Study of the interaction between endophytic actinobacteria and lentil crop

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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.

Gurpreet Kaur

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Abbreviations

ul, ml	microlitre, millilitre
uM, mM	micromolar, milimolar
%	percent
ANOVA	Analysis of Variance
approx.	approximately
bp	base pair
CFU	colony forming units
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
cDNA	Complementary Deoxyribonucleic acid
Ct	Cycle Threshold
exp.	experiment
IAA	Indole Acetic Acid
MQ water	Milli Q water
^{O}C	degree Celcius
OD600nm	Optical Density 600 nanometers
PCR	Polymerase Chain Reaction
PGPR	Plant Growth-Promoting Rhizobacteria
SARDI	South Australian Research and Development Institute
sp.	species (singular)
spp.	species (plural)

N-	no added nitrogen treatment
N+	unlimited nitrogen treatment
ROS	Reactive Oxygen Species

ABSTRACT

Lentil (*Lens culinaris*) is a winter crop and one of the most ancient foods having been cultivated in Southwestern Asia since 7000BC. Lentil crops play a vital role in the food and to complete nutritional security requirements of millions of people particularly in Asia. It is affordable highly nutritional food for those who cannot afford animal protein. Endophytic actinobacteria which colonize plant roots can increase the yield of legume crop and nitrogen fixation capacity by controlling the losses caused by the significant fungal root pathogens. The aims of this study were to isolate endophytic actinobacteria from leaves, roots and nodules of lentil plants which planted into seven different soils and screen their effects on the growth and nodulation of lentil. Endophytic actinobacteria which were isolated from different legumes were also examined on the lentil plants. The isolated endophytic actinobacteria were examined on lentils to investigate the drought stress.

Sixty two endophytic actinobacteria were isolated from roots, leaves, nodules and shoots of lentil and twenty endophytic actinobacteria were successfully recovered and purified. Most of the endophytic actinobacteria were isolated from lentil planted in Karoonda soil. Humic acid Vitamin B was the most effective isolation medium. Sixteen out of twenty endophytic actinobacteria produced spores and seven actinobacteria were examined on lentil plants in sand – vermiculite pot experiment to investigate their effect on the growth, yield and nodulation of lentil. The six existing endophytic actinobacteria from chickpea and lucerne were tested on lentil plants inoculated with rhizobium *Mesorhizobium ciceri* strain WSM-1455 to examine the growth and nodulation process of lentil. The endophytic actinobacteria CP200B, CP21 and CP56 were selected for further study as they increased the total dry mass and nodulation process.

The endophytic actinobacteria isolated in this study- LT1, LT5, LT6, LT10 and LT13- showed positive effects on lentil growth and were identified tote species level by 16S rRNA gene sequencing. In the drought stress experiment LT6 and LT10 endophytic actinobacteria assisted in overcoming the effects of drought stress in lentil plants. As a result the selected endophytic actinobacteria are beneficial bacteria for potential use as microbial inoculants with rhizobium *Mesorhizobium ciceri* strain WSM-1455 for increasing the yield and symbiosis of lentil.

CHAPTER -1
Introduction and Literature Review

1.1 Nitrogen fixation, legumes and rhizobia

1.1.1 Nitrogen Fixation and Biological Nitrogen Fixation (BNF)

Nitrogen is the most limiting nutrient in agriculture (Reinhold-Hurek and Hurek, 2007). The earth's atmosphere contains about 10^5 tons of N_2 gas, which cannot be used in this form by most living organisms unless it is reduced to ammonia. The nitrogen cycle involves the transformation of 3 x 10^9 tons of N_2 per year into a form that can be utilized by plants globally (Mabrouk and Belhadj, 2010). Inorganic nitrogen compounds ($NH_4^{+,}$ NO_2^-, and $NO_{3,}^-$) are absorbed by most plants and living organisms accounting for less than 5% of total nitrogen (Liu et al., 2014) and it impacts the crop productivity and species composition of plant communities. Economic use and environmental costs of heavy use of chemical N fertilizers in agriculture are a global concern. Biological nitrogen fixation (BNF) is an alternative method to the use of N fertilizers. It is a microbiological process which converts the atmospheric form of nitrogen into a plant usable form. About 80% of BNF come from symbioses formed between leguminous plants and various species of *Rhizobium* (Wani et al., 1995).

1.2 General information on legumes

Legumes have been an important component of agriculture since ancient times. Legumes are important for humans and animals as well as for the environment by fixing nitrogen from air. They are grown around the world in many developing countries with India, Turkey, China, Brazil, Australia and Canada, being the major producers. Legumes lag behind cereals in the terms of area of expansion and productivity gains. The reason for this lag is that legumes are considered secondary to cereals in terms of consumer use. There are between 44 to 66 million tonnes of nitrogen fixed from atmospheric nitrogen by symbioses of *Rhizobium* and legumes every year. There are approximately 700 genera and about 13,000 species of legumes, yet only 20% have been examined for nodulation and shown the ability to fix nitrogen (Zahran,

1999). Legumes benefit non-legumes by using a legume rotation in a cropping system, with an increase in the yield of a grain crop grown subsequently by at least 10 to 20 percent greater in terms of grain yield. Legumes improve the soil fertility and prevent soil erosion by enhancing the soil organic matter content and mineralizable nitrogen (Power, 1987). Legumes are being pushed to marginal areas of cultivation having low rainfall and poor soil fertility (Nedumaran et al., 2015). Grains legumes play a nutritional role in the diet of millions of people in developing countries. Legumes are a prominent source of protein, calcium, iron, phosphorus and other minerals, and form a significant part of the diet of vegetarians. Legumes provide fiber, carbohydrate, Vitamin B, zinc and iron. Legumes are naturally low in fat and free of saturated fat. Intake of legumes is linked to significantly lower risk of heart disease, high blood pressure, stroke and type2 diabetes (Polak et al., 2015).

1.3 Global Lentil production

1.3.1 History

Lentil (*Lens culinaris*) is a winter crop and one of the most ancient foods which were cultivated in south western Asia since 7000BC. Lentils are produced, preferably, in a temperate climate but now they are produced in different regions of the world. Lentils are placed in different categories based on cotyledon and colour of seed coat. Green and red colour seeds are predominant varieties of lentil grains (Bhatty, 1988) which are produced, consumed and traded globally (McNEIL et al., 2007, Williams et al., 1993).

1.3.2 Lentil production in world

Currently, the lentil crop is grown throughout the world in many developing and developed countries with a particular concentration in Asia where two major producing countries are Turkey and India. World lentil production for 2016 was 6.3 million tons led by Canada 51% and India 17% (Table1.1; FAO STAT 2016 report). Canada is the major leading producer and exporter country (Figure 1.1) followed by Austrasia, India, Turkey, USA, Nepal, Syria, China, Iran, and Bangladesh etc. India and Turkey produce mainly red lentils.

Table 1.1 Worldwide production of lentil crop (FAOSTAT 2016)

Country	Tonnes
Canada	3,233,800
India	1,055,536
Turkey	365,000
United States	255,061
Nepal	253,041
World	**6,315,858**
Source: FAOSTAT[9]	

Canadian production has shifted from13% red lentils in (1998-1999) to the present situation of approximately 56% red lentil production (Yadav et al., 2007a). In Europe, the three major producing countries are Spain, France and Russia. In North America, the Palouse region of eastern Washington and northern Idaho grow commercial lentil crops since the late 1930s. The other countries are Mexico, Peru, Argentina, Ethiopia, Pakistan, Colombia and Chile also contribute as producers (Yadav et al., 2007b). Australia and Turkey are dominant in trading of small-seeded red cotyledon whereas the large green lentil seed market is dominant in Canada and the USA. Due to its health benefits, per capita consumption of lentil grains has

been increased during the last five decades. Therefore, an increment was seen in the area sown by 155%, and the average yield of lentil crop has doubled from 529 to 1069 ha $^{-1}$ (FAOSTAT 2014).

Image removed due to copyright restriction.

Figure 1.1 – Major exporters of lentil as percentage of world exports (2001 – 2013 (FAO STAT 2014 report) (Joshi et al., 2017)

Lentil crop is an important food legume crop in the sector of farming and food systems of many countries. It plays a vital role in human and animal health improvement, but it is also beneficial for soil health with its ability in nitrogen fixation and carbon sequestration. It improves the nutrient status of the soil for cereal-based cropping systems. However, the current yields are still low as compared to other legume crops. The limiting factors of yield are lack of seedling vigour, slow growth, slow leaf area development, poor dry matter and abiotic and biotic stress (Erskine, 2009).

1.3.4 Lentil production in Australia

Lentils in Australia are grown predominantly in semi-arid regions of South Australia and Victoria with winter dominant rainfall patterns. The production of lentils industry is on average between 400 and 600 tonnes per year, most of which are exported. Victoria normally leads in lentil production but in 2016-17 the majority of the crop was grown in South Australia which increased its yield from 1.60 to 2.95 metric tonnes. There are some small areas of Western Australia and New South Wales where lentil is grown.

Table 1.1 The lentil area and production in Australia in 2018 and 2019(estimated)

State	2018 final		June 2019 estimate	
	Harvested hectares	Tonnes	Planted hectares	Tonnes
Qld	0	0	0	0
NSW	7,000	5,000	6,000	5,000
Vic	125,000	105,000	100,000	110,000
SA	160,000	200,000	124,000	208,000
WA	11,000	13,000	15,000	18,000
National total	**303,000**	**323,000**	**245,000**	**341,000**

(Source Pulse Australia, 2017)

1.4 Nutrition

Lentils crops play a vital role in the food nutritional security requirements of millions of people particularly in Asia. Lentil seeds are a rich source of proteins as compared to other plant foods such as cereals, grains, fruits, vegetables and root crops. The protein value of lentil seeds are reported from 15.9g to 31.5g / 100g dry matter, and it is an affordable and highly nutritional food for the poor (Grusak, 2009, Grusak and Coyne, 2009). Lentil grains are easily digestible with high amounts of macro and micronutrients (iron, zinc, calcium, phosphorous and potassium), vitamins, fibre and carbohydrate for balanced nutrition (Bhatty,

1988). The range of carbohydrate in lentils seeds is from 43.4 to 74.9 g/100g dry matter. The carbohydrate in lentils seeds has a low glycemic index, and it is suitable for diabetic patients. However, lentils seeds are lacking in sulphur containing amino acids such as methionine and cysteine although they are rich in lysine and tryptophan content. Therefore, it provides a balanced diet, and in essential amino acids for human nutrition when it is consumed with wheat or rice. Lentil crop is also beneficial for animals because lentil straw is a valuable feed for animals.

Table 1.2: Nutrient composition of dry lentil seeds (per 100g dry matter) (Grusak, 2009)

Component	Range	Mean
Energy (kJ)	1,418–2,010	1,638
Protein (g)	15.9–31.4	28.3
Carbohydrates (g)	43.4–74.9	67.1
Fat (g)	0.3–3.5	2.5
Total fibre (g)	5.1–26.6	12.2
Ash (g)	2.2–6.4	2.2

1.5 Rhizobia

As discuss above, *Rhizobium* is a genus of gram-negative bacteria that do not form spores. Rhizobia bacteria have the ability to establish a symbiotic relationship with leguminous plants, such as peas, alfalfa and lentil. It leads to the establishment of specialized structure called nodules which are able to covert atmospheric nitrogen into ammonia, via the process of biological nitrogen fixation (Parker, 2001). There are several species of Rhizobium, but each species can nodulate only a limited range of nodules (Downie and Brewin, 1992). The nitrogen fixation process starts when rhizobia form an endophytic symbiosis with legumes plants.

1.6 Plant Growth – Promoting Bacteria

A group of microbes called plant growth-promoting rhizobacteria (PGPR) offer a cleaner and greener approach for improvement of leguminous crop production. A group of heterogeneous bacteria found in the rhizosphere/ the plant tissue which promotes growth of the plant and enhancing the uptake of soil nutrients, phytohormones and resistance against phytopathogens (Sathya et al., 2017). The PGPR are grouped separately to the gram negative Rhizobia which form symbiotic association with leguminous plants, resulting in differentiated bacteria, or bacteroids, enclosed in intracellular compartments called symbiosomes within nodules on the root (Oldroyd et al., 2011). Recent studies have shown that the interaction of actinobacteria with rhizobia has increased growth of the rhizobia and also the plant and stimulates the positive the effect on the nitrogen fixation in plants (Le et al., 2016). In the presence of a fungal root disease, some *Streptomyces* together with rhizobial strains increased the vegetative growth of roots and shoot dry weight as compared to the infected control (Misk and Franco, 2011).

1.7 Legume- Rhizobium symbiosis

The plant partners of Rhizobia belong to the *Leguminosae/Fabaceae* family. The family *Leguminosae* contains a very large variety of plants. The majority of species are nodulated by rhizobia. Individual rhizobia species have a distinct host legume plant to interact for producing nodules and to nitrogen fixation. However, *Rhizobium* sp. NGR234 and *Rhizobium freddi* USDA257 have a broad host-range for nodulation and can establish symbiosis with 353 legumes species(Krishnan et al., 1992). Symbiosis of legumes and rhizobia is a selective process and highly specific interaction between two partners. The symbiosis is triggered by nitrogen starvation of the host plant which has selected its Rhizobium partner from billions of bacteria in the rhizosphere (Maróti and Kondorosi, 2014).

1.7.1 Nodulation Process

Nodulation by rhizobia is a complex and multistep process which is initiated by first step pre-infection in the rhizosphere before entry of rhizobia into root hairs. The other steps involve attachment root hair curling, formation of nodule meristem, infection thread formation, nodule organogenesis, bacteroids development and Biological nitrogen fixation (Gloudemans and Bisseling, 1989) (Figure 1.2)

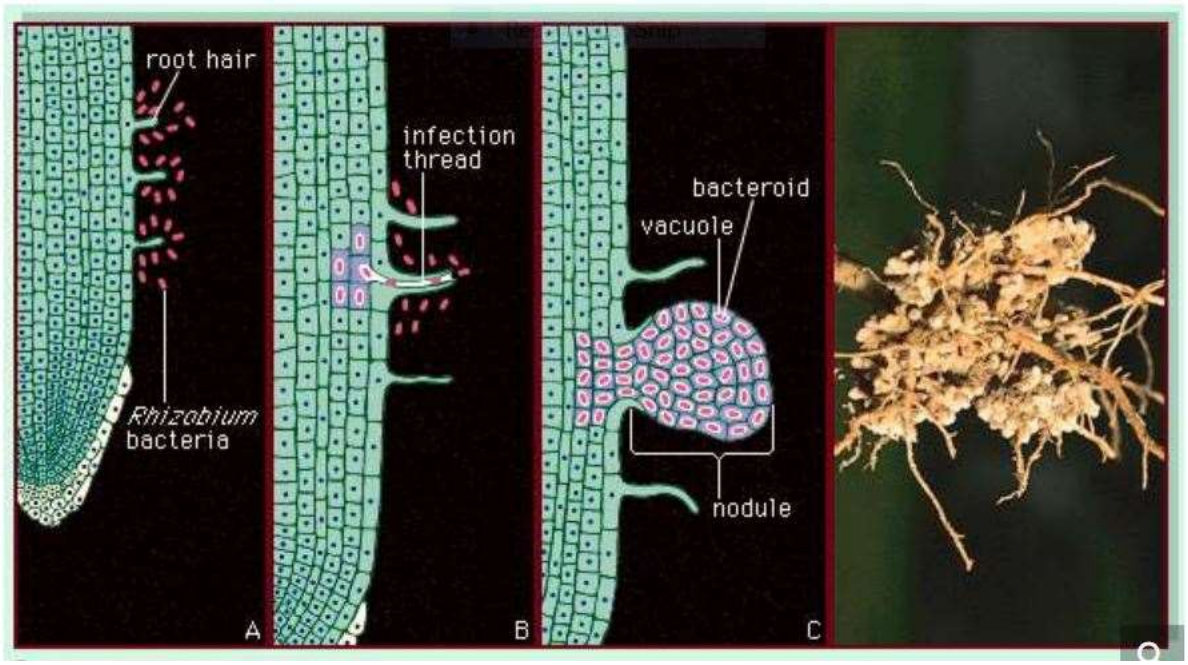


Figure 1.2 Steps of Nodulation Process a) Root hairs release chemical signals that attract rhizobia. b) Rhizobia proliferate and cause an infection thread to form and the infection thread grows into the cortex cell. c) The infection thread releases bacterial cells, which become bacteroids in the root cells (d) Enlarged the root cells form a nodule .

