Optimizing the sporulation of endophytes Actinobacteria on broth and agar media and their effect on growth and nodulation of chickpea

A thesis submitted for the award of degree

Master of Biotechnology

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Declarations

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

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Acknowledgement

I would first like to give special thanks to my thesis supervisor Prof. Christopher Franco. He has always supported me whenever I ran into a spot of trouble or had a question about my research or writing. He guided and gave me advice in the right the direction during my master's project. He also taught me everything from the basics such as preparing reagents, making media, to setting up experiments and making me think in critical way. I greatly appreciate his patience and suggestions for my writing.

I would also like to thank Srijan Shrestha, Yitayal Anteneh and Xu Ting for supporting me in the design of experiment and their experience in offering logical approaches in my experiments. I would like to thank Peng Su for assisting me in microscopy, and Gurpreet who help me with my pot experiments. I would like to express my appreciation to Dr. Ricardo Araujo who gave feedback during my progress seminar. I would like to thank Hanna Krysinska and Kushari Burns for their excellent assistance in laboratory. Thanks to Wendi, all PhD students, other students and staff in Department of Medical Biotechnology for helping me, sharing ideas and have a great time in the laboratory.

Finally, I must express my profound appreciation to my family for providing me with unlimited encouragement and support during my study and this thesis project. This accomplishment would not have been possible without them.

Thank you all.

Sita Ram Sapkota.

Abbreviations

ul, ml	microlitre, millilitre
uM, mM	micromolar, millimolar
%	percent
ABARES	Australian Bureau of Agricultural and Resource Economics
ANOVA	Analysis of Variance
approx.	approximately
CC1192 or R.	*Mesorhizobium ciceri* CC1192
CFU	colony forming units
exp.	experiment
FAO	Food and Agriculture Organization of the United Nations
hr	hour
min	minute(s)
MQ water	MilliQ water
N-	no added nitrogen treatment
N+	unlimited nitrogen treatment
OD600nm	Optical Density at 600 nanometers
PGPR	Plant Growth-Promoting Rhizobacteria
rpm	rotation per minute
SARDI	South Australian Research and Development Institute
sp.	species (singular)
spp.	species (plural)
temp.	Temperature

ABSTRACT

Chickpea is currently the second most important pulse crop produced in the world. It is a rich source of protein for human consumption and is cheaper compared to animal protein. Chickpea is a type of legume which can fix the atmospheric nitrogen via symbiosis with the rhizobial partner, *Mesorhizobium.* Recent research in our laboratory has found that the legume grain yield increased when the rhizobium inoculant was added together with selected endophytic actinobacteria in chickpea plants. The actinobacterium acts by colonising plant roots and increases the nitrogen fixation capacity of the rhizobial partner. Some strains have the added benefit of controlling the losses caused by fungal root pathogens. The actinobacterial inoculants are added as spores to the seed before sowing. However, spores normally grow on solid surfaces, but they are also able to be formed in liquid submerged culture. In this study, endophytic actinobacterial strains CP21A2, CP56, CP84B, and CP200B isolated from chickpea were evaluated for the sporulation rate in solid and liquid media. These resultant spores were evaluated for their stability at different pH and temperature. The spores and rhizobia were then inoculated in chickpea seeds in order to determine whether the spores produced on agar and in liquid culture had a similar symbiotic relationship on the growth and development of chickpea plants.

Calcium carbonate in the liquid broth and MS medium in solid agar media can be used to increase the sporulation rate in the actinobacteria. Additionally, we found out that almost all spore strains were stable at 70 °C but temperature greater than that were lethal to the spores obtained from both types of media. In addition, the tested spore strains were more sensitive and prone to lysis at alkaline pH rather than acidic. Furthermore, our study suggested that CP56 spores obtained from liquid media and CP84B from solid media were the best performers in promoting the overall growth of plants and nodules. However, further detailed investigations need to be carried out in order to determine their influence on the growth and

development of chickpea plant, which can be useful to increase the yield in the agricultural industry.

CHAPTER -1

1.1 Introduction and Literature Review

Actinomycetes, also known as actinobacteria are gram-positive fungus-like filamentous bacteria normally found in soil. Some actinomycetes are saprophytic, some are endophytic, and some are pathogenic. These bacteria have high a G+C content in their DNA, many of which are filamentous with substrate and aerial mycelia. Approximately 22,000 bioactive secondary metabolites of microbial origin have been reported so far, of which fifty percent are from actinobacteria only. Approximately 160 antibiotics are being currently used in human therapy and in agriculture (Berdy, 2012). Liquid medium is preferred over solid medium for the production of spore because of its rapidity and is less expensive than the solid medium. However, there has not been any reports till date. Therefore, in the present study some experiments were carried out with a higher inoculation rate to enhance longer time for the sporulation on the plates as well as to determine the maximal number of spores that could be added to one 9 cm diameter petri plate. Further some experiments were carried out in order to understand whether temperature, size of inoculum, and incubation time helps with the processes of sporulation on the agar medium and/or either the presence of mycelium. In Austraila, calcium carbonate costs is approximately AUD 50-60 per kg, compared to humic acid, which costs approximately AUD 40-50 per 10 g (Hartwigsen and Evans, 1996). The previous study inline that calcium chloride and humic acid are realted with the most effective in increasing sporulation. It was intresting to know that wether calcium chloride which is cheaper than the humic acid would be a better choice of reagent in the sporulation rates.Therfore, more research is required and drastic improvements has to be made in liquid and solid media for actinomycetes to increase sporulation. It was intresting to know that wether calcium chloride which is cheaper than the humic acid would be a better choice of reagent in the sporulation rates. Further, the sporulation of these kinds of bacteria

is studied in surface grown cultures, research on sporulatuion in submerged cultures is considered to be an attractive alternatives. The culturing time is much shorter, synchronous sporulation can be more readily achieved with spores maturing over a subsequent period of 10-12 h. with high yields under controlled sterile conditions as well as a simpler scale-up .Therfore, more research is required and drastic improvements has to be made in liquid and solid media for actinomycetes to increase sporulation.

1.1.1 Nitrogen fixation and biological nitrogen fixation

Nitrogen (N) is a key element present in many biochemical compounds (such as nucleotide phosphates, amino acids, proteins, and nucleic acids) of living cells (Rascio and LaRocca 2008). Some examples of organisms that carry out nitrogen fixation are shown in Table 1. N is one of the most important nutrients for agricultural production. The atmosphere contains around 10^{15} tonnes Nitrogen of which about 3×10^{9} tonnes is transformed into a nutrient annually on a global basis (Mabrouk and Belhadj, 2010, Postgate, 1982). Fixed nitrogen is the nitrogen gas which is transformed to nitrate, an ammonium ion, ammonia, or another nitrogen oxide, from which living organisms could use as a nutrient (Gilchrist and Benjamin, 2017, Smallwood et al., 2017). The transformations are provided by many sources, around 10% newly fixed nitrogen from lightning. The world fertiliser industry accounts for about 25% of the world's fixed nitrogen supply and around 60% of new nitrogen is fixed by biological processes (Sprent and Sprent, 1990, Mabrouk and Belhadj, 2010). The total global demand for fertiliser-N increased from 130 million tonnes in 2011 to 141 million tonnes in 2015 and is predicted to continue increasing (FAO, 2011). However, the current production and usage of fertilisers may result in various economic and ecological costs (Sprent and Sprent, 1990). Once

applied to soil, if all nitrogen fertilisers cannot be absorbed by plant, the excess nitrogen is transform to ionic or gaseous form and lost through denitrification, volatilisation, and leaching (Brady and Weil, 2008, Tamme et al., 2009). Wang et al. (2002) and Gastal and Lemaire (2002) stated that the excessive nitrogen fertilisers application could result in some environmental problems such as acid rain, the greenhouse effect and eutrophication.

Table 1. Some examples of organisms that carry out nitrogen fixation

N2-fixing prokaryotes	Genera
Aerobic bacteria	Azotobacter
	Azospirillum
Facultative bacteria	Klebsiella
	Bacillus
Photosynthetic	Chromatium
	Chlorobium
Non-photosynthetic	Clostridium
	Desulfovibrio
Cyanobacteria	Anabaena
	Nostoc
	Calotrix

To reduce the negative and unpredictable impacts, biological nitrogen fixation (BNF) is used as an alternative option for chemical fertilisers (Mabrouk and Belhadj, 2010). BNF has attached the attention of scientists and applied in agricultural practices extensively for 100 years. The BNF 's importance as a primary nitrogen source for agriculture may decline because of the increasing amounts of chemical nitrogen fertiliser and the slow effect on crops (Peoples et al., 1995, Dixon and Wheeler, 1986). However, the currently development of research about renewable resources, reforestation make attention on BNF researches and applications (Sprent and Sprent, 1990).

Table 2. The global area harvested and production of pulses between 1999 and 2010

(Source: FAO, 2013)

Country	Area Harvested (x 1000 ha)		Production (x 1000 tonnes)	
Year	**1999- 2001**	**2010**	**1999- 2001**	**2010**
India	19,998	26,574	13,676	17,236
Canada	2,188	2,928	3,754	5,347
China	3,461	2,786	4,843	3,891
Brazil	4,011	3,454	2,788	3,172
Australia	2,188	1,753	2,616	1,954
Others	34,554	40,816	28,571	37,229
World	**66,400**	**78,311**	**56,248**	**68,829**

1.1.3 Rhizobia.

The genus *Rhizobium* is well known as a bacterium that can infect the legume roots and induce nodule formation (Burdass, 2002, Baldwin and Fred, 1929). Rhizobia and their host plants create a symbiotic relationship that results in atmospheric nitrogen fixation; the nitrogen is only fixed when this symbiosis is formed. The enzyme system of the bacterium provides a constant nitrogen source to the host leguminous plant and the host plant supplies the energy and nutrients for the bacterium activities (Burdass, 2002). Rhizobia in soil are free living and can live on plant residues or endophytes (Mohammadi and Sohrabi, 2012, Anwer, 2013). Rhizobia are gram negative, aerobic and non - spore forming bacteria. They are straight and rod shaped (around 0.5-1.0 x 1.2-3.0 μm); however, they appear as irregular cells (club and Y-shaped) when in the nitrogen-fixing form in root nodules (called bacteroids) (Burdass,

2002). The optimal temperature for rhizobial growth is from 25^0C to 30^0C, some species can grow up to 40^0C. The range of pH for rhizobia growth is between 4 and 10 but the optimal is from 6 to 7 (Simon et al., 2014). The slow or fast growing Rhizobia are classified based on their ability to fix nitrogen (Chen et al., 1988). Yeast Mannitol Agar (YMA) is the best medium to grow Rhizobia that produce 2 to 4 mm diameter colonies after 3 - 5 days (Kuykendall et al., 2005). The genomes of Rhizobia vary in size from 6.5 Mb (*Sinorhizobium* sp.) to 9.0 Mb (*Bradyrhizobium* sp.) (Kaneko et al., 2000)

1.1.4 Legume-Rhizobia symbiosis

In legumes and rhizobia symbiosis, individual *Rhizobium* species only interact with one or a limited number of host plant species (Nap and Bisseling, 1990). Therefore, it can be said that this symbiosis is a selective and highly specific interaction (Sharma et al., 1993). However, there are some *Rhizobium* strains, for example NGR234 that can interact with a wide range of host plant (353 legume species) (Stougaard, 2000).

