Combined effects of endophytic actinobacteria and symbiotic *Rhizobium* on N_2 fixation, growth and development of Chick pea (*Cicer arietinum*)

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DECLARATION

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree.

To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person except where due references is made in the text.

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ABBREVIATIONS

ul, ml	microliter, millilitre
uM, mM	micromolar, millimolar
ug, mg, g	microgram, milligram, gram
%	percent
approx.	approximately
bp	base pair
hr.	hour
PCR	polymerase chain reaction
exp.	experiment
DNA	deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
MQ water	milli Q water
CFU	colony forming unit
min	minute (s)
^{O}C	degree Celsius
OD600nm	optical density 600 nanometres
PGPR	plant growth promoting rhizobacteria
SARDI	South Australia Research and Development Institute
sp.	species (singular)
spp.	Species (plural)
temp.	temperature
N-	no added nitrogen treatment
N+	unlimited nitrogen treatment
CC1192 or R.	*Mesorhizobium cicero CC1192*
FAO	Food and Agricultural and Resource Economics
ABARES	Australian Bureau of Agricultural and Resource Economics and sciences.

ABSTRACT

Chickpea (*Cicer arietinum*), being the second most significant pulse crop produced stands as a rich protein source for human consumption while less expensive . Improving the health of Chick pea plant roots and enhancing nitrogen fixation can conceivably improve its productivity and yield interms of quantity. Endophytic actinobacteria colonizing leguminous plant have been accounted for development, growth, nodulation of legumes and providing resistance against plant diseases. The aim of this investigation was to isolate endophytic actinobacteria from Chick pea (roots and nodules) and study their consequences in the development, growth and nodulation of Chick pea. In short, the Combined effects of endophytic actinobacteria and symbiotic *Rhizobium* on N2 fixation, growth and development of Chick pea (*Cicer arietinum*) was intended to be studied.

The endophytic actinobacterium strains of the following species viz., ***Streptomyces* sp. LuP30, *Streptomyces* sp. LuP47B, *Streptomyces* sp. CP21A2, *Streptomyces* sp. CP200B, *Microbispora* sp. CP56 and *Actinomadura* sp. CP84B** were used for the study**.** Two strains of *Streptomyces* sp. were isolated from Lucerne roots and Chick pea roots. The strain of *Microbispora* sp. CP56 and *Actinomadura* sp. CP84B showed improvement by legume-rhizobia symbiosis.

Six different endophytic actinobacteria were taken for investigating the impact on growth, development and nodulation of Chick pea in sand - vermiculite pot tests. Subsequently, four endophytic actinobacteria LuP30*,* LuP47B (isolated from Lucerne) CP56*,* CP21A2, CP200B, CP84B (isolated from Chick pea) were chosen for further examination as they upgraded the nodulation and total mass.

Two strains which were isolated from Chick pea roots CP200B and CP21A2 indicated the beneficial outcome on Chick pea development and was distinguished by RT-qPCR quality sequencing and amplification.

Isolated endophytic actinobacteria from Chick pea and lucerne substantiated the potential of effecient microbial inoculants for improving the development, growth and beneficial interaction of Chick pea via symbiosis. The six variants of endophytic actinobacteria that were isolated and screened delivered the most beneficial co-inoculation

with their *Mesorhizobium ciceri* strain CC1192 (rhizobial partner) and Chick pea. Screening of endophytic actinobacteria and further trials and experiments rendered a highly beneficial partner for co-inoculation with various rhizobium.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1. Nitrogen fixation, legumes and rhizobia

1.1 Nitrogen fixation:

The development of all organisms depends upon the availability and utility of mineral nutrients like nitrogen. Nitrogen is required in huge amounts as it is an essential component found in nucleic acids other cellular constituents and proteins. In atmosphere, there is an abundant supply of nitrogen-79% in the form of gaseous N_2 (Boddey et al. 1991). Though for most of the organisms N_2 in this form is unavailable because of the three bonds within the 2 nitrogen atoms, which it inert. For the growth of plants, the nitrogen must be converted or fixed in the form of ammonium or nitrate ions.

Biological Nitrogen fixation (BNF) is the phenomenon which involves the vital role of microbes in providing nitrogen availability for living organisms on earth. Bacteria convert nitrogen into ammonia as they are capable of associating symbiotically with other organisms or plants.

- The transformation of ammonia and nitrate are form of nitrogen or nitrogen gas is carried out by yet other bacteria.
- Release of nitrogenous compounds are carried out by organic matter degrading fungi and bacteria .

The table below depicts the estimation of amount of fixed nitrogen on a global scale. The total biological nitrogen fixation estimated is shown to be twice as compared to that of total nitrogen fixation by non-biological processes (Hardy et al. 1973). The data given below was compiled and studied by Bezdicek and Kennedy, (1998)

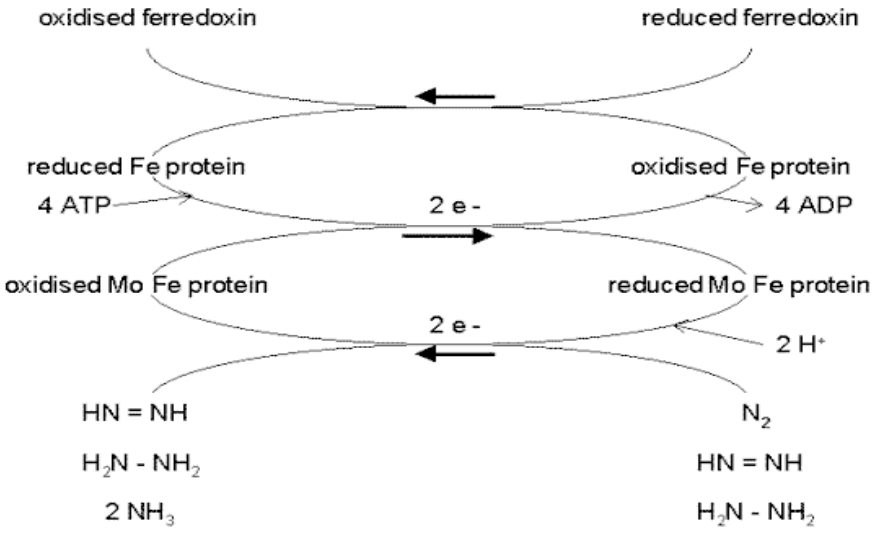
Table 1.1. has been removed due to Copyright restrictions.
List of Amounts of Fixed Nitrogen obtained from various sources. DF Bezdicek & AC Kennedy, (1998)

Mechanism of biological nitrogen fixation:

Biological nitrogen fixation is represented as follows

$$\mathbf{N_2 + 8H+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi}$$

From the above equation it is evident that 2 moles of ammonia is generated from 1 mole of nitrogen gas and 16 ATP moles. Prokaryotes carry out this reaction with a catalyst, nitrogenase that contains Fe protein and a Mo-Fe protein. Initially nitrogen gets bound to the enzyme complex i.e. nitrogenase which leads to reduction of Fe protein by electrons of donated ferredoxin. Later, the reduced Fe protein binds ATP and again reduces the Mo-Fe protein donating electrons to Nitrogen, which produces HN=NH which is then reduced to H2N-NH2 and finally to 2NH3 (Lee et al. 1942)



The functional communication within the nitrogenase proteins of all nitrogen-fixing bacteria, is remarkable. (Mahato G. 2011).

Studies demonstrates that maximum yield of crops is the major concern (Peoples et al. 1995). Currently, the vitality for production is coupled with a desire for sustainability. In agricultural perspective, , the Effective utilization of soil nitrogen is significant for sustained development of plants devoid of denitrification issues (Mabrouk et al. 2010)

While BNF, throughout the world has been a long component of the farming system. In recent decades nitrogen as a primary source in agriculture has reduced, as enhancing the amount of nitrogen fertilizer has gained momentum due to the improvement it has proved in production of cash crops and food (Postgate. 1982). International importance on sustainable environmental development with the utilization of renewable sources in perspective way to achieve the focus on the potential role of BNF providing N for agriculture system (Peoples et al. 1995).

1.2 General information on legumes

Legumes are flora categorized under the family of Fabaceae otherwise termed as Leguminosae. Agriculturally, Legumes are grown as a primary source for human consumption of grain seeds, for livestock forage and silage and as soil-enhancing green manure (Evans et al. 2001). Legumes are significant which shows the symbiotic nitrogen-fixing bacteria in structures called root nodules. So, in crop rotation, they play a key role.

Most of the legumes contain symbiotic bacteria called rhizobia living within the root nodules of their root systems. These bacteria provides insights on the special ability for fixing nitrogen from atmosphere and its conversion to ammonia.

The chemical reaction is: $N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$

In Legumes, plant proteins that contains amino acids are synthesized in root nodules. Crop rotation in legumes are more common in many organic and traditional farming practices (Brockwell et al. 1995)

As the 3rd largest terrestrial plant family in terms of a number of species, legumes are widely distributed, Orchidaceae and Asteraceae contain 751 genera and other 19000 known species which contains about 7% of flowering plant species. India is the world's largest producer and consumer in the case of pulses as huge demand for pulses are reflected due to increased consumption (FAO 2011).

Table 1.2. has been removed due to Copyright restrictions.

Worldwide Pulses Producers

In all states of Australia, Legumes are cultivated for various purposes and it witnesses two markets, one for livestock and another for export purposes. Although, globally Australia and India are one of the largest exporters of pulses. Australia had 2.2 million ha of pulses and produced 2.5 million tonnes in 2011 (FAO 2014).

1.3 Production of Chick pea

Currently, Chick pea is the second most important pulse produced in the world. In Australia, since 1980s chickpeas have been a commercial winter crop and mostly production is exported. Prices alter, in 2016, AU$1,108 per tonne (high) although AU$350 to AU$500 per tonne allotted to the port which is cited as a more realistic price (ABARES) Production of

Chick pea is observed in Australia, Queensland, and New South Wales. Whereas, Chick pea is also grown in other regions that is Victoria, South Australia and Western Australia (Muehlbauer et al. 2017).

As a legume, Chick pea performs better nitrogen fixation in a cereal-based farming system. Increased soil nitrogen and reduced root diseases can provide an important boost to subsequent cereal yields. They provide a useful break crop for control of diseases, weeds, and pests.

Production of Chick pea in Australia is a well-established industry, with a producer Research & Development (R&D) levy and strategic R&D priorities. Pulse Australia represents the Chick pea growers.

Facts and figures

- Globally 66% of Chick pea production is from largest producer India.
- 65% of world exports in Australia, India, and Mexico
- In 2016, Australian production of Chick pea was over 2,000,000 tonnes
- 80% of all exported Australian Chick peas went to Pakistan, Bangladesh, and India. Mostly Australian Chick peas are exported.

Production status

Australia produced over 2,000,000 tonnes of Chick peas in 2016 (ABARES). Most Australian Chick peas are exported to India, Pakistan, and Bangladesh.

Figure 1.1. has been removed due to Copyright restrictions. Map of current and potential pulses growing regions (http://www.pulseaus.com.au/about/australian-pulse-industry)

1.4 Rhizobia

Rhizobia are gram-negative, non-sporulating rods and motile bacteria that fix the nitrogen as a diazotroph inside the root nodules of legumes. It requires a plant host, to express genes for nitrogen fixation as they cannot independently fix nitrogen (Burdass 2002). Falling under the category of soil bacteria, rhizobia colonise the leguminous roots

leading to the formation of nodules (Burdass 2002). Rhizobia that are present form nodules in the roots through infection (Baldwin & Fred 1929) . From the atmosphere, they fix nitrogen and convert into useful form. Nitrogen exported from the nodules are utilized for leguminous growth. Fixation of nitrogen can enhance the yield of the crop. Inoculation with rhizobia inclines to enhance yield (Somasegaran et al. 2012).

The inoculation process carried out in legumes has been an agricultural practice since time immemorial and constantly improved over time. It has been stated that the quality and quanity of cereals increased due to inoculation. Rhizobium inoculation can be beneficial to crops other than legumes. Cereals are major crops, integrated into a crop rotation with a legume (Layek et al. 2018).

The symbiotic relationship between a host plant and a rhizobium gets established through biochemical changes taking place in a sequential manner Flavonoids secreted by the roots of host plant which trigger the accumulation of huge population of cells as well as eventually attached to hairs of roots, Rhizobia is a free-living bacterium in a soil till rhizobia are able to sense the flavonoids (Samanta et al. 2011). After that flavonoids promote the DNA binding activity of Nod D which triggers the secretion of nod factors when bacteria entered into the root hairs. Due to nod factor, there are development changes seen inside the hairs of a root, initial with curling of root hairs and formation of infection thread in the roots, a tube shape cellulose line bacterium can use to travel into the root cells through root hair (Brewin & N.J, 2004). Formation of root nodule followed by continuous proliferation of cells (Libbenga et al. 1973).

The morphology of bacteria differentiates into bacteroids and fix atmospheric nitrogen into ammonium, by utilizing the nitrogenase enzyme. After which the ammonium is taken up by the plants, converted into amino acids and supplied as carbohydrates to the bacteria (Gupta et al. 2017). Oxygen scavengers like leghemoglobins, found in root nodules of legumes, aid in transportation of oxygen to symbiotic bacteroids for its cellular respiration (Appleby & C.A. 1984). Hence oxygen content is kept under check in the root nodules that helps in prevention of inhibitory effect of nitrogenase.

In Medicago, it has been observed that root nodules can be formed in the absence of rhizobia (Middleton et al. 2007). Thus indicating the significance of growth and development of the nodules entirely controlled by the plant and produced by the nod factor.

1.5 Legume-rhizobia symbiosis (The Nodulation Process)

A classic example of mutualism is that of the legume-rhizobium relationship in which rhizobia supply ammonia to the plants and receive organic acid as an energy source.
The legume-rhizobia symbiosis takes place in a stage-wise manner.

Infection of legume roots by rhizobia:

Rhizobia, capable of surviving in the soil infect plant roots during environmental favourable conditions. Microorganism survives in the soil, but owing to acidity, drought, high temperature or other stress conditions their number can get declined (Slattery et al. 2001).
Rhizobia interact with legume species, and multiply in the root zone and attach to the hairs of the root. When rhizobia gets attached, the root hair is used by rhizobia as an entry gate into the plant. Occasionally, rhizobia also enters via lateral roots that emerge through cracks in the root surface. Rhizobia enter via infection thread, throughout numerous cell layers to the site where a nodule will develop (van Rhijin et al. 1995).

Figure 1.2. has been removed due to Copyright restrictions. Stages of infection, nodule development and nodule formation (Pate, J.S., 1958)

Nodule development:

Development of nodules are initiated by early infection of the epidermal layer and subsequent mitotic divisions of the cortical cells, followed by entrapement of rhizobium inside the root hair curls, forming a patch like region. Infection expands from the basal patch forming nodules, in which rhizobial nitrogen fixation is carried out. Mutual transport of carbohydrates to rhizobia and fixed nitrogen to plants is executed through vascular tissues of the plant. As tissues develop, swollening roots and nodules becomes visible within 21 to 28 days. Nodules differ in size, shape, texture, shape, and location. Figure 1.3

depicts the shapes of nodules. Shapes and location of the nodules depend on the host legume (Schultze et al. 1998).

Figure 1.3. has been removed due to Copyright restrictions. Some characteristic shapes of leguminous nodules (Pate, J.S., 1958)

Nodule function:

While nodule develops, the rhizobia become swollen. At this stage, they are called Bacteroids. Figure 1.4. Shows where nitrogen gas from the soil reaches the bacteroids via pores in the nodule. Nitrogenase enzyme produced by the bacteroids, converts atmospheric nitrogen to ammoniawhich is assimilated into glutamine through Glutamine synthetase or Glutamic acid synthase pathway of the plants (White, Prell, James and Poole, 2007) The amino acids produced thereafter move throughout the nodules where they endure advance changes and are used to produce proteins.

A considerable amount of energy is required by bacteroids to support their activity of nitrogen fixing. Through photosynthesis, the plant provides energy in the form of sugar. Probably, Legume-Rhizobia symbiosis needs around 10 kg of carbohydrates for each kg of N_2 fixed (Udvardi et al. 2013). Obviously, to supply energy for supporting BNF, the plant should be healthy enough. In response to a few different strains of rhizobia, legume plants will produce nodules, but not all of the strains are competently effective in fixing nitrogen. Some strains induce nodulation but not at all really fix the nitrogen. A reputable inoculant should possess rhizobia strains which are highly effective for nitrogen fixing (Checcucci et al. 2017).

Nodules that are produced by effective rhizobia are comparatively large on the lateral and primary roots. The rhizobium tends to be located in the upper portion of the root system. During the annual session the legume, the size, and the number of each nodule, on the time of flowering influence a peak. At this time nitrogen fixation is also at its peak. On the other side, by ineffective rhizobia, the nodules produced are very small. They are scattered and quite numerous throughout the root system.

Figure 1.4. has been removed due to Copyright restrictions. The legume-rhizobia symbiosis (Pate & J.S, 1958)

Nodule Senescence:

Ultimately, Nodules get older and hence deterioration occurs. The life span of the nodules depends on four factors:

- Physiological condition of the legume,
- Total moisture content in the soil,
- Presence of parasites,
- Formation of nodules.

In Annual legumes, after attaining maturity, the seeds develop with storage compounds and nutrients. The nitrogen-fixing ability of the bacteroids decreases as the plant utilizes more energy in the production of seeds. Ultimately, the functioning of the nodules stop and starts releasing the bacteroids into the soil. In adverse condition, these rhizobia infect new plants and may survive during the upcoming cropping season (Fred et al. 2002). Whereas, In the agricultural system, rhizobial inoculant must be added to every crop.

Certain plants tends to shed nodules in the early stage, when affected by drought conditions. After heavy grazing, forage legumes may also shed their nodules, but species of forage legume produce new nodules. Definitively, some of the crops may be susceptible to the parasites, for instance, larvae of weevil which are fed on nodules of the root (Gutschick & Vincent, 2012).

Factors influencing nitrogen fixation:

Legumes are diverse in size, length and growth habit in the growing season. The way they fix amount of nitrogen differs . Mostly legumes obtain nitrogen from the soil and fixes it based on the longevity and abundance of the root nodules and the effectiveness of BNF within the nodules.

