EXPLORING GROWTH ENHANCING RHIZOSPHERIC MICROORGANISMS FOR SILVICULTURE OF *EUCALYPTUS PELLITA*

by

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Abstract

Eucalyptus pellita is a tree species originating from Australia. It has been planted in Sarawak for forest rehabilitation programme. The primary issues faced by these plantations, however, are poor progress caused by poor tropical soil conditions in some degraded lands. Such lands are subjected to intense tropical weathering process due to the heavy rainfall and high temperature. Besides that, the humus layer of the soil causes the soil to be acidic, thus further inhibit the availability of nutrients. In addition, the degraded lands left behind by shifting cultivation are generally already exhausted of nutrients. Chemical fertilizer has been used extensively to treat nutrients deficiency in plants. However, extensive use of chemical fertilizer has contributed to cumulative amount of chemical pollutants which cause environmental damages, as a result, causing irreversible damages to the overall environmental system. Inoculation of plant growth promoting rhizobacteria (PGPR) is the biological way to reduce the use of chemical fertilizer. PGPR facilitates plant growth elevation through the alteration of diverse group of bacteria living in the rhizosphere at plant root surface and associates with the roots, which promote the feature of plant growth direct or indirectly by secretion of various substances. PGPR facilitate plant growth by simplifying resource acquisition (phosphorus, nitrogen and crucial minerals) directly, tempering plant hormone level, or indirectly acts as biocontrol agents to inhibit various pathogens on plant. Application of biofertilizer is the way to reduce the use of chemical fertilizer and the negative impacts it causes to the environment. Biofertilizer has arisen as a significant element of the cohesive nutrient supply system in refining crop yields. The aims of this thesis were to isolate and characterise indigenous PGPR microorganisms from Sarawak soils, and evaluate their growth enhancing effects on *E. pellita*. The soil samples were collected from two national parks, two reserved forests and a planted forest in Sarawak. The soil textures, pH readings and plant available macronutrients were included to the soil physicochemical analyses for the background studies of soil samples. The soil textures range from sandy clay loam, sandy loam and clay. The pH of soils ranged from 3.86 to 5.46. These microorganisms were isolated through selective media, and undergo DNA identification to identify their identity. Then, they were evaluated for their efficiency of

plant growth promoting abilities (nitrogen fixing, Indole- 3-Acetic acid producing, phosphate and potassium solubilizing) through bioassay quantitative analysis. The top two microorganisms from each plant growth promoting abilities group were then tested for their antagonistic characteristic against each other. Finally, the four selected species were tested for their plant growth promoting ability in pot trial using *Eucalyptus pellita* as the plant host. The results showed that, pot trial using biofertilizer – PGPR microorganisms combining with 50% of chemical fertilizer (half the amount) is capable of achieving the same results as 100% chemical fertilizer (full amount). The height, stem diameter and root weight increment over 6 months period were just as good as positive control (100% chemical fertilizer). It was concluded that, these four microorganisms *Lysinibacillus sphaericus*, *Paenibacillus quercus*, *Tolypocladium* spp. 1 WL-2011, and *Lysinibacillus fusiformis*, were capable in promoting the growth of *Eucalyptus pellita.*

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Declaration

I, Chan Wen Loong, higher degree research student of Master of Science by Research from Faculty of Engineering, Computing and Science in Swinburne University of Technology (Sarawak Campus) hereby declare that the dissertation entitled "Exploring growth enhancing rhizospheric microorganisms for silviculture of *Eucalyptus pellita*" is original and contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome; to the best of my knowledge, this dissertation contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, the relative contributions of the respective workers or authors has been disclosed.

Chan Wen Loong

As the principal coordinating supervisor, I hereby acknowledge and verify that the above mentioned statements are legitimate to the best of my knowledge.

Assoc. Prof. Peter Morin Nissom

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List of Abbreviations

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Unit
IAA	Indole-3-Acetic Acid
ITS	Internal Transcribed Spacer
LPF	Licensed Planted Forest
E. pellita	*Eucalyptus pellita*
NA	Nutrient Agar
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Broth
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SD	Standard Deviation
SE	Standard Error
SFC	Sarawak Forestry Corporation
SUTS	Swinburne University of Technology Sarawak Campus
PGPR	Plant Growth Promoting Rhizobacteria

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CHAPTER 1:
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Eucalyptus species are second to pines in global importance as plantation trees, as they are the most planted genus in the tropics and subtropics lands. Study showed that there were at least 1.4 million ha of formal *Eucalyptus* plantations in the Southeast Asian region (Table 1) (197). This statistic was testified that the data did not include the equivalent of about 2.0 million ha growing as scattered tress and boundaries around fields. A number of countries, particularly Thailand, China, Vietnam and Laos had enhanced planting programs since 1995 (198). It is estimated that *Eucalyptus* plantation had exceeded 2.0 million ha now in Southeast Asia.

Table 1: Estimated area of *Eucalyptus* plantation in Southeast Asia in 1995 (197).

Country	Area (ha)
China	670 000
Indonesia	80 000
Laos	62 000
Malaysia	8 000
Myanmar	40 000
Philippines	10 000
Thailand	195 000
Vietnam	350 000
Total	1 415 000

Eucalyptus are extremely prized in countryside communities for an extensive range of uses as well as large scale plantation and farm planting grown to supply fibre for industrial use. *Eucalyptus* was used for poles, furniture, essential oils, fuel, tannins and small lumber. The main species grown were *Eucalyptus grandis*, *E. urophylla*, *E. tereticornis* and *E. camaldulensis*.

Eucalyptus pellita is a forest tree occurring in north Queensland, Autralia and in New Guinea (both Papua New Guinea and Irian Jaya, a province of Indonesia). Evaluation of *E. pellita* from Queenland provenances in species were carried out in Brazil, India, China, and many other tropical and sub-tropical countries over the years. The species was not taken up for operational planting in large scale even though growth of the plant has been good. *Eucalyptus pellita* was introduced into Sarawak and Sabah, Malaysia, with provenances from Queensland and Papua New Guinea. Mainly on the island of Sumatra, Indonesia, this species is well recognized as a pulp species, but it is also well matched to solid wood end-use. Productivity, growth, and genetic gains' data for plantation grown *E. pellita* in Southeast Asia has been published (1)(2), however there are relatively little data published on the solid wood end use of *E. pelita*, apart from some work in Queensland, Australia (199) and Vietnam (3).

Forest grower in Borneo are showing interest in expanding yield, as plantation grown properties are increasingly available. However, nutrients deficiency was observed in most of the established plantation of *Eucalyptus*. Insufficient in nutrients affected the growth of *Eucalyptus*, which also affected the financial turnover indirectly. Therefore, chemical fertilizer was introduced to solve the nutrient deficiency in *Eucalyptus* plantation. Unfortunately, use of chemical fertilizer has amplified drastically throughout the world in recent years, which causes serious environmental issues. Accumulation of heavy metals from chemical fertilizer affects soil and plant ecosystem, which directly links to air, soil and water pollution in the long run.

Biofertilizer was then introduced to reduce the use of chemical fertilizer. Biofertilizer is becoming a critical feature of organic farming and play a major role in the economy and for general agricultural production on a worldwide scale. Biofertilizer is a product that contains living microorganisms that colonize the rhizosphere of the plant, and promote plant growth by improving the availability of primary nutrients to the plants, when being applied to the plants (4). Biofertilizer is a combination of living microorganisms that have the ability to fix nitrogen solubilize phosphate or cellulolytic microorganisms used for applications to roots, soil, composting areas or seeds with the resolve of improving the number of those mutualistic beneficial microorganisms and hastening those microbial processes, which enhance the availability of nutrients that can then be

integrated effortlessly and engaged by the plants (200). Biofertilizer consists of plant growth promoting rhizobacteria (PGPR). Studies have shown that PGPR as the main composition in biofertilizer, has contributed to increased crop production and soil fertility throughout the world. Therefore, the potential contribution of PGPR has led to sustainable agriculture and forestry (5). In conclusion, biofertilizer will be the key to improving the crops quality, as well as reducing the use of chemical fertilizer at the same time.

1.2 Literature Review

1.2.1 *Eucalyptus pellita* and its industry in Asia

Eucalyptus pellita, which is also known as Red Mahogany, is a hardwood species that was originated in the warm, high rainfall zones of northern Queensland. *Eucalyptus pellita* is a fast growing hardwood species that is capable of adapting to humid tropical environment with a dry season up to 6 months. It is a medium or tall size tree that is capable to grow up to 40m in height and about 1m in girth, with a straight trunk to about half the tree height, and a large profoundly branched crown, at its best condition (171). Given the right soil and climate, it could grow up to a mean height of 15m at 4 years of age (173)(174). *Eucalyptus pellita* could endure a wide range of soil textures from sandy loam to clay loam, and soil with fairly little fertility, provided that the soil is not drenched for extended periods (more than a few days at a time (172).

An annotated bibliography of *Eucalyptus pellita* was compiled to summarise its known natural forest distribution, including genetic disparity and seed sources of early plantings proceeding to significant plantation assessment (176). *Eucalyptus pellita* is in the subgenus of *Symphyomyrtus*; section Traversaria (171), where about 20 species of *Eucalyptus* were grouped into including some of the tallest and commercially most valuable species. *E. pellita* is much more easily propagated compared to other *Eucalyptus* species (176), which has quicker initial growth in plantations, and is much less susceptible to damage from pests and diseases (6)(7)(177)(178)(179).

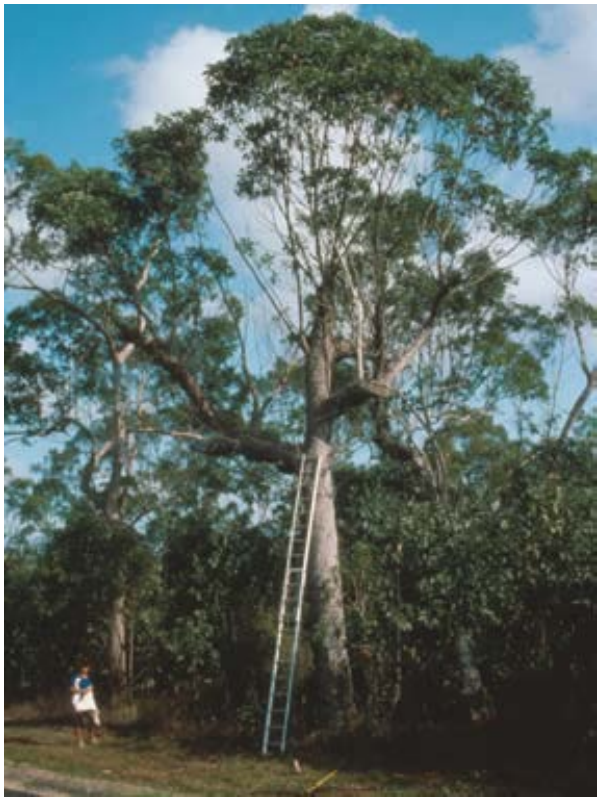


Figure 1: A mature *Eucalyptus pellita* in Mission Beach, Queensland (8).

Eucalyptus pellita has two different natural occurrences; In Queensland it covers from Iron Range, Cape York Peninsula to Ingham, and in the southern part of Papua New Guinea lowlands, where it covers from around Morehead and Keru in the western province, across the international border up to as far north as Muting in Irian Jaya, Indonesia (176). In its occurrences, annual rainfall is between 900 – 2200nm with warm humid climate where frost are rare to absent. It grows mostly on minor to moderately slanted lands; even to a limited extend, on steep, well-drained hills of large ridges and even alongside small rivers in drier and hotter parts of its existence (175). Soils vary from shallow sands on sandstone ridges to shallow sandy podsols (Queensland) and deep forest loams (Irian Jaya and New Guinea) (176).

In countries such as Malaysia, Philippines, Cuba, Indonesian Brazil, *Eucalyptus pellita* thrive as a role in reforestation. It has also been identified to be suitable for establishing plantation on the coastal sites of northern Queensland[180]. The characteristic structures of *E. pellita* are its fissured, fibrous, discolour leaves, brown bark to the small branches, broad peduncles and strongly exerted valves on large fruit with broad rims (171)(172).

A valuable dark-red timber with a basic density about 764 – 960kgm^3 is the product of *E. pellita* that can be used for appearance grade uses and flooring (171)(172)(181). Sawn timber of *E. pellita* is used to make decent furniture and in construction(8). Furthermore, it is also grown in many countries (9)(182) as an important source of raw materials for fibre cellulosic pulp and paper production, which is used for high quality tissue products or writing and printing paper (10)(11)(12). Its Kraft pulping and paper-making properties are comparable to plantation-grown *Eucalyptus globulus* and *Eucalyptus urophylla* (8).

Suitability for diversity of wood products, extensive adaptation to a series of environmental conditions, fast growth, higher resistance to diseases and pests, and decent stem straightness are the attractive attributes that make *Eucalyptus pellita* the choice for plantation establishment (8). Subsequently, several countries are choosing *E. pellita* as one of the main species for afforestation(14)(15)(8)(176). In Sarawak, Malaysia, *Eucalyptus* appeared to be one of the top planted timber species, where it occupied 7.5% of the planted land in 2011.

Table 2: Species composition in Sarawak, Malaysia as of 2011 (13)**.**

Species	Area (hectares)	Percentage	Remarks
Acacia mangium	213,442	73.6	Exotic
Eucalyptus	21,669	7.5	*E. deglupta* & *E. pellita*
Neolamarckia cadamba	19,260	6.6	Native
Paraserianthes falcataria	34,146	11.8	Native
Hevea brasiliensis	1,368	0.4	Clones
TOTAL	**289,885**		

1.2.2 Nutrient Deficiency in *Eucalyptus pellita*

Throughout most the established commercial plantation of *Eucalyptus* in practically all geographical regions, nutrients disorders have been documented (Table 1). Insufficient supplies of macronutrient nitrogen (N), phosphorus (P) and Potassium (K) are the most stumble upon syndromes causing premature leaf drop and reduction in wood volume. As shown in Table 1 below, yield could also be restricted by micronutrients, mainly B and Cu. Application of chemical fertiliser containing only macronutrients (for example; N, P, K and Mg) would also often prompted the disorder of micronutrients (B, Cu, Fe, Mn and Zn). Cases of Cu deficiency in Western Australia and B deficiency in China have been reported recently.

Table 3: Occurrence of nutrient deficiencies in *Eucalyptus* plantations (183).

	B	Ca	Cu	Fe	K	Mg	Mn	N	P	S	Zn
Australia	x		x	x	x		x	x	x	x	x
Brazil	x	x			x	x		x	x	x	x
Chile	x		x		x			x	x		
China	x			x	x			x	x	x	x
India					x			x	x		
Indonesia	x		x		x	x		x	x		x
New Zealand	x			x				x	x		
Philippines	x			x	x	x		x	x		x
Portugal	x	x						x	x		
South Africa		x	x		x	x		x	x		x
Thailand	x				x			x	x		
Zambia	x										

The volume of micronutrients to maximize productivity has not been effectively acknowledged earlier with the increasing numbers of micronutrient syndromes in *Eucalyptus* plantations (183). In China and India, the productivity of many *Eucalyptus* plantations is only one third of the world average. Comparatively, insufficient fertiliser treatments and soil constrains attributed to the low productivity (Table 2)(184). For instance, in parts of Guangdong Province, tree growth for more than three years was inadequately maintainable even with the routine application of 100kg of NPK fertilizer per hectare of planting, resulting in depressing cambial activity due to declining of canopy density. Soil erosion and removal of organic nutrient reserves resulted in declining of soil fertility (Figure 2). The branches and leaves of *Eucalyptus* were collected for fuel while remaining litter was collected and burnt. If only the nutrients reaped are refilled at the same time, then the regular reaping of foliage is justifiable (16).

Figure 2: Removal of organic nutrient reserves through soil erosion decreases the number of land to support commercial *Eucalyptus* plantations. Danzhou, Hainan (184).

1.2.3 Effects of chemical fertilizer to the environment

Chemical fertilizer has been used extensively to treat nutrient deficiency in *Eucalyptus* plantation(17)(18)(19). The use of chemical fertilizer has been increasing exponentially all over the world, which causes serious environmental issues. Chemical fertilizers are manufactured from petroleum products, rocks and organic sources. Even though chemical fertilizer improve yield production, overusing of it causes the accumulation of heavy metals in soil and plant system, which indirectly hardened the soil, decreasing soil fertility, released greenhouse gases, polluting air and water, therefore conveying hazards to human health and environment.

Chemical fertilizer contains acids, including hydrochloric and sulphuric acids. Soil crumbs, material that holds rock particles together were dissolved by these acids in the long run. These acids affect the structure of soils by damaging the cementing material, resulting in a compacted surface that prevents rain water from entering soil. Hence, causing trace nutrients not to be replenished in the soil. Continuous use of the chemical fertilizer diminishes indispensable soil nutrients and minerals that are naturally establish in fertile soil. Only nitrogen, phosphorus and potassium were left in the soil. Likewise, phosphorus does not dissolve in water and it will cause hardening of soil while nitrogen would develop alkalinity in the soil, thus decreasing its fertility and making it barren.

Soil pH is also unpleasantly affected by the acidity of chemical fertilizer, thus affecting the microorganisms' ecosystem in the soil. Prolonged use of chemical fertilizer upsets the pH of soil, which drives away the beneficial microorganisms present in soil. These microorganisms are beneficial to the plant by providing natural immunity to disease. When overabundance of nitrogen is found in the soil, plants are susceptible to mosaic infections. Repetitive use of chemical fertilizer cause toxic chemicals such as cadmium, arsenic and uranium to build up in the soil. The chemical will ultimately crawl their way into the fruits and vegetables growing on that particular soil, which could cause health issue to humans when consumed.

Highly soluble chemical fertilizer dissolves into the soil quite rapidly. Most of the fertilizer simply leaches away since plants can only absorb a certain amount of nutrition at a time. Not only is this leaching detrimental to the ground water, these chemicals also seep into the subsoil. There they interact with clay, forming impermeable layers called hardpan. Moreover, chemical fertilizer also causes fertilizer burnt. When nitrogen in the fertilizer was absorbed by the soil too rapidly, it causes osmotic imbalance which dehydrate and dry up the plant.

1.2.4 Biofertilizer as the substitute for chemical fertilizer

Biofertilizer is microbiological fertilizer that contains selected, highly efficient bacterial and fungal strains isolated from soil. Soil microorganisms act a vital role in varying the dynamic of organic substance breakdown and the availability of plant nutrients. Proper microbiological processes are activated with the involvements from these microorganisms, which allow enhanced and more constant supply of nitrogen, potassium and phosphorus, as well as other trace elements to the plants, which increase crop productivity (20). Biofertilizer vary from chemical fertilizer by does not supplying the crops with any nutrients, but instead are cultures of bacteria and fungi that create a healthy rizhosphere with the plant.

One of the most significant mechanisms in the natural nitrogen cycle with importance to agriculture is the binding of atmospheric nitrogen through biological nitrogen fixation[185]. Study has shown that *azotobacter* sp. could fix 50 to 80kg of nitrogen per hectare per year denpending on the strain and environment conditions (21). Aside from fixing nitrogen, *azotobacter* spp. could also produce plant growth promoting substances, such as auxin, gibberellin, pyridoxine, biotin and nicotinic acid (185). Other than nitrogen, plants also need phosphorus to grow, which is usually present in unavailable forms in the soil. Bacteria genera of *Bacillus* and *Azotobacter* have the ability to synthesized organic acid and phosphate, and convert the unavailable form of phosphorus into available form for plants. Researches have shown that *Bacillus* genus give rise to crop and growth of various plants (22)(23).

1.2.5 Plant Growth Promoting Rhizobacteria and Its Mechanisms

Plant growth promoting rhizobacteria (PGPR) are free living bacteria that can be found among microflora in the rhizosphere of plant. The term “plant growth promoting bacteria” refers to endophytes that are competent to colonize the roots of the plant (rhizosphere) which enhance plant growth (24)(25). Rhizosphere refers to the soil environment that a region of concentrated microbial activity resulting in a restricted nutrient pool at where the plants root is which vital micro- and macronutrients are extracted. The root exudates function as a source of nutrients for microbial growth which makes the microbial population in the rhizosphere relatively different from its surrounding (186). Compared to the bulk soil, it was proven that the thin rhizosphere zone is abundant in nutrients for microbes; in general, bacteria present in the rhizosphere are 10 times to 100 times higher in quantity compared to bulk soil (26).

Acticomycetes, fungi, bacteria, algae and protozoa are microbes colonizing the rhizosphere. Nevertheless, the most plentiful microbial existing in the rhizosphere is bacteria (187). Application of these microbial populations was proven and well known to enhance plant growth (27)(28). PGPR also have optimistic effects on regulating phytopathogenic microorganisms other than wielding beneficial effects on plant development (29)(30). Thus, PGPR is selected for biofertilizer formulation. There are two type of collaborations of PGPR with the plants; free living rhizobacteria that live outside the plant cells, and symbiotic bacteria that live inside the plants and exchange metabolites with the plant directly (31).

PGPR enhance plant growth over an inclusive variation of mechanisms. The mode of action of PGPR that promotes plant growth includes (i) plant growth regulators; (ii) abiotic stress tolerance in plants; (iii) production of siderophores; (iv) nutrient fixation for easier plant uptake; (v) production of protective enzyme such as glucanase, and ACC-deaminase for prevention of plant diseases; and (vi) production of volatile organic compounds (32)(33). Though, the types of host plants determine the mode of action for different PGPR (33).

1.2.5.1 Plant Growth Regulator

Productions of different phytohormones like indole-3-acetic acid (IAA), gibberellic acid and cytokinins were reported from PGPR that could modify root architecture and encourage plant development (33). IAA and gibberellic acid production in the rhizhopsheric soil by several PGPRs play a vital role in improving root surface area and number of root tips in many plants (33). Research on auxin synthesizing rhizobacteria as phytohormone creator illustrated that rhizobacteria has the ability to produce IAA from tryptophan through different pathways. Indole-acetamide pathway was used by the phytopathogenic bacteria to synthesize IAA that has been associated earlier in the tumor induction in plant (34). Studies have shown that *Bacillus* spp., *Azotobacter* spp. and A*zospirillum* spp. could produce high amount of IAA resulting in increase in root and shoot growth (35)(188)(189). Root biomass, root hair number and root surface area were consequently increased in the IAA-mediated ethylene production of PGPR inoculated tomato plants (36). Cell division, root initiation, cell enlargement and increase in root surface area of crop plant through enriched formation of lateral adventitious root were witnessed with the participation of PGPR formulated cytokinins(37).

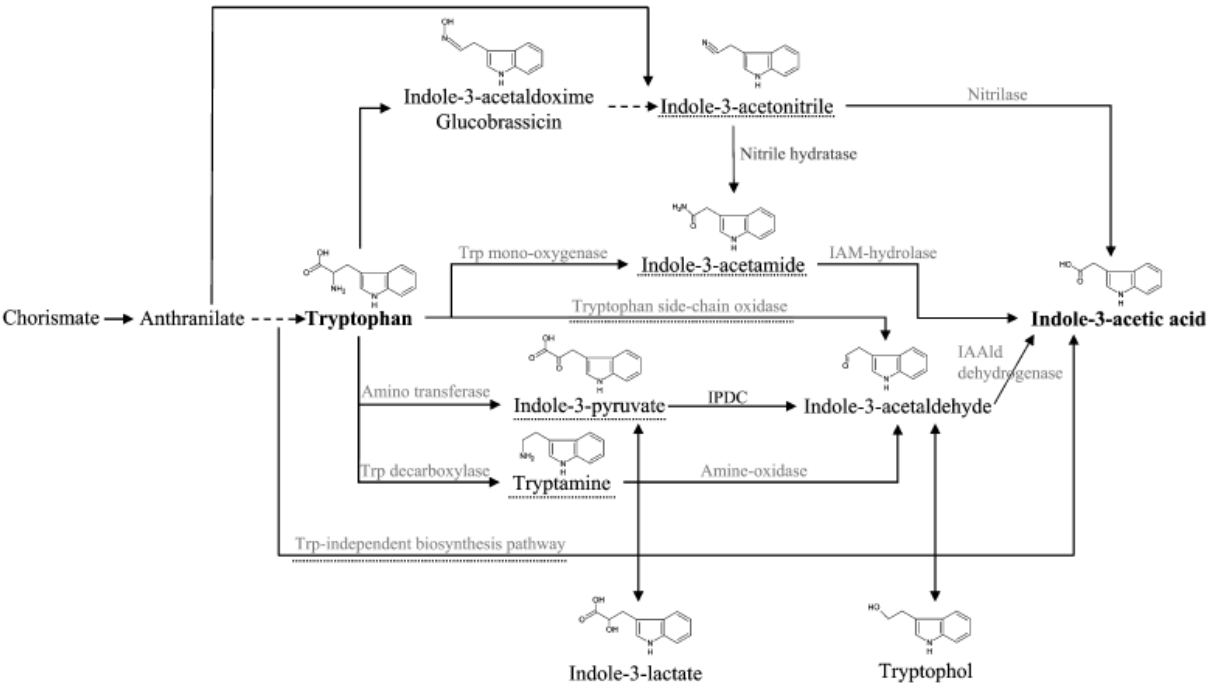


Figure 3: Overview of the different pathways to synthesize IAA in bacteria (33).

1.2.5.2 Abiotic stress tolerance in plant

The key cause of agricultural crop reduction is considered to be due to abiotic stresses. Though, the type of soils (nutritional imbalance) and plant factors (susceptible to diseases due to physiological disorders, abscission, etc) affects the intensity of abiotic stress (38). Studies have been done on the PGPR mechanisms in plant towards abiotic stress. PGPR have been observed to decrease the availability of cadmium in the soil (190), increase antioxidant capacity as well as increase growth (39), improvement of leaf water status under salinity and drought stress by regulating the stomata aperture to balance water content in the leaf and water uptake by the roots(40)(41), and increased in the root ability to absorb water under salinity conditions(42)(43)(44).

1.2.5.3 Production of siderophores

Even though iron is inaccessible in the soil for plants, yet iron is among one of the major minerals that exist on earth. Iron is normally exists in nature in the form of Fe^{3+}, which is highly insoluble. Siderophores secreted by PGPR are low molecular weight iron binding protein compounds that tangled in chelating ferric iron, Fe (III), from the environment. Many siderophores are non-ribosomal peptides (45), while several are biosynthesized independently (46). A siderophores-producing *Phyllobacterium* strain was shown to promote the growth and quality of strawberries (191). The predicted flow of mode of action is shown below

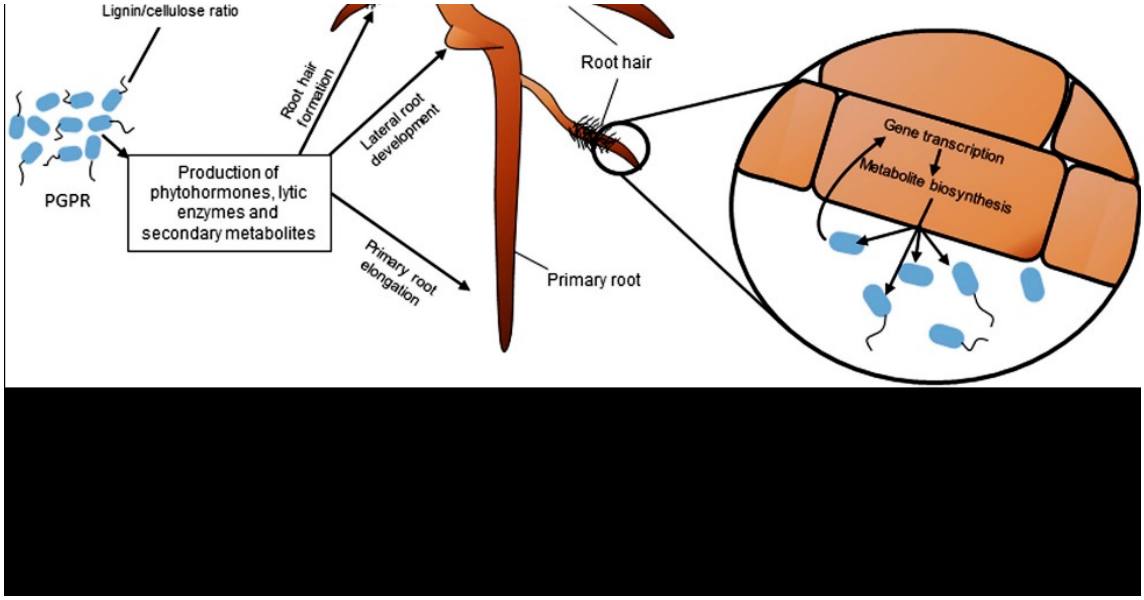


Figure 4: The possible mode of action used by plant growth promoting rhizobacteria (PGPR) towards growth promotion in plants (47).

1.2.5.4 Nutrient fixation for easier plant uptake

Fixing nutrients to raise the accessibility of nutrient concentration in the rhizosphere is one of the functions of PGPR (32). One of the key nutrients for plant growth is nitrogen (N). N is rich in earth atmosphere, however most the tropical soils are lacking in available N. Plants obtain nutrients from biological fixation (192) or through atmospheric inputs (48) when the ecosystems are low in inputs and without any fertilization or soil amendments. One way of converting elemental nitrogen into plant usable form is through biological nitrogen fixation (BNF). Nitrogenase enzyme which is released by BNF is accountable for fixing atmospheric dinitrogen into soil, hence refining soil fertility. Consequently, it is considered as an economically and environmentally friendly source to substitute for the use of chemical fertilizer N (49). An estimation of about 180 x 106 metric tons of nitrogen per year globally is contributed by BNF and about 80% of them came from symbiotic associations with the plants. Non symbiotic nitrogen fixers are also significant in supplying to the agronomic.

Another vital nutrient required for plants is phosphorus (P). Ironically, large reservoirs of total phosphorus are present in soil; however, the amount available to plants are so tiny in quantities. The majority of soil P is found in insoluble form, while plants are only capable of uptake P in two soluble forms, the monobasic ($H_2PO_4^-$) and the diabasic (HPO_4^{2-}) ions, which is why the low availability of P to plants (50). Several phosphate solubilizing microorganisms are capable of solubilizing the insoluble form of phosphorus into soluble form through the secretion of acid or proton (51) and chelatin- and exchange reactions (193) to solubilize P in soil. Phosphate solubilisation can also be induced by phosphate starvation (52), and by the release of plant root exudates such as organic ligands which alter concentration of P in soil (53). A common sketch of phosphorous solubilisation is shown below.

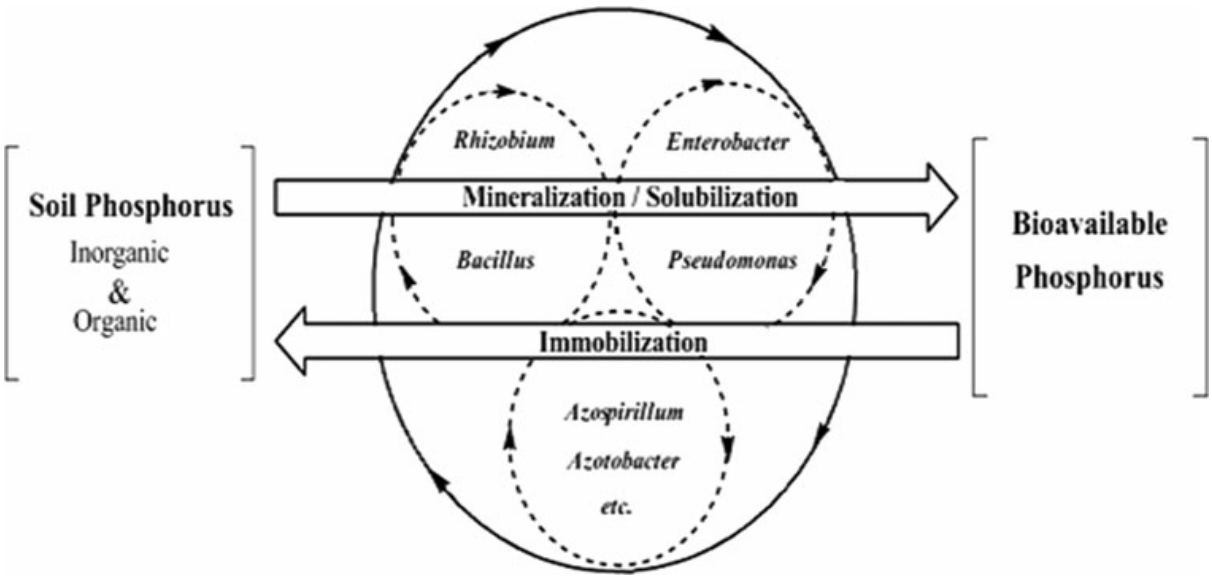


Figure 5: Schematic representation of solubilisation of soil phosphorus by rhizobacteria (54).

1.2.5.5 Production of Enzymes

Production of metabolites contributing to the antifungal and antibiosis properties by controlling the phytopathogenic agents through PGPR is used as defence system for plant growth. Glucanase and chitinase are examples of two hydrolytic enzymes produced by the mechanisms. Beta-glucanase and chitinase producing bacteria limit fungal growth because major fungal cell wall mechanisms are made up of beta-glucan and chitin. Beta-glucanase and chitinase produced by *Sinorhizobium fredii KCC5* and *Pseudomonas fluorescens* dictate the *fusarium* wilt produced by *Fusariumudum* (55). *Pseudomonas* spp. also has the ability to inhibit *Rhizoctonia solani* and *Phytophthoracapsici*, two of the most damaging crop pathogens in the world (56).

1.2.6 Borneo, Sarawak, Malaysia

The third largest island in the world and one of the largest in Asia, Borneo is located in South East Asia covering a total area of approximately 745,567 square kilometres. Borneo is famous for its oldest and mega biodiversity in the world. Large portion of diverse forest habitats exist in Borneo which include peat swamps, lowland dipterocarp forests, mangrove areas, ironwood forests, freshwater swamp and hill dipterocarp forests. Borneo also has the largest open area of uncultivated land forest (kerangas) in Southeasst Asia. Furthermore, Borneo has assorted rainforest which consist of estimated of more than 2000 species of orchids, 15,000 flowering plants species and at least 50 species of the carnivorous pitcher plant. Two of the largest flowers in the world, *Rafflesia* and *Amorphophallus* can be found in Borneo (194).

Ideal climate and enormous habitats areas that supports the growth of different plant species are the reason why Borneo is rich in biodiversity. Humid tropical climate with sunshine could be spotted all year long in Borneo. An evenly distributed annual rainfall of 3861mm with temperature ranges around 22ºC to 33ºC are ideal for the development and growth of flora and fauna found in Borneo. Three nations are currently occupying the land of Borneo, which are Brunei, Malaysia (Sarawak and Sabah) and Indonesia. Around 10 million hectares, 80% of the total land Borneo are still covered with forest (natural and also secondary forest), which makes Sarawak, which is a part of Borneo, an ideal ecosystem perfecting for new discovery research (195).

Sarawak, which consists of 37.5% of Malaysia total land area with 124,449.51 square kilometres, is the largest state in Malaysia that is located on the Equator and stretches out to around 800 kilometres along the northwest coast of Borneo island. Three different regions of land are found in Sarawak; a huge region of undulating hills (vary to about 300 meters), coastal lowlands conceding peat swamp as well as narrow deltaic and alluvial plains, and also mountain highlands which stretch out to Kalimantan border. The largest peatland area in Malaysia is also found in Sarawak with about 1.5 million hectares. Besides, Sarawak also has an extensive protected area which compromise of 10 natural reserves, 30 national parks and 4 wildlife sanctuaries with a total protected area of 837,553.80 hectares (602,035.8 hectares for land area and 229,789 hectares for water body). Sarawak has one of the most studied tropical karst area and the largest known cave chamber in the world, which is located in the south of Sarawak – Gunung Mulu National Park (The Sarawak Chamber, 600 metres by 415 metres and 80 metres high). Moreover, Gunung Mulu National Park which composed of high biodiversity and karst features is named as one of the UNESCO (United Nations Educational, Scientific and Cultural Organization) World Heritage site. The epitome bionetwork combine with the high diversity of plant species in Sarawak makes it an ideal site for the discovery of novel or potential species to be exploited in industry and agricultural regions to enhance the economics of the country (194).