The legumes and rhizobia symbiosis process can be outline according to the main steps: rhizobia attach to the root tip and enter via the root hairs causing hair curling, infection pocket, infection thread formation, nodule initiation, nodule formation and nitrogen fixation (Laranjoa et al., 2014, O' Hara et al., 1988, Glloudemans and Bisseling, 1989, Sharma et al., 1993). The nodule process is shown in figure 1.

Image removed due to copyright restriction.

Figure 1. The nodulation process (Laranjoa et al., 2014)

Firstly, a variety of chemicals are released from the cells of root into the soil by interaction between free-living rhizobia and the host plant. These chemicals can promote the bacteria growth in population in rhizosphere area. The rhizobia can recognize their correct host plant by the reactions between some compounds in the root surface and the cell wall of bacterial, and then attach to the root hair. After that, the Nod genes in the bacteria are activated by the flavonoids secreted by the root cells, this can induce the formation of nodule (Cooper, 2007). The highly complex chemical communications between the bacteria and the plant will fregulate the whole process of nodulation. The bacteria will expel nod factors and stimulate the hair curling when bound to the root hairs (Cooper, 2007). An infection thread formation is induced in the hair tip where rhizobia invade the plant root. This thread formation only responds to the infection and is constructed by the root cells. The infection thread grows and penetrates through cells of plant root with the thread branching. The network of tubes is expanded with multiplication of bacteria, the nod factors continue to be produced which stimulates the proliferation of the root cells and then root nodule formation. Small nodules are visible within a week. Thousands of living rhizobia form bacteroids packed into each

root nodule. The bacteroids are surrounded by plant cell membrane. These create the symbiosome structures in which the nitrogen fixation occurs (Nap and Bisseling, 1990).

In legumes, the conversion of atmospheric nitrogen to ammonia within the bacteroids is catalysed by nitrogenase (Dighe et al., 2010). Hydrogen and energy from ATP are required in this reaction. When the nitrogenase is exposed to air, it is inactivated because of the oxygen sensitiveness. In the nodule, leghaemoglobin, a protein containing iron, supports rhizobial control of the level of oxygen. This protein has an oxygen-binding function similar to haemoglobin. Leghaemoglobin supplies sufficient oxygen for the bacteroid metabolic functions and controls the free oxygen accumulation which inhibits the nitrogenase activities. The efficiencies of Rhizobium can be evaluated by the colour inside the nodules. The nodules are active and fix nitrogen for the host plant if they are pink or red inside (K¨astner, 2004).

1.1.5 Chickpea production globally

Chickpea is currently the second most important pulse crop produced in the world after soybean. It is rich source of protein for human consumption and is cheaper compared to animal protein. According to FAO statistics, global chickpea production was estimated at 13.7 million tonnes in 2014 with around 14 million hectares under chickpea cultivation. India is the biggest producer and consumer of chickpea in the world. The chickpea production in India is approximately at 9.9 million tonnes, accounting for 71.9 percent of total world chickpea production, and covering 9.9 million hectares. The other four major chickpea production countries are Pakistan, Iran, Australia, and Turkey estimated around 6.79, 4.25, 3.63 and 2.78 percent of total chickpea cultivation worldwide, respectively (Table 2) (FAOSTAT, 2014). Australia is presently the number one exporter of chickpea in the world, over 90 percent Australian chickpea are exported (Ziebell, 2016). Chickpea production has increased rapidly in Australia. In 2016 – 2017, the production of chickpea in

Australia was estimated at 1.4 million tonnes, an increase of 33 percent over last season (ABARES, 2017). In Australia, chickpeas are grown mainly in Queensland and New South Wales, and becoming more widely cultivated in Victoria, South Australia and Western Australia regions (Table 3) (ABARES, 2017). Chickpea is a cool season crop. The best temperatures for chickpea growth are between 21°C and 29°C during daytime and from 18°C to 21°C at night (Table 4). Chickpea grows best with rainfall from 150 to 250 mm of seasonal rainfall and neutral pH drained soils. The expectation of plant maturity is from 110 to 130 days (Saskatchewan Pulse Growers, 2010).

Table 3. The global area harvested and production of chickpea between 2012 and 2014

Countries	2012			2013			2014		
	Area (ha)	Product Quantity (Tons)	Yield (Tons/ha)	Area (ha)	Product Quantity (Tons)	Yield (Tons/ha)	Area (ha)	Product Quantity (Tons)	Yield (Tons/ha)
India	8,320,000	7,700,000	0.93	8,522,000	8,832,500	1.04	9,927,000	9,880,000	1.00
Australia	456,070	673,371	1.48	573,600	813,300	1.42	507,800	629,400	1.24
Pakistan	1,007,445	284,304	0.28	991,951	751,223	0.76	949,513	399,030	0.42
Iran	593,014	258,879	0.44	595,326	261,154	0.44	594,489	261,616	0.44
Turkey	416,242	518,000	1.24	418,889	506,000	1.21	388,169	450,000	1.16
World	**12,464,770**	**11,585,157**	**0.93**	**12,698,039**	**13,307,760**	**1.05**	**13,983,877**	**13,741,001**	**0.98**

(Source: FAOSTAT, 2015)

Table 4. The estimated Chickpea area and production in Australia between 2014 and 2016

States	2014		2015		2016	
	Area	**Production**	**Area**	**Production**	**Area**	**Production**
New South Wales	209,300	282,200	290,800	438,500	521,800	808,300
Queensland	165,000	201,100	338,000	555,000	570,000	933,000
South Australia	21,100	16,000	16,900	11,000	17,000	18,800
Victoria	26,400	14,600	12,900	5,300	15,500	18,800
Western	3,300	3,600	2,700	3,200	4,300	9,500
Total	**425,100**	**517,500**	**661,300**	**1,013,000**	**1,128,600**	**1,788,400**

(Source: Pulse Australia, 2016)

Table 5. The global chickpea production calendar

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(Source: Pulse Australia, 2017)

1.1.6 Chickpea symbiosis

Chickpea is a type of legume which can fix the atmospheric nitrogen via symbiosis with the rhizobial partner, *Mesorhizobium* (Laranjoa et al., 2014). A field trial in Tunisia showed that the shoot dry weight and number of nodules increased when chickpeas were inoculated with highly effective rhizobia strains (Ben Romdhane et al., 2007). Içgen et al. (2002) and

Alexandre et al. (2009) showed that the plant growth is promoted and the total nitrogen content increased when chickpeas were inoculated with rhizobia in plant growth chamber trials. Moreover, rhizobia could be used as plant growth promoting bacteria in plant parasites biocontrol (Siddiqui and Akhtar, 2009). In Turkey and India, there were field trials showing that the nitrogen and phosphorus fertilizers could be alternated in chickpea crops by the co-inoculations of rhizobia and *Bacillus megaterium* or *Bacillus subtilis (*phosphorus solubilizing bacteria) (Rudresh et al., 2005, Elkoca et al., 2008). A researcher in India showed that 32% in grain yield increased when *Mesorhizobium* sp. inoculant together with *Pseudomonas aeruginosa* in chickpea plant (Verma et al., 2013). In addition, some chickpea genes code for phenolic compounds or phytoalexins production is activated when rhizobia are inoculated in chickpea. These compounds support the prevention of disease development in plants (Arfaoui et al., 2007).

Chickpea plants are able to work symbiotically with known *Mesorhizobium* spp.*: Mesorhizobium ciceri* (Nour et al., 1994), *Mesorhizobium tianshanense* (Rivas et al., 2007), *Mesorhizobium huakuii* (Alexandre et al., 2009), *Mesorhizobium amorphae (Rivas et al., 2007), Mesorhizobium muleiense* (Zhang et al., 2012), *Mesorhizobium mediterraneum* (Nour et al., 1994), *Mesorhizobium. opportunistum* (Laranjo et al., 2012) *and Mesorhizobium loti* (Laranjo et al., 2008). All the genes involved in the symbiosis including nodC and nifH, which are required for effective chickpea nodulation are harbored in *Symbiovar ciceri*. These *Mesorhizobia* have a high diversity in effectiveness of symbiosis (Laranjo et al., 2001, Laranjo et al., 2008, Laranjo et al., 2002, Alexandre et al., 2009), stress condition tolerances such as acidity, salinity and heat (Alexandre and Oliveira, 2011, Brigido et al., 2007, Brigido et al., 2012). Each species cluster has the significant differences of tolerance to heat and acidity.

Genetic modification has also been applied to improve chickpea *Mesorhizobia*. The acdS gene from *Pseudomonas* sp. UW4 was transferred into *M. ciceri* LMS-1 by the pRK415 expression vector (Nascimento et al., 2012a). The transformed strain supports the chickpea nodulation and promotion of chickpea growth, compared to the wild-type strains. In addition, the *M. ciceri* LMS-1 (pRKACC) displayed susceptibility to root rot disease in chickpea (Nascimento et al., 2012b). For several decades, the commercial inoculant for chickpea has been *Mesorhizobium ciceri* strain CC1192 (Bullard et al., 2005, Drew et al., 2012).

1.1.7 Biocontrol of chickpea root rot using endophytic actinobacteria

Previously, eleven actinobacterial strains were isolated from different plants, lentil (Lens esculentus), chickpea (Cicer arietinum L.), pea (Pisum sativum), faba bean (Vicia faba) and wheat (Triticum vulgare) from Paskerville, South Australia. In their greenhouse experiment, actinobacteria with the highest biocontrol capabilities were tested for their ability to control Phytophthora root rot on chickpea. Both Streptomyces sp. BSA25 and WRA1 successfully suppressed Phytophthora root rot when inoculated with either Mesorhizobium ciceri WSM1666 or Kaiuroo 3. Streptomyces sp. BSA25 with either rhizobial strain enhanced vegetative growth of root (7–11 fold) and shoot dry weights (2–3 fold) compared to infected control, whereas Streptomyces sp. WRA1 increased root and shoot dry weights by 8- and 4-fold, respectively when inoculated with *M. ciceri* WSM1666. *Streptomyces* sp. BSA25, BSA26 and WRA1 inhibited growth of the three pathogens. *Streptomyces* sp. BS26 effectively inhibited *P. irregular*, where the survival percentage of the pathogen was less than 5%. Streptomyces sp. WRA1, WRA20 and BSA26 significantly inhibited 70–95% of Pythium growth at P B 0.01, while the rest of isolates moderately inhibited its growth and sporulation from 30–45%. *Streptomyces* sp. EN16 and BSA7 inhibited only 20% of its growth. *Streptomyces* sp. BSA7, BSA25 and BSA26 were the most powerful isolates

inhibiting growth of *B. cinerea*. *Botrytis cinerea* responses to actinobacterial treatments divided the isolates into two groups. One group of 11 actinobacterial isolates, ten of which belonging to streptomyces actinomycetes (SA), was able to suppress its growth to up to 75%; while the second group, mostly NSA, affected only 50% of *B. cinerea* survival percentage. *Microbispora* sp. EN2 as well as *Streptomyces* sp. strains CSA10 and BSA26 significantly suppressed *B. cinerea* at P B 0.01. (Misk and Franco, 2011).

1.2 Classification of actinobacteria

The major classification and taxonomy of actinobacteria was principally determined on polyphasic characteristics including phenotypic, genotypic and pylogenetic analysis (Wlink, Mohammadipanah and Hamedi 2017). This class of bacteria comprises of 6 classes, 6 orders, 14 suborders, and 56 families. The number of presented genera of this taxonomy and their physiological diversity proposes that taxonomical identification of this group will be based on active process. This bacterium are autochthonous inhabitants of soil and marine and often among the dominant population of their ecosystems and may occur in life-threatening environments (Wlink, Mohammadipanah and Hamedi 2017).