1.6. Chickpea symbiosis

Since ancient times the growth of Chick pea is recorded worldwide and presently it is being produced in various countries. Chickpea is the third most widely used leguminous crop grown around the globe. Traditionally Chick pea was known to resist nodulation. There are two species explaining the legume nodulation (Laranjo et al. 2004; Rivas et al. 2007) *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* (Broughton & Perret, 1999). From

a recent study, it was estimated that *Mesorhizobium* species can successfully produce nodules in the Chick pea plant. (Nour et al. 1994, 1995).

The setting up of an effective symbiosis is essential and best explained by bacterial symbiosis genes. Here they are divided into two main categories which are nodulation genes and nitrogen fixation genes (Mylona et al., 1995). The nodulation gene are found to be nod, noe and nol and are found to be responsible for nodulation (Arrighi et al. 2006). Whereas, the nitrogen fixation gene nif are responsible for atmospheric nitrogen fixation and are involved in the fixing function (Fischer & Hans-Martin, 1994).

Lipo-chito-oligosaccharides are a Nod factor, which is secreted and synthesized by rhizobia when the host plant releases the flavonoids. The dinitrogenase reductase subunit of the nitrogenase complex which codes for the *nifH* gene. A chitin synthase codes for the nodC gene, which initiate the stage in Nod factor, and which are essential for all rhizobia in nodulation and also determinant host as well. Nod genes are distinctive to rhizobia but the *nif* gene is easily found in bacteria.

The genes responsible for symbiosis are located on symbiosis plasmids and these plasmids transfer between the strains which has been determined in lab conditions (Young & Wexler. 1988; Laguerre et al. 1992; Louvrier et al. 1996) in cultivated soils (Hynes et al. 1986; Martínez et al. 1987; Kaijalainen & Lindström, 1989; Rogel et al. 2001) or transformation of saprophyte in a symbiont (Sullivan et al. 1995; Tan et al. 2001) and in natural soil as well (Wernegreen & Riley, 1999).

1.7 Endophytic Actinobacteria

1.7.1 Definition of Endophytes and Actinobacteria

An endophyte is an endosymbiont microorganism such as bacterium or fungus which live inside the plant without causing any disease (Brader et al. 2014). Endophytes are ubiquitous and may enhance the growth of host plant, nutrient acquisition. Hasegawa et al. 2006 described that endophytes are also isolated and cultured from host plant after surface sterilization.

Most of the endophytes and plant relationships are not understood well but some of the endophytes and plant engage in mutualism (Hardoim et al. 2015). By preventing parasitic organisms from colonizing them endophytes benefit the host plant (Kuldau et al. 2008). By excluding some other potential pathogens, endophytes are colonizing the plant tissue (Zabalgogeazcoa 2008). Some bacterial and fungal endophytes enhance the growth of a plant and improve the plant (Hardoim et al. 2008).

It is proven that endophytes and plants engage in communication and mutualism and provides benefit to each other which aids the symbiosis. For instance, activated gene expression by plant chemical signals have been shown by endophytes. The chemical signal produced by plant shows evidence for the hypothesis that signaling of a plant must induce expression of secondary metabolites of endophytes (Kusari et al. 2012).

Actinobacteria are a phylum of Gram-Positive bacteria is of high economic importance playing very essential role in sustaining agriculture through its contribution to the soil system.

Actinobacteria is a dominant bacterial phylum that comprises of the largest bacterial geneus *Streptomyces* (Ningthoujam et al. 2009) and also a source of antibiotics. Most of the actinobacteria are in subclass Actinobacteridae categorized under Actinomycetales order. *Streptomyces* spp (Atta et al. 2009) especially predicted as a producer of bioactive metabolites which are useful for human health in medicine such as antibacterials, antifungal, antiviral, antitumor drugs, enzyme inhibitors, immunomodifier and antithrombotics (Mahajan, G.B. and Balachandran, L., 2012) and also in agriculture which includes fungicides, herbicides and growth promoters for animals and plants (Bressan. 2003).

Actinobacteria play an essential role in medicine as actinobacteria-derived antibiotics that includes aminoglycosides, anthracyclines, chloramphenicol, macrolide, tetracyclines, etc.

Actinobacteria rich in GC content in their DNA, 70% of the GC content in the DNA (Ventura et al. 2007) By doing an analysis of glutamine synthetase sequence which suggested the phylogenetic analysis of the actinobacteria (Hayward et al. 2009).

1.7.2 Endophytic actinobacteria

The uniques characteristics of endophytic actinobacteria are found to be specific growth and colony formation within the plant tissue without causing any damage or negative impacts on the host plant (Hasegawa et al. 2006).

The invasion of endophytic actinobacteria occurs via the roots and spreads to other parts of the host plant. Promotion of plant growth by endophytes by producing the phytohormones, by degrading enzyme-polymer and secondary metabolites (Dinesh et al. 2017). Indirectly endophytic actinobacteria increase plant growth by enhancing the availability of nutrients and via induction of systemic resistance. Broad range of structurally diverse secondary metabolite are produced. Endophytic actinomycetes shows evidence of production of bioactive metabolites (Yadav et al. 2018).

These microbes produce a wide range of novel compounds that are exploited in agricultural, pharmaceutical and in other industries (Golinska et al. 2015).

1.7.3 General Characteristics of Actinobacteria and Endophytic Actinobacteria

Actinobacteria produces secondary metabolites, antibiotics, enzymes from microbial source and antitumor compounds (Hamashenpagam. 2011). Amongst varied biologically active compounds synthesised by microbial community , Actinobacteria produces 45%, other Bacteria synthesises 17% (Gebreyohannes et al. 2013) and 38% by Fungi. Whereas, Actinobacteria demonstrates utmost morphological distinction amongst the Prokaryotic Bacteria owing to the formation of numerous structures like mycelia, hyphae and numerous kinds of spores.

Plant growth promotion properties

Cattelan et al. 1999 demonstrated four possible mechanisms that promotes the plant growth to greater extent which are listed below;

- Synthesis of plant hormones viz., IAA, gibberellic acid, ethylene, and cytokinins,
- Fixation of nitrogen,
- Protection of plants from microbes that are phytopathogenic producing antibiotics, chitinase, siderophores, etc,
- Solubilizing mineral sources such as phosphate.

Production and secretion of numerous regulatory chemicals in rhizosphere, by plant growth promoting soil rhizobacteria that inhibits the surface of the root and via directly and indirectly involved in promoting plant growth development. Usually, plant growth promoting rhizobacteria assists the plant growth directly by acquisition resources such as phosphate, nitrogen, and essential mineral and helps in modulating the levels of plant hormone in the form of biocontrol agents indirectly decrease the inhibitory effect of pathogens on growth of plant and development.

Figure 1.5. has been removed due to Copyright restrictions. Plant growth promotion mechanism of rhizobacteria (Gupta et al. 2000).

PGPR, accomplishes multiple activities directed towards plant growth promotion exhibiting potential bioremediation of detoxifying pollutants such as pesticides, heavy metals and controlling a wide range of phytopathogens as biopesticides showing results in distinct studies of the crop. A specific PGPR enhance optimization and acclimatization in order to productive efficiency according to the soil conditions.

Antifungal activity

Worldwide fungal phytopathogens causes a serious problem in the food industry and in agriculture due to which there is need of a method for protection of plant, which is more environment friendly and less dependent on chemicals which further used as biocontrol agents (Pohanka. 2006, Shimizu et al. 2000, Yang et al. 2007).

Tinatin et al. (2006), revealed wide antifungal activity by Soil Actinomycetes that is proved to provide plant protection from wide variety of soil borne fungal pathogen. As a biocontrol agent, Actinomycetes produce a member of the antimycin class that is Urauchimycin. However, Actinomycetes also produces bio fungicides that controls soil born

plant pathogens (Schoenian et al. 2014, Seipke et al. 2011, Seipke et al. 2012) which causes root disease.

Occurrence and variety of Endophytic Actinobacteria in Plants

Actinobacteria are widely spread in nature and categorized under bacterial domain, showing largest taxonomic group. Their therapeutic applications have been extensively explored and they are abundant in soil (Tang et al. 2003; Hamedi et al. 2013). This kind of bacterial group is highly beneficial and adapt to various habitats. Thus, the scientist in recent studies considers attention that has opened up a major potential for novel metabolites that helps in resolving major challenges (Subramani and Aalbersberg, 2013). For instance, novel drugs for drug-resistant human pathogens , in several habitat ecological balance and several other sustainable agricultural practices.

Novel metabolites such as ectoin from xerophylic actinobacteria has shown protection against dry conditions. Studies has reported identification and isolation of biologically active metabolites from extremophilic actinobacteria (Mohammadipanah and Wink, 2016) thus highlighting the applications of Actinobacteria in agriculture, medicine and environment perspectives.

1.7.4 Actinobacteria legume benefits:

Grain legumes are cost-effective which are an alternative for animal protein which improved the diets in Africa. (Amarowicz et al. 2008). Through symbiotic nitrogen fixation, legumes play a major role for nitrogen demand and benefit the crops by enriching the soil supplies of nitrogen (Kloepper 1978). United Nations in 2016 had declared that the realization advantage of sustainability promoted pulse production as the “International year of pulses”. From the last five decades, the global yield of legumes has been inactive and has adopted a series of molecular breeding approaches and conventional approaches. However, cost enhancement and negative impact of fertilizers and pesticides for the protection and production of a crop are major concerns.

The plant growth promoting bacteria improves the soil and health of the plant and becomes a strategy for sustainable agricultural development system for their eco-friendliness, low-cost production and also minimize the non-renewable resources consumption (Sellsted et

al. 2013). The Actinobacteria and metabolites of Actinobacteria are essential in increasing the yield along with pathogen and pests control in grain legumes (Sathya et al. 2017).

1.8 Summary, potential for legume improvement and critical knowledge gaps:

Chickpea is the second most essential legume grown all over the world. Chickpea is a highly enriched source of protein for mankind for maintenance of health compared with that of the animal protein. In Australia, around 38, 500 tonnes of nitrogen gets fixed by nitrogen fixation in a plant per year. Chickpea is a legume which fixes the biological nitrogen fixation in the plants. Doughton et al. 1993 shows that when Chick pea was habitually sown on soil was found to increase the Nitrogen level resulting in reducing nitrogen fixation. However, the endophytic Actinobacteria possibly increase the nodulation, growth, and development of plants and also control the disease as well.

Thus, Endophytic Actinobacteria isolated from the root of legumes (chickpea) boosts the growth, development and nitrogen fixation potential in chickpea plant. Parameters measured for the analysis are number and weight of nodules, length, dry weight of roots and shoots, number and total mass of nodules per plant and nitrogen content in shoots and roots of the plant.

In this work, we have compared the morphology of the bacterial strains and amplified genes using RT-qPCR. The endophytes are are identified as *Streptomyces* sp. LuP30*,* *Streptomyces* sp. LuP47B*, Streptomyces* sp. CP21A2*, Streptomyces* sp. CP200B, *Microbispora* sp. CP56, *Actinomadura* sp. CP84B that nodulate the Chick pea. The symbiotic effeciency of the isolates along with a rhizobial partner will be determined, so as to analyse the nodulating potential of each and every isolate thereby determing their nitrogen fixing potential.

Quantitative PCR (qPCR):

Quantitative PCR (qPCR) is utilized to distinguish, describe and measure nucleic acids for various applications. RNA transcripts are reverse transcribed into cDNA first in RT-qPCR. In real-time PCR, DNA restricting, or binding dye is utilized as fluorescent correspondents to screen the continuous real-time PCR reaction. SYBR® Green is the most broadly utilized double-stranded DNA- specific dye revealed for real-time PCR. The absolute measure of a target sequence was evaluated with Real-time PCR (Maeda et al. 2003). The cycle quantification value (Cq value) is the number of the PCR cycle at which the reaction curve converges the threshold line. By utilizing a fluorescent correspondent in the reaction, it is conceivable to measure the generation of DNA in the qPCR test. In qPCR, amplification of DNA is checked at each cycle of PCR. The time when the fluorescence becomes quantifiable is known as the threshold cycle (CT) or intersection point. The key component in RT-PCR is the DNA amplification is identified continuously in real-time as PCR is in advancement by the utilization of fluorescent columnist. The fluorescent correspondent sign quality is straightforwardly relative to the number of amplified DNA particles (Chen et al. 2011).

1.9 Research plan

1.9.1 Aim of the research:

To study and analyse the combined effects of Endophytic Actinobacterial strains along with nitrogen fixing rhizobium on the growth and development of the Chick pea plant.

1.9.2 The Objective of research:

- To assess the effects of the 6 isolated strains **(*Streptomyces* sp. LuP30, *Streptomyces* sp. LuP47B, *Streptomyces* sp. CP21A2, *Streptomyces* sp. CP200B, *Microbispora* sp. CP56, *Actinomadura* sp. CP84B)** of Actinobacteria on the growth, nodulation potential and nitrogen fixing ability on chickpea plants and to evaluate the strains that have shown a statistically significant increase in one or more growth or nitrogen fixation in Chick pea.
- To study the most effective strains amongst the six strains isolated based on the results of the first experiment **(*Streptomyces* sp. CP21A2, *Streptomyces* sp. CP200B)**, for consistency and to acquire gene level information on their activity by using RT-qPCR.
- To examine and select the appropriate Actinobacteria strains which can increase the growth and nitrogen fixation in Chick pea plant.

1.9.3 Hypothesis:

Effective Endophytic Actinobacteria strains can increase the growth of the plant by inducing high nitrogen fixing ability by rhizobia in Chick pea plant. The effective Actinobacteria are present in the plant and rhizosphere through the life cycle of the Chick pea plant.

1.9.4 Summary of Research Plan

Isolation of 6 strains of Endophytic Actinobacteria on ISP2 media at 27^0 C for 14 days (stored at -20^0C in glycerol. Growth of rhizobia, *Rhizobium* streaked on YMA media and incubated at 27^0 C for a week to get pure rhizobia colonies (stored at -20^0C in glycerol). The germination rate of seeds were checked prior to starting the experiment.

Pot Assay 1: Plant growth media, nutrition, sowed and supply of water (6 treatments with three controls and 3 replicates) for 8 weeks of Chick pea plant. 8 Surface sterilized seeds in each pot, coated with 0.3% xanthan gum and were applied as 10^8 CFU per gram of seeds and after one-week Rhizobia were added for Pot Assay 1. Analysis and data collection for 8-weeks-old Chick pea plant. The six different Endophytic Actinobacterium strains of species treatment *Streptomyces* sp. **LuP30**, *Streptomyces* sp. LuP47B, *Streptomyces* sp. CP21A2, *Streptomyces* sp. CP200B, *Microbispora* sp. CP56, *Actinomadura* sp. CP84B. Harvest of 8-weeks-old Chick pea plant (-ve control, R^+, N^+).

Pot assay 2: Plants were transferred from 8 weeks Chick pea plant (small pot plant thinned into Big pot up to 15 weeks) (The number of plants was thinned from week 8 plant pots into big pots up to 15 weeks). Analysis and data collection for 15-weeks-old Chick pea plant. The six different Endophytic Actinobacterium strains of species treatment *Microbispora* sp. CP56, *Actinomadura* sp. CP84B, *Streptomyces* sp. CP200B, *Streptomyces* sp. LuP47B, *Streptomyces* sp. CP21A2, *Streptomyces* sp. LuP30. Harvest of 15-weeks-old Chick pea roots of R^+, N^+, N^-.

Pot assay 3: CP21A2 and CP200B treated seeds were sown again for 4 weeks with two replicates and three control which is untreated control, R^+ and, N^+.

From weeks 4, 8 and, 15 CP21A2 and CP200B treated Chick pea roots were sterilized.

DNA extraction, PCR amplification by using MaxyGene Gradient Thermal Cycler Gel electrophoresis and PCR amplification by using real-time qPCR (CFX96 real-time qPCR system BioRad) were performed.

CHAPTER 2
GENERAL MATERIAL AND METHODS

2. Materials and Methods

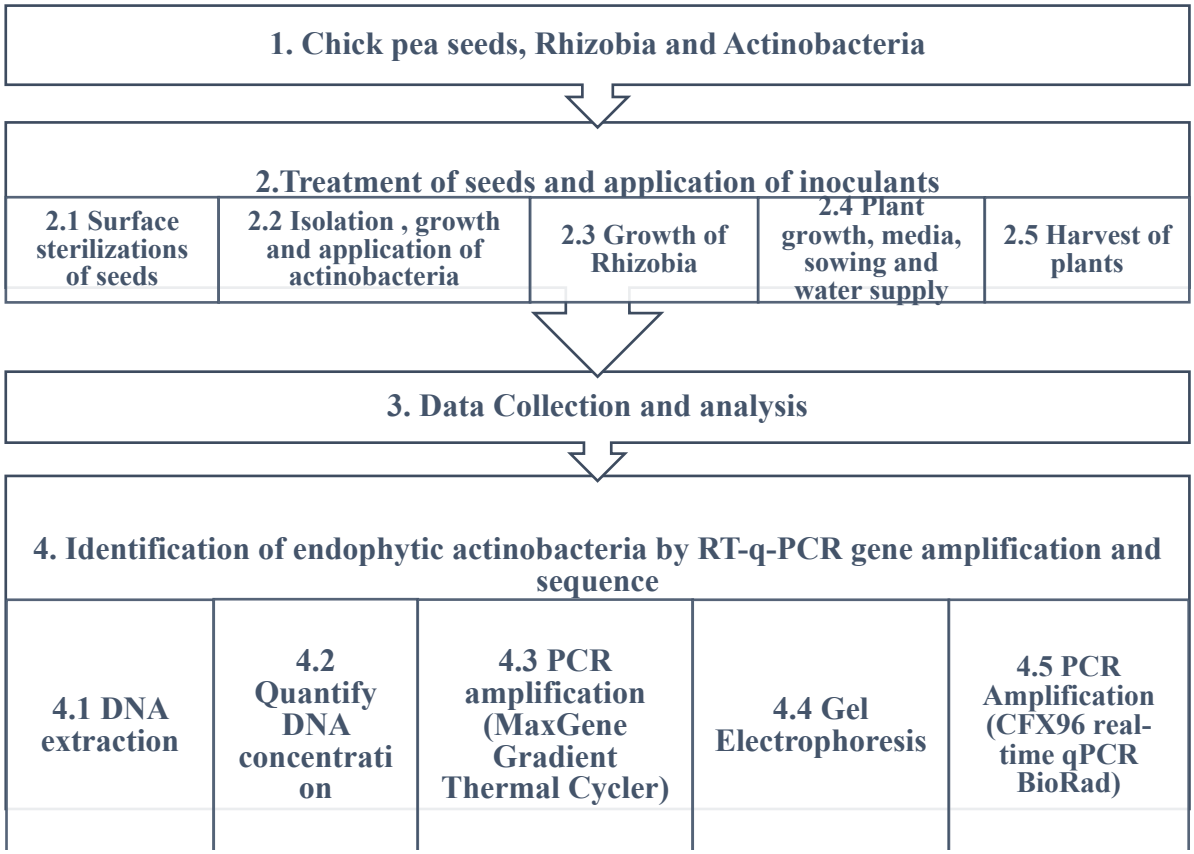


Figure 2.1 Outline of experimental design.

