concentration, the predominant colony colour was white in 55% of cases, and yellow in 36%. None of the colonies had the bright yellow pigment of *Xanthomonas*, a Pseudomonad which is often plant-pathogenic. Where the *Arthrobacter*-like colony forms were noted in the rhizosphere soil from the non-GM plants, they were also noted on the paired soil from the GM-plants, except in one instance where a yellow *Arthrobacter*-like colony was noted on the plant rhizosphere soil for the plant C312 but not its GM-counterpart (in the Waikerie soil at the 10^{-5} dilution). The t-test for the difference in occurrence of both the spreading colonies and the discrete colonies between the non-GM and GM paired plant rhizosphere soils resulted in a non-significant result of $p = 0.08$ and 0.47 respectively when compared over all trials.

There were differences in proportions of some colonies from the different sampling sites. More pink bacterial colonies occurred from the Waikerie soil samples than from Narrabri soil, which were absent from the Avon soil. No consistent difference was observed in the morphological appearance of the bacteria grown on the plates when the non-GM and GM plant soils were compared. Even so, the propensity of the bacteria for growth on protein and N-containing media was not different between the non-GM and GM plant rhizosphere soils from the three regions.

7.1.3.2 Actinomycetes

Actinomycetes were also observed on the general TSA medium, but only those cultured on ISP4 were used for colony counts. The common *Streptomyces* forms with white aerial mycelium and white-spores appeared most commonly in all sampled soils. There was no consistent trend for actinomycete colony forming units, for GM vs non-GM plants, within each sampled soil (Figure 7.1.3.2.1).

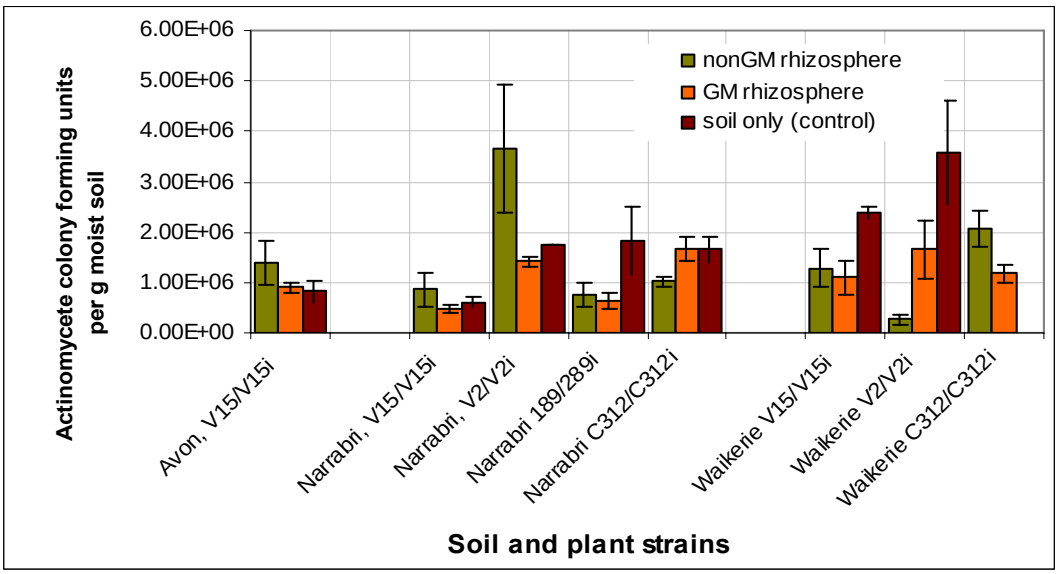


Figure 7.1.3.2.1 Actinomycete colonies cultured on ISP4 media, for paired soil and plant trials.

Most means for replicates of actinomycete colonies fell within the range of 1×10^5 and 1×10^6 cfu g^{-1} soil, and four of the eight averaged log-scaled estimates (V15/V15i in all soils, as well as 189/289i in Narrabri soil) were similar for the non-GM and GM plants. The V2/V2i counts were dissimilar between the non-GM and GM plants, but the colony density was reversed between Narrabri and Waikerie soils. In Narrabri soil estimates of actinomycetes for the C312 plant rhizosphere soil were lower than the GM C312i which had a count equal to the soil-only control. This was also the case for V2/V2i trials in the Waikerie soil. The count for the C312 plant grown in Waikerie soil was significantly greater than for the GM C312i.

The V15 and V15i plant trials compared over the three soils had slightly fewer numbers for the GM rhizosphere soils but the differences between the non-GM and GM individual trials were not significant. The V2 and V2i plant trials compared between the Narrabri and Waikerie rhizosphere soils showed a higher non-GM plant

colony count in the Narrabri soil, but this was less in the Waikerie soil. The C312 and C312i plant rhizosphere soil bacterial populations had lower averaged colony counts from the Narrabri soil and a higher colony count from the Waikerie soil. The numbers of Actinomycete colonies therefore appear to be affected by the plant and soil interactions of the individual trial.

7.1.3.3 Pseudomonads grown on King's B medium

The comparison of colony numbers developing on King's B (Fe-deficient) medium are shown in Figure 7.1.3.3.1.

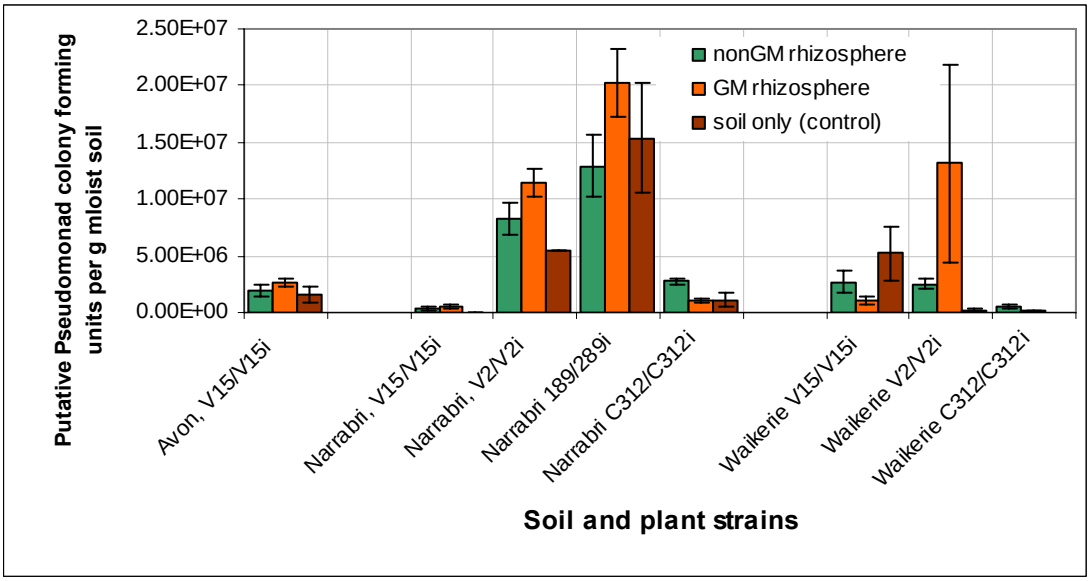


Figure 7.1.3.3.1 **Putative fluorescent Pseudomonads grown on King's B medium, for paired soil and plant trials.**

Pseudomonads varied with soil type and plant type of individual trial. The error bar is not shown for the Narrabri V2/V2i trial as the consistency of counts resulted in the symbol being too short to be illustrated. As with the actinomycetes, there was no indication that an effect was present in the rhizosphere soils of the paired plants that resulted in a consistent, significant difference in pseudomonad colonies.

The incidence of the different siderophore-producing pseudomonads (present or absent for each plate at the 10^{-5} concentration) appear in Figure 7.1.3.3.2.

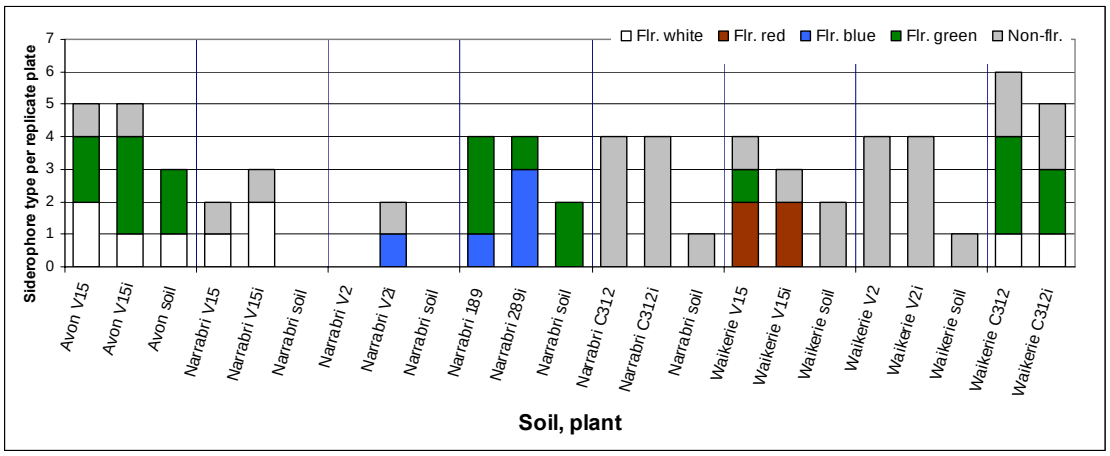


Figure 7.1.3.3.2 **Incidence of fluorescing colours from siderophores of pseudomonads harvested from non-GM, GM rhizosphere soils, and non-rhizosphere soils at the 10^{-5} concentration.**

Of the fluorescing colonies, pyoverdine, the yellow-green fluorescent pigment, appeared the most frequently, followed by white, blue and red over all trials. The most commonly occurring non-fluorescing colonies occurred in 7 of the 8 trials. The similar occurrences of the different siderophores, by paired trial can be clearly seen. Apart from the Waikerie V15/V15i trial, all fluorescing colonies were similar for individual treatment.

7.1.3.4 Comparison of *Bt* protein production from the plant root and the bacterial colony counts from $^{1}/_{10}$ TSA medium

The comparison between the amount of *Bt* protein in root tissue and bacterial colony counts grown on $^{1}/_{10}$-strength TSA medium, was insignificant ($p \leq 0.05$) for five trials where the *Bt* protein from root tissue was measured concurrently with the plating of the rhizosphere soil. The replicate-averaged, log-scaled comparison of both the quantified protein from root tissue and the bacterial colony forming units is shown in Figure 7.1.3.4.1.

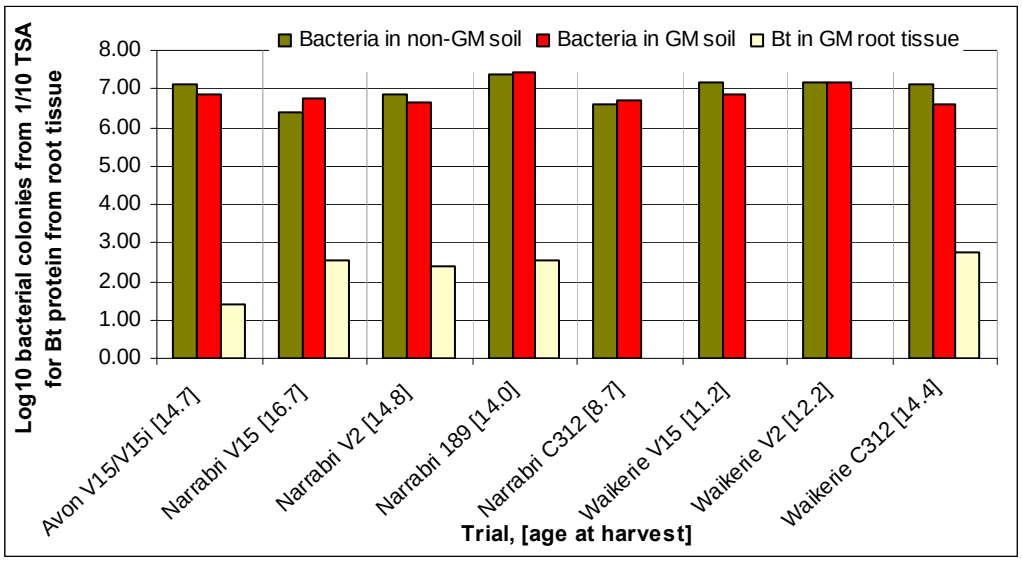


Figure 7.1.3.4.1 Comparison of *Bt* protein produced from the root tissue compared with the bacterial colony counts from $^{1}/_{10}$ TSA medium, for all plants and soils.

The bacterial colony count did not reflect a change in population number between the non-GM or GM rhizosphere soils in the presence or absence of the *Bt* protein.

7.1.3.5 Potential for soil microbial recovery after chloroform fumigation

The susceptibility of the soil microbiota to membrane lysing by chloroform, and ability to recover, was shown by a colony count six days after inoculation of the fumigated soil into $^{1}/_{10}$ TSA medium. The bacterial population from both the non-GM and GM plants had regrown to numbers of the same log scale (2×10^6) at this time. The non-plant associated soil populations had recovered to 1×10^6 colonies. A preponderance of transparent, spreading bacteria, and discrete white, cream and yellow colonies, similar to those present on the original plates (See p. 116) was also observed. Isolated cream and yellow bacterial colonies grew on the ISP4 plates, ie, bacteria not requiring protein were still able to grow. However, no actinomycetes grew from any of the soil dilutions after fumigation with chloroform. Only non-fluorescent colonies developed on King's B medium. Gram stains indicated that both +ve and –ve gram staining bacteria were present, as well as rods and cocci (data not shown).

7.1.4 DISCUSSION

7.1.4.1 Total number of colony forming units across all soils

7.1.4.1.1 Bacterial counts from the $^{1}/_{10}$-strength TSA general medium

There was no significant difference between the bacterial populations between paired non-GM and GM paired cotton plant strains grown in three different soils. Similarly,

the function of protein utilisation for growth by rhizosphere bacteria was illustrated using TSA medium which contains protein. The number of colony-forming units in the range of 10^6 to 10^9 units per gram of soil showed that protein is readily utilisable by the normal soil microflora. In addition, the difference in rhizosphere microflora was more influenced by the conditions of the individual trial than by the effect of plant modification. Similarity in numbers of rhizosphere bacteria between *Bt* producing plants and their non-genetically modified parental corn plants was shown by Saxena & Stotzky (2001). Cultivable colony forming units ranged from 1×10^6 to 1×10^7 per gram soil, which was in close agreement with the work described with cotton plants in this section.

Stotzky (2000) found that the *Bt*k and *Bt*t toxins, free or bound on clays, had no effect on the growth *in-vitro* of a spectrum of bacteria (both Gram-Positive and Gram-negative), fungi (both yeasts and filamentous forms) and algae, and the free toxins were readily utilized as sole sources of carbon and/or nitrogen. This is consistent with the results presented here, for the comparison of bacterial populations isolated from the Narrabri vertosol, where the *Bt* proteins would have been adsorbed onto clay particles. A similar study by Miller et al. (1989), removed the active, vegetative bacteria from rhizosphere soil populations of maize (a C_4 plant), wheat (a C_3 plant, as is cotton) and grass cultivars, and found that the colony numbers of *Bacillus* cultivated on $^1/_{10}$ TSA medium did not differ between rhizosphere soils, or between rhizosphere soils and root-free soil.

The greatest influence on bacterial colony numbers came from the conditions of the individual trials, where numbers generally had a more similar arithmetic mean between the non-GM and GM bacterial rhizosphere populations than across the separate trials, which showed the influence of both plant strain and soil type. Variability in agricultural soils can be expected because micro-aggregates may harbour different bacteria on the surface, than within the smaller and less aerobic pores (Grundmann & Debouzie, 2000), and the anaerobes are disadvantaged with culture plates incubated aerobically. Bacterial populations dependent on oxygen uptake (respiration) or nitrogen reduction which is an obligatory anaerobic process, will vary in number with the surface area of an aggregate in relation to its volume, or the density of the soil. Anaerobic or facultative anaerobes may therefore be higher in number in the denser Narrabri soil.

Given the inconsistent differences in bacterial colony numbers cultivated on a general medium, it can be concluded that there was no measurable effect on the cultivable bacteria from the *Bt* protein or other root exudates of the genetically modified plant.

These findings are also consistent with the results of Donegan et al. (1995), who compared the bacterial and fungal populations from a fine sandy loam similar to the sampled Avon soil in the presence of the HD73 protein. In that work, the same protein from Ingard cotton as used in this study was added to soil, and was compared with soil alone, soil + parental cotton, and soil + transgenic cotton. It was concluded that transient changes in bacterial species composition around the roots may not have resulted from the production of *Bt* protein, but may have come from an unassociated change in GM plant characteristics. They also found that actinomycetes and *Bacillus* species were the most commonly found bacteria Luria Bertani medium, a general bacterial medium.

The numbers of bacterial colony forming units are also generally in agreement with the findings of Ellingsøe & Johnsen (2002) who investigated the rate of growth of bacterial communities in soil, as a measure of community structure. Numbers of colony forming units of bacteria at the end of the trial ranged from 5.8×10^6 to 4.5×10^7 when grown on a general medium which included inorganic minerals and casamino acids, with Nystatin to inhibit fungi. There was a sharp initial increase of colony forming units after plating in all cases. Regardless of the large variability (Ellingsøe & Johnsen, 2002), all colony counts resulted in a log-scaled range of 1×10^5 to 1×10^6, across the four concentrations of 0.01 g, 0.1 g, 1.0 g and 10.0 g soils. The variability may suggest that within each sample, variance occurred through competition, where colonies growing on enriched media impeded neighbouring cell growth, or possibly resulted in higher colony counts through spore clustering.

Bacteria in the Waikerie soil may have been influenced by the previous crop of peas, as legumes are known to increase soil N, with retention until the next seasonal crop (Rochester, et al. 2001). Moreover, Waikerie soil had the highest amount of N (from the mid-infrared soil analysis (Section 2: page 55)) at the beginning of the trials. This, however, did not differentiate between the numbers of bacteria grown on the different soils, as organic nitrogen was available on the medium.

7.1.4.1.2 Actinomycete growth on ISP4 medium

The actinomycete counts were estimated on ISP4 medium which contained starch and inorganic salts, and the antifungal cycloheximide. Growth therefore occurred without the specific nutrient provided by a living plant root, and this may explain the lack of an obvious rhizosphere effect on colony numbers. Actinomycetes cultured from all three soils showed large variability between replicates, but with a non-significant difference in colony count from the Student's t-test at $p \leq 0.05$, between the non-GM and GM rhizosphere soils. Actinomycetes were the only categorised bacteria which did not grow on the ISP4 or $^{1}/_{10}$-strength TSA media after chloroform fumigation, which may indicate that actinomycetes are more susceptible to membrane-lysis than the *Bacillus*-like bacteria which recovered to approximately the same numbers after 7 days.

Wei-xiang et al. (2004) found that actinomycetes also grown on a similar medium ranged from about $Log_{(10)}{}^{4.1}$ to $^{5.1}$, so the order was similar. It is curious that the actinomycete counts described by Wei-xiang (2004) decreased from $Log_{(10)}{}^{4.7 \text{ to } 4.1}$ within the time period of the seven and thirty-five day counts, and this may reflect different counting criteria. It is also noted that more colonies of actinomycetes occurred in the soil without straw amendment, even though actinomycetes are known to be versatile in the breakdown of organic carbon compounds.

7.1.4.1.3 Fluorescent pseudomonads indicating different siderophores

The results of the soil acid digest analysis (Section 3) showed that Fe was not limiting for either Avon or Narrabri soil, so Fe-chelating bacteria would not have had a competitive advantage for survival. Even so, the growth of Fe-chelating bacteria on the King's B medium in competition with non-chelating bacteria showed that this propensity to chelate Fe was not lost from the population.

The growth of both non-fluorescing and fluorescing pseudomonads on the Fe-deficient King's-B medium showed that there was no consistently significant difference in the siderophores that would indicate a difference in the types of Fe-chelating pseudomonads within the rhizosphere soils of the paired non-GM and GM plants. The one trial which showed the reddish pigment pyorubrin may in fact have been produced by individuals that are also able to produce other pigments. This pigment (as with other melanins) is produced from aromatic amino acids such as tyrosine or

phenylalanine (Todar, 2004), but the reason for its appearance within this one trial and not others, is not known.

Grant & Long, (1981) reported a high proportion of Gram-negative bacteria, particularly *Pseudomonas* species in rhizosphere soil and the highest numbers of pseudomonads were cultured from the soil which had received a recent addition of nutrient solution just prior to harvesting. It may be of benefit to conduct further studies on the influence of rhizosphere microbiota under changing N content, particularly as *Pseudomonas* has been cited as having considerable commercial potential as soil inoculants (Metting, 1993, p. 589).

7.1.4.1.4 Observations from the method of comparison of bacterial populations by plate culture

A strong effect on numbers was found to occur from the addition of fertiliser within approximately 48 hours of plantharvest. While the duration of the influence on the growth of the rhizosphere microbial populations from the temporary increase in nutrients from the fertiliser is not known, the populations of both the non-GM and GM rhizosphere soils had increased similarly. The increase in bacterial numbers caused by the higher level of nitrogen or other substances of the genetically modified plant was therefore secondary to the effect of the fertiliser. The decreasing trend in microbial population which occurred with age of plant at harvest would suggest that as plant growth ceased, a lag would occur in population growth where the numbers may return to that of a bulk soil prior to the influence of the plant root. A similar trend of decreasing bacterial populations in soil without nutrient was shown by McLaren & Skujins (1968) who found that after 10 years the general population of soil microorganisms decreased about 100-fold. The higher bacterial cell numbers that occurred after the recent addition of nutrient solution, compared with soils that did not receive additional nutrient, showed a rapid response driven by the additional nutrient and a potential for rapid recoverability of population numbers depleted by nutrient deficiency.

The lack of consistent differences in the bacterial populations attributable to the exudates of the roots of the GM plants when compared to the effects of the exudates from the non-GM plants can be explained by one or more of the following:

1. there was no difference in the populations of the rhizosphere between the paired plants

2. any increase in bacteria from soil induced by different root exudates was compensated for by increased predation at a higher trophic level
3. differences in populations were not indicated by the method of plating.

Regarding the third point, the method of plating soil microflora onto general media demonstrated that the mixed soil populations of the non-GM and GM rhizosphere soil showed similar colony numbers when grown on the same nutrient source at the first colony count on day 3. With adequate nutrient, the discrete bacterial colonies (non-motile bacteria) were not different in size at this time, nor was there a significant difference in colony numbers. Clearly the bacterial colony numbers reflected the density of the original populations, for which a difference between the rhizosphere soils of the non-GM and GM bacteria was not observed.

Differences in colony phenotype were found within the individual soils, e.g. the subcultured bacterium illustrated in Figure 7.1.0, which was isolated from Narrabri rhizosphere and non-rhizosphere soils, but not from the Avon or Waikerie soils. This strain was isolated from the protein-containing King's-B medium, and was able to chelate Fe, but would not grow on ISP4, where starch was the only source of organic C, and nitrogen was available from the inorganic $(NH4)_2SO_4$. It appears to require one or more of the amino acids supplied by the King's B medium for growth. Rouatt et al. (1960) noted that the percentage incidence of amino acid requiring bacteria was much higher within the rhizosphere soil than in control soil. The rhizosphere is a temporary niche around the living plant root with its supply of multiple amino acids, and appears to support the survival of auxotrophic bacteria that would not be viable in bulk soil.

7.1.4.1.5 Limitations of the study

Classification of the estimated 4.1×10^6 to 4.5×10^7 individual cultivable bacteria per gram of soil by selective chemical agents was impossible within the constraints of this project. In addition to the high numbers of colonies per gram of soil, the multiplicity of bacterial types is 'greater than the diversity of any other group of organisms' (Kennedy, 1999).

The limitations of plate counts for estimating bacterial numbers are well documented, and one of the main criticisms is the development of organisms which grow under specific conditions far in excess of those normally occurring in the soil (Hawker et al, 1960). This artefact was discussed by Bowen & Rovira (1999) who reported that

fluorescent pseudomonads made up less than 10% of cultivated bacteria on non-selective media, after a number of other authors had reported that they were the major bacterial group in the rhizosphere. Because plating selectively enriches a portion of bacteria whose growth is suited by the medium, the microbial flora cultivated is biased, and should perhaps be interpreted as the **potential** activity of the soil microbiota, given these optimised conditions (Alef & Nannipieri, 1995, p. 1).

For practical purposes, the comparison of different bacteria was based here on observable differences in numbers, colony size, colour or colony morphology. The lack of difference between bacterial phenotypes cultured from those rhizosphere soils in the paired trials possibly masked genotype differences which could have become apparent under different growth conditions. Different methods of determining bacterial variability are now available, such as that used by Sessitsch et al. (2002). They used the method of terminal restriction fragment length polymorphism to determine the variability of microbial population structure within different soil particle size fractions, and found a greater bacterial diversity in soils of smaller particles. Either the finer size particles provided a protective habitat for microorganisms through pore size exclusion of predators (protozoa), or possibly higher nutrient availability in smaller size particles (resistance to percolation of nitrates through soil) caused higher bacterial diversity. However, the bacteria cultivated from the Narrabri soils with the finer pore sizes did not appear different in colony form from the Avon or Waikerie soils using the same medium for comparison.

7.1.4.1.6 Diversity of bacteria as a function of unit of soil sample

The variance of the replicates of the bacterial population numbers cultivated from 100µg of 1 in 5 soil:water dilution from 2 g soil was often greater than the arithmetic mean of the colony numbers, so the distribution between replicates occurred in a clustered, rather than a random distribution (Zar, 1984, p. 411). Uneven distribution of bacterial colonies does not mean that the plant type caused an aggregated colony distribution, as other studies, independent of plants that have been genetically modified, also showed this tendency. Ellingsøe & Johnsen (2002) pointed to the uneven distribution of heterotrophic and *Pseudomonas* communities, with standard deviations of one log scale less than the colony counts on soil extract agar. Rovira and Sands (1971) also found that the fluorescent pseudomonads were distributed unevenly

in soil. The variance of colony numbers reported in this section is in agreement with these findings.

The patchiness of distribution suggested that unless an adequate amount of soil sample was analysed, a perceived difference could occur where specific bacteria were observed in the one sample but not in the other. However this difference will not necessarily be resolved by using a more 'adequate' sample size where clusters are averaged over larger volumes. Ellingsøe & Johnsen (2002) reported that there was more bacterial population diversity within 0.01 g soil samples than in the 0.1, 1 and 10 g samples using microbial cultures on soil extract agar and patterns of DNA fragments using Denaturing Gradient Gel Electrophoresis. The apparent increased variability of the population decreased with increasing volume of sample and so the relatively larger number of different types of bacteria was a derivative of the low sample size. This relationship of bacterial variability to sample size may not be linear, but may conform to a diminishing rate of increase of different species.

The rate of increase of a soil community to carrying capacity depends on the nutrients available, and also to some extent on the competition strategies of similar guilds, or predation. A temporary amplification of microbial populations occurs during the presence of a root tip with its associated exudates (Semenov et al. 1998). Whether there is a different response to plant root exudates from non-GM or GM plants, which amplifies fast-growing bacteria, or whether there is a wider change in community microbial structure will be addressed in the section on phospholipid fatty acid analysis which is able to determine a shift in the soil community by a consensual trace.

As the *Bt*-producing cotton plants did not observably alter numbers or general morphology of bacteria, an investigation into the effect of genetically modified cotton plants on soil fungi within the rhizosphere was carried out. It follows in the next section.

Section 7 Microflora:

7.2 Fungi

Fig. 7.2.0 Diversity of fungi cultured from Narrabri rhizosphere soil at 7 days. Circle indicates a brown colony, with morphology unlike those observed in Avon or Waikerie soils.

7.2.1 INTRODUCTION

7.2.1.1 Complexity of soil fungi

Hawksworth (1991) conservatively estimated the actual number of fungal species in the world to be 1.5 million, and based on this estimate, only 1.6% of the fungi in the world have so far been categorised. Thus the biodiversity of fungi ranks second only to the insects within the eukaryotic kingdom (Hawksworth, 1991). Within each gram of a fertile soil it is estimated that there are about 400 metres of fungal mycelium (Hawker et al. 1960, p. 269).

All fungal genera including the zygomycetes, ascomycetes, basidiomycetes and deuteromycetes are represented by groups which proliferate in the soil and can degrade plant litter. The *Deuteromycetes* alone comprise approximately 17,000 species and are enzymatically extremely versatile. Soil fungi produce proteases, amylases, pectinases, cellulases, ligninases and xylanases, as well as chitinases, cutinases, phytases and phosphatases (Kjøller & Struwe 2002, pp. 267-281), so all plant organic matter, including plant-produced proteins (viz. the plant-produced *Bt* protein) can be degraded when these commonly occurring soil flora are present.

7.2.1.2 Fungal distribution in the cotton growing areas of Australia

Fungal diseases, particularly *Pythium* and *Fusarium*, have a major impact on cotton seedling mortality (Cotton Seed Distributors Ltd. 'Variety Trial Results' (2004, p. 76-77)). Within the cotton-growing areas of the Gwydir, McIntyre and Darling Downs regions, the pathogenic *Fusarium* spp. was recorded on soil of 20%, 36% and 75% of the farms surveyed for the 2003-2004 season, and the incidence is spreading.

Millions of fungal pathogen spores are carried in irrigation water. The cotton growing areas are supplied by different tributaries of the Murray-Darling river system, so the spread appears not to be from an upstream water-source, but from localised (district) infections.

As with bacteria, the growth of fungi depends on soil management, plant susceptibility and natural competition from other microbiota such as fungal-feeding nematodes. Soil management is implicated particularly with Verticillium-wilt, where disease severity reflects the prior cropping history (Cotton Seed Distributors, 2004). Overall field fertility is important in creating healthy, vigorous plants better able to fight off infections. Availability of nitrogen may also impact on the growth of fungi. Different soil microflora require different amounts of nitrogen, and can occur in proportion to the C:N ratio. Bacteria have a fast growth cycle, or rapid opportunistic growth with a low C:N ratio, and fungi tend to follow a slow cycle with a high C:N ratio (Ferris & Matute, 2003).

The analysis of leaf tissue (Section 4) showed that the C:N ratio was lower in the genetically modified cotton plants than the parental strains. As the *Bt* protein is expressed systemically, it is possible that different soil microbial populations may occur within rhizosphere soil adjacent to the plant roots through specific changes in the quantity and variety of root compounds from plants which have a higher N component.

It is not clear how much selective pressure occurs between the soil microflora under potentially different rhizodeposition and N component of root tissue. Here, the abundance of fungal colonies within the rhizospheres of paired plant trials (non-GM/GM) within the three sampled soils was compared, to determine whether the number of colony forming units was different. A comparison was also made of fungal morphology. This was categorised into six groups within the three soils, to determine whether consistent or significant differences in phenotype existed between the soils.

7.2.2 MATERIALS AND METHODS

The same dilution plate method was used for fungal growth as for bacteria, (described in Section 7.1). Czapek Dox agar was used to score and compare total

colony forming units from freshly harvested non-GM and GM rhizosphere soils from paired trials. Streptomycin at the concentration of 30 μg ml^{-1}, was added to inhibit bacteria (Alef & Nannipieri, 1995, p. 145).

Four replicate rhizosphere soil samples from the non-GM and four from the paired GM plant strains were serially diluted to 10^{-3} and 10^{-5}. Duplicates of each of these samples were spread-plated at the same time, using the same diluents as for the bacterial plates (Section 7.1). A more representative mix of microflora could then be observed as it was taken from the same 2 g of soil. The 100μL aliquots were taken from the middle of the shaken soil suspensions after enough soil had settled to avoid clogging the pipette. The fungal plates were not inverted, and were incubated at 25ºC in the dark.

7.2.2.1 Counts of fungal colonies

Counts of colonies were undertaken on the third and seventh day from Czapek Dox medium and an averaged count was taken of each of two duplicate dilution plates, and for each of the replicates from different plant pots kept under the same conditions for each trial.

7.2.2.2 Identification of fungi

Taxonomic inconsistency arises when fungi appear different in form. An example of this is shown on the plates in Figure 7.2.0. The commonly occurring blue-green spored fungus at 270º on the right plate is known as *Penicillium* when its growth occurs during the non-sexual stage, and as *Talaromyces* when it appears as the adjacent yellow fungal colony (300º on the plate) in the sexual stage. Even though both fungi appear to have predominantly the same spore structure under the light microscope, they are categorised into a different genus according to the presence of sexual spores.

It was too time-consuming to identify the fungi taxonomically. The isolates were therefore categorised into six major groups, five of which are illustrated on the two plates at the head of page 128. Colonies were checked under a light microscope for identification of septate/ non septate mycelium, spore size, type and presence of other identifying structures to most closely fit the categories. The categorised description follows as Table 7.2.2.2.1. This table has been constructed from the

mapped fungal plates that illustrated the most frequently cultivated fungi from the sampled soils, shown in Figure 7.2.2.2.1.

Table 7.2.2.2.1 Basis of grouped fungal morphology from fungi grown on Czapek Dox agar

Cate-gorisation	***Description***	***Example on plate***
Pythium-like	Profuse, long-stranded aerial hyphae, very fast growing that can fill the air space in a petri-dish to the lid within 3 days. Amorphous growth pattern.	Not shown
White, *Fusarium*-like	Dense white radial growth. Many different types, but the example illustrated had secondary branching at a perpendicular angle to the primary hyphae.	W
***Penicillium*-like**	Blue-green or green spores on white hyphae at maturity. Spore structure 'brush-like' under the light microscope. This is the predominant fungus on the right plate. Occasional red margin at the zone of contact with other fungi or bacteria, often with a yellow exudate.	P
Putative *Aspergillus niger*	Black heads of spores, with the spores arranged in chains external to the stipe.	A
Yellow	Compact and discrete. Showed a *Penicillium*-like spore structure under the light microscope. Probably *Talaromyces*.	Y
Brown, smooth colony	Multinucleate, wavy hyphae along upper surface of medium. Smooth, non-filamentous aerial structure. Example within the ring superimposed on the right-hand plate. Zygospores observed, therefore possibly of the genus *Mucorales*.	B
Orange	White hyphae with orange spores on the example plates.	O

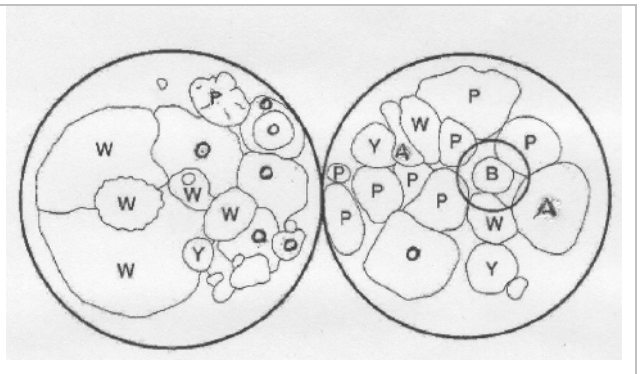


Figure 7.2.2.2.1 Map of fungal plates shown at the Section title, illustrating morphology used for grouping.

The soils and plant strains used to inoculate the plates and their duplicates, at the two concentrations for each of the treatments listed, are tabled below.

Table 7.2.2.2.2 Soil and plant treatments compared

Avon soil Total plates = 40	Narrabri soil Total plates = 164	Waikerie soil Total plates = 128
V15, V15i, soil-only	V15, V15i, soil-only V2, V2i, soil only 189, 289i, soil-only C312, C312i, soil only	V15, V15i, soil-only V2, V2i, soil-only C312, C312i

7.2.2.3 Statistical analysis

Counts of colony forming units were $\log_{10}+1$ transformed, to allow analysis by ANOVA. The guideline for homogeneity between sets to be compared was accepted if the standard deviation for one set did not exceed twice the value of the standard deviation of the other set being compared.

Warcup (1960) noted that a serious difficulty arises when 'counting' soil fungi because heavily sporulating species such as *Mucor, Aspergillus, Penicillium, Trichoderma* etc, isolated by the dilution-plate method, have a substantial competitive advantage in colonising available surface area through spore load, and statistics based on dominance may render the presence of sub-dominant species inconsequential. Accordingly, a binary diversity index was made by scoring each type of fungus as present if it appeared on a plate from either of the duplicates, for each different replicate pot, for one of the dilutions only.

7.2.3 RESULTS

7.2.3.1 Soil effect on fungal colony numbers

The comparison of the numbers of fungal colonies between the rhizosphere soils of non-GM and GM plants, compared by one cultivar across three soils, and for all cultivars within the Narrabri soil, is shown in Figure 7.2.3.1.1.

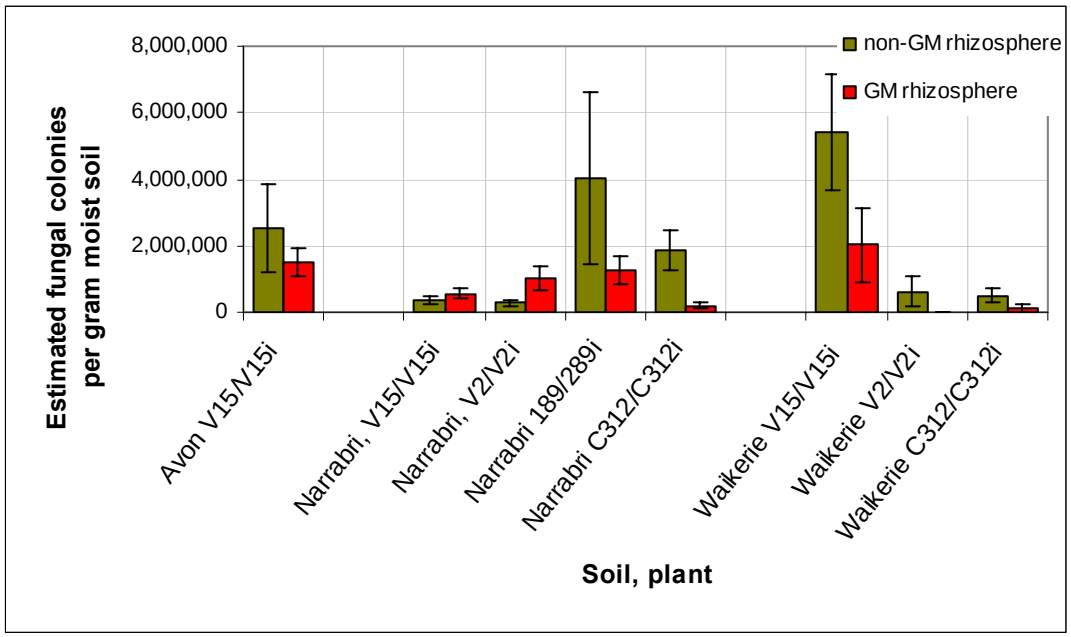


Figure 7.2.3.1.1 Fungal colony unit numbers for plant and soil type.

There were more fungal colonies overall, for the non-GM plants, but this trend was not consistent for plant type (V15/V15i) for each of the three soils, nor for the different plant strains within the same Narrabri soil. This lack of consistently observable difference indicates that the number of fungal colonies were not significantly affected by any difference in the micro-environmental conditions of the rhizosphere soils of the paired trials.

Many plates had relatively few colonies, but high colony counts occasionally occurred, including the non-rhizosphere samples. This resulted in high variability over the averaged scores, and can be particularly seen from the log-scale comparison, produced by SPSS® software (Figure 7.2.3.1.2) in the Waikerie V15/V15i trial. . The arithmetic mean is depicted as a bar within the boxplot of the quartiles, with the points of the highest and lowest outliers marked as extended bars.

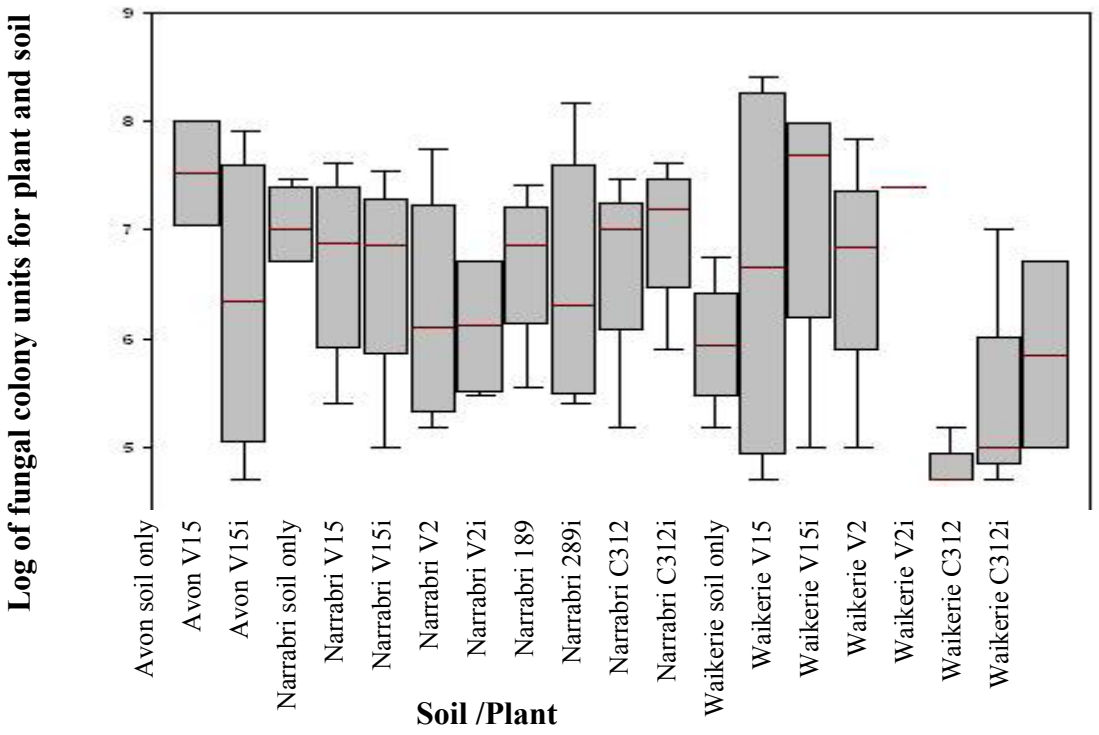


Figure 7.2.3.1.2 Boxplot of quartiles of log-transformed fungal colony units for plant and soil type.

According to the number of fungal colonies grown on Czapek Dox medium, a rhizosphere effect of a higher number of fungal colony forming units was not as evident as with bacteria on $^{1}/_{10}$-strength TSA medium. This may be because saprophytic fungi grow on dead plant tissue and do not need a living plant root. The ANOVA analysis showed that on the log-transformed data, all groups fell into one homogenous subset, that is, no groups were significantly different at a level of $p \leq 0.05$. Much of this lack of definition was due to variance between the samples taken from different replicate pots.

Excluding the soil-only samples, comparisons between the rhizosphere soils of non-GM and GM matched plants were made separately for each of the trials. As there were eight replicates in each case, independent t-tests were performed on non-transformed colony counts, assuming unequal variance. The number shown in Table 7.2.3.1.1 shows the probability of similarity between number of fungal colonies within the rhizosphere soils of the paired plants.

Table 7.2.3.1.1 Summary of independent t-tests comparing the number of fungal colony counts from the rhizosphere soil of non-GM and GM plant strains, for all sites. Nd = trial was not done for these plant strains. Significant differences are assumed at $p \leq 0.05$.

	Avon	Narrabri	Waikerie
V15/V15i	0.64 n = 8	0.25 n = 8	0.29 n = 8
V2/V2i	Nd	0.65 n = 8	0.31 n = 8
189/289i	Nd	0.38 n = 8	Nd
C312/C312i	Nd	0.12 n = 8	0.30 n = 8

The fungal colony counts for the V15 and V15i plant types grown in Avon and Narrabri were significantly lower than the numbers for the same plants in Waikerie soil, ie the fungal counts did not result from the influence of the plant strain alone. The lowest fungal counts were from other trials with Waikerie soil, ie, the colony numbers did not show consistency for plant type, or soil. They were more closely aligned by arithmetic mean for individual trials.

Of the eight trials averaged for four replicates, six had a higher fungal colony count for soil samples of the non-GM plants, and two had a higher count for soil with GM plants. When counts from soils with the same plant type V15 and V15i were compared across the three soils, there were higher counts with non-GM plants in Avon and Waikerie soils, but a higher count for the GM plant in the Narrabri soil. There was a non-significant negative correlation for number of fungal colonies with the age of harvest.

7.2.3.2 Effect of trial on colony phenotype

The dilution of 10^{-5} g^{-1} soil was too high as fungal colonies only grew on 37% of the Czapek Dox plates. The records of particular fungal colony phenotypes were therefore taken from the 10^{-3} dilution plates only.

The most dominant fungal phenotype across all soils was the *Fusarium*-like white form, which occurred in the rhizosphere soil for all plants on at least one of the soils. The next two most common fungal forms were the *Aspergillus*-like and *Penicillium* types, again occurring in all soils. The highest diversity of fungal phenotypes over all replicates for the rhizosphere and non-rhizosphere soils together occurred for the V2 and V2i plants grown in Narrabri soil, with all 6 phenotypes. The diversity of

fungal phenotypes within Waikerie soil was similar to the Avon soil, where four types were present. The yellow and orange phenotypes were found only in the Narrabri soil.

Comparing the fungal groups for the V15 and V15i paired plants across all three soils, the *Aspergillus* form was absent in cultures from V15i rhizosphere soil from both Narrabri and Waikerie, but was present in the corresponding Avon V15i soil. The *Penicillium* phenotype was present in the Narrabri V15 rhizosphere soil, V15i Waikerie soil, and in both V15 and V15i Avon plant rhizosphere soils. The dominant white *Fusarium*-like fungal strain occurred in both the non-GM and GM plant rhizosphere soils, from all three soils.

The frequency of different groups of fungi grown from each of the soils is summarised in Table 7.2.3.2.1.

Table 7.2.3.2.1 Incidence of fungal colony morphology from separate Czapek Dox replicate plates at 10^{-3} dilution n = 4 for the rhizosphere soils and n = 2 for the non-plant root associated soils

	Pythium-like	*Fusarium*-like White	*Penicillium*	*Aspergillus*	Yellow	Orange
Avon						
V15	1	2	3	0	0	0
V15i	1	2	1	1	0	0
soil only	1	0	1	0	0	0
Sum	***3***	***4***	***5***	***1***	***0***	***0***
Narrabri						
V15	0	2	2	2	0	0
V15i	0	3	0	0	0	1
soil only	1	1	1	1	0	0
V2	3	1	0	5	1	2
V2i	2	0	2	5	4	0
soil only	1	1	1	0	0	0
189	2	1	0	3	0	0
289i	0	1	1	1	0	0
soil only	0	0	0	1	0	0
C312	1	0	1	0	3	2
C312i	0	0	0	3	1	1
soil only	0	0	1	0	1	0
Sum	***10***	***10***	***9***	***21***	***10***	***6***

Waikerie						
V15	1	4	0	1	0	0
V15i	1	2	4	0	0	0
soil only	0	1	1	0	0	0
V2	0	1	0	0	0	0
V2i	0	0	0	0	0	0
soil only	0	1	0	0	0	0
C312	0	4	0	0	0	0
C312i	0	3	0	0	0	0
soil only	0	1	1	1	0	0
Sum	***2***	***17***	***6***	***2***	***0***	***0***
Dominance Ranking	**4**	**1**	**3**	**2**	**5**	**6**

Binary logistic analysis of dependence of fungal colony morphology showed that there is no higher likelihood of one of the particular fungal categories occurring within the non-GM than the GM plant rhizosphere soils, within the paired trials, grown under similar conditions.

7.2.4 DISCUSSION

Fungi need less N to degrade a unit of C than bacteria (Metting, 1993, p. 532). As GM plants contain more N in the tissues than non-GM plants (Section 5; Chen et al. 2004) their rhizosphere soils could promote higher populations of bacteria if the root exudates also contained more nitrogenous material. The results from plate cultures described here suggest no major effect on colonisation, or promotion of given phenotype with GM cotton plants grown under the conditions of the pot trials, when compared with their non-GM counterparts. Also, there was no difference (Section 6) in the rate of litter degradation, suggesting no difference in the saprophytic function of fungi in utilising *Bt*-containing litter.

The lack of difference in the microbial flora of non-GM and GM rhizosphere soils according to culture-based methods is in agreement with the work of Saxena & Stotzky (2001) and Koskella & Stotzky (2002) who used soils from America and genetically modified corn plants. Koskella & Stotzky (2002) found no effect of GM plants on nematodes, protozoa, bacteria or fungi. They concluded that the presence of the *Bt* toxin did not cause a shift in the microbial populations.

7.2.4.1 Observation on the method of colony estimation

All of the fungi grown on Czapek Dox had spore structures. As the soils had less than 3% moisture during storage, the dry conditions may have promoted spore-formation.

The effect of bacterial competition on fungal growth was seen from the $^{1}/_{10}$-strength TSA medium without antibiotics. Overgrowth by bacteria was also an occasional problem on the Czapek Dox medium, where the concentration of streptomycin, of 30 $\mu g\ ml^{-1}$ as recommended by Alef & Nannipieri, (1995, p. 145), was inadequate. In retrospect, as Czapek Dox medium contains 30g sugar l^{-1} this medium which selects for fast-growing sugar fungi may have inhibited other soil fungi with different nutrient requirements. Further studies may suggest the use of media containing the tissues of the specific plant being researched.

The culture plates (in Figure 7.2.0), showed many different phenotypes of fungi growing adjacent to each other, but competition between the cultured fungi did not result in antagonism, as there were no zones of clearance. This suggests that growth would only be limited by nutrient availability, rather than antibiotic inhibition. The *Penicillium* and *Fusarium*-like white forms were also the two most prolific fungi grown on STSM medium, purportedly specific for species of *Trichoderma* in the work of Dyer (1998).

7.2.4.2 Differences in fungal populations from sampled soils

Even though similar phenotypes were cultured from soils of both the non-GM and GM plants, a soil effect was apparent. All fungal phenotypes were cultured from the Narrabri soils, but only four from Avon and Waikerie soils. In addition, an unusual brown colony (emphasised by the red circle in the illustration at the head of the section) was cultured from the Narrabri soils but not from the Avon or Waikerie soils. This indicates a different population mix in the different soils. Increased diversity of microbiota in soils of small particle size fractions was found by Sessitsch et al. (2002). Particle density affected fungal diversity even more than the type of fertiliser applied.

The boxplot in Figure 7.2.3.1.1 shows that the largest variabilities did not always necessarily align with the highest arithmetic means. The V2 and V2i plant trials

from the Narrabri soil had the highest diversity of colony types in samples, but did not match with the highest arithmetic mean of colony numbers.

Comparison of cultures from the V15 and V15i plants, across the three different soils, showed that the *Pythium*-like fungus was present in non-plant associated soils, but not in rhizosphere soil in the Narrabri trials. It was present in each of the Avon non-plant associated soils as well as both rhizosphere soils, and within Waikerie soils the fungus was present in both rhizosphere soils, but not in the non-plant associated soils. It therefore did not occur consistently within rhizosphere soil, control soil, or with plant cultivar.

7.2.4.3 Redundancy of function between bacteria and fungi

The two most commonly occurring fungi, *Penicillium* and the Fusarium-like white form, both saprophytes and able to degrade proteins, were well represented in all soils sampled. Both bacteria and fungi isolated from the three sampled agricultural soils could grow on the protein-containing TSA medium. Regardless of the rate of growth, the boundaries between different microbial populations which utilise similar substrates were not sharply defined. Root (1967) originally coined the word 'guild' to describe a group of species that exploit the same class of environmental resources in a similar way. The *Bacillus thuringiensis* protein, if present in soil, and exposed to either bacteria or fungi, was apparently rapidly degraded by the indigenous populations from any of the three sampled soils (shown by the growth of bacteria and fungi on protein-containing media). Donegan et al. (1995) independently concluded that there were no significant environmental effects from three lines of GM-cotton (two of which contained the same Cry1A(c) gene insert as were tested in these trials), on total numbers of fungi. Saxena & Stotzky (2001) also found that there was no effect on the rhizosphere microbiota from the presence of the Cry1A(b) protein within the rhizosphere soils of corn, grown in similar soil types in the United States of America.

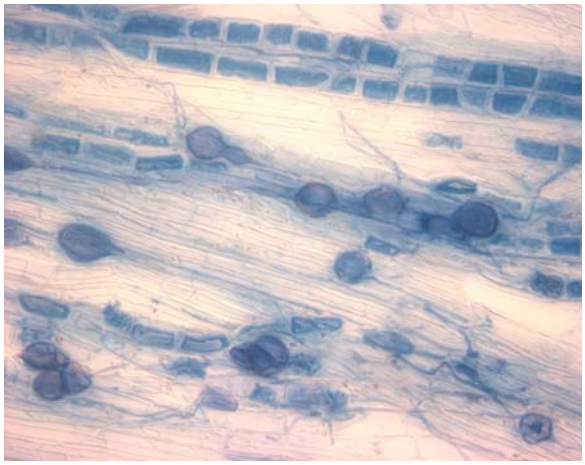
7.2.4.4 Effectiveness of the dilution method for harvesting fungal propagules

Dilutions were shaken by hand immediately before pipetting (Montégut, 1960) and the diluted soil sample taken from the middle of the shaken suspension. The fine clay particles in Narrabri soil, with its higher overall negative ionic charge, would have attracted the fungal spores and other organic matter, and may have contributed

to the more diverse harvest of fungal types from these samples. This selectivity of variable spore or hyphal load by sampling method was described by Warcup (1955), who noted more fungal species diversity in cultures from the residue of the dilution than from the suspension.

In future research it would be useful to include a surfactant such as Calgon to emulsify and disperse the soil solution before plating. This should increase the harvest of hyphal segments as some important pathogenic fungi (such as *Gaeumannomyces graminis* var. tritici) survive in soil and dead organic matter exclusively as hyphae (Cunningham 1981). The method of adding detergents has been used to select for A-M fungal spores in soils (Pattinson & McGee, 1997) but the effectiveness of the use of a surfactant to increase other soil fungi by differentiating spores and hyphae is not currently known.

An investigation of the Arbuscular-Mycohrrizal symbiotic fungi which grows internal to the plant root follows in Section 8.



Section 8

Arbuscular-mycorrhizal fungi

Fig. 8.0 Arum-type A-M fungal colonisation from a section of stained cotton root, showing longitudinal contiguous arbuscles, with hyphae and vesicles.

8.1 INTRODUCTION

The term Arbuscular-mycorrhizal (A-M) fungi describes a group of root colonising fungi which benefit both plant host and the fungus. These glomeromycotinous fungi cannot grow without a plant host, and to date have not been cultured in the absence of the host (Jarstfer & Sylvia, 1993, p. 349). Olsson (1999) considered that the A-M fungi were a separate functional group of microflora as they obtain carbon energy from their associated plants and do not compete with soil saprophytes for organic carbon.

The main benefit to the plant from the A-M fungi is increased uptake of P, Cu and Zn (Menge et al. 1977). The fungi extend from the root and allow exploitation for elements from a greater area of soil. They can translocate P from as far as 8 cm away from the root (Rhodes & Gerdemann, 1975). A-M fungi have also been documented to elevate the levels of P from soil where other nutrients are limiting (Jarstfer & Sylvia, 1993, p. 350). Because of this concentrating effect it has been argued that the elevation of P should be interpreted as a false positive effect of the A-M root association (Jarrell & Beverly, 1985).

A-M fungi are known to provide a partially protective effect against plant-pathogens such as the root-attacking nematodes for a broad range of host plants (Sikora 1992; Pinochet et al. 1996; Lopez et al.1997). The interaction between A-M fungi and nematode attack is of current importance because a variant of the *Bt* protein with nematicidal properties has been patented (Patents WO9319604-A1; EP633725-A1; US5378460-A).

Recent works by Gao et al. (2002) and Wegel et al. (1998) using plants with defective mycorrhizal colonisation suggest that the restricted colonisation patterns of A-M fungi in the mutant plants may have resulted from disrupted gene function which failed to produce a chemical signal required by the fungi to establish normal colonisation. In these cases A-M fungal colonisation was limited to the epidermal cells or minimally extended into the cortical cells, similar to the nodule-fixing myc$^-$ plants in rhizobia.

The recognition of a symbiotic partner is one of the first steps required for the initiation of colonisation by A-M fungi. In legumes, this partnership is established by plant lectins, the carbohydrate-binding proteins which are recognised by bacterial receptor molecules (McCardle et al 1993, p. 160). Using current methods, positioning of the inserted *Bt* gene within the cotton plant genome is largely random. It is not known whether the genes determining the facilitation of the recognition of the symbiont are altered, or whether the functioning of other genes involved in the establishment of A-M fungal colonisation is disrupted by the inserted genes in the GM-cotton plants. If this happens, the rate of A-M fungal colonisation could alter through reduced receptor specificity. A-M fungal growth along the GM-plant root may be less than the non-GM counterpart if colonisation within the root is affected.

Moreover, the rate of A-M fungi colonisation was affected by the host tissue P concentration, root exudates and CO_2 (Becard & Piche, 1989). If any of these factors were altered by the modified plant, then the A-M fungal assisted uptake of K, Ca, Cu, Zn and Fe (Marschner, 1997) could be affected.

The work described in this section compared the rate of colonisation and the appearance of the A-M fungal structures between paired non-GM and GM plant cultivars, in three different sampled soils, to determine if the signalling between the host and symbiont was disrupted as a possible secondary effect of the genetic modification of the GM plant.

8.2 MATERIALS AND METHODS

Negative controls to confirm that no A-M fungal spores were present on the seed at the time of planting were established by growing V15 and V15i plants in Waikerie triple-washed sand, with Hoaglands nutrient solution added. Surface-sterilised seeds were also germinated in agar to which 50% diluted Hoaglands solution was added.

All four paired cotton plant strains V15, V15i; V2, V2i; 189, 289i; C312 and C312i were cultivated as described in Section 3. At harvest the soil was washed from the roots which were then stored in ethanol until processing by a method modified from Phillips & Hayman 1970 (described in Appendix 8). Briefly, the freshly harvested roots were stored in ethanol and rinsed with distilled water and transferred to a 10% KOH solution for 5 days to clear pigmented tissue. The solution was then neutralised using 0.1N HCl. Following this the roots were stained in Trypan blue (0.65 g in 400 ml dH_2O; 300 ml glycerol and 325 ml lactic acid) for 30 minutes, rinsed, blotted dry and then stored in 50% glycerol until observation under a compound microscope (100x). Subsamples of fresh root were excised for each plant type, to determine the presence of Bt protein in GM varieties using ELISA (Section 5).

Trypan blue stain is not specific for A-M fungi, but for all fungi containing chitin, so either of the other two structures, vesicles or arbuscles, were used preferentially for scoring. The normal pattern of A-M fungi growth was to infiltrate the epidermis as well as the cortex, but not the stele, as occurs with the uncontrolled growth of some pathogenic fungi including *Fusarium* and *Verticillium*. The test for A-M fungi is destructive, and the same roots are not used for the later colonisation count.

Five microscope fields scan 1cm of root tissue, so it is possible to calculate the distribution of colonisation in very young roots before colonies merge, to determine the degree of clustering of the separate colonies. Accordingly the semi-subjective method of Allen & Allen (1980) was followed, which allows observation of A-M fungal colonisation along contiguous root lengths of 1cm. When *n* is greater than 30, the distribution approximates the standard normal curve (Larsen, 1975) and so six randomly selected root strands of at least 1 cm long were placed on a microscope slide between 1 cm markings and the root tissue was squashed under a cover slip. The slide was then observed under a light microscope by making five passes across the six root segments for a total of 30 microscope-field observations. This procedure was repeated for the roots grown within each of the replicate pots. The extent of colonisation was ranked from zero where no hyphae, arbuscles or vesicles occurred across the root segment, to a maximum of five observations, where all of the 2 mm root segments showed staining of hyphae or other A-M structures. This method satisfied the

statistical requirement of 30 observations for each replicate, where a normal approximation may be assumed (Zar, 1984, pp. 386-387).

Analysis of colonisation based on the variance being greater, equal to, or less than the population mean (Zar, 1984, p. 411) was used to determine whether the colonisation pattern was random, even, or clustered. Older (thicker) and younger roots were not differentiated in the scoring, nor was a distinction made between dead or live fungal structures, both of which absorb the stain.

8.3 RESULTS

Figures 8.3.1.1 and 8.3.1.2 show the rate of colonisation for all plants grown in the Avon and Narrabri soils respectively, for the plant age at harvest. The negative controls for the fungal stain resolved that there was no fungal colonisation in the roots of V15 and V15i plants grown in either washed sand or in Hoaglands medium. The ELISA test showed that all GM-plant roots expressed the Cry1A(c) protein, and the non-GM plants did not.

Arum-type colonisation occurred for all of the plants, from each of the three soils, with arbuscles in cortical cells and contiguous longitudinal colonisation. Vesicles were always present on at least one section of the root from the earliest harvest at day 10.

8.3.1 Time series analysis of colonisation

Arbuscles and hyphae were observed within the roots of both non-GM and GM cotton plants grown in both Avon and Narrabri soils at the first harvest (10 days after sowing). Arbuscles were observed in one or two layers of cells, with discrete fungal strands between the points of entry. Vesicles generally only occurred after the establishment of hyphae and arbuscles, but small clusters were observed occasionally in roots from all soils at the 10 day harvest. Varying thickness of fungal hyphae occurred in roots of plants grown in Avon soil, suggesting a simultaneous colonisation of different fungal strains, but the colonisation rate was not affected by the different thickness of the hyphae.

There was not enough root tissue available for 30 separate root segments for each replicate pot from the Narrabri harvest at less than four weeks so the averages of 30 pooled root strands from the replicate pots are shown as single data points in the early harvests.

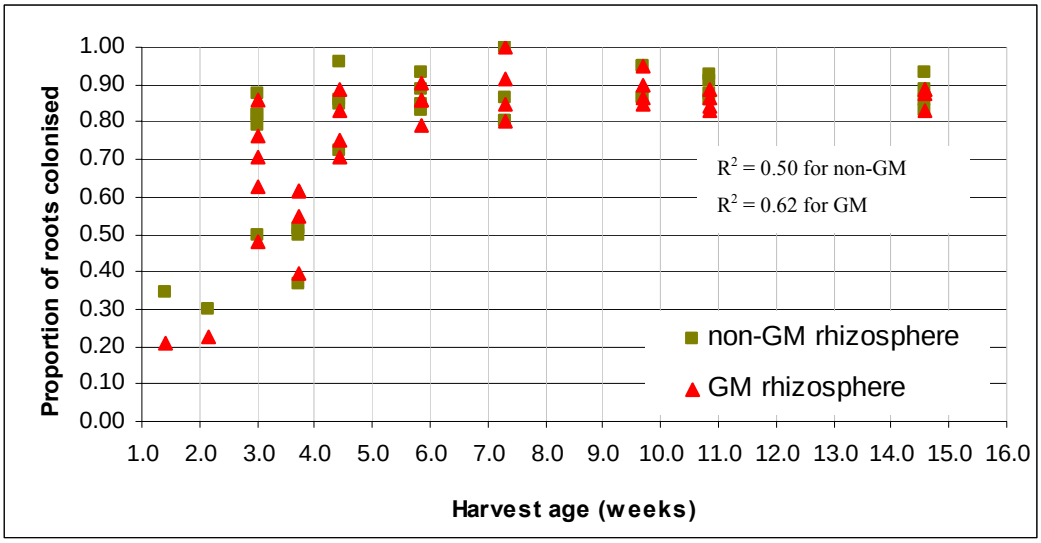


Figure 8.3.1.1 A-M fungal colonisation for non-GM and GM plants grown in Avon soil.

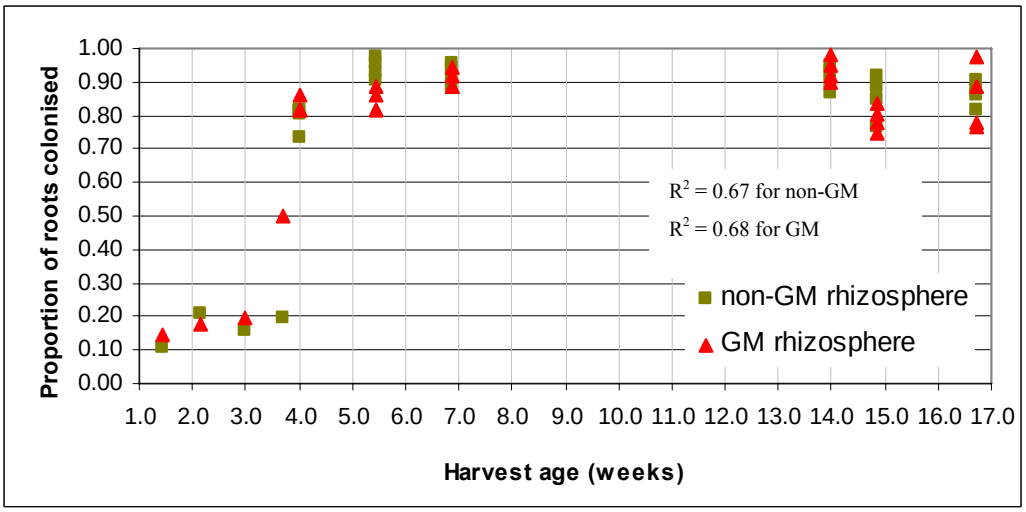


Figure 8.3.1.2 A-M fungal colonisation for non-GM and GM plants grown in Narrabri soil.

The colonisation curve is not shown for the Waikerie soil as observations of A-M fungal infections were not undertaken before 11 weeks. At this time colonisation in all V15/V15i, V2/V2i, 189/289i and C312/C312i plants ranged from 93% to 100%.

The early colonisation rate was lower for both the non-GM and GM plants in Narrabri than in Avon soil, until beyond the 26 day harvest where the highest colonisation rate was 50% for Narrabri and 60% for Avon soil. This trend of higher colonisation within the Avon soil continued until after the 4 week harvest, when the highest of the ranges was equivalent for both soils.

In Avon soil there was a higher colonisation rate of 4% for the V15 plants than the V15i plants at the 1.4 week harvest, and a higher rate of 3% for the non-GM plants for

the 2.1 week harvests. At the 3 week harvest the colonisation rate was the same for the V15 and V15i plants for both the Avon and Narrabri soils. The 3.7 week harvest from Narrabri soil showed a large difference in colonisation rates between the non-GM plants at 19% for non-GM, and 50% for GM plants. Within this trial germination was low for the V15i plants grown in Narrabri soil, and root tissue of these young plants was limited. Averages of the pooled roots from several pots were used but in order to obtain the 30 counts of 1 cm root tissue (the standardised method) independent data as true replicates (from different pots) could not be assumed. The V15i colonisation could therefore represent a high data point in a range which was similar to the Avon trial.

Nutrient solution including 1.5M K_2HPO_4 and 2.5M NH_4PO_4 was added at the rate of 10ml per week after the fourth week, as colonisation in all plants had already been established. Inconsistent dominance for either the non-GM or GM plants had shown that the rate of colonisation could not be seen as a function of the GM-status of the plant. This can be seen from the overlapping ranges of data points for the non-GM (green) and GM (red) symbols in Figures 8.3.1.l and 8.3.1.2. A non-significant result can also be seen by the R^2 values for both non-GM and GM colonisation rate, calculated as a 2nd degree polynomial.

After the four week harvest the arbuscles and fungal hyphae were continuing to spread longitudinally along the root cortical cells and the clusters of colonised cells were beginning to merge. Every cotton plant in every trial, regardless of strain or soil type, contained A-M fungi. The highest rate of increase in colonisation occurred between the 3 and 5 week harvests for both Avon and Narrabri soils, for all plant types. By 6 weeks, plants grown in the Avon soil, and by 7 weeks plants grown in Narrabri soil, showed A-M structures for nearly every contiguous centimetre of plant root.

At the four week harvest for the C312/C312i plant pairs, a higher rate of colonisation occurred in some of the finer roots than from some of the thicker roots, which showed that the incidence of infection was not proportional to root size. A contributing cause may have been the incidence of secondary infection from the hyphae of adjacent roots at the same depth in the pot.

By 6.9 weeks the plants grown in Avon soil had an average colonisation of 88% ± 2% for non-GM plants and 85% ± 2% for GM plants. Arbuscles were present 5 or more

rows deep, with 25 or more colonised cells along the length of the root, for both the non-GM and GM plants. The average colonisation of roots in Narrabri soil at 6.9 weeks was 93% ± 2% for non-GM plants and 91% ± 1% for GM plants. 100% colonisation did not occur in all trials as there were a few root strands close to the crown of the root structure in the older, woody tissue, just below the surface of the soil, where colonisation was not observed.

8.3.2 Spatial distribution of colonies within the Avon and Narrabri soils

Up to the 2.1 week harvest, the maximum count per microscope slide only occurred on two occasions for the V15 and V15i plants, and the rate of colonisation ranged from 37% to 51%. These individual clusters were still largely separate at this point. Clustering analysis was therefore applied to the data from Avon and Narrabri soils before adjacent colonies merged.

The incidence of the structures along the 1 cm lengths of roots showed that for both the non-GM and GM plants grown within Avon soil at the 1.4 and 2.1 week harvests, the variance exceeded the sample mean. Thus, the pattern of A-M colonisation occurred in a slightly clustered, rather than an even distribution (Zar, 1984, p. 411). At 1.5 weeks, the plants grown in Narrabri soil had a more random distribution for both non-GM and GM plants but by 2.1 weeks, the distribution was more clustered. A summary of the distribution of the A-M fungal colonies is shown in Table 8.3.2.1.

Table 8.3.2.1 Variation of A-M fungal colonisation rate for plant strains V15/V15i

Soil	Harvest age (weeks)	Mean of colonisation where max. = 5 A-M fungal structures per 1cm root	Variance	Number scored (*n*)	Distribution
Avon, V15	1.4	1.73	1.79	30	Random
Avon, V15i	1.4	1.03	1.41	30	Random
Avon, V15	2.1	1.50	2.67	30	Random
Avon, V15i	2.1	1.13	1.43	30	Random
Narrabri V15	1.4	0.53	0.53	30	Random
Narrabri V15i	1.4	0.92	0.69	30	Random
Narrabri V15	2.1	1.00	1.27	30	Clustered
Narrabri V15i	2.1	0.90	1.27	30	Clustered

The averaged rate of colonisation for the V15i plant grown from the Narrabri soil in the 15 week sample was less than that of the V15i plant grown in the same soil at 10 weeks. This effect was also seen in the work of Dickson (2004).

8.4 DISCUSSION

The results showed that the rate of colonisation and the appearance of the A-M fungal structures between paired non-GM and GM plant cultivars, in three different sampled soils, were equivalent. None of the plants had a restricted colonisation pattern, which could indicate no disruptions of gene function related to plant-assisted colony establishment. Other work described in the Literature Review found no observable effect of genetic modification on the architecture of the roots, and confirmed that morphology such as length of lateral roots was not affected by the presence of different colonising strains of A-M fungi.

8.4.1 Comparative rate of colonisation

Structures of A-M fungi were observed in root segments from the first harvest at 10 days after sowing, for both the V15 and V15i plants, in both Avon and Narrabri sampled soils. Over time, neither the non-GM nor the GM plants consistently had a higher rate of colonisation and no differences in the structural forms of vesicles, arbuscle and hyphae were observed between any of the paired plant strains. The early colonisation of cotton plants seen here concurs with Pattinson & McGee (1997) who recorded initiation of A-M fungal colonisation within cotton roots at 5 days within Narrabri soil, and with Brundett et al. (1985) who recorded colonisation of susceptible plant roots by A-M hyphae in two days.

Occasionally, thin fungal hyphae were observed without the definitive arbuscles or vesicles of A-M fungi in young roots. In this case the hyphae were not scored as A-M fungi unless other A-M fungal structures such as arbuscles or vesicles were present. This conservative scoring may have led to a slight underestimation of colony numbers. There is no doubt, however, that there was no difference in the later, high level of colonisation of both non-GM and GM cultivars.

Variability in the rate of early colonisation was apparent from 2 separate batches grown in Avon soil at 3 weeks, and the Narrabri soil for up to 4 weeks. Neither non-GM nor GM plants had a higher colonisation rate during this early period, and the

variability of early colonisation of cotton agrees with the work of Pattinson & McGee (1997). This may occur from the random distribution of propagules for primary infection of the plant root, or from secondary external infection from a closely positioned adjacent root (Jarstfer & Sylvia, 1993, p. 354), where colonisation spreads rapidly once it has been initiated. Variability in colonisation may also be due to spore numbers that vary with environment, season and year (Hayman, 1970).

The resilience of A-M fungi after adverse conditions has been shown by Dr Steven Allen of the Cotton CRC. Correspondence from Dr Allen (pers. comm. dated 21/07/03) stated that their research group assessed mycorrhizal infection in many fields, over many seasons, where there had been exposure to standard use of cotton insecticides and herbicides, and had never seen an impact of pesticides. The results of this section showed that the plant-produced insecticide, the *Bt* protein, also did not show any difference in the rate of colonisation, nor of the form of the A-M fungi.

McGee et al. (1999) found the amount of A-M fungal colonisation was proportional to the propagule density in the soil, and also found that the rate of colonisation of cotton plants decreased with increasing soil depth, but this could not be confirmed within the pot trials. Pattinson & McGee (1977), showed a colonisation curve of A-M fungi where the asymptote was approached from freshly harvested Narrabri rhizosphere soil at 4 weeks, but in severely disturbed soil the maximum had not been reached in 40 days (about 6 weeks). The work of this section showed that the asymptote was also reached at about 6 weeks for plants grown in both Avon and Narrabri soils. It is possible that the disruption due to passing the soil through a 5mm sieve prior to sowing may have disrupted the propagules and slowed the rate of colonisation.

Comparisons of colonisation rates were made from 3 to over 14 weeks, and included all of the four cotton plant strains used in the trials. The colonisation rate of A-M fungi from Avon was higher than from Narrabri soil in the early stages of plant growth, but by 4 weeks the plants grown in both soils averaged colonisation above 80%. Colonisation was similar for different plant strains. The increasingly rapid rate between 3 and 5 weeks can be attributed to both intercellular colonisation along the cortical cells, as well as secondary infection from an external hypha from an adjacent root (Jarstfer & Syliva, 1996). The high A-M root colonisation in cotton plants is

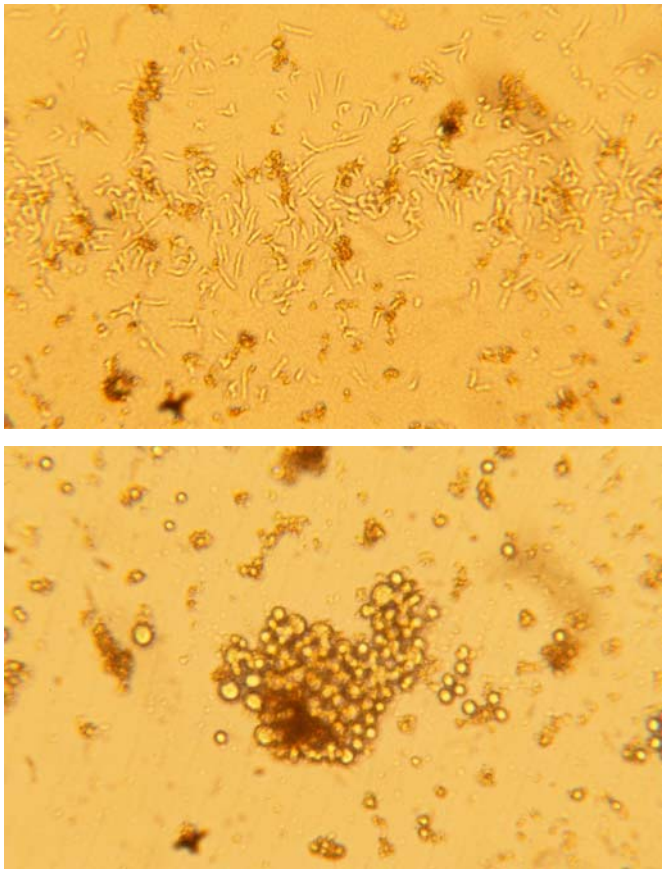
similar to the results of McGee et al. (1999), who reported colonisation of more than 80% of cotton roots within 36 days, from as little as 10 propagules.

As neither the rate of colonisation nor the establishment of normal growth form of the A-M fungus in the root differed in the GM or non-GM plants, presence of the *Bt* protein did not have a detrimental effect on the plant/fungal symbiosis. This observation agrees with the research of Lelmen et al. (2004) where the addition of 0.01, 0.1 and 50µg *Bt* toxin per gram of a vertisol soil did not affect A-M colonisation of sorghum plants. A similar rate of colonisation was also found by Vierheilig et al. (1995) with genetically modified protein and *Glomus mosseae* in tobacco.

8.4.2 Plant nutrient uptake determined by the leaf tissue content

Jarstfer & Sylvia (1993, p. 350) reported that elevated levels of P can occur through A-M fungi uptake from nutrient-depleted soils. Consequently the leaf tissue content for paired cotton plants grown under conditions of adequate nutrient was compared with plants grown under conditions of nutrient deficiency. The results of the tissue analysis of plants inoculated with A-M fungi (see Section 4.3) showed higher levels of P in the V15 and V15i plant nutrient-deficient trial in Avon soil, and also in the C312 and C312i plant trials under adequate nutrient supply in the Avon soil. These elevated levels of P for both trials were considered to be toxic to the plants according to Reuter & Robinson (1997) at 0.25% to 0.5%, and according to the Aust. Cotton CRC guidelines of 0.28% to 0.5%. However, the same plants (C312/C312i) grown in Narrabri soil under adequate nutrient conditions did not have elevated P levels. As both the non-GM and GM plants were similarly affected it suggests an A-M fungal response to nutrient-deficiency, and by the individual plant strain, rather than the genetic modification of the cotton plants.

The following section focuses on the soil microfauna. It investigates the possible impacts of plant-produced *Bt* protein on the higher order trophic groups – protozoa and nematodes.



Section 9

Protozoan populations within the rhizosphere of GM and non-GM cotton

Fig. 9.0 Light microscope images of a trail of *Vahlkampfia* amoebae isolated from Avon soil, top, and an aggregation of mixed protozoan cysts, below (×200). Using an optical graticule, cysts were slightly more than 3µm in width for flagellates, and around 10µm for the amoebae.

9.1 INTRODUCTION

Protozoa are predators of bacteria and fungi, and are prey to other soil-dwellers such as nematodes and tardigrades. They are thus an important intermediary trophic level in the soil microbial food web. Cowling (1994) reported that soil type, geometry of soil pores, organic matter content, soil pH, temperature, moisture and atmosphere, vegetation cover (above and below ground), litter and humus type derived from plants, root exudates and distribution of microbial food all influence the spatial distribution (and therefore sampled numbers in the population estimations) of protozoa. This sensitivity to environmental conditions make protozoa a useful index of changed soil conditions both by the abundance of the microflora which is their food source, and by the effect of the higher trophic group predators, which is directly reflected in their population numbers.

