EXPLORING GROWTH ENHANCING RHIZOSPHERIC MICROORGANISMS FOR SILVICULTURE OF *NEOLAMARCKIA CADAMBA*

by

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Abstract

Neolamarckia cadamba (*N. cadamba*) is an indigenous timber tree that was selected to rehabilitate degraded forest lands in Sarawak. Application of chemical fertilisers is a standard practice to support growth of *N. cadamba* on degraded forest lands. The negative impacts of chemical fertilisers on environments and human health are always raised up as public concerns. Thus, alternatives like plant growth enhancing microorganisms are introduced to agricultural and silvicultural sectors. Plant growth enhancing microorganisms were widely reported in the studies of agricultural crops but seldom presented in articles of silviculture. *N. cadamba* was reported to grow naturally in primary and secondary forests. It is possible that these microorganisms that co-exist in the natural rhizospheres of *N. cadamba* may enhance growth of host plants either by increasing the availabilities of nutrients for root uptake or promoting the root growth. The objectives of this study are to isolate and characterise such plant growth enhancing microorganisms from *N. cadamba* rhizospheric soils in Sarawak to formulate microbial consortia, and evaluate their plant growth enhancing effects through pot trial using *N. cadamba* seedlings. The *N. cadamba* rhizospheric soil samples were collected from Kubah National Park in Matang, Similajau National Park in Bintulu, Sabal Forest Reserve in Simunjan, Semenggoh Nature Reserve in Siburan and a planted forest in Bintulu, Sarawak. From the soil analysis, soil textures in these locations were ranging from sandy clay loam, sandy loam to clay. As for the soil pH, Semenggoh Nature Reserve had the lowest pH of 3.86 and Kubah National Park had the highest pH of 5.46. From these soil samples, 41 strains of plant growth enhancing microorganisms were successfully isolated and screened based on four functional traits namely nitrogen fixation, phosphate solubilisation, potassium solubilisation and plant hormone indole-3- acetic acid (IAA) production using Jensen's medium, Pikovskaya's agar, modified Aleksandrov's medium and tryptophan broth respectively. The isolated strains could render the availabilities of nitrogen, phosphorus and potassium which are essential macronutrients for plants, as well as produce IAA, a hormone generally known to stimulate root growth. These selected strains were genetically identified as *Methylobacterium* sp., *Pseudomonas* spp., *Stenotrophomonas* sp., *Streptomyces* sp., *Streptacidiphilus* sp., *Lysinibacillus* spp., *Serratia* sp., *Bacillus* spp., *Arthrobacter* sp., *Metarhizium* spp., *Phanerochaete* sp. and *Penicillium* sp. Their

efficiencies in nitrogen, phosphate, potassium and IAA production were evaluated using phenate method, vanadomolybdophosphoric acid colorimetric method, atomic absorption spectroscopy (AAS) and Salkowski's method. After that, four best strains namely *Streptomyces* N11 (nitrogen fixation), *Serratia* P8 (phosphate solubilisation), and *Bacillus* K5 (potassium solubilisation) and *Bacillus* I6 (IAA production) were selected for further in-vitro experiment. To ascertain plant growth enhancing properties of the microbial strains, a small scale pot experiment was conducted using *N. cadamba* seedlings in 10 replicates for each of the treatment. Each strain used for formulation of consortia would reach the recommended concentration range of 10^7-10^8 cfu/mL. The first consortium labelled as Consortium A contained nitrogen fixing, phosphate solubilising and potassium solubilising strains. The second consortium labelled as Consortium B included all the three strains as in Consortium A but with the addition of IAA producing strain. Besides using per se as treatments in the pot experiment, each consortium was also mixed with chemical fertiliser. The Consortium A-chemical fertiliser and Consortium B-chemical fertiliser were therefore the two integrated treatments in which each consortium was mixed with half regime of chemical fertiliser. The results showed that both consortia exerted significant plant growth enhancing effects on both the shoot and root growths comparing to negative control which was without any fertiliser. Among the consortial treatments, Consortium B containing IAA producing strain showed significantly better shoot and root growth results than Consortium A. Besides that, Consortium A-chemical fertiliser and Consortium B-chemical fertiliser were found to be as effective as full regime chemical fertiliser in promoting the shoot growths. Similar experience from Consortium B, Consortium B-chemical fertiliser showed significant plant growth enhancing effect on root growth to both Consortium A-chemical fertiliser treatment and full regime chemical fertiliser treatment. Consortium A-chemical fertiliser showed on par root growth result to full regime chemical fertiliser treatment. In conclusion, the study showed that indigenous microbial strains of Sarawak could enhance the growth of *N. cadamba* effectively either in consortial forms or in combination with half regime of chemical fertiliser. The integrated consortium-chemical fertiliser treatments were as good as full regime chemical fertiliser in enhancing the growth of *N. cadamba* seedlings in this study. As such, through using such microbial strains, the usage of chemical fertiliser could be substituted or reduced for silviculture of *N. cadamba*.

Acknowledgement

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Declaration

I, Alan Chua Yee Quan, candidate of Master of Science by Research from Faculty of Engineering, Computing and Science in Swinburne University of Technology Sarawak Campus hereby declare that my master thesis entitled "Exploring growth enhancing rhizospheric microorganisms for silviculture of *Neolamarckia cadamba*" is original writing outcome and contains no material or content which has been accepted for the award to the stated candidate of any other degree or diploma studies, except where due references are made in the text of the examinable outcomes; and where the work is based on joint research or publications, the disclosed relative contributions of the respective workers or authors.

Alan Chua Yee Quan

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

Dr Daniel Tan Lee Tung

Conference presentations

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Thesis Outline

The thesis contents were distributed in five chapters and listed accordingly as follow:

Chapter 1: Introduction and literature review

Chapter 1 would include a brief introduction on the importance of this study. The literature review would mention the relevant studies on forest plantations in Sarawak, *Neolamarckia cadamba*, tropical soil physicochemical properties, forest land degradation, chemical fertilisers and plant growth enhancing microorganisms. Besides these, the aim and objectives of this study would be stated.

Chapter 2: Isolation and characterisation of plant growth enhancing microorganisms from *Neolamarckia cadamba* rhizospheres

Chapter 2 would focus on the isolation of plant growth enhancing microorganisms from rhizospheric soil samples of *N. cadamba* mature trees and seedlings. The isolated strains were screened using selective media to identify their functions. In addition, the physicochemical properties of collected soil samples would also be discussed in this chapter.

Chapter 3: The genetic identification of selected strains and evaluation on their biological activities related to nitrogen fixation, phosphate and potassium solubilisation, and plant hormone IAA production

Chapter 3 would report the molecular identifications and microbial activity analyses of selected strains. The selected strains were identified using 16S rRNA and ITS genetic markers. The efficiencies of strains in nitrogen fixation, phosphate and potassium solubilisation, and IAA production were evaluated using selective liquid media and chemical reagents.

Chapter 4: Evaluating the plant growth enhancing effect of microbial consortia via small scale pot experiment on *Neolamarckia cadamba* seedlings

Chapter 4 would discuss the plant growth enhancing effect of the selected strains on the *N. cadamba* seedlings in small scale pot experiment. The selected strains were applied to the seedlings in the form of consortia, with or without combination with half regime of chemical fertiliser.

Chapter 5: Conclusion and further recommendations

Chapter 5 would present the important findings of this research. There were several future work recommendations to be discussed in this chapter as well.

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List of Abbreviations

$(NaPO_3)_6$	Sodium Hexametaphosphate
Al	Aluminium
Al_2O_3	Aluminium Oxide
ANOVA	Analysis of Variance
Ca	Calcium
$Ca_3(PO_4)_2$	Tricalcium Phosphate
$CaCO_3$	Calcium Carbonate
CFU	Colony Forming Unit
Fe	Iron
$FeCl_3$	Iron (III) Chloride
$FePO_4$	Iron Phosphate
FR	Forest Reserve
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulphuric Acid

IAA	Indole-3-Acetic Acid
ITS	Internal Transcribed Spacer
K	Potassium
KH_2PO_4	Potassium Dihydrogen Phosphate
LPF	Licensed Planted Forest
Mg	Magnesium
$MgSO_4 \bullet 7H_2O$	Magnesium Sulphate Heptahydrate
N. cadamba	*Neolamarckia cadamba*
N/N_2	Nitrogen
NA	Nutrient Agar
Na_2CO_3	Sodium Carbonate
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
NH_4Cl	Ammonium Chloride

NP(s)	National Park(s)
NR	Nature Reserve
P	Phosphorus
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PO_4^{3-}	Phosphate
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SD	Standard Deviation
SE	Standard Error
SFC	Sarawak Forestry Corporation
SiO_2	Silicon Oxide
SUTS	Swinburne University of Technology Sarawak Campus
USDA	United States Department of Agriculture

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CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In Malaysia, the total forest area coverage was 68.1% in 1990 but slightly decreased to 67.6% in 2015. As such, about two-third or 22.3 million hectares of land was still under forest coverage in Malaysia (D'enghien 2016). Sarawak, the largest state in Malaysia, plays an important role in maintaining the forest coverage. Sarawak, holding a landmass of 12.4 million hectares, had more than 80% of the land covered by forest in 2012. 335,049 hectares of these forest areas were created by replanting the trees on deforested lands (Wong et al. 2014). The major replanting activities were initiated under Reforestation Research Programme as early as 1965 (Krishnapillay 2002, p. 14).

Both Reforestation Research Programme and forest plantation projects in Sarawak used fast-growing species to rehabilitate the degraded forest lands. *Neolamarckia cadamba* was one of the indigenous species selected for tree planting activities. The indigenous species could have advantages in local environment adaptation, pest and disease resistance (Krisnawati, Kallio & Kanninen 2011). In 2014, 20,833 hectares or 6% of the total planted land was planted with *N. cadamba* in Sarawak (Wong et al. 2014). However, trees planted on the degraded forest lands would often not gain yield as high as nutrient-rich natural habitats (United States Office of Technology Assessment 1984, p. 201). This is because the degraded forest lands in general cannot fulfil the nutrient requirements of planted trees due to low available nutrient levels in the soils (Asio et al. 2009, p. 77). Malaysia had an estimated degraded forest area of 10.3 million hectares between 2004 and 2006 (Krishnapillay 2007, p. 9). Sarawak had also had more than 3.0 million hectares of degraded forest areas as recorded since 1985 (Sarawak Forestry Department 2016).

The common practice to provide nutrients for tree planting is the application of chemical fertilisers. According to Sarawak Forestry Corporation (SFC), chemical fertilisers could be applied in the planting hole or on the ground after planting of tree. The released nutrients are water soluble thus directly available for plant root absorption (Chen 2006, p. 2). Nonetheless, the heavy rainfall in tropical climate could also wash off these water soluble nutrients easily, thus depriving the plant of the nutrients, as well as polluting the environment. Due to this factor, the trees may actually receive less amount of nutrients from applied chemical fertilisers, and subsequent applications of fertilisers would be

required to maintain the tree growth (Adesemoye, Torbert & Kloepper 2009, p. 922). Thus, chemical fertiliser application may not be an ideal option to support the nutrient requirements of trees planted on the large area of degraded forest lands. Moreover, the chemical fertilisers are products derived from non-renewable resources which are not able to sustain the growing demand in agricultural and silvicultural productions in the future (Righi, Lucialli & Bruzzi 2005, p. 168; Lubkowski 2016, p. 72).

The plant growth enhancing microorganisms can be environmental-friendly alternatives to chemical fertiliser for sustainable silviculture. Plant growth enhancing microorganisms are a wide range of soil microorganisms found either in rhizosphere or associated with the host plants that demonstrated the abilities to enhance the growth of host plant (Vessey 2003, p. 571). They can form either rhizospheric or endophytic relationships with the host plant depending on the colonised organs and mechanisms of colonisation (Vessey 2003, p. 573). They were often referred as biofertilisers in research publications because biofertilisers are basically the beneficial microorganisms which can increase the availability of nutrients for the plants (Vessey 2003, p. 571).

The rhizospheric plant growth enhancing microorganisms are the main focus of this study. These microorganisms could be found in the rhizosphere of the soil near to the host plants where the soil are specifically influenced by the root system (Souza, Ambrosini & Passaglia 2015, p. 401). The plant roots provide sugars, amino acids and other small molecules in the forms of root exudates to increase the colonisation of plant growth enhancing microorganisms (Glick 2012, p. 2). In exchange, the plant growth enhancing microorganisms assist the plants in acquiring available nutrients from the environments (Abhilash et al. 2016, p. 847). As such, these microbes were said to be “rhizospheric microbial partner of plants” (Abhilash et al. 2016, p. 847). There are five mechanisms in enhancement of plant growth by microorganisms namely biological nitrogen fixation, increasing availability of nutrients in the rhizosphere, promoting root growth, assisting other beneficial symbioses of the host and multiple functions (Vessey 2003, p. 574). In this study, three of these five mechanisms were addressed namely biological nitrogen fixation, increasing availability of nutrients such as phosphate and potassium in the rhizosphere, as well as promoting root growth through IAA hormone produced by microorganisms.

In addition, these plant growth enhancing microorganisms were assessed if they can be applied together with chemical fertilisers. This integrated application was commonly practiced to fulfil the nutrient requirements of plants using a reduced amount of chemical fertiliser (Chen 2006, p. 3). The effect of integrated applications using biofertiliser and reduced amount of chemical fertiliser had been studied on woody plants such as, *Paulownia kawakamii*, and agricultural crops such as common bean *Phaseolus vulgaris*. The results showed that such integrated applications of biofertiliser and notable decreased amount of chemical fertilisers gained similar growth rate if compared to chemical fertiliser treatments (Farahat et al. 2014, p. 854; Kumar, Singh & Singh 2009, p. 135). Nonetheless, there was little research on application of effective plant growth enhancing microorganisms for sustainable silviculture, particularly for *N. cadamba* plantation in Malaysia.

1.2 Literature Review

1.2.1 The background of reforestation and forest plantations in Sarawak, Malaysia

Malaysia had two-third of the land area or 22,195,100 ha covered by forest in 2015. The forest area was 22,125,100 ha in the year 2010 and thus an increase at a rate of 14,000 hectares per year in between 2010 and 2015. The Malaysia forest area only slightly decreased by 0.5% or 111,533 hectares in the past 25 years (1990-2015) and continuous reforestation activities were ongoing (D'enghien 2016).

Malaysia is able to sustain forest coverage partly attributed to forest plantation. Forest plantation is also known as planted forest where forest was established by planting or seeding. The establishments of forest plantations are not merely for timber production but also for environmental conservation and wind protection. Forest plantation actually contributes to 5% or 187 million ha of the global forest cover in 2001 (Carnus et al. 2006, p. 65). Malaysia had 1,966,000 ha of forest plantations, representing 8.9% of the total forest cover in 2015 (D'enghien 2016). Sarawak is the biggest state in Malaysia with 12.4 million hectares landmass. Based on 2012 satellite imageries, there were more than 80% of the state land covered by forest. The Reforestation Research Programme was carried out to rehabilitate degraded forest lands since the year 1965. These degraded forest lands were estimated to be 3 million hectares. There were also 6 million hectares of Sarawak forest land designated as permanent forest estate for sustainable timber production (Figure 1) (Bohari 2010). Sarawak government proposed a target of 1 million hectares forest plantation in the year 2020 in order to produce 15 million m^3 timber annually for the timber industry. The timber industry was requested to plant approximately 90,000 hectares per year from the year 2013 to the year 2020 (Ngayop 2013). However, the area of forest plantations was established only up to 335,049 hectares in the year 2014 (Wong et al. 2014).

Sarawak's forest plantations initiatives were in fact started as early as 1920s in establishment of small *Shorea* spp. (Engkabang) plantation in Semenggoh Forest Reserve for illipe nut production. Since 1965, Reforestation Research Programme was initiated in

Sarawak to investigate fast-growing exotic tree species for rehabilitating the degraded forest lands due to repeated occurring shifting cultivation activities. There were five tropical conifer species selected for planting trial including *Pinus caribaea*, *Pinus insularis*, *Agathis macrophylla*, *Araucaria cunninghamii* and *Araucaria hunsteinii*. However, the growths of tropical conifers were poor and therefore not suitable for forest plantations. Since then, several fast growing exotic tropical hardwood species such as *Acacia mangium*, *Gmelina arborea*, *Paraserianthes falcataria*, *Swietenia macrophylla*, *Durio zibethinus* and *Shorea macrophylla* were also experimented for degraded forest land rehabilitation (Krishnapillay 2002, p. 14). These tree species were also chosen to establish forest plantations. *Acacia mangium* and *Paraserianthes falcataria* were two major species planted in forest plantations since the year 1985 (Krishnapillay 2002, p. 15). In 1985, *Eucalyptus* spp., *Neolamarckia cadamba* and other tropical hardwood species were also selected for forest plantations. The planting areas of these hardwood species in the year 2014 are listed in Table 1. Among the planted tree species, only *N. cadamba* is the most important indigenous species planted in forest plantations (Yusoff et al. n.d.). It was reported that planting indigenous species having advantages in local environment adaptation, pests and disease resistance (Vincent 2002).

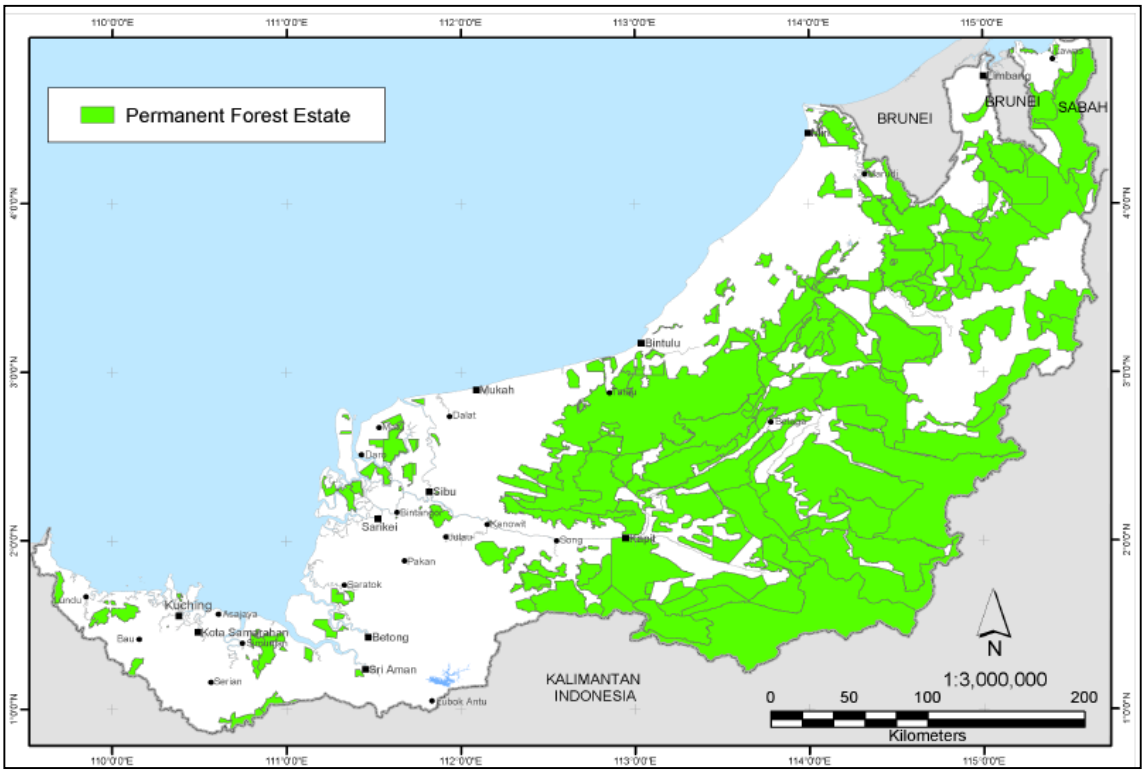


Figure 1 The designated permanent forest estate in Sarawak (Bohari 2010).

Table 1 The tropical species planted in forest plantations in the year 2014 (Wong et al. 2014).

Species	Area Planted (Hectares)	Percentage (%)
Acacia spp.	230,110	69
Batai (*P. falcataria*)	46,457	14
Eucalyptus spp.	26,527	8
Kelampayan (*N. cadamba*)	20,833	6
Others (Rubber, Meranti, Kapur, Keruing etc)	11,122	3
Grand Total	335,049	100

1.2.2 Introduction to *Neolamarckia cadamba*

Neolamarckia cadamba (*N. cadamba*) has another botanical name called *Anthocephalus cadamba* Miq. It belongs to family Rubiaceae. In Malaysia, its common local names are Kelampayan, Laran and Selimpoh. This tropical and subtropical species is native not only to Southeast Asia, but also to South Asia where it is known as Kadam. It is known to have fast growth characteristic, preference in alluvial regions and resistance to serious pests and diseases. Therefore, *N. cadamba* is suitable for both reforestation and industrial plantations (Krisnawati, Kallio & Kanninen 2011, p. 1). It is one of the selected indigenous species for Sarawak reforestation project and timber plantations.

N. cadamba is a pioneer species that could be found in the deep and moist alluvial regions. They are also found growing along the riverbanks in the secondary forest, permanently

and periodically flooded areas. It would not be unusual to find *N. cadamba* mother trees in the primary forests too. Although *N. cadamba* was reported growing on various types of soils, well-accelerated fertile soils would be preferred over the leached and poorly aerated soils (Krisnawati, Kallio & Kanninen 2011, pp. 2-3). The growth of *N. cadamba* is not only influenced by soil conditions but also the climate and landscape of its local environment. It is a light demanding species and sensitive to frost. The temperature in the natural habitat of *N. cadamba* ranges from 3°C to 42°C. Since it prefers moist soil environments, the annual rainfall should be in the range of 1500-5000 mm. However, some *N. cadamba* were reported to grow in regions with only 200 mm annual rainfall regions such as the central parts of South Sulawesi. *N. cadamba* usually grows at an altitude range of 300-800 m above the sea level. However, there are some exceptions that the trees were found to grow at 1000 m above sea level in highlands of the equator region (Krisnawati, Kallio & Kanninen 2011, p. 3). In South Asia region, *N. cadamba* is found in deep and moist deciduous forests of Maharashtra, India where can support the living requirements of *N. cadamba* (Maharashtra State Forest Department n.d., p. 2). Sarawak would fulfil the living requirements of *N. cadamba* with its local tropical climate and landscapes, except for the soil conditions that are generally poor in plant nutrients.

N. cadamba is a large tree with a broad umbrella-shaped crown and straight cylindrical stem (Figure 2). The branches are arranged in layers. It can grow to a height of 45 m with a trunk diameter of 100-160 cm. The bark appearance can differentiate the age of trees. Young trees have grey, smooth and very light bark while old trees have rough and longitudinal fissured bark (Krisnawati, Kallio & Kanninen 2011, p. 1). The heartwood is white in colour with a yellow tinge darkening to creamy yellow on the exposure that cannot be clearly differentiated from sapwood. The wood is fine to medium in texture and low lustre (Figure 3). It has a density range of 290-560 kg/m^3 at 15% moisture content (Krisnawati, Kallio & Kanninen 2011, p. 3). The wood is moderately strong in strength and can be treated with preservatives easily. The treated wood is durable but its durability does not cause an issue in wood processing operations (Bijalwan, Dobriyal & Bhartiya 2014, pp. 297-298). *N. cadamba* is a lightweight hardwood could be an important timber supply in the plywood industry. Sarawak is the largest plywood producer in Malaysia with a production amount of 2.74 million m^3 (Lissem 2013).

Besides its lightweight hardwood timber, the other parts of *N. cadamba* could also be useful products. Its dried bark can be used to relieve fever or to be consumed as a tonic (Orwa et al. 2009). The green, glossy leaves have the dimension sizes of 15-50 cm long by 8-25 cm wide (Krisnawati, Kallio & Kanninen 2011, p. 1). Fresh leaves can be treated as fodder to feed cattle. Shedded leaves and non-leaf litters are good soil improver. The decomposition process improves soil physical and chemical properties of the canopy (Orwa et al. 2009). *N. cadamba* has many fleshy fruitlets with four hollow or solid structures on each fruitlet upper parts. The fruitlets are small in size and the packed by fleshy capsules to form a fleshy yellow-orange infructescence. Each fruitlet may contain approximately 8000 seeds which are trigonal or irregular in shapes without wings attached (Krisnawati, Kallio & Kanninen 2011, p. 1). The ripened fruitlets and inflorescences are edible (Orwa et al. 2009).

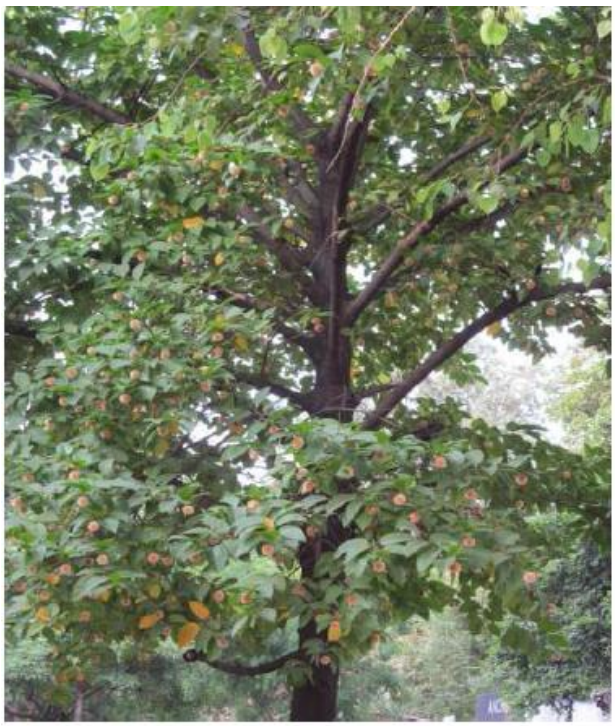


Figure 2 The flowering *Neolamarckia cadamba* (Bijalwan, Dobriyal & Bhartiya 2014, p. 297).

Figure 3 The wood texture of *Neolamarckia cadamba* (Krisnawati, Kallio & Kanninen 2011, p. 3).

1.2.3 Forest land degradation in Sarawak, Malaysia

The land degradation is an important global issue in the twenty-first century due to its negative impact on agriculture productivity, environment, food security and quality of life (Eswaran, Lal & Reich 2001). The degradation process causes a decline in soil productivity and its environment-regulating ability in long term (Asio et al. 2009, p. 70). Natural process and human activities are the two factors to cause land degradation. As the rate of natural degradation is slow, Global Assessment of Soil Degradation (GLASOD) emphasises on human-induced land degradation. Human activities is likely to make the land degradation to happen due to the drastic changes or disruption of normal soil formation process (Asio et al. 2009, p. 71).

The tropical forest lands have been cleared and degraded at an accelerating rate over the last few decades. The tropical forest land loss is described as the sixth global mass extinction in Earth's history. From the year 2000 to 2005, there were approximately 27 million hectares of tropical forest being cleared for timber extraction and agricultural purposes. The logging and slash-and-burn activities are the major causes of land degradation in tropical region (Bryan et al. 2013, p. 1). In Malaysia, although there were

32.9 million hectares of forest area in total, one-third or 10.3 million hectares of this area is actually degraded forest area (Krishnapillay et al. 2007, p. 9). The state of Sarawak held more than 3 million hectares of degraded forest area (Krishnapillay, Razak & Apanah 2007, p. 87). The tropical forest in Malaysia was disturbed by human activities including over-extraction of timber, shifting cultivation, slash-and-burn practice in forest land clearance and over-grazing (Krishnapillay, Razak & Apanah 2007, pp. 87-89). As a result, the exposed forest lands suffered from degrading process like soil erosion, nutrient loss and soil compaction.

The soil erosion process is common in tropical forest which causes removal of the fertile topsoil from soil surface and thus reducing the productive capacity of the forest lands (Asio et al. 2009, p. 72). The topsoil contains sand, silt, clay, soil organic matters and the plant growth enhancing microorganisms (Soil Science Society of America 2017). The removal of topsoil would cause the reduction of silt and clay contents which are responsible for plant nutrients retention, and thus tropical degraded forest lands have low storage capacity of plant nutrients in general (Sheard 1991, p. 4). The soil organic matters is a dominant reservoir of plant nutrients in strongly weathered tropical soils (Asio et al. 2009, p. 77). It represents 90-95% of nitrogen source and major sources of available phosphorus and available sulphur in unfertilised tropical soils (Schulte & Ruhiyat 1998, p. 15). Hence, the plant nutrient levels in the tropical degraded forest lands could be further lowered. In addition, the degraded forest lands are lacking of plant growth enhancing microorganisms to support the vegetation growth due to the removal of topsoils (Ramachandran & Radhapriya 2016, p. 1).

The essential plant nutrients including nitrogen, phosphorus, potassium, calcium and magnesium could be removed from tropical forest lands in the process of forest vegetation clearance (Asio et al. 2009, p. 77). Application of heavy machinery like bulldozers in logging and land clearing process could disturb the nutrient rich forest topsoils and thus exaggerate the removal of nutrients. The use of bulldozers can also cause soil compaction besides topsoil disruption. The compacted soils would have reduced soil porosity, low plant available water capacity, poor aeration and smaller rooting space. As a result, the seeds and seedlings established in degraded lands may not grow well and are susceptible to be washed away during heavy precipitation (Pinard, Baker & Tay 2000, p. 214). In

addition, the topsoils were often displaced by bulldozer blades during skid trail construction (Pinard, Baker & Tay 2000, p. 214). Hence, the degraded forest lands are both low in nutrient and poor in texture for new vegetation growth, and it is not uncommon to get low survival and yield for trees planted on such degraded forest lands. The degraded forest lands are in general known as unfavourable medium for plant growth. The plant nutrients in degraded lands are low that cannot support the normal growth rate of planted trees (United States Office of Technology Assessment 1984, p. 201).

1.2.4 The application of chemical fertilisers and the disadvantages

The current silviculture practice in Sarawak is highly dependent on the usage of chemical fertilisers. Based on the procedures carried out in SFC, chemical fertilisers would be applied in the planting holes or on the soil surfaces after tree planting. The chemical fertilisers have the advantage that mineral nutrient can be supplied to the plant in a fast rate. The mineral nutrients are water soluble thus could be directly absorbed by plant roots. Their nutrient contents are often high enough to support the optimum growth of plants (Chen 2006, p. 2).

Although chemical fertilisers are supportive for plant growth, there are a few downsides in their usage and application. The global population is growing and predicted to reach 7.5-8.0 billion people by 2020. To support such population growth, the demand of chemical fertiliser in agricultural and silvicultural production would rise rapidly and reach 200.2 million tonnes by 2020. The nitrogen fertilisers, phosphate fertilisers and potassium fertilisers are the three highly demanded chemical fertilisers (Lubkowski 2016, p. 72). These chemical fertilisers are the products derived from non-renewable resources like fossil fuels, rocks and ores (Righi, Lucialli & Bruzzi 2005, p. 168). Their manufacturing processes also incur consumption of huge amount of fossil fuels such as petroleum and natural gas (Rashid et al. 2016, p. 27). As a result, chemical fertiliser application is considered as an unsustainable practice.

Furthermore, the uptake efficiency of chemical fertilisers by the plants is relatively low as plants only absorb a small portion of nutrients (Adesemoye, Torbert & Kloepper 2009,

p. 922). The major portions of nutrients in chemical fertilisers are either washed off by heavy precipitation or converted into unavailable forms by the soil particles. These unavailable forms could not be absorbed by the plants. Hence, subsequent applications of chemical fertilisers may be required to maintain plant growth. On the other hand, chemical fertilisers may cause negative impacts to the environment. Commercial nitrogenous fertilisers such as urea, ammonia, ammonium salts and nitrate salts are soluble compounds. Some of these nitrogenous fertilisers can be easily oxidised to nitrate for plant root absorption. The nitrate compounds are not attached to the soil particles and present as solution in the soil (Food and Agriculture Organization of the United Nations 1972, p. 12). This soluble nitrate compound is thus vulnerable to heavy leaching process in the tropical climate and would contaminate the water bodies (Savci 2012, p. 288). In addition, the nitrate compound can be denitrified and escaped to the atmosphere in the forms of nitrogen gas or oxides of nitrogen such as nitrous oxide, N_2O (Food and Agriculture Organization of the United Nations 1972, p. 12). The release of nitrous oxide could pollute the air and create greenhouse effect (Savci 2012, p. 290).

As for phosphate fertilisers, they are easily converted to insoluble forms in the soil. The phosphate ions could render extremely immobilised due to binding to the surfaces of iron, aluminium and manganese oxides and hydroxides as well as the clay particles in the soil. The phosphate fertilisers could also be washed away by runoff if applied in excess, or heavy precipitation occurred shortly after their application (Food and Agriculture Organization of the United Nations 1972, pp. 10-11). The increased amount of phosphorous in water can lead to water eutrophication. The algae blooming would lead to deprivation the dissolved oxygen in water bodies, thus endangering the aquatic lives (Savci 2012, p. 289). Similar to nitrogenous fertilisers, potassium fertilisers are also highly soluble in water and the potassium ions could be absorbed by soil particles rapidly. The potassium ions would take part in cation exchange process. Equivalent amounts of calcium and magnesium ions are released into soil solution when the potassium ions are absorbed by clay or humus particles. If potassium fertilisers are added to increase the exchangeable potassium ions, a certain portion of the added exchangeable cations will be fixed by clay particles and less accessible to the soil solution (Food and Agriculture Organization of the United Nations 1972, pp. 11-12). The soil structure could be

deteriorated and the soil pH would be lowered if there is a heavy application of potassium fertilisers (Savci 2012, p. 290).

1.2.5 Tropical soil properties

Soils are unconsolidated mineral material on the earth surface and a growing medium for various plants. Soils consist of mineral matters, organic matters, water, gases and organisms (Igwe 2011, p.934). Soils are formed by the weathering process of rocks or materials deposited from water bodies and wind blows. The soil formation process is slow that forming a few centimetre of soil could cost thousands of years. The soil formation process is controlled by climate, organisms, parent material, topography and time. Hence, soil formation rates in different locations are variable due to these factors (Loganathan 1987).