1.2.1 Molecular mechanisms of morphological differentiation

Filamentous microorganisms involved into two main groups, filamentous fungi and filamentous actinomycetes, particularly the *Streptomycetes*. Eukaryotic fungi possess subcellular organelles and cytoskeletal structures directing growth while prokaryotic actinomycetes have no such cellular organization. Despite these fundamental differences, both groups exhibit similar morphologies, growth patterns, growth forms, hyphal and mycelial growth kinetics, spore, sporangia, and conidiospore. The previous studies reported that two groups have very similar molecular mechanisms of morphological differentiation (Prosser and Tough, 1991). *Streptomyces coelicolor* A3 is the most extensively

characterized actinomycete at the genetic level. These have been used to study various aspects of its biology, notably secondary metabolism and its life cycle (Chater, 1993). Genes required for aerial growth (*bld* genes) are often also needed for secondary metabolism. At least six further genes (*whiA, B, G, H, I, J*) are needed to initiate the subdivision of multigenomic aerial hyphal tips into unigenomic prespore compartments, while several more (including *sigF*, *whiD*, and the *whiE* spore pigment gene cluster) are in spore maturation.

1.2.2 Endophyte and Actinobacteria

"Endophyte" is defined as a microorganism such as a bacterium or fungus that lives inside plant tissues. They create a relationship with plant without any visible harm. They also could be isolated and cultured form plant tissues after surface sterilisation (Hasegawa et al., 2006). The term endophyte was named by (De Bary 1866), which includes the existence of microorganisms inside the infected plant tissues without having negative effects on host plant (De Bary 1866;Schulz and Boyle 2006).The biodiversity could be created from fundamental as symbiotic relationship between plants and microbes. Many endophytic microorganisms are novel so they can be screened for biocontrol agents or source of novel metabolites used in agriculture, medicine and industry (Bacon and James, 2000, Strobel and Daisy, 2003). Although it is claimed that each plant of about 300,000 currently described plant species can be host for one or more types of endophytes, only a few that studies have been undertaken about their relationship with their endophytes (Strobel and Daisy, 2003).

Actinobacteria are a kind of Gram-stain positive bacteria that have a high guanine and cytosine content in their genome. Actinobacteria can produce a slender and non-septate mycelium although some genera are unicellular like bacteria. They can be found in freshwater, soil and support the degradation of organic substances (such as chitin and,

organic acids, protein fats, polysaccharides and cellulose). Therefore, they play an important role in turnover of organic matter, carbon cycle and humus formation, provide the nutrients into the soil. They can survive in a broad variety of soil type from high to low pH (Anandan et al., 2016). Actinobacteria can produce various secondary metabolites which may have biological activity of interest to the pharmaceutical or agricultural industries.

1.2.3 Endophytic Actinobacteria

Endophytic actinobacteria are bacteria that inhabit the inside of the plant without causing any visible negative changes to their plant hosts. They could protect the host plants from diseases and insects. They are not only found in the rhizosphere but also inside plants. *Frankia* is an actinobacterial genus found in association with pine trees that form nodules which can fix nitrogen (Benson and Silvester, 1993b). The endophytic actinobacteria include *Streptomyces, Glycomyces Plantactinospora, Streptosporangium, Promicromonospora, Actinomadura, Kibdelosporangium, Nocardioides, Pseudonocardia, Actinopolyspora, Microbispora, Brevibacterium, Kitasatospora, Polymorphospora, Micromonospora, and Nocardia* that could be found in various plants (Anandan et al., 2016).

1.2.4 Direct contributions to Nitrogen fixation

Frankia is a kind of actinobacteria that can fix the nitrogen from the air by inducing root nodules formations on a diverse angiosperm group. They can inhabit twenty-four genera within eight families of host plants or live as free-living bacteria in soil (Chaia et al., 2010, Benson and Silvester, 1993a). *Frankia* have three natural morphological forms: spores, filaments, and vesicles. Vesicles and filaments have septae; however, vesicles are typically around 1–5 μm in diameter and spherical, while filaments are 0.5 μm thick and branched. Vesicles is unique cellular structures with lipid envelopment. Vesicles are specialised structures for nitrogen fixation that is formed under nitrogen-limiting conditions or inside

the nodules cells of plant (Newcomb and Wood, 1987, Chaia et al., 2010).

1.2.5 Plant growth promotion properties

The possible mechanisms by which microorganisms promote plant growth are: (1) the amount of the hormones in plant such as cytokinins, gibberellic acid, ethylene and indole-3-acetic acid (IAA); (2) by Nitrogen fixation; (3) by protecting the plant from phyto-pathogenic microbes by producing antibiotics, cyanide, siderophores, chitinase, and β-1,3 glucanase; and (4) by solubilising the mineral nutrients such as phosphates (Cattelan et al., 1999).

Various research has reported that the endophytic actinobacteria can produce a number of plant hormones such as siderophores and IAA, solubilise insoluble phosphate (Aráujo et al., 2000, Nimnoi et al., 2010, Yasmin et al., 2009). The indole-3-acetic acid (IAA) were produced in YMA media by *Streptomyces* species which were isolated from Thai medicinal plants rhizosphere soils. *Streptomyces* CMU-H009 produced IAA effectively, were isolated from soil of lemongrass (Khamna et al., 2010). *Streptomyces* sp. CMU-MH021 produced the high level of siderophores (26.0 $\mu g.ml^{-1}$) and IAA (28.5 $\mu g.ml^{-1}$). Actinobacteria also produce chitinase, catalase, and urease (Ruanpanun et al., 2010). Manulis et al. (1994) described the indole-3-acetic acid production and its biosynthetic pathway in a *Streptomyces* sp. Further, *Streptomyces* culture filtrates displayed an effect on the wheat growth through an increase in shoot dry mass, diameter, length and fresh mass. This activity was due to the production of exogenous auxins, as well as cytokinins and gibberellins (Aldesuquy et al., 1998).

Umezawa et al. (1965) showed that *Streptomyces kasugaensis* can produce a fungicidal and bactericidal metabolite, named Kasugamycin. This compound can inhibit the biosynthesis of protein in some microorganisms which cause of bacterial *Pseudomonas* diseases and rice

blast *Pyricularia oryzae*. *Streptomyces cacaoi* produced the Polyoxin B and D that can control the fungi. These metabolites can inhibit the synthase of chitin leading to interfere in the synthesis of fungal cell walls. Polyoxin B can control the fungal pathogens in ornamentals, vegetables and fruits. Polyoxin D is applied to against *Rhizoctonia solani* which cause of the rice sheath blight (Isono et al. 1965). In a greenhouse assay, validamycin from *Streptomycete* extracts with extremely strong trehalase inhibitor and against rice sheath blight was detected (Kameda et al. 1987).

1.3 Actinobacteria legume benefits

The growth, nodulation process and nitrogen fixation of the host leguminous plants as well as the properties of the rhizobial strain may be influenced by compounds and phytohormones that are released by endophytic actinobacteria either directly or indirectly. Because the auxin balance is important for the improvement of nodule formation and the root plant development, the IAA released from actinobacteria is a necessary factor to enhance the plant growth and nodulation as well as the growth of co-inoculated rhizobia (Nimmnoi et al., 2014, Solans et al., 2009). The leghemoglobin levels and nitrogenase activities also are significantly influenced by IAA released by actinobacteria (Ali et al., 2008). Actinobacteria can also increase the levels of iron in the plant by producing siderophores (Nimmnoi et al., 2014). A possible mechanism which was claimed by Tokala et al. (2002) is that *Streptomyces lydicus* WYEC108 increased the nodular assimilation of soil nutrients include iron, which leads to the enhancement of the nodules average size, longevity, vigor and nitrogen fixation ability of bacteroids. In addition, the rhizobia may infect the plant by using the *Streptomyces* colonisation sites on the root (Tokala et al., 2002).

There were 31 percent of 481 agricultural soil actinobacteria were good for clover or alfalfa growth but two *Rhizobium meliloti* strains S14 and A2 (Antoun et al., 1978). There were 35

percent and 53 percent nodules number on soybean were reduced when co-inoculated with actinobacterium E8 and *Rhizobium japonicum* 122 and 123 respectively (Damirgi and Johnson, 1966).

Le (2016) tested the effect of six effective endophytic actinobacteria from wheat and legumes on the growth *of Rhizobium* strains. The result showed that most of the actinobacteria did not negatively affect rhizobial growth, excepted for LuP10 which inhibited the growth of WSM 1115 and SARDI 736 but increased the growth of RRI 128. Strains LuP3, LuP30 and LuP47B also enhanced the growth of rhizobial strains.

Sathya et al. (2016) found 19 isolates of actinobacteria significantly enhanced the minerals (Fe, Zn, Ca, Cu, Mn and Mg) in chickpea plants compared to the uninoculated control treatments. Misk and Franco (2011) proved several endophytic actinobacteria isolated from chickpea could produce siderophores, cyanogens, soluble phosphate, and also had bio-control activities. Sreevidya et al. (2016) showed that actinomycetes had positive effect on chickpea plant growth by producing β-1,3-glucanase, IAA, hydrocyanic acid, chitinase, lipase, cellulase, protease, and siderophore. Actinobacteria isolated from medicinal plants (Singh and Gaur, 2016) or organic soils (Gopalakrishnan et al., 2016) also showed the chickpea plant growth promotion.

According to Coombs and Franco (2003) endophytic actinobacteria was first report of filamentous actinobacteria isolated from surface-sterilized root tissues of healthy wheat plants (Triticum aestivum L.). Wheat roots from a range of sites across South Australia were used as the source material for the isolation of the endophytic actinobacteria. Roots were surface sterilized by using ethanol and sodium hypochlorite prior to the isolation of the actinobacteria. Forty-nine of these isolates were identified by using 16S ribosomal DNA (rDNA) sequencing and found to belong to a small group of actinobacterial genera including

Streptomyces, *Microbispora*, *Micromonospora*, and *Nocardiodes* spp. Many of the *Streptomyces* spp. was found to be similar, on the basis of their 16S rDNA gene sequence, to *Streptomyces* spp. that had been isolated from potato scabs. Several isolates exhibited high 16S rDNA gene sequence homology to *Streptomyces caviscabies* and *S. setonii*. None of these isolates, nor the *S. caviscabies* and *S. setonii* type strains, were found to carry the nec1 pathogenicity-associated gene or to produce the toxin thaxtomin, indicating that they were nonpathogenic. Several isolation media were developed for the isolation of endophytic actinobacteria as described by (Crawford DL et al., 1993 and Hayakawa MT et al., 1987). However, the validation of the surface sterilization has been widely used for the isolation of endophytic fungi (Bills GF, 1996).