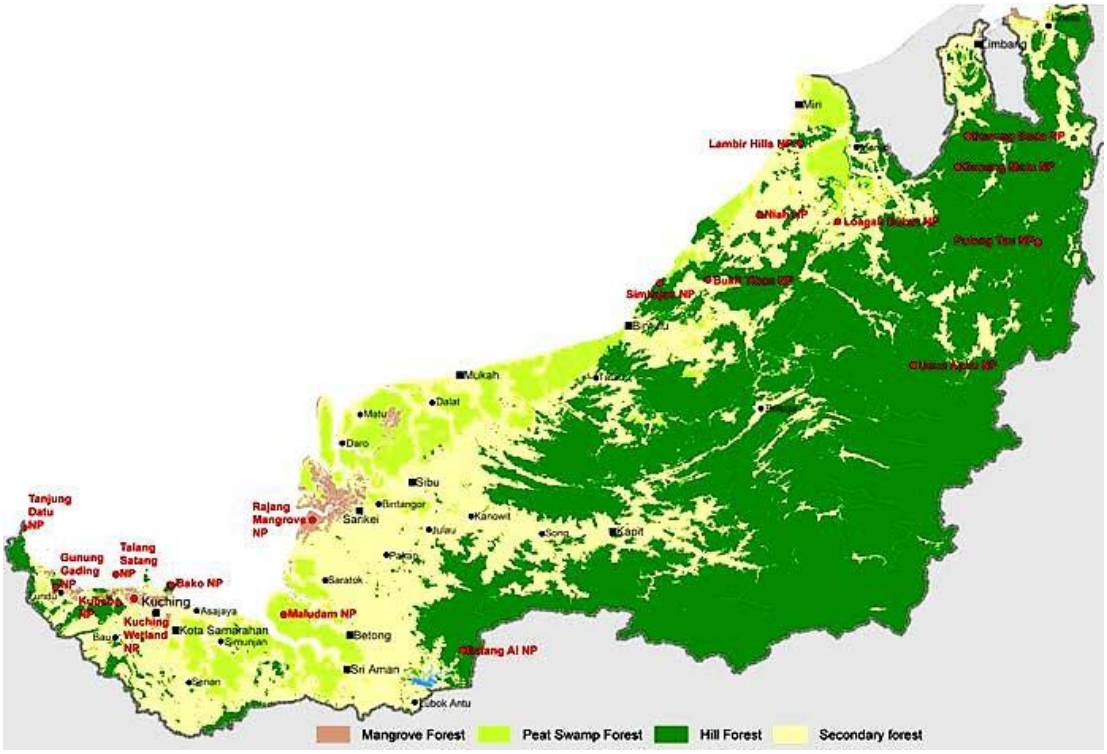


Figure 6: A brief statement of existing totally protected areas (TPAs) in Sarawak - Updated at 31 December 2014 (196).

1.3 Research Aim and Objectives

The low nutrient availability in degraded soil in *Eucalyptus pellita* plantation has led to the use of chemical fertilizer to improve tree growth. However, prolonged usage of chemical fertilizer causes negative impacts to the environment. The aim of this thesis was to introduce biofertilizer which contains plant growth promoting microorganisms that are beneficial to the plant and environment to be the substitution of chemical fertilizer. Several objectives were set to achieve the aim of this study; the objectives set were listed as follow:

- To isolate and identification of plant growth promoting rhizospheric microorganisms from the soil samples collected in Sarawak.
- To evaluate the efficiency of isolated strains in promoting plant growth through the mechanisms of nitrogen fixing, IAA producing, phosphate and potassium solubilizing.
- To evaluate the efficiency of the consortia isolated strains as plant growth promoting cultures on the silviculture of *Eucalyptus pellita* in pot trial condition.

1.4 Thesis Outline

This thesis study is reported in five chapters and is listed as follow:

Chapter 1 – Introduction and literature review

Chapter 1 provides the brief introduction on the background of the study. The literature review of the information related to the project which has been described in previous study by the other researchers. The aim and objectives of the research were described in this chapter.

Chapter 2 – Screening, isolation and characterization of plant growth promoting microorganisms isolated in Sarawak

Chapter 2 describes the detailed result of the study on the isolation, screening and characterization of the plant growth promoting microorganisms with the uses of selective media method. This chapter focuses on the isolation of nitrogen fixing, IAA producing, phosphate and potassium solubilizing microorganisms from soil ecosystem in Sarawak.

Chapter 3 – Evaluating the efficiency of microorganisms strains as potential plant growth enhancing inoculate

Chapter 3 presents the detailed results on the efficiency of the plant growth promoting microorganisms by using quantitative bioassay. Different assays were carried out to analyse the efficiency of the isolated strains. The focus on this chapter was to evaluate the effectiveness of the isolated strains as potential candidates for biofertilizer composition.

Chapter 4 –Pot trial evaluation of plant growth promoting rhizobacteria on silviculture of *Eucalyptus pellita*

Chapter 4 describes the efficiency of the selected plant growth promoting strains in pot trial experiment on *Eucalyptus pellita* saplings. This chapter focuses on the growth of *Eucalyptus pellita* saplings over 6 month's timeframe under different treatments.

Chapter 5 – General conclusions and recommendations

Chapter 5 presents the summary of the findings of this research study. The future work and recommendations of this study are described in this chapter as well.

CHAPTER 2:

SCREENING, ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING MICROORGANISMS ISOLATED IN SARAWAK

2.1 Introduction

The quality of soil environment, production of plant root exudates and growth of plants are associated to a great variety of abiotic and biotic factors that shape the soil and plant associated habitats which alter the structures and activities of these microbial societies (57). Abundance of microorganisms exists in the soil, particularly in the rizhosphere of plants. Among these budding soil microorganisms, there exist bacteria known as plant growth promoting rhizobacteria (PGPR) that have the potential use in enhancing plant health and promote plant growth rate without environmental contamination (58). These PGPR stimulate appropriate microbiological processes that facilitate enhanced and more constant supply of nitrogen, phosphorus and potassium, as well as some trace elements to the plants. The binding of atmospheric nitrogen in the process of biological nitrogen fixation - the binding of atmospheric nitrogen process is the most significant component in the nitrogen cycle in nature with distinct implication for agriculture. Phosphate and potassium solubilizing bacteria have the ability to improve mineral uptake of plants through solubilizing insoluble P and releasing K from silicate in soil (59). Studies have shown that PGPR has the potential to improve plant yield and growth (60)(61)(62). This has led to the development of this thesis to further explore the effects of plant growth promoting rhizobacteria and fungi.

Soil degradation plays a role in declining crops yield. Soil physical deterioration contributed to compaction of subsoil and surface layers, structural collapse, decline in size and percentage of water-stable aggregates, which accelerate soil erosion. Soil chemical deterioration caused leaching of bases, nutrient imbalance and also salinization in the root of plants. Soil fauna could minimize soil degradation by mediating the deterioration of soil quality. The physical and chemical properties of soil could be improved by colonization of these beneficial microorganisms (63).

This chapter focuses on the screening and isolation of plant growth promoting rhizobacteria that were associated to *Eucalyptus pellita* planted in Sarawak tropical soils. The PGPR that was focused on were nitrogen fixing, IAA producing, phosphate and potassium solubilizing. The PGPR was screened by using selective medium through nitrogen fixation, IAA production, and phosphate and potassium solubilisation. At the end of this chapter, the soil texture of soil samples was also discussed.

2.2 Materials and Methodology

2.2.1 Soil samples collection

Soil samples were collected from *Eucalyptus pellita* plantations and a protected forest in Sarawak in the period between year 2015 and 2016. Collected soil samples were kept in the cold room at 4ºC to preserve the nature of the samples prior to experimentation. The sampling locations are listed as of below;

1. LPF0014 Segan, Bintulu.
2. LPF0042 Sempadi, Lundu
3. Sabal Reserved Forest, Sri Aman

2.2.2 Media preparation

2.2.2 (a) Nutrient Agar (NA) preparation

Nutrient agar (NA) powder weighted 28g were added into 1L Schott bottle. Deionized water was added into the same Schott bottle to fill up the 1L mark. The bottle was then sterilized by autoclaving at 121ºC (100 kPa) for 15 minutes. After autoclaving, the media was left to cool down until about 60ºC. Then, the media was poured into 90mm petri dishes.

2.2.2 (b) Potato Dextrose Agar (PDA) preparation

Potatoes Dextrose Agar (PDA) powder weighted 39g were added into 1L Schott bottle. Deionized water was added into the same Schott bottle to fill up the 1L mark. The bottle was then sterilized by autoclaving at 121ºC (100 kPa) for 15 minutes. After autoclaving, the media was left to cool down until about 60ºC. Then, the media was poured into 90mm petri dishes.

2.2.2 (c) Aleksandrow's Medium Agar preparation

Aleksandrow's Medium Agar powder weighted 29.60g were added into 1L Schott bottle. Deionized water was added into the same Schott bottle to fill up the 1L mark. The bottle was then sterilized by autoclaving at 121ºC (100 kPa) for 15 minutes. After autoclaving, the media was left to cool down until about 60ºC. Then, the media was poured into 90mm petri dishes.

2.2.2 (d) Pikovskayas Agar preparation

Pikovskayas Agar powder weighted 31.30g were added into 1L Schott bottle. Deionized water was added into the same Schott bottle to fill up the 1L mark. The bottle was then sterilized by autoclaving at 121ºC (100 kPa) for 15 minutes. After autoclaving, the media was left to cool down until about 60ºC. Then, the media was poured into 90mm petri dishes.

2.2.2 (e) Jensen's Medium Agar preparation

Jensen's Medium Agar powder weighted 39.10g were added into 1L Schott bottle. Deionized water was added into the same Schott bottle to fill up the 1L mark. The bottle was then sterilized by autoclaving at 121ºC (100 kPa) for 15 minutes. After autoclaving, the media was left to cool down until about 60ºC. Then, the media was poured into 90mm petri dishes.

2.2.2 (f) Nutrient Broth Medium preparation

Nutrient Broth (NB) powder weighted 25g were added into 1L Schott bottle. Deionized water was added into the same Schott bottle to fill up the 1L mark. The bottle was then sterilized by autoclaving at 121ºC (100 kPa) for 15 minutes. After autoclaving, the media was left to cool down until about 60ºC. Then, the media was poured into 90mm petri dishes.

2.2.2 (g) Potato Dextrose Broth Medium preparation

Potatoes Dextrose Broth (PDB) powder weighted 24g were added into 1L Schott bottle. Deionized water was added into the same Schott bottle to fill up the 1L mark. The bottle was then sterilized by autoclaving at 121ºC (100 kPa) for 15 minutes. After autoclaving, the media was left to cool down until about 60ºC. Then, the media was poured into 90mm petri dishes.

2.2.2 (h) Bromothymol Blue solution

Bromothymol Blue powder weighted 0.05g was added into a 100mL Schott bottle together with 50ml of 95% ethanol. Fifty mL of deionized water was then added into the same Schott bottle to make up a 100mL solution.

2.2.2 (i) Salkowski's Reagent solution

Five mL of 0.5M $FeCl_3$ was added into a 500mL Schott bottle, together with 150mL of concentrated (96%) H_2SO_4. Finally, 250mL of deionized water was added into the same bottle.

2.2.3 Preliminary screening of potential plant growth inducing bacteria and fungi from soil samples

Aleksandrow's Medium Agar, Pikovskayas Agar and Jensen's Medium Agar, Bromothymol Blue solution and Salkowski's Reagent were used as agar plate assay and colorimetric technique to screen for potential plant growth inducing (nitrogen fixing, plant hormone indole-3-acetic Acid producing, phosphate and potassium solubilising) bacteria and fungi.

2.2.3.1 Serial dilution

In order to distinguish one colony from the other, serial dilution technique was carried out prior to the initial screening experiment. Direct screening of the soil samples would result in complex presence of microorganisms that are difficult to be identified.

A total of 6, 15mL falcon tubes were used to make a 10^{-6} dilution. Deionized water was filled into each of the falcon tubes until the 9mL mark. Approximately 1g of soil sample was then added into the first falcon tube, thus making it the dilution of 10^{-1}. Then the tube was mixed by using a vortex machine to even out the concentration of the sample in the solution. One L micropipettes was then used to transfer approximately 1mL of the mixture into the second falcon tube. This step was repeated onto the remaining 4 falcon tubes in order to achieve a dilution of 10^{-6}.

2.2.3.2 Preliminary screening of potential microorganisms by using selective media and colorimetric technique

Aleksandrow's Medium Agar, Pikovskayas Agar and Jensen's Medium Agar were used to conduct agar plate assay on the diluted solutions. Approximately 100µL of the diluted solution from 10^{-3} to 10^{-6} were transferred to these agar plates. On the other hand, Salkowski's Reagent was used to conduct colorimetric assay technique on the selected microorganisms.

2.2.3.2 (a) Nitrogen Fixing screening (64)

Jensen's Medium agar was used to screen for nitrogen fixing microorganisms. A hundred µL of the suspended soil samples from the selected dilutions were transferred onto each Jensen's medium agar. The suspended solution was spread evenly by using a sterile cotton swap. All the spread plates were then incubated at 30ºC ± 2ºC up to 4 days. After 4 days, 2mL of Bromothymol Blue solution were added onto the agar plates containing the microorganisms' colonies and incubated for 1 hour. The colonies with the surrounding in blue colour are noted as nitrogen fixer. These colonies were then isolated and kept in 4ºC cold room for further testing.

2.2.3.2 (b) Phosphate Solubilizing screening (65)

Pikovskayas agar was used to screen for phosphate solubilizing microorganisms. A hundred µL of the suspended soil samples from the selected dilutions were transferred onto each Pikovskayas agar. The suspended solution was spread evenly by using a sterile cotton swap. All the spread plates were then incubated at 30ºC ± 2ºC up to 3 days. After the incubation period, any colony with a clear zone formation surrounding the colony is considered as phosphate solubilizing microorganisms. These colonies were then isolated and kept in 4ºC cold room for further testing.

2.2.3.2 (c) Potassium Solubilizing screening (66)

Aleksandrow's Medium agar was used to screen for potassium solubilizing microorganisms. A hundred µL of the suspended soil samples from the selected dilutions were transferred onto each Aleksandrow's Medium agar. The suspended solution was spread evenly by using a sterile cotton swap. All the spread plates were then incubated at 30ºC ± 2ºC up to 3 days. After the incubation period, any colony with a clear zone formation surrounding the colony is considered as phosphate solubilizing microorganisms. These colonies were then isolated and kept in 4ºC cold room for further testing.

2.2.3.2 (d) Indole-3-acetic Acid (IAA) producing screening (67)

A hundred µL of the suspended soil samples from the selected dilutions were transferred onto each Nutrient Agar (NA) for bacteria, and onto Potato Dextrose Agar (PDA) for fungus. The spread plates were incubated at 30ºC ± 2ºC for 3 days. After that, isolation and purification were carried out for each colony that grew on the plates, in order to get a pure colony that does not interact with the other. These colonies were then transferred into Erlenmeyer flask with their respective liquid media and incubated at 30ºC ± 2ºC in incubator shaker, with constant shaking at 150rpm; Nutrient Broth (NB) for bacteria while Potato Dextrose Broth (PDB) for fungus.

After 3 days of incubation, the liquid cultures were then centrifuged at 13,400rpm by using microcentrifuge machine for 10 minutes. One mL of the supernatant was then added into 2mL of Salkowski's reagent in a test tube. The mixture was incubated in the dark at room temperature for 30 minutes. Pinkish or reddish colour observed on the mixture after 30 minutes incubation was the proof of IAA production.

2.2.4 Isolation of pure culture

Microorganisms with the potential to fix nitrogen, produce IAA, solubilize phosphate and potassium were identified from each of their respective screening. These microorganisms were then inoculated by using an inoculating loop onto Nutrient Agar (for bacteria) and Potato Dextrose Agar (for fungi) for pure culture isolation. These plates were incubated at 30ºC ± 2ºC for 3 days. Purity of the microorganisms was observed based on their morphology. Each streaking process eliminated the contaminated colonies. Repeated isolation was carried out until pure and uniformed culture was obtained. Proper label was recorded for every culture.

2.2.5 Storage and preservation

Pure cultures were isolated and preserved for further studies. Three methods were used for preserving the pure cultures. First off for the long term storage, glycerol stock culture technique was used. Cultures were inoculated in Nutrient Broth (NB) for bacteria and Potato Dextrose Broth (PDB) for fungi and incubated in an incubator shaker, at 150rpm and 30ºC overnight. Then, 400µL of the culture was transferred into a centrifuge tube containing 400µL of 50% glycerol. Thus end up with a final volume of 25% glycerol mixture. The mixture was then mixed well, sealed and kept in a -80ºC Freezer for future study. Second long term storage method was slant agar culture. Pure bacteria and fungus cultures were inoculated onto fresh slant NA and PDA, and incubated at 30ºC overnight. The cultures were then sealed and kept in cold room at 4ºC. While for short term storage, pure cultures were inoculated onto NA and PDA plates, incubated overnight and then stored in a refrigerator at 4ºC.

2.2.6 Molecular identification of microorganisms

2.2.6 (a) Crude DNA extraction

(a) DNA extraction by the using Phenol-Chloroform (68)

Phenol chloroform method was used to extract DNA from bacteria and fungus cultures. Below are the steps involved:

Pure bacteria and fungus cultures were cultured in Nutrient Broth (NB) and Potato Dextrose Broth (PDB) and incubated overnight at 30ºC, 150rpm. One mL of the overnight culture was then spun down at 13,400rpm for 5 minutes. This step was repeated for 2 more times in order to obtain enough culture pellets. TE buffer of 500µL were added to the culture pellet. Fifty µL of 10% SDS solution was added to re-suspend the culture pellet and incubated at 85ºC for an hour with inversion at 15 minutes interval. Fifty µL of proteinase-K were added to cool down the culture pellet and then incubated at 55ºC for 30 minutes. The, 600µL of phenol-chloroform-isoamyl alcohol with the ratio of 25:24:1 were added to the pellet suspension. The pellet suspension was centrifuged at

13,400rpm for 10 minutes. Aqueous layer of the suspension was transferred to a new 1.5mL centrifuge tube and chloroform-isoamyl alcohol with the ratio of 24:1 was added into it. The suspension was centrifuged at 13,400rpm again for 10 minutes. The latest aqueous layer was then transferred into a new 1.5mL centrifuge tube with 600µL of cold isopropanol with 30µL of 3M sodium acetate buffer. The suspension was then incubated at -20ºC for 2 hours. After 2 hours, the suspension was spun down at 13,400rpm for 10 minutes to obtain culture pellet. Isopropanol was then drained out and 600µL of cold 70% ethanol was added to wash the DNA pellet. The 70% ethanol was then drained and the pellet was left to air dried for 15 minutes. Fifty µL of cold sterile ultrapure water was then added to dissolve the DNA pellet. DNA solution was then stored at 4ºC overnight. The whole process was repeated for all fungi and bacteria culture.

(b) DNA extraction by the using Freeze and Thaw method (69)

Bacteria and fungus cultures were cultured as describe in method above. One mL of the broth culture was transferred to a centrifuge tube. The microbe culture was spun down at 13,400rpm twice for 3 minutes to obtain culture pellet. The supernatant was then drained off and 1mL of ultrapure water was added into the tube and vortex gently to suspend the pellet completely. The suspension was subjected to freeze and thaw cycles as follow;

1st cycle:

8 minutes	Freeze at -80ºC in -80ºC freezer
3 minutes	Thaw at 85ºC in water bath

2nd to 4th cycles

5 minutes Freeze at -80ºC in -80ºC freezer

3 minutes Thaw at 85ºC in water bath

After going through the cycles, the suspension was then spun down at 13,400rpm for 5 minutes. The supernatant was then transferred into a new sterilized microcentrifuge tube and stored at -20ºC. The cell pellet was discarded.

2.2.6 (b) DNA amplification by Polymerase Chain Reaction (PCR)

The extracted fungus and bacteria DNAs were then undergo amplification by using polymerase chain reaction (PCR) to increase DNA counts. Universal primer sets, 8F and 1541R were used for bacteria while ITS4 and ITS5 were used for fungus, targeting 16S rRNA sequences. The primer sequence and PCR master mix condition were listed as below;

Primer sequence used:

8F 5'-AGAGTTTGATCCTGGCTCAG- 3'

1541R 5' – AAGGAGGTGATCCAGCCGCA- 3'

ITS4 5'-TCCTCCGCTTATTGATATGC-3'

ITS5 5'-GGAAGTAAAAGTCGTAACAAGG-3'

Table 4: Mastermix compositions for bacteria.

Content	Amount per reaction	Amount per 25 reactions
Sterilized Ultrapure water	18µL	450µL
Bioline, MyTaq Red Mix	25µL	625µL
Forward primer, 20 µM (8F)	1µL	25µL
Reverse primer, 20 µM (519R)	1µL	25µL
Total	45µL	1125µL

Table 5: Mastermix compositions for fungi.

Content	Amount per reaction	Amount per 25 reactions
Sterilized Ultrapure water	21µL	525µL
Bioline, MyTaq Red Mix	25µL	625µL
Forward primer, 20 µM (ITS5)	1µL	25µL
Reverse primer, 20 µM (ITS4)	1µL	25µL
Total	48µL	1200µL

The master mixes were prepared in PCR tubes. Five µL of crude bacteria DNA extract were added into 45µL of bacteria mastermix while 2 µL of crude fungus DNA extract were added into 48µL of fungi mastermix. DNA amplification was performed in a PCR machine with following conditions for 35 cycles:

PCR setting:

• Initial denaturation: 95ºC for 5 minute

• Condition for the remaining 29 cycles:

- ❖ Denaturation process: 95ºC for 30 seconds
- ❖ Annealing process: 55ºC for 30 seconds
- ❖ Extension process: 72ºC for 30 seconds
- ❖ Final elongation at 72ºC for 5 minutes

• The final PCR products were on hold at 4ºC.

The PCR products was analysed on a 1 % (w/v) agarose gel with 3 µL of Midori stain (Nippon Genetics Europe GmBH, Duren, Germany) added to it for visualization under UV light. Bio-Rad Gel Documentation EQ System for DNA/RNA Gel Photos was used to visualize and capture the gel image of the PCR products.

2.2.6 (c) Sequencing analysis

The end products of PCR were sent to First Base Laboratories Sdn Bhd, Selangor, Malaysia, for DNA sequencing. The sequence results obtained were analysed by matching to the known 16S rRNA gene sequences in the Genbank database (70) using BLAST (Basic Local Alignment Search Tool) of the National Centre for Biotechnology Information (71). Mega 6 was used to create phylogenetic tree by the used of maximum likelihood method (72).

2.2.7 Soil texture analysis (206)

Soil samples were air-dried prior to the soil texture analysis. Ten grams of air-dried soil sample were transferred into a 2L form beaker. Fifty mL of 20% v/v hydrogen peroxide were added into the beaker and the beaker was covered with a watch glass. The mixture was left overnight. The overnight beaker was boiled for around 2 hours. Hydrogen peroxide was added until no further reaction was available to avoid formation of effervescence. The mixture was boiled vigorously until all hydrogen peroxide was removed. Calgon was prepared beforehand by dissolving 50g of commercial mixture of sodium hexametaphosphate and sodium carbonate to 1L of solution. The Calgon was added into the boiled mixture and left overnight. Then, the overnight culture was transferred into a 1L reagent bottle and made up to 500mL with water. The mixture was then stirred for 10 minutes with high-speed stirrer. The dispersed solution was then transferred into a 1L measuring cylinder and made up to the 1L mark. The temperature of the solution was measured. The suspension was mixed thoroughly with a special plunger. All the particles were allowed to settle down before the timing of settling was taken. Pipette was lowered into the cylinder 10cm below the suspension surface at appropriate sampling time to withdraw 20ml of suspension for silt and clay. The content in the pipette was drained in a weighed crucible and dried in an oven at 105ºC. The weight was collected after the content was dried and cooled in a desiccator. The same steps were repeated for clay content from the depth of 6cm below suspension surface.

Table 6: Sampling times for silt-clay and clay (206).

Temperature ºC	Silt – Clay (Settling time for sand)	Clay (Settling time for sand/silt)
26	4 min. 03 s	4 h 03 min
27	3 min 57 s	3 h 58 min
28	3 min 52 s	3 h 52 min
29	3 min 47 s	3 h 47 min
30	3 min 42 s	3 h 43 min

After that, supernatant liquid from the cylinder was decanted off. Sediment was transferred and washed into a 600mL beaker with a 10cm mark from the base. Water was added to allow sedimentation of sand to occur for a period required at the observed temperature (refer to Table 6). Most of the liquid was then slowly decanted off. This process was repeated until supernated liquid was perfectly clear. The sand was transferred into a weighed crucible and dried in an oven overnight at 105ºC. The weight of sand was recorded the next day. Five mL of Calgon solution was transferred into a weighed crucible and dried in an oven at 105ºC and weighted. Calculation of contents was recorded as below;

Weight of clay:

50 x (weight of oven dry residue from 2nd sampling) – weight of 5mL Calgon.

Weight of silt and clay:

50 x (weight of oven dry residue from 1st sampling) – weight of 5mL Calgon.

Weight of silt:

Weight of silt and clay – weight of clay.

Percentage of sand, silt and clay:

% sand = 100 x (weight of sand) / (weight of sand + silt + clay)

% silt = 100 x (weight of silt) / (weight of sand + silt + clay)

% clay = 100 x (weight of clay) / (weight of sand + silt + clay)

2.3 Results and Discussion

2.3.1 Preliminary screening of potential microorganisms by using selective media and colorimetric technique

Soil samples were collected from few plantations with *Eucalyptus pellita* within Sarawak, Malaysia. The microorganisms that were living in the rhizopheric atmosphere associated to the tree are believed be to plant growth promoting rhizospheric bacteria (PGPR) that could promote plant growth. Four characteristics of plant growth promoting microorganisms were targeted in this study, which were, nitrogen fixing, indole-3-acetic acid (IAA) producing, phosphate and potassium solubilizing. These plant growth prmoting microorganisms not only made available important nutrients for plant growth, they would also produce IAA that could stimulate plant hormone to improve growth. Studies have shown that these PGPR have vast potential in promoting plant growth (73)(74)(34)(75)(76). Hence, the focus of this study was on the isolation of microorganisms with the four characteristics mentioned above.

Selective media and colorimetric technique were used for the primary screening of plant growth promoting microorganisms. Pikovskaya's medium agar and Aleksandrow's medium agar were used to screen for phosphate and potassium solubilizing microorganisms. The clear zone formation surrounding the microorganisms colonies were the indication of phosphate and potassium solubilisation. For nitrogen fixation screening, Jensen's medium agar was used together with the addition of bromothymol blue solution. Colonies that formed blue colour zone within their surrounding were indication of nitrogen fixation. On the other hand, colorimetric technique was used to screen for IAA production. Culture broth that turned pink or red colour was the indication of IAA production. All the microorganisms that showed indications mentioned above were considered as positive results.

Table 7: total number of microorganisms with specific functions isolated from soil samples.

	Numbers of isolates
Nitrogen fixing	18
Phosphate solubilizing	5
Potassium solubilizing	4
IAA producing	19

A total of 46 potential isolates were isolated from the soil samples (Table 7). Five of the isolates were phosphate solubilizer while 4 of them were potassium solubilizer. Eighteen of the isolates were nitrogen fixer while the remaining 19 isolates were IAA producer. Studies have shown that microorganisms with the above capabilities could be isolated.

A study showed that 23 nitrogen fixing bacteria strains were successfully isolated from two plant species *Calligonum polygonoides* and *Lasiurus sindicus* (77). Eleven strains of nitrogen fixing strains were isolated from wild rice species (78). A study conducted on the rhizosphere of banana plant showed that 12 strains isolated were phosphate solubilizing and IAA producing bacteria (79). An isolation work on soil samples collected from different region of India showed that 14 isolates were potassium solubilizing (74).

These microorganisms were then isolated onto their respective agar medium for purification and identification purposes. Figure 9 showed the screening of microorganisms of interest.

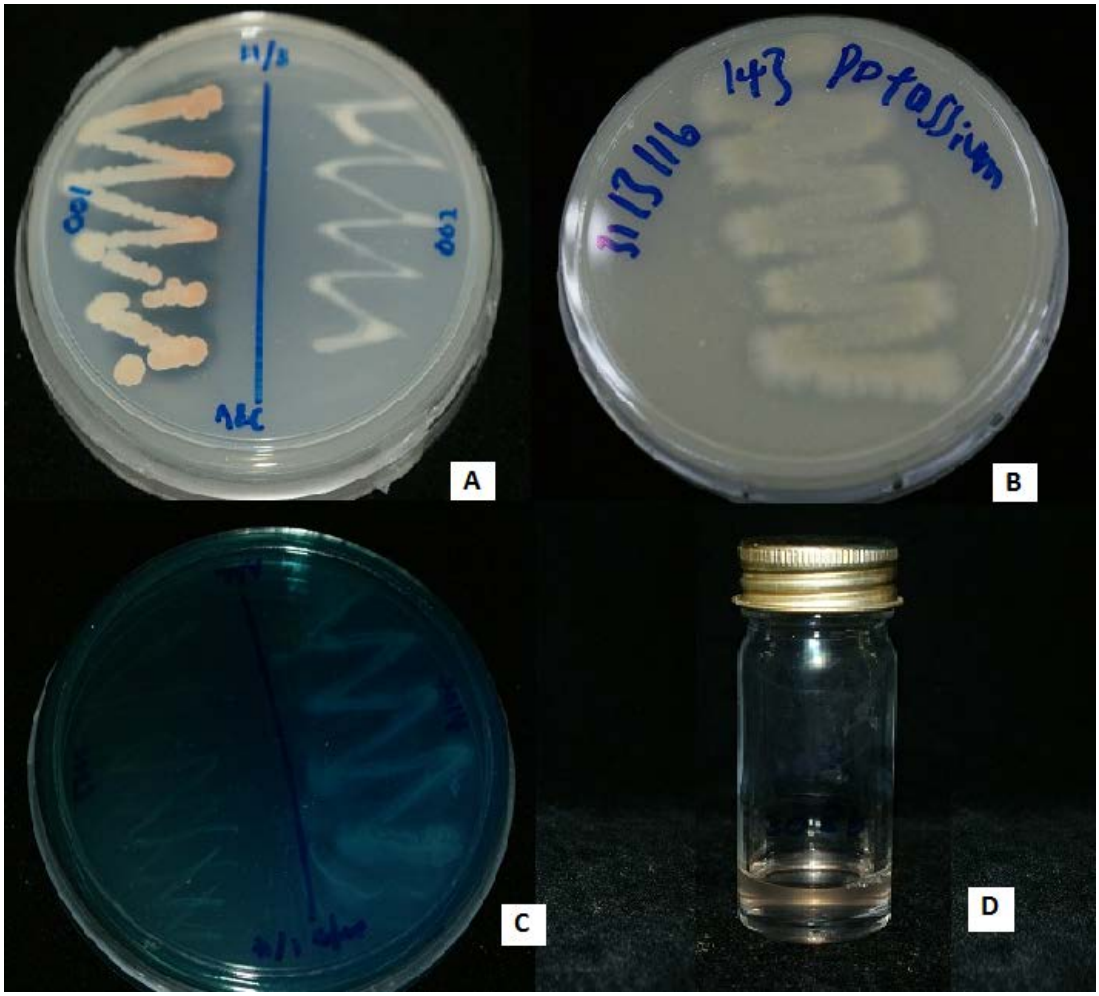


Figure 7: Screening for microorganisms with plant-growth-promoting characteristics. . The formation of clear zone by potential isolates on selective media Pikovskaya's medium (A) and Aleksandrow's medium (B) agar plates indicated phosphate and potassium solibilization. The blue colour zone formed around the colour on Jensen's medium (C) indicated nitrogen fixation while pinkish coloured solution formed with Salkowski's reagent (D) indicated IAA production.

2.3.2 Identification of the isolates

All 46 isolates were identified by using molecular identification method through DNA sequencing of the 16S rRNA sequence. DNA extraction method by Phenol-Chloroform and Freeze & Thaw were used to extract the isolates' crude DNA. Four universal primer sets were used to target the 16S rRNA sequence. Two sets; 8F (5'-AGAGTTTGATCCTGGCTCAG- 3') and 1541R (5' – AAGGAGGTGATCCAGCCGCA- 3') were used for bacteria sequence, while the other two sets, ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') were used for fungus sequence. Figure 10 showed the PCR products obtained. The PCR products were then sent for Sanger sequencing and the sequence results obtained were analysed by matching to the known 16S rRNA gene sequences in the Genbank database (70) using BLAST (Basic Local Alignment Search Tool) of the National Centre for Biotechnology Information (71).

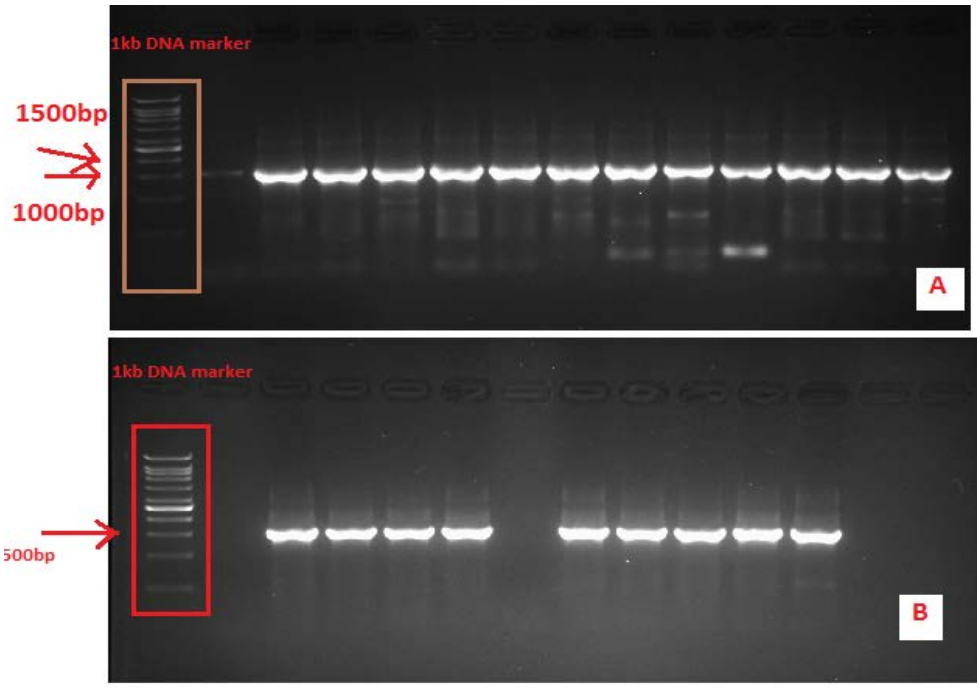


Figure 8: The visualization of PCR products for bacteria isolates (A) and fungal isolates (B) were observed on 1 % agarose gel by gel electrophoresis method. The gel image was viewed and captured by Bio-Rad Gel Documentation EQ system.

The BLAST results of all the sequences for each isolates were tabulated in Table 8 - 11. The matching species of isolates were selected based on the reference query cover and identity percentage that were not lower than 80% for both categories. The species with the highest possible percentage in both categories were selected together with accession number for the closest match species.

Table 8: The closest match obtained from Genbank database based on partial 16S rRNA sequence for phosphate solubilizing isolates. The closest match was selected based on query cover percentage (%) in base pairs and identity percentage (%).

Phosphate Solubilizing Isolates				
Isolates	Closest match	Accession number for closest match	Query cover (%)	Identity (%)
P1	*Tolypocladium 1 WL-2011*	gi|569534346|KF747259.1	99	97
P2	*Purpureocillium lilacinum*	gi|523713943|KC157751.1	99	99
P3	*Clonostachys rosea*	gi|639126856|KJ588219.1	97	99
P4	*Paramyrothecium roridum*	gi|429472640|JX867215.1	99	99
P5	*Talaromyces aculeatus*	gi|727366081|KM458839.1	98	99

The identities of five phosphate solubilizing microorganisms were identified. However, none of the strains listed above were reported to solubilize phosphate. Therefore, it would be a new discovery if all these five strains were to be proven as phosphate solubilizer. Gaur reported that other than these two genera of bacteria, *Achromobacter, Agrobacterium*, *Serratia* were also capable in solubilizing phosphorus in varying amounts (202). A study carried out showed that *Pseudomonas fluorescens* was one of the top isolated strains in phosphate solubilisation (80).

Table 9: The closest match obtained from Genbank database based on partial 16S rRNA sequence for potassium solubilizing isolates. The closest match was selected based on query cover percentage (%) in base pairs and identity percentage (%).

Potassium Solubilizing Isolates

Isolates	Closest match	Accession number for closest match	Query cover (%)	Identity (%)
K1	*Aspergillus flavus*	gi|951312099|KT067755.1	98	99
K2	*Rhizochaete filamentosa*	gi|817033641|KP135411.1	97	88
K3	*Lysinibacillus fusiformis*	gi|1061055476|KX444651.1	95	93
K4	*Pycnoporus coccineus*	gi|748042115|KJ862071.1	98	99

Four strains of potassium solubilizing microorganisms were identified. Researches were conducted and proven that *Aspergillus* spp. and bacillus spp. were capable of promoting plant growth by solubilize phosphate (25)(81). No information of plant growth promoting was discovered for the other two strains. However, *Fomitopsis meliae* and *Aspergillus tubingensis* was found to be able to solubilize potassium from a study carried out on isolation of potassium solubilizer from arid soil (82). *Bacillus mucilaginosus* was also reported to be potassium solibilizer from a study carried on the effect of potassium solubilizing microorganisms on the growth of cucumber and pepper (83).