A decline in numbers of predators of protozoa that are susceptible to *Bt* proteins could result in an increase in numbers of protozoans. This would diminish the numbers of bacteria, which are the first consumers of litter. Other effects of protozoa

leading to disturbances of the food web include a decrease of nitrifying bacteria, resulting in a larger nitrifying activity per bacterial cell (Verhagen & Laanbroek, 1992). Couteaux and Darbyshire (1997) also reported that protozoa play key roles in the carbon and nitrogen cycles of many soils by regulating both the decomposition rate of organic material and specific metabolic pathways. Old (1977) reported that giant amoebae could perforate fungal spores, so a change in protozoan populations could alter the bacterial:fungal ratio. The rate of C and N mineralisation can be an indicator of soil health, with degraded soil containing a higher C:N ratio which leads to different selection pressures of soil microbiota (Yeates & Boag, 2004). All of these influences affect the balance within the soil food web, the dynamics of which are still not widely understood.

The three protozoan groups, the amoebae, flagellates and ciliates, are not mutually exclusive in soil microhabitats, and compete for the bacteria which are their common food source. Singh (1945) documented protozoan feeding preferences and reported that "Amoebae are very selective in their choice of bacterial food; some bacterial species are readily eaten, others reluctantly, while some of them are inedible. Many soil bacteria are not only inedible to protozoa but produce secretions actively toxic to them". This preferential feeding shows that protozoans can detect differences in bacterial type, possibly by chemical cue. As all protozoa internalise their food, they may be susceptible to the *Bt* protein.

Protozoa were therefore chosen as a test of a potentially susceptible microfauna with which to determine any difference in the microbial populations of the rhizosphere of non-GM and GM plants, from the three sampled soils.

9.2 MATERIALS AND METHODS

9.2.1 Selection of consensual bacterial cultures as a food source prior to the estimation of protozoan populations from the sampled soils

In all trials, protozoans were categorised into three types: ciliates, amoebae and flagellates, which reflected their mode of locomotion. In order to avoid bias due to possible feeding preference of the protozoa for a type of bacteria (Singh, 1945; Casida, 1989), a bacterial strain was needed which achieved a feeding consensus. The three cultures used in the food trial are listed below.

1. *Enterobacter* soil isolate, strain E64, a Gram-negative bacterium, available as a live cell culture, kept at 4ºC.

2. *Bacillus thuringiensis*, subsp. Kurstaki, a Gram-Positive bacterium obtained in culture from the Insect Pathology Laboratory of the University of Adelaide. This type of bacterium was also used to test for possible toxic effects.
3. *Rhizobium meliloti,* a Gram-negative root-nodule bacteria on legumes, available as a live cell culture, stored at 4ºC.

Nutrient broth for culture of bacteria comprised 0.1% glucose and 0.1% yeast extract per 1 L, adjusted to a pH of 6.8, and 200 ml was transferred to three 1 L Erlenmeyer flasks and autoclaved. Each of the three bacterial cultures were inoculated into the (separate) sterile broths and shaken for 2 days on a rotating shaker at 25ºC. The bacterial cells were harvested by centrifuging at 6,000 rpm for 15 minutes and discarding the supernatant. One ml of sterile distilled water was added to the pellet and vortexed. The cultures were then transferred to sterilised McCartney bottles and stored at 4ºC.

Mixed populations of protozoa were harvested from the three sampled soils by dilution in phosphate buffer and extracted from the surface of a 3 micron filter to separate bacteria within 4 hours of harvest. The protozoan populations were separated into the three categories of amoebae, flagellates and ciliates by serial dilution in the culture boxes until single types were obtained. Pooled groups of the separated types of protozoa were then cultured on pure strains of the three selected bacteria for the food preference trials.

The bacterial cultures were each brought to an OD of 0.1 using the maximum λ of a spectrophotometric scan, to ensure that each type of bacteria had an equal density at the beginning of the trials, and that the food source for the protozoa was ample. Aliquots of 800µL of adjunct fluid plus bacterial mix were delivered into fifteen replicate cells, and 200µL of protozoan samples were added, to give a cross-tabulation of protozoan feeding preference for each of the three types of bacteria. All plates were incubated at 24ºC for 7 days. The density of each protozoan population was measured by random sample by optical graticule, as the numbers were too high for counting. The arithmetic mean of three counts from each of 8 wells per bacterial type was made for each type of protozoa (72 wells).

9.2.2 Cultivation of protozoa from the three sampled soils

A 1 ml aliquot of *Enterobacter* soil isolate, strain 64 (E64) was added to a sterile mixture of 92 ml saline phosphate buffer, 2 ml straw infusion and 5 ml soil extract (the detailed procedure for the preparation is in Appendix 9). An 800 µL aliquot was added to each of the 4 rows above the base row of the 5 × 5 cell tissue culture plates.

Within 24 hours of harvesting the rhizosphere soils, a 10^{-1} soil and water mixture was shaken. One ml of this was aliquoted to the baseline rows of the tissue culture plates. A 200 µL aliquot from each cell within rows was transferred to a higher row and thoroughly mixed with the 800 µL of E64 bacterial culture and nutrient solution. This procedure was repeated to make serial dilutions of 1 in 5.

Four replicates of each of the 5 × 5 plates were made for each rhizosphere soil from each individual paired plant (four non-GM and four GM rhizosphere soils in each trial). Two replicate plates were cultured from the soil-only plates for each of the individual trials. The plates were incubated in darkness at 24ºC in sealed plastic zip-lock bags and counted on 3, 6-7 and 10-11 days after plating using a Leitz bright-field and phase-contrast microscope (serial No. 520573) at 320× magnification.

9.2.3 Estimation of protozoan populations by Most Probable Number

A most probable number method was used to estimate protozoan populations, based on Darbyshire et al. (1974), and modified by Gupta et al. (1998). This was considered the best method for use in the opaque, fine particulate clay soils. Only the general classes of amoebae, flagellates and ciliates were scored on movement.

The protozoa were recorded as a presence/absence matrix and estimates were based on the presence of both live (motile) cells and cysts. After averaging the populations of each row of five cells, and for each dilution, MPN tables (Gupta et al. 1998) were consulted for the estimated populations.

9.3 RESULTS

9.3.1 Bacterial food preference trial

All groups of protozoa survived and increased in numbers on each of the three types of bacteria (Fig. 9.3.1.1). The most consistent growth occurred on *Enterobacter* strain 64, and so this bacterium was chosen as the food source in the most probable

number (MPN) estimations of protozoan population for the non-GM/GM rhizosphere soil trials.

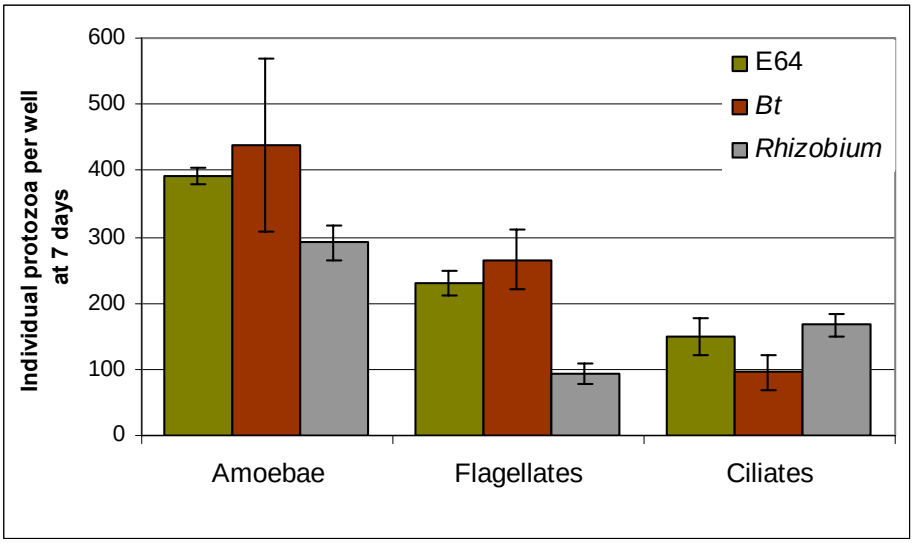


Figure 9.3.1.1 Comparison of protozoan growth on three types of bacterial food source after seven days, measured by optical graticule.

While the definition of a 'species' is difficult with protozoa that multiply asexually, there were identifiable differences within the groups. In the trials of mixed protozoa, Amoebae were nearly always present in many forms. The most common forms were smaller, rounded, multi-pseudopodial forms (similar to *Vahlkampfia*, illustrated as the top photograph at the heading of the section). Fast-moving 'slug' shaped forms, resembling the genus *Hartmanella* were also present, with a smaller proportion of *Naegleria*-like forms. Large, 'explosive' *Leptomyxidae* were also seen, but less frequently than the others. Usually there were at least three forms in any one sample. (Putative identification of the three forms of protozoa were made by reference to Kudo 1971, and by the personal observation of Gupta Vadakattu from photographs of the dilution series in culture).

The most common flagellates in all soils were of the genus *Bodo*. They were present in all soils, and in all samples. The most common form of the ciliates was from the genus *Colpoda*.

Where amoebae, flagellates and ciliates occurred in the replicates of rhizosphere soils of the one plant type, they also occurred in the other, which would suggest that the effect of the *Bt* protein was not lethal for these protozoans surrounding the root zone of the GM plants.

9.3.2 Estimation of the numbers of three protozoan groups within Avon soil

Figure 9.3.2.1 illustrates the proportions of protozoa for the separate pot trials. The three groups of amoebae, flagellates and ciliates have been shown as components of the total population, as all groups were competitors for the same bacterial food resource.

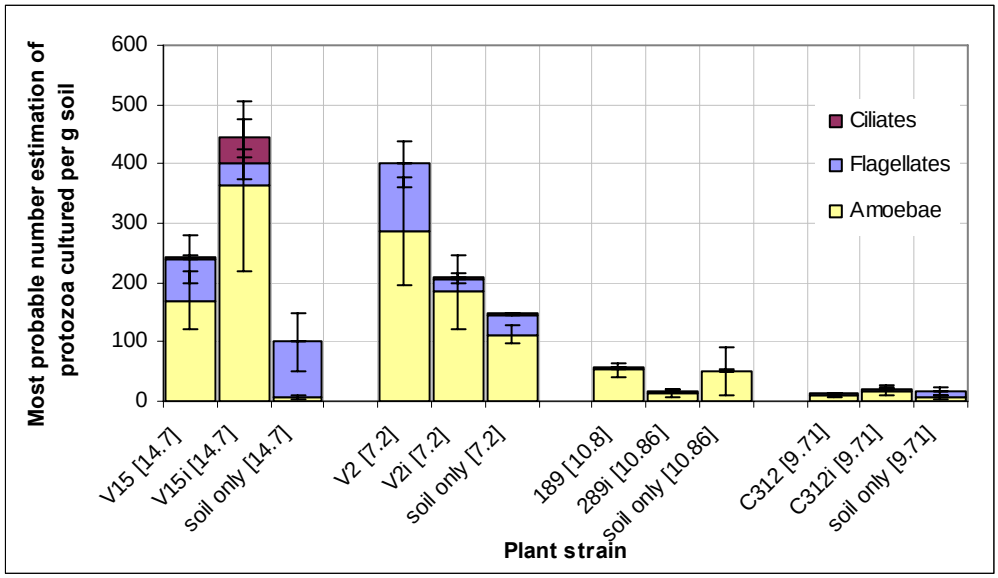


Figure 9.3.2.1 MPN estimate of total protozoa in Avon soil, n=4. Harvest age is shown in [weeks].

Figure 9.3.2.1 shows that protozoans extracted from the rhizosphere soils of the 4 paired trials showed more similarity by arithmetic mean within each separate trial than across the separate trials which differed by plant type and age of harvest. Two-way independent t-tests on the total grouped populations of each trial showed no significant difference between the non-GM and GM rhizosphere soils, for each of the different trials, however the C312/C312i trials approached a significant difference in total protozoa at $p = 0.05$. This significant difference for the C312/C312i trial is explained by the fact that both the variance and standard deviation, on which the t-test is based, depends on the magnitude of the data (Zar, 1984, p. 32). The coefficient of variation, which expresses the sample variability relative to the mean of the sample, does not depend on the magnitude of the data since the variance and the mean have identical units. When the coefficient of variation was calculated for each paired trial, neither soil type nor plant type explained consistently higher relative variability for amoebae or flagellates, that would suggest patches of sub-groups deleted from these populations.

When protozoan populations were compared the V15/V15i trial showed more variability (variance/mean) in total numbers of flagellates than amoebae, and numbers of flagellates from the V2/V2i plant comparison were marginally different at $p = 0.06$. Ciliates were not always observed in all replicates in Avon trials, and these occurred more frequently where the numbers of amoebae and flagellates were present in higher numbers (see Avon V15/V15i in Figure 9.3.2.1), which suggests a higher density-dependence for ciliates which does not occur with amoebae and flagellates.

9.3.3 Estimation of the numbers of three protozoan groups within Narrabri soil

The protozoan populations estimated from the Narrabri soils, from the four plant types is illustrated in Figure 9.3.3.1. The overall numbers of flagellates were similar across the V15/V15i, V2/V2i and 189/289i plant trials, using Narrabri soil, but amoebae varied with each treatment.

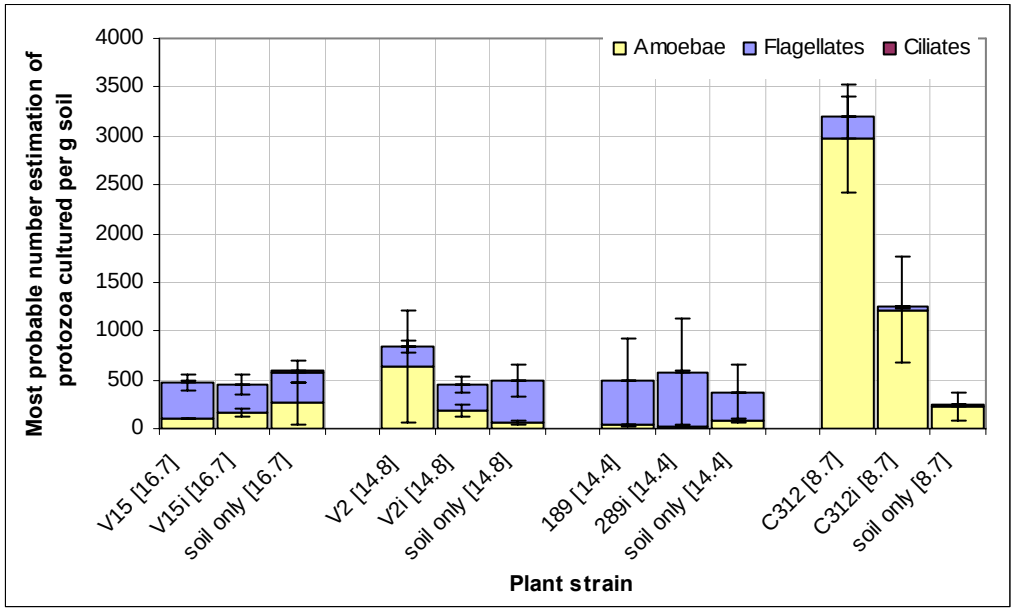


Figure 9.3.3.1 MPN estimate of total protozoa in Narrabri soil, n=4. Harvest age is shown as [weeks]

Flagellates generally predominated in samples with smaller total population numbers in Narrabri soil, whereas amoebae were better represented in the higher population samples, particularly the C312/C312i trial. The trend for higher numbers of flagellates than amoebae did not follow plant age at harvest, nor was there a consistent difference between the protozoan populations from rhizosphere soils of the non-GM or GM plants for any of the trials. The Narrabri C312/C312i trial

appears to differ between the non-GM and GM rhizosphere soil populations, and again between the rhizosphere and non-rhizosphere soil, however the result of the independent t-test between the C312 and C312i trials was 0.22, and between the C312 and non-rhizosphere soil was 0.12: neither was significant at $p \leq 0.05$. There was also no significant difference in numbers of protozoa for any of the remaining trials, V15/V15i, V2/V2i or 189/289i at $p \leq 0.05$.

Although not evident from Figure 9.3.3.1 because of the low numbers, where ciliates appeared in the non-GM soils, they were also observed in the paired GM soil in at least one of the replicates. Ciliates were present in three of the four Narrabri non-rhizosphere samples.

9.3.4 Estimation of numbers of the three protozoan groups within Waikerie soil

Figure 9.3.4.1 illustrates the results of the protozoan populations estimated from the pot trials for Waikerie soil.

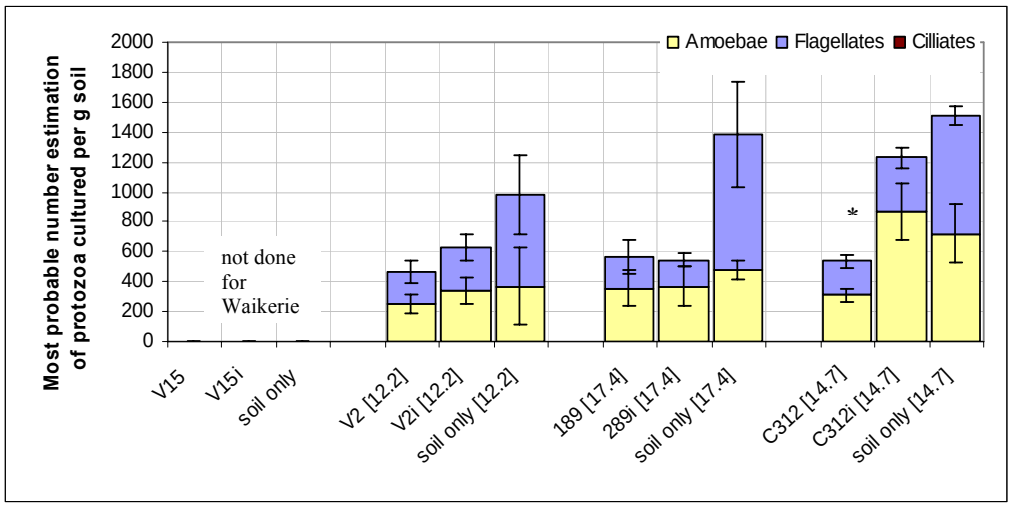


Figure 9.3.4.1 MPN estimate of total protozoa in Waikerie soil, n=4
The harvest age is shown as [weeks].

The independent t-tests from the amoebae populations in Waikerie rhizosphere soil showed a significant difference at $p = 0.02$ between the plant types C312 and C312i, but not from the total protozoan populations for this trial. The flagellates from Waikerie soil showed no significant differences for any of the three trials, using paired t-tests. Because of the absence of ciliates in some of the rhizosphere soil samples, the data was not statistically representative, and therefore is not conclusive for this group. Ciliates were not observed in any of the non-rhizosphere soils.

9.3.5 Comparison of protozoa across the three soil types

Larger numbers of amoebae occurred in the V15/V15i and V2/V2i trials in Avon soil than in Narrabri or Waikerie soil for these two paired plant strains. The 189/289i rhizosphere soils had more amoebae in Avon soil than Narrabri, but less than Waikerie. The Avon C312/C312i trial had fewest amoebae, intermediate for Waikerie and most for Narrabri in rhizosphere soils. There was no observable trend for amoebae by plant type across the three soils.

Flagellates were consistently higher in Narrabri soils for the plant strains V15/V15i, V2/V2i and 189/289i plants and were well represented in Waikerie rhizosphere soils for all plant strains. The populations of flagellates therefore showed numbers which varied by individual trial. A significant difference occurred between the protozoa in the rhizosphere soils of the C312/C312i paired trials in Waikerie rhizosphere soil, but the same trials from Avon and Narrabri showed no consistent difference for these paired plants. A difference is therefore not attributable to the GM plant strain.
The positively skewed data of the total protozoan populations within the rhizosphere soils of Avon, of 1.7; 3.7 for Narrabri and 1.5 for Waikerie showed a higher frequency of lower numbers of protozoa, than an even distribution over the whole range, for each of the three soils. This asymetry from the arithmetic mean shows patchiness within the rhizosphere populations, as it indicates the degree to which the data deviates from the normal distribution. It is interesting to note that the Narrabri soil had the highest asymetry from the mean, and this could indicate a soil effect on the protozoan populations at a micro-scale. It is not surprising then, that of the four trials, and 12 separate treatments, the spatial distribution as defined by Zar (1984, p. 411) was aggregated for amoebae in all 12 soil treatments, for flagellates in 11 soil treatments and for ciliates in 4 soil treatments. In 8 of 12 treatments ciliates were absent from the samples, so records for ciliates were statistically unrepresentative for the group. The absence of the ciliates was not related to the presence of the *Bt* protein because members of this group appeared in replicates of both non-GM and GM plant soils.

Six observations of ciliates appeared in the 20 non-plant associated Narrabri soil samples; 14 counts of ciliates occurred from 46 samples within non-GM soil, and 22 counts of ciliates occurred within the GM-plant rhizosphere from 44 samples (30%, 31% and 50% respectively). Amoebae and flagellates occurred in every replicate in

all soils, and for each different plant type, for both non-GM and GM plants. Relative comparisons of the two more abundant protozoan groups are shown in Table 9.3.5.1.

Table 9.3.5.1 Incidences of relatively higher populations of amoeba and flagellates within the three rhizosphere soils, for plant type

Soil	V15/V15i	V2/V2i	189/289i	C312/C312i
Avon	Amoebae higher for both plant strains	Amoebae higher for both plant strains	Amoebae higher for both plant strains	Amoebae higher for both plant strains
Narrabri	Flagellates higher for both plant strains	Amoebae higher for V2; flagellates higher for V2i	Flagellates higher for both plant strains	Amoebae higher for both plant strains
Waikerie	Flagellates higher for both plant strains	Amoebae higher for both plant strains	Amoebae higher for both strains	Amoebae higher for both plant strains

The cross tabulation showed that of 12 comparisons, the same type of protozoa were higher in numbers in 11 of the paired plant trials. This showed more similarity between the individual trials for plant type, than difference between groups for plant type. Amoebae dominated over flagellates in Avon soil, with all four trials showing amoebae numbers higher than flagellates. The Waikerie soil also showed a higher number of amoebae than flagellates, for the four paired plant types, and Narrabri soils differed across the plant types. These higher ratios of amoebae and flagellates showed more consistency between the paired plant types than soil type, which showed that neither the amoebae nor flagellates were susceptible to the different non-GM or GM plant strains.

9.3.5.2 Effect of individual pot trial protozoan populations

There was generally more similarity between the arithmetic means of the protozoan populations from the individual paired plant trials than between different soils of the same plant type. This was shown by the high amoebae populations for the C312 and C312i rhizosphere soils of Narrabri and Waikerie, compared to the low numbers from the Avon soil trials. The flagellate populations were also not consistent for plant type across different trials. The largest populations of flagellates occurred in the Waikerie rhizosphere soil and the smallest in the Avon soil. Ciliates were absent from many individual samples, but reached the highest numbers of all protozoans in samples of the Narrabri and Avon soils. This is not evident on the figures because of the scale of the numbers. Data on ciliate populations were not analysed by ANOVA

because of the number of replicates where these protozoans were not present, causing problems of log-constant values in calculation of standard deviation.

9.4 DISCUSSION

This study used cotton plant strains developed for Australian soil and climate conditions. The results of protozoan populations within the rhizosphere of cotton described in this section agreed with those of Donegan et al. (1995), who used an American soil ecosystem with three different types of transgenic cotton plants, and compared them with parental cotton, and soil with purified *Bt* toxin. Donegan et al. (1995) found no significant difference in the protozoa per gram dry weight of soil from the transgenic and parental cotton plants. It therefore suggests that the microbiota within the soils of both countries was not affected by the presence of the Cry1A(c) protein, or other influences of the modified plants.

The most notable aspect of the results were the relative similarity of the protozoan populations by individual trial, which included the influence of age of plant at harvest. Thus, factors other than plant cultivar and soil type had more influence on the protozoan population.

There high degree of variance between replicates within each of the separate trials. may be attributable to the uneven distribution of protozoa which related to the heterogeneous distribution of soil aggregates and the channels in between. Restriction to dispersion occurs in soil when amoebae meet a physical barrier such as large stones, suberised roots or densely packed particles in compacted soil clods. The clumping of protozoa found within small scale soil samples agrees with Kuikman et al. (1990) who reported that protozoa do not move more than a few millimetres in soil and may be confined to a particular soil aggregate. The uneven distribution of protozoa may also be caused by the localisation of bacteria as a food source. This follows from the works of Semenov et al. (1998) and Ellingsøe & Johnsen (2002) who found an uneven distribution of bacteria in soil.

Despite the increased microbial populations surrounding the plant root system (rhizosphere), Weller & Thomashow (1994) reported that only 7-15% of the actual root surface is covered with microorganisms, and Campbell (1985) estimated the area as less than 10%. These sites include grooves between epidermal cells, root hairs, lesions and sites where lateral roots break through cortical cells. Campbell

(1985) noted great variation between individual roots in the numbers of bacteria at different distances from the tip of the plant, and that some of the variability was due to both root architecture and antagonism between individuals. This unevenness of soil microbial distribution agrees with the findings of Mitchell et al. (2000), Rønn et al. (1996) and Wallace (1973).

It follows from the dependence of protozoa on bacteria as a source of food, that where there are localised patches of bacteria in the rhizosphere (Campbell, 1985) that protozoa, and their own predators, would be attracted to the areas of higher bacterial density. The distribution of the protozoa was shown to be clustered (Zar, 1984, p. 411) where aggregation occurred for amoebae in all 12 soil treatments, for flagellates in 11 soil treatments and for ciliates in 4 soil treatments. This occurred even in the open-pored, sandy soil of Avon, where protozoa would be more vulnerable to predators and possibly more mobile between pore channels. Aggregation can also be seen in Figure 9.0 for both amoebae and mixed protozoan cysts, in the fluid of cells without the restriction of physical barriers. Unevenness of population distribution in itself cannot be seen as being caused by difference in plant type, when clustering, rather than even distribution, is a naturally inherent pattern in soil communities driven by localised patches of nutrient and physical boundaries.

As all three soils contained clay-sized soil particles varying in proportion from 15.9% in Avon, 7.3% in Waikerie and 59.5% in Narrabri (Section 3) an additional factor in the clustered distribution of protozoa could be due to an adhesive substance near the border of the cysts, or an attraction by electrostatic charge or surface tension. The phosphate buffered saline in the protozoan mix and diluent ensured that the protozoa were effectively distributed in an electrolytic colloidal suspension and the cysts often clustered near organic matter in the wells.

Within arid desert litters, Bamforth (1984) found that flagellate populations ranged from 220 to 7930 g^{-1} dry weight for desert soil, and from 340 to 9480 g^{-1} in forest soil. It was found here that the carrying capacity of soil protozoa was not relatively consistent by soil type, but more closely aligned with individual pot trial. It was also found that the positively skewed data for each of the three soils showed a greater frequency of lower numbers of protozoa, but with a spatially clustered distribution overall, so degree of aggregation may be a useful additional descriptor of protozoan populations than range (high-low) alone, depending on the scale of measurements.

9.4.1 Comment on the method of MPN estimation of soil protozoa

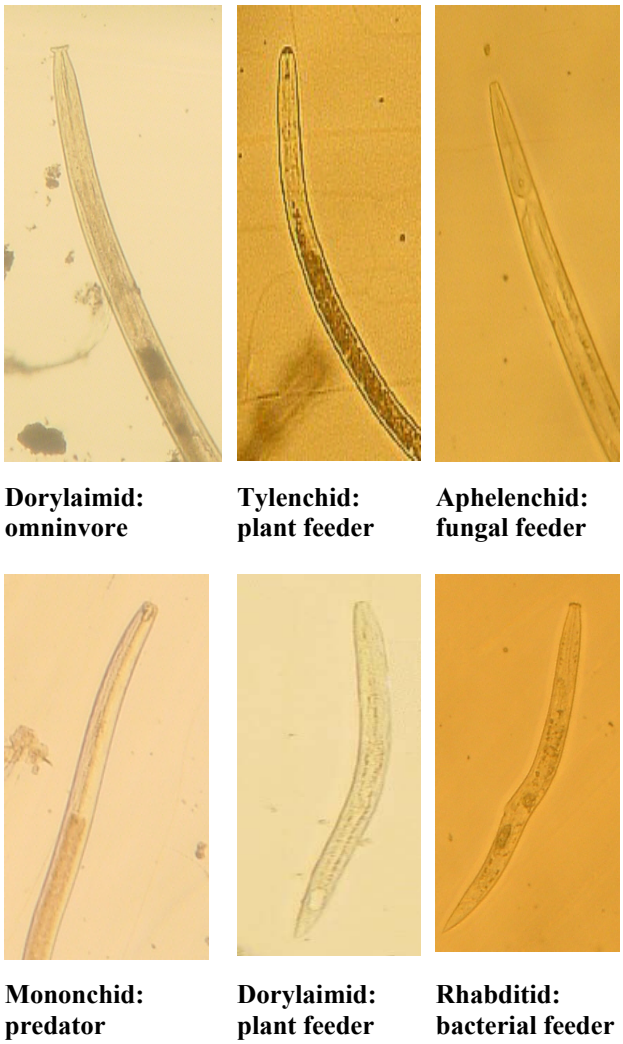
The MPN method favours growth of easily cultivable bacteriophagous protozoa such as *Ispumella, Bodo* and *Heteromita* at the cost of fungal feeders and detritivores (Fredslund et al. 2001). However, the rhizosphere is the site of active plant growth with high numbers of bacteria, and it could be argued that these bacteriophagous protozoa would naturally be higher in numbers in this region.

The difficulties in estimating overall protozoan populations in soil are similar to those of culturing microflora by plate method, where a source of nutrient is abundant, and predation pressure is removed. The preliminary trial which determined which bacteria to use in the trials showed that protozoan populations grew to uncountable numbers in 7 days with an ample food source, and without predation pressure. None of the freshly harvested protozoa were observed in such high numbers, and so these two factors, abundant nutrient availability and predation appear to impose major restrictions on the growth rate of soil protozoa.

Cowling (1994) noted the MPN method is not truly representative because the protozoa may not be evenly dispersed in the diluent, and that a restricted range of protozoa may develop in the soil dilution series. The absence of ciliates in many of the samples suggests that the dilution of 10^{-1} was too low for this class of protozoa.

However, within the limitations of the system, significant differences in numbers of the three protozoan groups between the rhizosphere soils of non-GM and GM cotton plants were not observed. It is notable also that the protozoan populations were not influenced by feeding on the pure culture of *Bacillus thuringiensis* for seven days, as was shown comparatively against the other types of bacteria in Figure 9.3.1.1. The populations continued to survive until encystment after the *Bt* bacteria were consumed. This occurred in spite of the fact that the *Bacillus* produces additional chitinolytic, phospholytic or other β-exotoxins in addition to the Cry1A(c) protein (Yamamoto & Powell, 1993). The pure *Bacillus* culture used as protozoan nutrient was added to the wells at the concentration of 1ml in 100ml adjunct fluid. The same culture measured by the ELISA test contained 5,000ppm Cry1A(c) at a concentration of 1 in 500 (Section 5). The 1% *Bt* culture added to the test wells as protozoan nutrient was therefore five times the concentration of the bacterial culture in the ELISA test. This is far in excess of the plant-produced protein, where the highest

levels of any plant-produced Cry1A(c) protein in the trials was 600 ppm in root tissue. In the next section the possible effects of the *Bt* protein on the nematodes, a next higher order of predators within soil mesofauna, are considered.



Section 10

Mesofauna: Nematodes

Fig. 10.0 Examples of body structures of nematodes isolated from trials, and used for categorisation of trophic groups. Not to scale.

10.1 INTRODUCTION

Bird (1999) examined the nematode population in soil taken from a wheat field at Avon, South Australia (34º 14' S, 138º 19' E) that was adjacent to the site from which the soil samples were collected for this project (34º 14' S, 138º 18' E). He found that nematodes were the most abundant metazoans in this soil. This agrees with other observations that nematodes constitute the major component of the mesofauna in many soils, and make up over 90% in numbers and about 10% of the total soil faunal biomass (Nicholas et al. 1992).

Of the estimated > 100,000 species of nematodes, roughly 10% are plant parasitic (Andrassy, 1992). Most of the plant parasitic nematodes are root feeders, and so are likely to be affected by roots and root excretions. They may influence root

development (Cohn et al. 1996) and the presence of some nematodes in host tissues can also cause profound metabolic disturbances throughout a plant. It is not surprising, therefore, that the exudates from plant tissues are altered by nematode infections (Dropkin, 1989). Nematodes can also indicate the N-mineralisation status of the soil as bacterial-feeders are more likely to be present in larger numbers where the C:N ratios are ~10-15, and fungal hyphae feeders are more likely to dominate where the C:N ratio is >15 (Yeates & Boag, 2004).

The rhizosphere has been defined previously as the soil closely associated with, and under the influence of exudates from plant roots (Literature Review, p. 8). In terms of nematodes, this definition may need to be modified as, according to Dusenbery (1987), volatile or gaseous compounds such as CO_2 given off by plant roots are attractive to phytonematodes, and can be detected as much as 1 metre away from a single long root and over 2 metres from a plant root mass. There would therefore be no soil from cotton plants grown in pot trials which would not be detected by phytonematodes after approximately 10 days (when the root reached the base of the pot).

As shown in Section 5, the *Bt* protein was not detected above background levels in the rhizosphere soil external to the root, so the most susceptible non-target organisms may therefore be nematodes that graze directly on the growing plant roots. Research by Ignoffo & Dropkin (1976) and Chen et al. (2000), showed nematode susceptibility to some *Bt* proteins (not necessarily the strains produced by the commercially developed GM-plants). Additionally, the detail in a patent application lodged at the time of the first commercialisation of *Bt* cotton by Narva et al. (1994) also cited nematicidal *Bt* proteins from a *Bacillus thuringiensis* isolate. Wei et al. (2002) confirmed that some *Bacillus thuringiensis* crystal proteins, particularly Cry21A, Cry5B and Cry14A, may be toxic to nematodes. The effect of plant-produced *Bt* on Australian nematode populations, the major predators of bacteria and other microflora, is not known.

If the plant-produced *Bt* protein is toxic to nematodes, it could alter the dynamics of the non-target soil microbial food web in two ways. First, there is a predator/prey relationship between nematodes and soil bacteria and between plant roots and plant-feeders. Secondly, some soil-dwelling mites and tardigrades are nematode predators.

If the nematode predators are affected by the *Bt* protein, their numbers may decline which could affect nematode populations. This, in turn, could result in a decrease in bacterial numbers due to increased nematode grazing, as found by Whitford et al. (1982). Subsequently, the fecundity of any nematode cohort susceptible to the Cry1A(c) protein would be affected.

Work by Saxena & Stotzky (2001) showed that root exudates of *Bt* corn expressing the Cry1A(c) protein were not toxic to nematodes, but the toxicity of the Cry1A(c) protein from the roots of cotton plants grown in Australian soil was not tested on root-feeding or free-living nematodes. Since the Avon soil sampled was from plots under wheat/wheat rotation and where genetically modified plants had never been grown, any effect on the soil microbial population would be a result of the interaction with the plant strain itself, uncomplicated by the effect of resistance in the nematode populations. The nematodes extracted from the Narrabri soil had built up after cotton, some of which may have included genetically modified crops.

The work in this section therefore compared the numbers and ratios of different trophic groups of nematodes from Avon and Narrabri rhizosphere soils, to establish if differences in the populations correlated with the non-GM and GM paired cotton plant cultivars developed for Australian conditions.

The ELISA tests (Section 5) on leaf and root tissue showed that the *Bt* protein occurred at the same concentration in root tissue from freshly harvested, and dried GM cotton plants. It was also shown from the photographs in Section 6 that the macroscopic rate of cotton plant tissue breakdown was effectively the same for both the non-*Bt* and *Bt*-protein producing cotton plants. The results from Section 3 showed that there was a higher content of nitrogen in the leaves, stems and roots of GM cotton plants. With a lower C:N ratio there is the possibility that the litter breakdown could have occurred by different guilds of detritivores, and this could be reflected by a difference in the proportion of bacterial-feeding nematodes.

As dried cotton plants are commonly ploughed into field soils after harvest, the work in this section continued the litterbag studies by specificially measuring the effect of degrading plant tissue on nematode populations (Bird 1999).

10.2 MATERIALS AND METHODS

10.2.1 Soils and plants

The growth conditions of the paired non-GM and GM cotton plants were described in Section 4. The plant strains V15 and V15i were used for rhizosphere soils in all cases, to obviate possible differences caused by different cotton plant strains. Each batch of nematodes was extracted from 200 g moist soil collected from around the roots of freshly harvested plants, and for comparison, from non-plant soil kept under the same conditions.

10.2.2 Method of nematode extraction

The Whitehead tray method (Whitehead & Hemming, 1965) was used to determine population diversity within the sampled soils, and the detailed procedure and apparatus is illustrated in Appendix 10.1. A 20µm mesh sieve was used to remove smaller nematodes which included some juveniles from the extracts, as the esophageal structures of the adult animals are easier to identify. After draining into plastic containers, the water containing the nematodes was allowed to settle for at least two hours, as nematodes are slightly denser than water. The upper layer of the water was removed by pipette to reduce the volume to 6 ml for all extracted populations and the water and nematodes were transferred to lidded 10 ml plastic graduated centrifuge tubes for storage at 4ºC. This volume allowed mixing via a 1000µL pipette tip by suction and replacement of the water (three times), before extracting 1 ml into a circular counting plate. The total numbers from each batch represent the nematodes from 1 ml of at least two averaged duplicates, for each of the replicate pots, and therefore represent the nematodes harvested from each 33g soil, calculated as 200g soil/6ml.

10.2.3 Identification and grouping

The nematodes were classified according to the characteristics listed in Table 10.2.3.1 and separated into trophic groups of omnivores, bacterial-feeders, fungus-feeders, plant-feeders or predators (Yeates et al. 1993). The groups were not subdivided into taxonomic order, as the environmental impact on functional groups, rather than taxon, was considered more relevant. Juveniles were not subdivided beyond trophic groups because species such as *Pratylenchus neglectus* and *P. thorneii*, are difficult to distinguish, even by experienced nematologists. A Leitz dissecting microscope using light field and differential interference contrast optics at

a magnification of 100× was used to identify the esophogeal structures. Male/female ratio was not scored.

Table 10.2.3.1 Characteristics used to group nematodes into feeding groups.

OMNIVORES	
Dorylamids	Omnivore adults much larger than all the other groups, excepting predators. Odontostyle present. Intestine dark. Procorpus sometimes winding and esophagous cylindrical. No median bulb. In unfixed specimens the movement is slower than the bacteriovores, but because of the larger size, the exploratory movement covers a wider span.
PREDATORS	
Mononchids	Adults roughly the same size as omnivores, but with a distinct buccal cavity present. Movement is rapid.
BACTERIAL FEEDERS	
Rhabditids	No stylet. Median and end bulbs present with crescent-shaped valves in end-bulb. Lip region sometimes pronounced and uneven. Intestine colour can vary. Width relative to length greater than other types. In unfixed specimens, movement within this group is often the most active, with a lateral, circular sweeping action.
Dorylamids	No stylet. Cylindrical esophagus, slender anteriorly; no bulbs. Several kinds of body shape, including long and slender. Much smaller than the dorylamid omnivore adults.
PLANT PARASITIC NEMATODES	
Tylenchids	Strong or weaker stylet with knobs at base. Median and end bulbs, but end bulb shape is less circular than that of the Rhabditids, and does not have crescent-shaped valves. Some unfixed specimens have a forward piercing movement, on contact with plant detritus.
Dorylamids	Stylet much less pronounced than in the Tylenchids, and without knobs. Head and tail bluntly rounded.
FUNGAL /LOWER PLANT FEEDERS	
Aphelenchids	Weak stylet. No, or small knobs. A more thinly tapering head region than the bacterial feeders. Large, rounded mid esophageal bulb present. These nematodes are often long and slender, with paler intestines than recently-fed tylenchids. The body of the nematodes moves relative to the fixed position of the head, when feeding.

10.2.4 Statistical analysis

Two methods of analysis were used. A comparison of averaged total numbers categorised by trophic groups – the bacterial feeders, fungal feeders, plant root feeders and omnivores – was used to show the trend over time between the nematode populations of non-GM and GM rhizosphere soils for each of the paired trials. A comparison of relative proportions of trophic groups by Chi-squared (χ^2) analysis was also used to compare the probability of independence of one group relative to the total extracted population, as per the procedure of Bezawada et al. (2003). This non-parametric method satisfied the test for independence of the nominal categories of the four groups of nematodes and treatment of soils for paired plant cultivars, and as a comparison of ratios, was independent of the total population numbers from each harvested batch. The standard method of calculation for $\chi 2$ was (O-E)/E, where O is an observed value and E is the expected value. As there were four groups, the data featured three degrees of freedom. A significantly changed population ratio was taken at $p \leq 0.05$.

10.2.5 Effect of soil type on microbial rhizosphere populations

It was possible that heavy clay soil which expands and shrinks dependent on water content could cause selection of different nematode ecotypes where the build-up of populations depends on the seasonal change of microhabitat. For that reason, two distinct and contrasting soils from Avon, South Australia, and Narrabri, New South Wales, were used to compare differences in nematode populations across soil type and remote sampling locations. The soils and storage conditions were described in Section 3.

10.2.6 Time series analysis of population diversification

Nematodes extracted from the air-dried soil from the Avon bulk storage bin were used as a benchmark population for calculating the change in nematode populations over time. The soil contained remnants of wheat stubble with attached roots which had remained after wheat had been grown in the previous season.

Nematodes were extracted from Avon rhizosphere soil and non-rhizosphere soil, respectively, with harvests at:

- 3.7 weeks when young cotton plants were actively growing, and had a well-established root structure;
- 6.4 weeks which was the mid-point of the active growth curve;
- 8 weeks which was the time-point at which the roots had approached the maximum volume in the pot (Section 4) before the plant became pot-bound; and
- 16 weeks, under nutrient deficient conditions when plant growth had stopped.

A corresponding comparison was made for the Narrabri soils at 3.7 weeks, 7 weeks, 8.1 weeks and under nutrient deficient conditions at 14.8 weeks. The two-day difference in harvesting allowed time to process one soil batch immediately after the other, as they were both grown in the glasshouse at the same time, and under the same conditions. All nematode tests of rhizosphere soil with living roots used the plant strains V15 and V15i.

10.2.7 Litterbag study

The litterbag procedure was described in Section 6. After ELISA tests for the presence of the *Bt* protein from the decomposing plant tissue, and after photographing (Section 6) the plant tissue in the litterbags and surrounding soil was collected for nematode extraction. The cotton plant strains used in the litterbag studies were the C312 and C312i cultivars in all cases.

10.3 RESULTS

The population counts of adult nematodes from the Avon and Narrabri soils are listed in Table 10.3.1 below.

Table 10.3.1 Total count of nematodes from four trials of Avon and four trials of Narrabri soils, for the V15/V15i plant trials (not averaged for duplicates).

	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders	Total count
Avon Soil					
Non-GM	1,095	7,233	637	2,227	11,192
GM	667	6,956	707	2,293	10,623
soil only	193	2,021	113	363	2,690
					24,505
Narrabri Soil					
Non-GM	358	2,341	368	603	3,670
GM	266	1,899	281	71	2,517
soil only	29	580	107	203	919
					7,106

Table 10.3.1 indicates that the bacterial feeders were the most highly represented group for both soils over all trials. The standard errors of the mean have not been shown as the table is a composite of 5 trials for Avon soil, and four trials from the Narrabri soil, and varied by individual trial. It can be seen that the total nematode count for the GM plants are lower than the non-GM plants in both Avon and Narrabri soils by a difference of 5% for Avon soil harvests, and 31% for Narrabri soil.

As each extraction was separate, a test of change in proportions of the whole populations was compared by χ^2 analysis, to verify that relative difference in numbers was not an artifact of the extraction process, but that a change had occurred for specific groups compared against the total population.

The comparisons of the trials are shown separately for Avon and Narrabri sampled soils, with a summation of averages based on the counts from the total extractions. This is followed by an illustration by individual trial and a commentary relating to the proportional change over time. A summation of the χ^2 tests follows, which compared the change in population proportions, shown as a flow diagram, and finally mirrored proportional time series graphs.

10.3.1 Extractions of nematodes from Avon soil

The time series for extracted nematodes from Avon soil is shown in Table 10.3.1.1.

Table 10.3.1.1 Time series of nematode populations extracted from Avon soil for paired V15 and V15i rhizosphere soil, compared with non-plant associated soil. (Averaged replicates ± SEM per 33g soil)

Harvest time (weeks) for V15/V15i plants	Rep-lic-ates (*n*)	Omni-vores	Bacterial feeders	Fungal feeders	Plant feeders	Sum of group aver-ages	% of harvest from bulk soil
Original bulk soil *	1	47 ± 12	646 ± 51	25 ± 4	77 ± 6	795	(100%)
V15 (3.7)	4	33 ± 8	340 ± 6	15 ± 5	80 ± 21	468	59
V15i (3.7)	4	26 ± 11	321 ± 117	10 ± 5	56 ± 23	413	52
V15 (6.4)	2	21 ± 7	201 ± 47	17 ± 7	39 ± 9	278	35
V15i (6.4)	2	10 ± 4	165 ± 20	24 ± 0	33 ± 6	232	29
V15 (8)	4	15 ± 5	183 ± 74	17 ± 7	33 ± 11	248	31
V15i (8)	4	31 ± 14	274 ± 92	53 ± 42	105 ± 63	463	59
soil only (8)	2	3 ± 1	5 ± 2	1 ± 0	2 ± 1	11	1
V15 (16) (nutrient deficient)	4	39 ± 8	70 ± 17	17 ± 5	87 ± 18	213	27
V15i (16) (nutrient deficient)	4	8 ± 2	51 ± 18	5 ± 2	74 ± 24	138	17
soil only (16)	2	5 ± 3	14 ± 1	8 ± 0	52 ± 6	79	10

* adjusted proportionately for soil moisture from 3% to 18% to bring all harvest to a wet-weight basis.

The ordinate scales of the figures for each of the paired trials from averaged counts of nematodes vary over the time series. Figure 10.3.1.1 shows the nematode population from the Avon soil, extracted directly from the bulk container at the commencement of the trials.

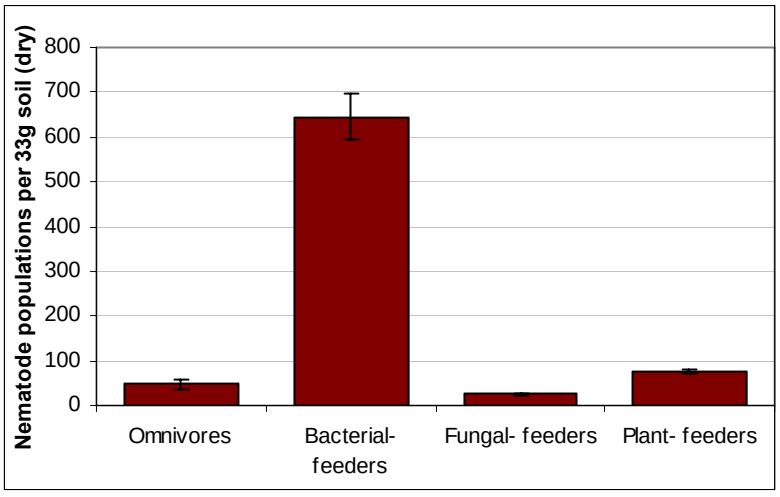


Figure 10.3.1.1 Nematodes harvested from (dry) Avon bulk soil before planting, after proportional adjustment of soil weight from 3% to 18% which was the field moisture capacity at which the Avon soils were kept.

A comparison at 3.7 weeks is shown in Figure 10.3.1.2.

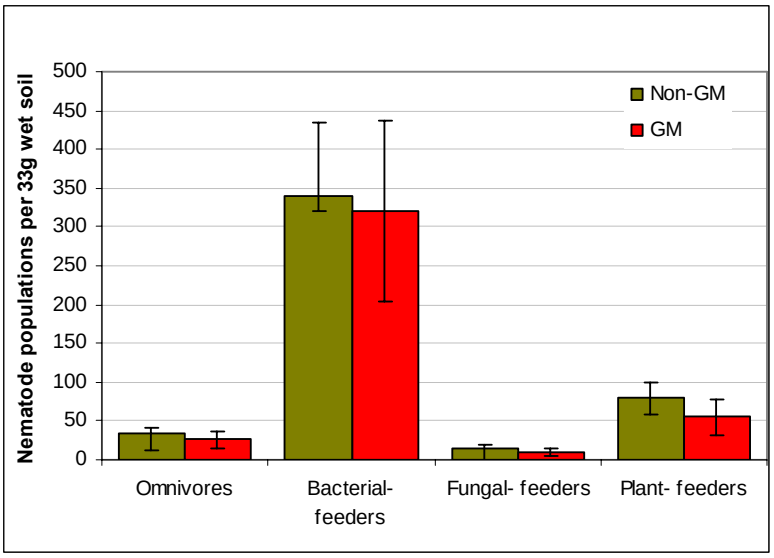


Figure 10.3.1.2 Nematode populations from rhizosphere soils of V15 and V15i plants grown in Avon soil, at 3.7 weeks harvest.

The χ^2 probability of independence, assuming normal distribution, of 0.34 between the rhizosphere of the non-GM plants compared with the GM plants at 3.7 weeks meant that the proportions of the groups in the population not changed significantly from those in the original soil sampled.

At 6.4 weeks (Figure 10.3.1.3) there was an increase in the proportion of bacterial feeders, while that of the fungal feeders and plant feeders remained similar. Because of this change in ratio, the χ^2 probability of 0.07 described a greater difference in nematode population proportions for both the non-GM and GM plant rhizosphere soils than was present at the 3.7 week harvest.

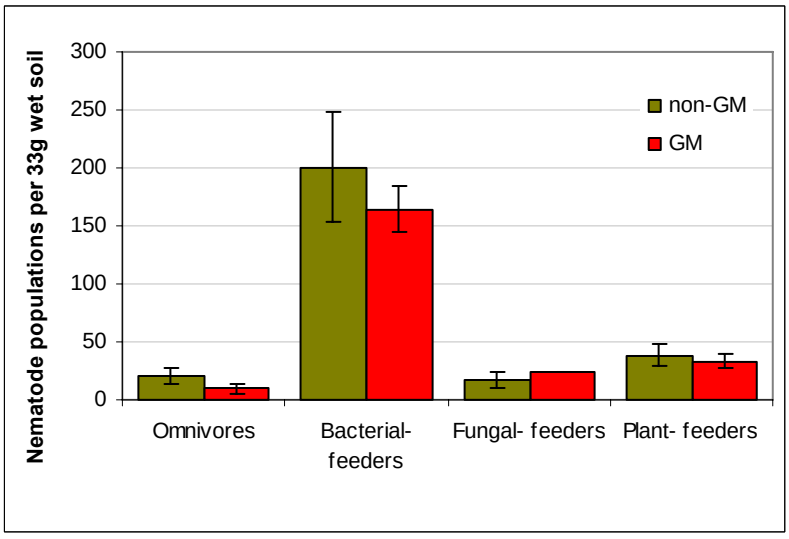


Figure 10.3.1.3 Nematode populations from rhizosphere soils of V15 and V15i plants grown in Avon soil, at 6.4 weeks harvest.

At 8 weeks, (Figure 10.3.1.4), there was a probability of independence of p = 0.001, indicating that the proportions of trophic groups between the non-GM and GM rhizosphere soils were significantly different. This difference was caused by a relative decrease in plant feeders in the GM rhizosphere soil.

The averaged plant mass of the two treatments at 8 weeks was 15.9 g for the non-GM plants, and 17.0 g for the GM plants. This was a non-significant difference from the univariate ANOVA tests on plant mass (as discussed in Section 4). As with the bulk soil, at the 3.7 and 6.4 week harvests, the bacterial feeders represented the highest nematode numbers for both the non-GM and GM plant types.

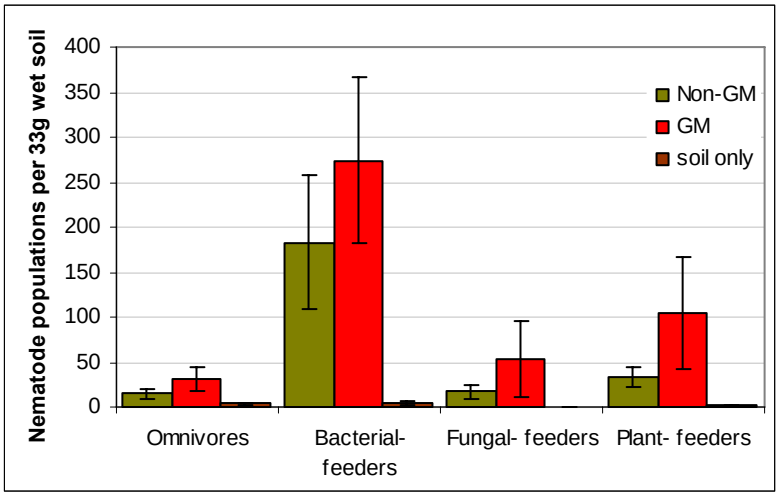


Figure 10.3.1.4 Nematode populations from rhizosphere soils of V15 and V15i plants grown in Avon soil, at 8 week harvest and comparison with non-rhizosphere soil.

Nematode numbers from non-rhizosphere soils were very low in comparison with those of the rhizosphere soils at the same time of harvest at 8 weeks. The χ^2 comparison between the non-rhizosphere soil and the non-GM rhizosphere soil at eight weeks resulted in a probability of 0.07 which indicated a marginally significant difference in population ratio resulting from the plant-driven rhizosphere effect.

Nematode populations, under nutrient deficient conditions are shown in Figure 10.3.1.5.

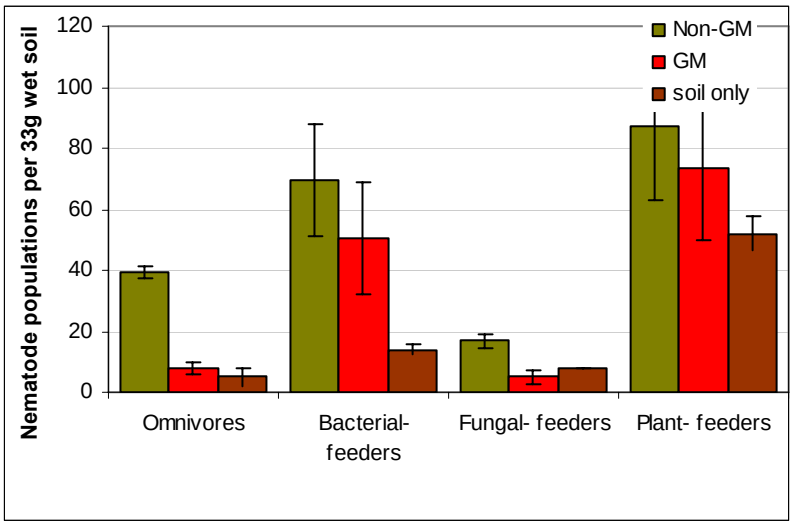
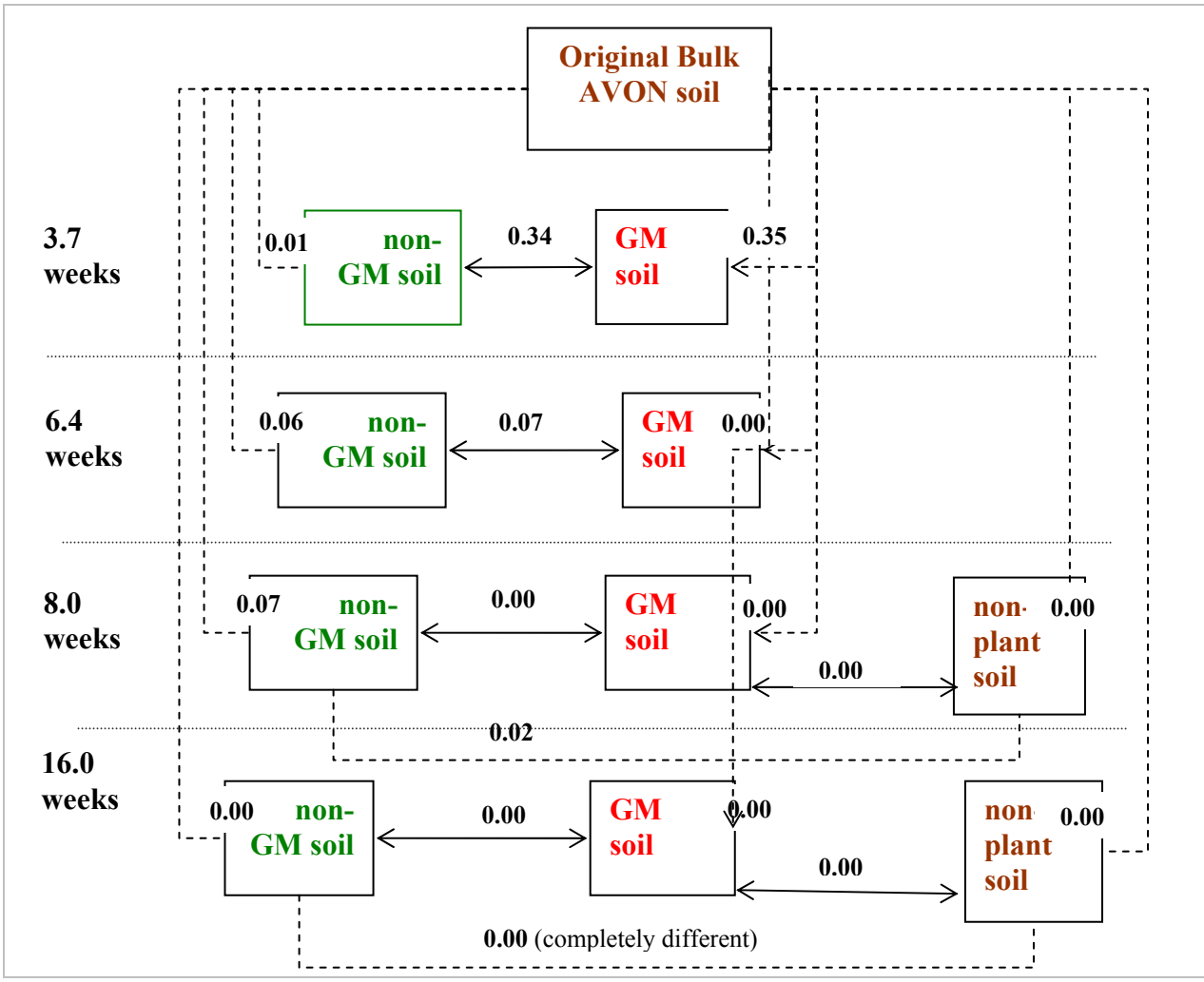


Figure 10.3.1.5 Nematode populations from rhizosphere soils of V15 and V15i plants grown in Avon soil at 16 weeks harvest, and comparison with non-rhizosphere soil.

It is notable that the plant feeders for both non-GM and GM plants had increased in number relative to the bacterial feeders.

The change in proportions of the original populations extracted from the bulk container of soil were compared with those of the extracted nematodes from the various trials, for both rhizosphere soils and non-rhizosphere pot soils kept under the same conditions. A summary of the comparisons of the χ^2 tests are shown in the flow-chart presented in Table 10.3.1.2. The difference in change of ratio between populations is shown as the number above the connecting lines between the batches, where 100% is an exact match (at $p \leq 0.05$), and 0.00 reflects a population with completely dissimilar proportions. Calculations are shown to 2 decimal places.

Table 10.3.1.2 **Comparison of change in proportions (χ^2) of nematode populations for V15 and V15i plants grown in Avon soil, and divergence of the original population over time (Significance taken at $p \leq 0.05$)**



A comparison of change in proportions of trophic groups in Avon soil at 3.7 weeks showed that the rate of change was greater for the rhizosphere of the non-GM plant compared to those of the GM plant rhizosphere, whereas proportions in the GM plant rhizosphere was closer to those of the original bulk soil. By 6.4 weeks, the rhizosphere soil populations were more similar to each other than the rhizosphere soils compared with the original bulk soil population. By the 8th week, all sets of nematode ratios in the rhizosphere soil had changed markedly. When compared with each other over time, the populations of nematodes within the non-rhizosphere pots, also quickly showed a marked degree of diversity.

Proportions of the nematode groups for the non-rhizosphere soil taken from the bulk storage bin and the soil kept damp in pots under similar conditions were completely

different at eight weeks, as shown by the χ^2 significance of independence (*p)* of 0.000. A further comparison of non-rhizosphere soils between the harvests at eight and sixteen weeks also showed dissimilar group ratios. The change in the ratio of different nematode groups could therefore occur inherently without the existence of a live plant root. While there is a divergence of populations between the non-GM and GM rhizosphere populations over the time of the trials, when the non-plant root associated soils were compared with the initial soil at the beginning of the trials, the non-plant associated soil population ratios showed more difference between each other as time elapsed, than the difference in population between the two plant types.

The rate of decline by correlation against date of harvest, averaged for the replicates per 33g soil, is shown in Table 10.3.1.3.

Table 10.3.1.3 . Correlation of nematode trophic groups as a function of harvest date, per 33g wet soil

AVON SOIL	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders
Non-GM plants (harvest date)				
V15, (3.7 weeks)	33.13	339.88	15.13	79.75
V15 (6.4 weeks)	21.00	200.75	16.75	38.50
V15 (8 weeks)	14.56	182.50	16.69	32.50
V15 (16 weeks)	39.38	69.50	16.88	87.00
Correlation over time	0.46	**-0.93**	0.67	0.38
GM plants (harvest date)				
V15i (3.7 weeks)	25.50	320.75	9.75	55.75
V15i (6.4 weeks)	10.00	164.50	23.75	33.25
V15i (8 weeks)	31.31	273.75	53.06	104.88
V15i (16 weeks)	7.58	50.71	5.04	73.71
Correlation over time	-0.56	**-0.87**	-0.26	0.33

Correlation data shows that the greatest effect came from the bacterial feeders (in bold) while the weak, positive increase in plant feeders showed that plant-feeders were unaffected by the changing rhizosphere conditions over time.

10.3.2 Extractions of nematodes from Narrabri soil

Nematodes were not extracted from the bagged soil from Narrabri prior to potting as was done for Avon, so comparisons between changes in trophic groups were made between numbers from the rhizosphere soils, with the non-plant associated soil at 3.7 weeks as the starting point.

The nematodes from the GM plant soils declined at both 7 and 8 weeks, relative to the non-GM plants such that numbers had fallen below the non-rhizosphere soil

count. However, the numbers were higher than the non-GM rhizosphere soil population at 14.8 weeks. A time series of categorised groups within the Narrabri soils is shown in Table 10.3.2.1.

Table 10.3.2.1 Time series of nematode populations extracted from Narrabri soil using the paired V15 and V15i plant rhizosphere soil, compared with non-plant associated soil. (Averaged replicates ± SEM).

Harvest time (weeks) for V15/V15i plants	Rep-licate No. (*n*)	Omni-vores	Bacterial feeders	Fungal feeders	Plant feeders	Sum of group ave.	% of Extraction from 3.7 week harvest
V15 (3.7)	4	4 ± 2	79 ± 10	10 ± 7	40 ± 19	133	} Taken as
V15i (3.7)	4	3 ± 2	70 ± 25	6 ± 1	47 ± 15	126	} 100%
soil only (3.7)	4	2 ± 1	52 ± 16	4 ± 1	34 ± 3	92	71
V15 (7)	2	22 ± 11	224 ± 111	25 ± 13	59 ± 29	330	329
V15i (7)	2	7 ± 2	271 ± 86	23 ± 5	6 ± 3	307	306
soil only (7)	2	2 ± 0	41 ± 7	5 ± 1	9 ± 1	57	29
V15 (8.1)	4	16 ± 3	205 ± 32	25 ± 13	35 ± 9	281	281
V15i (8.1)	4	24 ± 9	98 ± 12	13 ± 4	3 ± 1	138	138
soil only (8.1)	2	7 ± 0	104 ± 22	29 ± 4	10 ± 5	150	210
V15 (14.8) (nutrient deficient)	3	8 ± 3	31 ± 20	20 ± 10	5 ± 1	64	64
V15i (14.8) (nutrient deficient)	4	8 ± 1	19 ± 4	10 ± 2	3 ± 1	40	138
soil only (14.8)	1	1 ± 1	10 ± 6	6 ± 2	6 ± 2	23	31

The nematode population in Narrabri soil at 3.7 weeks is shown in Figure 10.3.2.1.

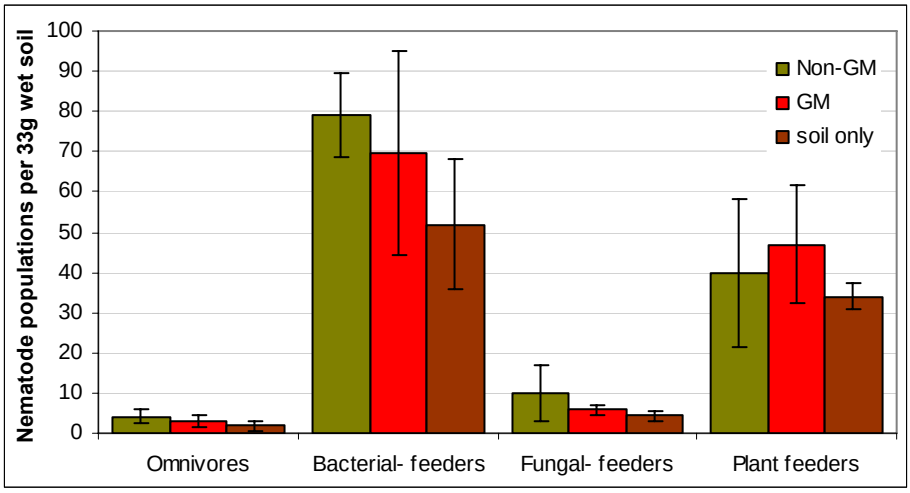


Figure 10.3.2.1 Nematode populations from rhizosphere soils of V15 and V15i plants grown in Narrabri soil, at 3.7 weeks harvest, and comparison with bulk soil.

The probability of trophic group independence by χ^2 analysis between the non-GM and GM rhizosphere soils was 0.51 at 3.7 weeks (not significant at $p \leq 0.05$).

At the 7 week harvest (Figure 10.3.2.2), the probability of independence for the group ratios of $p = 0.00$ described significantly different proportions between the non-GM and GM nematode populations. The change was due to a higher number of plant feeders and omnivores, and a relative increase in the bacterial feeders within the GM rhizosphere soil.

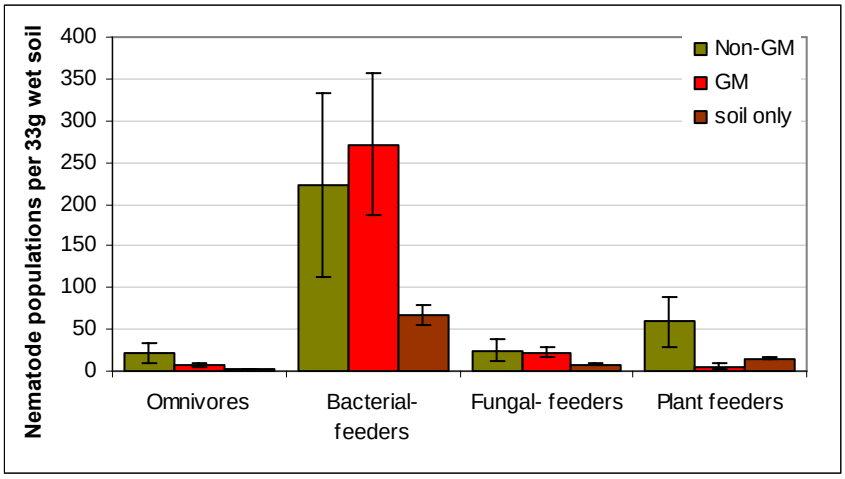


Figure 10.3.2.2 **Nematode populations from rhizosphere soils of V15 and V15i plants grown in Narrabri soil at 7 week harvest, and comparison with non-rhizosphere soil.**

Populations of nematodes in the soils at 8 weeks are shown in Figure 10.3.2.3.

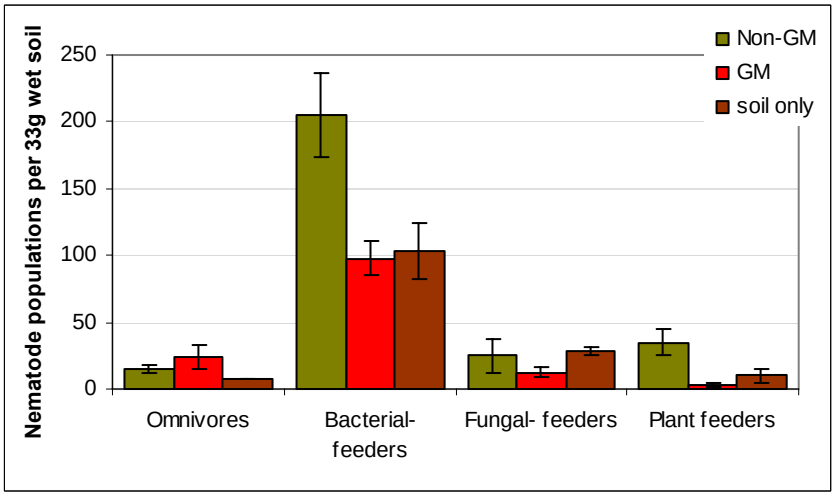


Figure 10.3.2.3 **Nematode populations from rhizosphere soils of V15 and V15i plants grown in Narrabri soil, at 8.1 weeks harvest and comparison with non-rhizosphere soil.**

At eight weeks, a large decrease in numbers of bacterial feeders appears, at first view, to differ from the non-GM bacterial feeders. The total harvest however was proportionately smaller over the bacterial feeders, fungal feeders and plant feeders, so the total harvest was less for this batch. Less noticable from the counts was the continued decline of GM plant feeders, relative to non-GM soils, with the χ^2

probability again being $p \leq 0.00$, caused by the change in proportion. The difference in plant feeders relative to the remaining population is addressed in the Discussion (10.4).

The 14.8 week harvest (Figure 10.3.2.4) showed the lowest numbers of nematodes of all trials. The Narrabri non-GM and GM plant nematode populations showed a probability of $p = 0.75$, showing more similarity between the ratios of the non-GM and GM populations. This is because the ratios were again similar, even though the total numbers had fallen. Table 10.3.2.2 provides a summary of the χ^2 tests.

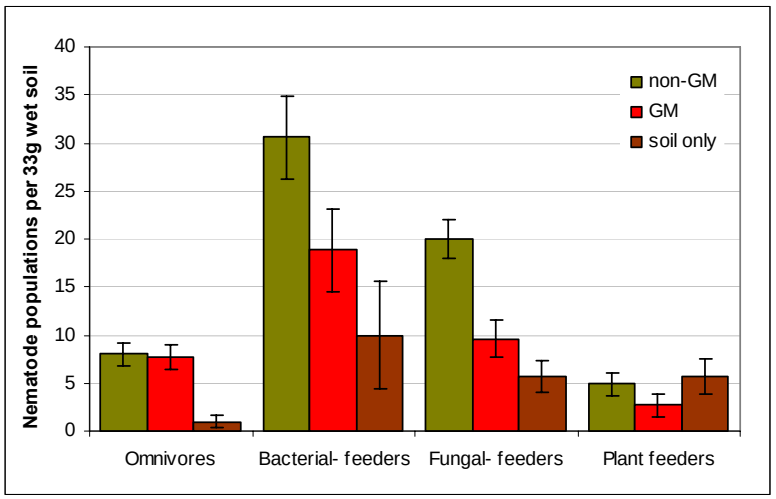


Figure 10.3.2.4 **Nematode populations from rhizosphere soils of V15 and V15i plants grown in Narrabri soil, with comparison of bulk soil, at 14.8 week harvest, grown under nutrient limited conditions.**

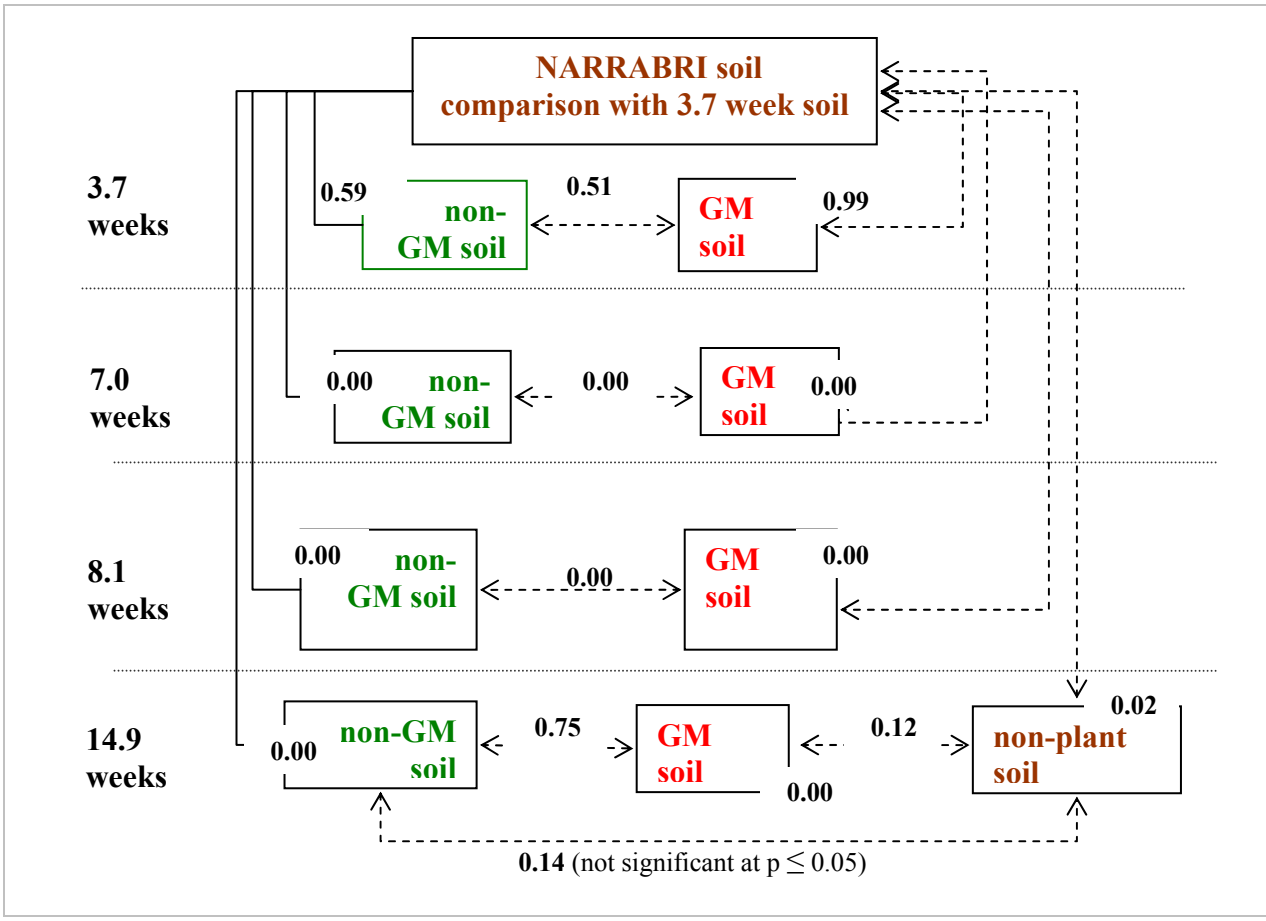
The rate of decline by correlation against date of harvest, averaged for the replicates per 33g soil, is shown in Table 10.3.2.2.

Table 10.3.2.2. **Correlation of nematode trophic groups as a function of harvest date, per 33g wet soil**

NARRABRI SOIL	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders
Non-GM plant (harvest)				
V15 (3.7 weeks)	4.13	79.13	9.88	39.75
V15 (7 weeks)	21.75	223.50	24.50	59.00
V15 (8 weeks)	15.50	205.33	25.00	34.83
V15 (14.8 weeks)	8.00	30.63	20.00	4.88
Correlation over time	-0.03	**-0.42**	0.40	**-0.79**
GM plant (harvest)				
V15i (3.7 weeks)	2.75	69.63	5.83	47.00
V15i (7 weeks)	7.00	271.25	22.50	5.50
V15i (8 weeks)	24.33	98.00	12.67	2.83
V15i (14.8 weeks)	7.67	18.83	9.58	2.67
Correlation over time	0.13	**-0.40**	-0.01	**-0.70**

Correlation data shows that the strong positive influence in bacterial feeders shown in Avon soil (Table 10.3.1.3) was quite different from the Narrabri soil trials. Figure 10.3.2.2 shows an insignificant negative effect in bacterial feeders, and a stronger decline in the plant feeders (both in bold). Both non-GM and GM plant rhizosphere soils were very similar, which indicates that the environmental conditions were stronger than the effect of the plant over the 14 weeks of the trial.

Table 10.3.2.3 **Comparison of change in proportions (χ^2) of nematode populations for V15 and V15i cotton plants grown in Narrabri soil, and divergence of the original population over time. (Significance taken at $p \leq 0.05$)**



A comparison of nematode trophic group proportions from Narrabri rhizosphere soil at 3.7 weeks showed a greater similarity between the GM-rhizosphere soil and the non-rhizosphere soil, than the non-GM and GM rhizosphere soils. This difference was not observed at seven weeks, when it was shown that significant differences (p <= 0.05) occurred between the nematode groups for both rhizosphere soils, shown by the χ^2 significance of independence (*p)* of 0.000. The independence of proportion between trophic groups within the non-GM and GM rhizosphere soils and the non-rhizosphere soils continued at 8.1 weeks, but at 14.9 weeks there was a more marked similarity between each of the non-GM and GM rhizosphere soils than the control

(non-rhizosphere) soils. This was possibly because of the senescence of the plant root, and a similarly changing series of saprophytes.

10.3.3 Comparison of trends for the time series trials for nematode populations from Avon and Narrabri soils, for V15/V15i plants

The comparison of the proportional change of the nematode populations over the time series is shown in Figure 10.3.3.1 for Avon soil, and Figure 10.3.3.2 for Narrabri soil. This allows comparison of separate groups relative to each other, rather than the χ^2 comparison, which takes all four groups into account. The groups are mirrored for comparison of the non-GM and GM soils.

