Effects of endophytic actinobacteria on lucerne growth and the development of its N_2-fixation symbiosis with rhizobia

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Declarations

I certify that this thesis does not contain material which has been accepted for award of any degree of diploma, and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis or in the notes.

Hoang Xuyen Le

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Abbreviations

µl, ml	microlitre, millilitre
ANOVA	Analysis of Variance
AON	Autoregualtion of Nodulation
approx.	approximately
bp	base pair
CFU	colony forming units
DNA	Deoxyribonucleic acid
exp.	experiment
hr	hour
IAA	Indole Acetic Acid
min	minute (s)
MPN	Most Probable Number
MQ water	Milli Q water
^{o}C	degree Celcius
OD_{600nm}	Optical Density 600 nanometers
ORF	Opening Reading Frame
P/O	Propylene Oxide
PCR	Polymerase Chain Reaction
PGPR	Plant Growth-Promoting Rhizobacteria
RO	Reverse Osmosis
rRNA	Ribosomal ribonucleic acid
SARDI	South Australian Research and Development Institute
SEM	Scanning Electron Microscopy
sp.	species (singular)
spp.	species (plural)
TEM	Transmission Electron Microscopy
temp.	Temperature
TLC	Thin Layer Chromatography

Abstract

Lucerne (*Medicago sativa* L.) is grown in Australia for hay and livestock production. Improving the root health of lucerne and nitrogen fixation capacity has the potential to improve its establishment, growth and persistence. Endophytic actinobacteria which colonise plant roots have been reported to increase the growth and nodulation of legumes. The main aims of this study were to isolate endophytic actinobacteria from the healthy roots of different legumes (lucerne, pea, clover and medic) and screen the well-sporulating actinobacteria for their effects on the growth and nodulation of lucerne. The antifungal and bio-control activity of endophytic actinobacteria against *Rhizoctonia solani* and *Pythium irregulare* which can cause root rot of lucerne roots were also examined.

Two hundred and twenty five endophytic actinobacteria were successfully recovered from roots and nodules of the four legumes. *Streptomyces* (56%) was the dominant genus amongst the isolates; humic acid vitamin B and tap water yeast extract were the most effective media for isolation, and at least half the isolates were obtained from plates incubated at 37°C.

The endophytic actinobacteria had positive effects on lucerne seeds germinated on agar plates and in a sandy loam soil. Forty nine of the 148 well-sporulating isolates (33%) enhanced the germination of lucerne in terms of the number of viable seedlings and improved root length on agar. These cultures appear to show host bias as 33 of the 49 were isolated from lucerne. In a sterile sandy loam soil, 22 isolates improved both germination and root length when co-inoculated with rhizobia strain RRI 128. Twelve actinobacteria which improved germination and early lucerne

growth were progressed to studies investigating their effect on lucerne growth, nodulation and nitrogen fixation in a series of pot experiments. As a result, two endophytic *Streptomyces* LuP30 and LuP47B were selected for further investigation as they increased the shoot weight by 87% and 88%, respectively, in a sandy loam medium. Shoot weight was increased by 25% and 35%, and the combined shoot and root weight by 26% and 30% by co-inoculation of *S. meliloti* RRI 128 with either LuP30 or LuP47B, respectively, in sand and vermiculite.

Streptomyces LuP30 and LuP47B were studied for their effects on lucerne growth and symbiosis at three different levels of nitrogen (3 mg, 25 mg and 50 mg NH_4NO_3 per kg soil). The actinobacteria had some effect independent of the rhizobial partner, as LuP30 increased the shoot dry weight of lucerne by 25% at 50 mg NH_4NO_3 while LuP47B increased the shoot dry weight by 30% and 23% at 25 mg and 50 mg NH_4NO_3, respectively, when applied the absence of rhizobia. Co-inoculation of either LuP30 or LuP47B with *Sinorhizobium meliloti* RRI 128 at 25 mg NH_4NO_3 per kg soil showed the greatest increases in plant growth, nodulation and symbiosis of lucerne. For example, co-inoculation of LuP30 with *S. meliloti* RRI 128 produced the largest increase in shoot weight of 46% with 25 mg NH_4NO_3. Co-inoculation with either LuP30 or LuP47B with the rhizobia increased the number of nodules by more than 100% compared with *S. meliloti* RRI128 alone after 4 weeks. In a labelled ^{15}N experiment, co-inoculation of *Streptomyces* spp. LuP30 or LuP47B has shown enhance the fixation of atmospheric nitrogen by 47% to 72%, respectively, rather than have any major effect on the uptake of soil N.

The actinobacteria has been also studied using different sources of lucerne rhizobia from two soil extracts and with two more legumes (sub-clover and serradella). The

application of either LuP30 or LuP47B to lucerne seeds inoculated with an extract of soil from Urrbrae High School that contained lucerne rhizobia increased significantly the number and mass of nodules and resulted in an increase of shoot dry weight. Shoot dry weight was increased by LuP30 and LuP47B by 16% and 36%, respectively. LuP47B significantly increased the number of nodules and the total nodule mass per plant. Moreover, LuP30 and LuP47B significantly increased the number of nodules and total nodule mass of sub-clover when they were inoculated with *Rhizobium* WSM 1325. LuP47B also increased the shoot weight and total mass per plant by 25% and 21% respectively 7 weeks after inoculation with the rhizobia.

The 148 well-sporulating cultures were evaluated for their ability to control the growth of fungal root pathogens *Rhizoctonia solani* AG8 and *Pythium irregulare in vitro.* Of the 62 cultures that were active against the *R. solani*, 4 showed strong activity, and 8 were moderately active. Twenty five cultures were active against the *P. irregulare,* of which 13 were moderately active. In the tube assay, the number of actinobacteria isolates from lucerne which reduced the damage of *R. solani* AG8 was more than double the number from the three other legumes; 47 isolates (32 from lucerne and 15 from other legumes) showed efficacy in reducing the damage symptoms associated with *R. solani* AG8. Six isolates (LuP10, LuP30, LuP44, LuP46B, LuP47B and LuP73B) of the 21 tested in pot assay increased by 34% to 47% the total weight of plants that had been inoculated with both rhizobia and the root pathogen *R. solani*.

All six isolates showing bio-control activity on lucerne were isolated from lucerne roots and have been identified as *Streptomyces* spp. by 16S rRNA gene sequencing. LuP30 and LuP47B increased the plant growth, nodulation and nitrogen fixation of

lucerne plants, and also reduced the root damage caused by *R. solani* AG8. Based on a full polyphasic taxonomic evaluation in which they were compared in side-by-side comparisons with their two closest type cultures, the conclusion is that both LuP30 and LuP47B are proposed as new species.

Endophytic actinobacteria isolated from roots and nodules of legumes have shown potential for use as microbial inoculants for improving the growth and symbiosis of lucerne. Careful screening and selection of endophytic actinobacteria has provided the most beneficial candidates for co-inoculation with different rhizobial partners and hosts. Field studies are needed to establish if the benefits measured in these studies are confirmed under conditions of more complex micro-flora interactions.

Chapter 1

Introduction and Literature review

1.1 Global importance of legumes and nitrogen fixation

1.1.1 General information on legumes

There are between 44 to 66 million tonnes of nitrogen fixed from atmospheric nitrogen by symbioses of *Rhizobium* and legumes every year. This amount is nearly half of the nitrogen used in agriculture around the world (Giller, 2001). Legumes are important plants for both animals and human beings. They provide essential proteins for people and animals as well as fix nitrogen from the air. Legumes are grown around the world in almost all countries with India, China, Brazil, Canada and Australia, being the major producers. Every year, the world produces around 60 million tonnes of legume grain harvested from about 70 million ha (Table 1.1) (FAO, 2010). If a comparison is made with 1986, the average gain yield of legumes was around 807 kg per ha and the total production of legumes is around 55 million tonnes (ACIAR Proceedings, 1986 cited in (Nieuwenhuis and Nieuwelink, 2005)). The average yield of legumes increased to 857 kg per ha in 2010.

Table 1.1 Production and area harvested of legumes in the world from 1999 to 2009

FAO Statistical Yearbook 2010	**AREA HARVESTED (1000 ha)**					**PRODUCTION (1000 tonnes)**				
COUNTRIES	**1999-2001**	**2003-2005**	**2007**	**2008**	**2009**	**1999-2001**	**2003-2005**	**2007**	**2008**	**2009**
World	66,400	70,570	74,213	71,807	70,598	56,248	59,974	60,594	61,498	61,506
Australia	2,188	1,698	1,683	1,512	1,449	2,616	2,200	1,545	1,705	1,806
Brazil	4,011	3,977	3,825	3,826	4,193	2,788	3,118	3,189	3,486	3,548
Canada	2,188	2,100	2,352	2,456	2,609	3,754	3,909	4,181	4,959	5,196
China	3,461	3,376	3,030	2,996	2,874	4,843	5,350	4,483	4,897	4,229
India	19,998	22,305	24,616	22,672	20,400	13,676	13,053	15,088	14,245	13,730
Myanmar	2,350	3,028	3,316	3,316	3,316	1,741	2,858	3,551	3,551	3,551
Niger	3,320	3,484	4,867	5,352	5,352	426	514	1,037	1,593	1,593
Nigeria	3,692	4,071	4,621	4,419	4,419	2,193	2,685	2,853	2,969	2,969
Others	25,192	26,532	25,904	25,258	25,986	24,211	26,287	24,667	24,094	24,885

Source: (FAO, 2010)

In Australia, legumes are grown in all states for different purposes such as livestock production and grain for export. The harvested area of pulses in 2009 was about 1.5 million ha, and the production about 1.8 million tonnes. It means that the yield per ha was about 1.2 tonnes per ha (FAO, 2010), which was much higher than the world average.

Legumes provide a range of essential nutrients including protein, low glycaemia carbohydrates, dietary fibre, minerals and vitamins (Munro, 2007). They are a good source of B vitamins, iron, zinc, calcium, magnesium, omega-3 fats and are rich in phytonutrients that can potentially reduce the risk of cancer, other chronic diseases (Lin and Lai, 2006), heart disease, osteoporosis and problems associated with menopause (Anderson *et al.*, 1999; Messina *et al.*, 2004). Legumes have about twice the protein content of cereal grains; generally low in fat and have no cholesterol. Soybeans and peanuts are the exception, with significant levels of mostly mono and polyunsaturated fatty acids, including alpha-linolenic acid (Williams, 2010).

1.1.2 Lucerne globally and in Australia

Lucerne is cultivated on about 35 million ha over 80 countries across the globe (Radovic' *et al.*, 2009), and is a valuable and important crop in Australia. It provides high quality forage for livestock grazing, silage, making hay and significant environmental benefits attributed to its deep root system and ability to reduce water leakage in farming systems (Peoples and Baldock, 2001; Robertson, 2006). Every year, lucerne is grown for hay production on about 200,000 ha, producing about 1 million tonnes that is mainly used for intensive livestock production (Australian

Bureau of Statistics). In 2001, the estimated value of lucerne hay was about $181 million and revised to $336 million in 2003 (Table 1.2) (Lattimore, 2008). It is also grown over approximately 3 M ha in extensive pasture systems.

There are many diseases affecting lucerne establishment, persistence and production. They are caused by fungi, viruses and mycoplasma. Common oomycetes diseases such as damping off (*Pythium* sp.), root rot (*Phytophthora* sp.) or a fingal disease, root canker (*Rhizoctonia solani*), significantly reduce growth and yield of lucerne (Lattimore, 2008).

Table 1.2 Australian lucerne hay production 2002

State	Area (ha)	Production (tonnes)	Average annual yield (tonnes/ha)
New South Wales	94390	389981	4.1
Victoria	39,124	215,607	5.5
Queensland	25,196	193,713	7.7
South Australia	30,741	101,663	3.3
North Australia	8,000	36,000	4.5
Western Australia	5,633	24,105	4.3
Tasmania	3,040	3,040	5.4
Australian Capital Territory	278	1,000	3.6
Australia	**206,124**	**977,538**	**4.7**

Source: Australian Bureau of Statistics 2002 cited in (Lattimore, 2008)

1.2 Importance of soil micro-flora to legume production

1.2.1 Rhizobia and nitrogen fixation

1.2.1.1 Rhizobium spp.

The genus *Rhizobium* was initially defined by the ability of these organisms to induce nodule formation in legumes (Baldwin and Fred, 1929), with species recognition biased toward agronomical important hosts (Graham, 2008). Rhizobia

are bacteria which have the ability to fix nitrogen in symbiosis with leguminous plants. However, nitrogen is only fixed when rhizobia form an endophytic symbiosis with leguminous plants (Sally, 2002), not when they are free living. Rhizobia are described as rod shaped (0.5-1.0 x 1.2-3.0µm), aerobic, gram negative bacteria and do not form spores. The optimal temperature for growth of rhizobia is between 25 to 30 degrees Celsius, but some species can grow at higher temperatures up to 40 degrees Celsius. Rhizobia can grow at a pH range of 4 to 10 but 6 to 7 is the optimal pH for their growth. The rhizobia for lucerne produce colonies that are usually white or beige, circular, convex, semi-translucent or opaque. They grow best on Yeast Mannitol Agar (YMA) producing colonies of 2 to 4 mm in diameter after three to five days growth (Kuykendall *et al.*, 2005). The rhizobia have large and complex genomes which range in size from 6.5 Mb (*Sinorhizobium* spp.) to 9.0 Mb (*Bradyrhizobium* spp.) (Kaneko *et al.*, 2000).

1.2.1.2 The nodulation process

Symbiosis of legumes and rhizobia is a selective process and highly specific interaction between the two partners (Sharma *et al.*, 1993). Individual rhizobia species have a distinct host legume plant allowing nodulation and nitrogen fixation to occur. However, *Rhizobium* sp. strain NGR234 is an exception which has a broad host-range and can nodulate 353 legume species representing 122 genera (Pueppke and Broughton, 1999) cited in (Stougaard, 2000).

The process of nodule formation is complex and involves some main steps: attachment root hair curling, formation of nodule meristem, infection thread formation, nodule organogenesis, bacteroids development and nitrogen fixation

(Figure 1.1) (Glloudemans and Bisseling, 1989; Sharma *et al.*, 1993). An important step in the nodulation process is attachment of rhizobia to root hairs of host plant. In general, rhizobia infect the host plants through the root hairs not in the body of epidermis cells. However, bacteria can also enter the plant through cracks or middle lamellae of legume roots (Caetano-Anolles and Gresshoff, 1991). In the second step, additional bacteria adhere to the root hair bound rhizobia, leading to the formation of bacterial aggregates at the root-hair tip. This step involves plant lectins (Smit *et al.*, 1987; Vesper and Bauer, 1986) cited in (Michiels and Vanderleyden, 1994) which are essential for infectivity as they are also involved in formation of tubular structures called infection threads (Sharma *et al.*, 1993). Mitotic activity is induced in the terminally differentiated root cortex during the development of the infection threads (Callaham and Torrey, 1981; Vijn *et al.*, 1993). Then, rhizobia are released into the plant cytoplasm of individual cells by infection thread branches. Inside the root cells, the bacteria continue to differentiate and synthesize proteins and compounds which are required for the nitrogen fixation processes and the maintenance of the mutualistic partnership. The plant plasma membrane, peribacteroid membrane which surrounds and converts the infected cells into bacteriods and begin symbiotic nitrogen fixation (Gage, 2004; Sharma *et al.*, 1993).

Different steps of the nodule development process require exchanges of signals between host legumes and rhizobia (Crespi and Ga'lvez, 2000; Schultze and Kondorosi, 1998; Spaink, 1996). At an early stage, plant host secretes into the rhizosphere different compounds such as (iso) flavonoids, stachydrines, or aldonic acids (Stougaard, 2000). Flavonoids are considered crucial for initiating the symbiotic programme in the bacterial partner and have multiple roles during legume

nodulation (Cooper, 2007; Moscatiello *et al.*, 2010; Reddy *et al.*, 2007). Different plants secrete different flavonoid inducers, and a single plant may make different inducers at different times during development. Besides their role in nod genes induction, some flavonoids cause growth enhancement of rhizobium (Fisher and Long, 1992).

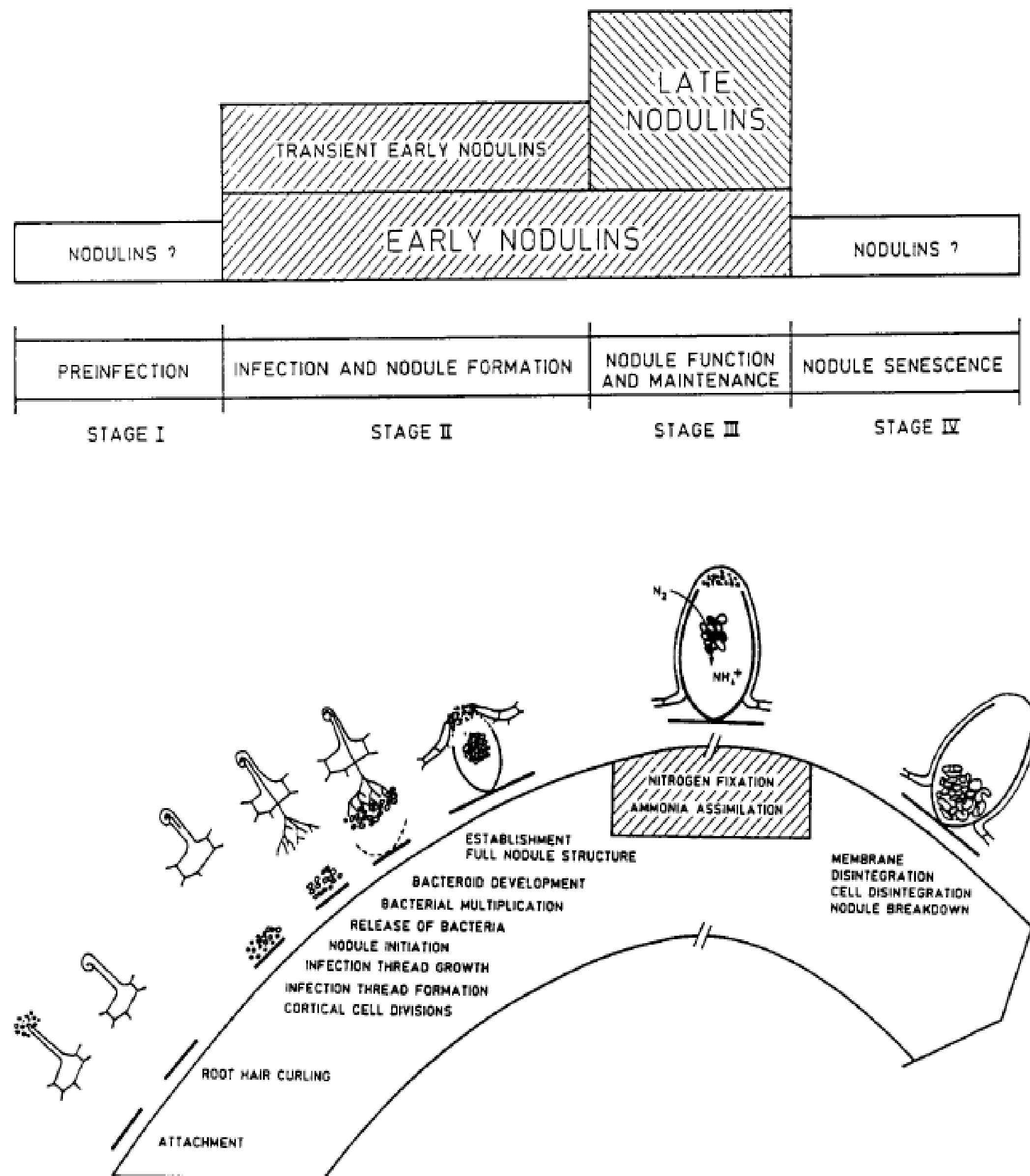


Figure 1.1 Different stages and sequence of events during nodule formation and nitrogen fixation in root nodule of legumes. Source: (Glloudemans and Bisseling, 1989)

Similarly to other soil saprophytes, rhizobia are chemotactic towards sugars, amino acids, unfractionated legume epidermal exudates, other nutrients and also to many individual flavonoids (Cooper, 2007). In the legume root rhizosphere rhizobia come under the influence of chemotactic, growth-promoting compounds and their combined effects to increase root colonization (Cooper, 2007), and only the correct flavonoid/s can induce symbiotic gene expression in a particular *Rhizobium* strain (Spaink, 1995). Rhizobia respond to the flavonoids by inducing *nod* genes which encode approximately 25 proteins required for the bacterial synthesis and export of Nod factors (Gage, 2004) which in turn influence regulation of many plant genes (Spaink, 1995).

Nod factors are essential signals for rhizobia to penetrate legume roots and in symbiotic development (Relic *et al.*, 1994). They elicit a range of responses from the plant, including: deformation of root hairs, plasma membrane depolarization, rapid fluctuations in levels of intracellular free calcium in root hairs (known as calcium spiking), alterations in the root hair cytoskeleton, pre-infection thread formation in deformed root hairs, cortical cell division at the sites of nodule primordial, inhibition of the reactive oxygen generating system, perturbation of auxin flow in roots (in conjunction with flavonoids), induction of plant genes (nodulins) at the pre-infection, infection, nodule development and nodule function stages of the symbiosis (Cooper, 2007; Gage, 2004).

Several other compounds are produced by rhizobia, some of which may be required for the successful progression from root colonization, a functioning root nodule or enhancement of plant growth prior to the onset of nitrogen fixation (Cooper, 2007).

Hopanoids, AHL (acylhomoserine lactone), bradyoxetin, lumichrome and phythohormones are synthesized by several rhizobia and could be involved in nodulation and nitrogen fixation process. Kobayashi et al. (2004) reported that hopanoid synthesis genes in *Rhizobium* sp. NGR234 are expressed in a flavonoid (daidzein), NodD1-dependent manner via nod box NB1, which could indicate a symbiotic function for these compounds. In rhizobia AHL, known as quorum sensors are responsible for inducing expression of genes encoding products that are required for host colonization and invasion. In addition to being able to influence rhizobial gene expression AHL can also elicit changes in protein accumulation in at least one legume host *Medicago truncatula* (Mathesius *et al.*, 2003). Bradyoxetin, a non-AHL quorum sensor, produced in *B. japonicum*, represses *nod* gene expression as population density in the rhizosphere increases (Loh *et al.*, 2002). Lumichrome, a riboflavin degradation product, produced by *S. meliloti*, enhances root respiration and improves the growth of *M. sativa* prior to the onset of nitrogen fixation (Matiru and Dakora, 2005; Phillips *et al.*, 1999) cited in (Cooper, 2007). Rhizobia also produce some phythohormones such as auxin, cytokinins, and gibberellins which are involved in nodulation (Glloudemans and Bisseling, 1989).

Nodulation genes

The development of symbiosis of rhizobia and legumes is a very complex process and controlled by various genes in the bacterium as well as in the plant. Molecular, genetic and cell biology studies have revealed three major independent series of events: (1) attachment of bacteria to the root hairs, followed by root hair deformation and curling; (2) formation and growth of infection threads in which the bacteria

multiply; and (3) induction of meristematic activity in the root cortex, leading to nodule development which culminates in N_2-fixing symbiosis (Megi'as *et al.*, 1993). Some genes of rhizobia involved in the process are *nod, nif* and *fix* genes, while host plants also have genes that are specific for nodulation called nodulin genes including early and late nodulin genes.

Rhizobial genes

A number of bacterial genes considered as nodulation genes are required for or involved in nodulation of legume hosts (Sharma *et al.*, 1993). Genomic organization of clusters of symbiotic nitrogen fixation genes can be divided into *nod*, *nif*, and *fix* genes (Fisher and Long, 1992). Later, Megi'as *et al.*, (1993) has grouped the bacterial genes involved in the nodulation process into three groups are: (1) the *nod* genes (genes involved in host recognition and nodule formation) and also *nol* genes (for nodulation related); (2) *ezo* genes, encoding exopolysaccharides, and lipopolysaccharide-encoding genes; and (3) *fix* and *nif* genes, involved in carrying out and supporting N_2 fixation. On the other hand, bacterial genes involving in the nodulation process have been grouped into two classes. One class includes several sets of genes involved in the formation of the bacterial cell surface, such as genes determining the synthesis of exopolysaccharides (*exo* genes), lipopolysaccharides (*lps* genes), capsular polysaccharides or K antigens, and b-1,2-glucans (*ndv* genes) (Breedveld and Miller, 1994; Gottfert *et al.*, 1992; Leigh and Coplin, 1992; Reuhs *et al.*, 1993). The second class consists of the nodulation (*nod* or *nol*) genes. In summary, *nod*, *nif* and *fix* are major genes of rhizobia involved in and studied intensively in the symbiosis of rhizobia and legumes.

Plant genes

The development of a legume nodule is accompanied by the expression of nodule-specific plant genes called nodulin genes in different steps of the process (Franssen *et al.*, 1992; van Kammen, 1984). Few genes that are involved in nodulation and nitrogen fixation have been studied in legumes (Nap and Bisseling, 1990; Verma, 1989; Verma and Fortin, 1989). Nodulin genes that are expressed during the early stages of nodule development are named early nodulin genes (or *ENOD*) genes whose transcripts were detected before nitrogen fixation starts, and late nodulin genes whose expression is induced during or after the nitrogen fixation process. The early genes are supposedly involved in infection thread formation and nodule organogenesis (Bladergroen and Spaink, 1998; Franssen *et al.*, 1992).

The expression of late nodulin genes starts around the onset of N_2 fixation. These genes are probably involved in nodule maintenance and functioning (Megi'as *et al.*, 1993). Apart from these nodulin genes, others related to nitrogen fixation and assimilation have been detected, such as sucrose synthase, carbonic anhydrase, and aspartate amino transferase (Shi *et al.*, 1997; Vance and Gantt, 1992). These latter genes are mainly expressed in the symbiotic zone and induced late in nodule development except for carbonic anhydrase whose transcripts accumulate specifically in the inner cortex cells (Coba de la Pena *et al.*, 1997); the same cells involved in controlling oxygen permeability. Other nodulins are leghemoglobins, constituents of the peribacteroid membrane (for example, Nod 26), peptide transporters, and a cytochrome P450 (Szczyglowski *et al.*, 1997) cited in (Crespi and Ga'lvez, 2000). Leghemoglobin, involved in protection of nitrogenase from

oxygen, is the classical example of this class of "late" nodulins (Cvitanich *et al.*, 2000).

1.2.1.3 Lucerne symbiosis

Lucerne, like all legumes, converts nitrogen from the air with the symbiosis due to the rhizobial partner, *Ensifer* "*Sinorhizobium*" *meliloti* and *Sinorhizobium medicae*. Successful inoculation of lucerne is important for optimum establishment (Gault *et al.*, 1995). The amount of fixed N depends on presence, density and effectiveness of *Sinorhizobium* spp. and on the soil type (Radovic' *et al.*, 2009). Lucerne is one of the best nitrogen fixation legumes (Angus, 2001); estimates of N_2 fixation by lucerne vary from 50 to 463 kg per hectare per year (average 200 kg) (Radovic' *et al.*, 2009).

1.2.2 Effects of other soil microorganisms on plant growth and nitrogen fixation by legumes

Many studies have been carried out to improve nodulation, nitrogen fixation process and plant growth of different legumes by using non-actinobacterial microorganisms such as fungi or bacteria (Mycorrhizae, *Trichoderma* and *Pseudomonas*)

1.2.2.1 Mycorrhiza spp.

Combinations of *S. meliloti,* arbuscular mycorrhizae (AM) and lucerne can improve the shoot dry weight and biological nitrogen fixation in organic farming under dry conditions. Arbuscular mycorrhizal fungi have fundamental effects on the ecophysiology of nodulated legumes (Bethlenfalvay and Newton, 1991). Inoculating lucerne in acid soils with *Rhizobium* and mycorrhiza has been shown to improve plant growth in glasshouse experiments (Guo *et al.*, 2010). Triple inoculation also

exhibited significant positive response on growth of *E. saligna* seedlings (Parkash and Aggarwal, 2011). The main effect of AM on rhizobia activity and nitrogen fixation is phosphate-mediated, and not much information is known about direct interactions between the AM and rhizobia. Arbuscular mycorrhizal endophytes improve the efficiency of phosphorus uptake and the efficiency of nitrogen fixation is dependant on adequate phosphorus availability. Therefore, the mycorrhizal fungi can play an important role in associative-symbiotic nitrogen fixation, especially in phosphorus deficient soils (Hayman, 1986; Mikola, 1986). Conversely, the rhizobia may influence the mycorrhizal establishment by producing polysaccharides which lead to increased synthesis of polygalacturonase at the infection site (Hayman, 1986). A strain AM increased yield and nitrogen fixation of lucerne when they applied with the *Sinorhizobium meliloti* partner (Ardakani *et al.*, 2009).

Several soybean flavonoids which accumulate in response to the acetylated Nod factors of rhizobia show a similar promoting effect on *Mycorrhiza*. These results suggest that plant flavonoids mediate the Nod factor-induced stimulation of mycorrhizal colonization in soybean roots (Xie *et al.*, 1995). Steinkellner *et al.* (2007) found that strigolactones are specific signaling compounds for the AM-plant interaction and are not involved in similar signaling events in other plant-fungus interactions.

1.2.2.2 Trichoderma spp.

Trichoderma spp. have been widely used as antagonistic fungal agents as well as plant growth enhancers (Verma *et al.*, 2007). The combination of *Rhizobium* spp. and *Trichoderma* spp. has the potential ability of controlling the fungi which cause the damping off and root rot in legume field crop *of Vicia fabae, Cicer arietinum* and *Lupines terms*. This resulted in an improvement in many plant growth parameters such as branches per plant, pods per plant, seeds per pod, mean seed weight and increased seed yield of the legume field crop broad bean, chickpea and lupine plants (Shaban and El-Bramawy, 2011). A combined application of *Rhizobium* and *Trichoderma harzianum* (ITCC – 4572) showed a decrease in stem rot disease of groundnut (*Arachis hypogaea* L.) (Ganesan *et al.*, 2007).

Trichoderma showed growth promoting activity in soybean plants with bio-control activity in a pot assay. The height of *Pythium* and *Fusarium* infected plants treated with *Trichoderma* was about 194% and 141% higher respectively, while the fruit yield was 5 and 1.6 times higher respectively than plants infected with pathogens alone (John *et al.*, 2010). Therefore, an application of inocula based on a mixture of *Rhizobium* and *Trichoderma* has provided encouraging results and shows their potential as bio-control agents as well as for plant growth promotion.

1.2.2.3 Pseudomonas spp.

The combination of some *Pseudomonas* species and rhizobia can promote plant growth and control pathogens of legumes. For example, co-application of either two strains *Pseudomonas putida* SP21 or SP22 with *Bradyrhizobium japonicum* TIIIB

significantly increased the growth of soybean under greenhouse conditions (Rosas *et al.*, 2006). The combined bio-inoculation of two fluorescent *Pseudomonas* R62 and R81 in a formulation increased the pod yield of *Vigna-mungo* by 300% in comparison to the control crop under field condition. There was also significant increase in terms of dry root weight, dry shoot weight, shoot length and number of branches per plant with treatment by two *Pseudomonas* species treatment (Sarma *et al.*, 2009).

Co-inoculation of some *Pseudomonas* species with rhizobial partners increased the number of nodules and nitrogen content of legumes. Fox *et al.* (2011) reported that *Pseudomonas fluorescens* WSM3457 significantly increased the nodulation and symbiotic effectiveness of *Medicago truncatula* by the total number of nodules and total N per plant co-inoculated with *E. medicae* WSM419 at low concentration (10^3 cells/plant). *Pseudomonas trivialis* 3Re27 the cellulase producing strain significantly increased nodule numbers and nitrogen content of the co-inoculated plants fodder galega (*Galega orientalis*) (Egamberdieva *et al.*, 2010). The production of IAA (Indole Acetic Acid) and/or cellulose by *Pseudomonas* strains may contribute to such positive effects and act as “*Rhizobium* helper bacteria” improving bean growth properties such as shoot and root fresh/dry weights (Egamberdieva *et al.*, 2010; Samavat *et al.*, 2011).

1.2.3 Endophytic actinobacteria

1.2.3.1 Definition of endophyte

Hasegawa *et al.* (2006) summarised and defined the term of “endophyte” as a microorganism that resides inside plant tissues without causing any visible harm and

can be culturable from surface sterilized plant tissues. Symbiotic associations between microorganisms and plants are fundamental to biodiversity, and many examples of complex and highly specific symbioses between plants and microbes have been described. Promising endophytic microbes have been applied as biological control agents, sources of novel metabolites for medicine, agriculture, and industrial uses (Bacon and James, 2000; Strobel and Daisy, 2003). There are nearly 300,000 described plant species, and it is claimed that each one is likely to be host to one or more types of endophytes. However, only a few plant species have ever been completely studied in terms of their association with endophytes (Strobel and Daisy, 2003).

1.2.3.2 General characteristics of actinobacteria and endophytic actinobacteria

The term 'Actinomycetes' was first used in 1890 by Gasperini to describe bacteria that have the ability to form a mycelium consisting of narrow hyphae (about 1µm diameter). In suitable conditions the mycelium sometimes produces spores of similar dimension. In nature, actinobacteria have different lifestyles such as plant commensals (*Leifsonia* spp.), nitrogen-fixing symbionts (*Frankia*), plant pathogens (*Streptomyces scabies*) and gastrointestinal tract (GIT) inhabitants (*Bifidobacterium* spp.) (Goodfellow and Williams, 1983). Actinomycetes are common in soil and produce a broad range of secondary metabolites, antibiotics and extracellular enzymes. They can protect plants against plant diseases as well as promote plant growth (Doumbou *et al.*, 2001).

1.2.3.2.1 Plant growth promotion properties

Cattelan *et al.* (1999) claimed four possible mechanisms by which microbes could be involved in plant growth promotion: (a) produce or affect the amount of the plant hormones, IAA, gibberellic acid, cytokinins, and ethylene; (b) fix N_2; (c) produce siderophores, β-1,3 glucanase, chitinase, antibiotics, and cyanide which could protect against phyto-pathogenic microorganisms; and (d) solubilise of mineral nutrients, for example, phosphates.

Several recent studies have found that endophytic actinobacteria produced plant growth-promoting compounds such as IAA and siderophores *in* (de Oliveira *et al.*, 2010; Ghodhbane-Gtari *et al.*, 2010; Nimnoi *et al.*, 2010). For example, Nimnoi *et al.* (2010) found that ten isolates (100%) produced IAA and eight of them produced siderophores. Similarly, fifteen rhizobacterial isolates were able to produce indole acetic acid (IAA), and six isolates (40%) were able to solubilize insoluble phosphate as evident by production of clear zone on calcium phosphate medium. Three of the isolates produced fluorescent pigment on agar plate indicated their abilities to produce siderophores (Yasmin *et al.*, 2009). Thirty-six actinobacteria of 445 isolates showed abilities to produce indole-3-acetic acid (IAA), and 75 isolates produced siderophores on chrome azurol S (CAS) agar. A collection of *Streptomyces* spp. isolated from the rhizosphere soils of 14 Thai medicinal plants were found to produce the plant growth hormone indole-3-acetic acid (IAA) in a yeast malt extract medium supplemented with 2 mg/mL L-tryptophan. *Streptomyces* CMU-H009 recovered from soil associated with lemongrass (*Cymbopogon citratus*) was very effective in producing IAA (Khamna *et al.*, 2010). *Streptomyces* sp. CMU-MH021

had high activity against tested fungi and high production of IAA (28.5 $\mu g.ml^{-1}$) and siderophores (26.0 $\mu g.ml^{-1}$) production (Ruanpanun *et al.*, 2010).

1.2.3.2.2 Antifungal activity

Studies on the mechanisms of antibiosis of actinobacteria have focused on *in vitro* assays (Crawford *et al.*, 1993; Getha *et al.*, 2005; Yuan and Crawford, 1995) which are used in screening of microbial antagonists (Getha *et al.*, 2005; Yuan and Crawford, 1995). In routine screening tests, antibiosis is determined by pairing colonies on agar plates (Cooper and Chilton, 1950; Johnson and Curl, 1973). Nutrient-rich media are often used for the *in vitro* assays to encourage antibiotic production although the availability of nutrients has a significant effect on the antagonistic activities of the microorganisms screened (Sivasithamparam and Parker, 1980; Whipps, 1987). In general, antifungal ability of actinobacteria can be tested by screening in nutrient media plates.

Actinobacteria isolated from various organs of different plants are antagonistic against plant pathogens *in vitro*. Twenty four of 131 endophytic actinobacteria strains isolated from surface-sterilized leaves and roots of banana inhibited the growth of pathogenic *Fusarium oxysporum* f. sp. cubense on banana tissue extract medium (Cao *et al.*, 2004; Cao *et al.*, 2005). Zhao *et al.* (2011) found that some actinobacteria isolated from medicinal plants can inhibit growth of pathogens *in vitro* assays. They concluded that medicinal plants are a potent source of endophytic actinobacteria with wide biological activity against pathogenic fungi as well as Gram-positive and Gram-negative bacteria. Mingma *et al.* (2014) found that sixty four isolates (20.2%) from roots and rhizospheric soils of leguminous plants

inhibited the growth of soybean pathogen *Xanthomonas campestris* pv. *glycine in vitro*.

1.2.3.3 Occurrence and diversity of endophytic actinobacteria in plants

Actinobacteria have been isolated successfully from a wide variety of plants including crop plants, medicinal plants and woody plants (Table 1.3). Where endophytic actinobacteria have been isolated from plant tissues using different culture media-dependent methods, *Streptomyces* and *Microbispora* species are most frequently isolated (Rosenblueth and Romero, 2006; Takahashi Y and Omura, 2003). Inderiati and Muliani (2008) reported that the majority of endophytic actinobacteria isolated from tobacco plants were classified as species of *Streptomyces*. However, Kizuka *et al.* (1998) noted that *Microbispora* spp. were isolated more frequently from plant leaves than soil, suggesting that they could have mutualistic associations with plants. The genera *Streptomyces* and *Microbispora* are both soil-inhabiting and endophytes with plants.

The majority of endophytic actinobacteria have been isolated from roots rather than other organs (Verma *et al.*, 2009; Zin *et al.*, 2010). The culturable population density of endophytic actinobacteria has been estimated in cucumber and lupin roots as approximately 10^5 CFU/g fresh root weight (El-Tarabily, 2003; El-Tarabily *et al.*, 2009). The diversity of actinobacteria is also relatively broader in roots (Shimizu, 2011). The relatively larger population density and broader diversity of endophytic actinobacteria in roots may be a function of their close proximity to the soil-inhabiting actinobacteria (Zinniel *et al.*, 2002). Conn and Franco (2004) demonstrated that the density and diversity of endophytic actinobacteria in wheat

roots detected by terminal restriction fragment length polymorphism (T-RFLP) was closely related to that of soil microflora. Several other factors such as cultivars and physiological status of plants may also influence the population and diversity of endophytic actinobacteria found in plant tissues (Conn and Franco, 2004).

Table 1.3 Examples of endophytic actinobacterial genera described within the past 10 years

Plant species	Actinobacterial taxa[a]	References
Crop plants		
Triticum aestivum	*Streptomyces, Microbispora, Micromonospora, Nocardioides*	Coombs and Franco (2003)
Cucumis sativus	*Streptomyces*	Shimizu *et al.* (2009)
Lupinus termis	*Actinoplanes*	El-Tarabily (2003)
Zea mays	*Microbispora, Streptomyces, Streptosporangium*	de Aráujo *et al.* (2000)
Lycopersicon esculentun	*Streptomyces, Streptoverticillium, Nocardia*	Cao *et al.* (2004)
Oryza sativa	*Streptomyces, Nocardioides*	Tian *et al.* (2007)
Brassica campestris	*Microbispora, Streptomyces, Micromonospora*	Lee *et al.* (2008)
Musa acuminate	*Streptomyces, Actinomadura, Streptoverticillium, Streptosporangium, Nocardia*	Cao *et al.* (2004)
Woody plants		
Taxus spp.	*Streptomyces, Micromonospora,* Nocardioforms, *Actinoplanes, Actinomadura, Kitasatospora,*	Caruso *et al.* (2000)
Acacia auriculiformis	*Actinoallomurus*	Thamchaipenet *et al.* (2010)
Rhododendron sp.	*Streptomyces*	Shimizu *et al.* (2000)
Kalmia latifolia	*Streptomyces*	Nishimura *et al.* (2002)
Aquilaria crassna	*Streptomyces, Nonomuraea, Actinomadura, Pseudonocardia, Nocardia*	Nimnoi *et al.* (2010)
Eucalyptus calmuldulensis; Eucalyptus microcarpa; Pittosporum angustifolus; Callistris preussii	*Streptomyces* and 17 other genera	Kaewkla and Franco (2013)
Medicinal plants		
Sambucus adnata	*Glycomyces*	Gu *et al.* (2007)

Alpinia crassna	*Streptomyces, Nocardia, Microbispora, Micromonospora*	Taechowisan *et al.* (2008)
Kennedia nigricans	*Streptomyces*	Castillo *et al.* (2002)
Maytenus austroyunnanensis	*Saccharopolyspora, Actinomadura*	Qin *et al.* (2008) Qin *et al.* (2009)
Thotttea grandiflora	*Streptomyces*	Ghadin *et al.* (2008)
Azadirachta indica	*Streptomyces, Streptosporangium, Microbispora, Streptoverticillium, Saccharomonospora, Nocardia*	Verma *et al.* (2009)
Others		
Monstera sp.	*Streptomyces*	Ezra *et al.* (2004)
Paphiopedilum appletonianum	*Streptomyces*	Tsavkelova *et al.* (2007)
Legumes		
Lupinus angustifolius	*Kribbella lupini*	Trujillo *et al.* (2006)
	Micromonospora lupini	Trujillo *et al.* (2007)
Leguminous plants	*Streptomyces*	Mingma *et al.* (2014)

[a]Genera are listed in order of reported abundance
Source: (Shimizu, 2011)and updated publications

1.2.3.4 Evidence of actinobacterial effects on agricultural plants

1.2.3.4.1 Non legumes

Endophytic actinobacteria are plant growth promoters (Shimizu, 2011). El-Tarabily *et al.* (2009) reported that some strains of endophytic actinobacteria produced IAA and IPYA (indole-3-pyruvic acid) that enhanced growth of cucumber plants significantly. The ability of streptomycetes to promote growth of tomato plants through the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase was reported under greenhouse conditions by El-Tarabily (2008). Meguro *et al.* (2006) reported an endophyte *Streptomyces* sp. MBR-52 that accelerated emergence and elongation of plant adventitious root. Positive effects of endophytic

actinobacteria have also been measured in non-legumes and associated with the colonisation of the internal parts of the plant and the production of a range of secondary metabolites which influence plant growth and/or protect the plant from pathogens (Coombs and Franco, 2003; Coombs *et al.*, 2003). A number of endophytic strains isolated from wheat plants have been shown to be effective in protecting wheat from fungal root pathogens in the field in Australia (Franco *et al.*, 2007).

Actinobacteria have been found to act as bio-control agents in various plants. Several plants such as pea, rice, raspberry, cotton seeds, wheat, lettuce seeds, banana, chilli, sugar beet, cucumber and medicinal plants were used to isolate endophytic actinomycete and/or test antifungal activity against pathogens of the host plants. Coombs *et al.* (2004) reported that actinobacteria isolated from healthy cereal plants included a number of *Streptomyces*, as well as *Microbispora* and *Nocardioides* spp. and were also able to control the development of disease symptoms treated plants exposed to *Gauemannomyces graminis* var. *tritici* and *Rhizoctonia* in the field soil. Eleven actinomycete strains that were previously shown to protect raspberry *(Rubus strigosus)* plants against *Phytophthora* infection were characterized (Toussaint *et al.*, 1997). Errakhi *et al.* (2009) found two strains *Streptomyces* J-2 and B-11 that showed potential for controlling root rot on sugar beet and against a diverse range of soil borne plant pathogens. Three endophytic actinobacteria strains *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* significantly reduced the impacts of *Pythium aphanidermatum* causing seedling damping-off, and root and crown rots of mature cucumber (*Cucumis sativus*) (El-Tarabily *et al.*, 2010).

1.2.3.4.2 Legumes – responses without delving into mechanisms

Actinobacteria have shown beneficial effects on nodulation and plant growth of legumes. The combination of *S. kanamyceticus* and *Bradyrhizobium japonicum* increased nodule occupancy and shoot nitrogen composition of soybean up to 55% and 41%, respectively (Gregor *et al.*, 2003). Similarly, the co-inoculation of soybean with *Streptomyces* sp. (Soe *et al.*, 2012) or *Nocardia* sp. (Nimmnoi *et al.*, 2014) and *Bradyrhizobium japonicum* has improved the growth of soybean plants. Tokala *et al.* (2002) found that the *Streptomyces lydicus* WYEC108 had a significant effect on *Rhizobium* spp. and the legume, *Pisum sativum,* by increasing the number of nodules, and height and weight of the shoot. They concluded that the *Streptomyces* was probably involved in one of the mechanisms of colonization and nodulation on pea. Studies specific to lucerne have shown improved growth associated with the application of *Micromonospora* spp. (Martínez-Hidalgo *et al.*, 2014) alone or as a co-inoculant with *Sinorhizobium* (syn. *Ensifer*) *meliloti* strain 1021. Solans *et al.* (2009) found that some actinobacteria isolated from the root nodule surface of *Discaria trinervis* promoted nodulation in the *Medicago sativa- Sinorhizobium meliloti* symbiosis in the presence of high nitrogen. Inoculation with antibiotic-producing *Streptomyces* at planting significantly reduced population densities of the root-lesion nematode population densities in roots of alfalfa varieties grown in either heat-treated or untreated soil (Samac and Kinkel, 2001). Misk and Franco (2011) reported that endophytic *Streptomyces* spp. either from wheat or legumes effectively suppressed *Phytophthora medicaginis* root rot on chickpea (*Cicer arietinum* L.).

1.2.3.5 Potential mechanisms of action

1.2.3.5.1 Direct contributions to N_2-fixation –Frankia plant associated actinobacteria

Frankia is defined as the N_2-fixing micro-symbiont of actinorhizal plants (Wall, 2000). The actinobacteria *Frankia* is a soil actinobacteria that can induce the formation of nitrogen-fixing root nodules on a diverse group of angiospermous plants and fixes atmospheric nitrogen symbiotically with its specific host plants in 24 genera within eight families (Chaia *et al.*, 2010; Chaia *et al.*, 2010; Pawlowski, 2009) but can also survive as free-living soil bacteria (Benson and Silvester, 1993; Chaia *et al.*, 2010). These actinobacteria have three morphological forms including: vegetative hyphae (or filaments), spores located in sporangia, and unique lipid-enveloped cellular structures called vesicles. Hyphae are typically 0.5 µm thick, septate and branched while vesicles are spherical, about 1–5 µm in diameter and septate (Chaia *et al.*, 2010; Newcomb and Wood, 1987). Vesicles are formed inside the plant cells of the nodules or in culture under nitrogen-limiting conditions and act as specialized structures for nitrogen fixation process. *Frankia* strains inhabit highly selective environments and often associate with distinct host ranges (Udwary *et al.*, 2011).

1.2.3.5.2 Effects on the legume rhizobia symbiosis

12.3.5.2.1 Interactions between rhizobia and actinobacteria within legumes

Effects of actinobacteria on rhizobia and symbiosis with legumes have been measured but not many studies have been complete to understand the basic of these outcomes. Antagonism tests between actinobacteria isolated from different soils and effective strains of rhizobia showed that some actinobacteria can inhibit growth of

rhizobia *in vitro* and *in planta*. Thirty one percent of 481 actinobacteria isolated from agricultural soils which were good for growth of alfalfa or clover, inhibited two efficient strains *Rhizobium meliloti* A2 and S14 (Antoun *et al.*, 1978). Moreover, Damirgi and Johnson (1966) reported that the number of nodules on soybean planted with *Rhizobium japonicum* strain 122 and 123 in autoclaved soil were reduced by up to 35 percent and 53 percent respectively by treatment with the actinomycete E8. They also isolated about sixty actinobacteria from one soil sample where poor nodulation of clovers was observed. However, twenty of twenty-four actinobacteria isolated from an experimental soybean field did not inhibit eight sensitive *R. japonicum* strains in *in vitro* tests. Antagonism was examined between the actinobacteria and 12 strains of rhizobia from five soil samples by Patel (1974). It was reported that about 23-70 percent of the actinobacteria inhibited rhizobia. Although there were large numbers of antagonists in the problem soils, there was no obvious evidence of antagonism by the actinobacteria; of the 279 actinobacteria tested none inhibited all twelve strains of rhizobia.

New evidence has been reported for actinobacteria living or colonizing the inside of the nodules of *Lupinus angustifolius* (Chen *et al.*, 2011; Trujillo *et al.*, 2010; Trujillo *et al.*, 2007). Based on the genotypic and phenotypic data, *Kribbella lupini* sp. nov., *Micromonospora lupini* sp. nov. and *Micromonospora saelicesensis* sp. nov were successfully isolated from root nodules of *Lupinus angustifolius* and proposed as new species of actinobacteria (Trujillo *et al.*, 2007). These findings proved that actinobacteria were present with of the rhizobia and therefore *Micromonospora* populations are probably natural inhabitants of nitrogen-fixing root nodules of *Lupinus* plants (Trujillo *et al.*, 2010).

1.2.3.5.2.2 Nodulation and regulation of nitrogen fixation

Tokala *et al.* (2002) claimed that one of the probable mechanisms by which *Streptomyces lydicus* WYEC108 increased in the average size of the nodules and improved the vigour, longevity and capacity of nitrogen fixation of bacteroids within the nodules is by enhancing nodular assimilation of iron and possibly other soil nutrients. By examining the nodulation of lucerne over time, Solans *et al.* (2009) claimed that the actinomycete operates at the beginning of the infection and nodulation of the plant roots, to sustain the initial nodulation process. Similarly, Tokala *et al.* (2002) also hypothesized that the rhizobia may use the root colonisation sites of the *Streptomyces* as preferential or opportunistic infection sites where *Streptomyces lydicus* WYEC108 just colonizes into young root hairs of germinating pea plants.