The tropical soils are mainly containing coarse particles because the fine particles like silt and clay would be removed by heavy rainfall and excessive leaching. Therefore, the soil textures are mostly sandy loam to sandy clay. Those soil textures are relatively low in silt and clay contents (Igwe 2011, p.934). Slit and clay are responsible for the storage of plant nutrients because the plant nutrients ins silt and clay are more resistant to leaching process by soil water infiltration. Meanwhile, sand is mainly responsible for soil water regulation and soil aeration. It has little effect on the retention of plant nutrients (Sheard 1991, p. 4). Hence, tropical soils do not have a large capacity to store plant nutrients due to lower silt and clay contents. When the organic matters are degraded by tropical climate, the tropical soils cannot retain the plant nutrients well and the rainwater would wash the plant nutrients away from soil particles easily (Loganathan 1987). Hence, the sandy soil texture that is low in silt and clay is one of the main cause of low plant nutrients in the tropical soils.

Besides that, there are three acidic soil orders identified from tropical soils including Alfisols, Oxisols and Ultisols (Igwe 2011, p.934). The pH of those three soil orders ranges from 4.0 to 5.5 (Schulte & Ruhiyat 1998, p. 14). In addition, the high precipitation rate in tropical climate leaches the basic ions into water bodies and causes soil acidification. Due to acidic condition, plant macronutrients including nitrogen, phosphorus, potassium,

calcium and magnesium are less available in tropical soils (Truog 1946, pp. 305-306). Therefore, low soil pH is another root cause of poor nutrients in the tropical soils.

Even though the tropical soils are low on plant nutrients, they can still support forestry and agriculture developments. The plant growth enhancing microorganisms in tropical rhizospheres may have played a key role. Dipterocarpaceae native to Southeast Asia are known to tolerate acidic tropical soils with low nutrients due to the plant growth enhancing microorganisms that help to provide available nutrients for plant growth (Fujii 2014, pp. 371-372). Besides that, a study in Malaysia showed that plant growth enhancing microorganism inoculated to rice seedlings would render the seedlings to gain significant height and biomass increments without any chemical fertiliser supplement (Tan et al. 2014, p. 352).

1.2.6 Interaction of rhizosphere and soil microorganisms

Plant roots are the crucial components in the soil ecosystem and a narrow region of soil influenced by plant roots is called rhizosphere (Fageria & Stone 2006, p. 1327; Hrynkiewicz & Baum 2011, p. 36). The term *rhizosphere* in Greek means "the effect of a root on its surrounding environment" (Fageria & Stone 2006, p. 1327). The term 'Rhizosphere' was first introduced by a German scientist, Lorenz Hiltner in the year 1904 who described the rhizosphere as the interaction between the soil microorganisms and the legume plant roots. After several studies over the decades by many scientists, the modern definition of rhizosphere would include plant-microbe interaction. The plant-microbe interaction can be beneficial, pathogenic and neutral. Rhizosphere is also an important fundamental study in crop production (Fageria & Stone 2006, pp. 1327-1328).

Rhizosphere was described as a soil ecological zone where the soil would be particularly affected by plant roots due to the release of exudates from plant roots and the presence of residues of dead root tissue (Manoharachary & Mukerji 2006, p. 4). The rhizosphere region can be differentiated into ectorhizosphere and endorhizopshere (Figure 4). The ectorhizosphere refers to the soil layer around the plant roots, while endorhizosphere is the root layer associated to the soil microorganisms (Fageria & Stone 2006, p. 1329). Those two areas are separated by the root surface called rhizoplane. The rhizoplane is the

contact surface of plant roots where the soil particles and debris are closely bound to it (Manoharachary & Mukerji 2006, p. 5). The rhizoplane has high nutrient contents to attract many fungal and bacterial species (Fageria & Stone 2006, p. 1329).

Rhizosphere zone is influenced by the studied host plants and soil environment conditions (Manoharachary & Mukerji 2006, p. 4). The rhizosphere zone was normally estimated at the range of 1 to 5 mm depending on the plant species (Fageria & Stone 2006, p. 1328). Nonetheless, a study on rhizosphere in 1990 indicated that the rhizosphere zone could extend up to 20 mm, indicating that rhizosphere zone could be influenced by the soil environment factors such as organic compounds, soil microorganisms, soil pH, oxygen concentration, carbon dioxide concentration and soil moisture content (Fageria & Stone 2006, p. 1328). Some scientists suggested that the extent of rhizosphere could not be defined precisely because the soil microorganisms would be manipulated by the soluble and volatile compounds in plant root exudates (Fageria & Stone 2006, p. 1328).

The interaction of rhizosphere and soil microorganisms as well as their ratio were described as rhizosphere effect (Manoharachary & Mukerji 2006, p. 4). Rhizosphere effect is expressed in the term of R/S (Root soil) ratio (Manoharachary & Mukerji 2006, p. 5). The R/S ratio is defined as the ratio of microbial population in the rhizosphere region to the microbial population in the bulk soil (Sukumaran, Anilkumar & Thanga 2015, p. 11). The rhizosphere effect is reflected by the abundance of fungi and bacteria in the rhizosphere. The fungal and bacterial abundances are 10-20 times and 2-20 times higher in the rhizosphere region than in the bulk soil (Hrynkiewicz & Baum 2011, p. 36). Since there are high microbial populations in the limited area of rhizosphere region, the competition of nutrients sources is uprisen and a range of antagonistic to synergistic interactions is formed within the rhizosphere microorganism community (Hrynkiewicz & Baum 2011, p. 37).

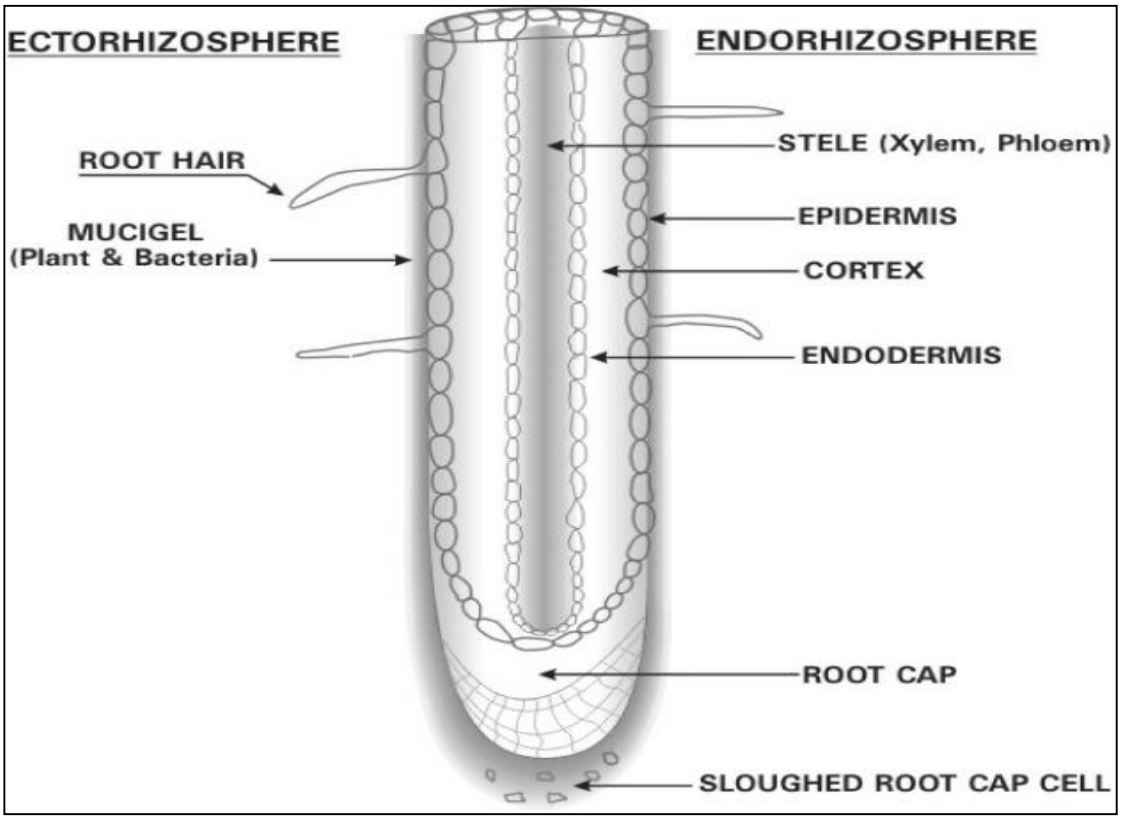


Figure 4 An overview of ectorhizosphere and endorhizosphere (Fageria & Stone 2006, p. 1329).

1.2.7 Plant growth enhancing microorganisms

To sustain the rapidly growing global population that would reach 8 billion people by the year 2020, the productions of food, energy and fibre would be among of the most crucial challenges in twenty-first century (Glick 2012, p. 1; Abhilash et al. 2016, p. 847). The usage of agrochemicals including chemical fertilisers, herbicides, fungicides and insecticides would increase along with the growing global demand of food and fibre. The increasing application of agrochemicals would lead to environmental pollutions and degradation of arable lands (Abhilash et al. 2016, p. 847). Thus, alternatives, such as plant growth enhancing microorganisms would emerge as an environmental-friendly substitute for chemical fertilisers.

The plant growth enhancing microorganisms refer to a wide variety of soil microorganisms found either in rhizosphere or associated to host plants which are able to promote their growth. They are also called often termed biofertilisers in research publications because biofertilisers could be defined as the application of soil microorganisms to increase the availability of nutrients for plant root uptakes (Vessey 2003, p. 571). There are rhizospheric and endophytic relationships formed by plant growth enhancing microorganisms and their host plants. The rhizospheric plant growth enhancing microorganisms are often found colonising the rhizosphere and plant root surface. The host plants release root exudates to attract rhizospheric plant growth enhancing microorganisms by changing the physical and chemical compositions of the soil in the rhizosphere including soil pH, soil moisture content and partial pressure of oxygen (Vessey 2003, p. 573). In addition, they also provide carbon sources, amino acids, organic acids and other small molecules in the forms of exudates to increase the microbial population around the root surfaces and in the rhizosphere (Glick 2012, p. 2). In the meantime, the rhizospheric plant growth enhancing microorganisms promote the growth of host plants by increasing the uptake of plant nutrients (Abhilash et al. 2016, p. 847).

There are five mechanisms used by rhizospheric plant growth enhancing microorganisms to enhance the nutrient uptake of host plants. These mechanisms are the biological nitrogen fixation, acquisition of the unavailable nutrients in the rhizosphere, increase of the root surface area, aiding effect on other beneficial symbioses of the host, and lastly a combination of these mechanism (Vessey 2003, p. 574). The nitrogen fixing microorganisms, phosphate solubilising microorganisms, potassium solubilising microorganisms and plant hormone IAA producing microorganisms were targeted in this thesis study. They covered the biological nitrogen fixation, fixed phosphate and potassium solubilisation as well as plant root growth promotion.

It was commonly practiced that the plant growth enhancing microorganisms were cultured artificially and mixed with suitable carrier materials to form biofertilisers or microbial inoculants (Pindi & Satyanarayana 2012, pp. 1-2). There are liquid based biofertilisers and solid carrier based biofertilisers. The existing carrier materials for solid carrier based biofertilisers include soils, plant waste materials and inert materials, while liquid medium for liquid based biofertilisers can be culture broth, sterile water, mineral

and organic oils (Bashan 1998, pp. 736-739). The liquid based biofertilisers have advantages in shelf life and handling technique over the carrier based biofertilisers. The plant growth enhancing microorganisms can remain viable for 6-24 months in suitable liquid medium without preservatives. In addition, the microorganisms are not clumping in the liquid medium after prolonged storage and can be directly used for resuspension (Pindi & Satyanarayana 2012, p. 4).

It was also common that the plant growth enhancing microorganisms was mixed with reduced amount of chemical fertiliser, without affecting sufficient nutrient supply to the host plants (Chen 2006, p. 3). This integrated applications of plant growth enhancing microorganisms and reduced amount of chemical fertilisers had been conducted on woody plants, agricultural crops and ornamental flowers. Those treated plant samples gained similar growth rate to plant samples treated with full strength chemical fertilisers (Kumar, Singh & Singh 2009, p. 135; Agamy, Mohamed & Rady 2012, p. 564; Farahat et al. 2014, p. 854). Hence, the effective plant growth enhancing microorganisms have the potential to be utilised to partly substitute the chemical fertiliser for the sustainable silviculture of *N. cadamba*.

1.2.7.1 Nitrogen fixing microorganisms

Nitrogen (N) is a key plant nutrient that promoting plant growth because it is an essential raw material for many important organic compounds. Plants need nitrogen to synthesise amino acids and form proteins, nucleic acids, alkaloids, chlorophyll, purine bases and enzymes (El-Ramady 2014, p. 464). The nitrogen element is abundant in the earth's atmosphere as the form of nitrogen gas. However, nitrogen gas is not the available nitrogen that plants can uptake. Plants can only uptake nitrogen in the forms of ammonium ion (NH_4^+) and nitrate ion (NO_3^-). The tropical soils are deficient in available nitrogen as high rainfall would wash off available nitrogen from the soil. There are two pathways to fix atmospheric nitrogen to available nitrogen. The first pathway is non-biological fixation that fixes atmospheric nitrogen to nitrate ion via thunder lightning. Non-biological fixation only fixes a small amount of atmospheric nitrogen. The second pathway is the biological fixation which contributes 60% of total nitrogen fixation (Buerdass & Hurst 2002). The biological fixation activities are carried out by symbiotic

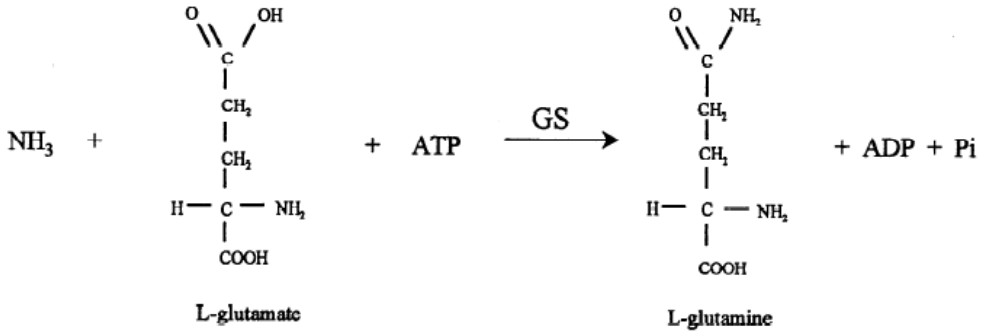
and free living nitrogen fixing microorganisms (Orr et al. 2011, p. 911). The nitrogen fixing microorganisms fix atmospheric nitrogen to form ammonia via nitrogenase catalysis.

Nitrogenase would utilise a metal-based catalyst and high input of adenosine triphosphate (ATP) to convert atmospheric nitrogen to ammonia molecules. There are four types of nitrogenases which are encoded by a set of specific genes and combined with different metals at their active sites. The molybdenum dependent (Mo-dependent) enzyme is the most abundant and widely reported nitrogenase group. It has a metallo-cofactor called FeMo-cofactor in its active site. The cofactor contains molybdenum, iron, sulphur, (R)-homocitrate and an unknown light atom, X (Seefeldt, Hoffman & Dean 2009, pp. 701-702). The nitrogen fixing reaction catalysed by Mo-dependent nitrogenase is shown in the equation below (Seefeldt, Hoffman & Dean 2009, p. 702):

$$N_2 + 8\ H^+ + 16\ MgATP + 8\ e^- \rightarrow 2\ NH_3 + H_2 + 16\ MgADP + 16\ P_i$$

The Mo-dependent nitrogenase consisted of two metalloprotein components which are known as iron (Fe) protein and molybdenum-iron (MoFe) protein. The nitrogen fixation process involves a series of reactions between the two metalloprotein components, electrons, MgATP and protons (Seefeldt, Hoffman & Dean 2009, p. 702). The Fe-protein is an exclusive electron donor with high reducing ability whereas the MoFe-protein receives the energy of electrons and convert atmospheric nitrogen molecule into two ammonia molecules. The nitrogen fixing microorganisms utilise organic molecules from the soil environment to support the high ATP input in the biological fixation process (Rashid et al. 2016, p. 29).

The ammonia molecules produced from biological nitrogen fixation are assimilated in the nitrogen fixing microorganisms by the enzyme glutamine synthase (GS). The ammonia molecules react with L-glutamate to yield L-glutamine as shown in the equation below (Colnaghi et al. 1997, p. 146):



However, the excess concentration of ammonia can inhibit the expression of *nif* gene and thus cause negative impacts on the synthesis and activity of nitrogenase (Colnaghi et al. 1997, p. 145). Therefore, the excess ammonia molecules are excreted from nitrogen fixing microorganisms to overcome the inhibitory effect on nitrogenase activity (Colnaghi et al. 1997, p. 146; Hartono et al. 2016, p. 11735). The first ammonia excretion experiment was carried out on mutated *Klebsiella pneumoniae*. The mutant strains were grown in the nitrogen free medium and found excreting excess amount of ammonia into the medium (Colnaghi et al. 1997, p. 147). On the other hand, a study of nitrogen fixing *Lysobacter* sp. strain suggested that the ammonia excretion was caused by the low carbon source in the environment and the bacteria cell lost its capability to retain ammonia (Iwata et al. 2010, pp. 417-418). The excreted ammonia molecules are either direct available for plant absorption or converted to nitrate as another source of available nitrogen by nitrifying bacteria via nitrification (Hartono et al. 2016, p. 11735).

There are a few nitrogen fixing strains widely used for biological nitrogen fertilisers namely *Rhizobium* spp., *Azotobacter* spp., *Azospirullum* spp., *Herbspirillum* spp., *Acetobacter* spp. and cyanobacteria. The biological nitrogen fertilisers would constitute approximately 80% of the global biofertiliser demand (Abhilash et al. 2016, p. 848). Nitrogen fixation trait of fungal strains was reported in Lipman (1911, p. 173) but it could not be validated by later research. As such, nitrogen fixing bacteria are more likely to be isolated from rhizosphere soil samples.

1.2.7.2 Phosphate solubilising microorganisms

Phosphorus (P) is another important plant nutrient besides nitrogen. It plays a central role in plant energy transfer and protein metabolism. It is also the component of RNA and DNA (El-Ramady 2014, p. 464). Although phosphorus is abundant in soil, most of it is in unavailable forms and could not be absorbed by the plant. In fact, only 0.1% of total phosphorus may exist as available forms (Sharma et al. 2013, p. 1). The available forms are soluble orthophosphate ions such as dihydrogen phosphate ion ($H_2PO_4^-$), hydrogen phosphate ion (HPO_4^{2-}) and phosphate ion (PO_4^{3-}). The most direct way to replenish soil available phosphorus is applying fertiliser. However, major available phosphorus released from the fertiliser could be promptly immobilised by highly reactive Al^{3+} and Fe^{3+} in the acidic soil, or Ca^{2+} in calcareous or normal soils (Yadav & Verma 2012, p. 93). Some microorganisms such as phosphate solubilising fungi and bacteria can extract phosphorus from this immobilised phosphorus pool in the soil. In the soil, there are organic and inorganic phosphorus sources which are equally important for plant growth. The phosphate solubilising microorganisms release mineral dissolving compounds to solubilise inorganic phosphorus sources. As for organic phosphorus sources, they could be released by extracellular enzyme secretion and phosphorus substrates degradation (Sharma et al. 2013, p. 5).

The solubilisation of inorganic phosphorus by microorganisms is one of the objectives in this study. The microorganisms were screened using Pikovskaya's medium which contained inorganic phosphorus source. There are two groups of inorganic phosphorus source in the soils. They are calcium phosphate from calcium dominant soils and iron and aluminium phosphates from iron and aluminium dominant soils (Yadav & Verma 2012, p. 94). These phosphorus sources are insoluble and unavailable for plant root absorption. Calcium phosphate is derived a mineral called apatite which is commonly found in calcareous and neutral soils. The phosphate solubilising microorganisms secrete different types organic acids to lower the pH of rhizosphere, and the solubility of calcium phosphate is thus enhanced via proton substitution or release of calcium ions. In addition, phosphate solubilising microorganisms could also solubilise calcium phosphate by releasing carboxylic anions to chelate the calcium ions due to its high affinity to calcium. Hence, the solubilisation of calcium phosphate could be accomplished by the combined

effect of lowering of rhizosphere pH and carboxylic acid synthesis (Yadav & Verma 2012, p. 99). Iron and aluminium phosphates are another inorganic phosphorus sources. The iron phosphate is originated from strengite whereas aluminium phosphate is derived from variscite. Those two minerals are abundant in the acidic soils (Yadav & Verma 2012, p. 95). The phosphate solubilising microorganisms release carboxylic acids to solubilise the iron and aluminium phosphates. The carboxylic acids can dissolve the mineral phosphate via anion exchange of phosphate ions by acid anions or chelation of both iron and aluminium ions (Yadav & Verma 2012, p. 99).

The solubilisation or mineralisation of organic phosphorus process plays an important role in recycling soil organic phosphorus. The organic phosphorus sources may contribute 4-90% of total soil phosphorus content depending on the soil types. Four types of enzymes are known to catalyse this process: phosphatases, phytase, phosphonatases and C–P lyases (Sharma et al. 2013, p. 7). The phosphatases can be classified into acid and alkaline phosphatases and are known to be released from the cell exterior (Khan, Zaidi & Ahmad 2014, p. 36). Acid phosphatases are commonly found in the acidic soils, while alkaline phosphatases are predominant in neutral and alkaline soils (Sharma et al. 2013, p. 7). These two phosphatases could release phosphorus from organic phosphorus compounds such as inositol hexaphosphate (Khan, Zaidi & Ahmad 2014, p. 36). Phytase is another widely studied enzyme in the mineralisation of phytate, the major component of organic phosphorus in soil (Khan, Zaidi & Ahmad 2014, p. 36). Lastly, phosphonatases and C-P lysases are another two enzymes known to cleave the carbon and phosphorus bonding in organophosphonates, thus releasing the phosphorus content (Khan, Zaidi & Ahmad 2014, p. 36).

The released phosphorus from both inorganic and organic sources would be available for plant absorption. Among the known phosphate solubilising strains being utilised as biofertilisers are *Bacillus* spp., *Burkholderia* spp., *Flavobacterium* spp., *Achromobacter* spp., *Agrobacterium* spp., *Aereobacter* spp., and arbuscular mychorrizal fungi (AMF) (Abhilash et al. 2016, p. 848).

1.2.7.3 Potassium solubilising microorganisms

Potassium (K) is usually considered as the third important plant nutrient that affects plant growth. It helps plants to maintain osmotic pressure and ionic concentration. It is also a cofactor or activator of many carbohydrate enzymes and protein metabolism (El-Ramady 2014, p. 464). There are four different forms of potassium in the soil: mineral potassium, non-exchangeable potassium, exchangeable potassium and soil solution potassium. Nonetheless, only 1-2% are available for plants in the forms of exchangeable potassium and soil solution potassium in the soils while the remaining 98% are unavailable forms such as mineral potassium and non-exchangeable potassium (Zarjani et al. 2013, p. 1713). Application of a low amount of chemical-based potassium fertilisers may slightly convert mineral potassium to available potassium but this method is considered not efficient (Meena et al. 2015, p. 341). Regular application of chemical-based potassium fertilisers may be necessary to support the mentioned conversion and could result high labour demand in plantations. Thus, application of chemical-based potassium fertilisers is not a good strategy to increase concentration of available potassium. On the other hand, potassium solubilising microorganisms can dissolve mineral potassium in the soils and release the soluble potassium into the soil solution.

Potassium solubilising microorganisms utilise organic acid production and acidic exo-polysaccharide secretion mechanisms to solubilise mineral potassium. Feldspar and mica are the major mineral potassium sources with high potassium content and abundant in most soils. They are in insoluble crystalline forms thus unavailable for plant root absorption. The weathering process of mineral potassium is time consuming and not able to immediately support the plant growth (Meena et al. 2016, p. 7). In the widely reported organic acid production mechanism by potassium solubilising microorganism studies, the organic acids would be produced from organic matter degradation and potassium solubilising microorganisms (Shanware, Kalkar & Trivedi 2014, p. 623). These organic acids acidify the microbial cells, rhizosphere and surroundings of mineral potassium. When the rhizosphere pH is lowered, the potassium solubilising microorganisms can dissolve mineral potassium by chelating the aluminium and silicon cations bound to potassium in the mineral potassium and thus the fixed potassium is solubilised into soil solution potassium (Rashid et al. 2016, p. 31). The secretion of acidic exo-polysaccharide

is the second mechanism to solubilise the mineral potassium. The exo-polysaccharides absorbs the organic acids in the soil and bound on the surface of mineral potassium. Hence, the exo-polysaccharides can absorb the silicon dioxide molecules in the mineral potassium and release the soluble potassium into soil solution (Jaiswal et al. 2016, p. 24).

The soluble potassium released from both mechanisms is directly available for plant root absorption. There are some potassium solubilising strains known to be used as biofertilisers: *Pseudomonas* spp., *Bacillus mucilaginosus*, *Bacillus circulans*, *Acidothiobacillus ferrooxidans*, *Paenibacillus* spp., and *Aspergillus* spp (Abhilash et al. 2016, p. 848).

1.2.7.4 IAA producing microorganisms

Phytohormones or plant hormones, are the chemical messengers that control plant growth and development. Besides the plant development, phytohormones are a principal agent to regulate plant response to changes in environmental conditions. There was a report as early as 1880 by Charles Darwin about a "matter" in plants that he claimed could regulate the plant growth responses and the effect could be transmitted from one part of the plant to another. The "matter" is called auxins nowadays, deriving from Greek *auxien* meaning 'to grow' (Spaepen, Vanderleyden & Remans 2007, p. 425). The most abundant naturally occurring auxin is indole-3-acetic acid (IAA), and IAA was reported involving to all the aspects of plant growth and development. Interestingly, IAA is found not only produced by plants but also synthesised by bacteria and fungi. The discovery of microbial IAA was reported in the study of *Agrobacterium* sp. where induced IAA containing tumours to support the growth of callus in the sterile plant tissue culture medium that was not added with IAA (Spaepen, Vanderleyden & Remans 2007, p. 425).

As much as 80% of isolated rhizosphere soil microorganisms were found to synthesise and secrete IAA as their secondary metabolites. The microbial IAA can influence the endogenous pool of plant IAA and the plant developmental processes. Hence, it plays an important role in rhizosphere plant-microbe interactions (Ahemad & Kibret 2014, p. 13). The microbial IAA has the effect of stimulating plant cell elongation. It helps plants to produce longer roots with a larger number of root hairs and lateral roots for nutrient and

water uptake (Mohite 2013, p. 639). Besides that, the microbial IAA could loosen plant cell wall to increase the secretion of root exudate. As a result, there are additional nutrients provided to boost the population of rhizosphere microorganisms (Ahemad & Kibret 2014, p. 13). In a nutshell, microbial IAA is widely known to promote plant root growth and indirectly increase the microbial population in the rhizosphere.

The microbial IAA can be synthesised from either tryptophan-dependent or tryptophan-independent pathways (Mohite 2013, p. 639). However, IAA producing microorganisms seemed to prefer tryptophan-dependent pathways to synthesise IAA for plant growth (Mohite 2013, p. 643). As such, IAA producing microorganisms can be screened and isolated using tryptophan containing liquid medium in this study. In the rhizosphere, IAA producing microorganisms would absorb the tryptophan containing root exudate and release IAA molecules in return to the plant root cells (Glick 2014, p. 32). There are five pathways for tryptophan-dependent IAA biosynthesis. The first pathway is indole-3-acetamide (IAM) pathway which mostly secreted by pathogenic strains to the infected plant tissues. The IAM pathway is a two-step pathway, the tryptophan is first converted by tryptophan monooxygenase to indole-3-acetamide, which then hydrolysed by IAM hydrolase to IAA and ammonia (Figure 5) (Spaepen & Vanderleyden 2011, pp. 2-3).

The second pathway is indole-3-pyruvate (IPA) pathway. The IPA pathway is the major IAA biosynthesis pathway in plants, but some beneficial bacterial strains also execute this pathway. Examples of these bacteria are *Azospirillum* spp., *Bacillus* spp., *Bradyrhizobium* spp., *Enterobacter cloacae*, *Paenibacillus* spp., *Pseudomonas* spp. and *Rhizobium* spp. The IPA pathway is a three-step IAA biosynthesis pathway. The tryptophan is first transaminated to indole-3-pyruvate by amino transferase, then decarboxylated into indole-3-acetaldehyde (IAAld) by indole-3-pyruvate decarboxylase (IPDC). After that, the indole-3-acetaldehyde is oxidised to IAA by IAAld dehydrogenase (Figure 5) (Spaepen & Vanderleyden 2011, p. 3).

The third pathway is tryptamine (TAM) pathway which occurs in both plants and microorganisms (Spaepen, Vanderleyden & Remans 2007, p. 427). The enzyme tryptophan decarboxylase converts tryptophan to tryptamine, which is then oxidised to IAAld by amine-oxidase. The IAAld is further oxidised to IAA by IAAld dehydrogenase

(Figure 5) (Spaepen & Vanderleyden 2011, p. 4). The fourth pathway is indole-3-acetonitrile (IAN) pathway. The pathway is still under study and the involved enzymes are still inconclusive. The current study would be based on the conversion of tryptophan to indole-3-acetonitrile, then the IAA could be formed via either IAM pathway or nitrilase conversion (Figure 5) (Spaepen & Vanderleyden 2011, p. 4). The fifth pathway is the tryptophan side-chain oxidase (TSO) pathway which was reported only in *Pseudomonas fluorescens* CHA0. The tryptophan can bypass the IPA pathway and directly converted to IAAld. After that, the IAAld is oxidised to IAA with a similar reaction as the IPA pathway (Figure 5) (Spaepen & Vanderleyden 2011, p. 4).

As an overview, the IAA can be produced by both plants and rhizosphere microorganisms. The IAA producing microorganisms convert the tryptophan to IAA via tryptophan-dependent pathways. The tryptophan would be provided by the plant root exudates. The microbial IAA has the effect on enhancing plant root surface and resulting in better plant nutrients and water absorption. There were some rhizosphere microorganism strains reported in the studies of agricultural crops and trees including *Bacillus* spp., *Paenibacillus* spp., *Rhizobium* spp. and *Streptomyces* spp. (Vejan et al. 2016, pp. 4-6). Although there were insufficient studies on IAA production by rhizosphere fungi strains, it is still possible to isolate IAA producing fungi from the rhizosphere as 80% of rhizosphere microorganism strains was reported producing IAA (Ahemad & Kibret 2014, p. 13).

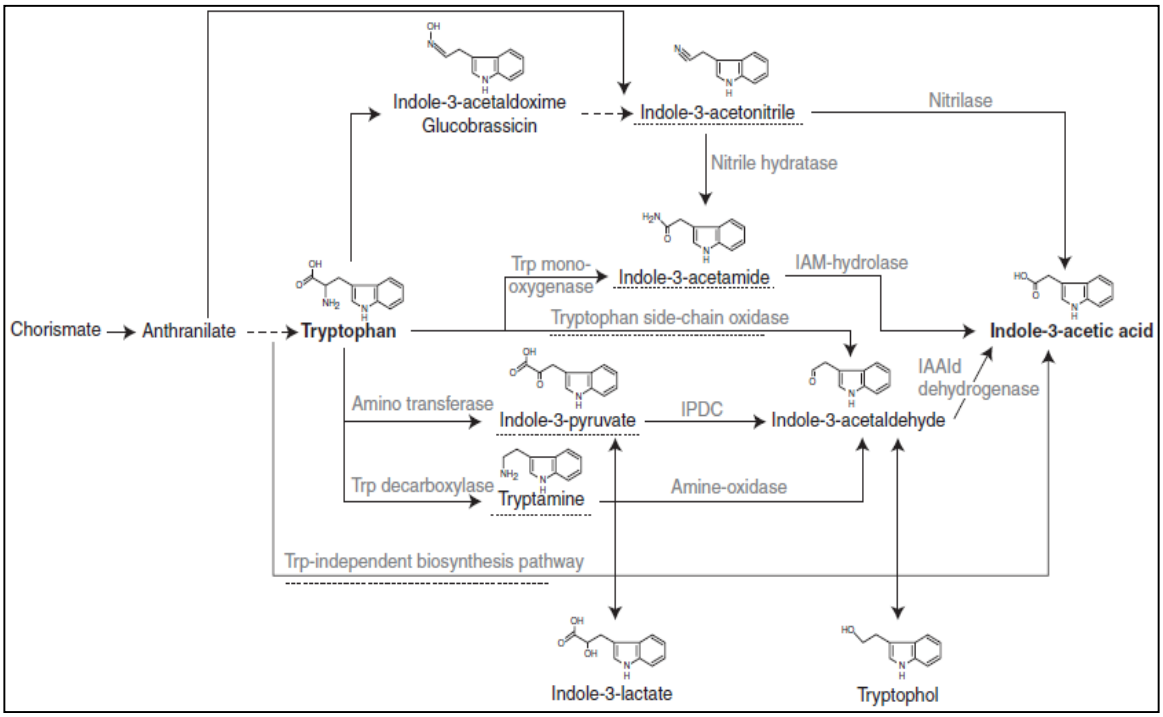


Figure 5 An overview of IAA biosynthesis pathways in IAA producing microorganisms (Spaepen & Vanderleyden 2011, p. 2).

1.3 Research Aim and Objectives

The aim of this study was isolating and characterising the plant growth enhancing microorganisms which act as environmental-friendly alternatives for chemical fertiliser application in the silviculture of *Neolamarckia cadamba*. With this aim, the following main objectives were set:

1. To isolate, screen and characterise the plant growth enhancing microorganisms from the *N. cadamba* rhizospheric soil samples collected in Sarawak.

2. To evaluate the efficiencies of selected plant growth enhancing strains in fixation of nitrogen, solubilisation of potassium and phosphate, and production of plant hormone IAA.

3. To investigate the effect of two consortial formulation using selected strains of plant growth enhancing microorganisms with or without integration with chemical fertiliser on the growth of *Neolamarckia cadamba* seedlings in small scale pot experiment.

CHAPTER 2:

ISOLATION AND CHARACTERISATION OF PLANT GROWTH ENHANCING MICROORGANISMS FROM *NEOLAMARCKIA CADAMBA* RHIZOSPHERES

2.1 Introduction

Soil is a mineral material formed by the weathering process of rocks or minerals by wind and water (Igwe 2011, p.934). The soil formation process can be affected by climate, organisms, parent material, topography and time. Tropical soil in Malaysia is an example of soil that is greatly influenced by tropical climate with high ambient temperature and rainfall.