2.1 Chickpea Seeds, Rhizobia, and Actinobacteria:

The seeds of Chick pea (*Cicer arietinum*) and Rhizobia (*Mesorhizobium cicero) were* procured from South Australia Research and Development institute (SARDI). The Endophytic Actinobacterium strains ***Streptomyces* sp. LuP30, *Streptomyces* sp. LuP47B, *Streptomyces* sp. CP21A2, *Streptomyces* sp. CP200B, *Microbispora* sp. CP56, *Actinomadura* sp. CP84B.** Two of the strains of *Streptomyces* sp. were isolated from lucerne roots and Chick pea roots.

S.No.	Microbes	Species	Strain identity
1.	*Rhizobium*	*Mesorhizobium cicero*	**CC1192**
2.	Endophytic Actinobacteria isolate from lucerne roots	*Streptomyces* sp.	**LuP30** **LuP47B**
3.	Endophytic Actinobacteria isolate from Chick pea roots	*Streptomyces* sp.	**CP21A2** **CP200B**
4.		*Microbispora* sp.	**CP56**
5.		*Actinomadura* sp.	**CP84B**

Table 2.1. List of microbes used in the project.

2.2 Treatment of seeds and application of inoculants

2.2.1 Surface sterilization of seeds

Healthy seeds were selected and surface sterilization was performed as described by Coombs and Franco 2003. For nearly 30 seconds, the seeds were submerged in 70% v/v ethanol , three minutes in 4% hypochlorite solution and was rinsed thrice with RO water . Seeds were taken after 10 min of the final rinse and placed in the laminar airflow hood for 4 hours or overnight for dry (Coombs and Franco 2003; Miche and Balandreau 2001).

2.2.2 Isolation, growth and application of Actinobacteria

Six strains of Endophytic Actinobacteria viz., *Streptomyces* sp. LuP30*, Streptomyces* sp. LuP47B*, Streptomyces* sp. CP21A2*, Streptomyces* sp. CP200B*, Microbispora* sp. CP56 *and Actinomadura* sp. CP84B were grown on ISP2 . The cultured strains were incubated for two weeks until they produced spores. The incubation temperature was maintained at 27 ^{0}C.

Later the spores were stored at -20^0 C in 50% v/v glycerol which was autoclaved twice. Then actinobacteria treatment were applied on surface-sterilized seeds as a seed coat. These spores of actinobacteria have been suspended in 0.3% xanthan gum which has been autoclaved and were applied 10^6 CFU per gram of seed.

2.2.3 Growth of *Rhizobium*

For pure single colonies, the rhizobia were streaked on YMA (Yeast Mannitol Agar) to get pure colonies of rhizobia. Later these single colonies were moved to new petri dishes and incubated for two weeks at 27 ^{0}C and good growth of the strains was observed. The culture was stored at 4 ^{0}C for consequent use.

A standard curve depicting the connection between cell number and OD @600nm was plotted for every *Rhizobium* strain to empower the utilization of a standard 10^8 CFU/ml over the experiment.

2.2.4 Plant growth media, nutrition, sowing and supply of water

Seeds were surface-cleaned as portrayed above and planted into purified pasteurized (via autoclaving) pottingmix by autoclaving equal volumes of sand and vermiculite (Décor WatermaticTM) in 1.25L pots. The pots used in the experiment were self-watering.

Before planting the seeds (8 seeds per pot) 100 ml of water was added to each pot. Six treatments, 3 control of each strain (-ve control, N^+, and R^+) and 3 replicates were randomly used along with changing position of the pot every week in the glasshouse.

At that point, 200 mL of nitrogen inadequate supplement solution (McKnight, 1949) enhanced with a modest quantity of nitrogen (300 mg NH_4NO_3/20L McKnight's solution) was carefully added to the pots. This was done prior to covering of the pots with plastic bags. Pots were the placed in glasshouse. Following 7-days period, the pack made of plastic were removed, and seedling number was reduced to 4 uniform plants for every pot before adding 1 ml of *Rhizobium* inoculant (around 10^8 CFU/ml). As required for the rest of the week's plants were watered with MQ water.

All treatment and control pots were totally randomized in the glasshouse with situation of the pots changed each week.

2.2.5 Plant harvesting

The plants were removed gently from pots and wrapped in a moist paper and were stored in a bag for further assessment. Roots were separately washed in order to wash out the rest residue of sand and vermiculite.

2.3 Analysis and data collection:

The data were collected and analyzed in an MS Excel spreadsheet. Parameters taken for the analysis were

a) Number of nodules and total mass of the nodules in a plant

b) Weight of nodules,

c) Length and dry weight of roots and shoots.

Prior to drying the roots, the number of nodules per pot was removed, dried and count was noted. The average of two plants measure per pot were made

$$\text{Average dry weight of the nodule} = \frac{\text{Total nodule dry weight}}{\text{Total nodule number}}$$

2.4 Identification of Endophytic Actinobacteria using RT-q-PCR gene amplification and sequencing

Extraction of DNA and amplification using **RT-q-PCR** of selected Actinobacteria were carried as described by Coombs and Franco 2003.

a) DNA extraction
b) Quantify the DNA concentration
c) PCR amplification by using MaxyGene Gradient Thermal Cycler
d) Gel electrophoresis

e) PCR amplification by using real-time qPCR (CFX96 real-time qPCR system BioRad)

2.4.1. DNA Extraction:

Surface sterilization was performed on Fresh roots using 90% ethanol for about a minute, after which the roots were immersed in 4% Sodium Hypochlorite for 6 minutes and again for 30 seconds the roots were rinsed with 90% ethanol. Finally the roots were rinsed with RO water five times for 5 minutes.These fresh roots were cut using a sterile blade into 1mm sized pieces approximately. Fresh root of 1g was ground in the presence of liquid nitrogen using a mortar and pestle that were already chilled .0.5-1 g of glass beads were added in 1g of crushed roots. 500 ul of CTAB extraction buffer was added to a 2ml screw-capped microcentrifuge tube and vortexed briefly.Add 500ul of mixture of Phenol, chloroform and isoamyl alcohol in the ratio of 25:24:1 and the tubes were shaken in a bead beating instrument for 5 minutes.

The tubes were incubated in a water bath for an hour at 65°C.The tubes were centrifuged at 16000 x g for 5 minutes at 40°CThe aqueous top layer was separated and taken in a new 1.5 ml microcentrifuge tube Chloroform and isoamyl alcohol mixture in the ratio of 24:1 was added and tubes were mixed well.The tubes were centrifuged at 16000 x g for 5 minutes at room temperature.

The aqueous top layer was separated and taken in a new 1.5 ml microcentrifuge tube. Two volumes of PEG solution was added and incubated in the fridge for 2 hours. The tubes were centrifuged at 18000 x g at 40°C for a period of 10 min. The supernatant was gently poured off without disturbing the DNA pellet.

The pelleted DNA was washed in 70% ethanol (ice-cold). It was centrifuged again at 18000 x g for about 5 minutes at 40°C.The supernatant was gently poured off without disturbing the DNA pellet. The DNA pellet was resuspended in 50ul of injection water.

2.4.2 Quantify DNA concentration:

The concentration of DNA was evaluated by Nanodrop molecular calculator. 2 ul of DNA sample was used for quantification. The purity of DNA was inspected by the proportion of 260/230, 260/280 and by using gel electrophoresis.

2.4.3 PCR amplification by using MaxyGene Gradient Thermal Cycler

Phase	Temp. (in ℃)	Time (min.)	Cycles
Pre-denaturation	95	5	1
Denaturation	94	1	30
Anealing	53	1	30
Extension	72	1	30
Final extension	72	10	1

Table 2.2. Protocol for PCR

PCR was done with the MaxyGene Gradient Thermal Cycler to amplify the gene in actinobacteria. The primer *(*CP21A2 and CP200B*)* were designated. CP21A2 forward primer (5'-TCCAGGCTCAGCGCGACCTT) and CP21A2 reverse primer (5'-CGGCATCGGCAGTCTGAAGG) with 87 bp's. CP200B forward primer (5'-ACCTTCTTCTGGGCCTCGCC) and CP200B reverse primer (5'-CATGCTCGTACTCGAACGGCTC) with 138 bp's. The PCR was run with the following reagents: 5ul of Taq 2X master mix (New England BioLabs), 0.2ul of $Mgcl_2$ (New England BioLabs), 3ul of injection water, 0.4 ul of forward and reverse primer each and 1ul of template DNA. The total reaction volume for PCR was 10- ul.

2.4.4. Gel Electrophoresis

Gel electrophoresis was used to confirm the correct size of DNA (PCR products). 400 mg of agarose gel was added in 40 ml of TBE buffer and melted for 1 minute and the gel was allowed to cool down for 2 minutes after which 4 ul of Gel red was added. The solution was poured into the electrophoresis chamber and dried. Lated 1ul of loading dye mixed with 5ul of PCR product was loaded and the gel was allowed to run in a 0.5X running TBE buffer at 70V for 45 minutes.

2.4.5. PCR amplification by using real-time qPCR (CFX96 real-time qPCR system BioRad)

Quantitative reverse transcriptase-polymerase chain reaction was performed by using BioRad 96 well plates in a CFX96 real-time qPCR BioRad system . The reaction was carried out for 10ul total volume by using , 1ul of the forward primer and 1 ul of reverse primer, 5ul of (applied biosystem) SYBR Green Master Mix, 2ul of injection water and, 1ul of DNA.

Temperature	Time
95^0 C	2 min
40x cycles	
95^0 C	15 sec
58^0 C	1 min
72^0 C	10 sec

Table 2.3. q-PCR Protocol

2.4.6. Statistical Analysis

The Collection and interpretation of data were collected from IBM software from 8 weeks, 15 weeks and, 4 weeks of plants from pot assay I, II, III, respectively. By performing Post Hoc Tests, in homogeneous subsets of shoot length, root length, number of nodules, dry weight of shoots, dry weight of roots and, dry weight of nodules.

CHAPTER 3
RESULTS

Six Endophytic Actinobacteria strains were isolated from Chick pea plants (CP200B, CP21A2, CP84B, and CP56) and lucerne plant (LuP30 and LuP47B) which were provided from a collection. Various parameters like growth of the Chick pea plant and its nodulating ability along with the symbiotic Nitrogen fixation capacity were studied using the above isolated strains along with a rhizobial partner in a sand-vermiculite system and the results were analysed.

These six cultures were selected for Pot assay I which is dependent on their performance on the germination paper test and the rhizobial interaction assay. This comprised few strains that showed higher sporulation, beneficial outcomes when combined with rhizobial partner, germination and early development of Chick pea on germination paper; while few other strains were selected as they negatively effect on at minimum one factor of seedling germination and development or had a negative function in rhizobial interaction assay. Similarly two Actinobacteria strain LuP30 and LuP47B with best performance in the pot assay I were selected as controls in Pot assay II.

During Pot assay III, two selected strains that were isolated from Chick pea plant CP200B and CP21A2 were used for further identification and amplification using thermal PCR and RT-q-PCR and compared with pot assay I and II.

3.1. Identification and characterization of the LuP30, LuP47B, CP200B, CP21A2, CP56 and, CP84B

Growth of *Streptomyces* sp. LuP30 was upright on ISP3 with pale grey colour. There was significant growth on all media utilized ISP3, HPDA and MS with pale grey, white yellow and grey in colour respectively as far as morphology and pigment produced is concerned. The details of morphological characterization of this strain are shown in Table 3.1.

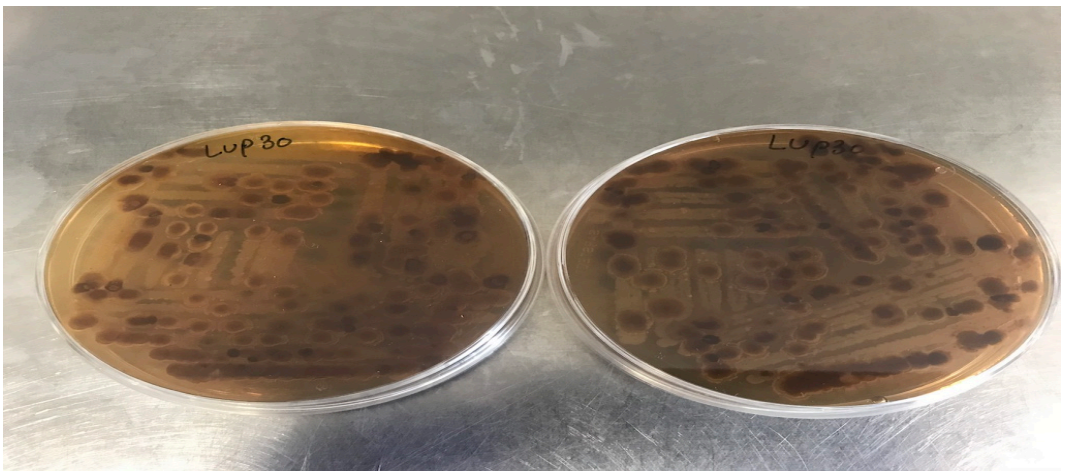


Figure 3.1. Morphology of *Streptomyces* sp. LuP30 on ISP3 media.

Streptomyces sp. LuP47B also showed a noteworthy growth on ISP3, MS and HPDA media. In terms of morphology (color property) and pigment produced from LuP47B produced Brownish Grey on MS media, Dark grey on HPDA and ISP3 media.

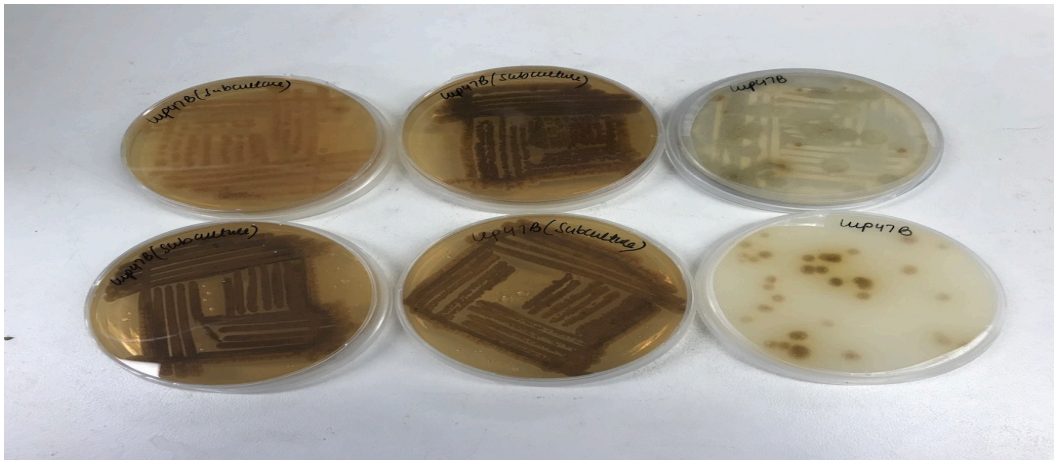


Figure 3.2. Morphology of *Streptomyces* sp. LuP47B on ISP3, HPDA and MS media

Streptomyces sp. CP200B showed varied colours of cultures on different media, viz., Dark Grey culture on MS media, Green colonies on HPDA media and, Dark grey cultures on ISP3 at temperature 27^0C. However, *Streptomyces* sp. CP21A2 produced dark grey cultures on MS and ISP3 media and dark white cultures on HPDA. Whereas, *Microbispora* sp. CP56 showed different colors on different media, viz., red colonies on MS media, Brown colonies on

HPDA and, light pink on ISP3 media. *Actinomadura* sp. CP84B showed brown pigmentation on MS media, dark brown on HPDA and, white on ISP3 (Table 3.1)

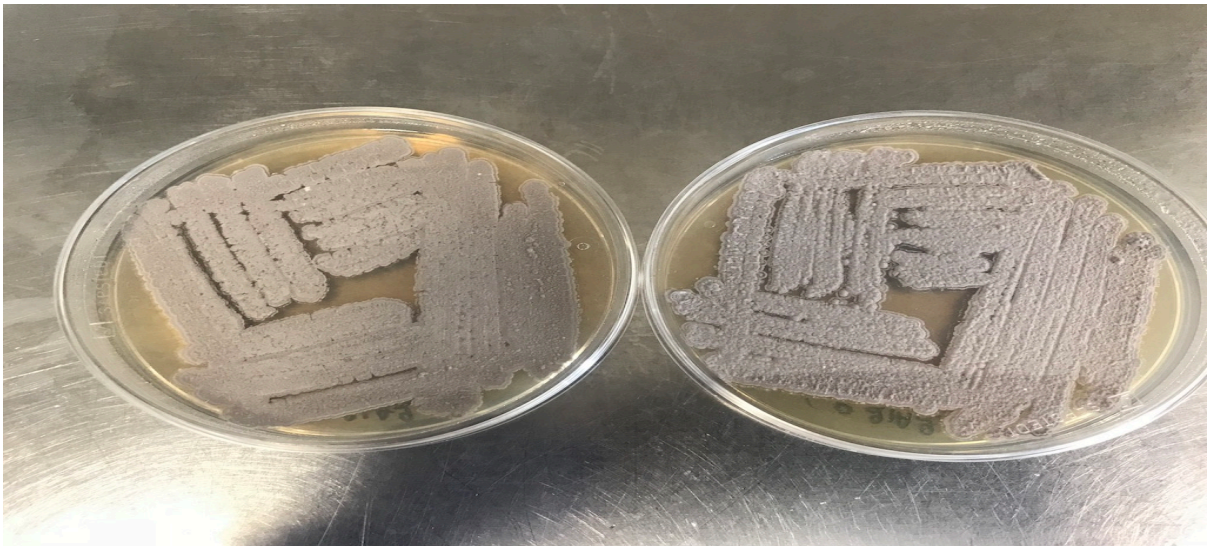


Figure 3.3. Morphology characterization of *Streptomyces* sp. CP200B on ISP3 media.