1.4 Spore formation

The division of a hyphae and the creation of a spore start with the development of a cross-divider. As a rule, there are three sorts of techniques for actinomycetes sporulation process when substrate hyphae are divided, the septum, which is known as a split septum, may happen and shape spore. Spores are framed by septation and disarticulation of prior hyphal components with a flimsy sinewy sheath. The spore divider is shaped, from divider layers of the parent hypha; this is named as holothallic advancement and is common for some other sporulating actinobacteria, like the genus *Streptomyces*. Globose spores are shaped in ethereal and substrate mycelium and item spore divider, for example, a few strains of *Thermoactinomyces*. The spores are traditional endospores with all the properties of bacterial endospores, ultrastructure, and physiology. Beside the mycelial development, spore arrangement is the most significant morphological basis that can be utilized to perceive an actinobacterium. Routinely, the arrangement of spores is confined to the morphological gathering of sporoactinomycetes, where sporulation happens in very much characterized

pieces of the mycelium. It is realized that various qualities are associated with spore development and that diverse development conditions can have an impact on the spore arrangement (Kim, 1999).

The attributes of spores have assumed a significant job in animal types portrayals for a long time. The spores created separately or in short chains are thicker than the hyphae, while those which are created in long chains for the most part have a similar measurement as the hyphae. Spores are around 1 to 2 µm thick and differ in surface morphology. Normal spore morphology is globose, ovoid, coliform, pole formed, allantoid, and reniform. The motile spores are outfitted with flagella such as *Kineococcus radiotolerans* SRS30216T; monotrichous spores have just a single flagellum. As in *Catenuloplanes japonica*, the spore is said to be peritrichous if various flagella are present over the entire spore surface. Polytrichous spores are described by a tuft of flagella, which can be embedded in one polar (monopolar polytrichous), as in *Actinoplanes regularis*, subpolarly (*Spirillospora*), or along the side (*Pilimelia*). Non-motile spores might be smooth or present a variety of surface ornamentation. They can be assembled into a few structures: smooth, rugose, warty, barbed, bumpy, verrucose, or unpredictable. In the variety *Micromonospora* which have nonmotile spores are borne independently, sessile, or terminally on short sporophores, which are monopodial or sometimes sympodial. Spores are circular to oval (0.7–1.5 µm) and in many species have prickly projections (Dhanasekaran and Jiang, 2016).

The morphological characteristics of actinobacteria due are generally quite stable, and it is an important basis for classification. The development and formation of some structures, like aerial mycelium, spore, and sporangia, are affected by culture conditions. In some media, strains produce many sporangia or spore, while in other media have little or none. The below figure is the diagram of some spore structures of some genera of actinobacteria.

Image removed due to copyright restriction.

Figure 2. Spore formation in some genera of Actinobacteria (Li et al., 2016)

1.6 Critical knowledge gaps

In general, spore production is optimal when the actinobacteria are cultured on a solid surface such as agar or a grain such as barley or soybean. However, scale up is a challenge a as a large surface area is required and the spores have to be washed off the surface. Spore production is not common in liquid submerged culture as the actinobacteria grow as mycelium or pellets. However, if liquid media could be used for the actinobacteria spore production at a high titer it would be less expensive and faster, thereby improving the economics of the process. This project aims to optimize the medium for the production of spores in submerged culture. The spores produced in this way may have slightly different properties therefore a comparison with spores produced in the traditional manner will also be carried out to gauge their stability to changes in pH and temperature and, more importantly, their efficacy as inoculants.

1.7. Biotechnological significance

Endophytic actinobacteria has been reported to enhance nodulation and chickpea growth and control disease. Herein, we used the different strains of endophytic actinobacteria in order to evaluate their influence in chickpea plant which can be used as a promising source for the boosting the chickpea growth, nodulation, nitrogen fixation or soil borne disease bio-control agents for chickpea production.

The study of sporulation in submerged media presents a better alternative to sporulation on solid medium or surface growth medium for several reasons. First, scale up can be carried out to boost yields of the inoculant, which can give immense economical advantage. The submerged system also differentiates between genes responsible for the control of hyphae formation and the gene involved in sporulation. Therefore, submerged sporulation can be an excellent model system to study sporulation than a solid-state medium.

1.8. Aims and objectives

The overall aim of this research is to make improvements in liquid media in order to increase sporulation while keeping the media production cost low thus making the process economically viable. The spores produced in this manner will be compare for stability and efficacy against spores produced on agar.

The specific objectives of this research are to:

1. Optimize the media for the production of spore in liquid media.

2. Compare the stability of spores grown in liquid versus solid media and their performance in influencing plant growth and nodulation with rhizobium of chickpea (*Cicer arietinum*).

1.9. Hypothesis:

Spore production with high titres are possible in liquid media and the spores produced are as stable and effective as spores produced on agar.

Chapter 2
Materials and Methods:

2.1 Organisms used

Chickpea seed (cv. Kabuli genesis 090) and the rhizobial culture *Mesorhizobium ciceri* CC1192 were provided by the South Australian research and development institute (SARDI) and endophytic actinobacteria *Streptomyces* sp. CP200B, CP84, CP21 and CP56 were obtained from previous research in our laboratory (Vo et al., 2017).

2.2 Spore production in liquid media

Galactose, at half the normal concentration, glutamic acid yeast extract medium (½ GGY) was used as liquid media (van Dissel et al., 2014). Initially, galactose (15 g), glutamic acid (1 g), yeast extract (5 g), anhydrous Iron (II) sulphate (0.001 g), anhydrous magnesium sulphate (0.25 g), and dipotassium hydrogen phosphate (0.2 g) was mixed in 1 liter of sterile water. Three flasks for each treatment one for each strain was prepared. 50 ml was transferred to each flask using 50 ml syringe and different concentration of humic acid, calcium carbonate, calcium chlorides, and chitin were added to each flask and the content was autoclaved. After autoclaving, the medium was allowed to cool down and each treatment was inoculated with the 2 loopful of the cultures CP56, CP84B, CP200B, and CP21A2. The inoculated medium was then placed on a 150-rpm shaker for 10 days at 27 °C.

The spore counting was done via the method developed by Miles and Misra (Miles and Misra, 1938). Serial dilution of the endophytic actinobacterial spore suspension was prepared by adding 1x spore suspension and 9x diluent and dilutions were made up to 10^{12}. Two drops of 10ul of suspension dilutions inoculated onto Mannitol Soy flour (MS) agar plates which were divided into 6 sectors. The plates were incubated at 27°C and the colonies

were counted in each sector which had less than 10 colonies (Bassi and Benson, 2007). Overgrowth was observed at the lower dilutions over the area of drop. The numbers of colony forming unit per ml was calculated by using following formula (Zhang et al., 2020). Collected spore were centrifuged at 3500 rpm for 5 minutes and the spores are stored in 20 % (v/v) glycerol at -20 °C until further use.

CFU per ml = Average number of colonies for a dilution x dilution factor x 102.

2.3 Visualization of spore-bearing structures of isolated endophytic actinobacteria

The visualization of endophytic actinobacteria was done in light microscope. Briefly, the actinobacteria was inoculated in 1/2 GGY media. One loop full spores were added on slides and observed under microscope (Bassi and Benson, 2007).

2.4 Preparation of inoculum

A loopful of four stains CP56, CP84B Cp200B and Cp21A2 in ½ Galactose-Glutamic Acid-Yeast extract liquid media were taken for inoculum. Culture was allowed to incubate at constant room temperature for 10 days at 150 rpm on a shaker. Culture was filtered using a sterile syringe half-filled with sterile cotton wool. The spores were counted by Miles and Misra technique. And the filtrate was centrifuged at 3500 rpm for 15 minutes. 4/5 of the total volume of the culture that has been spun down was removed. In this case, 30 ml was spun down and 24 ml was removed, leaving 6 ml of concentrated inoculum. The concentrated spores were resuspended in 8 ml 20 % (v/v) glycerol and stored.

2.5 Spores growth on solid media

The two media: Mannitol Soy flour agar (MS) and mannitol soy flour oatmeal agar (MSO) were prepared a few days before the experiment and checked for contamination. Inoculum

was prepared just before inoculation of the plates. Each treatment has 3 replicate plates. 0.1ml of inoculum was spread on to each plate. Plates were incubated at 27 ℃ for 6 days. Plates were harvested by scraping off spore and mycelium lawn on the surface of the media. Replicates were stored in the same manner. Mannitol Yeast extract agar was not harvested as no spore lawn was formed despite visible mycelium growth. Harvested spores and mycelium were resuspended in 12 ml 20 % glycerol to inhibit growth and to protect the spores during freezing. Total volume of suspension for all tubes was 14 ml. Spore suspensions stored at -20 ℃. The colony forming units of endophytic actinobacteria spores were counted using the drop-plate technique described by Miles and Misra (1938) as mentioned previously (Hedges et al., 1978).

2.6 Stability of spores obtained from liquid broth and solid media

2.6.1 Samples

Actinobacteria spores from four different cultures (CP56, CP200B, Cp84B, and CP21A2) optimized in liquid and solid media were used for the stability testing. The different parameters used were temperature and pH.

2.6.2 Temperature

The stability studies were done at the different time interval at 70 °C and 90 °C. Initially, each strain obtained from liquid and solid media were diluted in sterile water to get 1.4×10^{10} CFU/ml. 10 µl of each were added along with 4 ml 0.9 % NaCl to each glass tube in triplicate. The sample was incubated at 70 °C and 90 °C separately. The spore counting was done after 1, 3, 6, 12, and 24 hours using Miles and Misra method as described previously.

2.6.3 pH

Stability testing of the solid and liquid spore was done in the three different pH (6.0, 7.0,

and 8.0). Initially different pH was maintained in 0.9% NaCl by the addition of ammonium bicarbonate or 0.1N HCl. The dilution and spore count were added to each 4 ml 0.9% NaCl with different pH as mentioned previously.

2.7 Pot assay to compare performance of spores from agar versus submerged cultures

2.7.1 Surface sterilization of seeds

Similar size seeds of chickpea (cv. Kabuli genesis 090) were chosen and immersed in 70% (v/v) ethanol for 30 seconds, then in 4% (v/v) hypochlorite solution for 3 minutes. After that, the seeds were rinsed three times in sterilised reverse osmosis water (5 mins per time). At the end of process, the seeds were rinsed three times with 2% sodium thiosulfate solution, and then rinsed in sterilised water three times (10 mins per time). Finally, the seeds were removed from sterilized water and dried for at least 4 hours or overnight in a laminar flow cabinet (Coombs and Franco, 2003, Miche and Balandreau, 2001).

2.7.2 Growth and application of actinobacteria and rhizobia

Endophytic actinobacteria were grown on the media which best supported sporulation and incubated at 27^{o}C for 7-14 days until the culture produced spores. The actinobacterial spores were scrapped from plates and suspended in 0.3% autoclaved xanthan gum and were applied at the rate 10^{6} CFU per gram of seed. Single colonies of *Mesorhizobium ciceri* strain CC1192 were streaked onto Yeast Mannitol Agar (YMA) plates and incubated at 27 °C for 4-10 days until good growth was observed. The cultures were stored at 4 °C for subsequent use. A standard curve describing the relationship between cell number and OD600nm was developed for Rhizobium and each actinobacterium (spore) to enable the application of a standard CFU/ml across experiments.

2.7.3 Seed Coating with endophytic actinobacteria spores from liquid broth

The treatment of seeds was done by adding a known number of the actinobacterial spores from liquid broth as a suspension applied on the surface sterilized seeds as a seed coating. The suspension of actinobacterial spores were applied at the rate of 10^8 CFU per gram seeds. The actinobacterial spores were suspended in autoclaved 0.3% xanthan gum solution after washing off glycerol and applied on the seeds. The untreated control plants were from surface sterilized seeds treated only with 0.3% xanthan gum.