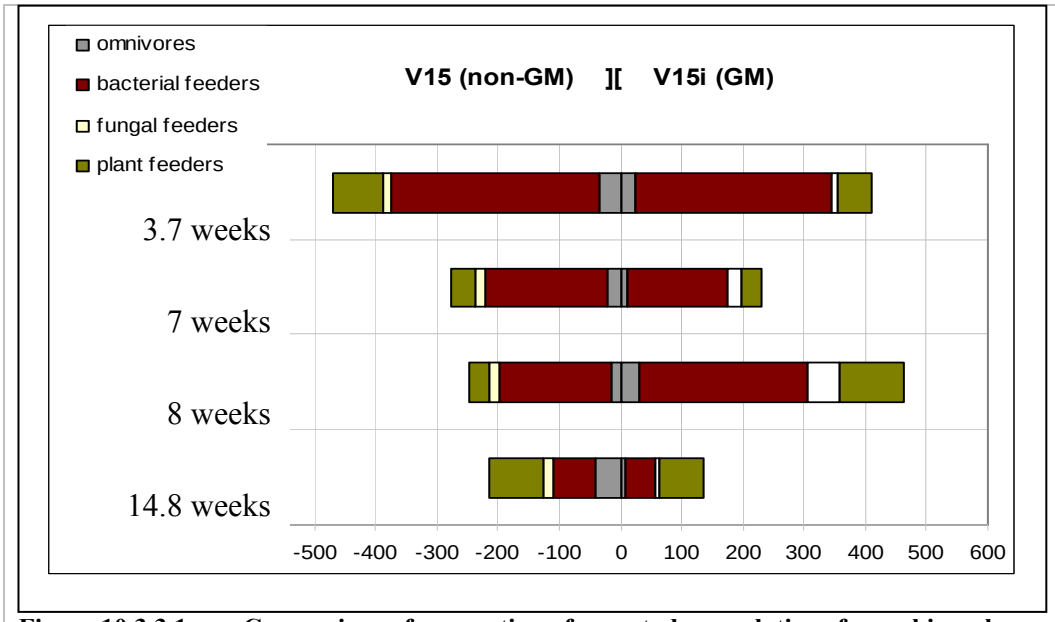


Figure 10.3.3.1 **Comparison of proportion of nematode populations from rhizosphere soils of V15 and V15i plants grown in Avon soil for 3.7, 7, 8 and 14.8 weeks.**

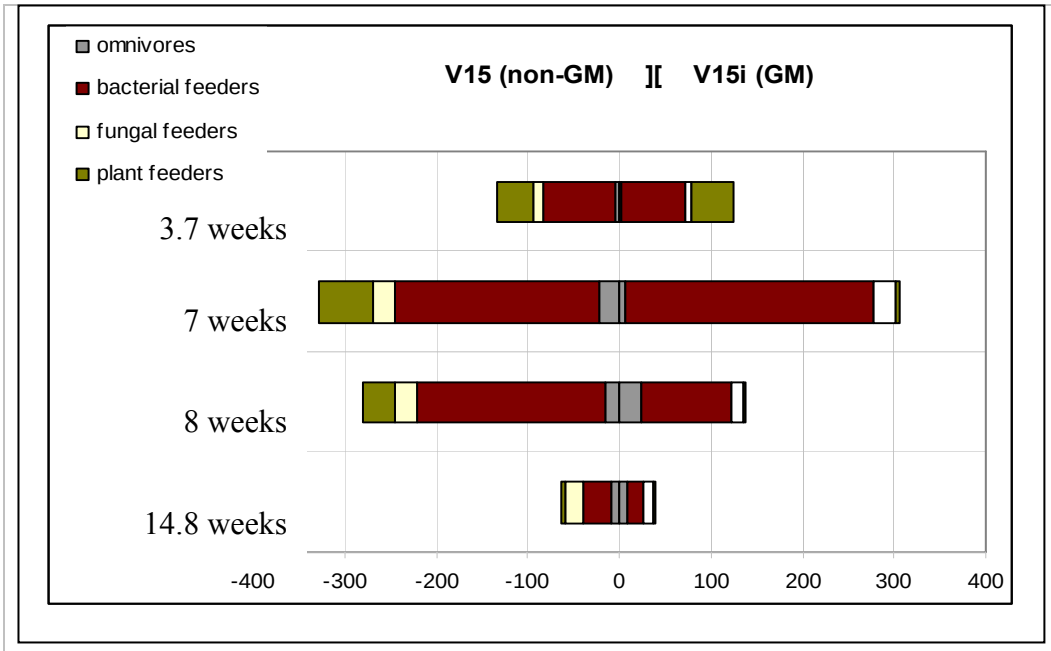


Figure 10.3.3.2 Comparison of proportion of nematode populations from rhizosphere soils of V15 and V15i plants grown in Narrabri soil for 3.7, 7, 8 and 14.8 weeks.

Allowance must be made for a differences in total harvests where all numbers are proportionally higher or lower. Even so, it can be seen from the proportional comparison that neither the omnivores, bacterial feeders nor fungal feeders were affected by the difference in plant cultivar in the Avon trial.

In comparison, there was a difference in proportion between the trophic groups of nematodes in the non-GM and GM rhizosphere soils. The population of plant-feeders from the non-GM plants remained constant from the original averaged 39 nematodes per 33g soil, to 59 at 7 weeks, and 34 at 8 weeks. This constant rate decreased at the 14.8 week harvest when the plants were depleted of nutrient and had stopped growing. The GM rhizosphere soils of Narrabri in comparison, showed a decline in root feeders, from the original averaged 47 nematodes per 33g soil, to 5 at 7 weeks, and 2 at 8 weeks respectively, before declining further (allowing for the modulus of the average) to 2 at 14.8 weeks. This is distinctly different from the Avon soil trials.

10.3.4 Extractions of nematodes from soil in and surrounding litterbags

The effect of *Bt* proteins on nematode populations in degrading leaf litter was investigated by growing C312 and C312i plants to nine weeks in Avon soil. (Note: this is a different cotton cultivar from the V15/V15i plants used for the time series.)

The result of the harvest from the rhizosphere soil (around the live roots before watering was stopped) showed the proportions of nematode groups as per Figure 10.3.4.1. The probability of independence of group ratios was 0.99, which described a very similar population between the C312 and C312i paired plants.

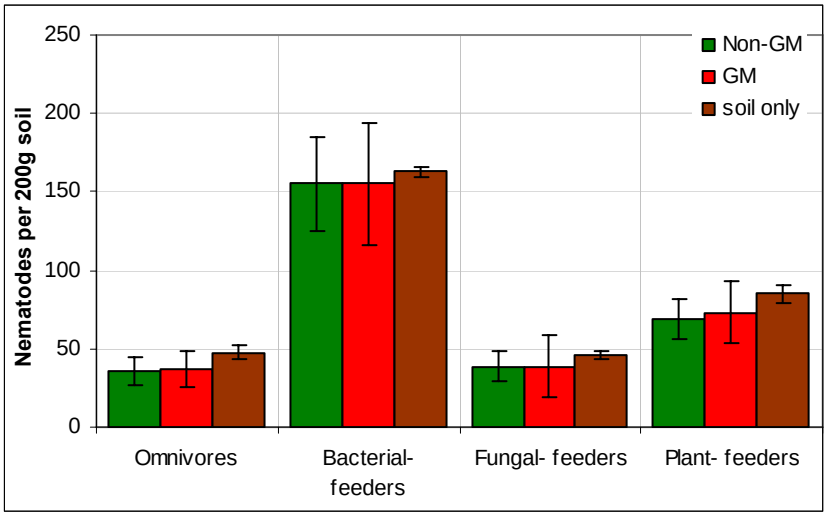


Figure 10.3.4.1 **Nematode populations from rhizosphere soils of C312 and C312i plants at 9 week harvest from Avon rhizosphere soil, and comparison with non-rhizosphere soil.**

The nematode populations from litterbag exhumations after two weeks showed a similarity between the C312 and C312i plant rhizosphere soils (Figure 10.3.4.2). The proportions of the groups between the non-GM and GM rhizosphere soils showed a probability of independence at p = 0.39.

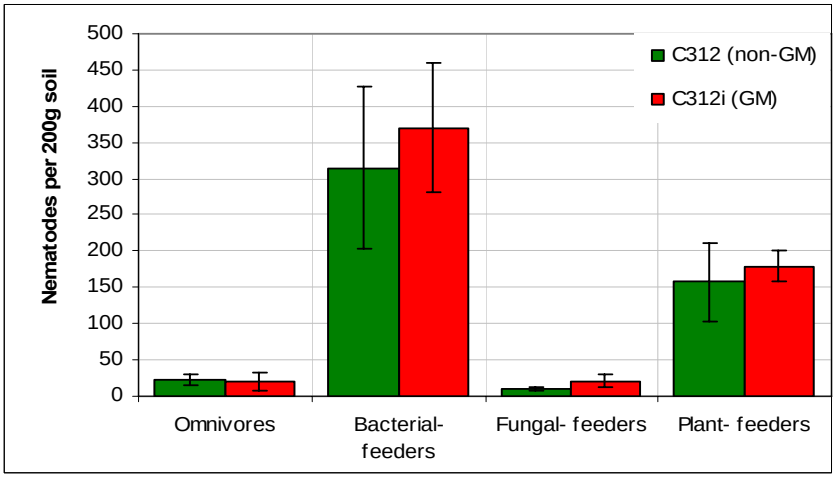


Figure 10.3.4.2 **Proportion of nematodes in leaf litter of C312 and C312i plants at 2 week exhumation from Avon soil.**

Figure 10.3.4.3 showed a similar trend at 4 weeks. The group ratios had a probability of similarity of 0.98 between the non-GM and GM nematode populations.

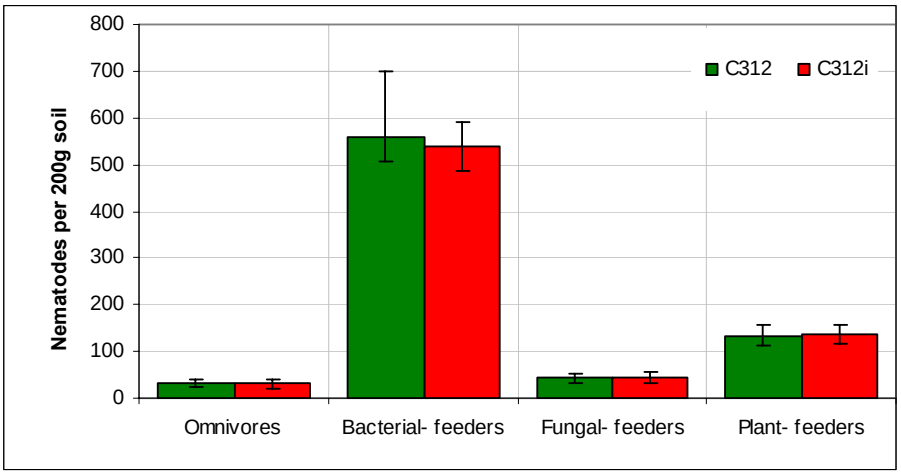


Figure 10.3.4.3 Proportion of nematodes in leaf litter of C312 and C312i plants at 4 week exhumation from Avon soil.

There was an increase in numbers of extracted nematodes compared with the two-week exhumation, and also an increase in the proportion of fungal feeders. Plant feeders decreased slightly as litter was broken down. The proportions were very similar between the two week extraction and the four week extraction.

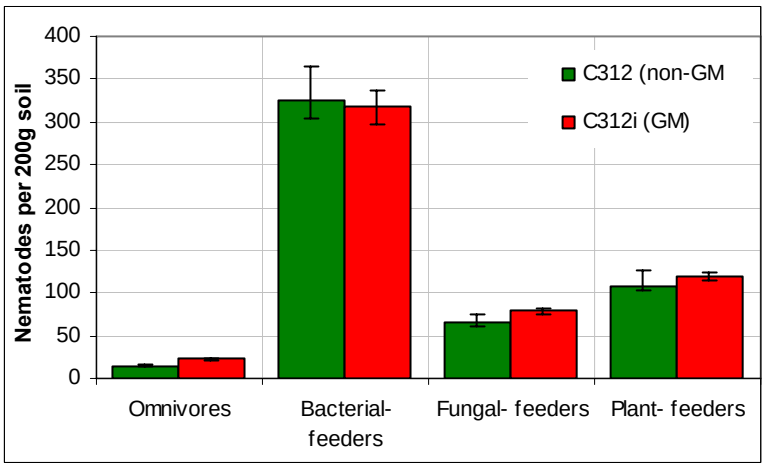


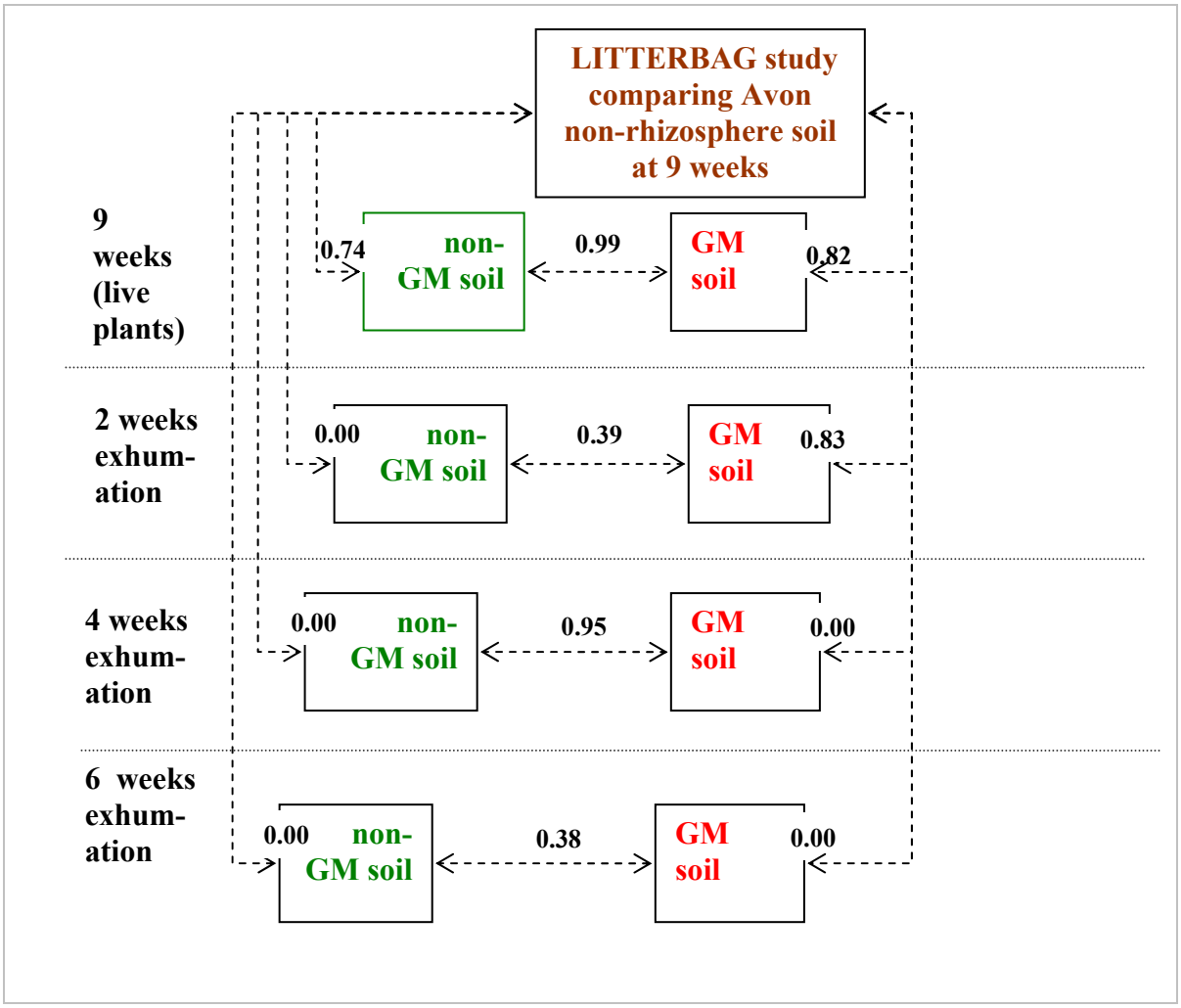
Figure 10.3.4.4 Proportion of nematodes in leaf litter of C312 and C312i plants at 6 week exhumation from Avon soil.

The trend of more similarity between the decomposing litter within the non-GM and GM plant litterbags continued from the original harvest of the live root until the 6 week exhumation (Figure 10.3.4.4).

The extracted numbers were again similar to the two-week exhumation, however the fungal feeders had increased from 10 and 20 for the non-GM plants, and from 60 and 80 for the GM plants respectively. The GM plant bacterial feeders decreased to less than the non-GM bacterial feeders from 330 to 320 at this extraction. The plant feeders had increased slightly in relation to the bacterial feeders. These changes

meant that the proportions had changed to a p value of 0.37 between the non-GM and GM rhizosphere soils, in and around the litterbags, which was not significantly different. The change in group ratios for the nematode populations from the litterbag trials is shown in Table 10.3.4.1.

Table 10.3.4.1 **Change in proportions (χ^2) of nematode populations for C312 and C312i plants grown in Avon soil, and comparison of the nematode population in, and surrounding, litterbags with non-GM and GM leaf litter. (Significant difference taken at $p \leq 0.05$)**



10.4 DISCUSSION

The numbers of nematodes obtained by Bird (1999) from a field at Avon was 1,400 per litre of moist soil, using the misting method. This compared with an averaged 23,242 nematodes per kg of dry Avon sampled soil described here, using the Whitehead tray method. To convert the averaged numbers of nematodes per 1kg of soil to the litre of Avon soil used by Bird, the numbers of nematodes from 1kg soil was divided by 1.03 which was the calculated density of Avon soil as per the mechanical analysis described in Section 3. The soil moisture content probably

differed, as Bird reported that the sampled soil was moist after rain, but even allowing for 16% which was the difference between the 18% moisture capacity of Avon soil (Section 2) minus the 2% measured moisture at time of extraction, the nematodes extracted by the Whitehead tray method resulted in an increase of 13 times that of Bird's method. This, however may have included the effect of nematode build-up after wheat in the sampled soil used here.

A comparison of the total numbers between the Avon total nematode population at 8 weeks showed 277 nematodes per 33g soil for the non-GM rhizosphere soil and 231 for the GM population, compared with the Narrabri harvest of 280 nematodes per 33g non-GM soil with 237 for Narrabri soil at 8 weeks. This similarity in numbers occurred in both soils even though the soil textures were quite different.

Total population numbers declined from the three week harvests to the 14.7 week harvests and 16 week harvests, for the time trials of both Avon and Narrabri soils. The major proportional change was caused by the bacterial feeders in relation to the omnivores, fungal feeders and plant feeders, with a slower, but simultaneous increase in the fungal feeders and plant-associated feeders. The decline in bacterial feeders possibly occurred because bacterial feeders reproduce faster than plant parasites, and reflect the r-strategist life cycle where population growth, as well as decline under adverse conditions, is faster. Similar results of rapid increase of bacterial feeders in response to low C:N plant materials fueled by resident soil organic matter, and increase of fungal feeders was found by Ferris & Matute (2003) who measured nematode populations in soil after the addition of mineral fertilizer and organic materials of varying nature, but without living plant roots. Ferris & Matute (2003) also found a succession of bacterivore to fungivore nematodes as the C:N ratio had changed from low to high, over the time of the decomposition of the soil organic matter. It was proposed that an enrichment index for soil could be used, based on the proportion of enrichment-opportunist bacteriovore nematodes.

However another notable trend occurred with a relative decline in the plant feeders within the GM rhizosphere soils of Narrabri compared with the non-GM plant rhizosphere soils. Whether the disproportionate decrease in the plant-feeding nematodes from Narrabri soil at the 7 and 8 week harvests is due to susceptibility of this group to the different plant roots of the non-GM and GM plants, is not known.

However non-GM and GM plant feeders were also lower in proportion at the 14.8 week harvest, so the effect may not have been due entirely to the different plant cultivar. Not all plant feeders were totally absent in the Narrabri rhizosphere soils at 7 and 8 weeks, so there may have been an effect at the species level, that was not represented across the trophic subset. Further research using different methods of identification at the species level of the nematode ecotype from Narrabri soil would be needed to confirm this.

It is possible that different ecotypes of nematode populations occurred between the Avon and Narrabri soils, given the different soil characteristics, the different histories of cropping and the isolation of the sampling sites. There were more pratylenchids in the Avon than the Narrabri soils, for both the non-GM and GM rhizosphere soils. This sub-group was not scored separately from the total plant feeders, but *Pratylenchus* spp. are known to be migratory endoparasites, with wide host ranges and all stages move between soil and roots, and species of this genus prefer coarse textured, sandy soils (Cohn et al. 1996). This would partly explain the higher plant feeders overall in the Avon soil. Additionally, within the class of Dorylaimids, Mononchids (predators) were observed at the 7 week harvest of Narrabri soils, for both non-GM and GM plants, but not for the Avon soils.

Susceptibility of the native populations to the *Bt* proteins, may also vary across geographical areas and control will require a knowledge of the individual ecotypes of nematodes within different agricultural areas, as well as the influences of other integrated pest management systems. Doucett (1988) noted that it is necessary to know the identity of the different nematodes present, since the role played by each species may vary considerably even though they may be present in similar numbers. The application of inorganic fertilisers and methods of irrigation also contribute to variability in soil microbial populations (Ferris, 1982). Each species, and sometimes even each population, has a set of peculiar characteristics that are frequently influenced by the environment, the host plant and by the cultivation system. On this point, Ferris (1982), found that plant parasitic nematodes comprised 21% of a nematode population in an undisturbed ecosystem compared to 35% in a disturbed environment.

Nematode bacterial-feeders are more likely to be present in larger numbers where the C:N ratios are ~10-15, and fungal hyphae feeders are more likely to dominate where the C:N ratio is >15 (Yeates & Boag, 2004). It follows from the greater decline in bacterial feeders and slight increase in fungal-hyphae feeders both for the non-GM and GM rhizosphere soils over time, that a greater effect was seen for the change in C:N ratios over time, than from the characteristics of plant cultivars.

Nematodes were documented by Rukmini et al. (2000) to be susceptible to the *Bt* Cry4D protein, but not Cry1A(c) which is expressed by GM cotton plants. Wei et al. (2002) documented nematode susceptibility for the Cry21A, Cry5B and Cry14A *Bt* crystal proteins. Given the different ecotypes of nematode populations it could be possible that different populations differ in susceptibility to the various *Bt* proteins. Even so, the slow increase in plant feeders over time in Avon soil for both non-GM and GM rhizosphere soils showed the adaptability of the plant feeders to cotton after wheat/wheat rotations, where cotton had never been grown in Avon soil, so adaptability and resistance is potentially problematic in their control.

The change in nematode trophic groups in the litterbag trials showed that the proportions of the nematode populations were more similar between the non-GM and GM (C312 and C312i) cotton plants at the 9 week harvest, than the non-rhizosphere soil, kept under similar conditions. This is not surprising, as at 6 weeks the photograph of exhumed litterbags showed cotton tissue was already largely degraded (Section 6). Any remaining organic matter from the non-plant associated soils at 9 weeks would contain less readily available organic matter, and it follows from the Avon and Narrabri trials that bacteriophores would decrease as bacteria were less abundant. The similarity in population ratios continued for the 4 and 6 week exhumations where there was more similarity between the soils associated with leaf litter for both the non-GM and GM plant detritus than the non-rhizosphere soils. This agrees with Donegan et al. (1995) who reported that a significant increase in bacterial and fungal numbers was nearly always found by the provision of nutrients from plant material in soil, and the carrying capacity of nematode populations would increase because of this. Any toxicity to the Cry1A(c) protein would not have existed at this time, as it was shown by ELISA that the *Bt* protein had degraded from the leaf litter previous to the 2 week exhumation, as soon as water was added. The differing nematode population ratios between litterbags and rhizosphere soils

adjacent to a living plant root implied that the microbial systems were sufficiently different to justify the separate analyses, and that the method of comparing soil microbiota from buried plant tissue does not necessarily correlate well to microbial population dynamics from rhizosphere soil.

At the end of the trials there were still live nematodes of all trophic groups within both soils, indicating that any susceptibility of nematodes to the plant-produced *Bt* protein did not remove of any of the categorised groups, taken from the indigenous population. Because the nematodes were not fixed (killed), the life-cycle of at least some nematodes could be observed over a longer period than the length of the trials. The remnants of the original bacterial-feeding cohort from both the Avon and Narrabri trials showed a few surviving free-living individuals after five months storage in water at 4ºC. Plant-feeders from both non-GM and GM soils however, had died and degenerated. If the bacterial feeders had been affected by *Bt* toxicity within the water, even at a lower concentration than within the plant root, the GM-soil should have contained lower nenatode numbers, but this was not the case.

Independent results from other investigations by Saxena and Stotzky (2001) showed no significant differences in the (uncategorised) total populations of nematodes between the soils of non-GM and GM rhizosphere soils in which biomass of *Bt* and non-*Bt* corn was buried. Recent work by Manachini & Lozzia (2002) and Manachini et al. (2004) also found that Cry1A(c) gene expressed by cotton leaves did not appear to have any significant influence on the nematofauna, either at the level of genus nor with regard to biodiversity, even though one of the regions under study recorded a change in trophic group composition. Whether there was any effect on the remaining annelids, archiannelids, crustaceans, insects, molluscs, nematodes, tardigrades and turbellarians described by Bird (1999) is not known. However, the results of the pot trials showed a slight yet significant relative decrease of the nematode populations in the GM cotton plant rhizosphere soil of Narrabri, (with no corresponding decrease in numbers for the same GM plant in the Avon soil trial). This suggest that the cohort, or their predators, may have differed between the two soils, as the Avon nematode population did not show a toxic effect from the roots of the same plant cultivars.

10.4.1 Additional general observations within this work

Colyer et al. (2000) revealed that some GM cotton cultivars such as Paymaster 1560BG are more susceptible to root-knot nematode damage than its non-GM parent, Paymaster 1560 in America, however McLeod et al. (1994) recorded cotton as a host plant of *Aphelenchus* and *Pratylenchus thornei* as well as other *Pratylenchus* species, but did not record the root knot nematode as a host plant in the (Australian) New South Wales study. There were no root knots on any of the harvested cotton roots grown in Avon or Narrabri soils, from either non-GM or GM plants. This shows that *Meloidogyne* was not present in the sampled soils, as cotton was shown as a host plant in the study by McLeod et al. (1994). Comment on susceptibility to the GM plant to the root-knot nematode from Narrabri and Avon soils therefore cannot be made.

The focus of this section was the nematode subset of soil mesofauna. Other soil microbiota which was observed opportunistically and is part of the microbial soil community which may have affected nematode populations follow.

Tardigrade observed in one of the Avon soils

Microscope observation during the nematode counts at 3.7 and 6.4 weeks showed tardigrades were present in all Avon soils sampled, for both non-GM and GM plants, but not from any of the Narrabri soils. Conversely, Mononchids were present in Narrabri sampled soils, but were not observed within the Avon samples, for either of the paired plants. This showed a predominance of different nematode predators from the two sampled sites. Where soil particle size limits dispersal it can be a controlling factor in the heirachy of a community (Rolstad, 1991). The length of adult Mononchid nematodes are almost as long as the tardigrades, but the body width approximates 35µm, compared to about 130µm for Acari (Begon et al. 1990, p. 368). The narrower channels from the smaller particles of the Narrabri soil may have given the nematode predators an advantage, even though the food source of microfauna was abundant in both Avon and Narrabri soils.

Tardigrades are also predators of protozoa, but the method of harvesting protozoa by dilution of 5g of soil did not result in observation of tardigrades. Tardigrades were probably present as the same sampled soil was used for extraction of all soil

microbial communities. The 20µm sieve used to harvest nematodes was effective in trapping tardigrades, so an effect of the method of harvest, and the concentration of animals from the 200g volume of soil used to harvest nematodes, revealed aspects of the community that predation from relative density of the prey species alone, did not show. In future studies, the water that passed through the 20µm nematode sieve could be subsequently passed through a 5µm sieve to collect protozoan populations. This would be especially helpful for the cilliates, which were not observed in some of the replicates from the 5g soil sample, possibly because the dilution was too high for these animals.

10.4.2 Effect of pot trials on change in nematode population

The pore space within Avon soil was determined in Section 3, and the calculation of volume from density of freshly harvested plant roots showed that while there was some pore space remaining, the roots within the pots were a thick mass. Some of the space through which nematodes could move would have been taken up by the accumulation of roots and root hairs between the soil interstices. As a nematode can move most effectively where the diameters of the soil particles are about one third of its length (Nicholas, 1975) movement may have become restricted within the limits of the space within the pot. Considering that soil type, change of moisture, restriction of space and presence of a plant root itself can all effect nematode populations, consistent difference was not explained by the presence or absence of the *Bt* protein for either Avon or Narrabri soils.

The arbitrary nature of categorising functional nematode groups by light microscopy according to mouthparts and esophagus is challenged by several *Tylenchidae* that can be reared on fungal hyphae (Okada & Kadota, 2003), and 'omnivores' and 'predators' adapt similarly to environmental perturbations (Yeates et al., 2003). This may be resolved in future by the use of molecular methods currently under development such as the sequencing and identification of bacteria, protozoa or fungi from nematode gut contents (Winkler & Adams, 2004).

Further development of a method used by Jones & Wang (2003) could be used in future studies to investigate plant response to nematode root attack, using Affymetrix Arabidopsis ATH1 GeneChips. These arrays contain 24,000 genes of the sequenced *Arabidopsis thaliana* plant. About 10% of the 24,000 genes arrayed changed in

expression by at least two-fold when uninfected root tissue and infested nematode gall tissue were compared. When similar micro-arrays become available for cotton plants, a plant response to root-invading pathogens will become possible and could give valuable information of infection and development of root disease.

As noted, other soil animals were also present in the rhizosphere soils that were not represented by methods used here. Additionally, the remains of soil inhabitants which could have been affected by *Bt* toxicity are disintegrated by bacteria and fungi after death, and so the microbial biomass may not alter as it would be converted to that of the saprophyte (Dropkin, 1989). Measurements of soil community function were therefore undertaken to examine some of the major respiratory and enzymatic functions of the whole soil community 'in situ'. These are detailed in the chapter following.

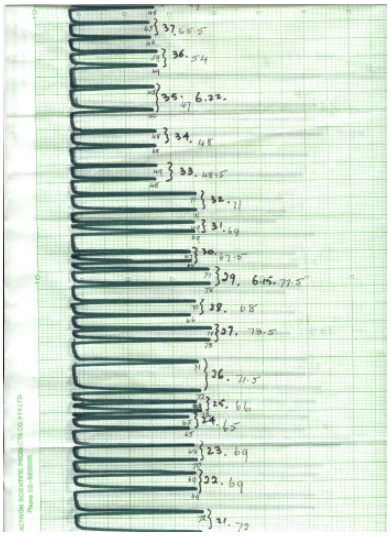
SUMMARY OF CHAPTER 3

This work covered the increasing complexity of soil microbiota from prokaryotes (bacteria), symbiotic and other soil eukaryotic microflora (fungi), single-celled motile microfauna (protozoa) and through multi-cellular eukaryotes (nematodes). No consistently significant differences between the rhizosphere microbial communities of the non-GM plants and their GM counterparts was found for the populations examined. The methods used were based on direct counting and estimated populations from stepwise dilution of the soils sampled. Growth of bacteria and fungi on enriched and selective media, and of protozoa under conditions of abundant nutrients, may not accurately simulate rhizosphere soil activity, as predation and competition are largely removed.

Functional diversity of microbiota present and active in the soil may be more illustrative of the processes of nutrient cycling than the characterization of selectively isolated populations that may not be expressed in soil and may have no bearing on soil processes (Lee & Pankhurst, 1992). Soil respiration, rate of nitrification, soil enzyme assays and other comparative tests for the whole soil microbial community follow in Chapter 4.

CHAPTER FOUR

TESTS TO DETERMINE COMMUNITY METABOLIC DYNAMICS



Section 11

Substrate-induced respiration

Fig. 11.1 Trace of CO_2 peaks from gas analyser

11.1 INTRODUCTION

All aerobic, active living cells respire CO_2 as a by-product of the transformation of organic matter (Alef & Nannipieri 1995, p. 214). The rate of rhizosphere soil microbial respiration is affected by the net photosynthesis according to plant type and nutrient status, and '… any perturbation of the biological equilibrium of the soil alters the nature and rate of the metabolic processes in action and the release of CO_2' (Golebiowska and Pedziwilk, 1984). If the non-GM or GM plants differ in their exudates due to different metabolic activities, a variability in soil microbial response may occur, and CO_2 as a measure of respiration, could be indicative of overall differences in the respiratory activity of the total rhizosphere soil population.

However, different microbial populations respire at different rates (Metting, 1993, p. 532), so the contribution of the different groups of microbiota in the soil cannot be differentiated by the measurement of CO_2. Devare et al. (2004) found no difference in soil respiration activity between *Bt* and non-*Bt* plots for corn grown in Freeville, New York, but did not use the substrate induced respiration method of measuring the opportunistic bacteria capable of rapid growth.

The method of temporarily increasing the pulse of CO_2 from respiration, due to readily available C from glucose, can measure the response of the r-strategists, or opportunistic bacteria. Respiration by rhizosphere soil microbial communities can then be compared for the effect of different plants. This method was used by Bowen and Rovira (1999) to detect differences in bacterial consortia between rhizosphere

soil samples of wheat and clover plants, which were found to be different from non-rhizosphere isolates.

To determine whether the microbiota in the rhizosphere soils differed in the rate of energy conversion, CO_2 was measured as an indicator of total microbial respiration, using readily utilisable glucose.

11.2 MATERIALS AND METHODS

11.2.1 Measurement of CO_2 from rhizosphere soils of non-GM and GM plants

A generalised procedure based on the method of Sparling et al. (1981) was used. Freshly harvested rhizosphere soils were stored in sealed glass containers, and kept at 8ºC overnight until processing next morning.

For each of the paired trials, either 4 g rhizosphere soil was weighed into 20 ml McCartney bottles, or 10 g soil was weighed into 100ml Ehrlenmeyer flasks. The precise volumes of each of the containers were determined by displacement of water. The containers with the weighed soil were sealed and stored at 8ºC overnight. Soil moisture was calculated after samples were oven-dried overnight, and all soils were brought to approximate field capacity by addition of RO water.

Five g glucose was diluted in 100 ml RO water and delivered at the rate of 5ml per 20 g soil to give 12.5mg glucose per g dry soil equivalent. The same volume of deionised water was added to the controls. The glucose solution and soils were vortexed until thoroughly mixed. After an initial 30 minutes pre-incubation for the Ehrlenmeyer flasks, and immediately for the McCartney bottles, suba-seals were applied. The soils were incubated for five hours at 25ºC, in a darkened room.

The CO_2 analysis was undertaken using a Gow Mac gas chromatogram, series 225. The internal gas reference was made by averaging three volumes of 0.5 ml standard CO_2. Five ml duplicate volumes of gas were measured for each of four replicates, for each of the non-GM and GM plant soils sample containers (eight measurements per paired plant type). Two non-rhizosphere soil samples, kept under the same conditions for each batch, were used to compare the rhizosphere effect. The units of measurement were calculated as μg CO_2 per hour, per gram dry soil equivalent.

A univariate ANOVA using SPSS® software was used to compare the variances between the efflux of CO_2 per hour, per g dry soil equivalent, for the paired trials

(Appendix 1). The CO_2 measured from the controls (water only) was subtracted from the amount released from the glucose-amended samples to give a measure of the difference due to the increased respiration from the addition of glucose.

11.3 RESULTS

When the respiration rates of rhizosphere soils within the paired trials were compared against the non-rhizosphere soils, all had a higher efflux of CO_2, confirming an increased rate of activity within the plant-associated micro-environment.

11.3.1 Comparison of soil microbial respiration for different plant strains within rhizosphere soils

The comparisons of CO_2 efflux are shown in Figure 11.3.1.1 for Avon soil.

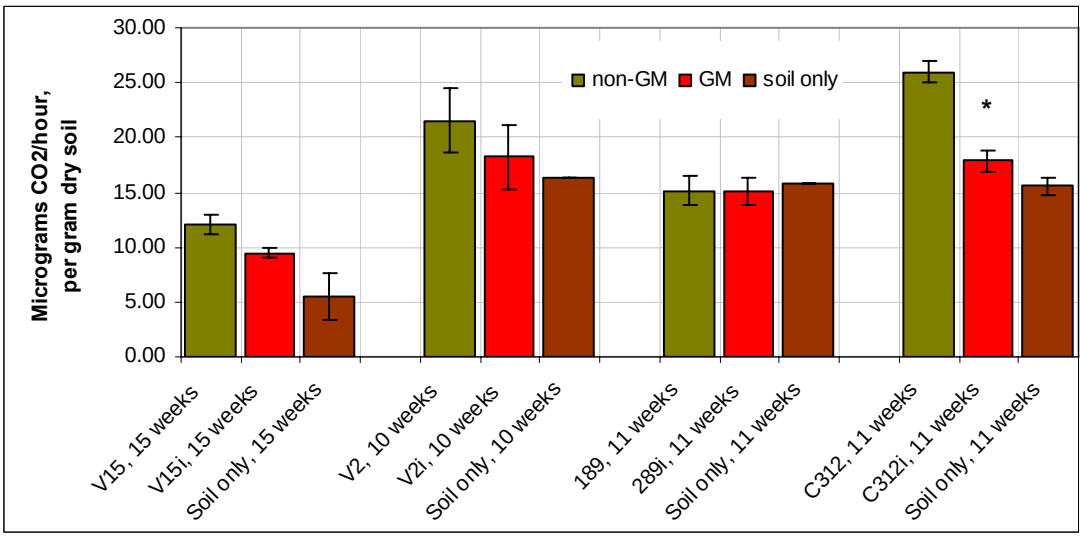


Figure 11.3.1.1 Substrate-induced respiration in Avon soil for the four paired cotton plant rhizosphere soils and non-plant associated soils, measured by the difference in CO_2 efflux after addition of glucose, minus the paired control soil, after 5 hours.

Within the Avon rhizosphere soils, the V15/V15i; V2/V2i and 189/289i showed more similarity between the arithmetic means for the pared plants than between the trials of different strains. This suggests that the conditions of the individual trials have more influence on the metabolic activity of the soil microbiota than the effect between the paired plant strains. Rhizosphere soils from Avon, comparing the plants V15/V15i, V2/V2i and 189/289 had a slightly higher, but non-significant difference in CO_2 efflux in the non-GM plants than the GM plants. There was a significant difference between the soils of the C312/C312i plants with a t-test result of 0.03. This difference was not repeated between the C312/C312i rhizosphere soils for either Narrabri or Waikerie.

The difference in efflux of CO_2 for the Narrabri soil is shown in Figure 11.3.1.2.

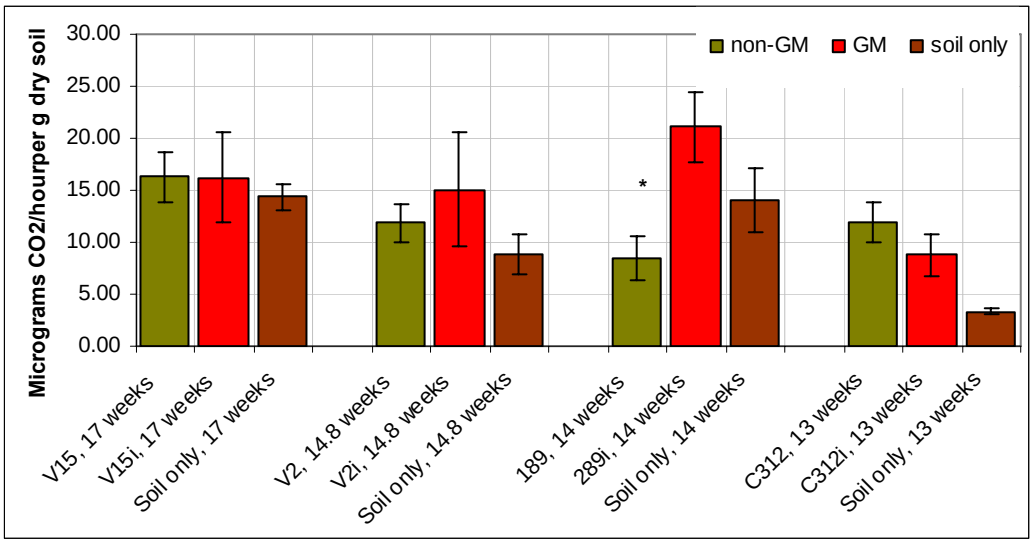


Figure 11.3.1.2 **Substrate induced respiration in Narrabri soil for the four paired cotton plant rhizosphere soils and non-plant associated soils, measured by the difference in CO_2 efflux after addition of glucose, minus the control, after 5 hours.**

The significant difference between the 189 and 289i plants at 14 weeks for the Narrabri rhizosphere soil did not occur for the same plant type in either the Avon or Waikerie soils, and can be seen as a difference attributed only to this particular trial.

In the 189/289i trials in Narrabri soil, the CO_2 efflux from both the duplicate rhizosphere soil controls (water only) and glucose-induced values were numerically higher from the rhizosphere soils, indicating a plant-root effect. The rhizosphere microbial response from the 289i plants was significantly higher than the other trials, and the non-plant root associated soil also showed an increase in CO_2 efflux. However, the plants in this trial were fertilised close to harvest, and the higher level of respiration in the GM plant rhizosphere could have been caused by the addition of nutrient, as neither the 189 nor 289i plants were different within the Avon or Waikerie soils.

The comparison of the substrate-induced respiration for the four plant types and the non-rhizosphere soils from Waikerie soil is shown in Figure 11.3.1.3. There was no significant difference between the paired trials of non-GM and GM plants at $p \leq 0.05$, but there was a rhizosphere effect for all trials.

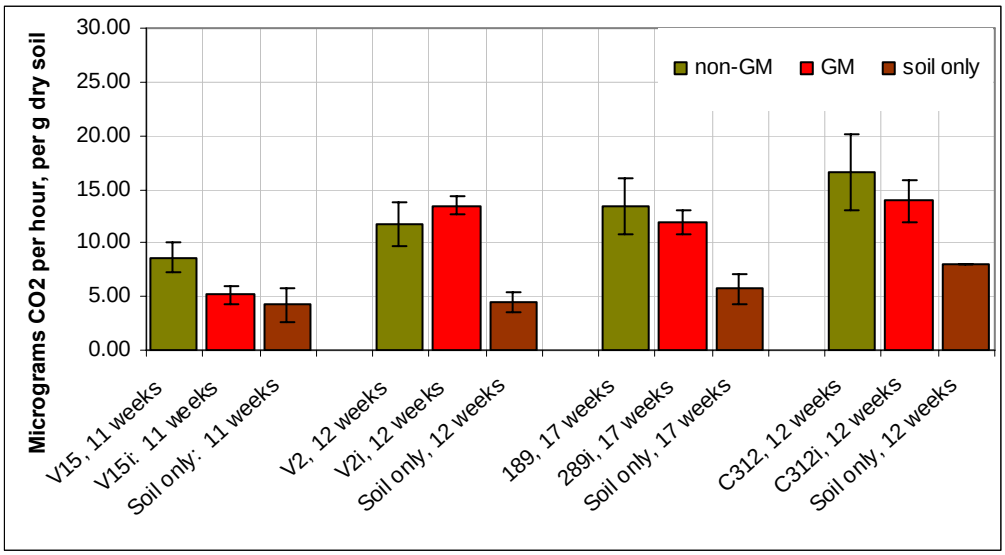


Figure 11.3.1.3 **Substrate induced respiration in Waikerie soil for the four paired cotton plant rhizosphere soils and non-plant associated soils, measured by the difference in CO_2 efflux after addition of glucose, minus the control, after 5 hours.**

The comparison of CO_2 efflux by plant types across all soils was V15 plant strain (non-GM) > V2i (GM) > 289i (GM) > C312 (non-GM).

11.3.2 Proportional rate of increase in CO_2 soil microbial efflux

In every trial, the CO_2 efflux was greater in rhizosphere soils than in non-rhizosphere soils before subtraction of the soil-only controls, indicating a plant-root effect. To compare the rate of increase with the addition of glucose between the rhizosphere soils and non-rhizosphere soils, the proportional increase was calculated by measuring the CO_2 efflux after the addition of glucose, divided by the measurement from the controls. The result is given in Figure 11.3.2.1.

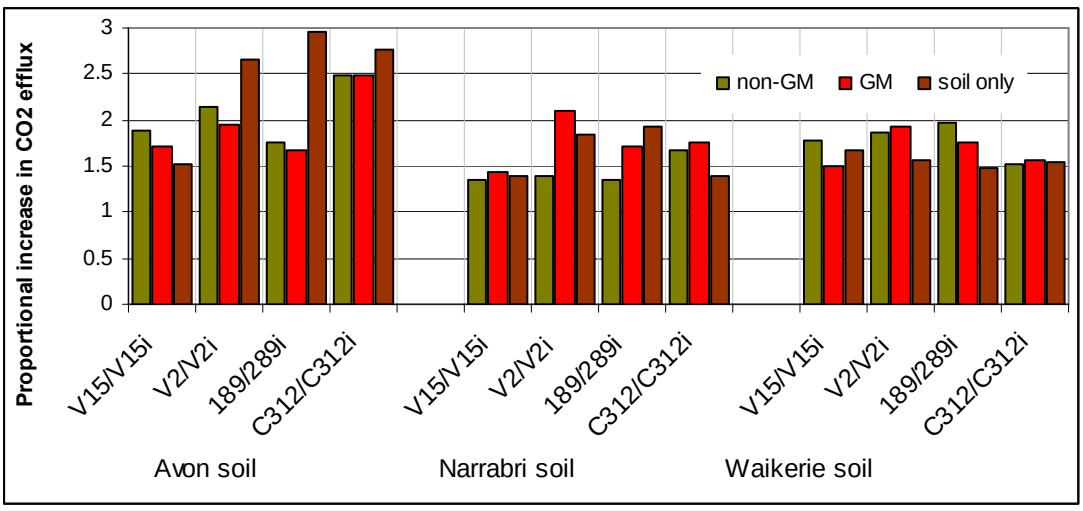


Figure 11.3.2.1 Rate of proportional increase in CO_2 efflux measured as the amount of CO_2 effluxed after the addition of glucose divided by that of the paired controls.

The rate of increase in respiration was higher in three of the non-rhizosphere soils from Avon and one from Narrabri, than the rhizosphere soils, even though the initial baseline respiration was lower than that of the rhizosphere soils. This underlines the individual response of each separate microbial group at the aggregate scale, where there may be a higher proportion of opportunistic bacteria, for example, that are able to respond more quickly to the availability of glucose. The response of the soil microbiota within the Avon soil to the addition of glucose was slightly higher than for the Narrabri and Waikerie soil microbiota, over the 5 hours of the assay: change was not consistent for plant type.

Averaged results of 12 paired trials over the three soils showed a rate of increase in CO_2 efflux of 1.77 times for non-GM plant soils; 1.76 for the GM plant soils, and 1.99 times for the non-rhizosphere soils. This very close result indicated a similar proportional increase of microbial activity in all soils, regardless of the numbers of microbiota present. This indicates a similarity of response by the opportunistic fraction of the microbial populations in the three soils.

CO_2 efflux of the soil microbiota after addition of glucose was not affected by age of harvest. Regression analysis showed that the r^2 value for non-GM was 0.12, for GM, 0.01, and for non-rhizosphere soil 0.07, ie, there was no significant difference.

11.3.3 Efflux of CO_2 in soil as a factor of the plant production of *Bt* protein

A regression analysis on the amount of the Cry1A(c) protein from plant tissue, as detected by ELISA, and substrate-induced respiration within the paired trials resulted in an r^2 value of 0.14 for Avon rhizosphere soil, 0.05 for Narrabri soil and 0.28 for Waikerie. The boxplots shown in Figure 11.3.3.1 illustrate the difference in CO_2 efflux between two harvests of the same plant grown in the same soil, harvested within two weeks of each other. The arithmetic mean is shown as a black bar, with 75% variance above and below, and with outliers as bars.

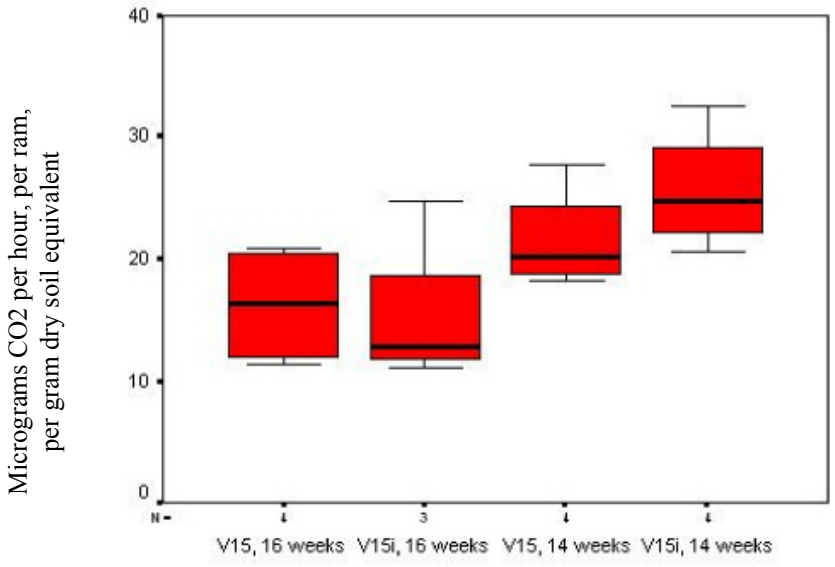


Figure 11.3.3.1 **Separate trials for V15/V15i cotton cultivars grown in Narrabri soil, with different ages at harvest.**

The observed differences in the efflux of CO_2 can therefore also be attributed to the individual trial, as the only differing factor was harvest age.

11.4 DISCUSSION

The results of the mid-infrared analysis (Section 3) indicated that biologically available carbon in the form of particulate organic carbon as leaf litter was available in the soils before the start of the experiments. Thus, C itself was not limiting. However, the form of the organic C is not always readily utilisable by soil microbiota. For example, cellulose is the most abundant organic compound on the surface of the Earth (Campbell, 1990, p. 70), but its β1→ 4 glycosidic linkage and high insolubility makes it a refractory compound for degradation. Addition of glucose allowed measurement of CO_2 efflux from the increase in microbial respiration after its addition to the soil. From the response of a fraction of soil microbiota and using regression analysis, no significant differences occurred between the non-GM and GM plant rhizosphere soils. Thus, plant types were not a major cause of difference in microbial respiration.

The levels of CO_2 efflux varied with each individual trial, even when the same plant and soil were used, with harvest dates differing by two weeks. Any difference in microbial activity can therefore be seen as being more influenced by the effects of the individual trial, rather than as a consistent and lasting effect. The results described here, using three Australian soils, and four cotton plant strains and their

GM-counterparts, differed from those of Stotzky (2000). He found significantly lower gross metabolic activity (measured as CO_2 evolution) of the microbiota from soil in which transgenic corn plants were grown than where non-transgenic plants were grown.

The variance of the CO_2 induction in the non-rhizosphere soils was not explained by different soil moisture content, as all pots were kept at the same water content by weighing, and adjustment before the assays, and was taken into account in the calculations. The differing responses by individual trials may be partly explained by differing micro-environments surrounding the pockets of plant residue, and possibly from different levels of protozoa within the microenvironments.

11.4.1 Observation on the method of measuring soil microbial respiration by substrate induction

Badalucco & Hopkins (1997) argued that the method of calculating respiration of soil microbiota by emission of CO_2 did not represent the metabolic potential of virtually all heterotrophic microorganisms as alternative energy pathways can be utilised. Sato et al. (1995) found that supplementing the growth medium with D-glucose led to an increase in growth rate of 82% of oligotrophic bacteria but of only 52% eutrophic bacteria isolated from soil. L-glutamine had a pivotal role in N assimilation by microorganisms, and may be a more sensitive indicator of biomass than glucose.

As soil microbial respiration measured by CO_2 was found to be inconsistent for the non-GM and GM rhizosphere soils, a direct measurement of the enzymes involved in six key metabolic cellular functions are investigated in the next section.

Section 12

Soil enzyme analysis

Fig. 12.0 Urease activity for rhizosphere soils of three GM-plant strains grown in Narrabri soil. Left lanes of the three groups are controls.

12.1 INTRODUCTION

Extracellular enzymes of microflora are induced by substrate availability within the immediate environment, and enzymatic activity can thus provide information on the soil environment of the microbiota at particular points in time. Voets & Dedeken (1966) showed that invertase, urease, β-glucosidase and phosphatase were more abundant within the rhizospheres of barley, rye and wheat, than in non-rhizosphere soil. After four weeks of growth, however, proteolytic activity was lower than in the non-rhizosphere soil, although the proteolytic microflora were proliferating at a high rate. Enzymes are proteins, susceptible to degradation from competing microbiota, and so there would be a dynamic equilibrium of production and repression within the microbial cell depending on substrate availability, and breakdown external to the cell. A difference in the metabolism of individual plants may have corresponding differences in the enzymes extruded from the surrounding microbiota.

Degradation of the polymers of carbohydrate, protein and nucleic acids that make up the exudates and broken cells of plant roots occurs mainly by the action of catabolic enzymes external to the microbial cell. The monomeric subunits are imported across the microbial cell membrane and then metabolised.

Of the many enzymes produced by soil microbiota, six of the enzymes considered to have a major impact on the rhizosphere microbiota are discussed below. The aim of the work in this section was to determine whether the function of these enzymes measured by C-transformation (cellulase), organic S-transformation (aryl sulphatase), P-transformation (acid phosphatase) overall microbial activity (dehydrogenase) and N-transformation (protease and urease) were expressed at

different rates in the rhizosphere soil of non-GM and GM plants which could reflect differences in the amounts of root exudates.

12.1.1 Cellulase

Cellulose forms the bulk of the cell wall material of all higher plants and is by far the most abundant organic material on earth (Cowling, 1963, p. 1; Campbell, 1990, p. 70). Cotton (the fibre) is almost pure cellulose (Lehninger et al. 1997, p311) and most animals, including soil animals, are unable to utilise cellulose as a source of food because of the β1→4 linkages which are not hydrolysed by α-amylases. Nearly all degradation of cellulose is the result of the activity of microorganisms such as wood-rot fungi and bacteria (Lehninger et al. 1997, p. 311; Rhee et al. 1987) which are able to produce cellulase. Microbial degradation of cellulose plays a significant role in the carbon cycle, returning to the atmosphere an estimated 85 billion metric tons of carbon each year (Cowling 1963, p. 2). It is not known if the extra carbon used in the construction of the *Bt* protein affects the amount of cellulose deposited in the plant cell walls, which could alter the ratio of cellulolytic microorganisms stimulated by the presence of this compound. Additionally, Whitford (1982) used a litter-bag study to determine the rate of litter decomposition after a pesticide was applied, and found the rate of litter decomposition decreased by nearly half, due to the toxic effect. Similarly, if the *Bt* protein affects soil microbiota, or their predators, it may result in the inhibition of carbon recycling. On the scale of increasing areas of genetically modified crop plants, this is an important question.

Cellulose is also more resistant to microbial attack than the plant sugars and starches because it is water-insoluble (Begon et al.1990, p. 365). The factors known to affect the hydrolysis of cellulose include the length of the cellulose polymer and water content of the surrounding environment (Cowling, 1963), and this in turn depends on soil structure and the potential for degradation by its microbial consortia. A comparison of cellulolytic activity by soil microbiota is therefore an important test for one aspect of the C cycle within the soil environment.

12.1.2 Aryl Sulphatase

Aryl sulfatase catalyses the hydrolysis of organic sulphate ester ($R{\cdot}O{\cdot}SO_3 + H_2O \rightarrow R{\cdot}OH + H+ + SO_4^{2-}$). It is found in plants, animals and microorganisms and was significantly correlated with soil organic carbon, total nitrogen and cation exchange capacity (Tabatabai & Dick, 2001, p. 581). Most of the S found in surface soils is

present in the form of organic sulphates (Tabatabai, 1982), and so this enzyme can be used to estimate sulphur activity between plant rhizosphere soils to investigate any differences between plant cultivars.

12.1.3 Acid Phosphatase

Kiss et al. (1974) noted that the presence and type of plants grown in a soil affected phosphatase enzyme activities. This agriculturally important enzyme cleaves the sulphate ester bond and has a major role in the P-mineralization process.

12.1.4 Dehydrogenase

Dehydrogenase activity is an intracellular process that occurs in every viable microbial cell. Measurement of the biological oxidation of organic compounds can be used to estimate the overall microbiological activity of soil (Nannipieri et al,1990) and is correlated with microbial biomass (McKenzie et al, 1995). The dehydrogenase enzyme can be a measure of the oxidation or reduction state of elements present in organic compounds (Lehninger et al, 1997, p. 387) and therefore of the level of chemical energy.

12.1.5 Protease

Proteases are a group of enzymes which hydrolyse the particular bonds for which they are specific. Here, bacteria characteristic of *Bacillus*, were isolated from all soil samples, and most species of this genus have proteolytic activity (Aslim et al, 2002).

ELISA tests showed that even though the *Bt* protein is still detectable at 50ppm, some detection was inhibited by clay soil by interference with the antibodies (Section 5). Additionally, leaves of *Bt*-protein producing plants contained more nitrogen than those of non-*Bt* producing plants (Section 4), and nitrogen was depleted in all sampled soils at the commencement of the experiments (Section 3). Measurement of protease activity in soils is important in determining how quickly proteins (including the *Bt* protein) are degraded in rhizosphere soils, and potentially have a toxic effect on susceptible microbiota.

Voets & Dedeken (1966) found proteolytic activity was lower in rhizosphere than in non-rhizosphere soil after four weeks of plant growth, even though proteolytic microflora were proliferating at a high rate. This could occur if the rate of turnover of protein degradation was faster in rhizosphere soils, and protease enzymes, which are also proteins, were also degraded. The comparative kinetics of the rhizosphere

zone of soil compared to the non-rhizosphere soil is not currently known. Even so, the presence of any proteins in the soil would be expected to stimulate protease activity by microbiota.

12.1.6 Urease

This enzyme is present in microbial, plant and animal cells. Urease catalyses the hydrolysis of urea to CO_2 and NH_3 with a reaction mechanism based on the formation of carbamate as an intermediate (Tabatabai & Bremner, 1972). It is described by the formula $H_2NCONH_2 + H_2O \rightarrow 2NH_3 + CO_2$. Tabatabai (1977) found that urease activity was not significantly correlated with microbial biomass and was affected by heavy metals, oxygen concentrations and nitrogen availability in different types of soils.

12.2 MATERIALS AND METHODS

The methods for these assays were followed from Alef & Nannipieri (1995) with reference to the original authors, and each is listed in detail in Appendix 12. Freshly harvested rhizosphere soils were used, and when not assayed within 24 hours, were frozen at point of harvest and stored in zip-lock plastic bags.

The growth of the plants, and method and time of harvesting was previously described in Section 3.

12.2.1 Cellulase

A method modified from Hope & Burns (1987) Nelson (1944) and Spiro (1966) was followed. The method is detailed in Appendix 12.1.

12.2.2 Aryl Sulphatase

The method of Tabatabai & Bremner (1970) which was followed, is detailed in Appendix 12.2.

12.2.3 Acid Phosphatase

The procedure of Tabatabai & Bremner (1969) was followed and is detailed in Appendix 12.3.

12.2.4 Dehydrogenase

The method of Thalmann (1968) was followed, and is detailed in Appendix 12.4.

12.2.5 Protease

The method of Ladd & Butler (1972) was followed, and is detailed in Appendix 12.5. The method was modified to use a 96 well plate (duplicates of 8 wells per sample, with an additional eight aliquots for the negative controls). As these assays were time critical, it was found that the times for reading the whole plates, which represented three different samples, much reduced the time difference between reading batches compared with measuring the OD of samples using single cuvettes.

12.2.6 Urease

The method was modified from that of Tabatabai & Bremner (1972). These assays were done with 96 well plates, using 8 wells for each of the 4 separate replicates, for each of the 2 negative and positive reactions (8 data points for each of 4 replicates, for a total of 32 data points for each replicate). The data was then pooled and averaged for each of the 4 replicates, for the two plant types. An illustration of the set-up of one of the urease plates is shown as Figure 12.0.

12.3 RESULTS

In the following figures, the means for homogenous subsets based on type III sum of squares from ANOVA analysis are represented by characters above the columns. Where columns share the same letter there is no significant difference at $p \leq 0.05$. The error term is Mean Square (Error) = $1.032E^{-2}$. Consistent with other figures in this thesis, the bars for the non-GM rhizosphere activity is shown in green, and the GM rhizosphere activity is shown in red. The error bars are ± the SEM.

12.3.1 Cellulase

Figure 12.3.1.1 showed the comparison of cellulase activity in Avon, Narrabri and Waikerie soils, for the paired plants listed.

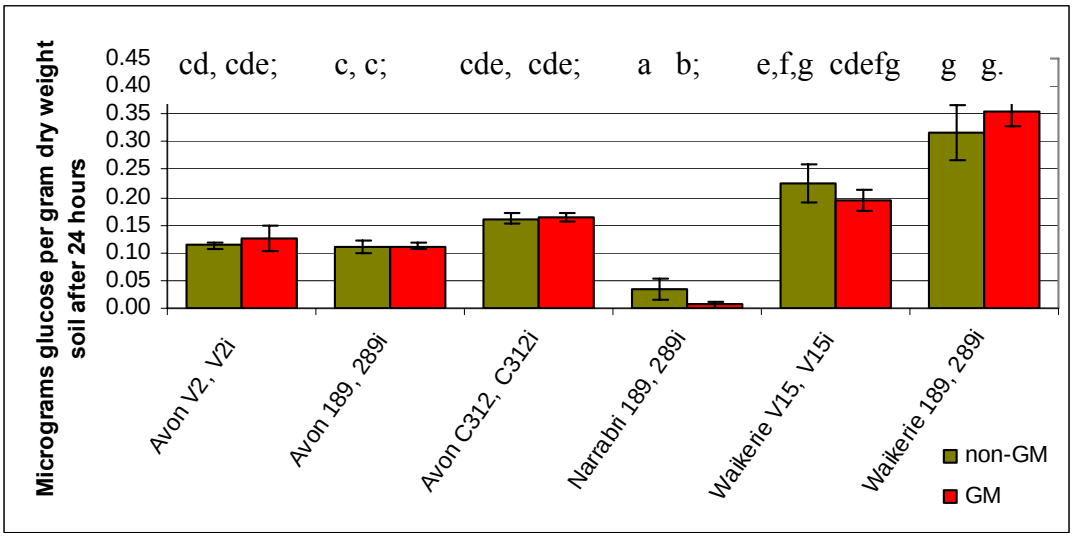


Figure 12.3.1.1 Cellulase activity (glucose equivalent) for three soils after 24 hours.

One significant difference in cellulase production occurred between the 189 and 289i plants in Narrabri soil. This significance may have been caused in part, by the low measurement of cellulase within this particular paired trial, as the GM equivalent rhizosphere soil was almost absent. It was shown that neither the 189 or 289i plants grown in the Avon or Waikerie soils showed a significant difference between the paired trials, so it was an indication of the particular environmental effects of this single trial.

When the Narrabri trial was excluded, the two sandier soils of Avon and Waikerie showed a correlation of cellulase activity by plant age at harvest, at 0.769, significant to $p = 0.01$.

12.3.2 Aryl sulphatase

The results of the univariate ANOVA for aryl sulphatase activity are shown in Figure 12.3.2.1. A comparison of the same rhizosphere soil from the 189 and 289i paired plant trials in Avon shows that the genetically modified rhizosphere soil was not different across the Avon, Narrabri and Waikerie soils, as the same subset groups (defined by characters across shared ranges of significance) was shared by all three cultivars across the different soils. The non-GM 189 plant had more variability for activity of aryl sulphatase, with Avon and Narrabri rhizosphere soils showing a significant difference. Expression in the 189 rhizosphere soil from Waikerie was more similar to that in Narrabri rhizosphere soil, in that two subsets (denoted ‘e’) were common to both. There is a general trend for the GM plant rhizosphere soils to show less sulphatase activity, but this was not consistent.

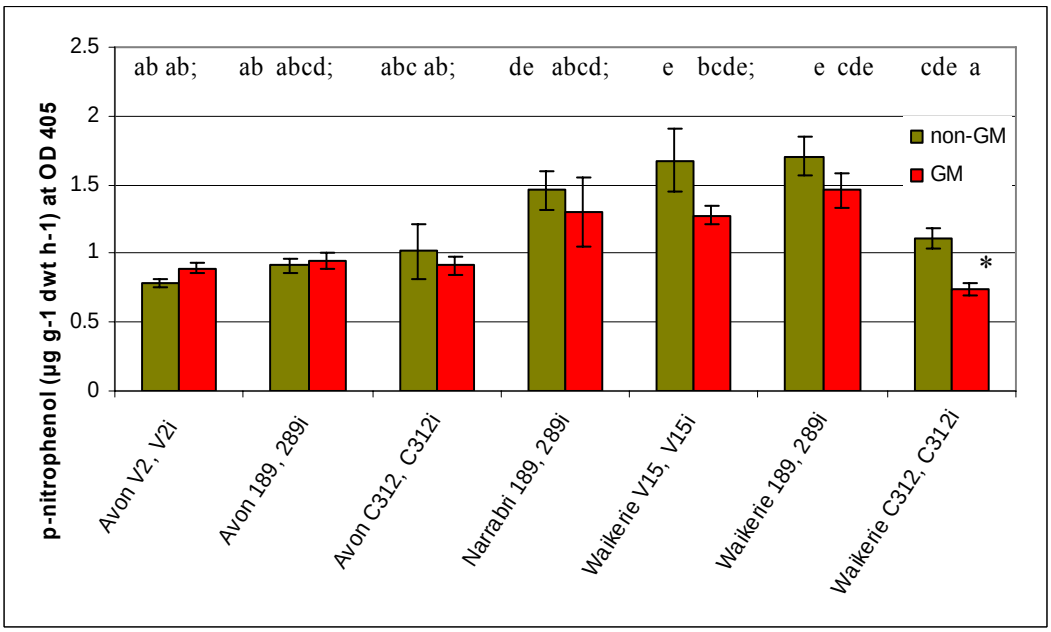


Figure 12.3.2.1 Aryl sulphatase activity measured by *p*-nitrophenol release after 24 hours.

12.3.3 Acid Phosphatase

Acid phosphatase activity ranged from 1.2 to 1.9 micrograms of *p*-nitrophenol per ml filtrate (Figure 12.3.3.1) over all soils. Univariate ANOVA could not be used to compare homogenous subsets because the standard deviation was too wide to justify the assumption of homogeneity, and so t-tests were used to compare each of the individual paired trials.

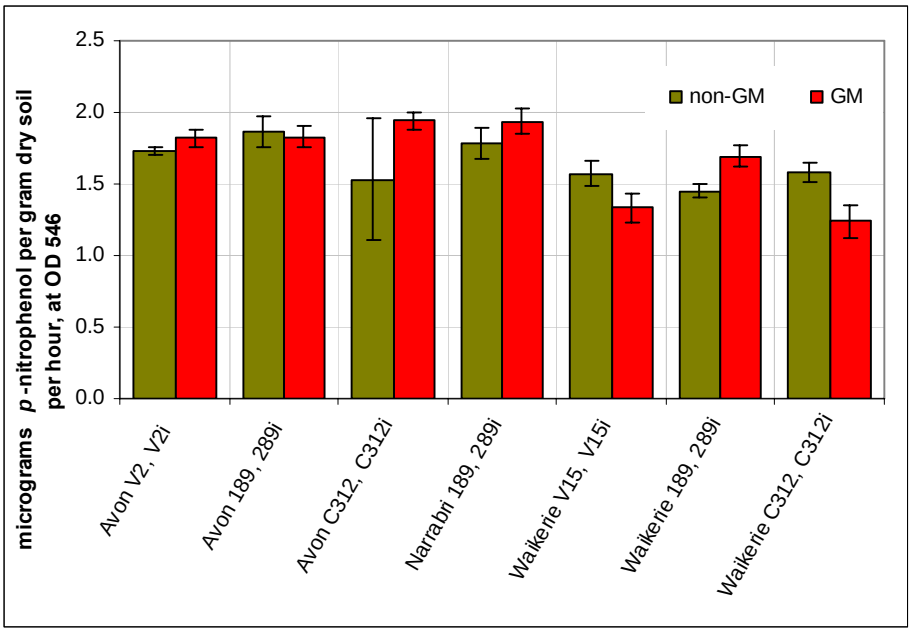


Figure 12.3.3 1 Acid phosphatase activity for paired plant rhizosphere soils. There are no compared homogenous subsets as the variability was too wide for an ANOVA test.

The results of the t-tests showed that within Avon rhizosphere soil there was no significant difference between each of the separate trials, at $p < 0.05$ (n = 11 and 12). Neither non-GM nor GM plant rhizospheres dominated consistently in the expression of acid phosphatase, for the three plant types tested. The 189 and 289i plant rhizosphere soils for Narrabri soil also had no significant difference, (n = 4). Within the Waikerie soil, there was a significant difference between the expression of acid phosphatase in the rhizosphere soil of the three plants tested (n = 12 for the 3 trials). However, the acid phosphatase activity was inconsistent for the non-GM and GM plants within this soil. Comparison of the 189 and 289i plant rhizosphere soils showed a slightly increased expression of the enzyme for the GM plant rhizosphere soils in Narrabri and Waikerie, but less for Avon soil, showing the effect of the individual trials, rather than a consistent difference for plant type.

12.3.4 Dehydrogenase

Figure 12.3.4.1 shows the dehydrogenase activity for three of the plant cultivars in Avon soil, one paired trial in Narrabri soil and three in the Waikerie soil.

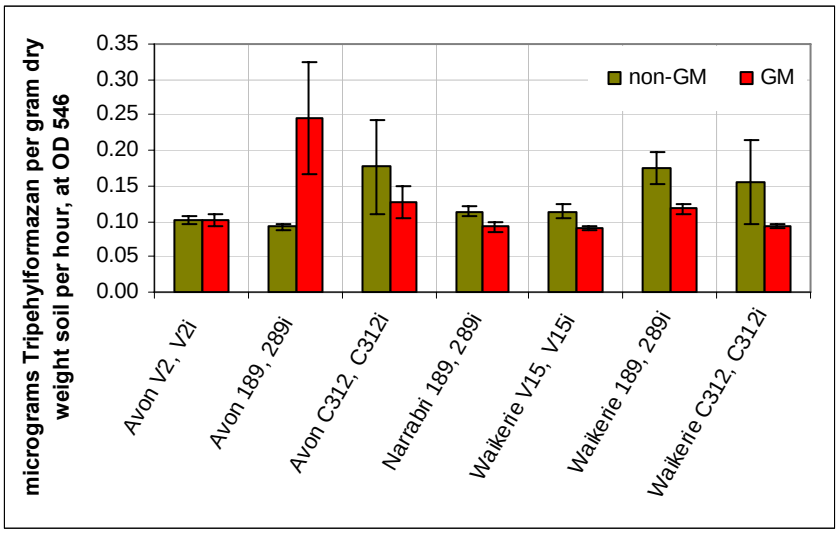


Figure 12.3.4.1 Dehydrogenase activity for paired plant rhizosphere soils.

The variance was too wide for a univariate analysis across all trials, so individual t-tests were done on each of the paired trials separately. The rhizosphere soils of the three paired plants V2/V2i, 189/289i and C312/C312i within Avon soils did not show any significant difference at $p \leq 0.05$ (n = 11 for the 3 trials), nor did the 189 and 289i plant rhizosphere soils show any difference within the Narrabri soil. With the Waikerie rhizosphere soils, there was a difference for the plants V15/V15i, 189/289i and C312/C312i, which indicated a difference in response of the soil

microbiota reflecting the individual conditions of the trials. Thus, the environment of the individual trials affected the rhizosphere dehydrogenase produced more than the effect of the paired plants.

The rhizosphere soil of the same paired plants (189/289i) showed a difference of 0.155, 0.022 and 0.057 µg tripehylformazan g^{-1} dry soil hr^{-1} for Avon, Narrabri and Waikerie soils respectively. The dehydrogenase expression for the non-GM and GM rhizosphere soils, for the 189/289i plants, was less in the Narrabri and Waikerie, but higher in the Avon soils.

12.3.5 Protease

Figure 12.3.5.1 shows the protease activity for the paired plant rhizosphere soils.

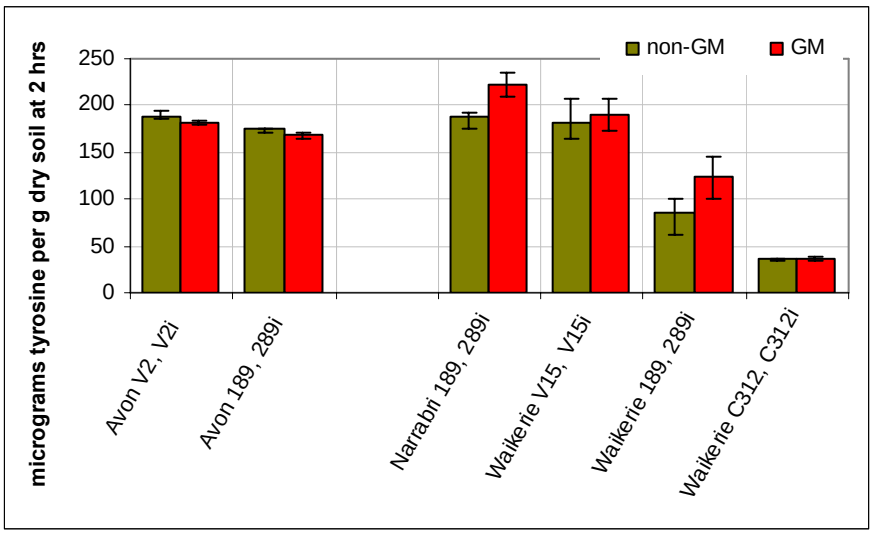


Figure 12.3.5.1 Protease activity for paired plant rhizosphere soils.

Comparison of the (189/289i) plants grown in all three soils, and harvested at 10, 14 and 12 weeks respectively, showed the Narrabri trial had the highest expressed values of protease for all soils, for this compared plant strain. The lowest protease activity was recorded from the rhizosphere of the C312/C312i plants grown in Waikerie soil (the oldest plants at harvest). Additionally, the Waikerie soils had the greatest variance between the plant treatments, for the same soil, so the environment of the individual trial influenced protease activity more than the paired non-GM and GM plants.

12.3.6 Urease

The results of the urease assays are shown in Figure 12.3.6.1.

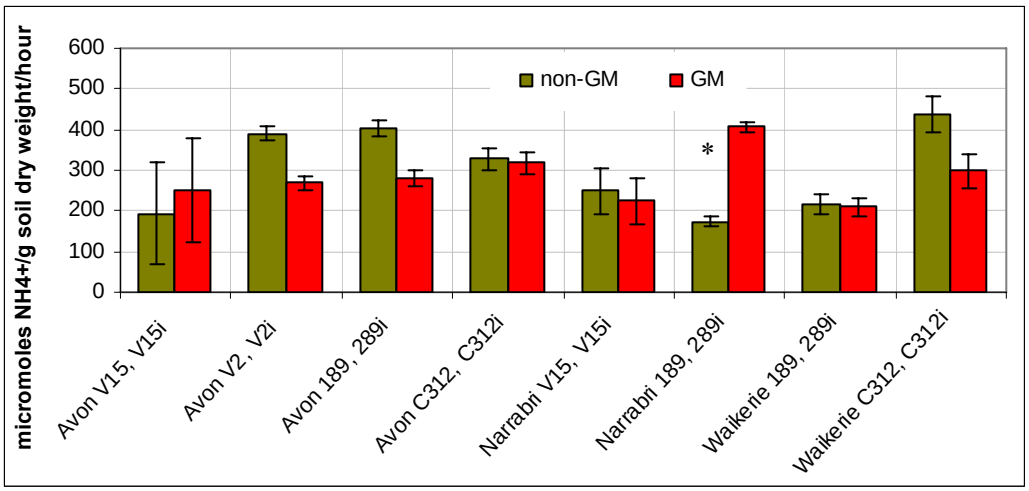


Figure 12.3.6.1 Urease activity for paired plant rhizosphere soils.

Comparison of the plant types 189 and 289i across the three different soils showed that the expression of urease was different between the paired trials, but not significantly so ($p \leq 0.05$) except in the case of the Narrabri 189/289i trial ($n = 3$). This was the rhizosphere soil where Ammonium oxidising bacteria (discussed in preceeding Section 13) and the ninhydrin estimate of microbial biomass was high due to the application of liquid nitrogen fertiliser just before harvest. Microbiota within the rhizosphere soil of the GM plant (289i) showed an immediate N-driven response in comparison with the non-GM 189 plant, which differered from the same paired plants grown in Avon and Waikerie soil, without the addition of nutrient. There the rhizosphere microbiota of the non-GM plant soil showed less activity than the GM plant.

12.3.7 Interrelationships of the six enzymes

Enzyme activities estimated from the different soils and plant types were plotted as radial graphs to compare the profiles of the six enzymes by soil type, and between the non-GM and GM paired plant rhizosphere soils. These are shown in Figures 12.3.7.1 (a,b,c) for Avon soil; 12.3.7.2 for Narrabri soil and 12.3.7.3 (a,b,c) for Waikerie soil. Green points show the arithmetic mean of the four replicate non-GM plant rhizosphere enzyme activities and red points show the paired equivalent GM plant rhizosphere soil enzyme activity. The scale of all figures was log-transformed to allow comparison of the ordinate axis. Where the symbols are superimposed at the co-ordinates there is no significant difference.