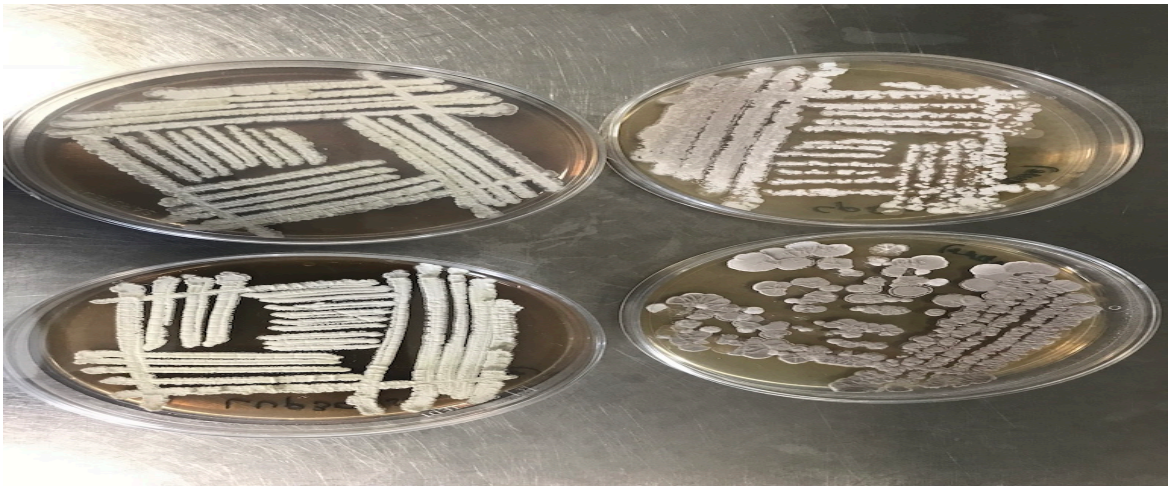


Figure 3.4. Morphology of *Streptomyces* sp. CP21A2 on ISP3 media.

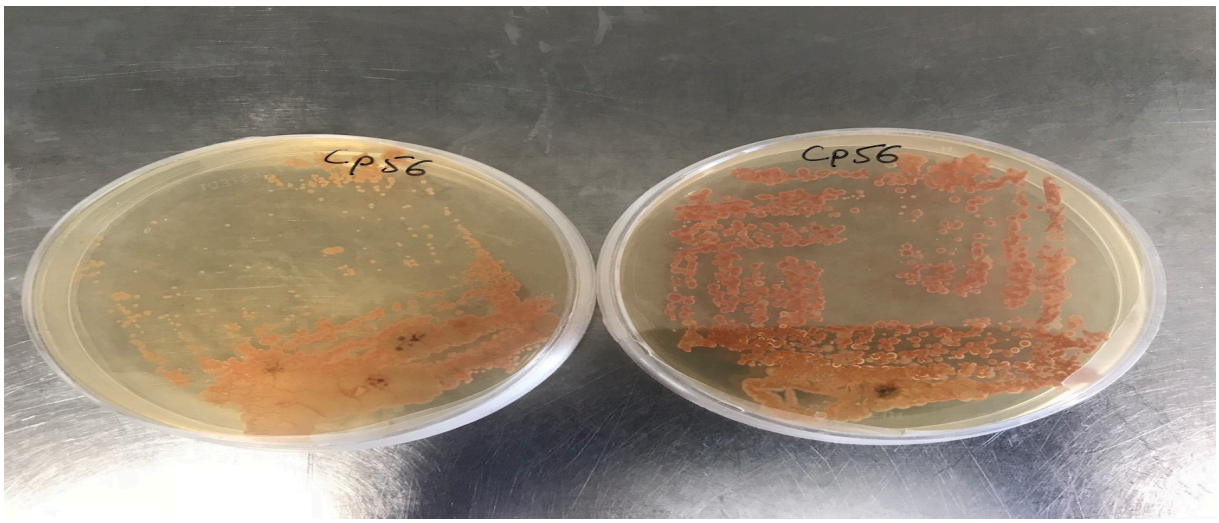


Figure 3.5. Morphology of *Microbispora* sp. CP56 on ISP3 media.

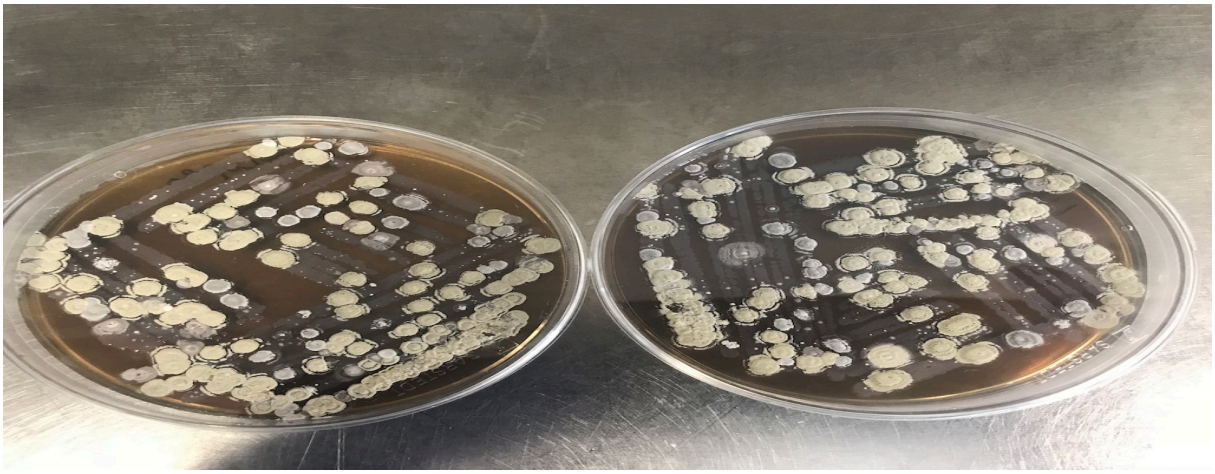


Figure 3.6. Morphology of *Actinomadura* sp. CP84B on ISP3 media.

No.	Strains	Plant soure	Temp. (in ^{0}C)	Morphology on the media (in terms of Color)			Proposed genus
				MS	HPDA	ISP3	
1	LuP30	Lucerne	27	Grey	White yellow	Pale grey	*Streptomyces*
2	LuP47B			Brownish grey	Dark grey	Dark grey	*Streptomyces*
3	CP200B	Chickpea		Dark grey	Green	Dark grey	*Streptomyces*
4	CP21A2			Dark grey	Dark white	Dark grey	*Streptomyces*
5	CP56			Red	Brown	Light pink	*Microbispora*
6	CP84B			Brown	Dark brown	White	*Actinomadura*

Table 3.1. Color Characteristics of the isolated strains on different media

3.2. Combined effects of Actinobacterium strains and *Mesorhizobium ciceri* (Rhizobial partner) strain CC1192 on growth of chickpea plant

3.2.1. Actinobacterium strains and rhizobial Interactions on YMA plate

The interaction between six Actinobacterial strains and *Mesorhizobium ciceri* strain CC1192 was studied using agar plate in an invitro test. Two Actinobacterial strains was found to be lacking influence on rhizobial development on the YMA medium. It was seen that the strains CP84B, CP56, CP21A2 and, CP200B enhanced the development of rhizobia. The strains CP84B and CP56 showed elevated rhizobial development.

Anti-toxins and Antibiotics produced by actinobacteria (Palaniyandi, et al. 2013) displayed antagonistic effect towards different microorganisms including rhizobia. In a study Solans, et al. (2016) established the fact, that Actinobacterial strains are a collection of spore-framing microbes linked with plants, known for their growth promotions and interaction with flora.

From the strains LuP30, LuP47B, CP200B, CP21A2, CP56, CP84B the growth of rhizobia (*Mesorhizobium cicero* strain CC1192) was shown by strains CP56, CP84B, CP21A2 and, CP200B on YMA medium. No interaction is shown by LuP47B and LuP30 strain.

3.2.2. Germination and preliminary growth of Chick pea due to Endophytic Actinobacteria in the absence of rhizobia

The impact of seed coated with Endophytic Actinobacteria on germination and early development of Chick pea was analysed using germination paper test. For each strain of Actinobacteria, there were 8 seeds on each paper, with significant outcomes for the 8 seeds introduced in Table 3.2 and, 3.3. The seeds covered with Endophytic Actinobacteria, R^+, N^+, N^- showed improved germination rate (Table 3.3). CP200B and CP84B strains showed germination rate as compared to the other four Actinobacteria strains (Table 3.2).

Furthermore, during the experiment in pot assays the rates of disease were also determined. This can be seen as there was no fungal or other parasitic infection on the germination paper or seed or during the growth or germination period. No parasitic growth were observed on a large portion of the seeds which were coated with Endophytic Actinobacteria and other three controls (R^+, N^+, and N^-). Even though, this data gave direction to choose strains on Chick peas development and nodulation in the pot measure.

Venkatachalam et al. (2010) and Postolaky et al. (2012) studied the beneficial impacts of Actinobacteria on the germination of seeds because of the generation of metabolites. *Streptomyces gibosonni* and *Streptomyces grieseoletus* produce polyamines to build the germination of maize, *Bromus lyticus*, black gram seeds, and radish.

Streptomyces segregated from soils in *Moldova* produce metabolites that upgrade the germination rate and invigorate root development of maize. Whereas, twelve *Streptomyces* strains improved the germination and early development of lucerne. The phytohormones which organisms can create, for example, auxins, gibberellins, cytokinins could likewise impact root development (Glloudemans and Bisseling, 1989). Some Actinobacteria can hinder pathogenic organisms separated from lucerne and clover by colonizing plant surfaces and delivering antibiosis against plant pathogens (Le 2016).

Strains taken for Treatment	Total no. of seeds sown	No. of diseased plants	No. of germinated seeds	Germination	Effect on rhizobia	Sporulation
CP84B (2 replicates)	(8.0+8.0) 16.0	7.0	9.0	56.25%	+	G
CP21A2 (2 replicates)	16.0	4.0	12.0	75%	+	G
CP56 (2 replicates)	16.0	3.0	13.0	81%	0	G
CP200B (2 replicates)	16.0	7.0	9.0	56.25%	+	G
LuP47B (2 replicates)	16.0	1.0	15.0	93.75%	0	G
LuP30 (2 replicates)	18.0	0.0	18.0	100%	+	G

Table 3.2. Germination assay, interaction assay and sporulation studies

Treatment	Total no. of seeds sown	No. of diseased plants	No. of germinated seeds	Germination
R^+	96.0	37.0	59.0	61.4%
N^+	96.0	40.0	56.0	58.3%
N^-	96.0	29.0	67.0	69.79%

Table 3.3. Study on germination rate of chickpea seeds treated with R^+, N^+, and, N^-

3.3. Chickpea plant growth due to Endophytic Actinobacteria in sand and vermiculite

3.3.1. Pot assay I

In pot assay I (8 weeks chickpea plant), six different Endophytic Actinobacteria strains (CP200B, CP21A2, CP56, CP84B, LuP30 and, LuP47B) which were isolated from Chick pea plant and lucerne plant was used with three controls R^+, N^+, N^- (with rhizobia, added nitrogen, absence of nitrogen respectively) with 3 replicates for each treatment. The 8 weeks old Chick pea which was treated with nitrogen and without nitrogen grew well in pot assay I. Whereas, CP200B and CP84B grew less than other treatments. In pot assay 1 the highest growth was seen in LuP30, CP21A2, CP56, and LuP47.

In trial with abundant nitrogen, the leaves of the chickpea plant showed a burnt appearance due to dehydration because when there was an excess amount of nitrogen

absorbed by the plant, the mineral salt content of soil gets increased and less amount of water in the plant which cause dehydration in leaves of the plant leading to color change from yellow or brown.

When eight weeks old Chick pea plants were harvested, various parameters were observed as provided in (Table 3.4).

At about two months after planting, chickpea plants that were just inoculated with *Mesorhizobium ciceri* strain CC1192 yielded an average of 0.044 g dry weight of nodules per plant and a total mass of 0.59 g per plant which was essentially more prominent than the total mass of the plant without Nitrogen N^- (0.36 g/plant), however not exactly the total plant mass in that of the N^+ (0.75g/plant) (Table 3.4). This implies that the symbiotic system provided a measure of the fixation of nitrogen, yet the total N_2 requirement of the plant was not met when supplied with rhizobia alone.

The co-inoculation with R^+, N^+, and N^- with total plant mass was 0.59g, 0.75g and 0.36g respectively. whereas, the different Endophytic Actinobacteria co-inoculated with rhizobia showed enhancement of total mass of the plant which was 0.7g of LuP30, 0.89g of LuP47B, 0.8g of CP200B, 0.93g of CP84B, 0.49g of CP56 and, 0.43g of CP21A2 (Table 3.4)

At about two months after planting, the number of nodules on the Chick peas with CP200B strain was 19.3 nodules per plant, with 0.27g dry weight of nodule which was altogether more noteworthy contrasted with those who treated with rhizobia only (17.6 nodules/plant with 0.044 g dry weight of nodule).

From LuP47B strain, 16.7 nodules per plant were measured with 0.26g dry weight of nodule. However, the number of nodules with R^+ and N^- was 17.6 with 0.04 g of nodules dry weight/plant and 18.4 with 0.08 g of nodules dry weight/plant. The reason behind this was that both two control nodule's sizes were smaller and tiny.

Notwithstanding, dry weight of nodules was most prominent with CP200B (0.27 g/plant), LuP47*B* (0.26 g/plant), CP84 (0.21 g/plant), CP56 (0.18 g/plant), LuP30 (0.16 g/plant), N^- (0.08 g/plant), CP21A2 (0.06 g/plant) and, R^+ (0.04 g/plant). (Table 3.4). Number of nodules in each treatment were measured as described in Figure 3.14 and Table

3.4 viz., CP200B (19.3 nodules/plant, N^- (18.4 nodules/plant), R^+ (17.6 nodules/plant), LuP47 (16.7 nodules/plant), CP21A2 (14 nodules/plant), LuP30 (5.8 nodules/plant), CP56 (3.5 nodules/plant) and CP84 (1.5 nodules/plant).

In spite of that, the dry weight of shoot and length of shoot were higher in CP200B treated Chick pea plant (0.71g and 40cm) which was less in unlimited nitrogen treated (0.47 g of dry weight and 34.8 cm of shoot length). whereas, dry weight of root and length of root were highly measured in LuP47B (0.29 g and 24.9 cm). However, it was observed that unlimited nitrogen treated dry weight of root was 0.28g and length of root was 22.7cm which was less as compared to other treated strains. Whereas, dry weight of shoots being higher, the length of roots of unlimited nitrogen treatment (22.7 cm/plant) was shorter than others with the exception of the rhizobia (28.8cm /plant) (Table 3.4). The shoot length of no nitrogen included treatment (31.4 cm/plant) were altogether not as much as rhizobia alone treatment (0.38cm/plant). Though, the shoot dry weight of Chick peas (0.47g/plant) with unlimited nitrogen was altogether expanded contrasted with chickpeas with just rhizobia included (0.38g/plant) (Table 3.4).

The parameters which demonstrate early chickpea development, for instance, root length, shoot, number of nodules were measured. Strains LuP30 and CP84B, presented the most higher positive outcome on Chick pea root length (35 and 29.5 cm/plant, respectively), while CP84B had a better impact on root length. Strains LuP47B, CP84B, LuP30 and, CP200B generated better stimulated shoot length of Chick pea (36.7, 36.5, 35 and, 40 cm/plant, separately). (Table 3.4)

Figure 3.7 Depicts the connection between plant total mass and dry weight of nodules in which Chick peas treated with included rhizobia. The correlation of pot assay 1 is 0.07, demonstrating a positive connection between nodulation and plant development.

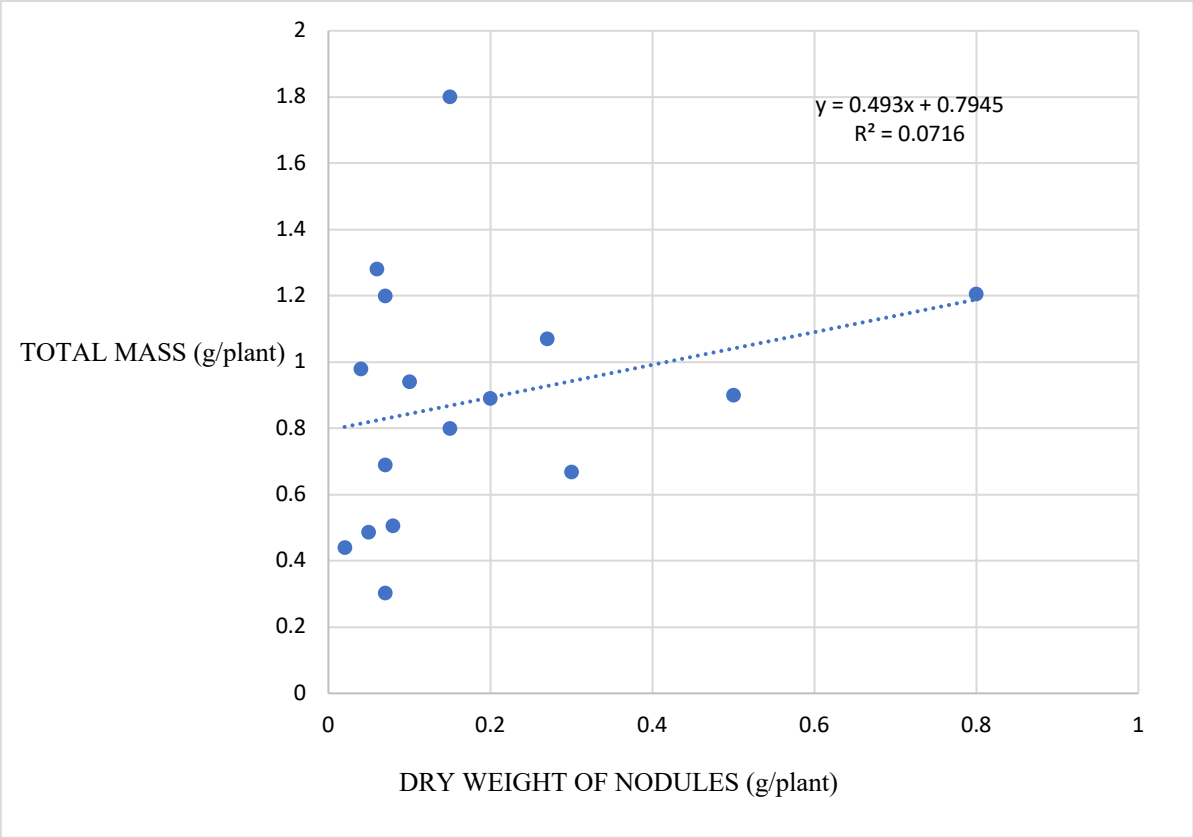


Figure 3.7 Standard curve for Total plant mass vs dry weight of nodules treated with six different actinobacteria strains

Treatment	Dry Weight of shoots (g/plant)	Length of Shoot (cm/plant)	Dry weight of roots (g/plant)	Length of root (cm/plant)	Dry weight of nodules (g/plant)	Total mass (g/plant)	No. of nodules/plant
R^+	0.38^{a}	22.6^{a}	0.21^{a}	28.8^{a}	0.044^{bcd}	0.59^{bc}	17.6^{ab}
N^+	0.47^{ab}	34.8^{ab}	0.28^{ab}	22.7^{b}	0.05^{cd}	0.75^{ab}	0.01^{cd}
N^-	0.22^{a}	31.4^{ab}	0.14^{bc}	23.5^{b}	0.086^{abc}	0.36^{a}	18.4^{ab}
LuP47	0.60^{c}	36.7^{b}	0.29^{ab}	16.2^{c}	0.26^{a}	0.89^{b}	16.7^{a}
LuP30	0.49^{ab}	35.0^{b}	0.21^{a}	24.9^{b}	0.16^{c}	0.7^{cd}	5.8^{c}
CP200B	0.71^{cd}	40.0^{bc}	0.09^{c}	14.0^{d}	0.27^{a}	0.8^{cd}	19.3^{ab}
CP84B	0.73^{cd}	36.5^{b}	0.2^{cd}	17.7^{c}	0.21^{b}	0.93^{a}	1.5^{c}
CP21A2	0.38^{a}	30.5^{a}	0.05^{c}	9.5^{cd}	0.06^{cd}	0.43^{bc}	14.0^{a}
CP56	0.41^{ab}	33.5^{ab}	0.08^{c}	16.0^{c}	0.18^{c}	0.49^{bc}	3.5^{c}

Table 3.4. Statistic analysis of the experimental data from 8 weeks of plants from pot assay I

3.3.2. Pot assay II

In the subsequent pot assay II the isolated Endophytic Actinobacteria contrasts within the six different Endophytic Actinobacteria which were treated with three different

controls, two replicates, and the plants developed superior to those in pot test I, with certain plants producing flowers and fruits, demonstrating that conditions were less constraining for development. Following two months, treatment with boundless nitrogen developed superior to others. Except for the boundless nitrogen treatment, the more seasoned and lower Chick pea leaves changed to yellow with yellow leaf veins.