In nodulation process, a variety of chemicals are released from the cells by interaction between free-living rhizobia and the host plant as shown in Figure 1.3. These chemicals promote the growth of bacteria in the rhizosphere area. An important step is attachment of rhizobia to root hairs of the host plant. Rhizobia can recognize correct host plant by chemical signals produced by the root and the cell wall of bacteria, such as flavonoids, aldonic acids in

the rhizosphere that attract and attach them to the root hair. The bacteria continue to differentiate and synthesize proteins and other substances in the root cells, which require for the process of nitrogen fixation. After attachment, nod genes in the bacteria are activated by the flavonoids and induce the formation of nodule (Cooper, 2007). The bacteria expel nod factors and stimulate the hair curling when bound to root hairs. The formation of an infection thread is induced in the hair tip where rhizobia invade the plant root. The infection thread grows and penetrates through cells of plant root with the thread branching. The formation of nodules is expanded with the multiplication of bacteria, and nod factors continue to be produced and stimulate the proliferation of root cells and root nodule formation. The nodule is usually visible within 7-10 days (Le et al., 2016).

Figure 1.3 - Overview process of nodulation formation and biological nitrogen fixation (Laranjo et al., 2014)

1.8 Endophytic actinobacteria

An endophyte is defined as a bacterium or fungus that colonises the host tissue internally without damaging the host or harming the host through symptoms of plant disease (Hasegawa et al., 2006). Endophytes are distributed throughout the host in all plant parts such as roots, stems, flowers, fruits and seeds. The bacteria can establish an endosymbiotic relationship with the plants from where the plant receives ecological benefits such as increased plant growth and stress tolerance (Hasegawa et al., 2006).

Actinobacteria are filamentous bacteria with high G+C content and belong to the gram-positive phylum, Actinobacteria. They are different from other phyla on the basis of their morphological structures which include germination of spores, elongation and branching of vegetative mycelium, formation of aerial mycelium, septation of hyphae and spore maturation (Miyadoh, 1997) (Champness, 2000). Actinobacteria are common in soil, rhizosphere, pond and lake water as saprophytes by degrading organic materials for nutrition. However, in the 19th century some actinobacteria were proven to closely associated with living plants, as a beneficial bacteria without adverse effects to the host plants (Hasegawa et al., 2006). There are different actinobacterial lifestyles such as Nitrogen fixing bacteria (*Frankia),* plant pathogens *(Streptomyces scabies*) and gastrointestinal tract inhabitants (*Bifidobacterium* spp.) (Goodfellow and Williams, 1983).

1.8.1 Direct Contribution to Nitrogen Fixation and plants

Streptomyces is the predominant genus followed by *Actinomadura, Micromonospora, Nocardia, Nonomurea, Mycobacterium, Frankia, Actinoplanes, Saccharopolyspora, and Verrucosispora (Sathya et al., 2017).* Endophytic actinobacteria work inside the plant and make them more effective in increasing crop yield and promoting plant growth by a range of mechanisms including nutrient acquisition, phytohormone production, and induction of plant defense response. Recent studies stated that actinobacteria have been shown beneficial effects

on the nodulation and legumes (Le et al., 2016). Actinobacteria *Frankia* is more versatile for nitrogen fixation in non- legumes under both symbiotic and free-living aerobic conditions. *Frankia* initiate the nitrogen fixation process via two mechanisms either intracellular root-hair infection or may be intracellular root invasion (Nimaichand et al., 2016). Beside the *Frankia,* other endophytic actinobacteria have ability for nitrogen fixation which include *Arthrobacter, Agromyces, Mycobacterium, Micromonospora and Streptomyces.* A Streptomyces strain, WYEC108, isolated from linseed rhizosphere soil in Great Britain was able to colonize the roots of *Pisum sativum*, increased the number and size of root nodules and enhanced the ssimilation of iron and other nutrients increase by plant (Tokala et al., 2002). Several actinobacterial strains isolated from wild plants, some of these strains produced auxin and indole acetic acid (IAA) and which influenced the seed germination and root elongation when tomato seeds were treated with bacterial supernatents (Trujillo et al., 2015).

1.8.2 Plant growth promoting properties

Microorganism promote plant growth with these possible mechanisms (a) the amount of hormones in the plant such as cytokinins, gibberellic acid, ethylene and indole-3-acetic acid (IAA); (b) Nitrogen fixation by nodulation process; (c) by protecting the plant from phyto-pathogenic microbes by producing antibiotics, siderophores and chitinase; (d) by solubilizing the mineral nutrients such as phosphates.

Several actinobacterial strains isolated from wild plants, some of these strains produced auxin and indole acetic acid (IAA) and which influences seed germination and root elongation when tomato seeds were treated with bacterial supernatants (Trujillo et al., 2015). Several actinobacterial strains isolated from wild plants produced auxin and indole acetic acid (IAA) which could influence seed germination and root elongation when tomato seeds were treated with bacterial supernatants (Trujillo et al., 2015). In the context of actinobacteria, *Arthrobacter, Rhodococcus, Gordonia, Streptomyces, and Micromonospora* have been

reported for phosphorus solubilization *in vitro* and glass house conditions (Sathya et al., 2017). A recent study found an endophytic actinobacteria *Nocardiopsis* associated with mandarin which recorded the highest reported IAA production (222.75ppm) (Sathya et al., 2017).

1.8.3 Secondary metabolites and Bio control agents

Actinobacteria contribute approximately 60% of new antibiotics among all other natural compounds which are derived from living sources. *Streptomyces* spp. are the major producers of secondary metabolites such as antifungals, antivirals, antitumoral, anti-hypertensives, and mainly antibiotics and immunosuppressives. Total, 80% of the antibiotics are sourced from the genus *Streptomyces*, actinomycetes being the most important (de Lima Procópio et al., 2012). Metabolites such as pteridic acids A and B produced by endophytic *Streptomyces hygroscopicus* TP_A045 were observed to produce activity due to an auxin and induced root elongation in common bean (Igarashi et al., 2002). *Streptomyces* spp. is well known for production of siderophores.

Actinobacteria have a major role as biocontrol agents and as inducers of plant immunization against different pathogens such as *Rhizoctonia, Fusarium, Pythium, Phytophthora* and *Colletot.*

1.9 Critical Knowledge gaps

Lentil is currently the fifth most important pulse crop produced in the world. It is a rich source of protein and nutrients for human consumption. Lentil contributes to a healthy diet which is cheaper than animal protein which people can afford easily in developing countries. According to the Food and Agriculture Organization statistics report in 2014, the global production of the lentils was primarily cultivated and harvested by Canada and India, which were estimated to be 1.99 million and 1.1 million metric tons, followed by Turkey (0.34

million), Nepal (0.22 million) and China (0.125 million) (Ganesan and Xu, 2017). In recent years, the production of lentils has declined and lags behind the other legumes production and cereals. Like other legumes, lentil has also known for its ability to fix nitrogen naturally as biological nitrogen fixation. Lentils can be grown in rotation to cereals crops which promote sustainable cereal-based production systems with a potential of fixing free nitrogen reached up to 107 kg ha^{-1} which increases the subsequent growth of cereal crop and production. The application of endophytic actinobacteria is a new approach for increasing the yield of legume crop by controlling the losses caused by the significant fungal root pathogens and developing sustainable agriculture. Endophytic actinobacteria have the potential to enhance nodulation, lentil growth and control disease. Recent studies stated isolated endophytic actinobacteria from nodules and roots of legume has a positive effect on the growth of same legumes and other crops. Endophytic actinobacteria isolate from lentil may be a promising source to increase the growth, nodulation, nitrogen fixation and act as bio-control agents for lentil production.

1.10 Research Plan

1.10.1 Aims of research

Study of the interaction between endophytic actinobacteria strains and lentil crop

1.10.2 Objectives of Research

- To isolate endophytic actinobacteria from lentil plants growing in different soils and screening the effect on lentil plants
- To evaluate the effect of endophytic actinobacteria isolated from legumes on the growth of lentils.

Select the appropriate actinobacterial strains which can interact with selected rhizobia and increase plant growth and nitrogen fixation.

- To examine the effect of endophytic actinobacteria from lentil and chickpea on drought stress in lentil plants.

1.10.3 HYPOTHESIS

- Some endophytic actinobacteria strains can increase the production, yield of lentil crops when co-inoculated with rhizobium and improve tolerance to drought stress.
  - Strains from Endophytic actinobacteria strains from lentil will work better for improving the growth of lentil crop.

CHAPTER – 2
General Materials and Methods

OBJECTIVE 1

2.1 Isolation of endophytic actinobacteria from lentil plants

2.1.1 Planting in seven types of soil

Similar sized seeds were selected and surface sterilized followed by the protocol of Coombs and Franco (2003). Seeds were immersed for 2 minute in 70% (v/v) ethanol, then 6 minutes in 4% (v/v) Sodium hypochlorite solution. Then seeds were immersed in 70% ethanol for 1 minute and rinsed with water 3 times with sterile water for 5 minutes. After surface sterilization, the seeds were placed in a laminar flow cabinet to dry for at least 4 -5 hours.

Lentil seeds were planted in seven different soils for isolation of endophytic actinobacteria from the growing plants. The soils collected from six different areas of South Australia and one from Queensland. The pots of 0.7 liter size were filled with the soil and six surface sterilized seeds planted in each pot. The pots were placed in a glass house at 22℃ and plants grown for six weeks to enable their colonization. 200 ml of N deficient nutrient solution (McKnight, 1949) supplemented with a small amount of nitrogen (300 mg NH_3NO_3 per 20 L McKnight's solution) was added to each pot. One ml of Rhizobium inoculant *Mesorhizobium ciceri* strain WSM-1455 around 10^6 CFU/ml added in each plant at the base of seedlings after sowing the seeds. The pots were watered every two days to maintain the 70% - 80% moisture content. Three seed germinated in all pots except four pots of two different soils. The plants were harvested after six weeks from pots and the roots gently shaken to remove the soil. The plants were stored in zip lock bags.

2.1.2 Surface Sterilization Procedure

The lentil plants were washed under running tap water to completely remove the soil from roots and nodules. The roots which contain the nodules were surface sterilized following the protocol of Coombs and Franco (2003). The roots were separated from the shoots and both

samples air dried for 4 – 5 hours by placing them in laminar flow cabinet. First, the air dried roots were immersed for 1 minute in 70% (v/v) ethanol, then 6 minutes in 4% (v/v) Sodium hypochlorite solution and then rinsed three times with sterile water for 5 minutes. The roots with nodules were immersed in 70% ethanol for 30 seconds and rinsed with water 5 times with sterile water for 5 minutes. The nodules were extracted from surface sterilized roots and leaves separated from shoots. The leaves and shoots were surface sterilized following the protocol described above and placed in air laminar flow for 2-3 hours for air dried. The leaves were air dried after separating in two sections young - leaves and mature leaves.

Some dried nodules were crushed in a sterilized pestle and mortar and spread onto the surface of isolation media. The surface sterilized roots and shoots were cut into fragments approximately 1 cm and placed onto isolation media plates. The young leaves were placed as is and mature leaves were cut or crushed and spread onto the surface of isolation media plates.

2.1.3 Media for Isolation

There were four different types of media selected for isolation of endophytic actinobacteria. The isolation media were Humic Vitamin B Acid (HV); Tap Water Yeast Extract (TPYE); VL70 with Carboxymethyl Cellulose, and VL70 with amino acids media all at 7.2 pH (Composition shown in the Appendix). These media were prepared following the specific recipes and one ml per liter of Benomyl (5g/100ml) was added to each agar medium after autoclaving to control the fungal growth and protect from fungal contamination. The isolation plates were placed in air – tight plastic containers containing wet towel papers to maintain the moisture content and incubated at 27°C.

2.1.4 Purification of endophytic actinobacteria

The agar plates were checked twice a week until no new colonies emerged on plates. The filamentous actinobacterial colonies appeared after four weeks. These colonies were excised from the plates and transferred to Half-strength Potato Dextrose Agar (HPDA) which was used as the purification medium.

2.2 Characterization of endophytic actinobacteria

2.2.1 Identification on the basis of Morphological characteristics

There were four media ISP3, Mannitol Soy Flour (MS), Mannitol Soy flour Oatmeal (MSO) and ISP 2 selected to distinguish between endophytic actinobacteria on the basis of their morphological characteristics. After purification, single colonies of purified culture was transferred onto the four media and incubated at 27℃. After characterization, the actinobacterial spores were grown on MS media and placed at 27℃ for 7 -14 days. The two loops of actinobacterial spores were extracted and stored at -80°C in 50% (v/v) glycerol.

2.2.2 Microscopic view of spore-bearing structures of isolated endophytic actinobacteria

Endophytic actinobacteria were streaking on MS media plates for the slide culture process. The autoclaved cover slips were put in the streaked agar plates an angle. Half of the coverslip was stuck into the agar and the other half above the surface. The slide culture plates were incubated at 27℃ for 7-10 days until the culture produced spores. When spores appeared, the coverslip was removed from the agar plate and placed on a glass slide. The structure and characteristics of aerial mycelium observed using a phase – contrast microscope.

2.2.3 Identification of actinobacterial isolates from lentil by 16S rRNA gene sequencing

2.2.3.1 DNA Extraction from actinobacteria strains

Prepared MS media plates and covered with sterile round shaped cellophane. The actinobacteria were cultured on cellophane plates to avoid contact with agar during extraction of DNA. The plates were incubated at 27℃ for 10 days. DNA was isolated from pellet of bacteria which has grown on media plates. Two loops of actinobacteria approximately 0.5g were excised from cellophane media plates and transferred into 2 ml screw cap tubes and 0.5-1 g of 100 Micron Zirconium / glass beads added. 500ul of modified CTAB extraction buffer (250ul of 240mM Potassium Phosphate Buffer and 250ul of CTAB buffer was added to tubes. Subsequently, 500ul of Phenol: chloroform: isoamylalcohal (25:24:1) was added to suspension. The tubes were shaken in a bead – beating instrument for 5 minutes followed by incubation for 1 hour at 65℃ in water bath. After that, tubes were centrifuged at 16,000 rpm for 5 minutes at 4℃ and the aqueous top layer removed to a new 1.5 ml microcentrifuge tube. Equal volumes of chloroform: isoamylalcohal (24:1) were added and mixed well. In the next step, the centrifugation was done at 16,000 rpm for 5 minutes at room temperature and the aqueous top layer removed to a clean 1.5ml microcentrifuge tube. Two volume of PEG/NaCl precipitate solution was added and incubated at 4℃ for 2 hours. The solution was centrifuged at 16,000 rpm for 15 minutes at 4℃. The supernatant was gently poured off without disturbing the DNA pellet. Then, pelleted DNA was washed twice using ice-cold 70% ethanol and centrifuged at 16,000 for 10 minutes at 4℃. The supernatant was removed, without disturbing the DNA pellet and dried overnight. The DNA pellet was resuspended in 50ul of DNAse – free water.

2.2.3.2 Quantify DNA concentration

The concentration of DNA was quantified by Nano drop molecular calculator. The purity of DNA was examined by the ratio of 260/230nm and 260/280 nm. The quality of DNA was checked on agarose gel electrophoresis.

2.2.3.3 PCR of the 16S rRNA gene

Ready PCR Master Mix was used for PCR reaction. PCR Master Mix is a batch mixture of PCR components at optimal concentrations that can be prepared and added into PCR tubes.

Table 2.1 Composition of components in Ready PCR Master Mix (New England BioLabs)

Components in Master mix	Final Conc.	Volume
10X PCR Buffer	1X	5ul
dNTPs (10 mM)	200uM	1ul
Taq DNA Polymerase (5U/ul)	2.5-5U	1ul

PCR Master Mix and other PCR reagents were added in PCR tubes for 50ul PCR reaction. Primers sequences were 27 forward primer (5'-AGAGTTTGATCCTGGCTCAG) and 765 reverse primer (5'- TACGGYTACCTTGTTACGACTT). DNA samples were diluted by adding nuclease free water to 100-200ng/ul.