Table 10: The closest match obtained from Genbank database based on partial 16S rRNA sequence for nitrogen fixing isolates. The closest match was selected based on query cover percentage (%) in base pairs and identity percentage (%).

Nitrogen Fixing Isolates

Isolates	Closest match	Accession number for closest match	Query cover (%)	Identity (%)
N1	*Hyphomucor assamensis*	gi|409185613|JN206211.1	94	99
N2	*Lysinibacillus xylanilyticus*	gi|1044894537|KX254351.1	100	98
N3	*Lysinibacilus sphaericus*	gi|822620069|KP347685.1	98	98
N4	*Lysinibacillus xylanilyticus*	gi|985484541|KU597545.1	100	99
N5	*Lysinibacillus xylanilyticus*	gi|985484541|KU597545.1	99	99
N6	*Bosea robiniae*	gi|566084974|NR_108516.1	99	97
N7	*Lysinibacilus sphaericus*	gi|822620069|KP347685.1	99	98
N8	*Lysinibacillus xylanilyticus*	gi|985484541|KU597545.1	100	99
N9	*Bacillus altitudinis*	gi|1043221193|KX230132.1	100	98

N10	*Lysinibacillus xylanilyticus*	gi|379975141|JN999887.1	99	95
N11	*Bacillus cereus*	gi|411172654|JX847612.1	99	99
N12	*Lysinibacilus sphaericus*	gi|557042157|KF598850.1	100	98
N13	*Bacillus cereus*	gi|954050468|KT719719.1	100	98
N14	*Lysinibacilus sphaericus*	gi|530758759|KF527213.1	100	98
N15	*Lysinibacillus boronitolerans*	gi|513136792|KF025654.1	99	98
N16	*Alcaligenes faecalis*	gi|1037234700|KX118706.1	99	99
N17	*Rhodococcus agglutinans*	gi|1024974913|NR_136860.1	99	97
N18	*Bacillus vireti LMG 21834*	gi|631252898|NR_114096.1	98	96

Eighteen of the isolated strains were identified. Out of the 18, most of the strains were connected to the family of *Bacillus* genus. *Bacillus* spp. was not only discovered to be nitrogen fixer. They were capable of solubilizing phosphate and IAA production (25)(81). *Azospirillum* spp. were proven of fixing nitrogen from a review which studied on this particular strain, thus classifying this bacteria species to be nitrogen fixer (84).

Table 11: The closest match obtained from Genbank database based on partial 16S rRNA sequence for IAA producing isolates. The closest match was selected based on query cover percentage (%) in base pairs and identity percentage (%).

IAA Producing Isolates

Isolates	Closest match	Accession number for closest match	Query cover (%)	Identity (%)
I1	*Peinibacillus quercus*	gi|961555147|NR_134116.1	98	94
I2	*Phomopsis sp. 122AC/L*	gi|283856803|GU066685.1	99	99
I3	*Bacillusci vireti LMG 21834*	gi|631252898|NR_114096.1	98	96
I4	*Lysinibacilus sphaericus*	gi|745286365|KJ767311.1	89	87
I5	*Lysinibacilus sphaericus*	gi|151579992|EF690423.1	100	97
I6	*Lysinibacillus fusiformis*	gi|756118028|CP010820.1	93	97
I7	*Rigidoporus vinctus*	gi|1026358864|KU194316.1	97	99
I8	*Penicillium citrinum*	gi|1050550526|KX664347.1	97	99
I9	*Lysinibacillus fusiformis*	gi|1012638202|KU179364.1	96	92

I10	*Paenibacillus lautus*	gi|254682024|GQ284372.1	93	98
I11	*Lysinibacilus sphaericus*	gi|530758759|KF527213.1	97	94
I12	*Lysinibacilus sphaericus*	gi|33468787|AB116123.1	96	96
I13	*Bacillus pseudomycoides*	gi|605060080|KJ188740.1	98	99
I14	*Bacillus thuringiensis*	gi|940376846|KR809376.1	97	97
I15	*Lysinibacilus sphaericus*	gi|530758759|KF527213.1	94	92
I16	*Lysinibacillus pakistanensis*	gi|1024638643|KU983851.1	99	91
I17	*Aspergillus flavus*	gi|763868845|KM115164.1	100	99
I18	*Bacillus luciferensis*	gi|219878372|NR_025511.1	100	96
I19	*Streptomyces psammoticus*	gi|268529004|GU166432.1	96	97

A review showed that various studies conducted using *Azospirillum* spp. were proven to be able to produce IAA. They were classified as IAA producing bacteria (84). Another study on isolation of rhizospheric microorganisms showed that *Pseudomonas aeruginosa* was one of the major IAA producers.

Table 12: The closest match of strains identity obtained after comparison with Genbank database.

No.	Strain Identity Obtained	Number of isolates	Strain species
1	*Tolypocladium 1 WL-2011*	1	*Tolypocladium* spp.
2	*Purpureocillium lilacinum*	1	*Purpreocillium* spp.
3	*Clonostachys rosea*	1	*Clonostachys* spp.
4	*Paramyrothecium roridum*	1	*Paramyrothecium* spp.
5	*Talaromyces aculeatus*	1	*Talaromyces* spp.
6	*Aspergillus flavus*	2	*Aspergillus* spp.
7	*Rhizochaete filamentosa*	1	*Rhizochaete* spp.
8	*Pycnoporus coccineus*	1	*Pycnoporus* spp.
9	*Hyphomucor assamensis*	1	*Hyphomucor* spp.
10	*Bosea robiniae*	1	*Bosea* spp.
11	*Alcaligenes faecalis*	1	*Alcaligenes* spp.
12	*Rhodococcus agglutinans*	1	*Rhodococcus* spp.
13	*Phomopsis sp. 122AC/L*	1	*Phomopsis* spp.
14	*Rigidoporus vinctus*	1	*Rigidoporus* spp.
15	*Penicillium citrinum*	1	*Penicillium* spp.
16	*Streptomyces psammoticus*	1	*Streptomyces* spp.

17	*Peinibacillus quercus*	1	*Paenibacillus* spp.
18	*Paenibacillus lautus*	1	
19	*Bacillus pseudomycoides*	1	*Bacillus* spp.
20	*Bacillus vireti LMG 21834*	2	
21	*Bacillus thuringiensis*	1	
22	*Bacillus luciferensis*	1	
23	*Bacillus cereus*	1	
24	*Bacillus altitudinis*	1	
25	*Lysinibacillus fusiformis*	3	*Lysinibacillus* spp.
26	*Lysinibacillus xylanilyticus*	5	
27	*Lysinibacilus sphaericus*	9	
28	*Lysinibacillus boronitolerans*	1	
29	*Lysinibacillus pakistanensis*	1	

Out of 46 strains of microorganisms that were identified, 29 different species were obtained. Some of the strains were isolated more than once. Nine strains of *Lysinibacilus sphaericus* were isolated from the soil samples. *Lysinibacillus xylanilyticus* was also isolated for 5 times while *Lysinibacillus fusiformis* was isolated for 3 times. The other two strains *Bacillus vireti* and *Aspergillus flavus* were each isolated twice. More than one strain of species was isolated from the following 3 bacteria species; *Paenibacillus* spp., *Bacillus* spp., and *Lysinibacillus* spp.

Mega 6 was used to create phylogenetic tree by the used of maximum likelihood method based on the Tamura-Nei model (72). The phylogenetic tree for phosphate solubilizing, potassium solubilizing, nitrogen fixing and IAA producing isolated were tabulated in figure 11 - 14. The phylogenetic tree is a diagram that portrays the lines of the evolutionary origin of different species, organisms or genes which shared a common ancestor (85).

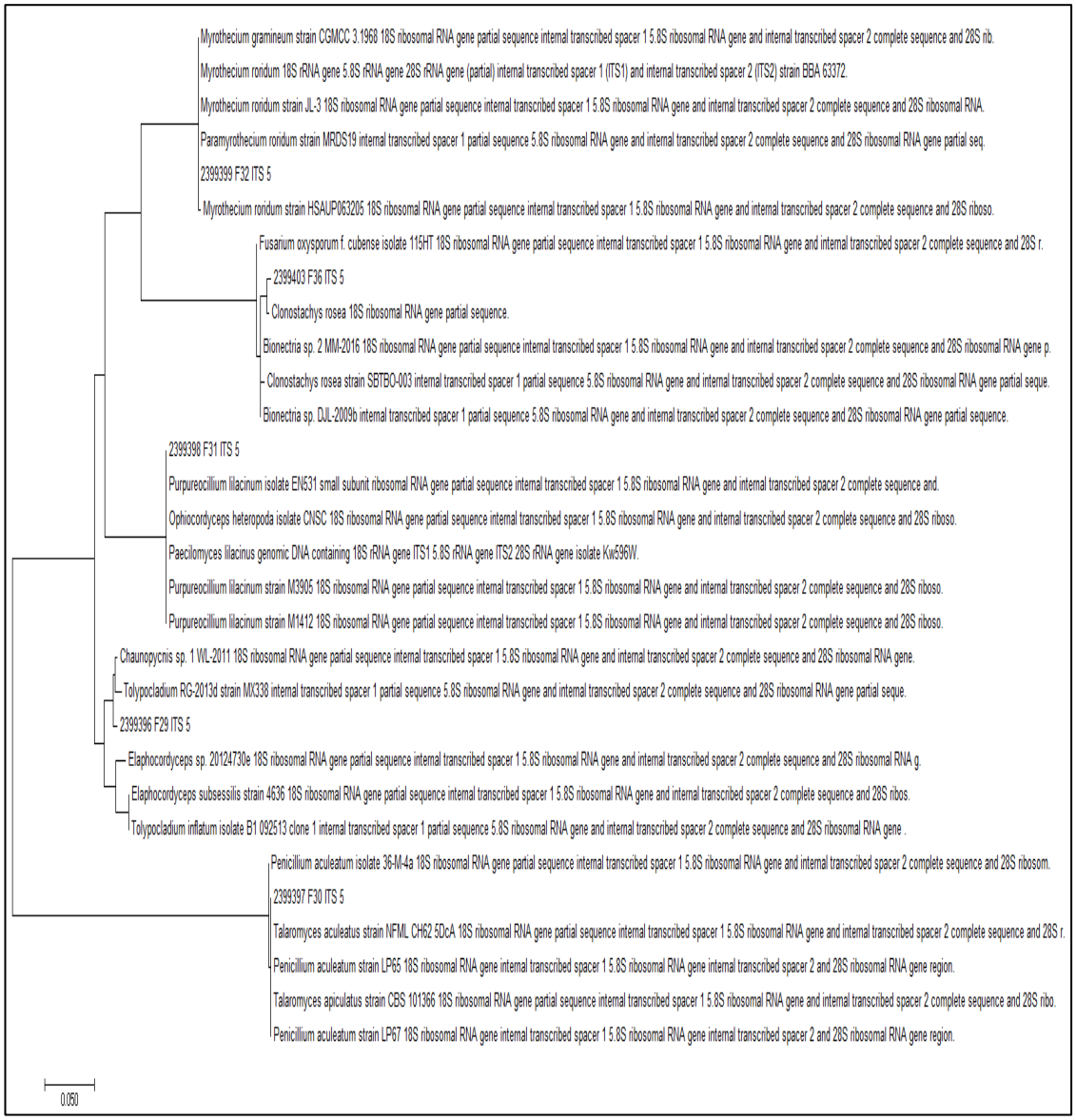


Figure 9: Phylogenetic tree shows the position of the phosphate solubilizing isolates, based on the partial 16S rRNA sequence comparison. The phylogenetic tree was constructed based on Maximum Likelihood method.

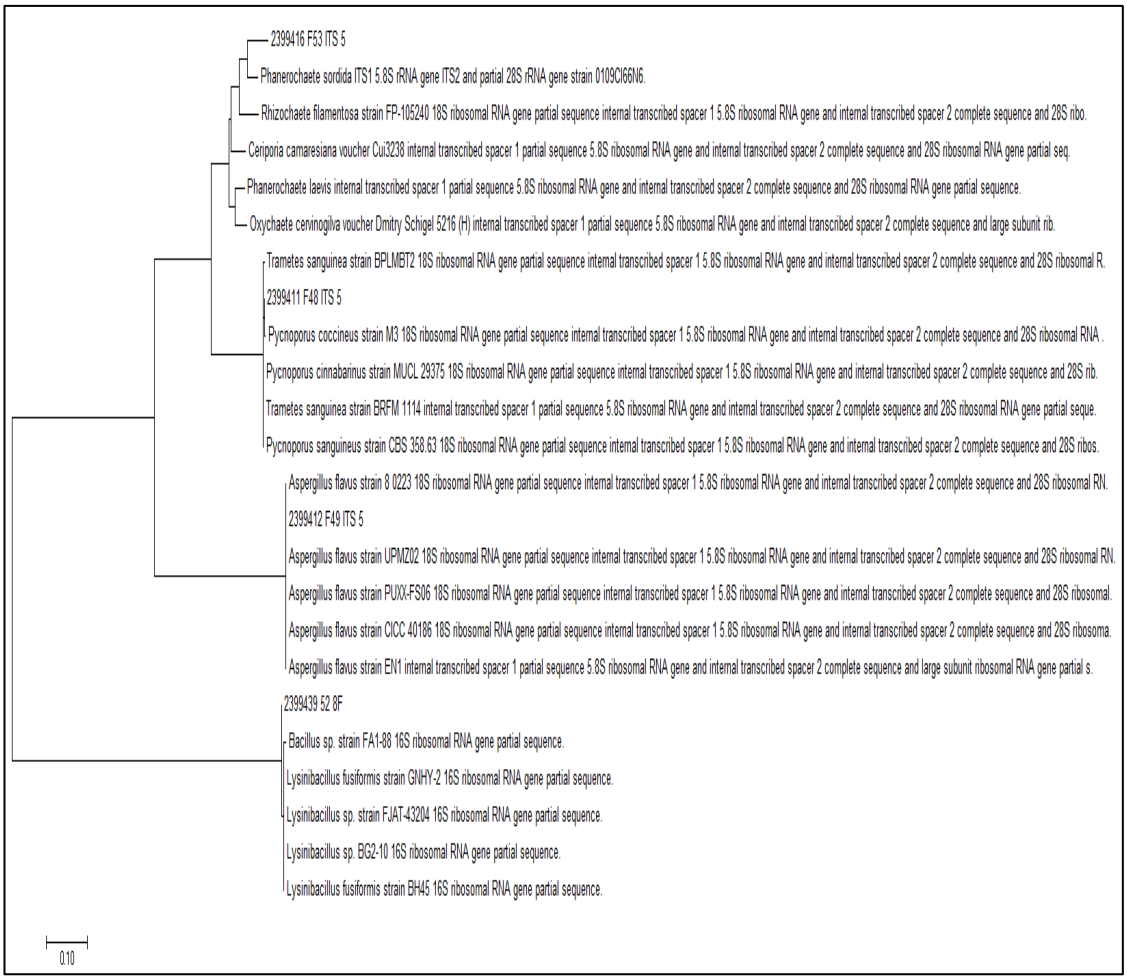


Figure 10: Phylogenetic tree shows the position of the potassium solubilizing isolates, based on the partial 16S rRNA sequence comparison. The phylogenetic tree was constructed based on Maximum Likelihood method.

Figure 11: Phylogenetic tree shows the position of the nitrogen fixing isolates, based on the partial 16S rRNA sequence comparison. The phylogenetic tree was constructed based on Maximum Likelihood method.

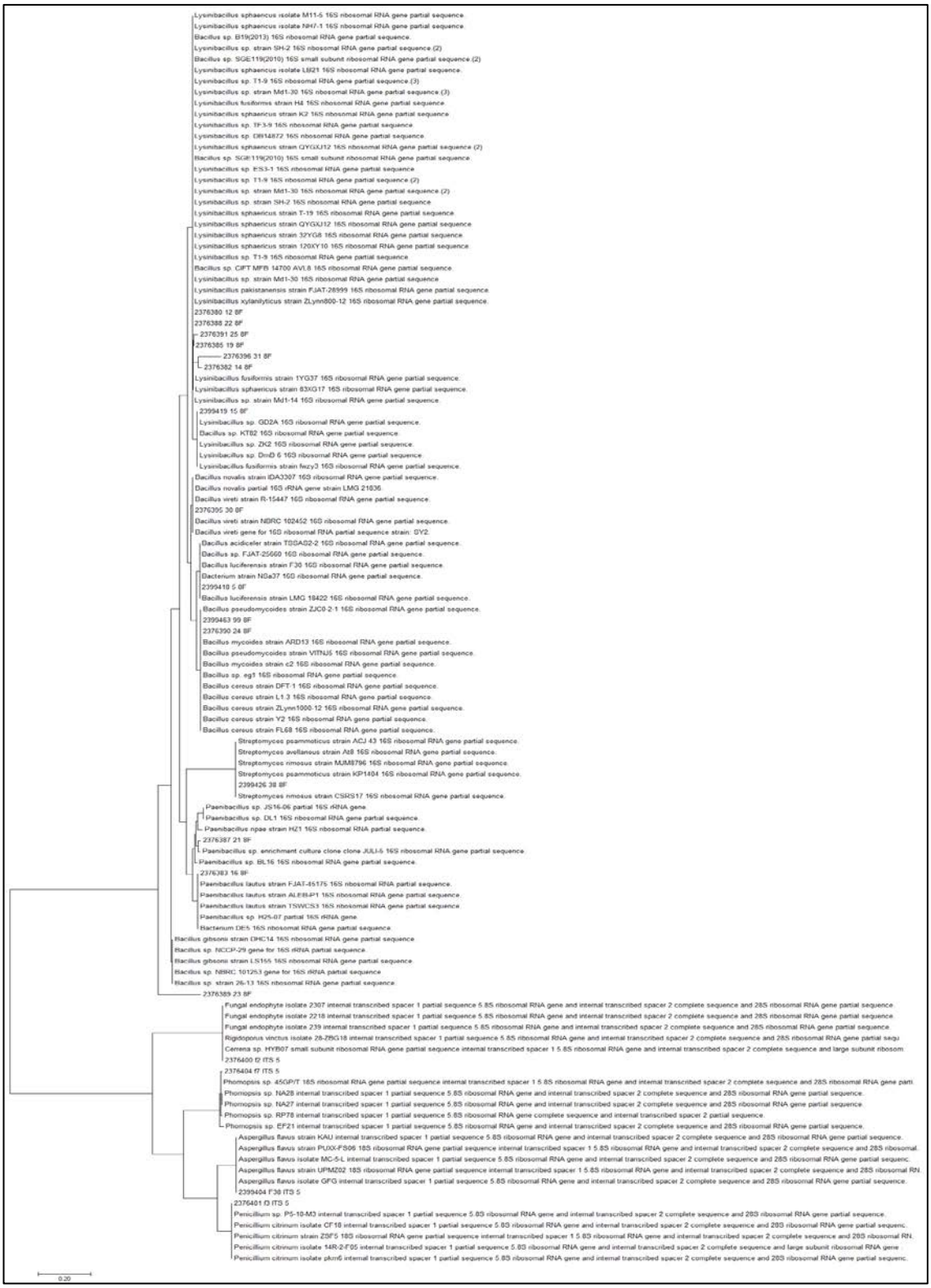


Figure 12: Phylogenetic tree shows the position of the IAA producing isolates, based on the partial 16S rRNA sequence comparison. The phylogenetic tree was constructed based on Maximum Likelihood method.

2.3.3 Soil texture analysis and microorganisms isolated

Soil samples collected were analysed for their texture characteristics according to the practise carried out in Sarawak Forestry Corporation, Sarawak, Malaysia. Figure 15 below showed the texture of soil samples collected from various sites.

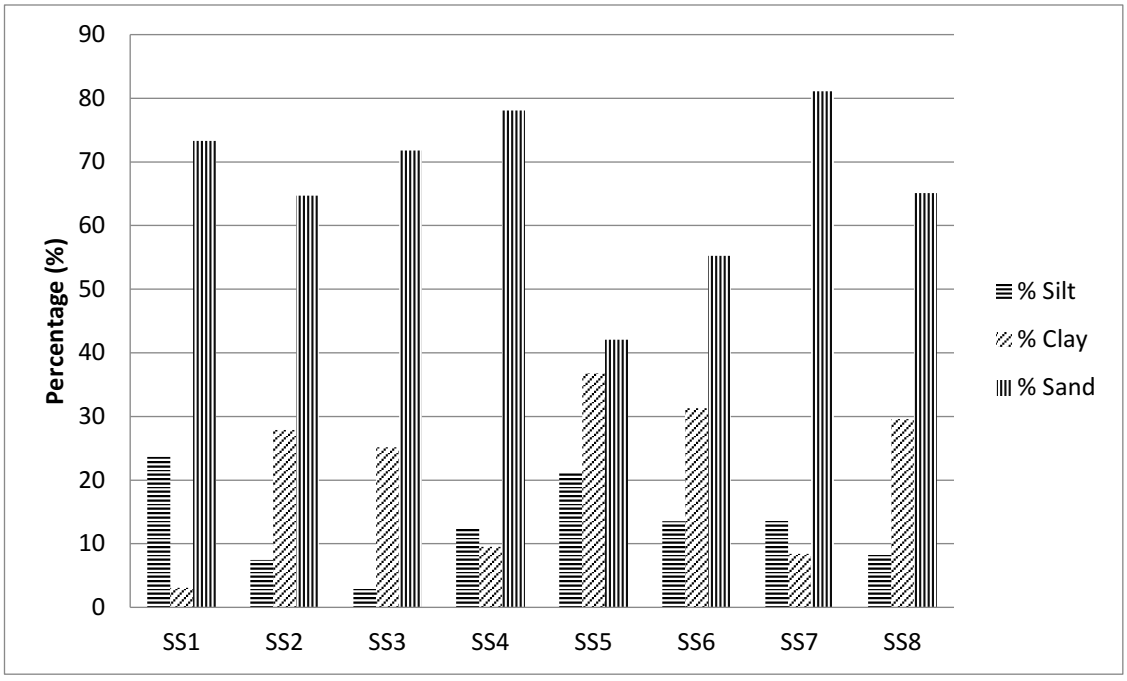


Figure 13: Texture of soil samples obtained from various locations within *Eucalyptus pellita* plantation.

The calculation for percentage of sand, clit and clay were based on the formula mentioned in section 2.2.7. The figure showed that the soil texture for soil sample 2, 3 6 and 8 were Sandy clay loam, while sample 4 and 7 were sandy loam. Soil sample 1 had more sand percentage in its texture, as it was categorized as loamy fine sand. On the other hand, soil sample 5 was categorized as clay loam. Almost all of the soil samples were considered as sandy soil texture except one, which was more of a clay texture due to higher clay concentration. Studies have shown that *Eucalyptus pellita* prefers to grow on sandy soils (86)(87). Most of the soil sample textures were sandy. The soil texture results served as a reference for soil texture in experimental pot design in chapter 4.

Cultivation of new lands for planting after slash-and-burn where uncultivated periods were shorten contributed to irreversible soil degradation and destruction of natural forests (205). High rainfall also caused loss of mobile nutrients from top soil (88). Sandy soil texture is the main issue of nutrient deficient found in *Eucalyptus* plantation. Most of the nutrients responsible for plant growth are absent in sandy soil found in *Eucalyptus* plantation (184).

2.4 Summary

The study of chapter 2 focused on the preliminary screening and characterization of the plant growth promoting rhizobacteria isolated from soil samples collected in Sarawak, Malaysia. Nitrogen fixing, indole-3-acetic acid producing, phosphate and potassium solubilizing microorganisms were successfully isolated by using selective mediums. Out of the 46 isolates, 4 isolates were potassium solubilizing microbes, 5 isolates were phosphate solubilizing microbes, 18 isolates were nitrogen fixing microbes while the remaining 19 isolates were IAA producing microbes. The isolates were identified. Out of the 46 isolates, 19 different members of genus were found with 29 different strains in total. The efficiencies of these isolates in promoting plant growth were evaluated in chapter 3.The soil texture of the soil samples collected was also evaluated. The soil textures were found to be the cause of nutrient deficiency in *Eucalyptus* plantation.

CHAPTER 3:

EVALUATING THE EFFICIENCY OF ISOLATED MICROORGANISMS AS POTENTIAL PLANT GROWTH PROMOTING INOCULANT

3.1 Introduction

Chemical fertilizers are use in agriculture to offer nutrients to the plants. Nonetheless, community apprehension regarding the prolonged use of chemical fertilizer is accumulating. Overusing of chemical fertilizer could lead to major negative impact to the environment (89). A practically dead community happened in the “dead zone” in the Gulf of Mexico across the Mississippi Basin where oxygen starvation caused by the nutrients washing from fertilized farm, was an example of the impacts caused by over application of chemical fertilizer (90). Inoculation of plant growth promoting rhizobacteria (PGPR) is the biological way to reduce the use of chemical fertilizer. PGPR facilitated plant growth through the alteration of diverse group of bacteria living in the rhizosphere at plant root surface and associates with the roots, which promote the feature of plant growth direct or indirectly by secretion of various substances (91). PGPR facilitate plant growth by simplifying resource acquisition (phosphorus, nitrogen and crucial minerals) directly, tempering plant hormone level, or indirectly acts as biocontrol agents to inhibits various pathogens on plant (92). A large community of bacteria species such as *Arthrobacter, Pseudomonas, Enterobacter, Azospirillum, Bacillus, Azotobacter, Klebsiella, Serratia, Alcaligenes* and *Burkholderia* have been discovered to improve plant growth (25)(81).

Earth atmosphere accounted for 78% of nitrogen, but unfortunately, this nitrogen is not available to plants. Nitrogen is known to be the most crucial nutrient for plant in terms of growth and yield. Biological nitrogen fixation changes the nitrogen in the atmosphere into ammonia by nitrogen fixing microorganisms by undergoing a multifaceted enzyme system known as nitrogenase in order to convert it into plant-utilizable form (76).

Soil in the ground is a bulky reservoir of Phosphorus. Phosphorus is available lavishly in soils in both inorganic and organic form. However, the amount available forms that is acceptable for plant uptake is in general insufficient (93). The low availability is due to plants consume phosphorus through two soluble forms, while most of the available phosphorus are in insoluble forms (75). Phosphorus is regarded as the second most important nutrient for plant after nitrogen. Phosphate solubilizing bacteria are able to solubilize the insoluble form of phosphorus into soluble form phosphate for plant intake.

Potassium is the third most crucial nutrients for plant, which is essential in the growth and development of plants. Potassium helps in plant photosynthesis, transportation of compounds in plants, adjusting plant cellular osmotic pressure and activation of enzymes (94). In general, lacks of potassium affects the plants in the leaves, slow growth and affected root development (95). Naturally, the amount of available potassium for plant is very in soil. Potassium solubilizing microorganisms release organic acid to transform the complex form of potassium from minerals into simple form that is available for plant intake (74)(96).

Indole-3-acetic acid (IAA) plays an important role in the interaction between rhizobacteria and plants (34). IAA functions as a mutual indicating molecule that affects the gene expression in numerous microorganisms. In general, IAA affects plant cell complexities, stimulate tuber and seed germination, increase root development, biosynthesis for metabolites and resistance to stressful condition (97)(98). For a long time, microorganisms with the abilities to synthesis IAA had been discovered. It was found that 80% of microorganisms isolated from the rhizosphere have the ability to synthesize and secrete IAA as secondary metabolites (99).

A very common phenomenon that occurs in soil is the antagonism between microorganisms. The survivability of plant pathogenic bacteria and fungi are affected by bacteria and fungi antagonists (100). Over the years, researches had shown that plants are having an intimate interaction with bacteria and fungi, and these bacteria and fungi are able to promote plant growth, at the same time, suppress plant pathogens as well (101)(100). High abundance of microorganisms were colonizing in the plant associated microenvironment, especially the rhizosphere (102). Studies had shown that between 1% and 35% of microbial isolated from plant associated habitats showed antagonistic ability to inhibit the growth of pathogens (102)(103). It was found that up to two thirds of the cultivable inhabitants were the isolates that exhibit plant growth promoting traits (104).

Chapter 3 will focus on the effectiveness of the isolates from chapter 2 in promoting plant growth. These isolates were screened to be plant growth promoting microorganisms that have the ability to facilitate plant growth. Different quantitative bioassay will be conducted to evaluate the effectiveness of the isolates as plant growth

facilitators. The results obtained from the studies will be presented and discussed in this chapter.

3.2 Materials and Methodology

3.2.1 Microorganism strains preparation

Pure cultures isolated from step 2.2.3.2 (refer above) were grown in Nutrient Agar (NA) for bacteria and Potato Dextrose Agar (PDA) for fungus. All the culture plates were incubated in an incubator at 30ºC ± 2ºC for 4 days prior to the start of the experiments. Growths of the culture on the plates were observed.

3.2.2 Screening of potential phosphate and potassium, nitrogen fixing and IAA producing microorganism isolates

3.2.2 (a) Phosphate solubilizing (65)

Pure cultures isolated with the ability to solubilizing phosphate were grown on Pikovskaya's Agar and were then incubated at 30ºC ± 2ºC up to 3 days. Each culture was replicated into 3 different plates. Clear zone formations around the microorganism culture were recorded after 4 days.

PSE (Phosphate Solubilization Efficiency) =

Z / C x 100

Z - Clearance zone including bacterial growth
C - Colony diameter

3.2.2 (b) Potassium solubilizing (105)

Pure cultures isolated with the ability to solubilizing potassium were grown on Aleksandrow's Agar and were then incubated at 30ºC ± 2ºC up to 3 days. Each culture was replicated into 3 different plates. Clear zone formations around the microorganism culture were recorded after 3 days.

KSE (Potassium Solubilization Efficiency) =

Z / C x 100

Z - Clearance zone including bacterial growth
C - Colony diameter

3.2.2 (c) Nitrogen fixing (77)

Pure cultures isolated with the ability to fix nitrogen were grown on Jensen's Medium Agar and were then incubated at 30ºC ± 2ºC up to 24 hours. Each culture was replicated into 3 different plates. Subsequently, 2mL of bromothymol blue solution were added onto all the culture plates and left for 30 minutes. Stains were washed off after 30 minutes with deionized water and the blue colouring zones below the cultures were recorded.

NFE (Nitrogen Fixing Efficiency) =

Z / C x 100

Z - Clearance zone including bacterial growth
C - Colony diameter

3.2.2 (d) IAA producing (106)(67)(107)

Microorganism strains with the ability to produce indole-3-acetic Acid were inoculated into Nutrient Broth (NB) or Potato Dextrose Broth (PDB) with Tryptophan (1g/L). The broth cultures were incubated at 28ºC ± 2ºC and shaken at 150rpm up to 4 days. After 4 days of incubation, the liquid cultures were then centrifuged at 13,400rpm by using microcentrifuge machine for 10 minutes, and 1 mL of the supernatant was then added into 1.5mL of Salkowski's reagent in a universal bottle. The mixture was incubated in the dark at room temperature for 75 minutes. The mixture was then read at 525nm by using a spectrophotometer.

3.2.3 Evaluating the efficiency of microorganism strains' abilities through bioassay quantitative analysis

3.2.3 (a) Phosphate – vanadate molybdate test (107) (108)

Standard was prepared as comparison for microorganism's sample. 219.5mg of anhydrous KH_2PO_4 were dissolved in 1L of deionized water to obtain 50.0 mg of PO_4^{3-} - P. This stock solution was then directly used for standard preparation. The standard solution was diluted into different concentration of PO_4^{3-} - P, which were 2mg/L, 4mg/L until 20mg/L subsequently. The standard solutions were treated with vanadate molybdate reagent, where yellow colour solution formed after 10 minutes of incubation. Microorganisms strains that passed though earlier screening were inoculated into Pikovskayas Broth and incubated at 30ºC ± 2ºC and shaken at 150rpm for 3 days. The broth cultures were then spun down at 13,400rpm for 5 minutes and the supernatants were mixed with vanadate molybdate reagent and deionized water. Absorbance readings at 420nm were taken after 10 minutes incubation by using a spectrophotometer. The following are the setup for test;

Broth supernatant : 1.4mL
Vanadate molybdate reagent : 0.4mL
Deionized water : 0.2mL

Standard solution : 2.8mL
Vanadate molybdate reagent : 0.8mL
Deionized water : 0.4mL

3.2.3 (b) Potassium

Due to shortage of lab equipment, potassium solubilizing test was not able to be carried out. Samples were sent to T&T Lab for potassium analysis. Selected microorganism strains were inoculated into Aleksandrov's Broth and incubated at 30ºC ± 2ºC and shaken at 150rpm for 4 days. The broths were then centrifuged at 12,000rpm for 10 minutes. A syringe was used to aspirate 20mL of the supernatant. The supernatants were then filter sterilized through 0.45µm PTFE syringe filter. The filtrates were then sent to T&T Lab for potassium content analysis by using Atomic Absorption Spectroscopy.

3.2.3 (c) Ammonia Nitrogen Detection Test

Standard was prepared as comparison for microorganism's sample. Overnight dried NH_4Cl of 3.918g were dissolved in 1000mL deionized water to obtain 1000mg/L NH_3 – N. The solution was then diluted into the 0.2mg/L, 0.4mg/L until 2.0mg/L and 4mg/L until 20 mg/L subsequently. Microorganism strains were inoculated into Jensen's Medium Broth and incubated at 30ºC ± 2ºC and shaken at 150rpm for 4 days. Ammonia nitrogen detection was carried out by using the ammonia standards and cultures through spectrophotometer at 640nm after 1 hour incubation. The following are the setup for the test;

2mL of ammonia standard or culture + 0.08mL phenol + 0.08mL sodium nitroprusside + 0.2mL oxidising solution + incubation for 1 hour.

Solution	**Composition**
Phenol	1.11mL of 89% liquefied phenol + 8.89mL of 95% ethanol
Sodium nitroprusside	0.05g sodium nitroprusside + 10mL deionized water
Oxidising solution	10mL alkaline citrate solution + 2.5mL Chlorox
Alkaline citrate	20g trisodium citrate + 1g sodium hydroxide + 100mL deionized water

3.2.3 (d) Indole-3-Acetic Acid test with Salkowski's reagent (106)(67)(107)

Standards (Blank Nutrient Broth and Potato Dextrose Broth) were prepared as comparison for microorganism's sample. Microorganism strains with the ability to produce indole-3-acetic Acid were inoculated into Nutrient Broth (NB) or Potato Dextrose Broth (PDB) with Tryptophan (1g/L). The broth cultures were incubated at 28ºC ± 2ºC and shaken at 150rpm up to 4 days. 1.5mL of the culture was spun down at 13.400rpm for 5 minutes. One mL of the supernatant was then mixed with 1.5mL of Salkowski's reagent and incubated in the dark for 75 minutes at room temperature. Absorbance readings were taken with spectrophotometer at 525nm.

3.2.4 Antagonist test - antimicrobial and antifungal activities of selected strains (109)

A total of 8 microorganism strains were selected, listed as below;

1) *Lysinibacillus sphaericus*
2) *Bosea robiniae*
3) *Paenibacillus quercus*
4) *Lysinibacillus sphaericus*
5) *Paramyrothecium roridum*
6) *Tolypocladium sp. 1 WL-2011*
7) *Lysinibacillus fusiformis*
8) *Rhizochaete filamentosa*

In order to find out the inhibition effect of microorganisms against each other, cross streaking method was chosen to be carried out. Nutrient agar was used in this method. Two strains of microorganisms were chosen from each function (nitrogen fixing, phosphate solubilizing, potassium solubilizing and IAA production). Only one of the strains from the two strains from each function was tested against other strains from other function at the time, without overlapping with each other. The main motive was to select only one strain from each function that does not inhibit the other microorganisms. So the selected strain was streaked in the middle of nutrient agar plate. The other strains from other function were streaked in a triangle covering the main strain in the middle. Triplicates were carried out for each test. The inoculated plates were incubated at 30ºC ± 2ºC, up to four days. The inhibitions of the selected strains against other strains were observed from day to day depending on the growth rate of each culture.

3.2.5 Growth rate and total plate count of microorganism strains

(I) Indirect growth measurements – Optical density by the use of the Spectrophotometer

Strains that passed through the bioassay quantitative analysis were selected for growth measurement. Strains were prepared by inoculated into 10mL of nutrient broth and incubated overnight in the incubator shaker at 30ºC and 150rpm. Visual density (OD) of the microorganism cultures were observed by using Spectrophotometer with the wavelength of 600nm. Prior to the experiment, an empty nutrient broth was used as a blank solution to standardise the readings. The overnight cultures were then transferred into shake flasks containing 90mL of nutrient broth and mixed well. At time, $t = t_0$, where the moment the cultures were transferred, the initial OD reading for each culture was taken immediately. One mL from each of the broth culture was pipetted into a cuvette for readings and then discarded after each reading. Triplicates readings were conducted for each culture. At every hour interval subsequently, the OD reading was taken until $t = t_8$, which was the 8^{th} hour, and then skipped until the 24^{th} hour interval, where $t = t_{24}$, which was the final OD reading.