Various plants had dark roots, probably a side effect of parasitic disease starting from the seeds. Furthermore, the roots of boundless nitrogen treatment were changed to dark shading and a few leaves changed to the yellow shading.

Chickpea roots show the effects by Endophytic Actinobacteria which were isolated from lucerne strains and Chick pea strains in sand and vermiculite after 15 weeks (which were treated with N- control, N+ control, R+ and 6 different Endophytic Actinobacteria with R+ (R= *Mesorhizobium cicero* strain CC1192).

At about two months after planting, Chick pea plants with just rhizobial *Mesorhizobium ciceri* strain CC1192 included, yielded a normal of 0.060 g of nodules per plant with a total shoot and root mass of 0.99 g for each plant. This was altogether more prominent than the total plant mass in the no additional Nitrogen treatment (0.68 g/plant), however essentially not exactly the total plant mass of Chick pea with boundless nitrogen treatment (0.67 g/plant of N+ control) (Table 3.7). Therefore, the fixed nitrogen delivered by Chick peas with rhizobia as control didn't fulfill the potential nitrogen need of plants.

As far as complete total plant mass was concerned, it contrasted with plants with just rhizobia included, the Chick pea plants co-inoculated with rhizobia and one of the strains LuP30, CP200B, CP84B, CP200B, CP21A2, CP56 indicated an essential increment. (Figure 3.7).

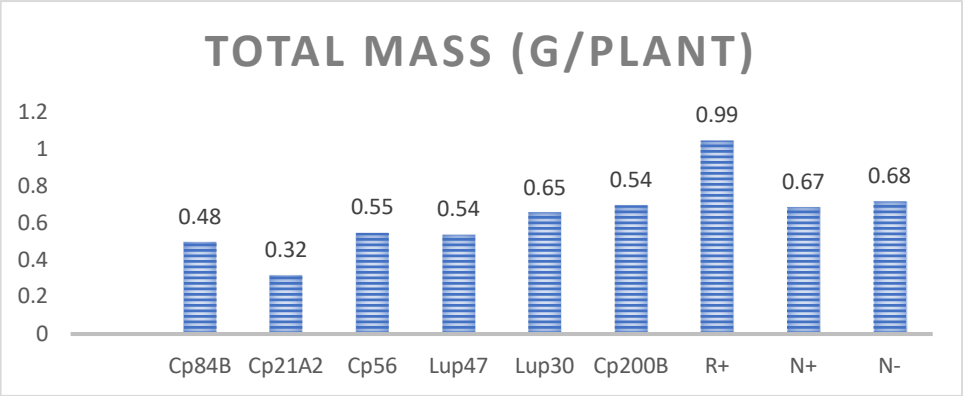


Figure 3.8. Effects of Endophytic Actinobacteria isolated from Lucerne and chickpea strains on 15 weeks old total mass g/plant in sand and vermiculite.

The strains isolated from lucerne and Chick pea inoculated with Chick pea seeds with rhizobia and has found to show an average nodule weights of plants while using CP84B, CP21A2, CP56, LuP47, LuP30, CP200B to be 0.022, 0.00, 0.004, 0.003, 0.016 and 0.16 g/plant respectively. Whereas, average nodule weights of three controls were 0.060, 0.026 and 0.041 for R^+, N^+ and N^- respectively as shown in Table 3.7

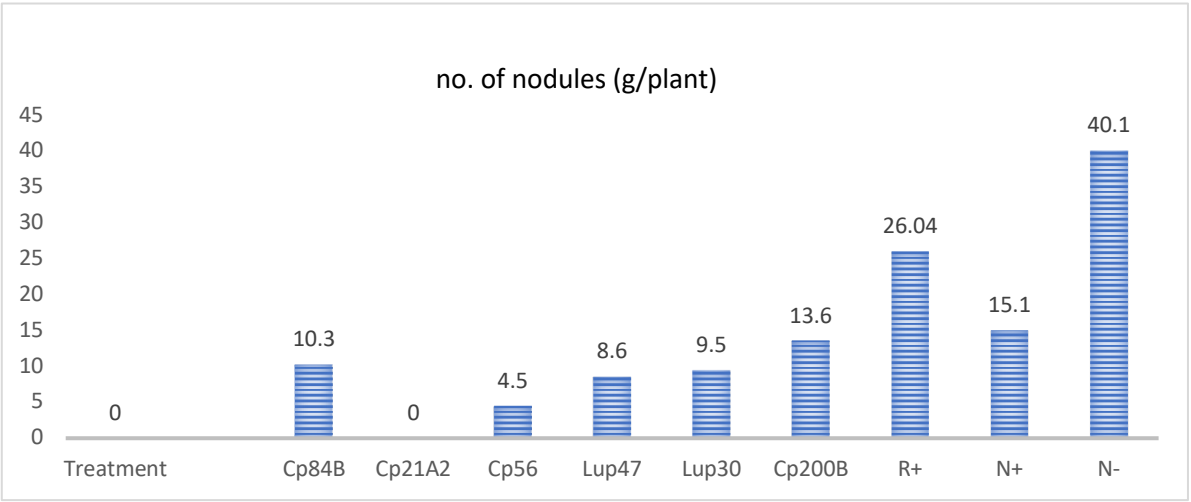


Figure 3.9. Endophytic Actinobacterial strain effects on nodulation (dry weight of nodules g/plant) isolated from lucerne and Chick pea on 15 weeks old Chick pea plant in sand and vermiculite.

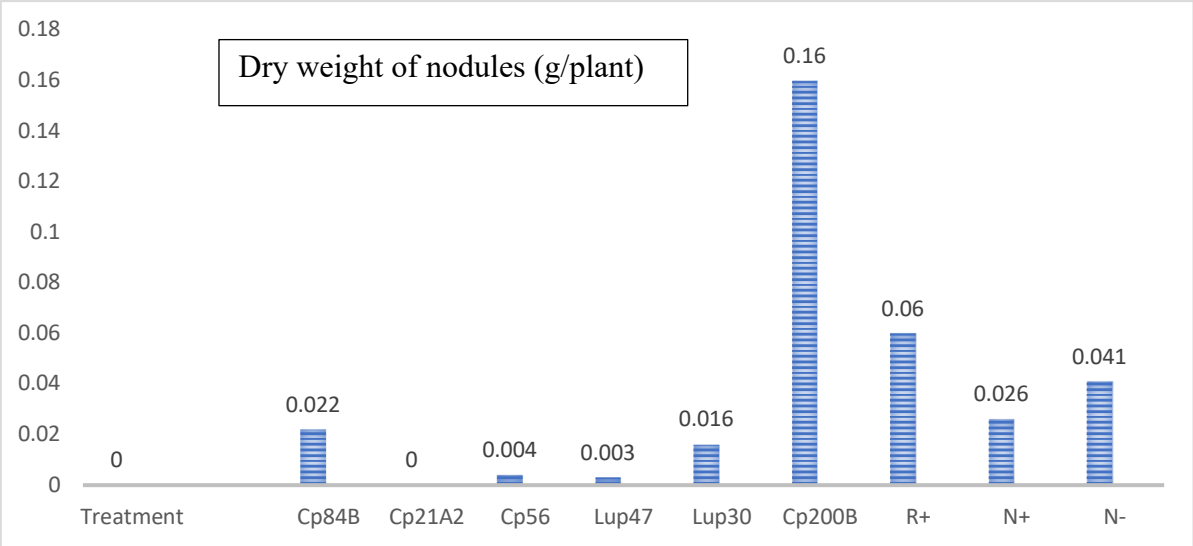


Figure 3.10. Endophytic Actinobacterial strain effects on nodulation (number of nodules g/plant) isolated from lucerne and Chick pea on 15 weeks old Chick pea plant in sand and vermiculite.

These plants altogether does not produce higher quantities of nodules in contrast to that of the treatment with rhizobia which yielded 26.04 nodules/plant. Similarly, the number of nodules produced within 15 weeks was 10.3, 0.00, 4.5, 8.6, 9.5, 13.6, 26.04, 15.1 and 40.1g/plant respectively by CP84B, CP21A2, CP56, LuP47, LuP30, CP200B, R^+, N^+, and N^-. The Strain CP200B, yielded increased number of nodules compared to other strains. Whereas, number of nodules shown by three different controls were higher as compared to strains which were 26.04g/plant for R^+, 15.1g/plant for N^+ and 40.1g/plant for N- (Figure. 3.7) The strain CP200B produced the highest number of nodules 13.6 nodules /plant as well as the highest dry weight of nodule as compared to other strains (0.16 g/plant).

It was seen that the lack of nitrogen affected the root development of the Chick pea plants in this examine. The root length of Chick peas provided with nitrogen (N^+ control,) was shorter than others. In contrast to the treatment using only rhizobia and N^- showed higher yields than others (Table 3.7). Average length of plants roots of CP84B was found to be 25.2 cm, CP21A2 - 31.0 cm, CP56 -33.08 cm, LuP47- 31.3 cm, LuP30- 46.0 cm, CP200B -26 cm, 34.4 cm by R^+, 31.5 cm by N^+ and 34.7 cm by N^- . The formation of seeds from 15 weeks old Chick pea plant was higher in R^+ (6.3) and N^+ (6.2) whereas, untreated control shows only 4.8. On the other hand, the formation of seeds in CP56 (5.6), CP84 (5.1), CP200B (5.0),

Lup47B (4.8), Lup30 (4.7) and Cp21A2 (3.7) (Table 3.7). But Chick pea plants treated with Endophytic Actinobacteria showed less formation of seeds as compared to R+. which showed that effects were negligible in treated strains Chick pea.

From the graph plotted (Figure 3.11), the correlation of pot assay II was 0.5 demonstrating a positive connection among nodulation and plant development.

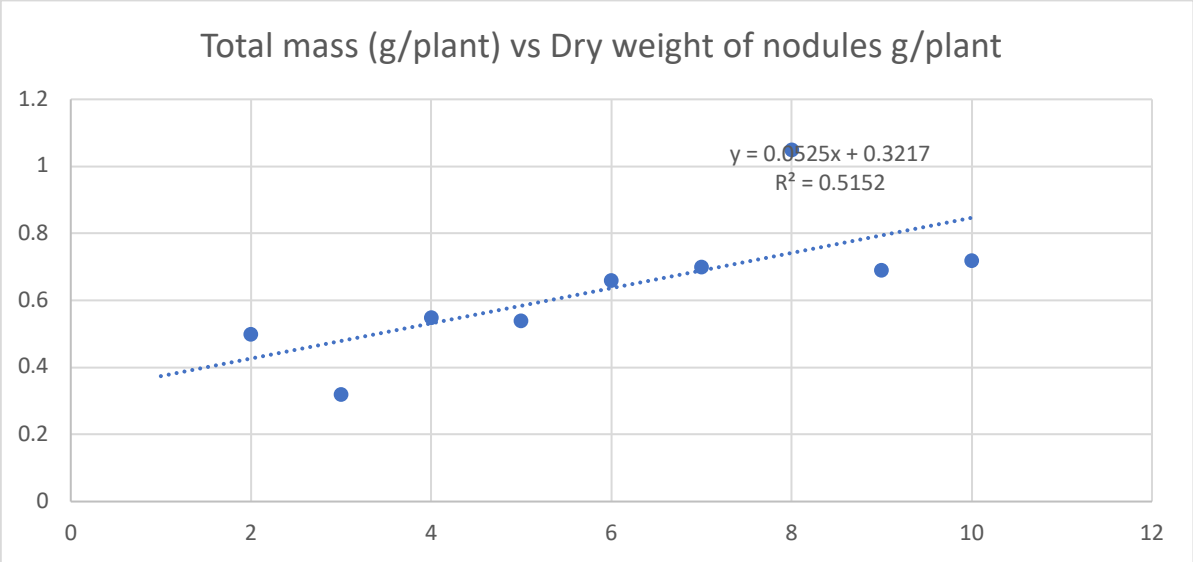


Figure 3.11. Standard curve of Total plant mass and Dry weight of nodules of Chick pea treated with six Actinobacterial strains isolated from lucerne and Chick peas.

Analysis of the observed data from 8 weeks to 15-week plants

Treatment	Total No. of plants	Total No. of seeds from plant	Total No. of flowers from plants	Sum of both seeds and flowers	Average
Untreated	21	51	50	101	4.8
R^+	21	90	43	133	6.3
N^+	24	52	99	151	6.2
CP56	6	14	20	34	5.6
CP200B	6	14	16	30	5.0
CP84	6	11	20	31	5.1
LuP30	4	11	8	19	4.7
LuP47*B*	6	2	27	29	4.8
CP21A2	4	0	15	15	3.7

Table 3.5 Formation of seeds and flowers from 15 weeks old chickpea plants

Treatment	Total number of seeds from plants	Fruits used for DNA extraction	Dry weight analysis	Dry weight of seeds(in g)
Untreated	51	10	41	0.35
R^+	90	10	80	0.74
N^+	52	10	42	0.33
CP56	14	12	2	0.0018
CP200B	14	13	1	0.0085
CP84	11	11	0	0
LuP30	11	11	0	0
LuP47B	2	2	0	0
CP21A2	0	0	0	0

Table 3.6 Data analysis for 15 weeks old Chick pea seeds

Treatment	Dry weight of shoots (g/plant)	Length of Shoot (cm/plant)	Dry weight of roots (g/plant)	Length of Root (cm/plant)	Dry weight of nodules (g/plant)	Total mass (g/plant)	No. of nodules /plant
R^+	0.65^{bc}	46.8^{b}	0.34^{b}	34.4^{ab}	0.060^{a}	0.99^{d}	26.04^{bc}
N^+	0.37^{a}	26.0^{a}	0.30^{b}	31.5^{ab}	0.026^{a}	0.67^{bc}	15.1^{b}
N^-	0.45^{ab}	35.5^{ab}	0.23^{ab}	34.7^{ab}	0.041^{a}	0.68^{bc}	40.1^{abc}
LuP47B	0.43^{ab}	35.0^{ab}	0.11^{a}	31.3^{ab}	0.003^{a}	0.54^{b}	8.6^{ab}
LuP30	0.40^{ab}	34.5^{ab}	0.25^{ab}	46.1^{b}	0.016^{a}	0.65^{bc}	9.5^{ab}
CP200B	0.34^{a}	30.3^{a}	0.20^{ab}	26.0^{a}	0.16^{b}	0.54^{b}	13.6^{b}
CP84B	0.26^{a}	29.3^{a}	0.22^{ab}	25.2^{a}	0.022^{a}	0.48^{ab}	$10.^{3ab}$
CP21A2	0.21^{a}	27.5^{a}	0.11^{a}	31.0^{ab}	0.00^{a}	0.32^{a}	0.00^{a}
CP56	0.38^{a}	39.2^{ab}	0.17^{a}	33.08^{ab}	0.004^{a}	0.55^{b}	4.5^{ab}

Table 3.7 Statistic analysis of the experimental data of 15 weeks Chick pea plant from pot assay II

3.3.3. Pot assay III

In Pot assay III, Endophytic Actinobacteria CP21A2 and CP200B treated seeds were sown again for 4 weeks with two replicates and three control viz., untreated control, R^+ and, N^+. The germination rate of Chick pea plant was analaysed as given below

Observed data and analysis for 4-week-old plants (CP200B and CP21A2 treated with R^+, N^+ and Actinobacteria + Rhizobia (R^+)

For CP200B and CP21A2, 4 replications were performed with three different treatments R^+, N^+ and, Actinobacteria + Rhizobia (R^+) in each replicate 6 seeds were grown.