Table 2.2 Composition of components in 50ul PCR reaction

Volume	Component	Final concentration
24 µL	2X Master Mix	1X
1 µL	MgCl2 (50mM)	1 mM
1 µL	Forward primer	5uM
1 µL	Reverse primer	5uM
2 µL	Template DNA	1-200ng
21 µL	Water	To make volume up to 50 µL
50 µL	Total volume	

After all the reagents were added, the reaction was mixed gently and all liquid collected to the bottom of the tube. Amplification was carried out with 50ul reaction in Axygen Maxygen II PCR machine and the thermocycler PCR profile was set up according to protocol in Table

Table 2.3 – Thermocycler PCR profile for PCR (Polymerase Chain Reaction)

STEP	TEMPERATURE	TIME (Minutes)	Cycle
Initial Denaturation	94℃	3	1
Denaturation	94℃	1	35
Annealing	56℃	1	35
Extension	72℃	2	35
Final extension	72℃	10	1
Hold	4℃		

The PCR products were run on gel electrophoresis to check the size of DNA products. A 1% of agarose gel containing 4ul of Gel red in 40 ml 1% TBE buffer was used to separate the PCR products on the basis of molecular weight. The samples were prepared by adding 1ul loading dye mixed with all 4ul of each PCR products. Then, samples were loaded into each well of the gel which was run in a running buffer 0.5% TBE at 70V for 40 minutes.

2.2.3.4 Sequencing the products

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

OBJECTIVE - 2

2.1 Overview of lentil seeds, Rhizobia and Endophytic actinobacteria

Seeds of Lentil cultivar PBM Jumbo (*Lens culinaris*) and rhizobial strain *Mesorhizobium ciceri* WSM 1455 were provided by the South Australian Research and Development Institute (SARDI). PBA Jumbo is protected under Plant Breeder's Rights (PBR) legislation. PBA Jumbo seeds are large sized red lentil seeds. It has proven to be consistently high yielding in all lentil growing regions of southern Australia. *Mesorhizobium ciceri* strain WSM-1455 is used as commercial inoculants for faba bean and lentil, and sometimes it is also used for field pea.

The endophytic Actinobacteria *Streptomyces* sp. Lup30 and Lup47 were isolated from roots and nodules of lucerne legume and use as a microbial inoculant for growth of lucerne (Le et al., 2016a, b) (Carvalho et al., 2017). The endophytic actinobacteria *Streptomyces* sp. CP200B, CP84, CP21 and CP56 were isolated from chickpea to improve the growth and symbiosis of chickpea (Vo et al., 2017).

2.3.1 Isolation and growth of endophytic actinobacteria

Endophytic actinobacteria *Streptomyces* sp. Lup30, Lup47, CP200B, CP84, CP21 and CP56 were grown on three media: Mannitol Soy flour agar (MS), International Streptomyces Project medium #2 (ISP2) and International Streptomyces Project medium #3 (ISP3). The agar plates were incubated at 27℃ for producing the spores. The culture produced spores after 7-14 days. The spores were stored at -20℃ in 50% (v/v) which had been autoclaved twice. The colony forming units of endophytic actinobacteria spores were counted using the drop-plate technique described by Miles and Misra (1938) (Hedges et al., 1978). Serial dilution of endophytic actinobacterial spore suspension was prepared by adding 1x spore suspension and 9x diluent and dilutions were made up to 10^{-12}. Two drops of 10ul of

suspension dilutions inoculated onto Mannitol Soy flour (MS) agar plates which were divided into 6 sectors. The plates were incubated at 27°C and the colonies were counted in each sector which had less than 10 colonies. Overgrowth was observed at the lower dilutions over the area of drop. The numbers of Colony forming unit per ml was calculated by using formula.

CFU per ml = Average number of colonies for a dilution x dilution factor x 10^2.

2.3.2 Growth of Rhizobium

The rhizobia culture was streaked onto Yeast Mannitol agar (YMA) media to provide pure single colonies. One colony was transferred to fresh YMA plates and incubated at 27°C for 5-10 days for good growth. Time period of incubation depend on each rhizobial strain. A standard curve described the relationship between cell number and $OD_{600\ nm}$ was developed for each Rhizobium strain to enable the application of a standard CFU/ml. (Appendix 2). Cell number was obtained using the Miles and Misra technique described earlier.

OBJECTIVE – 2

2.4 Effects of endophytic actinobacteria strain of different legumes on lentil plants

2.4.1 Surface sterilization of seeds

Undamaged and similar sized seeds were surface sterilized followed by the protocol of Coombs and Franco (2003) described in Section 2.1.1

2.4.2 Seed Coating with endophytic actinobacteria strains

The treatment of seeds was by adding a known number of the actinobacterial spores as a suspension applied on the surface sterilized seeds as a seed coating. The suspension of actinobacterial spores were applied at the rate of 10^8 CFU per gram seeds. The actinobacterial spores were suspended in 0.3% autoclaved xanthan gum after washing off glycerol and

applied on the seeds. The untreated control plants were from surface sterilized seeds treated with only 0.3% xanthan gum.

2.4.3 Pot assay #1, plant growth media, nutrition, sowing and water supply

Thirty six 1.25 liter sized pots were washed and dried and then a mixture of sand and vermiculite (50:50 by volume) was filled into the pots with mixture. The pots were covered with aluminum foil and autoclaved in a dry cycle. One hundred ml MQ water was added to each pot before planting the six seeds. The seeds were planted in each pot in mid of May, 2019, and covered with a thin layer of washed granulated plastic beads to reduce evaporation. The granulated plastic beads were also used to minimize the transfer of microbes between pots. Then, 200 mL of N deficient McKnight Solution with a small amount of nitrogen (300 mg NH_3NO_3 per 20 L McKnight's solution) was added to each pot before covering the pots with plastic bags and placing in the glasshouse. The temperature of the glass house was 22℃, which is suitable for lentil crop. The plastic bags were removed from the pots after 5 days. The number of seedlings was trimmed to four plants per pot and one ml of 10^6 cfu ml^{-1} *Mesorhizobium ciceri* inoculant was added around the base of each seedling near roots by using a pipette. Plants were watered with MQ water after every four days or as a required to a constant weight for the remaining weeks. The position of pots of all treatments with six endophytic strains and control plants were changed in a randomized manner every week.

2.4.4 Harvesting of plants

After six weeks, all the plants were removed from the pots and sand and vermiculite were removed from the roots. The plants were wrapped in moisture paper and stored in the zip lock bags per treatment and the bags labeled. Rhizosphere soil was collected by brushing off and sand attached to the roots and stored at -20^{o}C until required. The plants were washed with running tap water and to remove the remaining sand and vermiculite mixture

2.4.5 Data collection and analysis

The growth parameters of the lentil plants were collected by measuring the length and dry weight of shoot and root, and number, size, colour and dry weight of nodules per plant. Length of roots and shoots of each plant was measured with a ruler and separated the roots from shoots. The nodules were extracted from the roots, counted and observed the size and colour of nodules was recorded according to treatment. The roots and shoots were placed in paper bags and nodules stored in eppendorf tubes. The root, shoot and nodule samples were oven dried at 60℃ for 48 hours. After 48 hours, the dry weight of all samples was measured and calculated the average of length, dry weight of all samples and number of nodules was analysed statistically to find significant figures.

The data was entered into an MS Excel spreadsheet and calculated the average of length and dry weight of all samples. The data was statistically analyzed by using the IBM SPSS Statistics.

OBJECTIVE -1: POT EXPERIMENT #2

2.5 Effect of isolated endophytic actinobacteria on the growth of lentil

2.5.1 Pot Experiment #2

The methodology used for this experiment was similar to that described in the first experiment except for the following variations. The red lentil seeds used were of Pattu brand which is a product of Sabi Foods Company, Australia. The seeds were medium sized and the germination rate of seeds in petri dish checked. Twenty lentil seeds each were placed in petri dishes and sterile water added. The petri dishes were placed in an incubator at 22℃ to check for germination. After two days, 99% seeds had germinated. The surface sterilized seeds were treated with seven isolated endophytic actinobacteria and three actinobacteria strains which were selected from the first experiment. The 2.5 liter pots were filled with 50:50 sand and

vermiculite mixture and autoclaved. The method of sowing, nutrient supply and watering was similar as described in section 2.4.3. The position of pots were changed randomly and incubated at 22℃ for six weeks in the glass house in the first week of August.

2.5.2 Harvesting the lentil plants and collection of data

After six weeks, lentil plants were harvested and sand and vermiculite were removed from the roots. The plants were stored in zip lock bags. Fifteen plants were selected for dry weight and other plants stored at -20℃. The method for data collection and data analysis was similar to that described in Section 2.4.5

2.6 Quantification of actinobacteria DNA in lentil plants

2.6.1 DNA extraction from soil, shoots and roots

DNA was isolated from rhizosphere soil (attached with roots), soil (at bottom of pot), roots and shoots of lentil plants at different period of growth to determine the quantification of actinobacterial strain CP200B in sample. The lentil plants were planted in glasshouse for 6 weeks as is described in pot experiment #2. The plants which treated with CP200B actinobacteria strain with Rhizobium and rhizobium only (control) were harvested after three and six weeks. The rhizosphere soil and soil were collected from pots with the plants samples. The plants were washed under running tap water completely remove sand from the roots. DNA was isolated from samples by using a modified Cetyltrimethylammonium bromide (CTAB) method. The roots and shoots were crushed using a sterile pestle and mortar with liquid nitrogen and ground to a fine powder. The samples included soil approximately 0.5g was transferred into 2 ml screw cap tubes and followed the CTAB method process described in 2.2.3.1. The concentration of DNA was quantified by Nano drop molecular calculator. The purity of DNA was examined by the ratio of 260/230nm and 260/280 nm. The quality of DNA was checked on agarose gel electrophoresis.

2.6.2 Quantification of CP200B by using qPCR

Quantitative Polymerase Chain Reaction (qPCR) is a technique that couples amplification of a target DNA sequence with quantification of the concentration of that DNA species in the reaction. Quantitative PCR was performed with 96 well plates (BioRad) in a CFX96 real-time qPCR. The reaction was performed with 1ul DNA, 2ul of diluted forward and reverse primer mix (5uM), 5ul Power SYBER® Green PCR Master Mix and 2ul water.

The qPCR reactions were performed as initial denaturation at 95℃ for 2 minutes, 40 cycles for 95℃ for 15 seconds, 60℃ for 1 minute. The melt curve started at 58℃for 10 seconds and 95℃for 15 seconds.

2.6.3 Data Analysis

The qPCR reaction of all samples was performed with one replicate. Improved precision in a qPCR experiment enables to discriminate smaller differences in nucleic acid copy numbers or fold changes. The reproducibility and assess precision can measure with replicates in experiments. All values entered in MS Excel spreadsheet. The data was statistically analysis to detect the significant result.

OBJECTIVE - 3

2.7 The effect of isolated endophytic actinobacteria on Drought stress in lentil plants

2.7.1 Pot Assay #3

In this Pot experiment, the aim was to put the plants under drought stress. Initially the same procedure described for pot assay #2 was followed. Treated surface sterilized seeds were planted into 1.25 liter size pots as 4 pots per treatment and 4 plants per pot in first week of August, 2019. The method of nutrient supply and watering was same as the second

experiment till four weeks. After that, the pots were not watered next ten days and then watered and the plants harvested fifteen days later.

2.7.2 Harvesting and data analysis

Seven and a half weeks after sowing (and fifteen days after the last watering) the plants were harvested. The plants were stored in zip lock bags. Ten plants were selected for dry weight and the other plants stored at -20℃. The method for data collection and data analysis was similar to that described in Section 2.4.5

2.7.3 RNA Extraction

Chemicals, materials and equipment (Appendix 4)

The frozen plant tissue (100mg) was added to a pre-chilled microcentrifuge tube to the mortar and grinded with pestle until no visible debris remained.

In the fumehood, 400 μL of TRIzol reagent was added to an Eppendorf tube. Then, frozen powdered plant was transferred to the TRIzol. Then 600μL of TRIzol reagent was added into eppendorf tube and vortex so that plant cells make contact with TRIzol. After that, the tubes were placed on ice on ice until all samples have been ground. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. The next step was centrifuged the solution at 12,000 x g for 10 min at 4ºC and transferred the supernatant to fresh Eppendorf tube. Chloroform 100ul per 1 mL of TRIzol reagent was added into tubes and shaken vigorously by hand for 15 seconds.

The tubes were placed at room temperature for 2-3 minutes, then centrifuged at no more than 12,000g for 15 minutes at 4ºC. After centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA has remained exclusively in the aqueous phase. The volume of the aqueous phase was about 60% of the volume of TRIzol reagent used for homogenization. The aqueous phase was transferred

to a fresh tube without disturbing the semi-solid interphase containing DNA and added 250µL of isopropanol and 250µL of high-salt precipitation solution to precipitate the RNA, mixed gently. Then, tubes were placed for 10 minutes at room temperature and after 2-3 minutes centrifuged at no more than 12,000g for 10 minutes at 4ºC.The supernatant was removed and washed the RNA pellet by adding 1 mL of 75% ethanol and vortex briefly (at least 1 mL 75% ethanol for 1 mL TRIzol reagent). Centrifuged was done at no more than 7,500g for 5 minutes at 4ºC. The RNA pellet was air dried for 5-10 minutes in the fume hood. It is not necessary to dry completely as this will make resuspending the RNA more difficult. At last, RNA pellet was dissolved in RNase-free water to the desired concentration and store at -80ºC. RNA was prepared for next process DNase treatment

2.7.4 DNase treatment

RNA was treated with DNase 1 (Sigma) (1unit per 2ug RNA) prior to cDNA synthesis following the manufacturer's protocol.

RNA (8ul), 10X Reaction Buffer (1ul) and DNase I (1ul) were added into RNase free PCR tubes and mixed gently. Then the mixture was incubated for 15minutes at room temperature. After that, 1ul of Stop solution was added into mixture to bind calcium and magnesium ions, and to inactivate the DNase I. Then, the tubes were incubated for 10 minutes at 70℃ to denature both DNaseI and RNA and tubes placed on ice.

2.7.5 cDNA synthesis

The ProtoScript First Strand cDNA synthesis kit was used to transcribe the DNase treated RNA into cDNA. A reaction mix is shown in Table 2.4

Table 2.4 Composition of reagents for one cDNA synthesis reaction

Reagents	Volume (ul)
10X RT Buffer	2.0
25X dNTP Mix(100mM)	0.8
Reverse Transcriptase	1.0
10X Random Primers	2.0
RNase Inhibitor	1.0
Nuclease free Water	3.2
RNA (DNase treated)	10

All reagents were added into tubes and mixed 10ul master mix into PCR eppendorf using pipette. Then 10ul of DNase treated RNA was added into each tubes and mixed the solution.

The cDNA reaction was carried out with 20ul reaction in Axygen Maxygen II PCR machine and the thermocycler PCR profile was set up according to protocol for reverse transcription reaction.

Table 2.5 PCR profile of cDNA synthesis

Temperature	Time (minutes)
25℃	10
37℃	120
85℃	5

The cDNA product was diluted 1:10 DEPC treated water and stored at -20℃

2.7.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to assess RNA quality: gels were made at 1%(w/v) agarose using 1X TBE buffer containing GelRed and the samples were prepared and loaded same as that described in Section 2.2.3.3

2.9.7 qRT-PCR

Quantification Reverse Transcription Polymerase Chain Reaction (qPCR) process The expression of gene of interest in treated and control lentil tissue was assessed using qRT-PCR. The method for qPCR described in 2. was followed in qPCR.

The thermal cycle conditions are 3 minutes of 95℃, followed by 40 cycls of: 95℃ for 5 seconds: 60℃ for 15 seconds. This was followed by a melt curve consisting of 5 second increaments of 0.5℃ from 65℃ to 95℃. Ct values were compared against that of the standard curve and normalized against the reference gene GADPH (Table 2.6) to calculate the normalized concentration of cDNA. All samples and standard were run in duplicate to measure the error, reproducibility and assess precision in experiment.

The reference gene primer and candidate gene primer for abiotic stress is showing in Table 2.6

Table 2.6 The candidate and reference genes used gene expression

No.	Genes	Abbreviations	Function
1	Glutathione peroxidase	GPX	ROS scavenging
2	Superoxide dismutase	SOD	ROS scavenging
3	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Reference gene

ROS scavenging

CHAPTER – 3
RESULTS

3.1 Isolation of endophytic actinobacteria from lentil plants.

The lentil plants were planted in seven different types of soil for isolation of endophytic actinobacteria. The growth of plants in all soils was good except Loxton and Roseworthy soils as no plants grew in either soil. The presence of residual herbicides in these soils may have prevented the growth of plants and lentil is extremely sensitive to residual herbicides. The water drainage capacity of both soils was very poor and lentil does not tolerate flooding or waterlogged soils.