Phytohormones and compounds produced by endophytic actinobacteria may influence the growth, nodulation and nitrogen fixation of host legumes and rhizobial partners. IAA produced by actinobacteria is considered as a factor which increases the plant growth and nodulation of legumes co-inoculated with the rhizobial partner (Nimmnoi *et al.*, 2014; Solans *et al.*, 2009) as the changes in auxin balance are necessary for nodule formation (Frankenberger and Arshad, 1995). Moreover, IAA is also involved with nitrogenase activity. Ali *et al.* (2008) found that IAA significantly influences the level of leghemoglobin and activity of nitrogenase in legume nodules. Siderophore-producing actinomycetes were able to increase Fe levels within the plant (Nimmnoi *et al.*, 2014). The ability to produce cellulases by *Micromonospora* spp. led to an increase in the number of lucerne nodules after co-inoculation with *E. meliloti* 1021 (Martínez-Hidalgo *et al.*, 2014). Solans *et al.*

(2009) hypothesised that the actinomycetes may have an effect on the autoregulation of the plant nodulation mechanism by increasing the nodulation of lucerne at high N concentration (7mM).

1.2.3.5.2.3 Bio-control effects

Several properties associated with endophytic actinobacteria might explain their ability to act as bio-control agents. These properties are the ability to colonize plants internally and on their root surfaces, their antibiosis against plant pathogens, the synthesis of plant growth hormones, and the degradation of phytotoxins. Evidence indicates that actinobacteria are quantitatively and qualitatively important in the rhizosphere (Crawford *et al.*, 1993; Doumbou *et al.*, 2001), where they may influence plant growth and protect plant roots against invasion by root pathogenic fungi (Lechevalier, 1988). Endophytic actinobacteria colonize within plant roots, therefore, they are ideal for use as bio-control agents against soil-borne diseases (Coombs *et al.*, 2004; Weller, 1988). Shekhar *et al.* (2006) purified a bioactive compound from endophytic *Streptomyces violaceusniger* that showed a strong antagonism towards various wood-rotting fungi, and chitinase enzymes were associated with this inhibition. In general, the higher chitinase activity was correlated with higher fungal inhibition and chitinase produced by endophytic *Streptomyces* which has the potential for control of plant pathogenic fungi (Gupta *et al.*, 1995). A genetic correlation between chitinase production and pathogen inhibition was demonstrated, suggesting that this control could occur inside the host plant (Quecine *et al.*, 2008). Ningthoujam *et al.* (2009) reported that an indigenous actinomycete isolate, LSCH-10C isolated from Loktak lake sediment on chitin agar, was found most promising to be developed as bio-control agent for rice.

1.3 Summary, potential for legume improvement and critical knowledge gaps.

There are numerous potential constraints to lucerne establishment and nitrogen fixation. Lucerne may have low germination rates caused by fungal pathogens in the seeds and soils which lead to sub-optimal lucerne establishment. High nitrogen in the soil can also reduce the promptness of nodulation and subsequent nitrogen fixation of lucerne.

Previous studies found that actinobacteria were able to be isolated from nodules of lupin (*Lupinus angustifolius*), field pea (*Pisum sativum* L.) and lucerne (*Medicago sativa* L.) (Carro *et al.*, 2007; Martínez-Hidalgo *et al.*, 2014; Trujillo *et al.*, 2007; Trujillo *et al.*, 2006). *Micromonospora* spp. have been found in nodules of legumes and it is believed they are common and natural inhabitants of nitrogen fixing nodules. Endophytic actinobacteria have been shown to positively effect plant growth, the nodulation process and the nitrogen fixation of some legumes. They have been shown to increase plant growth, nodulation and control some fungal diseases on pea and soybean. Specifically, endophytic *Micromonospora* spp. isolated from the nodules of lucerne increased the growth, nodulation and the concentration of some plant nutritents when they were co-inoculated with the rhizobial partner (Martínez-Hidalgo *et al.*, 2014). Further work is needed to understand how the endophytic actinobacteria affect the nodulation process including nodule formation and/or functioning of the symbiosis. Some endophytic actinobacteria may also enhance plant growth through the production of growth promoters that in terms of enhancing nitrogen fixation via improved uptake of nutrientsor acting as bio-control agents. Therefore, endophytic actinobacteria from legume roots and nodules may provide a

promising source of isoaltes for enhancing lucerne growth, nodulation, nitrogen fixation or reducing the impacts of soil borne disease.

1.4. Research plan (Objectives)

1.4.1 Aims of research

To isolate endophytic actinobacteria from four different legumes lucerne, pea, sub-clover and medic.

To examine and select the appropriate actinobacteria strains which can increase plant growth and/or nitrogen fixation and/or reduce damage by a common pathogen such as *R. solani* on lucerne.

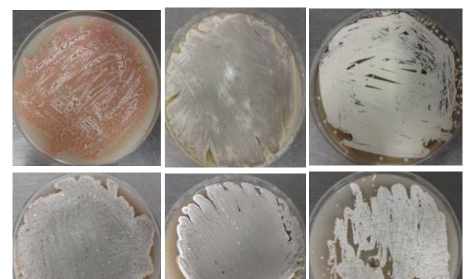
To understand the physical and morphological changes in a host plant, as well as the interactions between the selected actinobacteria, lucerne plant and rhizobia.

1.4.2 Hypothesis

Some endophytic actinobacteria can interact with selected rhizobia to improve plant growth and increase the nitrogen fixation of the lucerne plant host and/or also control disease due to fungal root pathogens.

1.4.3 Research plan

Details are presented in Figure 1.2



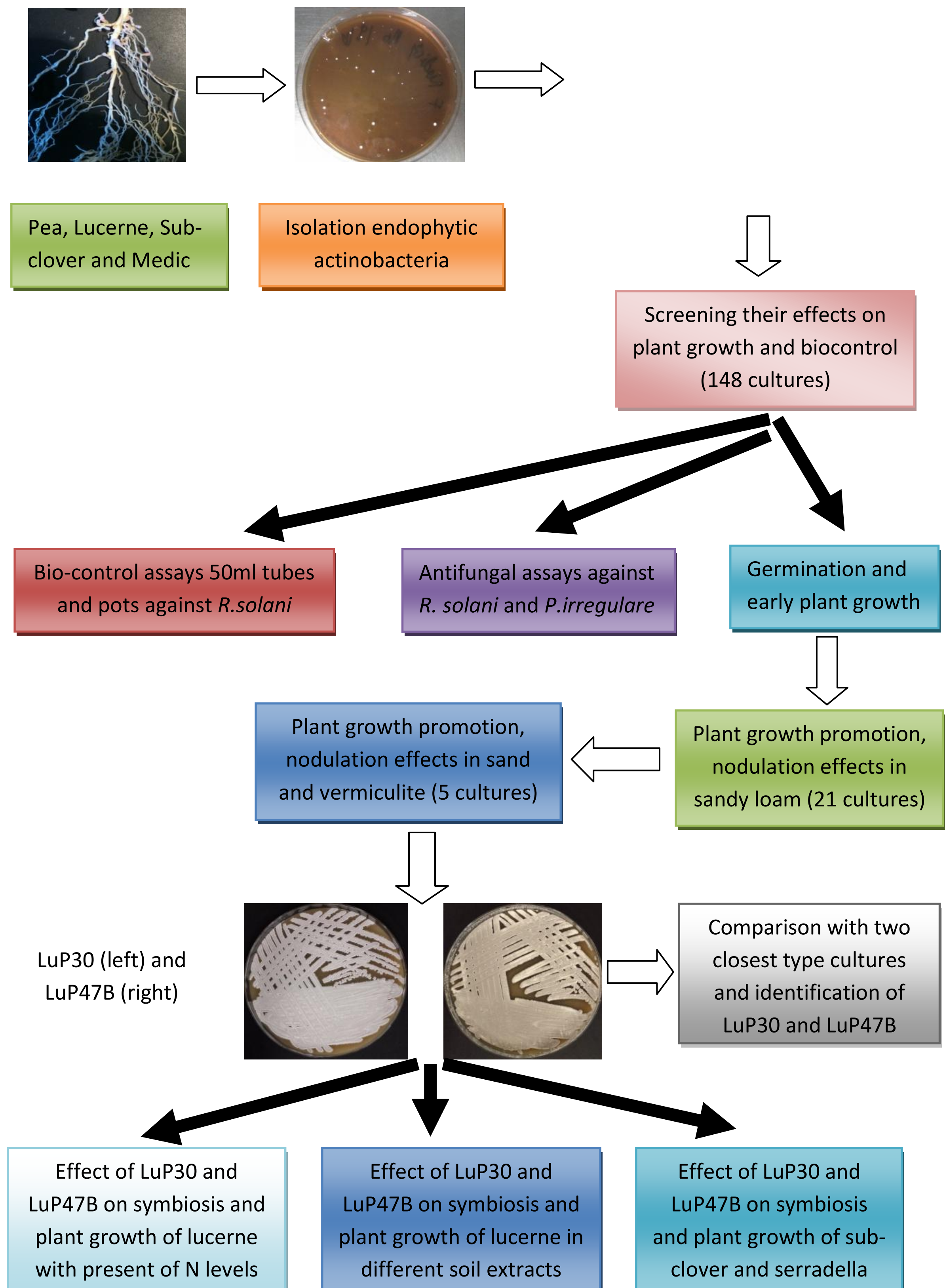


Figure 1.2 Summary of research plan

Chapter 2

General materials and methods

2.1 Legume seeds, rhizobia and actinobacteria

Seeds of the lucerne cultivar SARDI Ten (*Medicago sativa* L.), sub-clover cultivar Campeda (*Trifolium subterraneum* L.), pink serradella cultivar Cadiz-bare (*Ornithopus sativus* Brot.), wheat and rhizobial strains were provided by the South Australian Research and Development Institute (SARDI). Rhizobial strain RRI 128 is currently used as a commercial N_2 fixing inoculant for lucerne in Australia. Strain SARDI 736 has been selected as a potential replacement for strain RRI 128, and strain WSM 1115 is currently used to produce a commercial rhizobial culture for the inoculation of annual medics in Australia, but also nodulates lucerne. Strain WSM 1325 is used to produce commercial inoculants for sub-clover while strain WSM 417 is used for serradella (Table 2.1).

Table 2.1 Details of microbes used in the experiments

Microbes	Species	Strain identity
Rhizobium	*Sinorhizobium meliloti*	RRI 128
	Sinorhizobium meliloti	SARDI 736
	Sinorhizobium medicae	WSM 1115
	Rhizobium leguminosarum bv. *trifolii*	WSM 1325
	Bradyrhizobium lupini	WSM 471
Actinobacteria: 2 isolates from roots of wheat.	*Streptomyces* spp.	EN16 and EN23
148 well-sporulating strains	71 isolates from lucerne (LuP), 25 isolates from pea (P), 29 isolates from clover (CM) and 23 isolates from medic (M).	

Two endophytic actinobacteria, namely *Streptomyces* sp. EN16 and *Streptomyces* sp. EN23 previously isolated from healthy wheat roots and found to benefit plant growth

of cereals crops (Franco *et al.*, 2007) and lucerne (Le, 2010), and 148 endophytic actinobacteria isolated from different legumes were tested both *in vitro* and *in planta* (Table 2.1). The 148 strains were initially selected from 225 actinobacterial endophytes isolated from legumes and observed to sporulate well; a trait required for the commercial production of actinobacteria inoculants.

2.2 Treatment of seeds and application of inoculants

2.2.1 Surface sterilisation of seeds

Undamaged seeds of similar size were chosen and surface sterilised following the protocol by Coombs and Franco (2003). Seeds were immersed for 30 seconds in 70% (v/v) ethanol, 3 minutes in 4% (v/v) hypochlorite solution, and then rinsed three times in autoclaved R.O. water. Seeds were removed from the final rinse after 10 minutes, and placed in a laminar flow cabinet to dry for at least 4 hours or overnight.

2.2.2 Isolation, growth and application of actinobacteria

Endophytic actinobacteria were grown on Mannitol Soy flour agar (MS), International *Streptomyces* Program 2 (ISP2) or half strength potato dextrose agar (HPDA) plates and incubated at 27°C for 7-14 days until the culture produced spores. The spores were stored at -20°C in 50% (v/v) glycerol which has been autoclaved twice. The colony-forming units of the actinobacterial spore suspensions were counted as described by Miles and Misra (1938), after washing off the glycerol. Actinobacterial treatments were applied as a seed coat to surface-sterilised seeds. The actinobacterial spores had been suspended in 0.3% autoclaved xanthan gum and were applied at the rate 10^8 CFU per gram of seed.

2.2.3 Growth of Rhizobium

The rhizobia were dilution streaked on Yeast Mannitol Agar (YMA) to provide pure single colonies. The single colonies were transferred onto new YMA plates or slants and incubated at 27°C for 4-10 days until good growth was observed. Incubation time varied with the rhizobial strain. The cultures were stored at 4°C for subsequent use. A standard curve describing the relationship between cell number and OD_{600nm} was developed for each *Rhizobium* strain to enable the application of a standard CFU/ml across experiments.

2.2.4 Plant growth media, nutrition, sowing and water supply

Seeds were surface-sterilised as described above and sown into a pasteurised (by autoclaving) potting mix (50:50 by volume of sand: vermiculite) contained in 1.25 litre self-watering pots (Décor Watermatic^{TM}). One hundred ml MQ water was added to each pot before planting the ten seeds. The potting mix surface was covered with a thin layer of washed granulated plastic beads to reduce evaporation and minimise the transfer of microbes between pots. Then, 200 mL of N deficient nutrient solution (McKnight, 1949) supplemented with small amount of nitrogen (300 mg NH_4NO_3 per 20 L McKnight's solution) was gently added to each pot before covering the pots with plastic bags and placing them in the glasshouse. After 5 days, the plastic bags were removed and the number of seedlings was thinned to four uniform plants per pot before adding 1 ml of appropriate *Rhizobium* inoculant (around 10^8 CFU/ml). Plants were watered with MQ water as required for the remaining weeks. All treatment and control pots were completely randomised in the glasshouse with the position of the pots changed every week.

2.2.5 Harvest of plants

Plants were removed from the pots and gently shaken to remove most of the sand and vermiculite. The plants were wrapped in moisture paper tissue and stored in zip lock plastic bags at 4°C prior to assessment. Roots were subsequently washed under running tap water to remove remaining sand and vermiculite residues.

2.3 Data collection and analysis

The parameters commonly measured were length and dry weight of the shoot, length and dry weight of the root, and the number and dry weight of nodules per plant. Shoot and root lengths of individual plants (four plants per pot) were measured by ruler to 1 mm accuracy and then the shoots were separated from the roots and the samples placed into paper bags and dried in an oven at 60°C for 48 hours until constant weight. Prior to drying, the number of nodules on the root of two plants per pot was counted, removed and dried. The average dry weight of each nodule was calculated by dividing total nodule dry weight by total nodule number to provide and average for the two plants measured in each pot. Shoot, root and nodules dry weights were measured to five significant figures.

The data was entered and collated in a MS Excel spreadsheet and subsequently analysed using the IBM SPSS Statistics 20 package.

2.4 Identification of endophytic actinobacteria by 16S rRNA gene amplification and sequencing

DNA extraction and PCR of the 16S rRNA gene of selected actinobacteria was carried out as described by (Coombs and Franco, 2003).

2.4.1 DNA extraction

In a sterile 1.5 ml eppendorf tube, 10 µl lysozyme was added into 500 µl of Tris-EDTA (TE) pH 7.4 before re-suspending 2-3 loops of actinomycetes cells in the mixture which was then vortexed. The eppendorf tube was incubated at 37°C for 60 minutes before adding 10 µl of proteinase K and 32.5 µl of 10% SDS and incubating at 55°C for 60 minutes. Next, 100 µl of 5 M NaCl and 65 µl of CTAB/NaCl which was pre-incubated at 55°C were added and also incubated at 55°C for 10 minutes. The tube was left at room temperature for 30 minutes with intermittent shaking every 10 minutes after adding 600 µl of phenol:chloroform:isoamyl alcohol (25:24:1) 10 mM Tris, pH 8.0, 0.1 mM EDTA. After centrifuging at 12,000 rpm for 15 minutes, the supernatant was transferred to a new sterile 1.5 ml eppendorf tube containing 500 µl of chloroform. It was left at room temperature for 15 minutes with the tube inverted every 7-8 minutes before centrifuging at 12,000 rpm for 15 minutes. The aqueous phase was transferred into a new sterile 1.5ml eppendorf tube before adding 20 µl of 10 mg/ml of RNAse, which was activated earlier at 95°C for 30 min, and incubated at 37°C for 60 minutes. More chloroform (500 µl) was added, and left at room temperature for 15 minutes. After centrifuging at 12,000 rpm for 15 minutes, the supernatant was transferred to a new sterile 1.5 ml eppendorf tube containing 500

µl of chloroform, shaken and left at room temperature for 15 minutes (inverse mix every 7-8 minutes). After centrifuging at 12,000 rpm for 15 minutes, the supernatant was transferred to a new sterile 1.5 ml eppendorf tube (steps repeated). To the tube was added 0.1x volume of 3 M Na Acetate (50 µl) and 3x volume of 100% ethanol (1 ml) before leaving at -20°C overnight. The supernatant was removed carefully so as not to disrupt the pellet after centrifuging at a maximum speed of 16,000 rpm for 15 minutes. The pellet was washed twice with 70% ethanol and dried by placing the tubes in a heating block at 55°C with the lids open until no liquid was left. Finally, the pellet was re-suspended in 50 µl of sterile water and stored at -20°C for later use.

2.4.2 Quantify DNA concentration

The concentration of DNA was quantified by GeneQuant *pro* RNA/DNA calculator. Five micro litters of DNA extract was mixed with 95 µl of injection water while 100 µl of injection water were used as reference. The purity of DNA was examined by the ratio of 260/230, 260/280 and electronic agarose gel.

2.4.3 PCR 16S rRNA

The PCR process was run with the following protocol: start at 94°C for 2 minutes, 40 cycles of 94°C for one minute, 52°C for one minute and 72°C for two minutes, and end with 72°C for 10 minutes. A master mixture was prepared comprising 1 µl dNTPs 10 mM, 1 µl DNA Taq polymerase 5U/µl, 5 µl ThermoPol buffer, 2 µl 27f primer 10 mM, 2 µl 1465r primer 10 mM, 2 µl DNA simple and 37 µl injection water. Primer sequences were: 27f primer (5$^{'}$-AGAGTTTGATCCTGGCTCAG) and 1492r primer (5$^{'}$-TACGGYTACCTTGTTACGACTT). A 1.2% of agarose gel containing 3 µl of Gel red in 40 ml agarose was used to separate the PCR products

based on molecular weight. One micro litter of loading dye was mixed well with 2 µl of each PCR product before loading them into each well of the gel which was run in a running buffer 0.5xTBE at 70V and 400mA for 60 minutes.

2.4.4 Clean up and sequencing the PCR products

The PCR products were cleaned up by using a UltraClean^{TM} PCR Clean-Up, DNA purification Kit (Catalog # 12500-100 MoBio Laboratories, Inc.). Five times the volume of Spind bind solution was mixed well with the PCR products, and the mixture was added into a spin filter column which was inside a tube. The tubes were centrifuged at 13,000 rpm for 30 seconds in a microcentrifuge. The liquid flow-through from the tube was discarded, and the spin filter unit was put back into the same tube. Each spin filter was treated with 300 µl SpinClean buffer and centrifuged at 13,000 rpm for 30 seconds and the liquid flow-through was discarded. The tubes were centrifuged for 2 more minutes at 13,000 rpm before transferring the spin filters to clean collection tubes. Injection water (50 µl) was added into the spin filters and incubated at room temperature for 5 minutes before centrifuging for 2 minutes at 13,000 rpm. The clean PCR products were collected in the collection tubes and stored at -20°C while the spin filters were discarded.

The products of PCR were sequenced by Macrogen Inc., Korea. The resultant 16S rRNA sequences were compared to the GenBank database by using the National Center for Biotechnology Information database (NCBI), BLASTN program including the results of the highest matches for each isolate and the corresponding bit score and percentage of identity.

Chapter 3

Isolation and characterisation of actinobacterial endophytes from pasture legumes

3.1. Introduction

In Australia, agricultural crop and pasture legumes similarly provide a large amount of the nitrogen required for agricultural production (Peoples and Baldock, 2001). The introduction of legume specific root nodule bacteria (generically known as rhizobia) to Australian soils (Brockwell *et al.*, 1980; Dudman and Brockwell, 1968) has been critical to these fixed N contributions. The ecology of rhizobia, occupancy of root nodule structures and nitrogen fixation activity have been extensively studied (Drew *et al.*, 2012; Graham, 2008; Sharma *et al.*, 1993).

Other soil inhabiting organisms have also been isolated from legume roots and nodules and been identified as potential ‘helpers’ in the establishment and functioning of the N fixation process with some proposed for use as co-inoculants with rhizobia. For example, Fox *et al.* (2011) used *Pseudomonas fluorescens* WSM 3457 as co-inoculants with *E. medicae* WSM 419 significantly increased the nodulation and symbiotic effectiveness of *Medicago truncatula*. Whilst actinobacteria are common soil inhabitants with about 30 kg biomass/ha in the top 10 cm (Reid, 2013) in Australian soils and have been isolated from legume nodules (Chen *et al.*, 2011; Trujillo *et al.*, 2010; Trujillo *et al.*, 2007), their significance to the production of the agricultural legumes grown in Australia remains unknown.

A successful sterilisation and isolation of endophytic actinobacteria from healthy roots of wheat in Australia was published by Coombs *et al.* (2003). Different isolation media such as humic acid vitamin B (HV), tap water yeast extract (TWYE) and yeast extract-casein D-glucose agar (YECD) for endophytic actinobacteria were also reported. A number of new endophytic actinobacteria species has been isolated

from native trees growing in South Australia (Kaewkla and Franco, 2011, 2011, 2010, 2010, 2011). The endophytic actinobacteria have shown increases in plant growth and protection from the pathogens in wheat and cereal crops (Franco *et al.*, 2007).

Actinobacteria have been recovered from the inside of nodules of *Lupinus angustifolius* (Chen *et al.*, 2011; Trujillo *et al.*, 2010; Trujillo *et al.*, 2007). Based on the genotypic and phenotypic data, isolates identified as *Kribbella lupini* sp. nov., *Micromonospora lupini* sp. nov. and *Micromonospora saelicesensis* sp. nov occupied the lupin nodules and have been proposed as new species of actinobacteria (Trujillo *et al.*, 2010; Trujillo *et al.*, 2007). *Micromonospora* spp. have also been found in both rhizosphere and nodules of pea using MLSA (Multilocus sequence analysis) and ARDRA (Amplified Ribosomal DNA Restriction Analysis) methods (Carro *et al.*, 2012). Mingma *et al.* (2014) isolated 77 endophytic actinobacteria from the roots of healthy *leguminosae* plants with indications based on their rich content of the LL-isomer of diaminopimelic acid, that *Streptomycetes* dominated the isolates.

In this chapter roots and nodules of four different legumes (lucerne, pea, sub-clover and medics) collected from fields around South Australia were used to isolate the endophytic actinobacteria. Later on these actinobacteria were examined their effects on plant growth, nodulation and for their ability to protect lucerne plants from fungal pathogens.

3.2 Materials and methods

3.2.1 Source of legumes

Endophytic actinobacteria were isolated from the roots and nodules of four legume species: lucerne (*Medicago sativa* L.), field pea (*Pisum sativum* L.), sub clover (*Trifolium subterraneum* L.) which had been collected from the fields in South Australia and annual medic (*Medicago* spp.) which was collected at the main campus of Flinders University, Bedford Park, South Australia. Isolation and subsequent identification of the isolates followed the methods described by Coombs and Franco (2003).

3.2.2 Isolation of endophytic actinobacteria

3.2.2.1 Surface-sterilization procedure

The plants were washed under running tap water until soil attached to the roots and nodules was completely removed. The dry roots containing nodules were surface sterilised as per Coombs and Franco (2003) by immersing in 90% ethanol for 1 minute, followed by 6 minutes in 4% NaOCl, 30 seconds in 90% ethanol and followed by rinsing in sterile R.O. water to wash off all the sterilising agent. The surface-sterilised nodules were removed from the roots and crushed in a small aliquot of 0.9% saline until they formed a homogenous mixture. The nodule suspension was spread onto the surface of at least three different isolation media mentioned below (section 3.2.2.2). The surface sterilised roots were air dried before being cut into approximately 1 cm fragments and placed onto the plates of the four isolation media. Duplicate plates were incubated at 27°C or 37°C and checked regularly.

Water from the last wash was retained to check for microbial growth to confirm the effectiveness of surface sterilisation protocol. Validation of surface sterilisation of legume material was assessed by dropping 200 µl of the sterile water wash used as the final rinse onto TSA medium and incubated as described above. Effective surface sterilisation was considered to be successful when there were no microbial colonies on the TSA medium.

3.2.2.2 Media for isolation

Media for isolation were prepared following the specific recipes and one ml of Benomyl (5 g/100 ml) was added to 1 litre isolation media (50 µg ml^{-1}) to control the growth of (endophytic) fungus. Different media used for isolation endophytic actinobacteria were Humic acid-Vitamin B (HV), Tap water yeast extract (TWYE), yeast extract-casein hydrolysate agar (YECD), Tryptone Soy Agar (TSA) and all at pH of 7.2 (Appendix 1) (Coombs and Franco, 2003).

3.2.2.3 Purification of actinobacterial endophytes

The plates were checked at least once a week for up to 7 weeks or until no new single colonies emerged. When colonies appeared, they were transferred to half-strength potato dextrose agar (HPDA, Oxoid, UK) for purification.

3.2.3 Characterisation of endophytic actinobacteria

3.2.3.1 Morphology (frequency and distribution)

Single colonies after purification were transferred into three different media (HPDA, ISP3 and MS) to distinguish them based on their different morphologies, colour and pigments produced. The colour and pigments of the isolates were recorded based on the Methuen Handbook of Colour (Kornerup and Wanscher, 1978). Cultures were initially assigned to broad groups based on their different colony morphologies, aerial mycelium, substrate mycelium and spore colour and diffusible pigment production following the Bergey's Manual of Determinative Bacteriology (Whitman *et al.*, 2012).

3.3 Results and discussion

3.3.1 Isolation of endophytic actinobacteria from legumes

The surface sterilisation was effective indicating that the method used was effective for isolation of bonafide endophytic bacteria. Endophytic actinobacteria were successfully isolated from roots and nodules of the four types of legume plants (Figure 3.1). Two hundred and twenty five actinobacteria cultures were isolated from roots and nodules of lucerne (86), pea (64), clover (38) and medic (37) plants. Seventy three were from nodules and one hundred and fifty two were from roots. One hundred and twenty five isolates were isolated on HV medium, 72 cultures were from TWYE and 26 from YECD. Only 1 was isolated from TSA (Table 3.2). The results are similar to previous studies that found nutrient-poor-media were the most effective in isolation of endophytic actinobacteria from wheat (Coombs and Franco, 2003) and HV has been proven to be very effective by others (Otoguro *et al.*, 2001; Taechowisan *et al.*, 2003). Qin *et al.* (2009) also found that the relatively simple nutrient media, such as TWYE, sodium propionate agar, and HV were most effective for the isolation of endophytic actinobacteria.

Table 3.1 Number of endophytic actinobacteria isolated from roots and nodules of four legumes using different media and incubation temperatures

Plant	Parts of plant		Temperature		Media			
	Root	Nodule	27°C	37°C	HV	TWYE	YECD	TSA
Lucerne	86	0	40	46	44	31	10	1
Pea	42	24	35	31	45	20	1	0
Sub-clover	12	24	9	27	12	14	10	0
Medics	12	25	16	21	25	7	5	0
Total	**152**	**73**	**100**	**125**	**126**	**72**	**26**	**1**

The total number of cultures that were isolated at 27° Celsius and 37° Celsius were not too different, 100 and 125 isolates, respectively (Table 3.1). Around 27°C is the most common temperature used for isolation of endophytic actinobacteria, although there are many examples of successful isolation between 25°C to 30°C (Cao *et al.*, 2004; Mingma *et al.*, 2014; Qin *et al.*, 2009; Sardi *et al.*, 1992; Taechowisan *et al.*, 2003; Trujillo *et al.*, 2006). Widiantini (2012) found about 40% isolates recovered from 27°C and 60% at 37°C from rice plants while in this study about 45% were isolated at 27°C and 55% at 37°C. Therefore, 37°C is preferable for isolation of endophytic actinobacteria from plants that grow over the summer months.

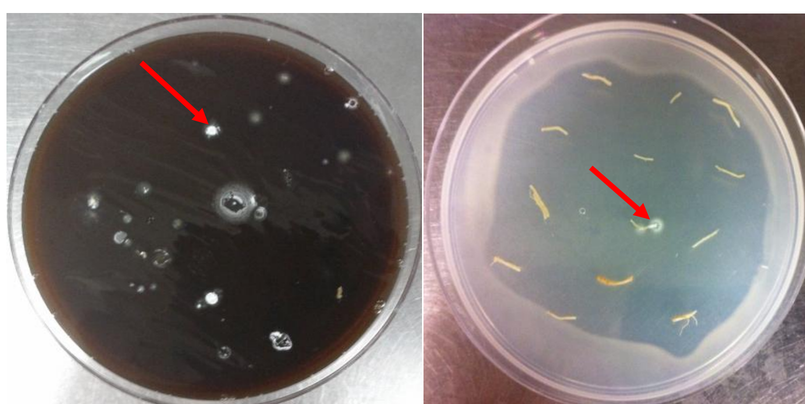


Figure 3.1 Colonies of endophytic actinobacteria being isolated from a nodule extract on HV medium (left) and from surface sterilised root fragments on TWYE medium (right) after 2 weeks incubation.

In this study, although 41% of the isolates were isolated during second week of incubation, a further 2 weeks incubation was needed to recover the majority (87%) of isolates (Figure 3.2) and highlights the importance of long incubation times for the recovery of actinobacteria. Although some studies used shorter times of 3 weeks (Mingma *et al.*, 2014; Sardi *et al.*, 1992; Trujillo *et al.*, 2006) and 4 weeks (Taechowisan *et al.*, 2003). There are also examples where incubations times of up to 16 weeks have been used to isolate actinobacteria from medicinal plants (Qin *et al.*, 2009), and endophytes from trees (Kaewkla and Franco, 2013). As the nodules were crushed four weeks incubation time was long enough to recover the endophytic actinobacteria. However, the intact roots of legumes need to be incubated up to six weeks to isolate the endophytic actinobacteria which could have small population inside the roots.

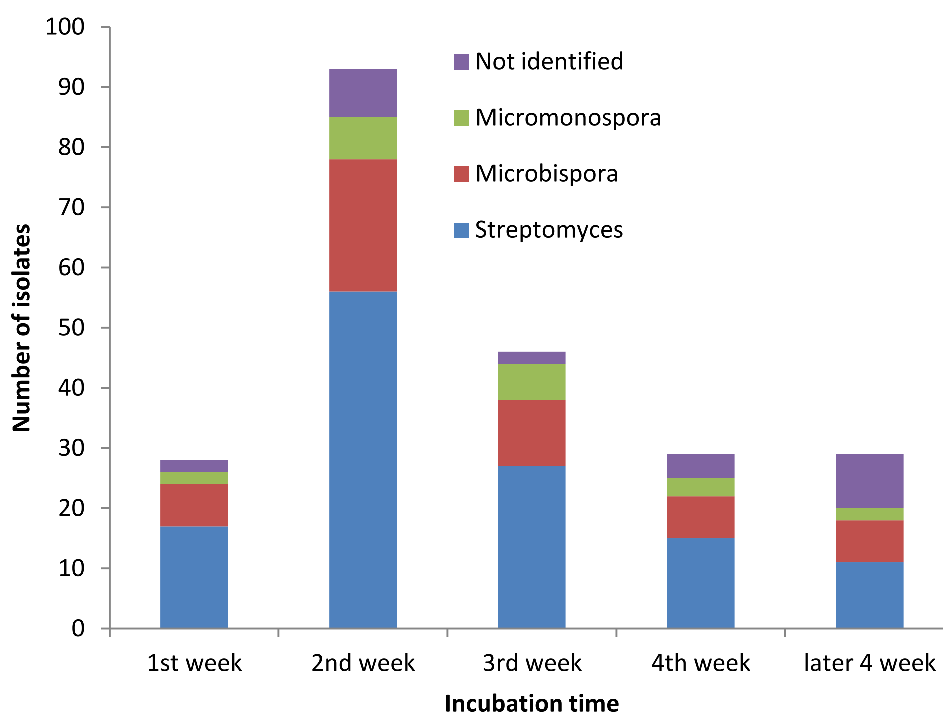


Figure 3.2 Number of isolates of endophytic actinobacteria recovered over incubation time

3.3.2 Morphological characterisation of actinobacteria

Based on their morphology, 126 cultures (56%) were characterised as *Streptomyces*, 54 cultures (24%) as *Microbispora* and 20 cultures (9%) as *Micromonospora*. Twenty five cultures (11%) were not readily identified (Appendix 2). *Streptomyces* and *Microbispora* were recovered with the highest number of isolates at both 27°C and 37°C by 53% and 58% isolates, respectively (Figure 3.3). Mingma *et al.* (2014) successfully recovered 91.2% *Streptomyces* sp. of 317 endophytic actinomycetes from roots (77) and rhizosphere (240) of leguminous plants. However, Trujillo *et al.* (2010) found that 73% *Micromonospora* strains were recovered from the nodules sampled of *Lupinus angustifolius* and 136 *Micromonospora*–like were recovered from the nodules. In contrast, in this study only 7% *Micromonospora* (5 isolates) out of 73 isolates were recovered from nodules of three legumes pea, clover and medics while *Streptomyces* was 44% (32 isolates) *Microbispora* 23% (17 isolates), and 33% (19 isolates) were unidentified. There was no nodule material of lucerne avaible. Particularly, in medics and pea nodules the number of *Streptomyces* and *Microbispora* were similar 28% and 32% for medics and 25% and 29% for pea while *Micromonospora* was only 4% and 17%, respectively. In addition, in clover nodules no obvious *Micromonospora* were found and up to 79% isolates were *Streptomyces*, followed by 8% *Microbispora* and 13% unidentified.

Table 3.2 Number of *Streptomyces* and non-*Streptomyces* isolated from roots and nodules of four different legumes in South Australia. No nodule materials from lucerne for isolation of endophytic actinobacteria.

Plant	Root		Nodules	
	Streptomyces	**non-*Streptomyces***	***Streptomyces***	**non-*Streptomyces***
Lucerne	65	21	0	0
Field Pea	20	22	7	17
Sub-clover	2	10	19	5
Medics	6	6	7	18
Total	**93**	**59**	**33**	**40**

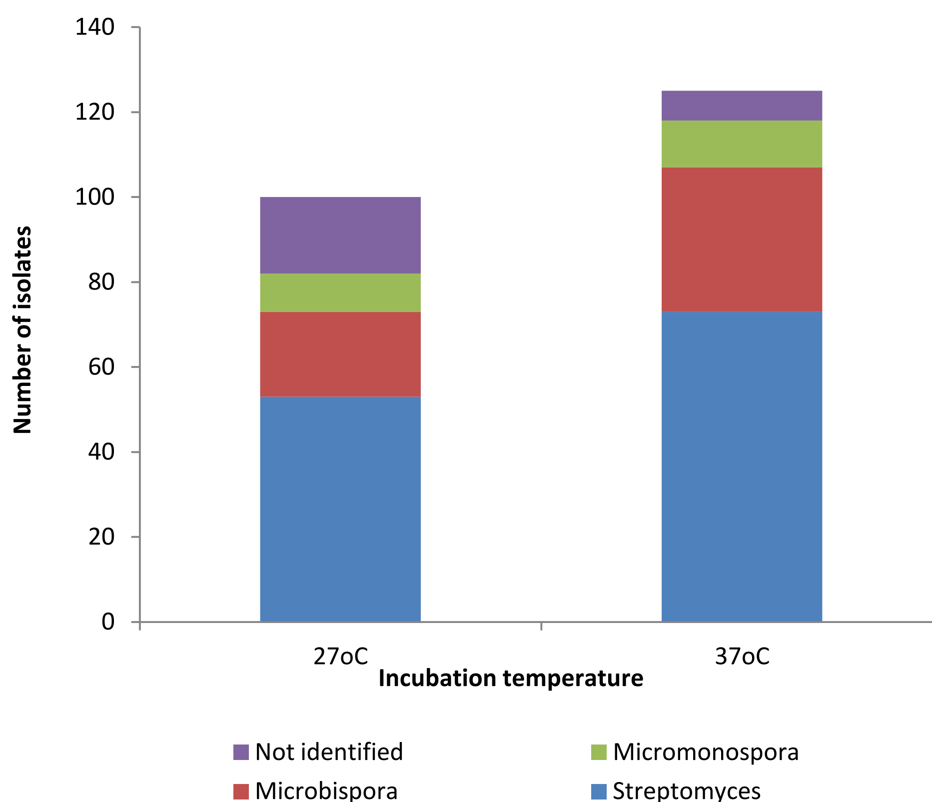


Figure 3.3 Number of endophytic actinobacteria recovered at different temperatures

The amount of root and nodule material for the isolation might have influenced the variation on the number of isolates and genera of actinobacterial endophytes. More plant fragments used for isolation increases the chances of getting different types of cultivable endophytic actinobacteria. In this study, the amount of roots and nodules of different legumes used was not equal which has a large influence on how the

numbers are interpreted. The mass of nodule materials is much lower than roots though there were 73 endophytic actinobacteria from nodules and 152 from roots (Table 3.2). *Streptomyces* were the most common isolates in the roots. In contrast, in nodules the number of non-*Streptomyces* isolates was higher than *Streptomyces* isolates, 40 and 33, respectively (Table 3.2).

3.4 Conclusion

The protocol of surface sterilisation was successful for isolation of endophytic actinobacteria. Endophytic actinobacteria were successfully isolated from roots and nodules from a range of legumes growing on Australian soils. Endophytic actinobacteria recovered from roots and nodules of each legume belonged to different genera. The appropriate media for isolation of endophytic actinobacteria from roots and nodules of the legumes were HV and TWYE. The incubation temperature of 37°C increased the number of cultivable actinobacterial endophytes recovered. The majority of actinobacterial isolates were recovered after 4 weeks of incubation and *Streptomyces* were the dominant genus isolated in this study.

Chapter 4

Effects of actinobacteria on lucerne germination, seedling growth, and tolerance of soil-borne disease

4.1 Introduction

A number of fungi are associated with seedling diseases of alfalfa (*Medicago sativa* L.). Depending on soil types, locations and environmental conditions, *Rhizoctonia*, *Pythium, Aphanomyces, Phytophthora* and *Fusarium* spp. cause seed rot, pre-emergence, and post emergence damping-off (Altier and Thies, 1995; Handelsman *et al.*, 1990; Holub and Grau, 1990; Schmitthenner, 1964) . They can lead to significant losses during plant establishment and also be detrimental to plant yield. *Rhizoctonia solani* affects not only on cereal crops but also pasture species and lucerne. The fungus not only depresses seedling germination but also causes damping off, as well as crown bud rot, stem blight and crown necrosis and results in poor crop growth and low yields in lucerne (Stuteville and Erwin, 1990). *Rhizoctonia solani* was first reported in lucerne as a root canker disease in the USA by Smith (1943). A number studies about the effects of *R. solani* on lucerne in Australia has been reported (Anderson *et al.*, 2004; Irwin, 1977; Larkin *et al.*, 1994; You *et al.*, 2008). These cankers attach and damage the root, and the disease often results in plant death (Irwin, 1977). *Pythium* spp. have also been implicated as pathogens of lucerne although on occasion can infect roots without causing obvious damage symptom (Larkin *et al.*, 1994).

Actinobacteria have been shown to colonise plant tissues and produce a range of secondary metabolites which influence plant growth or protect the plant from pathogens (Coombs and Franco, 2003; Franco *et al.*, 2007). Actinobacteria have been found to act as bio-control agents in various plants (Doumbou *et al.*, 2001). For example, three endophytic actinomycetes identified as *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* significantly improved plant

growth by reducing seedling damping-off, and root and crown rots of mature cucumber (*Cucumis sativus*) caused by *Pythium aphanidermatum* (El-Tarabily *et al.*, 2010). Similarly, Coombs *et al.* (2004) reported that actinobacteria (*Streptomyces, Microbispora* and *Nocardioides* spp.) isolated from healthy cereal plants controlled the development of disease symptoms in treated plants exposed to *Gauemannomyces graminis* var. tritici (Ggt) and *Rhizoctonia* in field soils. Eleven actinomycete strains that were previously shown to protect raspberry (*Rubus strigosus*) plants against *Phytophthora* infection were characterized (Toussaint *et al.*, 1997). Two *Streptomyces* strains J-2 and B-11 isolated from rhizosphere soil of sugar beet showed potential for controlling root rot caused by *Sclerotium rolfsii* on sugar beet and could be useful in integrated control against diverse soil borne plant pathogens (Errakhi *et al.*, 2009).

Actinobacteria might also have a role as bio-control agents in interaction with rhizobia and legumes. Two *Streptomyces* sp. BSA25 and WRA1 successfully suppressed *Phytophthora* root rot in chickpea when co-inoculated with either *Mesorhizobium ciceri* WSM1666 or Kaiuroo 3. *Streptomyces* sp. BSA25 with either rhizobial strain enhanced root (7–11 fold) and shoot dry weights (2–3 fold) compared to an infected control, whereas *Streptomyces* sp. WRA1 increased root and shoot dry weights by 8- and 4-fold, respectively when inoculated with *M. ciceri* WSM1666 (Misk and Franco, 2011). The yield of pea has similarly been shown to be increased with the addition of *Streptomyces* WYEC108.

Endophytic actinobacteria have shown the ability to improve the growth of cereal plants (Franco *et al.*, 2007; Sharma, 2014). An endophytic *Streptomyces* sp. GMKU

3100 isolated from roots of rice significantly increased the shoot and root biomass of rice and mungbean after 2 weeks and 4 weeks respectively (Rungin *et al.*, 2012). A number of studies about the ability to improve the plant growth of actinobacteria have been reported (Doumbou *et al.*, 2001; Strobel *et al.*, 2004; Venkatachalam *et al.*, 2010). IAA and siderophores production, and phosphate solubilisation ability have been found commonly from actinobacteria and endophytic actinobacteria (Caruso *et al.*, 2000; El-Tarabily *et al.*, 2010; El-Tarabily *et al.*, 2008; Sharma, 2014; Shimizu *et al.*, 2009).

This chapter examines the ability of actinobacteria isolated from legumes to improve the germination and early plant growth of lucerne. The ability of the actinobacteria to inhibit the mycelial growth and damage from lucerne root pathogens is also examined.

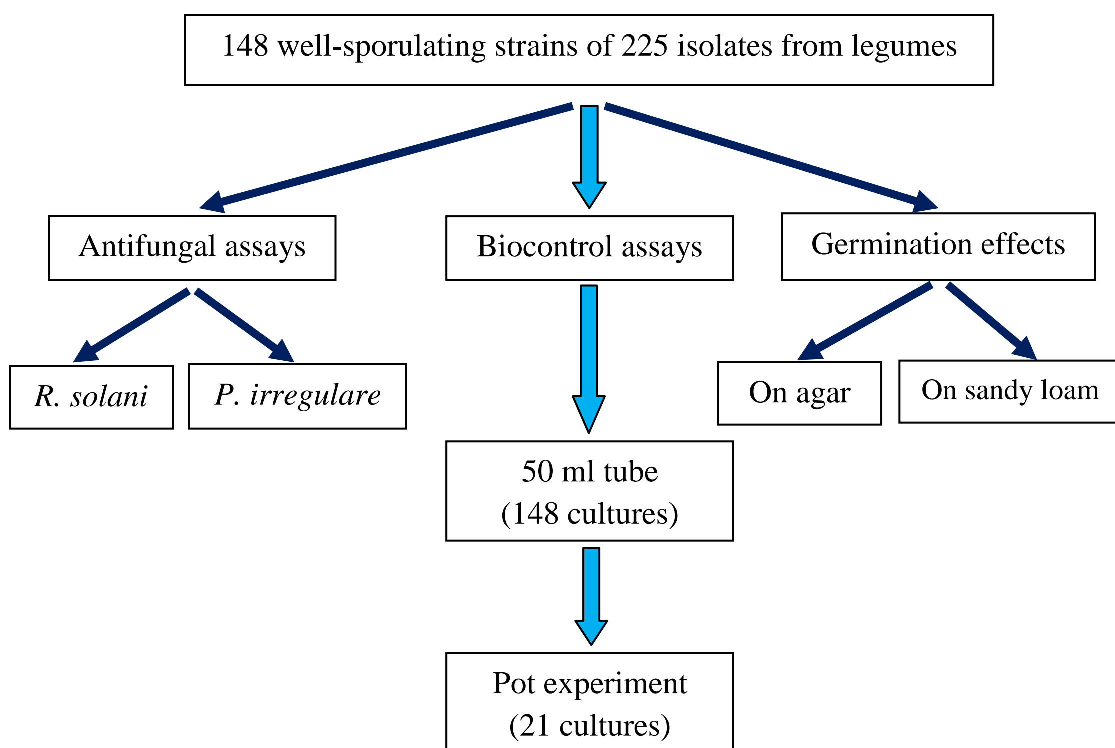


Figure 4.1 Screening the plant growth, symbiotic effects, antifungal and bio-control ability of endophytic actinobacteria on lucerne

4.2 Materials and methods

4.2.1 Effects of actinobacteria on lucerne germination

Two experiments, using different plant growth media, were completed. Lucerne seeds were surface sterilised prior to inoculation as described in 2.2.1 of Chapter 2. One hundred and forty-eight isolates of actinobacteria that had sporulated well on agar media, and thus considered good candidates for commercial spore production, were tested for their effects on lucerne germination and early growth.

4.2.1.1 Lucerne germination on agar

In the first experiment, uniform lucerne seeds (5 per Petri plate) were placed onto the surface of 1% agar medium supplemented with plant nutrient solution. Actinobacteria isolates were applied to lucerne seed as spore suspensions in 0.9% saline at a rate of one drop per seed (~ 2000 spores per seed). Control (un-inoculated) seeds received one drop of 0.9% saline. Treatments were replicated 3 times. After inoculation with actinobacteria, plates were placed in a plant growth cabinet (14/10 hr day/night cycle, ambient temperature 24 to 30°C) for 7 days.

4.2.1.2 Lucerne growing in a sandy loam

In the second experiment, seeds were sown into a sandy loam soil (20 seeds per punnet) that had been autoclaved at 121°C for 15 minutes and adjusted to 12 % moisture content with nutrient solution before being dispensed (300 g) into small punnets (10 × 8 cm diameter × 20 cm deep). Twenty surface sterilised seeds were sown as 5 seeds in each of 4 lines. After inoculation with actinobacteria suspensions, punnets were placed in a plant growth cabinet (conditions as above) for 14 days.

Sinorhizobium meliloti strain RRI 128 was added to germinated seedlings (250 µl; ~ 10^8 CFU ml^{-1} per seedling at 4 days after sowing). The number of germinated seeds, root length and number of nodules were measured.

4.2.2 Inhibition of the growth of pathogenic fungi on agar

This assay was based on the protocol of Crawford *et al.* (1993). Two common pathogens of legumes were used namely *R. solani* (isolate W19) and *P. irregulare* (isolate 89) to test the antifungal and biocontrol activity of the actinobacterial isolates. Five endophytic actinobacteria isolated from healthy wheat roots and one hundred and forty eight isolated from legumes were used in the assays. *R. solani* (W19) and *P. irregulare* (89) were grown on HPDA (Half strength potato dextrose agar) medium while the actinobacteria were grown on CMA (corn meal agar) plates for about 7 days. A plug of the pathogen was transferred onto the centre of the CMA plates containing a culture of actinobacteria. The plates were incubated at 27°C and the growth of the pathogen mycelia were measured after 3 days for *P.irregulare* (89) and 5 days for *R. solani* (W19), when the pathogens had completely covered the surface of the control plates in the absence of the actinobacteria. The inhibition zone was recorded from the plug of the actinobacteria on the plate to the furthest of pathogen growth. The distance between the actinobacteria and the pathogen was measured to estimate the inhibition of the pathogen growth by the actinobacteria. The width of inhibition zone between the pathogen and the actinomycete isolates was measured and classified as follows: +++ (strong), 15 mm <; ++ (moderate), 11-14 mm; + (weak), 2-10 mm; ±, ≤mm; -, 0 mm. The survival percentage of each pathogen was also determined, by using the formula T/C x 100, where T is the

growth diameter of the pathogen for each treatment, and C is the growth diameter of the pathogen of the control plate (Misk and Franco, 2011).

4.2.3 Effect of actinobacteria on the development of root damage symptoms caused by Rhizoctonia solani

4.2.3.1 Preliminary screening in Falcon tubes

Three moisture levels 8%, 10% and 12% of autoclaved sandy loam were used to test for the growth of lucerne and root damage by impacts of *R. solani*. The characteristics of the sandy loam which is high N is described in the supplementary data (Appendix 4). The moisture was made by adding McKnight's solution plus N starter (300 mg per 20 L) to the autoclaved sand. Four levels 0, 2, 4 and 6 millet seeds infested with *R. solani* in each tube were examined for their effects on lucerne root and growth. Forty five grams of autoclaved sandy loam were used at three moisture levels mentioned above, added to each tube. Number of millet seeds infested with *R. solani* AG8 strain W19 was added as mentioned and followed by 10 g of soil with the same moisture level to cover the millet seeds. The tubes were incubated with the millet seeds 2 weeks at 15°C to allow the fungi to develop. Lucerne seeds were surface-sterilized and pre-germinated on autoclaved moisture filter paper. When the roots grew to about 1-3 mm length, about 5 ml (that covered all the seeds) of the rhizobial suspension (approximately 10^8CFU/ml) was dropped onto the seedlings. These co-inoculated pre-germinated seedlings were transferred into each tube straight and covered with 5g of soil with the same moisture level and a layer of plastic beads. The tubes were kept at 15°C in a growth chamber for 3 weeks. Three replicates were prepared for each treatment and MQ water was added as

required. The number of seedlings that appeared, and length of root and root damage were recorded.