There are three soil orders namely Oxisols, Ultisols and Entisols commonly found in the tropical areas of Africa, Asia, North and South America (Igwe 2011, p.934). In Sarawak, Oxisols, Ultisols and Entisols can be found in the western and northern regions (Padmanabhan, Eswaran & Reich 2010, p. 173; Paramananthan & Lim 1978, p. 100; Momose et al. 1998, p. 1478; Maru, Haruna & Primus 2015, p. 2). These soil orders are particularly fragile and have low water holding capacity (Igwe 2011, p.934). The annual precipitation in tropical region is normally higher than 1500 mm, and this could cause heavy leaching of finer soil particles such as silt and clay, leaving only coarser sand particles on the sites (Schulte & Ruhiyat 1998, p. 11, Igwe 2011, p.934). As a result, most of the tropical soils contain high level of coarser sand particles which are not ideal for water retention. In addition, the coarse sand particles are also poor in retaining plant nutrients, and the plant nutrients are vulnerable to leaching process under heavy rainfall condition (Sheard 1991, p. 4). Besides that, Oxisols, Ultisols and Entisols are acidic with soil pH values fall within the range of 4.0-5.5 (Schulte & Ruhiyat 1998, p. 14; Bhat et al. 2017, p. 397). The acidic condition is also not ideal for the release of plant macronutrients like nitrogen, phosphorus, potassium, calcium and magnesium (Truog 1946, pp. 305-306). Hence, the poor nutrient levels of tropical soils are mainly attributed to higher level of sand particles and low soil pH.

Many tropical plant species are adapted to survive in poor soil condition. *N. cadamba* trees for example, were found to be able to grow on various poor soil conditions in the tropical region (Krisnawati, Kallio & Kanninen 2011, p. 1). The adaptation of tropical trees to poor soil conditions could be attributed to their relationships with rhizospheric plant growth enhancing microorganisms (Fujii 2014, pp. 371-372). For example, dipterocarp trees are known to have symbiotic association with ectomycorrhizal fungi in

the rhizosphere which may help in provision of nutrient to the plants through extensive mycelial network (Fujii 2014, pp. 375-376). In general, rhizospheric plant growth enhancing microorganisms have five mechanisms that help the host plants in acquiring the nutrients in tropical soils (Vessey 2003, p. 574). These five mechanisms are biological nitrogen fixation, solubilising the unavailable plant nutrients in the rhizosphere, promoting root growth, assisting other symbiotic microorganisms to the host plants and multiple mechanisms (Vessey 2003, p. 574). In this study, the first three mechanisms were studied. This includes microorganisms that perform nitrogen fixation, solubilising of unavailable phosphate and potassium, as well as microorganisms that promote root growth, which was IAA producing microorganisms. These would cover the first three mechanisms stated by Vessey (2003, p. 574) in promoting the growth of host plants. In addition, while applying these microorganisms together as consortia, the last mechanism pertaining to combination of various mechanisms, was also partly explored in this study and would be reported in Chapter 4.

This chapter focus on the isolation and screening of plant growth enhancing microorganisms from rhizospheric soil samples of *N. cadamba* mature trees and seedlings in several locations of Sarawak. For screening, several selective media were used to identify nitrogen fixation, phosphate and potassium solubilisation, and IAA production traits. Besides that, the physicochemical properties of collected soil samples were also discussed.

2.2 Materials and Methodology

2.2.1 Soil samples collection from *N. cadamba* mature trees and planted seedlings

The soil samples were collected about 10-20 mm from the root collar of *N. cadamba* mature trees located in Kubah National Park (01°36'46.1" N, 110°11'48.8" E), Semenggoh Nature Reserve (01°23'59.9" N, 110°19'26.8" E) and Sabal Forest Reserve (01°04'12.5" N, 110°57'0.87" E). Each soil column was sampled until the depth of 20 cm from the ground level using a 20 cm cylinder auger and kept in a zip lock bag. In addition, soil samples were also collected from the rhizospheres of *N. cadamba* seedlings located in the forest of Similajau National Park (03°20'39.9" N, 113°09'39.3" E) and, a riparian buffer zone of licensed planted forest (LPF) 0014 at Segan, Bintulu (02°59'17.9" N, 113°02'15.6" E). The soils attached to the seedling roots were collected and kept in zip lock bags. They were stored at 4°C in Swinburne University of Technology Sarawak Campus (SUTS) Research Laboratory cold room.

2.2.2 Soil samples physicochemical analyses

2.2.2.1 Soil samples preparation

Approximately 200 g of soil was air-dried for 14 days. The soil was turned over regularly in order to speed up drying process. Then, the dried soil was ground into fine particles using a stainless steel rod and filtered with a 2 mm sieve (Sabang & Bujang n.d., p. 3). The fine soil was stored for further analyses. pH measurement and soil texture analysis were conducted at Sarawak Forestry Corporation (SFC) Seed Bank Soil Laboratory. Soil samples were sent to a certified external laboratory (SP Lab, Sarawak Plantation Services Sdn. Bhd., Malaysia) for the analyses of soil chemical properties as stated in section 2.2.2.4.

2.2.2.2 pH

Fine soil (10 g) was mixed with 25 mL of distilled water and stirred at high speed for 5 minutes. It was left overnight before pH analysis. pH meter (Mettler Toledo, USA) was used for the measurement of soil suspension pH (Sabang & Bujang n.d., p. 10).

2.2.2.3 Soil texture analysis

Fine soil (10 g) was mixed with 50 mL of 20% v/v H_2O_2 solution (prepared from 30% H_2O_2, ChemAR, Classic Chemicals, Malaysia). The beaker was left overnight covered with watch glass cover. Then, the soil suspension was boiled for 15 minutes with minimal water spraying in order to bring down the frothing. Suitable amount of H_2O_2 was added until no effervescence observed. The soil suspension was boiled vigorously for 2 hours to remove excess H_2O_2. 5 mL of Calgon [a commercial mixture of $(NaPO_3)_6$ and Na_2CO_3] was added to the suspension and left overnight. After that, the suspension was topped up to 500 mL with distilled water. It was stirred at high speed for 10 minutes. The dispersed sample was transferred to 1 L measuring cylinder and the volume was made up to 1 L with distilled water. The temperature of soil suspension was measured. It was mixed thoroughly with a metal plunger. After the mixing process, soil suspension were kept at room temperature to allow the settlements of sand, silt and clay. The first timer was set for the settlement of sand according to Table 2. After that, 20 mL of silt-clay mixture was withdrawn with a glass pipette at a depth of 10 cm below the suspension surface. Then, the second timer was set for the settlement of silt according to Table 2. Same volume of clay was withdrawn at a depth of 6 cm below the suspension surface. Next, supernatant was drained and the sediment was washed into a 1 L beaker using 800 mL distilled water. The temperature was measured and settling time for sand at observed temperature was set (Table 2). After that, most of the supernatant liquid was slowly decanted off. The sand settlement process was repeated as many times as necessary until the supernatant liquid was perfectly clear. The silt-clay mixture, clay, sand and 5 mL Calgon were oven-dried overhight 105°C. Their weight readings were taken afterwards. The percentages of sand, silt and clay were calculated based on the formulae in Table 3 (Sabang & Bujang n.d., pp. 7-9). The soil texture was determined by matching the percentages of sand, silt and clay to soil texture triangle (Figure 6).

Table 2 Sampling times of silt-clay and clay

Temperature	Silt-Clay (Settling time for sand)	Clay (Settling time for silt)
26°C	4 minutes 3 seconds	4 hours 3 minutes
27°C	3 minutes 57 seconds	3 hours 58 minutes
28°C	3 minutes 52 seconds	3 hours 52 minutes
29°C	3 minutes 47 seconds	3 hours 47 minutes
30°C	3 minutes 42 seconds	3 hours 42 minutes

Table 3 List of formulae for the calculation of sand, silt and clay contents.

Weight of clay (g)	50 × (Weight of oven-dried clay) - Weight of oven-dried Calgon
Weight of silt-clay mixture (g)	50 × (Weight of oven-dried silt-clay mixture) - Weight of oven-dried Calgon
Weight of silt (g)	Weight of silt-clay mixture - Weight of clay
Percentage of sand (%)	$\frac{\text{Weight of sand}}{\text{Weight of sand+Weight of silt+Weight of clay}} \times 100$
Percentage of silt (%)	$\frac{\text{Weight of silt}}{\text{Weight of sand+Weight of silt+Weight of clay}} \times 100$
Percentage of clay (%)	$\frac{\text{Weight of clay}}{\text{Weight of sand+Weight of silt+Weight of clay}} \times 100$

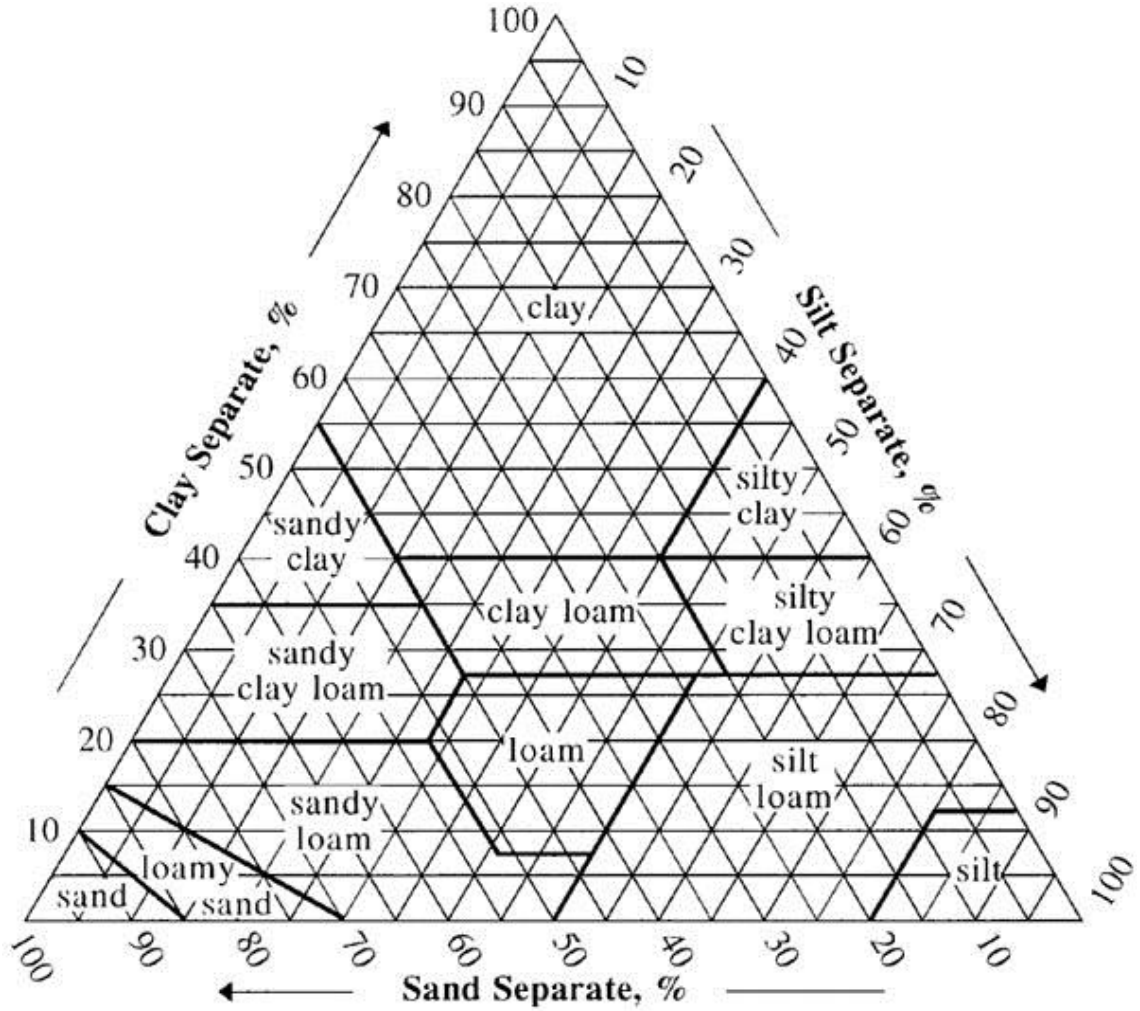


Figure 6 Soil texture triangle (National Resources Conservation Service, United States Department of Agriculture n.d.).

2.2.2.4 Soil chemical analyses

The soil chemical analyses included total N, available P, exchangeable K, exchangeable Ca and exchangeable Mg based on Malaysian Standards (MS) 678:1980 (Table 4).

Table 4 SP Lab soil chemical analysis techniques

Types of analyses	Techniques
Total N	MS 678:Part II:1980-Kjeldhal Digestion & Distillation method
Available P	MS 678:Part VIII:1980
Exchangeable K	MS 678:Part IV (3):1980
Exchangeable Ca	MS 678:Part IV(4):1980
Exchangeable Mg	MS 678:Part IV (5):1980

2.2.3 Media and reagent preparation

2.2.3.1 Nutrient agar (NA) preparation for general isolation of bacteria

NA powder (28 g) (HiMedia, India) was suspended in 1 L distilled water. It was autoclaved at 121°C for 15 minutes. It was supplemented with 7 mL of 0.1% cycloheximide solution (Sigma-Aldrich, USA) for bacteria isolation after autoclaving.

2.2.3.2 Potato dextrose agar (PDA) preparation for general isolation of fungi

PDA powder (39 g) (HiMedia, India) was suspended in 1 L distilled water. It was autoclaved at 121°C for 15 minutes. It was supplemented with 100 mg of chloramphenicol powder (Acros Organics, Belgium) for fungus isolation before autoclaving.

2.2.3.3 Jensen's medium preparation for isolation of nitrogen fixing microorganisms

Jensen's medium was prepared by adding 39.1 g of Jensen's medium powder (HiMedia, India) to 1 L distilled water. It was autoclaved at 121°C for 15 minutes. It was supplemented with cycloheximide solution for bacteria isolation and chloramphenicol powder for fungus isolation.

2.2.3.4 Pikovskaya's agar preparation for isolation of phosphate solubilising microorganisms

Pikovskaya's agar was prepared by adding 31.3 g of Pikovskaya's agar powder (HiMedia, India) to 1 L distilled water. It was autoclaved at 121°C for 15 minutes. It was supplemented with cycloheximide solution for bacteria isolation and chloramphenicol powder for fungus isolation.

2.2.3.5 Modified Aleksandrov's medium preparation isolation of potassium solubilising microorganisms

The modified Aleksandrov's medium was prepared by following recipe shown in Table 5 (Parmar & Sindhu 2013, p. 26). It was autoclaved at 121°C for 15 minutes. It was supplemented with cycloheximide solution for bacteria isolation and chloramphenicol powder for fungus isolation.

Table 5 Recipe of modified Aleksandrov's medium

Materials	Amount required
Glucose (Sigma-Aldrich, USA)	5 g
$MgSO_4 \bullet 7H_2O$ (Fisher Scientific, USA)	0.5 g
$CaCO_3$ (Fisher Scientific, USA)	0.1 g
$FeCl_3$ (Fisher Scientific, USA) *	0.006 g
Mica powder (Fisher Scientific, USA)	3 g
Bacteriological agar (HiMedia, India)	20 g
Distilled water	1 L

* $FeCl_3$ was dissolved in small amount of distilled water and added to the medium before autoclaving

2.2.3.6 Tryptophan broth preparation for screening of IAA producing microorganisms

The tryptophan broth was made from nutrient broth (NB) (HiMedia, India) or potato dextrose broth (PDB) (HiMedia, India) and L-tryptophan (Acros Organics, Belgium). For bacteria screening, 13 g of NB powder and 1 g of L-tryptophan dissolved in 1 L distilled water. For fungus screening, 24 g of PDB powder and 1 g of L-tryptophan dissolved in 1 L distilled water. The broth was autoclaved at 121°C for 15 minutes (Dasri et al. 2014, p. 269).

2.2.3.7 Bromothymol blue reagent preparation for Jensen's medium staining

There was 0.5 g of bromothymol blue (Nacalai Tesque, Japan) dissolved in 500 mL of 95% ethanol (Fisher Scientific, USA). Then, 500 mL of distilled water was added to the reagent (Gothwal et al., p. 103). The reagent was filter-sterilised with sterile 0.22 µm syringe filter (Catalog no. SLGP033RS, Merck Millipore, USA) before use.

2.2.3.8 Salkowski's reagent preparation for IAA detection

The salkowski's reagent consisted of 15 mL of 0.5 M $FeCl_3$ (Fisher Scientific, USA), 300 mL of concentrated H_2SO_4 (Fisher Scientific, USA) and 500 mL of distilled water (Meudt & Gaines 1967, p. 1395).

2.2.4 Screening and isolation of microorganisms from soil samples

The screening experiments were carried out using different selective media. The selective media were Jensen's medium, Pikovskaya's agar, Aleksandrov's medium and tryptophan broth.

2.2.4.1 Serial dilution

The concentration of soil microorganisms should be diluted in order to obtain single colonies. The dilution method was ten-fold serial dilution. The concept is theoretically scaling down the concentration by ten times in every dilution process.

There were six universal bottles (Favorit, Malaysia) filled with 9 mL of sterile distilled water. Approximately 1 g of fresh soil sample was added to one universal bottle and suspended in sterile distilled water by vortexing at high speed. A 1000 µL micropipette (Eppendorf, Germany) was used to transfer 1 mL of soil suspension to new universal bottle with sterile distilled water. The diluted soil suspension was mixed well by vortexing at high speed. The dilution process was performed until a dilution of 10^{-6}. The bottles with 10^{-4}, 10^{-5} and 10^{-6} dilution were selected for screening experiments.

2.2.4.2 Nitrogen fixation screening

The nitrogen fixing microorganisms were screened using Jensen's medium. A micropipette (Eppendorf, Germany) was used to transfer 100 µL of diluted soil suspension to Jensen's medium plate (Kayasth et al. 2014, p. 171). The spread plate step was performed using a sterile stainless steel spreader. The agar plates were sealed with parafilm (Bemis, USA) and incubated at 35°C for 7 days. Then, the plates were stained with 2 mL of bromothymol blue reagent for 1 hour and the excessive stains were washed away by sterile distilled water (Ahmad et al. 2013, p. 54). The morphologically distinct colonies with observed blue colouring zones were further isolated on Jensen's medium plates. After five-day incubation, the pure colonies were screened again using bromothymol blue reagent. The colonies with blue colouring zones were kept in Jensen's medium plates and stored at 4°C for further selection.

2.2.4.3 Phosphate solubilisation screening

Pikovskaya's agar was used to screen phosphate solubilising microorganisms. Diluted soil suspension was transferred to Pikovskaya's agar plate at a volume of 100 µL and spread thoroughly with a sterile stainless steel spreader (Ranjan, Mahalakshmi & Sridevi 2013, p. 30). The agar plates were sealed with parafilm and incubated at 35°C for 7 days. The colonies with observed clear zones were further isolated on NA plates or PDA plates. After five-day incubation, the pure colonies were inoculated on Pikovakaya's agar plates for confirmative screening. The colonies with clear zones were kept in NA or PDA plates and stored at 4°C for further selection.

2.2.4.4 Potassium solubilisation screening

The potassium solubilising microorganisms were screened using modified Aleksandrov's medium. One hundred microlitre of diluted soil suspension was added to modified Aleksandrov's medium plate and spread until dry with a sterile stainless steel spreader (Prajapati & Modi 2012, p. 9). The agar plates were sealed with parafilm and incubated at 35°C for 7 days. The colonies with observed clear zones were further isolated on NA plates or PDA plates. After five-day incubation, they were transferred to modified Aleksandrov's medium and incubated at 35°C for 7 days. The colonies with clear zones were kept in NA or PDA plates and stored at 4°C for further selection.

2.2.4.5 IAA biosynthesis screening

Tryptophan broth was used to screen IAA producing microorganisms. One hundred microlitre of diluted soil suspension was transferred to NA and PDA plates and spread thoroughly with a sterile stainless steel spreader. The agar plates were sealed with parafilm and incubated at 35°C for 7 days. All the colonies were isolated to new NA or PDA plates and incubated at the same temperature for 5 days. After that, they are inoculated to tryptophan both in the form of agar plugs and incubated at 28°C, 150 rpm for 4 days (Mohite 2013, p. 639). The broth cultures were spun down at 12000 × g for 5 minutes. The screening experiment was set up in a universal bottle with 1 mL of supernatant and 1.5 mL of Salkowski's reagent. The universal bottles were kept in dark environment for 75 minutes (Gordon & Weber 1951, p. 194). The colonies with pink colour formation were isolated and kept in NA or PDA plates. They were stored at 4°C for further research usage.

2.2.5 Selection of isolated strains

The isolated bacterial and fungal strains were coded accordingly based on their traits (Table 6). Each of the nitrogen fixing strains, phosphate solubilising strains and potassium solubilising strains were selected based on the respectively effective zone diameters, whereas IAA producing strains were selected based on the absorbance readings at 525 nm. The experiment procedures were identical to the screening protocols. The strains were introduced to respective medium in the form of agar plugs. The diameter and absorbance data were analysed using SPSS ver. 23.0 (IBM corporation, USA). The five best strains in each trait were kept on slant agars and in glycerol stocks.

Table 6 The codes of pure bacterial and fungal strains

Pure strains	Codes
Nitrogen fixing microorganisms	N1~N12, N14 & N15 (Bacteria); N13 (Fungus)
Phosphate solubilising microorganisms	P1~P4, P6, P8~P10 (Bacteria); P5 & P7 (Fungi)
Potassium solubilising microorganisms	K1, K2, K4 & K7 (Fungi); K3, K5 & K6 (Bacteria)
IAA producing micoorganisms	I1~I9 (Bacteria)

2.2.6 Storage and preservation

For short-term storage, bacterial strains were maintained on NA plates and fungal strains were maintained on PDA plates. Nitrogen fixing bacterial and fungal strains were exceptions that were stored on Jensen's agar plates. All the plate cultures were stored at 4°C. Slant agar culturing and preservation in glycerol were the two techniques used for long-term preservation of fungal and bacterial strains. The slant agar mediums were prepared in sterile universal bottles. The isolation methods were same as short-term storage. All the slant agar cultures were stored at 4°C. The bacterial strains were inoculated to 10 mL of nutrient broth and incubated at 35°C, 150 rpm for 1-3 days. The fungal strains were inoculated to baffled flasks (Duran, Germany) with 100 mL of potato dextrose broth. They were incubated at 35°C, 150 rpm for 3 days. The glycerol stocks were set up in 1.8 mL cryovial tubes with 500 µL broth cultures and 500 µL 50% glycerol solution. All the cryovial tubes were kept in ultrafreezer at -80°C (Thermo Scientific, USA).

2.3 Result and Discussion

2.3.1 Physicochemical properties of collected soil samples in Sarawak

The soil samples were collected from the rhizospheres of *N. cadamba* for isolation of plant growth enhancing microorganisms. It is important to study the physicochemical properties of these soil samples.

2.3.1.1 Soil texture analysis

Table 7 The soil texture of soil samples collected from different locations of Sarawak.

Soil samples	Particle size distribution			Classifications
	Sand (%)	Silt (%)	Clay (%)	
Kubah NP	76.46	3.36	20.18	Sandy Clay Loam
Similajau NP	76.15	3.33	20.52	Sandy Clay Loam
Semenggoh NR	22.88	35.10	42.02	Clay
Sabal FR	50.66	25.33	24.01	Sandy Clay Loam
LPF 0014 Segan	64.69	17.82	17.49	Sandy Loam

Based on the analysis, the soil texture was sandy clay loam for soil samples collected from Kubah NP, Similajau NP and Sabal FR, while clay texture and sandy loam texture were recorded for soil samples collected from Semenggoh NR and LPF 0014 Segan respectively. The soil samples from Kubah NP, Similajau NP and Sabal FR shared the sandy clay loam texture with different sand, silt and clay proportions. They contained sand, silt and clay components in the ranges of 50-77%, 3-26% and 20-25% respectively. The sandy loam soil samples have lower clay content of about 18%, otherwise similar to sandy clay loam soil samples for sand and silt contents. Clay soil sample on the contrary contains 23% sand, which is lower, while possessing 35% silt and 42% clay (Table 7).

The sand, silt and clay contents in soil may affect the retentions of plant nutrients and water in the soils. Due to large particle size, sand is known to be poor in retaning both plant nutrients and water. Silt particles size is moderate thus able to retain both plant nutrients and water. Clay is the finest in particle and mainly responsible for retention of plant nutrients (Sheard 1991, p. 4). Among the five soil samples, soil sample from Semenggoh NR had the highest content of silt and clay that could potentially hold high levels of plant nutrients and water. Soil samples from Sabal FR and LPF 0014 Segan contained moderate levels of silt and clay which is good in providing both plant nutrients and water. The remaining soil samples from Kubah NP and Similajau NP had lower content of silt and clay that would contribute to relatively poorer plant nutrients and water retention.

2.3.1.2 pH analysis of soil samples

Table 8 The pH readings of soil samples from different locations of Sarawak.

Soil samples	pH
Kubah NP	5.46
Similajau NP	4.00
Semenggoh NR	3.86
Sabal FR	4.51
LPF 0014 Segan	4.14

In Table 8, five soil samples showed pH values that fell within the range of 3.8 to 5.5. In the previous studies of Sarawak soils, they showed the similar range of pH (Katagiri, Yamakura & Lee 1991, p. 42; Ishizuka et al. 2000, p. 259). Acidic soils were expected as heavy rainfall in tropical climate washes alkaline cations away from tropical soils (van Vleit, Slingerland & Giller 2015, p. 9).

Based on USDA classification, the soil samples from Similajau NP, Semenggoh NR and LPF0014 Segan would be considered extremely acidic soils, while soil samples from Sabal FR and Kubah NP would be very strongly acidic soil and strongly acidic soil respectively (National Resources Conservation Service, United States Department of Agriculture 1988).

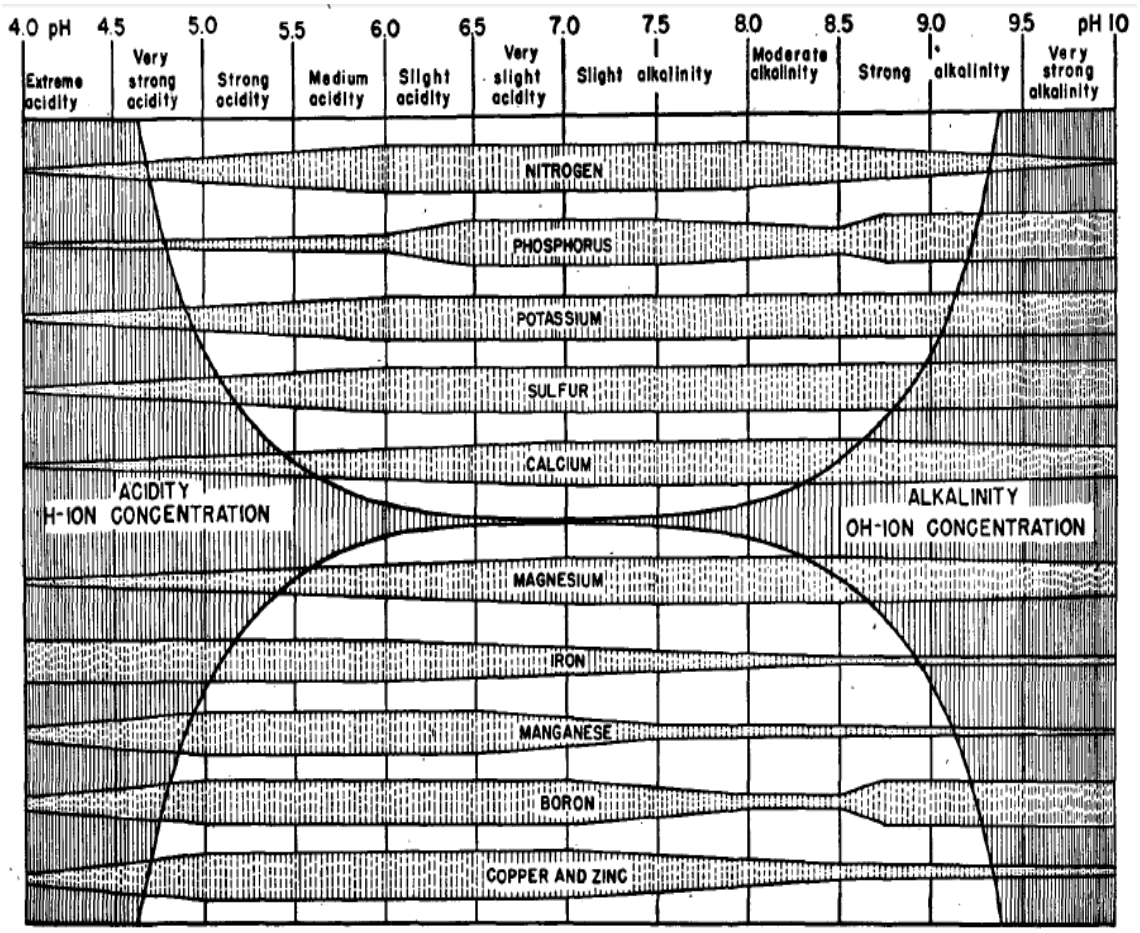


Figure 7 The impact of soil pH on the availability of plant nutrients. The width of the bar indicates the intensity of available plant nutrients in relation to the soil pH. It does not represent the actual amount of available plant nutrients in the soils (Truog 1946, p. 305).

As shown in Figure 7, soil pH had a strong relationship with availability of plant nutrients in soil. The availabilities of N, P, K, Ca and Mg were narrowing gradually below the pH value 5.5. Soil sample from Kubah NP had a pH of 5.46 would still provide reasonable environment for availability of N, P, K, Ca and Mg. Soil samples from the other locations had pH ranging from 3.86-4.51 would all be considered poor for available plant nutrients as the availabilities of these five elements would be low below pH 5.5 (Figure 7).

2.3.1.3 Chemical analyses of soil samples

Table 9 The nutrient values of soil samples from different locations and reference values of fertile soil.

Soil samples	Total N (%)	Available P (ppm)	Exchangeable (meq%)		
			K	Ca	Mg
Kubah NP	0.180	1.000	0.140	4.490	0.350
Similajau NP	0.095	1.000	0.120	0.355	0.185
Semenggoh NR	0.250	1.000	0.195	1.460	0.485
Sabal FR	0.195	1.500	0.170	3.820	1.405
LPF 0014 Segan	0.115	1.000	0.140	0.260	0.320
*Fertile Soil	0.200-0.300	20.000-30.000	0.600-1.000	10.000-20.000	3.000-8.000

* The reference values of fertile soil were obtained from Food and Agriculture Organization of the United Nations (Loganathan 1987).

The analysed nutrient parameters were total N, available P, exchangeable K, exchangeable Ca and exchangeable Mg. Among the macronutrients, N, P and K are classified as primary macronutrients for plants, while Ca and Mg are classified as secondary macronutrients for plants (Tucker 1999, p. 1). According to Table 9, soil sample from Semenggoh NR with about 42% of clay content had the highest level of total N at 0.250%. This would fall within the range of total N between 0.200-0.300% of typical

fertile soil. Soil samples from other locations with clay range of 17-25% contained total N in the range of 0.095-0.195%. This range would not meet the total N range of 0.200-0.300% in typical fertile soil. The total N content could possibly be influenced by clay content in soil. Clay contains soil organic matter that could represent 90-95% of nitrogen content in unfertilised tropical soils (Schulte & Ruhiyat 1998, p. 15). Besides that, all the soil samples did not reach the available P, exchangeable K, exchangeable Ca and exchangeable Mg ranges of typical fertile soil respectively. Available P, exchangeable K, exchangeable Ca and exchangeable Mg are the available plant macronutrients that could be absorbed by plant roots. The low concentrations of these available plant macronutrients could possibly be induced by the pH readings of soil samples which ranged from pH 3.8 to pH 5.5. The most favourable pH environments for available P are pH 6.5-7.5 and above pH 8.5 (Truog 1946, p. 306). For exchangeable K, Ca and Mg, pH above 7 is the favourable environment for their availabilities (Truog 1946, p. 306).

Lower level macronutrients were expected from typical tropical soil due to the tropical climate (Snoeck et al. 2009, p. 106). Nonetheless, many large tropical plant species such as *N. cadamba* would have adapted to grow to maturity on these soils (Krisnawati, Kallio & Kanninen 2011, p. 1). The growth of these tropical trees would have been supported by the adaptation of the trees, albeit the plant growth enhancing microorganisms in tropical rhizospheric soils may also play important role to sustain the tree growth (Fujii 2014, p. 378). For instance, it is known that both the plant roots and the plant growth enhancing microorganisms can release organic acids to extract P from Al and Fe oxides in soils with limited available P supply (Fujii 2014, p. 374).

2.3.2 Preliminary screening of plant growth enhancing microorganisms from Sarawak soils

The tropical soil microorganisms were known to promote the growth of tropical trees like Dipterocarpaceae in Southeast Asia. (Fujii 2014, pp. 371-372). Nonetheless, not much research was done on the plant growth enhancing microorganisms from Sarawak soils. As stated earlier, the strategy was to isolate four functional groups of plant growth enhancing microorganisms namely nitrogen fixing microorganisms, phosphate solubilising microorganisms, potassium solubilising microorganisms and IAA producing microorganisms.

Nitrogen fixing microorganisms were screened through Jensen's medium. The Jensen's medium was stained with bromothymol blue to screen the nitrogen fixing microorganisms. The Pikovskaya's agar was chosen to screen the phosphate solubilising microorganisms, while Aleksandrov's medium was used to screen potassium solubilising microorganisms. Lastly, IAA producing microorganisms were screened using tryptophan broth with the assistance of Salkowski's reagent (Figure 8).