(II) Direct growth measurements – Serial dilutions and total plate counts

The same strains as method above were selected for direct growth measurements method by measuring the colony-forming unit (CFU) and total plate count. In order to make the colonies of cultures countable, ten-fold serial dilution was carried out. Seven 15mL falcon tubes were prepared beforehand. Nine mL of deionized water were transferred into each of the falcon tubes. One litre micropipettes was used to transfer 1mL of the broth culture from the shake flask where the culture was undergoing exponential growth phase (method above) into the first falcon tube, which the dilution of 10^{-1} was obtained. The tube was overturned a few times to completely mix the solution. Then, 1mL of the solution was transferred into the second tube. These processes were repeated until obtaining the dilution of 10^{-7}. The solution with the dilution factors 10^{-1}, 10^{-2} and 10^{-3} (fungus culture) and 10^{-5}, 10^{-6}, $10^{-7}$was spread on nutrient agar and incubate in the incubator at 30ºC ± 2ºC for one day. The individual

colonies on each plate were counted on the next day. Below is the the calculation for the colony forming unit per millitre (CFU/mL);

CFU/mL = number of colonies per mL plated / Total dilution factor

3.2.6 Data analysis

Experiments results were analysed by using analysis of t-Test (IBM SPSS Statistics v24): Two-sample assuming unequal variances, with statistical significance taken as $p \leq 0.05$. Microsoft Office Excel software was also used for data analysis.

3.3 Result and Discussion

Plant growth promoting rhizobacteria are the main focus in this study. These PGPR wield beneficial properties to plant growth and development. Enhanced nutrient availability and efficiency of nutrients uptake are those important mechanisms for these beneficial effects that PGPR could possess (200). Studies have shown that PGPR have the ability to influence plant growth by facilitating uptake of nutrients from the soil through various direct mechanisms and also synthesizing plant hormones, such as solubilisation of phosphorus and potassium, atmospheric nitrogen fixation, and synthesis of IAA plant growth hormone in order to make nutrients more available to the plants (74)(99)(75)(76). These PGPR isolated from Sarawak, Malaysia, were evaluated for their beneficial properties to plant growth.

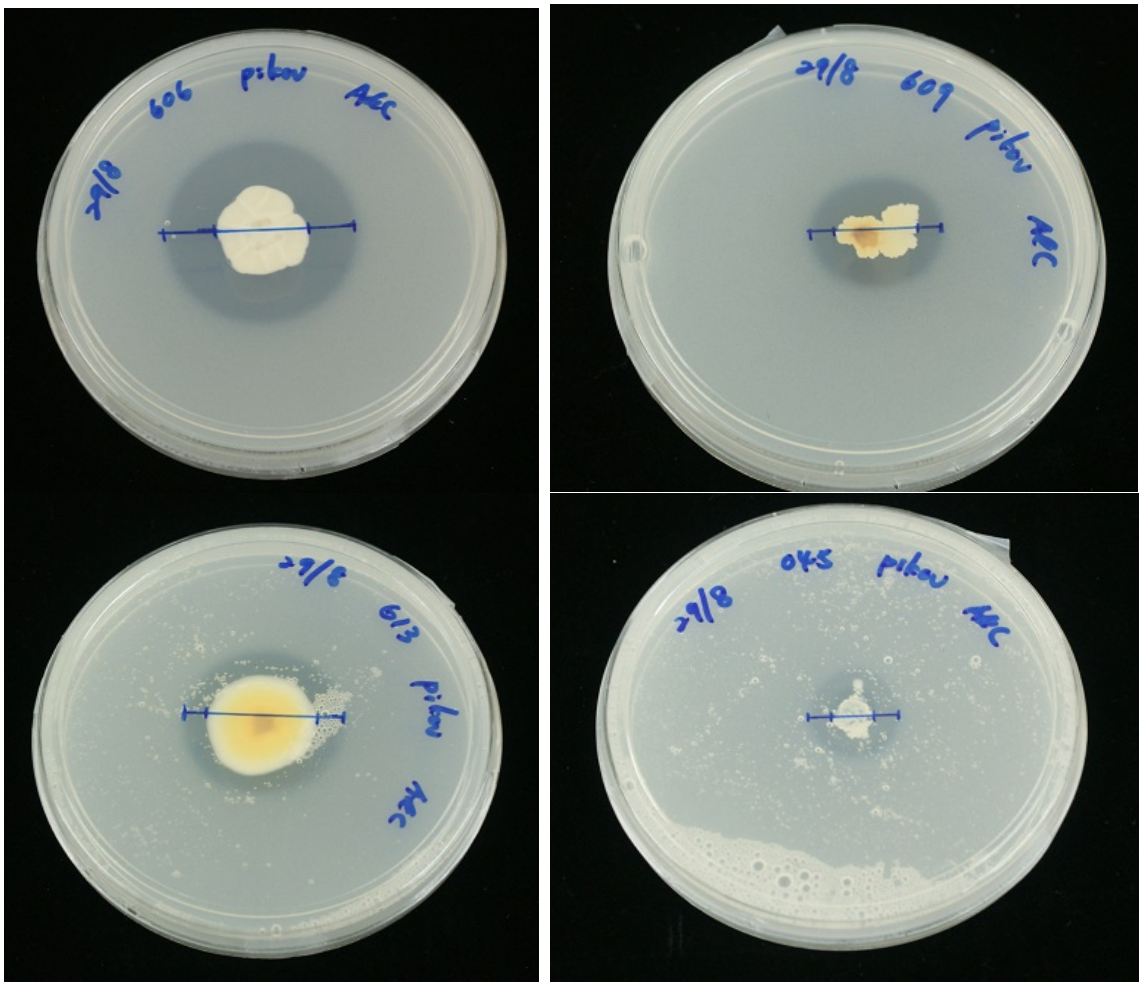
The growth rates of all the selected microorganism strains were studied. The growth rate of the microorganisms is an important factor that needed serious consideration due the implementation of pot trial experimental design that will be conducted in chapter 4. The different growth rate of microorganisms will affect the population of selected strain cultures added into the plants. In order to minimize the over population of certain strain, cultures population were needed to be analysed to grasp for the necessary preparation.

The ecological role of these PGPR and the interactions in the plant rhizospheres are essential for biotechnology applications. Study on their antagonistic characteristics is important to create a balance ecosystem of PGPR associated with the plant (110)(100). Colonization of sites, nutrients and minerals were the main focus of this study as they will affects the selection of strains needed for pot trail experimental design.

3.3.1 Screening of potential phosphate and potassium, nitrogen fixing and IAA producing microorganism strains

3.3.1 (a) Phosphate Solubilizing

Selective medium, Pikovskaya's Medium Agar was used in this test. A clear zone formation could be observed when phosphate solubilizing microorganisms were cultured on the medium due to the phosphate solubilisation in the neighbourhood of the colony (111). The diameters of clear zones were recorded according to the calculation mentioned in section 3.2.2 (a) after 4 days of incubation. The results of clear zone formation were presented in figure 16 below.


29/8 606 pibou ACC
29/8 609 pibou ACC
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29/8 045 pibou ACC

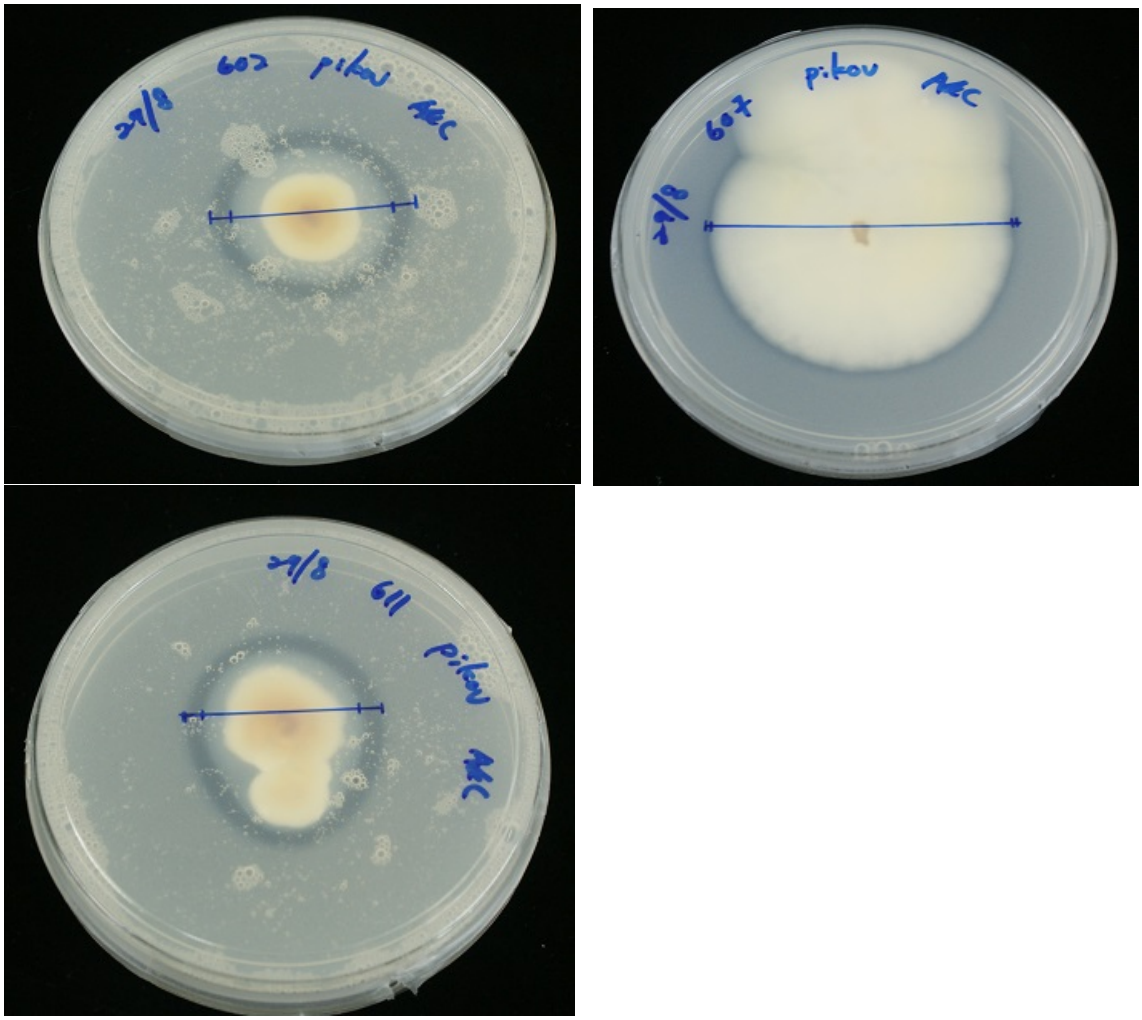

Figure 14: Selective medium Pikovskaya's Medium plate assay to screen for the potential phosphate solubilizing microorganisms. The comparison of the clear zone formations for two differents strains after 4 days of incubation.

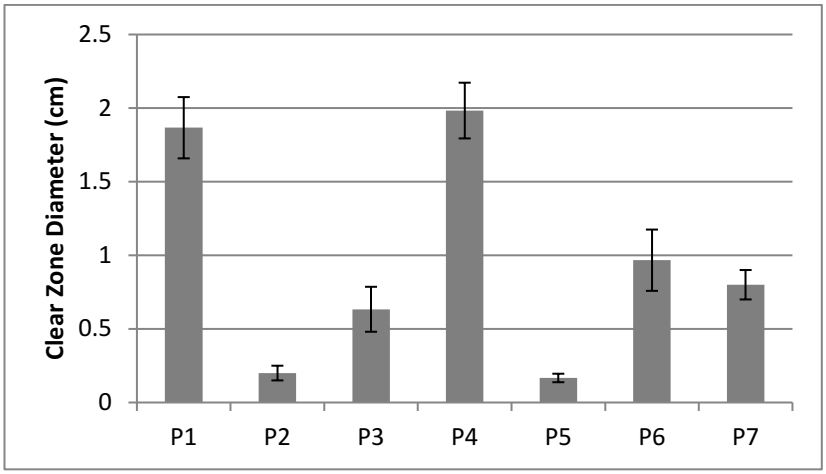

Figure 15: Clear zone diameter of phosphate solubilizing microorganisms on the 4th day of the experiment.

Table 13: Clear zone diameter of phosphate solubilizing on the 4th day of the experiment.

Code	Clear Zone Diameter (cm)
P1	1.8667
P2	0.2000
P3	0.6333
P4	1.9833
P5	0.1667
P6	0.9667
P7	0.8000

The results of clear zone diameter of phosphate solubilizing by the used of selective medium were shown on Figure 17 and Table 13. Two additional strains, *Bacillus* spp. that were identified as nitrogen fixer were also tested together with the initial 5 isolated phosphate solubilizing strains. Table 17 showed that seven strains displayed phosphate solubilizing ability. Two strains, P1 and P4, were showing exceptionally high phosphate solubilizing ability. The clear zones formed by these two strains were 1.8667cm and 1.9833cm respectively. They were about double the value of the next third highest clear zone formation, which was 0.9667cm by strain P6, followed by 0.8000cm by strain p7, 0.6333cm by strain P3, 0.200cm by strain P2 and lastly 0.1667 by strain P5. Six strains with the higher phosphate solubilizing ability were selected for further evaluation by bioassay quantitative analysis.

A study conducted showed that isolates of fluorescent *Pseudomonas* and *Bacillus* shown positive colonizing zones on Pikavskaya's agar (79). Another study carried out showed that 60 strains isolated from *Panax ginseng* were capable of forming clear zone on Pikavskaya's agar (80).

3.3.1 (b) Potassium Solubilizing

Selective medium, Aleksandrow's Medium Agar was used in this test. A clear zone formation could be observed when phosphate solubilizing microorganisms were cultured on the medium due to the phosphate solubilisation in the neighbourhood of the colony (105). The diameters of clear zones were recorded according to the calculation mentioned in section 3.2.2 (b) after 3 days of incubation. The results of clear zone formation were presented in figure 18 below.

Figure 16: Selective medium Aleksandrow's Medium plate assay to screen for the potential potassium solubilizing microorganisms. The comparison of the clear zone formations for two differents strains after 3 days of incubation.

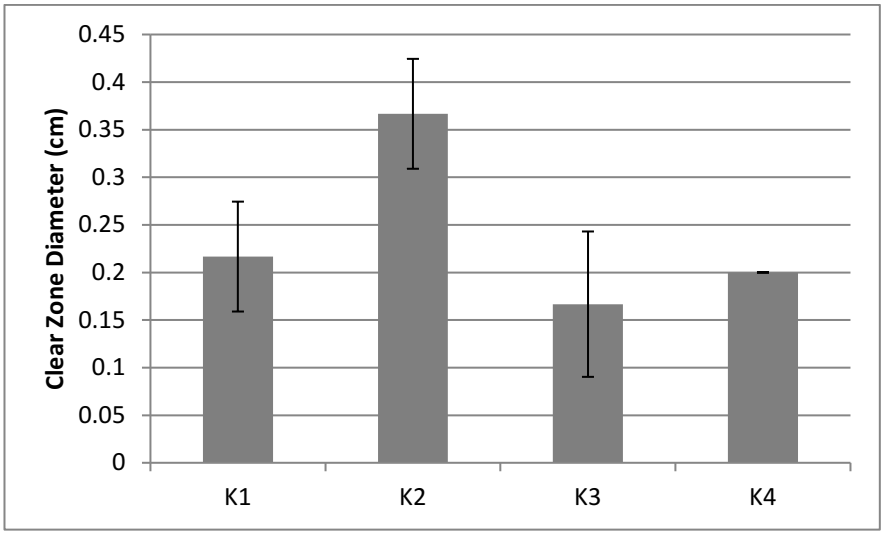

Figure 17: Clear zone diameter of potassium solubilizing on the 3rd day of the experiment.

Table 14: Clear zone diameter of potassium solubilizing on the 3rd day of the experiment.

Code	Clear Zone Diameter (cm)
K1	0.2167
K2	0.3667
K3	0.1667
K4	0.2000

The results of clear zone diameter of potassium solubilizing by the used of selective medium were shown on Figure 19 and Table 14. Only four strains were successful in solubilizing potassium. The other isolates were unable to solubilize potassium unfortunately. Diameters of clear zone formations were not as impressive as phosphate solubilizing. The results of the four strains were close to each other, with strain K2 emerging as the lead with 0.3667cm, followed by K1 with 0.2167cm, K4 with 0.2000cm and lastly K3 with 0.1667cm. All four strains were selected for further evaluation by bioassay quantitative analysis. A study on isolation of bacteria from soil samples across different region of India showed that 14 isolates were showing signs of clear zone around the colonies on Aleksandrov's medium agar (74).

3.3.1 (c) Nitrogen fixing

Selective medium, Jensen's Medium Agar and bromothymol blue solution were used in this test. A blue coloured zone formation could be observed when nitrogen fixing microorganisms were cultured on the medium (77). The diameters of clear zones were recorded according to the calculation mentioned in section 3.2.2 (c) after 3 days of incubation. The results of clear zone formation were presented in figure 20 below.

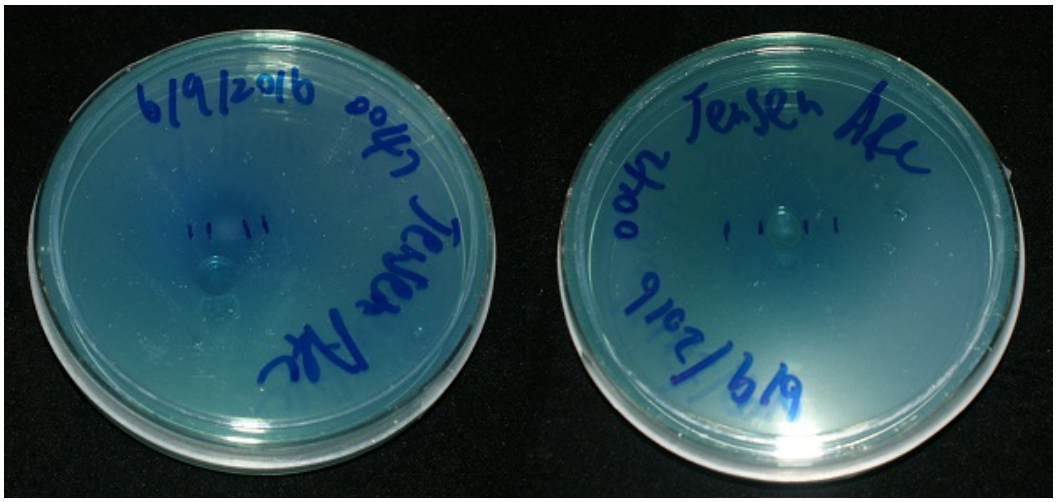

Figure 18: Selective medium Jensen's Medium plate assay to screen for the potential potassium solubilizing microorganisms. The comparison of the clear zone formations for two differents strains after 24 hours of incubation.

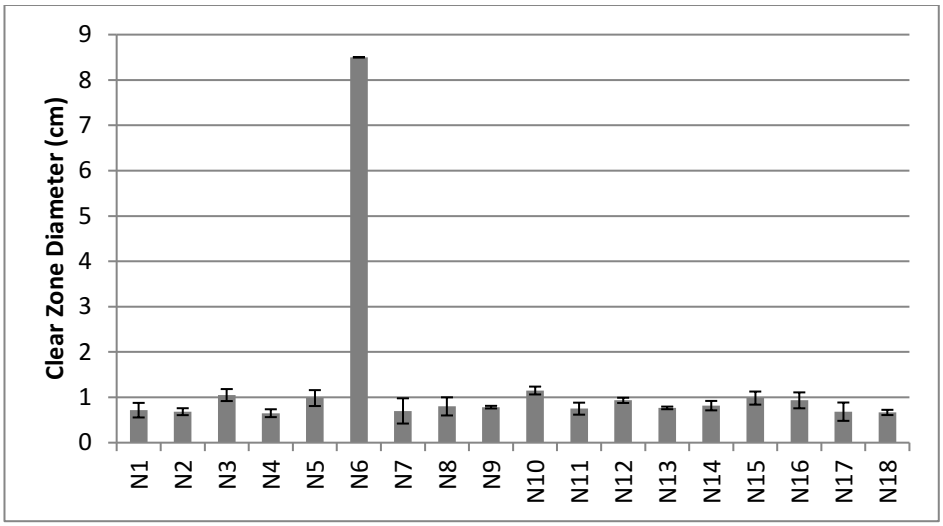

Figure 19: Clear zone diameter of nitrogen fixing on the next day of the experiment.

Table 15: Clear zone diameter of nitrogen fixing on the next day of the experiment.

Code	Clear Zone Diameter (cm)
N1	0.7167
N2	0.6830
N3	1.0500
N4	0.6500
N5	0.9833
N6	8.5000
N7	0.7000
N8	0.8000
N9	0.7833
N10	1.1500
N11	0.7500
N12	0.9333
N13	0.7667
N14	0.8167
N15	0.9833
N16	0.9333
N17	0.6833
N18	0.6667

The results of clear zone diameter of nitrogen fixation by the used of selective medium were shown on Figure 21 and Table 15. Eighteen strains isolated were considered as nitrogen fixer. Out of the 18 isolates, one strain, N6, showed particularly high nitrogen fixing ability, with 8.500cm, which is 800% more than the second highest value. In order to find out why N6 expressed such a high value, a detailed study of that microorganisms are required, which could be carried out in the further works after this experiment. The rest of the isolates had values from 1.1500cm to 0.6500cm. The six strains with the highest nitrogen fixing value were selected for further evaluation by bioassay quantitative analysis. They were N6 with 8.500cm, N10 with 1.1500cm, N3 with 1.0500cm, N6 & N15 both with 0.9833cm, and lastly N16 with 0.9333cm. A study conducted showed that a total of 23 strains of bacteria were able to form zone of coloration on nitrogen free malate agar isolated from two plant species, *Calligonum polygonoides* and *Lasiurus sindicus* (77).

3.3.1 (d) IAA production

Colorimetric estimation method with the use of Optical Density (OD) by spectrophotometer was used in this test. Pinkish or dark red colour of the solution will form when the peroxidase enzyme reacts with Salkwoski's reagents due to oxidation (106). The absorbance readings from spectrophotometer were recorded after 75 minutes of mixing and incubation in the dark. The pinkish and red coloured formation was presented in figure 22 below.

Figure 20: Broth cultures of isolates after Salkowski's reagent were added. Two isolates with colour in different density for comparison.

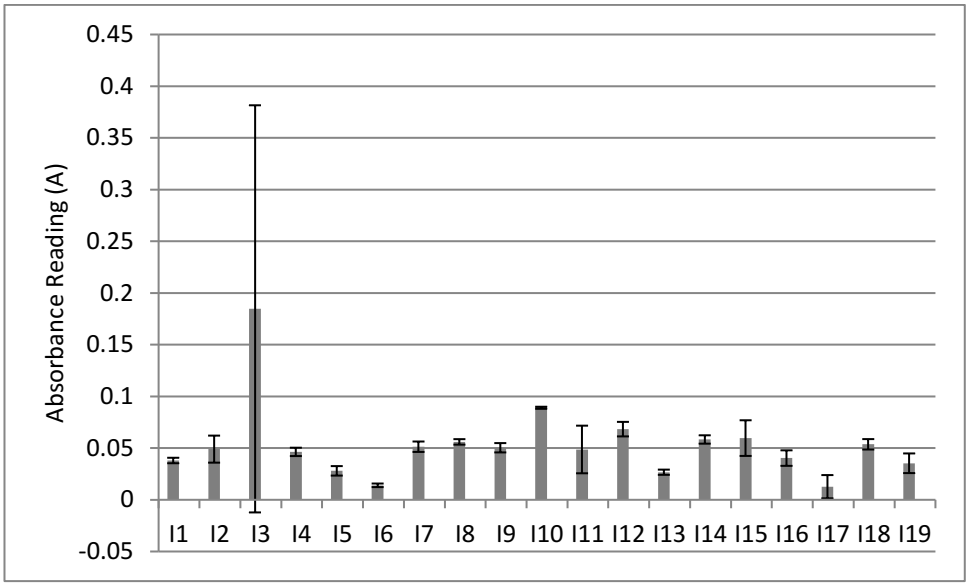

Figure 21: Absorbance reading of IAA production after incubation.

Table 16: Absorbance reading of IAA production after incubation.

Code	Absorbance Reading (A)
I1	0.0380
I2	0.0490
I3	0.1847
I4	0.0463
I5	0.0280
I6	0.0140
I7	0.0513
I8	0.0560
I9	0.0503
I10	0.0890
I11	0.0487
I12	0.0683
I13	0.0267
I14	0.0583
I15	0.0597
I16	0.0403
I17	0.0127
I18	0.0537
I19	0.0353

The results of Optical Density (OD) value by the used of spectrophotometer were shown on Figure 23 and Table 16. Nineteen isolates were found to have the ability to produce IAA. One individual strain, I3, once again showed very high value of reading, with 0.1847A, but it also had a very huge standard deviation. Detailed experiements had to be carried on I3 in order to fully understand its ability to secrete such a high reading. The rest of the 18 isolates had value ranges from 0.0890A to 0.0127A. Nevertheless, strain I3 with the highest absorbance reading but with huge standard deviation was chosen together with the 5 next strains with following highest value; I10 with 0.0890A, I12 with 0.0683A, I15 with 0.0596A, I14, with 0.0583A and lastly I8 with 0.056A, for further evaluation of IAA production through bioassay quantitative analysis. Ten isolates from the rhizosphere of banana plant were shown to be able of producing IAA from a study conducted (79). These ten isolates had varying degree of IAA produced. A study carried out on the isolation of rhizosperic bacteria from *Panax ginseng* showed that 50 isolated strains were IAA producer.

3.3.2 Evaluating the efficiency of microorganism strains' abilities through bioassay quantitative analysis

3.3.2 (a) Phosphate – vanadate molybdate test

Phosphate molybdate test was carried out to analyse the concentration of phosphate solubilized by the selected strains. Molybdophosphoric acid formed when soluble phosphate reacted with ammonium molybdate. With the presence of vanadium, vanadomolybdophosphoric acid formed in a yellow colour. Thus making the absorbance reading visible at the wavelength of 420nm (112).

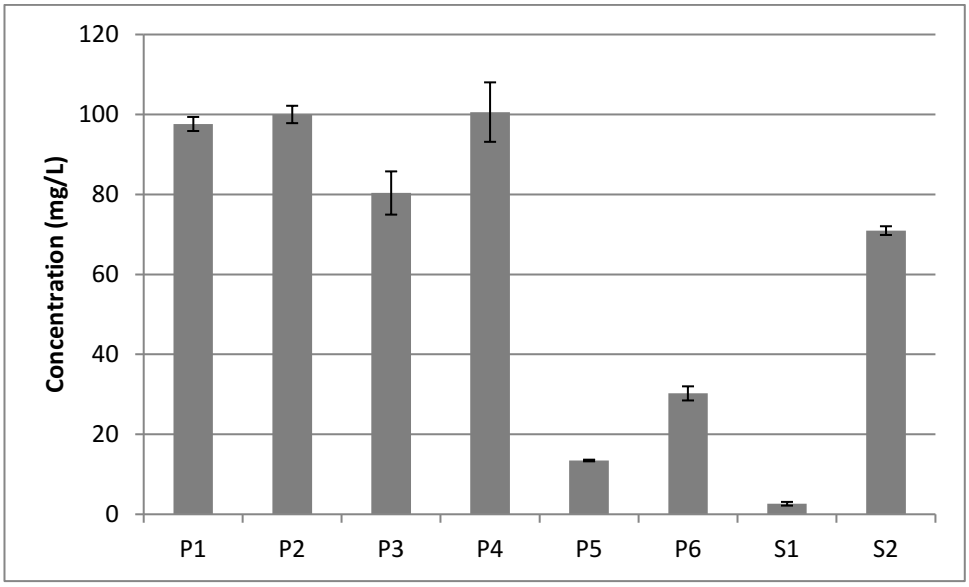

Figure 22: Concentration of phosphate solubilized by the selected strains through vanadate molybdate test. Six strains and 2 standard cultures were tested.

Table 17: Concentration of phosphate solubilized by the selected strains through vanadate molybdate test. Six strains and 2 standard cultures were tested.

Code	Mean (mg/L)
P1	97.62
P2	100
P3	80.3567
P4	100.593
P5	13.45
P6	30.236
S1	2.6333
S2	70.95

Two standard cultures with known phosphate solubilizing ability were tested in this vanadate molybdate test. These two microorganisms were *Arthrobacter ureafaciens* and *Aspergillus niger*. Both of them were known to have the ability to solubilize phosphate (113)(73)(80)(114)(115)(116)(117)(118)(119). They were used as a comparison for the other isolated strains in phosphate solubilizing concentration. Four strains showed very high concentration compared to standard strains. Strain P4 was the highest with 100.593mg/L. Followed closely was P2, with 100mg/L. Strain P1 and P3 showed 97.62mg/L and 80.3567mg/L respectively. The last two remaining strains P6 and P5 were quite low in phosphate concentration, with 30.236mg/L and 13.45mg/L respectively. The strains with the highest concentration to the lowest are as follow; *Paramyrothecium roridum, Tolypocladium sp. 1 WL-2011, Clonostachys rosea, Purpureocillium lilacinum, Talaromyces aculeatus,* and *Streptomyces gramineus*. Studies had shown that *Paramyrothecium roridum* has the ability to solubilize phosphate (120)(121). *Paramyrothecium roridum* and *Tolypocladium sp. 1 WL-2011* were chosen for further evaluation due to their ability to solubilize such high concentration of phosphate.

A study showed that *Bacillus* spp. and *Burkholderia* spp. were the best strain in solubiliing tricalcium phosphate among the 13 strains isolated from the rhizosphere of maize in Brazil, which ranges from 1.8 mg/L to 200.0 mg/L (122). A study conducted on rhizosphere of rice in Bangladesh indicated that 14 strains were capable of solubilizing phosphate in the range of 2.0 mg/L to 524.0 mg/L. Among the strains, *Acinetobacter* and *Klebsiella* spp. were found to be the best phosphate solubilizer (123).

3.3.2 (b) Potassium – Atomic Absorption Spectroscopy

Atomic Absorption Spectroscopy test was carried out by T&T laboratory Sdn. Bhd. (Kuching, Sarawak, Malaysia) to determine the soluble potassium amount. Potassium atoms absorbed light source emitted from a certain wavelength which transitioned into excited atoms. Atomic absorption spectroscopy could then detect the wavelength passing though the atoms (112).

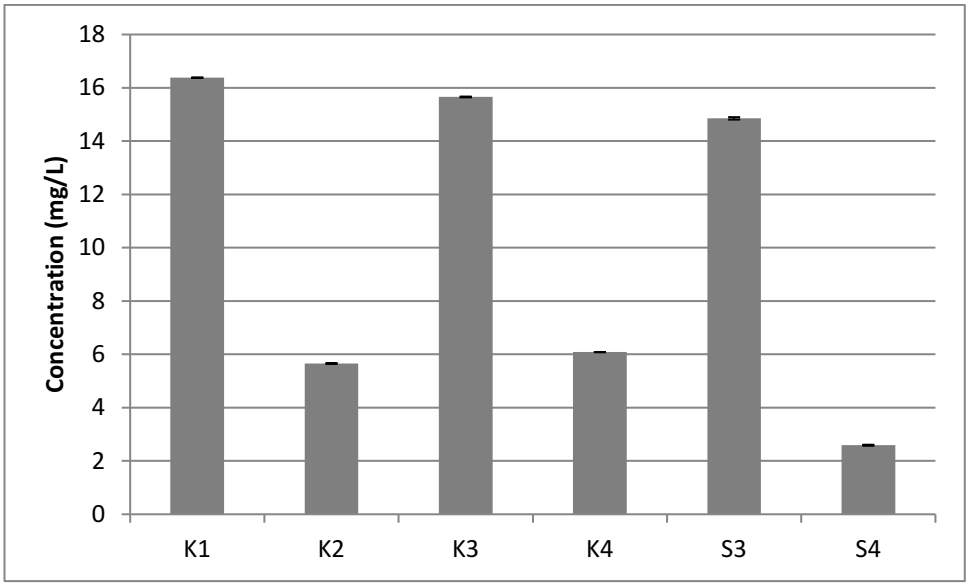

Figure 23: Concentrations of available potassium produced by the selected strains through analysis of atomic absoption spectrophotometer. Four strains and 2 standard cultures were analysed.

Table 18: Concentrations of available potassium produced by the selected strains through analysis of atomic absoption spectrophotometer. Four strains and 2 standard cultures were analysed.

Code	Mean (mg/L)
K1	16.380
K2	5.6567
K3	15.657
K4	6.0833
S3	14.853
S4	2.5900

The two standard cultures, *Enterobacter hormaechei* and *Aspergillus terreus* were known to be solubilize potassium were also tested for potassium solubilizing ability (124)(125)(74). All six strains were tested for available potassium by T&T Laboratory Sdn. Bhd. (Kuching, Sarawak, Malaysia). The test results were shown on Table 18 and Figure 25 above. Two of the isolated strains, K1 and K3, were showing higher available potassium content compared to standard culture, S3. They were both 16.380mg/L and 15.657mg/L respectively, which was higher than 14.853mg/L by standard culture. The identities of these two strains were *Lysinibacillus fusiformis* and *Rhizochaete filamentosa*. Strain K2 and K4, *Aspergillus flavus* and *Pycnoporus coccineus* were showing lesser available potassium compared to the rest. They were 5.6567mg/L and 6.0833mg/L. Studies have shown that *Lysinibacillus fusiformis* was potassium solubilizing microorganisms (126)(127), while *Aspergillus flavus* was a phosphate solubilizer (128)(129). Nevertheless, Strain K1 and K3 were selected for further evaluation.

A study conducted on Kharif crops (tobacco, sugarcane, maize, potato, banana, and pigeon pea) showed that 12 strains were able to solubilize potassium from waste mica in the concentration range of 2.86 mg/L to 13.31 mg/L. *Agrobacterium tumefaciens* and *Rhizobium pusense* had the best potassium solubilizing capacities (130). Four isolated strains from a study on the growth of tobacco plant in China showed potassium solubilisation activity more than 2.0mg/L (66).

3.3.2 (c) Ammonia Nitrogen Detection Test

Ammonia nitrogen test – phenate method was carried out to analyse the concentration of ammonia nitrogen produced by the selected strains. Blue indophenol compound formed when phenol and hyprochlorite reacted with ammonia (131). Absorbance reading of ammonia production was able to be measured at 640nm due to the formation of blue indophenol (112).

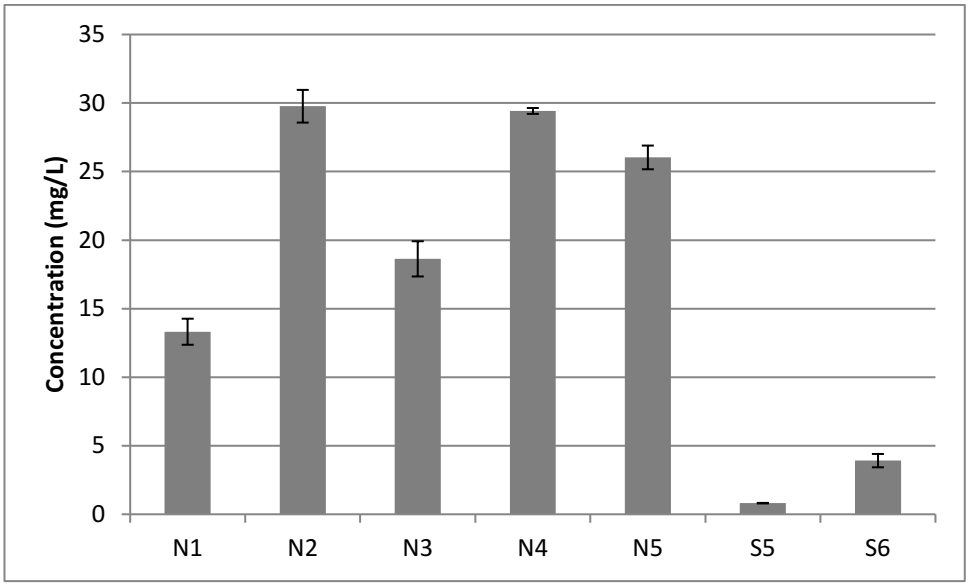

Figure 24: Concentration of ammonia nitrogen produced by the selected strains through ammonia nitrogen detection test. Five strains and 2 standard cultures were tested.

Table 19: Concentration of ammonia nitrogen produced by the selected strains through ammonia nitrogen detection test. Five strains and 2 standard cultures were tested.

Code	Mean (mg/L)
N1	13.317
N2	29.767
N3	18.633
N4	29.423
N5	26.033
S5	0.8100
S6	3.9133

Two standard cultures known to have the ability in nitrogen fixation were also tested. These two standard cultures were *Azotobacter salinestris* and *Azotobacter chroococcum*. Studies had shown that they were nitrogen fixer (132)(133)(134)(135)(136)(137)(138). Initially, 6 strains were selected for ammonia nitrogen test, however, one of the strains was not producing ammonia, therefore, it was discarded from the results. Five of the selected strains showed that they were producing ammonia better than those standard cultures. Strain N2 and N4 were leading the way with 29.767mg/L and 29.423mg/L respectively. The third highest ammonia producing strain was N5 with 26.033mg/L, followed by N3 with 18.633mg/L and N1 with 13.317mg/L. The two leading strains were identified to be *Lysinibacillus sphaericus* and *Bosea robiniae*. Studies showed they were both capable of producing ammonia (139)(140). These two strains were selected for further evaluation in inhibition of other microorganisms.