Treatment	Total no. of seeds sow	No. of germinated seeds	Number of diseased plants	Germination	No. of Plants used for DNA extraction
N^+ (2 Replicates)	24.0	21.0	3.0	87.5%	8
R^+ (2 Replicates)	24.0	16.0	18.0	66.6%	3
CP200B + R^+ (2 Replicates)	24.0	9.0	15.0	37.5%	1
CP21A2 + R^+ (2 Replicates)	24.0	16.0	8.0	66.6%	5

Table 3.8 Germination rate of Chick pea plant in Pot assay III

An average of the dry weight , shoot length, root length, dry weight of nodules , total mass and number of nodules per plant were calculated from which it is evident that CP21A2 produced a higher number of nodules within 4 weeks whereas CP200B yielded 4.5 number of nodules. Hence in the period of 4 weeks CP21A2 shows the development in the Chick pea plant as compared to other treatments. Given below is the table in which the average of the parameters are tabulated.

Treatment	Dry weight of Shoots (g/plant)	Length of shoot (cm/plant)	Dry weight of roots (g/plant)	Length of root (cm/plant)	Dry weight of nodules (g/plant)	Total mass (g/plant)	No. of Nodules/ plant
R^+	0.12^{a}	29.5^{a}	0.056^{a}	13.6^{a}	4.6^{bc}	0.17^{a}	0.038^{a}
N^+	0.21^{a}	27.5^{a}	0.11^{ab}	31.0^{bc}	0.00^{a}	0.32^{ab}	0.00^{a}
CP200B	0.38^{a}	39.2^{ab}	0.17^{ab}	33.08^{bc}	0.004^{a}	0.55^{b}	4.5^{ab}
CP21A2	0.43^{a}	35.0^{ab}	0.11^{ab}	31.3^{bc}	0.003^{a}	0.54^{b}	8.6^{b}

Table 3.9 Statistic analysis of observed data of 4 weeks old chickpea plant from pot assay III.

Figure 3.12 depicts the correlation between plant total mass and dry weight of nodules in chickpeas treated in N^- control, N^+ control, R^+ and 6 different Endophytic Actinobacteria with R^+ (R is *Mesorhizobium cicero* strain CC1192). The correlation factor of pot assay 3 was found to be 0.9 giving a positive demonstration among nodulation and plant development.

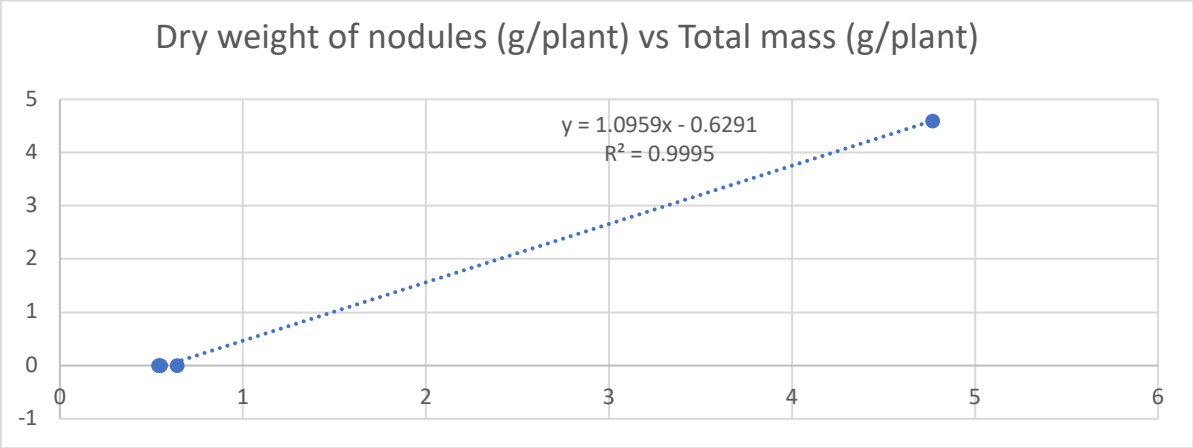


Figure 3.12 Standard curve of Dry weight of nodules vs Total plant mass of Chick peas treated by two Endophytic Actinobacteria isolated from Chick peas.

3.4. Identification of Actinobacterial strains using RT-q-PCR

Selected strains of CP200B and CP21A2 Actinobacteria from weeks 4, 8 and 15 were sterilized and prepared for extraction of DNA.

After the extraction of DNA, quantification of DNA concentration was performed using nanodrop molecular calculator method (Appendix table 2.1, 2.2, 2.3, 2.4 and, 2.5).

Quantified DNA showing better result was used for PCR amplification to obtain PCR product.

DNA product of CP200B, CP21A2 and PCR product of CP200B, CP21A2 were run in the gel electrophoresis from which desired bands in the gel were noted which shows the quantification of DNA and interaction with primers.

To check the quantification of DNA of CP200B and CP21A2, gel electrophoresis was run with extracted DNA. Whereas, the base pair of CP21A2 and CP200B was 87 and 138 respectively. From week 4 samples, wells 1, 2, 3, 4, 7 and 8 shows the bands of CP21A2, however, wells 5, 6, 9 and 10 depicts the bands for CP200B.

From week 8 samples, wells 1,2,3 and 4 highlight the bands for CP200B and rest of the wells 5,7,9 and 10 depicts the bands for CP21A2 (figure 3.13).

From week 15samples wells 1,2,7,8,9 and 10 shows the bands for CP21A2 however wells 3,4,5,6,11,12,13 and 14 shows the band for CP200B (figure 3.14).

MaxyGene Gradient Thermal Cycler the PCR amplification was used for CP200B and CP21A2 to get the PCR product. The PCR product was run in the gel electrophoresis to check that primer is working with the particular Chick pea strains.

Figure 3.15 gave confirmation, that primers worked and showed their interaction with CP200B and CP21A2. In well 1 mixing of DNA CP21A2 and Primer CP200B, well 2 mixing of DNA CP200B and primer CP21A2 and in well 3 same DNA and primer which was used for CP21A2 and they were found to lie nearer to the base pair which was 87. However, well 4 and 5 showed the interaction with primer and DNA in which the same DNA and primer were added for strain CP200B whereas, well 7 and 8 depict that primer was working with strain CP21A2 from week 4.

PCR product of CP200B and CP21A2 from week 8 shows the bands in gel electrophoresis which shows that primers were worked with the particular strains. In well 1, 2 and 5 same DNA and primer were added for strain CP200B. Considering wells 3, 4, 6 and 7 for strain CP21A2 in which same DNA and primer were added which shows exact band near to the base pair for this strain which was 87. Nevertheless, in wells 10 and 11 different primers were used from DNA which was in well 10, DNA from strain CP200B and primer from CP21A2 and in well 11, DNA from strain CP21A2 and primer from CP200B (figure 3.16).

On the contrary, the PCR product of CP200B and CP21A2 from week 15 shows the bands in gel electrophoresis in which the same number of DNA and primers were used for strain CP21A2 in well 1 to 4. In wells 5 to 10 the same number of DNA and primers were used for strain CP200B, but in wells 13 and 14 different DNA and primers were used in which DNA used from CP200B and primer from CP21A2. In wells 16 and 17 DNA were used from CP21A2 and primer from CP200B (figure 3.17)

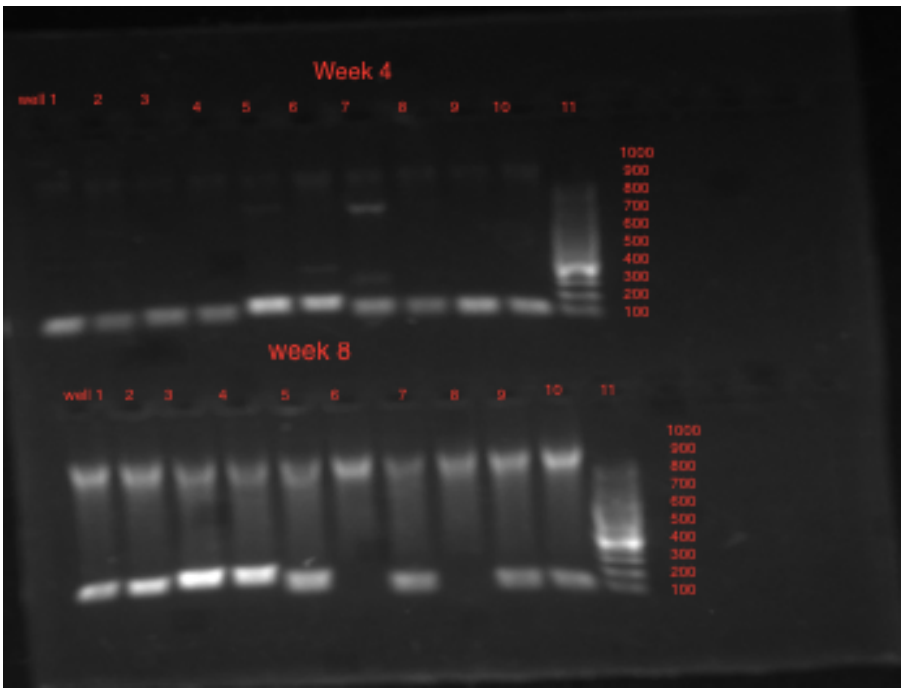


Figure 3.13 Gel electrophoresis of Extracted DNA from strain CP200B and CP21A2 from week 4 and 8 .

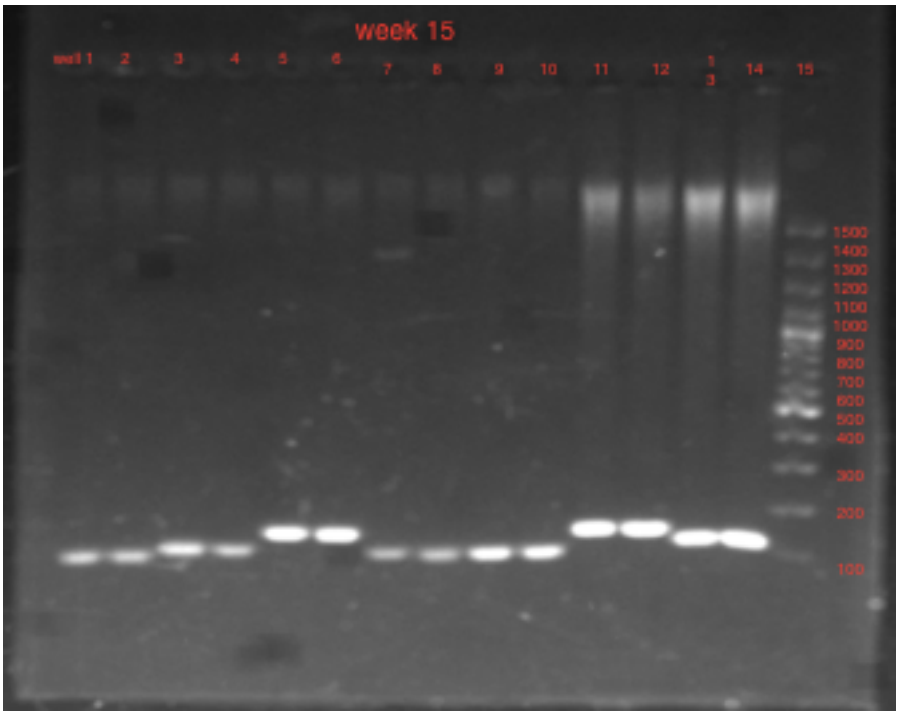


Figure 3.14 Gel electrophoresis of Extracted DNA from strain CP200B and CP21A2 from week 15 .

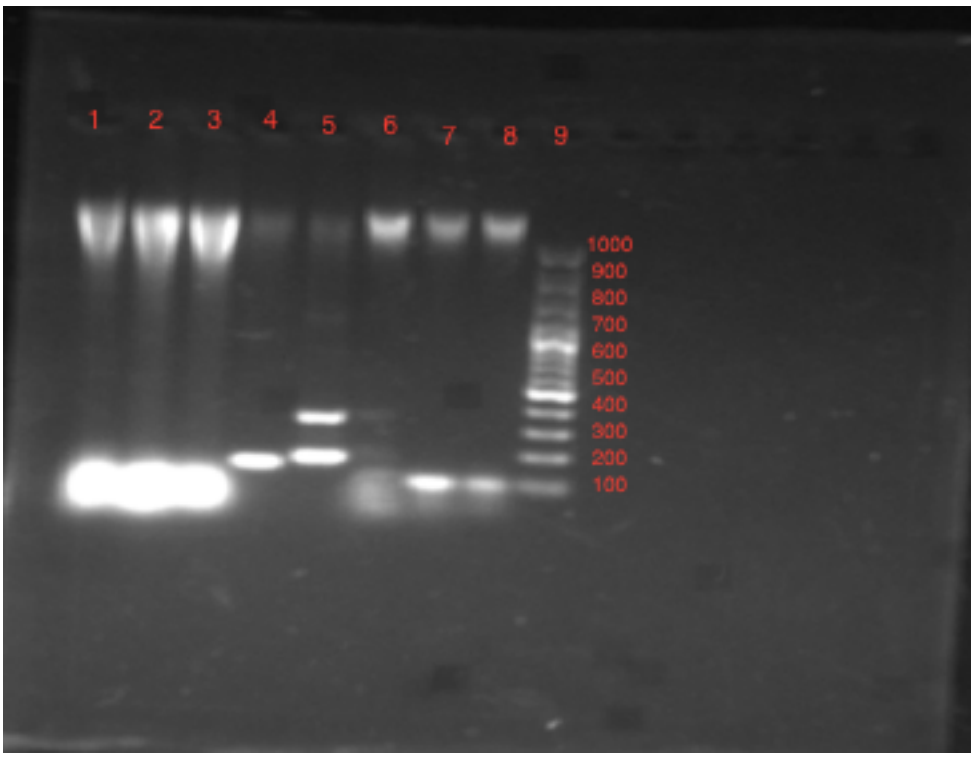


Figure 3.15 Gel electrophoresis of PCR product of CP200B and CP21A2 from week 4.

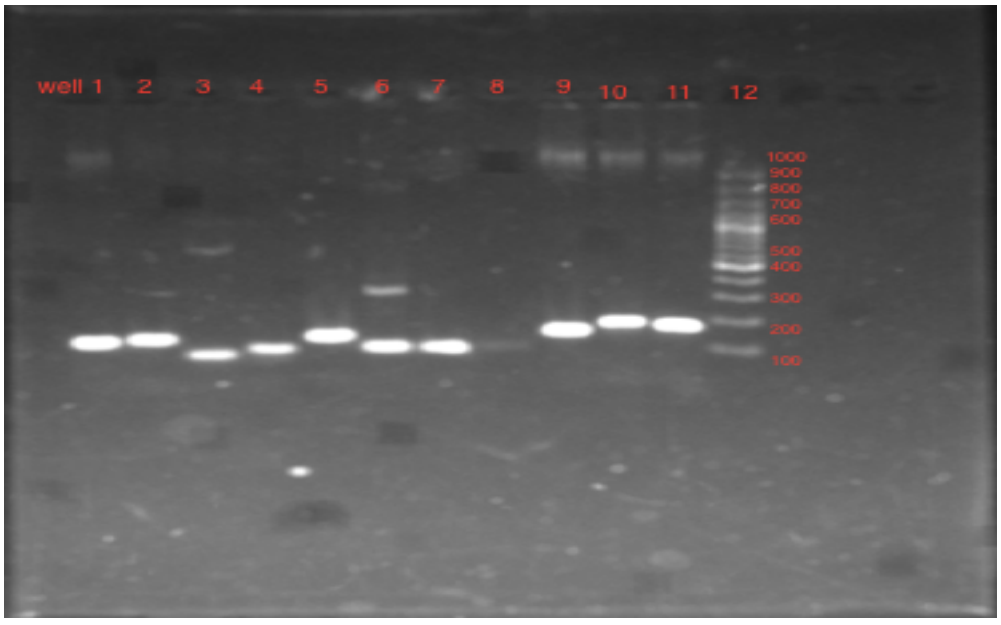


Figure 3.16 Gel electrophoresis of PCR product of CP200B and CP21A2 from week 8.

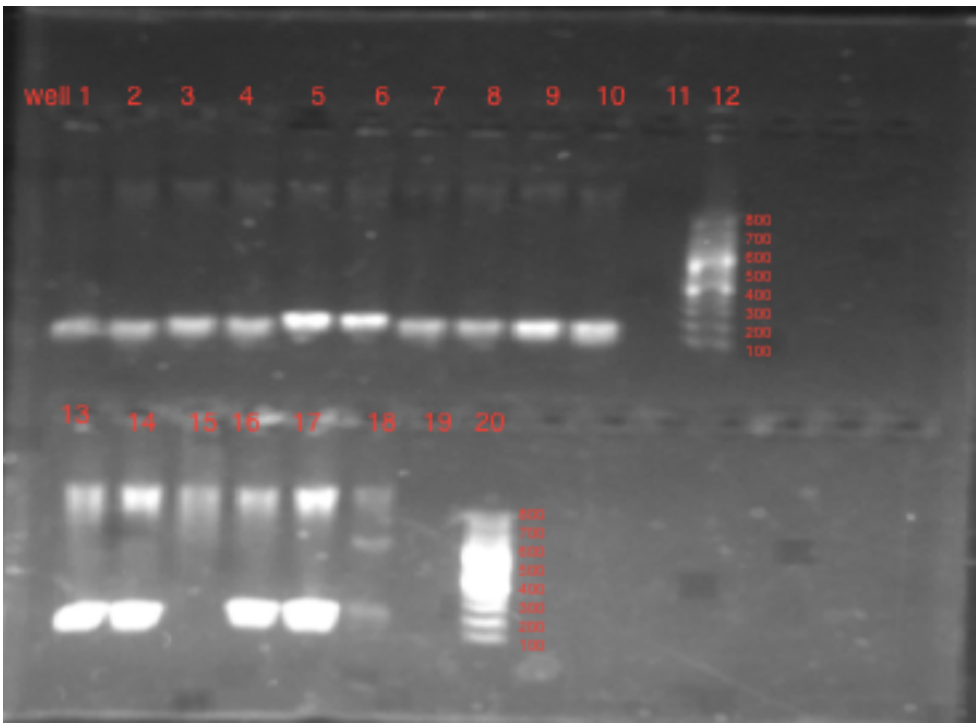


Figure 3.17 Gel electrophoresis of PCR product of CP200B and CP21A2 from week 15.

PCR amplification by using real-time qPCR (CFX96 real-time qPCR system BioRad) shows the amplification, standard curve and melting peak between CP200B and CP21A2. However, mixing the DNA with the opposite primer also depicts the gene amplification. Which was CP200B (DNA) + CP200B (Primer), CP21A2 (DNA) + CP21A2 (Primer), CP200B (DNA) + CP21A2 (Primer) and CP21A2 (DNA) + CP200B (Primer). CP200B had 10^5 CFU whereas CP21A2 had 10^6 CFU. By using CFX96 real-time qPCR both DNA shows the positive result.