Based on the above the high throughput assay is: forty five grams of autoclaved sandy loam were added to 50 ml Falcon tubes and adjusted to 12% moisture content using McKnight's nutrition with the addition of starter nitrogen (300mg of NH_4NO_3/20L) solution. Two millet seeds infested with *R. solani* AG8 strain W19 were added to each tube and then covered with 10 g of soil adjusted to 12% moisture. Millet seeds that had not been infested with the pathogen were added to tubes allocated to control treatments. The tubes were placed in a rack covered with aluminium foil to exclude light and placed in a plant growth chamber for two weeks at 15°C to promote growth of the pathogen. At the end of the incubation period, the aluminium foil was removed and two pre- germinated lucerne seedlings sown into each tube and covered with 5g of soil and a layer of plastic beads. The lucerne seedlings were treated with the individual actinobacterial spore suspensions which were added to moistened autoclaved filter paper. A 5ml rhizobial suspension containing approximately 10^8CFU/ml was added to the seedlings when the root radicals were about 2 mm in length. The tubes containing the lucerne seedlings were returned to the plant growth chamber and plants were grown for a further 3 weeks at 15°C (14/10 hrs day/night). There were two replicate tubes for each treatment and MQ water was added as required. The number of seedlings that emerged, root length and severity of root damage symptoms were recorded.

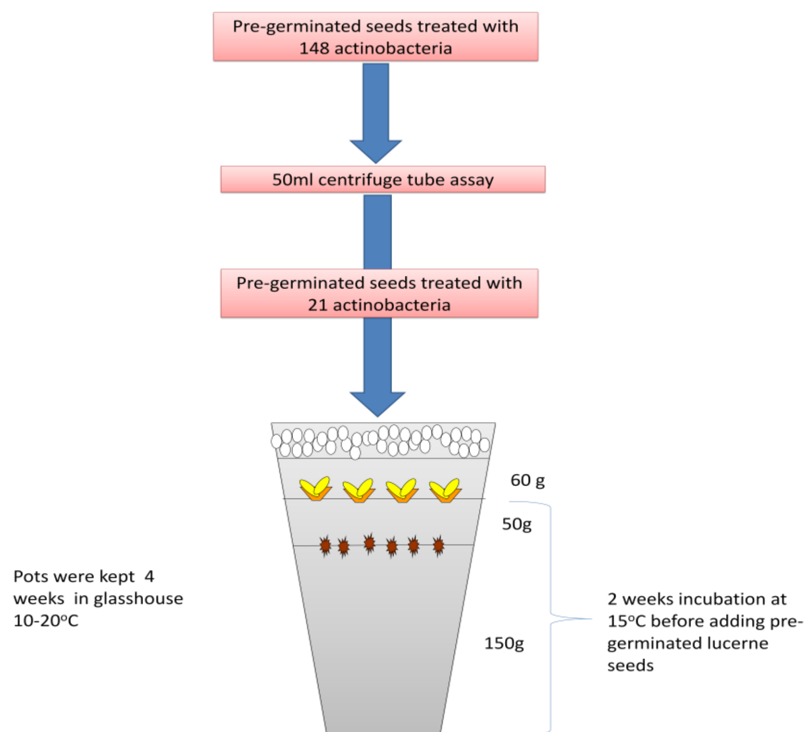


Figure 4.2 Screening bio-control ability of actinobacteria against *R. solani* AG8

4.2.3.2 Pot experiment in glasshouse

The methodology used was similar to that described above (4.2.3.1) for the tube experiment, with the exception of the following variations. Confirmation of the bio-control activity of the most 21 promising isolates (14 lucerne isolates, 1 pea isolate, 3 clover isolates and 3 medic isolates) of actinobacteria was undertaken using small pots containing approximately 200 grams of sandy loam that had been inoculated with 6 millet seeds infected with *R. solani* AG8 (except control treatments without the pathogen) (Figure 4.2). The pots were incubated for 2 weeks in a glasshouse maintained below 20°C to encourage colonisation by the pathogen. Six seeds of lucerne cultivar SARDI Ten were sown in each pot covered with 60 grams of sandy loam on top. The seeds had been surface sterilised, germinated and treated as in the previous section with one of 21 isolates of actinobacteria and rhizobia. Four plants per pots and each treatment was replicated four times. Pots were arranged in a

completely randomised design in the greenhouse and their positions changed once a week. Plants were grown for 4 weeks after sowing. Shoot height and root and shoot dry weights were determined.

4.2.4 Detection of plant growth promotion properties

4.2.4.1 Indole acetic acid (IAA) production

The IAA production ability of the endophytic actinobacteria was checked following the method by Khanmna *et al.* (2009). Five plugs (5 mm diameter) of the actinobacterium which was grown on ISP2 for 5-7 days were transferred into 5 ml of YME (Yeast Malt Extract) containing 0.2% L-Tryptophan. The broth was shaken at 125 rpm for 7 days at 27^oC before centrifuging 1 ml of broth at 11,000 rpm for 15 minutes. The mixture of 0.5 ml of supernatant and 1 ml of Salkowski reagent (12 g of $FeCl_3$ per litre of 7.9 M H_2SO_4) was mixed well and kept in dark for 30 minutes. The IAA production activity was measured using a spectrophotometer at OD_{530nm}. YME broth without L-tryptophan was used as the baseline and pure indole-3-acetic acid (Sigma) with different concentrations were used to make a standard curve.

4.2.4.2 Phosphate solubilisation

The ability of selected isolates for phosphate solubilisation was detected following the method performed by Beneduzi *et al.* (2008). The actinobacteria isolates were spot inoculated on the GY (glucose yeast) media which contained of 10 g of glucose, 2 g of yeast and 1.5% agar in 1L of distilled water. Two solutions were added to the medium, the first was 5 g K_2HPO_4 in 50 ml distilled water and the second solution was 10 g of $CaCl_2$ in 100 ml distilled water. These two solutions were autoclaved

separately and added into the GY medium before pouring into plates. These two solutions changed the colour of the GY medium to white opaque showing the presence of insoluble calcium phosphate. A positive reaction is demonstrated by the presence of clear area surrounding the isolates.

4.2.4.3 Siderophore production

The siderophore production of the actinobacterial isolates was determined using the chrome azurol S (CAS) assay by Alexander and Zuberer (1991). Four solutions were prepared for making the CAS media. Solution 1, an indicator solution, was made by consecutively adding 10 ml of 1 mM $FeCl_3.6H_20$ (in 10 mM HCl), 50 ml of CAS 1.21 mg/ml and 40 ml of Hexadecyl-trimethylammonium bromide (CTAB) 1.82 mg/ml. The mixture gave a dark blue colour. Solution 2, a buffer solution, was prepared by dissolving 30.24 g PIPES (piperazine-N,N'-bis 2-ethanesulfonic acid) in 750 ml salt solution containing 0.3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl. The pH was adjusted to 6.8 with 50% KOH. Fifteen grams of agar were used as solidifying agent and more RO water was added to make up the solution to 800 ml. Solution 3 consisted of 70 ml water, 2 g glucose, 2 g mannitol, 493 mg $MgSO_4.7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4.4H_2O$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4.5H_2O$, 1.2 mg $ZnSO_4.7H_2O$, and 1.0 mg $Na_2MoO_4.2H_2O$. Solution 4 contained 30 ml of 10% (w/v) casamino acids and was filter sterilized. The reagents used were freshly prepared. All the solutions except for solution 4 were autoclaved and cooled to 50^oC. All the solutions were mixed by pouring solution 3 and 4 into the buffer solution (solution 2), and solution 1 was added last. The solution was mixed well prior pouring to plates. The isolates were spot inoculated and a positive reaction was indicated by the presence of orange halo surrounding the colony.

4.3 Results and discussion

4.3.1 Effects of actinobacteria on germination

Forty nine of the 148 isolates (33%) enhanced the germination of lucerne in terms of the number of seedlings that emerged from the sandy loam soil and the improved root length of seedlings growing on agar (Figure 4.3). Most of these cultures (33 of forty nine isolates) had been sourced from lucerne (Table 4.1). In the sterile sandy loam, 22 isolates improved both germination and root length when co-inoculated with strain RRI 128 (Figure 4.3). Actinobacteria did not affect nodule number in the first two weeks of growth as the time period was probably too short. Twelve isolates improved germination on both agar and in sandy loam, of which nine were isolated from lucerne (Appendix 3). In contrast, sixteen isolates adversely affected germination and early growth of lucerne in both systems.

Other studies have similarly described beneficial effects of *Streptomyces* and their secondary metabolites on germination of black gram, maize, radish and *Bromus lyticus* (grass) seeds (Postolaky *et al.*, 2012; Venkatachalam *et al.*, 2010). *S. gibosonni* and *S. grieseoletus* induce seed (black gram, maize, radish and *Bromus lyticus* (grass) germination by the production of polyamines while S. *viridochromogenes* and *S. clavifer* inhibited the germination of seeds by the production of phosphinothricin (Venkatachalam *et al.*, 2010). In addition, the metabolites of *Streptomyces* isolated from soils of Moldova significantly improved the germination of maize and stimulated the growth of roots and coleoptiles (Postolaky *et al.*, 2012).

Table 4.1 Effect of different isolate sources on germination of seeds and early growth of lucerne

Source of isolates	Number of isolates tested	Germination		Early growth	
		Positive	Negative	Positive	Negative
Lucerne	71	33	9	9	4
Field Pea	25	6	4	3	6
Subclover	29	7	9	5	8
Annual medics	23	3	5	4	6
Total	**148**	**49**	**27**	**22**	**24**

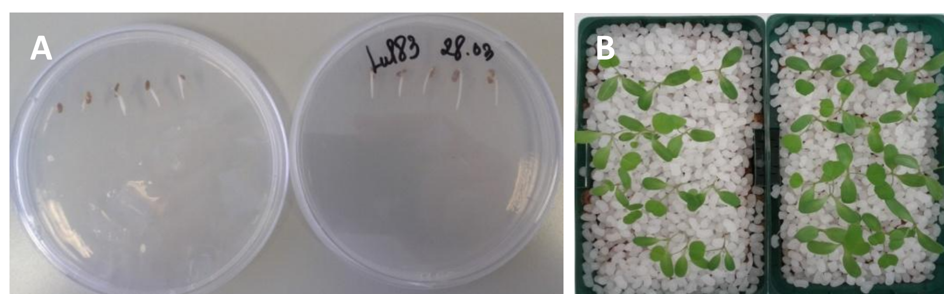


Figure 4.3 Effects of actinobacteria on germination of lucerne seeds after 36 hours on agar (A); Left is control and right is treated with LuP83. Effects of actinobacteria on germination and early growth of lucerne in sandy loam after 10 days planting (B); Left is control, right is treated with LuP30.

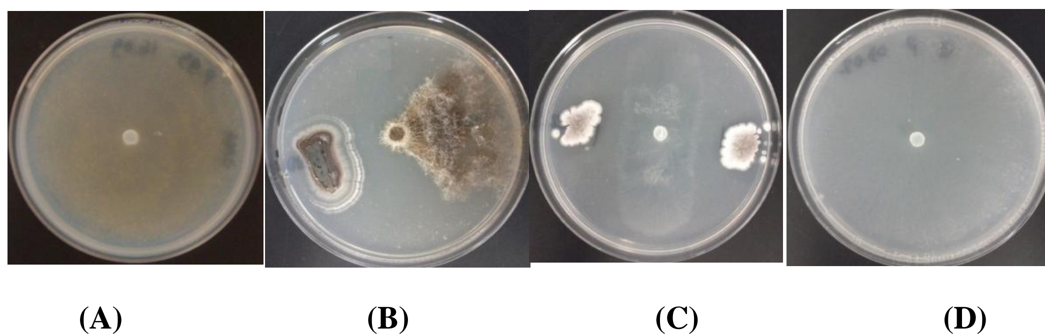
4.3.2 Inhibition of pathogen mycelial growth on agar

The actinobacteria tested were more effective at inhibiting the mycelial growth of *Rhizoctonia solani* than *Pythium irregulare.* Of the 62 cultures that were active against the *R. solani*, 4 showed strong and 8 showed moderate activity against the pathogen. Twenty five cultures were active against the *P. irregulare,* of which 13 were moderately active (Table 4.2). Eight isolates of actinobacteria (LuP35, LuP49, CM7, CM23B, PP1, PP3, PG7 and PP9) showed moderate to strong inhibition of

both pathogens. The antifungal activity of these eight isolates was displayed through the survival percentage of the two pathogenic fungi. The actinobacteria that was most effective against both fungi was CM23B which was isolated from clover (Figure 4.4) and reduced the colonisation of *R. solani* and *P. irregulare* by 12% and 44% respectively (Table 4.3).

Table 4.2 Number of endophytic actinobacteria showing antifungal activity

Source of isolates	Number of isolates tested	No. of actinobacteria isolates inhibiting growth of pathogen mycelia	
		***R. solani* AG8**	***P. irregulare* 89**
Lucerne	71	40	15
Pea	25	7	5
Clover	29	12	4
Medic	23	3	1
Total	**148**	**62**	**25**



(A) **(B)** **(C)** **(D)**

Figure 4.4 Antifungal activity of an endophytic actinobacteria against fungal pathogens. (A)- *R. solani* AG8 only; (B) CM23B against *R. solani* AG8; (C) CM23B against *P. irregulare* 89 and (D) *P. irregulare* 89 only.

Table 4.3 Effect of selected actinobacteria isolates on pathogen mycelial growth, IAA, phosphate solubilisation and siderophores.

Culture	Antimicrobial activity (survival percentage of pathogen)		Indole acid (IAA) µg/ml	Phosphate solubilisation (mm)	Siderophores (mm)
	R. solani	*P. irregulare*			
8 cultures medium to strong antifungal activity against *R. solani* and *P. irregulare*					
LuP35	31	57	0.97	0	3.5
LuP49	33	71	0.61	0	1
CM7	37	64	0.99	0	4
CM23B	12	44	1.64	0	5
PP1	63	78	0.65	0	2
PP3	57	71	2.20	0	3
PP9	39	51	0.00	0	2
PG7	71	89	0.84	0	2
9 cultures benefited to early growth and bio-control against *R. solani* in lucerne plants					
LuP3	29	100	0.05	0	1
LuP10	47	100	0.00	0	1.5
LuP12A	77	100	2.22	3.5	1.5
LuP30	47	100	1.86	1	2.5
LuP44	22	90	0.12	6	0
LuP46B	36	98	0.81	0	1.5
LuP47B	43	77	1.94	0	3
LuP73B	45	88	0.40	0	1.5
LuP83	52	100	0.05	0	1.5

4.3.3 Effect of actinobacteria on root damage from R. solani

4.3.3.1 Preliminary screening in Falcon tubes

Optimal moisture content for infection of *R. solani* on root and growth of lucerne was 12% while with 8% and 10% moisture the plants were unhealthy. Two millet seeds infected with *R. solani* AG8 in each pot were chosen for the assay as 4 and 6 millet seeds totally inhibited the germination of lucerne seeds; almost no seedlings were observed after 3 weeks (Appendix 5). In the Falcon tube assay, more of the actinobacteria isolated from lucerne isolates reduced the damage to the root caused by *R. solani* than isolates from the three other legumes; 47 isolates (32 from lucerne

and 15 from other legumes) showed efficacy in reducing the damage symptoms associated with *R. solani* AG8 (Table 4.2). About 45% of the lucerne isolates and 19.5% other legume isolates reduced lucerne root damage and although the amount of lucerne isolates (71) and other leguminous isolates (77) were as similar, the origin of cultures might give more benefits to their host plant (Table 4.4). The endophytic actinobacteria treatments had more plants surviving in each tube as well as lessened root damage due to infection by the *R. solani* AG8 (Figure 4.5).

Table 4.4 Number of endophytic actinobacteria showing biocontrol activity

Source of isolates	**50ml tube**		**Pot assay**	
	Number of isolates tested	**No. of isolates reducing lucerne root damage**	**Number of isolates tested**	**No. of isolates reducing lucerne root damage**
Lucerne	71	32	14	6
Pea	25	3	1	0
Clover	29	7	3	0
Medic	23	5	3	0
Total	**148**	**47**	**21**	**6**

Figure 4.5 Biocontrol ability of actinobacteria in reducing damage of *R. solani* AG8. Left plant, RRI 128 + *R. solani* AG8; right plants, RRI 128 + *R. solani* AG8 + LuP30.

4.3.3.2 Pot assay

Six isolates (LuP10, LuP30, LuP44, LuP46B, LuP47B and LuP73B) of the 21 tested increased plant weight by between 34% and 47% when compared to plants that had only been inoculated with rhizobia and the root pathogen *R. solani* (Table 4.5). This was associated with increases in lucerne shoot weight by LuP46B and root weight by three of the isolates (LuP30, LuP44 and LuP73B). All six isolates showing bio-control activity on lucerne were isolated from lucerne roots and have been identified as *Streptomyces* spp. by 16S rRNA gene sequencing (Table 4.6).

Table 4.5 Effect of isolates of 21 selected actinobacteria applied to seed on the shoot and root weight of lucerne seedlings growing in soil infected with *Rhizoctonia solani* AG8. Rhizoc = *Rhizoctonia solani* AG8.

Seed treatment	Rhizoc. in soil (+/-)	Source of isolate	Shoot length (cm)	Shoot weight (mg DM/plant)	Root weight (mg DM/plant)	Total Plant weight (mg DM/plant)
RRI 128 only	-		3.74^{a}	10.01^{a}	11.19^{a}	21.20^{a}
Nil	-		2.2^{d}	2.67^{d}	4.27^{d}	6.74^{d}
RRI 128 only	+		2.95^{c}	6.60^{c}	4.9^{cd}	11.50^{c}
RRI 128 + EN16	+	wheat	3.07^{c}	6.82^{bc}	7.31^{abc}	14.13^{bc}
RRI 128 + LuP8	+	lucerne	2.97^{c}	6.84^{bc}	4.75^{cd}	11.59^{c}
RRI 128 + LuP10	**+**	**lucerne**	**3.59**^{ab}	**8.09**^{bc}	**7.75**^{abc}	**15.84**^{b}
RRI 128 + LuP12A	+	lucerne	3.12^{bc}	7.67^{bc}	6.45^{abc}	14.12^{bc}
RRI 128 + LuP15	+	lucerne	3.03^{c}	6.53^{c}	4.82^{cd}	11.35^{c}
RRI 128 + LuP25	+	lucerne	2.96^{c}	6.85^{bc}	5.78^{bc}	12.63^{bc}
RRI 128 + LuP30	**+**	**lucerne**	**2.95**^{c}	**7.32**^{bc}	**8.52**^{ab}	**15.82**^{b}
RRI 128 + LuP44	**+**	**lucerne**	**2.97**^{c}	**6.85**^{bc}	**9.34**^{ab}	**16.19**^{b}
RRI 128 + LuP46B	**+**	**lucerne**	**3.37**^{abc}	**8.51**^{ab}	**7.78**^{abc}	**16.29**^{b}
RRI 128 + LuP47B	**+**	**lucerne**	**3.36**^{abc}	**7.27**^{bc}	**8.11**^{abc}	**15.38**^{b}
RRI 128 + LuP65	+	lucerne	2.89^{c}	7.01^{bc}	5.81^{bc}	12.82^{bc}
RRI 128 + LuP69	+	lucerne	3.06^{c}	6.56^{c}	4.92^{cd}	11.48^{c}
RRI 128 + LuP73B	**+**	**lucerne**	**3.08**^{bc}	**7.33**^{bc}	**9.65**^{ab}	**16.98**^{b}
RRI 128 + LuP74	+	lucerne	3.09^{c}	6.62^{c}	4.87^{cd}	11.49
RRI 128 + LuP83	+	lucerne	3.12^{bc}	7.16^{bc}	7.69^{abc}	14.75^{bc}
RRI 128 + PL23	+	pea	2.88^{c}	7.10^{bc}	5.72^{bc}	12.32^{c}
RRI 128 + CM14	+	clover	2.93^{c}	6.53^{c}	5.45^{bc}	11.98^{c}
RRI 128 + CM23B	+	clover	2..98^{c}	7.12^{c}	5.32^{bc}	12.44^{c}
RRI 128 + CM28	+	clover	2.97^{c}	6.60^{c}	5.54^{bc}	12.14^{c}
RRI128 + MF18	+	medic	3.02^{c}	6.57^{c}	4.98^{cd}	11.55^{c}
RRI 128 + MF19	+	medic	3.04^{c}	6.61^{c}	5.47^{bc}	12.08^{c}
RRI 128 + MF22	+	medic	2.89^{c}	7.03^{bc}	5.65^{bc}	12.68

Values within a column that do not contain the same letter in the post script are significantly different ($P < 0.05$). Data was analysed using one-way ANOVA and differences in means determined using Duncan's Multiple Range test.

Several properties associated with actinomycetes might explain the ability of several of them to act as biocontrol agents. Those properties are the ability to colonize plant surfaces, their ability for antibiosis against plant pathogens, the synthesis of particular extracellular proteins, and the degradation of phytotoxins. Evidence indicates that actinomycetes are quantitatively and qualitatively important in the rhizosphere (Crawford *et al.*, 1993; Doumbou *et al.*, 2001), where they may influence plant growth and protect plant roots against invasion by root pathogenic fungi (Lechevalier, 1988). Endophytic microorganisms that colonizes the internal parts of roots are ideal for use as a biocontrol agent against soil-borne diseases (Coombs *et al.*, 2004; Weller, 1988).

In these experiments, the six actinobacteria which reduced the damage by *R. solani* were able to produce IAA and siderophores. The selected cultures were able to produce IAA in the presence of 0.2 % L–tryptophan, with the exception of isolates Lup10 and PP9. The highest IAA producing actinobacteria were LuP12A and PP3 with 2.22 µg/ml and 2.20 µg/ml respectively, followed by LuP47B and LuP30. LuP3 and LuP83 produced at low concentrations of IAA 0.05 µg/ml (Table 4.3). Indole acetic acid (IAA) is the main auxin in the plant, a common natural auxin and is also a product of L-tryptophan metabolism by microorganisms (Bhavdish *et al.*, 2003). The auxins are able to improve plant growth by stimulating cell elongation, root initiation, seed germination and seedling growth (El-Tarabily *et al.*, 2008). The interaction between the IAA producing bacteria and plants results in various effects on plant growth and varies from pathogenesis to phytostimulation (Spaepen *et al.*, 2007). Therefore, it is possible that the bacteria producing IAA are not only able to

stimulate the plant growth but also compete in use of tryptophan with microbial pathogens in the rhizosphere.

Table 4.6 The 16S rRNA gene sequence similarity of the six actinobacteria with their closest type cultures

No.	Isolate	Closest type strain	Similarity (%)
1	LuP10	*Streptomyces drozdowiczii* NRRL B-24297	99.63
		Streptomyces drozdowiczii NBRC 101007	99.63
2	LuP30	*Streptomyces rishiriensis* NRRL B-3239	99.85
		Streptomyces rishiriensis NBRC 13407	99.90
3	LuP44	*Streptomyces ciscaucasicus* NBRC 12872	99.7
		Streptomyces canus NBRC 12752	99.7
4	LuP46B	*Streptomyces bluensis* NBRC 13460	100
5	LuP47B	*Streptomyces ciscaucasicus* NBRC 12872	99.49
		Streptomyces canus NBRC 12752	99.49
6	LuP73B	*Streptomyces lucensis* NBRC 13056	99.03
		Streptomyces myxogenes NBRC 13793	98.96

Phosphorus is one of major plant nutrients involved in various metabolic processes such as respiration, photosynthesis, cell division and development, energy transport, signal transduction, macromolecular and biosynthesis (Ahemad *et al.*, 2009; Khan and Doty, 2009; Shenoy and Kalagudi, 2005). Phosphate-solubilizing microbes are able to transform the insoluble phosphorus to soluble forms by acidification, chelation, exchange reactions, and polymeric substances formation (Khan *et al.*, 2010). Three isolates LuP44, Lup12A and LuP30 have phosphate solubilisation ability while almost all cultures produced siderophores except LuP44 (Table 4.3). Microbes are able to secret siderophores, low molecular weight compound which acts as an iron chelator during conditions of iron deficiency. Iron is involved in

catalytic activity during respiration, photosynthesis, DNA synthesis and defence mechanisms (Dellagi *et al.*, 2009). Although iron is often abundant in soils, it can have low asolubility at neutral or alkaline pH. Therefore, it is considered as a limiting nutrient for microbial and plant growth in many soils. Siderophore production from the endophytic actinobacteria has been shown directly suppress the development of pathogens by iron competition (Loper and Buyer, 1991).

However, eight cultures showed medium to strong antifungal ability against *R. solani* AG8 and *P. irregulare* 89 *in vitro* but they did not significantly reduce the root damage of lucerne by *R. solani in planta*. In contrast, the six isolates which showed reducing of damage by the infection of *R. solani* had weak to medium antifungal activity against *R. solani* and *P. irregulare in vitro*. Two possibile reasons to explain these results are the actinobacteria might induce the plant defense system to protect the damage by fungal pathogens; or the actinobacteria produce antifungals after colonising the roots and protect the host plants.

4.4 Conclusions

Twenty two actinobacteria promoted germination and early growth of lucerne growing on agar plates and in a sandy loam soil. Several isolates of actinobacteria demonstrated antifungal activity against to *R. solani* and to a lesser extent against *P. irregulare in vitro*. For the biocontrol assay against *R. solani* AG8 although the mechanisms have not been elucidated, several of the actinobacteria isolates from lucerne provided a level of protection against the pathogen. Six isolates have been short listed (LuP10, LuP30, LuP44, LuP46B, LuP47B and LuP73B) as potential biocontrol agents and for more detailed investigation of their effects on lucerne.

The next chapter will be focused on the interactions with the most promising actinobacteria, which have shown a positive result in our studies on germination, on early growth promotion, on the nodulation and nitrogen fixation, with lucerne and *Rhizobium* in the absence of pathogenic fungi, and biocontrol activity.

Chapter 5

Effects of selected actinobacteria on the nodulation and growth of lucerne and its rhizobial partner (*Sinorhizobium meliloti* strain RRI 128)

5.1 Introduction

Sharma *et al.* (2005) found a high abundance of actinobacteria in the rhizosphere of three different legumes namely faba beans (*Vicia faba* L., cv. Scirocco), peas (*Pisum sativum* L., cv. Duel) and white lupin (*Lupinus albus* L., cv. Amiga) by using actinobacteria-specific primers. New evidence also showed actinobacteria living or internally colonizing the nodules of *Lupinus angustifolius*. *Kribbella lupini* sp. nov. and *Micromonospora* spp. were isolated from root nodules of *Lupinus angustifolius* (Trujillo *et al.*, 2010; Trujillo *et al.*, 2007; Trujillo *et al.*, 2006) and rhizosphere and nodules of pea (*Pisum sativum*) (Carro *et al.*, 2007; García *et al.*, 2010). Based on the genotypic and phenotypic data, the novel species *Micromonospora lupini* sp. nov. and *Micromonospora saelicesensis* sp. nov were also proposed from six actinobacterial strains isolated from root nodules of *Lupinus angustifolius* (Trujillo *et al.*, 2007). These findings show that actinobacteria are often present in the rhizospheres and roots of legumes (Trujillo *et al.*, 2010) and therefore may have some impact on the development function of their symbioses. Whether actinobacteria can be exploited and used as co-inoculants may depend on their compatibility with the rhizobia that are used. Antagonism studies between rhizobia and actinobacteria have reported deleterious effects of the actinobacteria on rhizobial growth, which may be problematic to their use as co-inoculants with rhizobia. For example, Patel (1974) and Rangarajan *et al.* (1984) found that actinobacteria isolated from soil inhibited the growth of some rhizobia *in vitro*. There is evidence to indicate actinobacteria may be significant to legume symbioses. Studies are limited and their compatibility with Australian inoculant strains of rhizobia is unknown.

Different genera of actinobacteria have been found to benefit the growth and N_2-fixation of several legume symbioses. Tokala *et al.* (2002) showed that *Streptomyces lydicus* strain WYEC108 increased the growth, nodulation, nitrogen fixation of pea when it was co-inoculated with rhizobia. Actinobacteria have similarly been reported to improve the nodulation, plant growth, and N_2-fixation of soybean (Gregor *et al.*, 2003; Nimmnoi *et al.*, 2014; Soe *et al.*, 2012), common bean (*Phaseolus vulgaris* L.) (El-Tarabily *et al.*, 2008) and lucerne (*Medicago sativa* L.) (Martínez-Hidalgo *et al.*, 2014; Samac *et al.*, 2003). Nimmnoi *et al.* (2014) found that some *Nocardia* sp. increased plant growth and nutritional status of soya-bean when plants were treated either solely with actinobacteria or together with *Bradyrhizobium japonicum*. Martínez-Hidalgo *et al.* (2014) showed that at least two *Micromonospora* sp. improved growth and nutritional status of lucerne when they were applied alone or together with *Ensifer meliloti* 1021. This study examines the effects of selected actinobacteria (*Streptomyces* sp.) isolated from roots of wheat and lucerne on the nodulation and growth of lucerne and its rhizobial partner *S. meliloti* strain RRI 128.

5.2 Materials and methods

5.2.1 Actinobacteria, rhizobia and lucerne

Three strains of rhizobia were used. *Sinorhizobium* (syn. *Ensifer*) *meliloti* strain RRI 128 which is currently used as the commercial N_2 fixing inoculant for lucerne in Australia was used in all experiments. *Sinorhizobium* (syn. *Ensifer*) *meliloti* strain SRDI 736 which has been selected as a potential replacement for strain RRI 128, and *Sinorhizobium* (syn. *Ensifer*) *medicae* strain WSM 1115 which is currently used to produce a commercial rhizobial culture for the inoculation of annual medics in

Australia (and also nodulates lucerne) were used in the rhizobial growth study. Lucerne (*Medicago sativa* L.) cultivar SARDI Ten was used in all experiments. Thirteen isolates of endophytic actinobacteria, one isolate of actinobacteria was from wheat (strain EN23) and 12 isolates from legumes (Table 5.1).

Table 5.1 Summary of some general properties of thirteen cultures used in the first experiment

No.	Cultures	Part of plant	Germination effects	Early growth effects	IAA µg/ml	Bio-control efficacy
1	LuP3	lucerne root	+	+	0.05	0
2	LuP5B	lucerne root	+	+	0.0	0
3	LuP10	lucerne root	+	+	0.0	+
4	LuP12A	lucerne root	+	+	2.22	0
5	LuP13	lucerne root	+	+	ND	0
6	LuP30	lucerne root	+	+	1.86	+
7	LuP32	lucerne root	+	+	ND	0
8	LuP35	lucerne root	+	+	ND	0
9	LuP46B	lucerne root	+	+	0.81	+
10	LuP47B	lucerne root	+	+	1.94	+
11	LuP86	lucerne root	+	+	ND	0
12	CM23B	clover nodule	+	+	1.64	0
13	EN23	wheat root	+	+	0.0	0

+: positive response; 0: neutral; ND: not done

Two screening experiments were completed. In the first preliminary screening experiment, 12 actinobacteria that had increased early lucerne growth and shown potential to moderate soil borne disease (Chapter 4) were tested for their effects on the nodulation and growth of lucerne, growing in a pasteurised N rich sandy loam soil, in the glasshouse. The five most promising strains (LuP3, LuP10, LuP12A, LuP30 and LuP47B) were progressed to further assessments of their efficacy in N deficient autoclaved sand and vermiculite, in order to clarify the extent to which growth increases were due to N_2-fixation, with increased levels of replication (Figure 5.1). The most promising strains (LuP3, LuP10, LuP12A, LuP30 and LuP47B) were

further assessed for their effect on the growth of the rhizobial partner, growing in different media.

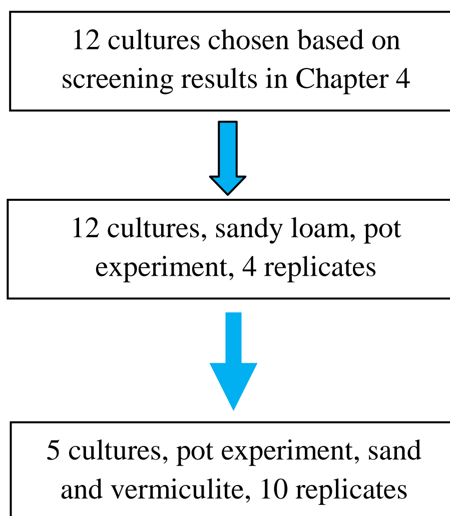


Figure 5.1 *In planta* experiments examining the efficacy of endophytic actinobacteria on the nodulation, growth and N_2-fixation of lucerne

5.2.2 Interaction of twelve endophytic actinobacteria with symbiosis of lucerne and rhizobia

5.2.2.1 In sandy loam

The sandy loam which contained a moderate level of N was collected from Sand and Metal (Netherton) (Appendix 4) and autoclaved. The planting inoculation processes are described in section 2.2 of Chapter 2. Details of the twelve endophytic actinobacteria used in this experiment were LuP3, LuP5B, LuP10, LuP12A, LuP13, LuP30, LuP32, LuP35, LuP46B, LuP47B, LuP56, LuP86, and EN23 are provided (Table 5.1). EN23 which had previously increased the growth and nitrogen fixation of lucerne in sand and vermiculite (Le, 2010) was included as positive control in this experiment. Treatments comprised i) an uninoculated control ii) inoculation with *Sinorhizobium meliloti* strain RRI 128 alone or iii) in combination with one of the 12 actinobacteria. There were four replications for each treatment. The plants were

grown in a glasshouse from September to October 2012 (approx. mean 12h/12h day/night temp. range 15-25°C). Plants were harvested after 7 weeks inoculation with rhizobia strain RRI 128.

5.2.2.2 Assessment of promising actinobacteria in sand and vermiculite media

The experiment examines the efficacy of the best five actinobacteria (LuP3, LuP10, LuP12A, LuP30 and LuP47B) from the above experiment (5.2.5.1) and EN23 was also used as a positive control. The planting and inoculation processes and nutrition are described in section 2.2 of Chapter 2. Treatments comprised i) an uninoculated control ii) inoculation with *Sinorhizobium meliloti* strain RRI 128 alone or iii) in combination with one of the six actinobacteria. A nitrogen solution (1.2g of NH_4NO_3/1L sterile RO water) was supplied for N treatment. Every week 50ml of nitrogen solution was supplied to each lucerne pot and MQ water as required. The plants were grown in a glasshouse from November 2012 to January 2013 (approx. mean 12h/12h day/night temp. range 15-30°C). There were ten replications for each treatment and the plants were harvested 7 weeks after inoculation with the rhizobial strain *S. meliloti* RRI 128. The measurement of shoot, root and nodules are described in section 2.3 of Chapter 2.

5.2.2.2.1 Microscopy

Transmission electron microscopy (TEM) of lucerne nodules collected from plants grown in sand and vermiculite were prepared following the protocol of (Tokala *et al.*, 2002) with some minor modifications. Two top nodules of roots from each pot and four pots were selected for the TEM analyses. Eight nodules were washed

several times with distilled water before being fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer. They were then washed three times (10 minutes per each) with 0.1M PBS buffer before fixing in 1% aqueous osmium tetroxide (150 µl of 2% Osmium and 150µl of PBS buffer) for 1 hour at room temperature. The tubes containing nodules and solutions were rotated at a slow speed. The samples were rinsed in distilled water three times (10 minutes each). The tissues were dehydrated by sequential treatment with increasing concentrations of ethanol 50%, 75%, 85%, 95% (5 minutes each) and twice with 100% ethanol (10 minutes each). The samples were put into 100% propylene oxide for 10 minutes (twice) before transferring to a mixture of two parts of 100% propylene oxide (1ml) and one part of 100% resin (500µl) and left for four hours. After that, 1ml of 100% resin was added to each sample tube before the tubes were embedded in flat molds for 2 days. Finally, only two nodules of each treatment were cut by a diamond knife and examined under a JEOL 1200EX TEM machine at Adelaide Microscopy, The University of Adelaide, South Australia.

5.2.2.2.2 Elemental composition and biochemical analyses

Whole lucerne shoots from the second experiment (section 5.2.2.2) were sampled from the control treatment (rhizobia strain RRI 128 only) and from each co-inoculation with *S. meliloti* RRI 128 and *Streptomyces* spp. EN23, LuP30 and LuP47B. The shoots were dried at 60°C for 48 h and ground using a clean mortar and pestle to about 1mm in size. Ground samples were sent to CSBP in Western Australia (Soil and Plant Analysis Laboratory) to determine N content (%), P, K and other macro and trace elements.

5.2.3 Interaction of five actinobacteria on the growth of three rhizobial strains

Antagonism by six actinobacteria on the growth of rhizobia growing on solid agar media was examined. Three rhizobial strains RRI 128, SARDI 736 and WSM 1115 (Previous described in Chapter 2.1) were grown on yeast mannitol agar (YMA) plates for 3-5 days. The rhizobia were harvested and serially diluted in 0.9% saline and the OD_{600nm} of each suspension measured and the number of colony-forming units counted (Miles and Misra, 1938) to develop a standard curve for each strain of rhizobia.

One hundred microlitres of the rhizobial suspensions containing 10^4 to 10^7 CFU/ml or alternatively $\geq 10^8$ CFU were spread onto YMA plates and allowed to dry in a laminar flow cabinet. Two plugs of 25 mm^2 of each actinobacterial strain grown on International *Streptomyces* Project 2 (ISP2) medium for 7 days were placed on the surface of the inoculated YMA plates. The six selected cultures of actinobacteria were tested in duplicate. The plates were incubated for 5-7 days at 27°C and checked daily for the growth of rhizobia around the plugs. Rhizobia and the actinobacteria that were grown separately as pure cultures on YMA plates were used as controls. One drop (10 µl) each of streptomycin, vancomycin and penicillin (0.1 mg/ml) were added onto the YMA plates containing the rhizobial suspensions as positive antibacterial controls. Effects on rhizobial growth were measured as (++) where increased rhizobial growth (thick culture) occurred in the 3-4 mm surrounding the actinobacterial plug; (+) as above with increased rhizobial growth evident within 1-2 mm; (0) no visible effect; (-) a zone of rhizobial inhibition extending no further than 1-2 mm from the actinobacterial plug; (--) a zone of rhizobial inhibition extending >2 mm from the actinobacterial plug.

5.2.4 Effects of actinobacteria LuP30 and LuP47B on the growth of lucerne nodulating rhizobia strain RRI 128

5.2.4.1 Growth in Yeast Mannitol Broth

A pure single colony of *S. meliloti* RRI 128 formed by streaking on YMA plate for 3-4 days was transferred into 5 ml of YMB (McKinney vial) and grown overnight (27°C and shaken at 125 rpm). One hundred µl of the rhizobia suspension from the McKinney vial was subcultured into a new YMB (5ml in a McKinney vial). Five plugs (25 mm^{2}) of actinobacteria that has been grown on ISP2 medium for 7 days were added to each rhizobial McKinney vial, incubated at 27°C and shaken at 125 rpm. Control treatments only had five plugs of ISP2 medium (without actinobacteria) added. Each treatment was replicated three times. CFU of rhizobia in the vials was measured (Miles and Misra, 1938) at 2h, 4h, 6h, 8h and 10h after the addition of the plugs.

5.2.4.2 In sand and vermiculite

McKinney vials containing 10 g of sand and vermiculite (50:50) were autoclaved at 121°C for 15 minutes. One ml of actinobacterial spores suspension (around 10^{8} CFU) was added into each tube, mixed well by vortex and allowed to settle overnight at 27°C. One ml of an aqueous suspension of rhizobia RRI 128 (around 10^{8} CFU) was added into each vial and incubated at 27°C. The number of actinobacteria and rhizobia was determined at 0, 2, 7, 17, 24, 31, 50, 150 and 240 hours after inoculating RRI 128. At each sampling time, 1 g of sand and vermiculite media was suspended in 9 ml of 0.9% saline, vortexed well for 1 minute and CFU was determined (Miles and Misra, 1938).

5.3 Results and discussion

5.3.1 Effects of actinobacteria on lucerne growth and symbiosis

5.3.1.1 Preliminary screening in a sandy loam

The sandy loam soil contained 94 mg nitrate and 10 mg ammonium per kg soil and had $pH_{(water)}$ 7.0 (Appendix 4). In the absence of actinobacteria, rhizobial strain RRI 128 produced 29 nodules per plant and a total plant weight of 185 mg/plant, which was significantly less than plant weights in the N treatment (350 mg/plant) indicating that plant N demand was not met by rhizobia alone (Table 5.2).

Table 5.2 Effect of endophytic actinobacteria on the growth (shoot and root length and weight) and nodulation (number and weight) of lucerne plants inoculated with RRI 128 and harvested 7 weeks after planting. Seeds were coated with one of thirteen different actinobacteria applied in 0.3% xanthan gum one day before planting. DM= Dry matter. R= rhizobia strain RRI 128.

Treatment	Shoot length (cm)	Root length (cm)	Shoot weight (mg DM/ plant)	Root weight (mg DM/ plant)	Total weight (mg DM/ plant)	Nodule Number (#/plant)	Total nodule mass (mg)
Untreated*	8^{a}	30.2^{a}	44^{a}	39.5^{a}	54.3^{a}	0^{a}	0^{a}
R only	17.8^{b}	29.7^{a}	117.3^{b}	67.8^{abc}	185.2^{b}	29^{b}	10.1^{b}
R + EN23	20.6^{bcd}	24.6^{a}	142.3^{bc}	72^{abc}	214.3^{bc}	31.3^{b}	8.6^{b}
R + LuP3	23.1^{bcd}	30^{a}	165.3^{bc}	63.7^{abc}	229^{bcd}	41.5^{b}	13.9^{b}
R + LuP5B	21.4^{bcd}	29.3^{a}	132.3^{bc}	61.3^{abc}	193.6^{b}	25.8^{b}	11.7^{b}
R + LuP10	21.5^{bcd}	28.4^{a}	142.4^{bc}	92.3^{abc}	234.7^{bcd}	24.9^{b}	7.1^{b}
R + LuP12A	23.3^{bcd}	30.8^{a}	192.2^{bc}	80.2^{abc}	272.4^{bcd}	33.4^{b}	12.4^{b}
R + LuP13	22.2^{bcd}	28.8^{a}	145.4^{bc}	71^{abc}	216.4^{bc}	37.7^{b}	9.3^{b}
R + LuP30	27.5^{cd}	27.1^{a}	219^{cd}	112.2^{c}	331.2^{cd}	33.3^{b}	12.3^{b}
R + LuP32	21.2^{bcd}	27.3^{a}	137.2^{bc}	76.7^{abc}	213.9^{bc}	35.8^{b}	9.7^{b}
R + LuP35	19.1^{bc}	24.4^{a}	105.2^{b}	78^{abc}	198.2^{b}	24^{b}	10.7^{b}
R + LuP46B	21.1^{bcd}	26.5^{a}	153.2^{bc}	78.5^{abc}	231.7^{bcd}	31.2^{b}	11.2^{b}
R + LuP47B	27.0^{cd}	31.2^{a}	220.3^{cd}	92.3^{abc}	312.6^{cd}	35^{b}	13.8^{b}
R + LuP86	19.4^{bc}	23.2^{a}	121.1^{bc}	48.7^{ab}	169.8^{b}	28.3^{b}	9.6^{b}
R + CM23B	17.3^{b}	28.7^{a}	107.3^{b}	67^{abc}	174.3^{b}	32^{b}	11.2^{b}
N supply	29.6^{d}	34.5^{a}	240.8^{d}	109.3^{bc}	350.2^{d}	0^{a}	0^{a}

Values within a column that do not contain the same letter in the post script are significantly different ($p \leq 0.05$).

Co-inoculation with rhizobia and LuP30 or LuP47B increased plant growth compared to inoculation with only rhizobia. Shoot weight of plants co-applied with either LuP30 or LuP47B with rhizobia were significantly increased by 87% and 88%, respectively (Table 5.2 and Figure 5.2A). The combined weight of roots and shoots was increased by 79% and 69% when co-inoculation with rhizobia and LuP30 or LuP47B, respectively. None of the twelve actinobacteria increased root length or weight, number and mass per plant. Five actinobacteria (LuP3, LuP10, LuP12A, LuP30 and LuP47B) that showed increases in plant weight were chosen for further testing in sand and vermiculite (low N) media, in order to provide more opportunity for N fixation response to develop. The effects of the short listed strains on the *in vitro* growth of several rhizobial strains which nodulate with lucerne is also examined.

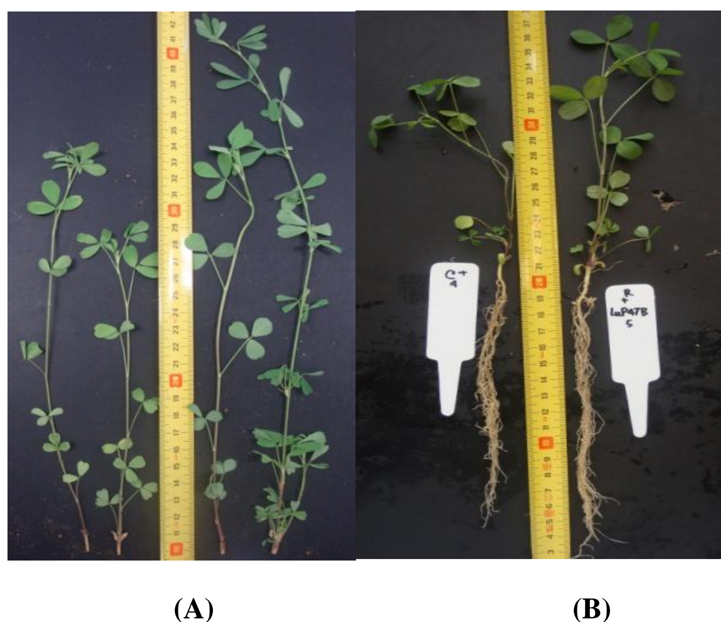
5.3.1.2 Validation of best actinobacteria in sand and vermiculite (low N) media

Actinobacteria isolates LuP30 and LuP47B increased the growth and nodulation of lucerne, compared to plants only inoculated with *S. meliloti* RRI 128 (Table 5.3 and Figure 5.2B). Shoot weight was increased by 25% and 35%, and the combined shoot and root weight by 26% and 30% in the R + LuP30 and R + LuP47B treatments, respectively. Shoot length was also increased by 26% in the R+LuP47B treatment. Similar to the previous experiment, none of the actinobacteria increased the number of nodules. However, in contrast to the previous experiment LuP47B enhanced total nodule weight/plant.

Table 5.3 Effect of endophytic actinobacteria on the growth (shoot and root length and weight) and nodulation (number and weight) of lucerne plants inoculated with RRI 128 and harvested 45 days after planting. Seeds were coated with one of six different actinobacteria applied in 0.3% xanthan gum one day before planting. Rhizobial strain RRI128 was applied five days after planting. (n = 10 pots, 4 plants per pot). DM= Dry matter. R= RRI 128.

Treatment	Shoot length (cm)	Root length (cm)	Shoot weight (mg DM/ plant)	Root weight (mg DM/ plant)	Total weight (mg DM/ plant)	Nodule Number (#/plant)	Total nodule mass (mg)
Untreated*	4.9^{a}	18.0^{a}	11.8^{a}	15.3^{a}	27.1^{a}	0^{a}	0^{a}
R only	12.2^{b}	18.8^{ab}	78.4^{b}	35.6^{b}	114.0^{b}	22.1^{b}	2.28^{b}
R + EN23	13.3^{bc}	23.8^{ab}	89.7^{bc}	43.6^{b}	132.3^{bc}	21.9^{b}	2.71^{bc}
R + LuP3	13.0^{bc}	20.5^{ab}	83.3^{bc}	44.2^{b}	127.5^{bc}	22.5^{b}	2.39^{b}
R + LuP10	14.2^{bc}	21.3^{ab}	80.4^{bc}	58.9^{c}	139.8^{bc}	18.2^{b}	2.30^{b}
R + LuP12A	14.0^{bc}	22.3^{ab}	81.9^{bc}	40.3^{b}	122.2^{bc}	18.3^{b}	1.96^{b}
R + LuP30	13.8^{bc}	32.6^{c}	97.9^{cd}	45.6^{bc}	143.5^{c}	20.8^{b}	2.06^{b}
R + LuP47B	15.4^{c}	25.9^{bc}	106.1^{d}	42.0^{b}	148.1^{c}	20.5^{b}	3.38^{c}

Values within a column that do not contain the same letter in the post script are significantly different ($P < 0.05$). Data was analysed using one-way ANOVA and differences in means determined using Duncan's Multiple Range test.



(A) **(B)**

Figure 5.2 Increase by endophytic actinobacteria on the growth and symbiosis of lucerne when co-inoculation with RRI 128 after 7 weeks. (A) Shoot plants grown in sandy loam: (left) RRI 128 alone and (right) LuP30 + RRI 128; (B) plants grown in sand and vermiculite: (left) RRI 128 alone and (right) LuP47B + RRI 128.

In this experiment the wheat isolate EN23 did not affect any of the measured parameters, except for increases in concentration of P, total N and total N per plant.