A total of 165 plates of four isolation media were incubated for isolation of endophytic actinobacteria. Five endophytic actinobacteria colonies were observed during the third incubation week, 30 were collected in the fourth week and 19 in the fifth week of incubation and 8 colonies were obtained during eighth week (Figure 3.1). A total of 62 cultures were isolated from lentil plants over an eight-week period. However, due to time constraints fifty four cultures collected by week five were used for further investigation in this study.

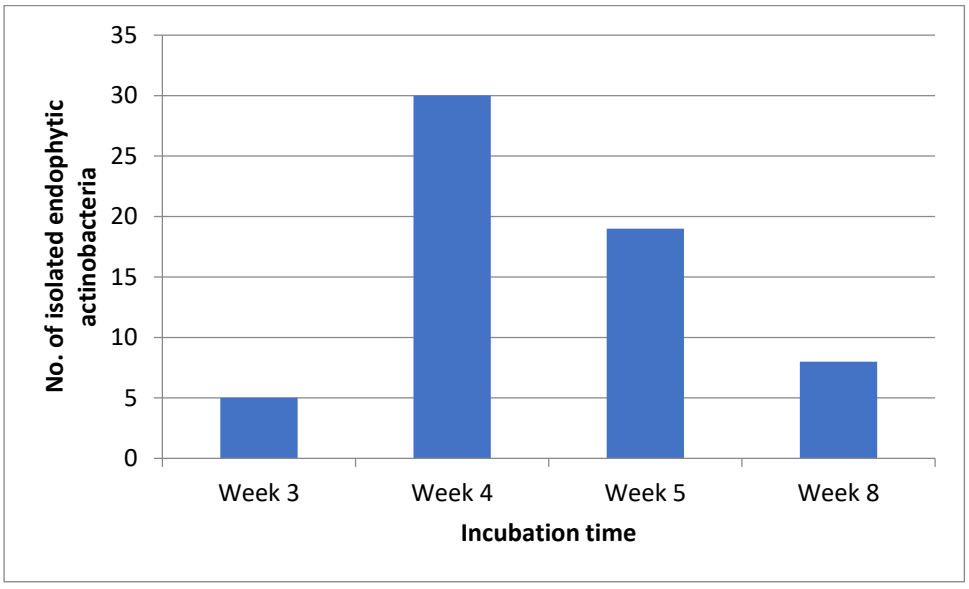


Figure 3.1 Number of total isolated endophytic actinobacteria colonies with incubation time.

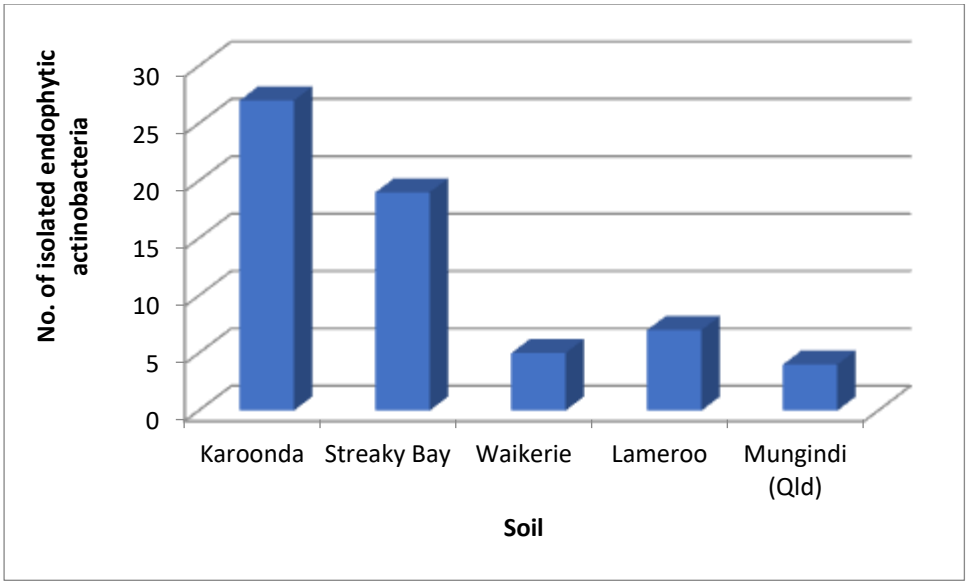


Figure 3.2 Number of total isolated endophytic actinobacteria colonies from lentil plants planted in different soils.

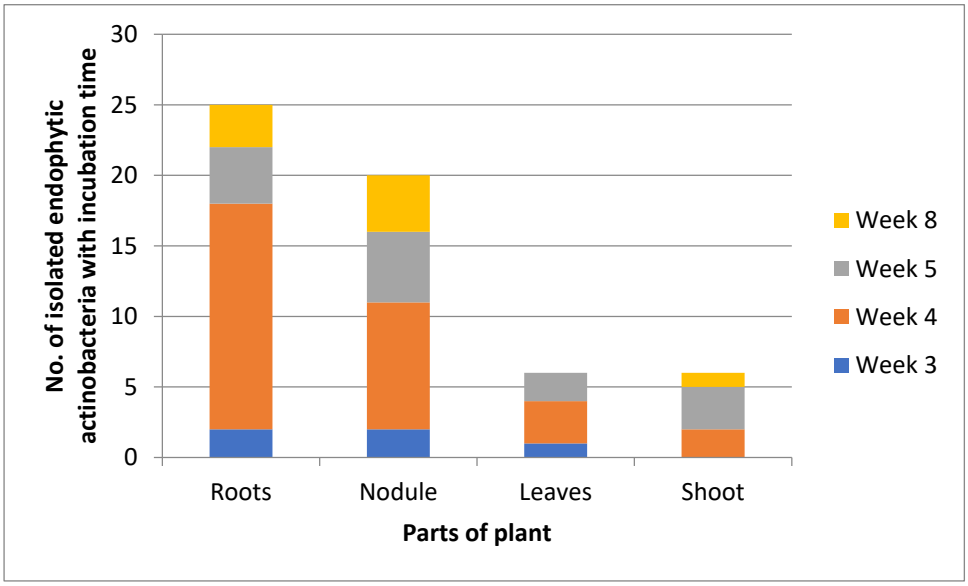


Figure 3.3 Number of total isolated endophytic actinobacteria colonies from parts of lentil plants with incubation time.

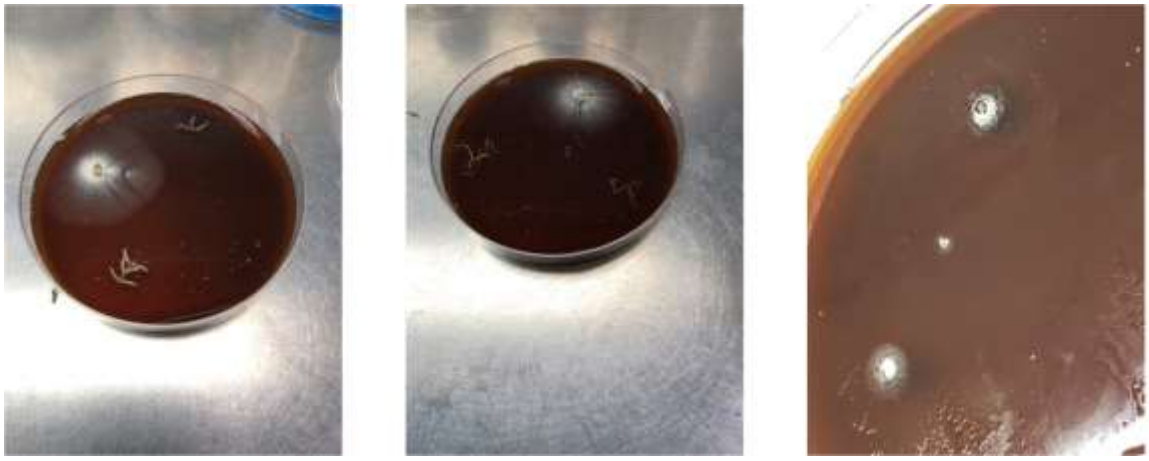


Figure 3.4 Isolated endophytic actinobacteria colonies from leaves, roots and nodules on HVA medium

Most of the endophytic actinobacteria were isolated from roots and nodule of lentil plants were 25 and 20 with respectively (Figure 3.3). Only six cultures were isolated from leaves and shoot which were very less as compare to roots and nodules.

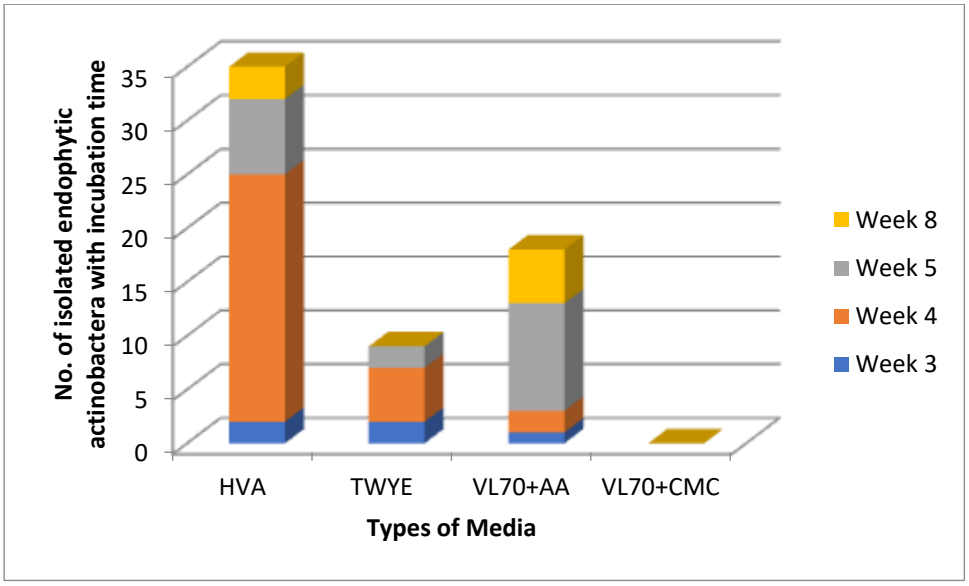


Figure 3.5 Number of isolated endophytic actinobacteria on different Isolation media with incubation time.

The majority of endophytic actinobacteria (n=35) were observed in HVA (Humic Acid

Vitamin B agar medium) and 9 and 15 cultures were isolated from TWYE (Tap Water Yeast Extract) and VL70+AA (VL70+Amino Acid) medium, respectively (Figure 3.5). No cultures grew on VL70 + Carboxy methyl Cellulose during the eight week incubation period.

3.2 Identification and characterization of isolated endophytic actinobacteria

Twenty one cultures were successfully recovered from fungal contamination and effect of non actinobacterial growth. Fifteen cultures out of twenty one produced spores after transferring to purification medium HPDA (Half-strength Potato Dextrose agar).

3.2.1 Morphology characterization

Based on their morphology, most cultures (66%) resembled *Streptomyces* sp. and four (26.6%) culture were *Propionibacteriales. Streptomyces*, strains were isolated during the third and fourth week of incubation. *Propionibacteriales* were isolated from the leaves and nodules during fifth week of incubation time. The population of endophytic actinomycetes was varied between tissue samples and incubation time. Mycelial fragmentation can be regarded as a special form of vegetative reproduction. Actinobacteria exhibit a wide variety of morphologies; differentiate on the basis of substrate mycelium or aerial mycelium, the colour of mycelium, the production of spore, and pigments.

Table 3.1 Growth and characterization of 19 endophytic actinobacteria on different medium (Figure 3.6)

No.	Culture	Growth, colour of spores /mycelium on different medium			
		HPDA	ISP3	ISP2	MS
1	LT 0	Moderate Pale Yellow/ Yellow	Week Off white/ Pale yellow	Good White/Dark Brown	Good White/ Dark Brown
2	LT1	Good White/Pale yellow	Moderate Grey/Black	Moderate Grey/Black	Good White/Orange
3	LT2	Good No spores /Light Brown	Moderate No spores/ off white	Moderate No spores/Light Brown	Good No Spores/ Light Brown
4	LT4	Week White/Red	Moderate Brown/Red	Week Brown/Red	Moderate White/Red
5	LT5	Good Brown/Red	Good Grey/ Dark red	Moderate Grey/Dark Red	Good Brown/Red
6	LT6	Week Light Red/White	Week White/White	Week White/White	Moderate White/Off White
7	LT8	Week Brown/Pale Yellow	Week White/Pale Yellow	Week White/Pale Yellow	Moderate Grey/Dark Grey
8	LT9	Good White/Pale Yellow	Moderate Pale yellow/ Whitish	Moderate Grey/Black	Good Grey/Black
9	LT10	Moderate Yellow/Yellow	Week White/Grey	Week White/Grey	Moderate White and Grey/Black
10	LT12	Good Pink / pale Yellow	Moderate White/Pale Yellow	Moderate White/Pale Yellow	Good Pure White/Yellow
11	LT13	Good White/Dark Grey	Good Brown/Off White	Moderate Grey/ Black	Good Pale Yellow/Brown

12	LT14	Week Red/Brown	Moderate Brown/Brown	Moderate Brown/Brown	Moderate White/Red
13	LT1B	Moderate Yellow/Orange	Moderate White/Pale Yellow	Week White/Orange	Moderate Pale Yellow/Orange
14	LT27	Moderate Pale Yellow/Pale Yellow	Week Pale Yellow/Pale Yellow	Week Pale Yellow/Pale Yellow	Moderate White /Pale Yellow
15	LT15	Good Grey/Black	Moderate White/Brown	Moderate White greyish /Brown	Good White/Grey
16	LT16	Moderate No spores/Pale Yellow	Week No spores/Pale Yellow	Week No spores/Pale Yellow	Moderate No spores/Pale Yellow
17	LT19	Week Pale Yellow/Pale Yellow	Week Pale Yellow/Pale Yellow	Week Pale Yellow/Pale Yellow	Moderate White/Pale Yellow
18	LT20	Week Pale Yellow/Pale Yellow	Week Pale Yellow/Pale Yellow	Week Pale Yellow/Pale Yellow	Moderate White /Pale Yellow
19	LT21	Week Pale Yellow/Pale Yellow	Week Pale Yellow/Pale Yellow	Week Pale Yellow/Pale Yellow	Moderate White/Pale Yellow

Figure 3.6 Morphological characterization of isolated endophytic actinobacteria from lentil plants on different medium

3.2.2 Characterization based on spore morphology

The microscopic studies of endophytic actinomycetes spore-bearing structures and structure of mycelium on agar plates provided a useful and rapid method for identification of their genus.