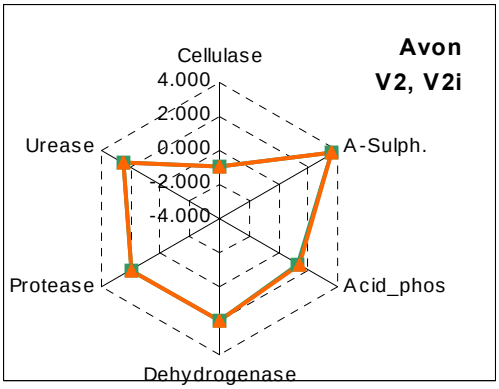


Figure 12.3.7.1 (a)

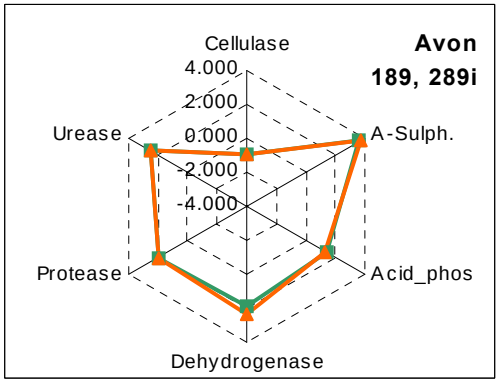


Figure 12.3.7.1 (b)

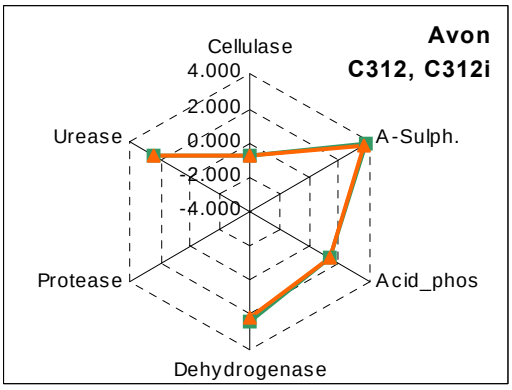


Figure 12.3.7.1 (c)

Figures 12.3.7.1 (a,b,c) **Comparison of six enzymes measured in Avon soil for 3 plant types. One data point is missing because of insufficient rhizosphere soil sample.**

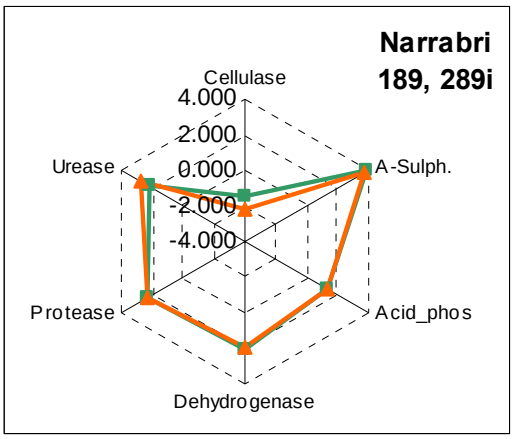


Figure 12.3.7.2 **Comparison of six enzymes measured in Narrabri soil for the 189/289i plants.**

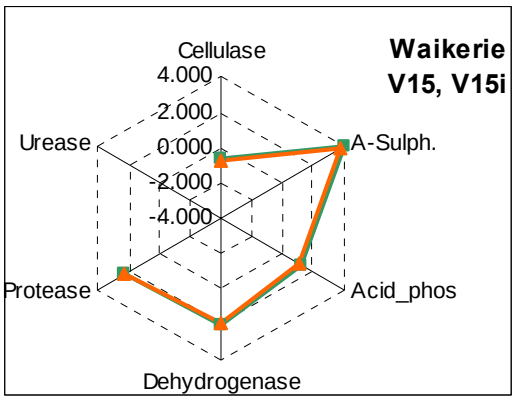


Figure 12.3.7.3 (a)

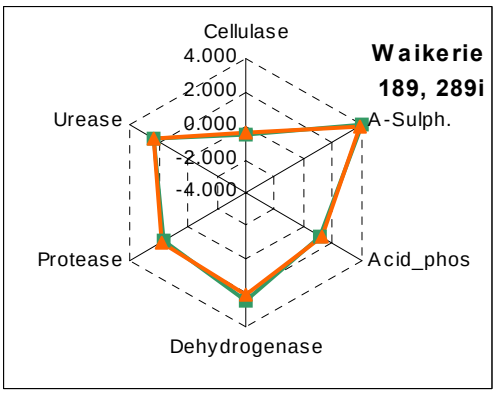


Figure 12.3.7.3 (b)

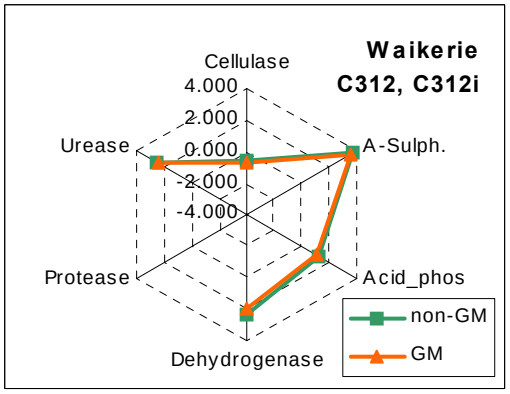


Figure 12.3.7.3 (c)

Figure 12.3.7.3 (a,b,c) Comparison of six enzymes measured in Waikerie soil for the V15/V15i, 189/289i and C312/C312i plants.

Figure 12.3.7.1 shows that the averaged activity for the groups of enzymes adjacent to the roots of cotton plants were extremely similar for each of the three strains of cotton plants tested by the plot. This can be seen from the proximity of the data points, where those of the GM-plants are generally superimposed over those of the non-GM parent. The simlarity of activity of each enzyme is also shown by comparison of the similar angles between the points on the same scale of axis. The most notable exception to similar enzyme activity occurred with dehydrogenase in the 189/289i trial, and this was explained on p. 211. Here the averaged enzyme activity of the rhizosphere surrounding the GM-plant was notably higher, however the variance of the replicates precluded a significant difference in the t-tests.

Figure 12.3.7.2 showed the enzyme activity within the Narrabri soil for the 189/289i cotton plant strains. With the notable exception of cellulase the results were similar to the enzyme profiles of the Avon and Waikerie trials. Here (Figure 12.3.7.2) the cellulase activity was lower in Narrabri than either Avon or Waikerie soils, and activity was also significantly different between the non-GM and GM plant

rhizosphere soils. The respective averaged plant root mass was 5.46 g (non-GM) and 5.11g for the GM plants, so the result may have been partly due to extra bulk of roots in the non-GM plants. While this may possibly reflect a difference in the function of cellulose degraders within this particular trial, results of the same plant strain grown in Avon or Waikerie soils, or for cellulase expression of the other plants grown in other trials were not consistently low for the rhizosphere soils

Figure 12.3.7.3 showed a similar enzyme expression profile to those of Avon and Narrabri for the plant strains tested. Urease expression was not tested in the V15/V15i Waikerie plant trial, but was done for the V15/V15i paired plants grown in Avon soil (results Figure 12.3.6.1). Comparing the results for urease in Figure 12.3.6.1 for the V15/V15i trials in Avon with those of the same plant strain grown in Narrabri soil showed no statistically significant difference in either trial.

12.4 DISCUSSION

Six major enzymes involved in C-transformation (cellulase), organic S-transformation (aryl sulphatase), P-transformation (acid phosphatase) overall microbial activity (dehydrogenase) and N-transformation (protease and urease), were profiled from the rhizosphere soil of paired non-GM and GM cotton plants grown in Avon, Narrabri and Waikerie soils. Results of the respective enzyme tests are discussed below.

12.4.1 Cellulase

It was shown in Section 6 that the rate of degradation of non-GM and GM cotton plant tissue was the same at a macroscopic level in both Avon and Narrabri soil in litterbag studies. The leaf, stem and root tissue had lost the tensile strength which is provided by cellulose, before the exhumation at week 8 (Section 6, page 105). However, cellulase activity was shown to be significantly different by ANOVA analysis both between the Narrabri soil, and the two sandier soils of Avon and Waikerie; and additionally, between the 189 and 289i plant strains grown in the Narrabri rhizosphere soil, over the 24 hours of the enzyme assay. However, of 6 paired trials, 5 showed that each was separated further from the other by soil type, rather than non-GM or GM plant strains (Figure 12.3.1.1). This showed that the environment had a greater effect on the cellulose-degrading rhizosphere microbiota than the influence of the plant genetic modification

A possible reason for the differences in the rate of activity between the sampled soils is that enzymic activity is proportionate to the amount of substrate (in this case cellulose) and the kinetics of the enzyme. The amount of glucose equivalent remaining at the end of the assay determines cellulase activity, and this enzyme is produced in response to the availability of cellulose. The lower cellulase activity measured in the Narrabri soil in comparison with the sandier soils of Avon and Waikerie showed that any available cellulose had already largely been hydrolysed in the Narrabri soil. Cowling (1963) noted that available moisture is possibly the most important factor in the hydrolysis of cellulose because of the highly insoluble nature of the polymer. The fibre saturation point for wood or cotton is 24-32% (Cowling, 1963), and this would depend on the water holding capacity of the soil surrounding the root. Narrabri soil was shown to have a water holding capacity of 34.5% (Section 2) which is approximately double that of Avon at 18.1%, and four times that of Waikerie at 8.2%. Cellulolytic activity would therefore be predicted to occur faster in soils of higher water holding capacity because of the higher particle density and the associated surface tension in the smaller pore sizes. This was shown by the results of the assays which indicated the least cellulase activity in the Narrabri soil, with an intermediate amount in the Avon soil and the most in the Waikerie soil. That is, the enzyme activity reflected the amount of cellulose remaining in the soils after degradation had occurred.

There are three possible reasons for the difference in rate of cellulolytic activity between the non-GM and GM plants. The first reason is that the structure of cellulose, or proportion of cellulose in the cell walls differs between the two plant types. This has already been discounted, as the same paired plant types showed the same cellulolytic activity within each of the Avon and Waikerie soils, albeit as a different rate of degradation between the two soils. The second reason could be attributed to the difference in the N content of the plant tissue between the non-GM and GM plants, leading to a difference in the proportion of fungi to bacteria. The wood destroying fungi are remarkable in their ability to effectively decompose carbohydrates in a material usually containing less than 0.05% nitrogen (Cowling, 1963), but bacteria have been shown to require a lower C:N ratio for their activity (Ferris & Matute, 2003). Possibly the degradation of the cellulose occurred by different ratios (guilds) of bacteria and fungi, where cellulose-degrading bacteria dominated over fungal-degraders because of the higher ratio of N in the plant tissue.

Older plant roots contain more cellulose than younger, actively growing roots. Cellulose activity would therefore also depend on the age of the plants. Correlation of cellulase with age of harvest for the soils of Avon and Waikerie, was confirmed in this work by a value of 0.77.

A third possible reason for the difference in cellulolytic activity is that the microbial populations differed in the proportion of cellulase-producers and their symbionts and antagonists. In the work of Cowling (1963, pp. 197-234) on wood-rotting fungi, half of all fungi screened were capable of degrading the β-1,4 glucanase (cellulase) linkage, although the rate may vary between genera. Actinomycetes were observed in all rhizosphere soils (Section 7.1) as well as *Penicillium* (Section 7.2), and these are two of the populations of microbiota that are able to degrade cellulose (Stutzenberger, 1972; Reese & Mandels, 1963 respectively). However, while cellulase-producing microbiota were abundant in all rhizosphere sampled soils, in a mixed soil microbial population, monopolistic conversion by one specific type of fungus or bacteria cannot be assumed. In some cases the metabolic energy load required to convert a relatively recalcitrant substance such as cellulose is shared between the adjacent microbial community. Kjöller & Struwe (2002, pp. 267-281) demonstrated that mixed communities of cellulose- and lignin-degrading fungi exhibited higher rates of decomposition than single strains of efficient degraders. It has also been suggested that pseudomonads flourish in soil in association with actinomycetes of the genus *Streptomyces* (Todar, 2004). The streptomycetes, which decompose organic compounds aerobically, could provide pseudomonads with monomeric carbon sources which they require. Competitive strategies of soil microbes are also combined with the myxobacteria (Hawker et al. 1960, p. 269), as this group includes both competition from active cellulase decomposers and species that destroy other bacteria. The difference in the rate of degradation could, therefore, have occurred through the difference in function which came about from a different mix of soil microbiota between the different plants. Further tests using PCR with primers specific for microbiota known to hydrolyse cellulose could possibly explain the relative abundance of the microbes that were responsible for the differing levels of cellulose-degrading activity.

From the perspective of an environmental toxicity study, while the rate of degradation of cellulose was confirmed to differ across the three different soils, potential cellulase activity was confirmed in each of the soils which meant that the

cellulose would not persist in the environment, and therefore it cannot be concluded that the effect of the plant-produced *Bt* protein affected the microbiota that degrade cellulose. This finding is in accordance with the work of Naseby et al. (1999), who compared non-GM and GM oil seed rape which expressed anti-fungal proteins. In that study, differences in soil enzyme activities were not attributable to plant genetic modification but rather to environmental variation and to differences in plant variety.

12.4.2 Aryl Sulphatase

The one significant difference for Aryl sulphatase occurred between the C312 and C312i plants in Waikerie soil, but the same plants compared in the Avon soil did not show a significant difference for the expression of the enzyme. The difference in organic S content of the two paired plants was less than 1% (Section 3) so there was no potential for differential induction of the sulphatase enzyme based on plant tissue content alone. This close association in S content was also found for the V15/V15i, V2/V2i and 189/289i plants in the paired trials, and included V15/V15i plants grown under nutrient-limited conditions. The difference in aryl sulphatase is not consistent across all trials, as a lower, but non-significant expression of aryl sulphatase in the Narrabri and Waikerie trials for GM plant rhizosphere soil was reversed in Avon. The work was conducted on the rhizosphere soil of living cotton plant roots, but the finding is in accordance with the work of Stotzky (2005) who found no consistently significant differences between the soils amended with biomass of *Bt* and non-*Bt* corn for aryl sulphatase in American soils of similar texture.

12.4.3 Acid Phosphatase

From the leaf tissue analysis (Section 3) in all cases where the non-GM plant showed deficiency or excess of P, the GM plant also showed the same result. All results fell within 1% for all paired plants, reflecting a similar plant response to the effect of the individual paired treatments, including the conditions of growth under nutrient deficiency. It follows, that as there was no difference in the content of P in the plant tissue, any differential induction of the phosphatase enzyme would be caused by influences other than the availability of the P content. This was not the case however, as the enzyme assays of this work confirmed that there was no significant differences in the expression of acid phosphatase in any of the paired trials, from any of the soils. Stotzky (2005), who measured acid phosphatase activity in the rhizosphere soils of *Bt* and non-*Bt*-corn in American soils, also found no difference. Similar results were found by Wei-Xiang et al. (2004) who reported that there were

no apparent differences in soils of flooded paddy fields to which non-*Bt* and *Bt* rice straw had been added. These three results confirm that similar patterns of phosphatase activity occur in both agricultural and flooded soils.

Under the conditions of nutrient depletion, elemental analysis of both non-GM and GM leaf tissue showed an increased concentration of phosphate had been taken up by the plant (Section 4). This elevation of the levels of P from soil where other nutrients are limiting were previously reported by Jarstfer & Sylvia, (1993, p. 350). The additional depletion of phosphate by the nutrient deficient plants did not appear to disrupt the growth of the soil microbiota as the inclusion of phosphatase in the principal components (Section 15) resulted in a difference of approximately 1% when this single enzyme was included. The possible reason for this is that common soil bacteria and fungi such as *Pseudomonas* spp. and *Penicillium* spp. are able to solublise inorganic phosphate (Illmer & Schinner, 1992; Burford et al, 2003), and the presence of both types of microflora was abundantly reported within all soils from the plate cultures. *Micrococcus* and *Aspergillus* are also capable of solubilizing rock phosphate (Goenadi, 1995) although the addition of cultures grown in mixtures of clay minerals and humic substances or peat increased the level of available phosphate, suggesting that P solubilization requires an input of energy.

12.4.4 Dehydrogenase

The widest variance in dehydrogenase activity for individual replicates occurred from the assays also showing higher expressed values. This variance suggests the occurrence of different oxidation states in localised patches within rhizosphere soils, possibly from root junctions, where temporary sources of energy are present. The apparent difference in dehydrogenase activity between the 189 and 289i plants for the Avon rhizosphere soil suggests differences in microbial activity within some of the different micro-habitats, but no statistically significant differences could be attributed to either plant strain because of the variance of the replicates. A similar energy-response of the microbiota in the rhizosphere soil of the paired plants was shown by the substrate-induced respiration activity, which measured CO_2, produced from the metabolism of glucose after its addition to the soil (Section 11). The results are similar to those reported by Stotzky (2005) who compared the dehydrogenase activity in the soil amended with the biomass of *Bt* and non-*Bt* corn plants in American soils, and who also found no difference.

12.4.5 Protease

The 189/289i plants compared over the three trials showed higher protease activity in Narrabri and Waikerie rhizosphere soils, but lower protease activity for Avon. Comparing the means, however, protease activity was more similar between the non-GM and GM paired trials than across the different trials. The lowest protease activity was recorded in Waikerie soil, but this was also the oldest trial, harvested at 17.4 weeks, and under the pot trials these plants had stopped growing.

When a protein substrate was added to the soils in the assays, there was abundant proteolytic activity, if water was available. This explains why the plant-produced *Bt* protein was not degraded when kept in dry soil in the litterbag trials, but was not recorded after the first watering (Section 6). It also follows from the negative results of Stotzky (2000) where purified *Bt* protein in moist soil was not detected after 6 hours. Stotzky (2005) also found no significant difference between the protease activity in soils amended with GM and non-GM corn plants in American soils.

12.4.6 Urease

The results of five of the six paired trials for urease did not show a significant difference in N-activity, even though the plant tissue contained a higher nitrogen content than the non-GM plants (Section 3). The Narrabri rhizosphere soil did show a significant difference however, (Fig. 12.3.6.1), although protease, another measure of nitrogen-containing compound utilisation did not show consistent difference for either plant strain, or soil type (Figure 12.3.5.1). Quiquampoix et al. (1989) showed that the binding of enzymes to clay depends on the interfacial area of the surface and the secondary structure of the proteins with clays, so the two differing results could possibly be explained not by N-availability, but by the type of enzyme reaction, and its interaction within the high clay-content soil of Narrabri. If the N-utilisation in the GM-plants was driven by a different set of enzymes, then this may be seen as being produced by a different population of microbiota. This will be tested further in Section 13 with other comparisons of N-utilisation.

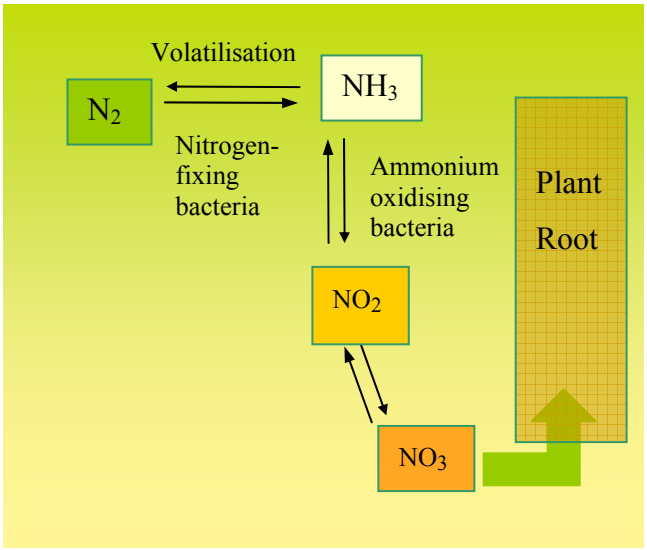
12.4.7 Observations

All hydrolytic enzymes depend on available soil moisture for catalysis and solubility, (Lehninger et al, 1997, p. 102). Microbial enzymatic activity is consequently dependent on the amount of organic matter, the soil type and its water retention. The results of commonly used soil enzyme tests which rely on mixing soil to a

slurry, should be considered to give the **potential** activity in soils, where water is rate-limiting, and not an actual measurement, unless the soils are continually moist. All assays in this work were measured under the same moisture levels, so enzymatic activity in an agricultural field with pronounced wetting and drying cycles may therefore be different from the results given here.

Combined profiles of the enzymes illustrated that all enzymes from the paired plant trials showed a closer alignment within the individual trial, than between GM and non-GM cotton plants. Some differences were seen in the 189/289i plants from the Narrabri trial for cellulase and urease, and dehydrogenase for the 189/289i plants in Avon soil. Comparisons of the differences in these enzymes (cellulase, urease and dehydrogenase) showed that significant differences did not occur in the rhizosphere soils of the same plants from similar trials from the other soil tests done, suggesting that the effect was one of the particular conditions of the individual trial.

Because the N-content of the GM plants was shown to be higher in the leaf tissue than non-GM plants, and a nitrogen-driven response was seen in the urease response between the non-GM and GM plants grown in Narrabri soil, further comparison of N-related activity of soil microbiota from non-GM and GM plants was tested, and follows in Section 13.



Section 13

Nitrogen dynamics

Fig. 13.0 Illustration of change in oxidation state of nitrogenous compounds. Diagram modified from Campbell (1990) p. 730.

13.1 INTRODUCTION

The acquisition and assimilation of nitrogen is second in importance only to photosynthesis for plant growth and development (Vance 1996, p. 723), and nitrogen is the major limiting nutrient for most plant species (Bohlool et al. 1992). For every bale of cotton grown, 11kg of nitrogen is removed from the field in seed and lint (Gibb & Rochester 1994).

The balance between adequacy and excess soil nitrogen is important in optimising crop yield, and opinions differ on the optimal amount. Boquet et al. (1991) showed that excess N fertilization increased the incidence of cotton pathogens such as boll rot, but Chen et al. (1994) reported that much of the excess fertiliser N is lost from the system, primarily through denitrification and volatilisation. Rochester et al. (2001) found that if legume crops are grown in the fallow period before cotton is grown, less nitrogen fertiliser is needed. This suggested that plant-derived nitrogen may be more beneficial in the longer term as it is mineralised slowly over extended periods. On the other hand, the choice of non-legume inter-season crop can adversely affect the soil nitrogen. Cotton crops planted into standing wheat stubble suffered nitrogen stress because the decomposition of wheat stubble led to the immobilisation of nitrogen. This was very evident in fields where high levels of trash on the ground required nitrogen for its breakdown (Waters & Kelly, 2004).

According to Begon et al. (1990, p. 375), the carbon:nitrogen ratio for most plant material is normally 40-80:1. Both bacteria and actinomycetes have a 5:1 protoplasmic C:N ratio, but fungi have a 10:1 ratio (Miller, 1992). This higher C:N ratio of plant material in contrast to that of soil microorganisms suggests that N is the more limiting element in the rhizosphere. However, Ferris and Matute (2003) suggested that C may be the rate determining component in microbial successions, and pointed to the well known fact that disturbance of soil after tillage, for example, increases microbial biomass. This may be explained by an altered C:N microbial biomass after soil disruption as plant-derived organic C becomes more available after being turned into the soil.

There are differing opinions on the effect of nitrogen on rhizosphere microbiota. Marschner (1997) reported that, despite a high supply of organic carbon compounds, rhizosphere microorganisms can be nutrient limited, particularly from nitrogen. While both soil type and nitrogen fertilization affected plant growth, canonical correspondence analysis showed that nitrogen had no significant effect on eubacterial community structures (Marschner et al. 1999). Again, Johansen & Binnerup (2002) investigated C turnover via the enzymes amylase, cellulase, mannanase, xylanase and chitinase and found that these enzymes were not stimulated by the growing plant, although protease and nitrate and nitrite reductase were.

Soil microflora are the prime decomposers of organic substrates, contributing more than 90% of the net energy flux in soil, and are the most important mediators of metabolic turnover of nitrogen (Wilson, 1987). In Section 4 it was shown that GM cotton plants contained about the same amount of N than non-GM plants in the green leaves under nutrient deficiency, but the yellow leaves of the GM plants were higher in N content (Table 4.3.8.2). This suggests that the N which had been utilised by the plant for the production of the extra protein, could not be released. In the event that the N-content of the rhizosphere associated with GM plants is higher than for non-GM, a different microbial community within the rhizospheres of GM/non-GM plants may exist.

It is therefore important that the soil microbial and plant interactions are investigated in the presence/absence of genetically modified cotton which contains a higher nitrogen content than the unmodified plants (Chen et al. 2004).

13.1.1 Measurement of C and N in the sampled soils

One of the strengths of mid-infrared analysis in soil is the high correlation of C (r^2 value of 0.94) when compared against laboratory chemical analysis. The mid-infrared analysis of the bulk soils at the start of the trials gave estimates for total carbon of 1.5% in the Avon soil, 1.2% in the Narrabri soil and 1.3% in Waikerie soil. However, not all soil carbon is utilisable by soil microbiota. Charcoal is highly inert, and is not used by soil microbiota. When the charcoal content was taken into account, the biologically available carbon (in the form of carbonates and leaf litter) was in a C:N ratio of 19:1 for Avon soil, 11:1 for Narrabri soil and 13:1 for Waikerie soil. Particulate organic carbon (carbon as litter) in Avon soil was measured at 1.0%, compared with 0.16% for Narrabri and 0.31% for Waikerie soil.

For estimation of nitrogen, mid-infrared analysis estimates the percentage of total soil nitrogen content, whether organic or inorganic, by diffuse reflectance on a spectrometric analyser. At present it cannot be used to measure nitrate and ammonia-nitrogen. Because of this, alternative tests were needed to establish whether the available nitrogen in the rhizosphere reflected different microbial populations between the different plants. Bacteria, fungi, protozoa and nematodes vary in their nitrogen content, so the effect of N availability on whole interactive soil microbiota is not estimated by counting separate individual subsets of soil microbiota. Chemical-based assays were therefore required to measure the product of the whole rhizosphere community.

13.1.2 Known problems with measuring rhizosphere soil N

The nitrogen cycle does not operate within a closed system. N in the form of ammonium (NH_4^+) can (and does) volatilise to the atmosphere, and being soluble, leaches, together with nitrate (NO_3^-), down through soil. It has been estimated that cotton crops recover about 33% applied N, 25% of which remains in the soil at crop maturity and the remainder (42%) is assumed lost from the system through volatilisation, denitrification and leaching. Nitrite (NO_2^-) and NO_3^- are similarly lost from the system by plant uptake, and are also immobilised by soil microbiota. The system is one of dynamic equilibrium, where soil microbes oxidise available inorganic nitrogen, and use the NO_2^- and NO_3^- to produce cellular components, which are in turn degraded upon the death of the organism.

Nitrogen fertilisers may not be dispersed evenly within soil aggregates. Grundmann & Debouzie, (2000) found a clustered distribution at a millimetre scale along a soil transect for NO_2^- oxidising bacteria, possibly because the proportion of NH_3^- or NO_2^- utilising bacteria may be dependent upon the redox potential within the microsite of the plant root.

The aim of the work was to determine differences in properties related to N cycling within the rhizosphere soils of non-GM and GM plants.

13.2 MATERIALS AND METHODS

The conditions under which the plants were grown, and the harvesting of the rhizosphere soils are detailed in Section 4. Hoaglands nutrient solution (Appendix p. 12) was added at the rate of 10 ml, applied once weekly to each pot and watered in. For the plants grown under conditions of nutrient-deficiency, only water was added to the plants, ie, the only nutrients were gained from the soil.

Three methods were chosen to compare differences in properties relating to N cycling within the non-GM/GM plant rhizosphere soils used, and are described below.

13.2.1 Rate of nitrification by measurement of nitrite

The measurement of nitrite (NO_2^-), the unstable intermediate compound between reduced and oxidised states of N, was used to measure chemotropic microbial activity in soil, in its transition from NH_3 to NO_3^-. The procedure of Berg & Rosswall (1985) was followed, which detects the presence of nitrite in the presence of ammonium sulphate by Griess-Ilosvay reagent. It is detailed in Appendix 13.1.

13.2.2 Estimation of ammonium oxidising bacteria

A most probable number method, based on the methods from Belser & Schmidt (1978); Matulewich et al. (1975) and Schmidt & Belser (1982) was used, and is detailed in Appendix 13.2.

13.2.3 Measurement of Ninhydrin-N, as an indication of microbial biomass

The reaction of ninhydrin with amines, amino acids, peptides and proteins was used in quantitative biochemical investigation. Diketohydrindylidenediketohydrindamine (also called Ruhemann's purple) occurs with the nucleophilic-type displacement of a hydroxy group of ninhydrin hydrate by a non-protonated amino group (Friedman &

Sigel, 1966). All components of nitrogenous organic matter, eg α-amino acids, imino acids, amino alcohols, primary amides etc. react with ninhydrin to yield a purple colour which is measured colorimetrically against known standards (Rosen, 1957). The detailed procedure for estimating microbial biomass from the amino and imino acids by Ninhydrin-N is outlined in Appendix 13.3, from the method of Amato & Ladd (1988).

The difference between the N-content in soil extracts for 10-day fumigated soils and the same for unfumigated soils (time zero) gave an indication of the N-component of all of the soil microbiota.

As noted by Wilson (1987), all bacterial cells are not lysed by the 10-day chloroform fumigation. Extraction efficiency of the microbial-N would depend on the penetration of the chloroform into the soil aggregates, and this would depend on the soil porosity, the nature of its aggregation, and depth of the soil in the container and soil moisture content. Non-GM and GM plant trials were therefore compared separately by soil type.

13.3 RESULTS

13.3.1 Rate of nitrification by measurement of nitrite

Figure 13.3.1.1 shows the amount of NO_2^- produced from the rhizosphere soils of the V15 and V15i plants grown in Narrabri soil, for three time points: T_0, T_5 and T_{24} hours. Each value represents the arithmetic mean of 2 duplicates of each test, for each of four replicates.

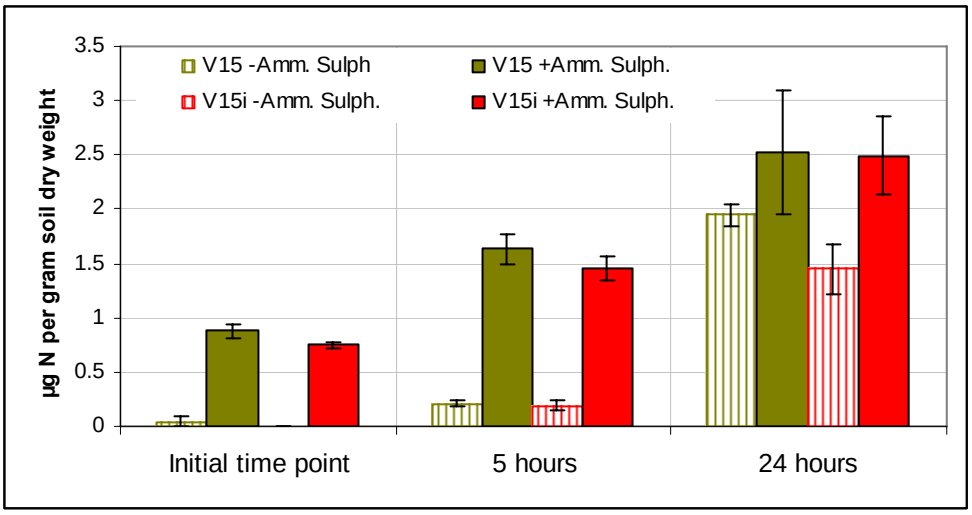


Figure 13.3.1.1 Rate of nitrification (measured as the amount of NO_2 produced per hour, per gram soil) in Narrabri soil for rhizosphere soils of plant strains V15 and V15i, harvested at 11 weeks.

Insignificant amounts of NO_2^- were detected from the rhizosphere soils, measured without the addition of $(NH_3)_2SO_4$ at the initial time point. It increased approximately 19 and 15-fold for non-GM and GM rhizosphere soils respectively, immediately after the addition of ammonium sulphate. For the soils with ammonium sulphate added, the measured NO_2^- continued to increase and at the five-hour timepoint showed a 1.8 and 1.9 fold increase over the initial timepoint, and a further 1.5 and 1.7-fold increase of NO_2^- for the non-GM and GM soils respectively, at the 24 hour timepoint. That is, the rate of increase had slowed and the K_{max} had been passed.

The rhizosphere soils from the V15 and V15i plants without added ammonium sulphate showed a 4-fold increase in NO_2^- at the 5-hour timepoint, and the rate of NO_2^- continued to increase until the 24 hour timepoint, when the rates of increase were 9 and 7-fold above that at 5 hours for non-GM and GM soils respectively. That is, the rate continued to increase up to the 24 hour timepoint. This differed from the soils that had NH_3SO_4 added initially, where the rate of increase had decreased. Very similar rates of nitrification over time were also found for the V2 and V2i plants, also grown in Narrabri soil, under the same pot trial conditions.

13.3.2 Comparison of ammonium oxidising bacteria for Avon, Narrabri and Waikerie soils

Comparisons were made for each soil type separately, as the buffer composition and concentration in enzyme extractions may not be equally efficient for all soils (Vepsäläme, 2001) and comparisons in enzyme activity between the different soils

may not be applicable. For each of the rhizosphere soils tested for ammonium oxidising bacteria there were four replicates for each plant strain. There were two replicate non-rhizosphere soils for the V15/V15i trials, and one each for the every other trial. Error bars therefore do not appear above the non-rhizosphere soils in Figure 13.3.2.1.

The non-rhizosphere soils had wide variance between each trial and were excluded from the same paired tests of the non-GM and GM plant soils after a rhizosphere effect was shown. A Tukey post-hoc homogeneous subset annotation based on Type III sum of squares is shown as letters above the columns for the rhizosphere soils. Columns that share the same letter are not significantly different at $p \leq 0.05$ (Figure 13.3.2.1). Non-rhizosphere soils were also not included in the subsets of the non-GM/GM comparisons for the post-hoc tests.

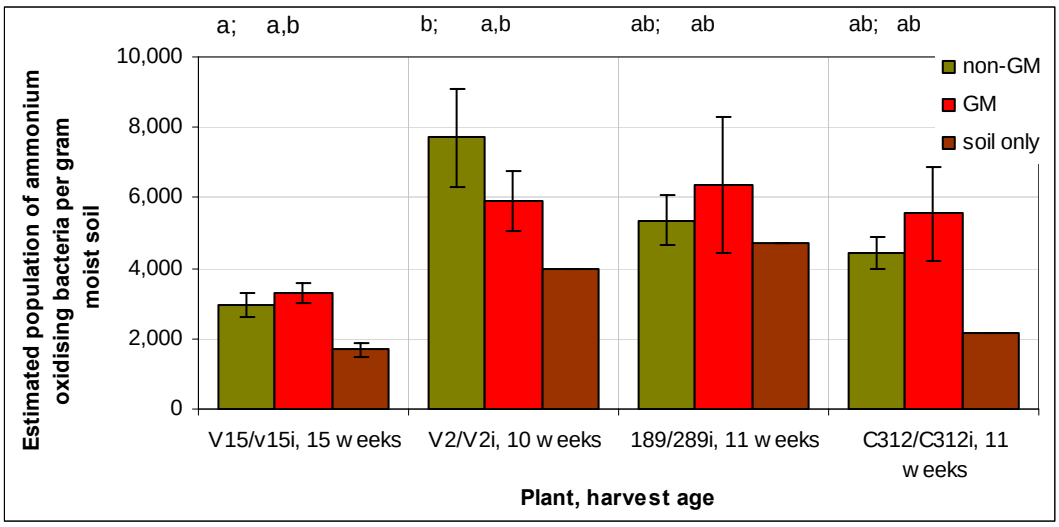


Figure 13.3.2.1 Comparison of Ammonium oxidising bacterial populations in Avon soil for four different cotton plant strains and their paired GM counterparts.

Each of the comparisons of the subsets show that the non-GM and GM plants within the same trials share the same range of subsets and are therefore not significantly different from each other.

The tests were repeated for the four different plant strains, using Narrabri soil. The estimates of ammonium oxidising bacteria are shown in Figure 13.3.2.2.

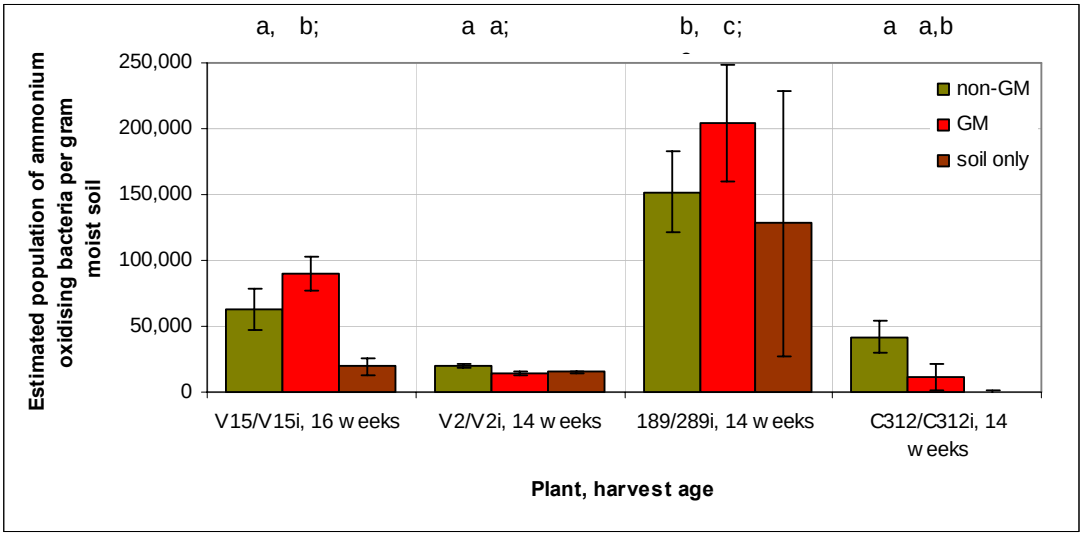


Figure 13.3.2.2 Comparison of Ammonium oxidising bacterial populations in Narrabri soil, for four different cotton plant strains and their paired GM counterparts.

The means of ammonium oxidising bacteria show that the individual paired trials are more similar in numbers than the separate trials. A check of the harvesting notes showed that the increased values for the 189 and 289i plant trial and associated non-rhizosphere Narrabri soil could have occurred as a result of the addition of Hoaglands nutrient solution within 48 hours of harvesting.

A comparison of Ammonium oxidising bacteria for two plant trials grown in Waikerie soil is shown in Figure 13.3.2.3.

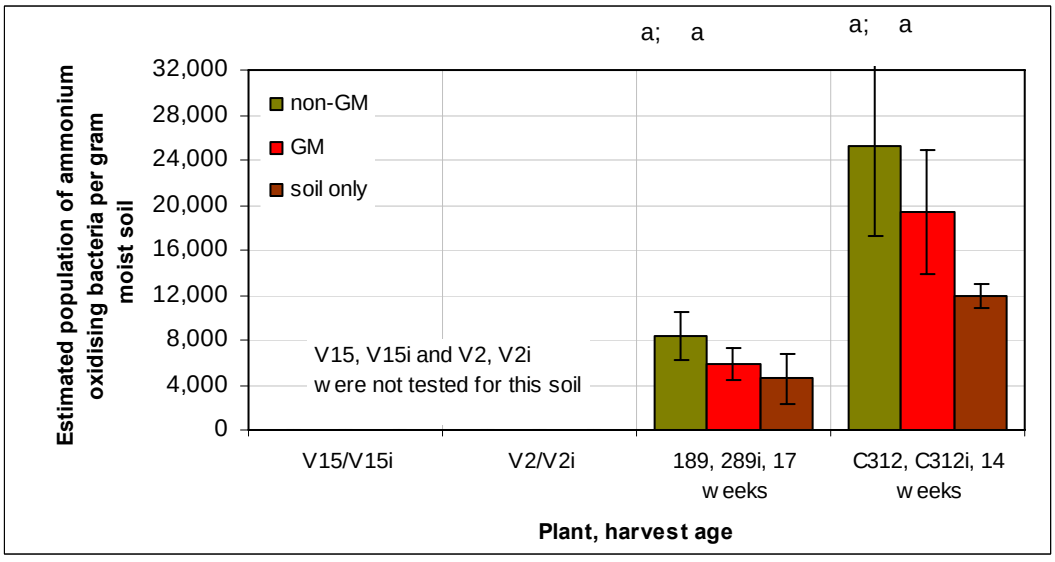


Figure 13.3.2.3 Comparison of Ammonium oxidising bacterial populations in Waikerie soil, for two different cotton plant strains and their paired GM counterparts

The ammonium oxidisers for the 189, 289i, and C312 and C312i Waikerie trials appear significantly different ($p \leq 0.05$) but the variance in the residuals of the ANOVA is in fact within the homogeneity subsets.

13.3.3 Measurement of Ninhydrin-N, as an indication of microbial-N

Figures 13.3.3.1, 13.3.3.2 and 13.3.3.3 show the estimated ninhydrin-detected N for the N-associated organic compounds from soils of the paired plant trials for the three sampled soils. This is expressed as the subtracted difference between the values for the unfumigated soils from the fumigated soils.

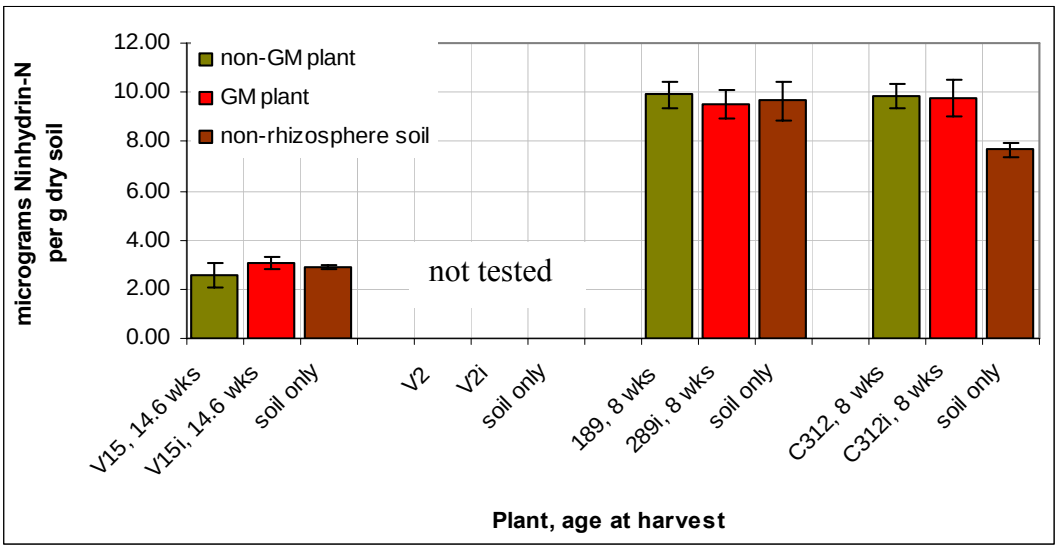


Figure 13.3.3.1 Microbial N-content estimated by Ninhydrin-N concentration of fumigated – unfumigated soils in Avon soil.

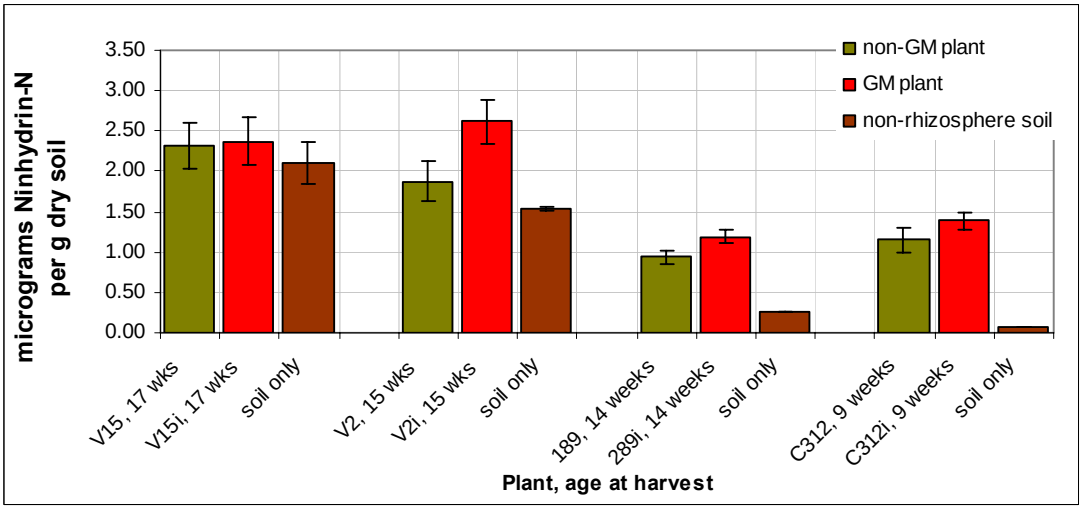


Figure 13.3.3.2 Microbial N-content estimated by Ninhydrin-N concentration of fumigated – unfumigated soils in Narrabri soil.

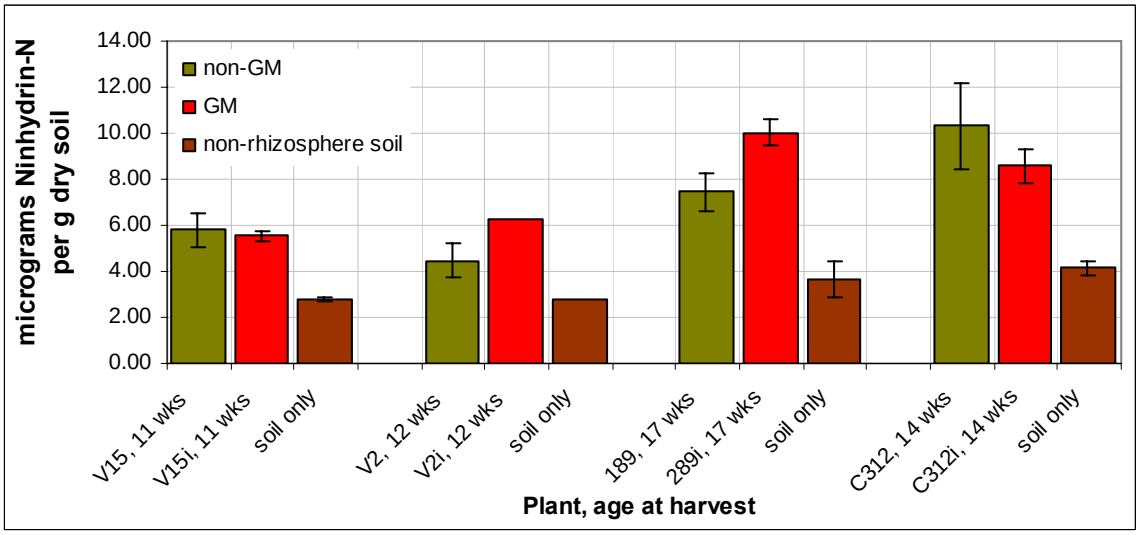


Figure 13.3.3.3 Microbial biomass of Waikerie soil estimated by Ninhydrin-N concentration of fumigated – unfumigated soils.

According to 2-tailed independent t-tests for every paired trial, there were no significant differences, ie, no difference was found between the non-GM and GM rhizosphere soils using the method of ninhydrin-N detection. The means were more similar within the paired non-GM and GM plant rhizosphere soils than across the different trials, showing that the greater influence resulted from the environmental factors of the individual trial.

When the plants were grown under nutrient-limiting conditions, the effect of the individual trial had a greater impact on the nitrogen detected by Ninhydrin than the plant strain, as shown in Figure 13.3.3.4. The low standard error of the mean between replicates is not visible against the bar in the graph.

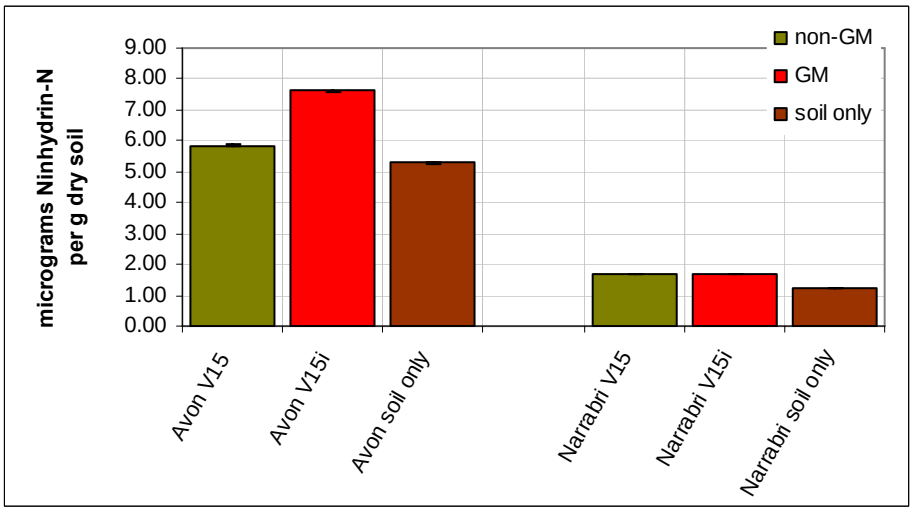


Figure 13.3.3.4 Ninhydrin-N reactive N for nitrogen deficient rhizosphere soils.

The values representing the subtracted difference between the fumigated and the unfumigated soils showed that the N-compounds detected using Ninhydrin ranged from 10.31µg N per gram soil for the non-GM rhizosphere soil in the C312/C312i Waikerie trial (Figure 13.3.3.3) to 0.06µg g-1 from a soil-only comparison for a Narrabri non-rhizosphere soil (Figure 13.3.3.2). While the non-rhizosphere soils nearly always showed a lower amount of ninhydrin-detected N than the rhizosphere soils, the relative amounts closely aligned with the separate trials, reflecting the response to N addition of the individual pots.

13.4 DISCUSSION

13.4.1 Rate of nitrification by measurement of nitrite

Wheatley et al. (2003) showed that soil potential nitrification rates were significantly different between and within three separate arable fields, and that differences in temporal pattern also occurred within each field. From his studies, using eubacterial primers in polymerase chain reaction denaturing gel gradient electrophoresis (PCR DGGE) he concluded that it was possible that soil potential nitrification rates (PNRs) are determined by the size and structure of both the eubacterial and nitrifier populations dependent on natural variability of soils and their location.

When compared within the pot trials using the same sampled Narrabri soil for the paired plants V15/V15i and V2/V2i, the rates of nitrification measured by the concentration of NO_2 after incubation with NH_3SO_4, were not significantly different ($p \leq 0.05$). Begon et al. (1990, p. 375) reported that if material with nitrogen less than 1.2-1.3% is added to soil, available ammonium ions are absorbed. If the material has a nitrogen content greater than 1.8%, ammonium ions tend to be released. The measurement of the ammonium sulphate at the rate of 1mM did not approach the levels at which a difference was found between the rhizosphere soils, so it can be concluded that the rhizosphere of both paired plants was effectively the same within the test range.

13.4.2 Estimation of ammonium oxidising bacteria

Soil type had a major influence on the estimated population of ammonium oxidising bacteria, with rhizosphere soils from Narrabri showing higher populations than any of those from Avon, and with the lowest range of Narrabri approaching the highest for Waikerie soils. It is known that NH_4^+ is attracted to negatively charged smectitic soils such as Narrabri (Crecchio & Stotzky, 2001) so it is possible that volatilization

or leaching of N compounds through the soil may have been inhibited, making them more available to the microbial populations.

The increased N in the fertiliser added just before harvest of the 189/289i trials in the Narrabri soil led to similar increases in numbers of ammonium oxidising bacteria for both non-GM and GM plants. The increase in ammonium oxidising bacteria for this particular trial was also reflected in increased substrate induced respiration for the 189/289i 14-week trial, showing both an increase in activity (microbial respiration) as well as N-induced activity. It can therefore be seen that while readily available glucose can be utilised as a temporary source of energy (Section 11), the significant response of the soil microbiota in the Narrabri soil after the addition of nitrogenous compounds showed the importance of this element.

Whether the N-content of the soil microbiota of the plants may have differed at an earlier harvest, when the roots were more active, is not known, as the earliest harvest occurred at 8 weeks, when the plants were fully grown under pot conditions. The non-significant difference between the plant cultivars at, and beyond eight weeks would suggest however, that the N-complement of the soil microbiota would equilibrate to similar levels dependent on the N-input of the soil system, and that a longer-term higher level of microbial N does not persist for genetically modified cotton plants.

Wheatley et al. (2003) showed by analysis of eubacterial primers in polymerase chain reaction denaturing gel gradient electrophoresis (PCR DGGE) that the bacterial components of microbial soil communities changed seasonally, but PCR DGGE analyses specific to ammonium oxidizers showed that the populations in the three fields tested were similar in types and did not vary with time. It follows that the subset of the soil population that can oxidise ammonium, can respond to ammonium-nitrogen as it becomes available, without altering the structure of the soil microbial population.

13.4.3 Measurement of Ninhydrin-N as an indication of microbial biomass

The relative measurement of Ninhydrin-detected N for the non-GM and GM paired plant rhizosphere soils of Avon, Narrabri and Waikerie soils was more closely aligned by individual trial than age of plant at harvest or soil type. The Narrabri soils showed a lower ninhydrin-N component than Avon or Waikerie for the same plant

types. This suggests that the soil microbiota in Narrabri have a more rapid rate of N-assimilation for the same microbial biomass-N, as was shown by the response of the ammonium-oxidising potential, and it could therefore be seen that individual environment had a greater effect than plant type. The lack of significant difference between the non-GM and GM plants in every trial showed that the ninhydrin-detected N component of the microbial population, plus fine root hairs and other organic compounds were similar in the non-GM and GM plant rhizosphere soils, despite the higher complement of N in the plant tissue of the GM plant cultivars (Section 4).

13.4.4 Observations of the difference in Nitrogen-containing compounds on soil microbiota for the paired trials

Regarding the effect of available organic N by depletion of a subset of the soil microbiota through toxic effect of the *Bt* protein, there is potential for compensatory effects such as the temporary increase in bacteria or fungi during decomposition of plant material (Dropkin, 1989).

Measures of NO_2^- to determine the rate of nitrification, estimation of the numbers of ammonium oxidising bacteria, and the ninhydrin-detected N compounds were all similar between the non-GM and GM plant rhizosphere soils. Any effects of the plant modification are therefore not shown by estimation of a limiting element (N). Further investigation into differences in rhizosphere microbial populations were carried out by measurement of a component of the membranes of active cells, the phospholipids (Section 14).

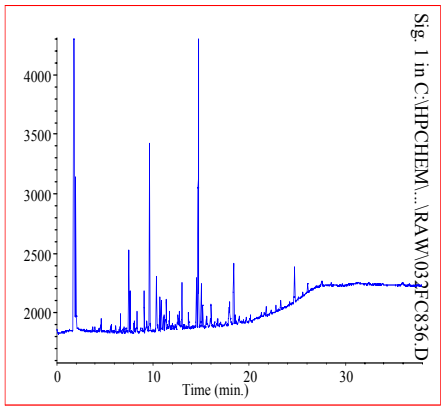


Fig. 14.0 Phospholipid Fatty Acid (PLFA) Chromatogram

Section 14

Phospholipid fatty acid analysis

14.1 INTRODUCTION

Environmental conditions greatly influence root exudation, which varies with plant nutrition and plant species (Watkins, 1981). Plant genotype controls the composition and amounts of carbohydrate-rich exudates and the chemical signalling from plant root exudates influences microbial populations in the rhizosphere (Hawes et al. 1994). The rhizosphere region not only contains an increased population density, but also a community structure distinct from that of the bulk soil (Curl and Truelove, 1986).

Where plants were grown under low fertility conditions, the rhizosphere biomass was higher than for plants maintained by regular nitrogen (N) additions (Bardgett et al. 1996). This would suggest a higher fungal to bacterial ratio in the rhizosphere of plants grown under conditions of limited N, as bacteria need a lower C:N ratio to degrade a unit of C than fungi (Metting, 1993, p. 532). GM plants contain more N than non-GM plants (Chen et al. 2004; Section 3). Exudates of the genetically modified plants, that constitutively produce an additional protein, may harbour a different ratio of microbial flora around the root zone, when compared with non-GM plants if grown on limited N.

Guckert et al. (1986) found that there was an increase in the ratio of saturated to unsaturated fatty acids for starving Gram-negative bacteria. Additional energy of 615 kJ/mol is needed to create the unsaturated C=C bond compared with the 348 kJ for a single C-C bond (Brady & Holum, 1988, p. 290). An increased ratio of *trans*- to *cis*-isomers of the fatty acids 16:1ω7 and 18:1ω7 also indicates increased environmental stress for several Gram-negative bacteria, especially under nutrient starvation (Guckert et al.1991). The cyclopropyl groups are also known to increase during the bacterial

stationary growth phase, indicating starvation conditions (Law et al. 1963). Çakmak & Marschner (1988) postulated that the decrease in plant lipid levels, especially unsaturated fatty acids, might be a consequence of peroxidative membrane injury resulting in increases in membrane permeability and zinc ions interfering with both the generation and peroxidative attraction of oxygen radicals. If both bacterial and plant changes occur, a compensating effect in the ratios of saturated to unsaturated fatty acids would mask the environmental effect of the closely associated plant roots and microbiota.

Phospholipid Fatty Acid (PLFA) analysis has not previously been used to investigate differences in rhizosphere soil microbiota between non-GM and GM plants by comparing the effect of four plant strains in two different soils, with and without the stringency of limited nutrients. Investigation is needed in this area, to understand whether a shift in the dominance of fungi to bacteria may affect higher order predators within the soil food web. This is known to occur with nematodes, as bacterial-feeders are more likely to be in large numbers where the C:N ratios are ~10-15, and fungal feeders are known to dominate where the C:N ratio is >15 (Yeates & Boag, 2004).

The present study therefore investigated whether changes in the phospholipid fatty acid abundance indicated divergences within the rhizosphere soils of genetically modified cotton plants compared with the paired parental unmodified plants. A shift in fatty acids extracted from the living fraction of the microbiota within rhizosphere soil, compared to bulk soil was also investigated. These comparisons were made under nutrient adequacy as well as nutrient deficiency, and for different plant ages at harvest.

14.2 MATERIALS AND METHODS

14.2.1 Nomenclature

The convention of naming fatty acids is as follows. The number of carbon atoms is denoted as an integer, followed by a colon, the position of the carbon atom where an unsaturated bond follows, and if known, the isomers *cis* or *trans*. This numbering takes the origin from the methyl or aliphatic end of the chain and in some literature this is referred to as the 'ω', or omega end. Branching is denoted as "i" or "a", where "i" denotes the iso-branched (a methyl branch on the second carbon from the methyl end), and "a" is an anteiso-branched (methyl branch on the third carbon from the

methyl end). "c" and "t" denotes cis- or trans orientation of the unsaturated bonds respectively, and the prefix "cy" designates the cyclopropane fatty acids.

14.2.2 Equipment and procedure

The method of White (1988), and the acid methylation procedure (Christie, 1989), is detailed in Appendix 14.1. Briefly, 10 g moist soil was weighed into a teflon centrifuge tube and phosphate buffer was added to bring the soil moisture to 30% by weight. Chloroform and methanol were then added to bring the ratios of phosphate buffer: chloroform: methanol to 0.8:1:2, a miscible triple point, in which lipids dissolve quickly. After this monophasic system was rotatory-shaken for two hours, it was centrifuged at 2,000 rpm for 5 minutes and the supernatant removed. Phosphate buffer and chloroform were added to bring the proportions to 0.9:1:1 to force separation of the aqueous and organic phases. The total lipid fraction separated to the lower chloroform layer, and the more polar proteins, cell walls and nucleic acids and other components remained in the upper methanol-buffer phase or at the chloroform-buffer interphase. The lipid-containing phase was transferred to a silicic acid column saturated with chloroform. The neutral, polar and charged lipids were separated by elution using the increasingly polar solvents of chloroform and acetone, and the charged phospholipids were collected by elution with methanol and retained for analysis.

The phospholipids were then dried under nitrogen and methylated by heating overnight at 60ºC with 1% H_2SO_4 in distilled methanol. The solution was dried again and 200µL hexane and 10µL nonadecanoic acid methyl ester was added (at the concentration of 0.037 g in hexane and making up to 50 ml). This was the internal standard against which fatty acid concentration for each sample was measured. Care was taken not to fully desiccate the concentrate because of the volatility of the short-chain fatty acids (Wollenweber & Rietschel, 1990).

The amounts and identification of peaks from the sampled fatty acids were resolved using a Hewlett Packard 5890 Series 2 gas chromatograph. The apparatus was calibrated and runs were made by Bruce Hawke, Senior Technical Officer, CSIRO Land and Water, Urrbrae. Two software packages were used for this analysis:

1. Hewlett Packard HP 3365 Series II Chemstation Version A.03.34, which integrates the area under the peak, and calculates retention time.

2. MIDI (Microbial Identification Inc) Sherlock Version 1.06, using the EUKARY program (Sasser, 1990), which converts the retention time of the fatty acid on the column to equivalent chain length based on the calibration of the standard run.

Each run was calibrated using standard calibration mixes of various fatty acids (straight chain and hydroxyl) at known concentrations. For the system to proceed with unknown samples, the calibration mix had to meet two criteria: concentrations of the fatty acids in the mix had to be present in the correct proportions, and the fatty acids had to elute from the column at the correct retention time (or ECL) within 0.01 minute frequencies. The system was required to successfully complete 2 calibrations before the start of a run and again after every 11 samples.

The tolerance of retention times for this equipment was within 0.01 minute, and this time was transcribed to an estimated chain length (ECL) against the standard run. The value of micrograms of fatty acid per gram of dry soil was calculated as (Area of peak/Area of 19:0 peak) x $210^{a}/2^{b}$ x 0.07^{c} x (1/dry weight of soil) (adjusted for moisture), where
19:0 is the internal standard,
[a] volume of solution of phial (µL)
[b] volume of solution injected (µL)
[c] µg of 19:0 injected.

After deleting the peak value of the 19:0 internal standard the remaining fatty acids between 10:0 and 20:0 were normalised as weight percentage values. This range was chosen because the equipment and software consistently detected straight and branched carbon chains and hydroxyl groups within the range. It was rare that peaks occurred below 9:0, except for the hexane solvent peak at approximately 7.3. The estimated chain length of 20:0 was retained as it was considered a biomarker for protozoa (Lechevalier & Lechevalier 1988). Above 20:0, a number of overlapping peaks confounded analysis.

To test the reproducibility of the detection of the fatty acids within rhizosphere soil for the equipment and software, the same fatty acid extracts were run through the gas chromatogram again within respective runs for Avon and Narrabri soils.

14.2.3 Detection of a rhizosphere effect

Cyclopropane rings have been detected in a number of bacteria, and certain plants contain both cyclopropane and cyclopropene fatty acids (Bishop & Stumpf, 1971). 7-(2-octyl-cyclopropen-1-yl)-heptanoic acid, ('malvalic acid'), occurs in roots, stems and leaves of cotton plants (Christie, 2005). If this cyclopropene fatty acid occurred in the rhizosphere soils from fine roots or abraded cells it would indicate plant tissue. In comparison, the non-rhizosphere soil may only contain traces of the fatty acid containing a cyclopropane ring from bacteria.

14.2.4 Analysis of community composition

Comparison of extracted fatty acids between a carbon chain length of 10:0 and 20:0 was made between the paired plant types for each separate trial. An initial investigation of the data showed that the correlation of the number of fatty acid peaks detected as a function of the total peak area for each treatment showed a highly significant value for all trials. This meant that with less peaks being detected for a lower total fatty acid volume, some fatty acids were possibly below a level of detection. Accordingly, where the total peak area was found to be less than 50,000 $\mu g\ g^{-1}$ dry soil equivalent, the sample was considered too dilute, and the record was rejected.

Principal component analysis was chosen to reduce the dimensionality in the data as the datasets contained more variates than samples. High and low factor loadings were investigated for correlation with plant type. Following the convention of this thesis, green symbols were used to depict measurements of rhizosphere soils of non-GM plants, red was used for soils from GM plants and brown showed the soil-only replicates within each trial.

14.2.5 Nutrient deficiency

Both trials used Narrabri soil, in which the paired V15 and V15i plants were grown under conditions of nutrient adequacy, and nutrient deficiency, and harvested within 8 days of each other.

14.3 RESULTS

14.3.1 Reproducibility of within-run and between run fatty acid components

Figures 14.3.1.1 and 14.3.1.2 show the results of tests for reproducibility within the same analytical run. While the principal components (or the most significant differences) have changed by approximately 1%, the signature overall is very similar.

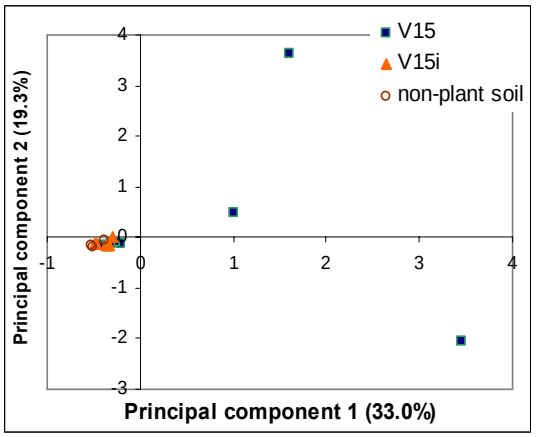


Fig. 14.3.1.1 Principal component plot of Avon rhizosphere soil at 8 week harvest, from the V15 and V15i plants, and non-rhizosphere soil control.

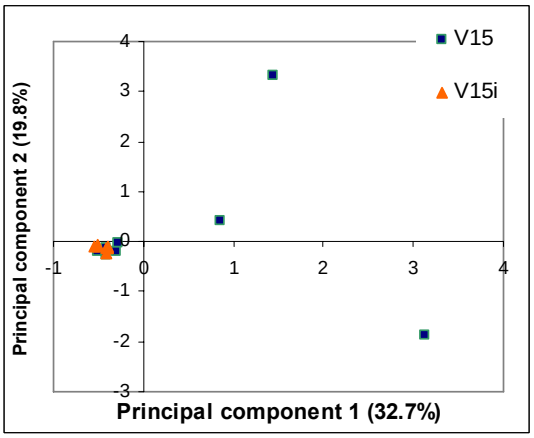


Fig. 14.3.1.2 Principal component plot of Avon rhizosphere soil at 8 week harvest, from the V15 and V15i plants, repeated within the same gas chromatogram run, but without control soil.

The largest positive factor loading was attributable to a cluster of seven datapoints close to the universal C14. The remaining high values were not aggregated around any single fatty acid.

The test for within-run precision was also conducted on the rhizosphere of the Narrabri soil using the same V15 and V15i plants compared above, and is shown in Figures 14.3.1.3 and 14.3.1.4.

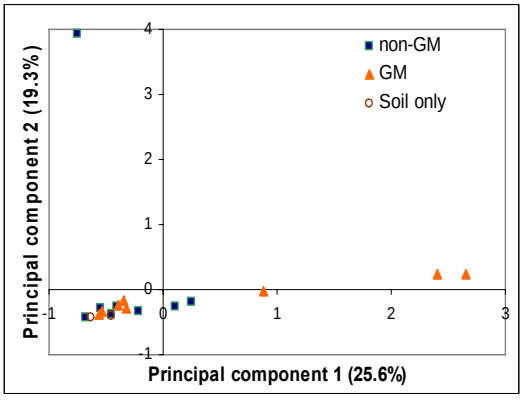


Fig. 14.3.1.3 Original analysis from Narrabri rhizosphere soil using V15 and V15i plants, with control (non-rhizosphere) soil.

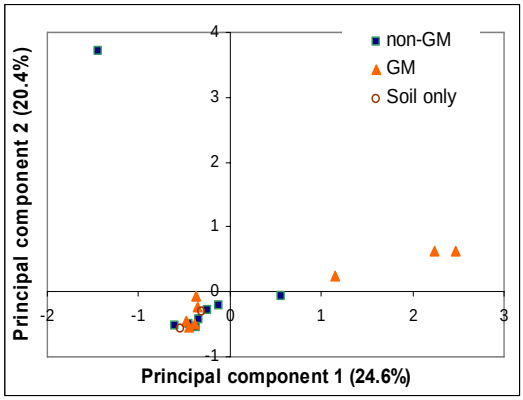


Fig. 14.3.1.4 Repeat within run for Narrabri rhizosphere soil using V15 and V15i plants, with control (non-rhizosphere) soil.

Comparisons of the original data and the repeat-within-run showed Principal component 1 had decreased by 1% from the original, and Principal component 2 increased by 1%. Also, 81 fatty acid peaks appeared in the original trace, and 93 were detected in the repeat run. This may have occurred through a concentration effect as the vial septum had been pierced by the needle of the autoanalyser and some fatty acids may have approached a detectable threshold. A comparison of the concentrations of the 19:0 internal standard for a within-run comparison over 16 samples showed a concentration effect which ranged between 1.24 and 2.07 times the original concentration. This did not detract from the similarity of the overall signature of the plot for the within-run repeat.

14.3.2 Rhizosphere effect

All files were searched for 7-(2-octyl-cyclopropen-1-yl)-heptanoic acid, 'malvalic acid' by the nomenclature of the software 'cy'. The three cyclic fatty acids identified by the software were '17:0 cyclo', '19:0 cyclo C11-12' and '19:0 cyclo 11-12 20'. The last two indicated the same chain length by the library identification software, suggesting that the variation was due to a structural difference of the same fatty acid. The incidence of each of the three cyclic rings is shown in Table 14.3.2.1. Because of the space restrictions of the column width, the cy 17:0, cy 19:0 11-12 and cy 19:0 11-12 20 have been truncated to C17, C19a and C19b. 'A/' denotes Avon soil, and 'N/' denotes Narrabri soil.

Table 14.3.2.1 Incidence of cyclopropane and cyclopropene ring structures from rhizosphere and non-rhizosphere soils, for all tests

	non-GM rhizosphere soils			GM rhizosphere soils			non-rhizosphere soils		
Soil/ Plant/ harvest age (weeks)	**C17**	**C19a**	**C19b**	**C17**	**C19a**	**C19b**	**C17**	**C19a**	**C19b**
N/ V15/V15i, (14.8)	✓	✓	✓	✓	✓	✓	✓	✓	×
A/ V15/V15i, (8)	×	×	×	×	×	×	×	×	×
N/ 189/289i, (12.1)	✓	✓	×	✓	✓	✓	×	✓	×
N/ V15/V15i (16.7)	✓	✓	×	✓	✓	✓	×	×	×
A/ V15/V15i (16.8)	✓	✓	×	✓	✓	×	×	✓	×
A/ V15/V15i, (3.7)	✓	×	×	×	×	×	×	×	×
A/ V15/V15i, (8)	✓	✓	✓	✓	✓	✓	nd	nd	nd
N/ V2/V2i, (16.7)	✓	✓	×	✓	✓	×	✓	✓	×
A/ V15/V15i, (6.4)	✓	✓	×	✓	✓	×	✓	✓	×

where ‘nd’ means the soil-only sample was not done for this run.

The 19:0 cyclo 11-12 20 was not found in any of the non-rhizosphere soils, but was detected in some of the non-GM and GM plant rhizosphere soil replicates.

14.3.3 Soil effect

The effect of soil type was investigated using the V15 and V15i plants, grown in the glasshouse in Avon and Narrabri soils, and both harvested at eight weeks. The principal component plot shows a separation between the same plants grown in the different soils, but not within the non-GM and GM plant strains.

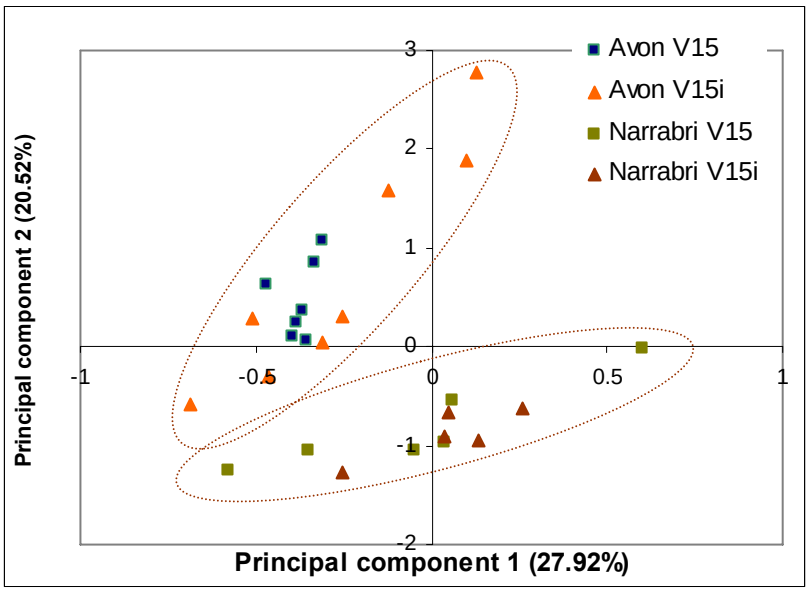


Figure 14.3.3.1 The effect of soil shown by V15 and V15i plants both harvested at 8 weeks.

14.3.4 Principal Component Analysis by individual trial

In total, thirteen separate plant trials were analysed, including four parental plant types and their modified GM counterparts. Comparisons were made of the same plant strain over the two soil types of Avon and Narrabri under different conditions of individual treatment. In the plots of principal component shown in Appendix B, nearly every one of the symbols for soil-only samples were positioned to the left, and below the plant rhizosphere soils, indicating sufficient percentage difference in the fatty acids to group the non-rhizosphere soils together. The principal component plots illustrating the comparisons of the trial results of the rhizosphere soils of non-GM plants, their genetically modified counterparts, and in the cases where the non-rhizosphere soil for the paired trials was also analysed, are illustrated in Appendix B.

14.3.5 Non-GM and GM fatty acid profiles exclusive to the one set, over all trials.

All datasets were interrogated to select the fatty acids that occurred in one of the pairs (eg non-GM plant rhizosphere soil) but not in the other (eg GM) for at least one of the replicates within each individual trial. The summary is shown as Table 14.3.5.1.

Table 14.3.5.1 Summary of individual fatty acids appearing exclusively in the one set of rhizosphere soil replicates, or both. Incidence indicates a match of the particular fatty acids.

Soil	Plants	Incidence of fatty acid present only in non-GM rhizosphere soil	Incidence of fatty acid present only in GM rhizosphere soil	Incidence of fatty acids present in both soils
Narrabri	189, 289i	37	44	31
Avon	V15, V15i	26	26	26
Narrabri	V15, V15i	57	61	69
Avon	V15, V15i	63	25	31
Narrabri	V2, V2i	56	67	50
Avon	V15, V15i	75	79	68
Avon	V15, V15i	57	55	46
Narrabri	V15, V15i	64	48	44
Narrabri	V15, V15i	75	67	60
Avon	V15, V15i	37	51	38
Narrabri	V15, V15i	42	49	38
Column sum		**589**	**572**	**501**

Excluding the unnamed fatty acids, and 'sum in feature …', 62 fatty acids named by the software occurred only in the non-GM or GM rhizosphere soil for each of the individual trials. These exclusively occurring fatty acids formed 39 groups. Excluding the 23 single cases, 14 fatty acids occurred in the one, but not the other rhizosphere soil, and are recorded in Table 14.3.5.2.

Table 14.3.5.2 Grouped fatty acids occurring exclusively in the one, but not the other of the compared rhizosphere soils

Fatty acid	Occurrence	plant type	Occurrence in both non-GM and GM soils, in other trials?
13:0 iso 3OH	2	non-GM	Yes
15:0	2	non-GM	Yes
15:0 2OH	4	GM	Yes
15:0 anteiso	5	non-GM	Yes
15:1 iso F	2	non-GM	Yes
15:1 ω3c	2	GM	Yes
16:0	2	non-GM	Yes
19:0 iso	2	GM	Yes
20:2 ω6c	2	GM	Yes
20:3 ω6c	2	non-GM	Yes
20:4 ω6c	3	non-GM	Yes
21:1 ω3c	3	non-GM	Yes
C20 N alcohol	3	GM	Yes
Iso 17:1G	2	non-GM	Yes

The record of the presence of each fatty acid type, therefore, showed a variable distribution, rather than a consistent exclusion from one or other of the paired plant strains.

14.3.6 Most significant fatty acids

The most frequently occurring fatty acids having concentrations above 3% of the total volume for that batch, are summarised in Table 14.3.6.1.

Table 14.3.6.1 Incidence of the same significantly occurring fatty acids from 10 trials

Fatty acid	Frequency		Fatty acid	Frequency (cont'd)
16:0	10		18:2 w6c . .	3
ISO 17:1 G .	9		19:2 w6c	3
15:0 ISO	7		16:1 w9c . . .	2
18:0	7		19:0 cyclo c11-12	2
18:1 w9c	6		21:1 w7c	2
16:0 ISO	5		cis 9_10 epoxy 18:0	2
ISO 17:1 w5c	4		14:0 2OH	1
15:0 ANTEISO	3		16:1 w7c	1
16:1 w5c	3		16:1 w7t	1
17:0 ISO	3			

Regression analysis of change in ratio of unsaturated bonds, by age of harvest, indicative of plant environmental stress, did not show a trend.

Both C16:0 and 18:0 unsaturated fatty acids (considered by Cavigelli et al. (1995) to be universal fatty acids), occurred very commonly and cannot be used to differentiate prokaryotes from eukaryotes. Even so, the 16:0 fatty acid can be taken as a surrogate measurement of the living cells within the rhizosphere and used to compare the specific fatty acid for eubacteria, iso 17:1G (Cavigelli et al, 1995).

C18:0 was nearly always secondary in volume (area under the peak) compared to that of the 16:0 saturate. A third commonly occurring fatty acid occurred as a 'sum in feature 8', normally linked to the retention time of the mono-unsaturated 18:1ω9t fatty acid. This fatty acid has a trans-configuration on the 9th carbon from the methyl end of the chain, and elutes closely to the isomer with a cis-configured double bond on the same carbon. There was some doubt, however, that the gas chromatograph used in the processing would have separated the cis- and trans- 18:1ω9 fatty acid consistently (B. Hawke, pers. comm).

The most highly expressed fatty acids were then considered separately within each trial. Where significant differences occurred at $p \leq 0.05$ from a Student's t-test, an asterisk appears above one of the paired bars. Figures 14.3.6.1 to 14.3.6.4 for Avon soil, and Figures 14.3.6.5 to 14.3.6.9 for Narrabri soil trials follow.

AVON SOIL

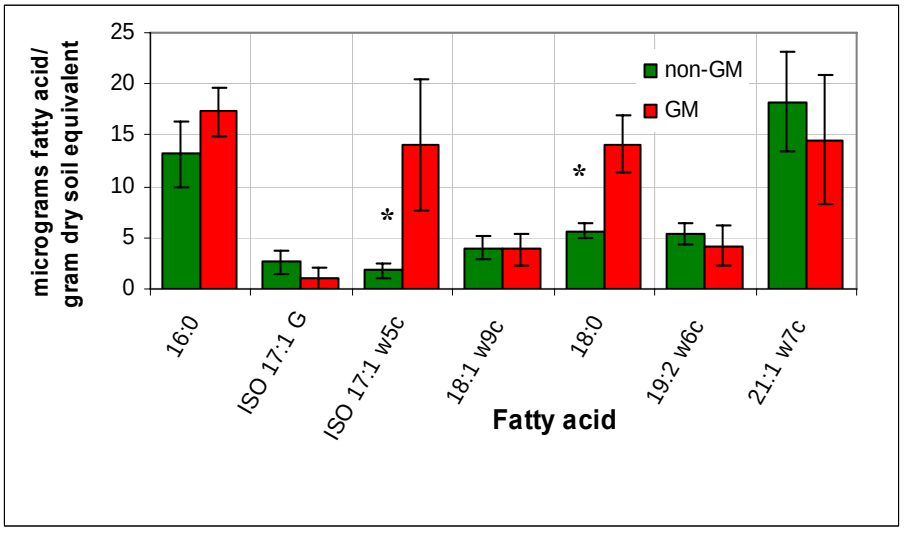


Figure 14.3.6.1 **Significant (> 3% of total volume) fatty acids for Avon rhizosphere soil, for plants V15, V15i, at the 3.7 week harvest.**

There was an increase in the 16:0 and 18:0 fatty acids in GM plant rhizospheres, in Avon soil, as shown in Figure 14.3.6.1. The Iso17:1G data points, indicative of eubacteria, although present in significant amounts, did not contribute as much as

other fatty acids in this soil mix. Notably, there was a significant increase in Iso 17:1ω5c for the rhizosphere soil of the GM plant. This branched fatty acid has been described by several authors as indicating Gram-Positive bacteria (Zelles, 1999, Haack et al. 1994). The increase in Gram-Positive bacteria was offset by a decrease in the rhizosphere soil of 21:1ω7c which indicates eukaryotes such as protozoa.

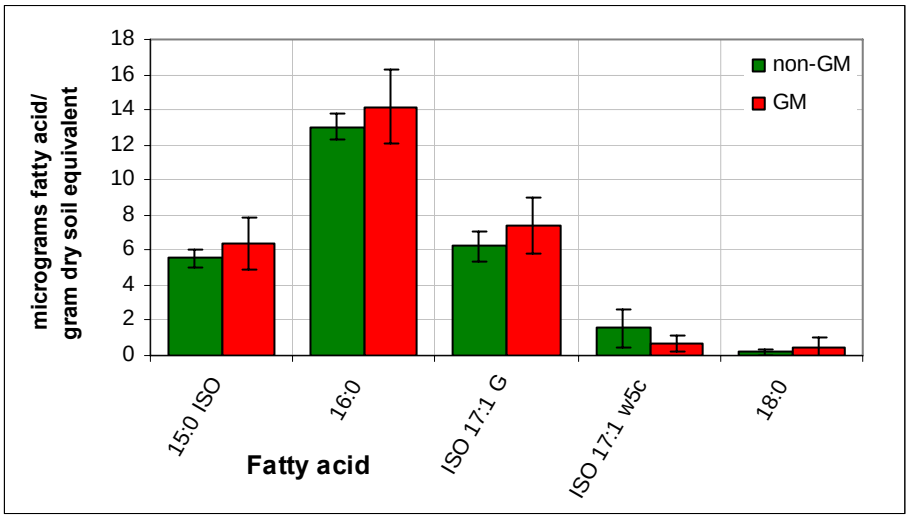


Figure 14.3.6.2 Significant (> 3% of total volume) fatty acids for Avon rhizosphere soil, for plants V15, V15i, at the 6.4 week harvest.