Co-inoculation with actinobacteria strain Lu47B increased total N accumulation by 28% (3.34 v. 2.60 mg N/plant) due to increased dry matter production rather than to changes in shoot N concentration, which remained relatively constant around 3.3% (mean 32,767 mg/kg) of shoot DM (Table 5.4). Strains LuP30 and EN23 also increased total N accumulation, but to a lesser extent. The shoot concentration of Ca, Mg K, Na, S, and Zn were not affected by the actinobacteria (data not shown). In contrast, the concentration of P, Bo, Cu, Fe and Mn in the lucerne shoots was affected by the actinobacteria (Table 5.4). Specifically, there was a consistent effect of LuP47B in reducing the concentration of Bo (-9%), Fe (-32%) and Mn (-13%), compared to the control (RRI128 only) treatment. LuP30 increased the concentration of both P and Cu. All tested actinobacteria had the ability to produce siderophores. *Streptomyces* sp. LuP47B was the best IAA producer with 1.94 µg.ml-1, followed by LuP30 and LuP12A as described in section 4.3.4 in Chapter 4.

Co-inoculation of RRI 128 with LuP30 or LuP47B significantly increased the dry weight of shoots, total shoot N, P and Bo. Other studies have shown that co-inoculation of actinobacteria with rhizobia can improve the nutrition of soya plants (Nimmnoi *et al.*, 2014), bean (El-Tarabily *et al.*, 2008) and lucerne (Martínez-Hidalgo *et al.*, 2014) and there is abundant evidence demonstrating the importance of both macro and trace elements for nodulation and the nitrogen fixation processes (Kiss *et al.*, 2004; Robson *et al.*, 1981; Yamagishi and Yamamoto, 1994). Changes in nutrient concentrations provides further evidence of the actinobacteria affecting plant physiology, in addition to changes observed in plant weight. In the N limited growing medium, the increase in total N must have been associated with improved N fixation.

There was no difference in the number of nodules when the lucerne plants were co-inoculated with EN23, LuP30 or LuP47B. Solans *et al.* (2009) similarly found no significant increase in the number of nodules, but an increase in nodule dry weight of up to 42% when lucerne was co-inoculated with *Actinoplanes* strain ME3 and *Micromonospora* strain MM18. In this study, LuP47B significantly increased nodule dry weight per plant by 48%. This suggests that the benefits of the actinobacteria likely occurred after nodule initiation and are more likely to be associated with nodule development or function.

Non-symbiotic responses are also possible. Studies of endophytic actinobacteria from non-legumes, such as strain EN27 which was isolated from wheat roots and improved growth of cereal crops (Franco *et al.*, 2007) indicate effects are also likely to be associated with non-symbiotic host plant genes. EN27 contains a plasmid that was completely sequenced and two of the 13 ORFs which were identified (ORF1 and ORF7) exhibited homology to a plant protein synthesis initiation factor and a plant transcriptional regulatory protein, respectively (Coombs *et al.*, 2003). Although LuP30 and LuP47B produced siderophores and high amounts of IAA *in vitro*, these strains did not affect any of the growth parameters of lucerne in the absence of rhizobial partner RRI 128 (data not shown), noting that responses would have been constrained by N limitation in the growing media.

Figure 5.3 shows differences between the presence of bacteroids in a nodule of control plant and plant treated with LuP47B after seven weeks planting. Presumptive starch granules or contents of vacuole were observed easily in the nodule of control plant while there were a few and very small in the nodule of plant treated with LuP47B. Tokala *et al.* (2002) found that the hyphae and spores of the *Streptomyces*

lydicus WYEC108 on the surface of pea nodules. They noted the presence of presumptive starch granules in the nodules which is considered to result in inefficient nodules (Nutman, 1959 cited in (Tokala *et al.*, 2002)). In this study, treatment plants with LuP47B showed an apparent reduction in starch granules in nodules compared to the nodules of control plants.

Table 5.4 Effects of endophytic actinobacterial isolates EN23, LuP30 and LuP47B on the concentration (mg/kg) and total accumulation of nutrients (ug) in lucerne shoots harvested 7 weeks after planting.

Treatment	[N] (mg/kg)	Total N (ug)	[P] (mg/kg)	Total P (ug)	[Fe] (mg/kg)	Total Fe (ug)	[Bo] (mg/kg)	Total Bo (ug)	[Cu] (mg/kg)	Total Cu (ug)	[Mn] (mg/kg)	Total Mn (ug)
RRI 128 only	33133^{a}	2600^{a}	1100^{a}	86.2^{a}	302^{b}	23.4^{a}	47.9^{b}	3.7^{a}	7.7^{ab}	0.60^{a}	160^{b}	12.5^{a}
RRI 128 + EN23	32867^{a}	2950^{b}	1233^{b}	110.6^{b}	236^{a}	20.0^{a}	43.8^{a}	3.7^{a}	8.3^{bc}	0.70^{ab}	144^{a}	12.2^{a}
RRI 128 + LuP30	32733^{a}	3160^{bc}	1233^{b}	120.7^{c}	249^{a}	21.2^{a}	47.2^{b}	4.5^{b}	8.8^{c}	0.85^{b}	155^{b}	15.0^{a}
RRI 128 + LuP47B	32333^{a}	3340^{c}	1133^{a}	120.2^{c}	206^{a}	21.9^{a}	43.7^{a}	4.6^{b}	7.0^{a}	0.72^{ab}	140^{a}	14.4^{a}

The different letters in the same column indicate a significant difference ($p \leq 0.05$). Data was analysed using one-way ANOVA and Duncan test.

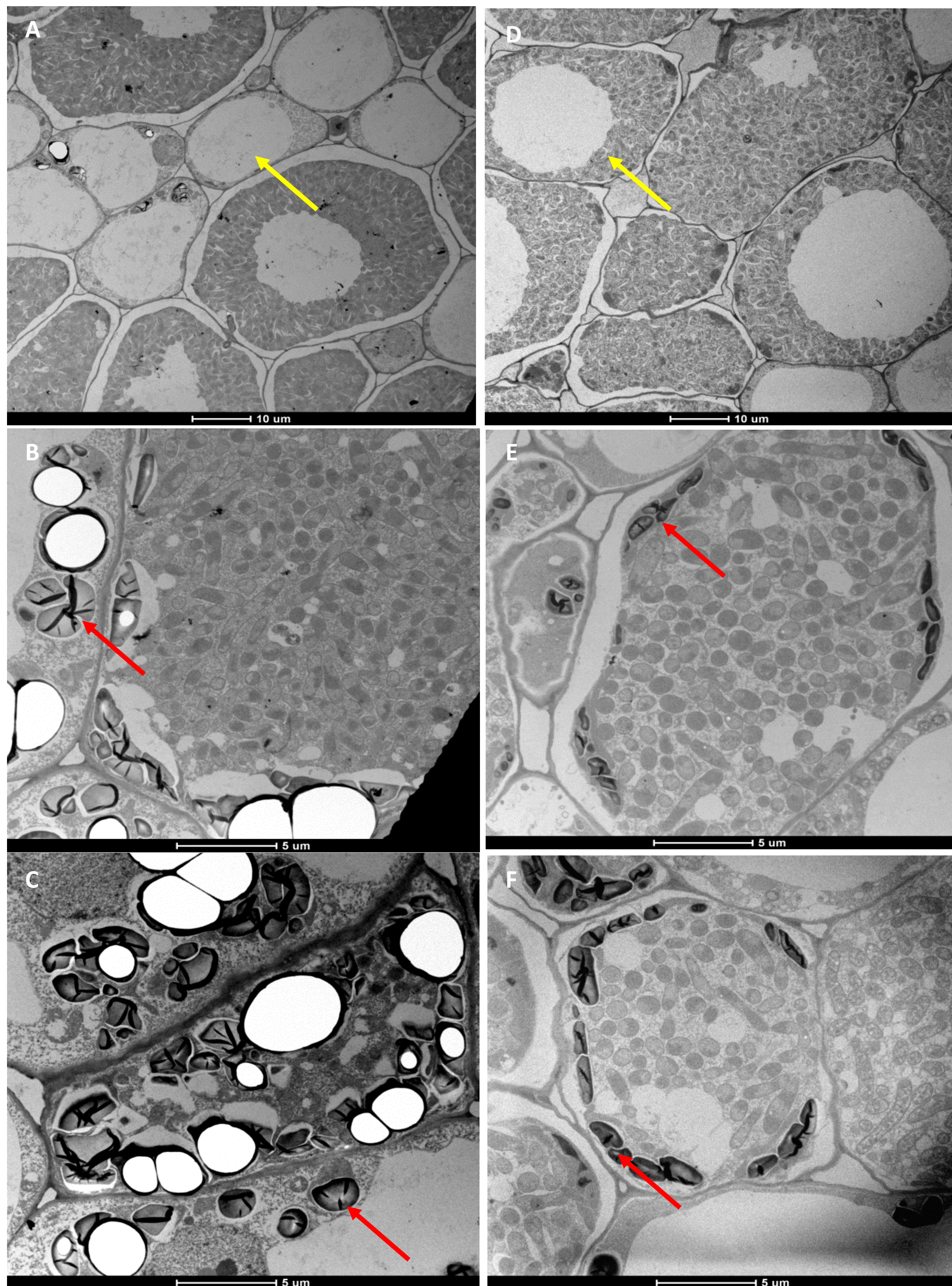


Figure 5.3 Transmission electron microscopy (TEM) of sections of nodules of untreated plants and plants treated with LuP47B 7 weeks co-inoculation with *S. meliloti* RRI 128 (bar = 5 and 10µm). (A, B and C) bacteroids from a nodule of plant treated with RRI128 only. (D, E and F) more bacteroids and smaller starch granules from a nodule of plant treated with LuP47B and RRI128. (yellow arrows: vacuoles and red arrows: starch granules)

5.3.2 Effect of actinobacteria on rhizobial growth

Six endophytic actinobacteria: One from wheat and five from legumes that had previously demonstrated positive effects on lucerne germination, plant growth and nitrogen fixation were tested for their effects on rhizobial growth on agar. Most of the actinobacteria had neutral or positive effects on the growth of the three strains of rhizobia, except for LuP10 which caused some inhibition of rhizobial growth (Table 5.5). The effects were dependant on the strain of rhizobia. For example, although LuP10 inhibited the growth of SARDI 736 and WSM 1115, it increased the growth of RRI 128. LuP3, LuP30 and LuP47B increased the growth of the three strains of rhizobia at both concentrations. EN23 also increased rhizobial growth, with the benefit limited to strain WSM 1115.

Table 5.5 Effect of endophytic actinobacteria on the growth of three strains of lucerne-rhizobia spread onto agar plates at two concentrations (CFU/ml).

	From 10^4 to 10^7 CFU/ml			**≥ 10^8 CFU/ml**		
Strains	**RRI 128**	**SARDI 736**	**WSM 1115**	**RRI 128**	**SARDI 736**	**WSM 1115**
EN23	0	0	++	0	0	+
LuP3	++	+	+	++	+	+
LuP10	++	--	-	+	--	-
LuP12A	0	0	0	0	0	++
LuP30	++	++	++	++	+	++
LuP47B	+	+	+	0	0	+

5.3.3 Effects of actinobacteria on the growth of the rhizobial strain RRI 128

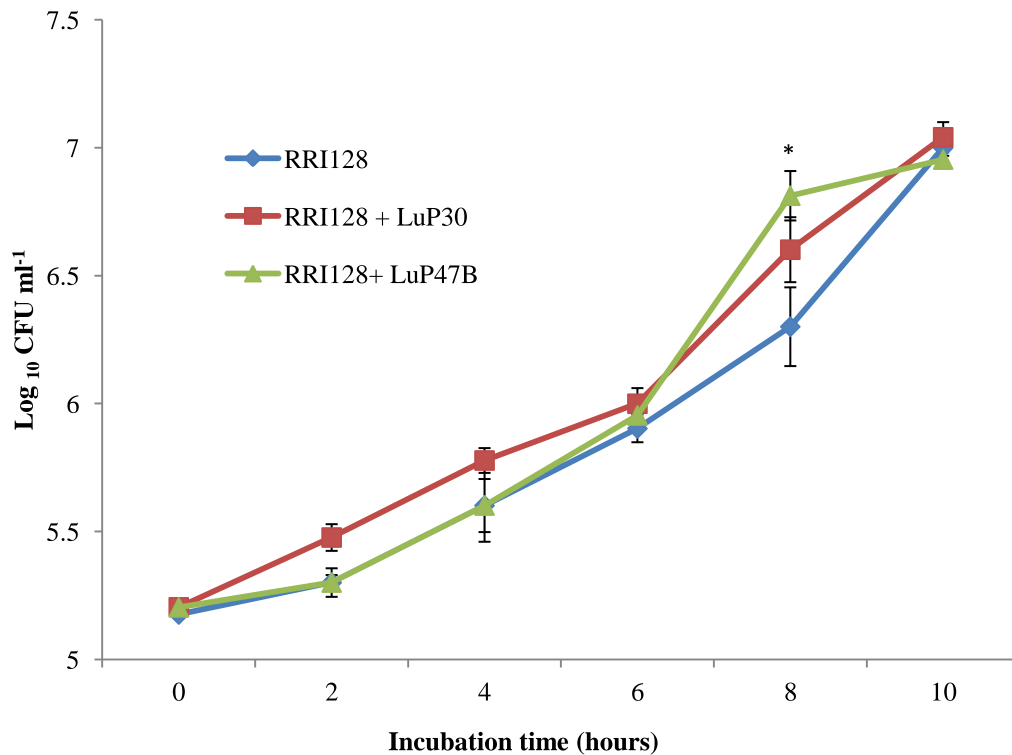


Figure 5.4 Growth (CFU) in Yeast Mannitol Broth medium of *S. meliloti* strain RRI 128 in the presence of actinobacteria cells. Asterisks indicate significant differences from RRI 128 treatment at $P < 0.05$ (*).Error bars: Mean ± S.E.

LuP30 and LuP47B did not generally affect the growth of rhizobia strain RRI 128 growing YMB except for where LuP47B increased the CFU/ml of the rhizobia at 8 hours incubation (Figure 5.4). However, the starting concentration of the *S.meliloti* RRI 128 may have been too high (around 10^6 CFU/ml) to allow for the effects of the actinobacteria to be measured. No effects of metabolites and/or nutrient competition from living cells of actinobacteria LuP30 or LuP47B to the growth of *S. meliloti* RRI 128 were found during the first 6 hours when the actinobacteria and *Rhizobium* were co-inoculated in YMB medium. In sterile sand and vermiculite media, the numbers of actinobacteria LuP30 and LuP47B were checked and not much variation was observed of around 10^8 CFU from day 0 to day 10 (data not shown). The actinobacteria LuP30

and LuP47B generally had no effect on the population of *S. meliloti* RRI 128 growing in soil media, except where LuP47B significantly increased the CFU.g^{-1} soil of the rhizobia at 17 and 168 hours when co-inoculated with the rhizobia (Figure 5.5). Overall, LuP30 and LuP47B are not antagonistic to the growth of *S. meliloti* RRI 128. Therefore, the actinobacteria LuP30 and LuP47B appear to be superficially suitable as co-inoculants with the *S. meliloti* RRI 128 strain and are therefore progressed for further interaction studies *in planta*.

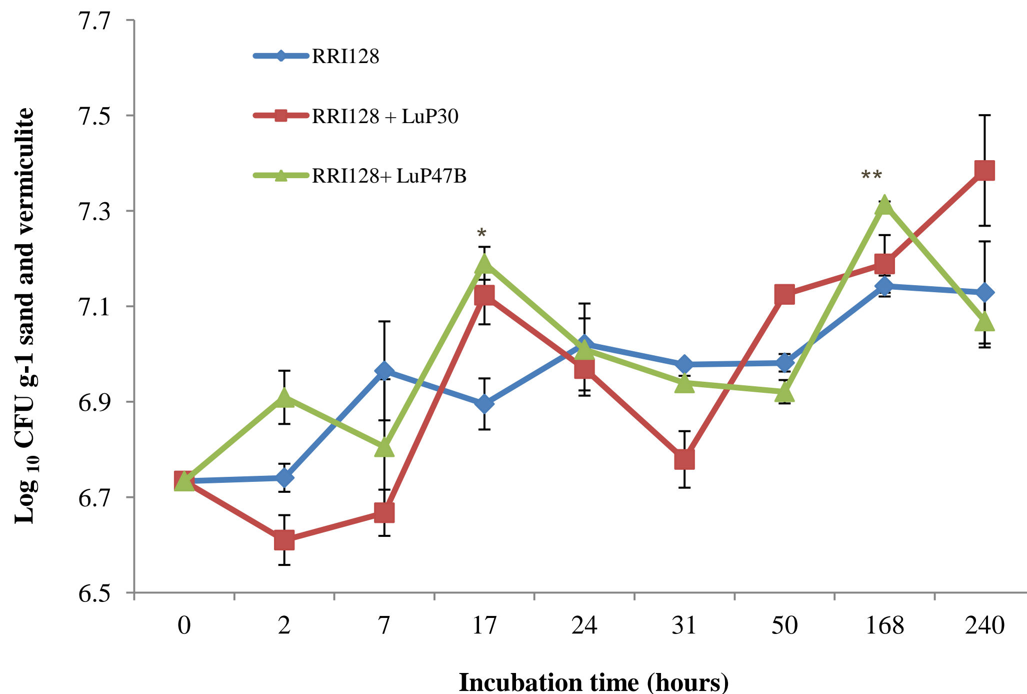


Figure 5.5 The population of *S. meliloti* strain RRI 128 when they were co-inoculation with actinobacteria (LuP30 and LuP47B) in sand and vermiculite. Asterisks indicate significant differences from RRI 128 treatment at $P < 0.05$ (*) or $P < 0.01$(**).Error bars: Mean ± S.E.

Actinobacteria are known as antibiotic producers (Doumbou *et al.*, 2001) and therefore are often antagonistic to the growth of rhizosphere microorganisms. In the case of

rhizobia, actinobacteria isolated from different soils have previously been shown to be antagonistic to growth of different rhizobial strains *in vitro* and *in planta* (Antoun *et al.*, 1978; Damirgi and Johnson, 1966; Patel, 1974). However, in this study, evidence of antagonism was limited, with only LuP10 inhibitory to the growth of two of the three strains of rhizobia. Several of the actinobacteria (LuP3, LuP30 and LuP47B) stimulated growth of all the three strains of rhizobia, a feature that may be favourable for their use as co-inoculation partners with *Rhizobium.*

5.4 Conclusions

The majority of actinobacterial strains tested did not show antagonism to the growth of rhizobia either on solid, or in liquid media. Strains LuP3, LuP30 and LuP47B stimulated growth *in vitro* of several strains of rhizobia that nodulate lucerne, reducing concerns about antagonism raised in other studies. LuP10 reduced the growth of rhizobial strains *in vitro*, however, did not appear to be detrimental to the nodulation or growth of lucerne plants when applied with rhizobial partner *S. meliloti* RRI 128. It follows that it should be possible to use these actinobacteria as co-inoculants without negative effects on growth of the rhizobia tested. It is recognised that the antagonistic effects will vary between rhizobial strains, but if this is an issue there may be inoculation strategies to overcome this.

Co-inoculation with endophyte strains from lucerne (*Streptomyces* spp. LuP30 and LuP47B) with the rhizobial partner RRI 128 significantly increased plant growth as well as total N accumulation in the shoots. They consistently improved the growth of lucerne and by inference its N_2-fixation in the two screening experiments, which used different growth media and provided a range of experimental conditions. The benefits of

the lucerne actinobacteria isolates were greater than the best wheat isolate (EN23). Improved understanding of how *Streptomyces* spp. LuP30 and LuP47B improve plant growth and interact with the nitrogen fixation symbiosis is needed to develop the case for their further development as co-inoculants for use on legumes. They appear to be able to promote nodulation and N_2 fixation with a variety of nitrogen levels in soils. The different concentrations of N in soils could be an opportunity which might allow the actinobacteria such as LuP30 and LuP47B to show increased benefits for the growth and symbiosis of lucerne.

Chapter 6

Effects of endophytic Streptomyces and mineral nitrogen on lucerne (Medicago sativa L.) growth and its symbiosis with rhizobia

6.1 Introduction

The establishment of nodulation and nitrogen fixation in legumes is affected by different soil factors such as pH, availability of nitrogen, calcium, and interactions with rhizosphere microorganisms (Solans *et al.*, 2009). Although N is required in significant amounts (~30 kg/t DM) to optimise plant growth (Pattison *et al.*, 2010), larger amounts (>50 kg/ha) of available N in soil generally have a negative effect on legume nodulation and nitrogen fixation (Herridge *et al.*, 2005; Lucínski *et al.*, 2002; Puiatti and Sodek, 1999; Streeter, 1988, 1985), with many stages of the symbiotic process were inhibited (Dogra and Dudeja, 1993; Dusha, 2002; Mortier *et al.*, 2012). However, a low, static concentration of ammonium (0.5 mM NH_4^+ or 7 mg/kg N) can result in the stimulation of nodulation in pea (*Pisum sativum*) (Fei and Vessey, 2003; Gudden and Vessey, 1997).

Plant mutation and breeding approaches have been used to produce nitrate tolerant soybean (Carroll *et al.*, 1985, 1985; Reid *et al.*, 2011), but the results of that work have not been widely adopted due to other agronomic shortcomings. Manipulation of the soil microbiology and in particular the application of bacteria, including actinobacteria, is another strategy that is currently being investigated (Le *et al.*, 2014; Li and Alexander, 1988, 1990). The potential for actinobacteria to modify legume symbioses was raised by (Sharma *et al.*, 2005) who found a high abundance of actinobacteria in the rhizosphere of three different legumes, faba beans (*Vicia faba* L., cv. Scirocco), peas (*Pisum sativum* L., cv. Duel) and white lupin (*Lupinus albus* L., cv. Amiga). Recent studies on the endophytic actinobacteria showed that they also colonise and live within the root nodules of *Lupinus angustifolius* (Trujillo *et al.*, 2010; Trujillo *et al.*, 2007; Trujillo *et al.*, 2006) and rhizosphere and nodules of pea (*Pisum sativum* L.) (Carro *et al.*, 2007;

García *et al.*, 2010). A number of actinobacteria have been shown to improve the growth, nodulation and N_2-fixation of soybean (Gregor *et al.*, 2003; Nimmnoi *et al.*, 2014; Soe *et al.*, 2012), pea (*Pisum sativum* L.) (Tokala *et al.*, 2002), bean (*Phaseolus vulgaris* L.) (El-Tarabily *et al.*, 2008) and lucerne (*Medicago sativa* L.) (Martínez-Hidalgo *et al.*, 2014; Samac *et al.*, 2003). With regard to overcoming N inhibition of the symbiosis, Solans *et al.* (2009) found that some actinobacteria isolated from the root nodule surface of *Discaria trinervis* promoted the nodulation (42% increase in nodule mass) of lucerne in the presence of high levels N (7 mM or 280 mg NH_4NO_3) of soil nitrogen.

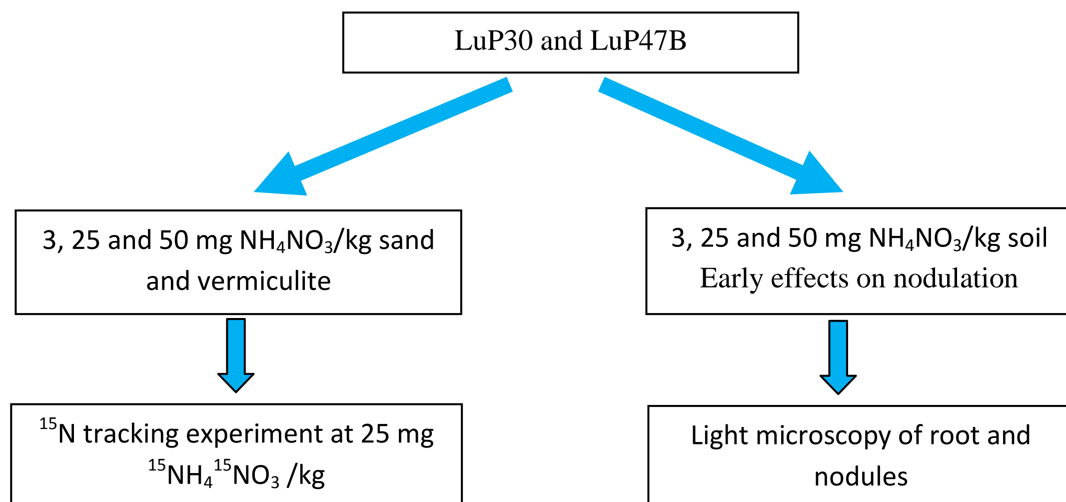


Figure 6.1 Experiments designed to examine the effects of LuP30 and LuP47B on the plant growth and symbiosis of lucerne with presence of different levels of nitrogen

This chapter presents the application of three actinobacteria (*Streptomyces* spp.) on the nodulation and growth of lucerne plants growing in potting media supplied with three levels of NH_4NO_3, a ^{15}N experiment and stidues of their early effects on nodulation (Figure 6.1). Details of experiments are in section 6.2.

6.2 Materials and methods

6.2.1 Actinobacteria, Rhizobium meliloti and lucerne seeds

Seeds of the lucerne cultivar 'SARDI Ten' and *Sinorhizobium meliloti* strain RRI 128 were introduced as described in section 2.1 in Chapter 2 and used to all experiments. The three actinobacteria used were *Streptomyces* sp. EN23, isolated from healthy wheat roots, and *Streptomyces* spp. LuP30 and LuP47B isolated from healthy lucerne roots. All three actinobacteria had been shown to increase the growth of lucerne nodulated with the *S. meliloti* strain RRI 128 in previous studies, under low soil N conditions (3 mg NH_4NO_3/kg soil)

6.2.2 Growth of actinobacteria and rhizobia

The process and protocol were described in section 2.2.2 and 2.2.3 in Chapter 2.

6.2.3 Plant growth media, nutrition, sowing and water supply

The process and protocol were described in section 2.2.4 in Chapter 2

6.2.4 Effects of actinobacteria on the growth and symbiosis of lucerne

Seeds of lucerne cultivar 'SARDI Ten' were surface sterilised following the protocol of Coombs and Franco (2003). Seeds were removed from the final water rinse after 10 minutes, and placed in a laminar flow cabinet overnight to dry. Actinobacteria were applied to the surface of the sterilised lucerne seeds as a suspension in 0.3% xanthan gum to provide 10^8 CFU/g seed and the seeds sown (10 seeds/pot) into a pasteurised (by autoclaving) potting mix (1 kg/pot of 50:50 by volume of sand:vermiculite) contained in

1.25 litre self-watering pots (Décor Watermatic^{TM}). Seedlings were thinned to 4/pot after 6 days, before inoculation with rhizobia strain RRI 128.

Experiment 1:

The factorial experiment comprised (i) 3 strains of actinobacteria, (ii) ± inoculation with *S. meliloti* RRI 128 and (iii) 3 levels of soil NH_4NO_3 (3, 25 and 50 mg/kg) which were added with 200 mL of McKnight's solution on planting day. MQ water was added to the pots as required in the following weeks. Plants designated to rhizobia treatments were inoculated with an aqueous suspension (1ml per plant containing 10^8 CFU) of rhizobia 6 days after sowing. Each treatment was replicated eight times. Pots were arranged in a completely randomised design in a greenhouse and moved weekly to reduce spatial effects. Four pots of each treatment were harvested at 4 and 7 weeks after inoculation. The experiment was carried out in the winter of 2013 (approx. mean 10h/14h day/night temp. range 5-20°C).

Elemental analyses

Four shoots per pot and four pots for each treatment from experiment 1 were sampled. The whole shoots were dried at 60°C for 48 h and ground using a clean mortar and pestle to about 1mm in size and sent to the Waite Analytical Services, University of Adelaide, Australia to determine N content (%), P, K and other macro and trace elements.

Light and electron microscopy

Nodules of three treatments (RRI 128 only, RRI 128 + LuP30 and RRI 128 + LuP47B) at 25 mg N after 4 weeks inoculation with *S. meliloti* RRI 128 were washed several

times with distilled water before fixing within 2.5% glutaraldehyde in 0.1M PBS buffer. The samples were stored in a fridge until used. The preparation process for light and TEM was followed as described in section 5.2.3 in chapter 5.

Experiment 2: ^{15}N experiment

The ^{15}N experiment to determine the relative contribution of soil and fixed N sources at different stages of plant growth was carried out as described in the above experiment except that only two strains, LuP30 or LuP47B, were applied separately to seed prior to inoculation with rhizobia strain RRI 128. A control treatment (rhizobia strain RRI 128 only) was also included. Plants were supplied with 25 mg $^{15}NH_4{}^{15}NO_3$ (98%, Cambridge Isotope Laboratories, Inc.), per kg of soil mix with 200 mL of McKnight's solution (McKnight, 1949) on planting day. The experiment was carried out in the winter of 2014 (approx. mean 10h/14h day/night temp. range 5-20°C). Plants were harvested at 1, 3 and 5 weeks after inoculation with rhizobia strain RRI 128.

The N content of the shoots and roots was analysed by mass spectrometry to determine the proportions of plant N derived from the atmosphere and soil. All four shoots and roots from each pot were ground separately to about 1mm in size. About 3 mg of each sample were analysed for the total N and ^{15}N on an isotope ratio mass spectrometer (Serco, Crewe, Cheshire, UK).

The At% of ^{15}N has been calculated taking account of the ^{15}N in air with the formula below:

At% = [100 x (0.3663/(100-0.3663)) x ((deltaN/1000)+1)]/1+[(0.3663/(100-0.3663)) x ((deltaN/100)+1)]

Experiment 3: Early assessment the effects of LuP30 and LuP47B on nodulation of *S. meliloti* RRI 128 strain and lucerne

Early effects of two actinobacteria LuP30 and LuP47B was investigated in 15 ml tubes in a growth chamber. Twenty grams of autoclaved sand and vermiculite in 15 ml tube were added with 4 ml of McKnight's solution plus 25 ppm nitrogen supply. Seeds were pre-germinated on 1% agar plates overnight then one seed was sown into each tube. All the rhizobial treatments were then inoculated with 1ml of RRI 128 (around 10^8 CFU/ml) after 5 days sowing. The tubes were kept inside a growth cabinet (14/10 day/night cycle) at room temperature (24-30^oC). The plants were harvested after 4, 7, 10 and 13 days after inoculation with the *Rhizobium*.

Microscopy

The experiment was set up in the same way as in experiment 3 and the three levels of NH_4NO_3 were 3 mg, 25 mg and 50mg, with and without the *S. meliloti* RRI 128 strain were conducted in 15 ml tubes. The number of nodules and root hair were assessed after 3, 4, 5, 7 and 10 days after inoculation with the *S. meliloti* RRI 128 strain. Three replications were harvested for each time. The root and nodules of plants were assessed under the Olympus Fluorescence IX71 microscope at Flinders Microscopy Facilities, Flinders Medical Centre, Flinders University, Adelaide, Australia.

6.2.5 General parameters measured and statistical analyses

After harvest, the height of shoots, dry weight of shoots and roots, and the number and dry weight of nodules per plant were measured. The height of the shoots was measured as the distance from the base of the dominant shoot at sand level to the youngest leaf,

and was averaged from four plants per pot. Shoot and roots were dried separately in an oven at $60^{o}C$ for 48 hours to constant weight and the dry weights of both were averaged from four plants per pot. The number and total mass of nodules per plant were determined from sampling 2 plants per pot. Nodules in the top 5 cm of roots and large nodules (diameter ≥1mm) were counted. There were four pots for each treatment.

All data were assessed for normality and log transformed prior to ANOVA where necessary. Data was analysed using ANOVA and significant differences between means determined using Duncan's Multiple Range test. All references to significance in the text imply statistical significance at $P < 0.05$, unless otherwise stated.

6.3 Results and discussion

6.3.1 Effect of actinobacteria on lucerne growth in absence of rhizobia

In the absence of the rhizobial partner, the three actinobacteria EN23, LuP30 and LuP47B significantly increased the shoot dry weights by between 19% to 33% in the 25 mg/kg NH_4NO_3 treatment; and 23% to 24% in the 50 mg/kg NH_4NO_3 treatment (Table 6.1). In contrast, root dry weight was reduced significantly by LuP30 (-21%) and LuP47B (-29%) at 25 mg and EN23 (-18%) at 50 mg NH_4NO_3 after 7 weeks. The actinobacteria significantly increased the shoot: root ratio at 25 mg/kg and 50 mg/kg NH_4NO_3 while there were no effects at 3 mg/kg NH_4NO_3. Treatment with all 3 actinobacteria significantly reduced the iron (~50%), copper (~40%) and manganese (~30%) concentrations in the plant shoots (Table 6.2). None of the actinobacteria increased shoot N concentration. However, total shoot N was increased (+28%) by LuP47B at 50 mg NH_4NO_3 compared with the control treatment (Table 6.2).

Table 6.1 Effect of endophytic actinobacteria (*Streptomyces* spp. EN23, LuP30 and LuP47B alone or in combination with *S. meliloti* strain RRI 128) and soil N on lucerne shoot and root weight at 4 and 7 weeks after inoculation (n=4). Different letters in the same column indicate means are significantly different ($P < 0.05$).

Treatment	Shoot weight DM (mg /plant)			Root weight DM (mg /plant)			Shoot:root		
	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3
Inoculation with *Streptomyces after 7 weeks*									
Not inoculated	20[a]	79[a]	173[a]	17.8[a]	56[b]	91[b]	1.1[a]	1.4[a]	1.9[a]
+ EN23	20[a]	105[b]	216[b]	17.3[a]	49[ab]	75[a]	1.2[a]	2.1[b]	2.9[b]
+ LuP30	20[a]	94[b]	216[b]	19.6[a]	44[a]	84[ab]	1.0[a]	2.2[b]	2.6[b]
+ LuP47B	20[a]	103[b]	212[b]	17.1[a]	40[a]	79[ab]	1.2[a]	2.6[b]	2.7[b]
Inoculation with *Sinorhizobium* and *Streptomyces after 4 weeks*									
Rhizobia only	43[a]	65[a]	88[a]	8.6[a]	20.5[a]	47.0[b]	5.0[c]	3.2[a]	1.9[a]
Rhizobia + EN23	38[a]	84[bc]	88[a]	8.4[a]	34.4[c]	43.2[ab]	4.6[bc]	2.4[a]	2.0[a]
Rhizobia + LuP30	43[a]	89[c]	98[b]	12.0[a]	36.6[c]	42.8[ab]	3.8[ab]	2.4[a]	2.3[a]
Rhizobia + LuP47B	35[a]	78[b]	91[ab]	9.6[a]	26.4[b]	39.4[a]	3.6[a]	3.0[a]	2.3[a]
Inoculation with *Sinorhizobium* and *Streptomyces after 7 weeks*									
Rhizobia only	229[a]	268[a]	348[a]	129[c]	100[a]	169[b]	1.8[a]	2.7[a]	2.1[a]
Rhizobia + EN23	238[a]	340[bc]	394[c]	114[bc]	151[b]	172[b]	2.1[ab]	2.3[a]	2.5[a]
Rhizobia + LuP30	283[b]	392[d]	379[bc]	76[a]	141[b]	142[a]	3.7[bc]	2.8[a]	2.7[b]
Rhizobia + LuP47B	284[b]	365[c]	331[a]	101[ab]	109[a]	163[b]	2.8[b]	3.5[b]	2.0[a]

Table 6.2 Effect of inoculation with *Streptomyces* spp. (alone or in combination with rhizobia) on the concentration (mg/kg) and total accumulation of nutrients (mg or ug) in lucerne shoots at 4 and 7 weeks after inoculation (n=3). Asterisks indicate significant differences from control treatment at the same soil N level at $P < 0.05$ (*) or $P < 0.01$(**).

Treatment	NH_4NO_3 level (mg/kg)	[N] (mg/kg)	Total N (ug)	[P] (mg/kg)	Total P (ug)	[Fe] (mg/kg)	Total Fe (ug)	[Bo] (mg/kg)	Total Bo (ug)	[Cu] (mg/kg)	Total Cu (ug)	[Mn] (mg/kg)	Total Mn (ug)
Inoculation with *Streptomyces* (7 week harvest)													
Not inoculated	25	9220	793	1845	148	102	8.2	70	5.6	18.0	1.4	260	20.8
EN23	25	8000	834	1807	189	59*	6.2*	62*	6.5	10.8*	1.1*	170*	17.7
LuP30	25	8620	823	1883	180	66*	6.3	68	6.5	11.5*	1.1*	182*	17.4
LuP47B	25	8200	845	1810	187	62*	6.5	67	6.9	10.6*	1.1*	175*	18.2
Not inoculated	50	9530	1647	1077	186	123	21.2	63	10.9	14.3	2.5	134	23.1
EN23	50	9110	1984	1027	224	63*	13.6*	48*	10.4	9.09*	2.0*	97**	21.0
LuP30	50	9250	2028	1050	229	64*	14.1*	52*	11.5	9.04*	2.0*	108*	23.7
LuP47B	50	9910	2110*	1040	222	39**	8.3**	53*	11.3	8.68*	1.8*	99*	21.2
Inoculation with *Sinorhizobium* and *Streptomyces* (4 week harvest)													
Rhizobia only	25	30900	2037	2000	132	135	8.8	60	3.9	12	0.8	97	6.4
Rhizobia +EN23	25	30600	2568*	2133	180*	127	10.6	59	5.0*	11	0.9	116	9.7*
Rhizobia +LuP30	25	33640	3049**	1877	170*	202*	18.2**	57	5.2*	13	1.2**	119*	10.8**
Rhizobia +LuP47B	25	32540	2595*	2250*	180*	145	11.6*	59	4.7*	12	1.0*	108	8.6*
Rhizobia only	50	24950	2178	1523	134	149	13.0	59	5.1	12	1.1	103	9.0
Rhizobia +EN23	50	28230	2494	1730**	152*	137	12.1	58	5.1	12	1.1	95	8.4
Rhizobia +LuP30	50	26910	2612*	1630*	158*	198*	19.3*	59	5.7	11	1.1	109	10.6
Rhizobia +LuP47B	50	27560	2477	1733**	156*	184	16.5	63	5.7	13	1.2	105	9.4
Inoculation with *Sinorhizobium* and *Streptomyces* (7 week harvest)													
Rhizobia only	3	22640	5198	823	189	130	29.8	45	10.4	8.7	2.0	98	22.4
Rhizobia +EN23	3	24270	5799	883	211	267*	63.8**	45	10.9	12.8*	3.0*	113*	26.9
Rhizobia +LuP30	3	21990	6530*	767	220*	167	48.1*	40*	11.5	7.7	2.2	86	24.7
Rhizobia +LuP47B	3	20590	7295**	743	223*	202	60.4**	41*	12.2	7.7	2.3	90	27.3
Rhizobia only	25	19410	5194	690	185	147	39.2	49	13.0	8.0	2.1	88	23.6
Rhizobia +EN23	25	16270**	5516	637*	216	101	34.3	40*	13.4	6.4*	2.2	69*	23.4
Rhizobia +LuP30	25	17740	6952*	687	269*	119	46.8	41*	15.9	6.9	2.7	71*	27.9
Rhizobia +LuP47B	25	16550**	6059	723	264*	182	67.2*	48	17.4*	8.6	3.2*	90	32.9
Rhizobia only	50	25120	8440	887	298	182	61.2	44	14.9	9.6	3.2	103	34.7
Rhizobia +EN23	50	25400	9934*	977	384*	156	60.9	46	18.0*	9.0	3.4	97	38.1
Rhizobia +LuP30	50	21490	8191	983*	375*	113*	43.3	39	14.8	7.4*	2.8	93	35.3
Rhizobia +LuP47B	50	21350*	7016*	913	298	117*	38.5*	40	13.1	7.8*	2.5*	102	33.4

In the absence of rhizobia, the three strains of *Streptomyces* increased lucerne shoot weight from 19% to 33% compared to untreated plants after 7 weeks at the two higher N levels, indicating some of the benefits were independent of the rhizobial symbiosis. Non-symbiotic benefits of actinobacteria have been reported for non-legumes such as cucumber (El-Tarabily *et al.*, 2010) and have been attributed to the solubilisation of phosphate, and in wheat to the control of soil borne disease (Franco *et al.*, 2007; Hamdali *et al.*, 2008). LuP30 and LuP47B have been shown to produce siderophores and IAA, and LuP30 has the ability to solubilise phosphate reported in section 4.3.4 in Chapter 4. Nimmnoi *et al.* (2014) found that the siderophore producing actinobacteria increased the iron content of plants. Martínez-Hidalgo *et al.* (2014) conducted a similar experiment with lucerne plants and concluded that *Micromonospora* spp. enhanced nitrogen uptake efficiency and/or improved nitrogen availability in soil. EN23, LuP30 and LuP47B have characteristics that are consistent with those reported in other actinobacteria that improved the growth of non-legumes, and so some non-symbiotic benefits are likely to contribute to their efficacy.

6.3.2 Effect of actinobacteria on lucerne growth when applied with rhizobia in presence of different levels of nitrogen

The application of actinobacteria with rhizobia significantly increased the shoot weight of lucerne (inoculated with rhizobia) at the 4 and 7week harvests at 25mg/kg NH_4NO_3 by 19% to 36% and 27% to 46%, respectively (Table 6.1). The effect of LuP30 was consistent across N levels (7 week harvest). LuP47B had no effect on shoot weight at 50 mg/kg NH_4NO_3, while EN23 had no effect at 3 mg/kg NH_4NO_3 (Table 6.1). Effects of the actinobacteria on root weight were variable. Whilst EN23 and LuP30 increased the root dry weight in the 25 mg/kg NH_4NO_3 treatment at 7 weeks, LuP30 reduced the root

dry weight at 3 and 50 mg/kg NH_4NO_3 (Table 6.1). LuP30 and LuP47B both significantly increased the shoot: root ratio at 3 mg/kg NH_4NO_3 but only LuP47B showed an increase at 25 mg/kg NH_4NO_3 while LuP30 increased the ratio at 50 mg/kg NH_4NO_3 at 7 weeks.

Application of actinobacteria affected the nutrient status of the lucerne shoots (Table 6.2). In general, the application of actinobacteria with rhizobia increased or had no effect on the accumulated amount of several nutrients (N, P, Fe, Bo Cu and Mn). Other nutrients (e.g. K) were never affected (data not shown). At 4 weeks after inoculation with rhizobia, the *Streptomyces* spp. EN23, LuP30 and LuP47B significantly increased the total N (µg), P (µg), Bo (µg) and Mn (µg) at 25 mg NH_4NO_3. For example, co-inoculation with EN23, LuP30 or LuP47B and rhizobia RRI 128 increased total N in shoot by 26%, 50% and 27%, respectively though the *Streptomyces* spp. did not significantly increase the N concentration of shoots (Table 6.2). Moreover, where actinobacteria were applied, the numbers of positive/neutral/negative responses (across all NH_4NO_3 treatments N at the 7 week harvest) were for N (4/4/1), for P (6/3/0), for Fe (4/4/1), for Bo (2/7/0), for Cu (2/6/1) and for Mn (0/9/0). Of the 18 positive responses, 7 were attributable to inoculation with Lup47B, 6 to Lup30 and 5 to EN23.

Nutrient accumulation is the product of shoot weight and nutrient concentration. In general, nutrient concentration in the lucerne shoots was not affected or decreased, with the application of actinobacteria. The numbers of positive/neutral/negative responses (across all NH_4NO_3 treatments N at the 7week harvest) were for N (0/6/3), for P (1/7/1), for Fe (1/6/2), for Bo (0/5/4), for Cu (1/5/3) and for Mn (1/6/2). Of the 15 negative

responses, 5 were attributable to each of the actinobacteria. Three of the 4 positive responses (not P) were attributable to EN23 in the 3mg/kg NH_4NO_3 treatment.

Nodulation impacts

The number of nodules per plant increased with the increasing N supply. This was associated with improved plant growth at the higher N levels. However, number of nodules per mg of root decreased significantly from 2.6 to 1.2 to 0.9 nodules in the 3, 25 and 50 mg/kg NH_4NO_3 treatments at 4 weeks, respectively, but they were similar at the 7 week harvest (Table 6.3). The promptness of nodulation (proportion of nodules on the top 5 cm of the root) was also decreased from 14.5/22, 12.3/24 and 2.5/40 in the 3, 25 and 50 mg/kg NH_4NO_3 treatments.

The addition of actinobacteria increased nodule number per plant at each level of N compared to the rhizobia treatment alone, after 4 weeks of plant growth. For example, at 25 mg/kg NH_4NO_3, all three actinobacteria increased the number of nodules per plant by more than 75%. EN23 and LuP30 also increased the number of nodules at 50 mg/kg NH_4NO_3 (Table 6.3). By 7 weeks, the increased nodulation associated with the actinobacteria only persisted in the 3 mg/kg NH_4NO_3 treatment for LuP30 and LuP47B. Inoculation with actinobacteria and N level also affected the position of nodules on the root system (Table 6.4). The number of nodules in the top 5 cm of the root was reduced by increasing the amount of N, but the effect was moderated by the application of EN23 or LuP30 especially in the 3 mg/kg NH_4NO_3 treatment after 4 weeks. Nodule size was also increased with actinobacterial treatment (Table 6.4). There were several examples where total nodule mass/plant increased with the addition of actinobacteria (Figure 6.2). At the 4 week harvest with 25 mg/kg NH_4NO_3, strains EN23, LuP30 and LuP4B increased nodule mass per plant by 125%, 140% and 78% respectively. At 50 mg/kg

NH_4NO_3, strains EN23 and LuP30 increased nodule mass/plant by 63% and 42%, respectively. At the 7week harvest, EN23 at 50 mg/kg NH_4NO_3 was the only treatment that increased nodule mass.

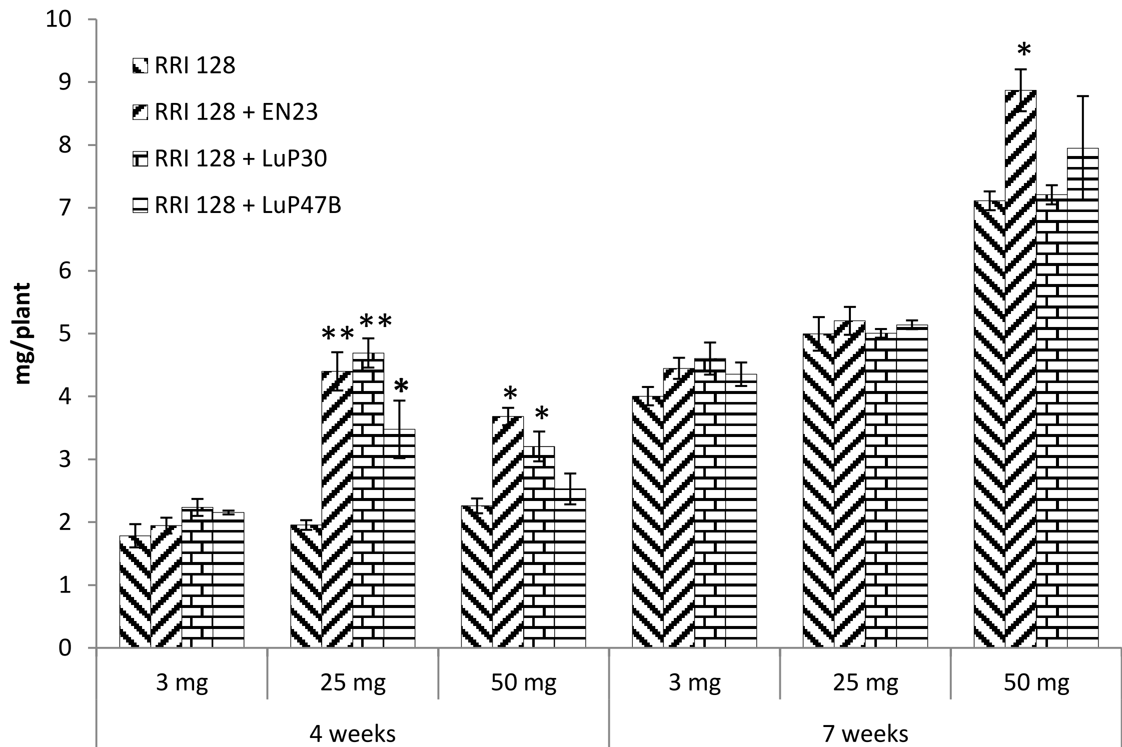


Figure 6.2 Effect of actinobacteria and soil N (NH_4NO_3) on lucerne nodule weight (mg DM/plant) at 4 and 7 weeks after inoculation (n=4). Asterisks indicate significant differences from RRI 128 treatment at $P < 0.05$ (*) or $P < 0.01$(**).Error bars: Mean ± S.E.

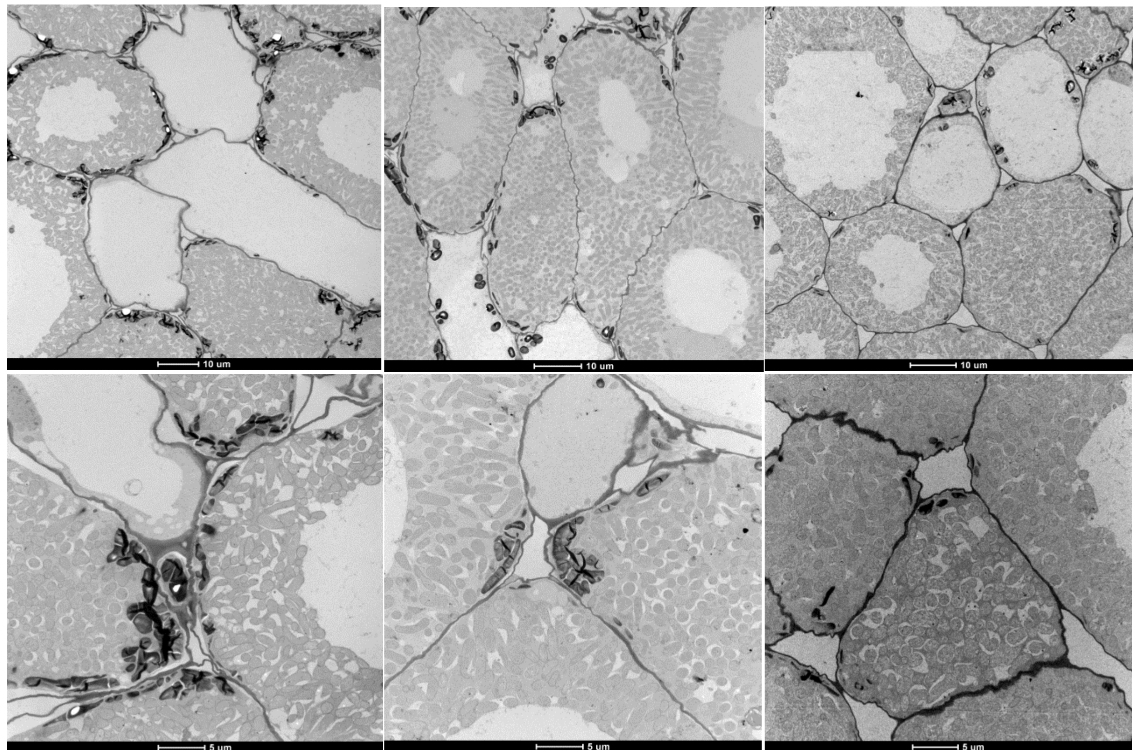
Table 6.3 Effects of actinobacteria and soil N on nodule number per plant and number of nodules per mg of root at 4 and 7 weeks after inoculation with rhizobia (n=4). Different letters in the same column indicate means are significantly different ($P < 0.05$).