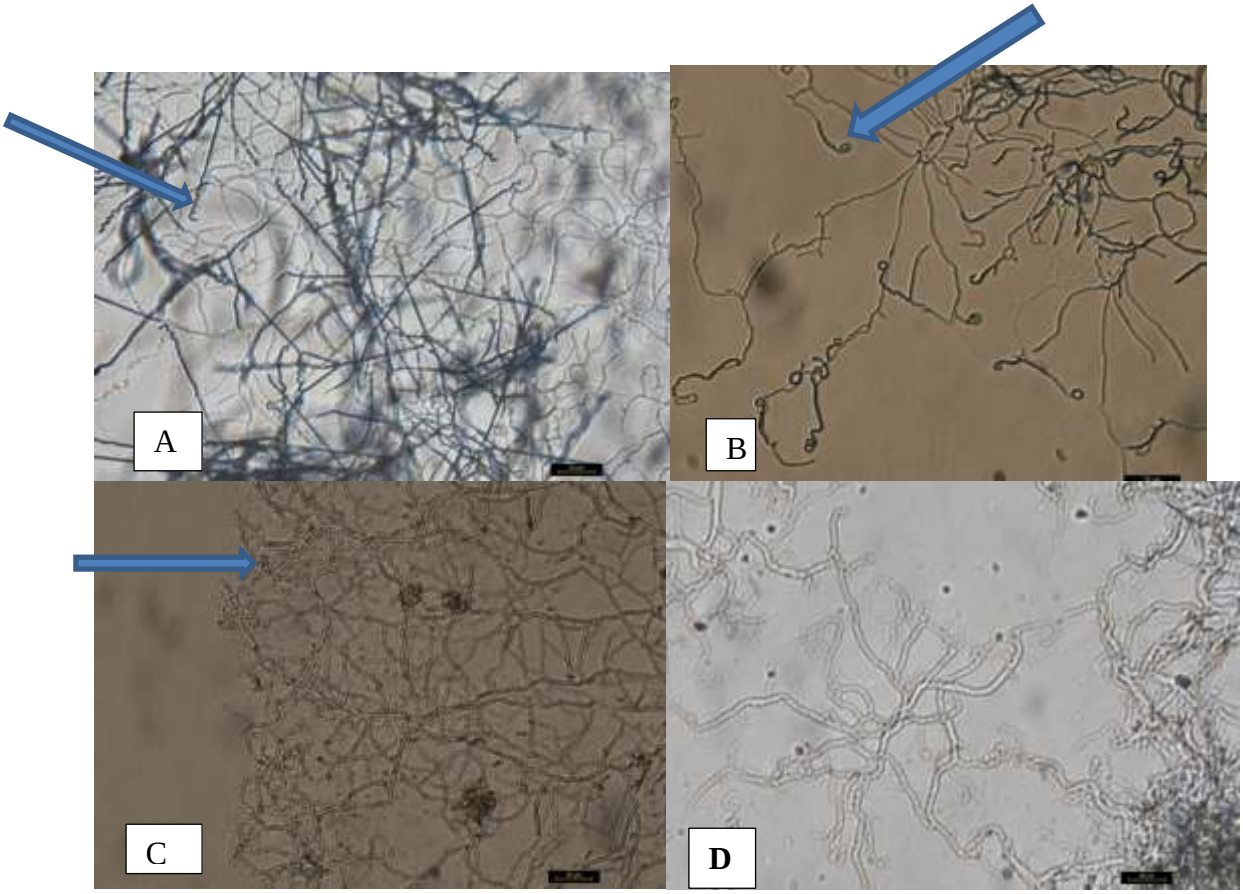


Figure 3.7 Endophytic actinobacteria strains observed under 40X magnification with light microscope and shown structure of mycelium and spore chain. (A) Open spiral spore chain observed in *Streptomyces* (LT5) and (B) Closed spiral shown in *Streptomyces* LT1; (C) *Micromonospora* LT 9; (D) *Streptomyces* LT8

Actinobacteria exhibit a wide variety of morphologies, including coccoid (*Micrococcus*) and rod-coccoid (*Arthrobacter*), as well as fragmenting hyphal forms (*Nocardia* spp.) and also forms with permanent and highly differentiated branched mycelia (e.g., *Streptomyces* spp., *Frankia*. The number of spores per spore chain varies widely from genus to genus. The genera *Micromonospora, Salinispora, Thermomonospora, Saccharomonospora,* and *Promicromonospora* produce isolated spores, while *Microbispora* produces spores in longitudinal pairs. Members of the genera *Actinomadura, Saccharopolyspora, Sporicthya,* have short spore chains, while members of the genera *Streptomyces, Nocardioides, Kitasatospora, Streptoverticillium,* and some *Nocardia* spp. produce very long chains of up to 100 spores. s. In contrast, *Frankia* species produce sporangia, which are essentially bags of spores. *Streptomycetes*' spore chains can be classified as being straight to flexuous (Rectus-Flexibilis) (Barka et al., 2016). The spore chain hooked to looped; straight to flexuous, long chains of smooth sores and spiral spore chain are morphological characteristics of *Streptomyces* (Lima et al., 2017)

3.2.3 Identification of endophytic actinobacteria isolated from lentil by 16S rRNA gene amplification and sequencing.

Table 3.2 shows the BLASTN results of the partial sequencing of well performing strains. Strain LT0 and LT5 and LT10 are Streptomyces spp. and have 16S rRNA gene sequence similarities to their closest type strains at over 99.50%. LT19 and LT22 are closest to *Kribbella catacumbae* strain BC631 with 98.05 and 99% respectively. LT11 has the closest similar to *Streptomyces globisporus* strain MAR12_4A (100%). *Streptomyces yeochonensis* strain is closest type strain of LT7.

Table 3.2 The 16S rRNA gene sequence similarity of selected actinobacteria with their closest type cultures.

No.	Isolates	Close type strain	Similarity %	Accession no.
1	LT0	*Streptomyces lienomycini* NBRC15425	99.70%	NR_112464
3	LT5	*Streptomyces galilaeus* strain NBRC 13400	99.5%	NR_112389
5	LT7	*Streptomyces yeochonensis* NBRC100782	98%	NR_112596
6	LT10	*Streptomyces libani* strain DSM 40555	99%	NR_117952
8	LT11	Streptomyces globisporus strain MAR12_4A	100%	MN419232.1
9	LT19	*Kribbella catacumbae* strain BC631	98.5%	NR_042657
10	LT22	*Kribbella catacumbae* strain BC631	99%	NR_042657

3.3 Effects of endophytic actinobacteria strains on the growth of lentil plants (Pot Experiment # 1)

The plants germinated within a week during the winter weather and were harvested 6 weeks after sowing. The number and colour of nodules were also recorded for the treated plants (Figure 3.8).

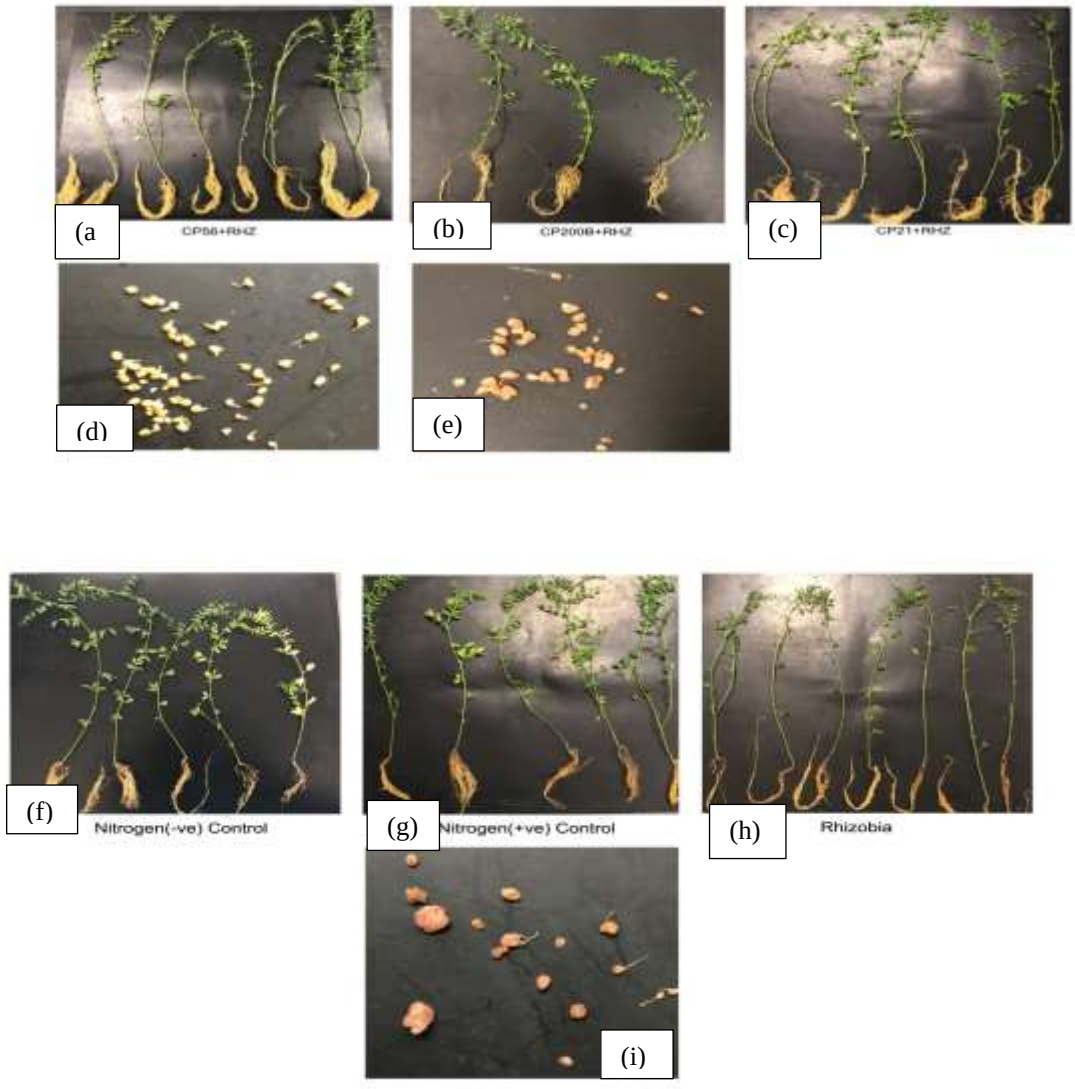


Figure 3.8: Washed lentil plants treated with actinobacteria, rhizobium and control after 6 weeks growth and their nodules. (a) CP56 + Rhz: Washed lentil plants and roots. (b)

CP200B + Rhz: Washed lentil plants and roots. (c) CP21 + Rhz: Washed lentil plants and roots. (d) CP56 + Rhz: Medium sized and white nodules. (e) CP200B + Rhz: Large and red nodules. (f) Nitrogen (-ve) control: Washed lentil plants and roots (g) Nitrogen (+ve) control: Washed lentil plants and roots. (h) Rhizobia: Washed lentil plants and roots. (i) Nitrogen (+ve) control: Large and red nodules.

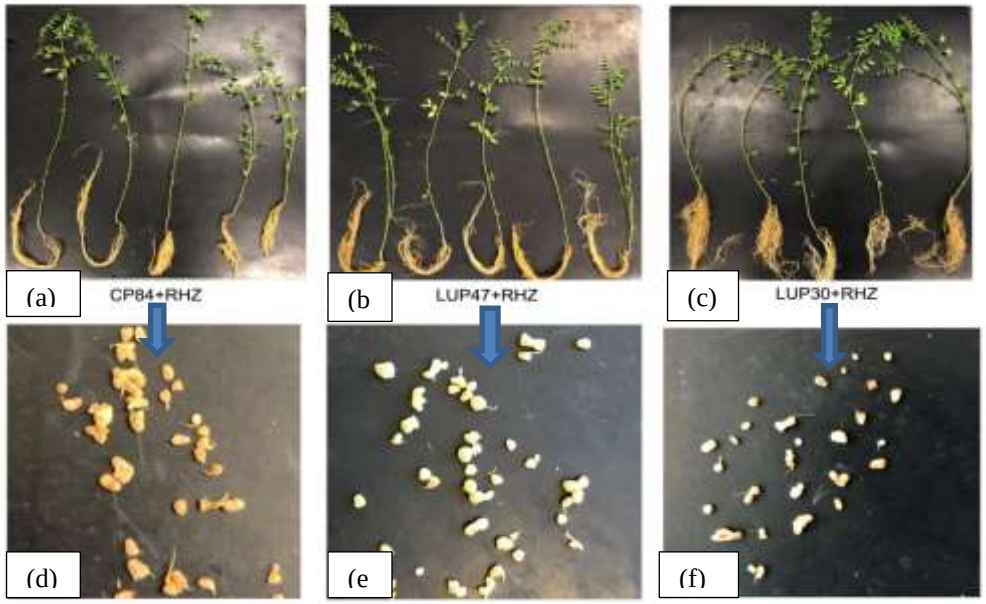


Figure 3.9: Washed lentil plants treated with actinobacteria and rhizobium after 6 weeks growth and their nodules (a) CP84 + Rhz: Washed lentil plants and roots. (b) LUP47 + Rhz: Washed lentil plants and roots. (c) LUP30 + Rhz: Washed lentil plants and roots. (d) CP84 + Rhz: Medium sized and red nodules. (e) LUP47 + Rhz: Medium sized and white nodules. (f) LUP30 + Rhz: small and white nodules

The treatment of lentil plants by different actinobacterial strains affected the number and colour appearance of nodules which was observed different in all treated plants. As shown in Table 3.1

the lentil plants treated with strains CP56 treatment (52/plant), Lup30 (46/plant), and CP21 (32/plant) had a high number of nodules but the nodules were medium sized. In contrast, the N positive control and CP200 treated plants produced bigger nodules which were red colour but with a lower number of nodules. However, the dry weights of their nodules were significantly higher than all the other treatment plants. The nodules produced by CP 84-treated plants were light red and medium sized but dry weight of the nodules (0.13 mg/plant) was much lower compared to N the positive treatment. Rhizobia, Lup30 and CP21treated plants produced very small sized and white colour nodules. Nodules of all treated plants were hard and rigid structure but nodules of Lup30 were small, round and composed with milky substance type liquid.

Table 3.2 Effect of endophytic actinobacteria strains on the growth (shoot and root length and weight) and nodulation (number and dry weight) of lentil plants inoculated with *Mesorhizobium ciceri* strain WSM 1455; harvested after six weeks. (n = 4 pots/treatment, 4 plants/pot). DW=Dry weight, RHZ = Rhizobium strain *Mesorhizobium ciceri* WSM 1455, N(-ve) = Nitrogen negative control without added nitrogen supply, N+ve = Nitrogen positive control added nitrogen supplement after week.

Treatment	Shoot length (cm)	Root length (cm)	Shoot Dry Weight (mg DW/plant)	Root Dry Weight (mg DW/plant)	Nodule No. (No./plant	Nodule Dry Weight (mg DW/plant)
N(-ve)	30.7^{b}	21^{b}	110^{d}	38.4^{b}	21.5^{de}	0.12^{b}
N(+ve)	33.5^{ab}	17.7^{bc}	263^{a}	48^{ab}	12.1^{e}	0.22^{a}
R only	33.5^{ab}	16.1^{bc}	168^{bcd}	46^{bc}	12.1^{e}	0.10^{b}
R+CP56	32.5^{ab}	17.9^{bc}	179^{bcd}	48^{ab}	52.1^{a}	0.20^{b}
R+CP200B	31.5^{ab}	16.1^{bc}	220^{abc}	43^{b}	30.5^{cd}	0.20^{b}
R+CP84	31.0^{b}	17.7^{bc}	147^{cd}	54^{ab}	$36.\ 8^{bc}$	0.13^{b}
R+CP21	36.2^{a}	19.8^{bc}	240^{ab}	60^{a}	34.2^{bcd}	0.12^{b}
R+Lup47	32.6^{ab}	28^{a}	207^{bc}	57^{ab}	37.0^{bc}	0.17^{b}
R+Lup30	33.1^{ab}	14.2^{c}	181^{bcd}	31.7^{b}	46.1^{ab}	0.15^{b}

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 difference in means determined using Duncan test.

In the pot experiment, the shoot length of all treatment and rhizobium only were greater than N negative control plants but the root length of N negative control plants was greater than the rhizobium only and N positive control (added N supplement after every week). The shoot dry weight of all treatment and rhizobium only were greater than N negative control plants but lower than N positive control plants. In Figure 3.4 average shoot dry weight of CP200B (220 mg/plant) and CP21 (240 mg/plant) was greater than other treatments and close to dry weight of N positive (263 mg/plant). Root dry weight of CP21 (60 mg/plant) was also the highest among all treatments.

Endophytic actinobacteria strains of chickpea have shown positive effect on the nodulation process and growth of lentil. CP200B, CP21 and CP56 were selected for further investigation as they increased the dry weight of shoot, root and nodules.

3.4 Effect of isolated endophytic actinobacteria on the growth of lentil

3.4.1 Pot Experiment #2

In the second pot experiment, seven isolated endophytic actinobacteria were examined for the growth of lentil in sand and vermiculite experiment. There was no significant difference observed visually with all treatments as shown in Figure 3.10 and 3.11 of lentil plants except the result of endophytic actinobacteria L9 strain. The growth of L9 treated plants was poor and roots were very short and diseased as black colour. The moisture content of sand and vermiculite mixture was reduced as compared to Pot Assay #1 after one week of planting which was suitable for lentil plants. All the treated plants germinated within three days of planting. The growth of plants was much better than those in Pot Experiment #1. In this experiment, with newly isolated strains, the treated plants produced flowers and pods. The colour of nodules was red, light red and pink but the number of nodules was less than observed in the first experiment. Nitrogen negative control plants produced a small number of nodules in Pot Experiments 1 and 2 without inoculating with rhizobial suspension. A

Rhizobial suspension was inoculated in most pots in the glass house and cross contamination may have been the cause of producing nodules.