Twelve nitrogen fixing *Azotobacter* spp. that were isolated from wheat rhizosphere in Pakistan showed production of ammonia nitrogen in the concentration of 11.0mg/L to 80 mg/L (141). Anotheer study conducted on ammonia excretion ability of 71 nitrogen fixing isolates from soils in India showed excretion of 0.018mg/L to 1.540mg/L (142).

3.3.2 (d) Indole-3-acetic Acid test with Salkowski's reagent

Salkowski's reagent with tryptophan test was carried out to analyse the concentration of IAA produced by the selected strains. Iron (III) chloride and sulphuric acid caused IAA to form chelate with ferric ions at acidic pH. IAA was oxidized by ferric ions which then turned into coloured oxidation compound that could be detected by using visible wavelength[203]. The coloured compound was then measured at 525nm (143).

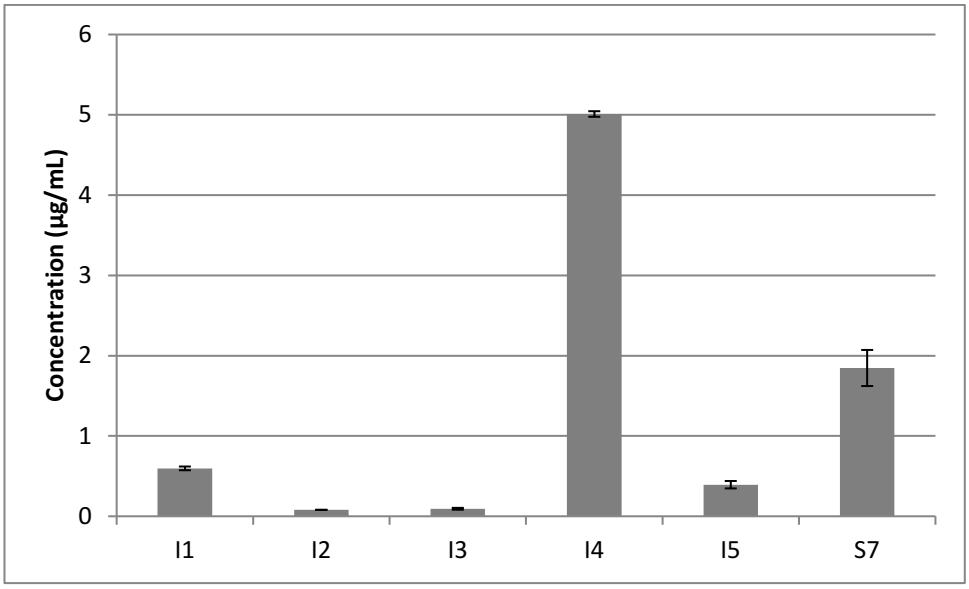

Figure 25: Concentration of IAA produced by the selected strains through Salkowski's reagent with tryptophan test. Five strains and 1 standard culture were tested.

Table 20: Concentration of IAA produced by the selected strains through Salkowski's reagent with tryptophan test. Five strains and 1 standard cultures were tested.

Code	Mean (µg/mL)
I1	0.5967
I2	0.08
I3	0.09333
I4	5.01
I5	0.3933
S7	1.8467

One standard culture that was known to produce IAA was tested together with the selected strains. Studies showed that *Pseudomonas putida* was capable of producing IAA (140)(144)(58). One of the initially selected strains was not showing any sign of IAA production, thus, it was casted off from the results. Only one strain was showing value higher than the standard culture, which was strain I4, with 5.01µg/mL. The other strains were producing lesser IAA compared to standard culture. Strain I1 was the next in line with 0.08µg/mL, followed by I5, I3 and I2, with 0.3933µg/mL, 0.09333µg/mL and 0.08µg/mL correspondingly. Eventhough strain I1 was producing very little IAA, but it was still the second highest producing IAA strain isolated. So strain I4 and I1 were selected for further evaluation. The identities for both strains were *Paenibacillus quercus* and *Lysinibacillus sphaericus*. Both species were identified to be IAA producer (145)(140)(58)(146)(147).

Fourteen bacteria strains isolated were IAA producing in a study of ginseng plant (*Panax ginseng* C.A. Meyer) in Korea. Among these bacteria strains, *Micrococcus luteus* and *Lysinibacillus fusiformis* are considered the better producer which produced IAA concentration from 0.31µg/mL to 13.93µg/mL (148). Another study on isolation of IAA producing microbes from root of *Prosopis strombulifera* carried out in Argentina showed that *Pseudomanas putida*, *Bacillus pumilus* and *Bacillus subtilis* produced IAA in the range from 0.5µg/mL to 2.2µg/mL (204).

3.3.3 Antagonist test - antimicrobial and antifungal activities of selected strains

Antimicrobial and antifungal test were carried for the following 8 strains;

1. *Paramyrothecium roridum,* P4
2. *Tolypocladium* sp. *1 WL-2011,* P2
3. *Lysinibacillus fusiformis,* K1
4. *Rhizochaete filamentosa,* K3
5. *Lysinibacillus sphaericus,* N2
6. *Bosea robiniae,* N4
7. *Paenibacillus quercus,* I4
8. *Lysinibacillus sphaericus,* I1

These strains were selected based on their quantitative bioassay analysis in section 3.3.2 and the growth properties of the strains. Cross-streaking technique was used to screen for the antimicrobial activity in this study due to the ease and relatively express method for screening. Two of the selected strains, *Tolypocladium sp. 1 WL-2011,* P2 and *Rhizochaete filamentosa,* K3 were fungus, while *Paramyrothecium roridum,* P4 was yeast and the others were bacteria. The antimicrobial and antifungal activities for each of the isolates were investigated in this study. The inhibitions among each isolates were also tested. However, no obvious antifungal or antimicrobial activity was observed. The results of inhibitions for each isolates were recorded as shown in figure 28. The results obtained here functioned as a parameter for experimental design in pot trial section that will be further discussed in chapter 4.

**Note: The label for isolates shown in figure 28.

S - *Lysinibacillus sphaericus,* N2

T - *Bosea robiniae,* N4

U - *Paenibacillus quercus,* I4

V - *Lysinibacillus sphaericus,* I1

W - *Paramyrothecium roridum,* P4

X - *Tolypocladium* sp. *1 WL-2011,* P2

Y - *Lysinibacillus fusiformis,* K1

Z - *Rhizochaete filamentosa,* K3

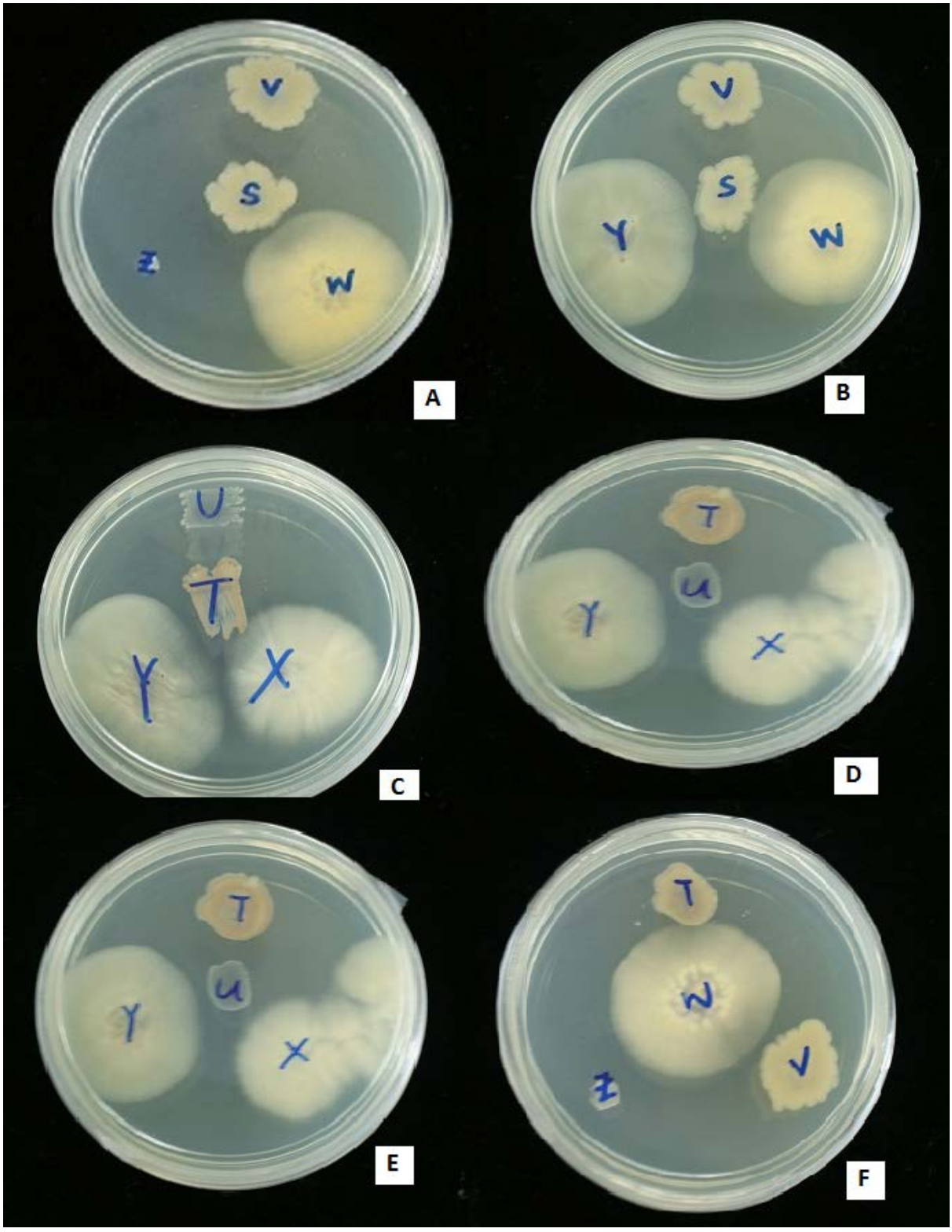

Figure 26: Evaluation of growth inhibition for all 8 selected strains against each other. Cross streaking of all 8 isolates against each other on Nutrient Agar plates (NA) at 28°C.

Table 21: Evaluation of growth inhibition for all 8 selected strains against each other. Cross streaking of all 8 isolates against each other on Nutrient Agar plates (NA) at 28°C.

Inhibition Test								
	P2	P4	K1	K3	N2	N4	I1	I2
P2	N/A	N/A	++	-	-	-	-	-
P4	N/A	N/A	-	-	-	-	-	-
K1	++	-	N/A	N/A	-	-	-	-
K3	++	++	N/A	N/A	++	++	++	++
N2	-	-	-	-	N/A	N/A	-	-
N4	-	-	-	-	N/A	N/A	+	-
I1	-	-	-	-	-	+	N/A	N/A
I4	-	-	-	-	-	-	N/A	N/A

Inhibition characteristic: - no inhibition + partial inhibition ++ strong inhibition

From the results, it was found out that strain K3, *Rhizochaete filamentosa*, was inhibited by all the other strains. From the figure 28, section (A) and (F), it was observed that culture Z was not growing. Besides, strain P2, *Tolypocladium* sp. *1 WL-2011,* was also inhibited by strain K1, *Lysinibacillus fusiformis*. On the other hand, a slight inhibition was observed on strain I1, *Lysinibacillus sphaericus*. The inhibition was caused by strain N4, *Bosea robiniae*. The rest of the strains were showing no sign of inhibition. Therefore, strains that were showing signs of inhibition were excluded for further testing. Since strains P4 and K3 were excluded, strains P2 and K1 were selected to represent their own function group for further testing. Strain N2 and I4 were selected to represent their own group due to their nitrogen fixing and IAA production ability were

the highest from their own respective group. The four selected strains were tested for growth rate and total plate and later on accounted for pot trial experimental design that will be discussed in chapter 4. The following were the selected strains;

Lysinibacillus sphaericus, N2
Paenibacillus quercus, I4
Paramyrothecium roridum, P4
Lysinibacillus fusiformis, K1

3.3.4 Growth rate and total plate count

Eight selected isolated strains from section 3.3.3 were chosen for the evaluation of growth kinetics. The selected strains were. All the strains went through indirect growth measurement technique with the use of spectrophotometer to get the optical density reading, and also direct growth measurement technique, which is also known as the plate count technique. The growth kinetics characteristics of all the 8 selected strains were summarized in Table 22 below.

Table 22: Growth kinetics characteristics of the selected isolated strains grown in Nutrient broth at 28 °C, 150 rpm.

Strains	Specific growth rate, k	Doubling time, td	Absorbance value of microbe at 24 hours	Colony forming unit at 24 hours
	[hr^{-1}]	[hr]	OD_{600}	CFU/mL
Paramyrothecium roridum, P4	0.117	4.08	0.018	3.72×10^3
Lysinibacillus fusiformis K1	0.322	2.15	1.552	4.36×10^7
Lysinibacillus sphaericus N2	0.297	2.33	1.631	5.43×10^7
Paenibacillus quercus I4	0.301	2.30	1.589	5.12×10^7

The growth kinetics characteristic of all the four strains, *Paramyrothecium roridum*, *Lysinibacillus fusiformis*, *Lysinibacillus sphaericus*, and *Paenibacillus quercus* were evalueated based on Optical Density (OD) through spectrophotometer assay and total plate count techniques. The specific growth rates (k) for all the isolates were presented. The doubling time for each isolate were calculated based on the growth rate obtained.

The absorbance values (OD) at 24 hours of all isolates were taken. The colony forming units per millilitres (cfu/mL) at 24 hours were also noted. All these values were essential as a reference for the experimental design of pot trial in chapter 4.

3.4 Summary

This chapter focused on the efficiency of the isolates obtained from chapter 2 as potential plant growth promoting microorganisms. The clear zone formations and optical density reading of nitrogen fixation, IAA production, and phosphate and potassium solubilisation were calculated in section 3.3.1. The selected strains from each prospect were then further evaluated through quantitative bioassay analysis. Their respective efficiencies were recorded in section 3.3.2. Out of the 20 strains, two strains with the highest efficiency from each respective group were selected for antagonistic test to check for inhibition among each strain.

The strains were *Lysinibacillus sphaericus,* N2*, Bosea robiniae,* N4, *Paenibacillus quercus,* I4, *Lysinibacillus sphaericus,* I1, *Paramyrothecium roridum,* P4, *Tolypocladium sp. 1 WL-2011,* P2, *Lysinibacillus fusiformis,* K1, and *Rhizochaete filamentosa,* K3. It was found out that strain *Rhizochaete filamentosa*, K3, was inhibited by all the other strains. At the same time, another strain, *Paramyrothecium roridum*, P4, was also inhibited by strain *Lysinibacillus fusiformis*, K1. Due to inhibitions, strains that were not affected by inhibition and had the best efficiency from each respective group were then selected for growth rate and total plate count. The four strains were *Lysinibacillus sphaericus,* N2, *Paenibacillus quercus,* I4**,** *Paramyrothecium roridum,* P4, and *Lysinibacillus fusiformis,* K1. The growth kinetics of each strain was recorded and the results obtained served as the reference for experimental design of pot trial which will be further discussed in chapter 4. The results of the quantitative analysis conducted in this chapter proposed that the final four selected strains were potentially efficient in promoting plant growth. The efficiency of these 4 selected isolates will be further evaluated and discussed under pot trial condition in chapter 4.

CHAPTER 4:

POT TRIAL EVALUATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA ON THE GROWTH OF *EUCALYPTUS PELLITA* SAPLINGS

4.1 Introduction

Biofertilizer is playing a crucial role in general agricultural production in order to reduce the use of chemical fertilizer. Biofertilizers contained living microorganisms that colonize the rhizosphere or interior of the plants that forms a beneficial relationship to the plant itself through plant growth by increasing nutrient availability. Plant growth promoting rhizobacteria have been used globally as component in biofertilizer, which lead to improvement in crop yied and soil fertility (149).

The study of plant interaction with plant associated microorganisms and the environmental condition is a key objective in plant ecology. Unfortunately, due to the complication of natural plant populations, various logistic and analytical limitations ascend in studying the interaction and responses of plants in the field (150). The advantage of pot experiment is it allows direct measurement in under controlled conditions without the influence of distracting biotic and abiotic factors, such as heterogeneous environmental conditions (for example, light and soil properties), and species *in situ* vary in size, age and lavishness (151)(152)(153). Impact of root and shoot competition in species mixtures are worth studying through pot experiment. It would be helpful in separating both the above and below ground competition in identifying which type of competition has a better influence on plant growth(154). Nevertheless, pot experiment is not as effective as field experiment in the evaluation of belowground interaction due to destructive yields that quantify root productivity and distribution that would be hardly feasible in natural standpoints (151).

Based on the quantitative analysis studied in previous chapter, 4 microorganisms were selected to be used in this pot trial experiment study. The selected strains were *Lysinibacillus* spp., *Paenibacillus* spp. and *Paramyrothecium* spp. Studies have shown that these species were effective in promoting plant growth except *Paramyrothecium* spp. No information of plant growth promoting was available for *Paramyrothecium* spp. even though this strain was found to exhibit plant growth promoting ability.

4.2 Materials and Methodology

4.2.1 Preparation of bacteria and fungi consortia culture

The following microorganisms were selected for testing in the pot trials;

1) *Lysinibacillus sphaericus,* N2
2) *Paenibacillus quercus,* I4
3) *Paramyrothecium roridum,* P4
4) *Lysinibacillus fusiformis,* K1

Nutrient Broth (NB) and Potato Dextrose Broth (PDB) were prepared in 250ml Erlenmeyer shake flasks and sterilized at 121 °C for 15 minutes. The four selected plant growth promoting microorganisms were inoculated into sterilized NB and PDB (bacteria in NB while fungus in PDB). Cotton stoppers were used to cover the opening of the flask to prevent contamination. The flasks with the cultures were then incubated in an incubator shaker at 30°C with 120 rpm speed. Bacteria cultures were incubated for 3 days while fungus culture was for 4 days. After the incubation period, the growth cultures were mixed together to form as a starter consortia culture to be used in pot trials.

4.2.2 Preparation of *Eucalyptus pellita* seedlings and experimental design

Eucalyptus pellita seedlings and the sowing medium were provided by Samling Sdn. Bhd. (Bintulu, Sarawak, Malaysia). The sowing method, transplanting, fertilization, watering, and saplings hardening were carried out according to the practice of Sarawak Forestry Corporation, Kuching, Sarawak, Malaysia (SFC). The pot trials preparations were carried out in the planting compartment of SFC.

Seedlings of *Eucalyptus pellita* were germinated on sawdust compost without addition of fertilizer, in the green house of SFC. The seedlings were left in the greenhouse for germination up to 6 weeks. After 6 weeks, the seedlings were transplanted to 6” x 6” polybags. The seedlings were left for growth for another 6 weeks under shaded area.

After that, when the seedlings were strong enough for hardening, all the seedlings were moved to area that was exposed to sunlight for hardening process before transplanting to bigger polybag for pot trial experiment. The hardening process took 3 months with application of tiny amount of fast-released chemical fertilizer (basal fertilizer) depending on the health of the seedlings.

Figure 27: The process of pot trial preparation; germination of *Eucalyptus pellita* seeds (top left), seedlings transplanted to 6x6 polybags (top right), and 6 months old saplings prepared for pot trials (bottom).

4.2.3 Experimental design of pot trial by using saplings of *Eucalyptus pellita*

Finally, after 6 months, 180 saplings with the most uniform growth were chosen for pot trials. Top soils provided by SFC were crushed and mixed evenly with sand to obtain sandy clay texture, which is the preferable soil type for *Eucalyptus pellita*. The mixtures were then packed into 12" x 14" polybag. All the selected 6 months old saplings were transferred into the polybags. The 180 selected saplings were divided into 6 groups, each containing 30 saplings. Below is the setup of the pot trial's treatment for each group.

Table 23: The saplings divided into 6 different groups with the description of the treatment to each of them.

Treatment Group	Description
T1	Negative control – No fertilizer or consortia culture added
T2	Positive control – 15g of chemical fertilizer.
T3	Consortia of NPK cultures
T4	Consortia of NPK + IAA cultures
T5	Consortia of NPK cultures with 7.5g of chemical fertilizer
T6	Consortia of NPK + IAA cultures with 7.5g of chemical fertilizer

**Note:

*N – Nitrogen fixing microorganism

*P – Phosphate solubilizing microorganism

*K – Potassium solubilizing microorganism

*IAA – IAA producing microorganism

*Chemical fertilizer composition – N:18, P:13, K:13

Two treatments were chosen to be the control group of this pot trial experiment. Nothing was added to T1, the saplings of negative control's group while 15g of chemical fertilizer was added to T2, which acted as the positive control. The remaining 4 groups were treated with the prepared consortia cultures and chemical fertilizer. Four mL from each selected culture were transferred into a container and mixed well before added into their respective treatment groups. T3 was treated with only NPK cultures while T4 was treated with NPK and IAA cultures. The setup for T5 and T6 were the same as T3 and T4, but with the addition of 7.5g chemical fertilizer (which is half, 50%, compared to positive control, T2). A randomised block planting design was conducted for the pot trial.

Initial physical examination was carried out and recorded on all the saplings prior to the start of pot trial experimentation. The measurements and observation were as follow;

1) The height of saplings. Measured from the base of the saplings, from above the surface of soil to the end of top shoot.
2) The stem diameter. Measured from the stem just right above the soil surface.
3) The viability of the saplings. Physical appearances of the saplings were noted to avoid selection of unhealthy saplings.

The pot trials were carried out in the nursery of SFC. The duration of pot trials were 6 months long. No additional chemical fertilizer was added to the plant during this 6 months period no matter how unhealthy the saplings were. Standard watering to the saplings was carried out by SFC staffs on fixed timing every day. Physical examination was carried out on the saplings once a month throughout the 6 months experiment period. After the 6 months period, the saplings were uprooted and cleaned off any remaining soil attached to the root of the saplings. The wet and dry weights for all the saplings were analysed and recorded. The wet weights of the saplings were measured after the saplings were cleaned. After the wet weights of the saplings were recorded, the saplings were then oven dried by using an oven dryer for 4 days at 68°C. The, the dry weights of the saplings were recorded. The examinations were carried out in the soil laboratory of SFC.

4.2.4 Data analysis

The data collected were analysed by the mean ±SE (standard deviation) of 30 pots replicates from each test group. Experiments results were analysed by using analysis of t-Test (IBM SPSS Statistics v24): Two-sample assuming unequal variances, with statistical significance taken as $p \leq 0.05$. Microsoft Office Excel software was also used for data analysis.

4.3 Results and Discussion

This small scale pot trial was conducted in this study to evaluate the efficiency of the selected plant growth promoting microorganisms in supplying nutrients to the plant. Six months old saplings were chosen to be used in this study. Studies have shown that trees below the age of 12 months are most suitable for pot experiments (155)(156)(157)(158)(159).

In order to study on the growth of the plant, height, stem diameter, wet and dry weight of the plant were targeted. The efficiency of plant growth promoting mechanisms will be able to be observed through the growth of the plants in these segments throughout the whole 6 months period. Plant growth promoting microorganisms are associated to the root of plants, so these microorganisms were added to the soil surrounding the root of the plants.

The four isolated strains *Lysinibacillus sphaericus, Paenibacillus quercus, Paramyrothecium roridum,* and *Lysinibacillus fusiformis* were selected to be used in the pot trial experiment. Evaluation on the efficiency of these selected strains on the growth promoting of *Eucalyptus pellita* was conducted and discussed in this study. The selection of the isolated strains wer based on the evaluation of selective screening and quantitative analysis carried out in chapter 3. The inhibition test of these strains against each other was also part of the consideration in the selection.

The *Eucalyptus pellita* saplings were divided into 6 different groups as described in the methodology section above. Each group was treated with different treatments. Each group consisted of 30 saplings of *Eucalyptus pellita* in order to obtain replicate results. The results obtained from this study will be discussed and presented in the following sections.

**Note:

T1 - Negative control – No fertilizer or consortia culture added

T2 - Positive control – 15g of chemical fertilizer.

T3 - Consortia of NPK cultures

T4 - Consortia of NPK + IAA cultures

T5 - Consortia of NPK cultures with 7.5g of chemical fertilizer

T6 - Consortia of NPK + IAA cultures with 7.5g of chemical fertilizer

** The trend of growth with the results obtained was discussed throughout the sections below. However, supporting references and findings were used to further supports the outcome in the following sections; 4.3.1.1 (d) and 4.3.1.3 (b).

4.3.1 Biofertilizer with the presence of consortia cultures could help to reduce the use of chemical fertilizer by 50%.

The presence of consortia cultures with the application of 50% chemical fertilizer showed significant growth in *Eucalyptus pellita* saplings, which was as well as the application of 100% chemical fertilizer. The height and stem diameter increment on all the treatments were observed after 180 days. Both fresh weight and dry weight for the stems and roots of *Eucalyptus pellita* saplings were also analysed.

4.3.1.1 Growth increments in term of height observed in *Eucalyptus pellita* saplings for 6 different test groups.

The height increment of *Eucalyptus pellita* saplings was observed and measured throughout the 6 months period by using a measuring tape. The parameter in measuring the height increment was measured from the stem of the saplings just above the soil surface until the internode of the first leaves at the top of the stem of *Eucalyptus pellita*. The height of increments of the saplings was recorded in unit centimetre (cm). The average height increment for all 6 treatments of the saplings over the 6 months period were calculated and recorded in tables below. The height measurement was conducted once a month throughout the pot trial experiment.

4.3.1.1(a) Height increment after 30 days. Most of the saplings treated with consortia culture showed significant increment.

Table 24: Mean height of *Eucalyptus pellita* saplings for the 6 different treatments after 30 days.

Height (cm) of *Eucalyptus pellita* saplings in 30 days			
Treatment	Code	Height (cm)	S. D
Negative control	T1	121.53	16.80052
Positive control	T2	128.97	12.84787
NPK	T3	132.37	20.10058
NPK IAA	T4	120.40	19.70052
NPK + F	T5	137.03	20.02151
NPK IAA + F	T6	130.67	17.8699

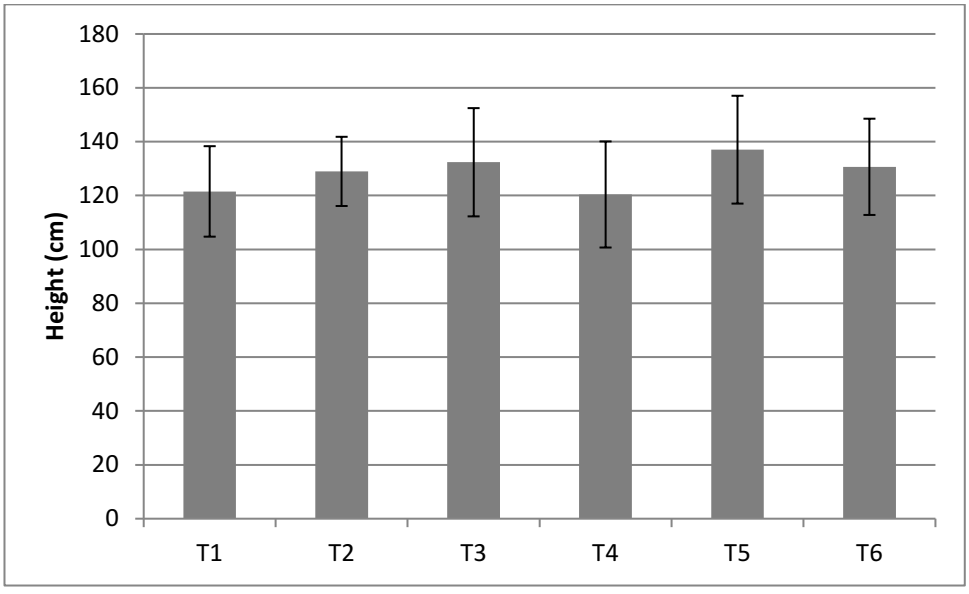

Figure 28: The mean height of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 30 days.

On the first 30 days after the initial addition of consortia cultures and chemical fertilizer to the saplings, 3 treatments group showed signs of higher height increment, compared to positive control. T5, which was treated with NPK consortia culture and 7.5g of chemical fertilizer, was the leading treatment group with 137.03cm, followed by T3, that was trated with NPK consortia culture, with 132.37cm, and T6, that was treated with NPK IAA consortia culture with 7.5g chemical fertilizer, with 130.67cm. Positive control, T2, which was treated with 15g of chemical fertilizer, was following closely behind with 128.97cm. Negative control, which was not treated with anything, T1 and T4, which were only treated with NPK consortia cultures, were showing lesser height increment, with 121.53cm and 120.40cm respectively.

Positive control, T2, which was treated with 15g of chemical fertilizer, was expected to have the highest growth increment due to more nutrient availability to the saplings. However, 3 other treatment groups T5, T3 and T6 surprisingly overtook T2. The reasoning behind for the higher surge of height increment for the 3 treatments with consortia culture and chemical fertilizer might be due to the overwhelming population of plant growth promoting microorganisms added to the saplings. Consortia cultures with plant growth promoting microorganisms were showing vital signs of improving plant growth in this stage of experiment. With the aid of chemical fertilizer, as for the case of T5, it proved to be much more effective. Even though the amount of chemical fertilizer added was only half compared to positive control.

IAA producing microorganisms might had altered the interaction of other microorganisms with the plant, which also focused more on the root of the plant, hence contributing to lower growth increment among the 3 leading treatment groups. As it can be seen for the case of T4, without the present of chemical fertilizer, it showed the least improvement. Therefore, it is believed that IAA producing microorganisms might have affected the interaction of other microorganisms and the plant.

4.3.1.1(b) Height increment after 60 days. A few treatment with consortia cultures showed a slight drop in efficiency.

Table 25: Mean height of *Eucalyptus pellita* saplings for the 6 different treatments after 60 days.

Height (cm) of *Eucalyptus pellita* saplings in 60 days			
Treatment	Code	Height (cm)	S. D
Negative control	T1	133.27	14.9895
Positive control	T2	149.03	13.97407
NPK	T3	142.27	19.04248
NPK IAA	T4	131.70	19.10795
NPK + F	T5	161.60	17.28384
NPK IAA + F	T6	149.83	19.09813

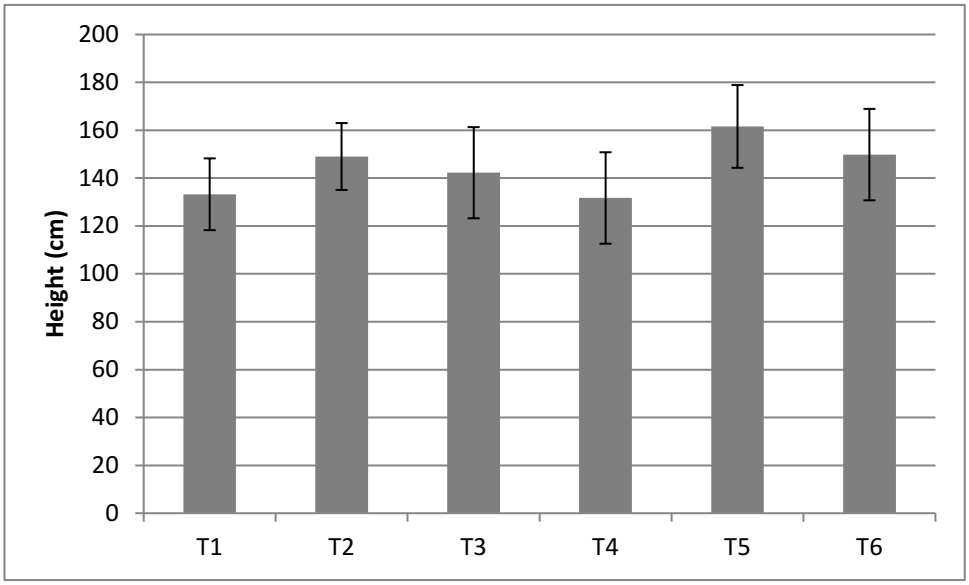

Figure 29: The mean height of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 60 days.

One the second month after the initial addition, only 2 treatment groups were showing signs of significant increment in height. T5 and T6 that were treated with consortia culture and chemical fertilizer had higher increment compared to positive control with 161.60cm and 148.83cm. Positive control was following closely behind with 149.03cm. T3 that overtook positive control in the previous month had lowered down in this month's increment, with 142.27cm. Nevertheless, it was still quite impressive that it had more increment compared to negative control. T1 and T4 showed the least sign of increment with 133.27cm and 131.70cm respectively.

Sudden dropped in growth efficiency was observed in the second month of experiment, even though it was not very significant as of yet. The reasoning of the sudden drop might be due to the constant watering applied to the saplings. The consortia cultures added to the saplings was without carrier materials that would made the cultures to attach to the roots of the plant, nor had the ability to remain in the soil for long term without being washed away. The constant watering might had washed away some of the cultures, which resulted in decreased in efficiency. Furthermore, the soil area that was packed to the root of the saplings was very limited. Consortia cultures could have been washed away easily.

4.3.1.1(c) Height increment after 90 days. Saplings treated with consortia cultures showed sign of comparable increment to their respective control group.

Table 26: Mean height of *Eucalyptus pellita* saplings for the 6 different treatments after 90 days.

Height (cm) of *Eucalyptus pellita* saplings in 90 days			
Treatment	Code	Height (cm)	S. D
Negative control	T1	147.97	18.76071
Positive control	T2	193.07	20.85737
NPK	T3	148.37	31.76474
NPK IAA	T4	143.13	18.03968
NPK + F	T5	200.77	17.57679
NPK IAA + F	T6	189.20	18.7771

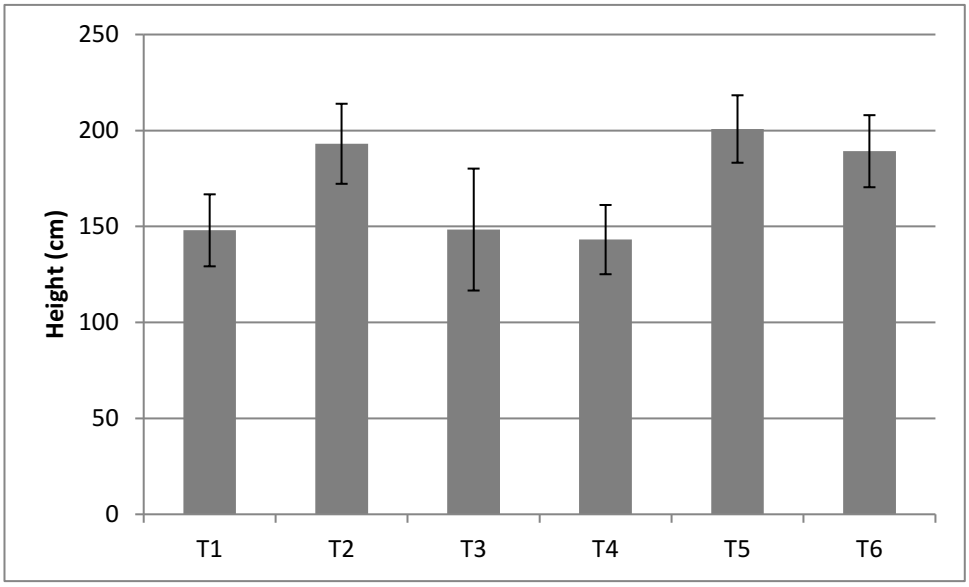

Figure 30: The mean height of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 90 days.

On the third month of the pot trial, the same declining trend to the second month was observed on all 4 treatment groups that were treated with consortia culture and chemical fertilizer. After 90 days of treatment, only one treatment group overtook positive control, which is T5 with 200.77cm. Followed by positive control T2, with 193.07cm. Positive control was catching up to T5. T6 which had better increment than positive control in the previous month now had lower increment, which is 189.20cm. T3, which had more increment in the previous month, was slowly being caught up by negative control. T3 was followed closely by T1 and lastly T4, with 148.37cm, 147.97cm and 143.13cm respectively.

Further signs of declining trends from the second month were observed in this month's increment. T3 which was slightly lowered than positive control in the previous month was now lowered by a lot. It was now slightly higher than negative control by just a little bit. Needless to say, T4 was declining even more compared to the first and second month. T6 that was slightly higher than positive control is now lower than positive control. T5 which had better gap of increment in the second month was also showing sign of closer gap in this month's increment. Signs of consortia cultures being washed away were continuing.

4.3.1.1(d) Height increment from day 120 to 180. Trend of parallel growth was observed for saplings treated with consortia culture to their respective control group.

Table 27: Mean height of *Eucalyptus pellita* saplings for the 6 different treatments after 120 days.