Treatment	**Number of nodules**						**Number of nodules per mg of root**					
	4 weeks			**7 weeks**			**4 weeks**			**7 weeks**		
	3 mg NH_4NO_3	**25 mg NH_4NO_3**	**50 mg NH_4NO_3**	**3 mg NH_4NO_3**	**25 mg NH_4NO_3**	**50 mg NH_4NO_3**	**3 mg NH_4NO_3**	**25 mg NH_4NO_3**	**50 mg NH_4NO_3**	**3mg NH_4NO_3**	**25 mg NH_4NO_3**	**50 mg NH_4NO_3**
Rhizobia only	22^{a}	24^{a}	40^{a}	27^{a}	47^{a}	69^{b}	2.6^{a}	1.2^{a}	0.9^{a}	0.2^{a}	0.5^{b}	0.4^{ab}
Rhizobia + EN23	21^{a}	53^{b}	52^{b}	30^{ab}	51^{a}	56^{a}	2.5^{a}	1.6^{b}	1.2^{b}	0.3^{a}	0.3^{a}	0.3^{a}
Rhizobia + LuP30	31^{b}	51^{b}	48^{b}	41^{c}	57^{a}	66^{ab}	2.6^{a}	1.4^{ab}	1.1^{b}	0.5^{b}	0.4^{ab}	0.5^{b}
Rhizobia + LuP47B	24^{a}	42^{b}	36^{a}	37^{bc}	49^{a}	71^{b}	2.5^{a}	1.6^{b}	0.9^{a}	0.4^{b}	0.4^{ab}	0.4^{ab}

Table 6.4 Distribution of total nodules in the top 5 cm of roots and large nodules (diameter ≥1mm) due to treatment with actinobacteria at different rates of NH_4NO_3. Data from harvests at 4 and 7 weeks after inoculation with rhizobia (n= 4). Different letters in the same column indicate means are significantly different ($P < 0.05$).

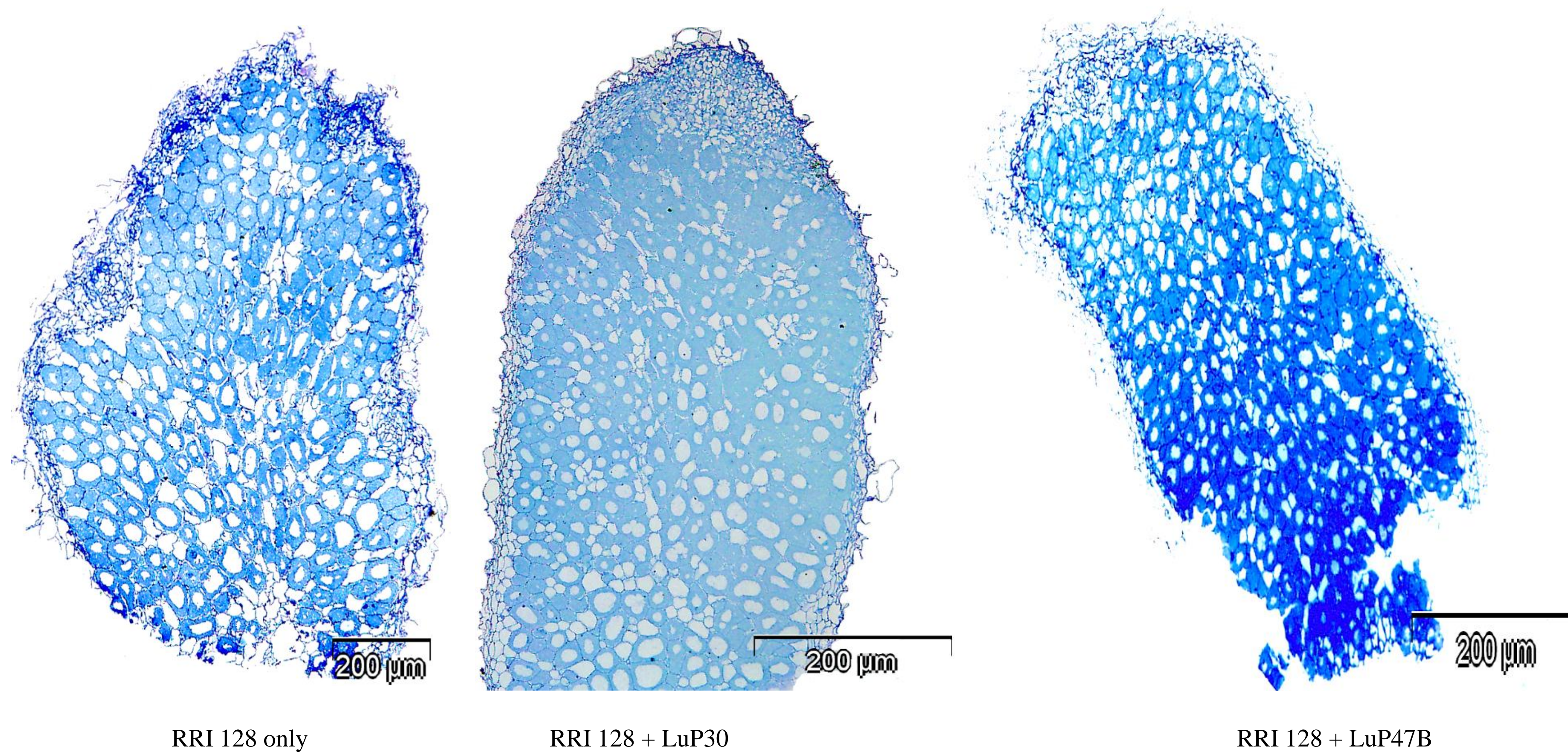
Treatment	**Nodules in the top 5 cm of roots**						**Large nodules (diameter ≥1mm)**					
	4 weeks			**7 weeks**			**4 weeks**			**7 weeks**		
	3 mg	**25 mg**	**50 mg**	**3 mg**	**25 mg**	**50 mg**	**3 mg**	**25 mg**	**50 mg**	**3 mg**	**25 mg**	**50 mg**
RRI 128 only	14.5^{b}	12.3^{ab}	2.5^{a}	14.3^{bc}	11.8^{a}	9.8^{b}	16.3^{a}	17.4^{a}	13.5^{a}	15.5^{a}	18.9^{a}	16.6^{a}
RRI 128 + EN23	10.0^{a}	15.5^{b}	4.3^{a}	16.0^{c}	13.1^{a}	5.5^{a}	14.1^{a}	24.8^{b}	16.4^{ab}	15.0^{a}	21.8^{b}	21.5^{b}
RRI 128 + LuP30	12.0^{a}	15.5^{b}	5.0^{a}	11.3^{a}	13.5^{a}	6.0^{a}	16.0^{a}	23.8^{b}	19.6^{b}	15.1^{a}	19.6^{ab}	14.8^{a}
RRI 128 + LuP47B	14.8^{b}	9.5^{a}	7.0^{a}	13.3^{ab}	15.3^{a}	9.6^{b}	17.1^{a}	21.1^{ab}	12.0^{a}	17.8^{a}	21.1^{ab}	22.0^{b}

(A)



(B)

Figure 6.3 Effect of LuP30 and LuP47B on the growth and nodulation of lucerne plants after 4 weeks inoculation with *S. meliloti* RRI 128 at different rates of nitrogen in sand and vermiculite. (A) Bigger plants co-inoculation with LuP30 and *S. meliloti* RRI 128. (B) TEM of nodules from left to right: RRI 128 only; RRI 128 + LuP30 and RRI 128 + LuP47B.



RRI 128 only RRI 128 + LuP30 RRI 128 + LuP47B

Figure 6.4 Sections of nodules after 4 weeks inoculation at 25 mg NH_4NO_3 with *S. meliloti* RRI 128 under light microscopy (scale bar 200 µm). From left to right: RRI 128 only; RRI 128 + LuP30; RRI 128 + LuP47B. Bigger vacuoles and fewer bacteroids in control treatment nodules compared to nodules co-inoculated with LuP30 or LuP47B.

Under light microscopy and TEM, the nodules of plants co-inoculated with LuP30 or LuP47B were observed to generally have smaller vacuoles and more infected cells than nodules of control plants (Figure 6.3 and Figure 6.4). The starch granules in nodule cells of the control plants were more dense and bigger than that of LuP30 or LuP47B plants. However, there were more starch granules in uninfected cells of plants treated with the actinobacteria, especially LuP30 (Figure 6.4).

6.3.3 ^{15}N experiment

The addition of LuP30 and LuP47B generally increased shoot weight and number of nodules per plant in the 3 and 5 week harvests, confirming previous measures of their efficacy (Table 6.5 and Table 6.6). None of the actinobacteria increased total plant N (roots and shoots) at 3 weeks, although there was a small increase in the amount of ^{15}N for the LuP47B treatment (Figure 6.5). At the 5 week harvest, the addition of LuP30 or LuP47B increased total plant N by 40% and 60%, respectively. This was mostly due to greater accumulation of ^{14}N (derived from N_2-fixation) which was increased by LuP30 or LuP47B by 47% and 72%, respectively. The total N and ^{14}N were distributed more in the shoots (about 70%) and roots (~30%) at the 5 weeks after inoculation with rhizobia (Table 6.7). LuP47B significantly increased the total amount of ^{15}N in the shoots at 3 weeks and roots at the 3 and 5 weeks harvest (Table 6.7).

Table 6.5 Effect of endophytic actinobacteria (*Streptomyces* spp. EN23, LuP30 and LuP47B in combination with *S. meliloti* strain RRI 128) on lucerne shoot and root weight at 1, 3 and 5 weeks at 25 mg $^{15}NH_4{}^{15}NO_3$ per kg sand and vermiculite after inoculation (n=4). Asterisks indicate significant differences $P < 0.05$ (*).

Treatment	**Shoot:root**			**Shoot weight (mg DM /plant)**			**Root weight (mg DM /plant)**		
	1w	**3w**	**5w**	**1w**	**3w**	**5w**	**1w**	**3w**	**5w**
Inoculation with *Sinorhizobium* and *Streptomyces*									
Rhizobia only	2.5	1.9	1.9	5.8	25.9	87.6	2.3	13.7	45.9
Rhizobia + LuP30	2.3	2.2	2.1	5.8	33.7*	110.2*	2.5	15.3	52.8
Rhizobia + LuP47B	2.3	2.1	1.9	5.4	34.3*	110.7*	2.3	16.1	59.6*

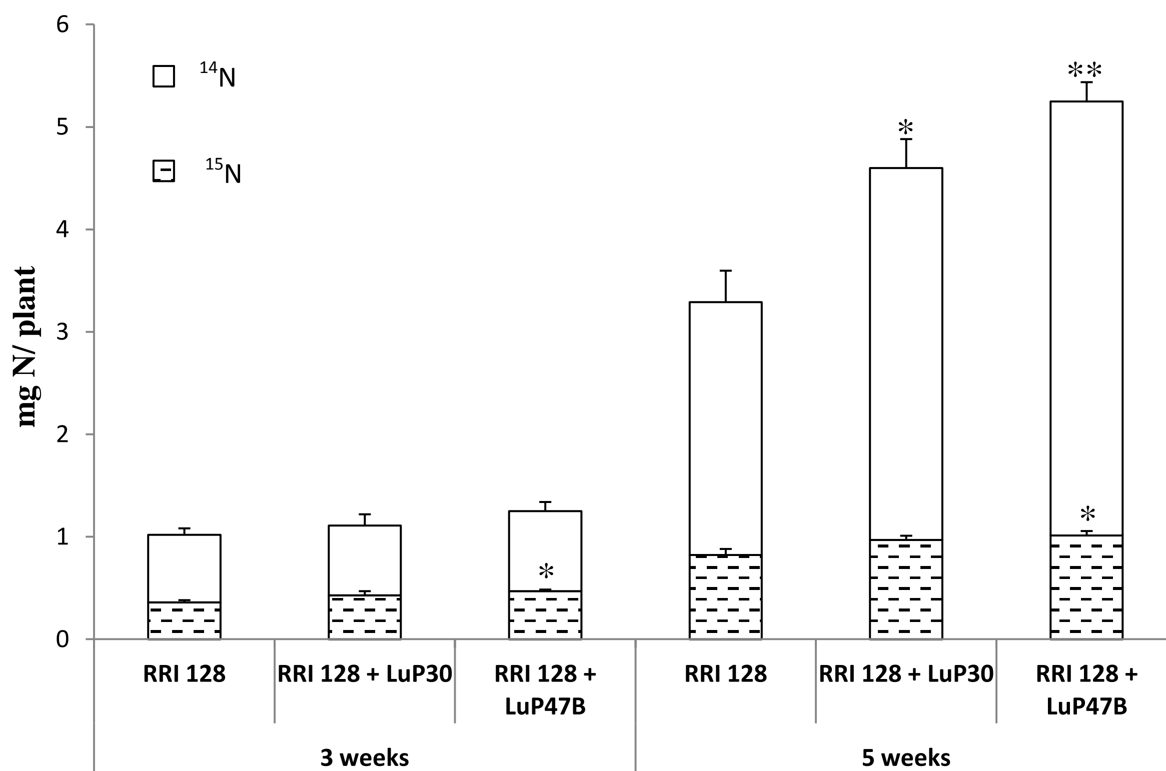


Figure 6.5 Accumulation of N (^{14}N and ^{15}N) in each lucerne plant (shoot and root) inoculated with rhizobia and actinobacteria. Asterisks indicate significant differences at $P < 0.05$ (*) or $P < 0.01$(**).Error bars: Mean ± S.E.

Table 6.6 Effect of co-inoculation of actinobacteria and *S. meliloti* RRI 128 on plant growth and nodulation of lucerne plant in time course in soil containing 25 mg $^{15}NH_4{}^{15}NO_3$. Different letters in the same column indicate means are significantly different ($P < 0.05$).

Treatment	Shoot length (cm)			Root length (cm)			Number of nodules			Nodules in 5cm depth			Nodules > 1mm		
	10d	21d	35d	10d	21d	35d	10d	21d	35d	10d	21d	35d	10d	21d	35d
Nil	2.8^{a}	5.9^{a}	7.2^{a}	7.3^{a}	22.7^{a}	30.3^{a}	0	0	0	0	0	0	0	0	0
RRI 128 only	2.9^{a}	5.4^{a}	8.4^{a}	7.3^{a}	21.6^{a}	35.8^{a}	0	18.6^{a}	30^{a}	0	12.4^{a}	12.8^{a}	0	2.5^{a}	8.8^{a}
RRI 128 + LuP30	2.8^{a}	6.1^{a}	12.7^{b}	9.6^{a}	27.4^{a}	47.5^{a}	0	24.6^{b}	48.6^{b}	0	10.8^{a}	20.7^{b}	0	4.8^{ab}	15^{b}
RRI 128 + LuP47B	3.0^{a}	6.3^{a}	12.9^{b}	10.0^{a}	25.6^{a}	43.1^{a}	0	22.8^{b}	37.2^{a}	0	13.2^{a}	21.1^{b}	0	5.5^{b}	9.8^{a}

Table 6.7 Accumulation of N (^{14}N and ^{15}N) in each plant inoculated with rhizobia and actinobacteria (n=4). Different letters in the same column indicate means are significantly different ($P < 0.05$).

Treatment	Shoot (mg)						Root (mg)					
	3 weeks			5 weeks			3 weeks			5 weeks		
	^{15}N	^{14}N	Total N	^{15}N	^{14}N	Total N	^{15}N	^{14}N	Total N	^{15}N	^{14}N	Total N
RRI 128 only	0.26^{a}	0.49^{a}	0.75^{a}	0.54^{a}	1.73^{a}	2.28^{a}	0.10^{a}	0.18^{a}	0.28^{a}	0.28^{a}	0.74^{a}	1.01^{a}
RRI 128 + LuP30	0.31^{ab}	0.50^{a}	0.82^{a}	0.66^{a}	2.56^{b}	3.22^{b}	0.12^{ab}	0.18^{a}	0.30^{a}	0.31^{ab}	1.06^{ab}	1.38^{ab}
RRI 128 + LuP47B	0.34^{b}	0.59^{a}	0.93^{a}	0.65^{a}	2.96^{b}	3.61^{b}	0.13^{b}	0.20^{a}	0.32^{a}	0.36^{b}	1.28^{b}	1.64^{b}

6.3.4 Early effects of actinobacteria on nodulation of lucerne plants

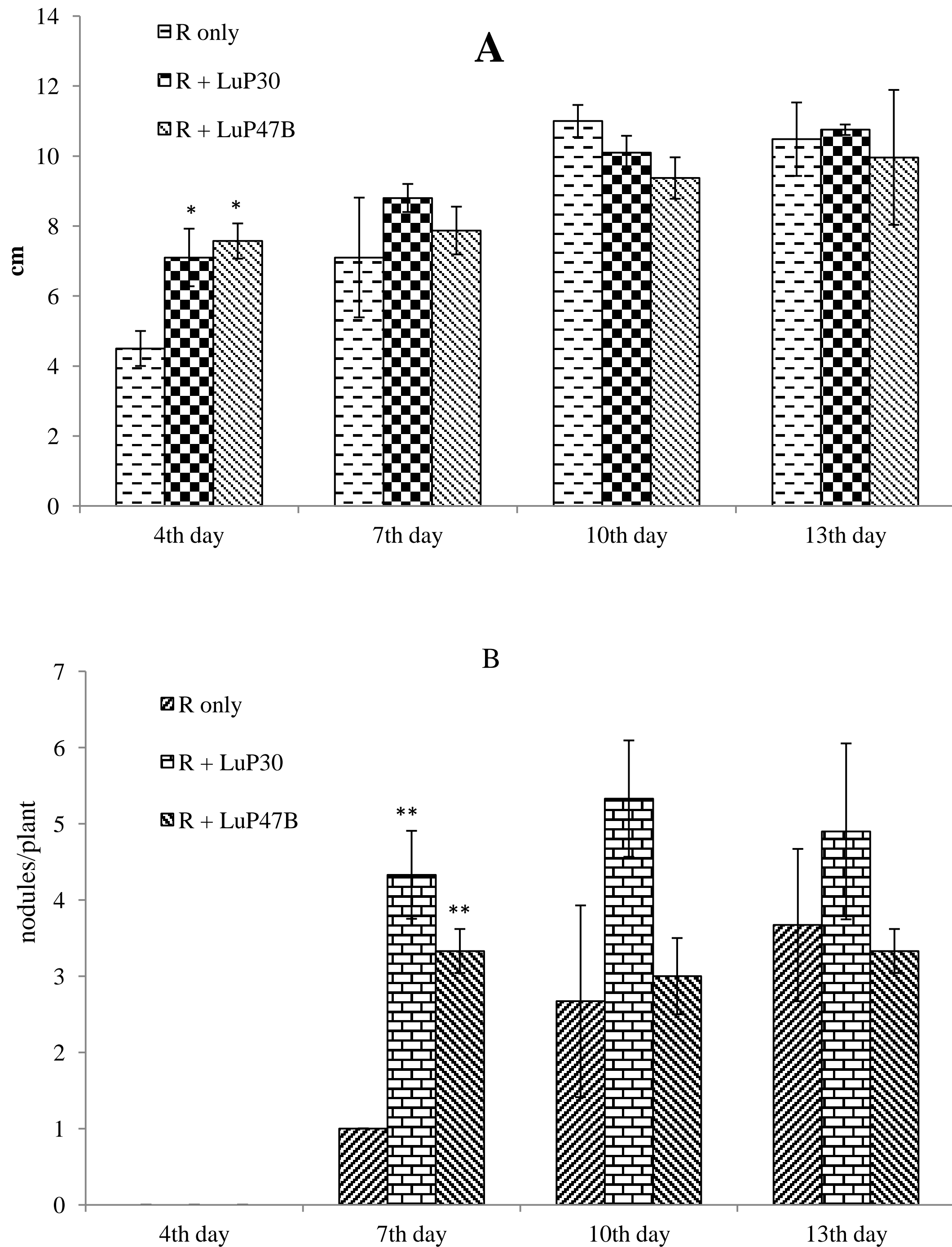


Figure 6.6 Early response of lucerene on root development and nodulation by impact of LuP30 and LuP47B at 25mg NH_4NO_3. (A) length of root and (B) number of nodules. Error bars: Mean ± S.E.

Co-inoculation of LuP30 or LuP47B with RRI 128 significantly increased the length of the root after 4 days and number of nodules 7 days after inoculation with the *S. meliloti* RRI 128 strain (Figure 6.6). Plants treated with either LuP30 or LuP47B significantly increased the length of root by 4 days after inoculation with the rhizobia. No nodules were found after 4 days but under light microscopy the root hairs of plants treated with LuP30 or LuP47B had started to curl by the third day. Plants co-inoculated with LuP30 and RRI 128 formed nodules on fourth day while the root hairs of control plants had not started to curl (Figure 6.7). Details of nodule development for individual plants are presented in Appendix 7. No nodules were found in any of the treatments at 3 days after inoculation with *S. meliloti* RRI 128. LuP30 was the most effective in reducing the effect of nitrogen on nodulation of lucerne at 25 mg NH_4NO_3; one nodule of lucerne plant coated with LuP30 was found after 4 days while no nodules were found on the roots of other treatments.

Plant growth and nodule number/plant were increased by the level of applied N and microbial treatment. Increases associated with N application were attributable to increased plant vigour. Although the larger plants had more nodules overall, they were observed to have delayed nodulation and fewer nodules/mg of root. N levels in the experiment were not high enough to reduce nodulation to the extent reported by others (Heichel and Vance, 1979), but were still sufficient to delay nodulation and provide the opportunity for improvement with actinobacterial inoculation.

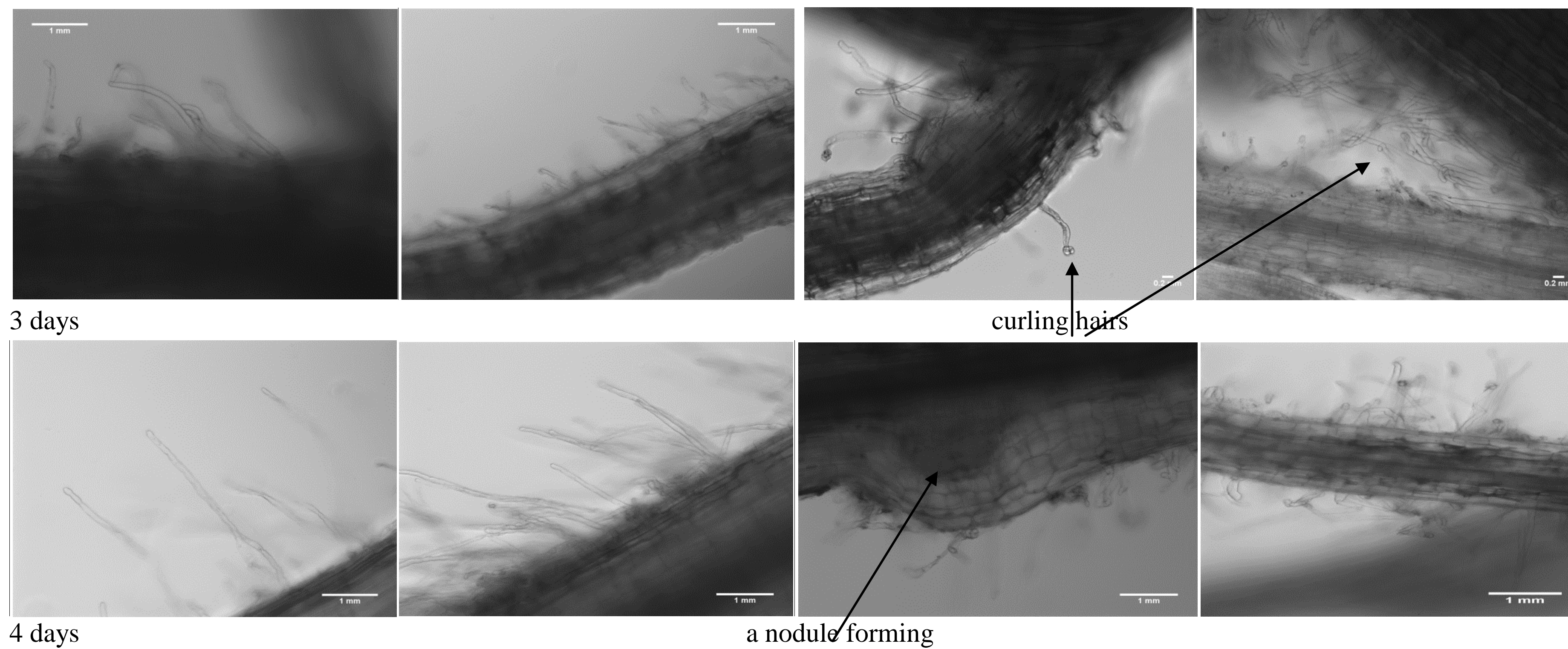


Figure 6.7 Root responses by co-inoculation of LuP30 and LuP47B after 3 days (top) and 4 days (bottom) with *S. meliloti* RRI 128. From left to right: Uninoculated, RRI 128 alone, RRI 128 + LuP30 and RRI 128 + LuP47B.

When LuP30 and LuP47B were co-inoculated with rhizobia strain RRI 128, lucerne growth and nodulation were increased. Most improvements in nodulation were measured in the first 4 weeks of plant growth, indicating early involvement of the actinobacteria in the regulation of nodulation. The isotope experiment provides evidence that actinobacteria strain LuP47B increased nitrogen (^{15}N) uptake from the soil, but this benefit was relatively small. By comparison, increases in early nodulation (number and density) were large. Martínez-Hidalgo *et al.* (2014) proposed that *Micromonospora* play a role as rhizobia helper bacteria (RHB) and since the actinobacteria were coated onto lucerne seeds prior to inoculation with rhizobia, they could have primed the plant root for rhizobial infection and nodulation (Tokala *et al.*, 2002). Measures of earlier root hair curling and reduced time to nodule appearance suggest the effects of the actinobacteria occur very early and therefore may be associated with rhizobial colonisation, plant/rhizobia signalling or *Nod* factor synthesis. The production of phyto-hormones such as auxins, cytokinins, and gibberellins could also be important (Glloudemans and Bisseling, 1989) because of their effects on root growth, even if they are not specifically involved in the complex signalling that regulates nodulation.

The large body of work on hyper- and super-nodulating soybeans that are tolerant of soil nitrate has shown nodulation in the presence of nitrate is strongly controlled by a feed-back signal from the plant shoot (Ferguson *et al.*, 2010; Li *et al.*, 2009; Reid *et al.*, 2011). Changes to the concentration of nutrients in the lucerne shoots when inoculated with actinobacteria suggests they had some effect on the broader plant physiology and so modification of the feedback loop for control of nodulation needs to be considered. Nimmnoi *et al.* (2014) proposed that co-inoculation with both

actinobacteria and rhizobia increased plant growth by improving the uptake of nutrients and El-Tarabily *et al.* (2008) also found similar results with actinobacteria in mungbean (*Phaseolus vulgaris* L.). Although changes to the nutritional status of the lucerne plants were measured, it is unlikely that nutrient levels *per se* were critical to the improvements in plant growth, because with the exception of N, they should not have been limiting to growth.

Equalisation of nodule number by the latter harvest at 25 and 50 mg NH_4NO_3 was probably the result of compensatory nodulation in the control treatment (RRI 128) as soil N declined through the course of the experiment. Whilst it shows that the effects of the actinobacteria may be quite transient, their potential value is not diminished, because early plant vigour is critically important to successful lucerne establishment and its subsequent persistence and production.

The nitrogen fixation costs a lot of energy and plants use carbohydrate as an energy resource besides other resources such as the photosynthesis (Minchin *et al.*, 1981). Lucerne nodules store starch in uninfected and infected cells (Tu, 1977; Vance *et al.*, 1979). Less starch in nodule cells might be a result of high consumption of carbohydrate for nitrogen fixation process of plants treated with LuP30 or LuP47B.

6.4 Conclusion

This study confirms that the selected actinobacteria can improve the growth, nodulation and nitrogen fixation of lucerne plants inoculated with rhizobia strain RRI 128. Both non-symbiotic and symbiotic effects are likely to be contributing to the improvement. The actinobacteria were most effective at 25 mg NH_4NO_3/kg of

soil and in the first four weeks of growth, indicating their use would be best targeted to encourage early plant vigour and aid pasture establishment in soils with low/moderate N levels. The efficacy and durability of the actinobacteria in field soils containing complex microflora including different strains of rhizobia is still to be determined, as are the mechanisms of action that promote early nodulation and the enhancement of the plant rhizobial symbiosis. The roles of LuP30 and LuP47B with root hairs curling need to be investigated for further study. Overall, actinobacteria strain LuP30 was the most promising to be used as a co-inoculant with rhizobia based on its benefits to nodulation and plant growth. Strain LuP30 provided the most consistent benefit to nodulation and lucerne growth across the three N levels in this study.

Chapter 7

Efficacy of LuP30 and LuP47B on other symbiotic associations (lucerne, sub-clover and serradella) and wheat

7.1 Introduction

There are two morphological types of nodules that form on legumes determinate and indeterminate (Ferguson *et al.*, 2010). Indeterminate nodules have a more persistent meristem, and the nodule shape is a cylinder, for example, nodules of lucerne (*Medicago sativa*) and clover (*Trifolium repens*), pea (*Pisium sativum*) and vetch (*Vicia sativa* L.) (Eckardt, 2006). Determinate nodules, on the other hand, are usually spherical, lack a persistent meristem, and do not display an obvious developmental gradient. Legumes that form determinate nodules are predominately tropical and subtropical species, including soybean (*Glycine max*) and bean (*Phaseolus vulgaris*), but also include other more temperate species such as *L. japonicus* (Eckardt, 2006).

The concentration of rhizobial partner in soil is crucial for nodulation of the host legume. Australian soils have a range of rhizobia populations varying from less than 10 to in excess of 10^6 CFU/g soil (Gibson *et al.*, 1974; Slattery and Coventry, 1999).

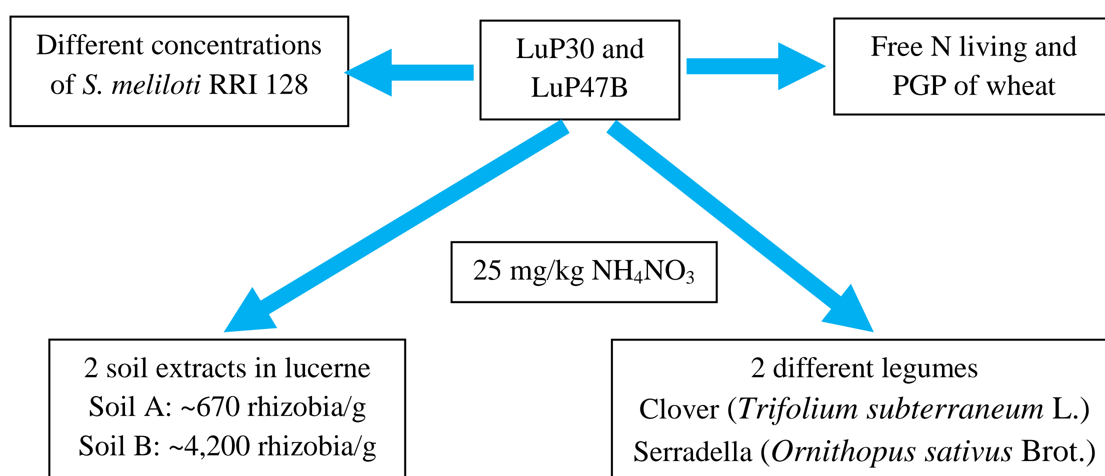


Figure 7.1 Experiments designed to understand the further beneficial ability of LuP30 and LuP47B on the growth of plants and symbiosis with different indigenous rhizobia

The aim of these experiments was to examine whether the beneficial effects of actinobacteria strains LuP30 and LuP47B with lucerne and rhizobial strain RRI128 extend to other legume symbioses, supplied with 25 mg NH_4NO_4 per kg of soil (the optimal level for responses previously measured in lucerne). Initially, this was investigated with lucerne inoculated with two soil extracts containing communities of rhizobia, and then extended to sub-clover (indeterminate nodules) and serradella (determinate nodules) which were chosen to provide contrasting types of nodules and species of rhizobial partner. In addition, the benefits to cereal plants for example wheat of the two actinobacteria were also studied (Figure 7.1). Details of experiments are in section 7.2 of this Chapter.

7.2 Materials and methods

7.2.1 Effects of LuP30 and LuP47B on plant growth of lucerne in two different soil extracts

7.2.1.1 MPN of two soil extracts

Two different soil extracts which were investigated for their MPN and the compatibility of LuP30 and LuP47B to interact with rhizobia in the soil extracts. The two soils used in this experiment were collected from an annual medic pasture at Rudall, Eyre Peninsula (Soil A) and from lucerne field plots at Urrbrae High School (Soil B). The MPN of lucerne nodulating rhizobia in each soil extract was estimated as described by Brockwell (1963) using SARDI 7 lucerne as the plant host. Ten grams of each soil was added into 90 ml of autoclaved 0.9% saline (NaCl) and shaken for 10 minutes at 125 rpm room temperature. Then serial dilutions from 10^{-1} to 10^{-6} were made by adding 1ml from stock into a tube containing 9ml of autoclaved 0.9% saline.

One ml of soil extract solution from each 10^{-1} to 10^{-6} dilution was added to the base of the lucerne seedlings growing in 3 replicate tubes. The presence of nodules at each dilution of soil extract was determined after 4 weeks of plant growth in the glasshouse, and the MPN of rhizobia in each soil was estimated.

7.2.1.2 Experimental design

The factorial experiment comprised (i) two strains of actinobacteria (LuP30 and LuP47B), (ii) ± inoculation with rhizobia strain RRI 128 or one of the two soil extracts and combined treatments where rhizobia strain RRI 128 and either soil A or soil B extract were applied together. Actinobacteria spores were applied to surface sterilised lucerne seeds (as a suspension in 0.3% xanthan gum to provide 10^8 CFU/g seed) and the seeds sown (10 seeds/pot and thinned down to 4 seedlings/pot before inoculating with *S. meliloti* strain RR I128 or soil extract solutions) into a pasteurised (by autoclaving) potting mix (50:50 by volume of sand:vermiculite) contained in 1.25 L pots. The pots had been watered with a nitrogen deficient nutrient solution supplemented with 25 mg/kg NH_4NO_3. Plants designated to rhizobia treatments were inoculated with a suspension (1ml per plant containing 10^8 CFU) of rhizobia 6 days after sowing while 1 ml of soil extract which was prepared by adding ten grams of soil into 90 ml of autoclaved 0.9 % saline for soil treatments. The rhizobia RRI 128 plus soil A or soil B extract treatments were inoculated with a rhizobial suspension (1ml per plant containing 10^8 CFU) before adding 1 ml of soil extract for each seedling. Each treatment was replicated eight times. Pots were arranged in a completely randomised design in a greenhouse from April to May 2014 (approx. mean 13h/11h day/night temp. range 25°C-35°C) and plants harvested twice

at 4 and 7 weeks after inoculation with the rhizobia. Four pots were harvested each time with the parameters measured including length and dry weight of root and shoot, nodule number and total nodule mass per plant.

7.2.2 Effects of LuP30 and LuP47B on nodulation of lucerne at different concentrations of rhizobia

Sinorhizobium meliloti strain RRI 128 w as examined at three different concentrations $5x10^2$, $5x10^4$ and $5x10^6$ CFU/ml in combination with actinobacteria strains LuP30 and LuP47B, and a non *Rhizobium* inoculant control treatment. Lucerne seed was surface-sterilised as described in 2.2.1 and coated with spores of the actinobacteria as described in 2.2.2. Fifteen grams of autoclaved sand vermiculite was added to each 15 ml tube and five ml of McKnight's solution applied before two surface sterilised seeds were sown in each tube. McKnight's solution was supplied as required in the following weeks. One seedling was kept in each tube 7 days after sowing and inoculated with 1 ml of rhizobial partner. The plants were kept in a controlled growth chamber (approx. mean 14h/10h day/night temp. range 20^oC-25^oC). There were four replications for each treatment and plants were grown up to three weeks after inoculation with the rhizobia. The plants were examined for the length and dry weight of shoots and roots, and number and total mass of nodules per plant.

7.2.3 Effects of actinobacteria on growth and symbiosis of sub-clover and serradella

7.2.3.1 Actinobacteria, rhizobia and seeds

Sub-clover cultivar Campeda (*Trifolium subterraneum* L.), and pink serradella cultivar Cadiz (*Ornithopus sativus* Brot.) were chosen for examining the effects of two actinobacteria LuP30 and LuP47B which showed increases in growth and nitrogen fixation of lucerne in previous experiments. Two rhizobial strains *Rhizobium* WSM 1325 and *Bradyrhizobium* WSM 471 were used to inoculate sub-clover (indeterminate nodules) and serradella (determinate nodules), respectively.

7.2.3.2 Interaction of LuP30 and LuP47B on the growth of two different species of rhizobia

The effect of LuP30 and LuP47B on the growth of *Rhizobium leguminosarum* bv. *trifolii* WSM 1325 and *Bradyrhizobium lupini* strain WSM 471 was described in 5.2.3.2. Three concentrations 10^4, 10^6 and 10^8 CFU/ml (or 10^3, 10^5 and 10^7 cells on each agar plate) of the two rhizobial strains were examined for their interaction with the two actinobacteria LuP30 and LuP47B. The method details of the test are described in section 5.2.3 in Chapter 5. The growth of the rhizobia was examined 5, 10 and 14 days after adding the actinobacterial plugs. Effects on rhizobial growth were measured as (++) where increased rhizobial growth (thick culture) occurred in the 3-4 mm surrounding the actinobacterial plug; (+) as above with increased rhizobial growth evident within 1-2 mm; (0) no visible effect; (-) a zone of rhizobial inhibition extending no further than 1-2 mm from the actinobacterial plug; (--) a zone of inhibition of inhibition extending >2 mm from the actinobacterial plug.

Diameter of colonies on plates at 10^3 CFU was measured to compare between the control (ISP2 plugs) and treatments (LuP30 or LuP47B plugs). Number and diameter of colonies on four sections on each of two plates were counted and measured (Figure 7.5).

7.2.3.3 Experimental design

The factorial experiment comprised (i) two strains of actinobacteria (LuP30 and LuP47B), (ii) inoculation with rhizobia strain WSM1325 for sub-clover and WSM 471 for serradella. Surface sterilisation of sub-clover and serradella seeds was followed as described as 2.2.1 in Chapter 2. The planting inoculation processes and nutrition are described in section 2.2 of Chapter 2. Actinobacteria spores were applied to surface sterilised sub-clover and serradella seeds (as a suspension in 0.3% xanthan gum to provide 10^8 CFU/g seed) and the seeds sown (10 seeds/pot and thinned down to 4 seedlings/pot before inoculating with the rhizobial partner) into a pasteurised (by autoclaving) potting mix (50:50 by volume of sand:vermiculite) contained in 1.25 L pots. Treatments comprised i) an uninoculated control ii) inoculation with *Rhizobium* WSM 1325 or *Bradyrhizobium* WSM 471 alone or iii) in combination with one of the two actinobacteria. The pots were watered with a nitrogen deficient nutrient solution supplemented with 25 mg/kg NH_4NO_3. Pots were arranged in a completely randomised design in a greenhouse from December 2013 to Febuary 2014 (approx. mean 12h/12h day/night temp. Range 20^oC-25^oC) and plants harvested twice at 4 and 7 weeks after inoculation day.

7.2.4 Nitrogen fixing ability, plant growth promotion and nitrogen use efficiency in non-legume, wheat

Wheat was used to check the N_2 fixing ability and N_2 use efficiency of the actinobacteria LuP30 and LuP47B in a non-legume plant. The factorial experiment comprised by 2 actinobacteria LuP30 and LuP47B and 2 levels of N supply, 0 and 25 mg/kg NH_4NO_3 (1kg of mixture of sand and vermiculite). Surface sterilisation of wheat seeds was followed as described as 2.2.1 in chapter 2. The planting inoculation processes and nutrition are described in section 2.2 of Chapter 2. Actinobacteria spores were applied to surface sterilised wheat seeds (as a suspension in 0.3% xanthan gum to provide 10^8 CFU/g seed) and the seeds sown (6 seeds/pot and thinned down to 4 seedlings/pot after 5 days) into a pasteurised (by autoclaving) potting mix (50:50 by volume of sand:vermiculite) contained in 1.25 L pots. There were five replicates for each treatment. The pots were randomised and kept in a greenhouse with natural light (14h day/ 10h night) and temperature (25-35°C). The plants were harvested after 7 weeks planting and assessed for the length, dry weight of shoot and root.

7.3 Results and discussion

7.3.1 Different soil extracts containing rhizobia

The lucerne plants treated with actinobacteria strains LuP30 or LuP47B alone were significantly bigger than the untreated control plants (Table 7.1). The non-symbiotic effects with rhizobia of LuP30 and LuP47B on the growth of lucerne plants were confirmed at 25 mg N per kg of sand and vermiculite which has been reported in Chapter 6. The results also confirm that co-inoculation either LuP30 or LuP47B with rhizobial strain RRI 128 increased the lucerne growth, number of nodules and nodule

mass per plant, compared to plants only inoculated with rhizobia. The current commercial inoculant strain (*S. meliloti* strain RRI 128) was the best rhizobial treatment in the interaction with LuP30 or LuP47B as they produced the biggest plants and more nodules per plant compared with plants inoculated with extracts of soil A or soil B which contained naturalised rhizobia.

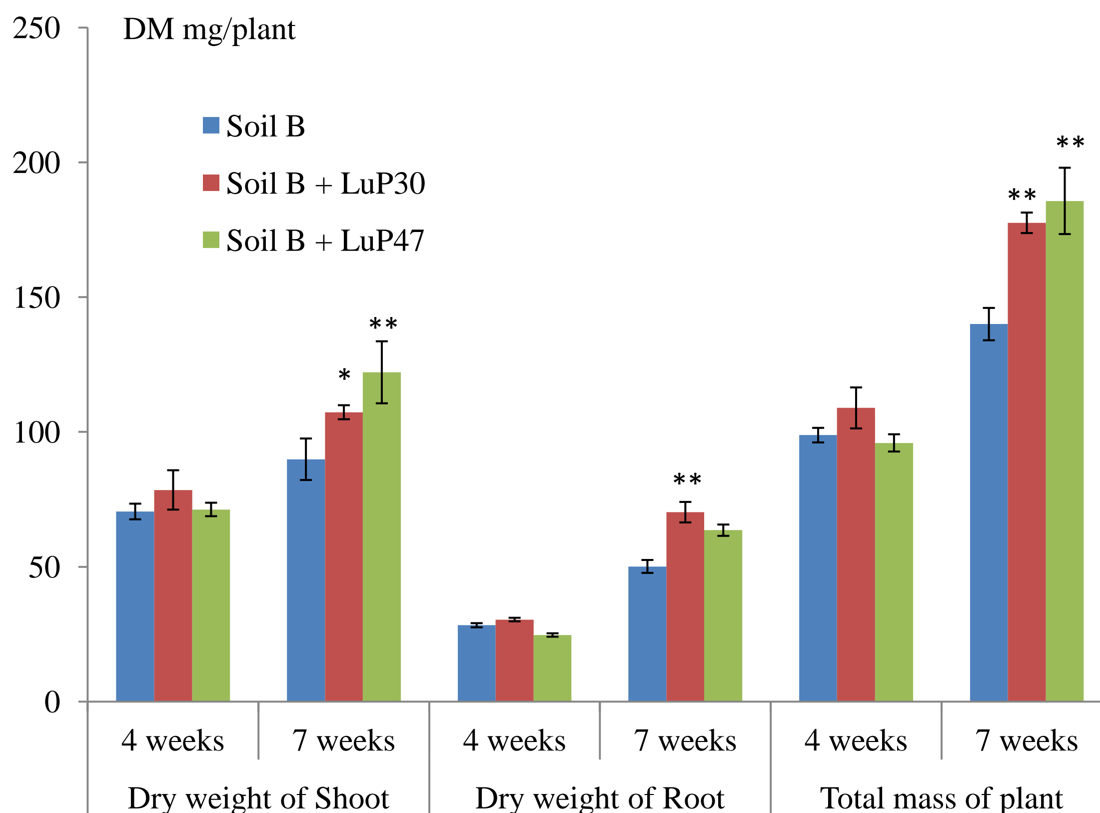


Figure 7.2 Effects of LuP30 and LuP47B on dry weight of shoot, root and total weight of lucerne after 4 and 7 week in extract of soil B. Asterisks indicate significant differences at $P < 0.05$ (*) or $P < 0.01$(**). Error bars: Mean ± S.E.

The MPN of lucerne-nodulating rhizobia in Soil A was estimated to be 4,200 rhizobia/ g and 670 rhizobia/ g in soil B. No significant effects on the growth and nodulation were noted when lucerne seeds coated with LuP30 or LuP47B were inoculated in the sand and vermiculite to which the soil A extract was added. LuP30 and LuP47B did not increase the number of nodules, nodule mass or plant growth

compared with control plants after 4 weeks. After 7 weeks growth, the number of nodules on plants treated with LuP30 (17 nodules) was significantly less than control plants (25) while LuP47B increased nodule number to 30 nodules per plant. It can be concluded that LuP30 and LuP47B had limited effect on the growth of lucerne when nodulated by the rhizobia in soil A.

In contrast, the application of seeds coated with LuP30 or LuP47B to soil inoculated with soil B extract significantly increased the number of nodules and nodule mass per plant and also increased shoot dry weight per plant. Shoot dry weight was increased by LuP30 and LuP47B by 16% and 36%, respectively (Figure 7.2). Both the effectiveness of the strains type and the number of rhizobia in soil extracts may have have affected the number of nodules on the lucerne plants, for example, 25 nodules compared with only 7 nodules per plant with soil A and B extracts, respectively, after 7 weeks (Table 7.1). LuP30 and LuP47B may assist the population of compatible rhizobia in soil B to form more nodules and hence increased the growth of lucerne plants. The symbiosis of legumes and rhizobia could also be influenced by other microorganisms, such as, for example, *Bacillus megaterium*, which has been reported to increase the population of rhizobia in the rhizosphere and subsequent nodulation of pigeon pea (*Cajanus cajan*) (Rajendran *et al.*, 2008). *Bacillus polymyxa* increased early rhizobial root population and final root population densities in the rhizosphere of *Phaseolus vulgaris*; and also increased lateral root formation and nodule number (Petersen *et al.,* 1996).

Table 7.1 Effects of actinobacteria LuP30 and LuP47B on growth and symbiosis of lucerne plants inoculated with two different soil extracts containing rhizobia (Soil A and Soil B), after 4 weeks and 7 weeks growth, (n=4)

Treatment	Shoot length (cm)		Root length (cm)		Shoot dry weigth (mg/plant)		Root dry weight (mg/plant)		Nodule number (#/plant)		Total nodule mass (mg/plant)	
	4 w	7 w	4 w	7 w	4 w	7 w	4 w	7 w	4 w	7 w	4 w	7 w
Single inoculation with actinobacteria												
Untreated*	12.8	15.4	26.4	35.3	66.9	88.5	31.3	74.0	0	0.0	0	0
LuP30	15.4*	18.6*	30.3	30.0	70.0	98.4**	31.1	76.0	0.0	0.0	0	0
LuP47B	15.3*	20.8**	27.9	28.8	80.9*	102.2**	33.1	79.7	0.0	0.0	0	0
Soil A only	19.5	28.0	30.7	36.8	89.2	181.1	32.0	93.1	11.5	25.4	2.3	5.6
Soil A + LuP30	18.0	25.4	36.0	38.6	80.5	153.3	27.6	92.9	14.8	17.3**	2.5	5.9
Soil A + LuP47B	17.4	26.2	31.9	35.2	74.3	179.1	29.5	105.7	11.3	29.9*	2.1	6.5
Soil B only	15.4	18.2	23.5	28.3	70.5	89.9	28.3	50.1	3.6	7.4	NA	1.2
Soil B + LuP30	17.9	20.0	31.2**	37.5	78.5	107.3*	30.4	70.3*	1.8**	11.2	NA	2.3*
Soil B + LuP47B	16.1	22.4**	27.9	31.2	71.3	122.1**	24.7	63.6	1.5**	17.3*	NA	3.8**
Co-inoculation with *S. meliloti* RRI 128												
RRI 128	21.8	25.6	23.4	28.1	95.3	238.2	33.5	124.9	30.1	32.2	3.8	4.0
RRI 128 + LuP30	21.8	29.8	30.2	30.6	120.3**	305.0**	36.1	136.7	43.3**	45.4**	6.0*	6.6*
RRI 128 + LuP47B	23.1*	30.9**	40.7**	40.5**	115.6*	313.8**	42.6*	132.2	47.8**	46.8**	7.4**	7.8*
RRI 128 + Soil A	22.7*	26.6	30.0	28.5	103.6	246.6	37.3	143.5	22.3	30.1	3.5	3.7
RRI 128 + Soil B	23.9*	27.9	29.0	30.7	112.8	230.5	38.2	156.0*	30.0	28.3	4.6	4.6

Asterisks indicate significant differences at $P < 0.05$ (*) or $P < 0.01$(**).