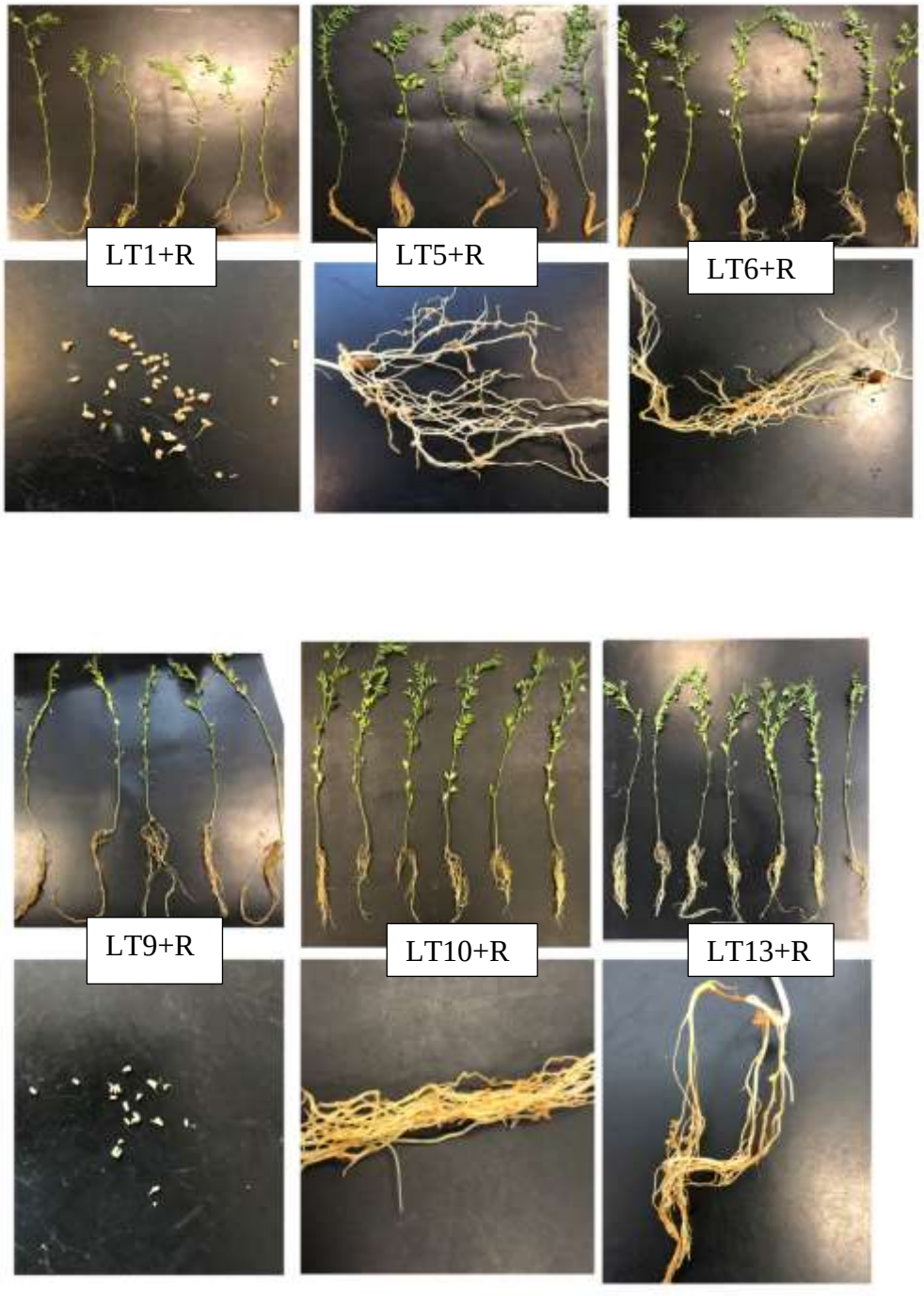


Figure 3.10 Washed lentil plants treated with actinobacteria, rhizobium and control after 6 weeks growth and their nodules and roots with nodules.

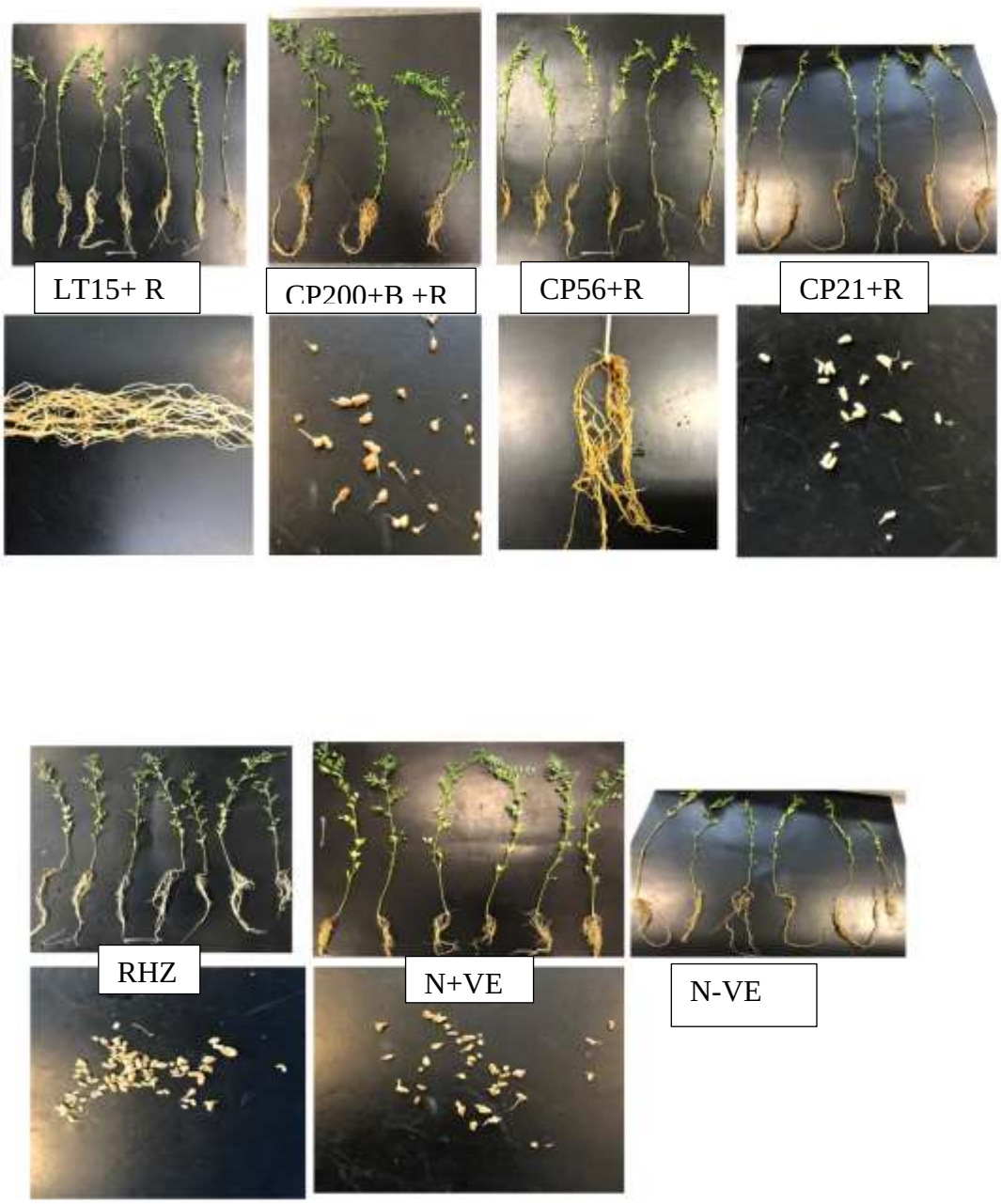


Figure 3.11 Washed lentil plants treated with actinobacteria, rhizobium and control after 6 weeks growth and their nodules.

Table 3.3 Effect of isolated endophytic actinobacteria strains on the growth (shoot and root length and weight) and nodulation (number and dry weight) of lentil plants inoculated with *Mesorhizobium ciceri* strain WSM 1455 and harvested after six weeks. (n = 5pots/treatment, 6 plants/pot). DW=Dry weight, RHZ = *Mesorhizobium ciceri* WSM 1455. The highlighted values indicated the significant value.

Treatment	Shoot Length (cm)	Root Length (cm)	Shoot Dry weight (mg)	Root Dry Weight (mg)	Nodule No. (no./plant)	Nodule Dry Weight (mg)
N +ve	27.13^{a}	10.67	88^{efg}	19^{b}	8.13^{fg}	1.31^{ef}
N –ve	20.90^{efg}	13.07	79.3^{fg}	17^{b}	3^{g}	0.23^{g}
LT1+Rhz	25.47^{abc}	10.40	110^{cde}	11.8^{c}	14.73^{cd}	3.22^{bc}
LT5+Rhz	25^{abcd}	8.13	141.3^{bc}	18.6^{b}	18.40^{bc}	4.6^{a}
LT6+Rhz	28^{a}	8.10	194^{a}	20.8^{b}	25.6$_{a}$	3.35^{bc}
LT9+Rhz	19.20^{g}	8.33	74^{g}	8.6^{c}	6.67^{fg}	0.74^{fg}
LT10+Rhz	25.33^{abc}	8.93	155.3^{b}	18.1^{b}	20.40^{b}	2.57^{cd}
LT13+Rhz	26.20^{ab}	10.27	196.6^{a}	18.4^{b}	19. 87^{b}	2.77^{bcd}
LT15+Rhz	26.20^{ab}	13	104.6^{cde}	22.6^{ab}	7.60^{fg}	1.21^{efg}
CP200+Rhz	22.13^{def}	11.90	132.63^{bcd}	19.8^{b}	15^{cd}	4^{a}
CP56+Rhz	23.27^{bcde}	12.60	111.4^{cde}	22.93^{a}	13.73^{de}	2.40^{cd}
CP21+Rhz	22.40^{cdef}	14.67^{a}	117.3^{cde}	21.2^{ab}	14.67^{cd}	1.92^{de}
R hz	25.67^{ab}	11.00^{bcd}	132.6^{bcd}	27.06^{a}	19.20^{b}	2.7^{bcd}

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

In comparison to the Rhizobium only control there was no significant difference observed in the length of shoot and root of all plants but the dry weight of shoot was different with some treatments. The dry weight of LT13 treated plants was the highest followed by LT6 whereas LT9 showed a significantly lower shoo dry weight (Table 3.3). Dry weight of root of all plants was greater than N+ve and N-ve control plants but less than Rhizobium HZ only treated plants. The highest number of nodules was produced by LT6 treated plants. LT10, LT5 and LT13 treated plants which produced large number of nodules as compare to other plants (Figure 3.10 and 3.11). Dry weight of nodules of LT5 and CP200 were 4.6mg/plant and 4mg/plant, respectively (Table 3.3). However, dry weight of all samples of LT9 treated plants was less than N-ve (no Nitrogen supply). Result for chickpea strain (CP) treated plants was similar to the previous pot experiment but growth of other plants treated with lentil strains except LT9 strain was better in this pot experiment. However, nodule dry weight of CP200B treated plants was higher than similarity treated plants in Pot Experiment #1. Lentil endophytic actinobacteria strains have shown potential effect on the growth of lentil plants.

Scatter plots of the nodule weight versus either shoot dry weight (Figure 3.7) or total plant dry weight (Figure 3.8) showed a poor correlation between these parameters.

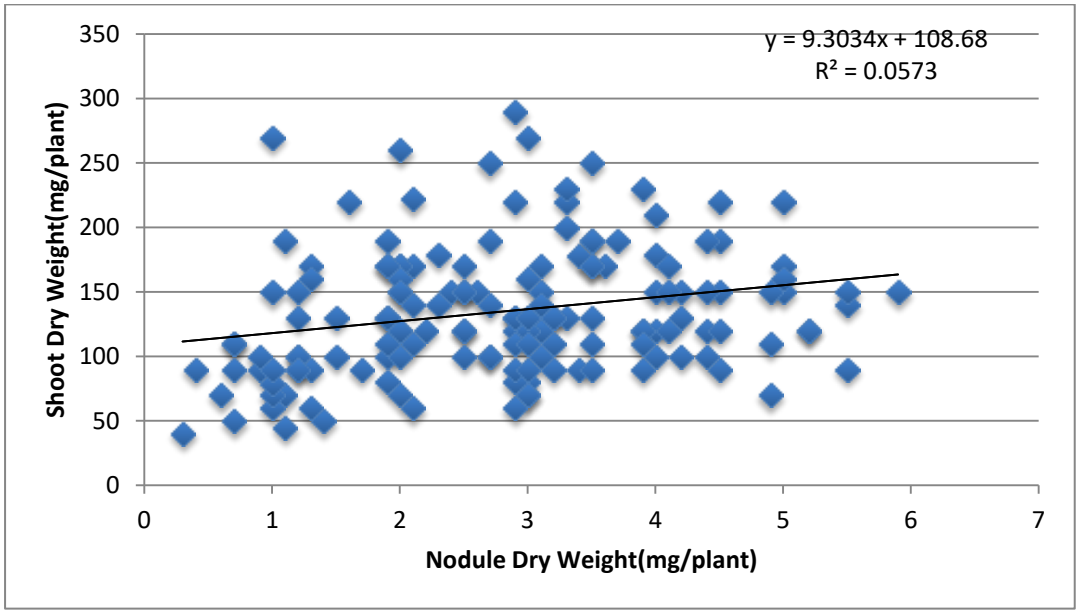


Figure 3.12 Correlation between Shoot dry weight and Nodule dry weight of isolated endophytic actinobacteria treated plants and Rhizobia only control

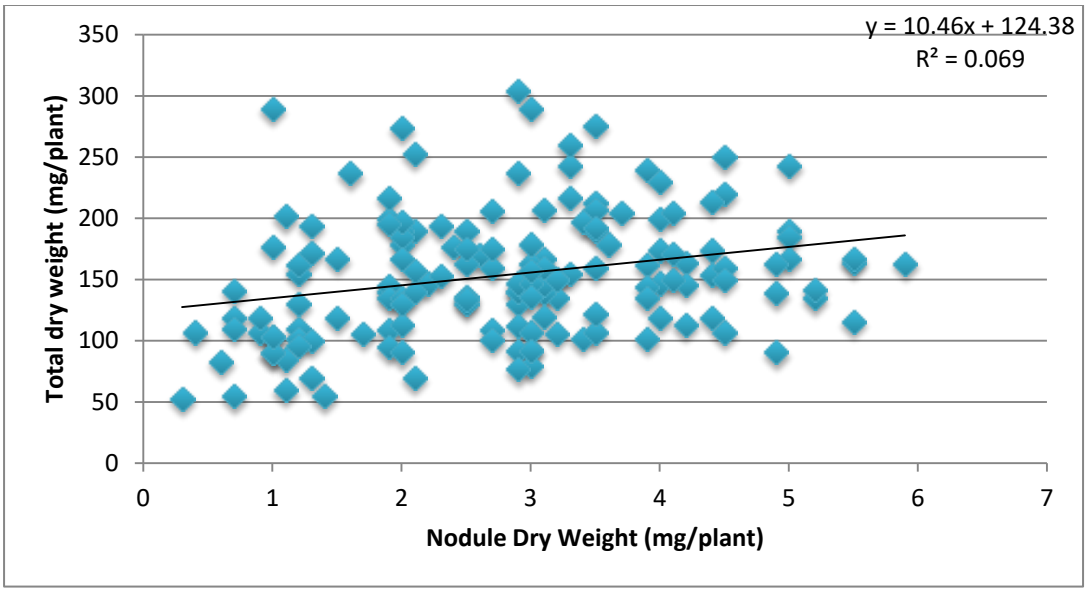


Figure 3.13 Correlation between Total dry weight of plant and Nodule dry weight of isolated endophytic actinobacteria treated plants and Rhizobia only control.