2.7.4 Seed Coating with endophytic actinobacteria spores from solid media

The treatment of seeds was by adding a known number of the actinobacterial spores from solid media as a suspension applied on the surface sterilized seeds as a seed coating at 10^8 CFU per gram seeds as mentioned previously.

2.7.5 Plant growth media, nutrition, sowing and water supply

The surface-sterilised seeds were sown into a pasteurised (by autoclaving) sand: vermiculite mix (50:50 by volume) contained in 1.25 litre self-watering pots. Ten seeds were planted into each pot for two separate set of experiment based on the source of the spore (liquid and solid media) and 100ml MQ water added. A thin layer of washed granulated plastic beads was covered onto the potting mix surface to reduce evaporation and minimise the transfer of microbes between pots. Then, 200 mL of McKnight solution supplemented with a small amount of nitrogen (300 mg NH4NO3 per 20 L McKnight's solution) was gently added to each pot before covering the pots with plastic bags and placing them in the glasshouse. After 5 days, the plastic bags were removed, and the number of seedlings were thinned to four plants per pot before adding 1 ml of rhizobium inoculant (around 10^8 CFU/gm) to the base of each plant. Plants were watered with MQ water as required for the remaining weeks. All treatment and control pots with four replicates each were completely randomised in the

glasshouse with the position of the pots changed every week.

2.7.6 Harvest of plants

Chickpea plants of pot experiment 1 (from liquid broth and solid agar media) were harvested in week 4, chickpea plants of pot experiment 2 (from liquid broth and solid agar media) were harvested in only week 6. Chickpea plants were removed from the pots and gently shaken to remove most of the sand and vermiculite. The plants were wrapped in moist paper tissue and stored in zip lock plastic bags at 4^{o}C prior to assessment. Roots were subsequently washed under running tap water to remove remaining sand and vermiculite residues.

2.7.7 Data collection and analysis

The harvested plants were measured for the following parameters dry weight and length of shoot, dry weight and length of root, and the number and dry weight of nodules per plant. Shoot and root lengths of individual plants (four plants per pot) were measured by ruler to 1 mm accuracy. After that, the roots and shoots had been separated from each other and dried in a 60^{o}C incubator for 48 hours until the weight was constant. Before drying, the number of nodules on the root of individual plants was counted, removed and dried. The average dry weight of nodules was calculated by the division of total nodule dry weight and total nodule number. Shoot, root and nodule dry weights were measured to three significant figures.

The data was entered and collated in a MS Excel spreadsheet and subsequently analysed using the IBM SPSS Statistics version 25.0.

Chapter 3

3. Results

3.1 Spore production in liquid broth

Table 1 demonstrated the treatment of endophytic actinobacteria isolated from chickpea

plant treated with 0.1% methyl jasmonate. It showed that methyl jasomanate increased the sporulation of CP84B strain significantly compared with other strain.

Table 6. Endophytic actinobacteria isolated from chickpea plant treated with 0.1% methyl jasmonate. The spores were counted in CFU/ml units in triplicates.

Culture strain	Methyl jasmonate 0.1%	½ GGY (control)
CP56	1.6×10^{13}	1.5×10^{13}
CP200B	1.4×10^{13}	1.4×10^{13}
CP84B	5.5×10^{11}	3.0×10^{11}
CP21A2	3×10^{11}	3.0×10^{11}

Table 7. Endophytic actinobacteria isolated from chickpea plant treated with different concentration of humic acid, calcium carbonate and chitin. The spores were counted in CFU/ml units in triplicates.

Culture strain	Humic acid 0.1%	Humic acid 0.25%	Humic acid 0.5%	Calcium carbonate 0.1%	Calcium carbonate 0.5%	Chitin 0.1%	½ GGY (control)
CP56	3.0×10^{13}	1.4×10^{12}	4.2×10^{13}	1.9×10^{9}	3.6×10^{13}	3×10^{12}	3.0×10^{13}
CP84B	3.0×10^{13}	2.8×10^{13}	3.4×10^{13}	3.0×10^{13}	4.0×10^{13}	1.4×10^{11}	3.0×10^{13}
CP200B	1.4×10^{11}	3.6×10^{11}	1.4×10^{11}	1.4×10^{11}	1.4×10^{11}	3.3×10^{11}	3.0×10^{11}
CP21A2	1.4×10^{13}	1.9×10^{12}	1.12×10^{13}	1.4×10^{12}	1.9×10^{11}	1.4×10^{11}	1.2×10^{11}

The strains were grown in presence of different concentration of humic acid, calcium carbonate and chitin in liquid broth in order to determine their influence on sporulation. Our results clearly demonstrated that humic acid (0.5 %), calcium carbonate (0.5 %), humic acid (0.25 %), and humic acid (0.1 %) increased the production of spore in CP56, CP84B, CP200B, and CP21A2, respectively compared with control in some extent. In case of CP56 strain, humic acid in the concentration of 0.5 % increased the spore up to 4.2×10^{13} CFU/ml. In contrast, calcium carbonate (0.1 %) decreased the sporulation as indicated in Table 7.

In the case of CP84B, Calcium carbonate (0.5 %) increased sporulation up to 4×10^{13}. However, chitin (0.1 %) showed negative effect in CP84B strain. It significantly decreased

the sporulation to 1.4×10^{11} CFU/ml. Interestingly, all reagents demonstrated negative effects on sporulation except humic acid (0.25 %) and chitin (0.1 %) in case of CP200B. Further, all reagent was found to have great influence in case of CP21A2 strain. Among the reagents, humic acid (0.1 %) was found to be superior and increased the production of spore up to 1.4×10^{13} CFU/ml. Overall, our result demonstrated that humic acid at different concentrations has a positive impact on sporulation for our tested strains.

Table 8. Endophytic actinobacteria isolated from chickpea plant treated with 0.1% and 0.5% of calcium chloride.

Culture strains	0.1% $CaCl_2$ (CFU/ml)	0.5% $CaCl_2$ (CFU/ml)	½ GGY (Control) CFU/ml
CP56	1.4×10^{10}	3.0×10^{11}	2.0×10^{12}
CP84B	2.4×10^{12}	1.4×10^{10}	1.4×10^{12}
CP200B	1.4×10^{10}	2.4×10^{10}	1.4×10^{10}
CP21A2	1.9×10^{12}	1.4×10^{12}	1.4×10^{12}

Table 8 showed the influence of sporulation in endophytic actinobacteria with different concentrations of $CaCl_2$. Our results demonstrated that treatment with different concentrations of $CaCl_2$ has negative influence in CP56. However, $CaCl_2$ (0.1%) increased the sporulation up to 2.4×10^{12} CFU/ml in CP84B. Further, $CaCl_2$ (0.5 %) significantly increased the production of sporulation in CP200B up to 2.4×10^{10}. In addition, 0.1 % $CaCl_2$ slightly increased the spore production in CP21A2 up to 1.9×10^{12} compared to control (1.4×10^{12}).

3.3 Optimizing the sporulation of actinobacteria in solid media.

The production of spores is dependent on several factors such as, pH, temperature, medium composition, humidity and amount of starting inoculum. The focus of this study was to determine the best media composition for the growth and sporulation of the four cultures.

As shown in Table 9, mannitol soy agar served as the best medium for the growth and production of spore of CP56 (1.2×10^{12} CFU/ml) and CP84B (1.2×10^{12} CFU/ml). On the contrary, mannitol soy oatmeal agar was found to be superior in the case of CP200B (2.7×10^{11} CFU/ml) and CP21A2 (2.7×10^{12} CFU/ml).

Table 9. Comparison of sporulation in different agar media

Culture strains	**Mannitol Soy agar** (CFU/ per plate)	**Mannitol Soy Oatmeal agar** (CFU/ per plate)
CP56	1.2×10^{12}	1.2×10^{11}
CP84B	1.2×10^{12}	2.2×10^{11}
CP200B	1.2×10^{11}	2.7×10^{11}
CP21A2	1.7×10^{12}	2.7×10^{12}

3.4 Microscopic observation of entophytic actinobacteria strain spores obtained from liquid and solid agar media

Microscopic representative images were taken from light microscope as shown in Figure 3. The images taken clearly showed that the morphological differences in the spores cultured in solid and liquid media.

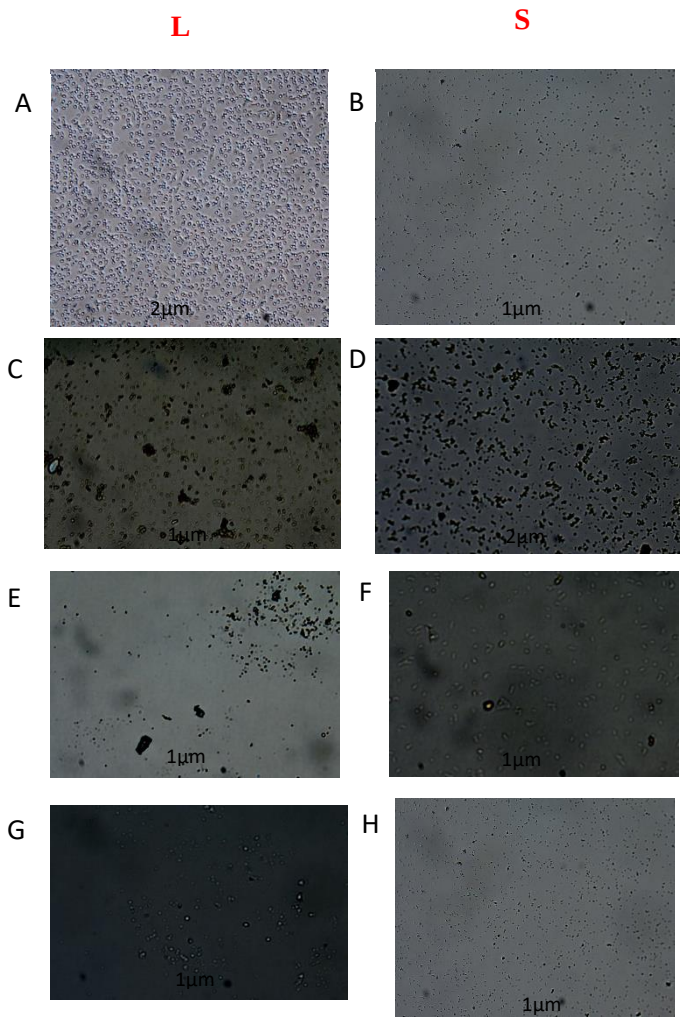


Figure 3. Endophytic actinobacteria strains A (CP56L), B (CP56S), C (CP84BL), D (CP84BS), E (CP200BS), F (CP200BL), G (CP21A2S), and H (CP21A2L) observed under 40X magnification with light microscope. S = solid; L = liquid.

3.5 Influence of temperature and pH on the stability of spores

3.5.1 Influence of temperature

The influence of temperature on the stability of spores of each of the 4 strains was exposed to various temperature until 24 hours as shown in Table 10 and Table 11. The spores obtained from the different sources (liquid and solid media) responded differently in our study. For instance, when CP56 spores obtained from liquid and solid media source were exposed to 70 °C, the both types of spores were stable until 12 h. However, after 24 h the spore from liquid broth showed exponential growth up to 0.5×10^8 CFU/ml but no spores from solid media were seen. In contrast, for the CP84B and CP21A2 strains, spores obtained from solid media were found to be more stable compared with the spore from liquid media. Further in case of CP200B, spores from both media were found to be stable for a fair amount of time as evident by the data shown in Table 10. Further, most of the spores were found to be thermolabile after 6 h under exposure at 90 °C. No germination nor growth of the spores was found after 12 h under exposure at 90 °C. Among the strains, CP56 from liquid media and CP21A2 from solid media were found to have more stable as 1.0×10^8 and 1.0×10^6 CFU/ml, respectively as shown in Table 11.