At the 6.4 week harvest Iso 15:0 appeared separately from 16:0, indicating an additional group of shorter-chain fatty acids compared with the 3.7 week harvest. This fatty acid also appeared at 8 weeks. The two unsaturated long chain fatty acids 19:2ω6c and 21:1ω7c detected at 3.7 weeks were not found at 6.4 weeks. Neither appear specifically in the referenced literature, but both of the longer-chain mono-unsaturated fatty acids indicate Gram-negative bacteria (Zelles 1999). The variance between replicates indicated by the error bars showed that no statistical difference could be determined between the significant fatty acids of the paired non-GM and GM plants.

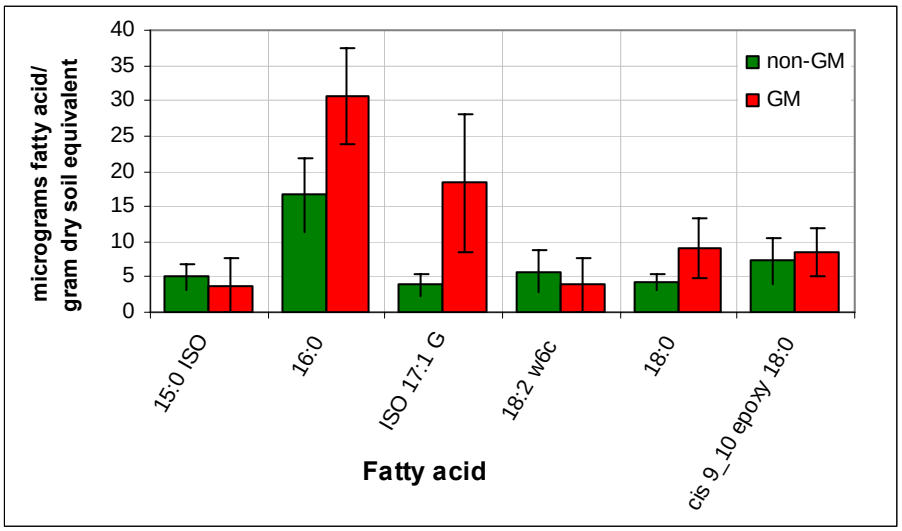


Figure 14.3.6.3 Significant (> 3% of total volume) fatty acids recorded for the V15, V15i plant, grown in Avon soil at the 8 week harvest.

The fatty acid Iso17:1G, an indicator of eubacteria, appeared to be more highly expressed in the GM plant soil than the non-GM plant soil (Figure 14 (Figure 14.3.6.3), but was unevenly distributed between the replicates, suggesting the effect of random sampling. The 18:2 ω6c (a fungal-specific fatty acid) was lower in the GM rhizosphere soil at this time, suggesting a lower fungal complement. The difference in expression was non-significant according to the t-test between the pairs.

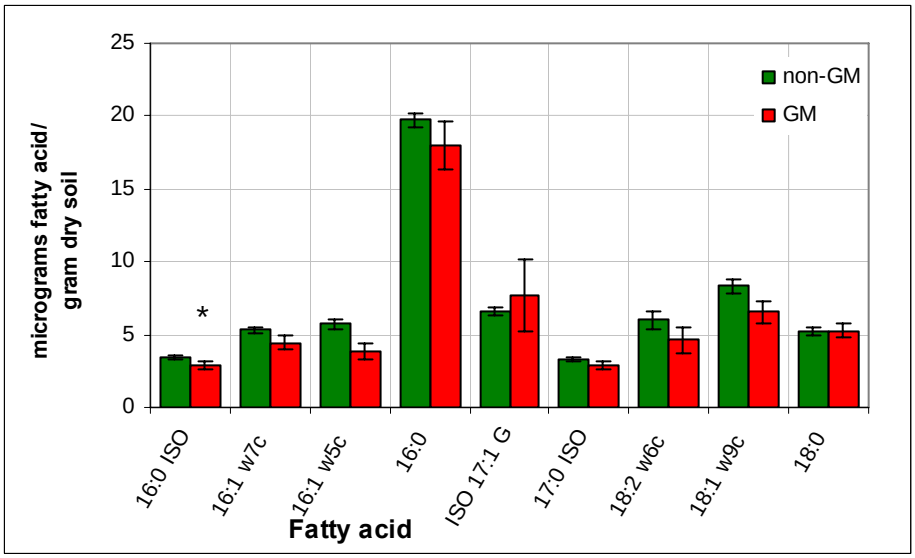


Figure 14.3.6.4 Significant (> 3% of total volume) fatty acids recorded for the V15, V15i plant, grown in Avon soil at the 16.8 week harvest, under nutrient limiting conditions.

Figure 14.3.6.4 showed a distinct change in the shift in fatty acids under conditions of plant nutrient deficiency in Avon soil, with four of the 9 fatty acids having unsaturated bonds. All fatty acids from both non-GM rhizosphere soil and GM rhizosphere soil were similar. Iso 17:1G, associated with gram positive bacteria, was the only bacterial fatty acid which had a higher fatty acid concentration within the GM rhizosphere soil

than non-GM soil. A t-test on these comparisons did not show significant differences, except for the universal 16:0 fatty acid. Guckert et al. (1991) reported a shift in the trans-to-cis configuration of the 16:1ω7 and 18:1ω7 fatty acids for several Gram-negative bacteria as an indication of nutritional stress, but this change in isomer was not detected within the rhizosphere soils in this study, nor was the 18.1ω7 detected in significant amounts from the Avon soil.

NARRABRI SOIL

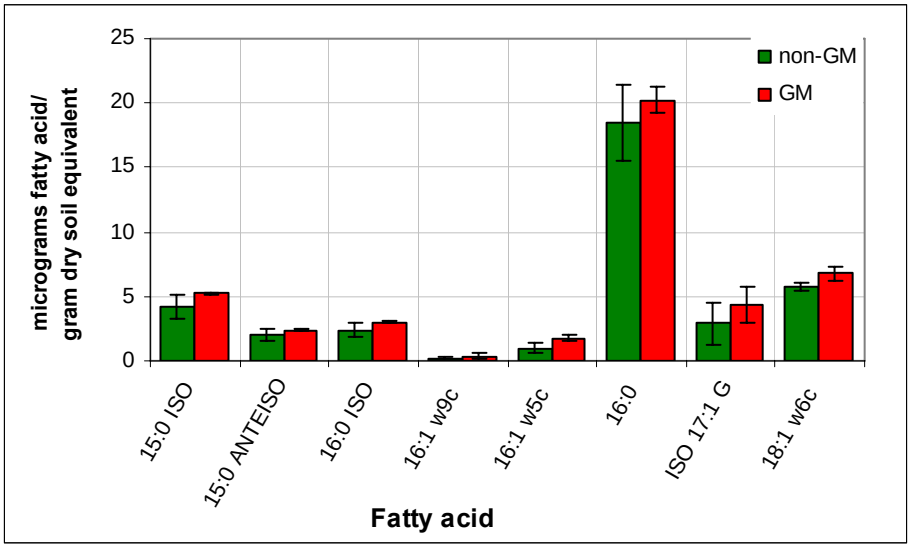


Figure 14.3.6.5 **Significant (> 3% of total volume) fatty acids for Narrabri rhizosphere soil, for plants V15, V15i, at the 7 week harvest.**

The Student's t-tests showed no significant difference between the individual fatty acids for the non-GM and GM plants grown in Narrabri soil, harvested at 7 weeks (Figure 14.3.6.5). The comparison of the 7 and 8 week harvest, as was done with Avon rhizosphere soil, was repeated using Narrabri soil (Figure 14.3.6.6).

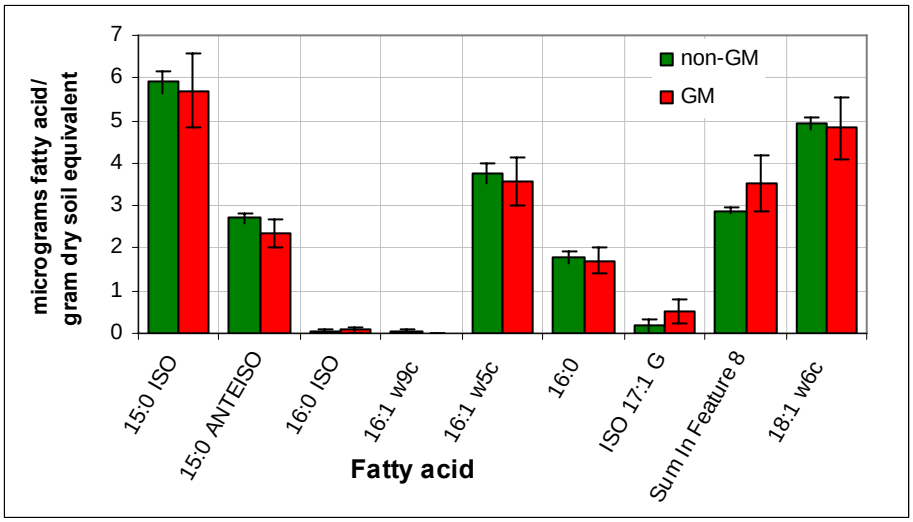


Figure 14.3.6.6 **Significant (> 3% of total volume) fatty acids for Narrabri rhizosphere soil, for plants V15, V15i, at the 8 week harvest.**

The largest difference between the Narrabri week 7 and week 8 samples was a decrease in levels of 16:0. All remaining significant fatty acids were at similar levels, apart from a sum in feature 8, indicating several overlapping peaks which could not be resolved into individual fatty acids. This fatty acid recovered in volume at the 16 week harvest.

A further comparison was made of the same plant type, within the same soil at twice the growing period (16 weeks), when the plant was pot-bound, but receiving adequate nutrient. The result of the analysis appears in Figure 14.3.6.7. Neither the non-GM nor GM significant fatty acids dominated within these paired trials, and a repeat-within-run analysis showed the same result. There was one replicate sample for the V15i plant soil where no Iso 17:1G fatty acid was detected, that resulted in a large variance for this fatty acid.

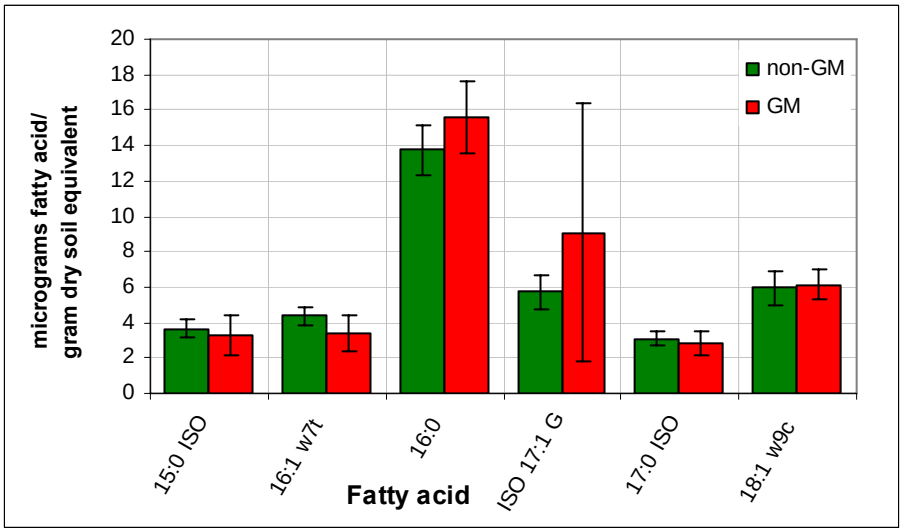


Figure 14.3.6.7 **Significant (> 3% of total volume) fatty acids for Narrabri rhizosphere soils, comparing the V15 and V15i plants at the 16.7 week harvest, when the plant was pot-bound, but receiving adequate nutrient.**

A comparison with the same plants V15, V15i was also made under nutrient deficient conditions. The result is shown in Figure 14.3.6.8.

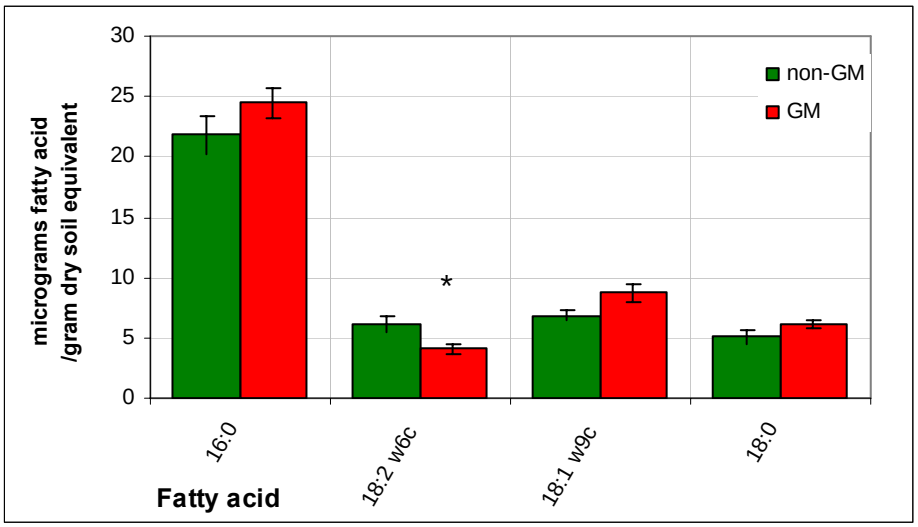


Figure 14.3.6.8 **Significant (> 3% of total volume) fatty acids from the V15, V15i plant rhizosphere soil from Narrabri soil, at the 14.8 week harvest, pot-bound and nutrient deficient.**

In Figure 14.3.6.8, the 17:1G peak (indicative of eubacteria) was missing, but the universal 18:0 appeared under conditions of nutrient deficiency. There were less fatty acids which reached significant levels within this run, but of those that did appear, neither the non-GM nor GM dominated in all cases. In both cases the 18:1ω9c and 18:2ω6c indicative of fungi were present in significant amounts from the nutrient deficient rhizosphere soils. However, with Narrabri soil there was a significant difference in the 18:2ω6c fatty acid between the non-GM and GM plant rhizosphere soils. This fatty acid (ergosterol) is a specific biomarker for fungi (Federle et al, 1986; Olsson, 1999; Ibekwe & Kennedy, 1998). Vestal & White (1989) reported it present in varying amounts in most other eukaryotes.

To compare the effect of plant strain against the V15/V15i plants discussed above, the significant fatty acids of the rhizosphere soils of the V2/V2i and 189/289i plant strains, harvested from the Narrabri soil at 16.7 and 12 weeks respectively, but grown under adequate nutrient, are shown in Figures 14.3.6.9 and 14.3.6.10.

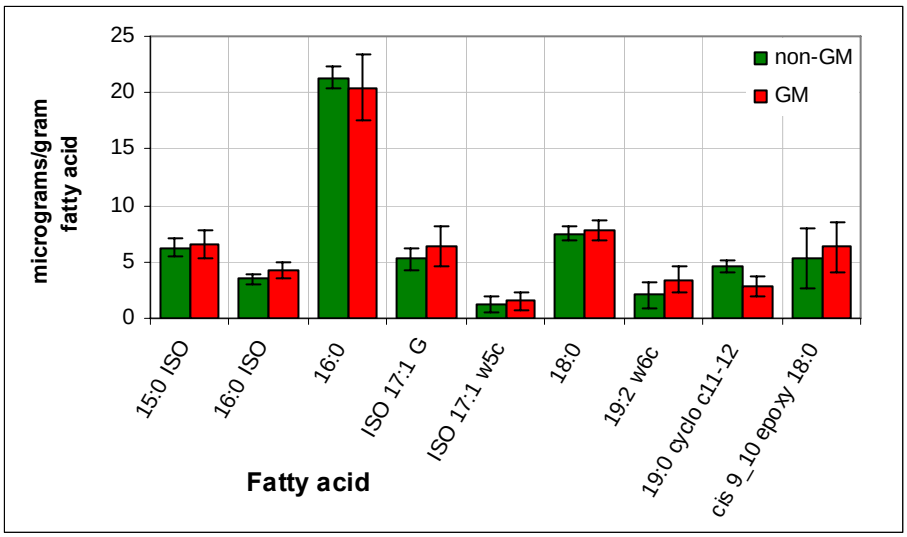


Figure 14.3.6.9 **Significant (> 3% of total volume) fatty acids from the V2/V2i cultivars from Narrabri soil at 16.7 weeks, grown under conditions of adequate nutrient.**

The universal 16:0 and 18:0 fatty acids dominated, but there was no significant difference between the other significant fatty acids for the V2/V2i cultivars.

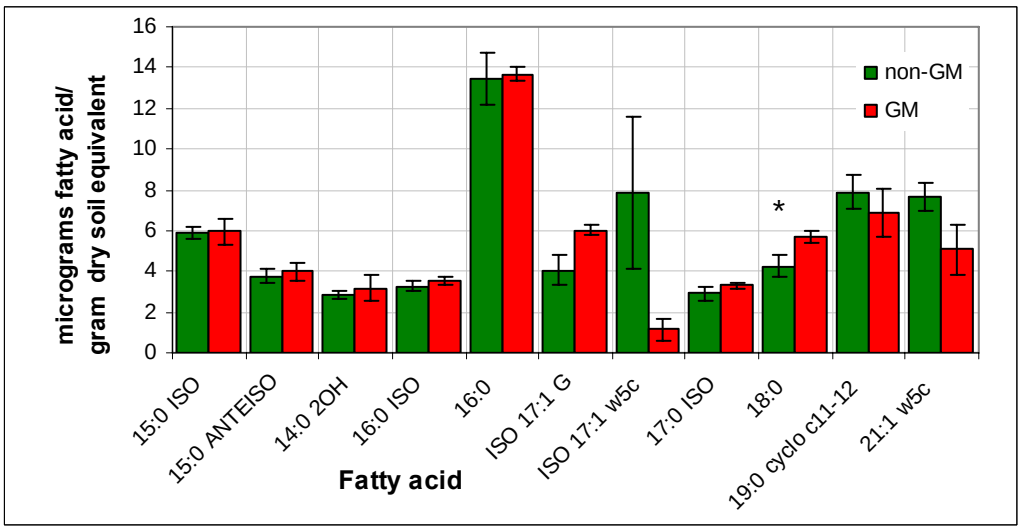


Figure 14.3.6.10 **Significant (> 3% of total volume) fatty acids from the 189, 289i cultivars from Narrabri soil at 12 weeks, grown under conditions of adequate nutrient.**

There was an increased number of shorter and intermediate chain length fatty acids of significant concentration for this plant cultivar compared with the V15/V15i fatty acids harvested at the same harvest date of 12 weeks, grown in the Avon soil. There were also more fatty acid peaks than the V15, V15i plants grown in Narrabri soil, for the harvest of 8 or 16 weeks. A significant difference in 18:0 fatty acid did occur between the non-GM and GM plants. However, as this is a universal fatty acid it could not be attributed to any one microbial entity. All of the remaining arithmetic

means for the non-GM and GM rhizosphere fatty acids were similar, and neither dominated consistently, with the exception of the universal 18:0.

14.3.7 Biomass Estimates

Iso 17:1 G occurred at significant levels in 9 of the 10 gas chromatograph runs. This fatty acid also occurred in six of eight non-rhizosphere soils in the paired trials and the peak concentration correlated to the universal 16:0 significantly, at r = 0.7. Independent t-tests on all Iso17:1G concentrations, averaged for the number of replicates for the paired trials, showed no significant difference between non-GM and GM rhizosphere soils for this fatty acid. This group of unsaturated fatty acids has been designated by Cavigelli et al. (1995) as 'eubacterial'. When compared in isolation, it does not explain any difference between non-GM and GM rhizosphere soils. A comparison of the proportions of C16:0/Iso17:1G, to compare the bacteria to total biomass, is shown in Table 14.3.7.1.

Table 14.3.7.1 Ratios of the C16:0 : Iso17:1G (universal:eubacterial) for trials where these fatty acids were significant (> 3% of total fatty acid volume).

Soil, plant	Non-GM	GM
Avon V15, V15i, 3.7 weeks	5.6	10.3
Avon V15, V15i, 6.4 weeks	2.1	2.0
Avon V15, V15i, 8 weeks	4.3	1.6
Avon V15, V15i, 16.8 weeks, nutrient deficient	6.6	9.2
Narrabri 189, 289i, 12 weeks	4.6	2.5
Narrabri V15, V15i, 16.7 weeks	2.7	1.8
Narrabri V15, V15i, 8 weeks	1.7	2.8
Narrabri V15, V15i, 14.8 weeks, nutrient deficient	3.0	3.4

The contribution of eubacteria to total fatty acids can be seen as varying with treatment, with neither non-GM nor GM plants dominating. This Iso 17:1G is not a test of definitive difference in the rhizosphere of cotton plants.

14.3.8 Gram-positive Bacteria

The iso and anteiso (branched) fatty acids were described by Haack et al. (1994) as indicative of Gram-Positive bacteria, and O'Leary (1989) noted that the major fatty

acids of members of the genus *Bacillus* are almost all of the branched-chain type. A summation of the total fatty acids with branching could be an indicator of this major subset of the bacterial community. The comparison of non-GM and GM rhizosphere soil fatty acids with branching (iso or anteiso) is shown in Figure 14.3.8.1.

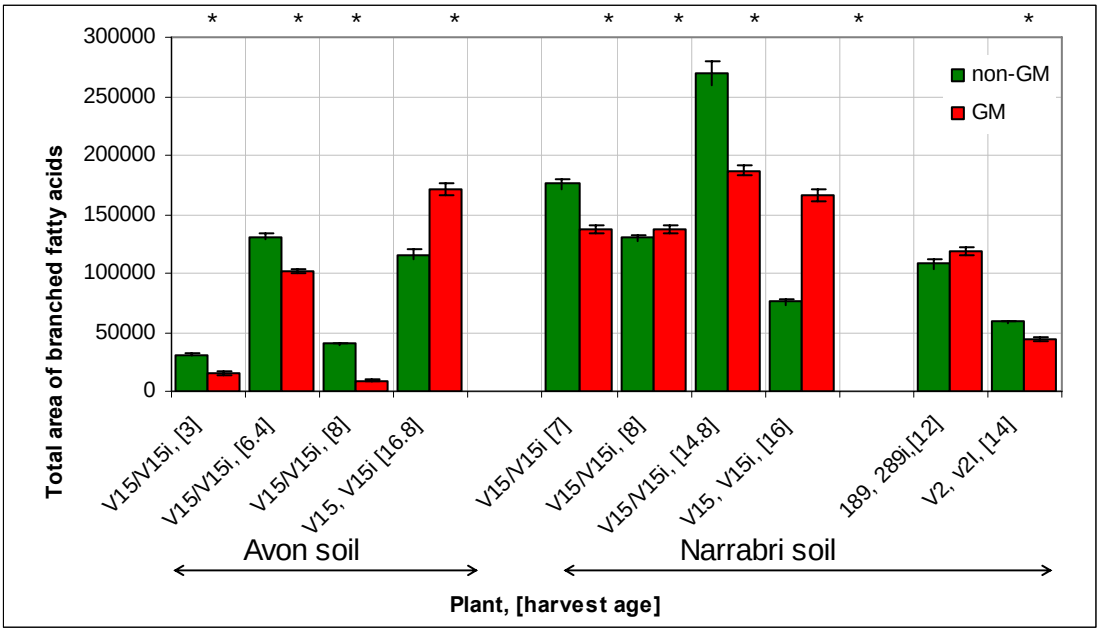


Figure 14.3.8.1 **Total branched fatty acids for all non-GM and GM trials grouped by soil, plant type, and [weeks at harvest].**

This suggests that *Bacillus* was present in significant numbers in each individual trial, from both soils. Small error bars contributed to the statistically significant differences between the non-GM and GM rhizosphere soils in 9 of the 10 paired trials. The arithmetic means of the paired trials of branched fatty acids varied both within and between runs, however for the V15 and V15i plants there was a trend for non-GM rhizosphere soils to contain more branched fatty acids than the GM plant rhizosphere soils, until about the 8th week.

14.3.9 Change in Community Profile with Variance by Nutrient Deficiency

The first and second principal components explained 27.65% and 22.07% of the difference between the microbial populations for plants grown in Narrabri soil, when the equivalent chain lengths of 10:0 to 20:0 were included for the plants grown with adequate nutrients. When the range of C chain lengths were reduced to between 14:0 to 20:0, the first two principal components changed to 32.39% and 15.05%. This meant that approximately 5% of the first principal component was attributable to undefined short chain fatty acids. With their elimination the differences between the replicates could be analysed with more precision. For comparison, the plant

rhizosphere soils from trials under nutrient deficiency showed that for C chain lengths between 10:0 to 20:0, the first two components were 27.6% and 13.5%, and for the 14:0 to 20:0 carbon chain lengths, the components were 31.2% and 24.4%. Comparison of the effect of nutrient deficiency was therefore made between the range of the 14:0 and 20:0 fatty acids. The two datasets generated in separate runs were merged, and retention times of less than or equal to 0.01 minutes were matched, where they occurred between the two sets, with particular alignment of the fatty acid peaks that had been named by the software. Figure 14.3.9.1 shows the environmental effect of the same paired plants under the constraints of nutrient supply.

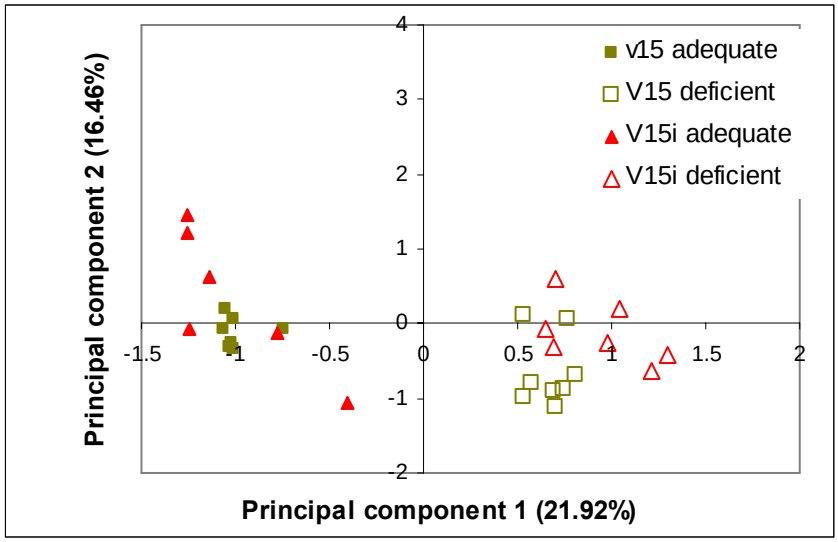


Figure 14.3.9.1 **Principal Component analysis of the comparison of treatment effect of nutrient on microbial community profile, for non-GM and GM rhizosphere soils, for plants grown in Narrabri soil.**

Principal component analysis showed demarcation between the trials of nutrient sufficiency and deprivation, but no difference between V15 and V15i. Thus environmental influence had more effect on community profile than did plant type.

To separate the factors (fatty acid peaks) which are the components of the PLFA profile, a graph was constructed with lines joining the discrete values for each replicate volume against retention time on the GC column. This was done to aid visual comparison of differences in volumes of each fatty acid between replicates. Different colours were used to clarify distinction between replicates. The result is shown in Figures 14.3.9.2 to 14.3.9.3.

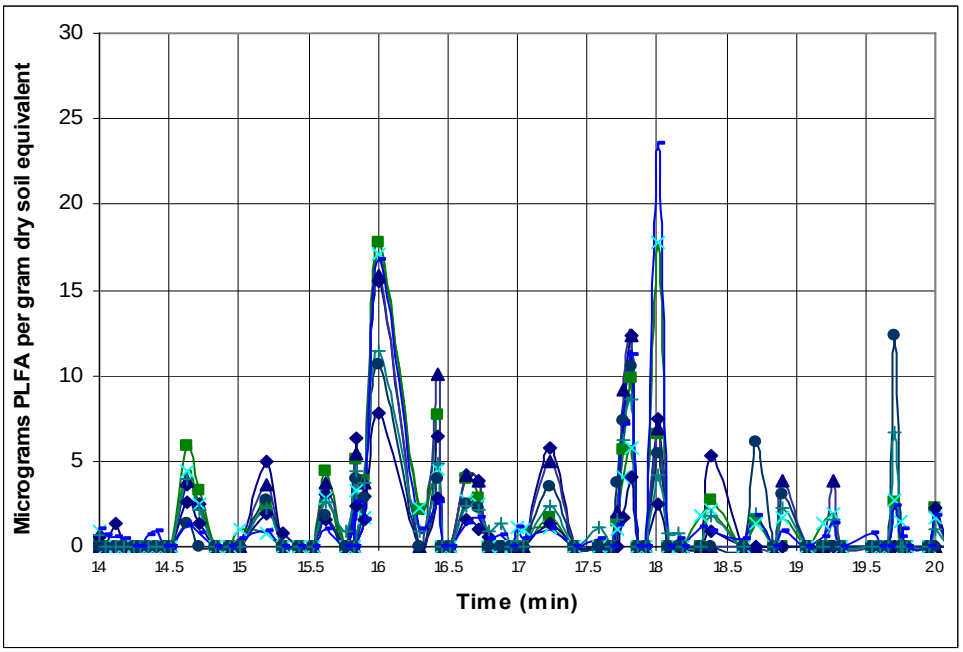


Figure 14.3.9.2 **PLFA profile of the V15 plant grown in Narrabri soil, and harvested at 16 weeks, grown under conditions of adequate nutrient.**

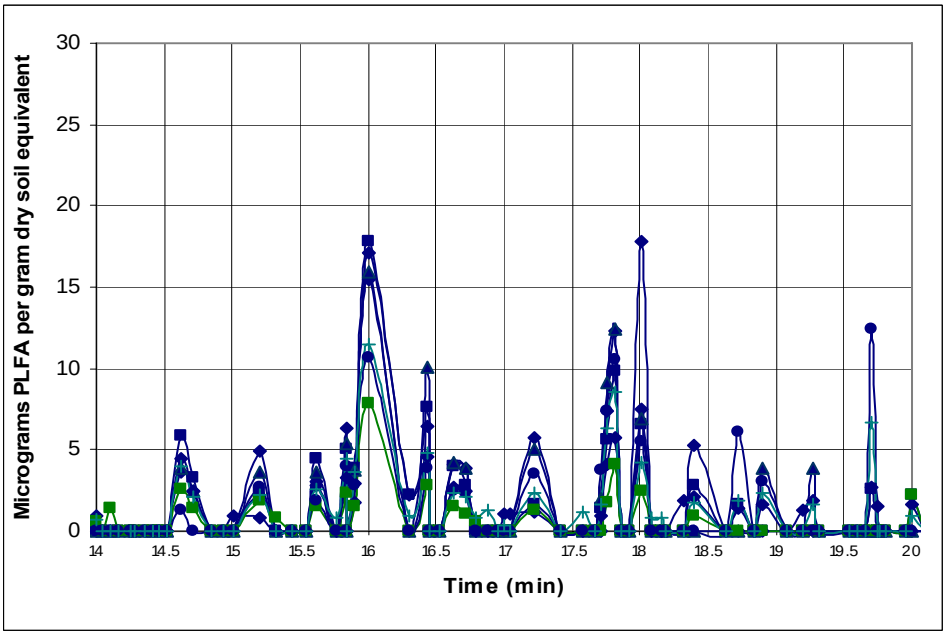


Figure 14.3.9.3 **PLFA profile of the V15i plant grown in Narrabri soil, and harvested at 16 weeks, grown under conditions of adequate nutrient.**

The same paired plant cultivars, grown in the same soil, but kept under different nutrient status, showed different fatty acid profiles, but showed more similarity within each of the treatments, than across the two sets, separated by nutrient status.

The traces for the non-GM and GM rhizosphere soils under nutrient deficiency are shown in Figures 14.3.9.4 and 14.3.9.5.

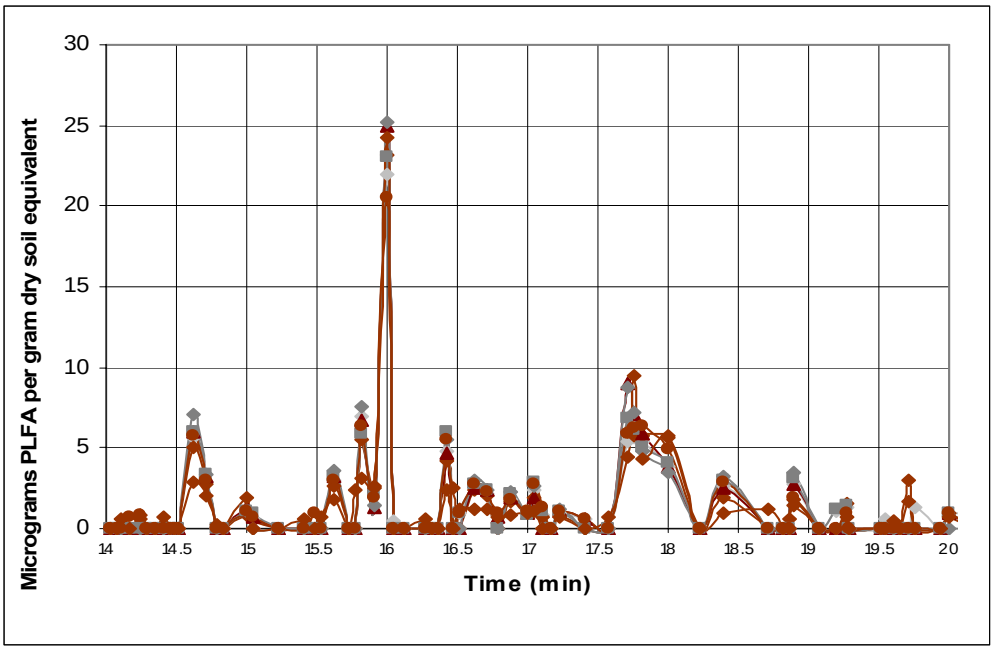


Figure 14.3.9.4 **PLFA profile of the V15 plant grown in Narrabri soil, and harvested at 14.8 weeks, grown under nutrient deficiency.**

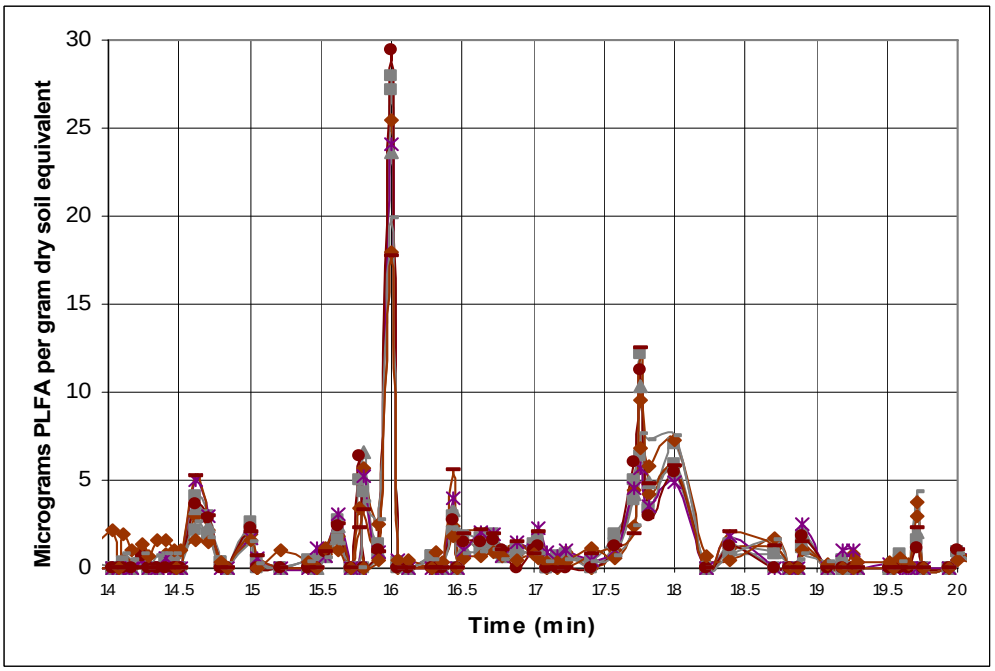


Figure 14.3.9.5 **PLFA profile of the V15i plant grown in Narrabri soil, and harvested at 14.8 weeks, grown under nutrient deficiency.**

Notable differences in the phospholipid fatty acid traces between the rhizosphere soils of the paired plants for adequate, and deficient nutrient conditions occurred in the area under the curve of the 16:0 and 18:0 peaks. Similar traces were seen for both the non-GM and GM plants. A more sharply defined peak (caused by the presence of fatty acids immediately before and after this major peak) was seen in both the nutrient deficient plant soils, but not in the nutrient-adequate soils. These two trends of peak definition were reversed for the 18:0 peaks in both the non-GM and GM plants.

Additionally, many more peaks approaching 5µg g-1 of soil occurred under adequate nutrient conditions, than under nutrient-deficiency, showing a higher active microbial cell mass under nutrient adequacy. The ancillary peaks at the retention time of 17:75 for both nutrient levels, show the grouping for 18:3ω6c, 18:2ω6c and 18:1ω9c and sum in feature 8; the first two of which define ergosterol, indicative of fungus.

The frequency of maximum concentration of a particular fatty acid within all replicates against the total fatty acids for each separate replicate (to test a concentration effect) resulted in an r^2 value of 0.97 for V15 plants, and 0.93 for the V15i plants under adequate nutrient. This indicates that the most numerous fatty acids in a sample nearly always came from the replicate with the highest overall volume. A possible 'loss' of minor peaks would therefore occur at low concentrations.

A comparison of fatty acids across treatments showed that under conditions of adequate nutrient, 41 fatty acids were common to both environments, 29 occurred exclusively in the nutrient-adequate rhizosphere soil and 23 different fatty acids occurred exclusively in the nutrient deficient set. The fatty acids that were named by the software, and found exclusively in only one of the two paired soils is tabulated in Table 14.3.9.1.

Table 14.3.9.1 Fatty acids differing in soils from plants grown in nutrient deficient, and nutrient adequate conditions.

Fatty acid	Only in nutrient deficient	Only in nutrient adequate
15:1 ISO G . . .	GM	
16:1 ISO G . .	GM	
16:1 w9c . . .	GM	
15:0 3OH	GM	
17:1 w8c . .		non-GM
18:1 w9c		non-GM
18:1 w9t Alcohol	GM	
19:0 ISO		non-GM
19:2 w6c	non-GM	
cis 9_10 epoxy 18:0	non-GM	
20:2 w6c	GM	
19:0 CYCLO 11-12 2O	GM	
21:0 .	GM	

Most fatty acids were found within both non-GM and GM rhizosphere soils under adequate nutrient. The effect of treatment is also shown by the comparison of the cyclopropyl groups indicative of nutrient deficiency (Law et al. 1963). These were summed for each of the replicates and are tabulated in Table 14.3.9.2.

Table 14.3.9.2 Difference in cyclopropyl groups between V15 and V15i plants grown in Narrabri soil, under adequate and deficient nutrient shown as summed µg fatty acid g^{-1} dry soil equivalent

	Non-GM rhizosphere soil	GM-rhizosphere soil	Non-plant soil
Adequate nurient	12.23 (n=7)	7.86 (n=6)	0.00 (n=2)
Nutrient deficient	30.82 (n=8)	19.83 (n=8)	9.73 (n=3)

This shows that different proportions of the cyclopropyl group were present in higher concentrations under the nutrient deficient conditions.

14.3.10 Change in microbial populations after a 'rhizosphere event'

Microbial rhizosphere populations may be altered mainly by amplification of a subset of opportunistic bacteria which grow rapidly under a temporary increase of readily utilisable nutrient, with the remainder of the microbial populations remaining the same. Alternatively, there may be an overall shift in the major components of the microbial population.

The Table 14.3.10.1 shows the likelihood of respective fatty acids appearing in the rhizosphere soil, that were also present in the bulk soil, kept under the same conditions, but without the effect of a plant root.

Table 14.3.10.1 The proportion of fatty acids which occurred in the rhizosphere soil, that were also present in the control soil

Soil	Plant type	Harvest age	In rhizosphere soil, if present in bulk soil
Avon	V15, V15i	3.7 weeks	16.90%
Avon	V15, V15i	8 weeks	42.30%
Avon	V15, V15i	16.8 weeks	48.10%
Narrabri	V15, V15i	7 weeks old	18.75%
Narrabri	V15, V15i	8 weeks	32.30%
Narrabri	189, 289i	12.1 weeks	43.40%
Narrabri	V15, V15i	14.8 weeks	51.30%
Narrabri	V15, V15	16.7 weeks	30.30%
Narrabri	V2, V2i	16.71 weeks	36.50%

The r^2 value of the increase in the same type of fatty acid for age at harvest was 0.85 for the Avon calcarosol, and 0.50 for the Narrabri vertosol, indicating different microbial soil dynamics in the different soil types.

14.4 DISCUSSION

The chromatographic analysis of the phospholipid fatty acids of the rhizosphere soil microbiota allowed comparison of the living component of the soil microbiota of parental cotton plants and the paired genetically modified plants under the conditions of pot trials. This method was repeatable to within 0.3% for a repeat within-run for an Avon trial and to within 1% for a Narrabri soil trial.

Comparisons of the phospholipid fatty acids from the rhizosphere soils of four parental cotton plant strains and their paired genetically modified counterparts showed that each of the samples of rhizosphere soil from four replicates of each of the pairs contained different mixes of rhizosphere microbial communities. The trace across all of the the fatty acids for each of the replicates showed different volumes for each of the separate fatty acids, even though the same plant, soil and conditions of growth were standardised. If the fatty acids were identical in composition and the only difference was one of concentration, the normalisation of peak volume to percentage would have resulted in a series of identical, overlapping traces, but this was not the case. This diversity of microbiota within soil samples is in agreement with several reports (Watkins,1981; Curl and Truelove, 1986). It is also shown by the comparison of the individual fatty acids which only occurred within the one set of the paired trials (for example non-GM rhizosphere soil) compared with the other set (GM) (Table 14.3.5.1). Approximately one third of all fatty acids appeared in one of the sets of paired trials, but not in the other, and approximately one third again, appeared in both, over the 11 trials. None of these exclusively occurring fatty acids appeared consistently at all times, for only one of the paired plant cultivars, over all trials. Therefore, each of the soil samples contained different ratios of microbiota across each replicate, even under similar conditions.

14.4.1 Rhizosphere effect

The consistent absence of cy 19:0 11-12 20 in the non-rhizosphere soils suggests that this may be the cyclopropene derivative of the cyclic ring structure, indicating the presence of malvalic acid from plant tissue. This fatty acid contains a chain length of 18 carbons (Christie 2005), but an offset in retention time which alters the

interpretation to equivalent chain length of 19 may possibly have occurred. The low concentration, however, limits its use as a rhizosphere indicator unless more root tissue is included. Further work regarding this indicator of plant tissue in the rhizosphere soil could be improved by avoidance of acid in the methylation procedure. Christie (2005) reported that the cyclopropene ring is highly strained and is therefore very reactive. In particular, it reacts readily with thiol groups and other sulphur compounds. The methyl acidification procedure of Christie (1989) followed here, used 1% H_2SO_4. Silver nitrate also reacts with cyclopropene fatty acids, so silver ion chromatography cannot be used for isolation purposes. A method which uses base methylation could be used safely.

14.4.2 The change in microbial population through a rhizosphere effect

Fine roots cannot be entirely eliminated from rhizosphere soil, and the putative identification of malvalic acid in some of the rhizosphere soils suggested some contribution of plant tissue to the total fatty acids. However, as phospholipids are mainly found in cell membranes, and as cell size decreases, membrane-to-biomass ratio increases. The system is therefore biased towards the microflora and the contribution by plant cells will be low.

Differences between the microbial communities of rhizosphere and non-rhizosphere soils could be seen from each principal component matrix which showed that the non-rhizosphere soils were positioned to the left, and below the rhizosphere soils on the cartesion coordinates in nearly all cases. The mix of fatty acids was therefore seen as having less overall factor loadings than the rhizosphere soils for both the first and second components.

Table 14.3.10.1 showed that over all trials, a range of 16.9% to 51.3% of fatty acids in the rhizosphere was also found in the soil from which the original microbial population was taken. In most cases, fatty acids were found that were not present in the bulk soil, so there was a shift by amplification of the original microbial inhabitants, and different fatty acids occurred within the ecosystem of the rhizosphere.

A higher incidence of Gram-negative bacteria in the rhizosphere was noted by Grant & Long (1981). With ageing and senescence of the root, the rhizosphere effect may be lessened if the amount of photosynthate material is less than from a younger, actively growing plant. Söderberg et al. (2004) found that the rhizosphere effect was stronger

in pot trials when comparing the root-associated soil with the bulk soil, than in field studies where there was no difference between bulk and rhizosphere soil. Additionally, in the field the rapidly dividing and sloughed-off root cells and exudates may have passed the probe where the sample had been taken, and the bacteria had resumed their normal respiration, akin to the travelling wave of bacterial activity described by Semenov et al. (1998). Opportunistic bacteria follow the abundance of photosynthate material along an expanding plant root system. However fatty acid methyl ester analysis, undertaken by Dunfield & Germida (2001) in field trials, indicated a variation in rhizosphere microorganisms according to genetically modified plant type but it was not known whether the difference in the rhizosphere microbiota occurred because of differences in the non-GM/GM cotton plant types, or whether the variability was attributable to the inherent complexity of the microbial communities.

14.4.3 Gas Chromatographic Analysis of the Rhizosphere Microenvironment

One difficulty in this method arises from comparison of fatty acids from separate runs of the gas chromatograph. The fatty acids to be compared were identified by the software based on equivalent chain length. Not all were identified, leaving unknown fatty acids which could not be compared when two datasets were to be merged. In manually comparing the two datasets from the Narrabri V15 and V15i rhizosphere soils under conditions of adequate and nutrient deficiency, the retention times had to be aligned to within 0.01 minutes. This was time intensive, and is a potential deterrent to investigation of soil microbial profiles.

The peak for 10me 18:OH a fatty acid unique for actinomycetes (Bååth et al. 1992; Kroppenstedt, 1985), was notably absent in all samples. This implied that actinomycetes were not present in the soil samples, but in fact they were cultivated on ISP4 media, at an estimated 10^5 and 10^6 per gram soil (Section 6.1). Previous work using fatty acid methyl ester analysis and the same equipment and software from sub-cultured actinomycete isolates (Walter, 1999) showed consistent and abundant detection of this fatty acid. However, the colonies harvested from the selective media would have been at much higher numbers than from a mixed soil dilution. The absence of this peak may indicate that a threshold for detection of actinomycetes, or it may be a consequence of the methylation procedure (B. Hawke, pers. comm). Additionally, the apparent absence of actinomycetes may also occur through the over-estimation of spore-forming bacteria which increase rapidly on selective media, but which are not represented in such high numbers *in situ*.

14.4.4 Comparison of analysis by Principal Component and by consensual replicate trace

The use of principal component analysis (PCA) to detect differences in the composition of microbial communities by the type of fatty acids served to identify the major influences through the loadings of the separate factors. No consistent differences between the paired plants for each of the trials could be attributed to a difference in function of the actively metabolising rhizosphere microbiota. As was pointed out by Zelles (1999), PCA of fatty acids does not specify which organisms account for the similarity or differences, unless large peaks (or their absence) are obvious and can be matched with a unique organism or metabolic pathway. Principal component analysis did, however, show a difference in microbial communities according to soil type and nutrient status.

The consensual trace of the fatty acids between 14:0 and 20:0 was more helpful for determining a population response to a particular effect, as individual fatty acid peaks could be identified, their relative magnitude assessed, and positioning of adjacent peaks observed. The sharper definition of the 16:0 peak caused by the existence of additional fatty acids proximal to this peak, and the wider C18 curve caused by less adjacent fatty acids under conditions of nutrient adequacy, and the exact reversal of this trend under nutrient deficiency, is one example of the information gained by the trace, but not revealed by PCA.

14.4.5 Biomass estimates

Olsson (1999) noted that the amount of phospholipids in an organism is correlated with membrane area. The amount of phospholipids per unit biomass is therefore not the same in bacteria, fungi or plants. Biomass of a mixed community may be biased towards the smaller organisms. Additionally, ergosterol is present in varying amounts in different fungi, and its quantity does not necessarily reflect the fungal biomass.

The results obtained from this analysis reinforced the wide difference in soil microbial populations during the growth of the plant roots by the differing ratios of significant fatty acids from each of the separate trials. No phospholipids indicative of Gram negative, Gram positive or fungi consistently dominated either the rhizosphere soils of the non-GM or GM plants. The measure of biomass, if related to the use of organic C

or N, may not reflect activity of the microbial community unless a disturbance in the community is extreme or prolonged.

14.4.6 Nutrient deficiency

There are no known unique signature fatty acids that can be used to identify one single microbial genus or species. The extrapolation of trends by observation is made difficult by the fact that membrane lipids are present as differing ratios of fatty acids across living communities, and are present in the cellular membranes of many dissimilar community members. Even so, a comparison of a single type of fatty acid, the branched iso and anteiso fatty acids indicative of eubacteria (O'Leary & Wilkinson, 1988; Kaneda 1991), and possibly *Bacillus* (O'Leary, 1989), is illustrated in Figure 14.3.8.1. Statistically significant differences were found in nine of ten analyses, with the indication of a higher ratio of branched fatty acids in the non-GM rhizosphere soils up to about 8 weeks, in both Avon and Narrabri soils, for the V15 and V15i plants.

Bacillus is ubiquitous in soils as was shown by its frequency of isolation from all soils on the $^{1}/_{10}$ TSA medium. These Gram-Positive bacteria respond quickly to available soil nutrients, as a direct response to root exudates. A lower C:N ratio in the GM plants through the presence of additional nitrogenous compounds could affect the balance of the *Bacillus* populations, but only when N was available to the plant. However, there seemed to be more *Bacillus* in the non-GM plant rhizosphere soils. Possibly the N locked up in the plant tissue in the form of the *Bt* protein and unavailable to the plant (Section 4), may have depleted the N as a source of root exudate compounds, and altered the balance in favour of other microflora that are able to survive with a wider C:N ratio.

The later harvests showed that the trend of non-GM plant rhizosphere soils containing a higher presence of eubacteria was reversed, with GM-rhizosphere soils having greater volumes of branched fatty acids. This was also shown by the different, compared plant strains 189/289i, harvested at 12 weeks. The trend was not universal for plant type, however, as the V2 and V2i plants had more *Bacillus* at 14 weeks. The 17:1G peak (indicative of eubacteria) was present in the 16.7 week harvest from Narrabri soil under adequate nutrient (Figure 14.3.6.7), but was missing in the 14.8 week harvest under conditions of nutrient deficiency (Figure 14.3.6.8).

After 8 weeks, when the plant had stopped growing, differences in microflora populations suggested that either the root exudates had diminished with the cessation of growth, or that a higher C:N ratio resulted in a different mix of microflora. This is supported by the increased fungal population under nutrient deficient conditions at 14.8 weeks (Figure 14.3.6.8) where both non-GM and GM rhizosphere soils had the 18:1ω9c and 18:2ω6c fatty acids indicative of fungi. There was a significant difference in both 18:2ω6c and 18:1ω9c. In the non-GM V15 there was more 18:2ω6c but 18:1ω9c was higher in the GM V15i plant. This fatty acid is a specific biomarker for fungi (Federle et al, 1986; Olsson, 1999; Ibekwe & Kennedy, 1998), but is present in varying amounts in most other eukaryotes (Vestal & White, 1989), so the difference cannot be guaranteed to have been due to the presence of fungi only.

The cyclopropyl groups can be an indication of starvation (Law et al. 1963) and were found to consistently increase under nutrient deficiency, for both non-GM and GM rhizosphere soils (Table 14.3.9.2). They also increase in the control soils (although not 19:0 cyclo 11-12 20), where Hoaglands nutrient solution was added, even though there was no plant root present. The volume of the cyclopropyl fatty acids did not increase to significant levels (> 3% of the total volume) for any of the rhizosphere soil trials, so while the frequency of this fatty acid increased relatively, the amount produced under starvation was limited by the diminishing nutrient resources available to bacteria for its production.

A comparison of the means of the branched fatty acids against plant age at harvest was more closely aligned to individual trial than to the plant type grown in different soils, indicating that the individual trials had more effect than harvest age alone. In future work, parallel time trials using *Bacillus* and plants grown under controlled conditions could be used to give information on the amount of root exudates and bacterial interaction. This would reduce random effects of competition from other microflora and predation from higher trophic groups present in agricultural soils.

Summit et al. (2000) showed that phospholipids derived from sediments did not disclose any *trans*-monounsaturated PLFAs and so an indication of the ratio *trans*- to *cis*- could not be calculated as an indicator of starvation-stress. They concluded that the *trans*- to *cis*- isomers were not a robust measure of stress in all environments. In this present work, 16:1ω7t was found in significant values from the Narrabri 16.7

week trial. In the nutrient-deficient Avon trial, however, the *cis*- isomer 16:1ω7c was found in significant amounts.

In conclusion, even though the fatty acid traces showed a consensual community response in soil microbiota for the conditions of nutrient status, there were still individual responses within the microbial populations. Allowing for the lower overall concentration of fatty acids within the rhizosphere of the plants grown under conditions of nutritional deprivation, there were other differences such as the occurrence of fatty acids of slightly greater chain length than the 18:0 equivalent fatty acid, and their absence in the nutrient deficient soils. In addition, fatty acids more closely distributed around the universal 16:0 fatty acid occurred under nutrient adequacy, but were lacking under conditions of nutrient deficiency. Thus community responses occurred, and each soil sampled contained its own discrete microbial populations.

14.4.7 Possible Future Research

The fatty acids from the rhizosphere of 'homogenised' soils (passed through a 5 mm sieve at the commencement of the trials) changed during the growth of the plant to show differing microbial communities were present at the time of harvest. A better understanding of the changes in soil microbiota could result in knowledge leading to optimisation of agricultural productivity through understanding symbiotic effects of beneficial rhizosphere microorganisms to enhance plant growth.

An example of a change in microbial flora in soil that has become problematic for farmers recently occurred at the Avon trial site. In the year 2000, the site (which had been sown with wheat after wheat annually, and was known to be suppressive to pathogenic fungi) began to lose its suppressive nature after an infestation of weeds, to the extent that antifungals now have to be applied. This change in microflora is as yet unexplained, but resulted in the loss of several crops.

Analysis of soil microbial fatty acids may allow comparison of the changing soil microbiota, including those that have adapted, or are able to tolerate increased soil salinity. Genetically modified plants are now including the attributes of salt-tolerance, and halophilic bacteria contain high proportions of ether lipids (Lehninger et al. 1997, p. 249). Fatty acid analysis could be used to compare the ratio of ether-linkage to

ester-linkage indicative of a change in proportion of halophytic microflora within the population.

In response to the marked effect of the soil microbiota to the addition of nutrient to rhizosphere soils, shown by the phospholipid fatty acid analysis, a further analysis to the changes in soil microbiota was undertaken by multivariate statistical analysis using factor loadings to determine the most important influence of change, for all factors tested. This follows in Section 15.

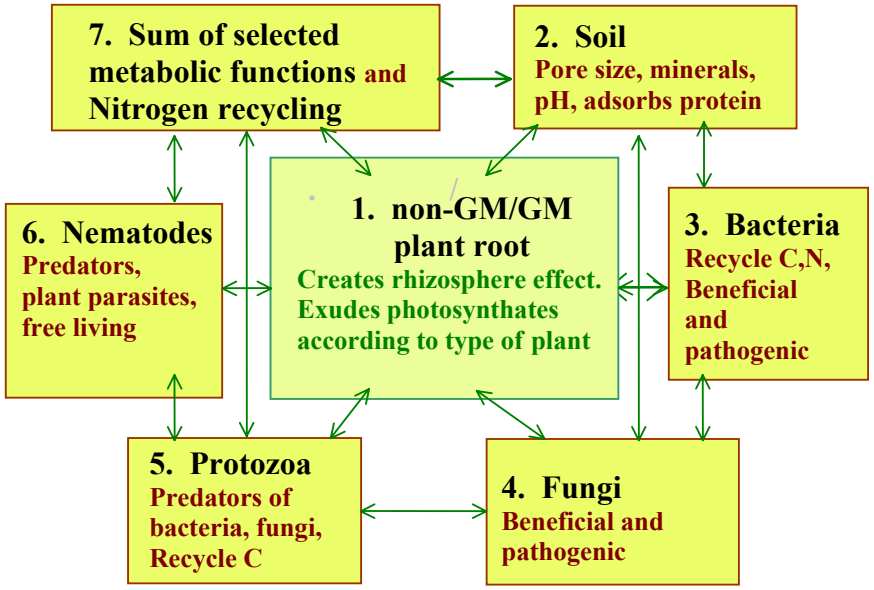


Fig. 15.0 Concept map of some of the rhizosphere plant/ soil/ microbial interactions considered in this work

15.1 INTRODUCTION

Measurements of community dynamics in soil seldom reflect simple positive or negative interactions, but changes within a soil microhabitat at any given time are the sum total of many interactions set in motion by the provision of a utilisable nutrient source and the absence of environmental extremes (Metting, 1993, p. 17). Within the rhizosphere, a combination of effects are not readily observed by the controlled addition of a single substance. Illustrative of this, the respiratory quotient of soil microbiota was increased by glucose, but increased again when NO_3 was added (Dilly, 2002).

The integration of nutrients and other effects among soil microbiota is not only 'vertical' where the impact is passed on to different trophic groups, but also horizontal, where an advantageous trait can be passed onto surrounding receptive microbiota after a change in environmental conditions. Metting (1993, p. 12) observed that the degree of phenotypic and genotypic plasticity that exists within microbial communities is facilitated by the widespread occurrence of infectious viral particles, plasmids and other mobile genetic elements. Their roles in transduction, transformation and conjugation led to the conclusion that microbial ecosystems are

genetically open communities (Terzaghi & O'Hara, 1990), and that the new genetic traits can permeate the community via panmixis (Richaume et al. 1989). This ensures that traits advantageous to the microbiota will continue to be carried in the population, and those with no benefit may be eventually lost.

However, in agricultural soil at the aggregate level, microbial populations remain distinct, as impediments to gene flow include physical barriers and antagonism through competition for localised nutrients. This occurs even with environmentally assisted dispersion such as water flow and via the movement of mesofauna and macrofauna. Grundmann & Debouzie (2000) found that soil micro-aggregates may harbour different bacteria on the surface from those within smaller, less aerobic, pores. For example, *Nitrobacter* bacteria were spatially aggregated over ranges of 2-4 mm. Localisation of microbial populations within soil microsites associated with plant roots was mentioned by Campbell (1985), who stated that the distribution of microorganisms varies over a short distance between different cells on the root and in general up the root as it ages, and that there is preferential colonisation at cell junctions, possibly because these are the sites of exudation of soluble material. On a fine scale, shifts in microbial ecologies in the rhizosphere are temporal as well as spatial. Semenov et al. (1998) described a 'travelling wave' of microbiota, following the growing root.

Marschner et al. (2001) pointed out that soil types and root zone effects contribute to the development of distinct local microbial communities. However the complex interaction between plant species, root zone and soil types may not be readily discerned by studying these parameters as fully independent variables. The interactions of the categories depicted in Figure 15.0 should not be seen as having rigid boundaries, or the same weighting of factors. For example, the breakdown of organic compounds within soil by multiple guilds adds to the complexity and precludes appropriation of activity to defined subsets of soil populations.

In the work presented here, the various analyses undertaken had particular biases and scales of measurement. Thus, individual bacterial and fungal populations were estimated by standard dilution plate-count methods, and protozoa by the method of Most Probable Number. Similarly, the enzyme and other chemical assays followed

methods of inverse regression estimated from optical density. Bacterial and fungal colonies had widely differing numbers and were reported on a log scale. Protozoan numbers ranged from single digits to 3,000 per gram of moist soil (small amoebae in the Narrabri soil). Nematode numbers extracted from 200 g soil reached thousands in the case of some bacterial feeders.

Apart from the phospholipid fatty acid analysis which used principal component evaluation, the comparisons of results in previous sections were based on ANOVA, t-tests and the simple arithmetic mean with standard error, and χ^2 for the different ratios of nematodes.

The aim of the work described in this Section was, therefore to compare the records of rhizosphere soil microbiota across the various methods of measurement. It attempted to determine the contribution of each group of microbiota, and to ascertain whether one effect could be correlated with the results of others, within each record. Each of these comparisons was made between the rhizosphere microbial populations of non-GM and GM cotton plants, grown under the conditions of the paired trials.

15.2 MATERIALS AND METHODS

15.2.1 Preliminary visual comparison by octile division

As a preliminary comparison between the individual trials and the factors tested, spreadsheets of the database were created, using percent-ranked divisions over the range of values measured under the conditions of the trials, to normalise the data to a common base. Eight divisions of 12.5% (octiles) were calculated from within the ranked range and colour coded according to the key in Figure 15.2.1.1. This method indicated the magnitude of response to the various treatments, across apparently unrelated factors.

The colour index ranges from low values indicated by light to darker blue, to intermediate values, indicated by shades of green and to high values, spanning from yellow to red. This is designed to remind the reader that gradients exist and rigid categorisation is not realistic (Hamilton, 1988). The sets of data were grouped separately by soil type, as the chemical analysis of leaf tissue (Section 4) was influenced by soil minerals taken up by the plant. Soil effects were also seen from

the microflora (Sections 7.1 and 7.2). An index of the rankings is shown in Figure 15.2.1.1.

Category	% Ranking	Colour	
1	0-12.4	1	pale blue
2	12.5-24.9	2	light blue
3	25-37.4	3	blue-grey
4	37.5-49.9	4	lime
5	50-62.4	5	dark yellow
6	62.5-74.9	6	yellow
7	75-87.4	7	light orange
8	87.5-100	8	red

Figure 15.2.1.1 Defined categories from percentage-ranked variables.

Figures 15.1(a), (b) and (c) show each of the three non-GM rhizosphere soils compared for all of the analyses undertaken. Figures 15.2 (a), (b) and (c) show the GM equivalent trials, and Figures 15.3 (a), (b) and (c) show the results of the non-rhizosphere soil.

Because of the variability seen previously between replicates of the individual trials (for example phospholipid fatty acids between micro-environments), averaged values for each trial were also compared. In the averaged sets of data, only the records of those with at least three replicates were used: any other records containing less than three replicates were not included.

15.2.2 Analysis by Principal Component

After the initial investigation of trend by octile ranking, principal component analysis was used to determine the factor weighting. The data were normalised to percent rankings for the scale of values obtained under the conditions of the pot trials, over all soils. Factor weightings were then investigated to determine which of the main components contributed most to a differentiating effect. Only the data from trials which incorporated all of the tests done simultaneously, within the conditions of the particular batch, were included in the comparisons.

	Table 15.1: non-GM rhizosphere soil analyses ranked using normalised data																								
Soil/ Plant	Harvest age (weeks)	ELISA Leaf	ELISA Root	General bacteria	Actinomycetes	Fungi	Pseudomonads	Amoebae	Flagellates	Cilliates	Amm. Ox. Bact	Subst. Ind. Resp	Ninhydrrin-N	Cellulase	Aryl_Sulph	Acid_Phosph.	Dehydrogenase	Protease	Urease	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders	
Figure 15.1(a) Avon non-GM rhizosphere soil																								
V15	15			8	8	1	3	5	5	1	1	2	1						1					
V15	15			3	5	3	1	7	6	8	2	1	2						4					
V15	15			1	3	5	8	3	8	1	3	1	3						1					
V15	15			5	1	8	5	7	5	8	1	1	1						2					
V15	8																			1	3	2	1	
V15	8																			1	3	3	1	
V15	8																			5	7	7	4	
V15	8																			1	2	1	1	
V15	4																			3	8	5	8	
V15	4																			2	1	1	2	
V15	4																			7	6	5	5	
V15	4																			7	7	2	4	
V15	6																			5	6	2	2	
V15	6																			1	5	4	3	
V15	17											5								3	1	1	2	
V15	17											5								6	2	5	5	
V15	17											2								7	1	3	5	
V15	17											4								8	3	4	7	
V2	7							8	8	1	7	3	1	3	3	1	7	8	1					
V2	7							6	7	1	7	8	1	3	1	4	5	7	6					
V2	7							7	4	1	6	7	1	4	2	4	4	2	4					
V2	7							5	7	1	8	1	1	2	5	5	3	6	7					
189	11							5	4	1	4	4	3	6	6	1	1	3	1					
189	11							3	1	1	5	4	8	4	3	8	5	3	5					
189	11							4	1	1	8	3	6	1	5	6	1	1	5					
189	11							4	1	1	5	5	4	1	8	3	3	5	8					
C312	9																			5	4	6	6	
C312	9																			4	4		3	
C312	9																			8	4	7	4	
C312	9																							
C312	10							1	4	1	3	8	7	7	8	2	2							
C312	10							2	1	1	4	8	5	8	4	8	8		2					
C312	10							1	1	1	2	7	5	8	7		6		8					
C312	10							1	3	1	5	6	8	5	1	7	8		3					
C312	9																			5	5	8	6	
C312	9																			4	2	4	3	
C312	9																			8	5	7	4	
C312	9																							
C312	37																			3	5	3	8	
C312	39																			6	8	6	7	
C312	39																			4	8	7	8	
C312	41																			2	7	8	7	

Table 15.2: GM rhizosphere soil analyses ranked using normalised data

Soil/ Plant	Harvest age (weeks)	ELISA Leaf	ELISA Root	General bacteria	Actinomycetes	Fungi	Pseudomonads	Amoebae	Flagellates	Cilliates	Amm. Ox. Bact	Subst. Ind. Resp	Ninhydrrin-N	Cellulase	Aryl_Sulph	Acid_Phosph.	Dehydrogenase	Protease	Urease	Omnivores	Bacterial feeder	Fungal feeders	Plant feeders
Figure 15.2 (a) Avon GM rhizosphere soil																							
V15i	15		2	8	1	1	1	8	8	1	1	1	1						1				
V15i	15		3	5	4	4	8	5	5	7	2	1	1										
V15i	15		2	7	6	7	5	8	2	8	3	1	1						5				
V15i	15		3	5	5	3	3	7	7	8	3	2	1						8				
V15i	8																			4	6	3	5
V15i	8																			2	5	3	1
V15i	8																			8	8	8	8
V15i	8																			5	2	2	1
V15i	4																			8	8	5	7
V15i	4																			3	4	1	2
V15i	4																			2	3	1	2
V15i	4																			3	7	2	2
V15i	6																			3	5	5	3
V15i	6																			1	3	4	1
V15i	17		6									8								2	1	2	7
V15i	17		6									4								1	1	1	3
V15i	17		5									6								1	1	3	5
V15i	17		5									4											
V2i	7							6	6	1	7	8	1	2	2	1	3	8					
V2i	7							5	7	1	6	3	1	3	3	2	5	6	6				
V2i	7							5	2	1	2	8	1	8	5	8	1	7	3				
V2i	7							7	8	7	8	3	1	1	6	5	1	4	3				
289i	11							4	2	1	8	7	7	5	5	5	4	3					
289i	11							2	5	1	1	2	3	1	8	3		2	5				
289i	11							1	2	1	5	5	4	4	2	7	7	5	1				
289i	11							3	1	1	4	5	5	3	7	1	8	1	7				
C312i	9																			6	3	4	3
C312i	9																			7	4	7	5
C312i	9																			5	2	6	4
C312i	9																						
C312i	10							4	1	1	4	5	6	6	1	6	2						
C312i	10							1	5	1	5	3	1	5	1	8	6		4				
C312i	10							2	1	1	5	7	8	8	4	4	4		2				
C312i	10							3	2	1	7	6	2	7	8	3	8		8				
C312i	9		7																	6	4	4	3
C312i	9		7																	7	5	8	5
C312i	9		7																	5	2	6	4
C312i	9		7																				
C312i	37																			5	7	4	8
C312i	39																			7	7	5	8
C312i	39																			4	8	7	7
C312i	41																			4	5	7	4
C312i	41																			5	6	8	6

Figure 15.3: non-rhizosphere soil analyses ranked using normalised data

Soil/ Plant	Harvest age	ELISA Leaf	ELISA Root	General bacteria	Actinomycetes	Fungi	Pseudomonads	Amoebae	Flagellates	Cilliates	Amm. Ox. Bact	Subst. Ind. Resp	Ninhydrrin-N	Cellulase	Aryl_Sulph	Acid_Phosph.	Dehydrogenase	Protease	Urease	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders
Figure 15.3 (a) Avon non-rhizosphere soil																							
Avon	15			8	8	8	1	1	8	1	1	3	2										
Avon	15			1	1	1	8	5	7	1	2	1	1										
Avon	8																			1	2	1	2
Avon	8																			2	1	1	1
Avon	4																			7	8	5	4
Avon	17											2								2	3	3	3
Avon	7							8	6	8	6	5											
Avon	7							6	4	1													
Avon	11							3	1	1	8	8	8										
Avon	11							6	4	1		7	5										
Avon	9																			4	5	7	7
Avon	10							2	2	1	4	6	4							1	1	1	1
Avon	10							4	3	1		4	7										
Avon	9																			7	7	4	5
Avon	9																			4	4	7	7

Soil/ Plant	Harvest age (weeks)	ELISA Leaf	ELISA Root	General bacteria	Actinomycetes	Fungi	Pseudomonads	Amoebae	Flagellates	Cilliates	Amm. Ox. Bact	Subst. Ind. Resp	Ninhydrrin-N	Cellulase	Aryl_Sulph	Acid_Phosph.	Dehydrogenase	Protease	Urease	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders	
Figure 15.1 (b) Narrabri non-GM rhizosphere soil																								
V15	17			3	7	7	4	5	5	7	7	5	6						8					
V15	17			4	2	1	1	5	4	6	4	5	3						1					
V15	17			5	5	4	4	5	5	4	4	8	4						5					
V15	17			1	1	5	3	4	5	4	6	8	5						7					
V15	4																			1	3	4	8	
V15	4																			2	4	1	3	
V15	7																			7	5	3	5	
V15	7																			8	8	7	8	
V15	8																			5	5	2	4	
V15	8																			8	7	4	5	
V15	8																			5	8	8	7	
V15	15											6								1	1	1	1	
V15	15											4								4	1	5	2	
V15	15											3								3	2	8	1	
V15	15											4												
V2	15			1	3	4	2	4	2	1	3	6	4											
V2	15			2	8	2	2	2	7	1	1	3	5											
V2	15			1	3	5	1	3	3	6	2	2	3											
V2	15			4	8	2	3	8	1	1	2	7	5											
189	14			7	7	3	7	1	5	5	5	7	8	1	8	8	5	1						
189	14			7	1	1	7	3	3	1	8	1	7	3	3	3	8	3	3					
189	14			6	2	5	8	2	1	7	8	1	8	8	5	1	1	8	4					
189	14			7	4	8	8	1	2	1	7	1	7	5	1	5	3	5	2					
C312	9			5	5	7	6	7	8	1	1	3	1											
C312	9			7	5	6	5	8	7	8	3	5	2											
C312	9			4	4	8	5	6	8	5	5	8	2											
C312	9			2	5	3	5	7	4	1	5	2	1											

Soil/ Plant	Harvest age (weeks)	ELISA Leaf	ELISA Root	General bacteria	Actinomycetes	Fungi	Pseudomonads	Amoebae	Flagellates	Cilliates	Amm. Ox. Bact	Subst. Ind. Resp	Ninhydrrin-N	Cellulase	Aryl_Sulph	Acid_Phosph.	Dehydrogenase	Protease	Urease	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders	
Figure 15.2 (b) Narrabri GM rhizosphere soil																								
V15i	17		3	5	8	2	3	3	5	5	5	7	5						3					
V15i	17		2	1	2	4	3	5	6	6	4		4						1					
V15i	17		5	1	1	1	4	4	7	1	7	4	2						4					
V15i	17			4	3	8	5	5	2	4	5	3	4						2					
V15i	4		3																	1	4	1	1	
V15i	4																			1	5	1	1	
V15i	7																			3	8	7	1	
V15i	7																			4	8	8	1	
V15i	8																			7	7	7	1	
V15i	8																			8	6	6	1	
V15i	8																			8	4	4	1	
V15i	15		5									3								4	1	3	1	
V15i	15		5									2								4	3	2	1	
V15i	15		5									5								4	1	4	1	
V15i	15		5									5								2	2	4	1	
V2i	15		3	3	7	5	2	6	1	1	1	6	5											
V2i	15		3	5	6	3	1	5	1	1	1	1	3											
V2i	15			5	5	5	1	3	2	1	2	5	5											
V2i	15		3	2	5	7	2	4	3	5	2	8	6											
289i	14		1	8	1	8	8	2	4	7	6	4	8	5	3	1	3	1						
289i	14		1	7	4	1	8	1	5	8	8	8	7	1	5	8	8	5	8					
289i	14			7	3	6	7	1	5	8	7	8	8	3	8	5	1	3	7					
289i	14			8	2	7	7	2	3	4	8	7	7	8		3	5	8	5					
C312i	9			6	5	3	5	8	8	3	5	2	2											
C312i	9			4	7	4	6	7	4	5	4	1	1											
C312i	9			2	8	5	4	8	8	7	3	4	1											
C312i	9			3	4	1	5	7	7	1	3	1	3											

Soil/ Plant	Harvest age	ELISA Leaf	ELISA Root	General bacteri	Actinomycetes	Fungi	Pseudomonads	Amoebae	Flagellates	Cilliates	Amm. Ox. Bac	Subst. Ind. Res	Ninhydrrin-N	Cellulase	Aryl_Sulph	Acid_Phosph.	Dehydrogenase	Protease	Urease	Omnivores	Bacterial feeder	Fungal feeders	Plant feeders
Figure 15.3 (b) Narrabri non-rhizosphere soil																							
Narrabri	17			1	1	6	4	8	7	8	6	8	7										
Narrabri	17			5	5	4	3	4	8	7	1	7	5										
Narrabri	4																			1	3	2	3
Narrabri	7																			1	5	4	2
Narrabri	8																			3	4	4	2
Narrabri	15											4								1	1	3	2
Narrabri	15											5											
Narrabri	15		0	3	6	3	1	5	5	1	3	3	4										
Narrabri	15		0	4	6	1	1	1	6	1	2	5	3										
Narrabri	14		0	8	3	8	8	2	2	5	8	2	8										
Narrabri	14		0	6	2	7	7	6	1	5	7	8											
Narrabri	9			7	4	2	5	3	3	1	5	1	2										
Narrabri	9			2	8	5	1	7	4	1	4	1	1										

Soil/ Plant	Harvest age (weeks)	ELISA Leaf	ELISA Root	General bacteria	Actinomycetes	Fungi	Pseudomonads	Amoebae	Flagellates	Cilliates	Amm. Ox. Bact	Subst. Ind. Resp	Ninhydrrin-N	Cellulase	Aryl_Sulph	Acid_Phosph.	Dehydrogenase	Protease	Urease	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders	
Figure 15.1 (c) Waikerie non-GM rhizosphere soil																								
V15	11			6	4	8	8					3	5	6	8	1	3	5						
V15	11			2	8	4	7					4	5	3		7	3	6						
V15	11			3	4	8	2					1	1	1	6	5	5	7						
V15	11			6	2	6	1					3	4	1	5	6	4	8						
V2	12			8	3	1	6	3	8	1		7	4											
V2	12			3	3	1	5	1	1	1		4	1											
V2	12			5	1	1	8	5	5	1		5	2											
V2	12			5	1	6	1	6	3	8		1	3											
189	17							2	2		1	2	5	8	5	5	8	3						
189	17							8	8		2	5	6		8	3	6	1	2					
189	17							1	1		5	7	7	4	7	1	5	4	5					
189	17							6	5		3	6	3	5	4	2	7	2	1					
C312	14			8	8	1	4	6	5	8	7	8	2	4	3	8	1							
C312	14			1	7	5	3	3	4	1	6	8	8	2	1	3	2		8					
C312	14			1	6	4	4	3	7	1	8	5	8	7	3	4	8		4					
C312	14			3	5	3	3	5	3	1	4	2	7	8	2	8	1		7					

Soil/ Plant	Harvest age (weeks)	ELISA Leaf	ELISA Root	General bacteria	Actinomycetes	Fungi	Pseudomonads	Amoebae	Flagellates	Cilliates	Amm. Ox. Bact	Subst. Ind. Resp	Ninhydrrin-N	Cellulase	Aryl_Sulph	Acid_Phosph.	Dehydrogenase	Protease	Urease	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders	
Figure 15.2 (c) Waikerie GM rhizosphere soil																								
V15i	11		3	3	7	7	8					1	1	1	5	2	1	4						
V15i	11		3	8	1	4	1					2	5	1	5	1	1	7						
V15i	11			2	5	8	7					2	2	4	6	4	4	8						
V15i	11			3	3	8	3					1	1	5	3	5	3	6						
V2i	12			8	8	3	5	1	8	5		7	4											
V2i	12			6	1	1	8	4	1	1		5	3											
V2i	12			5	2	3	4	2	3	5		4	2											
V2i	12			6	8	5	3	5	5	5		7	6											
289i	17		2					7	3	1	3	5	8	6	8	8	7	3						
289i	17		3					3	2	1	2	5	7	7	8	8	8	2	1					
289i	17		1					1	1	1	1	3	7	8	4	7	6	5	7					
289i	17		2					3	5	1	5	6	5	8	7	6	8	1	4					
C312i	14		1	5	6	1	2	6	6	5	4	3	8	3	3	5	5							
C312i	14		1	4	3	1	5	5	3	5	8	4	5	2	1	1	2		2					
C312i	14			1	5	4	5	8	8	1	6	8	4	3	1	3	5		5					
C312i	14			1	4	6	1	8	7	8	7	8	3	5	2	3	3		8					

Soil/ Plant	Harvest age	ELISA Leaf	ELISA Root	General bacteria	Actinomycetes	Fungi	Pseudomonads	Amoebae	Flagellates	Cilliates	Amm. Ox. Bact	Subst. Ind. Resp	Ninhydrrin-N	Cellulase	Aryl_Sulph	Acid_Phosph.	Dehydrogenase	Protease	Urease	Omnivores	Bacterial feeders	Fungal feeders	Plant feeders	
Figure 15.3 (c) Waikerie non-rhisophere soil																								
Waikerie	11			3	3	5	3			1		1	3											
Waikerie	11			5	5	8	8			1		5	8											
Waikerie	12			5	1	1	3	1	1	1		4	2											
Waikerie	12			1	8	3	1	7	7	1		1	1											
Waikerie	17		0					2	8	1	1	6	6											
Waikerie	17		0					5	2	1	3	2	4											
Waikerie	17		0									3												
Waikerie	14		0					8	5	1	5	8	5											
Waikerie	14		0					4	4	1	8	7	7											

15.3 RESULTS

15.3.1 Interactions of bacteria, fungi, protozoa, substrate induced respiration, ammonium-oxidising bacteria, ninhydrin-N extraction and enzymes

From the coloured spreadsheet matrices a clustering of similar scores was often seen within each of the trials even though there was some variability for replicates. A high response could be seen in the 189/ 289i plant trial from the Narrabri soil, with associated high scores in the ammonium-oxidising bacteria as well as ninhydrin-N, indicative of a nitrogen-driven response. In this trial, the general bacteria grown from TSA medium and pseudomonads (from King's B medium) were also relatively high in comparison with other trials, but high ranking scores were not also correlated to actinomycetes or fungi, nor was the trend consistent for urease, an indicator of response to nitrogen. This trial received nutrients just prior to harvest.