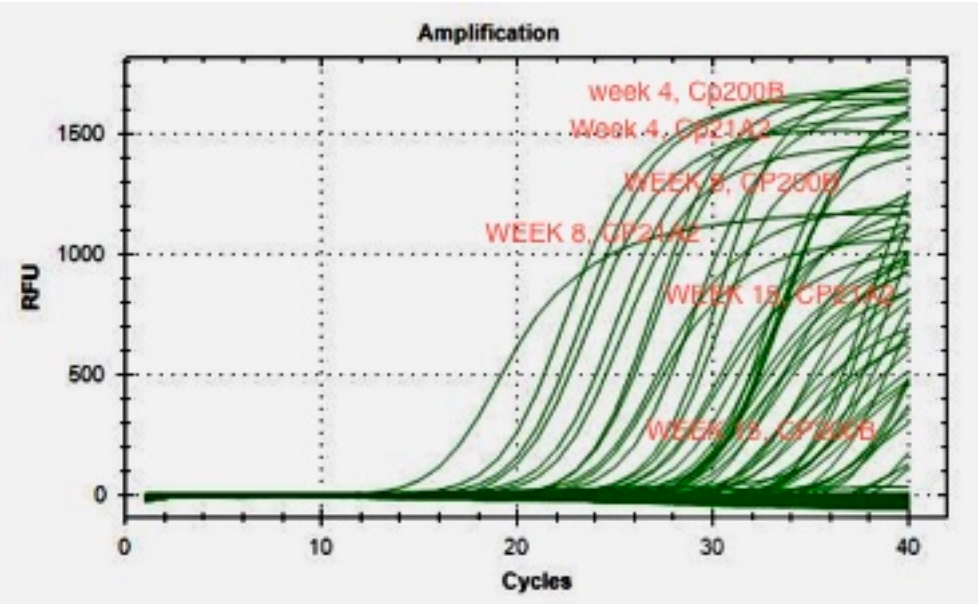
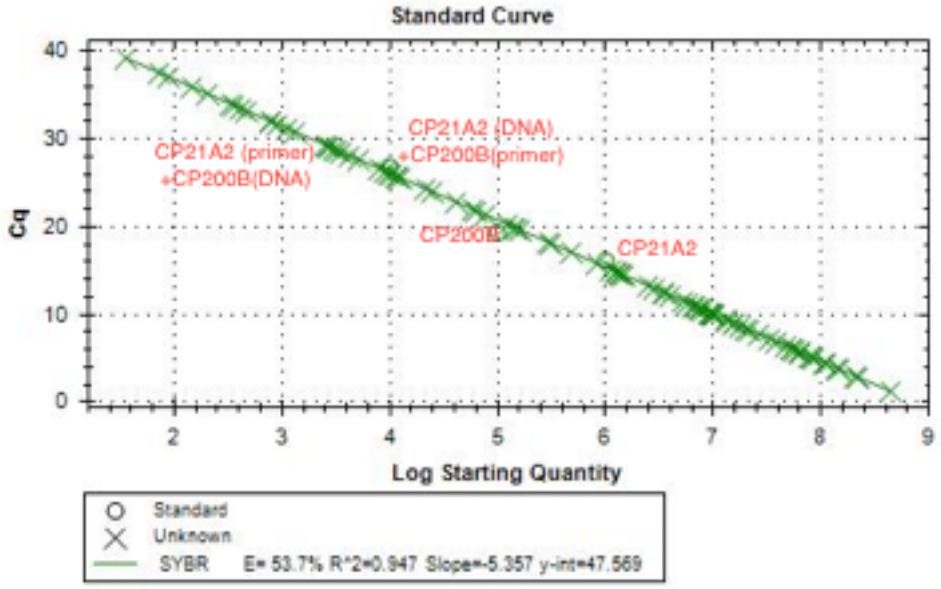


Figure 3.18 Gene amplification using real-time qPCR for selected actinobacteria from week 4, 8 and 15 CP200B (DNA) + CP200B (Primer), CP21A2 (DNA) + CP21A2 (Primer), CP200B (DNA) + CP21A2 (Primer) and CP21A2 (DNA) + CP200B (Primer).



----------CP21A2

$10^{1.}$ 10^{2} 10^{3} 10^{4} 10^{5} **10^{6}** 10^{7}

----------CP200B

$10^{1.}$ 10^{2} 10^{3} 10^{4} **10^{5}** 10^{6} 10^{7}

Figure 3.19 Standard curve using real-time qPCR for selected actinobacteria from week 4, 8 and 15 CP200B (DNA) + CP200B (Primer), CP21A2 (DNA) + CP21A2 (Primer), CP200B (DNA) + CP21A2 (Primer) and, CP21A2 (DNA) + CP200B (Primer).

CHAPTER

DISCUSSION

Discussion

In spite of the fact that this research has demonstrated positive effects of Endophytic Actinobacterial strains on nodulation, growth and development of Chick pea plant, the various factors involved underneath are yet to be resolved. Progressively detailed investigations of nodule advancement and functions, changes in gene expression and biochemical specifics of plants will provide the opportunity to furthermore comprehend the capability of Endophytic Actinobacteria on impact plant growth, development, and nodulation.

The Endophytic Actinobacteria were established to be increasing the fixation of nitrogen through the nodulation procedure and the mode the plant development was enhanced in limited nitrogen growth media. The measure of nitrogen fixed from the environment should be resolved in order to set, if the advantages are as well applicable to plants developed in field soil.

The Actinobacteria are moreover designated as bio-control specialists. In this investigation, it demonstrated the capability of diminishing seed-borne infection in the germination paper test. The antifungal activites and bio-control components of endophytic actinobacteria against the infectious fungal disease of chickpea ought to be portrayed in more detail since fungal diseases are a critical issue in Chick pea production. The hypothesis is sustained in which the result disclosed that six different endophytic actinobacteria against a fungal pathogen acts as a bio-control agent. CP200B, CP21A2, LuP30, and LuP47B also enhanced the growth, development, and nodulation via nitrogen fixation in Chick pea pea and lucerne strain with its rhizobial partner.

This work has specified the ability of endophytic actinobacteria to stimulate the development of Chick pea and its rhizobial partner. Nevertheless, the viability of endophytic actinobacteria should be checked in various field soils which encompass the complex small scale of micro-flora including pathogens and rhizobial networks.

Yandigeri et al, 2012 stated that from the districts of Rajasthan, India, where there was drought, *Streptomyces coelicolor* DE07, *S. olivaceus* DE10, and *Streptomyces*

geysiriensis DE27 were separated which are endophytic actinobacteria resistant to dry season. Wheat seeds which were treated with microbial cultures yielded good development of plants in contrast to control. Coating of cultures on seeds yielded better results and co-inoculation of microbes produced better yields compared to single culture inoculation.

From the results obtained in Pot assay I , it is evident that , the experimental Chick pea plants grown in abundant nitrogen manifested maximum growth and development of the plant whereas those grown in absence of nitrogen or rhizobia showed very less yield comparatively. Thus demonstrating the significance of nitrogen in plant development while using sterile sand-vermiculite medium and makes it appropriate to conclude the combined effects of actinobacteria and rhizobia on the nitrogen content.

Similarly, the results of Pot assay II and III suggests that, the total Chick pea mass generated in R^+ trials (treated only with rhizobia) yielded greater growth and development in Chick pea plants than those cultivated in unlimited and absence of nitrogen.

The Endophytic Actinobacterial strains CP200B, LuP47, CP84, CP21A2, and LuP30 possess capability to increase the nodulation, growth and development of the chick pea plants as stated in studies exemplifying the impact of microbes on legume-rhizobia symbiotic interaction. Smittle et al. 1979 established the fact that legume-rhizome interaction is favoured by the biochemical variations taking place during colony formation, and nodule development. A substantial amount of the phenolic mixes or their quinones resulted from enzymatic oxidation hinders the activity of cell which degrading the catalysts and phytohormones. It is hypothesized that comparable host reactions result in rhizobia intrusion and this reaction depicts the direct legume-rhizobia beneficial interaction. LuP30 and LuP47B strains improved the rhizobial populace and plant development of lucerne plant (Le et al. 2015).

Greater yield in the number of nodules and a low average dry weight of nodules in some trials and in others very lesser quantities with larger nodules was noted. An Actinobacteria, Mycorrhiza was referred to as rhizobia helper bacteria (RHB) in a research by Founoune et al. 2002. Smith and Read, 1997 demonstrated that Mycorrhizal organisms are a universal segment of most environments all through the world and assume a significant role in soil forms. Katznelson et al. 1962 examined the parasitic beneficial interaction which showed

changes in the function of the root and its microbial networks varied effectively from the uninfected and nearby soil (Ames et al. 1984). Linderman, 1988 exhibited that the compartment of microbes are largely mentioned as 'mycorrhizosphere'. Toro et al. 1996 and Secilia and Bagyaraj, 1987 established the fact, that microbes like mycorrhiza may supplement mycorrhizal exercises, viz., N_2 fixing microbes and phosphate-solubilizing microorganisms. Garbaye and Bowen,1989 specified negative or constructive outcome on mycorrhizal arrangement associated with few microbes.

From the experimental data of Pot assay I, it is obvious that LuP47B, CP200B, and CP21A2 enlarge the size of the nodule while other actinobacterial strains aids rhizobial infection. Hence the strains LuP30, CP84B, CP56, CP21A2 and CP200B influence both nodule size and rhizobial infection. However, in Pot assay II, strains CP84B, CP200B, LuP30, and LuP47 displays increased number of nodules.

Thies, 1991 stated that the upgrade of nodule number neither improves plant growth, nor nitrogen fixation, but adversely affect the function of nodules. Strains CP200B, LuP47, and CP21A2 substantially expanded nodule number while CP200B, LuP47, CP84B and LuP30 upraised nodule mass, dry weight of root and total mass.

The mild nitrogen insufficiency and adequate nitrogen supply improved the development of lateral and primary roots, while supply of inadequacy nitrogen hindered the lengthening of taproot and as well as elongation of lateral roo**t**. This clarifies the explanation that treatment with adequate nitrogen (abundant nitrogen treatment) and insufficiency nitrogen manifestation (no additional nitrogen, rhizobia + CP84B, Rhizobia + CP200B, Rhizobia + CP21A2 and so on.) yielded shorter roots than others.

Figure 3.11 and 3.12 depicts the correlation between the dry weight of nodules and the total mass of Chick pea (R2= 0.5 and 0.9) were high and positive. In the experimental system which used here demonstrates that more dry weight of nodules is linked to expanded development and growth of Chick pea.

The results of Pot assay II directed with the criteria of antagonism assay and germination assay. The total mass of plants treated by strains CP84B, CP21A2, CP56, LuP47B, LuP30, and CP200B were less compared to that of the rhizobial control, which

showed much reduced impact on germination, early plant development and growth and interaction with rhizobia. Trials other than CP200B revealed profitable and positive effects. In spite of the fact that CP21A2 had low activity on germination test, these strains still gave a good impact on Chick pea plant-advancement in pot assay which might be due to effect of the strain CP21A2 on rhizobia.

In both pot measures, side effects of Chick pea root rot infection. For instance, little roots, darkening of the plant crown and dim yellow leaves and negligible number of Chick peas in pot examine II. In the case of both trials with abundant nitrogen in pot examine II; the plants grown in absence of nitrogen had disease manifestations while the plants with nitrogen demonstrated only little impact of the disease.

In pot test II, the roots of the Chick pea plant from abundant nitrogen trial were dark due to overwatering. Chickpea shows a higher delicate tendency towards overwatering as a result of the nodulation procedure. In this experiment, the nitrogen solution was added in the subsequent day to every watering; these effects in the water level in two abundant nitrogen controls were constantly higher than others.

In pot assay III, among the CP200B and CP21A2 strains treated Chick pea plant with R^+, N^+ and Actinobacteria + Rhizobia (R^+) , the germination rate was higher in N^+, slightly lesser in R+ and CP21A2 treated and in much lesser in CP200B (table 3.8). The number of nodules and dry weight of shoots were greater in CP21A2 and increased total mass, dry weight of nodule, length and dry weight of root and length of the shoots were showed from CP200B treated plants (table 3.9).

The actinobacterial strains were identified using RT-q-PCR gene amplification and the sequencing results illustrates the positive effect of the strains CP200B and CP21A2 during PCR amplification and quantification of DNA by using gel electrophoresis which displayed specific bands for each strain. Bands were visible for CP200B under 138 and for CP21A2 near to 87. During PCR amplification, primers indicated positive effect with certain strains that proves primers were operational with the extracted DNA strains.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

5.1 Conclusions

This work supplements the theory and hypothesis that endophytic actinobacteria from the Chick pea can enhance nodulation and development of Chick pea plant when co-inoculated with rhizobia. Four endophytic actinobacterial strains isolated from Chick pea CP56, CP21A2, CP200B, CP84B in addition to two endophytic actinobacteria isolated from lucerne LuP30 and LuP47B, when co-inoculated with its rhizobial partner altogether improved the nodulation, growth and development of chickpea plant. The endophytic actinobacterium strains were *Streptomyces* sp. LuP30, *Streptomyces* sp. LuP47B, *Streptomyces* sp. CP21A2, *Streptomyces* sp. CP200B, *Microbispora* sp. CP56 and *Actinomadura* sp. CP84B. Four strains of *Streptomyces* sp. were isolated from lucerne roots and Chick pea roots two each. One strain of *Microbispora* sp. CP56 and one strain of *Actinomadura* sp. CP84B showed enhancement in legume-rhizobia symbiosis.

Surface sterilization of chickpea roots empowered the effective isolation of actinobacteria from chickpea. Undamaged seeds were chosen and surface sterilization was performed. Six strains of endophytic actinobacteria were grown on ISP2 and incubated at 27 ^{0}C for two weeks until the culture produced spores. For procuring pure single colonies, the rhizobia were streaked on YMA (Yeast Mannitol Agar). Later those single colonies were shifted to new plates and incubated for duration of two weeks at 27 ^{0}C and growth of the strains was observed. A standard curve depicts the connection between Cell number and OD @600nm was plotted for every rhizobium strain to empower the utilization of a standard CFU/ml slanting over the experiment. Seeds were surface-cleaned and planted into a purified pasteurized (via autoclaving) pot mix after autoclaving 50:50 volumes of sand and vermiculite.

Co-inoculation of endophytes CP56, CP21A2, CP84B, LuP30, LuP47 and CP200B with *Mesorhizobium ciceri* strain CC1192 increased the quantity of nodules in number, total mass and the dry weight of chickpea roots, shoots and nodules. Parameters like quantity of nodules by number and mass, length of nodules, dry weight and amount of nitrogen in shoots and roots were measured. Germination rate and effect on rhizobia were higher in CP56, LuP30, LuP47B, and CP21A2 as compared to other strains, whereas, dry weight of nodules

was higher in CP200B, CP84B, LuP47B, and CP21A2. However, the total mass was showed by LuP30, LuP47B, CP200B, and CP84B. Finally, the increased number of nodules was exhibited by CP200B, LuP47B, CP21A2, and CP84B. Overall, it is evident that the strains CP200B and CP21A2 had a good effect on the Chick pea plant.

In the second part of the investigation, RT-q-PCR gene amplification that was employed in identifying endophytic actinobacterial strains that have revealed positive effects by both strains CP200B and CP21A2. Both strains showed noteworthy effect with primers and from gel electrophoresis, it was proven that primers functioned effectively during RT-q-PCR gene amplification, standard curve and base pairs of CP200B (DNA) + CP200B (Primer), CP21A2 (DNA) + CP21A2 (Primer), CP200B (DNA) + CP21A2 (Primer) and CP21A2 (DNA) + CP200B (Primer) in which CP200B showed the ratio of 10^5 CFU and *CP21A2* presented 10^6 CFU.

5.2. Future directions

While this study has shown that endophytic actinobacteria can have a benefical impact on the nodulation and development of Chick pea, the mechanisms involved are yet to be established. More comprehensive research on nodule formation via microscopy and function, improvements in plant chemistry and expression will offer an opportunity to better understand the ability pf endophytic actinobacteria to affect nodulation and plant growth.

Actinobacteria have been shown to increase nitrogen fixation through the process of nodulation and to enhance growth of plant in nitrogen-restricted growth media. The emitted anount of nitrogen from the environment must be measuered in order to decide if the advantages still extend to plants growing in field soil.

This study has shown the ability of endophytic actinobacteria to promote the growth ofChickpea and its rhizobia, but the efficacy of endophytic actinobacteria must be tested in va rious field soils containing diverse microflora, including pathogens and naturalised rhizobian populations.

Actinobacteria are also act as a biocontrol agent. In this research, the ability to mitigate seed-borne disease was demonstrated in the paper germination test. The bio-control mechanisms and antifungal activities of endophytic actinobacteria against Chick pea fungal pathogen should be categorised in more depth as fungal diseases are a major problem in the cultivation of Chick pea.

Actinobacteria are broadly spread in nature and constitute the biggest taxonomic category within the Bacteria domain. They are common in soil and are widely explored for their therapeutic applications. This flexible group of bacteria has adapted to diverse ecological habitats, which has recently gained significant attention from the scientific community as because it has opened new possibilities for novel metabolites which could help to solve a number of the foremost challenging problems of the day, for instance, experimental medicines, novel drugs for drug-resistant human diseases, economical means to maintain ecological equilibrium in diverse ecosystem, and alternative approaches for sustainable agriculture.

Traditionally, free dwelling soil actinobacteria is a focus of intense study. Lately, symbiotic actinobacteria which live as endophytes within the plant tissues have created immense interest as potential source of novel compounds that can be used in medicine, agriculture, and environment. In the context of these possibilities, this study reflects on the spectrum of endophytic actinobacteria isolated from the plants of the highest environments and unique ecological niches (Singh et al.2018).

In addition, an effort has been made to assign chemical class to the compounds originating from endophytic actinobacteria. Endophytic actinobacteria and their host plants offer a exciting model for studying and interpreting their biology and chemistry in order to establish appropriate, non-deletrious applications for agriculture, human health and the environment.

Although, the *Streptomyces* genus has been recorded to be the most common, whereas, the non- *Streptomyces* group has also been commonly reported in endophytes of different types of plants from severe environments.