Height (cm) of *Eucalyptus pellita* saplings in 120 days			
Treatment	Code	Height (cm)	S. D
Negative control	T1	158.63	24.1125
Positive control	T2	225.97	33.15739
NPK	T3	161.67	19.83611
NPK IAA	T4	157.70	17.97345
NPK + F	T5	229.17	20.8477
NPK IAA + F	T6	225.79	18.72809

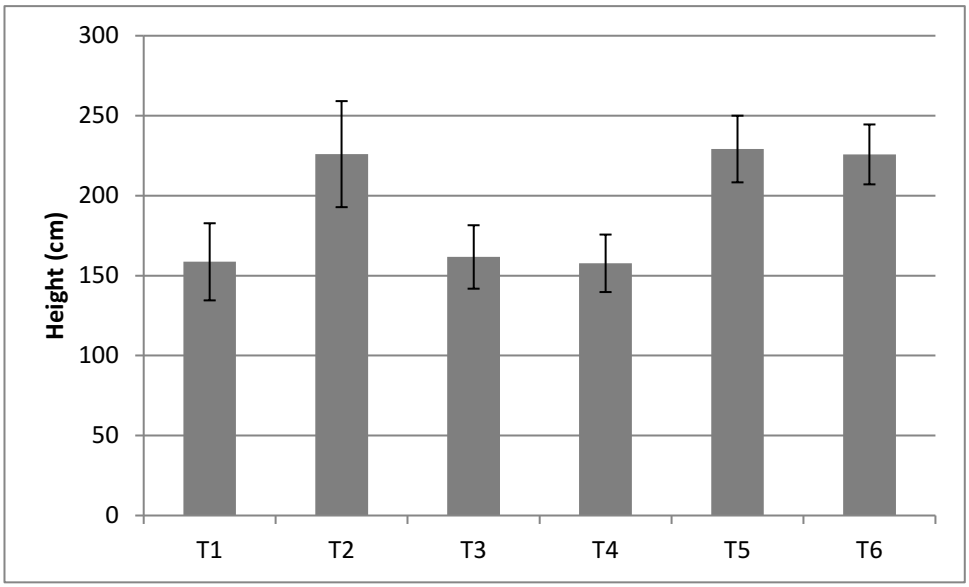

Figure 31: The mean height of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 120 days.

Table 28: Mean height of *Eucalyptus pellita* saplings for the 6 different treatments after 150 days.

Heigh (cm) of *Eucalyptus pellita* saplings in 150 days			
Treatment	Code	Height (cm)	S.D
Negative control	T1	169.77	28.83566
Positive control	T2	253.83	36.99216
NPK	T3	173.33	21.9456
NPK IAA	T4	167.00	34.9344
NPK + F	T5	257.17	22.27119
NPK IAA + F	T6	250.17	20.59311

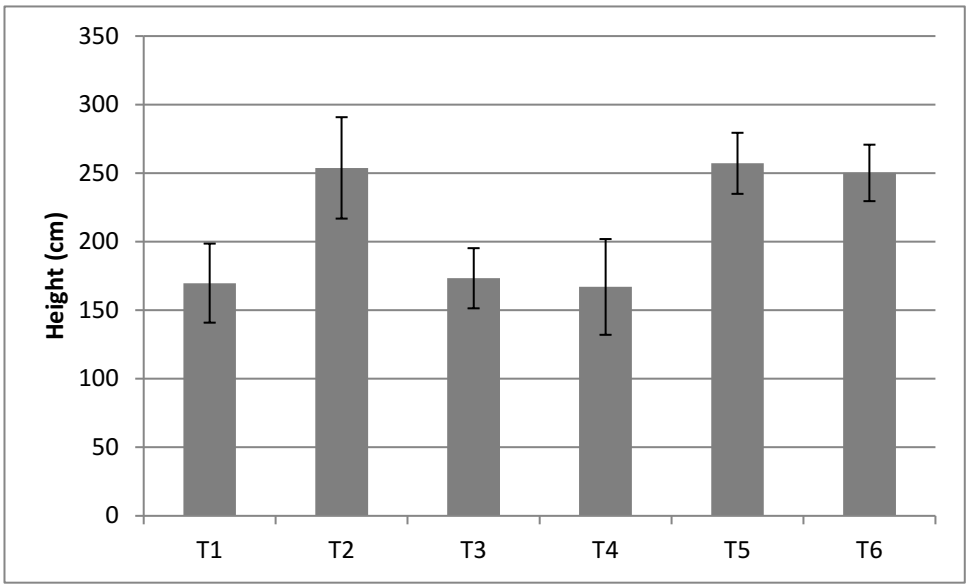

Figure 32: The mean height of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 150 days.

Table 29: Mean height of *Eucalyptus pellita* saplings for the 6 different treatments after 180 days.

Height (cm) of *Eucalyptus pellita* saplings in 180 days			
Treatment	Code	Height (cm)	S.D
Negative control	T1	178.00	31.82929
Positive control	T2	260.50	44.10704
NPK	T3	187.17	24.97182
NPK IAA	T4	184.17	27.38875
NPK + F	T5	279.50	21.94625
NPK IAA + F	T6	252.76	33.36985

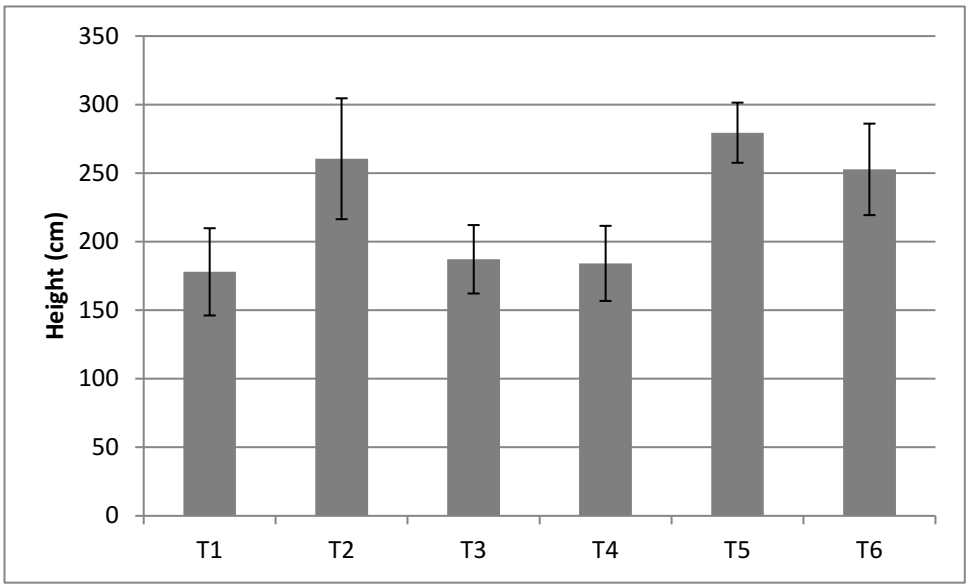

Figure 33: The mean height of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 180 days.

After the 90 days treatment, each treatment that had been treated with consortia cultures or also with chemical fertilizer, were starting to settle down in the fourth month. From Table 27, it could be observed that T3 and T4, which were treated with only consortia cultures, were showing more or less the same increment in height compared to negative control, T1. While on the other hand, T5 and T6, which were treated with consortia culture and chemical fertilizer, were showing more or less increment in height that was comparable to positive control T1. The same trend was carried over to the following month (the fifth month). Same trend in height increment was observed. More or less the trend values were observed on Table 28.

The consortia cultures that were not being washed away were getting acclimatized to the environment in the soil and also to the roots of saplings. The leftover cultures might be getting attached to the roots of the saplings during this period of time in order to continue surviving and also to supply nutrients to the plant at the same time. The height increments were quite proportional during this two month's period. The culture's microorganisms underwent multiplication process to increase their population during this period.

In the final month of pot trial, things turned out to be better. T3 and T4 which were treated with only consortia cultures showed sign of higher increment in height compared to T1, negative control. T1 had the lowest increment with 178cm while T3 and T4 were getting better, with 187.17cm and 184.17cm respectively. On the other hand, even though T6, with 252.76cm was still lower in increment compared to positive control T2 with 260.50cm, however, T5 was showing signs of significant improvement. T5 had the highest increment with 279.50cm.

After period of acclimatization, the consortia cultures were showing signs of improving plant growth. The leftover cultures might have increased in population, therefore resulting in increased plant height. More population lead to more nitrogen that had been fixed, more solubilized phosphate and potassium, and also production of IAA increased too, which were beneficial to the plant growth. Research showed that the dosage of response effect is affected by the size of the population of microbes (160). The reason for T3 and T4 to had lower height increment compared to T5 and T6 was because of the

initial minerals available in the pot was not sufficient enough. Study showed that inconsistent results yield when compared under different environment condition. Effects of plant growth promotion are directly correlated to the equilibrium of the environment (160). Therefore, the more the starting resources for these microbes to synthesize, the better the results yield. T5 and T6 were treated with 7.5g of chemical fertilizer. The consortia cultures were able to utilize the additional minerals to synthesize the required nutrients for the plant. The consortia culture in T3 and T4 had limited minerals from their surrounding in order to synthesize, therefore the progress was significantly slower. Nevertheless, T6 was still having lower height increment compared to positive control. As mentioned earlier, IAA producing microorganisms might had produced too much IAA for the plant, which affected more on the growth of root instead of what was on above the ground. Studies showed that amount of IAA provided to plants exceeding a certain dosage may be harmful to plants, which affect the growth of plants (161)(162). A study conducted showed that the number of flower produced, leaves, and dry weight increased in cowpea plant when IAA of 50mg and below was treated to the plant. Whereas the number decreased when more IAA was treated (161). Another study proven that maximum growth was observed at $10mgL^{-1}$ on Vinca (*Catharanthus roseus L.*) seedlings (162).

Nitrogen is responsible for plant growth and development (cell division that affect stem growth). Studies have proven that nitrogen fixing microorganisms are capable of promoting plant growth (163)(164)(23). Two bacteria strains, *Azospirillum* and *Bacillus* spp. showed association in the root of banana plant for nitrogen fixing, which resulted in the growth of shoot and photosynthetic activity of banana plant through minimal use of nitrogen fertilizer (33% of nitrogen content instead of 100%) (163). The same strains were also proven to contribute to 20 – 50% of total nitrogen requirement for oil palm seedlings. Inoculation of these strains improved the growth of the oil palm seedlings that were comparable to the use of inorganic nitrogen fertilizer (164). Another study showed that *Bacillus* spp. has the potential to prove the growth and yield of raspberry plant (23).

Phosphate is responsible for flower initiation, seed, roots and fruit development. Phosphate solubilizing microbe *Rhizobium leguminosarum* was reported to be able to

improve the dry matter of shoots and roots, in addition to the uptake of nitrogen and phosphate in both carrots and lettuce (165). A study found that inoculation of *Bacillus megaterium* with integrated rock phosphate increase the availability of phosphate in soil, uptake of phosphate in roots and the growth of cucumber and pepper (83).

Potassium does not form any important organic compounds in plants, unlike nitrogen and phosphate. Nonetheless, potassium acts as enzyme activator that promotes metabolism for early plant growth. *Bacillus edaphicus* was found to improve shoot growth of wheat significantly. Nitrogen, phosphate and potassium were also significantly higher in the plant component (166). *Bacillus mucilaginosus* promote growth of eggplant through increasing the availability of potassium in the soil, and also improving the uptake of potassium (167). *Bacillus mucilaginosus* was also found to increase the availability of potassium in soil, uptake of potassium in roots and the growth of cucumber and pepper (83)

4.3.1.1(e) Trend of growth in height (cm) throughout the 180 days period

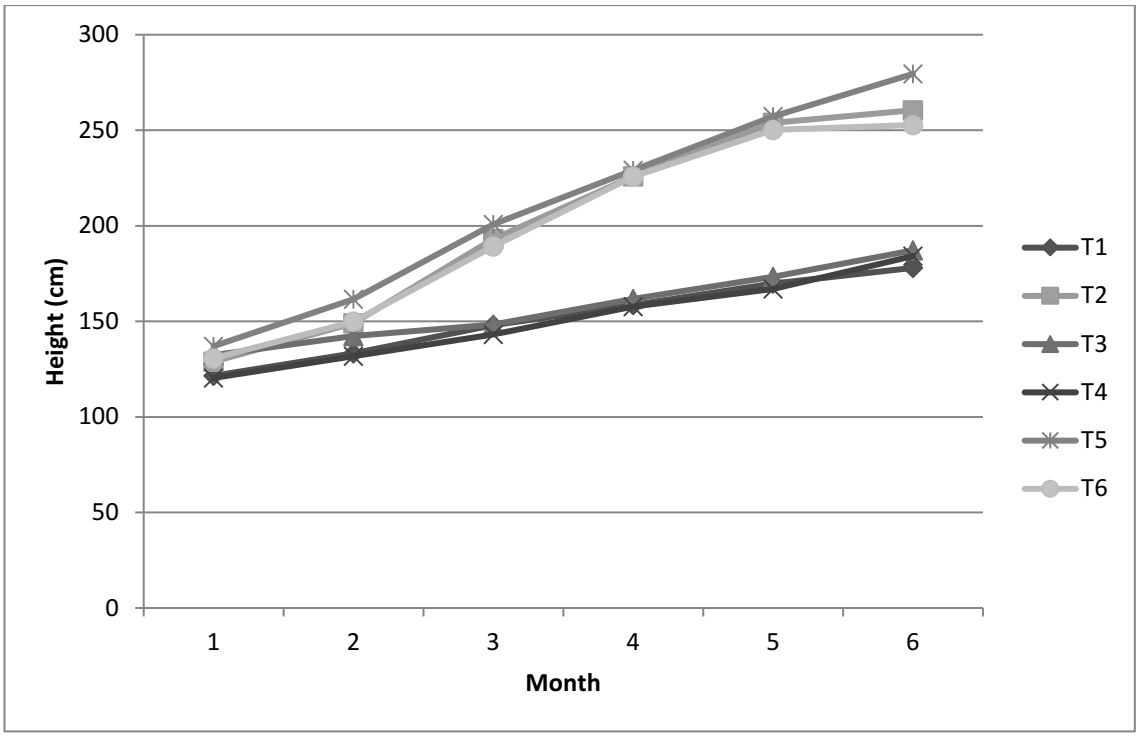

Figure 34: The trend of height increment of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments from day 30 to day 180.

As mentioned earlier, most of the treatments with consortia culture, with and without the addition of chemical fertilizer, were having better height increment in the starting month. T5 and T6 were leading the field compared to positive control, T1. In contrast, T3 and T4 were also performing better compared to negative control. The height increase was slower in the second and third month due to washing off of consortia cultures; until they were more or less the same with their respective control group (T5 and T6 were compared to positive control while T3 and T4 were compared to negative control). In the fourth and fifth month, all the remaining consortia cultures that were not being washed off, were acclimatizing to the environment and started reproducing to increase population. The height increase was proportional to their control group. In the final month, every treatment with consortia cultures, with or without addition of chemical fertilizer was producing better, except T6. T5, that was tread with consortia culture of NPK microorganisms and 7.5g chemical fertilizer was leading the way with significant increase in height. T3 and T4 were much higher compared to negative

control T1. T6 was slightly lower compared to positive control T2. Nevertheless, the trend of height increment was moving in the positive direction.

4.3.1.2 Growth increments in term of stem diameter observed in *Eucalyptus pellita* saplings for 6 different test groups

The stem diameter increment of *Eucalyptus pellita* saplings was observed and measured throughout the 6 months period by using a vernier caliper. The parameter in measuring the stem diameter increment was measured from the stem of the saplings just above the soil surface. The stem diameter increment of the saplings was recorded in unit centimetre (cm). The average steam diameter increment for all 6 treatments of the saplings over the 6 months period were calculated and recorded in tables below. The stem diameter measurement was conducted once a month throughout the pot trial experiment.

4.3.1.2(b) Stem diameter growth after 30 days. All saplings treated with consortia cultures showed significant growth.

Table 30: Mean stem diameter of *Eucalyptus pellita* saplings for the 6 different treatments after 30 days.

Stem diameter (cm) of *Eucalyptus pellita* saplings in 30 days			
Treatment	Code	Diameter (cm)	S. D
Negative control	T1	0.8317	0.071297
Positive control	T2	0.8917	0.121828
NPK	T3	0.9433	0.138174
NPK IAA	T4	0.8933	0.143679
NPK + F	T5	0.9617	0.117945
NPK IAA + F	T6	0.9533	0.113664

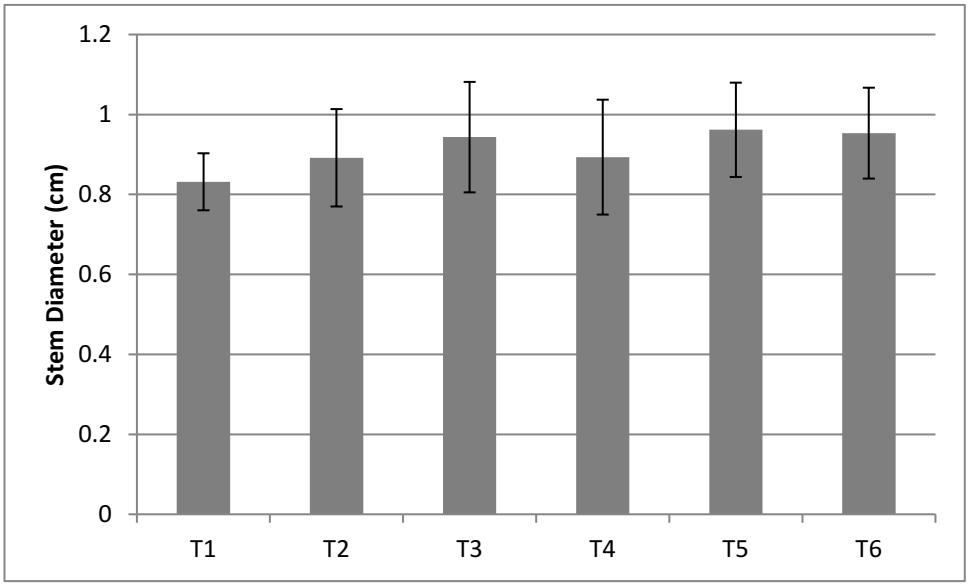

Figure 35: The mean stem diameter of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 30 days.

A very similar trend of increment was observed in stem diameter compared to height increment. On the first 30 days after the initial addition of consortia cultures and chemical fertilizer to the saplings, all 4 treatments group showed signs of higher stem diameter increment, compared to positive control. T5, which was treated with NPK consortia culture and 7.5g of chemical fertilizer, was the leading treatment group with 0.9617cm, followed by T6, which was treated with NPK and IAA consortia culture with 7.5g chemical fertilizer, with 0.9533cm. T3, that was treated with NPK consortia culture, with 0.9433cm, and also T4 that was treated with NPK and IAA consortia culture, at 0.8933cm. Positive control, T2, which was treated with 15g of chemical fertilizer, was following closely behind with 0.8917cm. Negative control, which was not treated with anything, T1 was showing the least stem diameter increment, with 0.8317cm.

As previously mentioned in 30 days height increment, a similar trend was observed, hence, the observation was also about the same. Positive control, T2, which was treated with 15g of chemical fertilizer, was expected to be leading the field due to more nutrient availability to the saplings. However, all 4 other treatment groups T3, T4, T5 and T6, surprisingly overtook T2. The reasoning behind for the higher surge of height increment for the 3 treatments with consortia culture and chemical fertilizer might be due to the overwhelming population of plant growth promoting microorganisms added to the saplings. Consortia cultures with plant growth promoting microorganisms were showing vital signs of improving plant growth in this stage of experiment. With the aid of chemical fertilizer, as for the case of T5 and T6, it proved to be much more effective. Even though the amount of chemical fertilizer added was only half compared to positive control.

IAA producing microorganisms might have altered the interaction of other microorganisms with the plant, which also focused more on the root of the plant. Both T4 and T6 treatments were showing lower increment compared to their counterpart, without IAA producing culture, T3 and T5. Therefore, it is believed that IAA producing microorganisms might have affected the interaction of other microorganisms and the plant.

4.3.1.2(b) Stem diameter growth after 60 days. A declining trend in growth efficiency was observed on saplings treated with consortia culture

Table 31: Mean stem diameter of *Eucalyptus pellita* saplings for the 6 different treatments after 60 days.

Stem diameter (cm) of *Eucalyptus pellita* saplings in 60 days			
Treatment	Code	Diameter (cm)	S. D
Negative control	T1	0.9033	0.074201
Positive control	T2	1.0883	0.16011
NPK	T3	1.0083	0.14208
NPK IAA	T4	0.9617	0.127768
NPK + F	T5	1.1350	0.158196
NPK IAA + F	T6	1.0757	0.235631

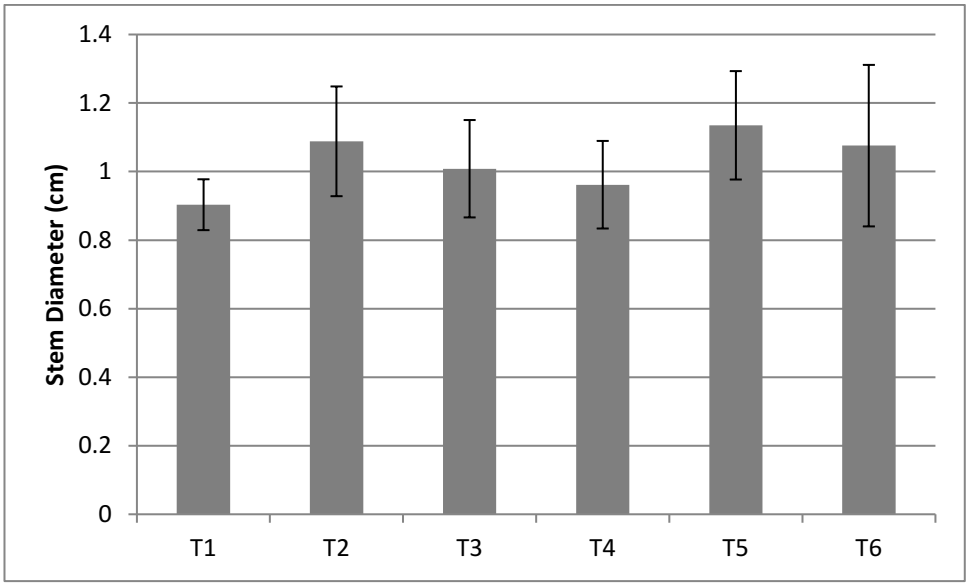

Figure 36: The mean stem diameter of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 60 days.

One the second month, only 1 treatment groups was showing signs of higher increment stem diameter compared to positive control. T5 that was treated with consortia culture and chemical fertilizer had higher increment with 1.1350cm. Positive control was the second highest with 1.0883cm and followed closely behind by T6, with 1.0757cm. T3 and T4, that overtook positive control in the previous month had lowered down in this month's increment, with 1.0083cm and 0,9617cm respectively. Nevertheless, it was still quite impressive that it had more increment compared to negative control. Finally, the negative control, T1 showed the least sign of increment with 0.9033cm.

Sudden dropped in growth efficiency was observed in the second month of experiment, even though it was not very significant as of yet. The same reasoning was observed in the sudden drop of height increment for other treatments in height increment. This might be due to the constant watering applied to the saplings. The consortia cultures added to the saplings was without carrier materials that would made the cultures to attach to the roots of the plant, nor had the ability to remain in the soil for long term without being washed away. The constant watering might had washed away some of the cultures, which resulted in decreased in efficiency. Furthermore, the soil area that was packed to the root of the saplings was very limited. Consortia cultures could have been washed away easily.

4.3.1.2(c) Stem diameter growth after 90 days. Further declining trend was observed in saplings treated with consortia cultures.

Table 32: Mean stem diameter of *Eucalyptus pellita* saplings for the 6 different treatments after 90 days.

Stem diameter (cm) of *Eucalyptus pellita* saplings in 90 days			
Treatment	Code	Diameter (cm)	S. D
Negative control	T1	0.985	0.111533
Positive control	T2	1.240	0.194679
NPK	T3	1.002	0.145912
NPK IAA	T4	0.9433	0.117248
NPK + F	T5	1.197	0.168632
NPK IAA + F	T6	1.172	0.133056

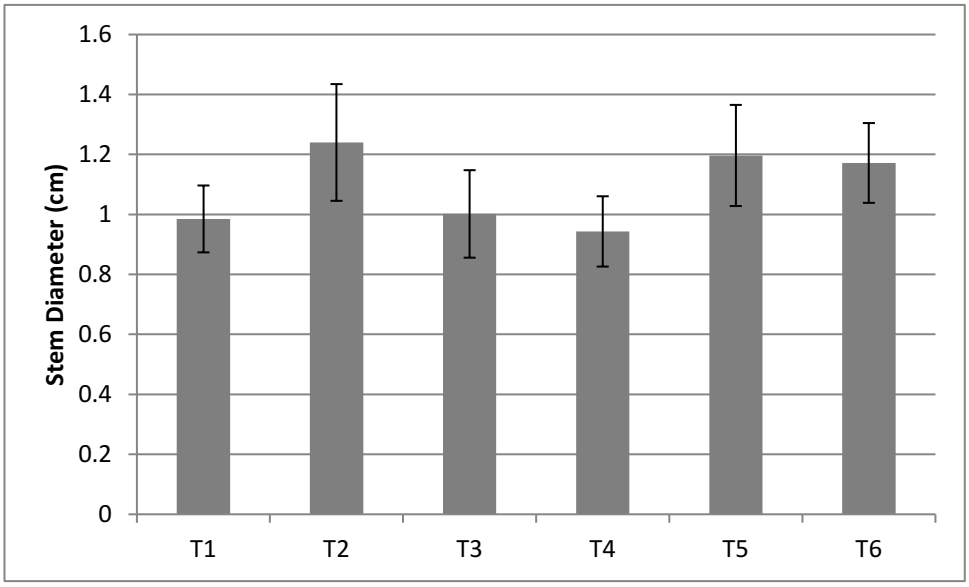

Figure 37: The mean stem diameter of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 90 days.

On the third month of the pot trial, the same declining trend to the second month was observed on all 4 treatment groups that were treated with consortia culture and chemical fertilizer. After 90 days of treatment, all four treatment groups were about the same or lower than the control groups that they were being compared to. Positive control T2 was the highest with 1.240cm, followed by T5 with 1.197cm and T6 with 1.172cm. Both these groups were treated with consortia culture and chemical fertilizer. T3 with 1.002cm was just slightly higher than positive control T2, with .985cm. T4 was the lowest among all treatment groups, with 0.9433cm.

Further signs of declining trends from the second month were observed in this month's increment. T5 and T6 were lower than positive control in this month's stem diameter growth. Positive control T2 overtook both of them and was leading the pack. T3 and T4 that were both higher in stem diameter growth for last month were now relatively comparable to the negative control T1. Signs of consortia cultures being washed away were continuing.

4.3.1.2(d) Stem diameter increment from day 120 to 180. Trend of parallel growth was observed for saplings treated with consortia culture to their respective control group.

Table 33: Mean stem diameter of *Eucalyptus pellita* saplings for the 6 different treatments after 120 days.

Stem diameter (cm) of *Eucalyptus pellita* saplings in 120 days			
Treatment	Code	Diameter (cm)	S. D
Negative control	T1	1.0983	0.236892
Positive control	T2	1.4083	0.291572
NPK	T3	1.1033	0.180006
NPK IAA	T4	1.0250	0.129155
NPK + F	T5	1.3883	0.158486
NPK IAA + F	T6	1.3741	0.112298

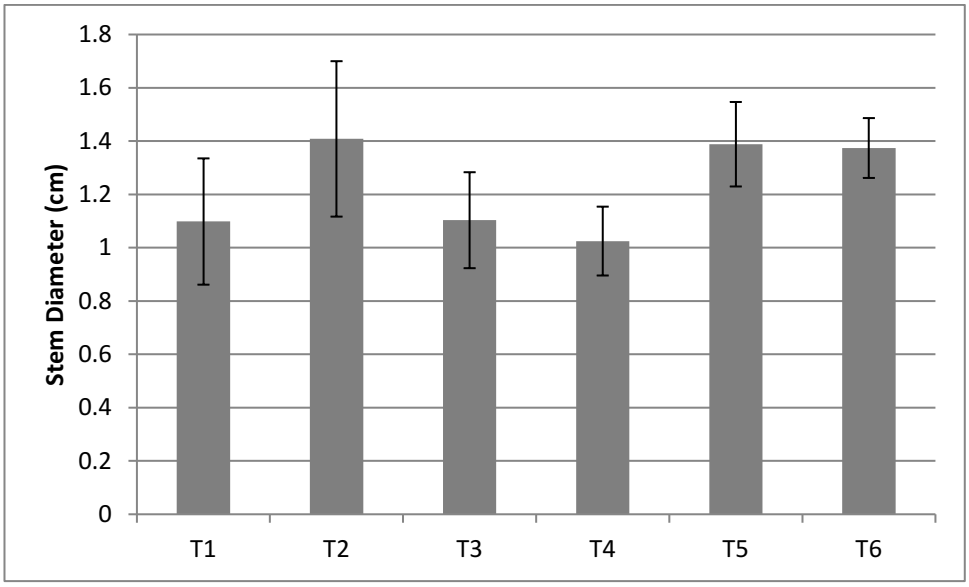

Figure 38: The mean stem diameter of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 120 days.

Table 34: Mean stem diameter of *Eucalyptus pellita* saplings for the 6 different treatments after 150 days.

Stem diameter (cm) of *Eucalyptus pellita* saplings in 150 days			
Treatment	Code	Diameter (cm)	S. D
Negative control	T1	1.0883	0.133703
Positive control	T2	1.4433	0.292355
NPK	T3	1.0750	0.130483
NPK IAA	T4	1.0367	0.128609
NPK + F	T5	1.4147	0.163835
NPK IAA + F	T6	1.4396	0.161676

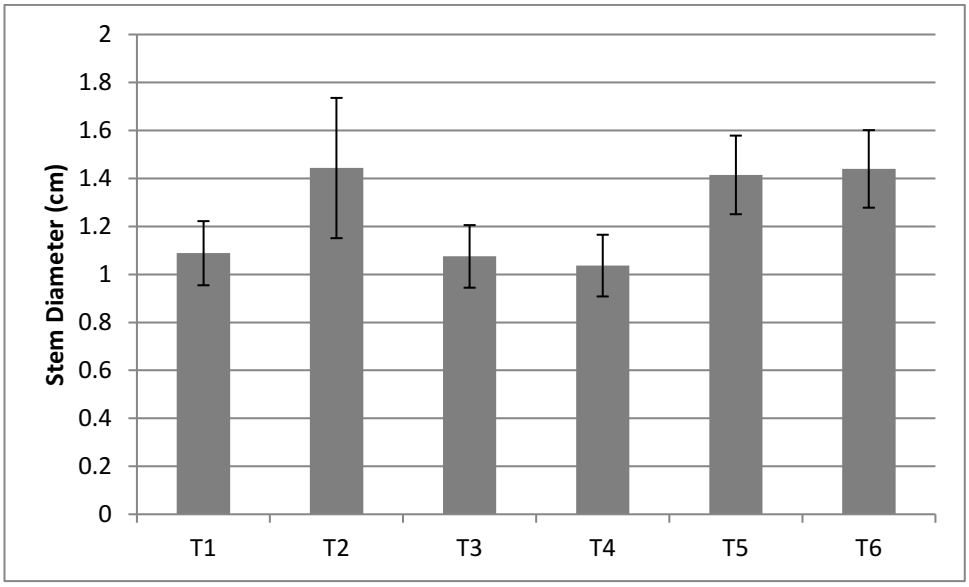

Figure 39: The mean stem diameter of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 150 days.

Table 35: Mean stem diameter of *Eucalyptus pellita* saplings for the 6 different treatments after 180 days.

Stem diameter (cm) of *Eucalyptus pellita* saplings in 180 days			
Treatment	Code	Diameter (cm)	S. D
Negative control	T1	1.1217	0.152385
Positive control	T2	1.4783	0.315596
NPK	T3	1.1317	0.201068
NPK IAA	T4	1.0750	0.185114
NPK + F	T5	1.4567	0.262853
NPK IAA + F	T6	1.4707	0.282374

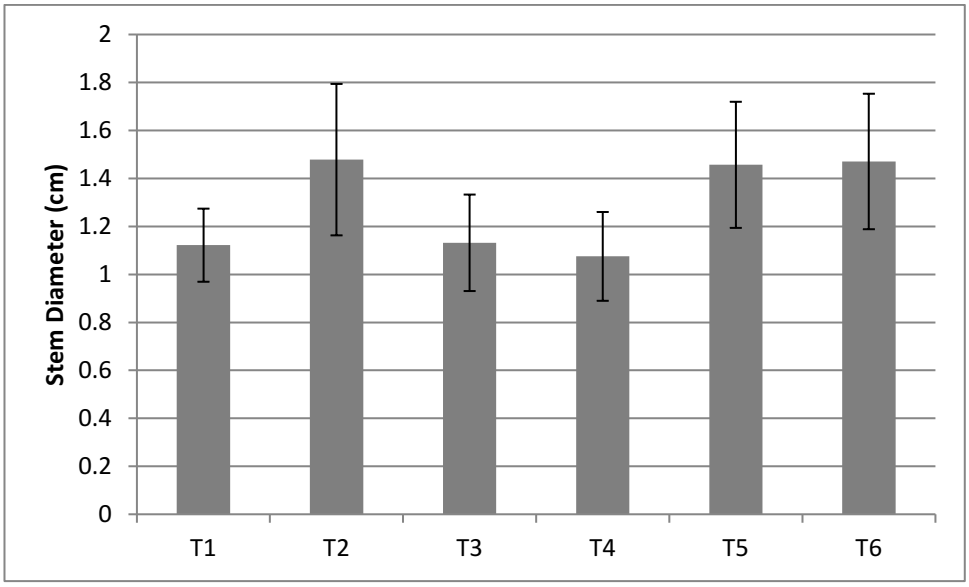

Figure 40: The mean stem diameter of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments after 180 days.

Yet again, a similar growth trend that was observed in height increment was also observed in stem diameter in the next coming 3 months. In these 3 months, All treatment with consortia cultures, with and without the addition of chemical fertilizer were growing at the same pace comparable to the control groups that they were compared to. From Table 33,34 and 35 showed that T3 and T4, which were treated with only consortia cultures, were showing more or less the same increment in stem diameter compared to negative control, T1. While on the other hand, T5 and T6, which were treated with consortia culture and chemical fertilizer, were showing more or less increment in height that was comparable to positive control T1. To be more specifics, T4 that was treated with consortia culture and IAA was a tab lower than the rest.

The same reasoning as height growth was deducted in the case of stem diameter increment. The consortia cultures that were not being washed away were getting acclimatized to the environment in the soil and also to the roots of saplings. The leftover cultures might be getting attached to the roots of the saplings during this period of time in order to continue surviving and also to supply nutrients to the plant at the same time. The stem diameter increments were quite proportional during this two month's period. Those microorganisms' cultures that were attached to the soil or root of saplings during

this period might also underwent replicating condition in order to increase their population.

After period of acclimatization, the consortia cultures were showing signs of improving plant growth. The leftover cultures might have increased in population, therefore resulting in increased stem diameter. More population lead to more nitrogen that had been fixed, more solubilized phosphate and potassium, and also production of IAA increased too, which were beneficial to the plant growth. The reason for T3 and T4 to have lower stem diameter increment compared to T5 and T6 was because of the initial minerals available in the pot was not sufficient enough. T5 and T6 were treated with 7.5g of chemical fertilizer. The consortia cultures were able to utilize the additional minerals to synthesize the required nutrients for the plant. The consortia culture in T3 and T4 had limited minerals from their surrounding in order to synthesize, therefore the progress was significantly slower. Nevertheless, T6 was still having lower stem diameter increment compared to positive control. As mentioned earlier, too much IAA produced by the microbes might affect the growth of plant above ground.

Stem growth is directly proportional to height growth. Therefore, the growth of stem was observed to be almost the same as height. The same explanation and supporting references were applicable to the stem growth.