Principal component analysis (PCA) was used to further investigate the factor loadings on the sets of data where the same complement of variables was present. The results of the actinomycetes and fungi populations were included, because while colony numbers did not correspond with other bacteria from the plate counts,

interactions including antagonism between bacteria are well known, especially with the production of antibiotics by streptomycetes. This is part of the sum of the interactions.

The first test examined the microflora, microfauna, ammonium-oxidising bacteria, substrate-induced respiration and ninhydrin-N (ie, the enzyme tests were not included). The results are shown in Figure 15.3.1.1 where the circles, colour coded for soil type, enclose the matching symbols for the co-ordinates for the individual trials.

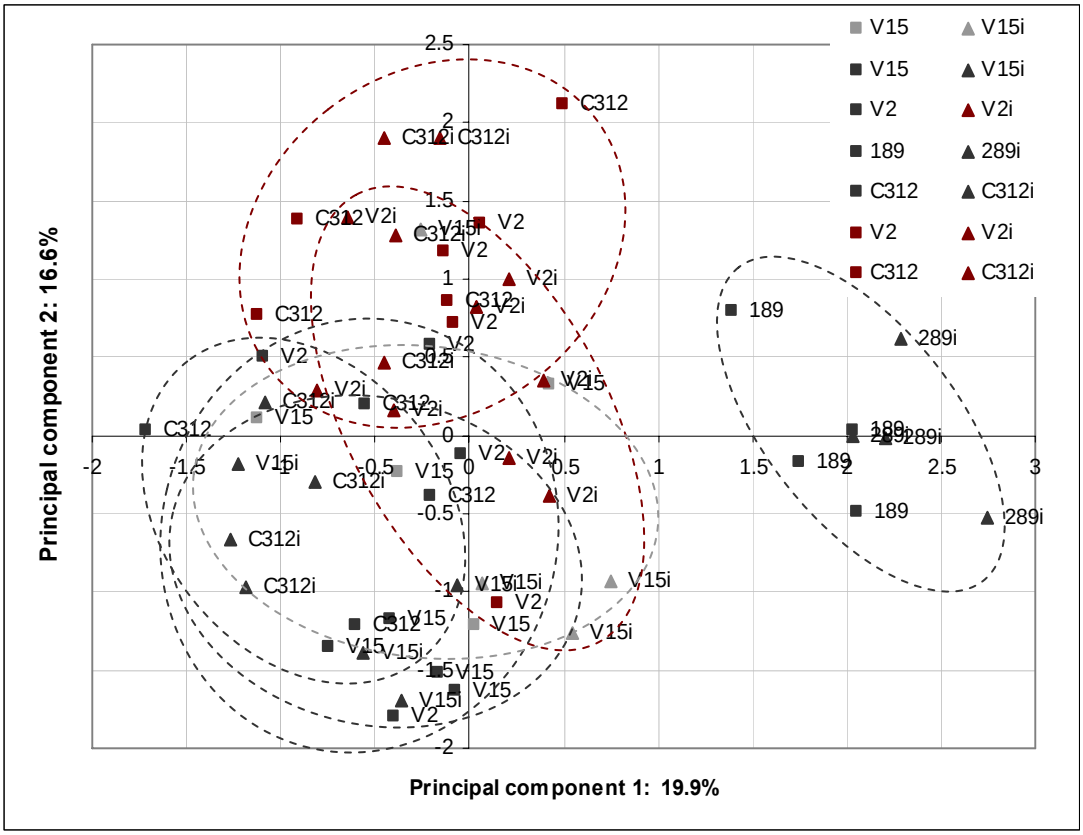


Figure 15.3.1.1 **PCA plot of microflora, microfauna, ammonium-oxidising bacteria, substrate-induced respiration and ninhydrin-N, for the tests done in Avon, Narrabri and Waikerie soils.**
The datapoints and enclosing circles have been colour-coded for each of the soils.
The symbols used to differentiate the paired plants are filled squares for non-GM, triangles for GM plants and open circles for non-rhizosphere soil.

The individual trials were found to group within intermixed sets, with the 189/289i trial from Narrabri soil showing the greatest separation from the remaining groups by the weighting factors attributed to the Ninhydrin-N component, and the general

bacterial numbers from the TSA medium for this trial. The highest contributor to the bacterial numbers was the Kings B medium from which Pseudomonads were cultivated. This suggested an effect driven by nutrients. There was no polarisation of the datapoints for non-GM vs GM plant rhizosphere soils.

To reduce the number of factors over many different trials, the second analysis compared two trials including the following factors: microflora, microfauna, substrate-induced respiration, ammonium oxidising bacteria, ninhydrin-N. In this analysis, the four major enzymes cellulase, arylsulphatase, acid phosphatase and dehydrogenase were included. The comparison was between two trials with Waikerie and Narrabri rhizosphere soils, plus the effect of recently added nutrient. The result is shown as Figure 15.3.1.2.

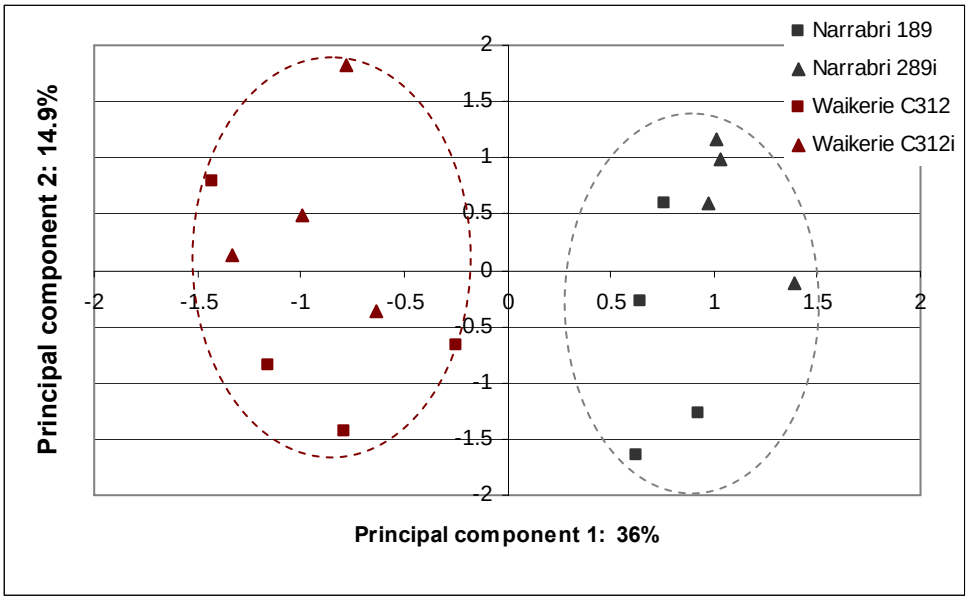


Figure 15.3.1.2 **PCA plot of microflora, microfauna, substrate-induced respiration, ammonium oxidising bacteria, ninhydrin-N and four major enzymes for Waikerie and Narrabri soils.**

While the separately grouped clusters of datapoints show that major differences can be attributed to the different trials, the first and second principal components account for about 50% of the difference. The two highest factor loadings were attributed to the numbers of bacterial colonies from the general TSA medium and the ammonium oxidising bacteria. Both of these factors are influenced by nutrition, and Narrabri included the trial where nutrient was added before harvesting. Cellulase was the least affected of all variables. (A major effect was also shown by the analysis of the

phospholipid fatty acids, with both adequate and nutrient deficiency, for the same paired plant types V15 and V15i shown in Section 14.)

The same two trials were then compared to investigate the contribution of the enzyme analyses. The result is shown in Figure 15.3.1.3.

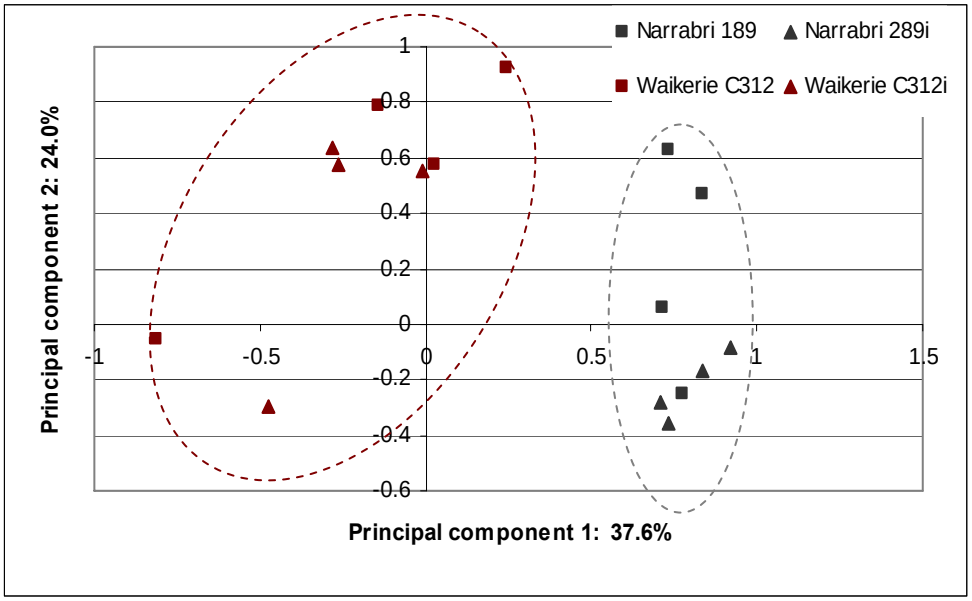


Figure 15.3.1.3 **PCA plot of microflora, microfauna, substrate-induced respiration, ammonium oxidising bacteria, ninhydrin-N, but without enzymes for Waikerie and Narrabri soils.**

The summed differences between the principal components 1 and 2, amounted to about 62% when the enzymes were excluded.

Because there was a higher concentration of phosphate in the leaves of the nutrient deficient plants (Section 4) and this is known to occur as a result of A-M fungal enhanced uptake (Jarstfer & Sylvia, 1993, p. 350), the same two trials were again compared, with the exclusion of all enzymes except phosphatase. This was to investigate the effect on the surrounding soil microbiota of increased phosphate uptake by the plant. This reanalysis accounted for about 1% of the difference.

To compare the environmental influence of different trials and soil types on the same paired plant strains, interactions between protozoa, substrate induced respiration, ammonium oxidising bacteria and five enzymes were then compared across the three soils, for the same plant type. This analysis is shown in Figure 15.3.1.4.

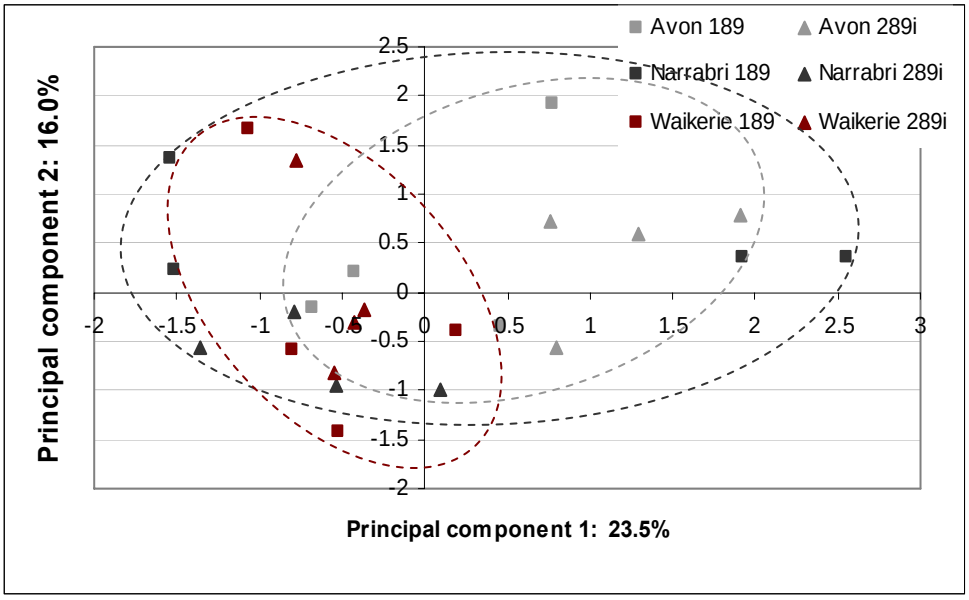


Figure 15.3.1.4 **PCA plot of V15 and V15i plants grown in Avon, Narrabri and Waikerie soil, comparing bacteria, fungi, protozoa, respiration, ammonium oxidising bacterial response and ninhydrin-N measurement.**

The two major influences from the first principal components were from the ammonium-oxidising bacteria and ninhydrin, ie, a nitrogen-driven and biomass differentiation. There was an overlapping association by soil type, but no distinct differentiation overall, nor between the non-GM and GM paired plants within each trial. Together, both principal components 1 and 2 only accounted for 39.5%, ie, no significant difference.

The final test, shown as Figure 15.3.1.5, demonstrates the response of the microbiota to addition of nutrient before harvesting, between the non-GM and GM rhizosphere soils. The comparison included the bacteria, fungi, protozoa, ammonium oxidising bacteria, ninhydrin response and all enzymes as factors. Non-rhizosphere soil was also included in this test. There was no indication that the soil microbiota of the non-GM nor GM rhizosphere soils differed with respect to changes in nutrient under the conditions of the same trial.

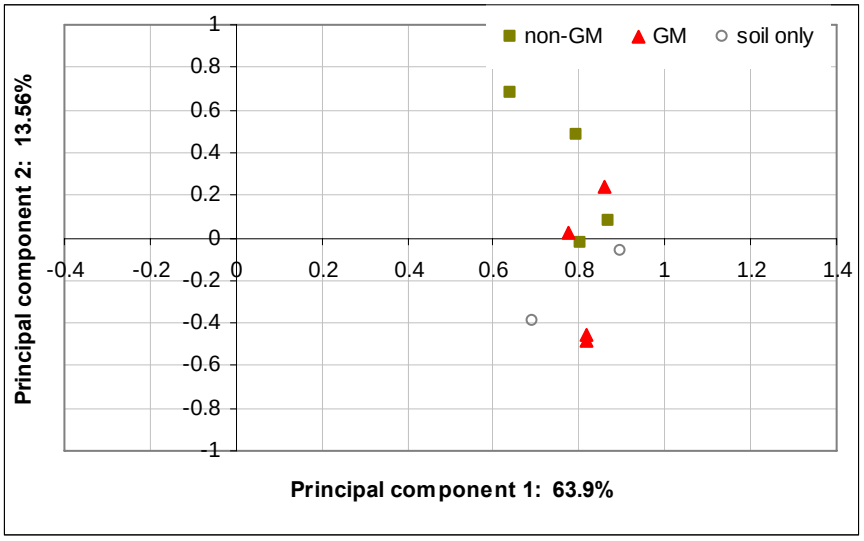


Figure 15.3.1.5 **PCA plot of 189 and 289i plants grown in Narrabri soil, comparing bacteria, fungi, protozoa, substrate induced respiration, ammonium-oxidising bacteria, ninhydrin-N and enzymes for response to recent nutrient addition.**

15.4 DISCUSSION

The analyses described in this section compared the response of microbial populations from three sampled soils to the effects of the plant root/soil environment between trials, and across different analyses from different scales of measurement. Across all tests discussed in this section, all of the outcomes of the mix of analyses were more closely correlated by individual trial, which showed that over all the tests of plant combinations, soil effects and nutrient effects, the non-GM and GM rhizosphere microbiota responded more similarly to the environmental conditions of each individual paired trial than through a plant-based effect.

The two different kinds of analytical procedures were used to answer different questions: the visual ranked index to assess the degree of variability between replicates for the unassociated factors, and principal component analysis to assess the strength of the correlation between the different factors.

From the positions of the non-GM and GM PCA co-ordinates within the polarised areas of the individual trials (Fig. 15.3.1.2), it could be seen that the effect of the environment, particularly nutrient depletion, exerted a stronger influence than the effect of plant genetic modification. This was reinforced by Figure 15.3.1.3 where all enzymes were eliminated from the PCA, and the difference between the total

complement of enzymes only accounted for about 4%. Most of the trials showed that the most significant factor loading was the number of bacteria grown on TSA medium, and the biomass measured by ninhydrin-N – again suggesting a nutrient-driven response – rather than a response to non-GM and GM cotton plants.

15.4.1 Observation on the method of analysis

Ettema & Wardle (2002) reported that spatial variability in soil organisms is largely considered random 'noise' and is problematic in understanding how highly species-rich soil communities such as in the rhizosphere, function. Previous studies of spatial ecology on a field scale suggested that spatial variability is the key, rather than the obstacle. The authors observed that spatial distribution on an aggregate scale may therefore reflect the zone of influence needed to understand the structure and function of soil biodiversity.

While living biota respond to stimulus, there will be a function of cause and effect, even at the microscopic level. The individually scored populations of bacteria, fungi, protozoa and nematodes in this work have shown degrees of clustering: an example can be seen by the amoebae illustrated in Figure 9.0 which were clumped, even in a dispersed electrolytic colloidal solution. Differences in microbial populations were shown in response to soil type (Sections 7.1 and 7.2), added nutrient (addition of Hoaglands solution particularly with regard to N responses) and age of plant (cycle of bacterial-feeding nematodes compared with fungal-feeders). If each sampled soil and its inhabitants were exactly matched, the distribution patterns would be random. Each record of soil microbiota has been shown to differ across different tests, with regard to replicate samples because the soil aggregates and the microbial populations that are harboured were not homogenous. Even though deviation from a trend line cannot always be accounted for by the analytical methods used, a 'misalignment' from a more comprehensible trend should not be perceived as 'random'.

A similar work by Gyamfi et al. (2002) was undertaken on the effect of microorganisms in the rhizosphere of genetically modified glyphosate-resistant oilseed rape. Glyphosate degrades in soil within 7-21 days with no toxic intermediate substance. The authors showed that the effects of the age of the plants in containment had a much greater effect on the microorganisms than the genetic

plant modification, and concluded 'we **assume** [my emphasis] that the observed differences were due to changes in exudation patterns.' No chemical analysis of the plant root exudates was conducted, and so the assumption is not supported by data. No consideration was given to the possibility of natural microbial variation between the replicates of the trials by ecological factors other than presumptive root exudate composition.

Other workers who have also documented microbial variability within rhizosphere soil while investigating non-GM/GM plant influence include Dunfield & Germida (2001) and Gyamfi et al. (2002) for *Brassica napus*; and Griffiths et al. (2000), Lukow et al. (2000) and Heuer (2002) for potato. All authors concluded that abiotic and biotic sources of variation such as season, weather, plant developmental state, location and plant genotype were all implicated as far more important drivers of microbial community structure in the rhizosphere than possible genetically modified protein-induced changes.

Given the variation in microbial populations at the aggregate scale, averaged measurements over a larger number of replicates still may not adequately measure a soil population microbial response for the following reasons:

1. The analysis was not sensitive or comprehensive enough to show any differences
2. Factors such as recycling by microbiota of N or other substances after the death of any susceptible organisms had compensating effects overall
3. There is no difference.

The first point is correct, insofar that it is impossible to test every facet of microbial life within even 1 g of soil. Principal component analysis explained the correlation between the tests that were done, but many other factors such as the effect of the macrofauna (earthworms, collembola), some microfauna (rotifers, tardigrades) and sub-microbiotic life forms such as rickettsia and bacteriophages, plus the effects of chemical signalling by plant and rhizosphere microbiota, could not be measured within the timeframe of the project.

Compensating effects whereby (for example) N or C can be degraded by many different soil microbiota regardless of the specific taxonomy were suggested by the high similarity of function, from the enzyme analyses for combined soil microbial populations. Additionally, populations of microflora from different genera such as the auxotrophic bacteria, or the brown algal colony isolated in the fungal populations from Narrabri soil, grew under the selective conditions of each of the cultures and from each of the soils in the trials. The propensity to degrade proteins was putatively shown by the log-scaled numbers of bacteria and fungi, able to grow on protein-containing media. Their ability specifically to degrade the *Bt* protein was shown by the degradation of GM-plant tissue in litterbags in soil. The same rate of breakdown of litter occurred, even though more or less fungal spores and/or bacterial colonies may have been present initially.

The third point was that there was no difference in microbial response. The principal component analysis showed that every replicate differed in some aspects, and each was different between trials. The variability between replicates for the same treatment of each individual test can be seen from the graphic spreadsheets. Several of the 12.5% divisions were spanned for individual variates (eg substrate induced respiration, protozoa and microflora) showing the different estimates of the microbiota resulting from different analyses. Moreover, from the principal component analyses there was no instance where two datapoints were positioned at the same point on the cartesian coordinates. Each microbial population differed from another in some respect, over the multiple tests. This can be expected, as the concept of *ceteris paribus*, or "all else being equal" (Cartwright, 1983) cannot be made with rhizosphere soil microbiota. Precise measurements of microbial responses to pre- and post-rhizosphere events cannot be made because microbial soil populations are not clonal, nor are the micro-habitats exactly the same over all replicates. Major differences must therefore occur to differentiate a real effect from 'noise' in small samples. This implies that given sufficient replicates, the function of the total soil biota on a larger scale would have the same potential for nutrient utilisation and regrowth after negative environmental effects, and that on a larger scale spatial variation would not exist, and 'no difference' is real.

If thousands of 'species' exist in each 1 to 10g of sample, with each species potentially responding differently to an event in its environment, then the screening of 100 clones is clearly insufficient to recover total diversity (Tiedje & Zhou, 1996). For practical purposes, the only measure of difference in microbial populations is by function; a communal response to rhizosphere microbiota that are able to respond to a different set of environmental conditions, and the cause of that response, from particular focus groups. Additionally, a post-event shift in community structure may retain similar functionality (Walker, 1992) as redundancy in bacterial substance utilisation is well known. It has been noted, for example, that one strain of *Pseudomonas* can break down over 100 different carbon compounds, and only a few strains utilise fewer than 20 (Brock & Madigan 1991, p. 754), so in each gram of agricultural soil with numbers of bacteria and fungi approaching billions, there will be opportunistic guilds able to adapt to new conditions. This redundancy makes analysis difficult because of the lack of sharply defined climax communities.

Even with the variability of microbiota in replicates from the gram unit of soil sampled it was found that there was no consistent pattern which would suggest that the exudates or other attributes of the genetically modified plants was a cause of a toxic effect on the soil microbiota.

The summary of the chemical tests and the analysis of interactions is summarised on the following page.

SUMMARY OF CHAPTER 4

The measurement of CO_2 efflux provided no evidence of different rates of respiration between the rhizosphere soils of the parental plant strains and their genetically modified counterparts. Measurement of nitrogen dynamics showed that the variability was not attributable to the extra metabolic requirement for the plant's production of the *Bt* protein.

The six commonly occurring soil enzymes tested provided no evidence of differences in function of the soil rhizosphere microbiota from the non-GM and GM paired trials.

Phospholipid fatty acid ratios did not reveal any differences in membrane lipids that would suggest a difference in living rhizosphere microbiota.

The total interactivity of all results was then checked for correlation across all tests undertaken, across all trophic groups, for both counted and estimated populations, and for all chemical-based tests done (excepting phospholipid fatty acid analysis and Arbuscular-Mycorrhizal rate of infection, because of the specificity of these tests).

The magnitude of the expressed results repeatedly showed that the greatest influence came from the conditions of the individual trial, and the age of the cotton plant at harvest. Within each of the soils, using the methods within the individual tests (with the acknowledgement of the technical limitations of the methodologies) a higher similarity for the non-GM and GM effects between individual trials was observed than there was across soils or comparisons of different trials. There was however no consistent difference between the non-GM and GM plant rhizosphere microbial populations themselves. The implication of this is that there is no detrimental effect to rhizosphere soil microbiota from the cotton plants, through the effect of the genetic modification.

A final analysis, expressed in terms of environmental risk, follows.

CHAPTER FIVE

DISCUSSION

Section 16
Summary of Results and Interpretation of Risk Analysis

The final outcome of the work described here, is the production of an environmental impact statement, with an associated risk analysis. To formalise the findings of this study 14 separate risk assessments were conducted in accordance with AS/NZS 4360: 1999. The format is based on the Australian/ New Zealand Standard™ 'Risk Management' Document No. AS/NZ 4360:1999©[5], reproduced as Table 16.1. The risk assessment matrix is shown as Table 16.2 and the tabulated results of these risk assessments from the work described in this thesis are shown in Table 16.3.

Within the concept of risk assessment[6] the term 'hazard' was used to describe a set of conditions which could potentially lead to harmful consequences in the environment. A substance such as a hormone present in nanomolar quantities can exert an influence on a cell, and in the soil environment the lack of quantification of some factor does not mean there will not be an effect. In deciding on the acceptability of an environmental risk, there should be awareness of the benefits to society of the technology and a balanced decision made between the potential disturbance (in this case on the soil microbiota) and the economic return on farmland investment.

An assessment of environmental risk is determined by the product of the consequences of harm to an ecosystem, multiplied by the likelihood of that event. In the case of the impact of the plant-produced *Bt* protein on soil microbiota, the increase in risk with increasing likelihood of an event is not necessarily proportional to a toxic effect through increasing concentration of the protein, if soil microorganisms are not affected. The *Bt* protein has been proven to be quickly

---

[5] Approved on behalf of the Council of Standards Australia on 2 April 1999 and on behalf of the Council of Standards New Zealand on 22 March 1999. Published on 2 April 1999

[6] Modified from Mount Isa Mines Limited, McArthur River Project Draft Environmental Impact Statement, Volume 2 – Appendices, prepared by Hollingsworth Dames & Moore, May 1992.

degraded, and becomes a small, ephemeral source of amino acids, to the soil microbiota that degrade plant tissue. The interpretation of risk analysis in soil microbiology differs from some other environmental toxicity analyses. In this environment even lethal effects on individuals within microbial populations are unmeasurable by classical computational approaches where populations are rapidly and continually changing, and numbers in tens of thousands may not make a significant difference.

Table 16.1 Risk Management protocol as per AS/NZ Australian/ New Zealand Standard™ AS/NZ Document No. 4360:1999©[7],

The protocol of risk assessment as per Section 3.2 'Main elements'	***Remarks***	***Corresponding section of thesis***
(a) Establish the context (b) Identify risks		Literature Review, and Introductions to Sections within Chapters 3 & 4.
(c) Analyse risks	For each listed hazardous incident, assess the likely severity of the consequences. This involves assessment of the impact, eg the elimination of a major microbial component in the soil food-web such as the breakdown of soil organic matter by primary detritivores, to a form which can be assimilated by plants.	Sections 3-13
(d) Evaluate risks	For each listed hazardous incident assess the likely frequency with which the incident can occur. By combination of the consequences and the likelihood of occurrence, calculate the risk to the function of the rhizosphere food web, to give an overall risk from the altered plant characteristics, compared with the cropping of non-GM plants.	Discussions of Sections 3-13 Section 14 Summary; Section 16, Table 16.11.1.
(e) Treat risks (f) Monitor and review (g) Communicate and consult		Not applicable to this work

[7] Approved on behalf of the Council of Standards Australia on 2 April 1999 and on behalf of the Council of Standards New Zealand on 22 March 1999. Published on 2 April 1999

Table 16.2 Risk assessment matrix based on AS/NZS 4360, 1999

Likelihood of causing an event of significant change to the soil microbiota	Consequences				
	Insig-nificant 1	Minor 2	Moderate 3	Major 4	Catas-trophic 5
A. (almost certain)	high	high	extreme	extreme	extreme
B (likely)	moderate	high	high	extreme	extreme
C (moderate)	low	moderate	high	extreme	extreme
D (unlikely)	low	low	moderate	high	extreme
E (rare)	low	low	moderate	high	high

Translated in terms of the findings of this study, the results of tests are summarised below.

Table 16.3 Risk evaluation matrix based on the work of this thesis

Characteristic compared for non-GM/GM	Risk assess-ment	Reference to Section
1. Plant physical attributes		
1.1 pH of root exudates	**Low**	Sect. 4
Plant growth in aseptic medium with bromocresol purple as a pH indicator showed both non-GM and GM root exudates were equivalent in volume and pH. NH_3 substances from the root exudates were therefore equivalent. As neither plant type differed in acidity of root exudates (Kennedy, 1992), microbial populations with different pH maxima would not have been influenced. The likelihood of a pH-driven effect on soil microbiota has been rated as unlikely (D) and the possible consequences estimated as minor (2) which gives a risk analysis of Low (D2).		
1.2 Root tissue mass and structure	**Low**	Sect. 4
Enquist & Niklas, (2002) showed root mass is proportional to above-ground plant mass. Non-GM and GM above-ground plant mass were equivalent within normal plant variability, for the same growing periods, under the conditions of the pot trials. This suggests that the total root mass between non-GM and GM plants		

were not measurably different. The dicotyledonous root structure of the non-GM and GM plants was not observably different, to provide advantageous habitats for microbial colonisation (Weller & Thomashow, 1994). The likelihood of differential microbial colonisation between the non-GM and GM soil rhizosphere under conditions of the pot trials has been rated as unlikely (D) and the possible consequences estimated as Minor (2) which gives a risk analysis of Low (D2).

2. Toxic effect through persistence of the *Bt* protein in the soil environment

2.1	**Environmental persistence**	**Low**	Sect. 5,6

The GM cotton plant material was illustrated to degrade at the same rate as the non-GM plants in litterbag experiments described in Section 6. ELISA tests showed degradation of the plant-produced *Bt* protein occurred as soon as moisture was present. The overall action of detritivores were therefore not affected by the presence of the *Bt* protein. Rapid breakdown of the protein by microbial action was confirmed by Saxena et al. (1999); ibid (2000).

However the possibility exists that the protein may remain bound to clay particles (Palm et al, 1994; Section 6), slowing degradation by microbiota, particularly when dry. In addition, the partial masking effect of clay on the ELISA test, may mean that an underestimation of the amount of protein in soil may occur.

Susceptibility of rhizosphere soil microbiota to the plant-produced *Bt* protein would depend on ingestion of soil particles which have been adsorbed to the protein, and kept dry, or away from the effect of abundant soil proteases (Section 12), from whatever source. It would also depend on the susceptibility of the organism feeding on the soil around the sloughed-off root cells of actively growing plants, as it has been shown by Stotzky (2005) that the *Bt* protein is not present in root exudates of cotton plants. It was shown that highly susceptible organisms such as *Helicoverpa* spp. and *Plutella* do not feed on root tissue (Section 5), nor do they feed below ground. The work by Head et al. (2002) showed no detectable Cry1A(c) protein occurred in soils which had grown transgenic cotton for 3-6 consecutive years, and no detectable biological activity from soil microbiota was found.

The assessment has been rated as unlikely (D) and the possible consequences estimated as Minor (2), which gives a risk analysis of Low (D2).

3. Individual counts and estimated populations of selected microbiota

3.1 General Bacteria	Low	Sect. 7.1

Numbers of colony forming units from mixed bacteria using a general-growth tryptone soya agar, ranged from $Log_{(10)}{}^{6.5}$ to $^{7.5}$ over three different soil types, taken from the rhizosphere soil around living plant roots. There was no significant difference in colony numbers between the non-GM and GM rhizosphere soils, for the paired trials. This indicates that bacteria, and their predators, either had not been affected by the presence of the *Bt* protein, or had recovered quickly under the conditions of plating and incubation. Bacterial morphology suggested that there was no colony type from replicates of the trials that did not also occur within the pairs of the treatment. Wei-xiang et al. (2004), investigated the effect of culturable microbiota on *Bt*- and non-*Bt*-producing rice straw in flooded paddy fields, and also concluded that no significant effect could be found. Donegan et al. (1995) added leaves of freshly harvested C312 and C312i cotton plants – one of the plant lines used in this project – to potting mix soil in laboratory flasks, and also found no difference in the culturable microbiota. From these studies, culturable microbiota also do not appear to be affected from the rhizosphere of living cotton plants.

Actinomycete counts described in Section 6.1, using a starch and inorganic salts medium resulted in a range of $Log_{(10)}{}^{5}$ to ^{6} per gram soil, and the orders were consistent within the paired trials. The commonly occurring white colonies typical of the *Streptomycete* group dominated the plates using the ISP4 medium, and there was no consistent difference between the non-GM and GM rhizosphere soils for this family of bacteria.

Under the experimental conditions imposed here, the assessment for difference in viable bacterial populations that colonise $^{1}/_{10}$ TSA and ISP4 media has been rated as unlikely (D) and the possible consequences estimated as Minor (2), which gives a risk analysis of Low (D2).

3.2 Fungi external to root	Low	Sect. 7.2
Fungal colonies ranged between 10^3 to 10^7 but there was no difference in morphological form or consistency of numbers between the non-GM and GM soils. Wei-xiang (2004) also found no difference between fungal colonies numbers from non-GM and GM plant soils. The phospholipid fatty acid analysis showed the presence of steric acid (ergosterol), through the peaks of the 18:3ω6c, 18:2ω6c and 18:1ω9c (which can be seen proximal to the retention time of 18 minutes) in both Figures 14.3.9.2/14.3.9.3 and 14.3.9.4/14.3.9.5. There was no observable difference between the non-GM and GM plant rhizosphere soils for this fungal-specific fatty acid. The lack of consistent, significant difference between fungal colony numbers and the presence of the specific fatty acid in the same concentrations in the phospholipid fatty acid analysis gives the likelihood of deleterious effects of the *Bt* protein on soil fungi a rating of unlikely (D) and the possible effect estimated as Minor (2) which gives a risk analysis of Low (D2).		
3.3 Fungi internal to root	**Low**	Sect. 8
The GM plants did not show a pattern of colonisation that would suggest inhibition by the plant (Gao et al. (2002); Wegel et al. (1998). The A-M Fungi followed a simple colonisation pattern of increase with time, even from soils that had never grown cotton previously. Arum-type colonisation pattern was also shown to be similar for the four paired plant strains (Dickson, 2004). The risk assessment from the lack of effect on A-M fungi by the presence of the *Bt* protein has been rated as unlikely (D) and the possible consequences estimated as Minor (2), which gives a risk analysis of Low (D2).		

4. Microfauna

4.1 Protozoa	Low	Sect. 9
Counts by Most Probable Number of three protozoan types from freshly harvested rhizosphere soils did not show a consistent difference between the non-GM and GM soils sampled. Additionally, it was shown in Figure 9.3.1.1 that the *Bacillus thuringiensis* bacteria was utilised as a food source by protozoan populations from Avon and Narrabri at a concentration far in excess of the plant-produced protein recorded by ELISA. This determined the likelihood of toxic effect as unlikely (D) and the possible consequences estimated as Minor (2), which gives a risk analysis of Low (D2). Donegan et al. (1995) also found no significant difference in protozoa from transgenic and parental cotton plant material.		

5. Mesofauna

5.1 Nematodes	Moderate	Sect. 10
There were no trophic groups found in the non-GM soil that were not also in the GM soil, however the ratios of nematode groups changed over time, indicating a shift in population structure. Although the methods employed were different from those in this study, the similarity of results obtained by Saxena & Stotzky (2001) corroborated the idea that the plant-produced *Bt* protein is quickly broken down in soil by microbial action, and has no detectable effect on the rhizosphere bacteria, fungi, protozoa **or nematodes** [my emphasis]. However, the number of plant-feeding nematodes in the Narrabri GM rhizosphere soil, was slightly different from the non-GM plants, so a doubt exists that an effect may have been caused by the root-feeders of this particular group. The proposed long-term effect would be negligible, as there were remnants of the cohort remaining at the end of the trials, which, in a field, would have survived to reproduce and recolonise any deficit in population numbers. However under the pot trial conditions, for the time of the trials, the likelihood has been rated as unlikely (D) and the possible consequences estimated as moderate (3), which gives a risk assessment of moderate (D3).		

6. Whole microbial soil community in-situ response

6.1	Difference in response by microbial populations to the addition of substrate	Low	Sect. 11

The response in the soil microbial populations to the addition of substrate was shown by four methods.

Respiration induced by the addition of glucose showed that the levels of CO_2 varied with each individual trial, and in 10 of 12 trials was statistically non-significant. The CO_2 efflux also varied for the rhizosphere soil from the same plant and soil grown under standardised conditions, with harvest dates differing by two weeks (Fig. 11.3.3.1). The C312/C312i trials grown in Avon soil, and the 189/289i trials grown in Narrabri soil both showed a significant difference in efflux of CO_2. Because these differences did not occur for the same plant types over the different soils, respiration was more influenced by the individual trial.

The addition of $(NH_4)_2SO_4$ to the rhizosphere soils of the V15/V15i plants grown in Narrabri soil showed that the ammonium sulphate was the driver of the difference in the increase in rate of NO_2, rather than the plant type. A similar trend for the rate of nitrification was shown for both non-GM and GM soils.

The fluidity with which microbial populations can change was particularly apparent with an N-driven response. Ammonium oxidising bacteria and numbers of cultivable bacteria increased markedly in the 189/289i trial in Narrabri soil, after the recent addition of nitrogen-containing fertilizer. This was seen from the weighting factors of the principal component analysis for this trial, compared with all other rhizosphere soils. The PCA plot of microflora, microfauna, ammonium-oxidising bacteria, substrate-induced respiration and ninhydrin-N, for the tests of Avon, Narrabri and Waikerie soils showed the 189/289i Narrabri trial co-ordinates were separated by the first principal component, through a higher weighting of factors than all of the other compared plant rhizosphere trials. It is notable that even though different trials showed overlapping clusters, all the non-GM and GM datapoints were grouped within the individual trials.

The likelihood of the whole soil microbiota differing between non-GM and GM plants from the effect of substrate availability is therefore unlikely (D), and the consequences of the effect are insignificant (1). The risk assessment is D1 (Low).

6.2 Enzyme functions	Low	Sect. 12
Both individual and grouped profiles of the six enzymes analysed, showed that the non-GM and GM plant rhizosphere soils were closely aligned in the amount of enzyme activity for the individual trials, with the exception of cellulase in one trial: the 189 and 289i plant trial from Narrabri soil. However, of 6 paired trials, 5 showed that each was separated further from the other by soil type, rather than non-GM or GM plant strains (Figure 12.3.1.1). This showed that the environment had a greater effect on the cellulose-degrading rhizosphere microbiota than the influence of the plant genetic modification. All enzymes are proteins, and are susceptible to breakdown and recycling of the peptides. The likelihood of toxic effect from enzyme build-up in the event of differential expression has been rated as unlikely (D) and the consequences estimated as Minor (2) which gives a risk of Low (D2).		

7. Interactions between microbial groups

7.1 Flow-on effect across tests	Low	Sect. 15
From the analysis of principal components, interaction between all variables tested indicated no flow-on effect in the microbiota of the rhizosphere between microflora, microfauna, protozoa, substrate-induced respiration, ammonium oxidising bacteria, biomass measured by ninhydrin-N or six major enzymes that could be attributed to the genetic modification of the four different cotton plants. All PCA plots showed clustering of datapoints representing non-GM and GM variables, within each of the trials. This indicates a non-significant difference in the interaction of all analyses undertaken, and that the factors were more heavily weighted by individual trial than plant type. Additionally, independent responses between replicates for each of the tests shown by the visual spreadsheets, shows that variability is inherent in the sampled soils, and is not driven by a GM-plant. The lack of consistent response between non-GM and GM plant rhizosphere soils for the individual tests therefore shows the likelihood of risk to be rare (E) and the consequences as Minor (2), which gives a risk factor of Low (E2).		

It is evident from the PCA analysis, from the surge of microbial activity after the addition of nutrient, and the relative similarity of the microbial populations after the

return to a 'resting' community activity level (depending on the soil type and individual conditions) that unless soil microbiota are tested shortly after the event that the effect will be lost. This return to population levels that are possible, dependent on the altered conditions (or perhaps in spite of it – see P. 16), was also shown by the samples of soil microbiota which were severely affected by 10-day exposure to chloroform. By the 7th day, the bacterial population had returned to a level similar to the colony counts from plates which had not undergone the fumigation (Section 6.1). Similar results had been shown by Gupta and Neate (1998) after the addition of the herbicides Logran, Hoegrass and Glean, where the soil microbiota had largely recovered after 7 days.

The comparison of the living microbiota by phospholipid fatty acid analysis showed a community response, and by amplification of similar phospholipids, a response by similar microbes, under the compared conditions of nutrient adequacy and nutrient deficiency (Table 14.3.9.1). This shows an adjustment to changing conditions which was more closely aligned with the environmental conditions of the trial, than the change in microbiota from the non-GM/GM plants.

In summary, seven of the risk assessments returned the lowest defined category of risk. The one risk which could be attributed as moderate arose from a result of a diminished plant-feeding nematode population from the GM plant time series trials, even though this did not occur for the same plant type grown in Avon soil. Whether this result can be duplicated under field conditions is not known, but the frequency and extent of the risk in this project is a result of the 200g soil samples under the conditions of the pot trials described here. From this translation of the risk index, the cotton plants which have been modified to produce the *Bt* protein do not present a significant risk to the microbiota of the rhizosphere.

Contemporary science is based on the assertion of two premises: that an effect must be observable and repeatable. The first premise, that the *Bt* protein was consistently detected (and quantified) within leaf and root tissue of the genetically modified cotton plant strains, and confirmed to be absent in the non-GM plant tissue, is satisfied by the work of Section 5. In 46 of the 50 ELISA tests, the *Bt* protein was either not detected, or less than 1ppm, within the rhizosphere soil around the living plant root. As there is no known method of guaranteeing an absence of fine root

tissue from the closely associated soil, the eight positive but very low results may have come from fine root hairs, and not from the rhizosphere soil. Independent work by Stotzky (2005) confirmed that the Cry1A(c) protein was not exuded from the roots of cotton plants, so apart from broken root cells from actively growing roots, the microbiota would not have had contact with the 'toxin' until degradation of the plant root after senescence.

The second premise for repeatability of lack of toxicity to soil microbiota is satisfied by other research, undertaken within independent laboratories. The studies included different plants (corn by Koskella & Stotzky, 2002), rice (Wei-Xiang et al, 2004), and cotton (Head et al, 2002). No impact on the microbiota of soils was found in any of the studies, and this work shows that no toxic effect was observed within the rhizosphere of living plants using Australian soils.

16.10 Risk analysis on rate of microbial recovery from the *Bt* protein in soil

It can be argued that bacteria and fungi are the fastest evolving living organisms on earth, as soil bacteria can inherit new traits within five hours via plasmid transfer. The rhizosphere microbiota has been shown to be functionally similar, even though the individual mix of population components may have varied. Given the tenet that there is no such thing as a stable microbial population, the question posed by Stotzky (1993) on how much of a response to a dose of an environmental perturbant is ecologically significant, in the case of microflora may never be known, as any possible temporary effect will become obscured by the establishment of new equilibria.

The null hypothesis that there is no significant difference within the soil microbiota for non-GM and GM crops, under the conditions of these trials, using Ingard cotton as a case study, is therefore accepted.

16.12 Multi-disciplinary project documentation

This project had a broad base, spanning the disciplines of soil analysis, plant physiology, bacteria and fungi, protozoa, nematodes and bioassay analysis of the soil/root system. At the point of writing there was no standardised protocol for environmental impact assessment of soil or its microbiota, nor of consensual methodology for some of the experiments used in this work. It will be difficult for

future researchers to compare the results of experiments in a common database if the procedures have not been standardised.

16.13 Recommendations for future work

In the pot trials, weeds were removed by hand so there was no record of the influence of herbicide on the soil. In addition, there was no herbivory by *Helicoverpa* which may have altered the defence responses of the plant, and possibly altered the level of phytoalexins in the root exudates. It may be that a more significant environmental impact on rhizosphere microbiota will be found from application of agricultural chemicals to suppress weeds and insects in the fields of non-genetically modified plants, compared with the GM-plants which do not require such frequent applications of these toxins. At present, this is not known.

It may also be of benefit to follow the effect of strains of single organisms, to eliminate the confounding effects of compensation by microorganisms that occupy a similar environmental and nutritional niche. This investigation may be more effectively expedited by the use of rhizoboxes, wherein the plant roots are relatively isolated from the bulk soil. This method may also use fluorescently tagged bacteria to detect 'hotspots of activity' with time exposure film, which can allow the microbiota surrounding the root to be visualised if the box has transparent sides.

The conclusion to the work follows as the final section of this thesis.

Section 17

Conclusion

**A flower of a (non-GM) cotton plant.
The structure is the precursor of the commercial product, and has been produced through many interacting effects of plant, soil and rhizosphere microorganisms.**

The aim of this research was to determine whether there was a significant difference in the rhizosphere microbiota that could be attributed to the genetically modified strains of cotton plants grown in three different soils. The paired trials of genetically modified and parental non-GM isolines were conducted under as near identical conditions as were practicable and followed the same protocols for each of the tests.

There was no evidence for the persistence of the *Bt* protein within the rhizosphere soil, nor of a significant alteration of the bacteria, fungi, protozoa or nematodes, nor of the tested community metabolic function that could not be explained by intrinsic variability within soil systems. This variability has been demonstrated through the individual population counts, bioassays for soil enzymes, gas analysis, the phospholipid component of microbial cell membranes, and plant physical and elemental components.

It has been shown in this work that the genetically modified cotton plants were just as capable of influencing the soil environment as non-genetically modified cotton plants. There was no evidence that the method of breeding caused any difference to the soil microbiota that could not have occurred by other methods of breeding (including hybridisation and selection) and so agricultural sustainability would not be affected any more than by the non-GM cotton plants.

Because ecosystems are ever-adapting biological systems, variance is dynamic and the changes in the microbial populations within the rhizosphere of living plants are no exception. Each microcosm differs temporally as successions of specialist feeders break down ephemeral nutrients in a continuing stepwise mineralisation process. The communities may not return exactly to their former components, but this altered state does not necessarily translate into an ecological impact of significance.

While the measurement of the multifactorial influences of the living plant/soil/ microbial interface are too complex to be completely understood, there is no doubt that the heterogeneity of each rhizosphere microcosm is not detectably altered by the single effect of the GM cotton plant, when compared with the non-GM cotton plants, grown under the same conditions.

The microbiota surrounding the non-GM and GM cotton plants were found to be functionally equivalent for all of the environmental effects tested. On the basis of the results presented here, it has been found that there is no significant environmental impact on the microbiology of the rhizosphere, which can be attributed to the presence or absence of the *Bt* protein from genetically modified cotton plants, grown under the conditions of these trials.

APPENDICES

APPENDICES

Appendix A	*Methods, apparatus and solutions*
Note:	**The integers of all numbered appendices correspond to the numbers of the related chapters**

APPENDIX 1
STATISTICAL ANALYSIS

The majority of the trials analysed had four replicates of each of the non-GM and four of the GM plant types, and two replicates of soil only controls. These were compared for significant differences, using an Analysis of Variance with three factors (soil only, non-GM plant and GM-plant) at a significance level of $p \leq 0.05$. SPSS[8] software for Windows Version 10 was used. Normality of the data for constant variance which is assumed with ANOVA, was interrogated using histogram and normal quantile plots. Homogeneity was assumed when the standard deviation of the highest value in the group was no more than twice that of the lowest value. The data was Log10(x + 1) transformed, where residuals increased with predicted dependent variables. The database was set up as a non-relational two-dimensional matrix. As SPSS V. 10 was not able to select subset data for processing on the basis of fields defined as string variables, the soils and plant identifiers were coded in Table Appendix 1.1.

Table Appendix 1.1 Coding for soil type and plant variables in SPSS

Soil type identifier: most significant digit of 3 (hundreds)	**Plant type identifier** 2 digits following soil type	**Comment**
Avon 100	V15 10 V15i 11 V2 20 V2i 21 189 30 289i 31 C312 40 C312i 41	All GM plants were odd numbers, non-GM plants were even, and soils only were zero, within the 'hundreds' defining soil type.
Narrabri 200	plant types as above	
Waikerie 300	plant types as above	

[8] SPSS Inc, 233 S Wacker Drive, 11th Floor, Chicago, Illinois. USA.

Interrogation of the database for specific subsets of data could therefore be selected by the functions:

Table Appendix 1.2 Algorithm for selection of entities in the database

Selection criteria	Function	Comment
Soil-only controls	Modulus: mod[numexpr,100][(1)], = 0	Baseline comparisons of difference in soil types
Comparison of plant type within soil type, including soil-only controls	Range: [numexpr] (≥100 <200 for Avon); (≥200 <299 for Narrabri); and ≥300 for <399Waikerie.	Rhizosphere effect and plant response to soil type
All plant isolines	Not mod[numexpr,100] = 0. (This eliminates the 'exact hundreds')	Plant response to different soil types
GM plants only/ non-GM plants only	Mod[numexpr,100] /2 = 1; Mod[numexpr,100] /2 = 0	Selects odd or even numbers

(1) where [numexpr] is the field name upon which the calculation is based.

Significant difference was taken as $p = 0.05$. Tukey's tests were used for post-hoc analysis.

The variability of seed germination and growth vigour of individual plants can differ between the four replicates pots and it can therefore be argued that there may be significant plant root growth factors between replicates, even though each trial is kept under equivalent conditions. The smallest statistical unit is therefore deemed to be each single replicate pot within plant type, within soil type. Error bars on all graphs denote the standard error of the mean, calculated as the standard deviation divided by the square root of the number of replicates.

APPENDIX 3.1

Procedure for calculating Soil Field Moisture Capacity

A funnel was set up as illustrated below, with saturated sintered glass above a column of water leading into a reservoir. A lid was kept over the samples to stop evaporation. The soil samples were placed on the surface of the sintered glass within metal rings, and allowed to saturate with RO water for 24 hours by positioning the water reservoir at the same height as the samples The reservoir was then lowered to 100cm below the samples, to induce a suction of 10kPa, and was again left for 24 hours. All samples were kept at 20ºC. Duplicates of the mass of the saturated soil (plus bottle of known weight) were weighed and then dried in an oven at 105ºC overnight. The samples were then taken out of the oven and allowed to cool in a desiccator jar above P_2O_5 for an hour at 20ºC. Lids were put on immediately.

Fig. Appendix 3.1.1
Apparatus for testing field capacity

The bottles plus dry weight of soil were then weighed (lids taken off momentarily before weighing), and the field moisture capacity calculated by the calculation:

$$\Delta \text{ Soil moisture} = \frac{(\text{wet weight of soil} - \text{container}) - (\text{dry weight of soil} - \text{container})}{(\text{dry weight of soil} - \text{container})}$$

(Blakemore, L.C., Searle, P.L. and Daly, B.K., 1987).

APPENDIX 3.2

MEASUREMENT OF SOIL PARTICLE DENSITY

Based on American Soc. of Agronomy, Inc. Soil Science Soc. of America, Inc. Publisher. Madison, Wisconsin. USA. 1986. from Methods of Soil analysis, Part 1. Physical and Mineralogical Methods. 2nd edn. Arnold Klute, Editor.

14-3 PYCNOMETER METHOD

(Method copied from ASTM, 1958, p. 80; U.S. Dep. Agric., 1954. p 122.)

A pycnometer (specific-gravity flask) is employed. A pycnometer is a glass flask fitted with a ground-glass stopper that is pierced lengthwise by a capillary opening. A thermometer is sometimes an integral part of the stopper, the glass-enclosed mercury reservoir being in contact with the fluid in the flask, with the stem extending above the ground joint. A 10-mL pycnometer has sufficient capacity.

14-3.2 Procedure

Weigh a clean, dry pycnometer in air. (Use tweezers to handle.) Add about 10g air-dry soil sieved through a 2-mm sieve. If a 100mL volumetric flask is used, add 50g of soil. Clean the outside and neck of the pycnometer of any soil that may have spilled during transfer. Weigh the pycnometer (including stopper) and its contents. Determine the water content of a duplicate soil sample by drying it at 105ºC.

Fill the pycnometer about one-half with distilled water, washing into the flask any soil adhering to the inside of the neck. Remove entrapped air by gently boiling the water for several minute with frequent gentle agitation of the contents to prevent loss of soil by foaming.

Cool the pycnometer and its contents to room temperature, and then add enough boiled, cooled, distilled water at room temperature to fill the pycnometer. Insert the stopper and seat it carefully. Thoroughly dry and clean the outside of the flask with a dry cloth, using care to avoid drawing water out of the capillary. Weigh the pycnometer and its contents, and determine the temperature of the contents after they have cooled to room temperature.

Finally, remove the soil from the pycnometer and thoroughly wash out. Fill the pycnometer with boiled, cooled distilled water at the same temperature as before,

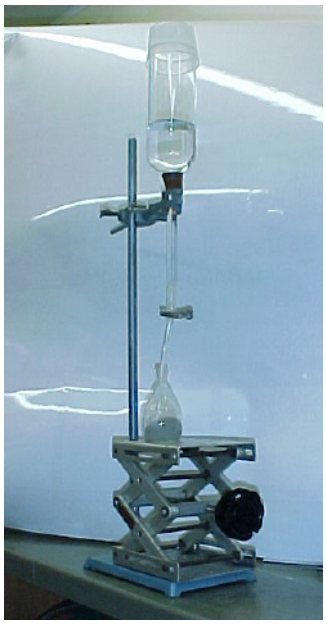
insert the stopper, thoroughly dry the outside with a cloth, and weigh the pycnometer and contents, being careful that the temperature remains the same as before.

Calculate the particle density as follows:

$$\rho_\rho = \rho_w (W_s - W_a)/[(W_s - W_a) - (W_{sw} - W_w)]$$

where ρ_w = density of water in grams per cubic centimetre at temperature observed,
W_s = weight of pycnometer plus soil sample corrected to oven-dry water content,
W_a = weight of pycnometer filled with air,
W_{sw} = weight of pycnometer filled with soil and water, and
W_w = weight of pycnometer filled with water at temperature observed.

Alternate method instead of boiling, used at Soil Engineering Section, Urrbrae.



The samples were placed into a desiccator jar under vacuum (lid of pycnometer off) with a glass beaker of RO water overnight. This sublimed moisture under vacuum enters the soil samples and helps remove occluded air.) The jars were removed from the vacuum and the jar was tipped so the soil sloped up one side of the jar, and slow drops of RO were added to the base of the slope, to allow capillary suction of water up the slope, to exclude air from the soil sample. Each day for 2 days the pycnometer jars were taken out, tapped gently to remove any air, and returned to the desiccator jar under vacuum.

Fig. Appendix 3.2.1 Setup of apparatus for removal of air from soil by capillary action

The jars together with the 'aerosoled' soil were filled with the water from the beaker from the desiccator jar to capacity, and the lids placed on. Excess water at the top of the pore in the lid was wiped away. Jars and soil and water were then weighed.

The jars were then cleaned and dried, and the 'air-evacuated' water added, and kept at 20°C for 1 hour and then reweighed for the known weight of air-evacuated water at this temperature.

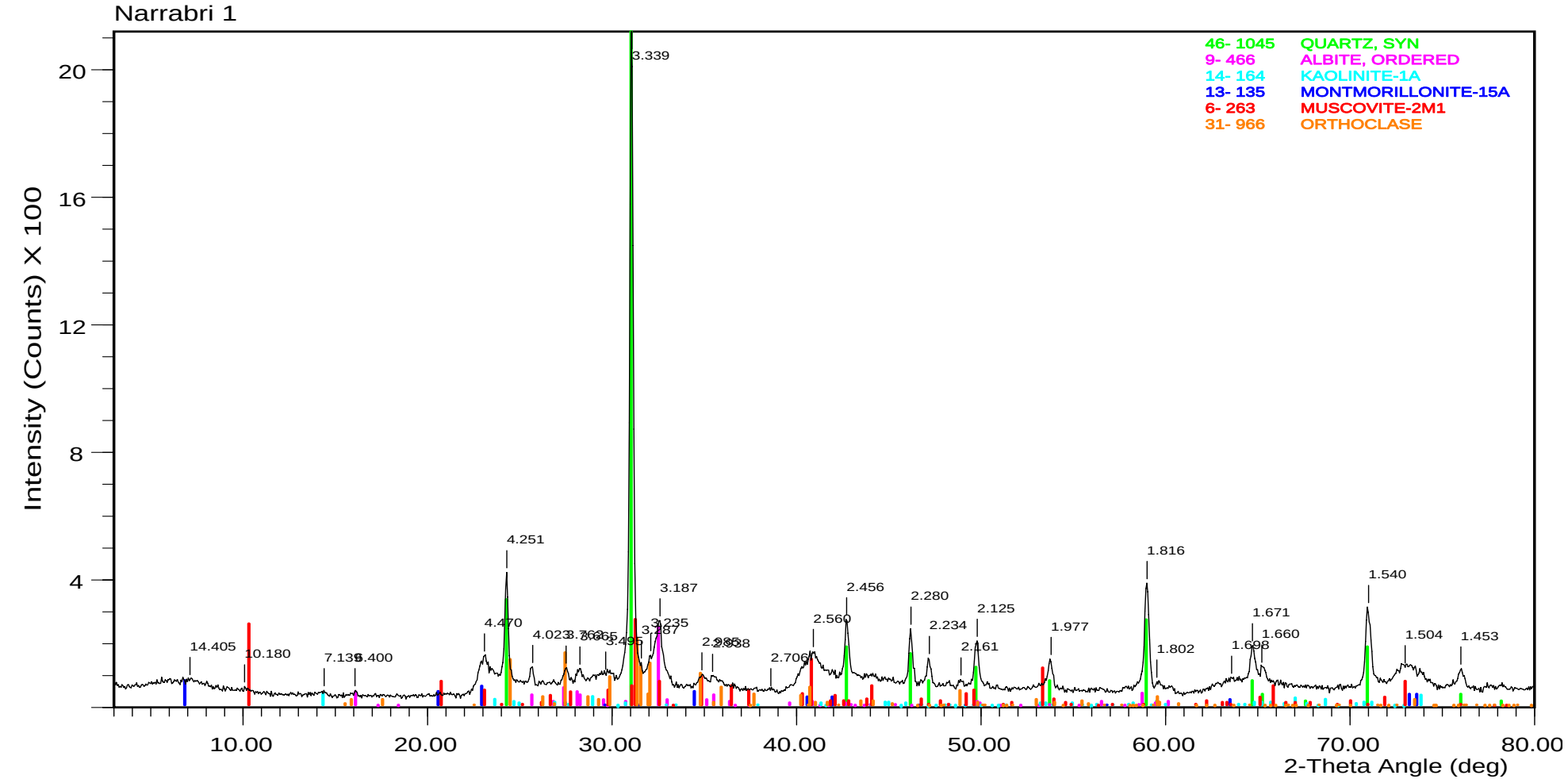
APPENDIX 3.3

The Pipette Method of determining Soil Particle Size Distribution

Weighing soil	10.00g air-dry < 2mm soil weighed into a 250ml plastic bottle. Water content of the soil is measured on a sample taken at the time of weighing out.
Dispersal	4ml 10% hexametaphosphate (Calgon) solution and 2 ml 1M NaOH, and 100 ml by measuring cylinder RO water are added and the bottle shaken for 64 hours (over weekend) on an end-over-end shaker.
Blank	4 ml 10% Calgon solution, 2ml, 1M NaOH and 100 ml RO water shaken in a 250ml plastic bottle alongside the samples, then diluted to 200 ml in volumetric flask and duplicate 20ml aliquots pipetted into weighing bottles, dried overnight at 105ºC and cooled in a desiccator (over P_2O_5) for weighing on the 4-place balance.
Dilution of samples	Samples are washed through a 70-mesh (200µ) sieve into a 500ml measuring cylinder and diluted to almost 400 ml, all with RO water. Each is diluted to 400ml just before <20µ aliquots are taken.
Tempera-ture of suspensions	All sedimentation work, (pipette sampling and decanting) was carried out at 20ºC.
<20µm particle extraction	2ml bulb pipette aliquots were taken from 5cm depth 2min, 24 seconds, after stirring.
<2µ particle extraction	5ml bulb pipette aliquots are taken from 5 cm depth 4 hours after stirring.
Decanting <20µm	A clean dry tall-form 600ml beaker was taken and its weight, to the nearest milligram, recorded. The contents of the cylinder are transferred to the beaker. On a retort stand, a 'J-tube' is clamped at such a height that its lower extremity is 1-2mm above the top of the residue. The supernatant is decanted via the 'J-tube' from 10cm depth 4 minutes 48 seconds after stirring of the suspension. 5 decants are made, fresh water being added to the beaker to bring the suspension surface to 10cm above the mouth of the 'J-tube' before each

Coarse Sand	A clean dry 250ml beaker is taken and its weight, to the nearest milligram, recorded. The sand caught on the 70 mesh (200 micron), sieve in the 'Dilution of Samples' step, is rinsed into the beaker. As much water as possible is pipetted off and the sand dried in the beaker at 105°C, cooled in air and weight of beaker plus sand, to the nearest milligram, recorded.
Fine Sand	The fine sand is left behind in the 600ml tall-form beaker after decantation of <20µ. It is dried and weighed as is the coarse sand.
Dry sieving of sand	After dry weights of coarse and fine sands have been recorded, the coarse sand is re-sieved on the (dry), 70 mesh (200 micron), sieve over the fine sand beaker and the fine sand plus beaker re-weighed. Weight of fine sand after dry sieving is considered correct and weight of coarse sand corrected by subtraction of difference between weights of fine sand before and after dry sieving.

APPENDIX 3.4
X-Ray Diffraction trace of Narrabri soil



File Name: d:\xrddat~1\narabri1.101

Appendix 4.1
Method of determining Plant Tissue Content
by inductively coupled plasma optical emission spectra

The elemental plant tissue content was analysed for Ca, Mg, P, K, S as percent content; and Al, B, Cu, Fe, Mn, Mo, Na and Zn as mg kg^{-1}

Reagents HNO_3

Apparatus
40-hole digestion block with programmable controller (Autostep 1012) capable of reaching 150ºC.

Metal digestion racks, 40 hold, with draught shields

Digestion tubes with 20mL graduations

Variable 10mL Brand dispenser mounted on a 500 mL reagent bottle

Variable speed vortex mixer (Chilton MT19).
Whatman No. 42 filter papers.

Filter funnels and rack.
ICP tubes, acid washed.
ICP-AES.

1g plant tissue was heated until 1ml of digest solution remained, then diluted with 0.1% HNO_3.

The solution was then analysed by ICP- optical emission spectra.

Each element tested is compared against known standards.

mg/kg = mg/L x (vol/wt).

Appendix 4.2
Method of detection of soil contamination on plant leaf tissue

Mid-infrared spectronomic scans of leaf tissue from plants (both grown in Narrabri soil) with the highest recorded concentration of Al (plant C312 at 3740mg/kg) was compared with the leaf tissue with the lowest concentration (V15i at 53 mg/kg) and the scans were superimposed to compare overall characteristics. The signatures of the two superimposed plant tissue spectra with defined peaks are shown in Figure 4.2.1.

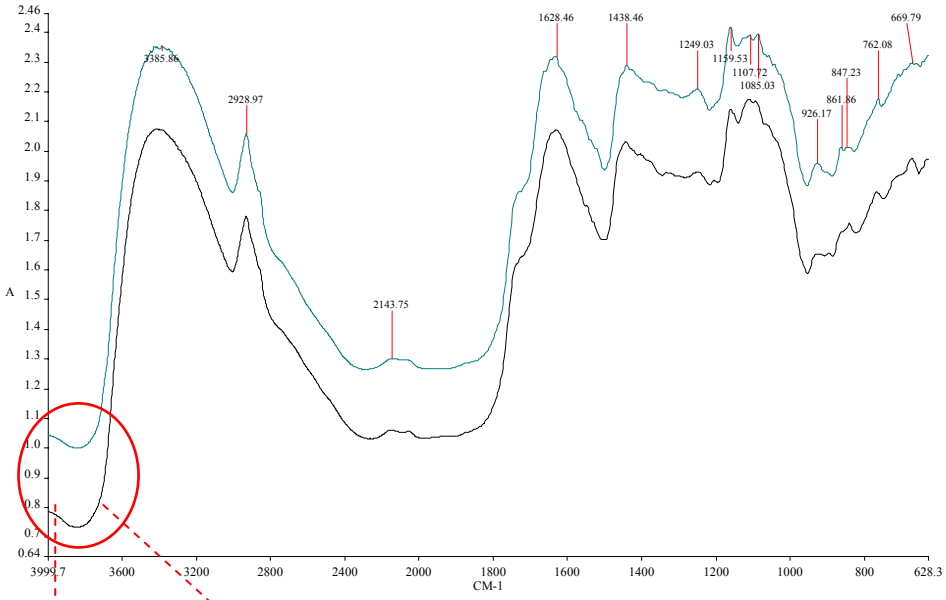


Figure 4.2.1 **Superimposed trace of plant tissue showing high and low Al content, both grown in Narrabri soil.**

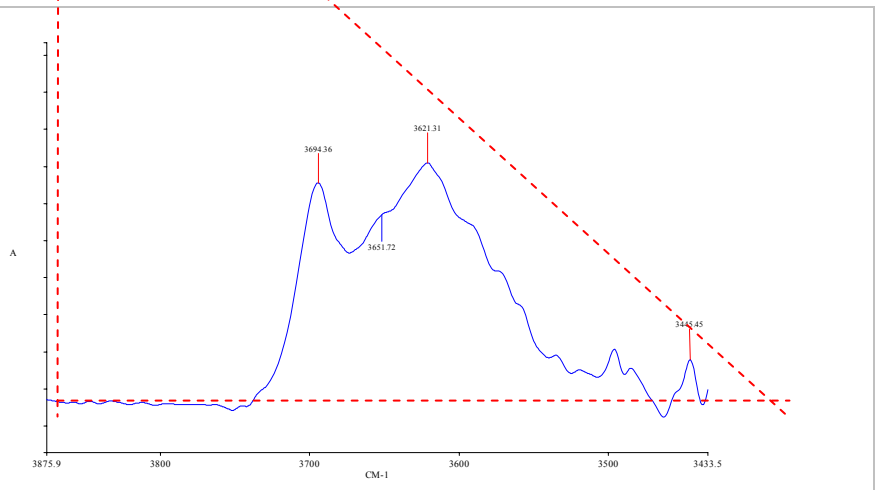


Figure 4.2.2 **High-low subtraction scan of compared plant tissues showing differences in position coincident with Kaolin (indicating a possible clay mineral content)**

The low values were subtracted from the high values, to give a subtraction scan, and the area of the frequency between 3433 and 4000 was selected. Slight differences were detected at the frequency units 3694cm^{-1}, 3651cm^{-1} and 3621cm^{-1}: these are consistent with the distinct peaks of kaolinite clay minerals. The major differences in the spectra were therefore only due to the Al of clay soils.

APPENDIX 5.1

Detection and quantification of the *Bt* Protein: ELISA method, using the Envirologix Cry1A(b) antibody coated plates

An Envirologix Cry1Ab/Cry1Ac Plate Kit (AP 003, EnviroLogix Inc. Portland, USA) was used for all assays. Extraction/dilution and wash buffers were diluted in accordance with the instructions on the kit.

Freshly harvested, or fresh-frozen leaf tissue was taken from mature leaves from new growth and 0.02 g was weighed into free standing rib screw tubes, (Catalogue No. 23400-00, with cap with O-ring, Cat. 2001-00: Interpath Services Pty Ltd), with two steel ball-bearings.

600µL extraction buffer was added to the tubes and the tissue and buffer homogenised in a Biospec mini-bead-beater, model. 3-15-1301 on maximum speed for 1 minute, and the homogenate was immediately put onto ice.

Soil was prepared similarly, except that 0.2g was weighed.

100µL of homogenate was added to the coated wells of the kit, and the plates were mixed on a rotatory shaker, setting 3, for 15 minutes.

100µL of conjugate solution was then added and the plates were rotated for 1 hour, then washed four times with the wash solution, and the plates inverted onto absorbant paper, and shaken to clear the liquid from the wells.

100µL of substrate was added and the wells were again shaken for 30 minutes.

Stop solution was added and the plates were then read on a Biolog Microstation plate reader with $\lambda 1$ set at 450 nm with a reference $\lambda 2$ at 650nm.

The standards of 0, 0.5, 2.5, and 5 ppb were used to calculate the Cry1A(c) protein by interpolation from a standard linear curve by estimating the value of x from the known value of the optical density of y.

Allowance was made for leaf and soil moisture.

APPENDIX 5.2
Helicoverpa Larval Diet and Incubation

Recipe for the Insect Diet supplied from the Aust. Cotton CRC

1 litre hot RO water	3.3g nipagin
60g wheat germ	1.7g sorbic acid
53g brewers yeast	13.5ml (10%) formaldehyde
130g soybean flour	2 teaspoons sunflower oil

Microwave on high until the mixture begins to rise.
When the temperature of the diet drops below 63°C add 5.3g ascorbic acid.
Blend mixture for at least 45 seconds.

Keep warm in a waterbath to avoid setting while delivering into wells.
Deliver 200µL into each well and dry in a laminar flow cupboard overnight.
Add diluted leaf or soil mixture into each well.

Put the control mixture into petri-dishes and into the plastic bags with the filter papers on which the neonates and eggs have been stored as a temporary diet, until the neonates are transferred to the 96-well plates.

Leaf tissue overlay.

Freeze-dried leaves from freshly harvested plant leaves were ground in a mortar and pestle RO water added to reconstitute the plant tissue to the moisture content at harvest. 100µl was delivered to each well. Ascorbic acid only was added to the media as a negative control.

Transfer one larva per well with a fine soft artist's paintbrush, and seal each column (8 wells) with adhesive tape to temporarily keep them in, as the larvae's first instinct is to disperse, and cannibalism occurs at about the 4th instar stage.

When all 96 wells are filled, seal the plates with glad-wrap and punch holes with a needle, for air. Place the clear plastic lid, with air-holes fused with a fine pointed soldering iron, and positioned at the centres of the wells, for air. Hold the lids down firmly with four strong spring clips. Incubate at 25ºC.

Count at Day 3 and Day 7.
Larvae are considered dead if there is no movement when prodded with the paintbrush.

APPENDIX 7.1: Selective Media for plating microflora
All dry weight per 1 litre

$^1/_{10}$ strength Tryptone Soya Agar for protein degraders

Tryptone Soya Broth Mix	3g
Agar	15g

RO water pH to 7.2

KINGS B medium for Pseudomonads
Simon & Ridge 1974.
J. Applied Bacteriology 37: 459-460.

Containing magnesium, phosphate and sulphate ions, essential for fluorescent pigment production. Fe is omitted as it interferes with pigment production and selects for bacteria able to chelate iron.

Proteose Peptone No 3	20. g
Bacto Agar	20. g
Glycerol	10. g
(11.5g for 87% glycerol)	
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g

pH 7.2

(Ampicillin 50mg/ Litre)
(Chloramphenicol 12.5mg / Litre)

CZAPEK-DOX Agar for Fungi

Difco Czapek-dox broth 35g.,
to which 15g agar was added.

Streptomycin added at 30µg per ml.
(Alef & Nannipieri, 1995, p. 145.)

ISP4: for isolation of Actinomycetes
Variation on International *Streptomycetes* Project Medium 4, (Atlas, 1993, p. 461).

Starch	10g
$CaCO_3$	2g
$(NH_4)_2SO_4$	2g
K_2HPO_4	1g
$MgSO_4.7H_2O$	1g
NaCl	1g
$FeSO_4$	0.001g)
$MnCl_2$	0.001g) trace
$ZnSO_4.7H_2O$	0.001g)
Agar	20g

pH ~ 7.2

Add 75µg/ml cycloheximide.

HOAGLANDS nutrient solution

1.	A.	$CaCl_2$	11g	) in 2 litres
		KCl	220g	)
2.	B.	$MgSO_4.7H20$	220g	per 2 litres
3.	C.	K_2HPO_4	265g	per 2 litres
4.	D.	$FeCl_3$ (60% w/v $FeCl_3$)	16.8 mls	) per 1 litre
		Na_2EDTA	2.0 g	) RO water

Take 1 ml of each of the above stock solutions per litre of water.

5.	A-Z trace	in 1 litre
	H_3BO_3	2.86g
	$MnSO_4.4H_2O$	2.03g
	$ZnSO_4.7H_2O$	0.222g
	$CuSO_4.5H_2O$	0.079g
	$H_2MoO_4.H_2O$	0.09g

Add 0.1 ml of A-Z stock per litre

For added Nitrogen:

6.	E.	CaNO3	200g/ litre
7.	F.	NH_4H2PO_4	28g/ 1 litre

Add 4 mls of each stock per litre.

APPENDIX 8
STAINING OF ROOTS FOR MYCORRHIZAE
Modified from Phillips & Hayman (1970)
omitting phenol from the reagents

Solutions required:

50% ethanol; 0.1M HCl; 50% glycerol

10% KOH (W/v):
Dissolve 100g KOH in 1 litre RO water, volumetric.
Caution: solution gets hot (swirl under running water as it dissolves).

Stain **Caution:** carcinogenic.

0.65g Trypan blue
400ml RO H_2O
300mL glycerol
400ml lactic acid

Preparation of roots (at harvest time)

1. Harvest plants and transfer roots to a vial containing 50% ethanol until ready to examine.

Clearing roots (approx. 1 week before you want to examine them).

2. Wash roots in water and subsection in the event that the stain has to be redone. Put one section into the stain, and the other into ethanol.

3. Add KOH (w/v) to the roots to be stained.

4. Swirl and allow to stand at room temperature for 4-5 days. Generally 4 days is adequate, but 5 days is OK.

Staining roots (best done immediately before you want to examine, or clearing/restaining may be required).

5. Tip off KOH (through tea strainer so roots are not lost) and rinse vial and roots with tap water.
6. Swirl roots in strainer in a weigh boat filled with 0.1M HCl.
7. Pat dry on paper towel
8. Return to vial
9. Pour on stain. Swirl. Mix with tweezers to ensure stain gets to all roots. Leave approx. 30 minutes (30-35 minutes OK).
10. Tip off stain to a waste container.
11. Rinse roots and return to vial.
12. Cover with 50% glycerol and examine when time permits.

APPENDIX 9 PROTOZOA
Method based on Darbyshire et al. 1974

and modified by Gupta et al. 1998b

9.1 Cultivation of bacterial cultures

1. Straw infusion was made by finely chopping wheat stubble and added to 1L of saline phosphate buffer and shaken at approx. 200rpm for 24 hours. It was then filtered through a Whatman No. 42 filter to remove coarse organic material and later using a 0.22µm millipore filter. The filtrate was stored at 4ºC.

2. Soil extract was made by adding 10g Avon soil to 200ml cold sterile distilled water. Shake on low setting for 30 minutes. Centrifuge at 6000rpm for 15 min. First decant suspension through Whatman #2 into vacuum filtration system (45µm filter). Autoclave for 20 minutes at 121ºC before use. Store at 4ºC.

3. Bacterial cell culture liquid of 0.1% glucose, 0.1% yeast extract and 0.8% adjunct liquid was brought to a pH of 6.8 and autoclaved. The liquid was separated into three Erlenmeyer flasks, and the cultures were shaken for 2 days on a rotating shaker (setting 3), at 25ºC. Each of the three cultures of *Enterobacter* soil isolate strain No. 64, *Bacillus thuringiensis* and *Rhizobium melliotti* were transferred into sterile centrifuge tubes and spun at 6,000 rpm for 15 minutes, and the supernatant discarded. One ml sterile distilled water was added to the pellet and vortexed. The culture was then transferred to sterilised McCartney jars and stored at 4ºC.

The mixture of soil extract, straw infusion and cultured bacteria was made up in the proportions of 92ml phosphate buffer, 2 ml straw infusion, 5 ml soil extract and 1ml bacterial culture (per 100ml). 200µL was aliquotted to each of the wells before adding the dilution of harvested rhizosphere soil and phosphate buffer.

9.2 Preparation of 5 x 5 tissue culture boxes for serial dilution of protozoa

Two grams rhizosphere soil was weighed into a sterilised McCartney bottle, and 20ml autoclaved RO water or phosphate buffer was added, to make a 10^{-1} dilution. The McCartney bottles were capped and laid down, and mixed on a rotating shaker for at least 30 minutes at setting 3. One ml of the mix was then transferred to each of the 5 lowest wells of a 5 x 5 tissue culture box.

200µL of each of the lowest rows were transferred to the higher rows, to make a 1 in 5 dilution for each row. Each trial consisted of four replicates of each non-GM plant type, and four of its GM-isoline. Two non-plant soil controls, also kept under the same conditions as a benchmark for that soil type, were compared for a rhizosphere effect. Estimation of the total population from the serial dilutions was by most probable number.

APPENDIX 10.1

WHITEHEAD TRAY METHOD

FOR ESTIMATING NEMATODE POPULATION DIVERSITY

(Whitehead & Hemming (1965)

The Whitehead Tray method is a modification and improvement on the Baermann funnel. The advantage is that more air is available to the soil and the extraction procedure is less likely to become anaerobic as the soil surface area is increased in the tray.

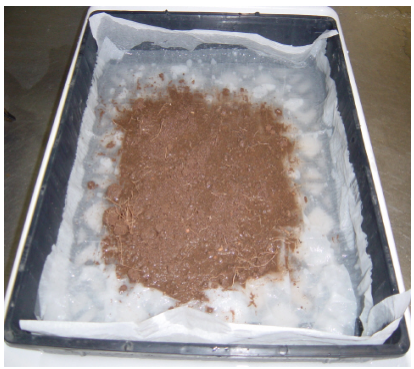


Fig. Append. 10.1 setup of tray with water level immediately below 200g soil.

A Yates seedling tray is placed inside a white plastic laboratory tray, and a large Kimwipe is placed on top of the seedling tray. A large Kleenex tissue is overlaid on top of the Kimwipe and both are wetted to saturation. 200g field-moist (or rhizosphere) soil is distributed on the moist tissues and water gently hosed into the bottom of the white tray, up to the base level of the tissue, to saturate the soil. The trays are left undisturbed for two days and the nematodes swim through the tissues and into the water in the tray.

The harvesting of nematodes involves removing the soil and filters together, and pouring the tray water through a 20µm filter, and into a lidded container. As nematodes have a higher density than water, the water was left undisturbed for at least 2 hours and the top layer of water was pipetted down to 6 ml. The live specimens were kept at 4ºC in between microscopy observations as the movement can be helpful in identification.

If specimen fixing is required, 4 ml of 4% formalin at 90°C can be used, and this liquid is then immediately cooled with 4 ml 4% cold formalin to retard the transparent cuticle from becoming opaque.

Hot and cold formalin:
10.8ml formaldehyde 37%, in 89.2ml DI water, one container warmed to 90ºC in a waterbath **in fumehood**, and one kept cold.