Table 10. Stability testing of different strain for temperature at 70 °C.

Treatment time (Hrs)	Strains							
	CP56		CP84B		CP200B		CP21A2	
	Liquid Spore (CFU/ml)	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml
0	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}
1	1.0×10^{10}	1.0×10^{12}	1.5×10^{10}	1.0×10^{10}	2.5×10^{10}	2.0×10^{10}	2.5×10^{10}	1.5×10^{10}
3	2.5×10^{10}	1.0×10^{10}	1.0×10^{10}	2.5×10^{8}	2.0×10^{10}	2.0×10^{10}	2.5×10^{8}	1.5×10^{10}
6	1.5×10^{8}	1.0×10^{10}	1.5×10^{8}	1.5×10^{10}	1.5×10^{10}	2.5×10^{8}	0.5×10^{8}	1.5×10^{10}
12	1.0×10^{6}	1.0×10^{8}	1.0×10^{7}	1.0×10^{9}	1.0×10^{6}	2.0×10^{8}	0.5×10^{4}	2.0×10^{8}
24	0.5×10^{8}	0	0	0.5×10^{6}	0.5×10^{6}	1.0×10^{6}	0	0.5×10^{6}

0 = $\leq 10^{6}$

Table 11. Stability testing of different strain for temperature at 90 °C.

Treatment time (Hrs)	Strains							
	CP56		CP84B		CP200B		CP21A2	
	Liquid Spore (CFU/ml)	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml
0	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}
1	1.5×10^{10}	1.5×10^{8}	1.0×10^{10}	1.5×10^{10}	1.0×10^{10}	1.0×10^{8}	2.5×10^{8}	1.5×10^{10}
3	0.5×10^{10}	0.5×10^{6}	0.5×10^{10}	0.5×10^{8}	0	0.5×10^{8}	2.0×10^{6}	1.0×10^{8}
6	1.0×10^{8}	0	0	0	0	0	0	1.0×10^{6}
12	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0

$0 = \leq 10^{6}$

3.5.2 Influence of pH

In order to determine the stability of the spores obtained from different strains, they were exposed to various pH (pH 6, pH 7, and pH 8). The results were shown in Table 12, 13, and 14. while comparing the stability of spores in different pH, our study clearly showed that the spores are most stable at neutral p^H. At pH 6.0, CP56 from liquid and CP84B from solid were found to be more stable than the other spores. In case of CP56 and CP21A2, the spores obtained from liquid media were more stable than the solid ones. These spores were stable more than 6 h as shown in Table 12.

As expected, the spores obtained from both solid and liquid medias for the 4 were stable until 24 hr of exposure under pH 7 as shown in Table 13. The CP56 strain spores from liquid media when exposed to pH 7, demonstrated fluctuation in the growth during the exposure in different time interval. The number of spores increases after the exposure in pH 7 at 1 h (3×10^{10} CFU/ml), 3 h (2.0×10^{10} CFU/ml), and 6 h (2.5×10^{10} CFU/ml). Further exposure of spores for 12 h and 24 h, demonstrated decline in the number up to 1.0×10^{8} and 1.0×10^{6} CFU/ml, respectively. Similar trends were observed with the CP56 spores obtained from the solid source. Further, CP84B was found to be stable until 12 h of exposure under pH 7. However, under 24 h of exposure spore from solid media were found to be more stable as evident by the number of spores 1×10^{6} CFU/ml. Similarly, in case of CP200B, the spores obtained from solid media were more stable than the liquid media. Interestingly, while exposing CP21A2 under pH 7, spores from liquid media were found to be more stable than the solid one. The number of spores were found to be higher than the spore before exposure until 6 h of exposure.

The spores were further exposed to pH 8 for the duration of 24 hours. We found out that none of the spores from both sourced were stable more than the 3 h of exposure except CP56

from liquid source as shown in Table 14. Further, no growth of the CP21A2 were found when exposed up to 3 h and is suggested that this spore from solid media was the Further, no growth of the CP21A2 were found when exposed up to 3 h and is suggested that this spore from solid media was the more prone to higher pH than the other spores.

Table 12. Stability of different strains at pH 6.

Treatment time (Hrs)	Strains							
	CP56		CP84B		CP200B		CP21A2	
	Liquid Spore (CFU/ml)	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml
0	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}
1	1.5×10^{6}	1.0×10^{10}	2.0×10^{8}	1.2×10^{10}	1.0×10^{6}	1.0×10^{10}	1.0×10^{10}	1.0×10^{10}
3	2.5×10^{4}	0.5×10^{8}	1.0×10^{6}	1.0×10^{8}	0	2.0×10^{8}	1.0×10^{8}	0
6	1.5×10^{4}	1.0×10^{6}	0	1.0×10^{6}	0	0	1.0×10^{6}	0
12	0.5×10^{4}	0	0	1.0×10^{4}	0	0	0	0
24	0	0	0	0	0	0	0	0

$0 = \leq 10^{6}$

Table 13. Stability testing of different strain at pH 7

Treatment time (Hrs)	Strains							
	CP56		CP84B		CP200B		CP21A2	
	Liquid Spore (CFU/ml)	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml
0	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}
1	3×10^{10}	2.5×10^{14}	2.0×10^{10}	1.2×10^{12}	2.0×10^{10}	2.0×10^{14}	1.4×10^{10}	1.0×10^{12}
3	2.0×10^{10}	1.5×10^{12}	1.0×10^{10}	1.0×10^{12}	2.0×10^{10}	1.0×10^{12}	1.0×10^{10}	1.0×10^{10}
6	2.5×10^{10}	1.0×10^{12}	1.0×10^{8}	1.0×10^{10}	2.5×10^{10}	1.0×10^{12}	2.5×10^{10}	1.0×10^{8}
12	1.0×10^{8}	1.5×10^{10}	1.0×10^{6}	1.0×10^{8}	1.0×10^{8}	1.0×10^{10}	0	0
24	1.0×10^{6}	0.5×10^{6}	0	1×10^{6}	0	0.5×10^{6}	1.0×10^{4}	0

0 = $\leq 10^{6}$

Table 14. Stability testing of different strain at pH 8

Treatment time (Hrs)	Strains							
	CP56		CP84B		CP200B		CP21A2	
	Liquid Spore (CFU/ml)	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml	Liquid Spore (CFU/ml	Solid spore (CFU/ml
0	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}	1.4×10^{10}
1	1×10^{10}	1.0×10^{12}	1.0×10^{8}	1.0×10^{6}	1.0×10^{10}	2.0×10^{10}	1.0×10^{10}	1.0×10^{10}
3	1.0×10^{6}	1.0×10^{8}	1.0×10^{6}	1.0×10^{4}	1.0×10^{6}	1.0×10^{4}	1.0×10^{4}	0
6	1.0×10^{4}	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0

$0 = \leq 10^{6}$

3.6 Effects of endophytic actinobacteria strains on the growth of chickpea growing in sand and vermiculite media

3.6.1 Effects of actinobacteria on chickpea plant from solid media and liquid media source after 4 weeks (Pot experiment #1)

The plants were germinated within a week of period. Inoculated chickpeas were sown in each pots and growth pattern were monitored after 4 weeks. Plants with no nitrogen and rhizobia had minimal growth pattern rate in compared to other treatments and the example of visual differences are shown in Figure 4.

Figure 4 Pot experiment 1. Chickpea plants in pots treated with endophytic actinobacteria spores (CP56, CP200B, CP84B, and Cp21A2) from solid and liquid after 4 weeks growth . N- control = no added nitrigen treatment, N+ control = unlimited added nitrogen, R = *Mesorhizobium ciceri* strain CC1192

Table 15. Effect of endophytic actinobacteria strains on the growth (shoot and root length and weight) and nodulation (number and dry weight) of chickpea plants inoculated with *Mesorhizobium ciceri* CC1192; harvested after four weeks. (n = 4 pots/treatment, 4 plants/pot). DW=Dry weight, RHZ = Rhizobium strain *Mesorhizobium ciceri* CC1192.

Treatment	Length of shoot (cm)		Length of root (cm)		No. of nodules		Dry weight of shoot (mg)		Dry weight of root (mg)		Dry weight of nodules (mg)	
	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid
N+ (control)	24.22^{abc}	24.22^{abc}	11.53^{ab}	11.53^{ab}	1.40^{b}	1.40^{b}	30.62^{bc}	30.62^{bc}	14.38^{c}	14.38^{c}	2.34^{a}	2.34^{a}
N- (control)	22.56^{bc}	22.56^{bc}	11.34^{b}	11.34^{b}	1.16^{b}	1.16^{b}	23.00^{a}	23.00^{a}	8.04^{c}	8.04^{c}	2.32^{a}	2.32^{a}
R only (control)	20.41^{c}	20.41^{c}	11.50^{ab}	11.50^{ab}	1.6^{a}	1.6^{a}	43.44^{a}	43.44^{a}	9.53^{c}	9.53^{c}	2.84^{a}	2.84^{a}
CP56 + R	23.81^{abc}	29.44^{a}	12.63^{ab}	17.13^{a}	1.81^{b}	1.25^{b}	67.00^{a}	83.13^{a}	20.25^{b}	30.18^{b}	5.45^{a}	4.11^{a}
CP84B + R	26.75^{a}	26.19^{abc}	13.19^{ab}	13.38^{bc}	1.56^{b}	1.88^{b}	93.43^{a}	105.13^{a}	29.75^{a}	65.18^{a}	4.62^{a}	2.76^{a}
CP200B+R	26.00^{ab}	26.75^{ab}	14.25^{a}	15.38^{ab}	2.31^{ab}	2.25^{b}	46.00^{a}	48.19^{b}	20.31^{b}	35.62^{b}	9.35^{a}	4.70^{a}
CP21A2 + R	23.06^{abc}	22.13^{de}	11.13^{b}	10.19^{d}	1.31^{b}	1.06^{b}	36.91^{a}	30.50^{bc}	20.68^{b}	19.25^{bc}	8.39^{ab}	2.66^{a}

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

Effect of endophytic actinobacteria strains on the growth (shoot and root length and weight) and nodulation (number and dry weight) of chickpea plants inoculated with *Mesorhizobium ciceri* strain CC1192 and different strains obtained from solid and liquid media is shown in Table 15. Our result clearly showed that the length of shoot treated with CP56 and rhizobium significantly greater than the any of the other treatments obtained from solid media ($P < 0.05$). Further, treatment with CP84B and CP200B yielded similar result (around 26 cm). However, in case of spores obtained from liquid, plant treated with CP200B demonstrated longer shoot length (26.00 cm) than the others. In contrast, treatment with rhizobium only yielded smallest shoot length obtained from both media.

Interestingly, the dry weight of shoot was significantly higher (105.13 mg from solid media and 93.43 mg from liquid media) in the chickpea treated with CP84B. The lowest dry weight of shoot was obtained in the chickpea treated with N negative control as shown in Table 15.