4.3.1.2(e) Trend of growth in stem diameter (cm) throughout the 180 days period

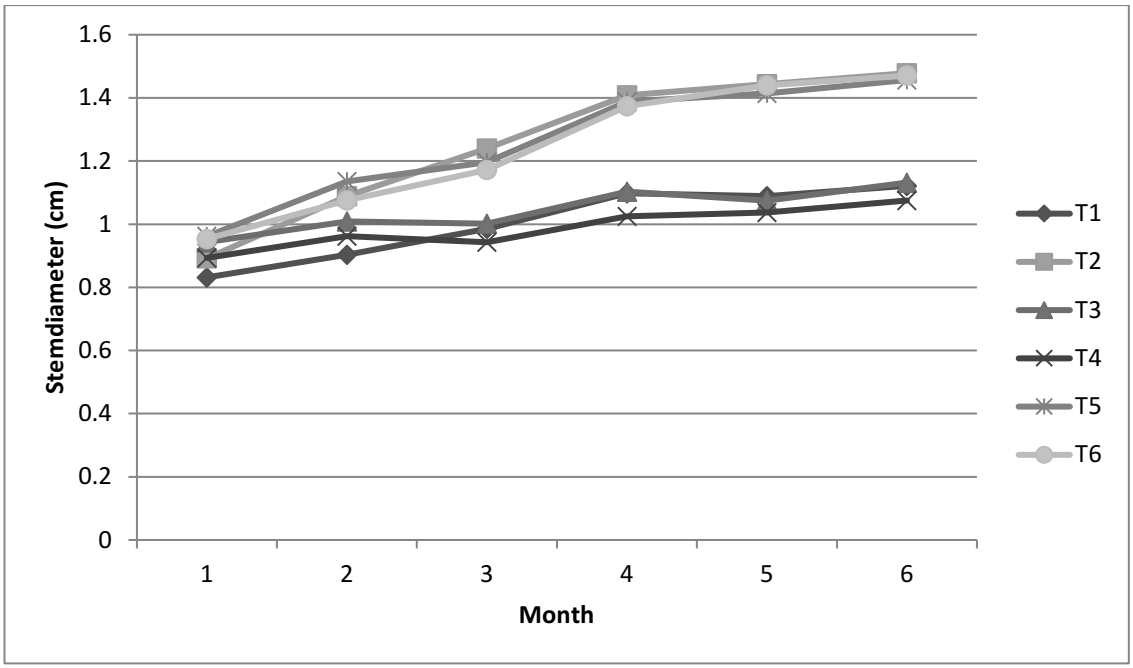

Figure 41: The trend of stem diameter increment of *Eucalyptus pellita* saplings in centimetre (cm) for all 6 different treatments from day 30 to day 180.

The trend of stem diameter increment throughout the 6 months period was comparatively with the height increment's trend, but in a lesser extent. Most of the treatments with consortia culture, with and without the addition of chemical fertilizer, were having better stem diameter increment in the starting month. T5 and T6 were leading the field compared to positive control, T1. In contrast, T3 and T4 were also performing better compared to negative control. The height increase was slower in the second and third month due to washing off of consortia cultures; until they were more or less the same with their respective control group (T5 and T6 were compared to positive control while T3 and T4 were compared to negative control). In the fourth and fifth month, all the remaining consortia cultures that were not being washed off, were acclimatizing to the environment and started reproducing to increase population. The height increase was proportional to their control group until the end of pot trial experiment, except T4 that was treated with consortia culture with IAA. T4 was a tab lower in stem diameter increment.

4.3.1.3 Growth increments in term of wet weight and dry weight observed in *Eucalyptus pellita* saplings for 6 different test groups

The wet weight and dry weight of *Eucalyptus pellita* saplings was observed and measured tat the end of the pot trial, right after the 6 months period. The wet weight of the saplings was obtained by weighing the weight of the saplings right after they were being uprooted from the pot. After the wet weights were obtained, the saplings were wrapped nicely and oven dried in in an oven incubator for 4 days. The dried up saplings was then weighed to obtain the dry weight. The weight was recorded in unit, gram (g). The average wet and dry weight of all the saplings were calculated and recorded in tables below.

4.3.1.3(a) Wet weight

Table 36: Mean wet weight of root for *Eucalyptus pellita* saplings for the 6 different treatments.

Wet weight (g) of root for *Eucalyptus pellita* saplings			
Treatment	Code	Root (g)	S. D
Negative control	T1	43.797	11.9646979
Positive control	T2	54.897	21.0966407
NPK	T3	44.209	12.5083923
NPK IAA	T4	44.568	7.00571101
NPK + F	T5	53.971	17.3831942
NPK IAA + F	T6	61.498	30.6107496

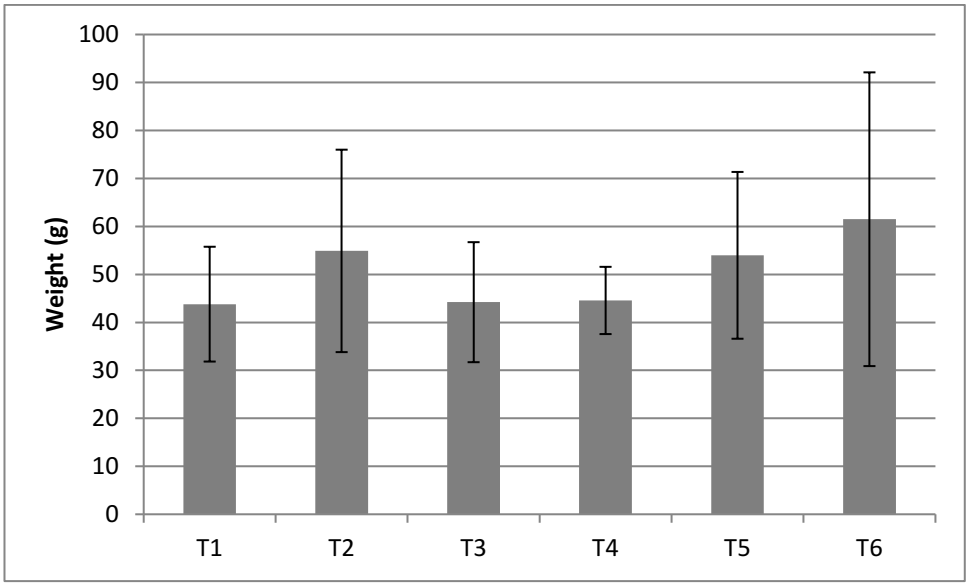

Figure 42: The wet weight of root for *Eucalyptus pellita* saplings in gram (g) for all 6 different treatments at the end of the pot trial experiment.

At the end of the pot trial experiment, the roots of all the saplings were measured for their wet weight. T6 that was treated with consortia culture of NPK IAA and chemical fertilizer showed the highest weigh among all the treatments, with 61.498g. Positive control T2 was the second highest with 54.897g, followed closely by T5 that was treated with NPK consortia culture and chemical fertilizer, with 53.971g. The remaining 3 treatments were much closed to each other. T4 that was treated with NPK IAA consortia culture was leading with 44.568g. Followed by T3 that was treated with NPK consortia culture with 44.209g, and finally, negative control T1 with 43.797g.

T3 and T4 were being compared to negative control T1, while T5 and T6 were being compared to positive control T2. Both T4 and T6 that were treated with IAA culture had higher root mass compared to their respective control and also T3 and T5 that were without the addition of IAA culture. The results showed that IAA culture was actually contributing to the increment in root length or density. Longer root length helps plant in achieving better nutrient supply, which directly improve plant growth.

Table 37: Mean wet weight of stem for *Eucalyptus pellita* saplings for the 6 different treatments.

Wet weight (g) of stem for *Eucalyptus pellita* saplings			
Treatment	Code	Stem (g)	S. D
Negative control	T1	134.05	34.692853
Positive control	T2	363.88	127.16522
NPK	T3	133.50	45.498745
NPK IAA	T4	149.16	69.543992
NPK + F	T5	255.29	78.93081
NPK IAA + F	T6	363.94	124.4674

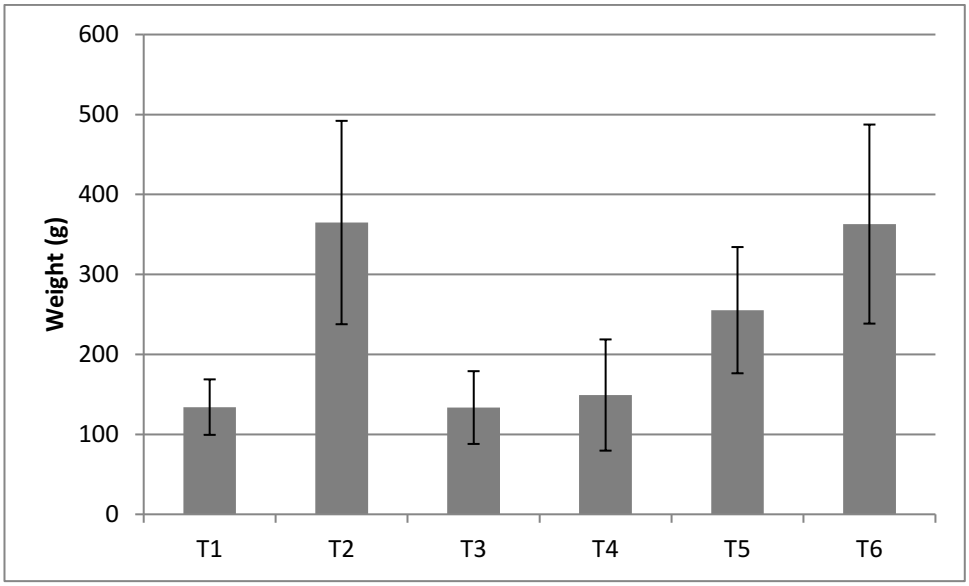

Figure 43: The wet weight of shoot for *Eucalyptus pellita* saplings in gram (g) for all 6 different treatments at the end of the pot trial experiment.

The wet weights of stem (including branches and leaves) for all saplings were also obtained. The wet weight of stem for T6 and positive control T2 were much closed to each other. They were 363.94g and 363.88g respectively. T5 was lower by roughly 100g with 255.29g. T4, again with IAA culture, was higher compared to T1 and T3. They were 149.16g, 134.05g and 13350g respectively.

The weight of stem for T6 was much closed to T2. Seem like a lot of water content was being transferred to the saplings. T5 had lower stem weight even though the weight of root for T5 was quite closed to T2. Perhaps the IAA cultures contributed to the root increment of the saplings, which also indirectly improved the water uptake of saplings at the same time. T4 also had higher stem weight compared to T3 and T1. Perhaps the same reasoning applied to both IAA culture.

4.3.1.3(b) Dry weight

Table 38: Mean dry weight of root for *Eucalyptus pellita* saplings for the 6 different treatments.

Dry weight (g) of root for *Eucalyptus pellita* saplings			
Treatment	Code	Root	S. D
Negative control	T1	21.761	7.210925
Positive control	T2	29.999	15.07448
NPK	T3	20.945	7.839484
NPK IAA	T4	22.012	5.526344
NPK + F	T5	27.655	7.239621
NPK IAA + F	T6	31.121	16.21524

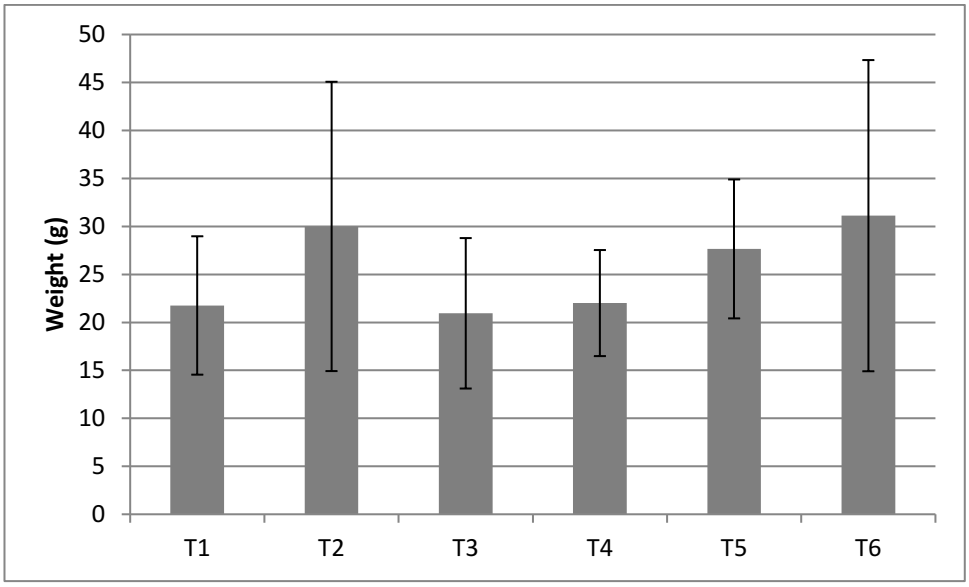

Figure 44: The dry weight of root for *Eucalyptus pellita* saplings in gram (g) for all 6 different treatments at the end of the pot trial experiment.

After getting the wet weights, dry weight for the roots of all saplings was also obtained. T6 that was treated with consortia culture of NPK IAA and chemical fertilizer showed the highest weight among all the treatments, with 31.121g. Positive control T2 was the second highest with 29.999g, followed by T5 that was treated with NPK consortia culture and chemical fertilizer, with 27.655g. The remaining 3 treatments were much closed to each other. T4 that was treated with NPK IAA consortia culture was leading with 22.012g, followed by negative control, T1 with 21.761g. Finally, T3 that was treated with only NPK consortia culture was the lowest, with 20.945g.

T3 and T4 were being compared to negative control T1, while T5 and T6 were being compared to positive control T2. Both T4 and T6 that were treated with IAA culture had higher root mass compared to their respective control and also T3 and T5 that were without the addition of IAA culture. The results showed that IAA culture was actually contributing to the increment in root length or density. Longer root length helps plant in achieving better nutrient supply, which directly improve plant growth. The same deduction observed in wet weight for root of saplings.

IAA is a phytohormone that is known to be responsible for root initiation, cell division and cell enlargement, which results in greater root surface that allows plant to access more nutrients from soil. Study showed that *Rhizobium leguminosarum* was able to colonize the root of lettuce and carrot. It promotes the update of phosphate and nitrogen in carrot and lettuce, and also improved the dry matter of shoots and roots (165). The amount of lateral root of pearl millet was found to be increased in number and thickly covered with root hair after inoculated with *Azospirillum brasilense*, which has the ability to produce IAA (168). Another research found that the fresh weight of tomato improved with the existence of increasing concentration of L-tryptophan through the inoculation of *Pseudomonas putida* and *Trichoderma atroviride* (169).

Table 39: Mean dry weight of stem for *Eucalyptus pellita* saplings for the 6 different treatments.

Dry weight (g) of stem for *Eucalyptus pellita* saplings			
Treatment	Code	Stem	S. D
Negative control	T1	63.359	26.31702
Positive control	T2	173.14	75.19042
NPK	T3	62.554	20.48341
NPK IAA	T4	67.494	24.62734
NPK + F	T5	171.58	30.63026
NPK IAA + F	T6	170.11	72.56079

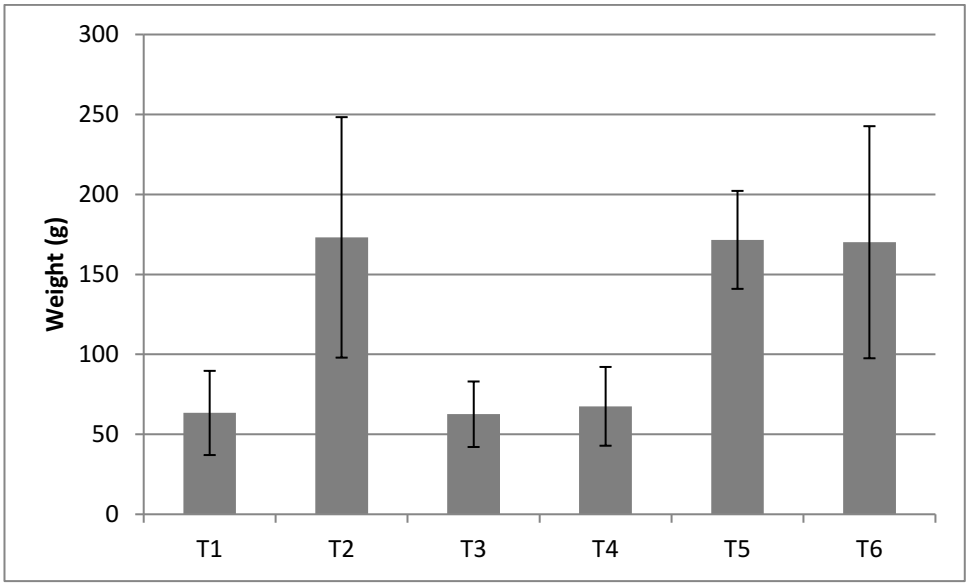

Figure 45: The dry weight of stem for *Eucalyptus pellita* saplings in gram (g) for all 6 different treatments at the end of the pot trial experiment.

The dry weights of stem (including branches and leaves) for all saplings were also obtained. The wet weight of stem for positive control, T5 and T6 were much closed to each other. They were 173.14g, 171.58g and 170.11g respectively. T4, again with IAA culture, was higher compared to T1 and T3. They were 149.16g, 134.05g and 133.50g respectively. Treatments that were not treated with chemical fertilizer had way lower dry weight compared to saplings that were treated with chemical fertilizer. T4 that was treated with NPK and IAA consortia culture had a slightly higher dry stem weight compared to T1 negative control and T3. They were 67.494g, 63.359g and 62.554g respectively.

The dry weight of stem for T2, T5 and T6 were much closed to each other. After getting rid of the excess water in the stem, all 3 of them showed relative firmness in the stem density. The same observation was also shown in the other remaining 3 treatments, T1, T3 and T4. But all 3 of these had lower weight compared to those treated with chemical fertilizer. Chemical fertilizer played a vital role in increasing the growth of the plants.

4.4 Summary

This chapter describes the efficiency of using plant growth promoting microorganisms as biological medium to substitute the use of chemical fertilizer in planting of *Eucalyptus pellita*. Four plant growth promoting microorganisms, which were nitrogen fixing, IAA producing, and phosphate and potassium solubilizing, were selected and used in this study. The selection of these microorganisms was based on the quantitative bioassay analysis that was discussed in Chapter 3. The microorganisms, *Lysinibacillus sphaericus,* N2, *Paenibacillus quercus,* I4, *Paramyrothecium roridum,* P4, and *Lysinibacillus fusiformis,* K1, were selected to be used in this pot trial experiment. The height and stem diameter increment throughout the 6 months period were recorded, as well as the wet and dry weight for the root and stem of the *Eucalyptus pellita* saplings. The aim of this pot trial experiment was accomplished at the end of the study. Overall, the *Eucalyptus pellita* saplings that were treated with NPK IAA consortia culture with 7.5g of chemical fertilizer (T6) had the most significant outcomes. It had the height and stem diameter increment that were comparable to positive control, which was treated with 15g of chemical fertilizer, despite that positive control had more nutrient availability due to higher amount of chemical fertilizer used. It also had the highest wet and dry root weight, and at the same time, had a very comparable wet and dry stem weight to positive control. T5, which was treated with NPK consortia culture and 7.5g chemical fertilizer, had the highest plant height among all treatments. The stem diameter, wet root weight and dry stem weight of T5 were slightly lesser than the positive control. However, the wet stem weight and dry root weight were more lowered compared to T6 and positive control. The remaining two treatments, T3 and T4 that were treated with NPK consortia culture and NPK IAA consortia culture respectively, had much lower growth compared to the treatments with chemical fertilizer. These two treatments had growth which was just comparable to negative control, which was not treated with anything at all. IAA producing microorganism was key in improving the root growth of the plant, which indirectly improve the overall growth.

From this experiment, it could be concluded biofertilizer that contain microorganisms with abilities of nitrogen fixing, phosphate solubilizing, potassium solubilizing and also IAA producing microorganisms had the ability to improve plant growth, thus reducing the use of chemical fertilizer. The aim of the experiment was accomplished. The biofertilizer containing PGPR showed that with the aid of 50% chemical fertilizer, the outcome obtained was as good as the use of 100% chemical fertilizer. The growth of *Eucalyptus pellita* saplings treated with consortia cultures and with 50% chemical fertilizer had the same outcomes as the saplings treated with 100% chemical fertilizer.

CHAPTER 5:

OVERALL CONCLUSIONS AND FURTHER RECOMMENDATIONS

5.1 General Conclusion

5.1.1 Aim of the thesis

The aim of this thesis was evaluate the use of biofertilizer which contains plant growth promoting microorganisms that are beneficial to the plant and environment to be the substitution of chemical fertilizer for the plantation of *Eucalyptus pellita*. The timber tree *Eucalyptus pellita,* was introduced into Sarawak in 2008 and became one of the main timber tree species planted here. The low nutrient availability in degraded soil due to the soil texture in *Eucalyptus pellita* plantation has led to the use of chemical fertilizer to improve tree growth. However, prolonged usage of chemical fertilizer causes negative impacts to the environment. Consequently, plant growth promoting microorganisms that were environmental and plant friendly were suggested as an alternative to improve the quality of soil and to provide the nutrients responsible for plant growth. Four type of plant promoting microorganisms were focused on in order to fulfil the objective of this thesis study. They were nitrogen fixing, IAA producing, phosphate and potassium solubilizing microorganisms which were responsible for the supplies of nitrogen, IAA, phosphate and potassium.

This thesis reports on the screening, isolation and characterisation of the plant growth promoting microorganisms isolated from Sarawak soil. Selective medium were used to screen and isolate for the respective microorganisms strains. The isolated strains from each group weas identified, further evaluated for their respective activities by using quantitative bioassay analysis, and also evaluated for the antagonistic characteristic against each other. The best growing strains were then selected for the formulation of consortia cultures that were then tested on the silviculture of *Eucalyptus pellita* for their growth promoting abilities. This chapter summaries the results obtained and finding in this research study that were described in details in Chapter 2, 3 and 4. Additionally, this chapter discusses on the relevant further works and provided recommendation.

5.1.2 Screening isolation and characterization of plant growth promoting microorganisms isolated from Sarawak soils

Soil samples were collected from 3 different regions in Sarawak. Selective mediums were used to screen and isolated the plant growth promoting microorganisms. Jensen's medium and bromothymol blue solution were used to screen for nitrogen fixing microorganisms. Tryptophan broth with Salkowski's reagent was used to identify IAA producing microorganisms. Pikovskaya's agar was used to screen for phosphate solubilizing microorganisms while Aleksandrov's medium was used to screen for potassium solubilizing microorganisms. The microorganisms with the respective traits produced clear halo zone surrounding the colonies.

The screening resulted in 18 nitrogen fixers, 19 IAA producer, 5 phosphate and 4 potassium solubilizers being successfully isolated. Molecular identification on the basis of partial 16S rRNA was carried out to classify the isolated microorganisms. Out of the 46 isolates, 19 different members of genus were found with 29 different strains in total. Most of the species were coming from *Bacillus* spp. and *Lysinibacillus* spp.

On the other hand, the collected soil samples were also evaluated for their respective soil textures. Most of the soil samples were considered as sandy soil texture except one, which was more of a clay texture due to higher clay concentration. The soil textures helped to further solidify the nutrient deficiency in *Eucalyptus pellita* plantation. The soil texture results also served as a reference for soil texture in designing pot trial.

5.1.3 Evaluating the efficiencies of isolated plant growth promoting microorganisms through quantitative bioassay analysis for their respective traits

Phenate method was used to determine the ammonia nitrogen produced by the nitrogen fixing microorganisms. Five of the selected strains showed better production of ammonia than the two standard cultures used for comparison. Two of the leader strains *Lysinibacillus sphaericus* and *Bosea robiniae* were producing ammonia concentration of 29.767mg/L and 29.423mg/L respectively.

Vanadomolybdophosphoric acid method was used to analyse the concentration of phosphate solubilize by the selected phosphate solubilizers. Four out of six selected strains showed higher concentration of tricalcium phosphate solubilized. The identities of these four strains were as follow; *Paramyrothecium roridum, Tolypocladium sp. 1 WL-2011, Clonostachys rosea,* and *Purpureocillium lilacinum*. They solubilized phosphate in the concentration as follow; 100.593mg/L, 100mg/L, 97.62mg/L and 80.3567mg/L respectively.

Atomic absorption spectrophotometer was conducted to determine the potassium released by the potassium solubilizers. Two isolates identified as *Lysinibacillus fusiformis* and *Rhizochaete filamentosa* showed higher available potassium content compared to standard culture. The released potassium concentrations were 16.380mg/L and 15.657mg/L respectively.

Finally, Salkowski's reagent with tryptophan test was carried out to analyse the concentration of IAA produced by the selected strains. Only one strain showed value higher than the standard culture with 5.01µg/mL. Another strain which was the second highest IAA producer only managed to produce 0.08µg/mL. The identities for both strains were *Paenibacillus quercus* and *Lysinibacillus sphaericus*. Nevertheless, these two strains were selected for antagonistic test together with the other top two selected species mentioned at the above characteristic.

The compatibility test conducted among the 8 selected strains showed that one of the strains was inhibited by the other strains. Therefore, the strains that were not inhibited and produced the best results in their respective quantitative analysis were chosen to be the strains for the formulation of consortia culture.

5.1.4 Evaluating the growth enhancing effects of the isolated strains on *Eucalyptus pellita* saplings

A pot trial experiment was conducted on *Eucalyptus pellita* saplings to test efficiency of plant growth promoting by the consortia cultures formulated based on the microorganisms selected. The period of the pot experiment was 6 months, by using six-months-old *Eucalyptus pellita* saplings. Four strains of microorganisms were selected; *Lysinibacillus sphaericus,* N2, *Paenibacillus quercus,* I4, *Paramyrothecium roridum,* P4 and *Lysinibacillus fusiformis,* K1. There were two consortia cultures that were formulated for the pot trial experiment, Consortium A and consortium B. Consortium A was formulated by strains N2, P2 and K1 while consortium B was formulated by all the four selected strains. The treatments that were applied to the saplings consisted of the following; negative control (no treatment add), positive control (100% or 15g of chemical fertilizer), Consortium A, consortium B, consortium A + 50% or 7.5g chemical fertilizer, and consortium B + 50% or 7.5g chemical fertilizer.

The growth increment in terms of stem height, stem diameter, stem wet and dry weight, and also root wet and dry weight were analysed during the six months period. At the end of the pot experiment, Consortium B + 50% chemical fertilizer had the most significant outcome. It had the height and stem diameter increment that were comparable to positive control, which was treated with 15g of chemical fertilizer, despite that positive control had more nutrient availability due to higher amount of chemical fertilizer used. It also had the highest wet and dry root weight, and at the same time, had a very comparable wet and dry stem weight to positive control. Consortium A + 50% chemical fertilizer had the highest plant height among all treatment, however the stem diameter, root wet weight and stem weight were slightly lesser than the positive control. The other two treatments, consortium A and consortium B had much lower

growth compared to the treatments with chemical fertilizer. Consortium B + 50 % chemical fertilizer was proven to be the most effective treatment, thus indicating that IAA producing microorganism was key in improving the root growth of the plant, which indirectly improve the overall growth.

From this experiment, it could be concluded that biofertilizers that contain microorganisms with abilities of nitrogen fixing, phosphate solubilizing, potassium solubilizing and also IAA producing microorganisms had the ability to improve plant growth when combined with lesser amount of chemical fertilizer, thus reducing the use of chemical fertilizer.

5.2 Further Recommendations

The plant growth promoting microorganisms isolated in this study displayed significant potentials in promoting the growth of *Eucalyptus pellita*. The four selected isolates *Lysinibacillus sphaericus, Paenibacillus quercus, Paramyrothecium roridum* and *Lysinibacillus fusiformis* showed their effectiveness as biofertilizer in the pot trial experiments described in this thesis. However, the application of this biofertilizer in the field on newly grown or maturing *Eucalyptus pellita* should be further investigated. The effectiveness of the isolated strains could be further evaluated based on the differences in nutrient availability, interaction between plants and microorganisms, environmental conditions, and also the competition of population of indigenous microorganisms in the soil.

Bioformulation of the biofertilizer is critical in determining the success or failure of the beneficial microbial strains in the inoculants. Suitable carrier materials are essentials in maintaining cell viability under hostile environmental conditions due to the use of these bioformulation in the field. Maintaining the survival and optimum population of beneficial microorganisms sufficient to radiate growth promoting effects on plant is one of the major requirements in formulation (208). A superior carrier material should be used to formulate the plant growth promoting microorganisms, such as high water

retention capacity, easily biodegradable, nontoxic to the environment, practically neutral in pH, chemically stable, and support bacterial growth and survival. Other synthetic or inert materials could also be used in the formulation, such as ground roch phosphate, calcium sulphate, alginate, polyacrylamide and vermiculite (170).

This study showed the effectiveness of the isolated strains in reducing the use of chemical fertilizer by 50%. However, additional tests could be conducted to further analyse the effectiveness of differences in amount of chemical fertilizer used. A study conducted on two bacteria strains that showed association in the root of banana plant for nitrogen fixing managed to improve the growth of shoot and photosynthetic activity of banana plant through minimal use of nitrogen fertilizer (33% of nitrogen content instead of 100%) (163). Another study showed that application of biofertilizer with only 25% of chemical fertilizer yield the same results as 100% chemical fertilizer on mustard seeds (207).

5.3 Concluding Statements

This thesis study aimed to reduce the use of chemical fertilizer by introducing biofertilizer that contains plant growth promoting microorganisms to promote the growth of *Eucalyptus pellita* on degraded soil with low nutrients in the plantation. Isolation of plant growth promoting microorganisms was screened through selective medium and the efficiencies of the promoting traits were evaluated by bioassay. The strains with the best attributes were selected for consortia formulation, that combined with the used of half amount of chemical fertilizer, showed significant growth promoting effects on *Eucalyptus pellita* saplings. Thus, the aim and objectives have been successfully achieved. The findings in this study proposed that isolated strains had the potential to be formulated as biofertiliser and to be used on *Eucalyptus pellita* plantations.

References

1. Luo J, Arnold RJ, Aken K. Genetic variation in growth and typhoon resistance in *Eucalyptus pellita* in south-western China. Aust For [Internet]. 2006;69(1):38–47. Available from: http://www.tandfonline.com/doi/abs/10.1080/00049158.2006.10674986

2. Leksono B, Kurinobu S, Ide Y. Realized genetic gains observed in second generation seedling seed orchards of Eucalyptus pellita in Indonesia. J For Res. 2008;13(2):110–6.

3. Hung TD, Brawner JT, Meder R, Lee DJ, Southerton S, Thinh HH, et al. Estimates of genetic parameters for growth and wood properties in Eucalyptus pellita F. Muell. to support tree breeding in Vietnam. Ann For Sci. 2015;72(2):205–17.

4. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers growth promoting rhizobacteria as biofertilizers. 2003;255(2):571–86.

5. Singh JS. Plant Growth Promoting Rhizobacteria. Ressonance. 2013;(March):275–81.

6. Sun D, Dickinson GR, Robson KJ. Growth of eucalyptus pellita and E. urophylla and effects on pasture production on the coastal lowlands of tropical northern australia. Aust For. 1996;59(3):136–41.

7. Bristow M, Vanclay JK, Brooks L, Hunt M. Growth and species interactions of Eucalyptus pellita in a mixed and monoculture plantation in the humid tropics of north Queensland. For Ecol Manage. 2006;233(2–3):285–94.

8. Clarke B, McLeod I, Vercoe T. Trees for Farm Forestry : 22 Promising Species. 2009;232.

9. Poke FS, Raymond CA. Predicting extractives, lignin, and cellulose contents using near infrared spectroscopy on solid wood in Eucalyptus globulus. J Wood Chem Technol. 2006;26(2):187–99.

10. Raymond CA, Schimleck LR. Development of near infrared reflectance analysis calibrations for estimating genetic parameters for cellulose content in *Eucalyptus globulus*. Can J For Res [Internet]. 2002;32(1):170–6. Available from: http://www.nrcresearchpress.com/doi/abs/10.1139/x01-174

11. Truţă E, Căpraru G, Surdu Ş, Zamfirache MM, Olteanu Z, Roşu CM, et al. Karyotypic studies in ecotypes of hippophaë rhamnoides l. from romania. Silvae Genet. 2010;59(4):175–82.

12. Schimleck LR, Kube PD, Raymond CA. Genetic improvement of kraft pulp yield in *Eucalyptus nitens* using cellulose content determined by near infrared spectroscopy. Can J For Res [Internet]. 2004;34(11):2363–70. Available from: http://www.nrcresearchpress.com/doi/abs/10.1139/x04-119

13. Yusoff MNM, Mahmudin Saleh, Yahya AZ. Potential utilization of fast growing species from planted forest. Potential Util fast Grow species from Plant For [Internet]. 2008; Available from: http://www.sarawaktimber.org.my/timber_issue/Paper 10 (International Conference 2013).pdf

14. Leksono B, Kurinobu S, Ide Y. Optimum age for selection based on a time trend of genetic parameters related to diameter growth in seedling seed orchards of Eucalyptus pellita in Indonesia. J For Res. 2006;11(5):359–64.

15. Brawner JT, Bush DJ, Macdonell PF, Warburton PM, Clegg PA. Genetic parameters of red mahogany breeding populations grown in the tropics. Aust For. 2010;73(3):177–83.

16. Dart P, Lamb D, Asia E, Delp B. Overcoming Impediments to Reforestation : Tropical Forest Rehabilitation in the Asia-Pacific Region Importance of Micronutrients for Productivity of Plantation Eucalypts in. 1998;(March).

17. Debell DS, Whitesell CD, Schubert TH. Mixed Plantations of Eucalyptus and leguminous Trees Enhance Biomass Production. United States Dep Agric For Serv. 1985;

18. Raymond CA, Muneri A. Effect of fertilizer on wood properties of Eucalyptus globulus. Can J For Res Can Rech For. 2000;30(1):136–44.

19. Washusen R, Baker T, Menz D, Morrow A. Effect of thinning and fertilizer on the cellulose crystallite width of Eucalyptus globulus. Wood Sci Technol. 2005;39(7):569–78.

20. Nastasija M, Mirjana J, Ivica D, Djordje J. Importance of PGPR application and its effect on microbial activity in maize rhizosphere. Ratar i Povrt [Internet]. 2012;49(3):335–44. Available from: http://scindeks.ceon.rs/Article.aspx?artid=1821-39441203335M

21. Tro IN, Tion DUC. BENEFITS OF INOCULATION WITH AZOTOBACTER IN THE GROWTH AND PRODUCTION OF TOMATO AND PEPPERS. 2010;(vi):71–6.

22. Cherr CM, Scholberg JMS, McSorley R. Green manure approaches to crop production: A synthesis. Agron J. 2006;98(2):302–19.

23. Orhan E, Esitken A, Ercisli S, Turan M, Sahin F. Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. Sci Hortic (Amsterdam). 2006;111(1):38–43.

24. Podile A, Kishore G. Plant growth-promoting rhizobacteria. Gnanamanickam SS Plant-Associated Bact Springer, Netherlands. 2007;(Table 1):195–230.

25. Kloepper JW, Lifshitz R, Zablotowicz RM. Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnology. 1989. p. 39–44.

26. Weller DM, Thomashow LS. Current Challenges in Introducing Beneficial Microorganisms into the Rhizosphere. Mol Ecol Rhizosph Microorg Biotechnol Release GMOs. 2007;(293):1–18.

27. Lugtenberg B, Kamilova F. Plant-Growth-Promoting Rhizobacteria. Annu Rev Microbiol [Internet]. 2009;63(1):541–56. Available from: http://www.annualreviews.org/doi/10.1146/annurev.micro.62.081307.162918

28. Bhattacharyya PN, Jha DK. Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. World J Microbiol Biotechnol. 2012;28(4):1327–50.

29. Kloepper JW, Leong J, Teintze M, Schroth MN. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria [Internet]. Nature. 1980. p. 885–6. Available from: http://www.nature.com/doifinder/10.1038/286885a0

30. Son JS, Sumayo M, Hwang YJ, Kim BS, Ghim SY. Screening of plant growth-promoting rhizobacteria as elicitor of systemic resistance against gray leaf spot disease in pepper. Appl Soil Ecol [Internet]. Elsevier B.V.; 2014;73:1–8. Available from: http://dx.doi.org/10.1016/j.apsoil.2013.07.016

31. Gray EJ, Smith DL. Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signaling processes. Soil Biol Biochem. 2005;37(3):395–412.

32. Choudhary DK, Sharma KP, Gaur RK. Biotechnological perspectives of microbes in agro-ecosystems. Biotechnol Lett. 2011;33(10):1905–10.

33. García-Fraile P, Menéndez E, Rivas R. Role of bacterial biofertilizers in agriculture and forestry. AIMS Bioeng [Internet]. 2015;2(3):183–205. Available from: http://www.aimspress.com/article/10.3934/bioeng.2015.3.183

34. Spaepen S, Vanderleyden J, Remans R. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol Rev. 2007;31(4):425–48.

35. Boiero L, Perrig D, Masciarelli O, Penna C, Cassán F, Luna V. Phytohormone production by three strains of Bradyrhizobium japonicum and possible physiological and technological implications. Appl Microbiol Biotechnol. 2007;74(4):874–80.

36. Ribaudo CM, Krumpholz EM, Cassán FD, Bottini R, Cantore ML, Curá JA. Azospirillum sp. Promotes root hair development in tomato plants through a mechanism that involves ethylene. J Plant Growth Regul. 2006;25(2):175–85.

37. Werner T, Motyka V, Laucou V, Smets R, Onckelen H Van, Schmuelling T. Cytokinin-Deficient Transgenic Arabidopsis Plants Show Functions of Cytokinins in the Regulation of Shoot and Root Meristem Activity. Plant Cell. 2003;15(November):2532–50.