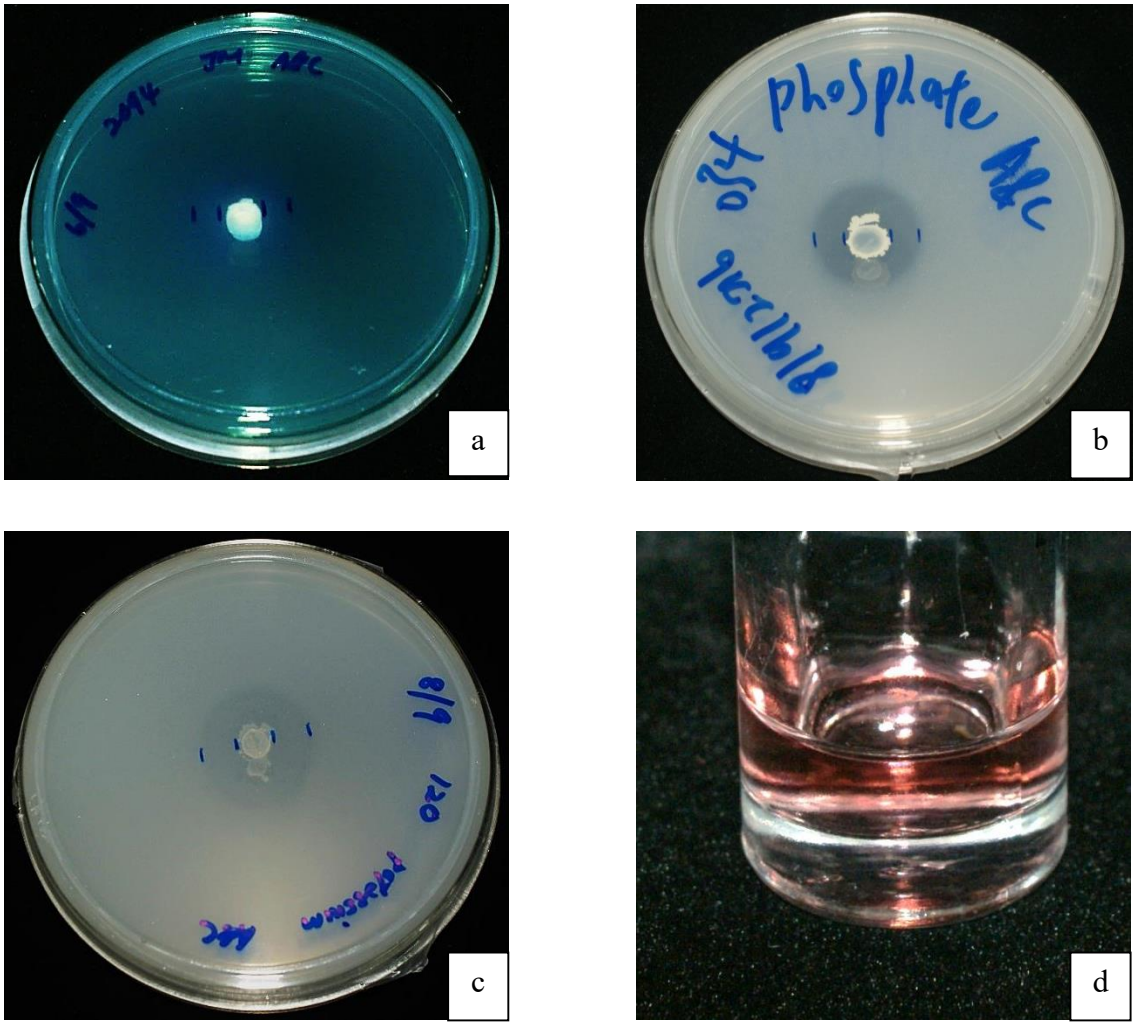


Figure 8 Screening of plant growth enhancing bacteria using selective media. The blue colour zone observed on Jensen's medium indicated nitrogen fixing activity of N11 (a). Clear zones observed on Pikovskaya's agar and modified Aleksandrov's medium showed phosphate and potassium solubilising activities of P1 and K5 (b and c). Red colour formation in tryptophan broth indicated IAA production of I6 (d).

Table 10 The number of plant growth enhancing microorganisms isolated from different locations of Sarawak.

Soil samples	*Traits				Total number of strains
	N_2	PO_4^{3-}	K	IAA	
Kubah NP	1	1	1	1	4
Similajau NP	2	2	2	2	8
Semenggoh NR	6	1	2	2	11
Sabal FR	4	4	1	3	12
LPF 0014 Segan	2	2	1	1	6
Total strain number	15	10	7	9	41

* N_2: Nitrogen fixing microorganisms; PO_4^{3-}: Phosphate solubilising microorganisms; K: Potassium solubilising microorganisms; IAA: IAA producing microorganisms

From the 41 strains isolated from 5 soil samples, there were 15 nitrogen fixing microorganisms, 10 phosphate solubilising microorganisms, 7 potassium solubilising microorganisms and 9 IAA producing microorganisms isolated from five soil samples (Table 10). Based on the morphologies, thirty-four bacterial strains and seven fungal strains were isolated from collected rhizosphere soil samples.

Soil samples from Semenggoh NR and Sabal FR had the two highest numbers of isolates (Table 10). These soil samples contained relatively high levels of macronutrients, silt and clay (Table 7, Table 9). The positive effects of plant macronutrients and soil textures on microbial community were reported in the previous studies. For examples, studies of coffee and maize plants showed larger microbial communities could be found in the soil samples with higher plant macronutrient contents (Shiva et al. 2014, pp. 4-5; Zhong et al. 2010, pp. 515-516). In addition, the silt and clay contents could retain soil moisture and organic matter for the growth requirements of soil microorganisms (Hamarashid, Othman & Hussain 2010, p. 62; Meliani, Bensoltane & Mederbel 2012, pp. 15-16).

2.3.2.1 Nitrogen fixing microorganisms screening and selection

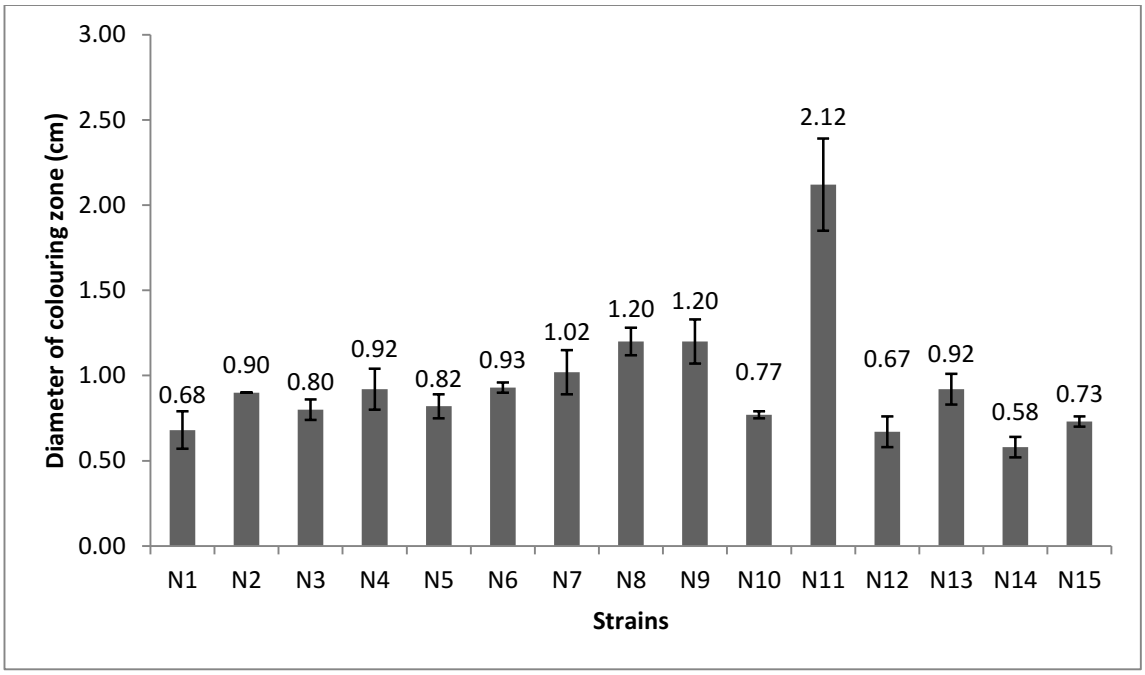


Figure 9 Diameters of colouring zones formed by nitrogen fixing microorganisms isolated from different locations of Sarawak. Each bar represents a mean ± SE from triplicated data.

The nitrogen fixing microorganisms were isolated from all the soil samples. Among the fifteen isolated strains, most strains were isolated from Semenggoh NR (six strains) and Sabal FR (four strains) (Table 10). The screening was based on a nitrogen free medium namely Jensen's medium. Jensen's medium contains sucrose as an energy source and sodium molybdate (Na_2MoO_4) for nitrogenase activity. After seven-day incubation, the colonies grew on Jensen's medium were the potential nitrogen fixing microorganisms. For further confirmation, a pH indicator called bromothymol blue was used to stain Jensen's medium and turns blue in the alkaline environment. As the atmospheric N_2 is reduced to ammonia, there is a pH shift to alkaline. Therefore, there was blue colour zone formed on Jensen's medium (Figure 8a). The diameters of blue colour zones fell within the range of 0.58 cm to 2.12 cm (Figure 9). The range of diameter was closely matched to the reported diameter range of 0.04-2.20 cm in a previous study of nitrogen fixing microorganisms associated with the rhizosphere of *Calligonum polygonoides*, a small shrub found in the desert in South Asia (Gothwal et al., p. 103). This study was probably

the only study that reported the diameters of blue colour zones. Based on the diameter readings, the five best strains selected were N6 (Sabal FR), N7 (Similajau NP), N8 (LPF 0014 Segan), N9 (LPF 0014 Segan) and N11 (Similajau NP) (Figure 9). They would be subjected to molecular identification and further quantification of nitrogen fixing activity.

2.3.2.2 Phosphate solubilising microorganisms screening and selection

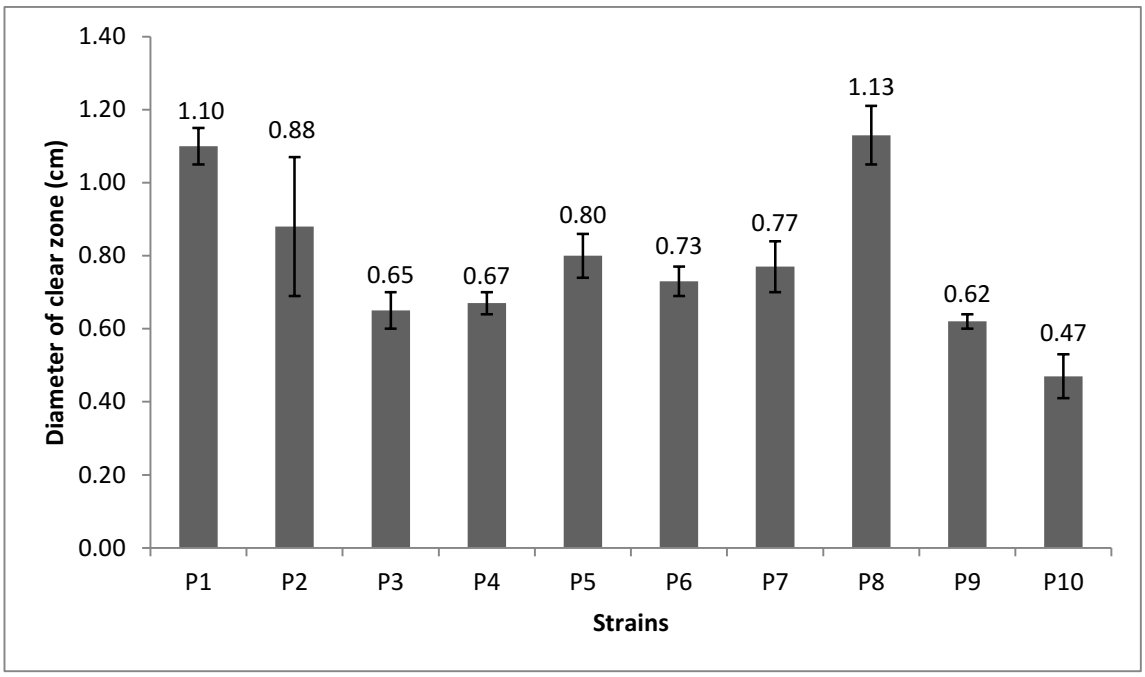


Figure 10 Diameters of clear zones formed by phosphate solubilising microorganisms isolated from different locations of Sarawak. Each bar represents a mean ± SE from triplicated data.

There were ten phosphate solubilising microorganisms isolated in total, from all collected soil samples. There were one strain isolated each from Kubah NP and Semenggoh NR, two strains each from Similajau NP and LPF 0014 Segan, and four strains from Sabal FR (Table 10). Pikovskaya's agar was used to screen phosphate solubilising microorganisms. The medium was formulated with $Ca_3(PO_4)_2$ as an insoluble PO_4^{3-} source. Clear zone was formed around the colony when phosphate solubilising microorganism grew on Pikovskaya's agar (Figure 8b). The isolated strains formed the diameters of clear zones in between 0.47 cm and 1.13 cm (Figure 10). This range is slightly wider than a previous study on isolating phosphate solubilising microorganisms from the rhizospheres of crops

such as tomato, jasmine, onion and maize which recorded the diameter range of 0.6 cm to 1.0 cm (Ranjan et al. 2013, p. 31). Another study of phosphate solubilising microorganisms in tomato plant rhizosphere also showed clear zone diameters of 0.6 cm to 2.3 cm (Karpagam & Nagalakshmi 2014, p. 607). Based on these previous studies, most of the isolated strains from this study would be considerable to demonstrate good phosphate solubilising effects because the clear zone diameters were above 0.60 cm, except for strain P10 with only 0.47 cm of clear zone diameter. Strains P1 (Sabal FR), P2 (Sabal FR), P5 (LPF 0014 Segan), P7 (LPF 0014 Segan) and P8 (Sabal FR) were selected based on their clear zone diameters for further experiments.

2.3.2.3 Potassium solubilising microorganisms screening and selection

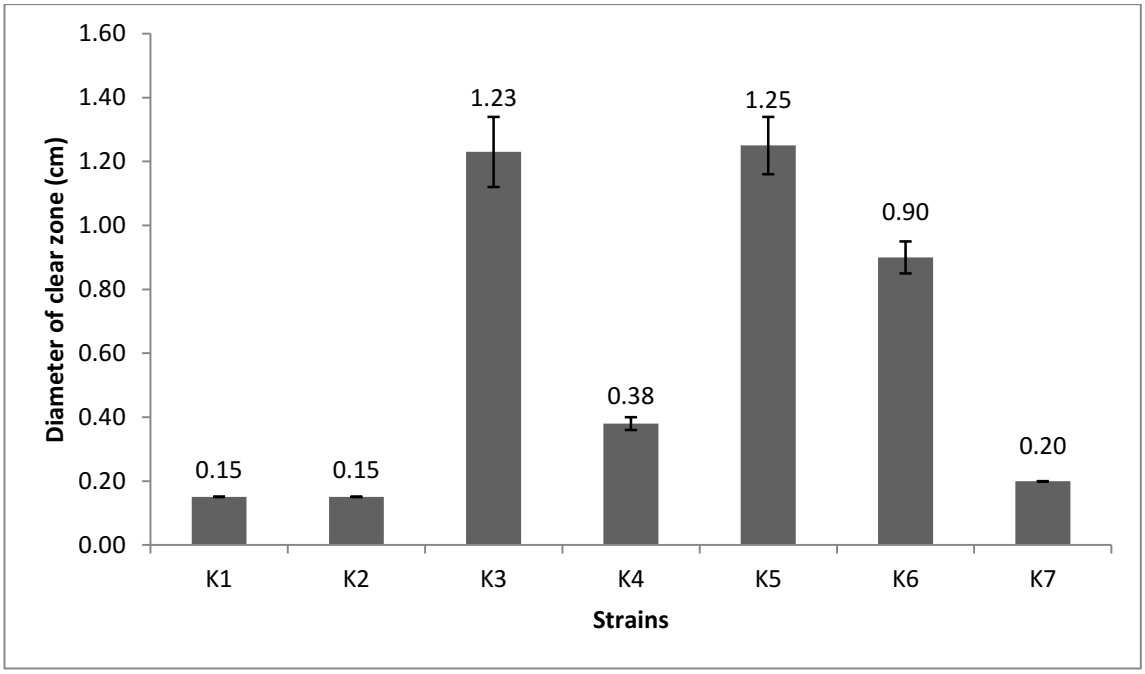


Figure 11 Diameters of clear zones formed by potassium solubilising microorganisms isolated from different locations of Sarawak. Each bar represents a mean ± SE from triplicated data.

There were seven potassium solubilising microorganisms in total isolated from all the soil samples: Kubah NP (one strain), Similajau NP (two strains), Semenggoh NR (two strains), Sabal FR (one strain) and LPF 0014 Segan (one strain) (Table 10). The screening was based on modified Aleksandrov's medium that contains mica powder as insoluble K

source. The mica powder could be solubilised by potassium solubilising strain, forming a clear zone around its colony (Figure 8c). The diameters of clear zones ranged from 0.15 cm to 1.25 cm (Figure 11). A study on isolating potassium solubilising microorganisms from tobacco rhizosphere also showed somewhat lower range from 0.11 cm to 0.30 cm (Zhang & Kong 2014, p. 21). Another study of potassium solubilising microorganisms in soils from ceramic industries showed the clear zone diameters of 0.6 cm to 1.3 cm which match the higher range of the current study (Prajapati & Modi 2012, p. 10). Strain K3 (Semenggoh NR), strain K4 (Kubah NP), strain K5 (LPF 0014 Segan), strain K6 (Similajau NP) and strain K7 (Sabal FR) were selected for further experiments.

2.3.2.4 IAA producing microorganisms screening and selection

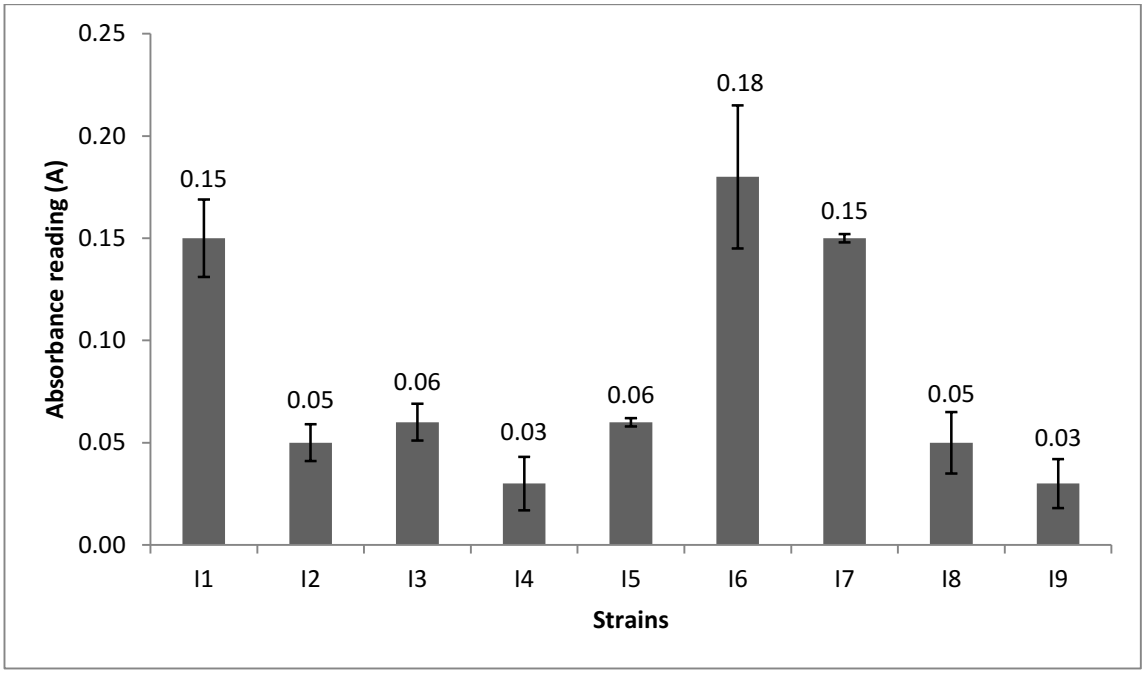


Figure 12 Absorbance readings of IAA formation from IAA producing microorganisms isolated from different locations of Sarawak. Each bar represents a mean ± SE from triplicated data. The blank was tryptophan broth without inoculation.

Strains isolated from all soil samples were found to demonstrate IAA production. There were one strain each from Kubah NP and LPF 0014 Segan, two strains each from Similajau NP and Semenggoh NR, and three strains from Sabal FR (Table 10). The IAA

producing strains were screened using tryptophan broth which contained tryptophan as a precursor for IAA biosynthesis. The presence of IAA was tested with Salkowski's reagent that would produce pink colour formation (Figure 8d). The level of IAA was detected by the spectrophotometry with absorbance at 525 nm. The range of absorbance readings was 0.03 A to 0.18 A (Figure 12). Even though there was no IAA producing microorganisms study reporting absorbance results, the absorbance readings are directly proportional to the concentrations of detected IAA (Mayer 1958, p. 1671). The five strains selected for further experiments were I1 (Sabal FR), I3 (Kubah NP), I5 (Sabal FR), I6 (Similajau NP) and I7 (LPF 0014 Segan).

2.4 Summary

This chapter reported the isolation of plant growth enhancing microorganisms from rhizospheric soil samples collected from *N. cadamba* mature trees and seedlings. The rhizospheric soil samples were collected from Kubah National Park, Similajau National Park, Semenggoh Nature Reserve, Sabal Forest Reserve and LPF0014 Segan. These soil samples were mostly sandy clay loam in texture (Kubah National Park, Similajau National Park and Sabal Forest Reserve), except for LPF0014 Segan which has sandy loam texture and Semenggoh Nature Reserve which has clay texture. All the collected soil samples were acidic in soil pH and fell within the range of 3.5-5.5. The chemical analyses showed the collected soil samples had lower plant macronutrient contents as expected in tropical soils due to the soil textures and pH values.

There were 41 strains isolated from the five collected soil samples. The soil samples from Semenggoh Nature Reserve and Sabal Forest Reserve held the two highest numbers of isolated strains at 11 and 12 strains respectively. These two locations have clay and sandy clay loam soil textures which were reported to have relatively high levels of plant macronutrients, silt and clay that may be conducive for microbial growth, thus a higher number of strains were actually isolated from the two locations. From the 41 strains isolated, there were 15 nitrogen fixing strains, 10 phosphate solubilising strains, 7 potassium solubilising strains and 9 IAA producing strains. It is interesting to note that the isolation process was rather successful and covered all the locations because at least

one strain was isolated in each location from each of the growth enhancing traits that is related to nitrogen, phosphate, potassium or IAA production.

In terms of the level of activities of each trait, for nitrogen fixation, the exhibited blue zones for the isolated strains on the bromothymol blue-stained Jensen's medium plates ranged from 0.58 to 2.12 cm were closely matched to a previous study of nitrogen fixing microorganisms isolated from *Calligonum polygonoides* rhizosphere. The five best strains were N6 (Sabal Forest Reserve), N7 (Similajau National Park), N8 (LPF 0014 Segan), N9 (LPF 0014 Segan) and N11 (Similajau National Park) which produced blue zone diameters within the range of 0.93 to 2.12 cm.

The phosphate solubilising microorganisms produced clear zones on the Pikovskaya's agar plates ranging from 0.47 to 1.13 cm. Except for 0.47 cm diameter, the range was agreeable to previous studies on phosphate solubilising microorganisms isolated from agricultural crop rhizospheres. The five best strains were P1 (Sabal Forest Reserve), P2 (Sabal Forest Reserve), P5 (LPF 0014 Segan), P7 (LPF 0014 Segan) and P8 (Sabal Forest Reserve) which showed clear zone diameters in the range of 0.77-1.13 cm.

The potassium solubilising microorganisms solubilised the mica powder in the modified Aleksandrov's medium plates and produced clear zone diameters in the range of 0.15 to 1.25 cm. The diameter range was comparable to previous studies of potassium solubilising microorganisms isolated from tobacco rhizosphere and soils from ceramic industries. The five selected strains were K3 (Semenggoh Nature Reserve), K4 (Kubah National Park), K5 (LPF 0014 Segan), K6 (Similajau National Park) and K7 (Sabal Forest Reserve) which presented the clear zone diameters ranging from 0.20 cm to 1.25 cm.

The IAA producing microorganisms released IAA into the tryptophan broth and the presence of IAA was detected by Salkowski's reagent. They showed absorbance readings in the range of 0.03 to 0.18 A. Although there was no previous study reporting absorbance results, the absorbance readings are directly propotional to the concentrations of detected IAA. The five chosen strains were I1 (Sabal Forest Reserve), I3 (Kubah National Park), I5 (Sabal Forest Reserve), I6 (Similajau National Park) and I7 (LPF 0014 Segan) which gave absorbance range in between 0.06 A and 0.18 A.

In conclusion, the soil samples collection and isolation strategy were successfully implemented in this study. There was at least one best plant growth enhancing strain isolated from each location. The five best strains from each plant growth enhancing trait were selected for further studies on their genetic identification and quantitative assay on the respectively plant growth enhancing trait.

CHAPTER 3:

THE GENETIC IDENTIFICATION OF SELECTED STRAINS AND EVALUATION ON THEIR BIOLOGICAL ACTIVITIES RELATED TO NITROGEN FIXATION, PHOSPHATE AND POTASSIUM SOLUBILISATION, AND PLANT HORMONE IAA PRODUCTION

3.1 Introduction

As the world population is increasing rapidly, the sustainable supplies of food, biofuel and fibre are the major challenges in the twenty-first century. On the other hand, the land is a limited resource and arable land is required to produce food, biofuel and fibre. In fact, more than one-third of arable lands are no longer suitable for agriculture and silviculture as they were already affected by pollution and other anthropogenic activities (Abhilash et al. 2016, p. 847). The extensive use of agrochemicals including chemical fertilisers, herbicides, fungicides and insecticides in agricultural and silvicultural activities can further deteriorate the quality of arable lands (Glick 2012, p. 1). Thus, it is crucial to seek for sustainable approaches in agriculture and silviculture that can fulfill such growing demands and avoid contaminating the arable lands. In addition, it is also important to restore these degraded arable lands through reforestation. The study focuses on *N. cadamba*, one of the tree species selected for reforestation in Sarawak (Krishnapillay 2002, p. 15).

The usage of plant growth enhancing microorganisms could be a good approach in sustainable agricultural and silvicultural practices. They are known as the rhizospheric microbial partner of plants because these microorganisms are found in the rhizospheric soil that contacting directly with the plant roots. The plant root secretes exudate containing sugars, amino acids, organic acids and other small molecules to attract such plant growth enhancing microorganisms (Glick 2012, p. 2). The microorganisms enhance the plant's growth in return via direct plant-growth promoting mechanisms such as the production of plant growth hormones, inhibition of stress hormone production and increasing the uptake of nutrients (Abhilash et al. 2016, p. 847). The IAA producing microorganisms that enhance nutrient uptake by the roots were part of this study. In addition, this study also included rhizospheric microorganisms that are important to enable nutrient availabilities such as nitrogen, phosphate and potassium. By using such microorganisms, it is hoped that *N. cadamba* could be planted with reduced usage of chemical fertiliser.

Plant growth enhancing microorganisms were widely mentioned in many research publications and commercial biofertilisers formulations. Microorganisms with the nitrogen fixation trait were often placed at the highest priority in biofertiliser research and development, such as various species of *Rhizobium*, *Azotobacter*, *Azospirillum*, *Herbaspirillum*, *Acetobacter* and cyanobacteria. *Rhizobium* spp. and *Acetobacter* spp. are symbiotic nitrogen fixing bacteria from legume plants and non-legume plants respectively, whereas *Azospirillum* spp., *Herbaspirillum* spp. and cyanobacteria are free living nitrogen fixing bacteria. Phosphate solubilising microorganisms were also widely studied for biofertiliser application. Examples are various species of *Bacillus*, *Burkholderia*, *Flavobacterium*, *Achromobacter*, *Agrobacterium*, *Aereobacter* and arbuscular mycorrhizal fungi. In addition, potassium solubilising microorganisms such as *Pseudomonas* spp., *Bacillus mucilaginosus*, *Bacillus circulans*, *Acidothiobacillus ferooxidans*, *Paenibacillus* spp. and *Aspergillus* spp. should not be neglected as potassium is one of the primary nutrients (Abhilash et al. 2016, p. 848). Besides that, IAA is known as plant growth promoting regulator and microbial IAA production trait is important for the plant. IAA producing microorganisms like *Bacillus* spp., *Paenibacillus* spp., *Rhizobium* spp. and *Streptomyces* spp. were reported in the studies of agricultural crops and trees (Vejan et al. 2016, pp. 4-6). For *N. cadamba*, very little research was conducted on plant growth enhancing microorganisms. Strains of *Glomus*, *Azospirillum*, *Azotobacter* and *Bacillus* were shown to be able to significantly improve growth of *N. cadamba* (Sreedhar & Mohan 2016, pp. 127-128). Besides that, strains of *Laccaria*, *Pisolithus*, *Boletus*, *Amanita* and *Frankia* were also reported as biofertilisers suitable for other tropical tree crops (Simarmata 2013, p. 9).

Albeit commonly applied in agriculture, research on plant growth enhancing microorganisms for silviculture application is relatively new. Silviculture, generally defined as the art and science of establishing forest, is rather different from agriculture (US Forest Service n.d.). It is necessary to investigate on how to establish trees with least use of chemical fertiliser and other chemicals. It is also ideal to explore the indigenous plant growth enhancing microorganisms that are suitable for silviculture application.

Chapter 3 includes the molecular identifications of selected microorganisms based on the genetic markers, and evaluation of their biological activities in N, P, K and plant hormone

IAA production. The bacterial strains were identified based on 16S rRNA genetic marker, while the fungal strains were identified based on ITS genetic marker. The selected strains were studied for their activities using designated media and analytical equipment.

3.2 Materials and Methodology

3.2.1 Media and reagent preparation

3.2.1.1 Tris-EDTA (TE) buffer preparation

1 M Tris solution (10 mL) (Vivantis, Malaysia) and 0.5 M EDTA solution (2 mL) (Vivantis, Malaysia) were then mixed in a volumetric flask (Duran, Germany) and the volume was made up to 1 L using ultrapure water.

3.2.1.2 10% SDS solution preparation

Sodium dodecyl sulphate (SDS) powder (10 g) (Fisher Scientific, USA) was added to 100 mL ultrapure water. The solution was heated and stirred to dissolve the SDS powder completely.

3.2.1.3 Proteinase-K preparation

Proteinase-K powder (100 mg) (Thermo Scientific, USA) was suspended in 5 mL of sterile ultrapure water to make 20 mg/mL proteinase-K stock solution. The working solution was made by diluting the stock concentration to 0.6 mg/mL.

3.2.1.4 Phenol-chloroform-isoamyl alcohol preparation

The reagent was prepared from liquified phenol (Amresco, USA), chloroform (Merck, USA) and isoamyl alcohol (Merck, USA) at a ratio of 25:24:1.

3.2.1.5 Chloroform-isoamyl alcohol preparation

The reagent was prepared from chloroform and isoamyl alcohol at a ratio of 24:1.

3.2.1.6 Sodium acetate buffer preparation

Sodium acetate anhydrous powder (24.6 g) (Fisher Scientific, USA) was dissolved in 100 mL ultrapure water to make a 3 M sodium acetate buffer. The pH of buffer was adjusted to 5.2 using glacial acetic acid (Merck, USA).

3.2.1.7 TAE buffer preparation

Tris (242 g), EDTA disodium dihydrate (18.61 g) and glacial acetic acid (57.1 mL) were mixed, and then made up to 1 L using ultrapure water. The mixture was autoclaved to form 50× TAE. The solution was then diluted 50 times to make 1× TAE working buffer.

3.2.1.8 6× Loading dye preparation

The loading dye was prepared by adding 25 mg of bromophenol blue (Sigma-Aldrich, USA) and 3 mL of glycerol (Fisher Scientific, USA) to 7 mL TE buffer.

3.2.1.9 Jensen's broth preparation

Jensen's broth powder (24.1 g) (HiMedia, India) was suspended in 1 L distilled water Afterand autoclaved at 121°C for 15 minutes.

3.2.1.10 Pikovskaya's broth preparation

The broth was prepared by adding 16.3 g of Pikovskaya's broth powder (HiMedia, India) to 1 L of distilled water and autoclaved at 121°C for 15 minutes.

3.2.1.11 Modified Aleksandrov's broth preparation

The recipe was similar to modified Aleksandrov's medium in chapter 2 with slight modification of exclusion of bacteriological agar powder. The broth was autoclaved at 121°C for 15 minutes and transferred to sterilised 250 mL baffled flasks (Parmar & Sindhu 2013, p. 26).

3.2.1.12 Phenol solution preparation

Phenol solution was prepared by diluting 11.1 mL of liquified phenol with 100 mL phenol solution with 95% ethanol (Fisher Scientific, USA).

3.2.1.13 Sodium nitroprusside solution preparation

The solution was prepared by adding 0.5 g of sodium nitroprusside (Fisher Scientific, USA) into 100 mL of deionised water. The solution should be kept away from light exposure and stored for not more than one month.

3.2.1.14 Alkaline citrate solution preparation

Trisodium citrate (200 g) (Merck, USA) and sodium hydroxide (10 g) (Merck, USA) were dissolved in 1 L deionised water.

3.2.1.15 Oxidising solution preparation

The solution was made up by mixing 100 mL alkaline citrate solution with 25 mL commercial 5% sodium hypochlorite solution (Clorox, USA). The solution were prepared in fresh daily.

3.2.2 DNA extraction

3.2.2.1 Freeze and thaw DNA extraction for bacterial cell

Bacterial cells were scraped from agar plate with a sterile toothpick and suspended in 100 µL sterile ultrapure water. The cells were dislodged by mixing with vortex at maximum speed. Then, they were precipitated at 12000 × g centrifugation for 5 minutes. The supernatant was discarded and bacterial cells were resuspended in 30 µL sterile ultrapure water. The DNA was released by breaking bacterial cells with multiple cycles of freeze-and-thaw (Table 11). After that, the cell debris was spun down at 12000 × g for 5 minutes (Higgins et al. 2001, p. 5321). The genomic DNA supernatant was transferred into a sterile 0.5 mL tube (Eppendorf, Germany) and stored at -20°C.

Table 11 Freeze-and-thaw cycles

Cycle	Activity	Temperature (°C)	Time (minute)
1st	Freeze	-80 (Ultrafreezer, Thermo Scientific, USA)	8
	Thaw	85 (Water bath, Memmert, Germany)	3
2nd to 4th	Freeze	-80 (Ultrafreezer, Thermo Scientific, USA)	5
	Thaw	85 (Water bath, Memmert, Germany)	3

3.2.2.2 Freeze and thaw DNA extraction for fungal mycelium

A small piece of fungal mycelium was cut using a sterile toothpick and transferred into a sterile 1.5 mL tube (Eppendorf, Germany) with 30 µL TE buffer. The tube was incubated at -80°C overnight. The DNA was released due to cell break at ultralow temperature. After that, the mycelium was thawed at room temperature (Higgins et al. 2001, p. 5321). The genomic DNA containing TE buffer was transferred into a sterile 0.5 mL tube and stored at -20°C.