7.3.2 Effects of actinobacteria on the growth and nodulation of lucerne plants inoculated with different concentrations of rhizobia

The increases of *S. meliloti* RRI 128 concentrations resulted in slight increases in the number of nodules and the growth of plant. The number of nodules per plant increased from 4.3 to 7.0 and 8.8 nodules when the concentration of rhizobia was increased from $5x10^2$ to $5x10^4$ and $5x10^6$, respectively (Table 7.2). The significant effects of LuP30 and LuP47B on plant growth and nodulation of lucerne plants were at $5x10^2$ CFU/ml of *S. meliloti* RRI 128 (Figure 7.4). The shoot dry weight and total mass per plant increased up to about 50% to 60% and was similar with plants treated with the rhizobia at 10^4 and 10^6 CFU/ml (Figure 7.3). In addition, co-inoculation either LuP30 or LuP47B individually with *S. meliloti* RRI 128 at $5x10^2$ CFU/ml increased the number of nodules up to 7 and 9, respectively compared to control plants that had 4.3 nodules per plant.

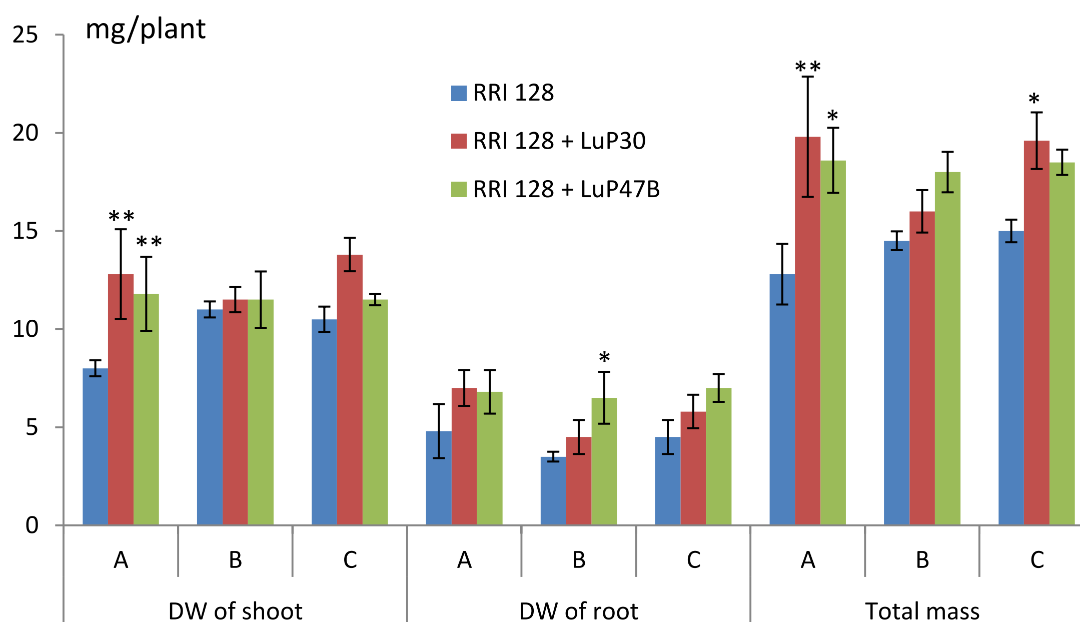


Figure 7.3 Response of lucerne plants under effects of LuP30 and LuP47B on different concentration of *S. meliloti* RRI 128 after 3 weeks inoculation with the *Rhizobium*: (A) $5x10^2$, (B) $5x10^4$, (C) $5x10^6$ CFU/ml. Error bars: Mean ± S.E.

Table 7.2 Effects of LuP30 and LuP47B on the number of nodules per plant of lucerne after 3 weeks inoculation with different concentrations of *S. meliloti* RRI 128, (n=4). Different letters in the same column indicate means are significantly different ($P < 0.1$).

Actinobacteria	*S. meliloti* RRI 128 concentrations (CFU/ml)		
	5×10^2	5×10^4	5×10^6
	Number of nodules per plant		
Nil	4.3^a	7.0^a	8.8^a
LuP30	7.0^b	7.3^a	8.5^a
LuP47B	9.0^b	7.8^a	9.8^a

Figure 7.4 Lucerne plants in tubes three weeks old after inoculation with *S. meliloti* RRI 128 at 5×10^2 CFU/ml. Left- *S. meliloti* RRI 128 alone; right- *S. meliloti* RRI 128 plus LuP30.

7.3.3 Effects of LuP30 and LuP47B on clover and serradella

7.3.3.1 Interaction tests between actinobacteria and the rhizobia

LuP30 and LuP47B increased or did not affect the growth of the two rhizobia namely clover nodulating strain WSM 1325 and serradella nodulating strain WSM 471. At low concentrations of rhizobia (between 10^7 CFU/ml and 10^5 CFU/ml) LuP30 and LuP47B were observed to visibly increase the growth of both rhizobia on YMA medium after 5 days incubation at 27°C (Table 7.3). When the concentration

of the rhizobia was more than 10^7 CFU/ml, the effects of the two actinobacteria LuP30 and LuP47B did not obviously affect the growth of the two rhizobia. These results are consistent with the effects on lucerne-nodulating rhizobia whose growth was also not inhibited by LuP30 and LuP47B. It provides more evidence of possible growth stimulation by the actinobacteria on different rhizobial strains: three strains were reported in Chapter 5, Table 5.1 in 5.3.1.

Table 7.3 Effects of two actinobacteria LuP30 and LuP47B on the growth of two rhizobial strains applied to agar plates at three concentrations and grown for 7 days. (++) positive effects on rhizobial growth visible as a zone of increased growth around the actinobacterial plug; (+) slightly positive effects, a smaller zone of increased growth around the actinobacterial plug; (0) neutral effect, n=8, different letters in the same column indicate means are significantly different ($P < 0.05$). NA=not applicable

Cultures	**≤ 10^3 CFU/plate**		**≤ 10^5 CFU/plate**		**≤ 10^7 CFU/plate**	
	WSM 1325	**WSM 471**	**WSM 1325**	**WSM 471**	**WSM 1325**	**WSM 471**
LuP30	++	++	++	++	+	+
LuP47B	++	++	++	++	+	+
Colony diameter (mm) after 2 weeks						
Control	0.8^a	0.6^a	NA	NA	NA	NA
LuP30	1.5^b	1.3^b	NA	NA	NA	NA
LuP47B	1.6^b	1.4^b	NA	NA	NA	NA

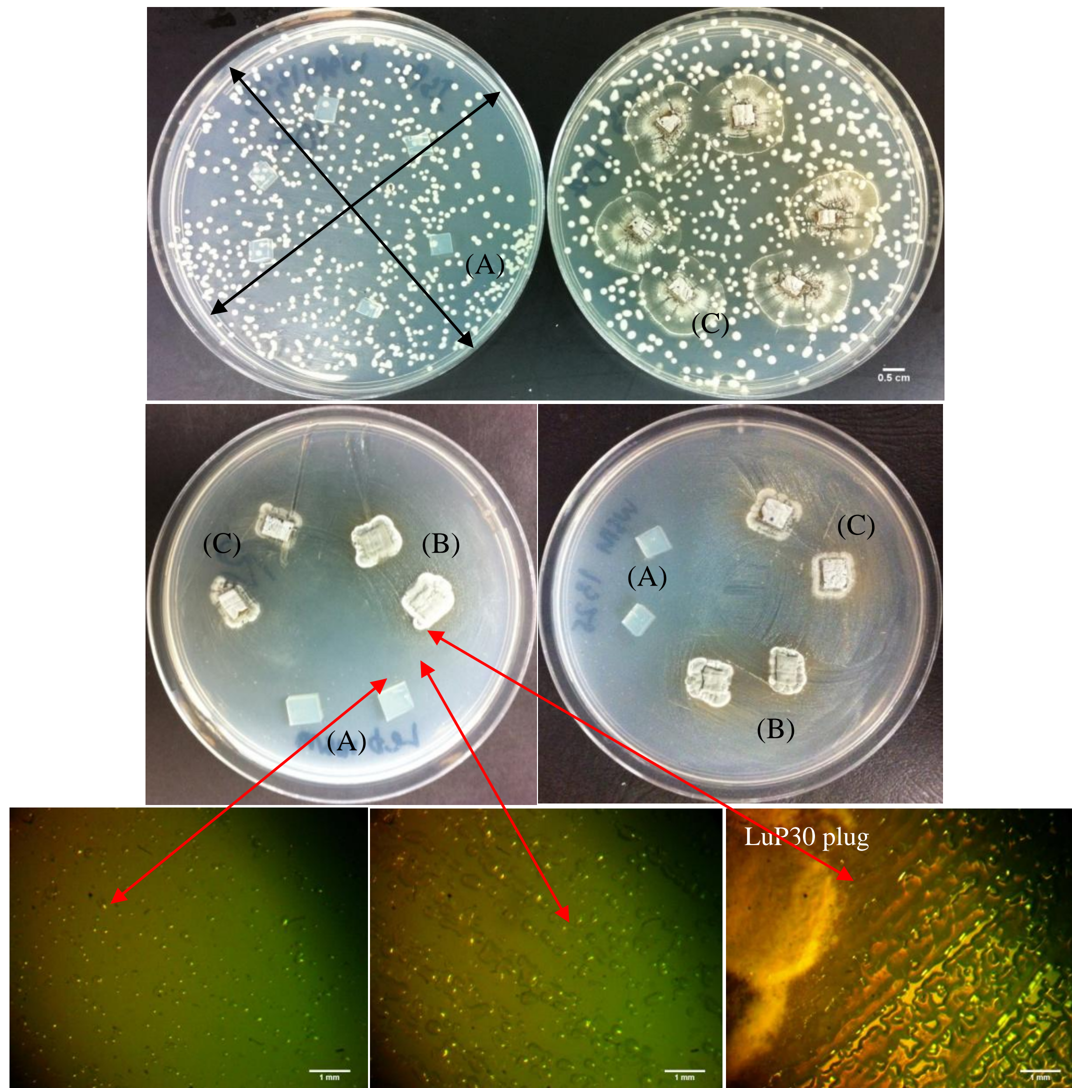


Figure 7.5 Stimulation of the growth of *Rhizobium leguminosarum* bv. *trifolii* strain WSM 1325 and *Bradyrhizobium lupini* strain WSM 471 by two actinobacteria LuP30 and LuP47B on YMA medium at different concentrations of rhizobium. From top to bottom and left to right: WSM 1325 at 2 weeks old with about 10^3 CFU per plate; LuP30 plugs on WSM 1325 with bigger colonies; WSM 471 at 7 days old with about 10^5CFU per plate (A) ISP2 control plugs, (B) LuP47B plugs and (C) LuP30 plugs; WSM 1325 at 7 days old with about 10^5 CFU per plate. From left to right images (bottom) the increase of the growth of two rhizobia when closer to the plug of LuP30 or LuP47B under a microscopy

At 10^5 or 10^7 CFU per plate, the rhizobial colonies close to the actinobacteria plugs of LuP30 and LuP47B were bigger and the zone of growth increased around the actinobacterial plugs, compared to the control treatment (ISP2 plugs alone) (Figure 7.5). At 10^3 CFU per plate, the diameter of rhizobial colonies on actinobacteria treatment plates (LuP30 or LuP47B plugs) were double the size of the colonies on the control plate (ISP2 plugs) (Table 7.3). Interaction between the actinobacteria and

rhizobia might result in the faster growth of strains of rhizobia which may be of advantage when the rhizobia were present in low concentrations. As presented in 5.3.1 in Chapter 5, both LuP30 and LuP47B did not have any significant effects on the growth of the *S. meliloti* RRI 128 in YM broth medium when the RRI 128 concentration was greater than 10^7 CFU/ml. Low concentrations of rhizobia may provide the conditions for some of the beneficial effects of the actinobacteria to occur.

7.3.3.2 Response of sub-clover growth symbiosis to inoculation with by LuP30 and LuP47B

Inoculation of sub-clover with LuP30 prior to inoculation with rhizobia strain WSM 1325 did not improve plant growth but it increased the number of nodules after 7 weeks and nodule mass after 4 and 7 weeks (Table 7.4). However, actinobacteria strain LuP47B significantly increased the dry weight of shoot, total mass and number of nodules per plant after 4 and 7 weeks inoculation with the *Rhizobium* while the nodule mass per plant was only increased after 7 weeks (Figure 7.6). Root weight responses varied between the two harvests, for example, LuP30 increased root dry weight after 4 weeks while LuP47B increased root dry weight after 7 weeks.

Table 7.4 Root growth and nodulation response of sub-clover to the application of LuP30 and LuP47B after 5 and 7 weeks inoculation with *Rhizobium* WSM 1325, (n=4). Different letters in the same column indicate means are significantly different ($P < 0.05$).

Treatment	Root length (cm)		Root weight (mg DM/ plant)		Nodule number (#/plant)		Total nodule mass (mg)	
	4 w	7 w	4 w	7 w	4 w	7 w	4 w	7 w
Untreated*	20^a	30^a	35.0^a	46.0^a	0^a	0^a	0^a	0^a
WSM 1325 only	30^b	34^{ab}	63.5^b	69.8^b	114^b	119^b	7.5^b	8.3^b
WSM 1325 + LuP30	36^c	39^b	71.7^c	70.6^b	127^{bc}	168^c	9.3^c	11.2^c
WSM 1325 + LuP47B	29^b	31^a	59.6^b	79.3^c	138^c	175^c	7.9^b	11.7^c

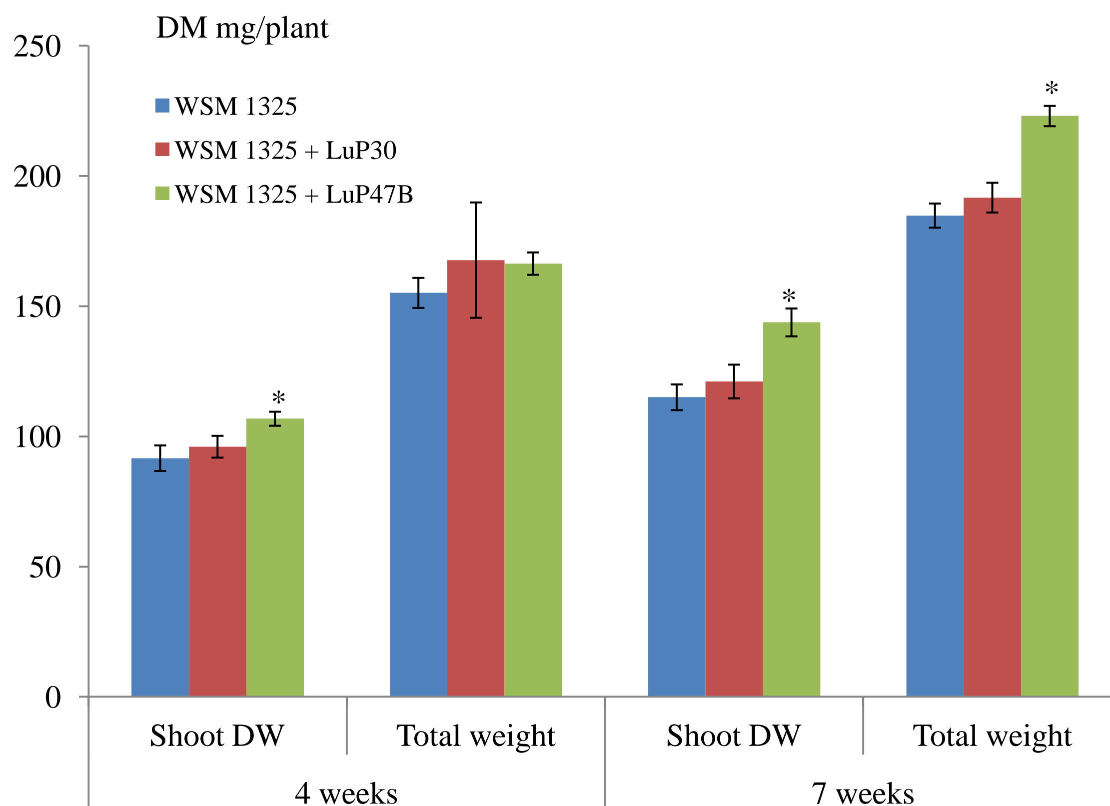


Figure 7.6 Effects of LuP30 and LuP47B on shoot DW and nodulation of clover when applied prior to inoculation with *Rhizobium leguminosarum* strain WSM 1325. Asterisks indicate significant differences at $p \leq 0.05$ (*) or $p \leq 0.01$(**) Error bars: Mean ± S.E.. Left pot and root: *Rhizobium* WSM 1325 only; Right pot and root: LuP47B + *Rhizobium* WSM 1325, showing bigger plants and more nodules after 7 weeks inoculation with the rhizobia.

LuP30 and LuP47B affected nodulation by increasing the number of nodules and nodule mass per plant (Table 7.4). These results were similar to their performance on lucerne when they were applied with the rhizobial partner, *S. meliloti* strain RRI 128. Although sub-clover forms indeterminate nodules similar to lucerne, the

actinobacteria LuP30 did not increase sub-clover growth, despite the increase in nodule number and mass. Singleton and Tavares (1986) found that the increase of nodule number does not necessarily increase of nitrogen fixation and plant growth, where nodule function is adversely affected. The formation of less effective nodules might be a reason that co-inoculation with LuP30 significantly increased the number and total mass of nodules per plant**.** In contrast, LuP47B also increased dry weight of root, number of nodules, and nodule mass per plant and led to an improvement of shoot and total weight of shoots and roots of plants (Table 7.4).

7.3.3.3 Response of serradella to inoculation with LuP30 and LuP47B

There were no significant effects of LuP30 and LuP47B on the growth, nodulation or N_2-fixation of serradella at 4 and 7 weeks after inoculation with *Bradyrhizobium lupini* strain WSM 471 (Table 7.5 and Figure 7.7). The actinobacteria LuP30 and LuP47B might not be appropriate for co-inoculation with the *Bradyrhizobium* strain WSM 471 or be able to improve the plant growth or symbiosis of serradella.

Table 7.5 Plant growth and nodulation response of serradella to effects of LuP30 and LuP47B with *Bradyrhizobium* WSM 471 after 5 and 7 weeks inoculation with the rhizobial strain, (n=4). Different letters in the same column indicate means are significantly different ($P < 0.05$).

Treatment	Root length (cm)		Root weight (mg DM/ plant)		Nodule number (#/plant)		Total nodule mass (mg)	
	4 w	**7 w**	**4 w**	**7 w**	**4 w**	**7 w**	**4 w**	**7 w**
Untreated*	25^{a}	33^{a}	53.2^{a}	78.3^{a}	0^{a}	0^{a}	0^{a}	0^{a}
WSM 471 only	30^{a}	38^{a}	89.4^{b}	113.8^{b}	35^{b}	45^{b}	9.8^{b}	13.4^{b}
WSM 471 + LuP30	32^{a}	31^{a}	84.5^{b}	92.1^{ab}	36^{b}	43^{b}	9.3^{b}	16.3^{b}
WSM 471 + LuP47B	30^{a}	36^{a}	91.2^{b}	104.9^{b}	39^{b}	43^{b}	10.2^{b}	17.9^{b}

Serradella forms determinate nodules and in this regard differs from both sub-clover and lucerne. Although LuP30 and LuP47B stimulated the growth of *Rhizobium* WSM 471 *in vitro* any response *in planta* is likely to be more complex. It can be initially concluded that the interactions of the actinobacteria are likely to be dependent on the species of host legume and/or its rhizobial partner.

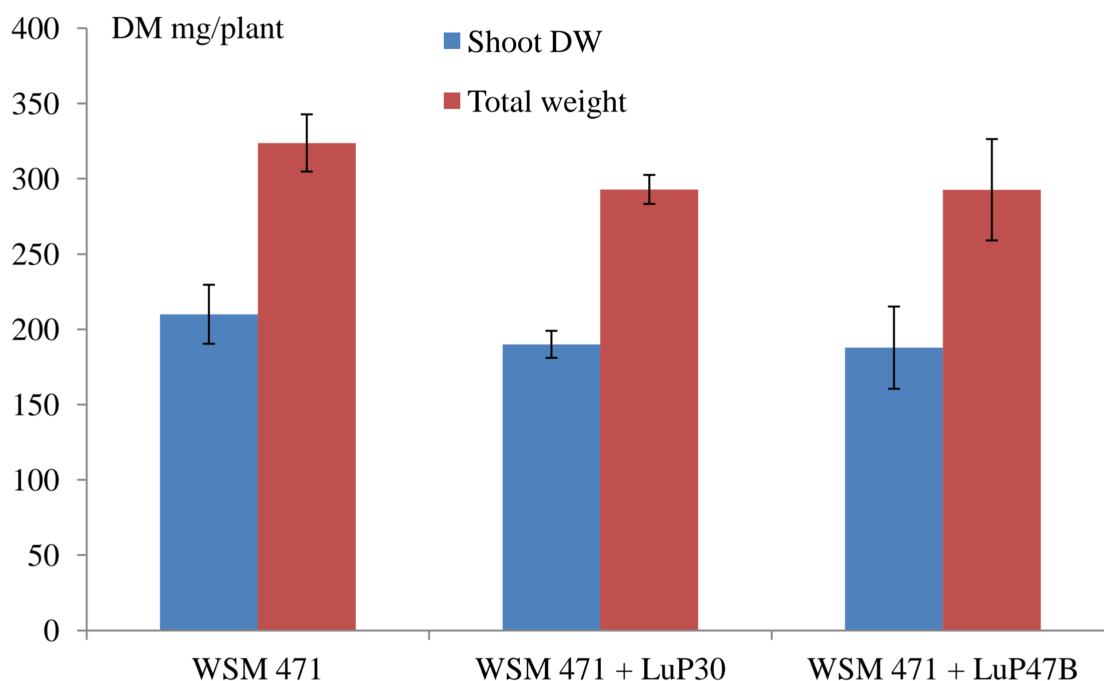


Figure 7.7 Effects of LuP30 and LuP47B on dry weight of shoot with *Bradyrhizobium* WSM 471 and total weight of serradella 5 and 7 weeks after inoculation with the rhizobial strain. Asterisks indicate significant differences at $P < 0.05$ (*) or $P < 0.01$(**), Error bars: Mean ± S.E.

7.3.4 Effects of LuP30 and LuP47B on the growth of wheat

LuP30 and LuP47B did not affect the growth of wheat in terms of the dry weight of shoots and roots produced in sand and vermiculite growing media supplied with 0 mg and 25 mg/kg NH_4NO_3. The results showed that the actinobacteria LuP30 and LuP47B did not provide any benefits when they were grown in sand and vermiculite with N deficiency. Therefore, the actinobacteria were not able to act as free living

nitrogen fixers or to produce an effect due to plant growth hormones on wheat production in the growing system, as the growth parameters of the plants were not different with the control plants (Table 7.6). Both LuP30 and LuP47B are IAA and siderophore producers as reported in Chapter 4 but they did not show an increase in the growth parameters measured for wheat. At 25 mg/kg NH_4NO_3 sand and vermiculite there were similarly no differences in the wheat plants treated with LuP30 or LuP47B compared with control plants. The actinobacteria were not able to use the available N better than control plants, or the growth conditions were not suitable to enable a response which increases the growth of wheat after 7 weeks when the N concentration in soil was 25 mg/kg NH_4NO_3. These results were obviously different to those of previous studies (6.3.1 in chapter 6 and 7.3.1 in chapter 7) where both LuP30 and LuP47B increased the growth of lucerne plants when the sand and vermiculite had 25 mg/kg NH_4NO_3 in absence of the rhizobial partner. LuP30 and LuP47B were endophytic actinobacteria from lucerne roots so they might not be endophytic within wheat roots and therefore not be able to benefits the growth of wheat in this study.

Table 7.6 Effect of LuP30 and LuP47B on growth of wheat plants at 0 mg and 25 mg N after 7 weeks plant growth, (n=4). Different letters in the same column indicate means are significantly different ($P < 0.05$).

Treatment	Length of shoot (cm)	Length of root (cm)	Shoot weight (mg DM/ plant)	Root weight (mg DM/ plant)	Total mass (mg DM/ plant)
Untreated*	26.7^{a}	55.7^{ab}	117.9^{a}	43.7^{a}	161.5^{a}
M only	28.0^{a}	56.7^{b}	162.0^{a}	46.5^{a}	208.5^{a}
M + LuP30	26.7^{a}	51.4^{ab}	155.6^{a}	52.8^{a}	208.3^{a}
M + LuP47B	27.3^{a}	57.0^{b}	162.1^{a}	51.6^{a}	213.6^{a}
M + 25 mg N	40.4^{b}	47.9^{ab}	398.2^{b}	101.6^{b}	499.8^{b}
M + 25 mg N + LuP30	38.0^{b}	43.1^{ab}	369.0^{b}	94.8^{b}	463.9^{b}
M + 25 mg N + LuP47B	36.3^{b}	35.1^{a}	341.8^{b}	94.9^{b}	444.1^{b}

Untreated*: only water supplied as required; M: 200 mL of McKnight's solution supplied at day 0 and water supplied as required following weeks.

7.4 Conclusion

LuP30 and LuP47B increased the growth of lucerne in the absence of *S. meliloti* RRI 128 when the concentration of N in soil was 25 mg/kg NH_4NO_3. The beneficial effects observed with *S. meliloti* strain RRI 128 were confirmed, but did not extend to plants which had unknown rhizobia present the soil. Therefore, the micro-symbiont is a very important consideration in the interaction with the host legumes. LuP30 or LuP47B re-displayed increases in the growth and nodulation of lucerne plants when they were applied together with strain RRI 128 in the presence of 25 mg/kg NH_4NO_3. At low concentration ($5x10^2$ CFU/ml) of *S. meliloti* RRI 128, LuP30 and LuP47B increased the growth and number of nodules per plant, but were not as effective at higher titres of rhizobia. With the soil extract A, many factors including the effectiveness of rhizobia and other soil microflora could also have been significant for this lack of response.

The benefit of the actinobacteria extended to other host legumes and rhizobial partners as they stimulated the growth of *Rhizobium* WSM 1325 and *Bradyrhizobium* WSM 471 *in vitro* and increased the growth and nodulation of sub-clover plants. The results are encouraging and need to be validated and further investigated to understand the mechanisms. The lack of response in serradella may mean that the effects of the actinobacteria may not be relevant to legumes that form symbioses with *Bradyrhizobium* spp. or possibly legumes that form determinate nodules. However, this conclusion will have to be validated.

Chapter 8

Identification and characterisation of the *Streptomyces* spp. LuP30 and LuP47B

8.1 Introduction

Streptomyces spp. LuP30 and LuP47B are beneficial to the plant growth, symbiotic function and the bio-control of disease affecting lucerne plants by *R. solani*. The aims are to identify and characterise these strains in order to determine if they are novel/new species. This was done by carrying out the full polyphasic taxonomy, including side-by-side comparison with their two closest type cultures to examine the presence of any pathogenic genes to plants.

Based on 16S rRNA gene sequence similarity, *Streptomyces* sp. LuP30 is closest to *Streptomyces rishiriensis* NRRL B3229 (99.85%) and *Streptomyces phaeofaciens* NRRL B1516 (99.1%) while *Streptomyces* sp. LuP47B has closest similarity with *Streptomyces ciscaucasicus* NRRL F7401 (99.49%) and *Streptomyces canus* NRRL B1989 (99.49%). However, the morphological characteristics of LuP30 and LuP47B are different from the two closest type cultures on different solid media. Kim *et al.* (2014) suggested that 98.65% 16S rRNA gene sequence similarity can be used as the threshold for differentiating two species instead of 98.2-99.0%. However, in many cases, two species share a high level of 16S rRNA gene sequence similarity (99%) but they can be clearly distinguished from each other by DNA-DNA hybridisation (Ash *et al.*, 1991; Rossello'-Mora and Amann, 2001). In addition, Wayne *et al.* (1987) recommended that the "cut off" value for the same species is 70% or higher DNA-DNA relatedness.

In this study, complete polyphasic taxonomic characterisations (except FAME analysis) of LuP30 and LuP47B were carried out with side-by-side comparison with

two nearest type cultures. The results show that the two *Streptomyces* endophytes are novel species.

8.2 Materials and methods

8.2.1 Morphological studies

8.2.1.1 Culture morphological characteristics

The actinobacterial candidates and their nearest type cultures were grown on different media such as MS, HPDA, and media from the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1996) namely ISP2, ISP3, ISP4, ISP5, ISP7, nutrient agar, Bennett's agar and Glycerol asparagine yeast extract agar. All plates were incubated at 27°C for 2 weeks to compare their growth and morphology. The colour of spore, aerial and substrate mycelium and soluble pigment of the cultures were recorded based on the Methuen Handbook of Colour (Kornerup and Wanscher, 1978). The *Streptomyces* type cultures were imported from the US Department of Agriculture's Agricultural Research Service Culture collection known as NRRL.

8.2.1.2 Scanning electron microscopy (SEM) of cellular and spore morphological characteristics

Streptomyces spp. LuP30 and LuP47B were grown on HPDA for 14 days at 27°C to get well sporulating and were cut in 5 mm x 5 mm plugs. The spores were firstly fixed for 1 hour by covering them with a fixative solution containing 4% paraformaldehyde and 1.2% glutaraldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.2. The tubes were shaken gently every 15 minutes to ensure the fixative solution covered the plugs. The plugs were washed twice with PBS 5 minutes for each wash before adding 2% Osmium in PBS for secondary fixation 1 hour. The dehydration process was started by washing in serial ethanol solutions of 70%, 90% and 100%, twice and 15 minutes for each ethanol concentration except 20 minutes

with 100% ethanol. The samples were subjected to critical point drying by using a Leica EM CPD300 Biosystem before mounting on a stub and coated with platinum. The cell and spore structures were observed under a scanning electron microscopy at Flinders Advanced Analytical Centre, Flinders University, Australia.

8.2.2 Physiological and biochemical characteristics

8.2.2.1 Growth at various temperatures, pH, sodium chloride and utilisation of carbohydrates

ISP2 was used as the base medium for a range of temperature 15°C, 27°C, 37°C, 45°C and 55°C, and two plates for each temperature. HPDA was used to check for tolerance with a range of pH 4, 4.5, 7, 9 and 10, and sodium chloride concentrations 2%, 3%, 4%, 5%, 10%, 15% and 20%. The pH of the medium was adjusted after autoclaving by using 1M NaOH and 1M HCl. The carbohydrates used for examining the carbohydrate utilisation were adonitol, L-arabinose, cellobiose, fructose, galactose, glucose, maltose, mannitol, mannose, methyl, sorbitol, sucrose, telibose and xylose. A basal inorganic medium was prepared following method by Gordon *et al.* (1974) containing 1g/L $(NH_4)_2HPO_4$, 0.2 g/L KCl, 0.2 g/L $MgSO_4.7H_2O$ and 15 g/L agar. The medium pH was adjusted to 7.0 and 15 ml of a 0.04% bromocresol purple solution added before autoclaving. A ten per cent solution of each carbohydrate was made and filter sterilised, and added into the basal inorganic medium to get a final concentration of 1% carbohydrate. The cultures were grown in 15 ml tubes which contained a slant of 4 ml of 1% of each carbohydrate in a basal inorganic medium. The isolate was streaked onto the surface of the slant and incubated at 27°C. The ability of the isolate to produce acid was scored by the changes of the medium colour from purple to yellow at 3, 7, 14 and 28 days after inoculation.

8.2.2.2 Hydrolysis of Gelatine

The method was described by Kurup and Schmitt (1973). Nutrient agar was used as base medium with the addition of 0.4% (w/v) of gelatine and the pH was adjusted to 7.0. Actinobacteria were grown on the medium plates by crossing them two lines in the middle of the plates for 14 days at 27°C. Each culture was prepared in duplicate. The plates were flooded with mercuric chloride solution ($HgCl_2$ 15 g, concentrated HCl 20 ml and R.O. water to make up 100 ml. The gelatine was hydrolysed with the presence of a clearing zone surrounding the growing colony.

8.2.2.3 Hydrolysis of Esculin

The method of hydrolysis of Esculin was described by Kurup and Schmitt (1973). The actinobacterial culture was inoculated into a 5 ml McKinney vial of Esculin broth containing 1 g/L Esculin, 10 g/L peptone and 5 g/L NaCl. The vials were incubated at 27°C for 4 weeks and 1 ml of each suspension was transferred into a 1.5 ml eppendorf tube. Esculin was hydrolysed following a colour change of culture suspension into black or brown fluorescence after adding 10 µl of 1% (w/v) ferric citrate. The same broth without esculin was also inoculated with actinobacterial cultures as negative controls.

8.2.2.4 Hydrolysis of Starch

The method for checking the hydrolysis ability of actinobacteria was described by Gordon *et al.* (1974). Nutrient agar was used as the basal medium and the pH was adjusted to 7.0 with the addition of 1.5% (w/v) starch. The starch and nutrient agar

were autoclaved separately and mixed thoroughly before pouring into plates. The actinobacteria were streaked across two lines in the middle of the plates and incubated at 27°C. At 7 and 14 days after incubation, the plates were flooded with the Lugol's Iodine (0.1% w/v iodine and 0.2% w/v KI) and placed at room temperature for 15 minutes. The positive actinobacteria showed a clearing zone surrounding the colony while negative actinobacteria plates turned to black as a result of the reaction of starch in the medium with Iodine.

8.2.2.5 Decomposition of Urea

The method of decomposition of urea was described by Gordon *et al.* (1974). The urea broth was made by adding 10 ml of filter sterilised urea into 75 ml of mixture (10 g KH_2PO_4, 9.5 g Na_2HPO_4, 1 g yeast extract, 20 ml of 0.04% phenol red solution and RO water 1 L, pH 6.7). The actinobacterial culture was inoculated in 5 ml of the broth in a McKinney vial and the colour change was observed after addition of alkali 28 days after incubation at 27°C.

8.2.2.6 Decomposition of Casein

The method of decomposition of casein was described by Gordon *et al.* (1974). Skim milk was used as a source of casein. The medium was made by mixing the skim milk 10% (w/v) solution thoroughly with agar 2% (w/v) solution after being autoclaved separately. The actinobacterial isolates were inoculated by streaking cross the agar plates and the plates were incubated at 27°C for 14 days. The positive cultures for decomposition of casein showed the presence of a clearing zone surrounding the growing colony.

8.2.2.7 Decomposition of adenine, xanthine and L-tyrosine:

The composition of adenine, xanthine and L-tyrosine was described by Gordon *et al.* (1974). Nutrient agar was used as a basal medium with pH 7.0. The solutions were made by dissolving separately 0.4 g of xanthine or 0.5 g of adenine and L-tyrosine in 10 ml RO water and autoclaved. Each solution was then added into the autoclaved nutrient agar, and mixed thoroughly before pouring into plates. The isolates were inoculated by streaking cross in the middle of the plates and incubated at 27^oC for 14 days. The clearing zone around the cultures indicated that the cultures were positive in decomposition of adenine, xanthine or L-tyrosine.

8.2.2.8 Catalase production

The catalase production of cultures was determined following the method by Kurup and Schmitt (1973). One full loop of actinobacteria grown on HPDA for 7-10 days was mixed with one drop of freshly prepared 5% H_2O_2 on a clean glass slide. The presence of effervescence was the indicator of the ability to produce catalase.

8.2.2.9 Use of organic acids

The utilisation of organic acids was examined following the method described by Kurup and Schmitt (1973). A basal medium was prepared as follows: 1 g/L NaCl, 0.2 g/L $MgSO_4.7H_2O$, 0.1 g/L $(NH4)_2HPO4$, 0.5 g/L KH_2PO_4, 18 g/L agar and 20 ml of 0.04% phenol red. The pH was adjusted to 6.8 and autoclaved. The actinobacterial cultures were streaked onto the agar medium containing 0.2% of each sodium salt of

the organic acids (acetate, benzoate, citrate, propionate, and L-tartrate) added separately into the medium. The colour of medium was checked at 3, 7 and 10 days after incubation at 27°C. The colour change of the basal medium from yellow to purple indicated the utilization of the organic acids.

8.2.3 Chemotaxonomy studies

8.2.3.1 DAP cell wall analysis

LuP30, LuP47B and type cultures *Streptomyces rishiriensis* NRRL B3229, *Streptomyces phaeofaciens* NRRL B1516, *Streptomyces ciscaucasicus* NRRL F7401 and *Streptomyces canus* NRRL B1989 were grown on cellophane ISP2 medium for 7 days. The cells and spores were harvested and added into 1 ml of 6 N HCl in 10 ml glass tube. The tubes were mixed through before autoclaved at 121°C for 15 minutes. Two ml autoclaved R.O. water was added to the solution before it was filtered through the Whatman No.1 filter paper and heated in a boiling water bath. The extracts were filtered again through the Whatman No.1 filter paper with 1 ml of R.O. water and drying. This was repeated twice before resuspending the DAP cell wall with 40 µl of autoclaved R.O. water. The suspension was stored at -20°C until further analysis.

The solvent system used to separate the sugars was pyridine: 6M HCl:RO-H_2O: MeOH (10:4:26:80) and equilibrated in the Thin Layer Chromatography (TLC) tank before developing the TLC for at least 1 hour. Serial dilutions of DAP extracts were made from 100%, 20% and 10%, and 2 µl of each solution was spotted onto a TLC (Thin Layer Chromatography) cellulose plate (Merck 5552, Victoria, Australia); which was heated up at 110°C for 1 hour prior to performing TLC. The plate was run

in the solvent for approximately 3 hours or until the solvent system reached 1.5 cm from the top of the TLC plate. The colour development of the DAP cell wall compounds was performed by air drying the plate in a fume hood then spraying with 0.2% ninhydrin in acetone spray reagent and left to dry. The TLC plate was kept in a dry heat oven at 100^oC for 5-10 min to enhance the colour development. The TLC plate was covered overnight to intensify the yellow DAP spot for analysis. Typically the DAP spots are olive green fading to yellow in colour.

8.2.3.2 Sugar cell wall analysis

The actinobacteria were grown on cellophane for 14 days before harvesting into a V glass shape containing 200 µl of 0.25 M HCl. The cells and spores were mixed well before autoclaving in a dry cycle (121^oC for 15 minutes). After cooling, the sugar cell wall of actinobacteria was determined by TLC. A volume of 2 µl of each actinobacteria hydrolysate including type cultures was spotted onto a cellulose TLC sheet (Merck 5552, Victoria, Australia) which was activated at 110^oC for 1 hour in an oven. Two µl of 1% different sugar solutions (arabinose, galactose, glucose, mannose, rhamnose and xylose) were used as positive controls. Thirty ml of the solvent system was n-butanol: RO H_2O: pyridine: toluene (10:6:6:1) and equilibrated in the TLC tank for 1 hr before developing TLC. The plate was run in the solvent system for approximately 3 hr or until the solvent system reached 1.5 cm from the top of the TLC plate. For visualization of sugar cell wall, the TLC plate was air dried in a fume hood and sprayed with aniline phthalic acid (aniline 2 ml; phthalic acid 3.3 g; water saturated n-butanol 100 ml) and allowed to dry. The TLC plate was kept in a dry heat oven at 100^oC for 10 min to enhance colour development.

8.3.3.3 Menaquinone analysis

The menaquinone analysis was conducted using the method by Alderson *et al.* (1985). The actinobacterial cultures were grown on HPDA until good growth was achieved. Two full loops of the cultures were inoculated to 50 ml of ISP2 broth in a 250 ml Erlenmeyer flask. After incubation at 27°C on a shaker at 150 rpm for 10 days, good growth of the actinobacteria was achieved. The biomass was collected and centrifuged at 3000 g, followed by 3 series of washes with sterile RO water. The biomass was kept at -20°C before being lyophilised using a freeze drier. Menaquinone compounds were obtained by extracting 50 mg of the dried cells with 40 ml of chloroform: methanol (2:1) and the mixture was shaken overnight at 50 rpm. The extract was filtered through a Whatman No. 1 filter paper and dried using a vacuum evaporator. It was resuspended in 50 µl of HPLC grade acetone.

Menaquinone was visualised by performing TLC in a solvent system of hexane: diethyl ether (85:15) which was equilibrated in a chamber for 1 hr prior to performing the TLC. The menaquinone extract was spotted across approximately 4 cm width on a 10 x 10 cm pre-coated silica gel 60 F_{254} plastic backed plate (Merck#5735, Victoria, Australia). The TLC was run until it reached 1.5 cm from the top of the TLC plate and then air dried in the fume hood. The plate was observed under UV (254 nm). Menaquinone was revealed as a dark brown band on a green fluorescent back ground (Rf approximately 8). The menaquinone band from the TLC plate was scraped and extracted with 1 ml acetone followed by vortexing and centrifuging at 8000 g for 5 min. The supernatant was left to dry overnight in a fume hood and then resuspended with 50 µl of HPLC grade acetone. This was then analysed using LC-MS employing UV detection and electrospray ionisation (ESI).

The Eclipse XDB-C18 4.6x150 mm, 5 µm (Agilent Part No. 99397-902, California, USA) was used as the column and the LC solvent system was isopropanol:methanol (1:1) at a flow rate of 1.0 ml/min. This was done at Flinders Advanced Analytical, Flinders University.

Streptomyces usually have MK-9 in their menaquinone but MK-8 and MK-10 were also found. In order to identify the different units of menaquinone of the cultures the mass of different MK was used as a key factor based on LC-MS (Goodfellow *et al.*, 2012).

8.3.3.4 Fatty acid methyl ester (FAME) analysis

The actinobacterial fatty acids were analysed using the method by Sasser (2001). The cultures were prepared by adding two full loops of spores or cells from ½PDA and inoculated into 250 ml flasks containing 50 ml of tryptone soy broth (TSB). The flasks were incubated at 27°C on a shaker at 150 rpm for 10 days to achieve good growth. The biomass was collected by centrifugation at 3000 g, washed twice with sterile RO water and stored at -20°C prior to further experiments. FAME samples were prepared in 4 steps: saponification, methylation, extraction and base washing.

In the saponification step, approximately 40 mg of wet cells were placed in 13x100 mm glass tubes with a Teflon-lined cap with 1 ml of reagent 1 (3.75 M NaOH in methanol prepared as follows: 45 g sodium hydroxide, 150 ml methanol and 150 ml distilled water) added. The tubes were vortexed briefly and heated in a boiling water bath at 95°C for 5 min followed by another vigorous vortexing for 10 sec prior to heating again in the water bath for another 30 min. Methylation was done by adding

2 ml of reagent 2 (325 ml 6 N hydrochloric acid and 275 ml methyl alcohol) to the tubes after it cooled down. The tubes were vortexed briefly and heated for 10 min at 80°C ± 1 °C. After the tubes were cooled, the extraction step was begun by adding 1.5 ml of reagent 3 (200 ml hexane and 200 ml methyl tert-butyl ether). The tubes were shaken gently for 10 min, allowed to stand and then the aqueous (lower) phase was discarded. For the base washing, 3 ml of reagent 4 (10.8 g sodium hydroxide dissolved in 900 ml distilled water) was added into the tubes and shaken again for another 5 min. FAME was collected by pipetting 2/3 of the organic phase (top layer) into a GC vial before sending for analysis to the HelmHoltz Institute, Braunschweig, Germany in a MIDI system. Waiting for the results

8.2.4 Genomic studies
8.2.4.1 Phylogenetic and genomic studies

The 16S rRNA gene sequences of *Streptomyces* spp. LuP30 and LuP47B were subjected to BLAST on the NCBI database and http://www.ezbiocloud.net/eztaxon to find the nearest neighbour type cultures. The phylogenetic trees were built by MEGA 6.0 version. Total genomic DNA of LuP30 and LuP47B were extracted as described in section 2.4.1 in Chapter 2, sequenced at the AGRF, Sydney and the sequence annotated in Manipal University, India.

8.2.4.2 DNA-DNA hybridisation

The homology between the representative *Streptomyces* spp. LuP30 and LuP47B isolates and their type strains was determined using DNA-DNA hybridization (DDH) following the methods developed by Ezaki *et al.* (1989) and Kusunoki *et al.* (1991) as described below. Total genomic DNA of all the cultures used in this

experiment was extracted and prepared as described in Section 2.4.1. The DDH method involved 3 steps; immobilization of reference DNA, photobiotinylation of the closest type strain DNA and hybridization.

Immobilization of the reference DNA (actinobacterial isolate and two type cultures) was conducted in a 96-microdilution flat well plate (Immunoplate, Nunc). The reference DNA was denatured at 95^oC for 10 min on a heating block and immediately put on ice prior dilution. A 100 µl unlabelled single stranded DNA with concentration of 5 µg/µl in 1x phosphate-buffered saline (PBS) with 0.1 M $MgCl_2$ was distributed into each microdilution well and four wells were for each reference DNA. The plates were incubated at 27-30^oC for 16-18 hr in order to immobilise the single stranded DNA on the wells. After 16-18 hr incubation at 27-30^oC, the solution was discarded and replaced with 300 µl of 1x PBS with 0.1 M $MgCl_2$ containing 50 µg/ml denatured single stranded DNA from salmon testes (Sigma). Genomic DNA from *Escherichia coli* JM109 was used as a negative control.

Labelling the type strain DNA was conducted using Photobiotin™Acetate (Sigma) as described by Ezaki et al. (1989). An equal volume (15 µl) of both photobiotin and type strain DNA with concentrations of 500-1,000 ng/µl were placed in a 1.5 ml Eppendoft tube and mixed well in a dark room with very dim light for activation of the photobiotin. The tube was kept open on crushed ice, and the mixture was irradiated using a UV light (365 nm) for 30 min with 2 cm distance from the light source. Photobiotin was cleaned up using 2-butanol extraction. To the tube, 50 µl of Tris-EDTA (pH 9.0) and 20 µl of water were added along with 100 µl of 2-butanol (Sigma). The mixture was mixed well by vortexing, and then spun at 1,000g for 1

min. After the top layer was discarded, the clean-up step with 2-butanol was repeated. Another 100 µl of 2-butanol and water was also added to make the volume up to 200 µl, followed by the vortexing and spinning. After discarding the top layer, the left over volume was approximately 40 µl. Labelled DNA was precipitated by adding 0.1x volume of sodium acetate pH 5.5 and 2x volume of 100% cold absolute ethanol and mixed well by vortexing. The DNA precipitation was visible at this stage by the presence of a light brown cloud in the mixture. DNA was precipitated at -20°C overnight or at -80°C for 2 hrs and was subjected to centrifugation at 4°C at maximum speed for 15 min. The supernatant was discarded and the pellet washed with 500 µl of 70% cold ethanol. It was centrifuged again at maximum speed for 5 min and the supernatant was discarded. DNA was dried using a heating block at 55°C for approximately 10 min before dissolving it in DNA/RNA free water. The labelled DNA concentration was then quantified.

The hybridization step was initialized by preparing pre-hybridization solution containing 2x SSC (Saline-sodium citrate, 0.3M NaCl [Merck] and 0.03M sodium citrate [Sigma]), 5x Denhardt solution (Sigma), 50% formamide (Sigma) and 200 µg/ml of denatured single stranded DNA from salmon testes (Sigma). Two hundred µl of pre-hybridization solution was added onto a microdilution plate which was coated by reference DNA as described previously. After 1 hr of incubation at 37°C, the pre-hybridization solution was discarded and replaced with 100 µl of hybridization solution containing 2x SSC, 5x Denhardt solution, 3% dextran sulphate (Sigma), 50% formamide, 50 µg/ml denatured single stranded DNA from salmon testes and 0.5 µg/ µl of photobiotinylated DNA which was denatured at 95°C for 10 min prior to dilution. The plate was then covered with vinyl tape and the

hybridization was carried out at 43°C for 2 hrs. The microdilution plate was then washed three times with 2x SSC. Furthermore, a 100 µl volume of 1x PBS containing 5000x dilution of streptavidin-horseradish peroxidase (Sigma) was added into each well and further incubated for 10 min at 37°C. Again the plate was washed 3 times with 1x SSC. Each well was filled with 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma). The colour changing reaction was measured by reading the optical density at 630 nm within 30 min after the addition of the substrate solution.

8.2.5 In vitro N free living ability

A nitrogen free and Jensen's medium (Appendix 1) were used to check the ability to live in nitrogen free medium of actinobacteria LuP30 and LuP47B. The growth of actinobacteria was observed after 7 days and 14 days after inoculation. *Streptomyces rishiriensis* NRRL B3229 and *Streptomyces phaseofaciens* NRRL B1516 were used as controls for LuP30 while *Streptomyces ciscaucasicus* NRRL F7401 and *Streptomyces canus* NRRL B1989 were grown to compare with LuP47B.

8.3 Results and discussion

8.3.1 Streptomyces strain LuP30

8.3.1.1 Morphological characteristics

Streptomyces sp. LuP30 grew very well on ISP2 with pale grey aerial mycelium and cinnamon substrate mycelium. It grew well on all media used ISP3, ISP4, ISP5, ISP7, HPDA and MS. There were obvious differences in terms of morphology, substrate mycelium and pigment produced from LuP30 and two cloest type cultures

S. rishiriensis and *S. phaeofaciens* (Figure 8.1). The morphological characteristic details of this strain are displayed in Table 8.1. Under scanning microscopy the spores are cylindrical and smooth and approximately 0.5µm wide and 1µm long (Figure 8.2).