38. Nadeem SM, Zahir ZA, Naveed M, Ashraf M. Microbial ACC-deaminase: Prospects and applications for inducing salt tolerance in plants. CRC Crit Rev Plant Sci. 2010;29(6):360–93.

39. Nautiyal CS, Govindarajan R, Lavania M, Pushpangadan P. Novel mechanism of modulating natural antioxidants in functional foods: Involvement of plant growth promoting rhizobacteria NRRL B-30488. J Agric Food Chem. 2008;56(12):4474–81.

40. Ahmad M, Zahir ZA, Khalid M, Nazli F, Arshad M. Efficacy of Rhizobium and Pseudomonas strains to improve physiology, ionic balance and quality of mung bean under salt-affected conditions on farmer's fields. Plant Physiol Biochem [Internet]. Elsevier Masson SAS; 2013;63:170–6. Available from: http://dx.doi.org/10.1016/j.plaphy.2012.11.024

41. Naveed M, Hussain MB, Zahir ZA, Mitter B, Sessitsch A. Drought stress amelioration in wheat through inoculation with Burkholderia phytofirmans strain PsJN. Plant Growth Regul. 2014;73(2):121–31.

42. Marulanda A, Azcón R, Chaumont F, Ruiz-Lozano JM, Aroca R. Regulation of plasma membrane aquaporins by inoculation with a Bacillus megaterium strain in maize (Zea mays L.) plants under unstressed and salt-stressed conditions. Planta. 2010;232(2):533–43.

43. Gonzalez AJ, Larraburu EE, Llorente BE. Azospirillum brasilense increased salt tolerance of jojoba during in vitro rooting. Ind Crops Prod [Internet]. Elsevier

B.V.; 2015;76:41–8. Available from: http://dx.doi.org/10.1016/j.indcrop.2015.06.017

44. Gond SK, Bergen MS, Torres MS, White JF, Kharwar RN. Effect of bacterial endophyte on expression of defense genes in Indian popcorn against Fusarium moniliforme. Symbiosis. 2015;66(3):133–40.

45. Miethke M, Marahiel MA. Siderophore-Based Iron Acquisition and Pathogen Control. Microbiol Mol Biol Rev [Internet]. 2007;71(3):413–51. Available from: http://mmbr.asm.org/cgi/doi/10.1128/MMBR.00012-07

46. Challis GL. A widely distributed bacterial pathway for siderophore biosynthesis independent of nonribosomal peptide synthetases. ChemBioChem. 2005;6(4):601–11.

47. Vacheron J, Desbrosses G, Bouffaud M-L, Touraine B, Moënne-Loccoz Y, Muller D, et al. Plant growth-promoting rhizobacteria and root system functioning. Front Plant Sci [Internet]. 2013;4(September):1–19. Available from: http://journal.frontiersin.org/article/10.3389/fpls.2013.00356/abstract

48. Calvaruso C, Turpault M, Frey-klett P, Arbres-microorganismes II, Nancy CI De. Root-Associated Bacteria Contribute to Mineral Weathering and to Mineral Nutrition in Trees : a Budgeting Analysis. 2006;72(2):1258–66.

49. Peoples MB, Herridge DF, Ladha JK. Biological Nitrogen-Fixation - an Efficient Source of Nitrogen for Sustainable Agricultural Production. Plant Soil. 1995;174(1–2):3–28.

50. 1989 New Biological Books 521. 1989;(December):2017.

51. Hameeda B, Harini G, Rupela OP, Wani SP, Reddy G. Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. Microbiol Res. 2008;163(2):234–42.

52. Gyaneshwar P, Parekh LJ, Archana G, Poole PS, Collins MD, Hutson RA, et al. Involvement of a phosphate starvation inducible glucose dehydrogenase in soil

phosphate solubilization by Enterobacter asburiae. FEMS Microbiol Lett. 1999;171(2):223–9.

53. Hinsinger P. hinsinger_2001_bioavailibility os soil inorganic P in the rhizosphere as effected by root-induced chemical changes A review.pdf. Plant Soil. 2001;237:173–95.

54. Khan AA, Jilani G, Akhtar MS, Saqlan SM, Rasheed M. Phosphorus Solubilizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production. J Agric Biol Sci. 2009;1(1):48–58.

55. Kumar H, Bajpai VK, Dubey RC, Maheshwari DK, Kang SC. Wilt disease management and enhancement of growth and yield of Cajanus cajan (L) var. Manak by bacterial combinations amended with chemical fertilizer. Crop Prot [Internet]. Elsevier Ltd; 2010;29(6):591–8. Available from: http://dx.doi.org/10.1016/j.cropro.2010.01.002

56. Arora NK, Khare E, Oh JH, Kang SC, Maheshwari DK. Diverse mechanisms adopted by fluorescent Pseudomonas PGC2 during the inhibition of Rhizoctonia solani and Phytophthora capsici. World J Microbiol Biotechnol. 2008;24(4):581–5.

57. Bever JD, Westover KM, Antonovics J. Incorporating the Soil Community into Plant Population Dynamics: The Utility of the Feedback Approach. J Ecol [Internet]. 1997;85(5):561. Available from: http://www.jstor.org/stable/2960528?origin=crossref

58. Vejan P, Abdullah R, Khadiran T, Ismail S, Nasrulhaq Boyce A. Role of plant growth promoting rhizobacteria in agricultural sustainability-A review. Molecules. 2016;21(5):1–17.

59. Wu SC, Cao ZH, Li ZG, Cheung KC, Wong MH. Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: A greenhouse trial. Geoderma. 2005;125(1–2):155–66.

60. Toši I, Goli Z, Radosavac A. Effects of the application of biofertilizers on the

microflora and yield of lettuce (Lactuca sativa L .). 2016;XXI:91–8.

61. Yazdani M, Bahmanyar MA, Pirdashti H, Esmaili MA. Effect of Phosphate Solubilization Microorganisms (PSM) and Plant Growth Promoting Rhizobacteria (PGPR) on Yield and Yield Components of Corn (Zea mays L.). Int J Biol Biomol Agric Food Biotechnol Eng. 2009;3(1):50–2.

62. Siddiqui ZA. PGPR: Biocontrol and biofertilization. PGPR: Biocontrol and Biofertilization. 2006. 1-318 p.

63. Lal R. Effects of macrofauna on soil properties in tropical ecosystems. Agric Ecosyst Environ. 1988;24(1–3):101–16.

64. Gothwal RK, Nigam VK, Mohan MK, Sasmal D, Ghosh P. Screening of nitrogen fixers from rhizospheric bacterial isolates associated with important desert plants. Appl Ecol Environ Res. 2008;6(2):101–9.

65. Chari KD, Reddy RS, Trimurtulu N. Screening and Characterization of Diazotrophic Bacterial isolates for Plant Growth Promoting Properties. Int J Curr Microbiol Appl Sci. 2015;4(9):704–10.

66. Zhang C, Kong F. Isolation and identification of potassium-solubilizing bacteria from tobacco rhizospheric soil and their effect on tobacco plants. Appl Soil Ecol [Internet]. Elsevier B.V.; 2014;82:18–25. Available from: http://dx.doi.org/10.1016/j.apsoil.2014.05.002

67. Rahman A, Sitepu IR, Tang S-Y, Hashidoko Y. Salkowski's reagent test as a primary screening index for functionalities of rhizobacteria isolated from wild dipterocarp saplings growing naturally on medium-strongly acidic tropical peat soil. Biosci Biotechnol Biochem [Internet]. 2010 [cited 2017 Jul 20];74(11):2202–8. Available from: https://eprints2008.lib.hokudai.ac.jp/dspace/bitstream/2115/45489/1/hashidoko1.pdf

68. Zhu H, Qu F, Zhu L-H. Isolation of genomic DNAs from plants,fungi and bacteria using benzy chloride. Nucleic Acids Res. 1993;21(22):5279–80.

69. Johnson BH, Hecht MH. Recombinant proteins can be isolated from E. coli cells by repeated cycles of freezing and thawing [Internet]. Bio/technology (Nature Publishing Company). 1994. p. 1357–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7765566

70. Geer LY, Marchler-Bauer A, Geer RC, Han L, He J, He S, et al. The NCBI BioSystems database. Nucleic Acids Res. 2009;38(SUPPL.1):492–6.

71. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. Nucleic Acids Res. 2008;36(Web Server issue):5–9.

72. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9.

73. Zaidi a, Khan MS, Ahemad M, Oves M. Plant growth promotion by phosphate solubilizing bacteria. Acta Microbiol Immunol Hung. 2009;56(3):263–84.

74. Shanware AS, Kalkar SA, Trivedi MM. Potassium Solublisers : Occurrence , Mechanism and Their Role as Competent Biofertilizers. Int J Curr Microbiol App Sci. 2014;3(9):622–9.

75. Sridevi M, Mahalakshmi M, Ranjan A. Isolation and characterization of phosphate-solubilizing bacterial species from different crop fields of Salem, Tamil Nadu, India. Int J Nutr Pharmacol Neurol Dis [Internet]. 2013;3(1):29. Available from: http://www.ijnpnd.com/text.asp?2013/3/1/29/106982

76. Kim J, Rees DC. Nitrogenase and biological nitrogen fixation. Biochemistry. 1994;33(2):389–97.

77. Gothwal RK, Nigam VK, Mohan MK, Sasmal D, Ghosh P. Screening of nitrogen fixers from rhizospheric bacterial isolates associated with important desert plants. Appl Ecol Environ Res [Internet]. 2008 [cited 2017 Jul 21];6(2):101–9. Available from: http://www.ecology.uni-corvinus.hu

78. Momose A, Ohtake N, Sueyoshi K, Sato TT, Nakanishi Y, Akao S, et al.

Nitrogen Fixation and Translocation in Young Sugarcane (Saccharum officinarum L.) Plants Associated with Endophytic Nitrogen-Fixing Bacteria. Mol Plant Microbe Interact [Internet]. 2013;67(3):5285–5293. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23352842%5Cnhttp://doi.wiley.com/10.1111/j.1365-2672.2006.02843.x%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/16629994%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/23177711%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/11952126%5Cnhttp://www

79. Apastambh AR, Taneever K, Baig MMV. Isolation and characterization of plant growth promoting rhizobacteria and their application in plant growth. Int J Curr Microbiol Appl Sci. 2014;37(1):72–8.

80. Hussein KA, Joo JH. Isolation and characterization of rhizomicrobial isolates for phosphate solubilization and indole acetic acid production. J Korean Soc Appl Biol Chem. The Korean Society for Applied Biological Chemistry; 2015;58(6):847–55.

81. Review Agronomic Applications of Azospirillum : an Evaluation of 20 Years Worldwide Field Inoculation. 1994;26(12):1591–601.

82. Kasana RC, Panwar NR, Burman U. Isolation and Identification of Two Potassium Solubilizing Fungi from Arid Soil. 2017;6(3):1752–62.

83. Han HS, Supanjani, Lee KD. Effect of co-inoculation with phosphate and potassium solubilizing bacteria on mineral uptake and growth of pepper and cucumber. Plant, Soil Environ. 2006;52(3):130–6.

84. Steenhoudt O, Vandereyden J. Azospirillum, fee-living nitrogen fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol Rev. 2000;24(4):487–506.

85. Felsenstein J. Phylogenies and the Comparative Method. Am Nat [Internet]. 1985;125(125):3–147. Available from: http://www.jstor.org/stable/2461605

86. Old KM, Wingfield MJ, Qing Yuan Z. A Manual of Diseases of Eucalypts in

South-East Asia. Cifor. 2003;106.

87. Eucalyptus pellita Myrtaceae Eucalyptus pellita. 2009;0:1–5.

88. Melgar RJ, Smyth TJ, Sanchez PA, Cravo MS. Fertilizer nitrogen movement in a Central Amazon Oxisol and Entisol cropped to corn. Fertil Res. 1992;31(2):241–52.

89. Adesemoye AO, Torbert HA, Kloepper JW. Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microb Ecol. 2009;58(4):921–9.

90. Valle M, Legault CM, Ortiz M. in the Gulf of Mexico. Fish Sci. 2001;55(July):174–7.

91. kloeppermicrolfore.pdf.

92. Glick BR, Glick BR. Plant Growth-Promoting Bacteria: Mechanisms and Applications. Scientifica (Cairo) [Internet]. 2012;2012:1–15. Available from: http://www.hindawi.com/journals/scientifica/2012/963401/

93. Halder AK, Mishra AK, Bhattacharyya P, Chakrabartty PK, Road APC. J. Gen. App!. Microbiol., 36, 81-92 (1990). 1990;92:81–92.

94. Potash I. Potassium dynamic in soil. :1–12.

95. Malavolta E. Potassium Status of Tropical and Subtropical Region Soils. Potassium Agric [Internet]. 1985;163–200. Available from: https://dl.sciencesocieties.org/publications/books/articles/acsesspublicati/potassiuminagri/1063?show-t-f=tables&wrapper=no%5Cnhttp://www.cabdirect.org/abstracts/19861908654.html

96. Bennett P, Choi W, Rogera J. Microbial destruction of feldspars. Miner Manag. 1998;8:149–50.

97. Dharmasiri S, Jayaweera T, Dharmasiri N. Plant Hormone Signalling: Current

Perspectives on Perception and Mechanisms of Action. Ceylon J Sci (Biological Sci [Internet]. 2013;42(1):1–17. Available from: http://www.sljol.info/index.php/CJSBS/article/view/5895

98. Westfall CS, Muehler AM, Jez JM. Enzyme action in the regulation of plant hormone responses. J Biol Chem. 2013;288(27):19304–11.

99. Patten CL, Glick BR. Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol [Internet]. 1996;42(3):207–20. Available from: http://www.nrcresearchpress.com/doi/abs/10.1139/m96-032

100. Cook RJ, Baker KF, others. The nature and practice of biological control of plant pathogens. 1983. 254-256 p.

101. Thakore Y. The biopesticide market for global agricultural use.. Ind Biotechnol. 2006;2(3):194–208.

102. Berg G, Krechel A, Ditz M, Sikora RA, Ulrich A, Hallmann J. Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. FEMS Microbiol Ecol. 2005;51(2):215–29.

103. Berg G, Opelt K, Zachow C, Lottmann J, Götz M, Costa R, et al. The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus Verticillium differs depending on plant species and site. FEMS Microbiol Ecol. 2006;56(2):250–61.

104. Cattelan AJ, Hartel PG, Fuhrmann JJ. Origin of Bacteria. 1996;1.

105. Sultana Q, Anjum A, Fathima N, Siraj M, Ishaq M. Seropositivity to anti-thyroid peroxidase and anti-thyroglobulin autoantibodies in hypo and hyper-thyroidism : Diagnostic and epidemiological significance. 2016;3(Singh 2010):368–72.

106. Meudt WJ, Gaines TP. Studies on the Oxidation of Indole-3-Acetic Acid by Peroxidase Enzymes. I. Colorimetric Determination of Indole-3-Acetic Acid Oxidation Products. Plant Physiol [Internet]. 1967 [cited 2017 Jul 20];42:1395–9. Available from:

http://www.plantphysiol.org/content/plantphysiol/42/10/1395.full.pdf

107. Glickmann E, Dessaux Y, Glickmann E, Dessaux Y. A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria . These include : A Critical Examination of the Specificity of the Salkowski Reagent for Indolic Compounds Produced by Phytopatho. Appl Environ Microbiol. 1995;61(2):793–6.

108. Bernhart N, Wreath AR. Co I or i m e t r ic Determination of Phosphorus by Modified Phosphomolybdate Method. :1–2.

109. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal [Internet]. Elsevier; 2016;6(2):71–9. Available from: http://dx.doi.org/10.1016/j.jpha.2015.11.005

110. Whipps JM. Microbial interactions and biocontrol in the rhizosphere. J Exp Bot [Internet]. 2001;52(suppl 1):487–511. Available from: http://jxb.oxfordjournals.org/content/52/suppl_1/487.short

111. GmbH HMC. Product Specification Sheet. 2011;49(0):2–3.

112. APHA/AWWA/WEF. Standard Methods for the Examination of Water and Wastewater. Stand Methods. 2012;541.

113. Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl Soil Ecol. 2006;34(1):33–41.

114. Leaungvutiviroj C, Ruangphisarn P, Hansanimitkul P, Shinkawa H, Sasaki K. Development of a New Biofertilizer with a High Capacity for N 2 Fixation , Phosphate and Potassium Solubilization and Auxin Production. Biosci Biotechnol Biochem. 2010;74(5):1098–101.

115. Reddy MS, Kumar S, Babita K, Reddy MS. Biosolubilization of poorly soluble rock phosphates by Aspergillus tubingensis and Aspergillus niger. Bioresour Technol. 2002;84(2):187–9.

116. Whitelaw M a. Growth promotion of plants inoculated with phosphate solubilizing fungi. Adv Agron. 2000;69:99–151.

117. Abd-Alla MH, Omar SA, Omar SA. SURVIVAL OF RHIZOBIA/BRADYRHIZOBIA AND A ROCK-PHOSPHATE-SOLUBILIZING FUNGUS *ASPERGILLUS NIGER* ON VARIOUS CARRIERS FROM SOME AGRO-INDUSTRIAL WASTES AND THEIR EFFECTS ON NODULATION AND GROWTH OF FABA BEAN AND SOYBEAN. J Plant Nutr [Internet]. 2001;24(2):261–72. Available from: http://www.tandfonline.com/doi/abs/10.1081/PLN-100001386

118. Sayer J a., Gadd GM. Solubilization and transformation of insoluble inorganic metal compounds to insoluble metal oxalates by Aspergillus niger. Mycol Res. 1997;101(6):653–61.

119. Vazquez P, Holguin G, Puente ME, Lopez-Cortes a., Bashan Y. Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. Biol Fertil Soils. 2000;30(5–6):460–8.

120. Sharma SB., Sayyed RZ., Trivedi MH., Gobi TA. Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. Springerplus [Internet]. 2013;2(1):1–14. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-84887302575&partnerID=40&md5=320f2008365d909d32c45fd3265ad043

121. Narsian V, Ahmed AA, Patel HH. Rock phosphate dissolution by specific yeast. Indian J Microbiol. 2010;50(1):57–62.

122. Oliveira CA, Alves VMC, Marriel IE, Gomes EA, Scotti MR, Carneiro NP, et al. Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. Soil Biol Biochem. 2009;41(9):1782–7.

123. Islama MT, Deora A, Hashidoko Y, Rahman A, Ito T, Tahara S. Isolation and identification of potential phosphate solubilizing bacteria from the rhizoplane of

Oryza sativa L. cv. BR29 of Bangladesh. Zeitschrift für Naturforschung C, J Biosci [Internet]. 2007;62(1–2):103–10. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17425114

124. Prakash J, Ram V, Meena S. Potassium Solubilizing Microorganisms for Sustainable Agriculture [Internet]. 2016. Available from: http://link.springer.com/10.1007/978-81-322-2776-2

125. Prajapati K, Modi HA. Growth Promoting Effect of Potassium Solubilizing Enterobacter hormaechei (KSB-8) on Cucumber (Cucumis sativus) under Hydroponic Conditions. Int J Adv Res Biol Sci. 2016;3(5):168–73.

126. Verma P, Yadav A, Kazy S. Evaluating the diversity and phylogeny of plant growth promoting bacteria associated with wheat (Triticum aestivum) growing in central zone of India. Int J Curr Microbiol Appl … [Internet]. 2014;3(5):432–47. Available from: http://www.ijcmas.com/vol-3-5/Priyanka Verma, ET AL.pdf

127. Wang Q, Wang RR, He LY, Lu JJ, Huang Z, Sheng XF. Changes in weathering effectiveness and community of culturable mineral-weathering bacteria along a soil profile. Biol Fertil Soils. 2014;50(7).

128. Khan MS, Zaidi A, Ahemad M, Oves M, Wani PA. Plant growth promotion by phosphate solubilizing fungi – current perspective. Arch Agron Soil Sci [Internet]. 2010;56(1):73–98. Available from: http://www.tandfonline.com/doi/abs/10.1080/03650340902806469

129. Chakraborty BN, Chakraborty U, Saha A, Sunar K, Dey PL. Evaluation of Phosphate Solubilizers from Soils of North Bengal and Their Diversity Analysis. World J Agric Sci. 2010;6(2):195–200.

130. Meena VS, Maurya BR, Verma JP, Aeron A, Kumar A, Kim K, et al. Potassium solubilizing rhizobacteria (KSR): Isolation, identification, and K-release dynamics from waste mica. Ecol Eng [Internet]. Elsevier B.V.; 2015;81:340–7. Available from: http://dx.doi.org/10.1016/j.ecoleng.2015.04.065

131. Park G eun, Oh H na, Ahn S. Improvement of the ammonia analysis by the

phenate method in water and wastewater. Bull Korean Chem Soc. 2009;30(9):2032–8.

132. Yoch DC, Arnon DI. Two Biologically Active Ferredoxins from the Aerobic Nitrogen-fixing Bacterium, Azotobacter vinelandii. J Biol Chem [Internet]. 1972;247(14):4514–20. Available from: http://www.jbc.org/content/247/14/4514.abstract

133. Bishop PE, Jarlenski DM, Hetherington DR. Evidence for an alternative nitrogen fixation system in Azotobacter vinelandii. Proc Natl Acad Sci. 1980;77(12):7342–6.

134. Page WJ, Shivprasad S. Microaerophilic , and Aeroadaptive Nitrogen-Fixing Bacterium. 1991;(July):369–76.

135. Kumar V, Singh KP. Enriching vermicompost by nitrogen fixing and phosphate solubilizing bacteria. Bioresour Technol. 2001;76(2):173–5.

136. Dalton H, Postgate JR. Effect of {Oxygen} on {Growth} of {Azotobacter} chroococcum in {Batch} and {Continuous} {Cultures}. J Gen Microbiol [Internet]. 1968;54(3):463–73. Available from: http://mic.sgmjournals.org/content/54/3/463

137. Drozd JW, Tubb RS, Postgate JR. A chemostat study of the effect of fixed nitrogen sources on nitrogen fixation, membranes and free amino acids in Azotobacter chroococcum. J Gen Microbiol [Internet]. 1972;73(2):221–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/4345960

138. Strandberg GW, Wilson PW. Formation of the nitrogen-fixing enzyme system in Azotobacter vinelandii. Can J Microbiol [Internet]. 1968;14(1):25–31. Available from: http://www.nrcresearchpress.com/doi/abs/10.1139/m68-005

139. De Meyer SE, Willems A. Multilocus sequence analysis of Bosea species and description of Bosea lupini sp. nov., Bosea lathyri sp. nov. and Bosea robiniae sp. nov., isolated from legumes. Int J Syst Evol Microbiol. 2012;62(10):2505–10.

140. Joseph B, Patra RR, Lawrence R. Characterization of plant growth promoting rhizobacteria associated with chickpea (Cicer arietinum L .). Int J Plant Prod. 2007;1(2):141–51.

141. Ahmad B, Nigar S, Abdul Malik N, Bashir S, Ali J, Yousaf S, et al. Isolation and characterization of cellulolytic Nitrogen fixing Azotobacter species from wheat rhizosphere of Khyber Pakhtunkhwa. World Appl Sci J. 2013;27(1):51–60.

142. Kayasth M, Gera R, Dudeja SS, Sharma PK, Kumar V. Studies on salinization in Haryana soils on free-living nitrogen-fixing bacterial populations and their activity. J Basic Microbiol. 2014;54(3):170–9.

143. Gordon SA, Weber RP. Colorimetric Estimation of Inodoleacetic Acid. Plant Physiol. 1951;26(1):192–5.

144. Ahemad M, Kibret M. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. J King Saud Univ - Sci [Internet]. King Saud University; 2014;26(1):1–20. Available from: http://dx.doi.org/10.1016/j.jksus.2013.05.001

145. Shokri D, Emtiazi G. Indole-3-acetic acid (IAA) production in symbiotic and non-symbiotic nitrogen-fixing bacteria and its optimization by taguchi design. Curr Microbiol. 2010;61(3):217–25.

146. Idris E, Iglesias DJ, Talon M, Borriss R. Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by Bacillus amyloliquefaciens FZB42. Mol Plant Microbe Interact. 2007;20(6):619–26.

147. Lebuhn M, Heulin T, Hartmann A. Produktion of auxin and other inolic and phenolic compounds by Paenibacillus polymyxa strains isolated from different proximity to plant roots. FEMS microbiology ecology. 1997. p. 325–34.

148. Vendan RT, Yu YJ, Lee SH, Rhee YH. Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion. J Microbiol. 2010;48(5):559–65.

149. Vessey J. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil [Internet]. 2003;255(2):571–86. Available from: http://link.springer.com/article/10.1023/A:1026037216893

150. Gibson DJ, Connolly J, Hartnett DC, Weidenhamer JD. Designs for greenhouse studies of interactions between plants [review]. J Ecol 87(1)1-16, 1999 Feb. 1999;386–91.

151. Haase JU. Biodiversity and ecosystem functioning: The effects of tree and litter diversity. 2009;135.

152. Kohout P, Sýkorová Z, Bahram M, Hadincová V, Albrechtová J, Tedersoo L, et al. Ericaceous dwarf shrubs affect ectomycorrhizal fungal community of the invasive Pinus strobus and native Pinus sylvestris in a pot experiment. Mycorrhiza. 2011;21(5):403–12.

153. Passioura JB. Viewpoint: The perils of pot experiments. Funct Plant Biol. 2006;33(12):1075–9.

154. McPhee CS, Aarssen LW. The separation of above- and below-ground competition in plants a review and critique of methodology. Plant Ecol. 2001;152(2):119–36.

155. Blair BC, Perfecto I. Successional status and root foraging for phosphorus in seven tropical tree species. Can J For Res [Internet]. 2004;34(5):1128–35. Available from: http://www.nrcresearchpress.com/doi/abs/10.1139/x04-004

156. Hata K, Kato H, Kachi N. Litter of an alien tree, Casuarina equisetifolia, inhibits seed germination and initial growth of a native tree on the Ogasawara Islands (subtropical oceanic islands). J For Res. 2010;15(6):384–90.

157. van Hees a FM, Hees A Van. Growth and morphology of pedunculate oak (Quercus robur L) and beech (Fagus sylvatica L) seedlings in relation to shading and drought. Ann des Sci For [Internet]. 1997;54(31):9–18. Available from: http://www.afs-journal.org/index.php?option=com_article&access=doi&doi=10.1051/forest:199

70102&Itemid=129%5Cn%3CGo to ISI%3E://A1997WL35800002

158. Wagner PA, Dreyer E. Interactive effects of waterlogging and irradiance on the photosynthetic performance of seedlings from three oak species displaying different sensitivities (Quercus robur, Q petraea and Q rubra). Ann des Sci For. 1997;54(5):409–29.

159. Society E. Forest Litter Decomposition in Relation to Soil Nitrogen Dynamics and Litter Quality Author (s): C . A . McClaugherty , John Pastor , J . D . Aber , J . M . Melillo Published by : Ecological Society of America Stable URL : http://www.jstor.org/stable/19. 2010;66(1):266–75.

160. Martínez-Viveros O, Jorquera M., Crowley D., Gajardo G, Mora M. Mechanisms and Practical Considerations Involved in Plant Growth Promotion By Rhizobacteria. J soil Sci plant Nutr. 2010;10(3):293–319.

161. El-Saeid HM, Abou-Hussein SD, El-Tohamy W a. Growth Characters, Yield and Endogenous Hormones of Cowpea Plants in Response to IAA Application. Res J Agric Biol Sci. 2010;6(1):27–31.

162. Van Iersel M. Auxins affect posttransplant shoot and root growth of vinca seedlings. HortScience. 1998. p. 1210–4.

163. Mia MAB, Shamsuddin ZH, Wahab Z, Marziah M. Rhizobacteria as bioenhancer and biofertilizer for growth and yield of banana (Musa spp. cv. “Berangan”). Sci Hortic (Amsterdam) [Internet]. Elsevier B.V.; 2010;126(2):80–7. Available from: http://dx.doi.org/10.1016/j.scienta.2010.06.005

164. Amir HG, Shamsuddin ZH, Halimi MS, Ramlan MF, Marziah M. N2 fixation, nutrient accumulation and plant growth promotion by rhizobacteria in association with oil palm seedlings. Pakistan Journal of Biological Science. 2003. p. 1269–72.

165. Flores-Félix JD, Menéndez E, Rivera LP, Marcos-García M, Martínez-Hidalgo P, Mateos PF, et al. Use of Rhizobium leguminosarum as a potential biofertilizer for Lactuca sativa and Daucus carota crops. J Plant Nutr Soil Sci. 2013;176(6):876–82.

166. Sheng XF, He LY. Solubilization of potassium-bearing minerals by a wild-type strain of *Bacillus edaphicus* and its mutants and increased potassium uptake by wheat. Can J Microbiol [Internet]. 2006;52(1):66–72. Available from: http://www.nrcresearchpress.com/doi/abs/10.1139/w05-117

167. Han HS, Lee KD. Phosphate and potassium solubilizing bacteria effect on mineral uptake , soil availability and growth of eggplant. Res J Agriulture Biol Sci [Internet]. 2005;1(2):176–80. Available from: http://www.aensiweb.net/AENSIWEB/rjabs/rjabs/176-180.pdf

168. Tien TM, Gaskins MH, Hubbell DH. Plant Growth Substances Produced by Azospirillum brasilense and Their Effect on the Growth of Pearl Millet (Pennisetum americanum L.). Appl Env Microbiol [Internet]. 1979;37(5):1016–24. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16345372

169. Gravel V, Antoun H, Tweddell RJ. Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with Pseudomonas putida or Trichoderma atroviride: Possible role of indole acetic acid (IAA). Soil Biol Biochem. 2007;39(8):1968–77.

170. Domenech J, Reddy MS, Kloepper JW, Ramos B, Gutierrez-Mañero J. Combined application of the biological product LS213 with Bacillus, Pseudomonas or Chryseobacterium for growth promotion and biological control of soil-borne diseases in pepper and tomato. BioControl. 2006;51(2):245–58.

171. Brooker MIH,, Kleinig DA. Field guide to eucalypts northern Australia: Volume 3. Inkata Press. 1994.

172. Boland DJ, Brooker MIH, Chippendale GM, Hall N, Hyland BPM, Johsnston RD, Kleinig DA, Turner JD. Forest Trees of Australia. CSIRO Publications. 1992.

173. Dickinson GR, Sun D. Species and provenance evaluation of *Eucalyptus* cloeziana, E. *pellita* and E. urophylla at age 4 years, in far north Queensland.

Queensland Department of Primary Industries (Forestry). 1995. p. 19.

174. Vigneron P. Creation and improvement of hybrid varieties of *Eucalyptus* in the Congo. Bois et Forets des Tropiques. 1992. 234, 29-42

175. Lott R, Sexton G, Novak M. Seed and seedling supply for farm forestry projects in the tropics and subtropics of eastern Australia. Rural Industries Research and Development Corporation, Canberra. 2005. p. 24-48.

176. Harwood CE. *Eucalyptus pellita*: an annotated bibliography. CSIRO Forestry and Forest Products. 1998.

177. Harwood CE, Alloysius D, Pomroy P, Robson KW, Haines NW. Early growth and survival of *Eucalyptus pellita* provenances in a range of tropical environments, compared with *E. grandis*, *E. urophylla* and *Acacia mangium*. New Forests. 1997. 14(3), p.203-219.

178. Annandale M, Keenan R. Ten-year growth performance of exotic and native species on the coastal lowlands of north Queensland. 1999. Queensland Forestry Research Institute.

179. Bristow M, Erskine PD, McNamara S, Annandale M. Species performance and site relationships for rainforest timber species in plantations in the humid tropics of Queensland. 2005. Erskine PD, Lamb D. and Bristow M.(eds), p.84-100.

180. Alcom PJ, Forrester DI, Smith RGB, Thomas DS, James RN, Nicotra AB, Bauhus J. Crown structure and vertical foliage distribution in 4-year-old plantation-grown *Eucalyptus* pilularis and *Eucalyptus* cloeziana. 2013. Trees. 27(3). p. 555-566.

181. Bootle KR. Wood in Australia. Types, properties and uses. 1983. McGraw-Hill book company.

182. Eldridge K. Davidson J. Harwood C. Wyk GV. Eucalypt domestication and breeding. 1994. Clarendon Press.

183. Xu D, Dell B. Importance of micronutrients for productivity of plantation eucalypts in east Asia. Kikkawa, J. et al. 1997. p. 133-138.

184. Xu D, Bai J, Dell B. Overcoming the constraints to productivity of plantation eucalypts in Southern China. China Forestry Publishing House. 2000.

185. Mrkovacki N, Milic V. Use of Azotobacter chroococcum as potentially useful in agricultural application. Annals of Microbiology, 51(2). 2001. p.145-158.

186. Burdman S, Jurkevitch E, Okon Y. Burdman S, Jurkevitch E, Okon Y. Recent advances in the use of plant growth promoting rhizobacteria (PGPR) in agriculture. Microbial interactions in agriculture and forestry. Vol (II). 2000. p. 229-250.

187. Kaymak DC. Potential of PGPR in agricultural innovations. Plant growth and health promoting bacteria. Springer Berlin Heidelberg, 2010. p. 45-79.

188. Swain MR, Naskar SK, Ray RC. Indole-3-acetic acid production and effect on yam (Dioscorea rotundata L.) minisetts by Bacillus subtilis isolated from culturable cowdung microflora. Pol J Microbiol. 2007;56. p. 103–110

189. Ahmad F, Ahmad I, Khan MS. Indole acetic acid production by the indigenous isolates of Azotobacter and fluorescent Pseudomonas in the presence and absence of tryptophan. Turk J Biol. 2005;29. p. 29–34.

190. Pishchik VN, Vorobyev NJ, Chernyaeva LI, Timofeeva SV, Kazhemyakov AP, Alexeev YV. Experimental and mathematical simulation of plant growth promoting rhizobacteria and plant interaction under cadmium stress. Plant Soil. 2002; 243, p. 173–186.

191. Flores-Felix JD, Silva LR, Rivera LP. Plants probiotics as a tool to produce highly functional fruits: The case of Phyllobacterium and vitamin C in strawberries. PLoS ONE. 2015

192. Postgate J. The origins of the unit of nitrogen fixation at the University of Sussex. Notes and Records of the Royal Society. 1998;52(2). p.355-362.

193. Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil. 2009;321. p. 305–339.

194. ASEAN Centre for Biodiversity. Heart of Borneo [Internet] 2011. p. 1–5. Available from http://chm.aseanbiodiversity.org/index.php?option=com_content&view=article&id=132&Itemid=219.

195. Sarawak Government. The Geography of Sarawak [Internet]. 2016. Available from http://www.sarawak.gov.my/web/home/article_view/159/176/

196. Sarawak Forestry. Sarawak National Park. National Parks and Reserves [Internet]. 2014. Available from http://www.sarawakforestry.com/htm/snp-np.html

197. FAO. Proceedings of the FAO Regional Expert Consultation on *Eucalyptus*. FAO Regional Office for Asia and the Pacific 1995.

198. Midgley SJ. Pinyopusarerk K. The role of *Eucalyptus* in local development in the emerging countries of China, Vietnam and Thailand. CSIRO. 1995. p. 4-10.

199. Baillères H, Hopewell GP, and McGavin RL. Evaluation of wood characteristics of tropical post-mid rotation plantation# *Eucalyptus* cloeziana# and# *E. pellita*#. Part (c) wood quality and structural properties. 2008.

200. Mishra DJ, Rajvir S, Mishra UK, Kumar SS. Role of bio-fertilizer in organic agriculture: a review. Res. J. Recent Sci. 2013;2, p.39-41.

201. Bakker PA, Raaijmakers JM, Bloemberg G, Höfte M, Lemanceau P, Cooke B. New perspectives and approaches in plant growth-promoting rhizobacteria research. Springer Science & Business Media. 2010.

202. Gaur AC. Microbial mineral phosphate solubilization-An overview. National Symposium On Mineral Phosphate Solubilization. Vol (I). 2002. p. 1-3.

203. Mayer AM. Determination of indole acetic acid by the Salkowsky reaction. Nature. Vol (182). 1958. p. 1670-1671.

204. Sgroy V, Cassán F, Masciarelli O, Del Papa MF, Lagares A, Luna V. Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte Prosopis strombulifera. ApplIed microbiology and Biotechnology. 2009. 1;85(2):371-81.

205. Vosti SA, Carpentier CL, Witcover J, Valentim JF. Intensified small-scale livestock systems in the western Brazilian Amazon. Agricultural technologies and tropical deforestation. 2001. p. 113 – 133.

206. Sabang J, Bujang HB. A laboratory manual of selected analytical methods for soil and plant, Soil Research Unit, Forest Research Centre, Forest Department Sarawak.

207. Banerjee A, Datta JK, Mondal NK. Impact of different combined doses of fertilizers with plant growth regulators on growth, yield attributes and yield of mustard (Brassica campestris cv. B 9) under old alluvial soil of Burdwan, West Bengal, India. Frontiers of Agriculture in China. 2010; 4(3). p.341 - 351.