3.2.2.3 Phenol-chloroform DNA extraction

1 mL of each bacterial or fungal broth culture was spun down at 12000 × g for 5 minutes to obtain cell pellet and cell pellet collection steps were repeated twice before proceeding to DNA extraction. The cell pellet was then suspended in 500 µL of TE buffer and 50 µL of 10 % SDS solution was added after that. The cell suspension was incubated at 85°C in a water bath for 1 hour with inversion at 15 minutes interval. 50 µL of proteinase-K was added to the cooled cell suspension and incubated at 55°C water bath for 30 minutes. An equal volume of phenol-chloroform-isoamyl alcohol was added to the cell suspension. The cell suspension was centrifuged at 12000 × g for 10 minutes. The 500 µL aqueous layer was transferred to a new sterile 1.5 mL tube. After that, an equal volume of chloroform-isoamyl alcohol was added to the aqueous solution. The same centrifugation step was carried out. The purified aqueous layer was added to a new sterile 1.5 mL tube with 600 µL of isopropanol (Fisher Scientific, USA) and 30 µL of sodium acetate buffer. The tube was inverted several times and incubated at -20°C for 2 hours. Then, the DNA pellet was precipitated at 12000 × g for 10 minutes. The supernatant was drained and the DNA pellet was washed with 600 µL of 70% ethanol (Fisher Scientific, USA). The process of DNA pellet precipitation remained as earlier. The 70% ethanol was drained and DNA pellet was air-dried for 15 minutes. Finally, the DNA pellet was dissolved in 50 µL sterile ultrapure water and incubated at 4°C overnight. The hydrated DNA was stored at -20°C for further usage (Kramvis, Bukofzer & Kew 1996, p. 2731).

3.2.3 DNA amplification and gel electrophoresis

3.2.3.1 Agarose gel preparation

For bacterial and fungal DNA samples, 1% agarose gel and a 1.5% agarose gel were prepared respectively. The agarose powder (Vivantis, Malaysia) was weighed and added to 100 mL 1× TAE buffer. The agarose solution was heated using a microwave for 1 minute and 30 seconds in order to dissolve the agarose powder completely. When it was cooled, 3 µL of RedSafe nucleic acid dye (iNtRON Biotechnology, Korea) was added and the solution was poured into a gel tank (Thermo Scientific, USA).

3.2.3.2 PCR reaction set up

The molecular genetic markers for identification of bacteria and fungi were based 16S rRNA and ITS molecular genetic markers. The primer sequences were listed in Table 12. Polymerase chain reaction (PCR) mix was set up using MyTaq™ Mix (Bioline, UK) (Table 13). Standard PCR and Touchdown PCR were used in DNA amplification (Table 14, Table 15).

Table 12 Primer Sequences (Macrae, Rimmer & O'Donnell 2000, p. 14; Ahmed et al. 1999, p. 3176)

Primers **(Integrated DNA Technologies, USA)**	**Sequences (5'→3')**
8F (Forward primer)	AGA GTT TGA TCC TGG CTC AG
1541R (Reverse primer)	AAG GAG GTG ATC CAG CCG CA
ITS 4 (Reverse primer)	TCC TCC GCT TAT TGA TAT GC
ITS 5 (Forward primer)	GGA AGT AAA AGT CGT AAC AAG G

Table 13 PCR reaction set up

Components	**Amount added**
MyTaq™ Mix	25 µL
Forward primer, 20 µM	1 µL
Reverse primer, 20 µM	1 µL
Genomic DNA template	200 ng
Sterile ultrapure water	up to 50 µL

Table 14 Standard PCR condition set up for 16S rRNA and ITS molecular genetic markers (Mastercycler® gradient, Eppendorf, Germany)

Step	Temperature (ºC)	Time (minute)	Cycle(s)
Initial denaturation	95	5	1
Denaturation	95	0.5	34
Annealing	55	0.5	35
Extension	72	0.5	34
Final extension	72	7	1

Table 15 Touchdown PCR condition set up for 16S rRNA and ITS molecular genetic markers (Mastercycler® gradient, Eppendorf, Germany) (Korbie & Mattick 2008, p. 1455)

Step	Temperature (ºC)	Time (minute)	Cycle(s)
Initial denaturation	95	5	1
Denaturation	95	1	9
Annealing	65 (Temperature increment: -1ºC, Ramp rate: -0.3ºC/s)	1	10
Extension	72	1	10
Denaturation	95	1	15
Annealing	55	1	15
Extension	72	1	14
Final extension	72	10	1

3.2.3.3 Gel electrophoresis

PCR product (5 µL) was mixed with 1 µL 6× loading dye and loaded into the agarose gel well. For bacteria, the agarose gel was run at 120 V for 30 minutes and the DNA ladder used was GeneRuler 1 kb DNA Ladder (Thermo Scientific, USA). For fungi, the agarose gel was run at 100 V for 50 minutes and the DNA ladder used was 100 bp DNA Ladder H3 RTU (GeneDireX, Taiwan). After that, the gel image was visualised via Gel Doc™ XR and Gel Documentation System (Bio-Rad Laboratories, USA).

3.2.4 DNA sequencing and BLAST analysis

The PCR products were sent to First Base Laboratories Sdn Bhd, Selangor, Malaysia for DNA sequencing service. The sequencing results were compared to the National Centre for Biotechnology Information (NCBI) GenBank database using Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed by MEGA 7 (Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets) for an overview of relationships between microorganisms (Kumar, Stecher and Tamura 2015).

3.2.5 Quantification of microorganism biological activities

3.2.5.1 Phenate method for measuring ammonia production in selected nitrogen fixing strains

The ammonia standards were prepared from analytical grade NH_4Cl (Merck, USA). The NH_4Cl was oven-dried at 100ºC overnight and weighed for 3.918 g. Then, it was dissolved in 1 L distilled water to make 1000 mg/L ammonia stock solution. The stock solution was diluted to 10 mg/L ammonia working solution. After that, ammonia standard solutions were made from the concentration range of 0.2 mg/L to 2.0 mg/L (American Public Health Association, American Water works Association & Waste Environment Federation 2005, p. 4–114).

The nitrogen fixing strains were inoculated to 250 mL of Jensen's broths using broth cultures. The Jensen's broth cultures were prepared in three replicates. The broth cultures were incubated at 30ºC, 150 rpm for 3 days (Iwata et al. 2010, p. 416; Hartono et al. 2016,

p. 11736). 50 mL of Jensen's broth culture was pipetted to a sterile 50 mL tube (Tarsons, India) from each flask and centrifuged at 21700 × g for 10 minutes.

The phenate reaction was set up by adding 25 mL of standard or culture sample, 1 mL of phenol solution, 1 mL of sodium nitroprusside solution and 2.5 mL of oxidising solution to a 50 mL Erlenmeyer flask (Duran, Germany). The flask was covered with a piece of parafilm (Bemis, USA) and incubated in the dark environment for 1 hour. The solution would turn blue and its absorbance reading would be measured at 640 nm. The concentration of ammonia in the sample was determined from the standard curve [Figure 35 (Appendix)] (American Public Health Association, American Water works Association & Waste Environment Federation 2005, p. 4–114). The obtained results were compared to a known nitrogen fixing strain namely *Azotobacter chroococcum* NBRC 102613.

3.2.5.2 Vanadomolybdophosphoric acid colorimetric method for measuring phosphate solubilisation in selected phosphate solubilising strains

The phosphate standards were prepared from analytical grade anhydrous KH_2PO_4 (Merck, USA). Briefly, 219.5 mg of KH_2PO_4 was dissolved in 1 L distilled water to make 50 mg/L PO_4^{3-} stock solution. Then, phosphate standards were created from the concentration range of 2.0 mg/L to 10.0 mg/L (American Public Health Association, American Water works Association & Waste Environment Federation 2005, pp. 4–151– 4–152).

The phosphate solubilising strains were added to 250 mL of Pikovskaya's broths using broth cultures. The Pikovskaya's broth cultures were prepared in three replicates. They were incubated at 30ºC, 150 rpm for 3 days (Hu, Chen & Guo 2006, p. 984). There was 50 mL of Pikovskaya's broth culture pipetted to sterile 50 mL tube from each flask and centrifuged at 21700 × g for 10 minutes.

The reaction was set up in a 50 mL volumetric flask. There were 35 mL of standard or culture sample and 10 mL of vanadate-molybdate reagent (Merck, USA) added to the flask. After topping up to the volume of 50 mL, the flask was incubated in the dark environment for 10 minutes. The solution turned yellow and its absorbance reading was measured at 420 nm. The concentration of phosphate in the sample was determined from

the standard curve [Figure 36 (Appendix)] (American Public Health Association, American Water works Association & Waste Environment Federation 2005, pp. 4–151–4–152). The obtained results were compared to two known phosphate solubilising strains namely *Paenarthrobacter ureafaciens* NBRC 12140 and *Aspergillus niger* NBRC 33023.

3.2.5.3 Atomic absorption spectroscopy (AAS) method for measurement of potassium solubilisation in selected potassium solubilising strains

The potassium solubilising strains were added to 250 mL modified Aleksandrov's broths using broth cultures. The modified Aleksandrov's broth cultures were prepared in three replicates. All cultures were incubated at 28ºC, 150 rpm for 7 days (Meena et al. 2015, p. 342). The cultures were centrifuged at 21700 × g for 10 minutes and filtered through 0.22 µm filter membrane (Merck Millipore, USA).

The filtrates were sent to T & T Laboratory (Sarawak) Sdn Bhd, Malaysia for the analysis of potassium concentrations. The laboratory utilised atomic absorption spectrophotometer to analyse the concentrations of potassium in the filtrates. The concentrations of soluble potassium in the samples were derived from the laboratory analysis results. The obtained results were compared to two known potassium solubilising strains namely *Enterobacter hormaechei* NBRC 105718 and *Aspergillus terreus* NBRC 33026.

3.2.5.4 Salkowski's method for measurement of IAA production in selected IAA producing strains

The IAA stock solution was prepared by dissolving 200 mg of IAA (Acros Organics, USA) in 2 mL of absolute ethanol (Fisher Scientific, USA) and topping up to 200 mL with distilled water. The 1000 µg/mL of IAA stock solution was diluted to 10 µg/mL of IAA working solution. The IAA standards were made in the concentration range of 0.1 µg/mL to 1.0 µg/mL.

The IAA producing strains were inoculated to 250 mL tryptophan broth using broth cultures and incubated at 28ºC, 150 rpm for 4 days (Mohite 2013, p. 639). The tryptophan broth cultures were prepared in three replicates. There was 50 mL of tryptophan broth culture from each flask centrifuged at 21700 × g for 10 minutes.

The standard or culture sample was added to a 100 mL Erlenmeyer flask (Duran, Germany) in a volume of 30 mL. Then, 45 mL of Salkowski's reagent was added to it. The flask was covered with parafilm and incubated in the dark environment for 75 minutes. After that, the solution became pink in colour and its absorbance reading was measured at 525 nm. The concentration of IAA in the sample was determined from the standard curve [Figure 37 (Appendix)] (Gordon & Weber 1951, p. 194). The obtained results were compared to a known IAA producing strain namely *Pseudomonas putida* NBRC 14164.

3.2.5.6 Data analysis

The collected quantitative data were analysed using ANOVA with Tukey HSD post-hoc test in SPSS ver. 23.0 (IBM corporation, USA). The significant difference was set at $p \leq 0.05$ level for the comparison of strain activities in each characteristic.

3.3 Result and Discussion

3.3.1 Molecular identification of bacteria and fungi strains

3.3.1.1 Analysing PCR results

There were 16 bacterial strains and 4 fungal strains selected for genetic identification based on their 16S rRNA and ITS molecular genetic markers respectively. The DNA sequences from the respectively genetic markers were amplified using PCR technology. The bands of PCR products was thick and bright in the agarose gel as shown in Figure 13 and Figure 14 in order to achieve high accuracy of sequencing results. In addition, in order to ascertain amplification fidelity, some DNA samples were amplified using Touchdown PCR which was claimed to be able to increase the specificity and sensitivity in PCR amplification (Korbie & Mattick 2008, p. 1452). As shown in Figure 13 and Figure 14, the estimated band sizes of bacterial and fungal PCR products were around 1500 bp and in the range of 700 bp to 800 bp respectively.

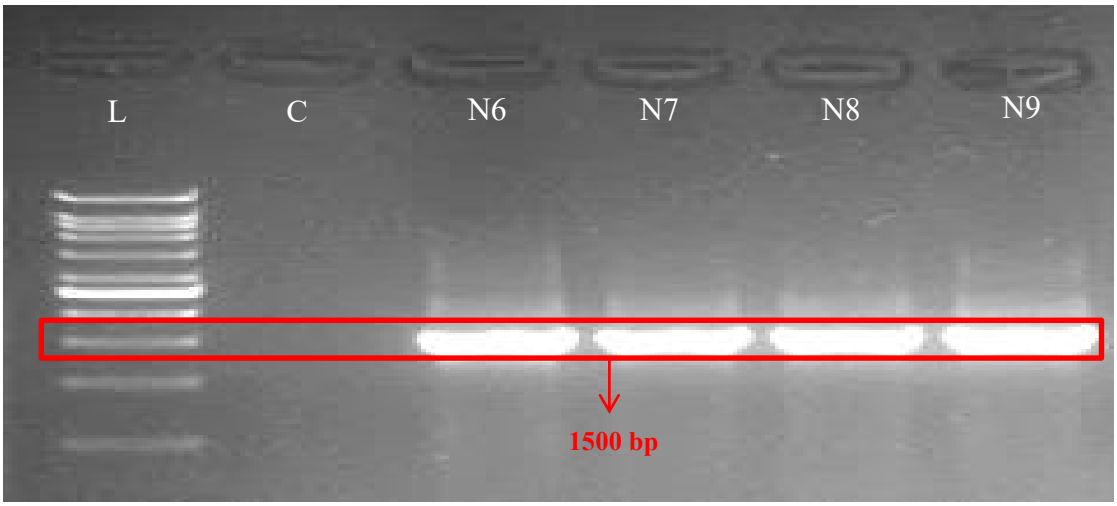


Figure 13 The size of the PCR product in 1% agarose gel. The PCR products were derived from bacteria genomic DNA samples. Label L represents 1kb DNA ladder; C represents negative control (Master Mix without genomic DNA templates); and N6-N9 represents the bacterial PCR products.

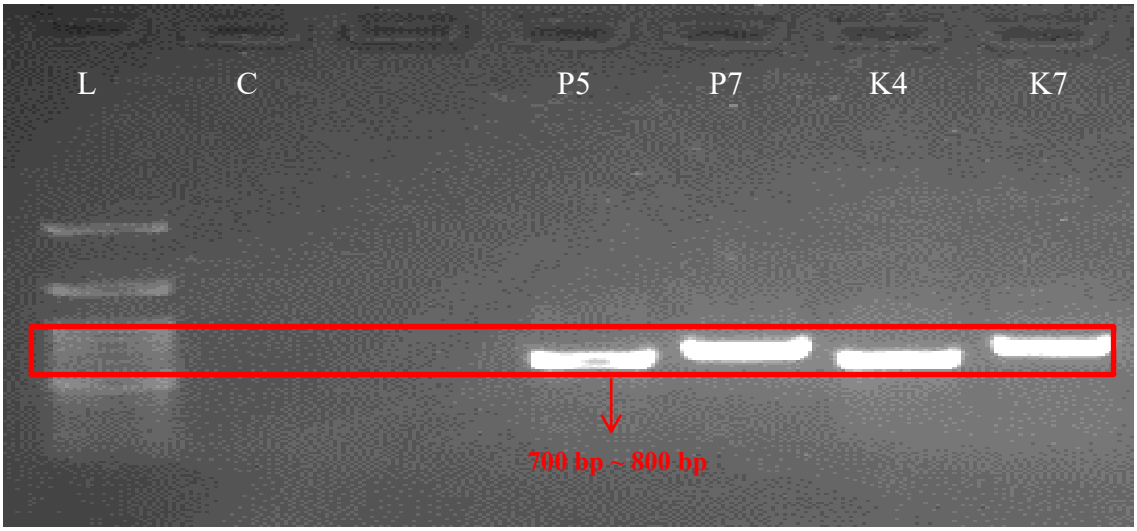


Figure 14 The size of the PCR product in 1.5 % agarose gel. The PCR products were derived from fungi genomic DNA samples. Label L represents 100 bp DNA ladder; C represents negative control (Master Mix without genomic DNA templates); and P5-K7 represents the fungal PCR products.

3.3.1.2 Analysis of sequencing results

After obtaining the sequencing results, the sequences were compared to NCBI Genbank database using BLAST. The BLAST webpage would show the matching information of 100 aligned sequences for each sample run. The microorganism strains were identified to the type strains with the highest percentage of query cover and identity. The designation of taxa level for each strain was based on Yuwa-Amornpitak et al. (2006, p. 1063) which recognised matching level of 99% or above for species level identification. As such, for the matching level below 99%, genetic identification would only be confirmed at genus level instead of species level. The DNA sequences of both the selected strains and matching sequences from NCBI Genbank database were analysed by MUSCLE (Multiple Sequence Comparison by Log-Expectation) alignments in MEGA 7. Then, phylogenetic trees were constructed by Maximum Likelihood method based on Tamura-Nei model.

3.3.1.2.a Genetic identification of nitrogen fixing strains

As shown in Table 16, for nitrogen fixing strains, there were *Methylobacterium* sp., two *Pseudomonas* spp., *Stenotrophomonas* sp. and *Streptomyces* sp. identified through comparison to the NCBI Genbank database. The percentages of query cover and identity were above 80% and 70% respectively. The genetic identification was further confirmed based on the phylogenetic tree generated from the 16S rRNA sequences of selected strains and their closely matched 16S rRNA sequences of strains derived from Genbank database (Figure 15).

Table 16 The closest match of type strains from NCBI Genbank database based on 16S rRNA genetic marker which were selected based on the percentages of query cover and identity.

Strains	Closest type strain	Accession No.	Query cover (%)	Identity (%)
N6	*Methylobacterium populi*	HM355721.1	86	76
N7	*Pseudomonas moraviensis*	LN714047.1	99	96
N8	*Pseudomonas migulae*	LK391510.1	98	95
N9	*Stenotrophomonas maltophilia*	GU170362.1	89	94
N11	*Streptomyces gramineus*	NR_109017.1	90	95

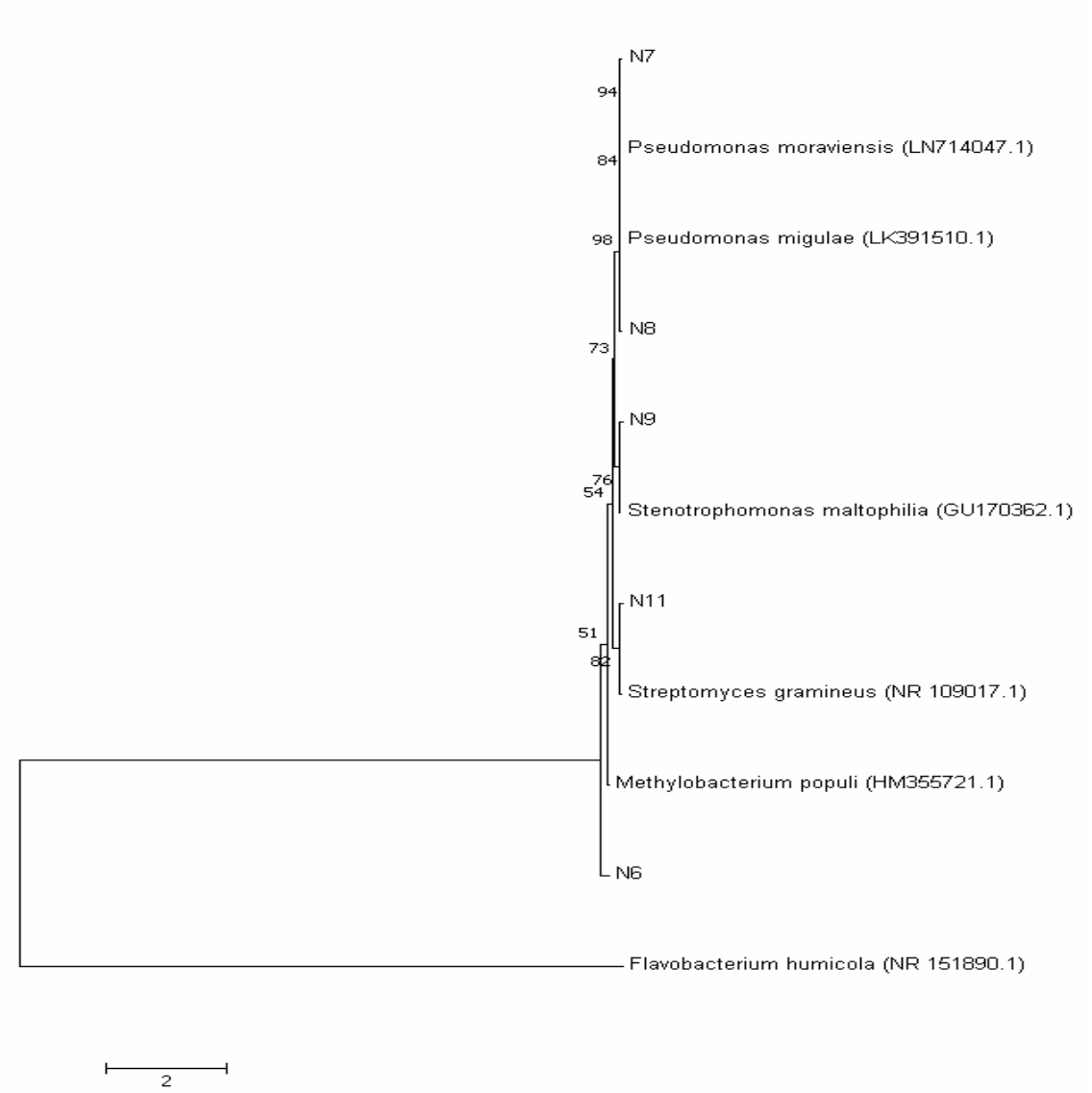


Figure 15 The phylogenetic tree showed the matching distance in between nitrogen fixing bacterial strain sequences and Genbank database sequences based on the comparison of 16S rRNA genetic marker. It was constructed by Maximum Likelihood method based on Tamura-Nei model. Bootstrap values based on 1000 replications are given on the nodes as precentages above 50%. *Flavobacterium humicola* was used as the outgroup. Bar: 2.00 substitutions per nucleotide position.

Strain N6 could be genetically identified as *Methylobacterium* sp. based on moderate level of 76% matching to *Methylobacterium populi*. It could be a novel strain based on this low matching level. *Methylobacterium* spp. belong to class Alphaproteobacteria. *Methylobacterium* sp. could be commonly found in many plant tissues albeit its symbiotic

relationship with plants could not be established (Jourand et al. 2004, p. 2269). However, a recent report also suggested that *Methylobacterium* sp. could be isolated from legume root nodules of *Crotalaria* spp. and *Lotononis bainesii* albeit their roles in nitrogen fixation could not be confirmed (Jourand et al. 2004, p. 2269). There was also a successful isolation of *Methylobacterium* spp. from the rhizoplane region of *Eucalyptus* sp. (Andreote et al. 2009, p. 88). *Methylobacterium* N6 strain isolated in this study could be the first nitrogen fixing *Methylobacterium* sp. to be isolated from *N. cadamba* and tropical tree species in general.

Strain N7 and N8 were both genetically identified as *Pseudomonas* spp. with identity of 96% and 95% respectively to *Pseudomonas moraviensis* and *Pseudomonas migulae* (Table 16). *Pseudomonas* spp. are classified under class Gammaproteobacteria. Nitrogen fixing *Pseudomonas* spp. were reported to be isolated from coniferous trees, sugar canes, rice, wheat, rye grass and compost. In the coniferous trees, *Pinus nigra* and Douglas-fir, nine nitrogen fixing *Pseudomonas* strains were isolated from the needle samples (Favilli & Messini 1990, p. 93). In sugarcane, Pimentel et al. (1991, pp. 62-63) isolated a total of six nitrogen fixing *Pseudomonas* strains from various locations in USA, Reunion, Jamaica, Mauritius and Australia. These strains could grow abundantly in N free semi-solid medium with sucrose as sole carbon source. In another report, two nitrogen fixing *Pseudomonas* spp. were isolated from sugar canes in Uruguay (Taulé et al. 2012, p. 42). In rice root, two *Pseudomonas* strains were isolated from the rhizospheres of wheat and rye grass showed good nitrogenase activities (Habibi et al. 2014, p. 59). Besides rhizospheres, *Pseudomonas* strain with nitrogenase activity was also isolated from compost pile in Japan (Hatayama et al. 2005, p. 1542). In this study, N7 and N8 strains were shown to be nitrogen fixing *Pseudomonas* spp.. The moderate level of matching of 96% and 95% to *Pseudomonas moraviensis* and *Pseudomonas migulae* respectively would suggest that both strains could be novel species or strains of *Pseudomonas* discovered from *N. cadamba*, a tropical timber species. Previously, nitrogen fixing *Pseudomonas* spp. were mostly discovered in temperate coniferous trees or crops.

Based on the genetic identification, strain N9 could be *Stenotrophomonas* sp. with 94% matching to *Stenotrophomonas maltophilia* (Table 16). *Stenotrophomonas* sp. belongs to class Gammaproteobacteria. They were reported either isolated from rhizospheres or plant tissues. Nitrogen fixing *Stenotrophomonas* strains were found in rhizospheres of cucumbers and showed high level of nitrogenous activity (Islam et al 2016, p. 6). The treated cucumber seedlings showed a significant increment in nitrogen content (Islam et al 2016, p. 7). Another report described an *Stenotrophomonas* isolate from wheat rhizosphere that could significantly increase the dry weight of maize seedlings (Neemisha & Gosal 2016, p. 10). Nitrogen fixing *Stenotrophomonas* strain was also shown to significantly improve grain yield in wheat (Bulut 2013, p. 548). *Stenotrophomonas* spp. were also isolated as nitrogen fixing endophytes from sweet potato explants, pear rootstock and stems of sugar cane cultivars in Uruguay (Khan & Doty 2009, p. 201; Liaqat & Eltem 2016, p. 6; Taulé et al. 2012, pp. 42-43). Strain N9 isolated in this study could be the first nitrogen fixing *Stenotrophomonas* sp. to be isolated from a tropical timber tree.

Strain N11 could be identified as *Streptomyces* sp. with 95% matching to *Streptomyces gramineus* (Table 16). *Streptomyces* sp. is categorised under class Actinobacteria. Nitrogen fixing *Streptomyces* spp. were previously isolated from rhizospheres and plant tissues. *Streptomyces* strain isolated from Egyptian rhizospheric soils could grow on nitrogen free medium and significantly improve shoot dry weight of wheat (EL-Shanshoury 1995, pp. 120-123). From the soil from arid land of South Dakota, USA, *Streptomyces* strains with nitrogen fixing functions were isolated (Dahal et al. 2017, p. 37). Nitrogen fixing *Streptomyces* sp. was also reported as an endophyte isolated from pea root at Kurima, Japan (Soe et al. 2012, p. 320). Its interaction with another nitrogen fixing bacteria *Bradyrhizobium japonicum* strain USDA110 had shown positive impacts on shoot nitrogen accumulation and seed weight in Hinthada (Myanmar) soybean (Soe et al. 2012, p. 322). In addition, nitrogen fixing *Streptomyces* spp. were reported as dominant endophytic bacterial genera in the *Eucalyptus* roots (Fonseca et al. 2017, p. 5). This could be the first report on nitrogen fixing *Streptomyces* strains to be isolated from the rhizospheres of tropical timber tree *N. cadamba*.

3.3.1.2.b Genetic identification of phosphate solubilising strains

Based on genetic identification, the phosphate solubilising strains were *Streptacidiphilus* sp., *Lysinibacillus* sp., *Serratia* sp. and two *Metarhizium* spp. (Table 17). As described earlier, the 16S rRNA and ITS sequences of isolated strains were analysed in comparison with sequences available in NCBI Genbank database (Figure 16, Figure 17).

Table 17 The closest match of type strains from NCBI Genbank database based on 16S rRNA and ITS genetic markers which were selected based on the percentages of query cover and identity.

Strains	Closest type strain	Accession No.	Query cover (%)	Identity (%)
P1	*Streptacidiphilus luteoalbus*	AY530187.1	77	94
P2	*Lysinibacillus fusiformis*	KP419697.1	89	87
P5	*Metarhizium anisopliae*	KX806656.1	100	100
P7	*Metarhizium anisopliae*	KX809521.1	100	99
P8	*Serratia marcescens*	KT887950.1	80	98

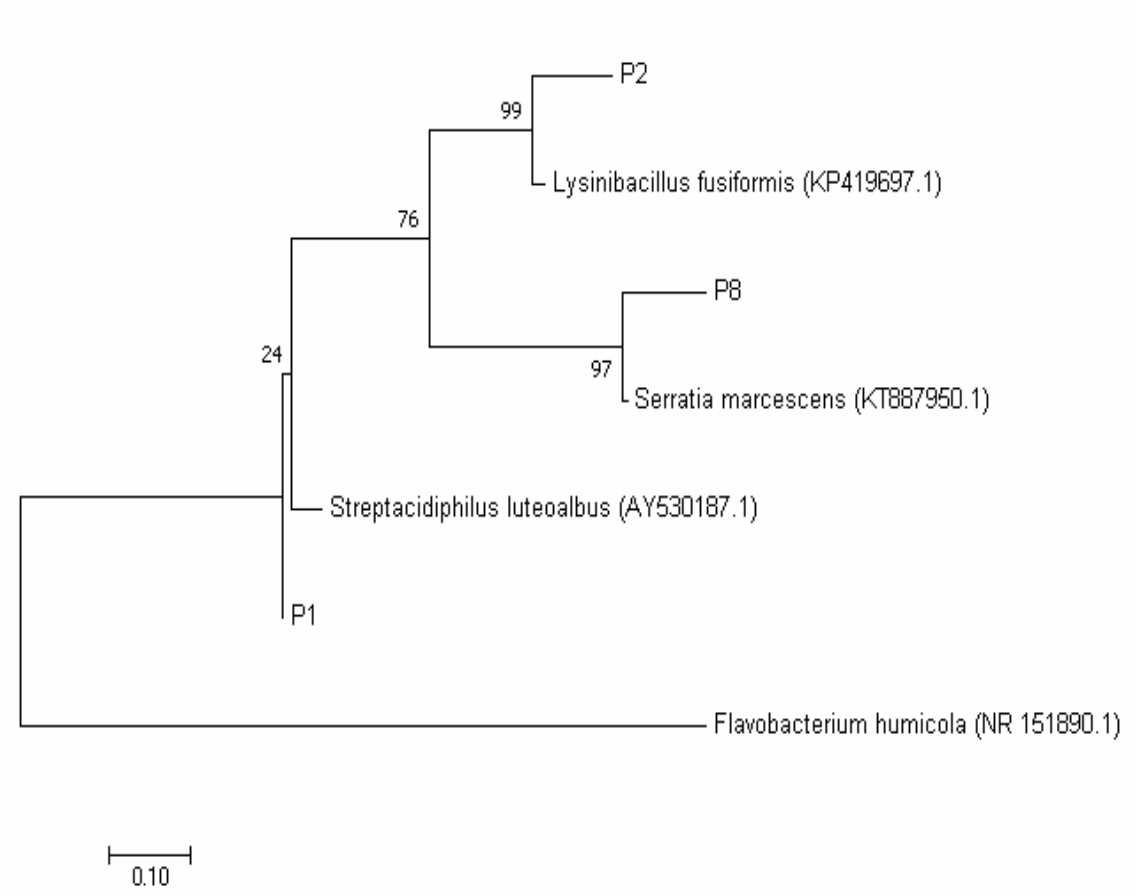


Figure 16 The phylogenetic tree showed the matching distance in between phosphate solubilising bacterial strain sequences and Genbank database sequences based on the comparison of 16S rRNA genetic marker. It was constructed by Maximum Likelihood method based on Tamura-Nei model. Bootstrap values based on 1000 replications are given on the nodes as precentages above 20%. *Flavobacterium humicola* was used as the outgroup. Bar: 0.10 substitutions per nucleotide position.

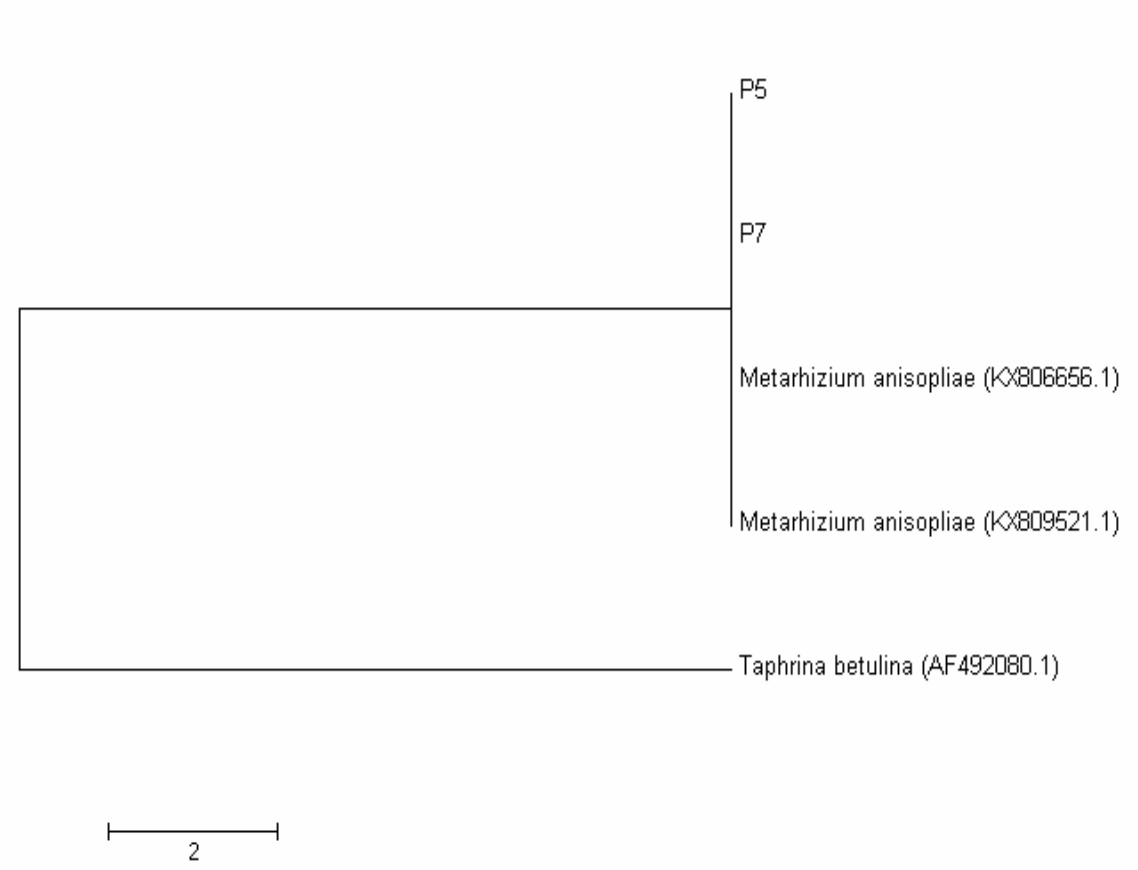


Figure 17 The phylogenetic tree showed the matching distance in between phosphate solubilising fungal strain sequences and Genbank database sequences based on the comparison of ITS genetic marker. It was constructed by Maximum Likelihood method based on Tamura-Nei model. Bootstrap values based on 1000 replications are not given on the nodes. *Taphrina betulina* was used as the outgroup. Bar: 2.00 substitutions per nucleotide position.