Table 8.1 Cultural characteristics of *Streptomyces* LuP30 at 2 weeks after incubation

Medium	Growth	Aerial mycelium	Substrate mycelium
ISP2	Good	Pale grey, greyish white	Cinnamon
ISP3	Good	Orange grey	Brownish orange
ISP4	Good	White –thin small spores	Yellowish white
ISP5	Good	Orange grey	Reddish blonde
ISP7	Moderate	Greyish brown	Greyish brown
MS	Good	Brownish grey spores	Dark blond yellowish
HPDA	Good	White grey	Dark brown whisky
Nutrient agar	Good	Brown	Light yellow
Bennett’s agar	Good	White	Dark greyish
Glycerol asparagine yeast extract agar	Medium	Brown	Dark brownish

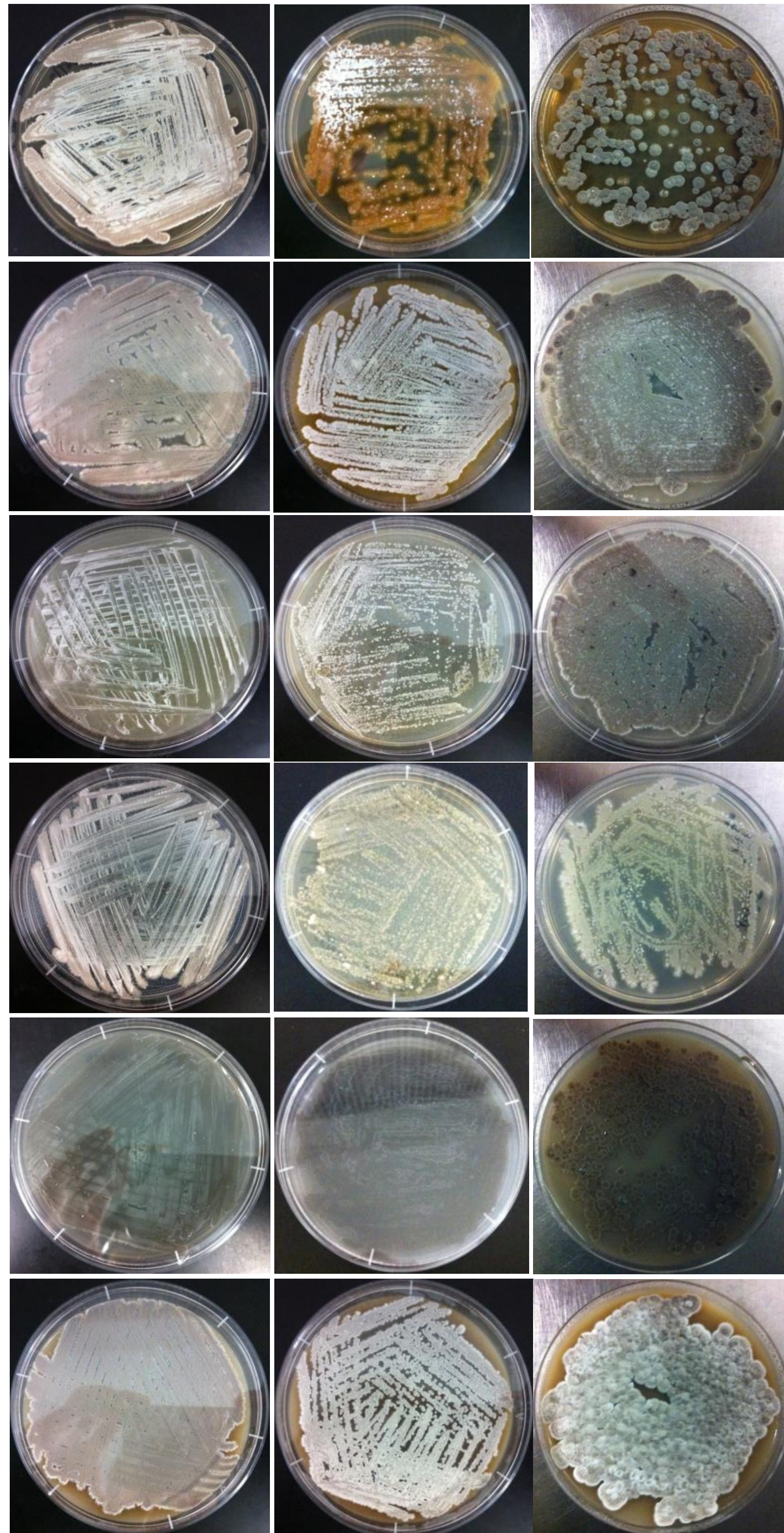


Figure 8.1 Morphological comparison of *Streptomyces* LuP30 and two closest type cultures. From left to right: *S. rishiriensis*, LuP30 and *S. phaeofaciens* grown on ISP2, ISP3, ISP4, ISP5, ISP7 and MS from top to bottom at 14 days after incubation at 27°C.

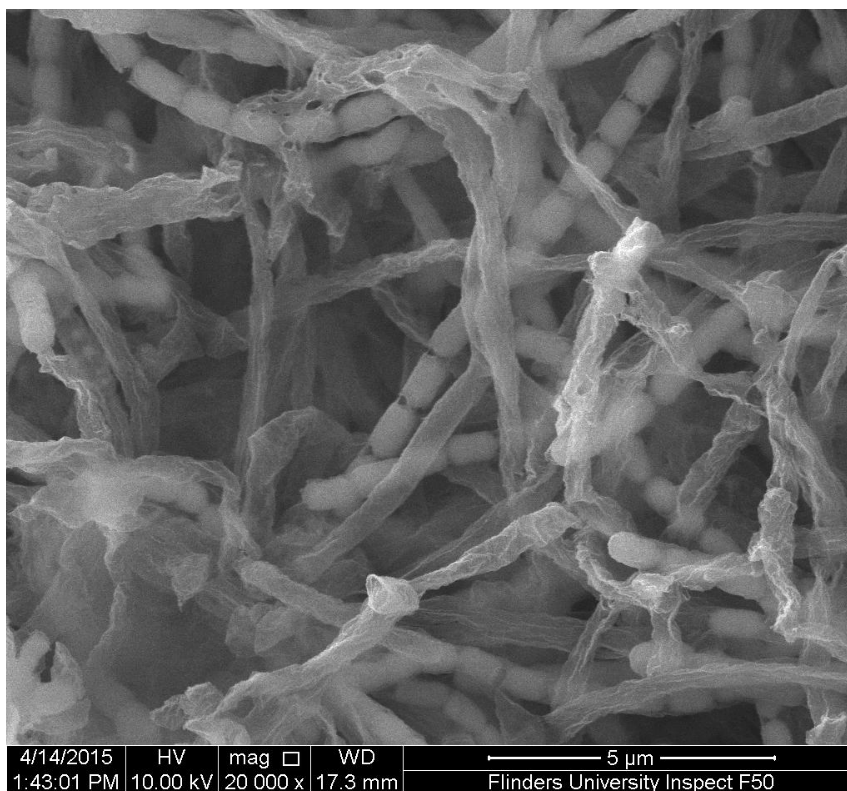


Figure 8.2 Scanning electron microscope of aerial mycelia and spore chains of *Streptomyces* LuP30 after incubation at 27°C on HPDA for 14 days, scale bar 5µm.

8.3.1.3 Physiological and biochemical characteristics

The physiological and biochemical characteristics of *Streptomyces* sp. LuP30 were compared side by side with its two closest type cultures *S. rishiriensis* and *S. phaeofaciens* (Table 8.2). LuP30 and *S. phaeofaciens* grew on a range of pH4-10 while *S. rishiriensis* could not grow at pH 4. *Streptomyces* sp. LuP30 grew well on 2% to 4% sodium chloride medium and temperatures from 15°C to 27°C. However, it grew weakly at 37°C and did not grow at 45°C. *Streptomyces* sp. LuP30 could decompose adenine, xanthine, urea but not casein while *S. rishiriensis* could not decompose xanthine. *Streptomyces* sp. LuP30 could hydrolyse esculin, starch, gelatine and produce catalase. In contrast, *S. rishiriensis* and *S. phaeofaciens* were not able to hydrolyse starch. *Streptomyces* sp. LuP30 was able to utilise the organic

acids acetate, benzoate, citrate, propionate and tartrate 14 days after inoculation. Interestingly, *S. rishiriensis* and *S. phaeofaciens* did not use benzoate while later on *S. phaeofaciens* was also negative for tartrate. In addition, LuP30 was able to use a range of carbohydrates such as arabinose, cellobiose, fructose, galactose, glucose, maltose, mannose, sucrose, telibose and xylose but it did not use adonitol, mannitol, methyl -α - D- pyranoside and sorbitol. In contrast, *S. phaeofaciens* was able to utilise methyl -α - D- pyranoside and did not use sucrose.

Table 8.2 Physiological and biochemical characteristics of *Streptomyces* LuP30 in comparison with two closest type cultures *S. rishiriensis* and *S. phaeofaciens*

Component	**Growth of isolates under the following conditions (14 days)**		
	LuP30	***S. rishiriensis***	***S. phaeofaciens***
pH			
4	+	-	+
4.5	+	+	+
7	+++	+++	+++
9	+++	+++	+++
10	++	++	++
Sodium chloride concentration (%)			
2	+++	+++	++
3	+++	+++	++
4	++	++	+
5	-	-	-
10	-	-	-
15	-	-	-
Temperature (^{o}C)			
15	++	++	+
27	+++	+++	+++
37	+	+++	+
45	-	-	-
55	-	-	-

- : no growth
+ : week/poor growth
++ : moderate growth
+++ : strong growth

Table 8.2 (*Continued)* Physiological and biochemical characteristics of *Streptomyces* LuP30 in comparison with two closest type cultures *S. rishiriensis* and *S. phaeofaciens*

Component	Utilization after 14 days		
	LuP30	***S. rishiriensis***	***S. phaeofaciens***
Decomposition of			
Adenine	+	+	+
Hypoxanthine	NA	NA	NA
L-tyrosine	-	-	-
Xanthine	+	-	+
Casein	-	-	-
Urea	+	+	+
Hydrolysis of			
Esculin	+	+	+
Starch	+	-	-
Gelatin	+	+	+
Catalase production	+	+	+
Carbohydrate			
Adonitol	-	-	-
Arabinose	+	+	+
Cellobiose	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Maltose	+	+	+
Mannitol	-	-	+
Mannose	+	+	+
Methyl	-	-	+
Sorbitol	-	-	-
Sucrose	+	+	-
Telibose	+	+	+
Xylose	+	+	+

Component	LuP30				*S. rishiriensis*				*S. phaeofaciens*			
Utilization of organic acids	3 d	7d	10d	14d	3 d	7d	10d	14d	3 d	7d	10d	14d
Acetate	-	-	W	+	-	W	+	+	-	W	+	+
Benzoate	-	-	-	+	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+	+	+	+
Propionate	-	-	W	+	-	+	+	+	-	W	+	+
Tartrate	-	-	W	+	-	-	-	+	-	-	-	-

NA: not available; +: positive or present; -: negative or absent; W: weakly positive

8.3.1.4 Chemotaxonomy studies

The Rf of DAP in methanol solvent is 0.26 and 0.34 for *meso*-DAP and LL-DAP, respectively (Harper and Davis, 1979). The cell wall of LuP30 has LL-DAP which is a typical DAP of *Streptomyces* as the Rf of diaminopimelic acid extract was 0.337 (Figure 8.3). The major menaquinones of LuP30 were MK-9 (H6) and MK-9 (H8). LuP30 and *Streptomyces rishiriensis* had the same sugar cell wall compositions which were galactose and xylose (Figure 8.3)

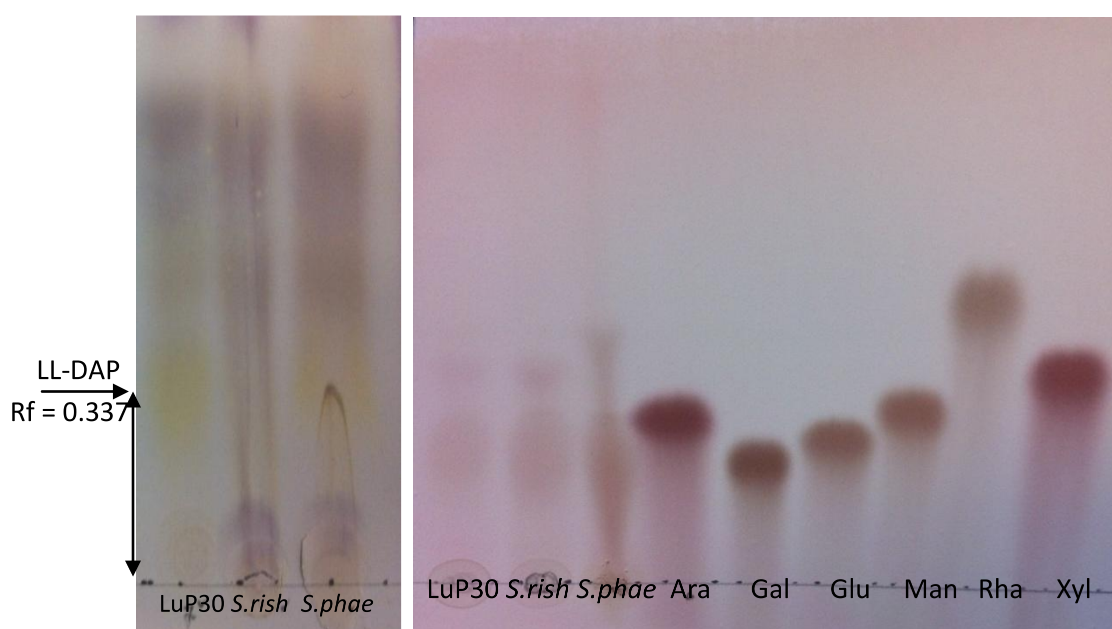


Figure 8.3 LL-DAP extraction and sugar cell wall of LuP30 and two closest type strains on TLC plates. *S.rish* (*Streptomyces rishiriensis*), *S.phae* (*Streptomyces phaeofaciens*), Ara (Arabinose), Gal (Galactose), Glu (Glucose), Man (Mannose), Rha (Rhamnose) and Xyl (Xylose).

8.3.1.5 Genomic characteristics

A complete 1372 bp nucleotide long of 16S rRNA gene sequence of LuP30 was aligned and compared with the 16S rRNA sequences of other *Streptomyces* members group and *Micromonospora sagamiensis* as the out group. Strain LuP30 (accession

no. for 16S rRNA gene: KP973994) has *Streptomyces rishiriensis* as its closest neighbouring species (99.9% sequence similarity), *Streptomyces humidus* (99.2% similarity) and *Streptomyces phaeofaciens* (99.1% sequence similarity). However, in the evolution of phylogenetic trees, the two closest type cultures of LuP30 are *Streptomyces rishiriensis* and *Streptomyces phaeofaciens* (Figure 8.4A and 8.4B). The G+C content of strain LuP30 is 71.6 mol% and the genome size is 9189785 bp. DNA-DNA hybridisation value of LuP30 with *S. rishiriensis* is 32.4% whereas its value with *S.phaeofaciens* is 23.9%. Based on the full genome sequence of LuP30 there are no *nec1* and thaxtomin biosynthesis genes in its genome.

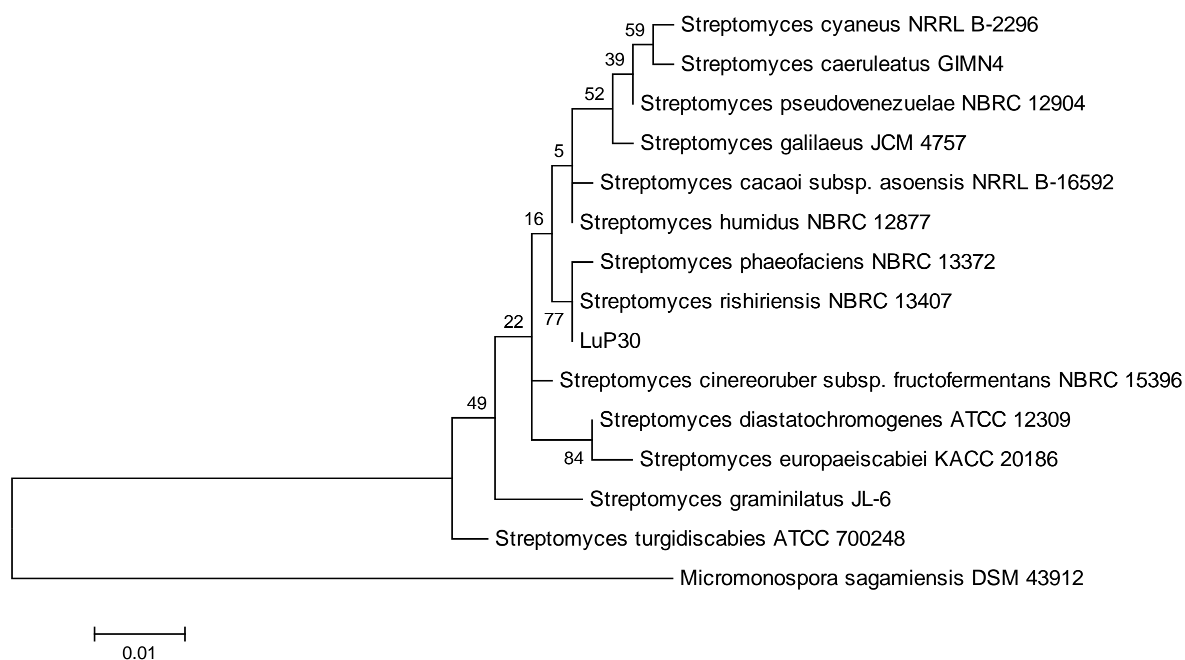


Figure 8.4A Molecular Phylogenetic analysis by maximum likelihood method tree based on the 16S rRNA gene sequence of *Streptomyces* LuP30 and valid neighbouring species. The evolutionary distances were computed using the Kimura 2-parameter. The analysis involved 14 nucleotide sequences and *Micromonospora sagamiensis* was included as an out group. Each number on the dendrogram is the percentage of the occurrence in 1000 bootstrap replications. The bar represents 0.01 substitutions per nucleotide position. Evolutionary analyses were conducted in MEGA6.

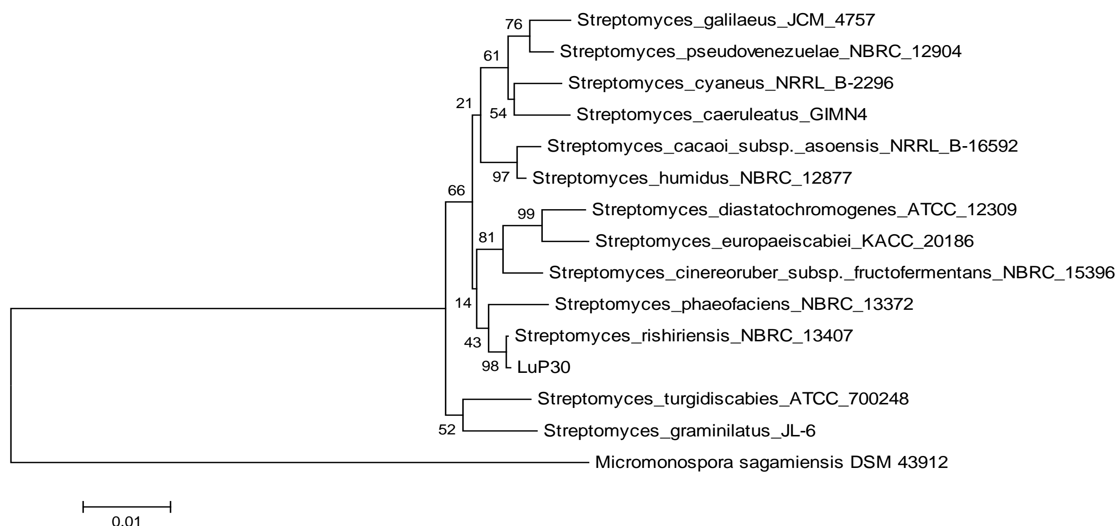


Figure 8.4B A neighbour-joining tree based on the 16S rRNA gene sequence of *Streptomyces* LuP30 and valid neighbouring species. The evolutionary distances were computed using the Kimura 2-parameter. The analysis involved 14 nucleotide sequences and *Micromonospora sagamiensis* was included as an out group. Each number on the dendrogram is the percentage of the occurrence in 1000 bootstrap replications. The bar represents 0.01 substitutions per nucleotide position. Evolutionary analyses were conducted in MEGA6.

Based on these studies, strain LuP30^T is differentiated from current *Streptomyces* type strains. Therefore, LuP30 is proposed as a new species of the *Streptomyces* genus and its name is *Streptomyces lucernesis* LuP30 sp. nov..

8.3.2 Streptomyces strain LuP47B

8.3.2.1 Morphological characteristics

Streptomyces sp. LuP47B grew well on different media such as ISP2, ISP3, ISP4, ISP5, ISP7, MS and HPDA. There were obvious differences in terms of morphology, substrate mycelium and pigment produced from LuP47B and two closest type cultures *S. ciscaucasicus* and *S. canus* (Figure 8.5). The morphological characteristics of this strain are displayed in Table 8.3. Under scanning microscopy, the spores of LuP47B are spiny and the spore size is about 0.8µm wide and 0.9-1.0µm long (Figure 8.6).

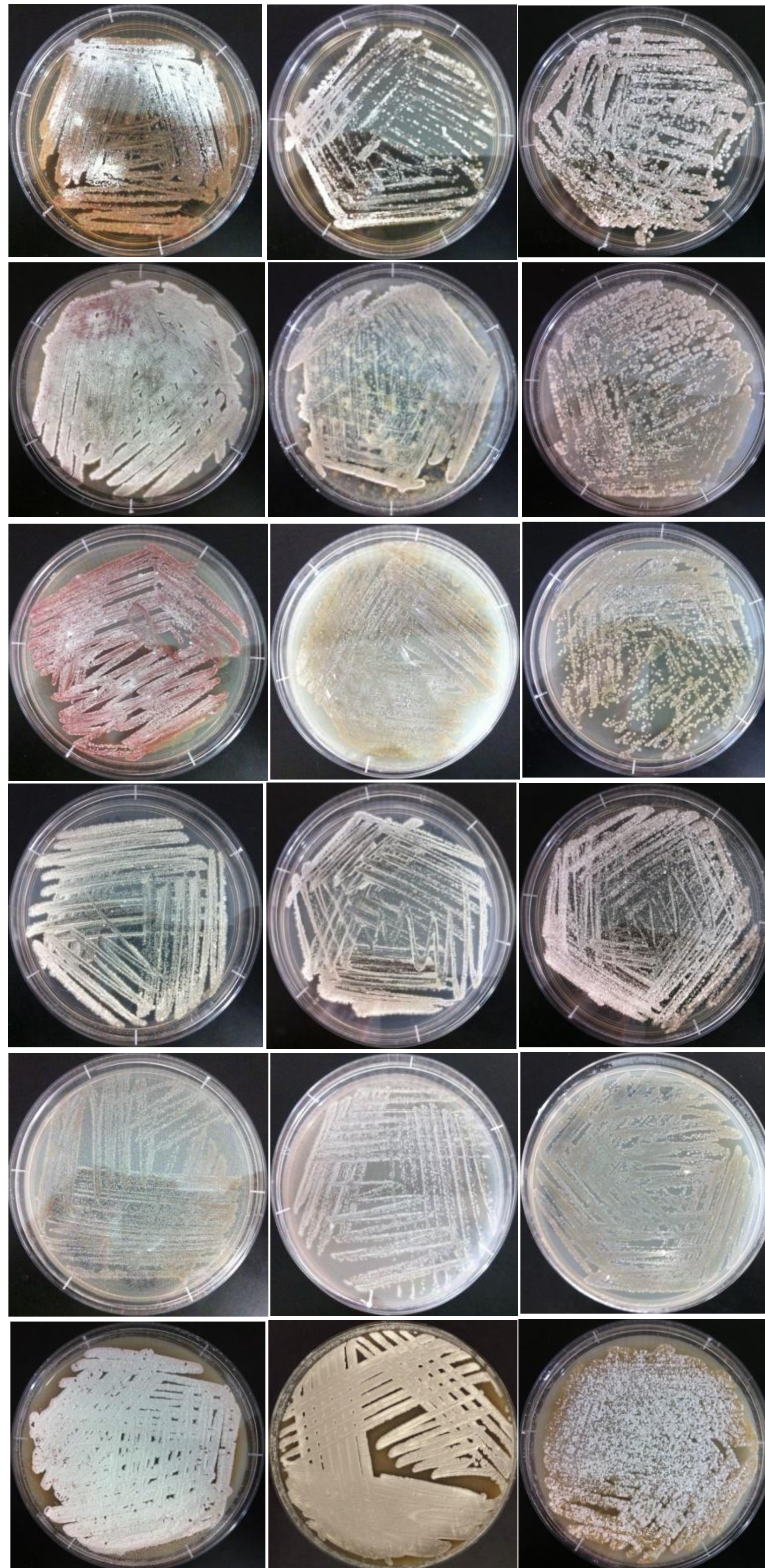


Figure 8.5 Morphological comparison of *Streptomyces* LuP30 and two closest type cultures. From left to right: *S. ciscausicacus*, LuP30 and *S. canus* grown on ISP2, ISP3, ISP4, ISP5, ISP7 and MS from top to bottom at 14 days after incubation at 27°C.

Table 8.3 Cultural characteristics of LuP47B on different media after 14 days incubation at 27°C

Medium	Growth	Aerial mycelium	Substrate mycelium
ISP2	Good	Light grey	Hair brown
ISP3	Good	Pale grey	Birch grey
ISP4	Good	Brown	Greyish brown
ISP5	Good	Pastel grey	Orange grey
ISP7	Good	Orange grey	Greyish orange
MS	Good	Dust brownish grey	Dark blonde
HPDA	Good	Grey black	Black
Nutrient agar	Moderate	Light brown	Light brown
Bennett's agar	Moderate	Light brown	Light cinnamon
Glycerol asparagine yeast extract agar	Moderate	Light brown	Light brown

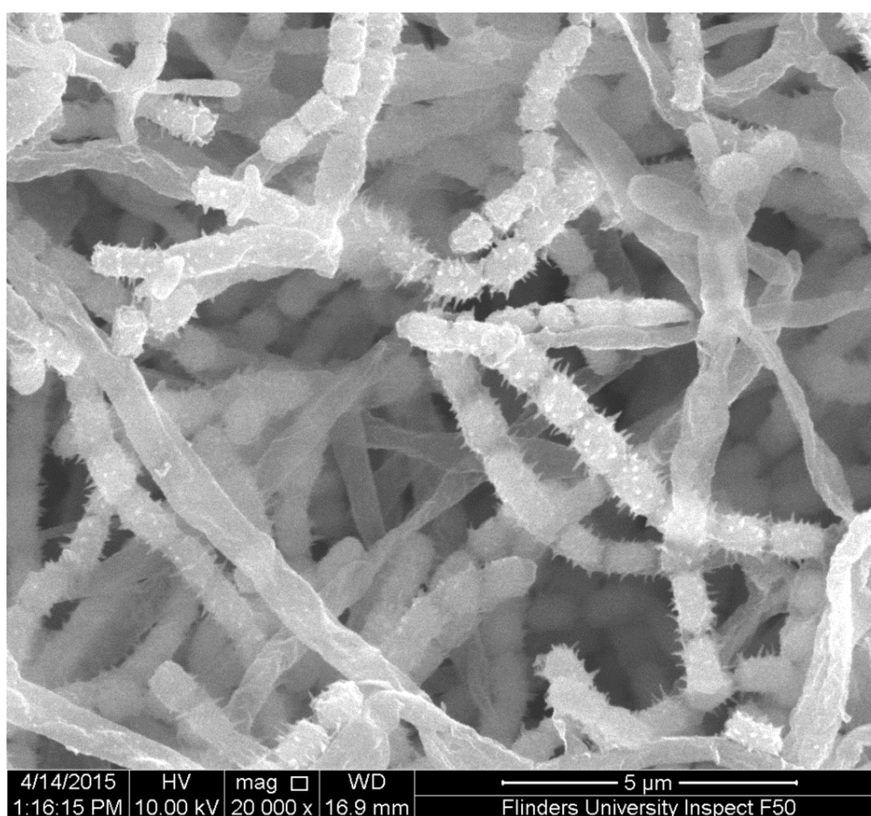


Figure 8.6 Scanning electron microscope of aerial mycelia and spore chains of *Streptomyces* LuP47B after incubation at 27°C on HPDA for 14 days; scale bar 5μm

8.3.2.2 Physiological and biochemical characteristics

LuP47B and *S. canus* grew at a range of pH tested from pH 4.5 to pH 10 while *S. ciscaucasicus* did not grow at pH 4.5. LuP47B could grow on 6% NaCl medium while *S. ciscaucasicus* was the most tolerant to sodium chloride where it could grow on 7% NaCl medium. LuP47B grew well at temperatures from 15°C to 37°C but it could not grow at 45°C.

Table 8.4 Physiological and biochemical characteristics of LuP47B in comparison with two closest type cultures *S.ciscaucasicus* and *S.canus*

Component	Growth of isolates under the following conditions (14 days)		
	LuP47B	***S. ciscaucasicus***	***S. canus***
pH			
4	-	-	-
4.5	+	+	-
7	+++	+++	+++
9	++	++	++
10	+	+	+
Sodium chloride concentration (%)			
2	+++	+++	+++
5	++	+++	++
6	++	++	+
7	-	+	-
10	-	-	-
15	-	-	-
50	-	-	-
Temperature (^{o}C)			
15	++	++	+
27	++	+++	+++
37	++	+++	++
45	-	-	-
55	-	-	-

- : no growth
+ : week/poor growth
++ : moderate growth
+++ : strong growth

Table 8.4 (***Continued)*** Physiological and biochemical characteristics of LuP47B in comparison with two closest type cultures *S.ciscaucasicus* and *S.canus*

Components	**Utilization after 14 days**		
	LuP47B	***S. ciscaucasicus***	***S. canus***
Decomposition of			
Adenine	++	++	+++
Hypoxanthine	NA	NA	NA
L-tyrosine	-	-	-
Xanthine	++	-	++
Casein	++	+	++
Urea	+	+	+
Hydrolysis of			
Esculin	+	+	+
Starch	++	+	++
Gelatin	++	++	+++
Catalase production	+	+	w
Carbohydrate			
Adonitol	-	-	-
Arabinose	+	+	+
Cellobiose	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Maltose	+	+	+
Mannitol	+	+	+
Mannose	+	+	+
Methyl	w	W	W
Sorbitol	-	+	-
Sucrose	+	+	+
Telibose	W	W	+
Xylose	+	+	+

Utilization of organic acids	3 d	7d	10d	14d	3 d	7d	10d	14d	3 d	7d	10d	14d
Acetate	-	-	w	+	-	W	+	+	-	+	+	+
Benzoate	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+	+	+	+
Propionate	-	+	+	+	-	W	+	+	-	+	+	+
Tartrate	+	+	+	+	-	-	-	-	-	-	-	-

NA: not available; +: positive or present; -: negative or absent; W: weakly positive

LuP47B and *S. canus* were positive for decomposition of adenine, xanthine, casein and urea while *S. ciscaucasicus* did not compose xanthine. In contrast, LuP47B and *S. ciscaucasicus* were able to hydrolyse esculin, starch and gelatine and were

positive on catalase production while *S. canus* was a very weak producer of catalase. LuP47B utilised acetate, citrate, propionate and tartrate while both *S. ciscaucasicus* and *S. canus* could not use tartrate and none of the actinobacteria could use benzoate. In addition, LuP47B was also able to use different carbohydrate sources such as arabinose, cellobiose, fructose, galactose, glucose, maltose, mannitol, mannose, methyl, sucrose, telibose and xylose. None of the actinobacteria used adonitol and sorbitol except for *S. ciscaucasicus* which could utilise sorbitol.

8.3.2.3 Chemotaxonomy studies

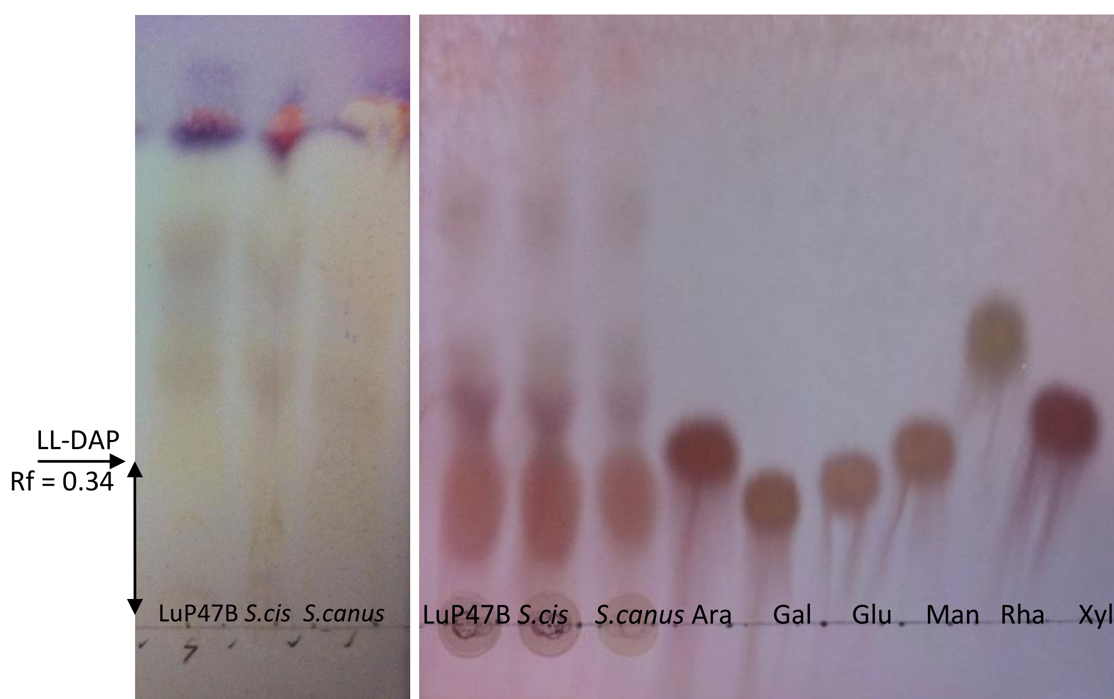


Figure 8.7 LL-DAP extraction and sugar cell wall of LuP47B and two closest type strains on TLC plates. *S.cis* (*Streptomyces ciscaucasicus*), *S.canus* (*Streptomyces canus*), Ara (Arabinose), Gal (Galactose), Glu (Glucose), Man (Mannose), Rha (Rhamnose) and Xyl (Xylose).

The Rf of diaminopimelic acid (DAP) in methanol solvent is 0.26 and 0.34 for *meso*-DAP and LL-DAP, respectively (Harper and Davis, 1979). The cell wall of LuP47B has LL-DAP which is a typical DAP of *Streptomyces* as the Rf of (DAP) extract was 0.34. The major menaquinone of LuP47B was MK-9 (H8). LuP47B, *S. ciscausicacus* and *S. canus* had the same sugar cell wall compositions which were galactose, rhamnose, xylose and an unidentified (Figure 8.7).

8.3.2.4 Genomic characteristics

A complete 1182 bp nucleotide long 16S rRNA gene sequence of LuP47B was aligned and compared with the 16S rRNA sequences of other *Streptomyces* species with *Micromonospora sagamiensis* as the out group. In the evolution of phylogenetic trees the two closest type cultures of LuP47B (Genbank Accession no. for 16S rRNA gene: KP973995) are closest to *Streptomyces ciscaucasicus* (99.49%), and *Streptomyces canus* (99.49%) based on sequence similarity (Figure 8.8A and 8.8B). The genome size of LuP47B is 11004536 bp with 70.2 mol% G+C content. DNA-DNA hybridisation value of LuP47B with *S.ciscaucasicus* is 23.2% while its value with *S.canus* is 29.3%. Based on the full sequence of LuP47B, there are no *nec1* and thaxtomin biosynthesis genes in its genome.

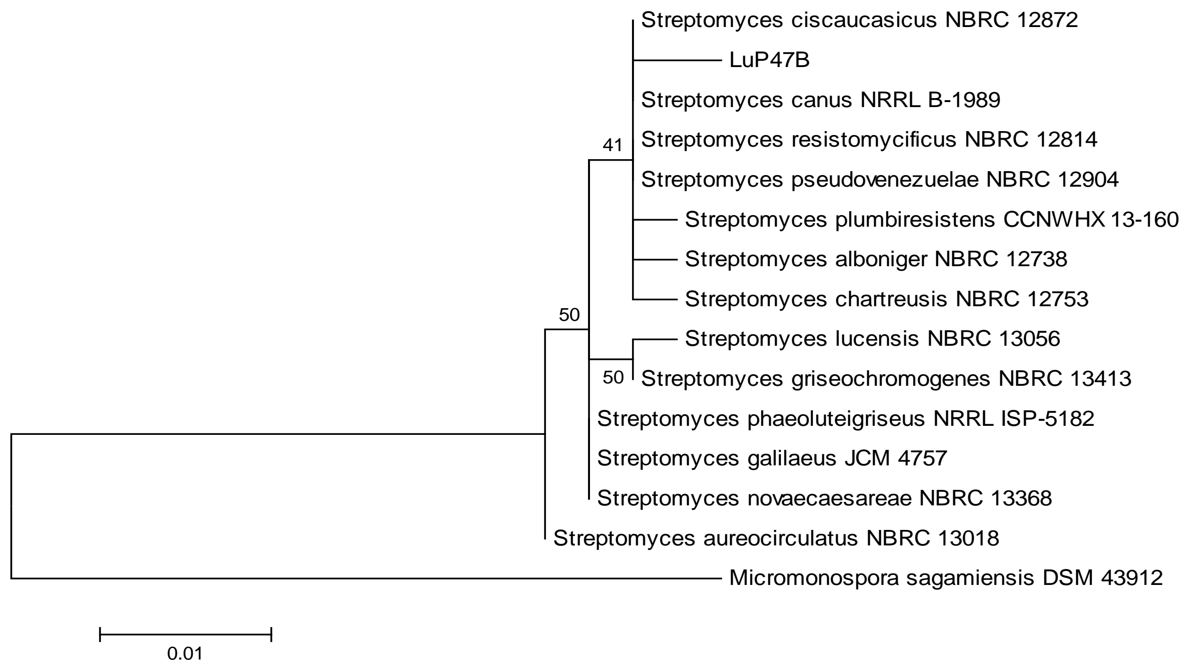


Fig 8.8A Molecular Phylogenetic analysis by maximum likelihood method based on the 16S rRNA gene sequence of *Streptomyces* LuP47B and the valid species. The evolutionary distances were computed using the Kimura 2-parameter. The analysis involved 14 nucleotide sequences and *Micromonospora sagamiensis* was included as an out group. Each number on the dendrogram is the percentage of the occurrence in 1000 bootstrap replications. The bar represents 0.01 substitutions per nucleotide position. Evolutionary analyses were conducted in MEGA6.

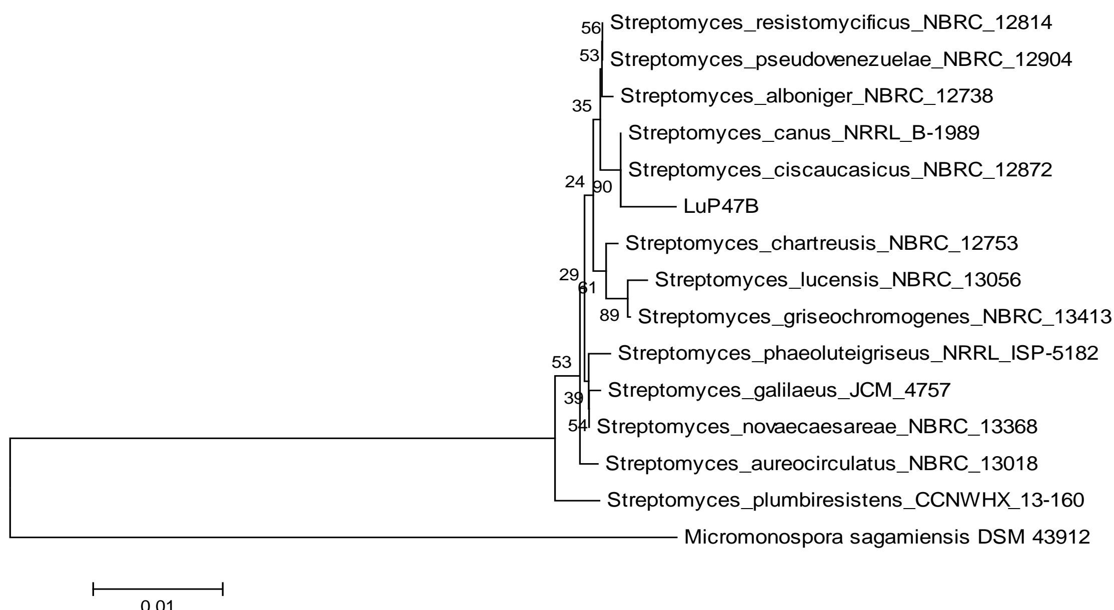


Figure 8.8B A neighbour-joining tree based on the 16S rRNA gene sequence of *Streptomyces* LuP47B and the valid species. The evolutionary distances were computed using the Kimura 2-parameter. The analysis involved 14 nucleotide sequences and *Micromonospora sagamiensis* was included as an out group. Each number on the dendrogram is the percentage of the occurrence in 1000 bootstrap replications. The bar represents 0.01 substitutions per nucleotide position. Evolutionary analyses were conducted in MEGA6.

Based on these studies, strain LuP47B^T is differentiated from current *Streptomyces* type strains. Therefore, LuP47B is proposed as a new specie of the *Streptomyces* genus and its name is *Streptomyces medicagensis* LuP47B sp. nov..

8.3.3 Growth of actinobacteria on nitrogen free media

LuP30 and LuP47B were able to grow on N free medium and Jensen's medium which is typical medium for testing the ability to live on a medium without N for bacteria. They displayed the growth from moderate to good compared to type cultures which also had visible differences in colour of spores (Table 8.5).

Table 8.5 Growth of LuP30 and LuP47B and closest type cultures on nitrogen free and Jensen's medium

Cultures	N free medium		Jensen's medium	
	Growth	Description	Growth	Description
LuP30	Moderate	White spores	Good	Brown spores
S. rishiriensis	Weak	Grey-black spores	Good	Grey spores
S. phaeofaciens	Moderate	Less grey spores,	Weak	Grey spores
LuP47B	Moderate	White grey spores	Good	White-grey spores
S. ciscaucasicus	Moderate	White pink spores	Good	Grey-pink spores
S. canus	Weak	No pigment, no spores	Good	White-grey spores

8.4 Conclusion

The morphological, physiological, biochemical, chemotaxonomy and genomic characteristics of strain *Streptomyces* LuP30 and LuP47B demonstrate that these two endophytic actinobacteria are new species of the *Streptomyces* genus. Both LuP30 and LuP47B were isolated from surface sterilised roots of lucerne (*Medicago sativa*

L.) grown in South Australia and are designated as *Streptomyces lucernesis* LuP30 sp. nov. and *Streptomyces medicagensis* LuP47B sp. nov..

Chapter 9

Major findings and future directions

9.1 Major findings

The hypothesis is supported. The results showed that six endophytic actinobacteria act as biocontrol agents against a fungal pathogen, *R. solani*, and two of the six actinobacteria, LuP30 and LuP47B, also increased the plant growth and nitrogen fixation of lucerne with its rhizobial partner.

9.1.1 Endophytic actinobacteria from legumes: genus, temperature, isolation media

Endophytic actinobacteria were recovered from lucerne, pea, sub-clover and medic plants collected from fields in South Australia. *Streptomyces* comprised the main actinobacterial genus that was isolated from the four legume species. Incubating the isolation plates at 27°C as well as 37°C resulted in a doubling of the number of strains isolated. Amongst the four isolation media used, HV was the best for the recovery of endophytic actinobacteria from legume roots and nodules with more than half of the 225 isolates recovered on this medium. TWYE medium was the second best, yielding approximately one third of isolates.

9.1.2 Effects of endophytic actinobacteria on the plant growth and symbiotic function of lucerne

Thirty three percent of isolates tested improved germination of lucerne seeds on agar and about fifteen percent enhanced the germination and root length when applied prior to inoculation with *S. meliloti* strain RRI 128. These results indicate that some endophytic actinobacteria have the potential to improve plant growth alone or with the presence of the rhizobial partner of lucerne. Two endophytic actinobacteria LuP30 and LuP47B increased the plant growth and symbiotic function of *S. meliloti*

RRI 128 and lucerne at different rates of NH_4NO_3 in soil. Co-inoculation of LuP30 with *S. meliloti* RRI 128 increased plant growth, number of nodules and nodule mass per plant at three rates of NH_4NO_3 (3, 25 and 50 mg/kg soil) while LuP47B only increased the plant growth and nodulation at 3 and 25 mg/kg NH_4NO_3. The increases were greatest when the soil N was increased to 25 mg NH_4NO_3/kg soil indicating their use would be best targeted to encourage early plant vigour and aid pasture establishment in soils with low/moderate N levels. The actinobacteria mostly improved the nodulation during the first 4 weeks, indicating the action of the actinobacteria might involve in the regulation of nodulation. The ^{15}N experiment indicated that the increases of nitrogen in plants co-inoculated with LuP30 or LuP47B and the rhizobial partner were due to improved N fixation rather than uptake of N from soil.

LuP30 and LuP47B did not inhibit and sometimes stimulated the growth of several strains of rhizobia belonging to the genera *Sinorhizobium*, *Rhizobium* and *Bradyrhizobium in vitro*. This result showed that these specific strains of actinobacteria are potential candidates for co-inoculants because they are unlikely to interfere with the growth of rhizobial partners.

The potential benefits of Lup30 and LuP47B extend beyond lucerne nodulated by RRI128. These actinobacteria also increased plant growth of lucerne when co-inoculated with a naturalised *Rhizobium* in soil collected from Urrbrae High School, Adelaide. LuP30 and LuP47B also improved the growth and nodulation with another legume symbiosis (sub-clover with *R. trifolii* WSM 1325) at 25 mg soil NH_4NO_3. The effects of actinobacteria were changed by the variations in the levels of N and *Rhizobium* concentrations in soils, rhizobium partners and host legumes.

9.1.3 Antifungal and bio-control properties of actinobacterial endophytes

The endophytic actinobacteria showed antifungal activity against the fungal pathogens *Rhizoctonia solani* AG8 and *Pythium irregulare*. Forty two and seventeen percent of 148 isolates showed ability to against the fungal pathogens respectively. Six isolates (LuP10, LuP30, LuP44, LuP46B, LuP47B and LuP73B) of the 21 tested (chosen from 47 positive isolates in tubes) increased the total weight of plants that had been inoculated with both rhizobia and the root pathogen *R. solani*. Two isolates (LuP30 and LuP47B) that increased total plant weight have been shown elsewhere to benefit the lucerne nitrogen fixation symbiosis. All six isolates showing bio-control activity on lucerne were isolated from lucerne roots (86 isolates). These results indicate that a significant percentage of endophytic actinobacteria potentially play important roles in protection the host plants against the fungal pathogens.

9.1.4 Novel species

Based on 16S rRNA sequence similarity data and the results of the other polyphasic taxonomy tests both LuP30 and LuP47B are proposed as novel species of *Streptomyces* and were given the names *Streptomyces lucernesis* LuP30 sp. nov. and *Streptomyces medicagensis* LuP47B sp. nov.

9.2 Future directions

9.2.1. Understanding the roles of LuP30 and LuP47B involved in nodulation and nitrogen fixation processes

LuP30 and LuP47B improved the growth and symbiotic function of *S. meliloti* RRI 128 and lucerne. The genomes of LuP30 and LuP47B have been sequenced to provide the opportunity to further understand the gene and protein expressions involved in nodulation and nitrogen fixation of rhizobial partner and lucerne that lead to increased nitrogen fixation. Although a significant data detailing the early affects of the actinobacteria on the nodulation of lucerne has been collected, understanding the pathways/metabolites involved is still need to be determined and clarified.

9.2.2 Mechanism of LuP30 and LuP47B in biocontrol activity

In this study, there was no correlation between antifungal activity measured *in vitro* and biocontrol activity detected using *in planta* assays. All eight isolates which showed medium to strong antifungal activity against both *R. solani* AG8 and *P. irregulare* did not significantly reduce the root damage caused by *R. solani in planta.* The actinobacteria may induce the plant defence system to protect the damage by fungal pathogens or the actinobacteria produce antifungal compounds after colonising the roots of lucerne. The mechanisms and pathways for biocontrol should be understood to save time and efforts due to screening large number of isolates. The 6 isolates showing bio-control activity provide the opportunity to study the mechanism/s of bio-control.

9.2.3 Efficacy of LuP30 and LuP47B on other symbioses and field trials

These actinobacteria stimulated the growth of rhizobial strains *in vitro*. It would be worthwhile to identify the pathways or compounds involved. In these plant experiments, the level of actinobacterial spores added as inoculant was high, but in order to improve the economics, it would be necessary to reduce the number of spores applied on seeds while still maintaining the benefits to the plant. *Streptomyces* are generally considered stable inoculants because they sporulate well and produce robust spores However, opportunity remains to optimise the spore production during manufacture as well as refine application methods to maximise the survival of the spores on the surface of seeds over time. The efficacy of the actinobacteria should be examined in different field soils where the micro-flora are complex and contain complex communities of naturalised rhizobia and often fungal pathogens. Therefore, the characterisation of other micro-organisms including fungal pathogens in the field soils may help with the interpration the symbiotic and bio-control effects.

9.3 Publications (Conferences and Journals)

A poster at 16th Australian Nitrogen Conference in Sydney, Australia.

Le H.X., Franco C.M.M., Ballard A.R. and Drew A.L. (2012) Endophytic actinobacteria from legumes: their antifungal activity and effects on plant growth. The 16th Australian Nitrogen Conference, Sydney, Australia, 24th – 27th June 2012.

Travel scholarship: one of the six recipients of Alan H. Gibson travelling scholarships.

Oral at Actinobacteria within soils in Munster, Germany.