3.4.2 N content in lentil plants

The dried plants of lentil which inoculated with CP200 and Rhizobia only were oven dried basis for 48 hours at 80℃. Then shoots were crushed into sterile pestle and mortor to make finely grounded powder. The grinded powder was added (2 gm) into 50ml eppendorf tubes with one replicate and send the samples to determine the nutrogen content to Apal Australian precision Ag laboratory. The finely ground dry samples were analysed by the Dumas method. The Dumas metho**d** in analytical chemistry is a method for the quantitative determination of nitrogen in chemical substances based on a method first described by Jean-Baptiste Dumas in 1826.

Table 3.7 Nitrogen content detected in the lentil plants inoculated with CP200 and Rhizobia (control) amd (R) = one replicate of sample

Sr. No.	Sample	N content %	Total Shoot N content
1	Rhizobia only	1.91	37.71
2	Rhizobia only (R)	1.85	36.63
3	CP200B+ Rhizobia	2.08	44.09
4	CP200B + Rhizobia (R)	2.04	43.24

There was no significant difference in the nitrogen content in CP200B and rhizobia only treated plants. The nitrogen content in CP200B treated plants was more than 2%. For analysis of N content, 2 gm sample require with one or two replicates. The amount of other treated plants was not enough for replicate to analysis the nitrogen content.

3.5 Quantification of CP200B in the root, shoot and soil of CP200B treated plants.

Dilution series were prepared using pure culture DNA of CP200 endophytic strain and quantified. A standard curve was potted using the log concentration of dilutions value and Ct value from qPCR. This standard curve was used to quantify the DNA concentration present in rhizosphere soil, soil, root and shoot.

Table 3.5 Log DNA concentration and Ct values of dilution series from CP200B

DNA Dilution	**Log10 DNA amount (ng)**	**Ct value**
1	0.99	19.31
2	0.67	24.42
3	0.39	31.4

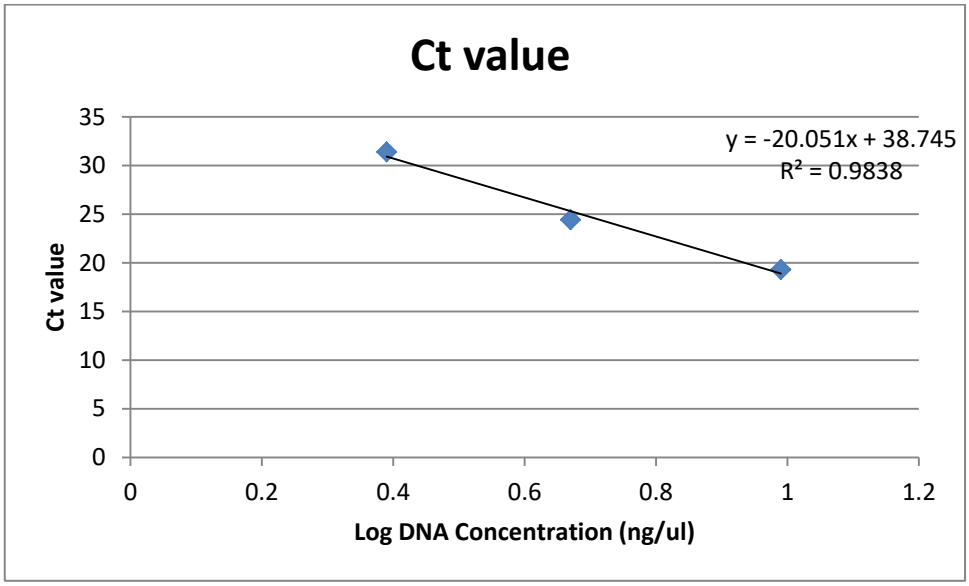


Figure 3.Standard curve of log DNA concentration ng/ul and Ct values for three dilution series.

DNA from eight samples: rhizosphere sand, bottom sand, shoot and root of CP200B treated lentil plants at different growth period were tested against standard dilutions in replicates using specific primer set of CP200B. Primer sequence: 139 bp size

Forword primer – 5'-TCCACTTCATCCCCGCCATGCT

Reverse primer - 5'-ACGTCGAGCAGGTCGCGGAAGT

The presence of strain CP200B in the roots and shoots of three week and six week were detected (Table 3.5). The shoots of three and six weeks resulted similar DNA concentrations. DNA concentration in roots of 6 weeks was more than roots of three weeks. DNA from Rhizobia treated plants was used as control and did not detect presence of strain CP200B.

Table 3.6 DNA concentration of CP200B endophytic actinobacteria strain in the plants treated with CP200B after 3 and 6 weeks.

Samples	Ct value	DNA concentration (ng/ul)
Rhz soil(3)	Nil	-
Soil (3)	Nil	-
Shoot(3)	26.77	0.77
Root(3)	24.26	0.87
Rhz soil(6)	Nil	-
Soil (6)	Nil	-
Shoot(6)	26.79	0.75
Root(6)	23.59	0.90

Note : Rhz soil (3) – Rhizosphere soil after 3 weeks and (6) = the samples collected after 6 weeks.

3.6 The effect of endophytic actinobacteria on drought stress in lentil plants

3.6.1 Pot Assay#3

The phenotypic apperance of the lentil plants was scored under drought stress treatment with 6 endophytic actinobacteria treated plants of perfect health and 4 representatives and control were shown dry plants. The leaves of LT1, LT5, LT6, LT10, CP200 and CP56 strains treated plants were still green and producing flowers and pods. Leaves of LT9, LT13, LT15 and CP21 treated plants turned pale yellow and mature leaves were turning to yellow. Mature leaves of N+ve and Rhizobia only control plants started to dry. The number of flowers. and pods in healthy plants were greater than LT9, LT15,CP21 and control plants.

RHZ

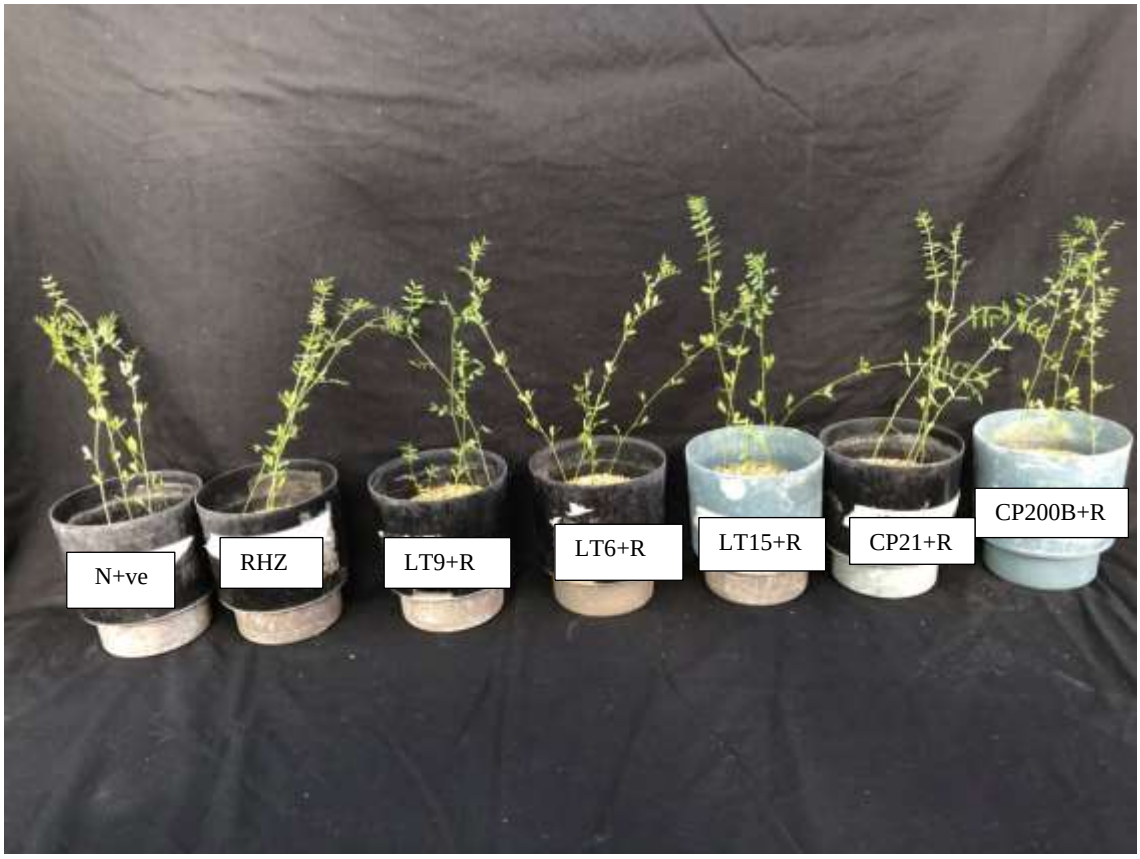


Figure 3.15 Lentil plants treated with endophytic actinobacteria after 10 days interval of watering

Table 3.9 Observed number and size of pods produced by lentil plants in pot experiment #3 and dry weight

Treatments	Average no of pods	Size of seeds	Average dry weight of pods (mg)
LT1	1.6	Large	21
LT5	1.26	Medium	15
LT6	1.13	Medium	13
LT9	1.13	Small	14
LT10	1.2	Large	25
LT13	1.73	Medium	24
LT15	0.9	Small	17
CP21	1.33	Medium	14
CP200B	1.53	Medium	17
CP56	1.32	Medium	16
N-ve	0.46	Small	9
N+ve	0.33	Small	12
RHZ	1.2	Medium	14

Two seeds per pod were observed in treated plants but there is a significant difference in the size and dry weight of pods. The size of LT1 and LT 10 were large and maximum dry weight than other plants.

Table 3.8 Observed drought stress effect on the growth of (shoot and root length and weight) endophytic actinobacteria strains treated lentil plants inoculated with *Mesorhizobium cicero* strain WSM 1455 and harvested after 7 weeks. (n = 5pots/treatment, 6 plants/pot). DW=Dry weight, RHZ = *Mesorhizobium cicero* WSM 1455**.** The highlighted values indicated the significant and maximunm value amont all treatment.

Treatment	Shoot Length(cm)	Root Length(cm)	Shoot Dry Weight (mg)	Root Dry Weight(mg)
LT1	29.20^{a}	14.20^{abc}	124^{ab}	17^{cd}
LT5	29.60^{a}	17.00^{a}	112^{abcd}	20^{bc}
LT6	29.60^{a}	19.20^{a}	120^{abc}	35^{a}
LT9	22.60^{b}	10.20^{bc}	90^{bcd}	12^{d}
LT10	30.20^{a}	18.00^{a}	130^{a}	37^{a}
LT13	29.70^{a}	9.80^{c}	124^{ab}	12^{d}
LT15	29.00 ^{a}	9.40^{c}	94^{bcd}	21^{bcd}
CP200B	28.60^{a}	17.40^{a}	120^{abc}	22^{bcd}
CP21	29.60^{a}	14.20^{abc}	118^{abcd}	27^{abc}
CP56	28.60^{a}	16.20^{ab}	106^{bc}	32^{ab}
N+ve	23.20^{b}	13.90^{abc}	82^{cd}	18^{bcd}
RHZ	26.20^{a}	17.40^{a}	115^{abc}	30^{abc}

Values with in column of different superscripts indicate a significant difference between groups (P <

0.05). Data was analysed using one-way ANOVA and difference in means determined using Duncan test.

In pot assay#3, pots were watered after 10 and 15 days interval. The growth of all plants was similar except LT9 treated and N+ve control plants as observed shoot and root length. However, a significant difference in dry weight of shoot and root was determined the effect of drought stress on endophytic actinobacteria strains treated plants (Table 3.6). The dry weight of root and shoot of L10 treated plants was the highest 130 mg/plant and 37 mg/plant with respectively among all treated plants. After that, LT 6 has maximum dry weight of root (35mg/plant) followed by CP56 (32mg/plant) and RHZ only (30mg/plant). As previous result of pot experiment, dry weight of shoot and root of L9 treated plants was similar to the N-ve control which was the minimum value among all plants. Overall, LT10 and LT6 strains have improved drought stress tolerance in lentil plants.

3.6.2 Quantitative Reverse Transcriptase PCR(qRT-PCR)

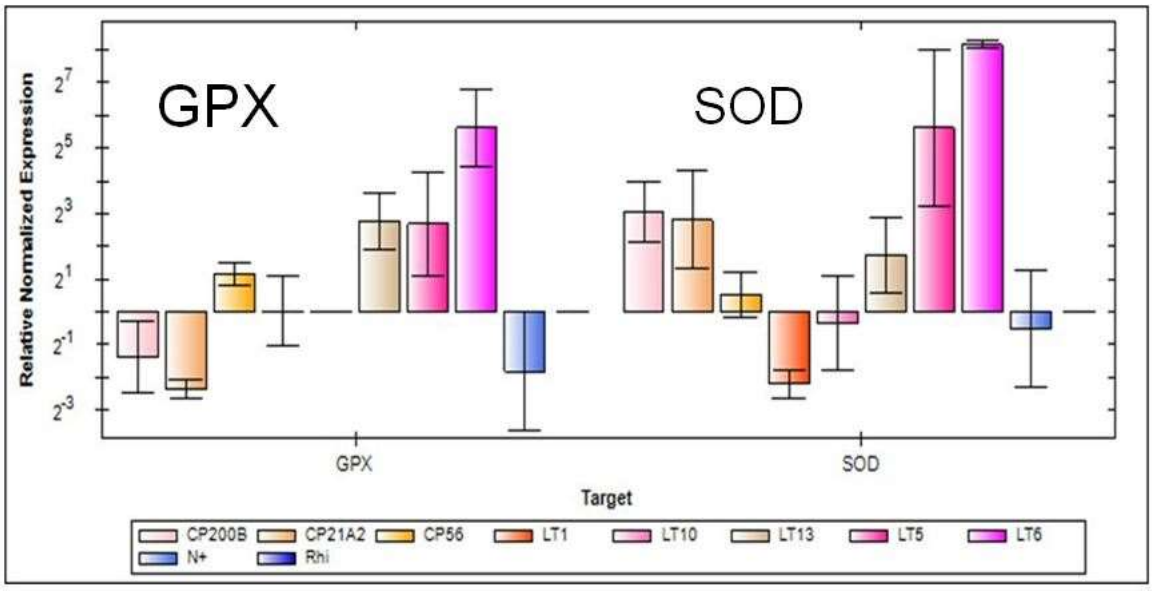


Figure 3.16 Normalised expression changes in candidate gene expression GPX and SOD against reference gene (GADPH) between control and actinobacteria strains treated lentil plants.Six weeks old lentil leaves treated with endophytic actinobacteria strain and two

control rhizobia and N+ve = Nitrogn added after week. Leaf sample were grinded in liquid nitrogen. RNA was extracted from frozen leaf tissue sample and cDNA synthesised prior to qRT-PCR analysis. Normalised expression has been compared to its control and actinobacteria treated lentil plants.

CHAPTER - 4
Discussions

Streptomyces was the dominant genus isolated among the endophytic actinobacteria during fourth week of incubation followed by and *Propionibacteriales.* Studies of isolation of endophytic actinobacteria from lucerne reported that 56% were classified as *Streptomyces* and 24% belongs to genus *Microbispora* and 9% was *Micromonospora*. *Streptomyces* was almost double the number of non-*Streptomyces* isolated from roots (Le et al., 2016). Ecologically, actinobacteria and, particularly, the *Streptomyces* spp. are generally saprophytic, soil-dwelling organisms that spend the majority of their life cycles as semi-dormant spores (Mayfield et al., 1972). It has also been demonstrated that actinobacteria inhabit the rhizosphere of many plant species, including cereal crops such as wheat.

Effects of *Propionibacteriales* strains were not treated on the lentil plants because these strains were obtained after the fourth week of incubation. Endophytic colonization by actinobacteria can occur in any part of plant niches such as leaves (phylloplane), fruits (carposphere), roots (rhizoplane) and seeds also. Reported studies stated that majority of endophytic actinomycetes were isolated from roots (52.38%) followed by stem (21%), leaf (14%) and flower (7%) of seven medicinal plants (Passari et al., 2015). Similarly, Kaur et al. reported that 50% endophytic actinobacteria out of 62 cultures were isolated from roots of seven different plants which were greater than other parts of plants (Kaur et al., 2013). These studies confirmed that roots are the most preferred niche for isolation of endophytic actinobacteria.