Based on the genetic identification, strain P1 could be *Streptacidiphilus* sp. with 94% matching to *Streptacidiphilus luteoalbus* (Table 17). *Streptacidiphilus* spp. belong to class Actinobacteria. This bacterium could grow in a wide range of pH 3.5 to 6.0, and therefore could be found in acidic soils (Golinska et al. 2013, p. 965). This acidophilic actinobacterium was reported from rhizospheres and river sediments. A study on acidic rhizospheres in Thailand showed that 93.9% of acidophilic actinobacteria were able to release soluble phosphate (Poomthongdee, Duangmal & Pathom-aree 2014, p. 6). Another study of yam rhizospheres indicated that most of the isolates could solubilise $Ca_3(PO_4)_2$ in the broth medium below pH 5.5 (Palaniyandi et al. 2013, p. 989). Besides

that, the research of river sediments showed *Streptacidiphilus* strains could demonstrate significant phosphate solubilising activities that the liquid medium in between pH 3.0 and 4.5 (Dastager & Damare 2013, p. 423). Since P1 strain was also isolated from soil sample of Sabal FR with pH value 4.51, this could also be the first report on isolation of phosphate solubilising *Streptacidiphilus* sp. found in the rhizosphere of *N. cadamba*.

Strain P2 could be identified as *Lysinibacillus* sp. which is from class Bacilli (Table 17). Based on the low matching rate of 87% to *Lysinibacillus fusiformis*, strain P2 could be a novel strain of *Lysinibacillus*. The phosphate solubilising *Lysinibacillus* strains were previously reported to be isolated from banana trees, rice, wheat and citrus fruit trees. Two *Lysinibacillus* strains were isolated from the roots of banana tree cultivar 'Prata Anã' in Brazil and showed phosphate solubilisation abilities (Andrade et al. 2014, p. 31). In Malaysia, phosphate solubilising *Lysinibacillus* strain was isolated from the rhizosphere of rice, and showed to significantly improve the height of rice seedlings (Tan et al. 2014, pp. 346-352). In wheat rhizospheres in India, two *Lysinibacillus* strains were isolated and showed to release soluble phosphate from $Ca_3(PO_4)_2$ (Verma et al. 2016, p. 49). In Florida, USA, a phosphate solubilising *Lysinibacillus* strain was isolated from root samples of Valencia orange (*Citrus sinensis*) trees (Trivedi, Spann & Wang 2011, p. 331). As such, most previous studies on phosphate solubilising *Lysinibacillus* strains were reported on fruit trees and agricultural crops. Thus, the isolation of phosphate solubilising *Lysinibacillus* sp. from rhizospheres of *N. cadamba* could be interesting.

Based on the genetic identification, strain P5 and P7 could be both *Metarhizium anisopliae* based on matching of 100% and 99% respectively (Table 17). *Metarhizium anisopliae* is classified as a fungus under class Sordariomycetes and family Clavicipitaceae. *Metarhizium anisopliae* is known as an effective biocontrol agent against pest insects in the plant rhizosphere. It could infect the insect host and deliver the insect-derived nitrogen to plant roots (Zimmermann 2007, p. 879; Tiago et al. 2014, p. 648). However, there was no previous report on phosphate solubilisation trait in *Metarhizium anisopliae*. Nonetheless, *Paecilomyces* spp., which is another different species under the same family Clavicipitaceae were reported to carry phosphate solubilisation trait based on studies of rhizospheric soils and nematodes. A study reported *Paecilomyces* strain isolated from rhizospheric soil samples in Argentina could solubilise $Ca_3(PO_4)_2$ in NBRIP

agar medium and showed clear halo zone (Arrieta et al. 2015, p. 150). Besides soils, a phosphate solubilising *Paecilomyces* strain was reported isolating from potato cyst nematode *Globodera rostochiensis* (Lima-Rivera et al. 2016, p. 508). It gave the highest level of soluble phosphate in Pikovskaya's broth after eighteen-day incubation (Lima-Rivera et al. 2016, p. 512). In conclusion, most previous studies on phosphate solubilisation were reported on *Paecilomyces* spp. under family Clavicipitaceae. There was little report on *Metarhizium* spp., albeit they are under the same family Clavicipitaceae. As such, the current isolation of phosphate solubilising *Metarhizium anisopliae* from the rhizospheres of tropical timber tree species would be an interesting discovery.

Strain P8 could be identified as *Serratia* sp. with 98% matching to *Serratia marcescens* (Table 17). *Serratia* sp. belongs to class Gammaproteobacteria. In previous studies, phosphate solubilising *Serratia* sp. could be found in soils and legume plants. The *Serratia* strain isolated from soil in mangrove forest in India was shown to secrete organic acids such as lactic acid, malic acid and acetic acid to mobilise inorganic phosphate source (Behera et al. 2017, p. 5). This *Serratia* strain was suggested as bio-inoculants to improve soil fertility (Behera et al. 2017, p. 9). Another study of rhizospheric soils in Argentina demonstrated that clear halo zone was observed around the *Serratia* strain colony after seven-day incubation on NBRIP agar medium (Viruel, Lucca & Siñeriz 2011, p. 492). In tropical region, *Serratia* sp. isolated from Venezuela acidic Ultisols would solubilise $Ca_3(PO_4)_2$ and $FePO_4$ efficiently in NBRIP liquid medium (Pérez et al. 2007, p. 2909). In addition, phosphate solubilising *Serratia* strain from India alkaline soils was proved to promote the growth of maize and improve uptake of phosphate (Lavania & Nautiyal 2013, p. 4410). In Pakistan, two phosphate solubilising *Serratia* strains isolated from root nodules of chickpeas significantly improved grain and straw yields of chickpea grown in the nutrient deficient area (Zaheer et al. 2016, p. 517). In this study, phosphate solubilising *Serratia* strain was isolated from tropical acidic soils.

3.3.1.2.c Genetic identification of potassium solubilising strains

There were three bacterial and two fungal strains selected for genetic identification (Table 18). The bacterial strains were *Lysinibacillus* spp. and *Bacillus* sp., while the fungal strains were *Phanerochaete* sp. and *Penicillium* sp. (Table 18). The genetic identification methods were as described earlier (Figure 18, Figure 19).

Table 18 The closest match of type strains from NCBI Genbank database based on 16S rRNA and ITS genetic markers which were selected based on the percentages of query cover and identity.

Strains	Closest type strain	Accession No.	Query cover (%)	Identity (%)
K3	*Lysinibacillus sphaericus*	KF151164.1	97	97
K4	*Phanerochaete chrysosporium*	KP771707.1	97	99
K5	*Bacillus cereus*	KY750689.1	99	97
K6	*Lysinibacillus fusiformis*	KF208487.1	99	99
K7	*Penicillium rolfsii*	KM458781.1	99	99

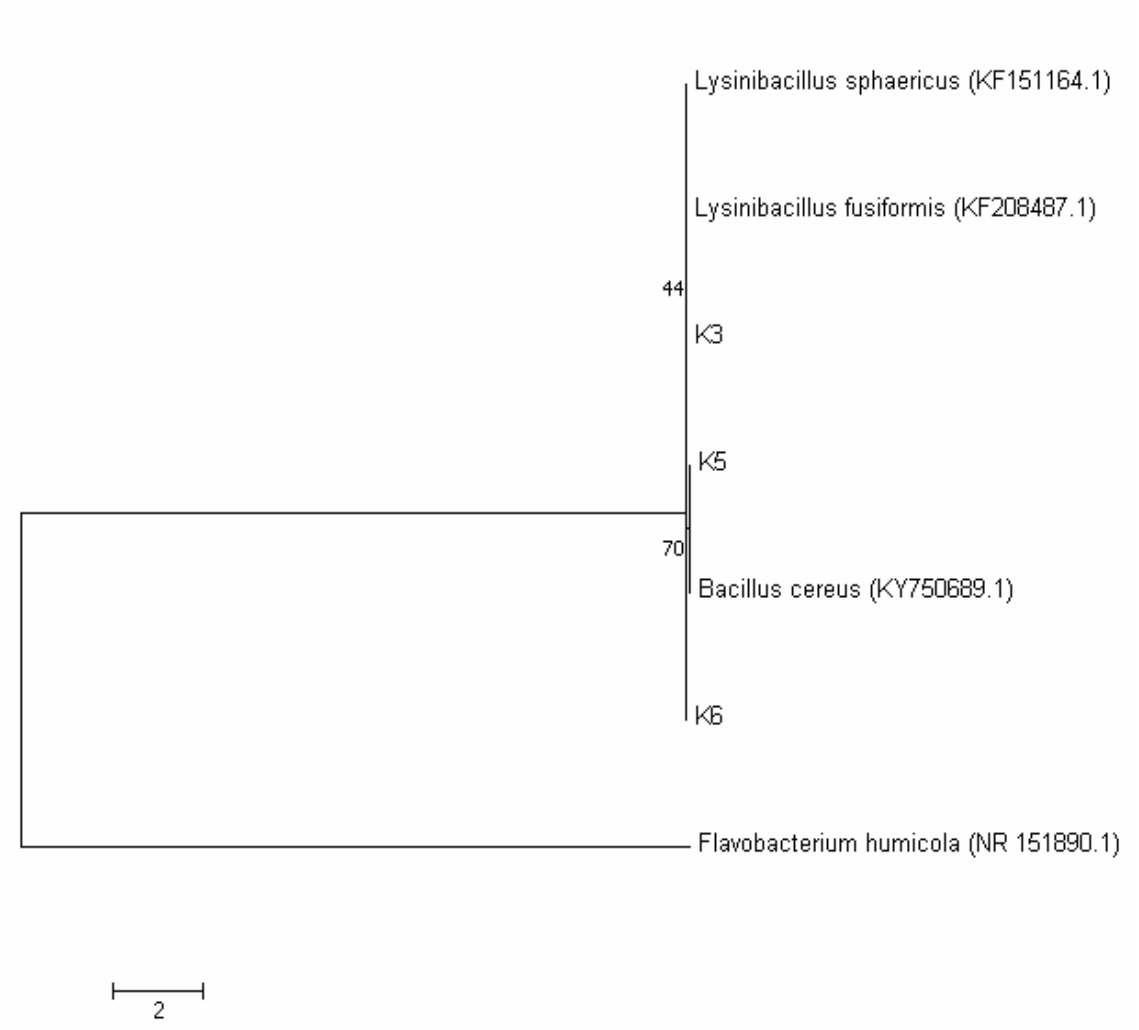


Figure 18 The phylogenetic tree showed the matching distance in between potassium solubilising bacterial strain sequences and Genbank database sequences based on the comparison of 16S rRNA genetic marker. It was constructed by Maximum Likelihood method based on Tamura-Nei model. Bootstrap values based on 1000 replications are given on the nodes as precentages above 40%. *Flavobacterium humicola* was used as the outgroup. Bar: 2.00 substitutions per nucleotide position.

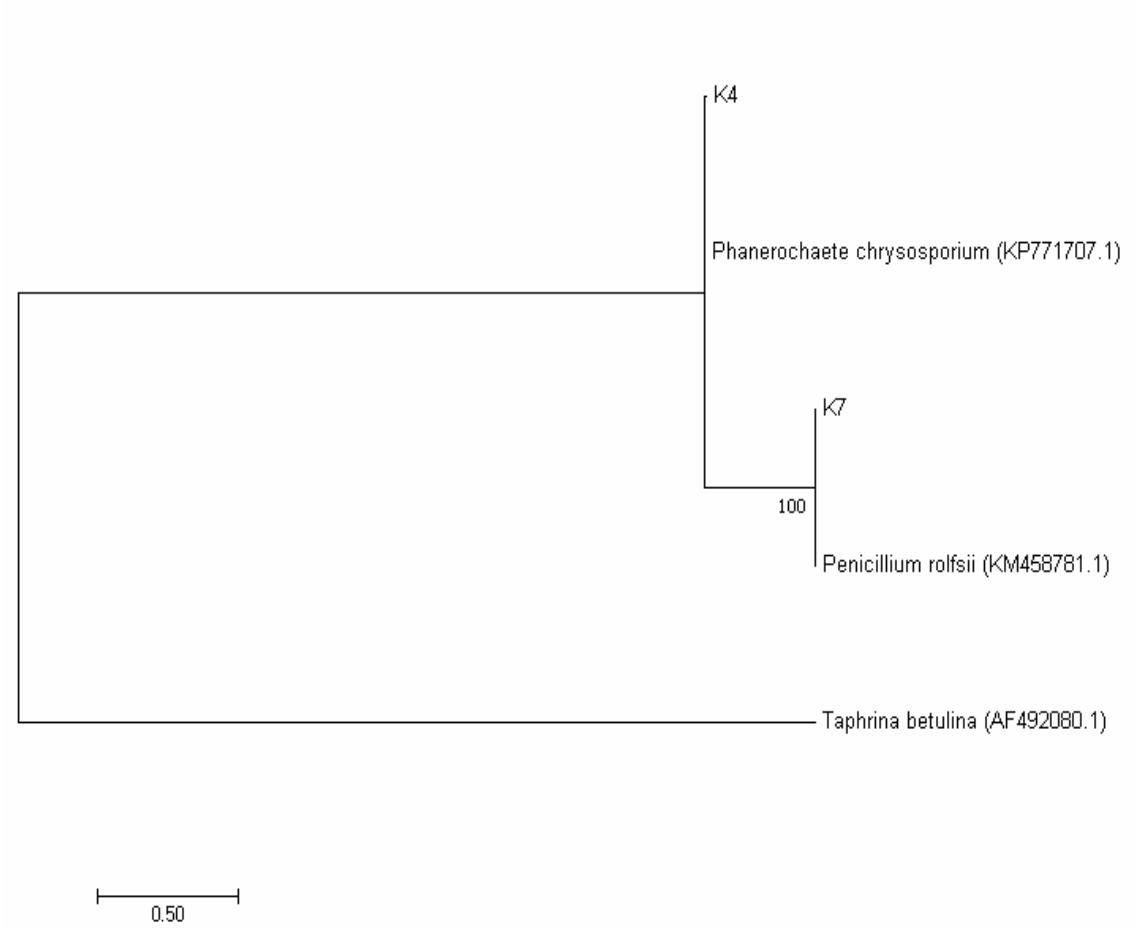


Figure 19 The phylogenetic tree showed the matching distance in between potassium solubilising fungal strain sequences and Genbank database sequences based on the comparison of ITS genetic marker. It was constructed by Maximum Likelihood method based on Tamura-Nei model. Bootstrap values based on 1000 replications are given on the nodes as precentage of 100%. *Taphrina betulina* was used as the outgroup. Bar: 0.50 substitutions per nucleotide position.

Strain K3 and K6 could be identified as *Lysinibacillus* sp. and *Lysinibacillus fusiformis* with 97% and 99% matching to *Lysinibacillus sphaericus* and *Lysinibacillus fusiformis* respectively (Table 18). The potassium solubilising *Lysinibacillus* strains were reported to be isolated from soil samples. A study in Malaysia showed successful isolation of potassium solubilising *Lysinibacillus* sp. from rice rhizosphere (Tan et al. 2014, p. 349). Another study of maize rhizosphere showed the isolated *Lysinibacillus* sp. could release organic acids to solubilise insoluble potassium source in Aleksandrov's agar medium and clear halo zone was observed around the colony (Naureen et al. 2017, p. 5). Besides the

warm rhizospheric soils, a *Lysinibacillus* strain from cold desert soil samples showed potassium solubilisation trait (Yadav et al. 2016, p. 146). This study would be the first report on the isolation of potassium solubilising *Lysinibacillus* strains from rhizospheres of tropical timber tree species.

Based on the genetic identification, strain K5 could be *Bacillus* sp. with 97% matching to *Bacillus cereus* under class Bacilli (Table 18). The potassium solubilising *Bacillus* strains were reported to be isolated from rhizosphere soils, weathered mineral surfaces and biofertilisers. In Malaysia, a *Bacillus* strain was isolated from oil palm rhizosphere and released the highest concentration of soluble potassium from muscovite mica (Tan et al. 2014, p. 349). A study in India showed nine *Bacillus* strains were isolated from rhizospheric soils of wheat, rice, banana, maize and sorghum. They could significantly increase the concentration of soluble potassium in modified Aleksandrov's broth (Bahadur et al. 2017, p. 458). Besides the agricultural crops, *Bacillus* strains were isolated from the rhizospheres of herbal plants in Tianmu Mountain, China and Ha Tien Mountain, Vietnam. They could solubilise potassium minerals such as montmorillonite, kaolinite and K-feldspar (Hu, Chen & Guo 2006, p. 985; Diep & Hieu 2013, pp. 89-91). Additionally, a potassium solubilising *Bacillus* strain was found in weathered feldspar samples from the mine area. It could solubilise three silicate minerals including feldspar, muscovite and biotite under the environments with wide ranges of pH, salinity and temperature (Sheng et al. 2008, p. 1065). For the study of a biological potassium fertiliser in China, a potassium solubilising *Bacillus* strain was shown to significantly improve the biomass yield and potassium uptake of sudan grass (Basak & Biswas 2009, p. 253). Besides the studies of agricultural crops and herbal plants, there was probably no previous study on potassium solubilising *Bacillus* strain found in tropical timber tree species.

According to Table 18, strain K4 could be identified as *Phanerochaete chrysosporium* at a matching rate of 99%. *Phanerochaete chrysosporium* belongs to class Agaricomycetes. *Phanerochaete chrysosporium* is a strain known as the model white rot fungus. It is capable of degrading lignin and leaves behind white crystalline cellulose (Singh & Chen 2008, p. 401; Martinez et al. 2004, p. 695). There was no report showed the potassium solubilisation trait in *Phanerochaete chrysosporium*. As reported in Chapter 2, strain K4 had showed clear zone diameter in modified Aleksandrov's medium which contains mica,

an insoluble potassium source. As such, this study had discovered potassium solubilisation trait of a *Phanerochaete chrysosporium* strain isolated from the rhizospheres of tropical timber tree species.

Strain K7 was genetically identified as *Penicillium rolfsii* at a matching rate of 99% (Table 18). *Penicillium rolfsii* belongs to class Eurotiomycetes. There were two studies mentioning potassium solubilisation ability of *Penicillium* spp. The first study showed the isolated *Penicillium* sp. from coniferous tree, Douglas-fir rhizosphere soils had potassium solubilisation effect on serpentine rock and muscovite (Crawford, Floyd & Li 2000, p. 317). The second study was carried out in two Spanish Cathedrals which was constructed of sandstone, limestone and granite (Torre et al 1992, p. 130). The isolated *Penicillium* sp. could release potassium from insoluble potassium sources such as sandstone, limestone and granite (Torre et al 1992, p. 142). In this study, we reported that potassium solubilising *Penicillium rolfsii* could also be isolated from rhizospheres of *N. cadamba*, tropical timber tree species.

3.3.1.2.d Genetic identification of IAA producing strains

For IAA production, five bacterial strains were selected for genetic identification based on the methods described earlier (Table 19). The bacterial strains were *Bacillus* spp., *Lysinibacillus* spp. and *Arthrobacter* sp. (Table 19).

Table 19 The closest match of type strains from NCBI Genbank database based on 16S rRNA genetic marker which were selected based on the percentages of query cover and identity.

Strains	Closest type strain	Accession No.	Query cover (%)	Identity (%)
I1	*Bacillus niacini*	KJ767356.1	99	98
I3	*Lysinibacillus sphaericus*	JN835502.1	100	86
I5	*Lysinibacillus fusiformis*	KJ767344.1	83	87
I6	*Bacillus cereus*	KT719719.1	99	98
I7	*Arthrobacter humicola*	AB648944.1	99	97

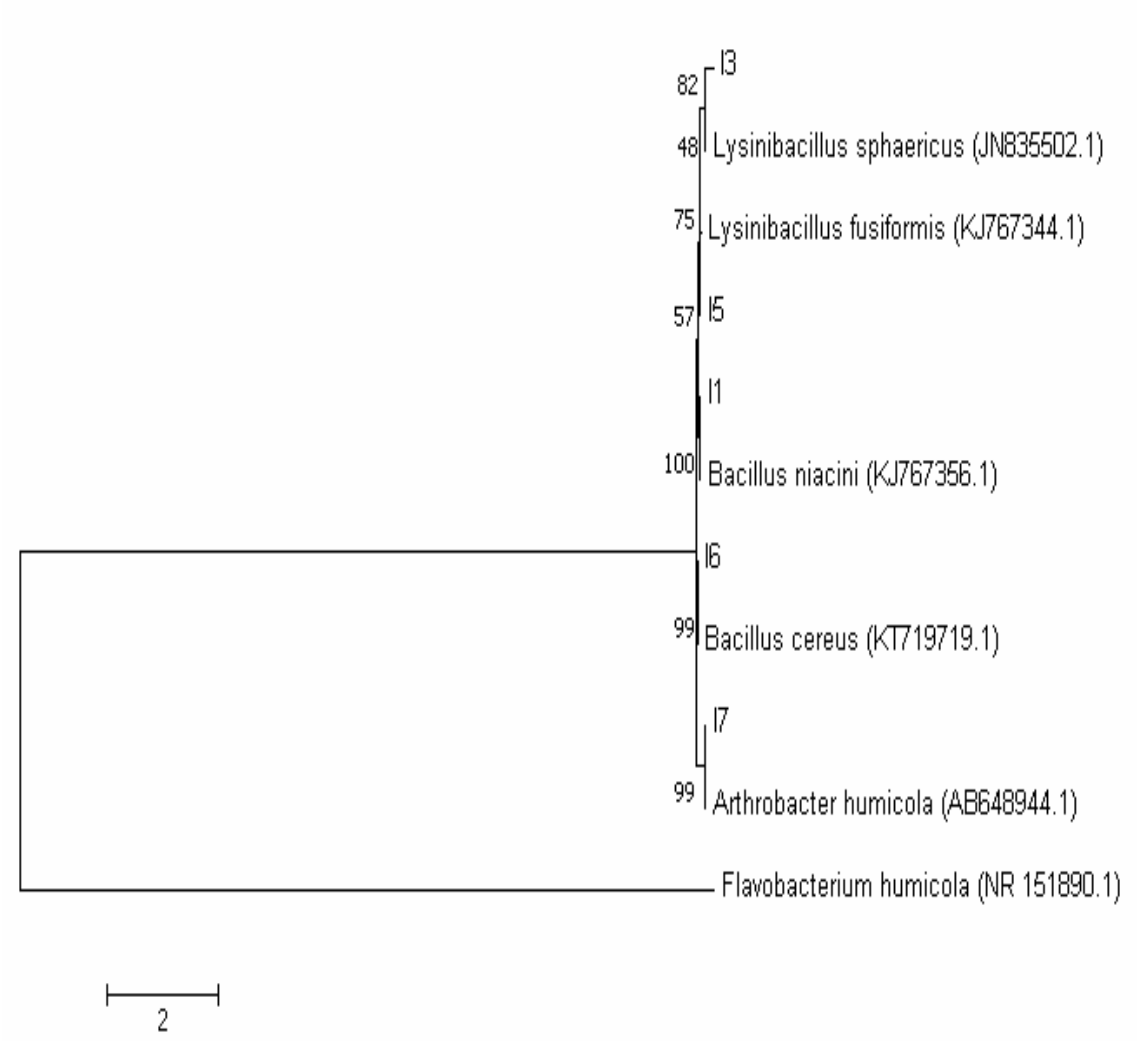


Figure 20 The phylogenetic tree showed the matching distance in between IAA producing bacterial strain sequences and Genbank database sequences based on the comparison of 16S rRNA genetic marker. It was constructed by Maximum Likelihood method based on Tamura-Nei model. Bootstrap values based on 1000 replications are given on the nodes as precentages above 40%. *Flavobacterium humicola* was used as the outgroup. Bar: 2.00 substitutions per nucleotide position.

According to the identification results in Table 19, strain I1 and I6 would be identified as *Bacillus* spp. with 98% and 98% matching to *Bacillus niacini* and *Bacillus cereus* respectively. IAA producing *Bacillus* strains were reported to be found in rhizosphere soils, plant tissues, cow dungs as well as *Eucalyptus* tree. Twenty *Bacillus* strains were isolated from Tocantins, Brazil based on positive IAA production with the presence of L-tryptophan (Junior et al. 2015, p. 286). There were two reports from Korea indicating that

Bacillus spp. from rhizospheres of agricultural crops could produce IAA in the presence of L-tryptophan. The first article reported isolation of two *Bacillus* strains from soybean and rice (Park et al. 2005, p. 128). They produced high concentrations of IAA in liquid medium supplemented with L-tryptophan (Park et al. 2005, p. 132). The second article studied the plant growth promoting effect of IAA producing *Bacillus* strains on rice seedlings. One of them showed the greatest plant growth promoting effect on shoots and roots (Ji, Gururani & Chun 2014, pp.87-89). Besides rhizosphere soils, orchid roots were targeted for IAA producing *Bacillus* strains. Three IAA producing *Bacillus* strains were isolated and showed positive effects on root formation (Tsavkelova et al. 2007, pp.74-75). In India, two IAA producing *Bacillus* strains were isolated from cowdung and showed significant effects on the growth of yam shoots and roots (Swain, Naskar & Ray 2007, pp. 104-108). There was very little report on IAA producing *Bacillus* strain isolated from trees except for *Eucalyptus* spp. Seven isolated *Bacillus* strains from *Eucalyptus* leaf and stem samples could release distinct levels of IAA in broth medium with L-tryptophan (Paz et al. 2012, p. 3717). In this study, IAA producing *Bacillus* strains were isolated from *N. cadamba*, a tropical tree.

Strain I3 and I5 could be identified as *Lysinibacillus* spp. based on 86% and 87% matching to *Lysinibacillus sphaericus* and *Lysinibacillus fusiformis* respectively (Table 19). The previous studies indicated that IAA producing *Lysinibacillus* strains were found in rhizospheres of agricultural crops, water, soil, sediment samples, plant roots and plant stems. A study in India showed that two efficient IAA producing *Lysinibacillus* strains were isolated from acidic rhizospheric soils of wheat plants (Verma et al. 2013, pp. 223-224). Besides warm farming lands, IAA producing *Lysinibacillus* strains were found in the cold desert of north western Indian Himalayas and produced distinct levels of IAA (Yadav et al. 2016, pp. 143-145). The IAA producing *Lysinibacillus* strains were also reported as endophytes. Two IAA producing *Lysinibacillus* strains were reported to be isolated from corn roots (Yu et al. 2016, p. 1382). In addition, the inoculated wheat and soybean seedlings gained significant improvements in the growth of shoots and roots (Yu et al. 2016, p. 1385). The ginseng plant in Korea was reported for isolation of two IAA producing *Lysinibacillus* strains (Vendan et al. 2010, pp. 561-563). In Colombia, the obtained *Lysinibacillus* strains from the Centro de Investigaciones Microbiológicas (CIMIC) collection produced different concentrations of IAA in Luria-Bertani broth with

L-tryptophan supplement (Martínez & Dussán 2017, p. 4). The consortium of *Lysinibacillus* strains could significantly increase the growth of *Canavalia ensiformis* (jack bean) shoots and roots (Martínez & Dussán 2017, p. 5). The previous studies mostly reported the isolation of IAA producing *Lysinibacillus* strains from agricultural crops and herbal plants. Therefore, this study could be the first report on isolation of IAA producing *Lysinibacillus* strains from a timber tree species.

Based on the identification result, I7 strain could be identified as *Arthrobacter* sp. at a matching rate of 97% (Table 19). *Arthrobacter humicola* belongs to class Actinobacteria. The IAA producing *Arthrobacter* strains were reported isolating from rhizospheric soils, coastal soils, plant shoots, aquatic ferns and vermicomposts. In India, IAA producing *Arthrobacte*r strains were isolated from rhizosphere soils of wheat plants (Verma et al. 2015, p. 1893). Other than farming lands, there was a halotolerant IAA producing *Arthrobacter* strain isolated from the saline coastal region of Yellow Sea in Korea (Siddikee et al. 2010, p. 1580). Besides soil samples, *Arthrobacter* strains were reported as endophytes in plant shoots and aquatic ferns. Three *Arthrobacter* strains were isolated from the shoots of sweet pepper and characterised as IAA producers (Sziderics et al. 2007, p. 1198). On the other hand, three *Arthrobacter* strains were reported to be isolated from aquatic ferns, *Azolla filiculoides* and *Azolla pinnata*. They produced distinct levels of IAA in mineral medium M9 with different concentrations of L-tryptophan (Forni et al. 1992, p. 378). In addition, *Arthrobacter* stain was isolated from straw and goat manure based vermicompost and could produce IAA in Luria-Bertani broth with 5 mM L-tryptophan (Jayakumar & Natarajan 2013, p. 39). There was no report on isolating IAA producing *Arthrobacter* spp. from rhizospheres of tropical timber trees. Therefore, isolation of IAA producing *Arthrobacter humicola* from the rhizospheres of *N. cadamba* could be an interesting finding.

3.3.2 Evaluating the efficiencies of microorganisms in plant macronutrients and hormone production

3.3.2.1 Ammonia production by nitrogen fixing microorganisms

The ammonia production of each strain was quantitatively measured using phenate method. The detection process involved the reaction of ammonia with phenol and hypochlorite to form blue indophenol compound in alkaline solution (Park, Oh & Ahn 2009, p. 2032). The formation of indophenol was detected at 640 nm (American Public Health Association, American Water works Association & Waste Environment Federation 2005, p. 4–114). The concentrations of ammonia were determined by referring absorbance reading of each strain to the linear curve of ammonia standards.

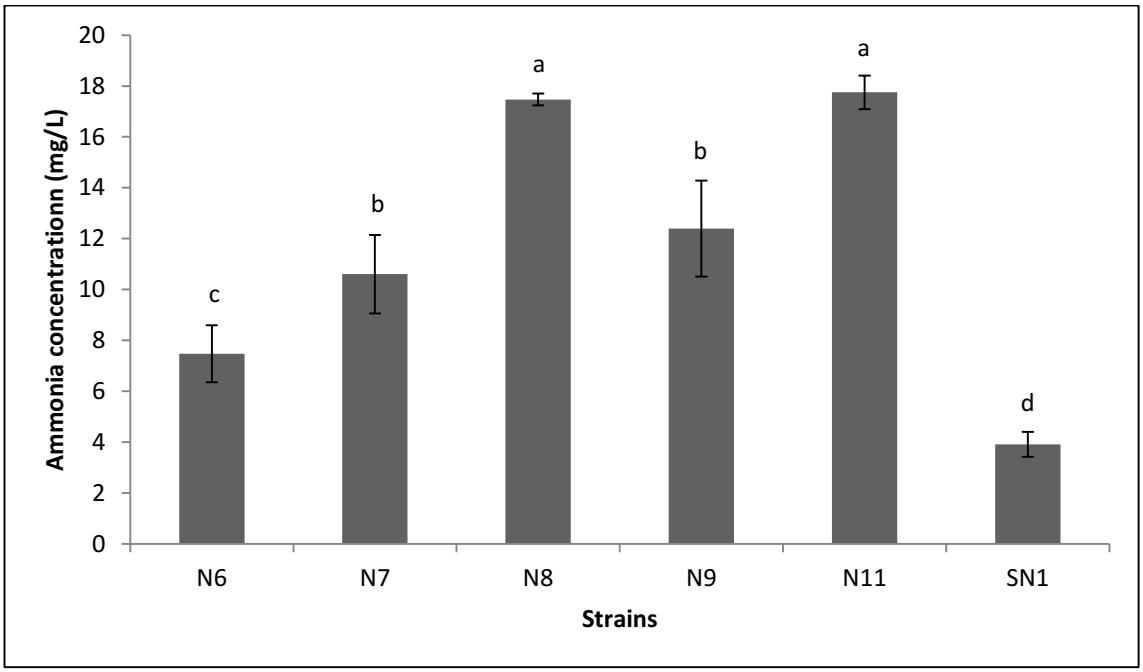


Figure 21 The concentrations of ammonia produced by nitrogen fixing microorganisms after three-day incubation at 30°C. The results are expressed as mean ± SE (n = 3). The value bars with the same letters are not significantly different from each other ($p \leq 0.05$) according to Tukey HSD test. Strain SN1 (*Azotobacter chroococcum* NBRC 102613) was served as a control strain.

There were a total of five isolated strains and one control strain inoculated to Jensen's broth. The five isolated strains were *Methylobacterium* N6, *Pseudomonas* N7, *Pseudomonas* N8, *Stenotrophomonas* N9 and *Streptomyces* N11. The control strain was *Azotobacter chroococcum* NBRC 102613, a known nitrogen fixing strain (Darbyshire 1972, p. 359; EL-Shanshoury 1995, p. 120). After three-day incubation, *Pseudomonas* N8 and *Streptomyces* N11 strains produced the two highest concentrations of ammonia at 17.47 mg/L and 17.75 mg/L respectively. *Stenotrophomonas* N9 strain had the third highest ammonia production at 12.39 mg/L, and followed by *Pseudomonas* N7 and *Methylobacterium* N6 with 10.60 mg/L and 7.47 mg/L respectively. *Azotobacter chroococcum* NBRC 102613 released the lowest concentration of ammonia at 3.91 mg/L. All the isolated strains thus produced significant concentrations of ammonia to the control strain ($p \leq 0.05$) (Figure 21). *Pseudomonas* N8 and *Streptomyces* N11 strains were the two leading strains which showed the highest levels of ammonia were selected for strain compatibility test and pot experiment in Chapter 4.