APPENDIX 12: Soil Enzyme Analysis
12.1 CELLULASE ACTIVITY

Using the modified method of Hope and Burns, 1987

Cellulase activity measurements with Avicel as the substrate and measuring the released reduced sugars using the Nelson and Somagyi method with some modifications, or glucose equivalents using Sigma Diagnostics Glucose (Trinder) reagent (Sigma Diagnostics, Procedure no. 315). This is based on the determination of the total cellulase (endoglucanase, exoglucanase and ß-gucosidase) activity which is converted to a reducing sugar and can be measured colorimetrically at OD_{490}.

Day 1: Avicellulase setup:

1. Weigh 2g field-moist soil into centrifuge (50ml) tubes. Close tubes to avoid moisture loss.
2. Add 0.2g Avicel to each tube (at 100mg/g soil)
3. Vortex 10-15 secs until Avicel is uniformly mixed with soil
4. Add 8 ml of acetate buffer (& 0.25% Azide) mixture (pH 5.5) to each tube. Vortex 30 seconds, full speed. Make sure all the soil is dispersed in the buffer.

Incubate 40°C for 24 hours in shaking water bath – 100-150 rpm) for 24 hours..

Day 2:
Take samples and Avicel and buffer mixture (and Na Azide) out of waterbath.
Centrifuge for 10 min at 7,000 rpm. (Centrifugation stops the process).
Collect 4 ml supernatant. Do not throw away centrifuge tubes until you complete analysis.

Glucose Equivalents Standards:
1. Turn on waterbath. Set temp. at 30°C – 30 mins ahead .
2. Pipette 2ml reagent into test tubes.
3. Allow the reagent to come to room temp (15 minutes). Keep reagent bottle refrigerated after use.
4. Pipette 0.5ml sample into long test tubes.
   Shake by hand. (note time, beginning to end).
5. Prepare standards and blanks (acetate/azide) same as samples.
6. Incubate for 15 minutes at 30°C
7. After 15 minutes take out samples from waterbath and record absorbance at 505nm (within 15-20 minutes) against blank.

Time difference between sample 1 to last is very important since colour development is time dependent.

APPENDIX 12: Soil enzyme analysis
12.2 ARYL SULPHATASE ACTIVITY
as per Tabatabai & Brenner, (1970)

The method is based on the determination of *p*-nitrophenol released after the incubation of soil with *p*-nitrophenol sulphate for 1 hour at 37ºC.

Materials and apparatus

Photometer
Incubator adjustable to 37°C
Filter paper (Whatman No. 2v)
Erlenmeyer flasks
Volumetric flasks (100 ml)

Chemicals and solutions.

Toluene.
Acetate buffer (0.5M, pH 5.8)

Dissolve 68g of sodium acetate trihydrate in 700 ml of distilled water, adjust the pH with concentrated acetic acid to 5.8 and bring up to 1000 ml with distilled water.

***p*-nitrophenol sulphate solution (25 mM).**

Dissolve 0.312g of potassium *p*-nitrophenol sulphate in about 40 ml acetate buffer and dilute the solution to 50 ml with buffer. Store at 4ºC.

$CaCl_2$ (0.5M)

Dissolve 73.5g of $CaCl_2.H_2O$ in dist. water and dilute with RO water to1000 ml.

NaOH (0.5M)

***p*-nitrophenol standard solution.**

Dissolve 1.0g *p*-nitrophenol in about 70 ml distilled water and dilute to 1000 ml with RO water. Store at 4ºC.

Procedure

Place 1g of moist sieved (2mm) soil in Erlenmeyer flask (50 ml), add 0.25 ml toluene, 4 ml of acetate buffer, 1 ml of *p*-nitrophenol sulphate solution and mix the contents. Cap the flasks and incubate for 1hour at 37ºC. After the incubation add 1 ml of $CaCl_2$ (0.5M) and 4 ml of NaOH (0.5M), mix the contents, filter the soil suspension and measure the optical density at 400 nm. To prepare the control, make the addition of 1 ml of *p*-nitrophenol sulphate after the addition of $CaCl_2$ and NaOH, ie immediately before filtration of the soil suspension.

Calibration curve

Dilute 1 ml of standard *p*-nitrophenol solution to 100 ml with distilled water into a volumetric flask. Then pipette 0, 1, 2, 3, 4 and 5 ml aliquots of this diluted standard solution into Erlenmeyer flasks (50 ml), adjust the volume to 5 ml by addition of RO water. Proceed as described for *p*-nitrophenol analysis of the incubated soil sample.

Calculation

Correct the results for the control and calculate the *p*-nitrophenol per millilitre of the filtrate by reference to the calibration curve.

$$p\text{-nitrophenol}(\mu g\ g\text{-}1\ dwt\ h\text{-}1) = \frac{C \times v}{dwt \times SW \times t}.$$

where C is the measured concentration of *p*-nitrophenol (µg ml-1 filtrate), dwt is the dry weight of 1 g moist soil, v is the total volume of the soil suspension in millilitres, SW is the weight of soil sample used (1g) and t is the incubation time in hours.

APPENDIX 12: Soil enzyme analysis
12.3 ACID PHOSPHATASE ACTIVITY
Based on Tabatabai and Bremner 1969

Materials and apparatus

Photometer
Incubator adjustable to 37°C
Filter paper (Whatman No. 2v)
Erlenmeyer flasks
Volumetric flasks (100 ml)

Chemicals and solutions

Toluene

Modified universal buffer (MUB) stock solution

Dissolve 12.1 g of Tris, 11.6g of maleic acid, 14 g of citric acid and 6.3g of boric acid (H_3BO_3) in about 500 ml of NaOH (1M), and dilute the solution to 1000 ml with distilled water. Store at 4°C.

Modified universal buffer, pH 6.5 and 11.

Titrate 200 ml of MUB stock solution to pH 6.5 under continuous stirring with HCl (0.1M) and dilute to 100 ml with distilled water.

***p*-nitrophenol phosphate solution (PNP, 15 mM).**

Dissolve 2.927 g disodium *p*-nitrophenol phosphate tetrahydrate in about 40ml MUB (pH 6.5 or 11) and bring up to 50 ml with the buffer of the same pH. Store at 4°C.

$CaCl_2$ (0.5m) solution

Dissolve 73.5g of $CaCl_2$.H2O in distilled water and dilute with distilled water to 1000 ml.

NaOH (0.5M) solution

Dissolve 20g of NAOH in distilled water and bring up to 1000 ml.

NaOH (0.1M) solution

Dissolve 4g of NaOH in distilled water and bring up to 1000 ml.

Standard *p*-nitrophenol in about 70 ml of distilled water and dilute the solution to 1000 ml with distilled water. Store at 4°C.

Procedure

Soil (1g) is placed in an Erlenmeyer flask (50 ml) and treated with 0.25ml of toluene, 4 ml of MUB (pH 6.5 for the assay of acid phosphatase, pH 11 for the assay of alkaline phosphatase), and 1 ml of *p*-nitrophenol phosphate solution made in the same buffer. After stoppering the flasks, the contents are mixed and incubated for 1 hour at 37°C. After the incubation, add 1 ml of CaCl2 (0.5M) and 4 ml of NaOH (0.5M). Mix the contents and filter the soil suspension through a Whatman No. 2 folded filter paper. Measure the absorbance at 400 nm. To perform the controls, add 1 ml of PNP solution after the additions of $CaCl_2$ (0.5M) and 4 ml of NaOH (0.5M) and immediately before filtration of the soil suspension. All measurements are performed in triplicate.

Calibration curve

Dilute 1 ml of standard *p*-nitrophenol solution to 100 ml with distilled water in a volumetric flask. Then pipette 0, 1, 2, 3, 4 and 5 ml aliquots of this diluted standard solution into Erlenmeyer flasks (50 ml), adjust the volume to 5 ml by addition of distilled water, and proceed as described for *p*-nitrophenol analysis of the incubated soil sample.

Calculation

Correct the results for the control and calculate the *p*-nitrophenol per millilitre of the filtrate by reference to the calibration curve.

p-nitrophenol (µg g-1 dwt h^{-1}) =

$$\frac{C \times v}{dwt \times SW \times t}.$$

where C is the measured concentration of *p*-nitrophenol (µg ml-1 filtrate), dwt is the dry weight of 1 g moist soil, v is the total volume of the soil suspension in millilitres, SW is the weight of soil sample used (1g) and t is the incubation time in hours.

APPENDIX 12: Soil Enzyme Analysis
12.4 DEHYDROGENASE ACTIVITY
as per Thalmann, 1968.

Dehydrogenase was measured as an estimation of triphenyl tetrazolium chloride (TTC) reduction rate to triphenyl formazan (TPF) in soils after incubation at 30ºC for 24 hours.

Materials and apparatus
Test tubes (60 ml volume, 2.5 cm diameter) and rubber stoppers resistant to solvents. Incubator adjustable to 30ºC.

Chemicals and solutions

Tris—HCl buffer (100 mM)
Dissolve 12.1g of Tris (hydroxy methyl)-aminomethane in 700 ml distilled water, adjust with HCl to pH 7.8 for acid soils with pH values less than 6, to pH 7.6 for neutral soils with pH values ranging from 6 to 7.5, and to pH 7.4 for alkaline soils with pH values higher than 7.5. Bring up with distilled water to 1000 ml.

TTC solution
Depending on the soil type, TTC quantities from 0.1 to 1.5g are dissolved in 80 ml Tris buffer and made up with the same buffer to 100 ml:

Extractant Acetone (analytical grade)

Tripehylformazan(TPF) standard solution.
Dissolve 50mg of TPF in 80 ml acetone (500 µg tpf $ml^{-1}$and bring up with acetone to 100 ml.

Procedure
Because of the light sensitivity of TTC and TPF, perform all procedures under diffused light. Weigh 5g field-moist soil into test tubes and mix with 5ml of TTC solution. Seal the tubes with rubber stoppers and incubate for 24 hours at 30ºC. The control contains only 5ml Tris buffer (without TTC). After the incubation, add 40 ml acetone to each tube, shake thoroughly and further incubate at room temperature for 2 hours in the dark (shaking the tubes at intervals). Filter the soil suspension (15ml) measure the optical density of the clear supernatant against the blank at 546 nm.

Calibration curve
Pipette 0, 0.5, 1.0, 2.0, 3.0 and 4.0 ml of TPF standard solution in a volumetric flask (50 ml), add 8.3 ml Tris buffer (pH 7.6) and bring up with acetone to 50 ml to obtain the following concentrations: 0, 5, 10, 20, 30 and 40 µg TPF ml^{-1}.

Calculation
Read the TPF concentrations (µg/ml) from the calibration curve, correct for the control value and calculate as follows:

$$\text{Dehydrogenase activity TPF} = \frac{(\mu g)/ml \times 45}{\text{dwt} \times 5}$$

where dwt is the dry weight of 1 g moist soil, 5 is the moist soil used (g) and 45 is the volume of solution added to the soil sample in the assay.

APPENDIX 12: Soil Enzyme Analysis
12.5 PROTEASE ACTIVITY
as per Ladd & Butler, 1972

The procedure measured the released amino acids after incubation of soil with sodium caseinate using Folin-Ciocalteu reagent. Two lots of four replicate samples were made for each of the four replicates of the non-GM and GM plant rhizosphere samples. The method is based on the estimation of the triphenyltetrazolium chloride (TTC) reduction rate to triphenyl formazan (TPF) in soils after incubation at 30ºC for 24 hours.

Materials and apparatus.

Spectrophotometer
Shaking water bath (adjustable to 50ºC)
McCartney bottles.
Folded filter paper,
Centrifuge tubes (25 ml),
No. 6 plastic tubes

Chemicals and solutions.

1. Trisma base (buffer) (50 mM, pH 8.1) Store at room temp.

05g Tris (hydroxy methyl) amino methane (Trizma base)	6.05g	2.42g
distilled water	700 ml	300 ml
	pH 8.1 with HCl.	pH 8.1
Distilled water up to	1000 ml volumetric	400 ml volumetric

2. Sodium caseinate (2%: 50mM) Store in fridge, or make up fresh.

Sodium caseinate (or **Casamino acids in Tris buffer**, pH 8.1 as above)	Suspend 10g in Tris buffer (as above) (warm to 50ºC)	2g in Tris buffer (50ºC)
	bring up with distilled water to 500mL (use a stirrer)	bring up with distilled water to 100mL (use a stirrer)

3. Trichloroacetic acid (15%) Room temp (Stop solution).

trichloroacetic acid (TCA)	75g	60g
(Stored in acid cupboard)	dissolve in about 300 ml dH_2O	dissolve in about 250 ml dH_2O
	dilute to 500 ml with dH_2O	dilute to 400ml with dH_2O

4(a) Alkaline reagent: mix 4(a), 4(b) & 4(c)

NaOH (1M) (1M NaOH = 40g/L)	60 ml. Dilute with extra dH_2O
Na_2CO_3 (water free)	add 50g into the solution
	bring up to 1000 ml with dH_2O

4(b) Dissolve 0.5g **$CuSO_4.5H_2O$** in distilled water and dilute to 100 ml with distilled water.

4(c). Dissolve 1g **potassium sodium tartrate** ($C_4H_4KNaO_6.4H_2O$) in distilled water and dilute to 100 ml with distilled water.

Mix 1000 ml (100ml) of $NaOH/Na_2CO_3$ solution **(4a)** with 20 ml (2 ml) of $CuSO_4$ solution **(4b)** and 20 ml (2ml) of potassium sodium tartrate solution **(4c)**

Folin-Ciocalteu reagent (33%) Keep in fridge 4°C.
Dilute 167 ml (33.4 ml) of **Folin** reagent to 500 ml (100 ml) with distilled water.

Tyrosine standard solution (500 µg ml^{-1}) Keep in fridge 4°C
Dissolve 50mg tyrosine in **Tris buffer** and dilute to 100ml with Tris buffer.

PROCEDURE.
1(a) Place 1g of moist, sieved soil (2 mm) in a large centrifuge tube, add 5 ml Tris buffer and 5 ml sodium caseinate solution. Stopper the tubes, mix the contents and incubate for 2 hours at 50ºC on a shaking water bath. At the end of incubation, add 5 ml of TCA solution and mix the contents thoroughly.

1(b) Filter the sample mixtures through paper filter into No. 12 tubes and transfer 5 ml of the clear filtrate into new tubes, mix with 7.5 ml of the alkaline reagent, and incubate for 15 min at room temperature. After adding 5 ml of the Folin reagent measure the absorbance after exactly 1 hour at 700 nm. Take three OD readings.

1(c) To perform the +ve controls, pipette 5 ml of Casamino Acid solution **(2)** into McCartneys at the end of the incubation and immediately before adding the TCA solution. Add 7.5 ml alkaline reagent as per the samples. Incubate with samples for 15 minutes, room temp. Add 5 ml Folin reagent as per samples, and measure absorbance after 1 hour.

Calibration curve (McCartney)	**1**	**2**	**3**	**4**	**5**	**6**
Tyrosine solution	0,	1,	2,	3,	4	5 ml
Casamino acid solution (2),	5 ml to all.					
Tris buffer up to 10 ml.	5,	4	3	2	1	0 ml
TCA solution	5ml to all					
Alkaline reagent (4)	7.5 ml to all. Incubate room temp. 15 minutes.					
Folin Reagent.	5 ml to all.					

Measure the absorbance after exactly 1 hour at 700 nm.

Calculation.
Correct the measured absorbance for the controls and calculate as follows:

Protease activity (µg tyrosine g^{-1} $dwt2h^{-1}$) =

$$\frac{C \times 15}{dwt.}$$

where dwt is the dry weight of 1g of moist soil, 15 is the final volume of solutions added to the soil in the assay (ml) and C is the measured tyrosine concentration (µg ml^{-1} filtrate).

APPENDIX 12: Soil Enzyme Analysis
12.6 UREASE ACTIVITY
as per Tabatabai & Bremner, 1972

The method is based on the colorimetric determination of released ammonia after the incubation of soil samples with urea solution for 2 hours at 37ºC.

Material and apparatus

Incubator adjustable to 37C	spectrophotometer
Shaker	volumetric flasks (100, 500, 1000, 2000 ml)
Filter paper	Erlenmeyer flasks (50, 100 ml)

Chemicals and solutions

Urea solution	Dissolve 2.4g urea in 400 ml distilled water and make up with distilled water to 500 ml (prepare daily).
KCl solution	Dissolve 74.6g KCl in distilled water, add 10 ml of 1M HCl (32% HCl is equal to 10M) and bring up with distilled water to 1000 ml.
NaOH (0.3M)	Dissolve 12g NaOH in distilled water and bring up with distilled water to 1000 ml.
Sodium salicylate solution	Mix equal volumes of the NaOH and sodium salicylate solutions, and distilled water (to be prepared daily).
Sodium dichloro-isocyanide solution (0.1%)	Dissolve 0.1g sodium dichloro-isocyanide in 100 ml distilled water (prepare the solution shortly before using).
Borate buffer (pH 10).	Dissolve 56.85g disodium tetraborate, or 30g disodium tetraborate (water free) in 1500 ml warm distilled water. After cooling, adjust to pH 10 with NaOH solution (20%) and bring up to 2000 ml with distilled water.

Ammonium standard solution.

Solution 1	Dissolve 3.82g ammonium chloride in distilled water and bring up with distilled water to 1000 ml (1000 µg NH4-N ml^{-1}). The solution is stable for several weeks at 4°C.
Solution II	Pipette 0.0, 1.0, 1.5, 2.0, 2.5 ml of solution I into volumetric flasks (100 ml) and bring up to 100 ml with KCl solution.

Procedure for the non-buffered method.
Place 5 g of moist soil in an Erlenmeyer flask (100ml) and add 2.5 ml urea solution. Then stopper the flasks and incubate for 2 hours at 37°C. After the incubation, add 50 ml of KCl solution and shake the flask for 30 min. After filtering the resulting suspension the filtrates are analysed for the ammonium content. Perform the blanks as

described above but with 2.5ml distilled water, and add the urea solution at the end of the incubation and immediately before KCl addition. It is recommended that at least three replications are carried out.

Ammonium determination.

Pipette 1 ml of the clear filtrate into an Erlenmeyer flask (50 ml), then add 9 ml of distilled water, 5 ml of the Na salicylate/NaOH solution and 2 ml of the sodium dichloro-isocyanide solution and allow to stand at room temperature for 30 min prior to measuring the optical density at 690nm.

Procedure for the buffered method.

Place 5g of moist soil samples in Erlenmeyer flask (100 ml), add 2.56ml urea solution and 20 ml borate buffer. Further types are performed as described for the non-buffered assay, with the addition of 30 ml KCl solution at the end of incubation.

Calibration Curve

Pipette 1 ml of the ammonium standard solution II in glass tubes, dilute with 9 ml distilled water, and determine the ammonium concentrations (0, 1, 1.5, 2, 2.5 µg NH_4-N ml-1).

Calculation

Correct the results to the blanks and calculate as follows

$$NH_4\text{-N g-1 dwt 2 h}^{-1}) = \frac{\mu g\ NH4\text{-}4\ ml\ -1 \times V \times 10}{dwt \times 5}$$

where dwt is the dry weight of 1 g moist soil, V is the total volume of the extract (52.5ml) 10 is the dilution factor and 5 is the weight of the soil used in the assay.

APPENDIX 13: NITROGEN DYNAMICS
13.1 RATE OF NITRIFICATION
BY MEASUREMENT OF NITRITE
by the method of Berg & Rosswall, 1985

The basal measurement detects any residual nitrite in soil and this is compared with the oxidised N which is converted by microbial activity after induction with excess ammonium sulphate. This test requires measurement of nitrite-N from soil + added Ammonium sulphate substrate in a 24h incubation assay.

Incubation and extraction

(1) Set up six 10ml centrifuge tubes for each soil sample – 2 tubes/ incubation time (ie, 2 tubes for each for T_0, T_5 and T_{24}). Mark each set as +AS and –AS respectively.

(2) Add 1g (SDW) of appropriate soil into both the +AS and –AS tubes.

T_5 incubation

(3) To all T_5 + AS tubes, add 4 ml of 1mM $(NH_4)2SO_4$ and 50µL of 1M $KClO_3$ and vortex.

(4) To all T_5 –AS tubes, add 1 ml of 450mM $KClO_3$ and vortex.

(5) Place both sets of tubes in constant temperature room to incubate for 5 hours.

T_{24} incubation

(6) Repeat steps (3) & (4) for another set of duplicate tubes (T_{24}). Place in constant temperature room to incubate for 24 hours.

T_0 incubation

(7) Repeat steps (3) & (4) for another set of duplicate tubes (T_0). To the +AS tubes, add 1 ml 2M KCl and to the –AS tubes, add 2 ml 2M KCl and 2ml dH_2O.

(8) Vortex and then centrifuge tubes at 3000rpm for 5-10 minutes. Transfer supernatant to labelled 5 ml serum tubes. Place in freezer until the colorimetric assay for nitrite-N.

(9) Repeat steps (7) and (8) for T_5 and T_{24} tubes, after the appropriate incubation time and store the extracts in freezer until the colorimetric assay. Immediate analysis of extracts for nitrite-N is recommended and long-term storage should be avoided.

Calibration standards

(10) A. for $+NH_4SO_4$, add 0 ml to 10ml of 10Mg/ml nitrite standard to appropriately labelled 100ml volumetric flasks.
B. Add 20ml 2M KCl solution, then make up volume with dH_2O.

(11) A. For $–NH_4SO_4$, add 0 ml to 10ml of 10g/ml nitrite standard to labelled 100ml volumetric flasks.
B. Add 40ml of 2M KCl solution, then make up volume with dH_2O.

Colorimetric assay

(12) Ten 10ml test tubes, add 1ml of each standard (6 tubes for each set).

(13) Pipette 1ml of soil nitrification extract/sample in duplicate, to 10ml test tubes.

(14) Add 600µl of Ammonium Chloride buffer, and 400µl of Nitrite Reagent to all tubes and vortex.

(15) Read absorbance at 520nm. Calculate µg N per gram SDW using the appropriate standard curve.

Reagents

1A	$KClO_3$-I (1M)	$KClO_3$ in 200 ml DW. Heat and dissolve, then bring up to 250ml.
1B	$KClO_3$ – II (450mM)	Dissolve 112ml of $KClO_3$ – I in a 200ml hot DW; make up the volume to 250ml.
2.	$(NH4)_2SO_4$ (1mM)	Dissolve 0.132g $(NH_4)2SO_4$ in 800ml DW. Bring up to 1 litre.
3.	KCl Soln. (2M)	Dissolve 149.12g of KCl in 700mL DW. Bring up to 1 litre.
4.	Buffer (0.19M, pH 8.5)	Dissolve 10g NH_4Cl in 500mL DW. Adjust pH to 8.5 with NH_4OH. Adjust to 1 litre with DW.
5.	Nitrite Reagent	Dissolve 2g Sulphanilamide + 0.1g N-1(Naphthyl)Ethyl-enediamine in 150ml DW. Add 20ml of orthophosphoric acid, and adjust volume to 200ml.
6A.	Nitrite stock soln (1000 µg N/ml)	Dissolve 4.92g $NaNO_2$ in 700ml DW. Bring up to 1 litre, then store in the fridge.
6B	Nitrite standard solution (10µg N/ml)	Take 5ml nitrite stock solution and bring up to 500ml with DW (for standards).

APPENDIX 13.2: Detection of Ammonium oxidising bacteria by a modified method (Gupta V.V.S.R. unpublished) based on the methods from:

Belser, L.W. and E.L. Schmidt (1978)
Matulewich, V.A., P. F. Strom and Finstein, M.S, (1975)
and Schmidt, E.L. & Belser, L.W. (1982)

An estimation by most probable number using a colorimetric indicator using the Griess-Ilosvay Reagent, a diazo dye, is used to measure the oxidised N-compounds.

MEDIA A: (500ml)		**MEDIA B: (500ml)**	
$(NH_4)_2SO_4$	0.25	NaCl*	
K_2HPO_4	0.5	$CaCO_3$*	0.15
$FeSO_4.7H_2O$	0.015	pH	3.75
$MgSO_4.7H_2O$	0.15		6.8-7.0

1. *Autoclave separately to minimise precipitation. Mix A and B solutions after they reach room temperature. Then add 75µL per well to all wells. Use multipipette dispenser and sterile pipette tips. 12 x 8 = 96 well /plate = ≅ 10 ml per plate.
2. Add to 10^{-1} soil dilution (with phosphate buffer) to first (bottom) row only.
3. With multichannel pipetter (8 tips) dilute 5 x by mixing up and down several times. Keep last row (top) as negative control (no soil).

———

At 21 days:
Griess – Ilosvay Reagent (G.I.R.), make up just before adding to plates.

Solution A. (200 ml)		**Solution B.** (200 ml)		**Solution C.** (200 ml)
1.2g Sulphanilic acid in		1.2g αNapthylamine (N-1Napthyl)-ethylene d-amine dihydrochloride)		32.8g Sodium Acetate trihydrate ($CH_3COONa.3H_2O$), in
140ml hot distilled H_2O. Cool solution and add		40ml RO H_2O with		200 ml RO H_2O in volumetric flask.
40 ml Conc. HCl and		2ml conc HCl		
20ml dd H_2O. Mix well.		158ml d H_2O		

Mix equal parts A, B & C in light-proof bottle. Add 75µL per well in fume hood [pink/red = positive, as per illustration, left].

The colour appears within one minute. The intensity of the colour is proportional to the quantity of nitrite formed.

Add 1-3 drops **phenyl amine solution**. (NO)
per well into plates that tested negative with G.I.R.

0.4g (up to 1.0g) Phenyl amine.) keep in light-

50mL H2SO4 (concentrated)) proof container

Plate showing positive reaction from Avon soil sample.

APPENDIX 13: THE NITROGEN CYCLE:
13.3 METHOD FOR MEASURING NINHYDRIN REACTIVE-N (NHD-N) IN SOIL EXTRACTS.

A variation on the procedure of Amato & Ladd (1988)

SOIL PREPARATION

Determine moisture content as per Blakemore et al. 1987.

1. Weigh 15g moist weight per sample in duplicate for both Time zero (T_0) and for ten-day fumigated soils (T_{10}). Immediately put into glass jars with screw caps to avoid drying.

   For the T_0 soils, add 2M KCl and close the jar tightly (1:3 soil:KCl ratio). Shake the soil + KCl mixture for 60 min and filter through Whatman No. 42 (12.5cm) into plastic tubes. Store the extracts in freezer immediately.

2. For the T_{10} soils, put the jars with weighed soils to be fumigated into desiccators with 40ml EtOH-free-chloroform into a beaker with a few anti-bumping granules. Moist absorbant paper at the bottom of desiccator avoids moisture loss from the soil samples.

   Evacuate the desiccator for 10 minutes after the start of bubbling of chloroform. Place the desiccator in the dark at 22-25ºC for 10 days. After ten days take the soils out of the desiccator (fume cupboard) and filter as for the T_0 samples.

Process all paired treatments concurrently.

REAGENTS:

1. Analysis for Ninhydrin-N:

2M KCl – Dissolve 149.1g of KCl in 750m ml of RO water and make volume up to one litre.

2. Acetate buffer (use at room temperature)

Add 272g of Sodium acetate trihydrate (AR) To 200 ml of distilled water and dissolve by stirring with heat, or heat in microwave (endothermic).

Allow the solution to cool to room temp.

Add 50ml of Glacial acetic acid (fume hood), mix and adjust pH 5.51 ± 0.03 with acetic acid and make volume up to 500 ml. Store at 4°C.

3. Diluent: **Ethanol** – 50% ethanol in d-distilled water. Store in a cool place. (5 ml for each sample required).

4. Ninhydrin reagent (Per 100ml) Make immediately before analysing.

	Dissolve		Dissolve
1. Ninhydrin	2g	3. Hydrindantin	0.2g
2. Methoxyethanol	50ml	4. Acetate buffer	50ml

Mix well before use. Store in a dark bottle since this reagent breaks down under light.

5. Standards. Leucine and Ammonium Sulfate.

1. Dissolve 0.164g of Leucine in 100ml 2M KCl (Leucine contains 10.67% N – 175ppm N) as stock solution
2. Dissolve 0.083g of Ammonium Sulfate in 100ml 2M KCl (ammonium sulfate contains 21.2% - 175ppm N) as stock solution
3. For working standards dilute 1 ml of stock solution in 100ml 2M KCl.

   One ml of working standard will give 1.75µg N/ml.

   Store the stock solutions and working standards at <= 4°C.

3. Place standards, blanks (2M KCl) and samples in 20ml test tubes
   Usually unfumigated filtrate is used in the ratio of 2ml, + 0ml 2M KCl, and 10-day fumigated filtrate is used at the rate of 1ml filtrate + 1 ml 2M KCl, however this will vary with soil, and concentration may need to be adjusted for the spectrophotometer.

4. For a standard curve use 0.25, 0.50, 0.75, 1.0, 1.5, 2.0 ml of working standard of ammonium sulphate to give 0.875, 1.750, 2.625, 3.500, 5.250, 7.000 g N/sample and add appropriate amount of 2M KCl to bring volume to 2 ml.

5. Add 2ml freshly prepared Ninhydrin reagent to all samples, blanks and standards and mix well using vortex mixer.

6. Water bath must be at boiling point before adding ninhydrin reagent.
   Place all samples in water bath and allow to boil for 15 min.

7. Immediately cool samples in cold water. Add 5ml diluent per sample.
   Vortex again.

8. At spectrophotometer, invert test tube several times using parafilm and read optical density (absorbance) at OD_{570} Blank against 2M KCl. OD measurements must be taken within 30min, since colour fades with time.

Calculations:

(a) From the OD readings for standards prepare a standard curve and extrapolate OD to µg N/g soil = g N/ ml * total volume of extract)/ wt soil used in extraction.

(b) Microbial biomass C/ g soil = (fumigated – unfumigated) * kC conversion factor, where kC conversion has been taken as 21 (Amato & Ladd, 1988).

APPENDIX 14.1

EXTRACTION OF PHOSPHOLIPID FATTY ACIDS FROM SOIL
A modification of the method of White 1988,
using the acid methylation procedure of
Dr Brian Siebert, University of Adelaide, from Christie, 1989

Reagents	**Equipment**
HPLC Grade Chloroform	Teflon centrifuge tubes
HPLC Grade Methanol	100ml glass tubes with teflon-lined lids
HPLC Grade Acetone	Glass test tubes
HPLC Grade Hexane	Long and short pasteur pipettes
K_2HPO_4	
1N HCl	
Silicic Acid	
1% H_2SO_4 in Methanol (dried and distilled)	
Petroleum Ether (distilled)	

Phosphate Buffer
Dissolve 8.7g of K_2HPO_4 in 1l H_2O and neutralise to pH 7.4 with 1M HCl

Preparation of One-Phase Buffer

0.8: Phosphate Buffer	**1.0:** Chloroform	**2** Methanol	proportions to obtain the volume required
420 ml	525 ml :	1050 ml.	suggested ~ 2 litres

Soil Preparation and Extraction
(1) Require moisture content of soil. 40°C oven overnight. Subsample.
(2) Weigh 10g of soil (ww) into a 50ml teflon centrifuge tube.
Add 1-phase buffer and/or components as indicated below:

			- Single phase solution -				
		Add (1)	(2)	(3)	(4)	(5)	(6)
Sample	Wt. of Soil	Wt. H_2O in soil	Phos.buffer to add ***(0.8):***	CHCl3 to add ***(1.0):***	MeOH to add ***(2)***	Extra 1-Phase to add	After Wash (1-phase) to add
1	10g	3.0g	0ml	3.8ml	7.5ml	4.0ml	5.0ml
2	10g	2.2g	0.8ml	3.8ml	7.5ml	4.0ml	5.0ml
Blank	0g	0g	3.0ml	3.8ml	7.5ml	4.0ml	5.0ml

ie, bring up all soil samples to the same moisture level (3 ml) with phosphate buffer, add $CHCl_3$, add MeOH, add extra 1 phase, then mix on rotating shaker for 2 hours.

Centrifuge at 2,000 rpm for 5 min 10-15°C, brake on.
Remove liquid phase with pasteur pipette into a clean 50ml culture tube. Record weight of liquid. Add 5ml of 1-phase buffer wash to the left-over soil, vortex for 15 sec, centrifuge again (2000rpm for 5 min.), remove liquid phase and add to previous. Record weight of liquid. Add a blank tube with reagents only – Extraction Blank

Phase Separation
Using the combined weights of liquid obtained after centrifuging, calculate the volume of chloroform and phosphate buffer to add to separate the phases. For example: If 17ml of extract (1-phase buffer) was obtained, this is composed of:

Phos. Buffer ***(0.8):***	CHCl3 ***(1):***	MeOH ***(2)***	Total weight (I-Phase Buffer – 0.8:1:2)
3.5	4.5	9.0	17.0
4.5	**4.5**		**TO ADD**
8.0	9.0	9.0	Final ratio of 0.9:1:1 is required

Add the volumes of chloroform and phosphate buffer to the 1-phase extract in the clean culture tube. Cap with a teflon lined cap, vortex for 1min and leave to settle overnight.

Remove bottom phase into a clean 100x15mm culture tube and blow down under N_2. (Can freeze overnight.) This is the lipid extract.

Lipid Fractionation: Silicic Acid Columns
Place sufficient silicic acid in a beaker and heat at 120°C overnight. Add approx 0.5g of Silicic Acid (or 30-40mm of column length) to small Pasteur pipette column after plugging with a small amount of glass wool. Tap column with spatula to consolidate silicic acid and flood with chloroform. Using long stemmed pasteur pipette pulse air bubbles out of column. Wash sides of column with chloroform. Allow chloroform to drain to silicic acid surface

↳ discard chloroform **↳ discard acetone** **↳ collect methanol fraction**

Resuspend lipid extract in 1ml of chloroform. Add lipid extract to the column to charge the silicic acid surface

Elute lipid fractions as follows:

(1) 5 ml of chloroform
and drain: Neutral Lipids.
Discard.

(2) 10 ml acetone
and drain: Glycolipids
Discard.

(3) 5 ml of methanol
and drain: Phospholipids
Keep.

Add a tube with reagents only passed through the column (ie no lipid extract) – Fractionation Blank. Collect the above fractions in culture tubes and blow down to dryness under nitrogen on a heating block set at ~40°C Dried fractions can be frozen or kept in a fridge (short term) prior to methylation

Acid Methylation Procedure
Add approximately 1.5ml (1 pasteur pipette full) acidified (1% H_2SO_4) methanol* (dried and distilled) to the phospholipid fraction in a 16x100mm culture tube. Screw the cap on tightly and heat the tubes in an oven at 100^oC for 45min or at 60ºC overnight.

Allow samples to cool to room temperature then add 3ml purified water and 5ml petroleum ether (40-60^oC b.pt., distilled). Vortex or shake the samples for 15 sec then transfer the upper ether layer to a clean dry culture tube taking care not to take up the aqueous layer. Add a further 5ml petroleum ether to the aqueous sample and repeat the extraction and removal of the ether layer. Pool the extracts then evaporate the ether solvent with a stream of nitrogen on a heating block at 40^oC.

* Reflux A.R. methanol over calcium hydride for 1 hour.
Filter through fine paper, distill and store in an air tight bottle.

Optional – to remove colours or complex lipids such as cholesterol.
Add 1.5ml hexane (1 pasteur pipette) to the dry esters to dissolve them, then transfer the solution to the top of a small column containing Florosil (approximately 20mm in pasteur pipette). Elute methyl esters in with 2ml 10% diethyl ether in hexane and collect eluate in clean culture tube. Dry with a stream of nitrogen at 40^oC.

Add 10µl of C 19 methyl ester standard to the evaporated methyl esters and dissolve in 200µl hexane and pipette solutions into GC phials.
(C 19 methyl ester standard prepared by dissolving 0.037g of Nonadecanoic Acid Methyl Ester (SIGMA N5377) in hexane and making up to 50ml)

Calculation: (ug/g) of PLFA

Area of peak/Area of C19 peak x $210^a/2^b$ x 0.070^c x 1/dry wt of soil

a: volume of solution in phial (µl);

b: volume of solution injected (µl)

c: µg of C 19 injected

13/02/01

Appendix B Additional computations of data and graphs

Phospholipid Fatty Acid Principal Component Analyses

The first analysis shown as Figure Appendix 14.3.3.1 showed the differences in the rhizosphere soils between the V15 and V15i plants harvested at three weeks, when the plants were still actively growing. The plot is scattered across both axes, without differentiation between non-GM and GM plants. At 3.7 weeks two of the three soil only samples have differentiated from the rhizosphere soil. The remaining symbol for the soil only is positioned directly underneath the cluster of GM points in the lower left quadrant.

AVON SOIL

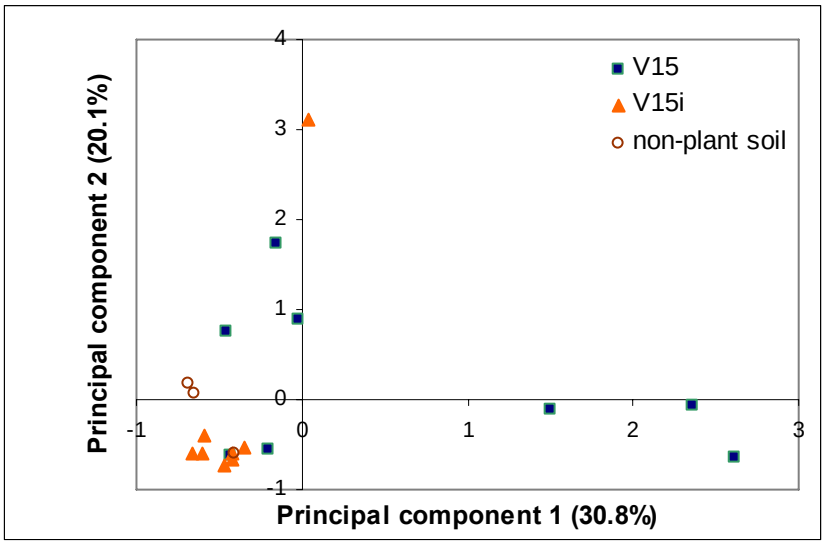


Figure Appendix 14.3.3.1 **Comparison of non-GM, GM and non-rhizosphere soil at the 3.7 week harvest from V15 and V15i plants grown in Avon soil**

The figure 14.3.3.2 shows the same plant and soil as per the previous graph, but at a harvest age of 6.4 weeks. The analysis showed that the phospholipids did not polarise into separate clusters for the non-GM and GM rhizosphere soils indicative of consistent overall difference between rhizosphere soil types. The range of the axis scale of the principal components had changed, suggesting a different population mix from the 3.7 week harvest, with principal component 1 explaining nearly half of the variance for all samples. A comparison of the fatty acids for the two treatments showed a great deal of similarity between the types of fatty acids present, with only the concentrations differing sufficiently to show differences in population component.

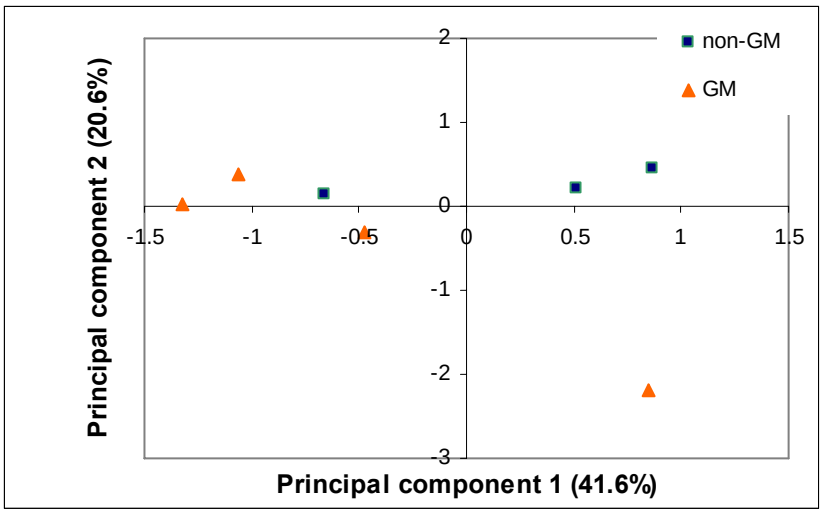


Figure Appendix 14.3.3.2 Comparison of non-GM, GM and non-rhizosphere soil at the 6.4 week harvest from V15 and V15i plants grown in Avon soil

The eight-week harvest of the V15, V15i and soil only controls shown in Figure 14.3.3.3 below showed a close clustering of GM plant rhizosphere fatty acid data points, with three non-GM replicates showing large variability. The major differences in profiles were attributable to a large amount of short chain fatty acids for the soil-only data point, and a large increase of multiple fatty acids in the non-GM data point, that were not present in the other samples. The remaining two soil-only data points were positioned beneath the GM symbols in the lower left quadrant.

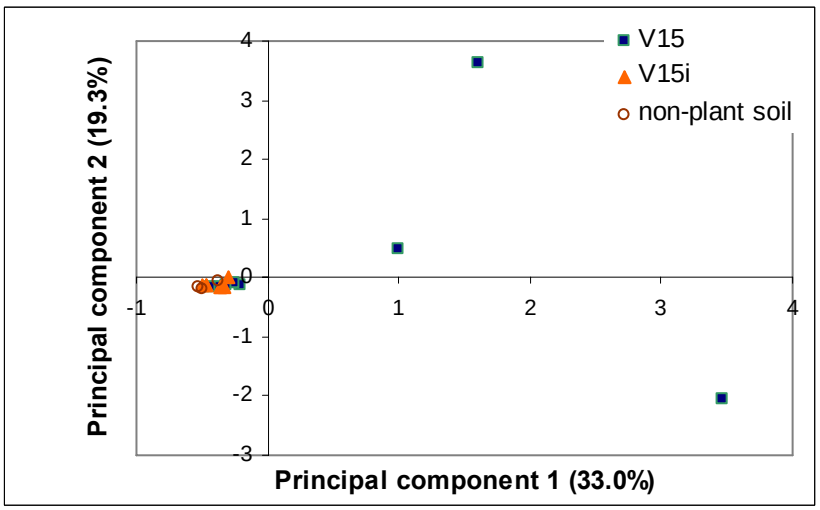


Figure Appendix 14.3.3.3 Comparison of non-GM, GM and non-rhizosphere soil at the 8 week harvest from V15 and V15i plants grown in Avon soil.

Figure 14.3.3.4 (below) showed the analysis of the fatty acids of the rhizosphere soils for plants V15 and V15i grown under nutrient deficiency. Difference in composition of fatty acids in non-plant soil fatty acids were distinct from the two rhizosphere soils but the fatty acids of the two plant rhizosphere soils were not positioned separately

within the first two principal components. All rhizosphere points fell within a very narrow band along the first principal component, showing similar weighting factors.

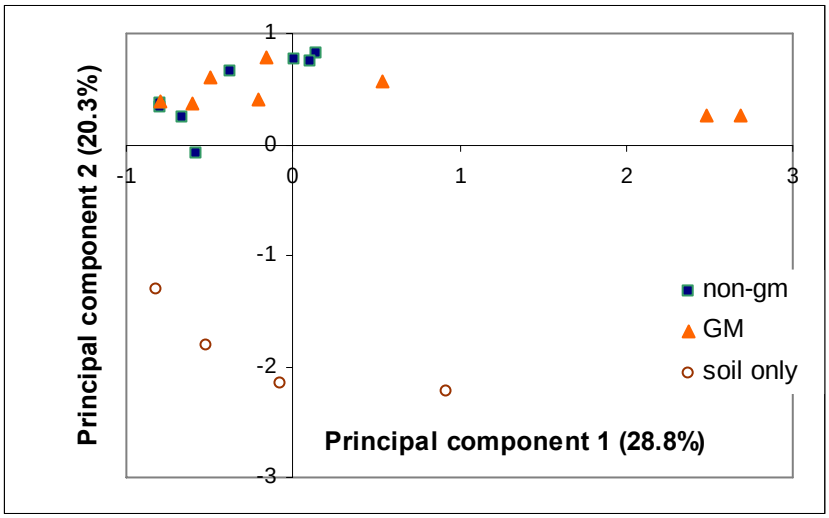


Figure 14.3.3.4 Comparison of non-GM, GM and non-rhizosphere soil at the 16.8 week harvest from V15 and V15i plants grown in Avon soil

The following figures show the analysis of fatty acids from the Narrabri soils. There were four data points (n = 4) for the non-GM replicates shown in Figure 14.3.3.5, as some replicates were deleted because the total volume of fatty acids was too low.

NARRABRI SOIL

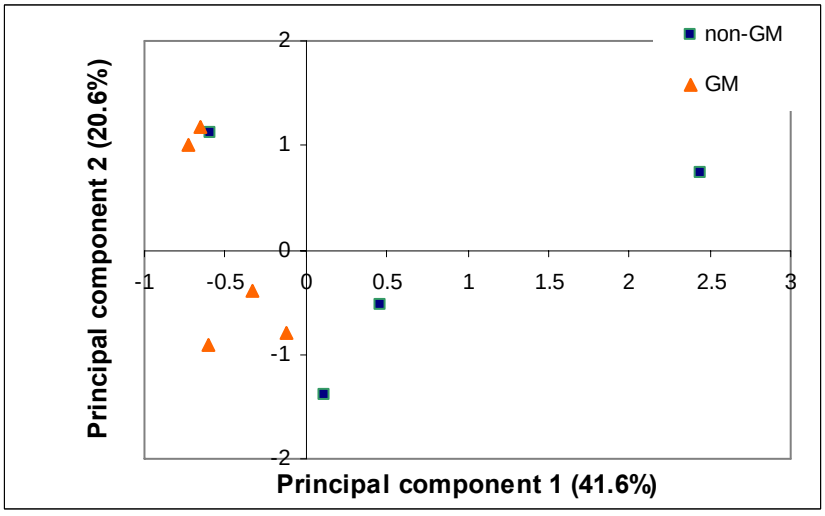


Figure 14.3.3.5 Comparison of non-GM, GM and non-rhizosphere soil at the 7 week harvest from V15 and V15i plants grown in Narrabri soil

The profiles of the same plant cultivar (V15/V15i) harvested at eight weeks from the Narrabri soil (Figure 14.3.3.6) were distinctly different between the non-plant soil and the plant-associated soils. The factor loadings were more similar within the Narrabri soil than occurred with the Avon soil, for this time plant type and sime of harvest.

However the non-GM and GM rhizosphere soil fatty acids did not polarise to indicate differences between the two.

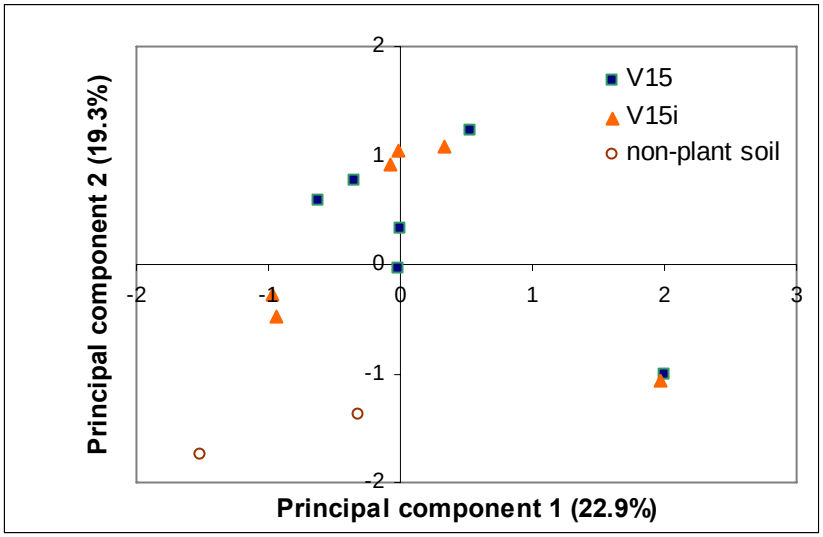


Figure Appendix 14.3.3.6 **Comparison of non-GM, GM and non-rhizosphere soil at the 8 week harvest from V15 and V15i plants grown in Avon soil.**

The fatty acids from the rhizosphere of the same plant strains in the Narrabri soil at 16.7 weeks, shown in Figure 14.3.3.7, showed more similarity between the replicates than the 8 week harvest, but both the non-GM and GM plants showed significant differences between some individual replicates. At this point the plant was pot-bound, but receiving adequate nutrient supplement.

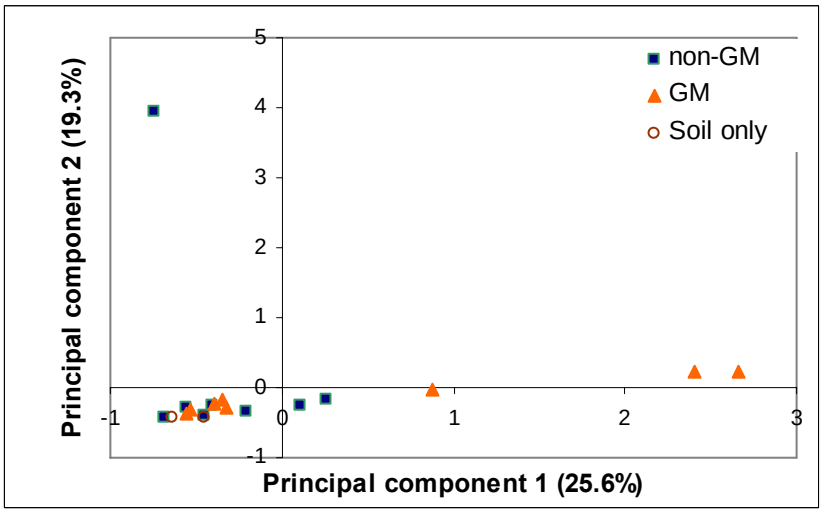


Figure Appendix 14.3.3.7 **Comparison of non-GM, GM and non-rhizosphere soil at the 16.7 week harvest from V15 and V15i plants grown in Narrabri soil, grown with adequate nutrient**

A trial was also conducted under nutrient deficient conditions for Narrabri soil, as was done for Avon, using the same plant strains. The plants were harvested at 14.8 weeks and the principal component analysis is shown in Figure 14.3.3.8 below.

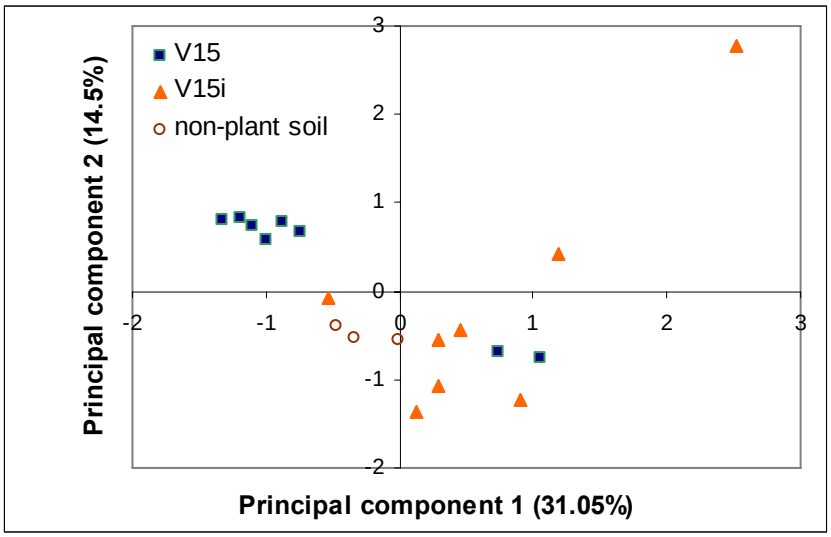


Figure Appendix 14.3.3.8 **Comparison of non-GM, GM and non-rhizosphere soil at the 14.8 week harvest from V15 and V15i plants grown in Narrabri soil nutrient deficient.**

Three of the four replicates for the non-GM plant fatty acids were similar but one replicate was different. Each of the fatty acids taken from separate 10 gram samples from the same pot do not necessarily align closely with each other, however the soil-only samples was distinct from the rhizosphere soils.

An analysis for the fatty acids from Narrabri rhizosphere soil, for the plants V2/V2i (and soil-only control), for a harvest age of 14.8 weeks, is shown in Figure 14.3.3.9.

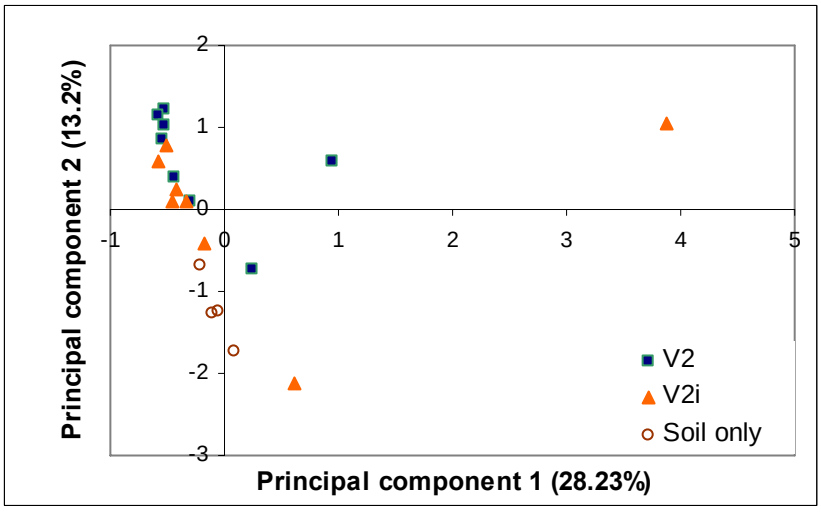


Figure Appendix 14.3.3.9 **Comparison of non-GM, GM and non-rhizosphere soil at the 14.8 week harvest from V2, V2i and soil-only controls for Narrabri soil.**

The fatty acids from the non-rhizosphere soils were different from the rhizosphere soils, but the overall combination of components for each of the non-GM and GM rhizosphere soils were mainly similar, but with occasional distinctly different single replicates.

A comparison of the rhizosphere soil from a different paired plant type, 189 and 289i grown within Narrabri soil was harvested at 12 weeks (Figure 14.3.3.10).

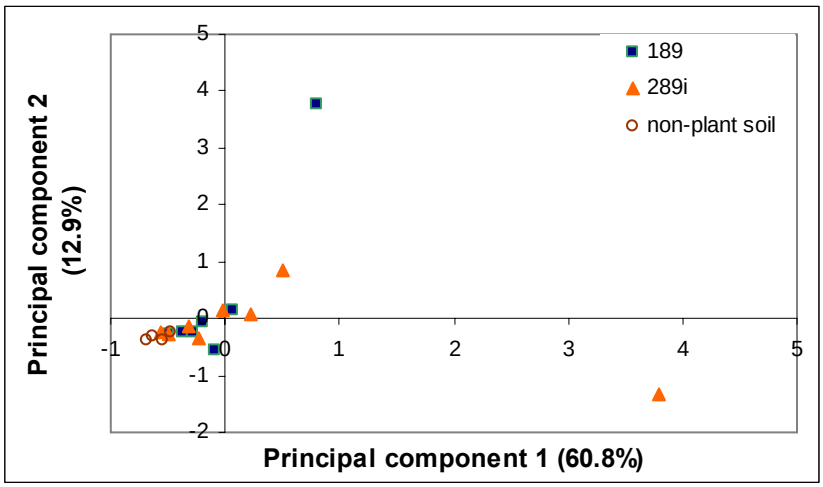


Figure Appendix 14.3.3.10 **Comparison of non-GM, GM and non-rhizosphere soil, at the 12 week harvest from 189, 289i and non-plant soils for Narrabri soil.**

At 12 weeks the plant had stopped growing, and was potbound. The profile showed a high degree of similarity between the rhizosphere soils of both the non-GM and GM plants, and both had become more similar to the non-rhizosphere soil. This 'control' soil, however, was showing different characteristics from the previous rhizosphere soil as the first principal component of the non-rhizosphere values remained the most negative of all samples. Even at this time, however, there were still a few individual exceptions in the microbial community, and this demonstrates that 'pockets' of microflora or microfauna may exist as distinct entities.

Comparison of organic substances between the paired plants and non-rhizosphere soil

The extracted lipids from the living cells of rhizosphere soils of paired V15 and V15i plants grown in Avon soil were dried and re-suspended in 1 ml chloroform to compare the difference between the organic substances within the rhizosphere soils. A multi-wavelength spectrophotometric scan determined that the maximum absorbance for the mix was 450nm. The concentration of the pigmented organic substances of the non-GM and GM-plant rhizosphere soils by measurement of optical density was equivalent. A repeated test for the same plants grown in Narrabri soil showed colour intensity similar to those of Avon soils. The pigmented substances were shown to be polar in nature for both non-GM and GM plant rhizosphere soils, as decolourisation occurred after elution of the solution through columns of packed, dried silicic acid by acetone. The photograph in Figure 14.3.3.1 shows the comparison of the non-GM, GM and non-rhizosphere soils.

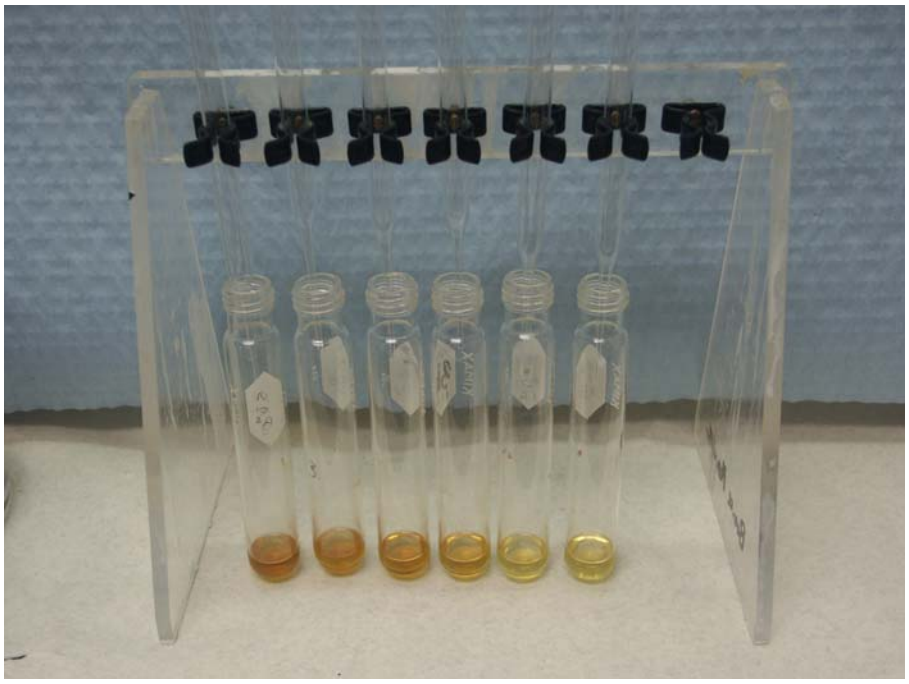


Figure 8.3.3.1 Comparison of pigmentation from plant organic matter and soil microbiota surrounding the roots of V15/V15i plants grown in Avon soil harvested at 9 weeks, dried and suspended in 1 ml chloroform.
Two tubes on the left are non-GM rhizosphere soil, middle two are GM rhizosphere soil, and the two on the right are non-rhizosphere soil.

APPENDIX 14.4

Overview of literature references for signature fatty acid peaks, or significantly altered ratios.

Structure	Signature, or significant increase in fatty acid	Reference & comment
12:0	eukaryotes	Cavigelli *et al* 1995.
*i*14:0	gram +ve bacteria	Baath *et al* 1992
14:0	universal	Cavigelli *et al* 1995
14:03 OH	gram –ve eubacteria	Cavigelli *et al* 1995.
15:0	eubacteria	Vestal & White, 1989
a15:0	gram +ve bacteria	Microbiology Aust. 2002 Journal 23(5): p. 7 catabolic product of isoleucine. Required by L. monocytogenes to survive and grow at low temperature, Ibekwe 1998. Also, high proportions associated with Arthrobacter (Haack, 1994).
*i*15:0	eubacteria	Vestal & White 1989
*i*17:0	gram +ve bacteria	Baath et al 1992
*a*17:0	gram +ve bacteria	Baath et al 1992 Ibekwe, 1998 high proportions associated with Arthrobacter (Haack, 1994).
*i*15:0	gram +ve bacteria	Baath *et al* 1992. Original ref. O'Leary, 1988 Ibekwe & Kennedy, 1998
a15:09	gram +ve bacteria	Ibekwe & Kennedy, 1998 original ref Ratledge, C & Wilkinson, S.G 1988.
cy15:1	clostridia	Vestal & White 1989
15:1 iso 3	eubacteria	Cavigelli *et al* 1995
15:1 iso 7	Eubacteria (2) gram +ve	Cavigelli *et al* 1995 (2) Ibekwe & Kennedy, 1998
15:1 at 8	eubacteria	Cavigelli *et al* 1995 original ref Ratledge, C & Wilkinson, S.G 1988.
15:1 at 6	eubacteria	Cavigelli *et al* 1995
10Me16:0	gram +ve bacteria (2) sulphate-reducing bacteria Desulfobacter spp. (3) eubacteria	Baath, *et al* 1992 Original ref. O'Leary, 1988 (2) Rajendran et al, 1992. (3) Cavigelli *et al* 1995
16:0 Palmitic acid	(1) Plant root (2) universal (3) eubacteria (4) fungus	(1) Wellburn *et al* 1994. (2) Universal fatty acid B. Hawke, pers. comm. (3) Cavigelli *et al* 1995 Also the precursor of membrane lipids, fats and waxes (Lehninger et al, 1993, p. 71). Nematodes. Chen et al. 2001 (4) Vestal & White, 1989
16 branched and straight	Gram +ve (2) eubacteria	Baath, *et al* 1992 (2) Cavigelli *et al* 1995
16: l in 7c Palmitoleic Acid	Plant root	(1) Wellburn *et al* 1994. Original ref. O'Leary, 1988
16:1ω5c	eubacteria (2) AM-fungi	Vestal & White 1989 (2) Olsson, 1999 Marker acid, under controlled conditions. Madan, *et al* 2001. Can also be background fatty acid for other microorganisms, eg bacteria, but may occur in some other fungi

	(3) eubacteria	Olsson, 1999 (3) Cavigelli *et al* 1995
16:1ω7	eukaryotes aerobes	Cavigelli *et al* 1995. Vestal & White 1989
16:1 ω 7t	eukaryotes, aerobes	Vestal & White 1989
16:1ω9	eubacteria	Vestal & White 1989
cy17:0	gram –ve bacteria (3) eubacteria (4) universal	(1) Baath *et al* 1992 Original ref. Wilkinson, 1988. (2) Ibekwe & Kennedy, 1998. Original ref. Wilkinson 1988 (3) Cavigelli *et al* 1995 (4) B. Hawke, pers. comm.
16:0 branched 10	eubacteria	Cavigelli *et al* 1995
10Me16:0	sulfate reducing bacteria (2) eukaryotes	Vestal & White 1989 (2) Cavigelli *et al* 1995.
16:1ω3t	diatoms green algae	Vestal & White, 1989
16:3ω6	microalgae	Vestal & White 1989
17:0 branched and straight Palmitoleic acid	eubacteria	Cavigelli *et al* 1995
cy17:0	anaerobes	Vestal & White 1989
10Me16:0	desulfobacter spp.	Rajendran *et al* 1992
10Me18:0	actinomycetes	Baath, *et al* 1992; Kroppenstedt 1985).
*i*17ω7	sulfate-reducing bacteria	Vestal & White 1989
*i*17	gram +ve bacteria	Ibekwe & Kennedy, 1998 Vestal & White 1989
17:1 ω6	eubacteria (2) sulfate reducing bacteria	Cavigelli *et al* 1995 (2) Vestal & White 1989
17:1 at 8	eubacteria	Cavigelli *et al* 1995
18:1 cis 11	eubacteria (2) strictly anaerobic bacteria, and gram –ve aerobes	Cavigelli *et al* 1995 (2) Zelles et al. 1991.
18:0 Stearate	universal	Cavigelli *et al* 1995 Nematodes. Chen et al. 2001.
18:0 me16	desulfobacter spp.	Rajendran *et al* 1992
18:1ω5	eubacteria	Vestal & White 1989
18:1ω7c *cis*Vaccenic Acid	gram –ve bacteria aerobic bacteria	Baath, *et al* 1992 Original ref. Wilkinson, 1998 Also a major component of AM fungi. Olsson, 1999 Madan, et al 2001 Nematodes. Chen et al. 2001. Vestal & White 1989
18:1ω9 Oleic acid	eukaryotes (2) higher plants (3) gram +ve bacteria (4) Nematodes (5) Fungi (6) green algae (7) AM-fungi marker acid under controlled conditions	Cavigelli *et al* 1995 (2) Vestal & White 1989; Lehninger et al, 1993 (3) Baath, *et al* 1992 Original ref. O'Leary, 1988 (4) Chen et al. 2001. (5) Vestal & White 1989 (6) Vestal & White 1989 (7) Madan, *et al* 2001

18:1ω11	higher plants (2) 18:1 cis 11	Vestal & White 1989 (2) major unsaturated acid in many bacterial species.
18:ln7c *cis*Vaccenic Acid		
18:2 cis 9,12 Linoleic acid	Most plant and animal tissues	The structure, Chemistry and Occurrence of Lipids
18:2	nematodes	Chen et al. 2001.
18:2ω6,9	ergosterol, basidiomycetes, ascomycetes and deuteromcetes.	a fungal-specific sterol, Olsson, 1999 Ibekwe, 1998 Also present in varying amounts of most other eukaryotes, Vestal & White, 1989 Olsson, 1999
18:2 ω cis 9, 12	Eukaryotes (2) ergosterol, basidiomycetes, ascomycetes and deuteromcetes. Also Rhizoctonia solani, fusarium oxysporum and trichoderma sp. As the major component (3) Also present in varying amounts of most other eukaryotes.	Cavigelli *et al* 1995 Plants, Lehninger et al, 1993. (2) a fungal-specific sterol Olsson, 1999. (3) Chen et al. 2001
18:3ω6,c	fungi	Ibekwe & Kennedy, 1998 Vestal & White, 1989
a 18:3 cis 9, cis 12, cis 15	Eukaryotes (2) major component of plant lipids	Cavigelli *et al* 1995 (2) The Structure, Chemistry and Occurrence of Lipids
18:3ω3	fungi green algae higher plants	Vestal & White, 1989
a 18:3 cis 9, cis 12, cis 15	eukaryotes (2) Plants	Cavigelli *et al* 1995 (2) Lehninger et al. 1993
y –18:3	microfauna	Cavigelli *et al* 1995
*i*19	eubacteria	Vestal & White 1989
cy19:0	(1) gram –ve bacteria (2) eubacteria (3) anaerobes	(1) Ibekwe & Kennedy 1998l. Original ref. Wilkinson 1988; (2) Cavigelli, 1995. (3) Vestal & White 1989
20:1	Nematodes	Chen et al. 2001.
20:2	Nematodes	Chen et al. 2001
20:3	AM-fungi	Madan, 2001
20:3ω6	protozoa	Cavigelli *et al* 1995 Vestal & White, 1989
20:4	Nematodes	Chen et al. 2001. AM-fungi; Madan, 2001
20:5	barophilic, psychrophilic bacteria	Vestal & White, 1989
20:5ω3	diatoms	Vestal & White, 1989
20:5ω5	Diatoms; higher plants	Vestal & White, 1989
20:4 at 6	protozoa	Cavigelli *et al* 1995
C20	algae and protozoa Rare in non-AM fungi. Not present in significant amounts in bacteria Eukaryotes (2) nematodes	Lechevalier & Lechevalier 1988. (2) Chen et al. 2001.
> C20 polyunsaturated	eukaryotes	Cavigelli *et al* 1995
22:6	barophilic, psychrophilic bacteria	Vestal & White, 1989

20:3 at 6	protozoa	Cavigelli *et al* 1995
30:4 at 6	protozoa	Cavigelli *et al* 1995
26	higher plants	Vestal & White, 1989

β-hydroxy, cyclopropane, and branched chain fatty acids,	bacteria	O'Leary, W.M., 1989. Also in The Structure, Chemistry and Occurrence of Lipids.
Low ratio of polyunsaturated fatty acids or molecules with acetylenic linkages or sterols	eubacteria	(Cavigelli *et al* 1995) O'Leary, W.M. 1989
Branched-chain fatty acids	Gram-positive bacteria	(Haack *et al* 1994) The Structure, Chemistry and Occurrence ol Lipids. (O'Leary and Wilkinson 1989; Kaneda 1991).

Additional Notes:

BACTERIA

B. thuringiensis also contain substantial amounts of unsaturated fatty acids. Gram positive bacteria characteristically contain odd-chain methyl-branched (eg iso- and anteiso-branched fatty acids (O'Leary and Wilkinson 1988; Kaneda 1991).

Even-number, straight chain and cyclopropyl fatty acids are from **gram-negative** bacteria; gram-negative bacteria lipids contain abundant hydroxylated fatty acids (Zelles et al. 1992).

Usually the straight-chain acids have the least taxonomic potential in terms of bacteria, because their production is ubiquitous (Zelles et al. 1992).

The biosynthesis of the critical component anteiso-C15:0 occurs from a branched chain CoA molecule provided by the catabolism (deamination and decarboxylation) of isoleucine.

Halophilic bacteria contain high proportions of ether lipids (Lehninger et al. p. 249). This could be a test for salinity in soils. -> Research proposal.

Cyanobacteria (and also eukaryotes) lipids contain polyunsaturated fatty acids

Cyclopropane fatty acids are found in bacterial lipds, particularly those of a number of gram negative and a few gram-positive families of the order eubacteriales. The Structure, Chemistry and Occurrence `of Lipids.

STARVATION

"An increased ratio of trans to cis isomers of the fatty acids 16:1w7 and 18:1w7 has, for several bacteria, been shown to indicate increased environmental stress, especially due to nutrient starvation (Guckert et al 1991)

"Changes that are typicaly found in PLFA profiles when gram-negative bacteria are starved include an increase in the ratio of saturated to unsaturated fatty acids (Guckert, J.B., Hood, M.A. & White, D.C. 1986. 'Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vicrio cholerae:* increases in the *trans-/cis* ratio and proportions of cyclopropyl fatty acids. *Appl. Environ. Microbiol.* **52:**794-801.

PROTOZOA

The membranes of ciliated protists and of certain inverterbrates contain high proportions of ether lipids (Lehninger et al. p. 249). The ether-linked chain may be saturated, as in the alkyl ether lipids, or may contain a double bond between C1 and C2, as in plasmalogens

NEMATODES

A. avenae and *A. composticola* included 16:0, 18:0; 18:1omega7, 18:1omega9, 18:2, 20:0, 20:1, 20:2, 20:3 and 20:4. Chen.et al. 2001.

PLANTS

The major plant fatty acids are 12:0, 14:0, 16:0, 18:0, 18:1(9 cis); 18:2 (9cis, 12cis); and 18:3 (9cis, 12cis and 15cis) Hitchcock, C. 1975. Structure and distribution of plant acyl lipids, in: T. Galliard, Q.I. Mercer (Eds.), Recent Advances in the Chemistry and Biochemistry of Plant Lipids. Academic Press, London. 1975, pp. 1-19. (So it is impossible to use them as plant biomass.)

The metabolic pathway of chain elongation:
C16:0 – palmitate; elongation to Stearate C18:0; desaturation to Oleate 18:1 delta 9; desaturation in plants only Linoleate 18:2(delta 9,12); desaturation in plants only alpha-Linolenate 18:3(delta 9,12,15); other polyunsaturated fatty acids. (Lehninger et al. p. 653.)

TECHNIQUE

Concentration to complete dryness should be avoided because of the volatility of short-chain fatty acid methyl esters (Wollenweber & Rietschel, 1990)

REFERENCES

REFERENCES

Adamczyk, J.J., Hardee, D.D., Adams, L.C. & Sumerford, D.V. 2001,(a), 'Correlating differences in larval survival and development of bollworms (*Lepidoptera: noctuidae*) and fall armyworms (*Lepidoptera: noctuidae*) to differential expression of Cry1A(c) δ-endotoxin in various plant parts among commercial cultivars of transgenic *Bacillus thuringiensis* cotton', *Journal of Economic Entomology* vol. 94, pp. 284-290.

Adamczyk, J.J. & Sumerford, D.V. 2001(b), 'Potential factors impacting season-long expression of Cry1Ac in 13 commercial varieties of Bollgard® cotton', *Journal of Insect Science*, vol. 1, Available online: http://insectscience.org/1.13.

Adang, M.J., Staver, M.J., Rocheleau, T.A., Leighton, J., Barker, R.F. & Thompson, D.V. 1985, 'Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. kurstaki HD-73 and their toxicity to *Manduca sexta*', *Gene,* vol. 36, pp. 289-300.

Addison, S. 2003, Report presented at the Cotton Consultants Australia 'Cotton Production Seminar 2003'

Alef, K. & Nannipieri, P. (Eds) 1995, *Methods in Applied Soil Microbiology and Biochemistry*, Academic Press Ltd., London.

Agnet 2000, 'All the difference in the world', *The Toronto Star*, editorial. Available: http://www.gefoodalert.org/News/news.cfm?News_ID=3187 Accessed 22/02/02.

Allen, E.B. & Allen, M.F. 1980, 'Natural re-establishment of vesicular-arbuscular mycorrhizae following stripmine reclamation in Wyoming', *Journal of Applied Ecology,* vol. 17, pp. 139-147.

Allen, S.J. 2000, 'Integrated disease management. How are we doing?' *Proceedings of the 10th Australian Cotton Conference, Queensland, Aust. 2000.* Australian Cotton Cooperative Research Centre, Narrabri, NSW

Allen, S.J. 2003 'Yield best indicator of plant and soil health', Video Interview, available http://www.csd.net.au/news.asp?news=1053

Altieri, M.A. 1999. 'The ecological role of biodiversity in agroecosystems' *Agriculture, Ecosystems and Environment* vol. 74, pp.

Altieri, M. 2000, Executive Summary, *Proceeedings of the International workshop on the Ecological Impacts of Transgenic Crops*, held March 2-4, CGIAR-NGO Committee, p.7.

Amato, M. & Ladd, J.N. 1988, 'Assay for microbial biomass based on ninhydrin-reactive nitrogen in extracts of fumigated soils', *Soil Biol. Biochem.* vol. 20, pp. 107-114.

American Academy of Microbiology, 2002, '100 years of *Bacillus thuringiensis:* a critical scientific assessment' Report based on a colloquium sponsored by the American Academy of Microbiology held in Ithaca, New York. Available: online at www.asmusa.org. Accessed November 2002.

Andrassy, I. 1992, 'A short census of free-living nematodes', *Fundamental and Applied Nematology* vol. 15, pp. 187-188.

Aslim, B., Sağlam, N. & Beyatu, Y. 2002, 'Determination of some properties of *Bacillus* isolated from soil', *Turkish Journal of Biology* vol. 26, pp. 41.

Atlas, R.M. 1993, *Handbook of Microbiological Media* ed. L.C. Parkes, CRC Press, Boca Raton, USA.

Auchincloss, A. 2004, Swiss-Prot Annotator, personal communication received by email 28/05/04.

Aust. Bureau of Agricultural Research Economics 2003, available: http:// abareconomics.com/australiancommodities/ commods/cotton.html

Aust. Cotton CRC., 2004, 'Nutripak', available online at www.cotton.crc.org.au/ Publicat/Agro/Nutrient/NUTRIpak.htm

Australian Cotton Seed Distributors (2004) 'Variety Trial Results', available www.csd.net.au.

Aust. Bureau of Statistics, *Year Book Australia 2003*, Ch. 16, 'Agriculture' p. 515.

Australian/New Zealand Standards Document AS/NZ 4360:1999, Appendix E; Risk Analysis Matrix.

Ayers, W.A. & Thornton, R.H. 1968, 'Exudation of amino acids by intact and damaged roots of wheat and peas' *Plant and soil*, vol. 28, pp. 193-206.

Azcón, R., Rubio, R. & Barea, J.M. 1991, 'Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N_2 fixation (^{13}N) and nutrition of *Medicago sativa*', *New Phytologist*. vol. 117, pp. 399-404.

Bååth, E., Frostegard, A. & Fritze, H. 1992, 'Soil bacterial biomass, activity, phospholipid fatty acid pattern, and pH tolerance in an area polluted with alkaline dust deposition.' *Applied & Environmental Microbiology*, vol. 58, pp. 4026-4031

Bacic, A., Moody, S.F. & Clarke, A.E. 1986, 'Structural analysis of secreted root slime from maize (*Zea mays*)', *Plant Physiology,* vol. 80, pp. 771-777.

Badalucco, L. & Hopkins, D.W., 1997, 'Available carbon in soil determined from substrate utilization kinetics: comparison of substrates and soil amendments', *Journal of Microbiological Methods*, vol. 30, pp. 43-47.

Bamforth, S.S. 1984, 'Microbial distributions in Arizona deserts and woodlands', *Soil Biology and Biochemistry*, vol. 16, pp. 133-137

Bange, M.P. & Milroy, S.P. 2000, 'Timing of crop maturity in cotton: Impact of dry matter production and partitioning', *Field Crops Research* vol. 68, pp. 143-155.

Bardgett, R.D., Hobbs, P.J., & Frostegård, A. 1996, 'Changes in soil fungal-bacterial biomass ratios following reductions in the intensity of management of an upland grassland', *Biology and Fertility of Soil* vol. 22, pp. 261-264.

Becard, G. & Piche, Y. 1989 'Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis', *Applied & Environmental Microbiology,* vol. 55, pp. 2320-2325.

Begon, B., Harper, J.L. & Townsend, C.R. 1990, *Ecology. Individuals, populations and communities.* 2nd edn, Blackwell Scientific Publications, Melbourne.

Belser, L.W. & Schmidt, E.L. 1978, 'Diversity in the ammonia-oxidising populations of a soil' *Applied Environmental Microbiology*, vol. 36, pp. 489-493

Berg & Rosswall, 1985, 'Ammonium oxidizer numbers, potential and actual oxidation rates in two Swedish arable soils.' *Biological Fertility Soils* vol. 1, pp. 131-140, in *Methods in Applied Soil Microbiology and Biochemistry*, eds. K. Alef & P. Nannipieri, p. 241. Academic Press, London.

Bergey's Manual of Systematic Bacteriology 2nd Ed. 2001

Besawada, C., Saha, S., Jenkins, N., Creech, R. & McCarty, J. 2003, *The Journal of Cotton Science*, vol. 7, pp. 179-184

Bird, A.F., 1999, 'A comparison of some soil microinvertebrate assemblages in Southern Australia', *Transactions of the Royal Society of S. Aust*, vol. 123, pp 69-75.

Bishop, D.G. & Stumpf, P.K. 1971, 'Fatty acid metabolism', in *Biochemistry and methodology of lipids*', p. 385, eds. A.R. Johnson & J.B. Davenport, Wiley-Interscience.

Blakemore, L.C., Searle, P.L. & Daly, B.K. 1987, '1: moisture factor', in *Methods for chemical analysis of soils*, N.Z. Soil Bureau Scientific Report 80, p. 7.

Bodman, B., Bauer, W.D. & Coplin, D.L. 2003, 'Quorum sensing in plant-pathogenic bacteria', *Annual Revew of Phytophatology*, vol. 41, pp. 455-482.

Bohlool, B.B., Ladha, J.K. Garrity, D.P. & George, T. 1992, 'Biological nitrogen fixation for sustainable agriculture: a perspective', *Plant Soil.* vol. 141, pp. 1-11.