Le HX, Franco CMM and Lacey E (2012) Are metabolites produced by endophytic actinobacteria influenced by their plant source. “Actinobacteria with soils: Capacities for mutualism, symbiosis and pathogenesis”, Munster, Germany, 25th – 28th October 2012.

Travel scholarship: FEMS Young Scientist travel scholarship.

Oral at 17th Australian Nitrogen Conference in Adelaide, Australia.

Le HX, Franco CMM and Ballard AR (2014) Effects of endophytic actinobacteria on the lucerne symbiosis at different levels of nitrogen. The 17th Australian Nitrogen Conference, Adelaide, Australia, 28th September – 2nd October 2014.

Proceeding of conference papers:

Le HX, Franco CMM and Ballard AR Effects of endophytic actinobacteria on the lucerne symbiosis at different levels of nitrogen. In: Gupta VVSR, Unkovich M, Kaiser BN (eds) The 17th Australian Nitrogen Fixation Conference, Adelaide, Australia, 2014. pp 66-67.

Le HX, Franco CMM, Ballard AR Isolation and characterisation of endophytic actinobacteria and their effect on the early growth and nodulation of lucerne (*Medicago sativa* L.). In: Gupta VVSR, Unkovich M, Kaiser BN (eds) The 17th Australian Nitrogen Fixation Conference, Adelaide, Australia, 2014. pp 134-135.

Le HX, Franco CMM and Ballard AR Selected nitrogen fixation–enhancing endophytic actinobacteria which control soil-borne diseases of lucerne (*Medicago*

sativa L.). In: Gupta VVSR, Unkovich M, Kaiser BN (eds) The 17th Australian Nitrogen Fixation Conference, Adelaide, Australia, 2014. pp 138-139.

Posters: 17th International Symposium on the Biology of Actinomycetes, 8th Oct. 2014-12th Oct. 2014, Kasudasi, Turkey.

Le HX, Franco CMM and Ballard AR Endophytic actinobacteria that enhance and control fungal pathogens in Lucerne (*Medicago sativa* L.).

Le HX, Franco CMM and Ballard AR Effects of endophytic *Streptomyces* on the lucerne (*Medicago sativa* L.) symbiosis at different levels of nitrogen.

Submitted papers:

Le HX, Franco CMM Ballard AR and Drew AL Isolation and characterisation of endophytic actinobacteria and their effect on the early growth and nodulation of lucerne (*Medicago sativa* L.) to special issue "Soil, Plants and Endophytes" in Plant and Soil Journal. (accepted pending minor corrections).

Le HX, Franco CMM and Ballard AR Effects of endophytic *Streptomyces* and mineral nitrogen on lucerne (*Medicago sativa* L.) growth and its symbiosis with rhizobia to special issue "Soil, Plants and Endophytes" in Plant and Soil Journal (Under review).

Australian patent

Franco CMM, Le HX and Ballard RA (20/6/2014) Inoculants and methods for their use thereof, Australian Patent Application 2014902374.

Appendices

Appendix 1: Media

1. **MS (Mannitol Soya)**

Mannitol	20 g
Soya flour	20 g
Agar	20 g
R.O water	1,000 ml

2. **CMA (Corn Meal Agar)**

Corn meal agar premix	17 g
R.O water	1,000 ml

3. **PDA (Potato Dextrose Agar)**

PDA (Oxoid)	39 g
R.O water	1,000 ml
Adjust pH 7.2	

4. **HPDA (Half strength Potato Dextrose Agar)**

PDA (Oxoid)	19.5 g
Agar	8 g
R.O water	1,000 ml
Adjust pH 7.2	

5. **Yeast extract-malt extract media (ISP2)**

Malt extract	10 g
Yeast extract	4 g
Glucose	4 g
Agar	18 g
R.O. water	1,000 ml
Adjust pH 7.2	

6. **Oat agar media (ISP3)**

Oatmeal	20 g
Trace salt solution (added after autoclaving)	1 ml
Agar	18 g
R.O. water	1,000 ml
Adjust pH 7.2	

Trace salt solution per 100 ml RO water (filter sterilised)

$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g

7. Inorganic salt starch agar (ISP4)

Soluble starch	10 g
$CaCO_3$	2 g
$(NH4)_2SO_4$	2 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	1 g
NaCl	1 g
$FeSO_4.7H_2O$	1 mg
$MnCl_2.7H_2O$	1 mg
$ZnSO_4.7H_2O$	1 mg
Agar	20 g
R.O. water	1,000 ml
Adjust pH 7.2	

8. Glycerol asparagine agar (ISP5)

Glycerol	10 g
L-asparagine	1 g
K_2HPO_4	1 g
Trace salt solution	1 ml
R.O. water	1,000 ml
Adjust pH 7.2	

Trace salt solution per 100 ml RO water (filter sterilised)

$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g

9. Tyrosine Agar (ISP7)

Glycerol	15 g
L-tyrosine	0.5 g
L-asparagine	1 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
NaCl	0.5 g
$FeSO_4.7H_2O$	0.5 g
Trace element solution HO-LE	0.01 ml
Agar	20 g
R.O. water	1,000 ml
Adjust pH to 7.3±0.2	

Trace element solution HO-LE (filter sterilised)

H_3BO_3	2.85 g
$MnCl_2.4H_2O$	1.8 g
	1.77 g
Sodium tartrate	
$FeSO_4.7H_2O$	1.36 g
$CoCl_2.6H_2O$	0.04 g
$CuCl_2.2H_2O$	0.027 g
$Na_2MoO_4.2H_2O$	0.025 g

$ZnCl_2$	0.020 g
R.O. water	1,000 ml

10. Bennett's medium

Glucose	10 g
Pancreatic digest of casein	2 g
Yeast extract	1 g
Beef extract	1 g
Agar	15 g
R.O. water	1,000 ml
Adjust pH to 7.3±0.2	

11. Nutrient agar

Peptone	5 g
NaCl	5 g
Yeast extract	2 g
Beef extract	1 g
Agar	15 g
R.O. water	1,000 ml
Adjust pH 7.2	

12. Glycerol asparagines yeast extract agar

Yeast extract	10 g
Glycerol	10 g
L-asparagine	1 g
K_2HPO_4	1 g
Trace salt solution	1 ml
Agar	20 g
RO water	1,000 ml
pH 7.4 ± 0.2	

Trace salt solution per 100 ml RO water (filter sterilised)

$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g

13. Humic acid Vitamin B Agar:

Humic acid	1 g
Na_2HPO_4	0.25 g
KCl	0.85 g
$MgSO_4.7H_2O$	0.025 g
$FeSO_4.7H_2O$	0.05 g
$CaCO_3$	0.01 g
Agar	18 g
Vitamin B 100x (added after media autoclaved)	1ml
RO water	1,000 ml

Prepare NaOH 0.2N then 1g of humic acid was dissolved in 10ml of NaOH 0.2N

Vitamin B (100x) per 100 ml RO water

Thiamine-hydrochloride	5 mg
Riboflavin	5 mg
Niacin	5 mg
Pyridoxine-hydrochloride	5 mg
Inositol	5 mg
Ca-panthotenate	5 mg
p-aminobenzoic acid	25 mg
Biotin	25 mg

Adjust pH to 4.5 and filter sterilised

14. Tryptic Soya Agar (TSA)

Tryptic Soy Broth (Oxoid)	17 g
Agar	18 g
RO water	1,000 ml

15. YMA (Yeast Mannitol Agar)

Yeast extract	0.5 g
Mannitol	5 g
Sodium glutamate	0.5g
Solution S	10 ml
Solution T	10 ml
Solution U	1 ml
Solution V	1 ml
Agar	20 g
R.O. water	960 ml

*** Solution S:**

Na_2HPO_4	1.33 g
RO water	100 ml

*** Solution T:**

$MgSO_4.7H_2O$	0.45 g
RO water	100 ml

*** Solution U:**

$CaCl_2.2H_2O$	0.53 g
RO water	10 ml

*** Solution V:**

$FeCl_3$	0.04 g
RO water	10 1

16. YECD (Yeast Extract Casamino D-glucose)

Yeast extract	0.3 g
Casamino acids	0.3 g
D-Glucose	0.3 g
K_2HPO_4	2.0 g
Agar	18.0 g
RO water	1,000 ml
Adjust pH 7.2	

17. TWYE (Tap Water Yeast Extract)

Yeast extract	0.25 g
K_2HPO_4	0.5 g
Agar	18.0 g
RO water	1,000 ml
Adjust pH 7.2	

18. N free medium

Glucose	5 g
$CaCl_2.2H_2O$	10 mg
$MgSO_4.7H_2O$	100 mg
K_2HPO_4	500 mg
$CaCO_3$	2.5 mg
$FeSO_4.7H_2O$	25 mg
$NaMoO_4$. $2H_2O$	0.5 mg
Agar	7.5 g
R.O. water	500 ml
Adjust pH 7.3±0.2	

19. Jensen's medium

Sucrose	10 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.25 g
NaCl	0.25 g
$FeSO_4.7H_2O$	0.05 g
$NaMoO_4$. $2H_2O$	0.0025 g
$CaCO_3$	1 g
Agar	7.5 g
RO water	500 ml

Appendix 2. General characterisation of 225 endophytic actinobacteria from four legumes

No.	Culture name	Part of plant	Temperature		Isolation medium	Morphology on medium			Proposed genus
			27°C	37°C		MS	HPDA	ISP3	
1	LuP1	Lucerne root		x	HV	Brown	White brown	Brown grey	Streptommycete
2	LuP2	Lucerne root		x	TWYE	Brown	White	Light brown	Streptommycete
3	LuP3	Lucerne root		x	TWYE	Greyish ruby	Greyish rose	Reddish grey	Streptommycete
4	LuP4	Lucerne root		x	TYWE	White grey	Brown grey	Grey	Streptommycete
5	LuP5	Lucerne root		x	TWYE	Dark brown	Brownish grey	Light brown	Streptommycete
6	LuP6	Lucerne root		x	HV	White grey	Yellowish brown	Grey	Streptommycete
7	LuP7	Lucerne root		x	HV	Grey	Yellow	White	Streptommycete
8	LuP8	Lucerne root	x		HV	Grey	Light grey	Grey	Streptommycete
9	LuP9	Lucerne root	x		HV	Bluish grey	Light grey	Light grey	Streptommycete
10	LuP10	Lucerne root	x		TWYE	Greyish ruby	Greyish rose	Grey	Streptommycete
11	LuP11	Lucerne root	x		HV	White grey	Bluish grey	grey	Micromonospora
12	LuP12A	Lucerne root	x		HV	Bluish grey	Bluish grey	Light grey	Micromonospora
13	LuP13	Lucerne root		x	TWYE	Light brown	Light yellow	Light grey	Streptommycete
14	LuP14	Lucerne root	x		HV	White	White brown	White	Streptommycete
15	LuP15	Lucerne root	x		TWYE	White brown	Yellow brown	White	Micromonospora
16	LuP16	Lucerne root	x		TWYE	Black	Grey black	Black pink	Streptommycete
17	LuP17	Lucerne root	x		TWYE	Grey black	Grey	Black	Streptommycete
18	LuP18	Lucerne root	x		HV	White	White brown	White brown	Streptommycete
19	LuP19	Lucerne root	x		HV	White pink	Black blue	Grey black	Streptommycete
20	LuP20	Lucerne root	x		HV	White	White	Light white	Streptommycete
21	LuP21	Lucerne root	x		HV	Grey black	Grey	Grey	Streptommycete
22	LuP22	Lucerne root	x		HV	White	Grey brown	Grey	Streptommycete
23	LuP23	Lucerne root	x		TWYE	Grey white	White	Grey black	Streptommycete
24	LuP24	Lucerne root	x		TWYE	White grey	White blue	Grey blue	Micromonospora
25	LuP25	Lucerne root	x		TWYE	Grey	White grey	Grey black	Streptommycete

No.	Culture name	Part of plant	Temperature		Isolation medium	Morphology on medium			Proposed genus
			27°C	37°C		MS	HPDA	Oatmeal	
26	LuP26	Lucerne root	x		TWYE	Grey	Grey	Light grey	Streptommycete
27	LuP27	Lucerne root	x		TWYE	White	White brown	Light grey	Streptommycete
28	LuP28	Lucerne root	x		TWYE	Grey blue	Grey dark	Grey	Streptommycete
29	LuP29	Lucerne root	x		TWYE	Blue green	Black brown	White green	Micromonospora
30	LuP30	Lucerne root	x		TWYE	Grey	White yellow	Grey	Streptommycete
31	LuP31	Lucerne root	x		HV	White green	White	White green	Streptommycete
32	LuP32	Lucerne root		x	HV	Light green	Grey	Blue dark	Streptommycete
33	LuP33	Lucerne root		x	TWYE	Grey dark	Dark grey	Grey black	Streptommycete
34	LuP34	Lucerne root		x	TWYE	White	White grey	Grey dark	Streptommycete
35	LuP35	Lucerne root		x	TWYE	White grey	White	White grey	Streptommycete
36	LuP36	Lucerne root		x	HV	White brown	Yellow brown	White	Streptommycete
37	LuP37	Lucerne root		x	HV	Grey brown	Light grey	White brown	Streptommycete
38	LuP38	Lucerne root		x	TWYE	Grey dark	Grey dark	Grey dark	Streptommycete
39	LuP39	Lucerne root		x	TWYE	Grey	Light grey	Grey	Streptommycete
40	LuP40	Lucerne root		x	HV	Pink	Pink	White pink	Microbispora
41	LuP41	Lucerne root		x	HV	White purple	Purple	White purple	Microbispora
42	LuP42	Lucerne root		x	HV	White	White brown	White grey	Streptommycete
43	LuP43	Lucerne root		x	HV	Light brown	White brown	White	Streptommycete
44	LuP44	Lucerne root		x	HV	Grey blue	Blue dark	Brown white	Micromonospora
45	LuP45	Lucerne root		x	TWYE	Grey dark	White	Grey white	Streptommycete
46	LuP46B	Lucerne root		x	TWYE	White green	Blue green	Blue green	Streptommycete
47	LuP47B	Lucerne root		x	TWYE	Grey brown	Grey dark	Grey dark	Streptommycete
48	LuP48	Lucerne root		x	HV	White brown	Light brown	White	Streptommycete
49	LuP49	Lucerne root		x	HV	White brown	Brown	White	Microbispora
50	LuP50	Lucerne root		x	TWYE	White	White	White grey	Streptommycete
51	Lup51	Lucerne root		x	TWYE	White	White brown	White	Streptommycete

No.	Culture name	Part of plant	Temperature		Isolation medium	Morphology on medium			Proposed genus
			27°C	37°C		MS	HPDA	Oatmeal	
52	LuP52	Lucerne root	x		TSA	White green	Light grey	Grey dark	Micromonospora
53	LuP53	Lucerne root	x		HV	Pink red	Pink	Pink red	Microbispora
54	LuP54	Lucerne root		x	HV	Purple orange	Purple	Purple orange	Microbispora
55	LuP55	Lucerne root	x		HV	Grey green	Grey	Grey black	Streptommycete
56	LuP56	Lucerne root	x		HV	Grey	Light grey	Grey	Streptommycete
57	LuP57	Lucerne root	x		HV	White red	Light orange	Orange	Microbispora
58	LuP58	Lucerne root	x		HV	Brown	White brown	White	Streptommycete
59	LuP59	Lucerne root	x		TWYE	Light brown	Brown	White	Streptommycete
60	LuP60	Lucerne root	x		HV	White brown	White	White grey	Streptommycete
61	LuP61	Lucerne root	x		HV	Grey green	Light green	White grey	Streptommycete
62	LuP62	Lucerne root	x		YECD	White green	White grey	White green	Streptommycete
63	LuP63	Lucerne root	x		TWYE	White grey	White grey	Grey	Streptommycete
64	LuP64	Lucerne root	x		HV	Grey green	Grey	Grey green	Streptommycete
65	LuP65	Lucerne root	x		TWYE	Grey	Grey black	Grey	Micromonospora
66	LuP66	Lucerne root		x	YECD	Brown	Brown yellow	White brown	Streptommycete
67	LuP67	Lucerne root		x	HV	Grey green	Grey green	Grey	Streptommycete
68	LuP68	Lucerne root		x	HV	Light pink	Grey	Grey dark	Streptommycete
69	LuP69	Lucerne root		x	HV	Grey white	White brown	Grey brown	Streptommycete
70	LuP70	Lucerne root		x	HV	Light pink	White pink	Pink	Microbispora
71	LuP71	Lucerne root		x	YECD	Pink	Pink red	Light red	Microbispora
72	LuP72	Lucerne root		x	YECD	Grey white	Light grey	Grey	Streptommycete
73	LuP73B	Lucerne root		x	HV	Grey brown	Grey	Grey	Streptommycete
74	LuP74	Lucerne root		x	YECD	Brown	White	White grey	Micromonospora
75	LuP75	Lucerne root		x	YECD	Brown	White green	Green	Not identified
76	LuP76	Lucerne root		x	YECD	White	Light grey	White	Micromonospora
77	LuP77	Lucerne root		x	YECD	Green	Light green	Grey green	Streptommycete

No.	Culture name	Part of plant	Temperature		Isolation medium	Morphology on medium			Proposed genus
			27°C	37°C		MS	HPDA	Oatmeal	
78	LuP78	Lucerne root	x		YECD	Grey green	White grey	Light green	Streptommycete
79	LuP79	Lucerne root	x		TWYE	Brown	White brown	Light green	Streptommycete
80	LuP80	Lucerne root		x	HV	Green dark	Light green	White grey	Streptommycete
81	LuP81	Lucerne root		x	YECD	Light brown	Grey	Grey brown	Streptommycete
82	LuP82	Lucerne root		x	HV	White	White green	Green	Streptommycete
83	LuP83	Lucerne root	x		HV	White grey	Grey	Light grey	Streptommycete
84	LuP84	Lucerne root		x	HV	Grey	White grey	Grey	Streptommycete
85	LuP85	Lucerne root		x	HV	Light pink	Grey brown	Grey pink	Microbispora
86	LuP86	Lucerne root		x	HV	White	White grey	Grey	Streptommycete
87	PL1	Pea root		x	TWYE	Brown	Light yellow	White brown	Microbispora
88	PL2	Pea root		x	TWYE	Pink red	Pink	Pink red	Microbispora
89	PL3	Pea root		x	TWYE	White pink	Pink	Pink	Microbispora
90	PL5	Pea root	x		YECD	White brown	Brown	Brown	Microbispora
91	PL6	Pea root	x		HV	Brown	Brown	White brown	Not identified
92	PL7	Pea root		x	TWYE	Brown	Brown	White	Streptommycete
93	PL8	Pea root		x	TWYE	Brown dark	Brown white	White	Streptommycete
94	PL9	Pea root		x	HV	Grey	Black dark	Brown dark	Streptommycete
95	PL10	Pea nodule		x	TWYE	Brown	Brown	White	Microbispora
96	PL11	Pea nodule		x	HV	White pink	White pink	Pink	Microbispora
97	PL12	Pea nodule		x	HV	White brown	White	White	Streptommycete
98	PL13	Pea nodule		x	TWYE	Brown	White brown	White	Microbispora
99	PL14	Pea nodule	x		HV	Pink	Red pink	White pink	Microbispora
100	PL15	Pea root		x	HV	Grey	Grey	Grey dark	Streptommycete
101	PL16	Pea nodule		x	HV	Brown	White	White	Not identified
102	PL17	Pea nodule		x	TWYE	Light grey	Grey black	Grey	Streptommycete
103	PL18	Pea nodule		x	HV	Brown	White	White	Not identified

No.	Culture name	Part of plant	Temperature		Isolation medium	Morphology on medium			Proposed genus
			27°C	37°C		MS	HPDA	Oatmeal	
104	PL19	Pea root	x		HV	Red pink	Light pink	Pink	Microbispora
105	PL20	Pea root	x		HV	Grey	Grey blue	White grey	Streptommycete
106	PL21	Pea nodule	x		HV	Grey	Grey	Grey	Streptommycete
107	PL22	Pea nodule	x		HV	Brown pink	Pink	White pink	Micromonospora
108	PL23	Pea root	x		TWYE	Grey	Brown	Grey	Streptommycete
109	PL24	Pea nodule	x		HV	Pink red	Light pink	Pink	Microbispora
110	PL25	Pea nodule	x		HV	Brown	Brown	White brown	Not identified
111	PL26	Pea nodule	x		HV	Grey	Grey dark	Grey	Streptommycete
112	PL27	Pea root		x	HV	Brown dark	White brown	White	Streptommycete
113	PL28	Pea nodule		x	HV	Pink	Pink red	Pink purple	Microbispora
114	PL29	Pea root	x		HV	White grey	Grey	Pink white	Microbispora
115	PL30	Pea nodule	x		HV	Light pink	Pink	Red	Microbispora
116	PL31	Pea nodule	x		HV	White pink	Pink	White pink	Microbispora
117	PL32	Pea nodule	x		HV	Brown	White brown	White brown	Not identified
118	PL33	Pea nodule		x	HV	Light brown	Yellow brown	Brown	Microbispora
119	PL34	Pea nodule	x		HV	White grey	Grey dark	Grey	Streptommycete
120	PP1	Pea root	x		TWYE	Green	Yellow brown	Yellow grey	Streptommycete
121	PP2	Pea root		x	TWYE	Pink brown	Pink	Pink	Microbispora
122	PP3	Pea root	x		TWYE	Grey dark	Pink red	Grey dark	Streptommycete
123	PP4	Pea root		x	TWYE	Orange	Orange	Orange	Microbispora
124	PP5	Pea root		x	TWYE	Brown	White	White brown	Streptommycete
125	PP6	Pea root		x	TWYE	Light brown	Grey	Green dark	Micromonospora
126	PP7	Pea root	x		HV	Orange	Pink orange	Orange red	Microbispora
127	PP8	Pea root	x		HV	White	White	White	Streptommycete
128	PP9	Pea root	x		TWYE	Grey dark	Green white	White grey	Streptommycete
129	PP10	Pea root	x		HV	White brown	White brown	White	Streptommycete

No.	Culture name	Part of plant	Temperature		Isolation medium	Morphology on medium			Proposed genus
			27°C	37°C		MS	HPDA	Oatmeal	
130	PP11	Pea root	x		HV	White	White	White	Streptommycete
131	PP12	Pea root	x		HV	White grey	White	White brown	Streptommycete
132	PP13	Pea root		x	HV	Pink	Light pink	Pink	Streptommycete
133	PG1	Pea root		x	HV	Brown	Light brown	White brown	Not identified
134	PG2	Pea nodule		x	TWYE	Grey pink	Grey purple	Light pink	Micromonospora
135	PG3	Pea nodule		x	HV	White grey	Grey	White grey	Streptommycete
136	PG4	Pea root		x	TWYE	Grey	Grey dark	Grey	Streptommycete
137	PG5	Pea root		x	HV	Grey pink	White pink	White brown	Streptommycete
138	PG6	Pea root		x	HV	Grey	Pink	White	Streptommycete
139	PG7	Pea root	x		TWYE	Green	Grey yellow	Green	Streptommycete
140	PG8	Pea root		x	HV	Green brown	Black	Black	Micromonospora
141	PG9	Pea root	x		HV	Brown yellow	Black	Pink red	Microbispora
142	PG10	Pea root	x		HV	Brown grey	Pink	Pink dark	Microbispora
143	PG11	Pea root	x		HV	White pink	Pink	White pink	Microbispora
144	PG12	Pea root	x		HV	White	White pink	Pink red	Microbispora
145	PG13	Pea root	x		HV	White grey	Pink grey	White pink	Micromonospora
146	PG14	Pea root	x		HV	White pink	Light pink	Pink	Microbispora
147	PG15	Pea root		x	HV	White brown	Brown	White	Micromonospora
148	PG16	Pea root	x		HV	White grey	Grey dark	Grey	Microbispora
149	PG17	Pea nodule	x		HV	White pink	Light pink	Pink	Microbispora
150	PG18	Pea root	x		TWYE	White brown	Brown	White	Not identified
151	PG19	Pea nodule	x		HV	Brown	White brown	White	Not identified
152	PG20	Pea nodule		x	HV	Brown	Light brown	Brown white	Not identified
153	CM1	Clover nodule		x	TWYE	Grey	Grey	Grey	Streptommycete
154	CM2	Clover nodule		x	TWYE	Grey white	Grey white	Grey	Streptommycete
155	CM3	Clover nodule		x	TWYE	Grey	Grey	Brown grey	Streptommycete

No.	Culture name	Part of plant	Temperature		Isolation medium	Morphology on medium			Proposed genus
			27°C	37°C		MS	HPDA	Oatmeal	
156	CM4	Clover nodule		x	YECD	Grey	White grey	Grey	Streptommycete
157	CM5	Clover nodule		x	HV	White grey	Grey	White grey	Streptommycete
158	CM6	Clover nodule		x	HV	White grey	White grey	White grey	Streptommycete
159	CM7	Clover nodule		x	HV	White grey	White yellow	Grey	Streptommycete
160	CM8	Clover nodule		x	YECD	White grey	Grey	Grey	Streptommycete
161	CM9	Clover nodule		x	TWYE	Grey	Light grey	Grey	Streptommycete
162	CM10	Clover root		x	TWYE	Grey	Grey	White grey	Streptommycete
163	CM11	Clover nodule		x	YECD	Grey dark	Grey	White grey	Streptommycete
164	CM12	Clover nodule		x	HV	White	Grey	Light black	Streptommycete
165	CM13	Clover nodule		x	TWYE	Black	grey	Black	Streptommycete
166	CM14	Clover nodule		x	TWYE	White	Light grey	Black	Not identified
167	CM15	Clover root		x	TWYE	Grey	Grey	Grey white	Streptommycete
168	CM16	Clover root		x	YECD	Orange	Light orange	White	Microbispora
169	CM17	Clover root		x	HV	Brown pink	Pink	Light pink	Microbispora
170	CM18	Clover root		x	TWYE	White pink	Pink	Pink	Microbispora
171	CM19	Clover nodule		x	HV	White pink	Pink	Light pink	Microbispora
172	CM20	Clover nodule		x	YECD	Brown white	White	White	Not identified
173	CM21	Clover nodule		x	HV	Brown	White	Grey dark	Streptommycete
174	CM22	Clover nodule	x		YECD	Grey	Grey green	Grey	Streptommycete
175	CM23B	Clover nodule	x		YECD	White grey	Grey	Grey	Streptommycete
176	CM24	Clover nodule	x		HV	White	Grey brown	White grey	Streptommycete
177	CM25	Clover nodule		x	HV	White grey	White	White	Streptommycete
178	CM26	Clover nodule		x	HV	Pink red	Grey	Pink white	Microbispora
179	CM27	Clover nodule	x		YECD	White brown	Brown grey	White	Streptommycete
180	CM28	Clover root	x		TWYE	White	Light pink	Pink	Microbispora
181	CM29	Clover root	x		TWYE	White	Light pink	White pink	Microbispora

No.	Culture name	Part of plant	Temperature		Isolation medium	Morphology on medium			Proposed genus
			27°C	37°C		MS	HPDA	Oatmeal	
182	CM30	Clover root		x	YECD	White	White pink	Pink red	Streptomyces
183	CM31	Clover root		x	TWYE	Red	Pink	White pink	Microbispora
184	CM32	Clover root		x	HV	Pink brown	Pink	White	Microbispora
185	CM33	Clover root		x	HV	Brown	White brown	Pink	Microbispora
186	CM34	Clover nodule	x		TWYE	Red	Pink red	White pink	Not identified
187	CM35	Clover root	x		YECD	White brown	Brown	Brown	Not identified
188	CM36	Clover root	x		TWYE	Red pink	Light pink	White	Not identified
189	MF1	Medic nodule		x	HV	Grey green	White green	Grey	Streptomyces
190	MF2	Medic nodule		x	HV	Brown	Pink	Light pink	Streptomyces
191	MF3	Medic nodule		x	HV	Brown dark	Pink	Light pink	Microbispora
192	MF4	Medic nodule		x	HV	Brown	White	White	Not identified
193	MF5	Medic nodule		x	HV	Brown	Light pink	Grey pink	Microbispora
194	MF6	Medic nodule		x	HV	Brown	Pink	White pink	Microbispora
195	MF7	Medic nodule		x	HV	Brown	White brown	Brown black	Not identified
196	MF8	Medic nodule		x	HV	White	White	White grey	Streptomyces
197	MF9	Medic root		x	HV	White brown	Brown grey	Grey	Streptomyces
198	MF10	Medic root		x	TWYE	Green dark	Brown	Green dark	Micromonospora
199	MF11	Medic nodule		x	TWYE	White pink	White pink	Pink	Microbispora
200	MF12	Medic nodule		x	TWYE	Grey	Grey	Grey black	Not identified
201	MF13	Medic root		x	HV	Grey red	Light red	White pink	Microbispora
202	MF14	Medic root			HV	Brown	White pink	White	Microbispora
203	MF15	Medic nodule		x	HV	Pink grey	White pink	White pink	Microbispora
204	MF16	Medic nodule		x	HV	Brown	Brown pink	White	Microbispora
205	MF17	Medic nodule		x	HV	Brown	Grey	White brown	Microbispora
206	MF18	Medic nodule		x	HV	White	Pink	White pink	Streptomyces
207	MF19	Medic nodule		x	HV	Pink red	Pink	White pink	Streptomyces

No.	Culture name	Part of plant	Temperature		Isolation medium	Morphology on medium			Proposed genus
			27°C	37°C		MS	HPDA	Oatmeal	
208	MF20	Medic root	x		HV	White grey	Grey	Grey	Streptomyces
209	MF21	Medic root	x		YECD	Grey	Grey	Grey brown	Streptomyces
210	MF22	Medic root	x		YECD	White green	Brown green	White	Streptomyces
211	MF23	Medic root	x		TWYE	Pink	Pink	White pink	Microbispora
212	MF24	Medic nodule		x	TWYE	Grey	Grey dark	Grey	Micromonospora
213	MF25	Medic nodule	x		HV	Grey	Grey	White	Streptomyces
214	MF26	Medic nodule	x		HV	Grey	Black	Grey	Microbispora
215	FM27	Medic root	x		HV	White grey	Grey white	Grey	Streptomyces
216	FM28	Medic nodule	x		HV	Pink	Pink white	White	Microbispora
217	FM29	Medic root	x		HV	Brown	Brown	White brown	Not identified
218	FM30	Medic nodule	x		YECD	White grey	Brown grey	Grey	Not identified
219	FM31	Medic nodule	x		YECD	Pink	Light pink	White pink	Not identified
220	FM32	Medic nodule	x		YECD	Pink red	Light pink	Pink	Not identified
221	FM33	Medic nodule	x		HV	Pink	Light pink	White pink	Not identified
222	FM34	Medic nodule	x		HV	White pink	Pink brown	Pink	Not identified
223	FM35	Medic nodule	x		HV	Pink	Pink red	Pink	Not identified
224	MS1	Medic root	x		TWYE	Brown	White brown	White	Streptomyces
225	MS2	Medic root		x	TYWE	White	White	White	Streptomyces

Appendix 3. Positive effects of twelve cultures on the germination of lucerne seeds on agar plates and in sandy loam. There were three replicates for each treatment on agar plate containing 5 lucerne seeds. There were 20 seeds sown in a small punnet containing sandy loam and control with 3 replicates.

Treatments	On agar plates with 5 seeds on each plate													In sandy loam with 20 seeds per one container													
	Germination (# seedlings/plate)									Length of root (cm)				Germination (# seedlings/plate)									Length of root (cm)			
	3th day			5th day			7th day			7th day			Ave.	3th day			5th day			7th day			2 weeks (cm)			Ave.
Control	3	4	5	5	4	5	5	4	5	6.5	4.8	5.4	5.57	11	10	7	16	15	11	19	17	17	10.7	9.3	10.1	10.03
LuP3	5	5	5	5	5	5	5	5	5	6.5	7	7	6.83	12			17			19			11.6			11.60
LuP5B	4	5	5	4	5	5	4	5	5	6.5	6.8	7	6.77	13			16			19			11.3			11.30
LuP10	5	4	5	5	5	5	5	5	5	7.5	7.5	7	7.33	18			18			19			14.2			14.20
LuP12A	4	5	5	4	5	5	4	5	5	7	7.5	8	7.5	20			20			20			11.5			11.50
LuP13	5	5	5	5	5	5	5	5	5	7	7	5.7	6.57	14			17			18			12.3			12.30
LuP30	5	5	5	5	5	5	5	5	5	5.3	7	7.2	6.5	15			17			18			14.05			14.05
LuP32	5	5	5	5	5	5	5	5	5	6.8	7	7.2	7	15			18			19			12.4			12.40
LuP35	5	5	5	5	5	5	5	5	5	7.2	7.3	7.3	7.27	15			18			18			12.5			12.50
LuP46B	5	5	5	5	5	5	5	5	5	5	7	7	6.33	14			17			18			12.3			12.30
LuP47B	4	5	5	5	5	5	5	5	5	7	6.4	7	6.8	19			19			20			11.1			11.10
LuP86	5	5	5	5	5	5	5	5	5	6.7	7	7.2	6.97	19			20			20			12.3			12.30
CM23B	5	5	5	5	5	5	5	5	5	7	7	7.4	7.13	19			20			20			12.1			12.10

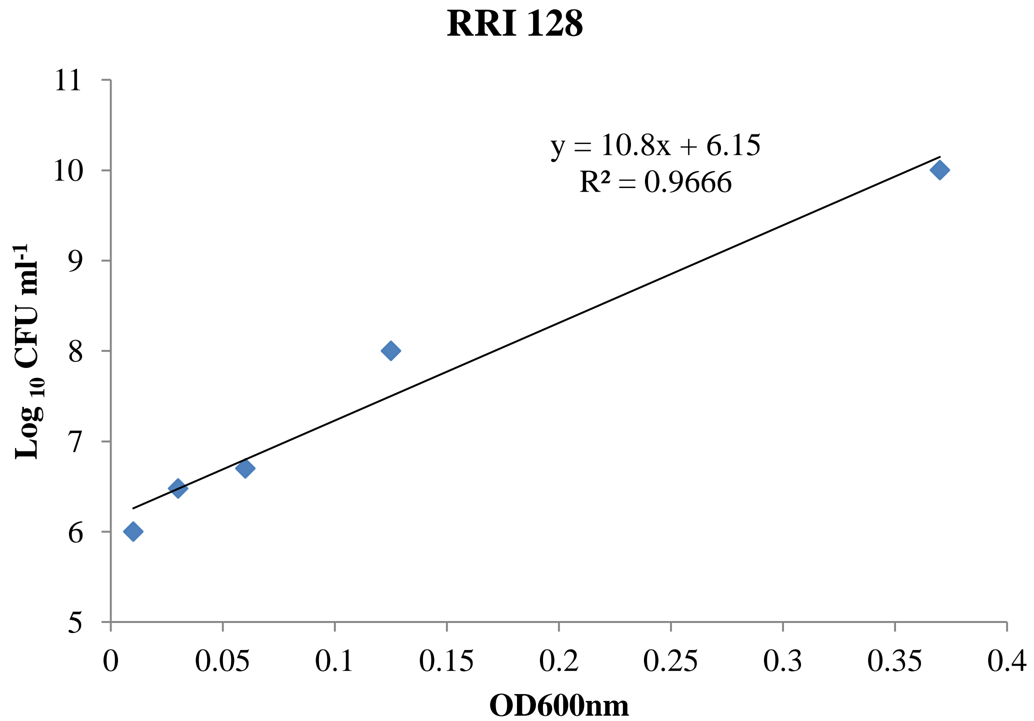
Appendix 4. Sandy loam analysis

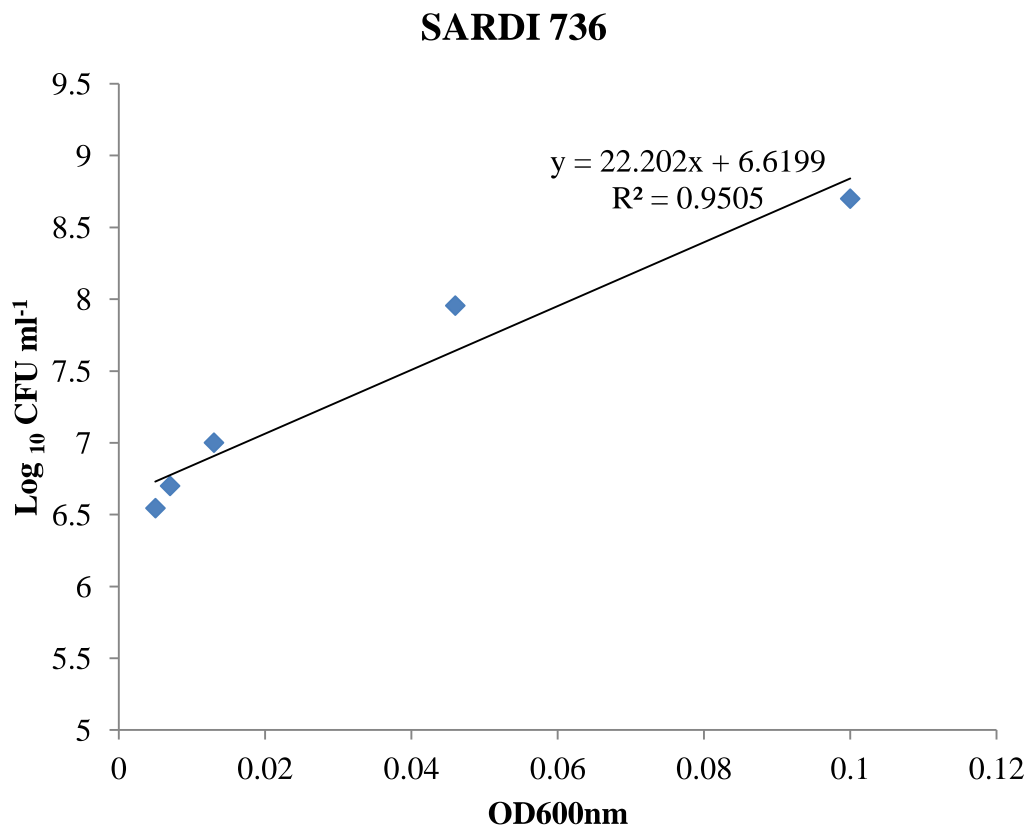
			mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	%	pH	pH
Soil	**Texture**	**Colour**	**Nitrate N**	**Ammonium**	**Phos**	**Potassium**	**Sulphur**	**Orgcarbon**	**PH_CACL2**	**PH_H2O**
Netherton	1.5	GR	94	10	34	164	11.3	1.65	5.7	7

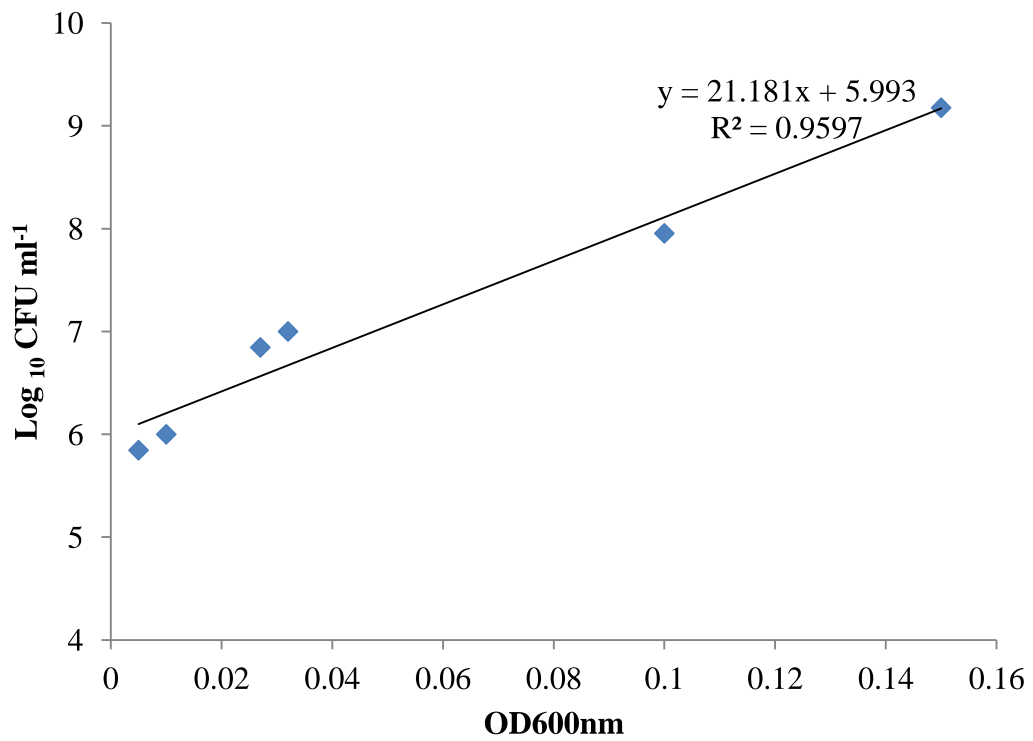
Appendix 5. Optimisation the number of millet seeds and moisture percentage of sandy loam for infection of *Rhizoctonia solani*

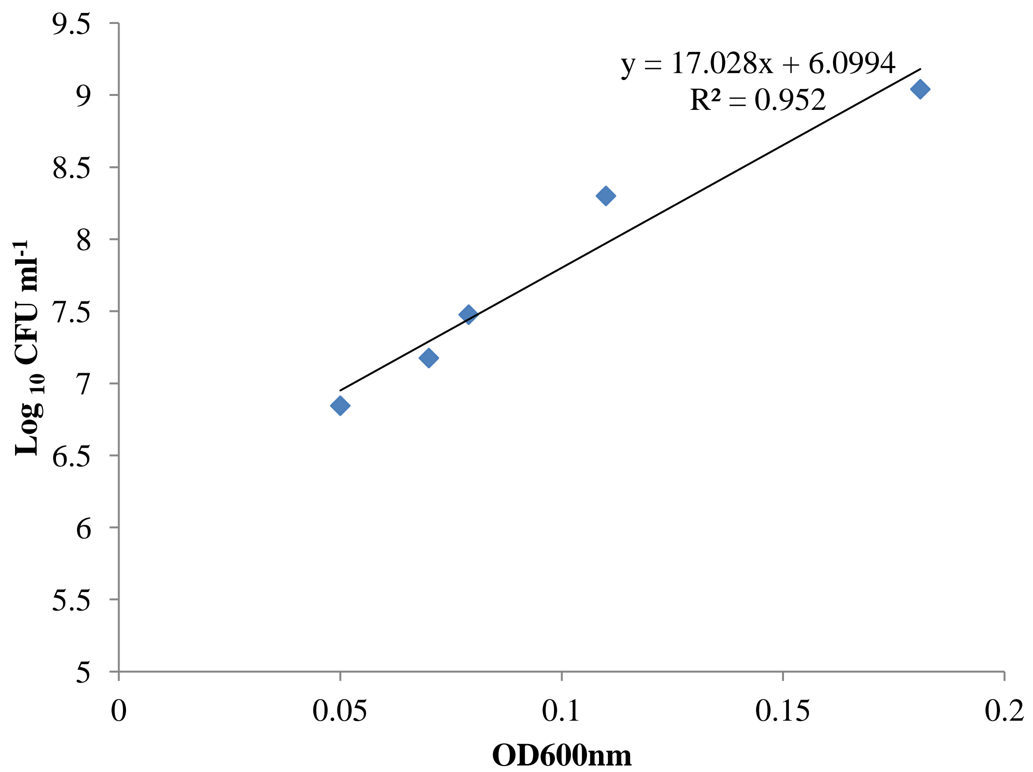
Number of millet seeds	**0**			**2**			**4**			**6**		
Percentage of moisture	8 %	10 %	12 %	8 %	10 %	**12 %**	8 %	10 %	12 %	8 %	10 %	12 %
Number of plants	2,2,2	2,2,2	2,2,2	1,2,1	2,1,0	**2,1,2**	1,0,0	0,1,1	2,0,0	1,0,0	0,0,1	0,0,0
Average of the length of root (cm)	15	16.4	17.8	1.3	1.5	**2.0**	0	0	0	0	0	0
Number of root	2,2,2	2,2,2	2,2,2	0,0,0	0,0,0	**0,0,0**	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Growth description of plants	Pale	Weak	Good	Pale	Weak	**Weak**	Pale	Pale	Pale	Pale	Pale	Pale

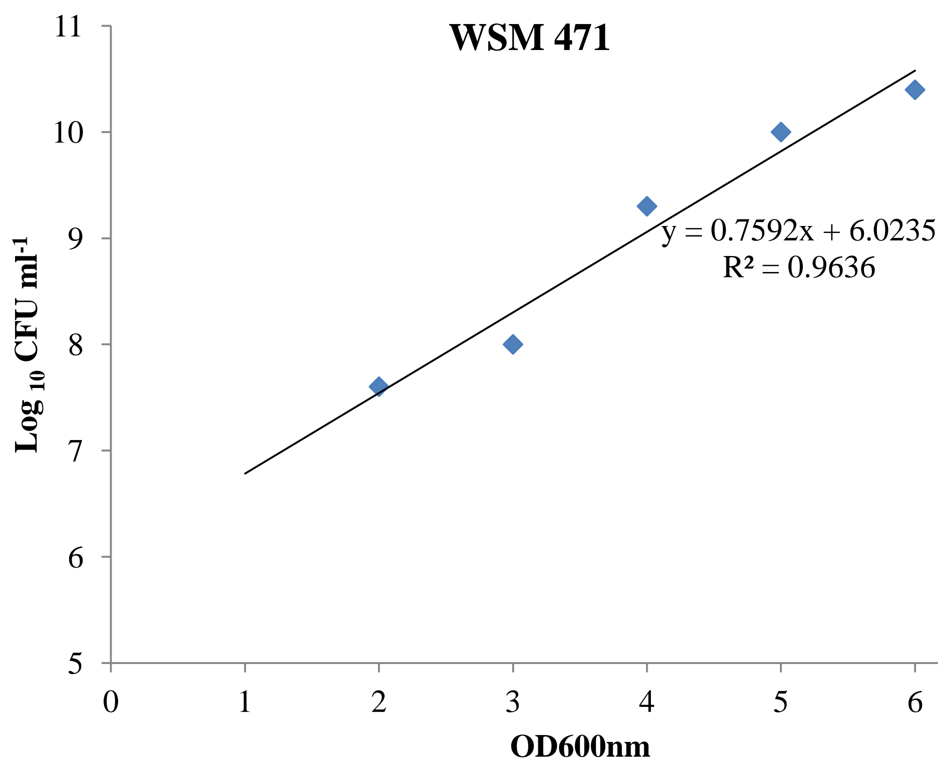
Appendix 6. Correlation between OD_{600nm} and CFU/ml of five rhizobial strains. (A) *S. meliloti* RRI 128; (B) *S. meliloti* SARDI 736; (C) *S. meliloti* WSM 1115; (D) *Rhizobium* WSM 1325; (D) *Bradyrhizobium* WSM 471






WSM 1115
y = 21.181x + 5.993
R² = 0.9597
Log 10 CFU ml-1
10
9
8
7
6
5
4
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
OD600nm


WSM 1325
y = 17.028x + 6.0994
R² = 0.952
Log 10 CFU ml-1
9.5
9
8.5
8
7.5
7
6.5
6
5.5
5
0
0.05
0.1
0.15
0.2
OD600nm



Appendix 7. Early effects on nodulation by actinobacteria at different levels of NH_4NO_3. One plant in each tube and three replications for each treatment. LN = 3 mg/kg NH_4NO_3, MN = 25 mg/kg NH_4NO_3 and HN = 50 mg/kg NH_4NO_3. R=RRI 128.

N levels	Treatments	Number of nodules per plant				
		3d	4d	5d	7d	10d
LN	Nil	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	R	0,0,0	1,0,0	3,0,2	3,4,3	6,3,2
	R + LuP30	0,0,0	0,0,0	0,3,0	3,2,3	6,2,3
	R + LuP47B	0,0,0	0,1,0	1,5,0	4,5,4	6,2,4
MN	Nil	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	R	0,0,0	0,0,0	0,0,0	1,2,1	6,3,1
	R + LuP30	0,0,0	0,1,0	2,1,0	4,3,4	5,4,4
	R + LuP47B	0,0,0	0,0,0	1,0,0	3,3,4	3,4,3
HN	Nil	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
	R	0,0,0	0,0,0	0,0,0	0,1,0	2,1,1
	R + LuP30	0,0,0	0,0,0	0,0,0	0,0,0	1,0,1
	R + LuP47B	0,0,0	0,0,0	0,0,0	0,1,0	2,1,1

Appendix 8.

Streptomyces lucernesis **sp. LuP30 16S rhibosomal RNA gene, partial sequence**

Genebank: KP973994

CAGTCGAACGATGAACACTTCGGTGGGGATTAGTGGCGAACGGGTGAGT
AACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGG
GTCTAATACCGGATAACACTTCCACTCGCATGGGTGGAGGTTAAAAGCTC
CGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAAT
GGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGG
ATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAG
TGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAG
CTCGTAGGCGGTCTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCC
GGGTCTGCATTCGATACGGGCAGACTAGAGTGTGGTAGGGGAGATCGGA
ATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTG
GCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTG
GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGG
GAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCAT
TAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCA
ACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCCAGAGA
TGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTT
CTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTG
CCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCC
CTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGCTGC
GAAGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGA
TCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT
CACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACC

Streptomyces medicagensis **sp. LuP47B 16S rhibosomal RNA gene, partial sequence**

Genebank: KP973995

GTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGG
CGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCC
GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAG
AAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGG
CGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGGTGTGAAAGCCCGGGG
CTTAACCCCGGGTCTGCATTCGATACGGGCTAGCTAGAGTGTGGTAAGG
GAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGA
ACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGC
GAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGGTGGGAACTAAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGC
AGCTAACGCATTAAGTTCCCGCCCGGGGGAGTACGGCCGCAAGGCTAAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTT
AATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACGCCGGAA
AGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATG
GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTC
ACAGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAA
GTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCAGGT
ACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAATAGCCTGTC
TCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTA
GTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTAC
ACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCC
CAAC

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