While comparing the length of root, plant treated with CP56 has the longer root compared with the others in case of spores obtained from solid media. While in the case of plants treated with spores obtained from liquid media CP200B, root length was slightly longer than the others. Plants treated with CP84B yielded highest dry weight of root i.e. 65.18 mg in solid media sourced spores and 29.75 mg in liquid media sourced spores, compared with the others. Surprisingly, the dry weight of root varied greatly in the plants with same strains but differ in the media used for sporulation.

Further, we compared the number of nodules among the different treatments. Very few plants with the smaller nodules were observed in our experiment. Spores obtained from liquid media demonstrated greater dry weight of nodules than the solid ones. This might be due to the duration of harvesting time which was only 4 weeks. Plant treated with CP200B sourced from both media demonstrated greater number and dry weight of nodules than the others.

3.6.2 Effects of actinobacteria on chickpea plant from solid media and liquid media source after 6 weeks (Pot experiment #2)

The plants were germinated within a week of period. The plants were harvested after 6 weeks after sowing and the example of visual differences is shown in figure 4. Chickpea with no added nitrogen and rhizobia (R) only grew less than the other treatments as shown in Figure 5.

Figure 5. Pot experiment 2. Chickpea plants in pots treated with endophytic actinobacteria spores (CP56, CP200B, CP84B, and CP21A2) from solid and liquid after 6 weeks growth . N- control = no added nitrigen treatment, N+ control = unlimited added nitrogen, R = Mesorhizobium ciceri strain CC1192.

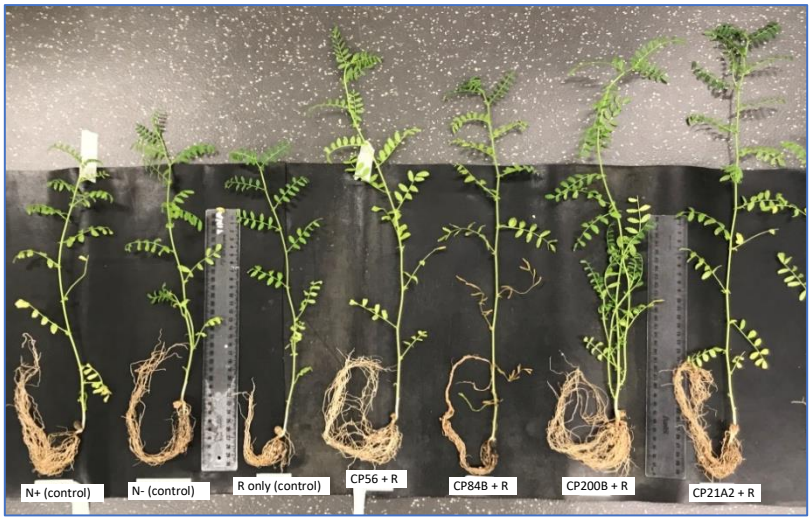


Figure 6. Chickpea plants treated with endophytic actinobacteria spore from solid with four treatment CP56, CP84B, CP200B, and CP21A2 after 6 weeks in sand and vermiculite system. N- control = no added nitrigen treatment, N+ control = unlimited added nitrogen, R = *Mesorhizobium ciceri* strain CC1192.

Figure 7. Visual representative images of nodules from chickpea plant treated with actinobacteria from solid media with rhizobium harvested after 6 weeks

Figure 3. Chickpea plants treated with endophytic actinobacteria spore from liquid media with four treatment CP56, CP84B, CP200B and CP21A2 after 6 weeks in sand and vermiculite system. N- control = no added nitrigen treatment, N+ control = unlimited added nitrogen, R = *Mesorhizobium ciceri* strain CC1192.

Table 16. Effect of endophytic actinobacteria strains on the growth (shoot and root length and weight) and nodulation (number and dry weight) of chickpea plants inoculated with *Mesorhizobium ciceri* CC1192; harvested after six weeks. (n = 4 pots/treatment, 4 plants/pot). DW=Dry weight, RHZ = Rhizobium strain *Mesorhizobium ciceri* CC1192.

Treatment	Length of shoot (cm)		Length of root (cm)		No. of nodules		Dry weight of shoot (mg)		Dry weight of root (mg)		Dry weight of nodules (mg)	
	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid
N+ (control)	29.00^{a}	29.00^{a}	18.12^{ab}	18.12^{ab}	2.30^{a}	2.30^{a}	88.18^{a}	88.18^{a}	17.81^{b}	17.81^{b}	0.73^{a}	0.73^{a}
N- (control)	26.91^{a}	26.91^{a}	14.85^{ab}	14.85^{ab}	2.78^{a}	2.78^{a}	102.90^{a}	102.90^{a}	12.14^{b}	12.14^{b}	0.72^{a}	0.72^{a}
R only (control)	29.49^{a}	29.49^{a}	15.76^{b}	15.76^{b}	2.44^{a}	2.44^{a}	45.05^{a}	45.05^{a}	13.07^{b}	13.07^{b}	0.90^{a}	0.90^{a}
CP56 + R	33.65^{a}	26.50^{a}	18.00^{a}	21.19^{ab}	1.13^{a}	1.13^{a}	38.17^{a}	98.44^{bcd}	21.68^{a}	74.81^{a}	2.19^{a}	0.17^{a}
CP84B + R	30.38^{a}	31.29^{a}	13.44^{abc}	26.65^{a}	1.44^{a}	2.59^{a}	33.81^{a}	191.94^{a}	18.65^{ab}	31.05^{b}	3.56^{a}	0.29^{a}
CP200B+R	25.88^{a}	31.81^{a}	12.35^{bc}	26.13^{a}	2.65^{a}	3.38^{a}	36.39^{a}	176.50^{ab}	18.50^{ab}	17.28^{b}	0.50^{a}	0.33^{a}
CP21A2 + R	32.63^{a}	27.69^{a}	16.81^{ab}	19.69^{ab}	0.94^{a}	1.56^{a}	43.38^{a}	46.38^{d}	14.81^{abc}	22.37^{b}	1.39^{a}	0.12^{a}

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

Effect of endophytic actinobacteria strains on the growth (shoot and root length and weight) and nodulation (number and dry weight) of chickpea plants inoculated with *Mesorhizobium ciceri* strain CC1192 and different strains obtained from solid and liquid media is shown in Table 16. Our results clearly suggested that CP84B and CP200B were better in comparison to other strains in case of strains from solid source for increasing the shoot length and root length. The length of the shoot and length of root were found to be around 31 cm and 26 cm respectively, strains obtained from solid media. However, in case of spores obtained from liquid source, CP56B was found to be superior where the shoot length and root length were found to be 33.65 cm and 18.00 cm, respectively. However, the overall length of roots obtained from liquid source strains were lower than strains (<18.00 cm) obtained from solid source. In addition, the number of nodules were higher in plants treated with CP200B obtained from solid source. In contrast, plants treated with strains obtained from liquid source were lower than the controls. Further, dry weight of shoot and root were also evaluated in plants treated with different strains obtained from liquid broth and solid media. We found out that dry weight of shoot in plants treated with CP84B was significantly higher (191.94 mg) than the plants treated with other strains obtained from solid source. Additionally, dry weight of shoot in plants treated with strains obtained from liquid source were significantly lower (<45.98 mg) than the strains obtained from the solid source. The dry weight of root in plants treated with CP56 were significantly higher which were 74.81 mg (solid sourced) and 21.68 mg (liquid sourced). While comparing the dry weight of nodules, we found out that plants treated with CP84 sourced from liquid media were higher (3.56 mg) than any other treated plants.

3.6.3 Correlation between dry weight of shoot and length of root between plants treated with spores obtained from liquid and solid media

Scatter plot of the dry weight of shoot and length of root treated with spores obtained from solid and liquid media after 6 week demonstrated poor correlation as shown in figure 11 and figure 12.

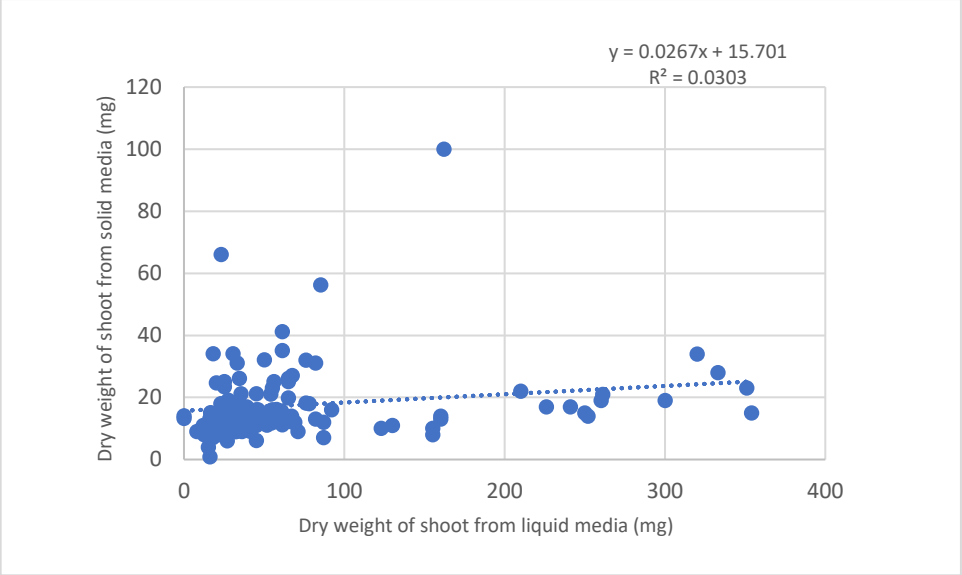


Figure 4. Correlation between dry weights of shoot treated with spores obtained from solid and liquid media harvested after six weeks

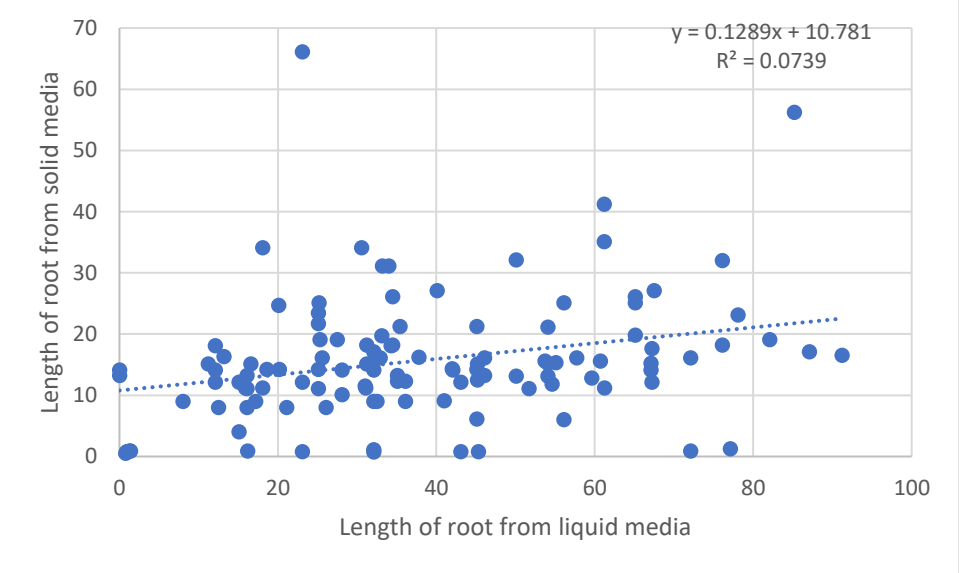


Figure 5. Correlation between length of root treated with spores obtained from solid and liquid media harvested after six weeks

Chapter 4

4. Discussion

In our present study, endophytic actinobacteria strains CP21A2, CP56, CP84B, and CP200B isolated from chickpea were evaluated for the sporulation rate in solid and liquid agar media. These obtained spores were evaluated for their stability and their symbiotic relationship with rhizobium for the growth and development of chickpea plant.