The production of ammonia by nitrogen fixing microorganisms was reported in some previous studies. In Indonesia, the isolated nitrogen fixing strains released ammonia in the concentration range of 4.60-19.00 mg/L (Hartono et al. 2016, p. 11737). Another study in Pakistan showed the produced ammonia fell within the concentration range of 11.00 mg/L to 80.00 mg/L (Ahmad et al. 2013, p. 57). The ammonia production by all the five isolated nitrogen fixing strains were comparable to these previous studies.

3.3.2.2 Phosphate solubilisation by phosphate solubilising microorganisms

The concentrations of soluble phosphate were determined by vanadomolybdophosphoric acid method. The soluble phosphate reacts with ammonium (VII) molybdate to form molybdophosphoric acid under acidic conditions. Then, molybdophosphoric acid reacts with vanadium to form yellow vanadomolybdophosphoric acid which can be detected at 420 nm (American Public Health Association, American Water works Association & Waste Environment Federation 2005, pp. 4–151). The absorbance readings of samples were referred to the linear curve of standard phosphate solutions.

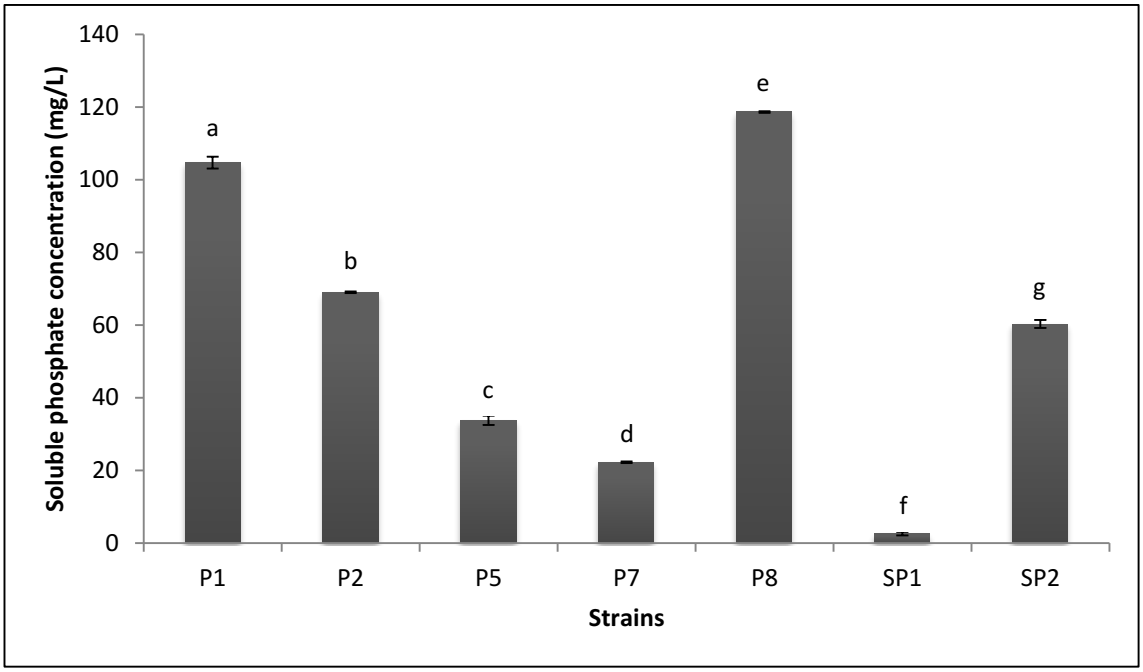


Figure 22 The concentrations of soluble phosphate produced by phosphate solubilising microorganisms after three-day incubation at 30°C. The results are expressed as mean ± SE (n = 3). The value bars with the same letters are not significantly different from each other (p ≤ 0.05) according to Tukey HSD test. Strain SP1 (*Paenarthrobacter ureafaciens* NBRC 12140) and SP2 (*Aspergillus niger* NBRC 33023) were served as control strains.

The studied strains consisted of *Streptacidiphilus* P1, *Lysinibacillus* P2, *Metarhizium* P5, *Metarhizium* P7, *Serratia* P8, *Paenarthrobacter ureafaciens* NBRC 12140 and *Aspergillus niger* NBRC 33023. The last two strains were control strains and their phosphate solubilising ability had been reported (Chen et al. 2006, p. 38; Fitriyanti, Mubarik & Tjahjoleksono 2017, p. 2; Sharma et al. 2013, p. 5; Mendes et al. 2015, p. 931). They were cultured in Pikovskaya's broth containing insoluble phosphate source and incubated at 30°C for 3 days. From the result shown in Figure 22, *Serratia* P8 strain showed the highest soluble phosphate value at 118.69 mg/L. *Streptacidiphilus* P1 and *Lysinibacillus* P2 strains released the second and third highest concentration of soluble phosphate at 104.76 mg/L and 69.05 mg/L respectively. Lastly, *Metarhizium* P5 and *Metarhizium* P7 had lower soluble phosphate value at 33.81 mg/L and 22.26 mg/L respectively. Meanwhile, *Aspergillus niger* NBRC 33023 showed only moderate value of 60.31 mg/L. The other control strain *Paenarthrobacter ureafaciens* NBRC 12140

nonetheless produced the lowest concentrations of soluble phosphate at 2.63 mg/L. Based on the results, *Streptacidiphilus* P1, *Lysinibacillus* P2 and *Serratia* P8 strain showed significant results to the two control strains ($p \leq 0.05$). *Serratia* P8 strain was the best strain in phosphate solubilisation which showed significant result in comparison to all the other strains and the two control strains ($p \leq 0.05$). Therefore, *Serratia* P8 strain was chosen for further study in Chapter 4.

A couple of phosphate solubilising microorganism studies in India showed similar phosphate solubilisation activities. In the first study, isolates from alkaline soils released 8.00 mg/L to 35.00 mg/L soluble phosphate in Pikovskaya's broth (Nautiyal 1999, p. 268). Another study conducted on tomato rhizospheres showed the concentrations of soluble phosphate in Pikovskaya's broth fell within the range of 31.00-143.00 mg/L (Hariprasad & Niranjana 2009, p. 19). The phosphate solubilising isolates in this study showed good phosphate solubilising activities as the results fell within these reported concentration ranges.

3.3.2.3 Potassium solubilisation by potassium solubilising microorganisms

The levels of soluble potassium were measured using atomic absorption spectrophotometer at 766.5 nm. This method measures the amount of light photon energy absorbed by the potassium atoms. The light source emits a specific wavelength that can be absorbed by potassium atoms. Then, the potassium atoms make transitions to become excited atoms. The detector measures the wavelength passing through potassium atoms in the sample. The obtained absorbance readings were referred to the linear curve of standard potassium solutions and the concentrations of potassium in the samples were determined (García & Báez 2012, p. 1; American Public Health Association, American Water works Association & Waste Environment Federation 2005, pp. 3–88-3-89).

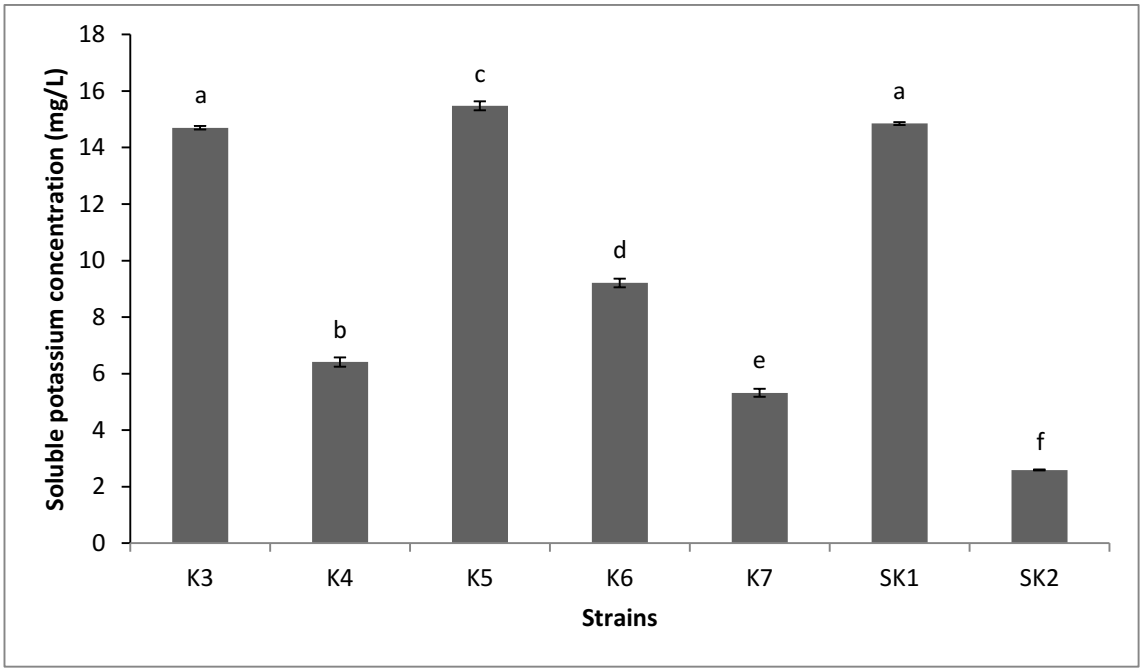


Figure 23 The concentrations of soluble potassium produced by potassium solubilising microorganisms after seven-day incubation at 28°C. The results are expressed as mean ± SE (n = 3). The value bars with same letters are not significantly different from each other ($p \leq 0.05$) according to Tukey HSD test. SK1 (*Enterobacter hormaechei* NBRC 105718) and SK2 (*Aspergillus terreus* NBRC 33026) were served as control strains.

The five isolated potassium solubilising strains were *Lysinibacillus* K3, *Phanerochaete* K4, *Bacillus* K5, *Lysinibacillus* K6 and *Penicillium* K7. There were two standard cultures, *Enterobacter hormaechei* NBRC 105718 and *Aspergillus terreus* NBRC 33026, served as control strains which were capable of releasing soluble potassium (Prajapati, Sharma & Modi 2013, p. 182; Meena et al. 2016, pp.10-11). The isolated strains and control strains were inoculated to modified Aleksandrov's broth which contained insoluble potassium source for the evaluation of potassium solubilising activities. *Bacillus* K5 strain had the highest concentration of released potassium at 15.48 mg/L and followed by *Lysinibacillus* K3 strain with 14.70 mg/L (Figure 23). *Phanerochaete* K4, *Lysinibacillus* K6 and *Penicillium* K7 strains produced lower concentrations of soluble potassium at 6.41 mg/L, 9.21 mg/L and 5.32 mg/L respectively. The control strain, *Enterobacter hormaechei* NBRC 105718 performed better than all the isolated strains except *Bacillus* K5. It showed the concentration of soluble potassium at 14.85 mg/L (Figure 23). The

other control strain *Aspergillus terreus* NBRC 33026 showed the lowest concentration of soluble potassium at 2.59 mg/L. *Bacillus* K5 strain was the best potassium solubilising strain which showed significant result to all the strains ($p \leq 0.05$). Thus, it was selected for further evaluating studies.

The potassium solubilising microorganisms with similar potassium solubilising activities were reported in the studies from Indonesia and India. The potassium solubilising isolates from Indonesia sugarcane rhizospheres showed the soluble potassium fell within the concentration range of 0.17-18.17 mg/L (Setiawati & Mutmainnah 2016, p. 113). The study in India stated the isolated potassium solubilising strains from crop rhizospheres produced soluble potassium in the concentration range of 2.86-13.31 mg/L (Meena et al. 2015, p. 346). For this study, the five isolated potassium solubilising strains showed results that were consistent to these previous studies.

3.3.2.4 IAA production by IAA producing microorganisms

The IAA concentrations were quantified using Salkowski's reaction method. The detection method was based on the reaction of IAA with Fe^{3+} ions. The reagent contains $FeCl_3$ and H_2SO_4. IAA can form chelate with Fe^{3+} ions at acidic pH. The Fe^{3+} ions slowly oxidise IAA at pH 2.6 in the solution. Then, the oxidation compound of IAA can be detected using visible wavelengths. Thus, IAA concentration is measured by the formation of its coloured oxidation compound at very acidic pH in the presence of Fe^{3+} ions (Mayer 1958, p. 1670). The coloured oxidation compound was detected at 525 nm and the IAA concentrations were determined based on the linear curve of standard IAA solutions (Gordon & Weber 1951, p. 194).

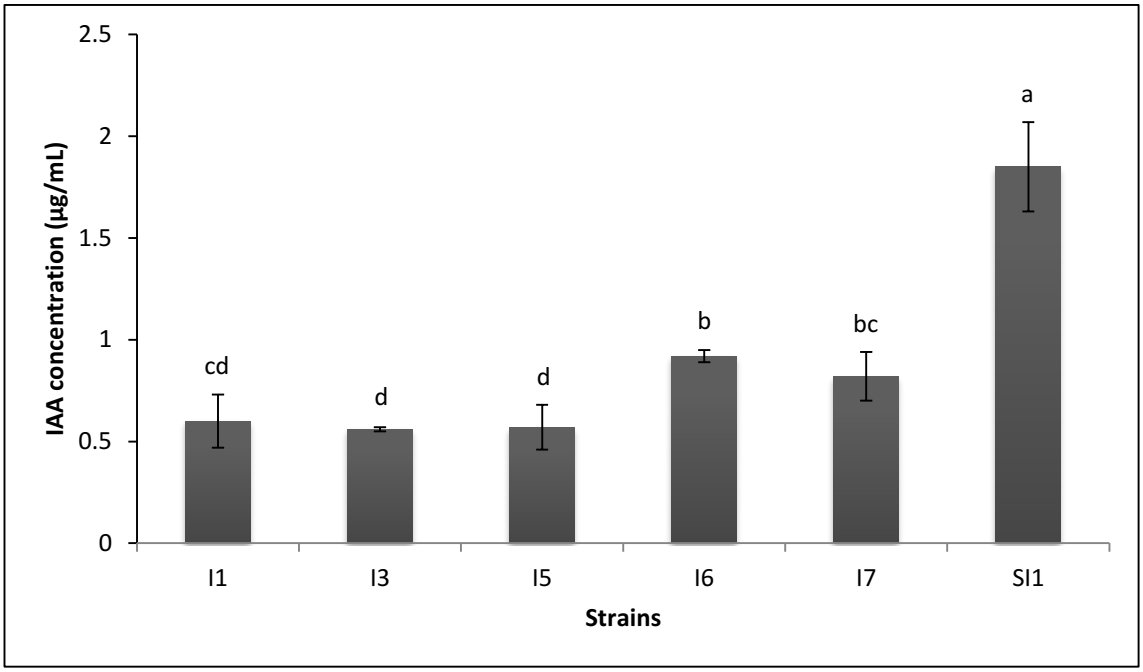


Figure 24 The concentrations of IAA produced by IAA producing microorganisms after four-day incubation at 28°C. The results are expressed as mean ± SE (n = 3). The value bars with the same letters are not significantly different from each other ($p \leq 0.05$) according to Tukey HSD test. Strain SI1 (*Pseudomonas putida* NBRC 14164) was served as a control strain.

There were five isolated strains and one control strain inoculated to tryptophan broth. The isolated strains were *Bacillus* I1, *Lysinibacillus* I3, *Lysinibacillus* I5, *Bacillus* I6 and *Arthrobacter* I7. The control strain was *Pseudomonas putida* NBRC 14164 which was a known IAA producing strain (Leveau & Lindow 2005, p. 2365; Sgroy et al. 2009, p. 375). After four-day incubation, *Bacillus* I6 and *Arthrobacter* I7 strains produced the two highest concentrations of IAA at 0.92 µg/mL and 0.82 µg/mL respectively among the isolated strains. Then, *Bacillus* I1, *Lysinibacillus* I5 and *Lysinibacillus* I3 demonstrated IAA biosynthesis levels at 0.60 µg/mL, 0.57 µg/mL and 0.56 µg/mL respectively (Figure 24). The control strain *Pseudomonas putida* NBRC 14164 showed the IAA concentration at 1.85 µg/mL which was significantly higher than all the isolated strains ($p \leq 0.05$).

A study of ginseng plant in Korea showed the IAA producing isolates produced IAA in the concentration range of 0.31-13.93 µg/mL (Vendan et al. 2010, p. 564). Another IAA producing microorganism study in Argentina stated the released IAA concentrations fell

within the range of 0.5-2.2 µg/mL (Sgroy et al. 2009, p. 375). The IAA biosynthesis levels of five isolated IAA producing strains would meet the overall reported range in the previous studies. *Bacillus* I6 and *Arthrobacter* I7 were selected for further study because they were the two best strains.

3.4 Summary

There were sixteen bacterial strains selected for genetic identification. Based on genetic identification, these strains could be classified into classes of Alphaproteobacteria, Gammaproteobacteria, Actinobacteria and Bacilli. Class Alphaproteobacteria had only one strain, a nitrogen fixing *Methylobacterium* N6 strain. Class Gammaproteobacteria contained three nitrogen fixing strains and one phosphate solubilising strain. The nitrogen fixing strains were *Pseudomonas* N7, *Pseudomonas* N8 and *Stenotrophomonas* N9 strains. *Serratia* P8 strain was the only phosphate solubilising strain under this class. Class Actinobacteria had one strain each for nitrogen fixation, phosphate solubilisation and IAA biosynthesis, namely *Streptomyces* N11, *Streptacidiphilus* P1 and *Arthrobacter humicola* I7 strains respectively. Class Bacilli had the most numerous strains in this study with five *Lysinibacillus* strains and three *Bacillus* strains. Among the five *Lysinibacillus* strains, there were one phosphate solubilising strain, namely *Lysinibacillus* P2 strain, two potassium solubilising strains, namely *Lysinibacillus* K3 and *Lysinibacillus fusiformis* K6 strains, and two IAA producing strains, namely *Lysinibacillus sphaericus* I3 and *Lysinibacillus* I5 strains. Among the three *Bacillus* strains, there were one potassium solubilising strain, namely *Bacillus* K5 strain, and two IAA producing strains, namely *Bacillus* I1 and *Bacillus cereus* I6 strains. The isolation of plant growth enhancing bacteria from *N. cadamba* was an interesting finding because most previous studies focused on agricultural crops, coniferous trees and *Eucalyptus* trees.

As for fungi, there were two phosphate solubilising strains and two potassium solubilising strains selected in this study for genetic identification based on ITS genetic marker. The two phosphate solubilising fungal strains, P5 and P7, were both identified as *Metarhizium anisopliae* which belongs to class Sordariomycetes. *Metarhizium anisopliae* is a known biocontrol agent against pest insect in plant rhizospheres but its phosphate solubilisation trait has rarely been reported. Hence, this study was probably the first report on phosphate

solubilising *Metarhizium anisopliae* strains. The two potassium solubilising strains, K4 and K7, were identified as *Phanerochaete chrysosporium* and *Penicillium rolfsii* respectively. *Phanerochaete chrysosporium* is a model white rot fungal strain that belongs to class Agaricomycetes but their potassium solubilisation trait had not been mentioned in previous studies. As such, it would be interesting to note *Phanerochaete chrysosporium* could also carry out potassium solubilisation activity, as demonstrated by strain K4 isolated in this study. *Penicillium rolfsii* belongs to class Eurotiomycetes and its potassium solubilisation trait was reported earlier in a study on conifer tree Douglas-fir.

For nitrogen fixation, all the isolated strains showed significantly higher ammonia concentrations than the control strain, *Azotobacter chroococcum* NBRC 102613. *Pseudomonas* N8 and *Streptomyces* N11 strains were found to produce the two highest concentrations of ammonia in the Jensen's broth. Except for *Metarhizium* P5 and *Metarhizium* P7 strains, the isolated phosphate solubilising strains were able to release significantly higher soluble phosphate concentrations than the control strains, *Paenarthrobacter ureafaciens* NBRC 12140 and *Aspergillus niger* NBRC 33023. *Serratia* P8 strain showed the highest level of soluble phosphate in the Pikovskaya's broth among the isolated strains. On the other hand, *Bacillus* K5 strain was the only isolated potassium solubilising strain to show significant potassium solubilisation result to control strains, *Enterobacter hormaechei* NBRC 105718 and *Aspergillus terreus* NBRC 33026. It could release the highest concentration of soluble potassium in modified Aleksandrov's broth. For IAA producing strains, all of them produced significantly lower levels of IAA than control strain, *Pseudomonas putida* NBRC 14164. Nonetheless, their results were comparable to the previous studies of ginseng plant and shrub. *Bacillus* I6 and *Arthrobacter* I7 strains produced the two highest concentrations of IAA among the five isolated strains.

Based on the overall results, *Pseudomonas* N8, *Streptomyces* N11, *Serratia* P8, *Bacillus* K5, *Bacillus* I6 and *Arthrobacter* I7 strains were selected for further studies to formulate consortia that could be used as biofertiliser for *N. cadamba*.

CHAPTER 4:

EVALUATING THE PLANT GROWTH ENHANCING EFFECT OF MICROBIAL CONSORTIA ON *NEOLAMACRKIA CADAMBA* SEEDLINGSVIA SMALL SCALE POT EXPERIMENT

4.1 Introduction

The current practice for planting of *N. cadamba* or other timber tree seedlings in the forest plantation in Sarawak would involve application of chemical fertilisers to replenish the soil nutrient. According to the Sarawak Forestry Corporation practice, chemical fertilisers would be applied in the planting hole or on the soil surface immediately after planting of each tree. The released nutrients are available for plant absorption to maintain the health of the plant (Chen 2006, p. 2). Nevertheless, there are a few downsides on the application of chemical fertilisers. For instance, the nitrogen element is in the form of nitrate ions which are good for plant absorption albeit they are hardly retained by soil particles, as such easily leached into underground therefore regarded as a form of water pollution (Food and Agriculture Organization of the United Nations 1972, p. 12). Likewise, the phosphorus and potassium elements are also required to be in the ionic forms for plant absorption, but the ionic forms are easily fixed by clay and humus particles in the soil thus rendered them unavailable for plant absorption (Food and Agriculture Organization of the United Nations 1972, pp. 10-11). As a result, the plant normally utilises only a small portion of the total phosphorus and potassium elements in the chemical fertilisers. Based on these considerations, the application of chemical fertilisers could be regarded inefficient and not environmental friendly. In addition, chemical fertilisers are produced from non-renewable resources that can be a challenge in the future. Therefore, an efficient alternative technique is required for future usage and environment protection.

Biofertilisers can be considered as environmental-friendly alternatives for sustainable silviculture of *N. cadamba*. Biofertilisers are generally referring to a substance that contains living microorganisms which may promote growths of plants. Such plant growth enhancing microorganisms can promote plant's growth through their biological activities, such as mobilisation of unavailable nutrients, or provision of plant growth enhancers such as hormones. As each microorganism has its respective specific biological activities, they would be ideally mixed together as a consortium for formulation of biofertiliser. Biofertilisers are also often formulated to deliver the microorganisms either in liquid formulations or carrier-based formulations (Pindi & Satyanarayana 2012, pp. 1-2). This study utilised liquid formulation to sustain the selected nitrogen fixing strains, phosphate solubilising strains, potassium solubilising strains, and IAA producing strains for a pot

trial using *N. cadamba* seedlings. The nitrogen fixing strain would fix the atmospheric nitrogen to plant available nitrogen compounds, while phosphate solubilising strain and potassium solubilising strain would dissolve the phosphate and potassium ions from soil particles. Besides that, IAA producing strain produces IAA hormone, a natural plant growth enhancer that could be transported to plant root cells and induce their elongation and growth. In addition, the liquid formulation can be combined to reduced level of chemical fertiliser as an integrated application. The integrated application would reduce the usage of chemical fertiliser without compromising on the plant growth (Chen 2006, p. 3).

In chapter 4, the compatibility of selected strains, consortial formulations and impact of consortia to the *N. cadamba* seedlings would be carried out. As the interaction among different species of microorganisms could be antagonistic, a compatibility test need to be carried out using agar plates to rule out antagonism among the selected strains. The compatible strains were further evaluated for their growth characteristics including specific growth rate, doubling time, absorbance readings and colony forming units. These would ensure that each strain would be in the concentration range of 10^7-10^8 cfu/mL when the selected strains were mixed into consortia.

The selected strains were then used for formulations of two consortia: Consortium A with nitrogen fixing, phosphate and potassium solubilising strains, while Consortium B with all the strains used in Consortium A and IAA producing strain. These two consortia would be added or integrated to half regime of chemical fertilisers and compared to full regime chemical fertiliser. These treatments were applied on 6-month *N. cadamba* seedlings for six-month pot experiment to study their plant growth enhancing effects.

4.2 Materials and Methodology

4.2.1 Compatibility test for high efficient strains

As microorganisms are known to have interactions among each other either mutualistic, synergistic or antagonistic, it is important to run a compatibility test to confirm that the selected strains are not antagonistic to each other. Based on Prasad & Babu (2017, p. 1028), a compatibility testing method was developed to assess the compatibility among the six selected strains (Table 19). These strains were earlier selected for optimal biological activities as discussed in Chapter 3, and it is important to ascertain they would be also compatible to each other so that they could be used as microbial consortia. Each strain was inoculated into 10 mL nutrient broth and incubated at 28°C, 150 rpm for overnight or 3 days. The broth cultures were streaked on nutrient agar plates in the method shown in Figure 25. A strain from each trait was streaked in the middle and treated as test strain. Only one strain from each trait was selected in the compatibility tests and the tests were not carried out to check the interactions between the strains from the same trait, for example *Pseudomonas* N8 and *Streptomyces* N11 or *Bacillus* I6 and *Arthrobacter* I7. The agar plates were incubated at 35°C for 7 days. The compatibility of each strain was checked by direct observation.

Table 20 The selected strains for compatibility test.

Strain	Trait
Pseudomonas N8	Nitrogen fixation
Streptomyces N11	Nitrogen fixation
Serratia P8	Phosphate solubilisation
Bacillus K5	Potassium solubilisation
Bacillus I6	IAA biosynthesis
Arthrobacter I7	IAA biosynthesis

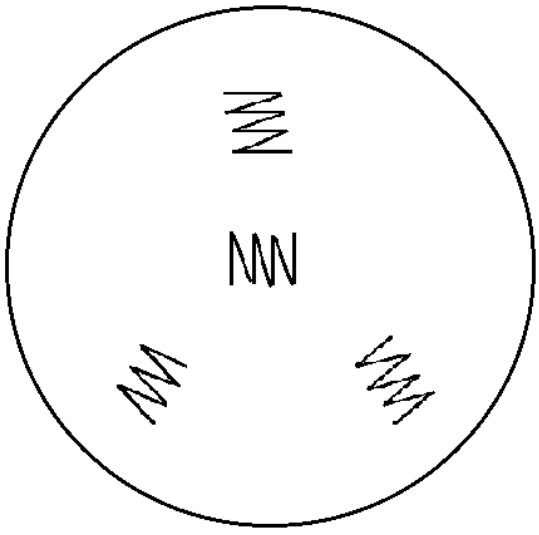


Figure 25 The streaking method carried out in compatibility test with one strain from each trait.

4.2.2 Growth rate and total plate count of selected strains

After undergoing compatibility test, each selected strain was inoculated into 10 mL of nutrient broth and incubated at 28°C, 150 rpm for overnight or 3 days. Then, the broth culture was added to a 250 mL baffled flask (Duran, Germany) with 90 mL nutrient broth. The 100 mL broth cultures were incubated at 28°C, 150 rpm. The growth kinetic information of each selected strain was measured using optical density measurement and total plate count.

For optical density measurement, the wavelength used was 600 nm and the blank solution was uninoculated nutrient broth. The first absorbance reading was taken after the 10 mL broth culture was mixed into 90 mL nutrient broth. There was 1 mL of upscaled broth culture added to a plastic cuvette and the optical density was measured using a spectrophotometer (Thermo Scientific Genesys™ 20, Waltham, MA USA). The procedures remained for the following every hour sampling interval up to the eighth hour sampling. The final optical density measurement was carried out after 24 or 72-hour incubation. The specific growth rate and doubling time of each strain were determined using absorbance readings and the following formulae:

$$\mu = \frac{\ln \frac{OD_{600T2}}{OD_{600T1}}}{T2 - T1}$$

$$T_d = \frac{\ln 2}{\mu}$$

μ: Specific growth rate
ln: Natural log (log e)
OD_{600T1}: Initial optical density measurement at 600 nm
OD_{600T2}: Final optical density measurement at 600 nm
T_1: Initial time
T_2: Final time
T_d: Doubling time

For total plate count, it followed the sampling timeline of optical density measurement up to the eighth hour sampling. There was 1 mL of upscaled broth culture diluted in 9 mL of sterile distilled water. The dilution process was conducted until 10^{-7}. The dilutions of 10^{-5} to 10^{-7} were selected for total plate count. A micropipette (Eppendorf, Germany) was

used to transfer 100 µL of diluted broth culture to nutrient agar and spread plate was performed. The agar plates were incubated at 35°C for overnight or 3 days. The workflow remained for the following every hour sampling. After the incubation period, the colonies were counted and the concentrations of broth cultures were calculated by the following formula:

CFU / mL = (Number of colonies per agar plate × 10) / Dilution factor

4.2.3 Preparation of microbial consortia from the selected strains

Each selected strain was inoculated to 25 mL of nutrient broth and incubated at 28°C, 150 rpm for overnight or 3 days. Then, the 25 mL broth culture was added to a 250 mL baffled flask with 225 mL nutrient broth for scale up production. The 250 mL broth cultures were incubated at 28°C, 150 rpm for overnight or 3 days. There were two consortial formulations created for pot experiments. The first formulation was named as Consortium A which included *Streptomyces* N11, *Serratia* P8 and *Bacillus* K5 strains, whereas the second formulation was named as Consortium B which included all the three strains in consortium A and *Bacillus* I6 strain. The microbial consortium for each *N. cadamba* seedling was prepared by adding 4 mL of each strain to a 250 mL beaker (Duran, Germany) and topping up to a volume of 200 mL with sterile distilled water.

4.2.4 Planting media preparation

4.2.4.1 *N. cadamba* seed germination media preparation

The germination media was prepared by mixing sand, compost and chemical fertiliser (NPK 15-15-15) in a ratio of 6:12:1 based on Sarawak Forestry Corporation general practices.

4.2.4.2 *N. cadamba* seedling potting media preparation

The potting media was prepared by adding forest topsoil, sand, compost and same chemical fertiliser to a concrete mixer in a ratio of 12:6:6:3 based on Sarawak Forestry Corporation general practices. After mixing, the potting media was filled in the black polythene bags (Width × Length = 15.24 cm × 25.40 cm).

4.2.5 Pot experiment design

4.2.5.1 *N. cadamba* seedlings preparation

The *N. cadamba* seeds [SFTJB(7)/01/02/0115, 00005(6), Germination rate: 94.5%, Seed source: Kuching] were provided by Sarawak Forestry Corporation (SFC) Seed Bank (Figure 26). The preparation procedures of seedlings were following the practice in SFC nursery. They were sown on germination media by SFC nursery officer and watered using water sprinkler system (Figure 27). The germination period took around four to six weeks in the greenhouse. When the height of seedlings reached around 1 cm, they were transplanted into the potting media for raising individual seedling (Figure 28). After transplanting, the seedlings were raised in the nursery shed and watered three times every day using water sprinkler system. They took around one to two months for raising under the shed. Then, they were moved to open area for hardening under the exposure of sunlight. During hardening period, they were watered three times every day with tap water and fertilised with little amount of chemical fertiliser (NPK 15-15-15) every month according to the SFC's nursery practice.

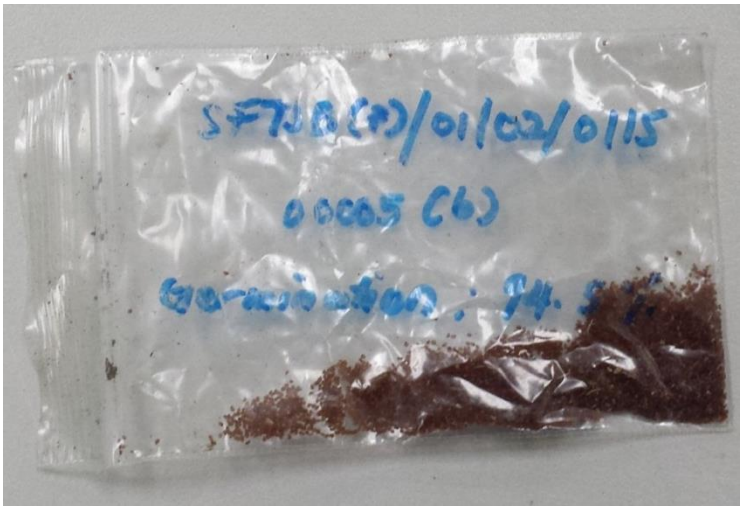


Figure 26 The *N. cadamba* seeds given by SFC Seed Bank.

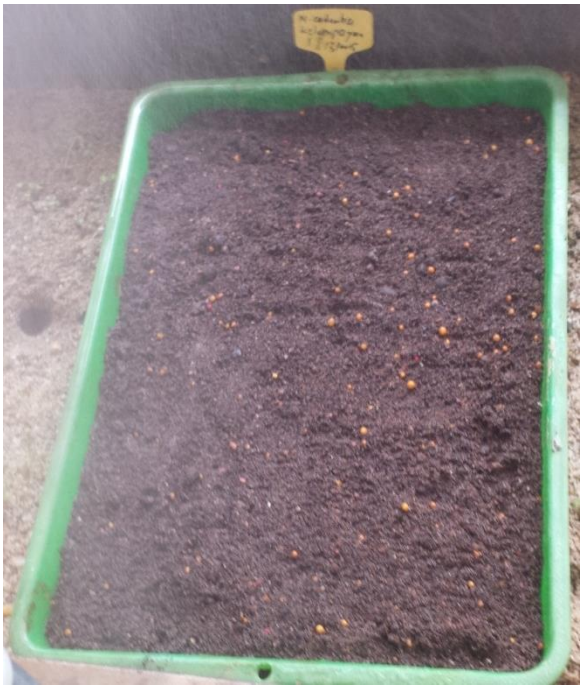


Figure 27 The *N. cadamba* seeds were moistened by water sprinkler system.

Figure 28 The *N. cadamba* seedling was planted on the potting media.