Boquet, D.J., Breitenbeck, G.A., Coco, A.B. & Aguillard, W., 1991, 'Fertilizer nitrogen rates to optimize cotton yield and fiber quality', *Louisiana Agriculture*, vol. 35, pp. 10-11.

Bowen, G.D. & Rovira, A.D. 1999, 'The rhizosphere and its management to improve plant growth' *Advances in Agronomy*, vol 66. CSIRO Land & Water publication, Academic Press, pp 2-102.

Brady, J.E. & Holum, J.R. 1988, *Fundamentals of Chemistry*, 3rd edn, John Wiley & Sons, Brisbane.

Brady, N.C. & Weil, R.R., 1999, *The Nature and Properties of Soils.* 12th edn, Prentice Hall of Aust. Pty Ltd, Sydney.

Brock, T.D. & Maddigan, M.T. 1991, *Biology of Microorganisms*, 6th edn, Prentice Hall, New Jersey.

Brundett, M.C., Piche, Y. & Petersen, R.L. 1985, 'Developmental study of early stages of vesicular-arbuscular mycorrhizae formation', *Canadian Journal of Botany*, vol. 63, pp. 184-194.

Burford, E.P., Fomina, M. & Gadd G.M. 2003, 'Fungal involvement in bioweathering and biotransformation of rocks and minerals' *Mineralogical Magazine*, vol. 67, pp. 1127-1155.

Buyer, J.S. & Drinkwater, L.E. 1997, 'Comparison of substrate utilization assay and fatty acid analysis of soil microbial communities', *Journal of Microbiological Methods* vol. 30, pp. 3-11.

Campbell, N.A. 1990, *Biology*, 2nd edn, The Benjamin/Cummings Publishing Co. Inc.

Campbell, R. 1985, *Plant Microbiology* Edward Arnold (Australia) Pty Ltd, Caulfield East, Vic. Aust.

Cartwright, N. 1983, *How the Laws of Nature Lie*, Oxford University Press.

Casida, L.E. 1989, 'Protozoan response to the addition of bacterial predators and other bacteria in soil' *Applied and Environmental Microbiology*, vol. 55, pp. 1857-1859.

Casarett, L.J. & Doull, J. 1975, *Ecotoxicology: The Basic Science of Poisons*, 6th Edn, Ed. C.D. Klaassen, McGraw-Hill, New York.

Cato-Smith, A. 1996, 'Gut flora and mucosal function', *Asia Pacific Journal of Clinical Nutrition* , vol. 5, pp. 36-38.

Cavigelli, M.A., Robertson, G.P. & Klug, M.J. 1995 'Fatty acid methyl ester (FAME) Profiles as measures of soil microbial community structure', *Plant and Soil*, vol. 170, pp. 99-113.

Chen, D.L., Freney, J.R., Mosier, A.R. & Chalk, P.M., 1994 'Reducing denitrification loss with nitrification inhibitors following presowing applications of urea to a cotton field', *Australian. Journal of. Experimental Agriculture.* vol. 34, pp. 75-83.

Chen, D.L, Ye., G., Yang, C., Chen, Y. & Wu, Y. 2004, 'Effect after introducing *Bacillus thuringiensis* gene on nitrogen metabolism in cotton', *Field Crops Research*, vol. 87, pp. 235-244. Available www.elsevier.com/locate/fcr.

Chen, J., Abawi, G.S. & Zuckerman, B.M. 2000, 'Efficacy of *Bacillus thuringiensis, Paecilomyces marquandii,*and *Streptomyces costaricanus* with and without organic amendments against *Meloidogyne hapla* Infecting Lettuce', *Journal of Nematology*, vol. 32, pp. 70-77.

Chen, J., Ferris, H., Scow, K.M. & Graham, K.J., 2001, 'Fatty acid composition and dynamics of selected fungal-feeding nematodes and fungi', Comparative Biochemistry & Physiology Part B: *Biochemistry & Molecular Biology*, vol. 130, pp. 135-144.

Chen J, Anderson J.B., DeWeese-Scott C., Fedorova N.D., Geer L.Y., He S., Hurwitz D.I., Jackson J.D., Jacobs A.R., Lanczycki C.J., Liebert C.A., Liu C., Madej T., Marchler-Bauer A., Marchler G.H., Mazumder R., Nikolskaya A.N., Rao B.S., Panchenko A.R., Shoemaker B.A., Simonyan V., Song J.S., Thiessen P.A., Vasudevan S., Wang Y., Yamashita R.A., Yin J.J., Bryant S.H. 2003, 'MMDB: Entrez's 3D-structure database', *Nucleic Acids Res*., vol. 31, pp. 474-477.

Chen, X.J. 1995, 'Mutations in Domain I of *Bacillus thuringiensis* δ-endotoxin CryIA(b) reduce the irreversible binding of toxin to *Manduca sexta* brush border membrane vesicles', *The Journal of Biological Chemistry*, vol. 270, pp. 6412-6419.

Christensen, M. 1989, 'A view of fungal ecology', *Mycologia*, vol. 81, pp. 1-19**.**

Christie, W.W, 1989, *Gas Chromatography and Lipids*, The Oily Press, 1989.

Christie, W.W. 2005, 'Fatty Acids: Natural Alicyclic', *The Lipid Library*, available online at www.lipidlibrary.co.uk

Churchman, G.J., 2002, 'The role of clays in the restoration of perturbed ecosystems', *Developments in Soil Science*, vol. 28A, pp. 333-350

Clarholm, M. 1981, 'Protozoan grazing of bacteria in soil – impact and importance', *Microbial Ecology*, Vol. 7, pp. 343-350.

Clark, F.E. & Paul, E.A. 1970. 'The microflora of grassland', *Advances in Agronomy*, vol. 22, pp. 373-435.

Clarke, P.H., 1984, 'The evolution of degradative pathways' in *Microbial Degradation of Organic Compounds,* ed. D.T. Gibson, Marcel Dekker, Inc. New York, pp. 11-27.

Cohn, E., Koltai, H., Sharon, E. & Spiegel, Y. 1996, 'Root-Nematode Interactions: Recognition and Pathogenicity', in *Plant Roots The Hidden Half*, 2nd edn, York, p. 783.

Conway, G. & Tonniessen, G. 1999, 'Feeding the world in the twenty-first century' *Nature*, vol. 402, pp. C55-C58.

Colyer, P.D., Kirkpatrick, T.L., Caldwell, W.D. & Vernon, P.R. 2000, 'Root-knot nematode reproduction and root galling severity on related conventional and transgenic cotton cultivars', *Journal of Cotton Science*, vol. 4, pp. 232-236.

Colyer, P. 2002, 'Meeting the Fusarium challenge', *The Australian Cottongrower* vol. 23, No. 3. Available www.greenmountpress.com.au/cottongrower/issues/233mjcot02/233fusarium.htm

Cooperative Research Centre for Soil & Land Management (CRCSLM) 1999, *The costs of soil acidity, sodicity and salinity for Australia: Preliminary estimate,* Co-operative Research Centre for Soil & land Management Report CRCSLM/CTT2/6/99 (ISBN: 1 876 162 02 3).

Corbett, J.R., Wright K & Baillie, A.C. 1984, 'Pesticides affecting biosynthesis' in: *The Biochemical Mode of Action of Pesticides*, 2nd edn, Academic Press, London.

Cotton CRC, 2004, available http://www.cotton.crc.org.au/Assets/PDFFiles/NUTRIpak/03N.pdf

Cotton Research and Development Corporation (CRDC) Annual Operating Plan 2004–2005, Australian Government Publication, Narrabri, NSW. (available http://www.crdc. com.au/ Resources/ CRDC%20AOP%2004-05.pdf).

Cotton Seed Distributors, 2002, Disease Survey Results 2003/04 by region. Available www.csd.net.au

Cotton Seed Distributors, 2004, *Variety Guide 2004*, available www.csd.net.au

Couteaux, M-M. & Darbyshire, J.F., 1997, 'Functional diversity amongst soil protozoa', *Journal of Applied soil ecology*, vol. 10(1998), pp. 229-237.

Cowling, A.J. 1994, 'Distribution and Adaptation' in *Soil Protozoa*, ed. J.F. Darbyshire, CAB International, UK.

Cowling, E.B. 1963, 'Structural features of cellulose that influence its susceptibility to enzymatic hydrolysis', in *Advances in enzymic hydrolysis of cellulose and related materials*, Ed. E.T. Reese, pp. 1-32, Pergamon Press, Oxford, London.

CRC Handbook of Chemistry and Physics 1968, (a) 48th edn, Chemical & Rubber Co. Ed. in Chief Robert C. Weast; co-ord. editor George W. Tuve.

Ibid, (b) *1978-79*, 59th Edition.

Crecchio, C. & Stotzky, G. 2001, 'Biodegradation and insecticidal activity of the toxin from *Bacillus thuringiensis* subsp. kurstaki bound on complexes of montmorillonite-humic acids-Al hydroxy-polymers. *Soil Biology & Biochemistry*, vol. 33, pp.573-581.

Cunningham, 1981, 'Isolation and culture', in *Biology and Control of Take-all*. Eds. M.J.C. Asher & P.J. Shipton. pp. 103-123. Academic Press, New York.

Curl, E.A. & Truelove, B. 1986, *The Rhizosphere*, SpringerVerlag, Berlin.

Çakmak, I. & Marschner, H. 1988, 'Zinc-dependent changes in ESR signals, NADPH oxidase and plasma membrane permeability in cotton roots', *Physiologia Plantarum*, vol. 73, pp. 182-186.

Dale, P.J., Clarke, B. & Fontes, E.M.G. 2002, 'Potential for environmental impact of transgenic crops', *Nature Biotechnology*, vol. 20, pp. 567-574.

Darbyshire, J.F., Wheatley, R.E., Greaves, M.P. & Inkson, R.H.E. 1974, 'A rapid method for estimating bacterial and protozoan populations in soil', *Revue d'Ecologie et de Biologie du Sol* vol. 11, pp. 465-475.

de Vries, M.C.P. 1980, 'How reliable are results of pot experiments?' *Communications in soil science and plant analysis*, vol. 11, pp. 895-902.

Devare, M.H., Jones, C.M. & Thies, J.E. 2004, 'Effect of Cry3Bb transgenic corn and tefluthrin on the soil microbial community: biomass, activity, and diversity', *Journal of Environmental Quality*, vol. 33, pp. 837 – 843.

DeRisi, J.L., Iyer, V. & Brown, P.O. 1997, 'Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale', *Science*, vol. 278, pp 680-686, available www.sciencemag.org.

Dickson, S. 2004, 'The Arum-Paris continuum of mycorrhizal symbioses', *New Phytologist,* vol. 163, pp. 187-200.

Dien, B.S., Bothast, R.J., Iten L.B., Barrios, L. & Eckhoff, S.R. 2002, 'Fate of *Bt* protein and influences of corn hydrid on ethanol production' *Cereal Chemistry*, vol. 79, pp. 582-585.

Dilly, O. 2002, 'Regulation of the respiratory quotient of soil microbiota by availability of nutrients', *Fems Microbiology Ecology* vol. 43, pp. 375, 381.

Donegan, K.K., Palm, C.J., Fieland, V.J., Porteous, L.A., Ganio, L.M. Schaller, D.K., Bucao, L.Q. & Seidler, R.J. 1995, 'Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin', *Applied Soil Ecology*, vol. 2, pp. 111-124.

Donegan, K.K., & Seidler, R.J. 1999, 'Effects of transgenic plants on soil and plant microorganisms', In *Recent Research Development in Microbiology*, Ed. S.G. Pandalai. Volume 3 - 1999 Part II. Research, available http://agroeco.org/fatalharvest/articles/gmo&_soil_organisms.html

Dropkin, V.H. 1989, *Introduction to Plant Nematology*, 2nd edn, Wiley, Brisbane.

Doucett, M.E. 1988, 'Identification of nematodes in areas where Nematology is little developed', in *Nematode Identification and Expert System Technology*', Ed. R. Fortuner, NATO ASI Series, Series A: Life Sciences, vol. 2, p. 254.

Dunfield, K.E. & Germida, J.J. 2001, 'Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*', *FEMS Microbiology Ecology*, vol. 38, issue 1, pp. 1-9.

Dusenbery, D.B. 1987, 'Prospects for exploiting sensory stimuli in nematode control', in *Vistas on Nematology*, J.A. Veech & D.W. Dickson, Eds. Society of Nematologists, Hyattsville, Md., pp 131-135.

Dyer, S. 1998, Thesis entitled 'The role of colonisation of soil and wheat roots by *Trichoderma koningii* in biological control of *Gaeumannomyces graminis* var. tritici.' University of Adelaide, Australia.

Edwards et al., U.S. United States Patent & Trademark Office, 1988, Patent No 4,948,734 on 'influence of *Bt* on nematodes'.

Ellingsøe, P. & Johnsen, K. 2002, 'Influence of soil sample sizes on the assessment of bacterial community structure', *Soil Biology & Biochemistry*, vol. 34, pp. 1701-1707.

Edwards, C. A. & Heath, G.W.1963, 'The role of soil animals in breakdown of leaf material', in *Soil organisms*, Eds. J. Doekson & J., Van der drift, pp. 26-85, North Holland, Amsterdam, Netherlands.

Enquist B.J. & Niklas, K.J. 2002, 'Global allocation rules for patterns of biomass partitioning in seed plants' *Science*, vol. 295, pp. 1517-1520.

EPA Biopesticides Registration Action Document, 2001, '*Bt* Plant-incorporated Protectants October 15, 2001', available at http://www.epa.gov/pesticides/ biopesticides/pips/bt_brad.htm, p. IIA3, accessed 1/04/04.

EPA Report, 2000, 'The United States Environmental Protection Agency Report April 2000', EPA Publication No. 730-F-00-007), available: http://www.epa.gov/pesticides/biopesticides/factsheets/fs006445t.html

Estermann, E.F., Peterson, G.H. & McLaren, A.D. 1959, 'Digestion of clay-protein, lignin-protein, and silica-protein complexes by enzymes and bacteria', Soil Science Society Proceedings 1959. *Procedings of the Soil Science Society of America*, vol. 23, 31-36.

Ettema, C.H. & Wardle, D.A. 2002 'Spatial soil ecology', *Trends in Ecology & Evolution* vol. 17, pp. 177-183.

Federle, T. W., Livingston, R. J., Wolfe, L. E. & White, D.C. 1986, 'A quantitative comparison of microbial community structure of estuarine sediments for microcosms in the field', *Canadian Journal of Microbiology* vol. 32, pp 319-325.

Food and Agricultural Organisation of the United Nations, 2003, 'Report of the FAO Expert Consultation on Environmental Effects of Genetically Modified Crops 16-18 June 2003, Rome, Italy'. Available http://www.gmo-guidelines.info/

Feitelson, J. S., 1993, 'The *Bacillus thuringiensis* family tree', in *Advanced engineered pesticides*, ed. L. Kim, pp. 63-71. Marcel Dekker, Inc., New York, N.Y.

Feitelson, J.S., Payne, J. & Kim, L. 1992, '*Bacillus thuringiensis*: insects and beyond'. *Bio/Technology*, vol. 10, pp. 271-275.

Ferris, H. & Matute, M.M. 2003, 'Structural and functional succession in the nematode fauna of a soil food web', *Applied Soil Ecology*, vol. 23, pp. 93-110.

Ferris, H. 1982, 'The role of nematodes as primary consumers', in *Nematodes in Soil Ecosystems* Ed. D.W. Freckman, University of Texas Press, Austin, pp. 3-13.

Fitt, G.P., Mares, C.L. & Llewellyn, D.J. 1994, 'Field evaluation and potential ecological impact of transgenic cottons (*Gossypium hirsutum*) in Australia', *Biocontrol Science and Technology*, vol. 4, pp. 535-548.

Fitt, G.P. 1998, 'Efficacy of Ingard® cotton – patterns and consequences', in *The Ninth Australian Cotton Conference Proceedings*, pp. 233-235. Australia, The Cotton Research and Development Corporation.

Fitt, G.P. 2000, 'IPM with two-gene cotton' in Proceedings of the 10th Australian Cotton Conference Queensland, Aust.

Foissner, W. 1999, 'Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples', *Agriculture, Ecosystems and Environment*, vol. 74, pp. 95-112

Fredslund, L., Ekelund, F., Jacobsen, C.S & Johnsen, K. 2001, 'Development and Application of a Most-Probable-Number-PCR Assay To Quantify Flagellate Populations in Soil Samples', *Applied & Environmental Microbiology*, pp. 16313-1618

French, R.J. 1995, 'Multi-disciplinary teams to conduct integrated research on farming systems: a challenge', *Australian Journal of Soil Research,* vol. 33, pp. 659-671.

Friedman M. & Sigel, C.W. 1966, 'A kinetic study of the ninhydrin reaction', *Biochemistry* vol. 5, pp. 478-485.

Gallori, E., Bazzicalupo, M., Dal Canto, L., Fani, R., Nannipieri, P., Vettori, C. & Stotzky, G. 1994, 'Transformation of *Bacillus subtilis* by DNA bound on clay in non-sterile soil', *FEMS Microbiology Ecology*, vol. 15, pp. 119-126.

Gao, L-L., Delp, G. & Smith, S.E. 2002, 'Colonization patterns in a mycorrhizal defective mutant tomato vary with different arbuscular mycorrhizal fungi', *New Phytologist*, vol. 151, pp. 477-491.

Galleguillos, C., Aguirre, C., Barea, J.M. & Azcon, R. 2000, 'Growth promoting effect of two *Sinorhizobium meliloti* strains (a wild type and its genetically modified derivative) on a non-legume plant species in specific interaction with two arbuscular mycorrhizal fungi. *Plant Science*, vol. 159, pp. 57-63.

Genetic Modification Advisory Committee 2000, Public information sheets on deliberate release proposals. Ref. 2000/005689.

Germida, J.J., Siciliano, S.D., de Freitas, R. & Seib, A.M. 1998, 'Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus*) and wheat (*Triticum aestivum* L.)', *FEMS Microbiology Ecology*, vol. 26, pp. 43-50.

Gibb, D. & Rochester, I.J. 1994, 'Nitrogen Fertiliser Efficiency – Denitrification', *CRC – Newsletter for the Research Extension Education Program*, Vol. 1 No. 2 April, 1994. Available http://cotton.pi.csiro.au/ Publicat/ Agro/ Nutrient/denitrif.htm.

Glandorf, D.C.M., Verheggen, P., Jansen, T., Jorritsma, J-W., Smit, E., Leeflang, P., Wernars, K., Thomashow, L.S., Laureijs, E., Thomas-Oates, J.E., Bakker, P.A.H. & van Loon, L.C. 2001, 'Effect of genetically modified *Pseudomonas putida* WCS358r on the fungal rhizosphere microflora of field-grown wheat', *Applied & Environmental Microbiology,* vol. 67, pp. 3371-338.

GMAC, 2001 Available at the URL: (http://health.gov.au.ga/gene/gmac website, accessed 14/05/01.)

Godoy, A.S., Moreno, A.L.E., Garcia, C.E.A., Dugger, P. (ed) & Richter D. (ed.), 1998, 'Plant growth analysis of transgenic Bt Cotton', *Proceedings Beltwide Cotton Conferences, San Diego, Calif. USA. 5-9 Jan. 1998.* National Cotton Council, Memphis. USA, vol. 2, pp. 1485-1487.

Goenadi, D.H. 1995, 'Suitability of selected mixtures of clay minerals with humic substances as carrier of phosphate-solubilizing microbes', *Menara Perkebunan,* vol. 63, pp. 102-113.

Golebiowska J. & Pedziwilk, Z. 1984, CO_2 release as an index of biological activity of cultivated soils. *Acta Mikrobiological Polonica,* vol. 33, pp. 249-256.

Grant, W.D. & Long, P.E. (eds) 1981, *Environmental Microbiology*, Blackie, London.

Griffiths, B.S., Geoghegan I.E., & Robertson W.M. 2000, 'Testing genetically engineered potato, producing the lectins GNA and Con A, on non-target soil organisms and processes', *Journel of Applied Ecology* vol. 37, pp. 159-170.

Griffitts, J.S., Haslam, S.M. Yang, T. Stephan F. Garczynski, S.F. Mulloy, B., Morris, H. Cremer, P.S., Dell, A., Adang, M.J., & R.V. Aroian 2005, 'Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin,' *Science*, vol 307, pp. 922-925.

Grundmann, G.L. & Debouzie, D. 2000, 'Geostatistical analysis of the distribution of NH_4^+ and NO_2^- oxidizing bacteria and serotypes at the millimeter scale along a soil transect', *FEMS Microbiology Ecology*, vol. 34, pp. 57-62.

Grundon, N.J. 1987, *Hungry Crops: a Guide to Nutrient Deficiencies in Field C rops*, Qld Dept. of Primary Industries. Brisbane, Qld. p. 229.

Guckert, J.B., Hood, M.A. & White, D.C. 1986, 'Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the trans-/cis ratio and proportions of cyclopropyl fatty acids. *Applied & Environmental Microbiology*, vol. 52, pp. 794-801.

Guckert, J.B. Ringelberg, D.B., White, D.C. Hanson, R.S. & Bratina, B.J. 1991, 'Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria', *Journal of General Microbiology* vol. 137, pp. 2631-2641.

Gupta, V.V.S.R. & Neate, S.N. 1998, 'Impact of agrochemicals on soil biota and biological processes', in *Synthetic organic chemicals as pollutants in the environment*, pp. 17-19. Aust. Soil Science Society. (SA Branch), Adelaide, South Australia.

Gupta, V.V.S.R., Rogers, S. and Naidu, R. 1998, 'Effects of secondary treated sewage effluent application on the populations of microfauna in a forest soil', *Geoderma* vol. 84, pp. 249-263.

Gyamfi, S., Pfeifer, U., Stierschneider, M. & Sessitsch A. 2002, 'Effects of transgenic glufosinate-tolerant oilseed rape (*Brassica napus*) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere', *FEMS Microbial Ecology,* vol. 41, pp. 181-190.

Haack, S.K., Garchow, H., Odelson, D.A., Forney, L.J. & Klug, M.J. 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities', *Applied & Environmental Microbiology*, vol. 60, pp. 2483-2493.

Hadwiger, L.A. & Loschke, D.C. 1981, 'Molecular communication in host-parasite interactions: Hexosamine polymers (chitosin) as regular compounds in race-specifric and other interactions', *Phytopathology*, vol. 71, pp. 756-761.

Hamilton, W.A. 1988, 'Microbial energetics and metabolism', in *Microorganisms in Action: Concepts and Applications in Microbial Ecology*, J.M. Lynch & J.E. Hobbie, eds. Blackwell Scientific, Oxford. pp. 75-100.

Harter, R.D. & Stotzky. G. 1973, 'X-Ray diffraction, electron microscopy, electrophoretic mobility, and pH of some stable smectite-protein complexes', *Soil Society of America Procedings*, vol. 37, pp. 116-123.

Hausenbuiller, R.L. 1973, *Soil Science: Principles and Practices*, Dubuque, Iowa, ed. W.C. Brown, 1973.

Hawes, M.C., Brigham, L.A., Nicoll, S.M. & Stephenson, M.B. 1994, 'Plant genes controlling the release of root exudates' in *Biotechnology and Plant Protection*, eds. D.D. Bils & S-D. Kung, pp. 61-84.

Hawker, L.E., Linton, A.H., Folkes, B.F. & Carlile, M.J. (eds) 1960, *An Introduction to the Biology of Microorganisms.* Edward Arnold (Publishers) Ltd.

Hawksworth, D.L. 1991, 'The fungal dimension of biodiversity: magnitude, significance and conservation', *Mycological Research*, vol. 95, pp 641-655.

Hayman, D.S. 1970, '*Endogone* spore numbers in soil and vesicular-arbuscular mycorrhizae in wheat as influenced by season and soil treatment,' *Transactions of the British Mycological. Soc.* vol. 45, pp 53-63.

Head, G., Surber, J.B., Watson, J.A., Martin, J.W. & Duan, J.J. 2002, 'No detection of Cry1Ac protein in soil after multiple years of transgenic Bt cotton (Bollgard) use', *Environmental Entomology*, vol. 31, pp. 30-36.

Henriksen, T.M. & Breland, T.A. 1999. 'Nitrogen availability effects on carbon mineralization, fungal and bacterial growth, and enzyme activities during decomposition of wheat straw in soil', *Soil Biology and Biochemistry* vol. 31, pp. 1121-1134.

Herman, R.A., Evans, S.L., Shanahan, D.M., Mihaliak, C.A., Bormett, G.A., Young, D.L. & Buehrer, J. 2002, 'Rapid degradation of Cry1F delta-endotoxin in soil', *Enviromental Entomology*, vol. 30, pp. 642-644.

Heuer, H., Kroppenstedt, M., Lottmann, J., Berg, G. & Smalla, K. 2002, 'Effects of T4 Lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors', *Applied & Environmental Microbiology*, vol. 68, pp. 1325-1335.

Hitchcock, C. 1975. 'Structure and distribution of plant acyl lipids', in: T. Galliard, Q.I. Mercer (Eds.), *Recent Advances in the Chemistry and Biochemistry of Plant Lipids*. Academic Press, London, pp. 1-19.

Holmes, L. & Thies, J. (eds) 1999, Molecular techniques for the analysis of *microbial community structure in soil and the rhizosphere*' Laboratory Manual, University of Western Sydney, Hawkesbury, Richmond, N.S.W.

Holt, H.E., 1998, 'Season-long monitoring of transgenic cotton plants – Development of an assay for the quantification of *Bacillis thuringiensis* insecticidal crystal protein' in Proceedings of the 9th Australian Cotton Conference, Queensland, Aust.

Hope, C.F.A. and Burns, R.G. 1987, Activity, origins and location of cellulase in a silt loam soil. *Biology & Fertility of Soils*. vol. 5, pp. 164-170.

Horst, W.J., Wagner, A. & Marschner, H., 1982, 'Mucilage protects root meristems for aluminium injury', *Zeitschrift für Pflanzenphysiologie*, vol. 105, pp. 435-444.

Heuer, H. & Smalla, K. 1999, 'Bacterial phyllosphere communities of *Solanum tuberosum* L. and T4-lysozyme-producing transgenic variants', *FEMS Microbial Ecology.* vol. 218, pp. 357-371.

Heuer, H., Kroppenstedt, R.M., Lottmann, J., Berg, G. & Smalla, K. 2002, 'Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors. *Applied & Environmental Microbiology* vol. 68, pp. 1325-1355.

Hurst, C.J. (Editor in Chief) 1997, *Manual of Environmental Microbiology*, p. 511. ASM Press

Ibekwe, A.M. & Kennedy, A.C. 1998, 'Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions', *FEMS Microbiology Ecology*, vol. 26, pp. 151-163.

Ignoffo, C.M. & Dropkin, V.H., 1976, 'Deleterious effects of the thermostable toxin of *Bacillus thuringiensis* on species of soil-inhabiting, myceliophagus, and plant-parasitic nematodes', *Journal of the Kansas Entomological Society*, vol. 50, pp. 394-398.

Illmer P. & Schinner F. 1992, 'Solubilization of inorganic phosphate by microorganisms isolated from forest soils', *Soil Biology & Biochemistry*, vol. 24, pp. 389-395.

International Service for the Acquisition of Agri-biotech applications (ISAAA) 2004, available: http://www.isaaa.org/ accessed 12/4/2002; 20/01/04.

IOGTR (Interim Office of the Gene Technology Regulator) 2000, Information Bulletin No. 6: 'Questions and answers on the commercial release of Roundup Ready® and Roundup Ready®/INGARD® cotton', URL: http://www.health.gov.au/ogtr/publications/

Isbell, R.F. 1996, *The Australian Soil Classification*, CSIRO Publishing, Collingwood, p. 143.

Janik, L.J., Merry, R.H. & Skjemstad, J.O. 1998, 'Can mid-infrared diffuse reflectance analysis replace soil extractions?' *Australian Journal of Experimental Agriculture*, vol. 38, pp. 681-96.

Jarrell, W.M. & Beverly, R.B. 1985, 'The dilution effect in plant nutrition studies', *Advanced Agronomy* vol. 34, pp. 197-234.

Jarstfer, A.G. & Sylvia, D.M. 1993, 'Inoculum Production and Inoculation Strategies for Vesicular-Arbuscular Mycorrhizal Fungi', in *Soil Microbial Ecology*, *Applications in Agricultural and Environmental Management*, ed. F. Blaine Metting Jr., Marcel Dekker, New York.

Jarstfer, A.G. & Sylvia, D.M. 1996, 'Isolation, culture, and detection of arbuscular mycorrhizal fungi', in *Manual of Microbiology*, C. J. Hurst, Ed. in Chief. ASM Press, Washington, D.C. pp. 406-411.

Johansen, J.E. & Binnerup, S.J. 2002 'Contribution of cytophaga-like bacteria to the potential of turnover of carbon, nitrogen, and phosphorus by bacteria in rhizosphere of barley (*Hordeum vulgare* L.)'.*Microbial Ecology*, vol. 43, pp. 298-306.

Jones, M. & Wang, Z. 2003, 'Differential display analysis of gene-expression in the cytoplasm of giant cells induced in tomato roots by *Meloidogyne javanica*', *Molecular Plant Pathology*, vol. 4, pp. 361-371.

Jordan, W.R. 1983, 'Cotton' In *Crop-water Relation*, eds. I.D. Teare and M.M. Peets. John Wiley & Sons, New York.

Kaneda, T. 1991, 'Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance', *Microbial Reviews* pp. 288–302.

Kapulnik, Y. 1996, 'Plant Growth Promotion by Rhizosphere Bacteria' in *Roots the Hidden Half*, 2nd edn, eds Y. Waisel, A. Eshel, & U. Kafkafi, Marcel Dekker, Inc, USA.

Keddie, R.M. 1975, 'Arthrobacter' in *Bergeys Manual of Determinative Bacteriology*, 8th edn, co-eds. R.E. Buchanan & N.E. Gibbons, part 17 Actinomycetes and related organisms, p. 618.

Kemp, A. & Alexander, F. 2000, 'Land care and degradation: a survey of natural resource management', *Australian Farm Surveys Report 2000,* ABARE, Canberra, pp. 37-39.

Kennedy, A.C. 1999, 'Bacterial diversity in agroecosystems', *Agriculture Ecosystems & Environment*, vol. 74, pp. 65-76**.**

Kennedy, I.R. 1992, *Acid Soil and Acid Rain*, 2nd edn, John Wiley & Sons Inc. Brisbane.

Kieft, T.L., Ringelberg, D.B. & White, D.C. 'Changes in ester-linked phospholipid fatty acid Profiles of subsurface bacteria during starvation and desiccation in a porous medium', *Applied & Environmental Microbiology,* vol. 60, pp. 3292-3299.

Kirby, G. Senior Lecturer, Flinders University, (*pers comm.* Received by email 20/02/01).

Kiss, S., Stefanic G. & Dreagan-Bularda M. 1974, 'Soil enzymology in Romania', (Part 1). *Contributii Botanice din Cluj* pp. 207-219.

Klappenbach, J.A., Dunbar, J.M. & Schmidt, T.M. 2000, 'rRNA Operon copy number reflects ecological strategies of bacteria', *Applied & Environmental Microbiology*, vol. 66, pp. 1328-1333.

Kjøller, A.H. & Struwe, S. 2002, 'Fungal Communities, Succession, Enzymes, and Decomposition' in *Enzymes in the environment, Activity, Ecology and Applications*', eds. Richard G. Burns & Richard P. Dick. Marcel Dekker, Inc. New York. pp. 267-281.

Kloepper, J.W., Liftshitz, K., Zablotowicz, R.M. 1989, 'Free-living bacterial inocula for enhancing crop productivity', *Trends Biotechnology*, vol, 7, pp. 39-43.

Kloepper, J.W., McInroy, J.A. & Bowen, K.L. 1992, 'Comparative identification of fatty acid analysis of soil, rhizosphere and geocarposphere bacteria of peanut (*Arachis hypogaea* L.) *Plant Soil,* Vol. 139, pp. 85-90.

Knight, B.P., McGrath, S. & Chaudri, A. 1997, 'Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc, *Applied & Environ. Microbiology*, vol. 63, pp. 39-43.

Kochian, L.V. 1995, 'Cellular mechanisms of aluminium toxicity and resistance in plants', *Annual Revue of Plant Physiology & Plant Molecular Biology*, vol. 46, pp. 237-260.

Koskella, J. & Stotzky, G. 2002, 'Larvicidal toxins from *Bacillus thuringiensis* subspp. *kurstaki, morrisoni (*strain *tenebrionis),* and *israelensis* have no microbicidal or microbiostatic activity against selected bacteria, fungi, and algae in vitro', *Canadian Journal of. Microbiology* Vol. 48, pp. 262-267.

Kostichka, K. & Warren, G.E. 1996, 'Cloning of a *CryV*-type insecticidal protein gene from *Bacillus thuringiensis* the *cryV*-encoded protein is expressed early in stationary phase', *Journal of Bacteriology*, vol. 178, pp. 2141-2144.

Kroppenstedt, R.M. 1985, 'Fatty acid and menaquinone analysis of actinomycetes and related organisms,' in *Chemical Methods in Bacterial Systematics*, eds. M. Goodfellow & D.E. Minnikin, pp. 173-199. Academic Press, London.

Kudo, R.R. 1971, *Protozoology*, 5th edition, Charles C. Thomas, Publisher, Springfield, U.S.A.

Kuikman, P.J., Van Elsas, J.D., Jansen, A.G., Burgers, S.L.G.E., Van Veen, J.A. 1990, 'Population dynamics and activity of bacteria and protozoa in relation to their spatial distribution in soil. *Soil Biology & Biochemistry* vol. 22, pp. 1063-1073.

Ladd, J.N. & Butler, J.H.A 1972, 'Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates', *Soil Biology & Biochemistry*, vol. 4, pp. 19-30.

Langmuir, I. 1918, 'Identical Monolayer adsorption', *The American Chemical Society*, vol. 40, p. 1361.

Lao, C., Heckel, D.G & Akhurst, R. 2002, 'Toxicity of *Bacillus thuringiensis* insecticidal proteins for *Helicoverpa armigera* and *Helicoverpa punctigera (Lepidoptera: Noctuidae*), major pests of cotton', (abstract) *Journal of Invertebrate Pathology*, vol. 80(1), pp. 55-63.

Larsen, R.J., 1975, Statistics for the health sciences Charles E. Merrill Publishing Co., pp. 210-211.

Laskin, A.I. & Lechevalier, H.A. (eds) 1974, *CRC Handbook of Microbiology Condensed edition*, CRC Press, Ohio. p. 669.

Law, J.H., Zalkin H. & Kaneshiro T. 1963, 'Transmethylation reactions in bacterial lipids', *Biochimica et Biophysica Acta.* Reviews on biomembranes. vol. 70.

Lawley, R.A., Campbell, R. & Newman, E.I. 1983, 'Composition of the bacterial flora of the rhizosphere of three grassland plants grown separately and in mixtures. *Soil Biology & Biochemistry*, vol. 15, pp. 605-607.

Lechevalier, H. & Lechevalier, M.P. 1988 'Chemotaxonomic use of lipids – an overview. In *Microbial Lipids*, Vol. 1, (C. Ratledge, & S.G. Wilkinson eds.) pp. 869-902. Academic Press, San Diego, CA.'

Lee, K.E. & Pankhurst, C.E., 1992, 'Soil organisms and sustainable productivity. *Australian Journal of Soil Research,* vol. 30, pp. 855-892.

Lehninger, A.L., Nelson, D.L. & Cox, M.M. 1997, *Principles of Biochemistry*, 2nd edn, Worth Publishers, New York.

Lelmen , E.K., Osir, E.O., Anyango, B., Jefwa, J. & Boga, H.I., 2004, 'effects of *Bacillus Thuringiensis* Cry1a(c) δ-endotoxin on Arbuscular mycorrhizal colonization in sorghum and spore germination in vitro', available www.academicjournals.org/AJB/Events/9th%20BSK%20Symposium/Lelmen_EK_et_al.pdf

Lemanceau, P., Corberand, T., Gardan, L., Latour, X., Laguerre, G. & Boeufgras, J.M. 1995, 'Effect of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.), on the diversity of soil-borne population of fluorescent pseudomonads', *Applied & Environmental Microbiology*, vol. 61, pp. 1004-1014.

Li, J., Caroll, J. & Ellar, D.J., 1991, 'Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5Å resolution'. *Nature,* vol. 353, pp. 815-821.

Lindsey, K. & Jones, M.G.K., 1989, *Plant Biotechnology in Agriculture*. Milton Keynes, Open University Press.

Liu, C-N, 1997, 'Nuclear genome sizes of important plant species' in *Plant Molecular Biology – a laboratory manual.* ed. M.S. Clark. Springer-Verlag.

Llewellyn, D.J., Brown, M., Cousins, Y., Hartweck, L., Last, D., Mathews, A., Murray F. & Thistleton, J. 1992, 'The science behind transgenic cotton plants'. Proceedings of the 6th Australian Cotton Conference Broadbeach, Qld.

Llewellyn, D.J. & Higgins, T.J.V. 2002, 'Future Biotechnologies'. Proceedings of the 11th Australian Cotton Conference Brisbane, Qld.

Lopez A., Pinochet, J., Fernandez, C., Calvet, C. & Camprubi, A. 1997, 'Growth response of OHF-333 pear rootstock to arbuscular mycorrhizal fungi, phosphorus nutrition and *Pratylenchus vulnus* infection. *Fundamental and Applied Nematology*, vol. 20, pp. 87-93.

Lukow, T., Dunfield, P.F. & Liesack, W. 2000, 'Use of T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiological Ecology* vol. 32, 241-247.

Lynch, J.M. 1982, 'Interactions in the root environment', in *Bacteria and Plants,* ed. M.E. Rhodes-Roberts & F.A. Skinner. Academic Press. London.

Madan, R., Pankhurst, C., Hawke, B. & Smith, S. 2002 'Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil.' *Soil Biology & Biochemistry*, vol. 34, pp. 125-128.

Manachini, B. & Lozzia, G.C. 2002, 'First investigations into the effects of Bt corn crop on Nematofauna', *Bolettino di Zoologia Agraria e di Bachicoltura* series II, vol. 34, pp. 85-96.

Manachini, B., Landi, S., Fiore, M.C., Festa, M. & Arpaia, S. 2004, 'First investigations on the effects of *Bt* transgenic *Brassica napus* L. on the trophic structure of the nematofauna', GMOs in Integrated Production, *IOBC wprs Bulletin,* vol. 27, pp. 103-108.

Marrone, P.G. 1993, 'Engineered plants and microbes in integrated pest management systems' pp. 233-247, in *Advanced Engineered Pesticides*, ed. L. Kim, Marcel Dekker, Inc., New York, N.Y.

Marschner, H., Romheld, V. & Ossenberg-Neuhaus, H. 1982, *Zeitschrift für Pflanzenphysiologie*, vol. 105, pp. 407-416.

Marschner, H. 1997, *Mineral Nutrition of Higher Plants*, Academic Press, 2nd edn, Sydney.

Marschner, P., Gerendás, J. & Sattelmacher, B. 1999, 'Effect of N concentration and N source on root colonization by *Pseudomonas fluorescens* 2-79RLI'. *Plant Soil* vol. 215, 135-141.

Marschner, P., Yang, C.-H., Lieberei, R. & Crowley, D.E. 2001, 'Soil and plant specific effects on bacterial community composition in the rhizosphere' *Soil Biology & Biochemistry.*, vol. 33, pp. 1437-1445.

Matulewich, V.A., P. F. Strom and M.S. Finstein, 1975, 'Length of incubation for enumerating nitrifying bacteria present in various environments', *Applied Microbiology*, vol. 29, pp. 265-268

Mauseth, J.D. 1988 *Plant Anatomy*, The Benjamin/Cummings Publishing Co. Inc

McCardle, A, Sadowsky, M.J. & Cregan, P.B. 1993, 'Genetics and Implementation of Biological Nitrogen Fixation', in Metting, F.B. Jr., (ed) *Soil Microbial Ecology: Applications in Agricultural and Environmental Management*, Marcel Dekker, New York.

McGee, P.A., Torrisi, V. & Pattinson, G.S. 1999, 'The relationship between density of *Glomus mosseae* propagules and the initiation and spread of arbuscular mycorrhizas in cotton roots', *Mycorrhiza*, vol. 9, pp. 221-225.

McKenzie, D. 1998, *SoilPak for Cotton Growers*, Third Edition', eds. A. Munroe and E. Roberts, NSW Dept. of Agriculture publication. Available http://cotton.crc.org.au/Assets/PDFFiles/acgra98/mckenzie.pdf

McKenzie, R.H., Dormaar, J.F., Schaalje, G.B. & Stewart, W.B. 1995, 'Chemical and biochemical changes in the rhizospheres of wheat and canola' *Canadian Journal of Soil Science*, vol. 75, pp. 439-447.

McLaren, A.D. & Skujins, J. 1968, 'The physical environment' in *The Ecology of Soil Bacteria, an International Symposium,* eds. T.R.G. Gray & D. Parkinson Liverpool University Press. p. 8.

McLeod, R., Reay, F. & Smyth, J. 1994, *Plant nematodes of Australia listed by Genus*, New South Wales Dept. of Agriculture.

McMichael, B.L. & Burke, J.J. 1996, 'Temperature Effects on Root Growth', in *Plant Roots The Hidden Half*, eds. Yoav Waisel, Amram Eshel & Uzi Kafkafi, Marcel Dekker, Inc., New York.

Menge, J.A., Lembright, H.W. & Johnson, E.L.V. 1977, 'Utilization of mycorrhizal fungi in citrus nurseries,' *Procedings of the International Society Citric*,. vol.1, pp. 129-132.

Metting, F.B. Jr., 1993, (ed) *Soil Microbial Ecology: Applications in Agricultural and Environmental Management*, Marcel Dekker, New York.

Meyer, J-M., Geoffroy, V.A., Baida, N., Gardan, L., Izard, D., Lemanceau, P., Achou, W. & Palleroni, N.J. 2002, 'Siderophore typing, a powerful tool for the identification of fluorescent and nonfluorescent pseudomonads', *Applied & Environmental Microbiology* , vol. 68, pp. 2745-2753.

Miller, F.C. 1992, 'Composting as a Process Based on the Control of Ecologically Selective Factors' in *Soil Microbial Ecology*, *Soil Microbial Ecology*, Marcel Dekker, New York.

Miller, H.J. Henken, G. & van Veen, J.A. 1989, 'Variation and composition of bacterial populations in the rhizosphere of maize, wheat and grass cultivars. *Canadian Journal of Microbiol.*, vol. 35, pp. 656-660.

Mitchell, E.A.D., Borcard, D., Buttler, A.J., Grosvernier, Ph., Gilbert, D. & Gobat, J.-M. 2000, 'Horizontal distribution patterns of testate amoebae (Protozoa) in a Sphagnum magellanicum Carpet'. Microbial Ecology, vol. 39, pp. 290-300.

Montégut, J. 1960, 'Methods for isolation and estimation of activity of fungi in soil' in *The Ecology of Soil Fungi, An International Symposium*, eds. D. Parkinson & J.S. Waid, Liverpool University Press.

Nannipieri, P., Ceccanti, B. & Grego, S. 1990, 'Ecological significance of the biological activity in soil', in *Soil Biochemistry.* eds. J-M Bollag & G. Stotzky, vol. 6, Marcel Dekker, New York pp. 203-355.

Narva, K.E., Schwab, G.E., Galasan, T & Payne, J.M. 1994, 'Nematode-active toxin from a *Bacillus thuringiensis* isolate' International classification C07K 1504. Patent No. 05322932, available: http://www.nal.usda.gov/bic/BTTOX/BT-patents/05322932.html

Naseby, D.C., Greenland, A. & Lynch, J.M. 1999, (Unpublished data) in *Enzymes in the environment. Activity, Ecology and Applications. 2001*, eds. Richard G. Burns & Richard P. Dick, Marcel, Dekker. Inc. New York. pp. 119-120.

Nelson, N. 1944, 'A photometric adaptation of the somagyi method for the determination of glucose', *Journal of Biology & Chemistry* vol. 153, pp. 375-380.

Nester, E., Thomashow, L.S., Metz, M. & Gordon, M. 2002, '100 years of *Bacillus thuringiensis*: a critical scientific assessment', *American Academy of Microbiol.* Washington, DC.

Nicholas, W.L., 1975, *The Biology of Free-Living Nematodes*. Clarendon Press, Oxford. pp. 42-43; 141-143.

Nicholas, W.L., Bird, A.F., Beech, T.A. & Stewart, A.C, 1992, 'The nematode fauna of the Murray River estuary, South Australia; the effects of the barrages across its mouth', *Hydrobiologie*, vol. 234, pp. 87-101.

Nielsen, K.M., Bones, A.M., Smalla, K. & Vanelsas, J.D., 1998, 'Horizontal gene transfer from transgenic plants to terrestrial bacteria – a rare event', *FEMS Microbiology Reviews.*, vol. 22, pp. 79-103.

Northcote, K.Y. 1974, *A Factual Key to classification of Australian Soils*, 4th edn, CSIRO Publication,

NPTN (National Pesticide Telecommunications Network) 2000 '*Bacillus thuringiensis* Technical Fact Sheet', U.S. Environmental Protection Agency, available http://NPIC.orst.edu/factsheets/ Bt tech.pdf.

Nye, P.H. & Tinker, P.B. 1977, *Solute movement in the soil-root system*, Blackwell Scientific Publications, Melbourne, p.178.

Oger, P., Petit, A. & Dessaux, Y. 1997. 'Genetically engineered plants producing opines alter their biological environment', *Nature Biotechnology*, vol. 15, pp. 369-372.

O'Leary, W.M., & Wilkinson, S.G. 1988, 'Gram-positive bacteria', in *Microbial lipids*, C. Ratledge and S.G. Wilkinson (eds), vol. 1. p. 117-185 Academic Press, London.

O'Leary, W.M. (ed) 1989, *Practical Handbook of Microbiology*. CRC Press, Inc., Florida. p. 456.

Office of the Gene Technology Regulator (OGTR) 2001, 'The Gene Technology Act' available: http://scaletext.law.gov.au/html/ pasteact/3/3428/0/ PA000170.htm, accessed 18 December 2003.

OGTR (Office of the Gene Technology Regulator) 2002, Email received regarding regulations on the disposal of Ingard cotton, Thursday, 28 February 2002.

OGTR 2003, Personal communication received by email on 18/12/03.

Old, K.M. 1977, 'Giant soil amoebae cause perforation of conidia of *Cochliobolus sativus*'. *Transactions of the British Mycological Society*, vol. 68, pp. 277-281.

Old, R.W. & Primrose, S.B. (eds). 1994, *Principles of Gene Manipulation. An introduction to genetic engineering*. Blackwell Science Ltd. Oxford.

Olsen, K.M. & Daly, J.C. 2000, 'Plant-Toxin interactions in transgenic *Bt* cotton and their effect on mortality of *Helicoverpa armigera* (*Lepidoptera: Noctuidae*)', *Journal of Economic Entomology*, vol. 93(4), pp. 1293-1299.

Olsson, P.A. 1999, 'Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil', *FEMS Microbiology Ecology*, vol. 29, pp. 303-310.

Otten, W., Gilligan, C.A., Watts, C.W., Dexter, A.R. & Hall, D. 1999, 'Continuity of air-filled pores and invasion threshold for a soil-borne fungal pathogen, *Rhizoctonia solani*', *Soil Biology and Biochemistry*, vol. 31, pp. 1803-1810.

Pagel-Wieder, S., Gessler, F., Niemeyer, J. & Schröeder, D. 2004, 'Adsorption of the *Bacillus thuringiensis* toxin (Cry1Ab) on Na-montmorillonite and on the clay fractions of different soils', *Journal of Plant Nutrition Soil Society* 2004, vol. 167, pp 184-188.

Palm, C.J., Donegan, K., Harris, D. & Seidler, R.J. 1994, 'Quantification in soil of *Bacillus thuringiensis* var. *kurstaki* δ-endotoxin from transgenic plants', *Molecular Ecology*, vol. 3, pp. 145-151.

Palm, C.J., Donegan, K.K. & Siedler, R.J. 1996, 'Persistence in soil of transgenic plant produced *Bacillus thuringiensis* var. Kurstaki δ-endotoxin', *Canadian Journal of Microbiology*, vol. 42, pp. 1258-1262.

Parkinson, D. & Waid, J.S. (eds) *Fungi*, An International Symposium. Liverpool University Press.

Patel, B. 1999, Microbial Habitats, [Online accessed 19 Jan 01] URL: http://trishul.sci.gu.edu.au/courses/2213bmm/microbial_habitats.html

Pattinson, G.S. & McGee, P.A. 1997, 'High densities of Arbuscular Mycorrhizal fungi maintained during long fallows in soil used to grow cotton except when soil is wetted periodically', *New Phytologist* vol. 136(4), pp. 571-580. Available http://www.blackwell-synergy.com/links/doi/10.1046/j.1469-8137.1997.00783.x

Paul, E.A. & Ladd, J.N. (eds) 1981, *Soil Biochemistry*, Vol. 5, Marcel Dekker Inc. New York

Perotto, S. & Bonfante, P. 1997, 'Bacterial associations with mycorrhizal fungi: close and distant friends in the rhizosphere', *Trends in Microbiology*, Review, vol. 5**.**

Phillips, J.M.& Hayman, D.S. 1970, 'Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection, *Transactions of the British Mycological Society*, vol. 55, pp. 158-161.

Phipps R.H. & Park J.R. 2002, 'Environmental benefits of genetically modified crops – global and European perspectives on their ability to reduce pesticide use'. *Journal of Animal and Feed Science*, vol. 11, pp. 1-18.

Pickup, R.W. & Saunders, J.R. (Eds). 1996, *Molecular Approaches to environmental microbiology.* Ellis Horwood Ltd. Hertfordshire, U.K.

Pierret, A., Moran, C.J., McLachlan, C.B and Kirby, J.K. 2000, 'Measurement of root length density in intact samples using x-radiography and image analysis', *Acta Stereotogica:* Special issue 10th International Conference for Stereology, Melbourne, 1-9 Nov. 1999.

Pinck, L.A., Dyal, R.S. & Allison, P.E. 1954, 'Protein-Montmorillonite complexes, their preparation and the effects of soil microorganisms on their decomposition', *Soil Science*, vol. 78, pp. 109-118.

Pinochet, J., Calvet, C., Camprubi, A. & Fernandez, C. 1996, 'Interactions between migratory endoparasitic nematodes and arbuscular mycorrhiza fungi in perennial crops: A review', *Plant and Soil,* vol. 185, pp. 183-190.

Press, C.M. & Kloepper, J.W. 1994, 'Growth chamber and field screening procedures for evaluation of bacterial inoculants for control of *Rhizoctonia solani* on cotton', in *Improving plant productivity with rhizosphere bacteria*, eds. M.H. Ryder, P.M. Stephens & G.D. Bowen, pp. 61-63.

Pyke, B. & Fitt, G.P., 1998, 'Field performance of Ingard cotton – the first two years', in *Pest Management – future challenges*, eds. M.P. Zalucki, R.A.I. Drew & G.G. White, University of Qld Press, Brisbane, pp. 230-238.

Quiquampoix, H., Chassin, P., Ratcliffe, R.G. 1989, 'Enzyme activity and cation exchange as tools for the study of the conformation of proteins adsorbed on mineral surfaces', *Progress in Colloid & Polymer Science* vol. 79, pp. 59-63.

Ranjard, L., Poly, F., Combrisson, J., Ichaume, A. Gourbiere, F., Thioulouse, J. & Nazaret, S. 2000, 'Heterogeneous cell density and genetic structure of bacterial pools associated with various soil microenvironments as determined by enumeration and DNA fingerprinting approach (RISA)', *Microbial Ecology*, vol. 34, pp. 263-272.

Raper, R.L., Reeves, D.W., Schwab, E.B & Burmester, C.H. 2000, 'Reducing soil compaction of Tennessee Valley soils in conservation tillage systems', *Journal of Cotton Science,* vol. 4, pp. 84-90.

Rasmussen, P.H., Knudsen, I.M.B., Elmholt, S. & Jensen, D.F. 2002, 'Relationship between soil cellulolytic activity and suppression of seedling blight of barley in arable soils', *Applied Soil Ecology*, vol. 19, pp. 91-96.

Rajendran, N., Matsuda, O., Imamura, N. & Urushigawa, Y. 1992. 'Variation in Microbial Biomass and Community Structure in Sediments of Eutrophic Bays as Determined by Phospholipid Ester-Linkes Fatty Acids.' *Applied & Environmental Microbiology* vol. 58, pp. 562-571.

Rayment, G.E. & Higginson, F.R. 1992, '*Aust. Laboratory Handbook of Soil & Water Chemical Methods*', Section 4, Inkata Press, Melbourne.

Ream, J.E., Berberich, S.A., Sims, S.R., Rogan, G.J. & Fuchs, R.L. 1993, 'Distribution and environmental fate of insect resistant cotton proteins', in *Planta* Suppl. *Plant Physiology*, vol. 99, p. 80.

Reece, E.T. & Mandels, M. 1963, 'Enzymatic hydrolysis of β-glucans', in *Enzymic hydrolysis of cellulose and related materials*, Ed. E.T. Reese, Proceedings of a symposium sponsored by The American Chemical Soc. Pergamon Press, Oxford, London.

Reid, R. (Assoc. Prof., University of Adelaide), 2004, 'The role of membrane transport to tolerance of abiotic stresses', Public Lecture, Plant Genomics Centre, Waite Campus, Adelaide.

Reuter, D.J. & Robinson, J.B. (eds) 1997, *Plant Analysis. An interpretation Manual.* CSIRO Publishing.

Rhee, Y.H., Hah, Y.C. & Hong, S.W. 1987, 'Relative contributions of fungi and bacteria to soil carboxymethylcellulase activity', *Soil Biology & Biochemistry* vol. 19, pp. 479-481.

Rhodes, L.H. & Gerdemann, J.W. 1975, 'Phosphate uptake zones of mycorrhizal and non-mycorrhizal onions', *New Phytologist*, vol. 75, pp. 555-561.

Richaume, A., Angle, J.S. & Sadowsky, M.J. 1989, 'Influence of soil variables on in situ plasmid transfer from *Escherichia coli* to *Rhizobium fredii*', *Applied & Environmental Microbiology*, vol. 55 pp. 1730–1734

Robson, A.D. 1989, *Soil Acidity and Plant Growth* Ch. 2. Academic Press, Sydney.

Rochester, I.J., Peoples, M.B. & Constable, G.A. 2001, 'Estimation of N fertiliser requirement of cotton grown after legume crops', *Field Crops Research*, vol. 3947, pp. 1-11.

Roget, D.K. '1995' Decline of root rot (*Rhizoctonia solani* AG-8) in wheat in a tillage and rotation experiment at Avon, South Australia. *Australian Journal of Experimental Agriculture* vol. 35, pp. 1009-1013

Rolstad, J. 1991, 'Consequences of forest fragmentation for the dynamics of bird populations: Conceptual issues and the evidence. *Biological Journal of the Linnean* Society, vol. 42, pp. 49-163.

Rønn, R. et al. 1996. 'Spatial distribution and successional pattern of microbial activity and micro-faunal populations on decomposing barley roots', *Journal of Applied Ecology*, vol. 33, pp. 662-672.

Root, R., 1967, 'The niche exploitation pattern of the blue-grey gnatcatcher. *Ecological Monographs,* vol. 37, pp. 317-350.

Rosen, H. 1957, 'A Modified Ninhydrin Colorimetric analysis for Amino Acids' *Archives of Biochemistry and Biophysics*, vol. 67, pp. 10-15.

Rosenthal, S.L. 1974, 'Comparison of two methods for the detection of fluorescent pigment production by *Pseudomonas* species', *Journal of Applied Bacteriology*, vol. 37, pp. 469-470.

Rouatt, J.W., Katznelson, H. & Payne, T.M.B. 1960, 'Statistical evaluation for the rhizosphere effect', *Soil Science Society of America Proceedings*, vol. 24, pp. 271-273.

Rovira, A.D.1959, 'Root excretions in relation to the rhizosphere effect: IV. Influence of plant species, age of plant, light, temperature, and calcium nutrition on exudation', *Plant Soil*, vol. 9, pp. 53-64.

Rovira, A.D. & Sands, D.C. 1971, Fluorescent pseudomonads – a residual component in the soil microflora? *Journal of Applied Bacteriology*, vol. 34, pp. 253-259.

Rukmini, V., Reddy, C.Y. & Venkateswerlu, G. 2000, '*Bacillus thuringiensis* crystal δ-endoxoxin: Role of proteases in the conversion of protoxin to toxin', *Biochimie*, vol. 82, pp. 109-116.

Sachs, E.S., Benedict, J.H., Stelly, D.M. Taylor, J.F., Altman, D.W., Berberich, S.A. & Davis, S.K. 1998, 'Expression and segregation of genes encoding Cry1A insecticidal proteins in cotton'. *Crop Science*, vol. 38, p. 1.

Salama, H.S., Foda, M.S., El-Sharaby, A., Matter, M. & Khalafallah, M. 1981, 'Development of some lepidopterous cotton pests as affected by exposure to sublethal levels of endotoxins of *Bacillus Thuringiensis* for different periods', *Journal of Invertebrate pathology,* vol. 38, pp. 220-229.

Sasser, M. 1990, 'Identification of bacteria through fatty acid analysis' in *Methods in Phytobacteriology*, Z. Klement, K. Rudolf & D. Sands, (eds) pp. 199-204 Akademiai Kiado, Budapest.

Sato, M., Ishii, M., Kimura, Y., Izumori, K. 1995, 'Evaluation of the ability of soil bacteria to utilize rare sugars', *Journal of General Applied Microbiology*, vol. 41, pp. 171-174.

Saxena D., Flores, S. & Stotzky, G. 1999, 'Insecticidal toxin in root exudates from *Bt* corn' *Nature*, vol. 402, p. 480.

Saxena, D. & Stotzky, G., 2001, '*Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil', *Soil Biology & Biochemistry*, vol. 33, pp. 1225-1230.

Saxena, D. Flores, S. & Stotzky, G., 2002, 'Bt toxin is released in root exudates from 12 transgenic corn hybrids representing three transformation events', *Soil Biology & Biochemistry*, vol. 34, pp. 133-137.

Saxena, D., Flores, S. & Stotzky, G., 2002, 'Vertical movement in soil of insecticidal Cry1Ab protein from *Bacillus thuringiensis*' *Soil Biology & Biochemistry*, vol. 34, pp. 111-120.

Schlumbaum, A., Mauch, F., Vogelli, V. & Boller, T. 1986, 'Plant chitinases are potent inhibitors of fungal growth', *Nature*, vol. 324, pp. 365-367.

Schmidt, E.L. & Belser, L.W. 1982, 'Nitrifying bacteria', in *Methods of Soil analysis* Part 2. Chemical and microbiological processes. A.L. Page et al. (eds.). pp. 1027-1042. ASA monograph No. 9. ASA, Wisconsin, USA.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., J. Feitelson, J., Zeigler, D.R. & Dean, D.H. 1998, '*Bacillus thuringiensis* and its pesticidal crystal proteins'. *Microbiology and Molecular Biology Reviews*, vol. 62, pp. 775-806.

Schulz, G.E. & Schirmer, R.H. 1979, '*Principles of Protein Structure*' Springer Verlag, New York.

Sessitsch, A., Weilharter, A., Gerzabek, M.H., Kirchmann, H. & Kandeler, E. 2002, 'Microbial Population Structures in Soil Particle Size Fractions of a Long-Term Field Experiment', *Applied & Environmental Microbiology*, vol. 67, pp. 4215-4224.

Shaw K.J., Rather, P.N., Hare, R.S., Miller, G.H. 1993, 'Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglyciside-modifying enzymes', *Microbiological Reviews*, vol. 57, pp 138-163.

Siminoff, P. & Gottlieb, D. 1951, 'The Production and role of antibiotics in the soil: I: The fate of streptomycin', *Phytopathology* vol. 41, pp. 422-429.

Stone, M. 2003, Report presented at the Cotton Consultants Australia 'Cotton Production Seminar 2003'

Schinner, F., Öhlinger, R., Kandeler, E. & Margesin, R. (Eds), 1995, '*Methods in Soil Biology*' Springer-Verlag, New York.

Straub, F.B. 1967, 'SH groups and SS bridges in the structure of enzymes', *Procedings of the 7th International Congress Biochemistry (Tokyo)*, pp. 42-50.

Semenov, A.M., van Bruggen, A.H.C. & Zelenev, V.V. 1998, 'Moving waves of bacterial populations and total organic carbon along roots of wheat' *Microbial Ecology*, vol. 37, pp. 116-127.

Sikora, R., 1992, 'Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes', *Annual Review Phytopathology*, vol. 30, pp. 245-70.

Simeoni, L.A., Lindsay, W.L. & Baker,R. 1987, 'Critical iron level associated with biological control of fusarium wilt', *Phytopathology*, vol. 77, pp. 1057-1061.

Simon, A. & Ridge, E.H. 1974, 'The use of ampicillin in a simplified selective medium for the isolation of fluorescent pseudomonads', *Journal of Applied Bacteriology.*, vol. 37, pp. 459-460.

Simpfendorfer, S., Kirkegaard, J.A. & Heenan, D.P. 1999, 'Soil microorganisms limit the early growth of direct drilled wheat in south eastern Australia', Proceedings of the First Australasian Soilborne Disease Symposium, ed. R.C. Magarey, Bureau of Sugar Experiment Stations, Brisbane.

Singh, B.N. 1945, 'The selectivity of bacterial food by soil amoebae, and the toxic effects of bacterial pigments and other products on soil protozoa'. *British Journal of Experimental Pathology*, vol. 26, p. 316.

Söderberg, K.H., Probanza, A., Jumpponen, A. & Bååth, E. 2004, 'The microbial community in the rhizosphere determined by community-level physiological profiles (CLPP) and direct soil- and cfu -PLFA techniques', *Applied Soil Ecology*, vol. 25, pp. 135-145.

Sparling, G.P., Ord, G.B & Vaughan, D. 1981, 'Microbial biomass and activity in soils amended with glucose', *Soil Biology & Biochemistry*, vol. 13, pp. 99-104.

Soil Conservation and Landcare Act (1993), as part of SA Consolidated Acts, Section 25, Part 44 General Environmental Duty, available http://www.austlii.edu.au/au/legis/sa/consol_act/epa1993284/s25.html.

Sperber J.I. & Rovira A.D. 1959, 'A study of the bacteria associated with the roots of subterranean clover and Wimmera rye-grass', *Journal of Applied Bacteriology*, vol. 22, pp. 85-95.

Spiro R.G. 1966, 'Analysis of sugars found in glycoproteins', in: *Methods in Enzymology*, vol 8. E.F. Neufeld, and V. Ginsberg, (eds). Academic press, pp 3-26.

Stotzky, G., 1993, 'Release of genetically engineered microorganisms', in *Advances in Applied Microbiology*, eds. Saul Neidleman & Allen I. Laskin, vol 38.

Stotzky, G. 2000, 'Persistence and biological activity in soil of insecticidal proteins from *Bacillus thuringiensis* and of bacterial DNA Bound on clays and humic acids', *Journal of Environmental Quality,* vol. 29, pp. 691-705.

Stotzky, G. 2005, 'Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants, *Plant and Soil*, vol. 266, pp. 77-89.

Stutzenberger, F.G. 1972, 'Cellulolytic activity of *Thermomonospora curbata* optimal assay conditions, partial purification and product of the cellulase', *Applied Microbiology*, vol. 24, pp. 83-90.

Sullivan, J.A., Shirasu, K. & Deng, X.W. 2003, 'The diverse roles of ubiquitin and the 26S proteasome in the life of plants', *Genetics* vol. 4, pp. 948-958

Summit, M., Peacock, A., Ringelberg, D., White, D.C & Baross, J.A. 2000,' Phospholipid fatty acid-derived microbial biomass and community dynamics in hot, hydrothermally influenced sediments from Middle Valley, Juan De Fuca Ridge', in R.A. Zierenberg, Y. Fouquet, D.J. Miller, W.R. Normark, (eds) *Procedings ODP, Sci. Results,* vol. 169, pp. 1019.

Sumitomo Chemical, 2003, Publication, available http://www.landmark.com/articlepics/Dipel_technote_Nov03.pdf

Style Manual for Authors, Editors and Printers, 1994, 5th edn. AGPS Press Publication, Aust. Govt. Publishing Service, Canberra.

Tabatabai, M.A. and Bremner, J.M., 1969, 'Use of r-nitrophenyl phosphate for assay of soil phosphatase activity', *Soil Biology & Biochemistry*, vol. 1, pp. 301-307. Soil Enzymes. In: Methods of Soil Analysis. Part 2. Microbiological and Biochemical Properties. p 775-833. 1994. R. W. Weaver et al., editors.

Tabatabai, M.A., & Bremner, J.M. 1970, 'Arylsulphatase activity of soils', *Soil Science Society Amemerican Procedings* vol. 34, pp. 225-229.

Tabatabai, M.A. & Bremner, J.M. 1972, 'Assay of urease activity in soil', *Soil Biology & Biochemistry*, vol. 4, 479-487.

Tabatabai, M.A. 1977, 'Effects of trace elements in urease activity in soils.' *Soil Biology & Biochemistry*, vol. 9, pp. 9-13.

Tabatabai, M.A. 1982 'Soil enzymes', in *Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties*. A.L. Page, E.M. Miller & D.R. Keeney (eds.) American Society of Agronomy, Madison, WI, pp. 903-947.

Tabatabai, M.A. & Dick, W.A. 2001, 'Enzymes in Soil' in *Enzymes in the Environment. Activity, Ecology, and Applications*, eds. Richard G. Burns & Richard P. Dick, Marcel, Dekker. Inc. New York.

Takahashi, K. Kasai, K. & Ochi, K. 2004, 'Identification of the bacterial alarmone guanosine 5'-diphosphate 3'-diphosphate (ppGpp) in plants', *Procedings of the National Academy of Science of the United States of America*, available http://www.pnas.org/cgi/content/full/101/12/4320.

Taiwo, L.B. & Oso, B.A. 1997, 'The influence of some pesticides on soil microbial flora in relation to changes in nutrient level, rock phosphate solubilization and P release under laboratory conditions', *Agriculture, Ecosystems & Environment*, vol. 65, pp. 59-68.

Tapp, H., Calamai, L. & Stotzky, G. 1994, 'Adsorption and binding of the insecticidal proteins from *Bacillus thuringiensis* subp. kurstaki and subsp. Tenebrionis on clay minerals', *Soil Biology & Biochemistry* vol. 26, pp. 663-679.

Tapp, H. & Stotzky, G. 1998, 'Persistence of the insecticidal toxin from *Bacillus thuringiensis* Subsp. *Kurstaki* in soil', *Soil Biology & Biochemistry* vol. 30, pp. 471-476.

Taylor, J.C. 1991, 'Computer Programs for the Standardless Quantitative Analysis of Minerals Using the Full Powder Diffraction Profile', *Powder Diffraction*, vol 6, pp 2-9.

Terzaghi, E. & O'Hara, M. 1990, 'Microbial plasticity. The relevance to microbial ecology', *Advanced Microbial Ecology* vol. 11, pp. 431-460.

Thalmann, A. 1968, 'Zur Methodik der Bestimmung der Dehydrogenaseaktivität im Boden mittels Triphenyltetrazoliumchlorid (TTC)', *Landwirtsch Forsch*, vol. 21, pp. 249-258.

The Cotton Foundation, 2001, *Beltwide Yield Losses* [Online, accessed 29 March 2001] URL:http://www.cotton.org/cf/seedlings/disease.cfm

Tiedje, J.M. & Zhou, J. 1996, 'Analysis of Non-Culturable Bacteria' in *Methods for the examination of organismal diversity in soils and sediment,* ed. G.S. Hall. CAB International.

Todar, K. 2004, 'The Genus *Pseudomonas*' in *Todar's Online Textbook of Bacteriology*', available online at http://textbookofbacteriology.net/Pseudomonas.etc.html.

Torsvik, V., Goksøyr, J. & Daae, F.L. 1990, 'High diversity in DNA of Soil Bacteria'. *Applied and Environmental Microbiology*, vol. 56, pp. 782-787.

Treves, D.S., Xia, B., Zhou, J. & Tiedje, J.M. 2002, 'A two-species test of the hypothesis that spatial isolation influences microbial diversity in soil', *Microbial Ecology,* vol. 45, pp. 20-28.

United Nations Environmental Program, Environment Canada and UNESCO 1975, 'Environmental Impact Assessment: Principles and Procedures' SCOPE Workshop on Impact Studies in the Environment (WISE), ed. R.E. Munn, SCOPE Report 5. Toronto, Canada.

Upchurch, D.R. & Taylor, H.M. 1990, 'Tools for studying Rhizosphere Dynamics', in *Rhizosphere Dynamics*, Ed. J.E. Box Jr. & L.C. Hammond, American Association for the Advancement of Science, Selected Symposium 113.

Van Rie, J., Jansens, S., Hofte, H., Degheele, D. & Van mellaert, H. 1989, 'Specificity of *Bacillus thuringiensis* ∂-endotoxins; importance of specific receptors on the brush border membrane of the mid-gut of target insects' *European Journal of Biochemistry*, vol. 186, p. 239.

Vance, C.P. 1996, 'Root-Bacteria Interactions: Symbiotic Nitrogen Fixation' in *Plant Roots The Hidden Half*, eds. Y. Waiel, A. Eshel & U. Kafkafi, 2nd edn, Marcel Dekker Inc. New York.

Vepsälämen, M. 2001, 'Poor enzyme recovery by extraction from soils' *Soil Biology & Biochemistry*, vol. 33, pp. 1131-1135.

Venkateswerlu, G. & Stotzky, G. 1992, 'Binding of the protoxin and toxin proteins from *Bacillus thuringiensis kurstaki* on clay minerals. *Current Microbiology*, vol. 25, pp. 225-233.

Verhagen, F.J.M. & Laanbroek, H.J. 1992, 'Effects of grazing by flagellates on competition for ammonium between nitrifying and heterotrophic bacteria in chemostats', *Applied & Environmental Microbiology,* vol. 58, pp. 1962-1969.

Vestal, J.R. & White, D.C. 1989, 'Quantitative approaches to the study of microbial communities.' *Bioscience* vol. 39, no. 8.

Vierheilig, H., Alt, M., Lange, J., Gut-Rella, M., Wiemken, A., Boller, T. 1995, 'Colonization of transgenic tobacco constitutively expressing pathogenesis-related proteins by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*, *Applied &. Environmental Microbiology*, vol. 61, pp. 3031-3034.

Vilas-Bôas, L.A., Vilas-Bôas, G.F.L.T., Saridakis, H.O., Lemos, M.V.F., Lereclus, D. & Arantes, O.M.N., 2000, 'Survival and conjugation of *Bacillus thuringiensis* in a soil microcosm', *FEMS Microbiology Ecology*, vol. 31, pp. 255-259.

Voets J.P. & Dedeken, M. 1966, 'Soil enzymes'. *Meded. Rijkafac. Landb-Wetterschappin Gent.,* vol. 31, pp. 177-190.

Waisel, Y., Eshel, A. & Kafkafi, U. 1996, *Plant Roots The Hidden Half*, 2nd edn, Marcel Dekker, Inc., New York.

Walter, D.J. 1999, Thesis entitled 'The morphological and metabolic diversity of *Actinomycetes* from cave soils across geographically distant locations', Flinders University, South Australia.

Warcup, J.H. 1955, 'Isolation of fungi from hyphae present in soil', *Nature*, vol. 175, pp. 953-954.

Warcup, J.H. 1960, 'Methods for isolation and estimation of activity of fungi in soil' in *The Ecology of Soil Fungi*, An International Symposium, eds. D. Parkinson & J.S. Waid, Liverpool University Press.

Walker, B.H. 1992, 'Biodiversity and ecological redundancy', *Conservation Biology*, vol. 6, pp. 18-23.

Walker, G.E. 1997, **'**Effects of *Meloidogyne* spp. and *Rhizoctonia solani* on the growth of grapevine rootings', *Journal of Nematology*, vol. 29, pp. 190-198.

Wallace, H.R. 1973, Nematode ecology and plant disease. Arnold, London.

Watkins, E.M. (ed) 1981, *Compendium of cotton diseases*. American Phytopathological Society, ISBN 0-89054-031-4.

Waters, D. & Kelly, D. 2004, 'Planting Cotton into Standing Wheat Stubble', Information sheet from the Aust. Cotton Research Centre

Webster, J.M. (ed) 1972, *Economic Nematology*, Academic Press, London.

Wegel E., Schauser, L. Sandal, N. Stougaard, J.& Parniske, M. 1998, 'Mycorrhiza mutants of *Lotus japonicus* define genetically independent steps during symbiotic infection', *Molecular Plant-Microbe Interaction,* vol. 11, pp. 933-936.

Wei, J-Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S-C & Aroian, R.V. 2002, '*Bacillus thuringiensis* crystal proteins that target nematodes' *PNAS,* vol. 100, pp. 2760-2765.

Wei-xiang, W., Qing-fu, Y., Hang, M., Xue-jun, D. & Wen-ming, J. 2004, '*Bt* transgenic rice straw affects the culturable microbiota and dehydrogenase and phosphatase activities in a flooded paddy soil', *Soil Biology & Biochemistry*, vol. 36, pp. 289-295.

Wellburn, A.R., Robinson, D.C., Thompson, A., Leith, L.D. 1994, 'Influence of episodes of summer O_3 on $\Delta 5$ and $\Delta 9$ fatty acids in autumnal lipids of Norway Spruce [*Picea abies* (L.) Karst] *New Phytologist* vol. 127, pp. 355-361.

Weller, D.M. & Thomashow, L.S. 1994, 'Current Challenges in Introducing Beneficial Microorganisms into the Rhizosphere' in *Molecular Ecology of Rhizosphere Microorganisms*. *Biotechnology and the release of GMOs*, eds F. O'Gara, D.N. Dowling & B. Boesten. Weinheim, New York.

West-Eberhard, M.J. 1989, 'Phenotypic plasticity and the origins of diversity' *Annual Revue of Ecological Systems,* vol. 20, pp. 249-278.

Westover, K.M., Kennedy, A.C & Kelleys, S.E. 1997, 'Patterns of rhizosphere microbial community structure associated with co-occurring plant species', *Journal of Ecology*, vol. 85, pp. 863-873.

Wheatley, R.E., Caul, S., Crabb, D., Daniell T. J., Griffiths, B. S. & Ritz 2003, 'Microbial population dynamics related to temporal variations in nitrification in three arable fields', *European Journal of Soil Science* vol. 54, p. 707.

White K. 1988, 'Validation of quantitative analysis for microbial biomass, community structure, and metabolic activity', *Archive für Hydrobiologie Beihefte*, vol. 31, pp. 1-18. after extraction of lipids originally described by Bligh and Dyer (1959) Bligh, E.G. and Dyer, W.J. 1959. 'A rapid method of total lipid extraction and purification', *Canadian Journal of Biochemical Physiology*, vol. 37, pp. 911-917.

White, D.C., Stair, J.O. & Ringleberg, D.B. 1996, 'Quantitative comparison on in situ microbial diversity by signature biomarker analysis', *Journal of Indian Microbiology*, vol. 17, pp. 185-196.

Whitehead, A.G., & Hemming, J.R. 1965, 'A comparison of some quantitative methods of extracting small vermiform nematodes from soil', *Annual Applied Biology* vol. 55, pp. 25-38.

Whitford, W.G., Freckman, D.W., Santos, P.F., Elkins, N.Z. & Parker, L.W. 1982, 'The role of nematodes in decomposition in desert ecosystems', in Nematodes in soil ecosystems, ed. D. Freckman, pp. 98-116. University of Texas Press. Austin. Tex.

Wikström, P., Hägglund, L. & Forsman, M. 2000, 'Structure of a natural microbial community in a nitroaromatic contaminated groundwater is altered during biodegradation of extrinsic, but not intrinsic substrates', *Microbial Ecology*, vol. 39, pp. 203-210.

Wildermuth, G. B. 1980, 'Suppression of Take-all by some Australian soils', *Australian Journal of Agricultural Research*, vol. 31, pp. 251-258

Wilkinson, S.G., 1988 Gram-negative bacteria, p. 299-457 in *Microbial lipids*, C. Ratledge and S.G. Wilkinson (eds), vol. 1, Academic Press, London.

Wilson, J.R. (ed) 1987, *Advances in Nitrogen Cycling in Agricultural Ecosystems* Proc. of the Symposium of Advances in Nitrogen Cycling in Agricultural Ecosystems. Brisbane, Aust. 11-15th May 1987.

Winkler, K.A & Adams, B.J. 2004, 'Identification of trophic relations for free-living microvivorous nematodes via DNA extraction, amplification and sequencing of nematode gut contents', Abstract No. 100 in Oral Presentation Abstracts, SON Annual Meeting, Estes Park, CO, 7-11 August 2004.

Wintzingerode, F., Göbel, U.B. & Stackebrandt, E. 1997, 'Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis', *FEMS Microbiology Reviews*, vol. 21, pp. 213-229.

Wollenweber, H-W. & Rietschel, E.T. 1990, 'Analysis of lipopolysaccharide (lipid A) fatty acids', *Journal of Microbiological Methods*, vol. 11, pp. 195-211.

Wünschke, L., Brüggermann, L. & Wolfgang, B. 1995, 'Determination of substrate utilization patterns of soil microbial communities. An approach to assess population changes after hydrocarbon pollution', FEMS *Microbiology. Ecology,* vol. 17, pp. 295-306.

Yamamoto T. & Powell, G.K. 1993, '*Bacillus thuringiensis* crystal proteins: recent advances in understanding its insecticidal activity', in *Advanced Engineered Pesticides*, ed. L. Kim, Marcel Dekker Inc., New York, N.Y

Yassir, A., Lagacherie, B., Houot, S. & Spoulas, G. 1999, 'Microbial aspects of atrazine biodegradation in relation to history of soil treatment', *Pesticide Science*, vol. 55, pp. 799-809.

Yeates, G.W. & Boag, B. 2004, 'Background for nematode ecology in the 21st century', Chapter 8, pp. 406-437 in Z.X. Chen, S.Y. Chen & D.W. Dickson (editors) *Nematology, Advances and Perspectives,* Vol. 1: *Nematode Morphology, Physiology & Ecology*. CAB International, Wallingford, UK.

Yeates, G.W., Bongers, T., de Goede, R.G.M., Freckman, D.W., Georgieva, S.S. 1993, 'Feeding habits in soil nematode families and genera – An outline for soil ecologists', *Journal of Nematology*., vol. 25, pp. 315-331.

Yeates, G.W., Newton, P.C.D. & Ross, D.J. 2003, 'Significant changes in soil microfauna in grazed pasture under elevated carbon dioxide', *Biology & Fertility of Soils* vol. 38, pp. 319-326.

Zar, J.H., 1984, *Biostatistical analysis* 2nd edn, Prentice Hall, London.

Zelles, L., Bai Q.Y & Beese, F. 1992, 'Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils', *Soil Biology & Biochemistry*, vol. 24**,** pp. 317-323.

Zelles, L., 1999, 'Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review', *Biological Fertility of Soils*, vol. 29, pp. 111-129.

Zhang, W-H., Ryan, P.R. & Tyerman, S.D., 2001, 'Malate-permeable channels and cation channels activated by aluminium in the apical cells of wheat roots', *Plant Physiology*, vol. 125, pp. 1459-1472.