Initially, the spore production of the endophytic actinobacteria strains CP21A2, CP56, CP84B, and CP200B on ½ GGY liquid broth treated were optimised using different concentration of humic acid, calcium carbonate, sodium chloride, chitin, and methyl jamsonate. Previously, various agar media were used for the non-actinobacterial microorganism (Kieser et al., 2000). YMA (a high nutrient media) was reported to have highest level of non-actinobacteria. However, we used GGY media for our study because of the presence of galactose in the media. Galactose has been reported to enhance the growth rate of sporulation of actinobacteraial strains. Previously, Rueda et al. (2001) also reported that the sporulation rate was higher in the media containing galactose. Galactose was responsible for efficient sporulation and synthesis of glycogen. However, no spores or poor sporulation were obtained when used glucose, mannitol, fructose, and maltose instead (Rueda et al., 2001). Humic acid and calcium carbonate were most effective in increasing sporulation. Therefore, calcium carbonate would be the choice of reagent because of its lower cost and its efficacy in sporulation rate. Calcium carbonate costs approximately AUD 50-60 per kg, compared to humic acid, which costs approximately AUD 40-50 per 10 g. Further, there has been some reports that suggested that addition of high concentration of calcium to liquid grown cultures induces the occasional formation of spore like compartments involving the reduction of phosphate pool. In addition, liquid media offers shorter culturing time, with high yields under controlled sterile conditions

as well as a simpler scale-up and spores maturing over a subsequent period of 10-12 h (van Dissel et al., 2014).

Further optimization of the four strains for sporulation on MS and MSO media were performed. The production of spores is dependent on several factors such as, pH, temperature, medium composition, humidity and amount of starting inoculum. The focus of this study was to determine the best media composition for the growth and cultures. As seen in Table 9, mannitol soy agar served as the best media for the growth and production of spore of CP56 (1.2×10^{12} CFU/ml) and CP84B (1.2×10^{12} CFU/ml). On the contrary, mannitol soy oatmeal agar was found to be superior in the case of CP200B (2.7×10^{11} CFU/ml) and CP21A2 (2.7×10^{12} CFU/ml). The obtained results were concurrent with the previous reports published by Bennett et al. More importantly, the number of spores in our study were higher than the previous one which might be due to the different factors that includes contamination, incubation time and temperature (Bennett et al., 2018).

The optimised spores obtained from both liquid and solid media were subjected to pot experiments in order to understand the influence of different spores from both media for the growth and development of shoot, root and nodules in chickpea plants. In both pot assays, it was noted that nitrogen deficiency affected root growth of the chickpea plants. This indicates that the sterile sand-vermiculite medium, provided a system where nitrogen availability was the primary limitation to growth and was, therefore, suitable to study impacts of the actinobacteria on the level of N provided by rhizobia. In both experiments, the total mass of chickpeas supplied with unlimited nitrogen produced the most growth, and plants without nitrogen or rhizobia the least growth. This indicates that the experimental system, using a sterile sand-vermiculite medium, provided a system where nitrogen was the primary limitation to growth and was therefore suitable to study impacts of the actinobacteria on the level of N provided by rhizobia.

Strains CP21A2, CP56, CP84B, CP200B were able to increase the nodulation and growth of chickpea plants in 4 and 6 weeks. Previous researchers have shown that the legume-rhizobia symbiosis can be affected by other microorganisms, such as *Bacillus megaterium* which could enhance the rhizobial population in pigeon pea rhizosphere and subsequent nodulation (Rajendran et al., 2008). In addition, Petersen et al. (1996) showed that *Bacillus polymyxa* increased the densities of rhizobial colonization of *Phaseolus vulgaris* rhizosphere as well as lateral root formation and nodule number.

The root and shoot length of added nitrogen control plants was significantly higher than that of the rhizobia only control (Table 13 and 14). Mardanov et al. (1998) showed that the retardation of shoot growth and the development of the root system seem to be a strategy of the plant to adapt to nitrogen deficiency. Compared with the CC1192 only control, actinobacterial strains CP56, CP200B and CP84B and CP21A2 produced plants with significantly higher root lengths (Table 13 and 14) in both liquid and solid treated plants after 4 week and after 6 weeks except their root dry weights (Table 13 and 14). Harris (1992) stated that growth of root must be enhanced sufficiently to supply adequate water for the larger crop and leaf surface. In this study, the root and shoot length of unlimited nitrogen treatments was significantlye higher than that of the rhizobia only control (Table 13 and 14). Surprisingly, CP200B treated plants had significantly higher dry weights both of shoot and root dry weight after 4 weeks from both liquid and solid (Table 13). This might be because for the shoot mass to reach to 191 mg/plant, the supply of adequate water for the crop was not sufficient. In addition, Coutts and Philipson (1980) realised that the root and shoot dry weight was reduced when increasing soil fertility; that means, root growth increases less in weight than shoot growth. Giehl and von Wiren (2014) stated that mild nitrogen deficiency enhanced the growth of primary and lateral roots compared to when sufficient nitrogen was supplied; while severe

nitrogen deficiency inhibited the elongation of the tap root as well as the emergence and elongation of lateral roots.

There were differences in the number and dry weight of nodules between the strains. Some treatments produced a high number of nodules but with a low average dry weight and others fewer but larger nodules. Martínez-Hidalgo et al. (2014) suggested that some *Micromonospora* can act as rhizobia helper bacteria (RHB) and Tokala et al. (2002) found that some actinobacterial strains can increase the nodular assimilation of soil nutrients, including iron, leading to the enhancement of the average nodule size, longevity, vigor and nitrogen fixation ability of bacteroids. Singleton and Tavares (1986) showed that the enhancement of nodule number does not necessarily boost plant growth and nitrogen fixation, because it is possible that when nodule function is reduced, greater numbers are produced by the plant to compensate. These strains CP21A2, CP84B, and CP56 significantly increased only nodule number, but strain CP200B enhanced nodule mass and also total mass (Table 13 and 14). Araujo et al. (2017) showed that shoot biomass had a stronger correlation with the size and weight of nodules than with the number of nodules.

Chapter 5

Conclusions and future directions

5.1 Conclusions

Overall, endophytic actinobacteria strains CP21A2, CP56, CP84B, and CP200B isolated from chickpea were evaluated for the sporulation rate in solid and liquid agar media. These obtained spores were evaluated for their stability and their symbiotic relationship with rhizobium for the growth and development of chickpea plant. Our result suggested that, calcium carbonate in the liquid broth and MS media in solid agar media can be used for increasing the sporulation rate in the actinobacteria. Additionally, we found out that spores of almost all the 4 strains were stable at 70 °C but temperature greater than that are not suitable and lethal to the spores obtained from both media. In addition, the tested spore strains were more sensitive and prone to lysis in alkaline pH rather than acidic. Further, our study suggested that Cp56 spores obtained from liquid media and CP84B from solid media are the best spores which promoted the overall growth of plants and nodules. However, liquid media offers shorter culturing time, with high yields under controlled sterile conditions as well as a simpler scale-up. Further detail investigations needed to be carried out in order to determine their influence in the growth and development of chickpea plant which can be useful to increase the yield in the agricultural industry. The Major conclusion is that the hypothesis is validated as the spores produced in liquid are as stable and effective spores' production on solid media.

5.2 Future directions

Although this study has suggested that, there is significant change in the morphology, stability, and influence of these spores from liquid and solid media in the growth and development of chickpea plant, detail micromorphological investigation (morphology of an organism, mineral or soil component visible through microscopy) must be done. Further, number of strains should be increased in order to validate our finding. In addition, the amount of nitrogen fixed from the

atmosphere needs to be determined and establish the benefits to plants grown in field soil. Different soil containing complex microflora and natural rhizobial communities can be used in order to stimulate the growth and development of chickpea plant and other lentils.

APPENDICES

Appendix 1: Media and solutions

1. ½ Galactose - Glutamic acid - Yeast extract media (½ GGY)

Per litters RO water	
Galactose	15g
Glutamic Acid	1g
Yeast Extract(oxide)	5g
K_2HPO_4	0.2g
$FeSO_4.7H_2O$	0.001g
$MgSO_4.7H_2O$	0.25g

Adjust pH to 7.2 ± 0.2.

2. Mannitol Soya (MS) agar

Per litters RO water	
Mannitol	20g
Soya Flour	20g

Agar	20g

Adjust pH to 7.2 ± 0.2

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

3. **Mannitol Oatmeal Agar (MSO)**

Per litters RO water	
Mannitol	20g
Soya Flour	20g
Agar	20g
Ground Oatmeal	20g

Adjust pH to 7.2 ± 0.2

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

4. **YMA (Yeast Mannitol Agar)**

Per litters RO water	
Yeast extract	0.5g
Mannitol	5g

Sodium glutamate ($C_5H_8NO_4Na$)	0.5g
Agar	20g
Solution S	10 ml
Solution T	10 ml
Solution U	1 ml
Solution V	1 ml
Congo Red solution	10 ml
* Solution S:	
$Na_2HPO_4.2H20$ (Disodium phosphate)	1.8g
RO water	1000 ml
* Solution T:	
$MgSO_4.7H_2O$ (Magnesium Sulphate Heptahydrate)	10g
RO water	1000 ml
* Solution U:	
$CaCl_2.2H_2O$ (Calcium chloride dihydrate)	53g
RO water	1000 ml
* Solution V:	
$FeCl_3$ (Ferric chloride)	4g
RO water	1000 ml
* Congo Red Solution:	
Congo Red solution	2.50g

RO water	1000 ml

5. **McKnight's solution:**

Mc Knight's solution (no autoclave)	**250 ml concentrate stock solution**
$CaSO_4.2H_2O$ (Calcium Sulphate)	6.75 g
$MgSO_4.7H_2O$ (Magnesium Sulphate)	1 g
KH_2PO_4 (Potassium dihydrogen orthophosphate)	1 g
KCl (Potassium Chloride)	1.5 g
A-Z trace elements	5 ml
D-solution	5 ml
Distilled water	make up to 250 ml

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

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

Start "N"	
NH_4NO_3 (ammonium nitrate)	266 mg
Distilled water	make up to 1000 mls

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

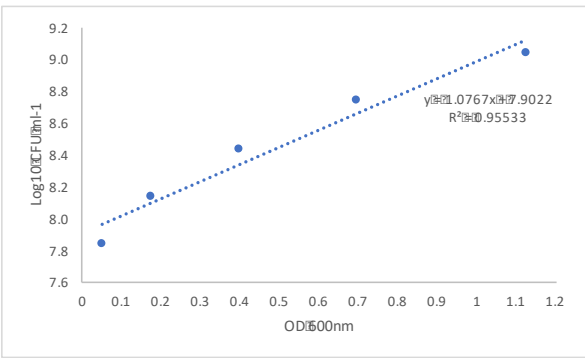
"Starter N" - add 266 mg of ammonium nitrate to the 20 ltr of nutrient solution above before applying to pots

6. **N solution for unlimited nitrogen treatments**

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

50 ml solution added into pot weekly.

Appendix 3. The correlation standard curve between OD600nm and CFU/ml of Rhizobium CC1192



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