Most endophytic actinobacteria were isolated from HVA medium during isolation of endophytes from chickpea root, wheat root tissues (Coombs and Franco, 2003) and lucerne (Le et al., 2016). These studies stated that HVA medium is most effective agar medium for isolation of endophytic actinobacteria. Moreover, HVA medium provided the least contamination and isolated actinobacteria were successfully recovered. Actinobacteria endophytes could grow out of tissues before fungi overgrew the samples and masked the

actinobacteria (Qin et al., 2009). However, VL70+Carboxy methyl cellulose yielded the highest number of *Streptomyces* and non-*Streptomyces* isolates from native apricot plant (Kaewkla and Franco, 2013). The highest contamination was observed in TWYE medium and VL70+AA. Non actinobacteria effected the growth of endophytic actinobacteria and therefore only few actinobacteria were recovered in both medium.

The growth of plants in pot experiment #2 (August, 2019) was better than pot experiment #1 (May, 2019). The plants grew above the surface of the sand after one week of planting in pot experiment #1. In pot experiments #2 and #3, the moisture content was reduced during the first week and the plants emerged in three days. Temperature and moisture content significantly influence the growth and developmment of crop. Solar radiation, temperature, soil mositure content and sunshine hours are important for crop life cycle (Satyendra et al., 2016). In three pot experiments, dry weight of N+ve control (added Nitrogen every week) produced more than no added nitrogn (N-ve control) which provided least growth. These results indicate the experimental system of sterile sand and vermiculite had nitrogen as primary limiting factor for the growth and development of lentil plants. The N+ve control produced red colour and bigger size nodules and N-ve produced a small number of nodules in both pot experiments even though no Rhizobia suspenion was added into either control plants. The difference between colour and number of nodules in both control plants described that rhizobia provided by another source such as seed but still does not explain the size of nodules.

Strain CP200B, LT1, LT5, LT6 and LT2 have shown better growth as maximum dry weight of plant and produced red and light pink colour of nodules. Red and pink colour nodules are effective and fixing nitrogen. These strains are able to incresase the nitrogen fixation in lentil crop. There was a difference between the numbers and dry weight of nodules of in all treated plants and varied with each treatment of endophytic actinobacteria strain. Some strains of

Streptomyces treated plants produced a lower number of nodules but the dry weight was higher than those nodules which were produced in larger numbers by other plants. Some strains treated plants have shown good growth as produced more dry weight of shoot. Recent study stated that actinobacterial genus, *Micromonospora* increase the nodulation process of alfalfa crop and some of their strains can significatnly promote the growth and nutrition of N_2 fixing plants (Martínez-Hidalgo et al., 2014). Correlation between total dry weight of plant and nodule presented that some strains increased the dry weight of nodules but dry weight of plant is minimum. However, LT6 isolated endophytic strain has shown good resullt in nodulation process and growth of plant.

CHAPTER - 5
Conclusions and Future Directions

5.1 Conclusions

The result of this hypothesis is that some endophytic actinobacteria strains from lentil and other legumes can increase nodulation and growth of lentil when co-inoculated with rhizobia. LT6, LT10, LT5 endophytic actinobacteria isolated from lentil and CP200B (previously isolated from chickpea), when co-inoculated with its rhizobial partner *Mesorhizobium ciceri* significantly increased the nodulation process in lentil plants. CP56 (chickpea endophytic actinobactria strain), LT13 and LT15 were able to increase plant growth. LT13 and LT1 produced more pods and the size of seeds were bigger. LT6 and LT10 endophytic actinobacteria strains has effected nodulation process and rhizobial fynction, and increased plant growth. The concentration of endophytic actinobacteria strain CP200B was detected in roots and shoot after 3 and 6 weeks. The concentration in both samples was observed similar after interval of two weeks.

In drought stress, LT6 and LT10 endophytic actinobacteria increased the plant growth of lentil plants. The dry weight of shoot and root of these two strains treated plants was higher than other plants. Increasing lentil dry weight were affected by some endphytic actinobacteria strains effected the nodulation process and other effected the plant growth. The result of LT6 and LT10 treated plants indicated the increase in nodule dry weight is associated with increased dry weight of lentil plant.

5.2 Future directions

Although this result has shown that endophytic actinobacteria can have a positive effect on nodulation and growth of lentil. The mechanisms invilved are still to be determined. Further detailed study of nodule development, changes in plant chimistry and gene expression and function will provide the opportunity to further understand the interaction and potential of endophytic actinobacteria to influence the plant growth and nodulation process.

In this study, some endophytic actinobacteria were reported that as increasing the nitrogen fixation with nodulation process and the plant growth was increased in a nitrogen limited growth media. The amount of nitrogen fixed from the atmosphere needs to be determined and establish the benefits to plants grown in field soil. In this study, isolated endophytic actinobacteria has shown the potential to stimulate the growth of lentil plant, effectiveness of these endophytic actinobacteria needs to be checked in different soils which contains the complex microflora and natural rhizobial communties.

Lentil crop is sensitive to water logging conditions, In addition, drought or heat stress effects the yield of lentil crop. Endophytic actinobacteria have the potential to alleviate abiotic stress in these conditions and improve the drought tolerance. The effectiveness on lentil yield should be determined an area of drought stress conditions.

APPENDICES

Appendix 1: Media and solutions

1. Humic acid Vitamin B Agar medium (HVA)

Per litre RO water	
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)	1 ml

Adjust pH to 7.2 ± 0.2

Dilute humic acid in 0.2 M NaOH before adding it into the media solution as it will not dissolve completely in water.

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 sterilized

2. Mannitol Soya (MS) agar

Per litre RO water	
Mannitol	20 g
Soya flour	20 g
Agar	20 g

Adjust pH to 7.2 ± 0.2

Keep Soya flour separate from the mannitol and agar for autoclaving. Once autoclaved, mix them together before pouring into plates.

3. Tap Water Yeast Extract (TWYE) agar

Per litre tap water	
Yeast extract	0.25 g
K_2HPO_4	0.5 g
Agar	18 g

pH adjusted to 7.2 ± 0.2

4. VL70 medium

Per liter RO water	
3-(N-morpholino) propanesulfonic acid	2.09 g
$MgSO_4$ / $MgSO_4 \cdot 7H_2O$	24.09mg / 49.3 mg
$CaCl_2$ / $CaCl_2 \cdot 2H_2O$	66.61 g / 88.2 g
K_2HPO_4	27.02 mg
Growth substrate*	
Selenite tungstate solution [a]	1ml
Trace element solution SL-10 [b]	1ml
Agar	18 g
Vitamin solution 1 [c] (add after autoclaving)	1 ml

Vitamin solution 2 [d] (add after autoclaving)	3 ml

Adjust pH to 7.2 ± 0.2

***Growth substrate (depends on desired)**

1. Amino acid (add 10ml per liter VL70 medium)

Per 100ml RO water (Stock solution)	
Aspartic acid	470 mg
Threonine	210 mg
Serine	320 mg
Glutamic acid	960 mg
Proline	690 mg
Glycine	150 mg
Alanine	200 mg
Valine	220 mg
Methionine	90 mg
Isoleucine	110 mg
Leucine	250 mg
Tyrosine	70 mg
Phenylalanine	140 mg
Lysine	350 mg
Histidine	100 mg

Arginine	150 mg
L-tryptophan	80 mg

Sterilize by using a 0.22 µm Millipore filter and add after autoclaving

2.Carboxy methylcellulose (CMC) 0.05% (W/V)

Add 0.5g CMC per litre VL70 medium

[a] Selenite tungstate solution

Per litre stock solution	
NaOH	0.5 g
Na2SeO3·5H2O	3 mg
Na2WO4·2H2O	4 mg
Distillated water	1 litre

Store in the fridge at 4°C

[b] Trace element solution SL-10

Per litre stock solution	
HCL (25%, 7.7 M)	10 ml
$FeCl_2 \cdot 4H_2O$ / $FeCl_3 \cdot 6H_2O$	1.5 g / 2.04 g
$ZnCl_2$	70 mg

$MnCl_2 \cdot 4H_2O$	100 mg
H_3BO_3	6 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$CuCl_2 \cdot 2H_2O$	2 mg
$NiCl_2 \cdot 6H_2O$(or $NiCl_2$ 13mg)	24 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
Distilled water	990 ml

Dissolve $FeCl_2 \cdot 4H_2O$ in HCL first, then dilute it in water. Add and dissolve the other chemicals.

Store in the fridge at 4°C

c <u>Vitamin solution 1</u> (filter sterilization)

Per litre RO water	
4-aminobenzoate	40mg
(+)-biotin	10mg
Nicotinic acid	100mg
Calcium D-(+) panthothenate	50mg
Pyridoxine hydrochloride	150mg
Thiamine hydrochloride	100mg

Cyanocobalamin	50mg

Store in the freezer at -20°C

[d] Vitamin solution 2 (filter sterilization)

Per litre RO water	
DL-6,8-thioctic acid	10 mg
Riboflavin	10 mg
Folic acid	4 mg

Adjust pH to 4.5

Store in the freezer at -20°C

Media for purifying cultures

1. Half strength Potato Dextrose Agar (HPDA)

per litre RO water	
PDA (Oxoid)	19.5 g
Agar	7.5 g

Adjust pH to 7.2 ± 0.2

2. International *Streptomyces* Project (ISP) 2 media

per litre RO water	
Malt extract	10 g
Yeast extract	4 g
Glucose	4 g
Agar	18 g

pH adjusted to 7.2 ± 0.2

3. International *Streptomyces* Project (ISP) 3 media

per litre RO water	
Oatmeal	20 g
Trace salt solution (added alter autoclaving)	1 ml
Agar	18 g

pH adjusted to 7.2 ± 0.2

Trace salt solution per 100 ml RO water	
$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g

4. Mannitol Soya Oatmeal (MSO) agar

Per litre RO water	
Mannitol	20 g
Soya flour	20 g
Oatmeal flour	20 g

Agar	20 g

Adjust pH to 7.2 ± 0.2

Keep Soya flour and oatmeal flour separate from the mannitol and agar for autoclaving. Once autoclaved, mix them together before pouring into plates.

3. YMA (Yeast Mannitol Agar)

Yeast extract	0.5g
Mannitol	5g
Sodium glutamate ($C_5H_8NO_4Na$)	0.5g
Agar	20g
Solution S	10 ml
Solution T	10 ml
Solution U	1 ml
Solution V	1 ml
Congo Red solution	10 ml
R.O. water	960 ml

* Solution S:

$Na_2HPO_4.2H20$ (Disodium phosphate)	1.8g
RO water	1000 ml

* Solution T:

$MgSO_4.7H_2O$ (Magnesium Sulfate Heptahydrate)	10g
RO water	1000 ml

* Solution U:

$CaCl_2.2H_2O$ (Calcium chloride dihydrate)	53g
RO water	1000 ml

* Solution V:

$FeCl_3$ (Ferric chloride)	4g
RO water	1000 ml

* Congo Red Solution:

Congo Red solution	2.50g
RO water	1000 ml

4. PBS Buffer (10X)

NaCl (Sodium Chloride)	80g
KCl (Potassium Chloride)	2g

Na2HPO4.2H20 (Sodium hydrogen phosphate)	18.1g
KH2PO4 (Potassium hydrogen phosphate)	2.4g
MQ water	1000 ml

1. Add the above chemicals to 900ml of MQ water in a 1L volumetric flask.

2. Adjust pH to 7.4.

3. Increase volume to 1L, add lid and mix well by shaking.

4. Pour into 1L glass Schott bottle.

5. Send PBS solution for autoclave.

6. Store at room temperature on bench.

Prepare 1L 1X PBS buffer from 10X PBS stock as follows:

Dilute 100 **ml** of the 10X PBS stock buffer in a total volume of 900m**L** dH_2O.

5. McKnight Solution

Mc Knight's solution (no autoclave)	**250 ml concentrate stock solution**
$CaSO_4.2H_2O$ (Calcium Sulphate)	6.75 g

$MgSO_4.7H_2O$ (Magnesium Sulphate)	1 g
KH_2PO_4 (Potassium dihydrogen orthophosphate)	1 g
KCl (Potassium Chloride)	1.5 g
A-Z trace elements	5 ml
D-solution	5 ml
Distilled water	make up to 250 ml

Trace elements

H_3BO_3	
(Boric acid)	2.86 g
$MnSO_4.H_20$ (Manganese sulphate monohydrate)	2.08 g
$ZnSO_4.7H_2O$ (Zinc sulphate)	0.222 g
$CuSO_4.5H_2O$ (Copper Sulphate pentahydrate)	0.079g
$Na_2MoO_4.2H_20$	
(Molybdic acid (Sodium molybdate))	0.1292 g
Distilled water	make up to 1000 mls

D solution

$FeCl_3$ (Ferric chloride)	10 g
Distilled water	make up to 1000 mls

Start "N"

NH_4NO_3

(ammonium nitrate)	300 mg
Distilled water	make up to 1000 mls

250 ml concentrate is diluted in 20 litres of water for application to pots at 200 ml/pot - note we use the smaller water well pots (125 mm diameter and 1 liter volume) .

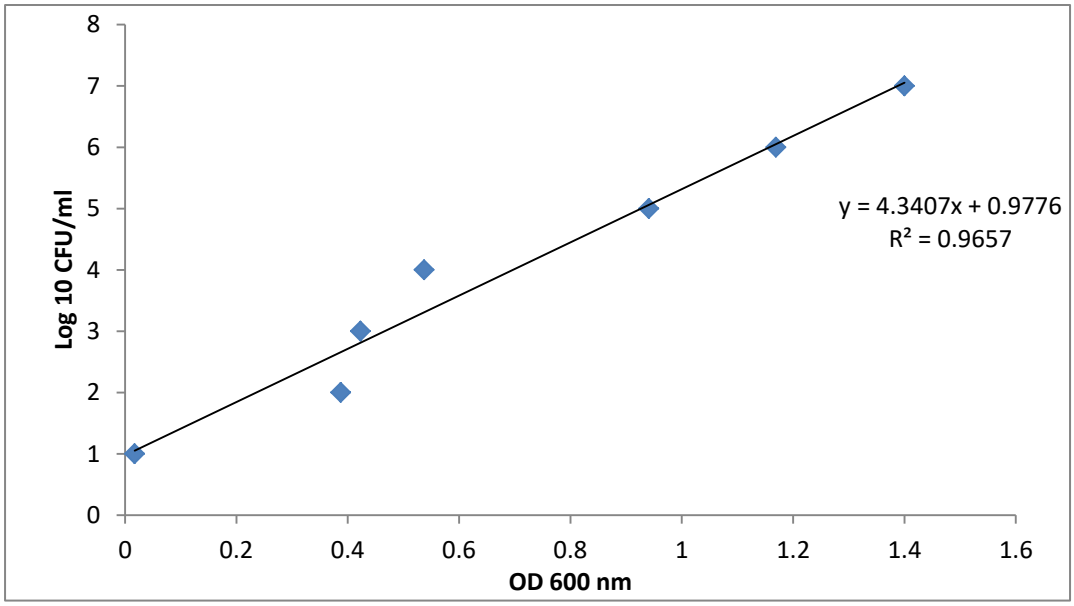
"Starter N" - add 300 mg of ammonium nitrate to the 20 liter of nutrient solution above before applying to pots

15. N solution for unlimited nitrogen treatments

NH_4NO_3 (ammonium nitrate)	12g
Distilled water	make up to 5000 mls

100 ml solution added into pot weekly

Appendix 2 The correlation standard curve between OD600nm and CFU/ml of Rhizobium *Mesorhizobium ciceri* WSM1455



Appendix 3- Chemicals, materials and equpment for RNA extraction

1. <u>Chemicals/reagents</u>

    - TRIzol reagent (Invitrogen)
    - Chloroform or chloroform:isoamyl 24:1 (filter sterilised)
    - Isopropanol (filter sterilised)
    - High salt precipitation solution (autoclaved)
        i. 0.8 M sodium citrate
        ii. 1.2 M sodium chloride
    - 75% ethanol (filter sterilized)
    - Liquid nitrogen (if extracting RNA from plant material)

- Water for injection or DEPC treated water

2. Equipment/materials

- Mortar and pestle (heated at 250 ºC for at least 2 hours)
- Spatula (sterilised as above)
- Centrifuge at 4ºC
- Autoclave
- Fumehood
- Eppendorf tubes (1.7 mL, 500 µL, autoclaved twice)
- Yellow and blue tips (autoclaved twice)
- Ice in esky
- Dewar (for transporting liquid nitrogen)
- Foam container (for liquid nitrogen bath)

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