Chickpea yields are typically vulnerable to drought or heat stress where the primary rainfall areas are grown in winter. Whether endophytic actinobacteria have the ability to relieve abiotic stress in these conditions is an area of potential enquiry (Hamedi et al. 2015).

Appendices

Appendix 1: Media and Solutions

1. Yeast Mannitol Agar (YMA)

Yeast extract	0.25g
Mannitol	2.5 g
Sodium glutamate ($C_5H_8NO_4Na$)	0.25g
Solution S	5 ml
Solution T	5 ml
Solution U	0.5 ml
Solution V	0.5 ml
Congo Red solution	5 ml
Agar	10 g
R.O. water	480ml

* Solution S:

$Na_2HPO_4.2H_20$ (Disodium phosphate)	0.9 g
RO water	500 ml

* Solution T:

$MgSO_4.7H_2O$ (Magnesium Sulfate Heptahydrate)	5g
RO water	500 ml

* Solution U:

$CaCl_2.2H_2O$ (Calcium chloride dihydrate)	26.5 g
RO water	500 ml

* Solution V:

$FeCl_3$ (Ferric chloride)	2 g
RO water	500 ml
Congo Red solution	1.25 g
RO water	500 ml

2. HPDA

Per litre R.O water	
PDA (Oxoid) (potato dextrose agar)	9.75 g
Agar	4 g
R.O water	500 ml
Adjust pH 7.2	

3. Mannitol soya (MS)

Per litre R.O. water	
Mannitol	40 g
Soya flour	40 g
Agar	40 g
R.O water	500 ml
Adjust pH 7.2	

4. ISP2

Yeast extract (g)	2 g
Dextrose (g)	2 g
Agar (g)	10 g
Malt extract (g)	5 g
Distilled water	500 ml
pH 7.2	

5. ISP3

Oatmeal	10 g
Trace salt solution	0.5 g

Agar	9.0 g
Distilled water	500 ml
pH 7.2	

Solution –Trace salt

FeSO4.7H2O (Iron(II) sulfate)	0.05 g
ZnSO4.7H2O (Zinc sulfate heptahydrate)	0.05 g
MnCl2.4H2O (Manganese(II) chloride tetrahydrate)	0.05 g
R.O. water	50 ml
(filter sterilised)	

Add oatmeal to 1L R.O water, boil and steam for 20 minutes. Filter through cheesecloth and add agar.

6. Yeast Mannitol Broth (YMB)

Yeast extract	0.5 g
Dipotassium phosphate (K2HPO4.3H2O)	0.38 g
Mannitol	5 g
Sodium chloride NaCl	0.05 g
Magnesium sulphate MgSO4.7H2O	0.20 g
Calcium carbonate CaCO3	0.5 ml
R.O. water	500 ml

7. Glucose Yeast media (GY)

Glucose (g)	5 g
Yeast (g)	1 g
Agar (g)	7.5 g
Distilled water	500 ml

8. PBS Buffer (10X)

NaCl (Sodium Chloride)	40 g
Na2HPO4.2H20 (Sodium hydrogen phosphate)	9 g
KH2PO4 (Potassium hydrogen phosphate)	1.2 g
KCl (Potassium Chloride)	1 g
MQ water	500 ml

1. Add the above chemicals to 500ml of MQ water in a 500 ml flask.
2. Adjust pH to 7.4.
3. Increase volume to 500 ml, add lid and mix well by shaking.
4. Pour into 500ml glass Schott bottle.
5. Send PBS solution for autoclave.
6. Store at room temperature on bench.

Preparation of 500 ml 1X PBS buffer from 10X PBS stock :

50 ml of the 10X PBS stock buffer is diluted in a volume of 500 mL dH_2O.

9. McKnight's solution

Mc Knight's solution (no autoclave) solution

KH_2PO_4 (Potassium dihydrogen orthophosphate)	1 g
$CaSO_4.2H_2O$ (Calcium Sulphate)	6.75 g
$MgSO_4.7H_2O$ (Magnesium Sulphate)	1 g
KCl (Potassium Chloride)	1.5 g
A-Z trace elements	5 ml
D-solution	5 ml
Distilled water	make upto 250 ml

Trace elements

Boric acid	1.43 g
$MnSO_4.H_20$ (Manganese sulphate monohydrate)	1.04 g

$ZnSO_4.7H_2O$ (Zinc sulphate)	0.11 g
$CuSO_4.5H_2O$ (Copper Sulphate pentahydrate)	0.03 g
$Na_2MoO_4.2H_20$ (Molybdic acid (Sodium molybdate)	0.06 g
Distilled water	500 ml

D solution

$FeCl_3$ (Ferric chloride)	5 g
Distilled water	make upto 500 ml

N solution

NH_4NO_3 (ammonium nitrate)	133 g
Distilled water	make upto 500 ml

10. N solution for unlimited nitrogen treatments

NH_4NO_3 (ammonium nitrate)	6.0 g

Distilled water make up to 5000 mls

50 ml solution added into pot weekly

Appendix 2:

2.1. Quantification of DNA concentration for surface sterilized roots (8 weeks chickpea plant)

Sample	Concentration of nucleic acid	Unit ng/ul	A260	A280	260/280	260/230	SAMPLE
Surface sterilized roots							
CP21A2-a	341.5	ng/ul	6.831	4.23	1.61	1.77	DNA
CP21A2-b	150.8		3.017	2.047	1.47	1.48	
CP200B-a	225.4		4.507	2.89	1.56	1.58	
CP200B-b	291.8		5.836	3.737	1.56	1.64	
Washed roots							
CP21A2-a	850.5	ng/ul	17.009	13.951	1.22	1.52	DNA
CP21A2-b	387.1		7.741	5.444	1.42	1.47	
CP200B-a	461.0		9.220	6.279	1.47	1.67	
CP200B-b	657.1		13.142	11.163	1.18	1.49	
Soiled roots							
CP21A2-a	551.2	ng/ul	11.025	7.276	1.52	1.82	DNA
CP21A2-b	501.1		10.023	6.783	1.48	1.79	
CP200B-a	855.2		17.103	13.907	1.23	1.56	
CP200B-b	539.5		10.790	6.580	1.64	1.81	

2.2. Quantification of DNA concentration for actinobacteria spores (serial dilution 1/10) of CP200B and CP21A2.

Sample	Concentration of nuclei acid	Unit ng/ul	A260	A280	260/280	260/230	SAMPLE
CP200B spore-1(a)	5167.0	ng/ul	103.34	65.44	1.58	1.92	DNA
CP200B spore-1 (b)	2857.6		57.151	30.63	1.87	2.09	
CP200B spore-2	636.0		12.71	7.20	1.77	2.01	

(a)							
CP200B spore-2 (b)	620.5		12.409	7.024	1.77	2.01	
CP21A2 spore-1(a)	2045.4		40.90	25.50	1.60	1.93	
CP21A2 spore-1 (b)	1844.0		36.88	23.209	1.59	1.93	
CP21A2 spore-2 (a)	1042.0		20.84	14.42	1.44	1.83	
CP21A2 spore-2 (b)	1037.7		20.753	14.298	1.45	1.83	

Table 2.3 Quantification of DNA concentration for 15 weeks Chick pea plant (Roots DNA):

Sample	Concentration of nuclei acid	Unit ng/ul	A260	A280	260/280	260/230	SAMPLE
Sterilized roots							
Untreated-a	58.7	ng/ul	1.17	0.97	1.21	1.20	DNA
Untreated-b	71.3		1.42	1.16	1.23	1.25	
N^+- a	808.2		16.05	12.80	1.25	1.40	
N^+- b	517.2		10.34	8.34	1.24	1.56	
R^+-a	76.8		1.53	1.23	1.24	1.34	
R^+-b	410.5		8.21	5.76	1.42	1.51	
CP84-a	608.7		12.17	8.05	1.51	1.82	
CP84-b	67.9		1.35	1.10	1.23	0.68	
CP200B-a	520.1		10.42	6.27	1.66	1.95	
CP200B-b	907.2		18.14	13.72	1.32	1.72	
CP21A2-a	852.2		17.04	12.44	1.37	1.72	
CP21A2-b	3162.5		63.24	57.93	1.09	1.54	
CP56-a	556.0		11.12	6.71	1.66	1.92	
CP56-b	587.5		11.75	8.14	1.44	1.68	
LuP47B-a	56.9		1.13	0.95	1.20	1.19	
LuP47B-b	1083.1		21.66	36.27	0.60	0.34	
LuP30-a	854.9		17.09	20.52	0.83	1.07	
Washed roots							
Untreated-a	171.8	ng/ul	3.43	5.39	0.64	1.22	DNA
Untreated-b	756.9		15.13	12.64	1.20	1.52	
N^+- a	802.6		16.05	12.80	1.25	1.40	
N^+- b	517.2		10.34	8.34	1.24	1.56	

R^+-a	673.3		13.46	8.48	1.59	1.89	
CP84-a	2292.6		45.85	33.48	1.37	1.57	
CP84-b	398.4		7.96	5.502	1.45	1.38	
CP200B-a	927.7		18.55	14.50	1.28	1.45	
CP21A2-a	286.2		5.72	5.13	1.11	1.40	
CP21A2-b	1234.7		24.69	21.30	1.16	1.57	
CP56-a	1137.8		22.75	19.14	1.19	1.55	
LuP47B-a	765.1		15.30	14.46	1.06	1.27	
LuP47B-b	5777.7		115.5	163.05	0.71	0.57	
LuP30-a	4442.0		88.83	71.25	1.25	1.64	
LuP30-b	336.3		6.72	6.718	1.00	1.27	
Soiled roots							
Untreated-a	1112.3		22.24	17.51	1.27	1.68	
Untreated-b	26.1		0.52	0.34	1.50	0.58	
N^+- a	639.4		12.78	7.87	1.62	1.94	
N^+- b	3285.7		65.71	45.90	1.43	1.82	
R^+-a	776.9		15.53	12.42	1.25	1.63	
R^+-b	357.5	ng/ul	7.14	5.25	1.36	1.70	DNA
CP84-a	523.3		10.46	8.21	1.27	1.67	
CP84-b	1917.4		38.34	29.10	1.32	1.72	
CP200B-a	20191.6		403.83	395.02	1.02	0.95	
CP200B-b	935.8		18.71	14.49	1.29	1.69	
CP21A2-a	705.8		14.11	13.47	1.05	0.98	
CP56-a	892.0		17.83	15.73	1.13	1.53	
LuP30-a	1291.5		25.83	38.86	0.66	0.39	

Table 2.4 Quantification of DNA concentration for 15 weeks Chick pea plant (seeds sample):

Sample	Concentration of nuclei acid	UNIT ng/ul	A260	A280	260/280	260/230	SAMPLE
Untreated seeds-a	1547.5		30.951	22.29	1.39	1.58	
Untreated seeds-b	3675.5		73.51	58.23	1.26	1.65	
N^+- a	536.5		10.73	6.69	1.60	1.50	
N^+- b	3050.6		61.01	41.26	1.48	1.91	
R^+-a	126.3		2.52	1.55	1.63	0.54	
R^+-b	2913.0		58.26	37.22	1.57	1.61	
CP200B-a	1872.8	ng/ul	37.45	26.35	1.42	1.66	DNA
CP200B-b	674.9		13.49	8.21	1.64	1.45	
LuP47B	2526.7		50.53	30.96	1.63	2.00	
LuP30-a	1423.0		28.46	20.09	1.42	1.66	
LuP30-b	922.5		18.45	13.18	1.40	1.64	
CP56-a	1730.1		34.60	23.27	1.49	1.81	
CP56-b	2945.4		58.90	35.60	1.65	2.02	
CP84-a	5096.6		101.93	78.79	1.29	1.72	

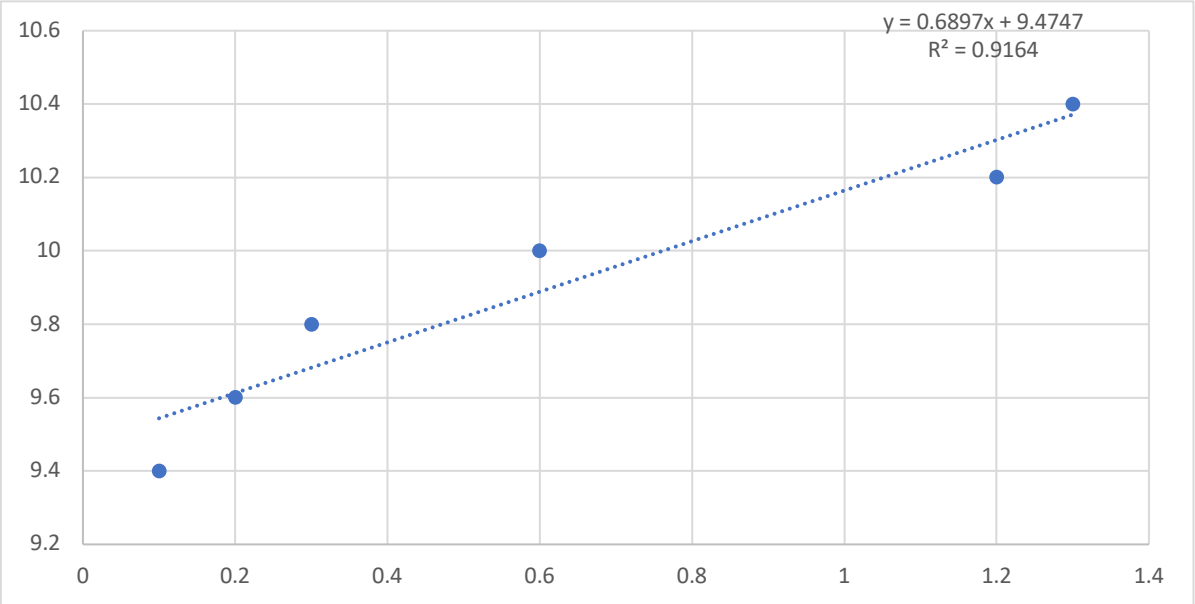
Cp84-b	613.7		12.27	7.48	1.29	1.72	

Table 2.5 Quantification of DNA concentration for 4 weeks Chick pea plant:

Sample	Concentration of Nucleic acid	Unit ng/ul	A260	A280	260/280	260/230	SAMPLE
Surface sterilized roots							
N^+-a	595.8	ng/ul	11.91	8.24	1.45	1.81	DNA
N^+-b	971.0	ng/ul	19.421	14.9	1.30	1.69	
R^+-a	752.3	ng/ul	15.04	10.0	1.50	1.79	
R^+-b	1161.1	ng/ul	23.22	16.00	1.45	1.84	
CP200B-a	157.7	ng/ul	3.15	2.00	1.58	1.04	
CP200B-b	3217.5	ng/ul	64.35	41.27	1.56	1.99	
CP21A2-a	1529.6	ng/ul	30.59	21.28	1.44	1.86	
CP21A2-b	163.3	ng/ul	3.26	2.64	1.23	1.40	
Washed roots							
N^+-a	443.6	ng/ul	8.87	5.34	1.66	1.73	DNA
N^+-b	896.6		17.93	14.94	1.20	1.58	
R^+-a	7351.9 2009.9		147.0 40.19	138.6 37.46	1.06 1.07	1.38 2.27	
R^+-b	9576.6		191.532	192.079	1.00	1.32	
CP200B-a	278.2		5.563	3.955	1.41	1.58	
CP200B-b	657.1		13.14	11.16	1.18	1.49	
CP21A2-a	1198.1		23.96	19.06	1.26	1.64	
CP21A2-b	1476.4		29.52	21.84	1.35	1.75	
Soiled roots							
N^+-a	242.7	ng/ul	4.85	3.7	1.31	1.38	DNA
N^+-b	597.3		11.947	7.646	1.56	1.79	
R^+-a	10080.8		201.61	204.34	0.99	1.32	
R^+-b	1620.3		32.40	22.204	1.46	1.84	
CP200B-a	1228.3		24.56	19.76	1.24	1.39	
CP200B-b	330.7		6.61	4.97	1.33	1.62	
CP21A2-a	823.4		16.46	13.75	1.20	1.58	
CP21A2-b	1040.9		20.81	15.77	1.32	1.66	

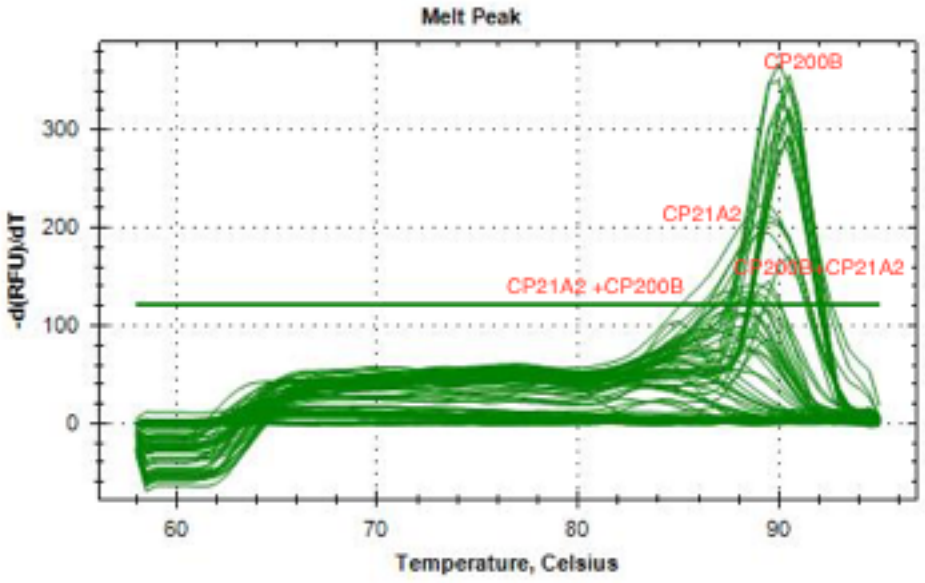
Appendix 3:

3.1. The correlation standard curve between OD_{600} vs $\log_{10}$ CFU ml^{-1} of rhizobia CC1192



Appendix 4:

4.1. Depicts the correlation standard curve between OD_{600} vs $\log_{10}$ CFU ml^{-1} of rhizobia CC1192



Appendix 4.1. Shows the melting point using real-time qPCR for selected actinobacteria from week 4, 8 and 15 CP200B (DNA) + CP200B (Primer), CP21A2 (DNA) + CP21A2 (Primer), CP200B (DNA) + CP21A2 (Primer) and CP21A2 (DNA) + CP200B (Primer).

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