4.2.5.2 Pot experiment set up

The pot experiment was set up with six-month-old *N. cadamba* seedlings (Figure 29). Instead of pot, these seedlings were transplanted to large polythene bags (Width × Length = 30.48 cm × 35.56 cm). The fresh forest topsoil was provided by SFC as planting medium. The topsoil samples were taken and sent to SP Lab (Sarawak Plantation Services Sdn. Bhd., Malaysia) for physicochemical analyses (Table 21). These large polythene bags were filled with fresh forest topsoil at around three-quarter full before transplanting and a layer of fresh forest topsoil was added after applying treatments. There were six treatments, each assigned to 10 seedlings, the duration of the experiment was six months (Table 22). The chemical fertiliser given was the same chemical fertiliser (NPK 15-15-15) given during the hardening period. The amount of chemical fertiliser given to each seedling in positive control treatment was 15 g based on the recommendation of Krisnawati, Kallio & Kanninen (2011, p. 5). The chemical fertiliser was applied around the seedling in a circle. For consortial treatments, each microbial consortium was applied to the seedlings by pouring around the seedling in a circle. The treated seedlings were placed in the nursery shed and watered three times every day with water sprinkler system.

Figure 29 The six-month-old seedling used in pot experiment.

Table 21 The physicochemical properties of fresh forest topsoil.

Parameter	**Result**
Soil texture (Sand-Silt-Clay)	Clay (41.6-17.7-40.7)
pH	4.8
Conductivity (μS)	58.4
Total nitrogen (%)	0.07
Total phosphorus (ppm)	717
Total potassium (ppm)	312

Table 22 Treatments given to six-month-old *N. cadamba* seedlings.

Treatment	Description
Negative Control	Neither microbial consortia nor chemical fertiliser was given.
Positive Control	Full regime chemical fertiliser was given.
Consortium A	The consortium included nitrogen fixing strain, phosphate solubilising strain and potassium solubilising strain.
Consortium B	The consortium included nitrogen fixing strain, phosphate solubilising strain, potassium solubilising strain and IAA producing strain.
Consortium A-chemical fertiliser	Consortium A and half regime chemical fertiliser were given.
Consortium B-chemical fertiliser	Consortium B and half regime chemical fertiliser were given.

4.2.5.3 Pot experiment seedlings growth data collection

The growth data of seedlings were collected in the beginning and end of pot experiment. There were non-destructive and destructive measuring methods. The non-destructive methods included height measurements, root collar diameter measurements and leaf area. The height and root collar diameter measurements were conducted in both beginning and end of pot experiment. The height and root collar diameter were measured using measuring tape and electronic digital calliper (Ted Pella Inc., California, USA). Leaf area was measured only at the end of experiment. All the leaves from every seedling were plotted on the 1 cm by 1 cm grid graph papers and the areas were measured based on the occupied square areas. Besides that, the destructive methods like shoot and root dry weight measurements were also carried out only at the end of experiment. Towards the sixth month, the seedlings were uprooted and the roots were rinsed with tap water. The uprooted seedlings were air-dried overnight in the laboratory before oven-drying them. The seedlings were separated into shoots and roots using a garden cutter. The shoots and roots were wrapped in old newspapers and oven-dried at 60°C for 72 hours. The dried shoots and roots were weighed afterwards.

4.2.6 Data analysis

The plant data were analysed to determine the effectiveness of the treatments. The data were processed using ANOVA with Tukey HSD post-hoc test in SPSS ver. 23.0 (IBM Corporation, USA). The significant difference was set at $p \leq 0.05$ level for the comparisons in between the treatments.

4.3 Result and Discussion

4.3.1 Compatibility test for selected strains

There were six bacterial strains selected for compatibility test. These strains were selected from high levels of biological activities as discussed in Chapter 3 for nitrogen fixation, phosphate and potassium solubilisation and IAA production respectively. Fungal strains were not selected because they did not show significant better results than these bacterial strains in quantitative assays. The strain compatibility test was carried out to avoid any mutual inhibitory activities which may affect the mixing of these organisms in the consortial formulation. Similar experience was conducted for a groundnut pot experiment using mixed cultures of *Azospirillum brasilense* and *Pseudomonas fluorescens* (Prasad & Babu 2017, p. 1028). The streaking method was reported to be effective in confirming the compatibility of each strain. This method was practical and the results could be assessed by direct observation (Lertcanawanichakul & Sawangnop 2008, p. 168). The inhibitory effects of strains from different traits on test strains were observed and tabulated in Table 23. The inhibited test strains may not show inhibitory effects to strains from different traits.

Table 23 The compatibility test of six plant growth enhancing bacterial strains.

	Test strains					
	N8	N11	P8	K5	I6	I7
N8	N/A	N/A	-	-	+	++
N11	N/A	N/A	-	-	-	+
P8	-	-	N/A	-	-	++
K5	+	-	-	N/A	-	++
I6	+	-	-	-	N/A	N/A
I7	-	-	-	-	N/A	N/A

Inhibition characteristic: - no inhibition; + partial inhibition; ++ strong inhibition; N/A not applicable.

N8: Pseudomonas N8; N11: Streptomyces N11; P8: Serratia P8; K5: Bacillus K5; I6: Bacillus I6; I7: Arthrobacter I7

As shown in Table 23, *Arthrobacter* I7 strain was inhibited by *Pseudomonas* N8, *Streptomyces* N11, *Serratia* P8 and *Bacillus* K5 strains. As such, *Arthrobacter* I7 strain was excluded from consortial formulation and *Bacillus* I6 strain was chosen to represent IAA producing strain in the consortial formulation. On the other hand, *Pseudomonas* N8 strain was partially inhibited by *Bacillus* K5 and *Bacillus* I6 strains. Meanwhile, *Pseudomonas* N8 strain also showed partial inhibition effect on *Bacillus* I6 strain. Thus, *Streptomyces* N11 strain was selected to represent nitrogen fixing strain in consortial

formulation. Since *Serratia* P8 and *Bacillus* K5 strains were the only phosphate solubilising strain and potassium solubilising strain, these strains were automatically assigned to represent their own traits in consortial formulation.

Streptomyces N11, *Serratia* P8, *Bacillus* K5 and *Bacillus* I6 strains did not show inhibition results to each other as shown in Table 23. Therefore, these strains proceeded to their growth rate evaluation and consortial formulation for pot experiment.

4.3.2 Growth rate and total plate count

Streptomyces N11, *Serratia* P8, *Bacillus* K5 and *Bacillus* I6 strains which had passed the compatibility test were further evaluated for their growth characteristics. Each strain was inoculated to nutrient broth and incubated at 28°C, 150 rpm. The growth was measured using a spectrophotometer, which is an indirect growth measurement technique. Total plate count, a direct growth measurement technique, was also conducted. The growth kinetics information of selected strains was presented in Table 24.

Table 24 The growth kinetic information of the compatible strains grown in nutrient broth at 28°C, 150 rpm.

Strain	Specific growth rate, K (hr^{-1})	Doubling time, td (hr)	Absorbance reading in the end of experiment (A, OD_{600})	Colony forming units per millilitre in the end of experiment (CFU/mL)
N11[a]	0.064	10.83	1.363	4.67×10^9
P8[b]	0.431	1.61	1.642	2.80×10^{10}
K5[b]	0.196	3.54	1.316	4.67×10^9
I6[b]	0.416	1.67	1.816	2.23×10^{10}

[a] represents 72-hour incubation, [b] represents 24-hour incubation.

N11: Streptomyces N11; P8: Serratia P8; K5: Bacillus K5; I6: Bacillus I6

The specific growth rate indicates the increment of bacterial cell biomass within a period. The specific growth rates of selected strains were calculated based on absorbance readings from optical density measurements. *Serratia* P8 strain had the highest specific growth rate at 0.431 hr^{-1}, and followed by *Bacillus* I6 and *Bacillus* K5 strains with specific growth rate at 0.416 hr^{-1} and 0.196 hr^{-1} respectively. Lastly, *Streptomyces* N11 strain showed the lowest specific growth rate at 0.064 hr^{-1}.

The doubling time is the time required to increase the bacterial cell population by a factor of 2. The doubling time of each selected strains was calculated based on its specific growth rate. The doubling time of each selected strains is inversely proportional to its

specific growth rate. Basically, it means a bacterial strain with higher specific growth rate requires a shorter time to double its cell population. Therefore, *Serratia* P8 strain had the shortest doubling time at 1.61 hours and *Streptomyces* N11 strain showed the longest doubling time at 10.83 hours among the four selected strains.

After 24-hour incubation, *Serratia* P8 and *Bacillus* I6 strains could reach higher concentrations at 2.80×10^{10} cfu/mL and 2.23×10^{10} cfu/mL respectively. *Bacillus* K5 and *Streptomyces* N11 strains gained the same concentration at 4.67×10^{9} cfu/mL after 24-hour and 72-hour incubations. As *Streptomyces* N11 strain is under class Actinobacteria that generally would require a longer time to reach over 10^{8} cfu/mL (Davis, Joseph & Janssen 2005, p. 827; Shepherd et al. 2010, p. 2). When the selected strains were mixed into consortia in equal proportions, each strain would be in the concentration range of 10^{7}-10^{8} cfu/mL. This concentration range in consortia was suitable for pot experiment set up according to several previous studies. For example, the pot study of pearl millet used consortia of several bacterial strains with 10^{8} cfu/mL each in equal proportion (Garima & Nath 2015, p. 50). In addition, a pot study of saffron also showed the preparation of consortia containing bacterial strains with 10^{7}-10^{8} cfu/mL each (Ambardar &Vakhlu 2013, p. 2273).

4.3.3 Evaluating the plant growth enhancing effect of isolated strains on *N. cadamba* seedlings

4.3.3.1 The stem height increment of *N. cadamba* seedlings in 6-month pot experiment

The height readings were taken starting from the soil surfaces until the shoot tips of seedlings using a measuring tape. The height readings were recorded in centimetre (cm) and the increment readings were calculated by subtracting the initial readings from the final readings. All the increment readings were exported to SPSS for calculating means, standard errors and significant differences. The mean of height increment readings with standard error from each treatment was plotted in Figure 30.

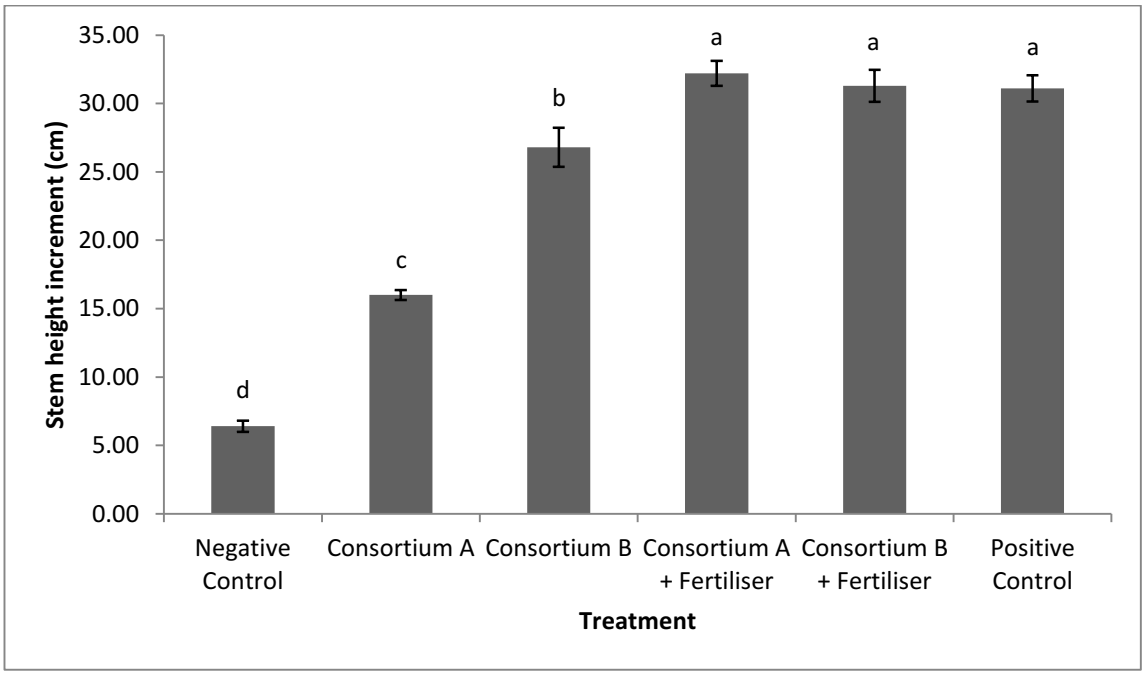


Figure 30 The stem height increment of *N. cadamba* seedlings over 6-month treatments. The results are expressed as mean ± SE (n = 10). The value bars with same letters are not significantly different from each other ($p \leq 0.05$) according to Tukey HSD test.

According to the results shown in Figure 30, both Consortium A and Consortium B treatments could significantly increase the stem height of *N. cadamba* seedlings over the six-month pot experiment if compared to negative control with reading at 6.40 cm ($p \leq 0.05$). Furthermore, microbial consortia studies on seedlings of *N. cadamba* and other tree species like *Azadirachta indica* also reported the significant effects of consortial treatments on their stem height increments (Sreedhar & Mohan 2016, p. 127; Banerjee et al. 2013, p. 589). Consortium B showed the height increment at 26.80 cm which was significantly higher than Consortium A with reading at 16.00 cm ($p \leq 0.05$). In general, plants with better root growth would absorb more nutrients and water, thus stem growth would be enhanced as well (Al-Khafaf et al. 1989, pp. 60-61).

The consortium-chemical fertiliser treatments were on par with chemical fertiliser positive control. The seedlings gained stem height increment at 32.20 cm and 31.30 cm for Consortium A-chemical fertiliser and Consortium B-chemical fertiliser treatments respectively, while the positive control showed stem height increment at 31.10 cm. Moreover, some integrated treatment studies on tropical tree species like *Schizolobium parahyba* and *Elaeis guineensis* also showed that the seedlings treated with integrated treatments could gain similar stem height increments to those treated with full rate chemical fertiliser treatments (Cely et al. 2016, p. 5; Astriani, Mubarik & Tjahjoleksono 2016, p. 152). Since IAA producing strain was known to promote root growth, major portion of plant nutrients from microorganisms and chemical fertiliser could be accumulated in root organ for growth enhancement. Thus, shoot organ like stem would received less plant nutrients from Consortium B-chemical fertiliser and resulting relatively less stem height increment than Consortium A-chemical fertiliser. However, seedlings with better root growth could promote shoot growth as well in long run (Al-Khafaf et al. 1989, pp. 60-61). Given period of study longer than 6 months, it would be interesting to note if seedlings treated with Consortium B-chemical fertiliser would demonstrate significant stem increment in longer term.

In conclusion, both Consortium A and Consortium B treatments could significantly increase the seedlings height if compared to negative control. In addition, they could even replace 50% amount of chemical fertilisers by showing on par height increment readings to the positive control with full rate chemical fertiliser.

4.3.3.2 The root collar diameter increment of *N. cadamba* seedlings in 6-month pot experiment

The root collar diameter increment readings were analysed by ANOVA with Tukey HSD test in SPSS ver. 23.0 The mean of root collar diameter increment readings with standard error from each treatment was plotted in Figure 31.

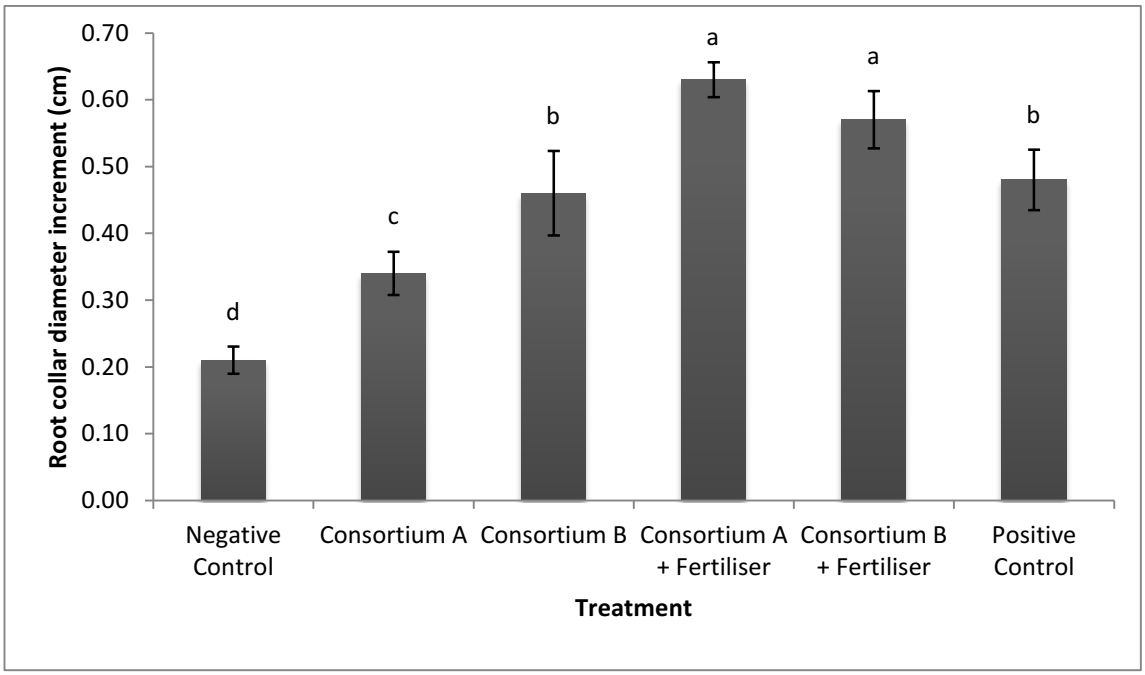


Figure 31 The root collar diameter increment of *N. cadamba* seedlings over 6-month treatments. The results are expressed as mean ± SE (n = 10). The value bars with the same letters are not significantly different from each other ($p \leq 0.05$) according to Tukey HSD test.

Based on the results shown in Figure 31, *N. cadamba* seedlings had significant root collar diameter increments from both Consortium A and Consortium B treatments if compared to negative control ($p \leq 0.05$). The negative control had a reading of 0.21 cm. In addition, pot studies of *N. cadamba* and other woody plants like *Corylus avellana* showed the significant effects of microbial consortia on their root collar diameter growths as well (Sreedhar & Mohan 2016, p. 127; Rostamikia et al. 2016, p. 1474). Consortium B showed the root collar diameter increment at 0.46 cm was also significantly higher than Consortium A with reading at 0.34 cm ($p \leq 0.05$).

When the consortia were integrated with half regime chemical fertiliser, it was surprising to note that both Consortium A-chemical fertiliser and Consortium B-chemical fertiliser, showing collar diameter increment readings at 0.63 cm and 0.57 cm respectively, both were significantly higher than full regime chemical fertiliser positive control with reading at 0.48 cm ($p \leq 0.05$). A few previous studies on tropical tree species, such as *Elaeis guineensis* and *Schizolobium parahyba* seedlings had also reported the significant effects of integrated treatments with half regime chemical fertiliser on the root collar diameter increments if compared to full regime chemical fertiliser treatments (Astriani, Mubarik & Tjahjoleksono 2016, p. 152; Cely et al. 2016, p. 5). Nonetheless, the current results could not differentiate whether Consortium B-chemical fertiliser is significantly better than Consortium A-chemical fertiliser because the two results were not significantly different from each other. As discussed earlier for stem height increment, Consortium B-chemical fertiliser was likely to show a relatively slower shoot growth than Consortium A-chemical fertiliser and resulting lower root collar diameter increment readings. As a result, a longer trial period would be needed to confirm if Consortium B-chemical fertiliser would be the same, or higher in root collar diameter increment if compared to Consortium A-chemical fertiliser.

In short, the microbial consortia and their integrated treatments showed to significantly promote the root collar diameter growth of *N. cadamba* seedlings if compared to negative control and positive control respectively. Meanwhile, both microbial consortia also showed their effectiveness in integration with half regime chemical fertiliser, and their results were actually significantly better than full regime chemical fertiliser positive control.

4.3.3.3 The leaf areas of *N. cadamba* seedlings over 6-month treatments

The leaf area is an important parameter for the studies of fertilisers and plant growth (Pandey & Singh 2011, p. 1). It can affect the carbon fixation rate in photosynthesis that provides carbon sources to plants (Weraduwage et al. 2015, p. 2). The average leaf area readings were taken from every seedling and recorded in centimetre square with two decimal places in centimetre square (cm^2). The average leaf area readings were exported to SPSS ver. 23.0 for ANOVA analysis with Tukey HSD post-hoc test. The means of average leaf area readings with standard errors from each treatment were represented by the column levels with error bars in Figure 32.

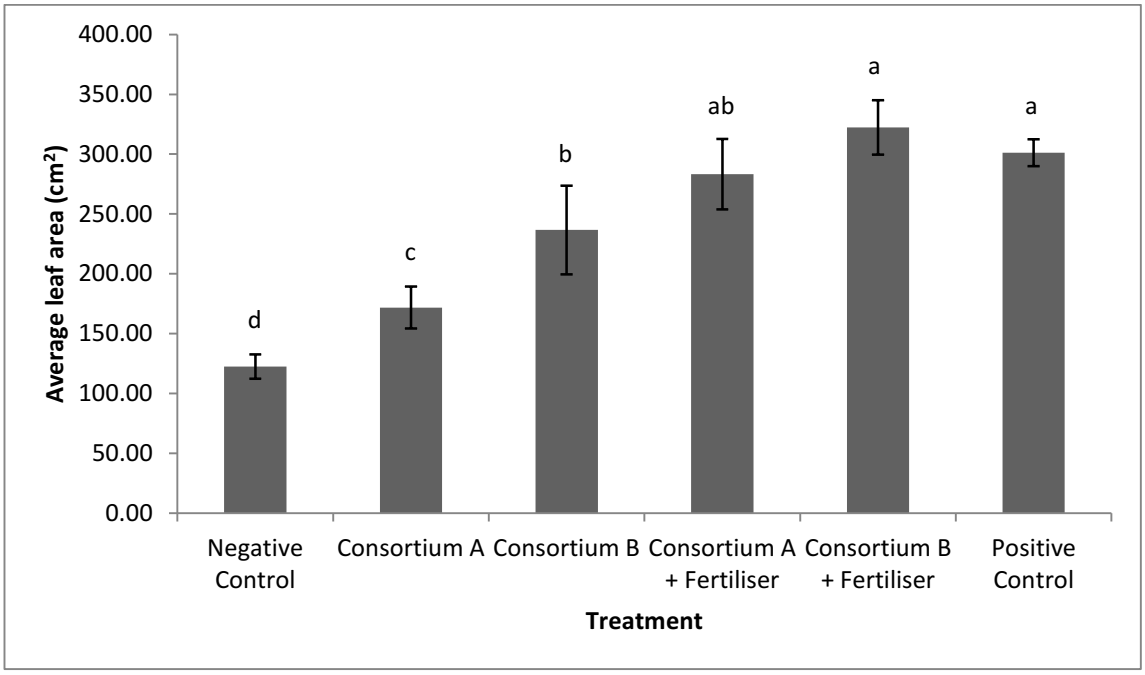


Figure 32 The average leaf area of *N. cadamba* seedlings over 6-month treatments. The results are expressed as mean ± SE (n = 10). The value bars with the same letters are not significantly different from each other ($p \leq 0.05$) according to Tukey HSD test.

According to the results shown in Figure 32, *N. cadamba* seedlings treated with Consortium A and Consortium B had significantly larger average leaf area to the seedlings under negative control ($p \leq 0.05$). The negative control showed average leaf area reading at 122.60 cm^2. Besides that, some microbial consortia studies also showed the significant effects of consortial treatments on leaf areas of woody plants like

Eucalyptus camaldulensis and *Corylus avellana* in pot experiments (Al-Hadad et al. 2014, p. 62; Rostamikia et al. 2016, p. 1474). Consortium B showed average leaf area reading at 236.60 cm^2 which was also significant to Consortium A with reading at 171.80 cm^2 ($p \leq 0.05$). Better root growth was reported to have positive impact on leaf growth as well (Al-Khafaf et al. 1989, pp. 60-61).

The integrated treatments of consortia and half regime chemical fertiliser showed to be on par with positive control in average leaf area readings. *N. cadamba* seedlings yielded average leaf area readings at 283.20 cm^2 and 322.40 cm^2 from Consortium A-chemical fertiliser and Consortium B-chemical fertiliser treatments respectively. The positive control had a reading of 301.20 cm^2. Previous studies on trees like *Paulownia kawakamii* and *Psidium guajava* also reported that seedlings treated with integrated treatments could gain similar leaf area readings to those treated with full regime chemical fertiliser treatments (Farahat et al. 2014, p. 854; Kumar, Jaganath & Guruprasad 2017, p. 316). Similar to the other parameters, the current study also could not confirm if Consortium B-chemical fertiliser treatment would have significantly larger average leaf area readings than Consortium A-chemical fertiliser.

In conclusion, both Consortium A and Consortium B could significantly increase leaf areas if compared to the negative control. In addition, both consortium-chemical fertiliser treatments showed similar plant growth enhancing effects on leaf areas to positive control treatment with full rate chemical fertiliser. As a result, the consortial treatments could replace 50% amount of chemical fertiliser without affecting the leaf growth of *N. cadamba* seedlings.

4.3.3.4 The shoot dry weight of *N. cadamba* seedlings over the 6-month treatments

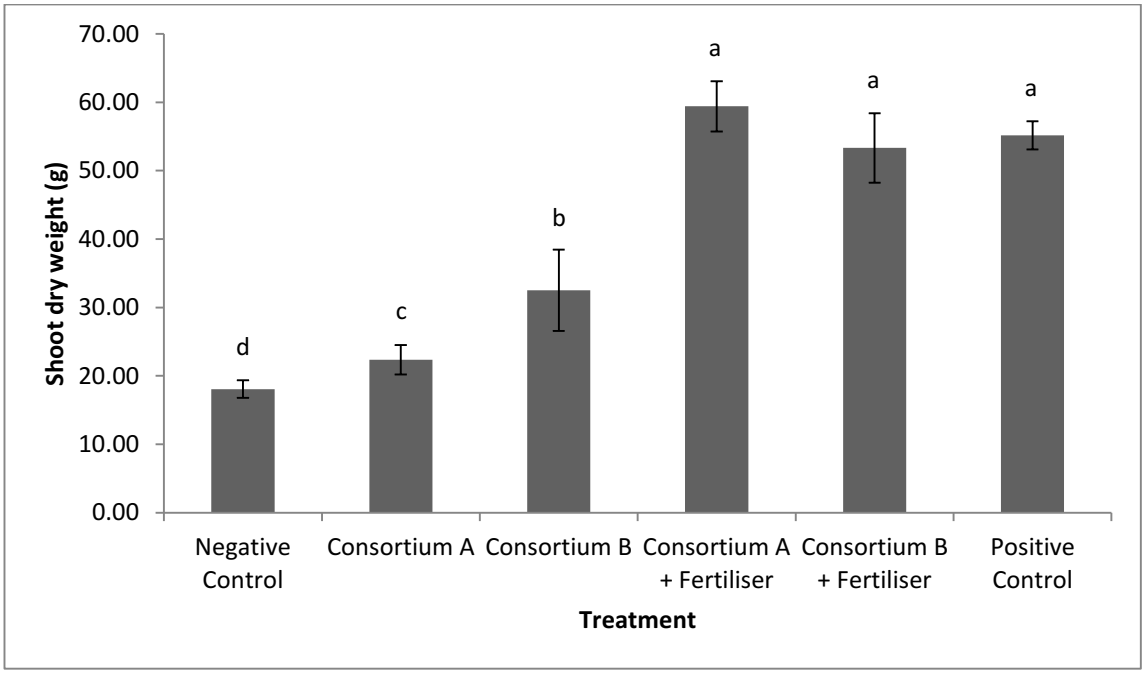


Figure 33 The shoot dry weight of *N. cadamba* seedlings over 6-month pot experiment. The results are expressed as mean ± SE (n = 10). The value bars with the same letters are not significantly different from each other ($p \leq 0.05$) according to Tukey HSD test.

According to the results shown in Figure 33, *N. cadamba* seedlings could gain significant shoot dry weight from Consortium A and Consortium B if compared to negative control with reading at 18.05 g ($p \leq 0.05$). Moreover, in pot experiments of woody plants like *Eucalyptus camaldulensis* and *Jatropha curcas*, there were reports about microbial consortia which could give significant plant growth enhancing effects on their shoot dry weight readings as well (Al-Hadad et al. 2014, p. 62; Jha & Saraf 2012, p. 593). Consortium B showed significant shoot dry weight reading at 32.53 g to Consortium A with reading at 22.35 g ($p \leq 0.05$). The effect of consortial treatments on shoot dry weight might be related to their root dry weight results because plants with better root growth could gain higher shoot dry weight readings in general (Figure 34) (Al-Khafaf et al. 1989, pp. 60-61).

While the consortia were integrated with half regime chemical fertiliser, Consortium A-chemical fertiliser and Consortium B-chemical fertiliser showed on par but not significant shoot dry weight readings at 59.41 g and 53.33 g respectively to positive control with reading at 55.17 g. Furthermore, integrated treatment studies on seedlings of *Paulownia kawakamii* and *Capsicum annuum* also showed the treated seedlings gained similar shoot dry weight readings to those treated with full regime chemical fertiliser treatments (Farahat et al. 2014, p. 855; Thilagar, Bagyaraj & Rao 2016, p. 30). As mentioned in stem height increment, it was possible for seedlings treated with Consortium B-chemical fertiliser to show relatively lower shoot dry weight readings. It would be interesting to study if Consortium B-chemical fertiliser would give significantly higher shoot dry weight readings at a longer pot experiment period.

In a nutshell, both Consortium A and Consortium B could significantly enhance the shoot dry weights of *N. cadamba* seedlings. Meanwhile, both consortium-chemical fertiliser treatments could reduce chemical fertiliser usage by 50% and show on par shoot dry weight readings to full regime chemical fertiliser treatment.

4.3.3.5 The root dry weight of *N. cadamba* seedlings over the 6-month treatments

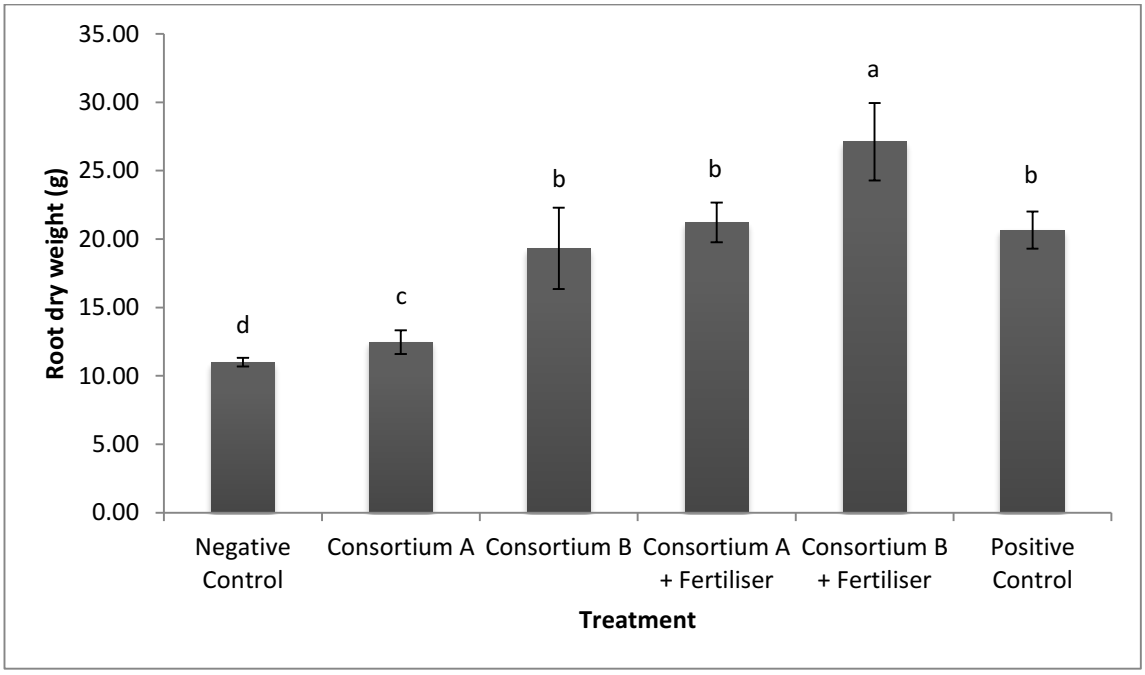


Figure 34 The root dry weight of *N. cadamba* seedlings over 6-month pot experiment. The results are expressed as mean ± SE (n = 10). The value bars with the same letters are not significantly different from each other ($p \leq 0.05$) according to Tukey HSD test.

Based on the results shown in Figure 34, seedlings treated with Consortium B and Consortium B-chemical fertiliser gained significant increase in root dry weight readings, compared to negative control and positive control which had readings of 11.00 g and 20.65 g respectively ($p \leq 0.05$). In addition, both treatments also showed significant root dry weight readings to Consortium A and Consortium A-chemical fertiliser respectively ($p \leq 0.05$). *N. cadamba* seedlings yielded root dry weight readings at 19.33 g and 27.11 g from Consortium B and Consortium B-chemical fertiliser respectively. The IAA producing bacteria, *Bacillus* I6 strain, in Consortium B could be a factor to significantly increase the root dry weight readings of *N. cadamba* seedlings. The studies of IAA producing bacteria on timber tree species, *Eucalyptus urograndis* and *Eucalyptus camaldulensis* showed significant plant growth enhancing effects on the roots of studied seedlings (Paz et al. 2013, p. 3718; Karthikeyan & Sakthivel 2011, p. 621).

Albeit inferior to Consortium B and Consortium B-chemical fertiliser, Consortium A also showed significant root dry weight reading at 12.47 g to negative control with reading at 11.00 g ($p \leq 0.05$), whereas Consortium A-chemical fertiliser showed on par root dry weight reading at 21.22 g to positive control with reading at 20.65 g. The plant growth enhancing effects of Consortium A on root dry weight might be related to its content of nitrogen fixing strain, phosphate solubilising strain and potassium solubilising strain which could provide available nitrogen, phosphorus and potassium for plant root growth (Fageria & Moreira 2011, p. 278). Furthermore, some previous studies of woody plants like *Corylus avellana* and *Paulownia kawakamii* also reported the significant effects of microbial consortia on the root dry weight readings of treated seedlings (Rostamikia et al. 2016, p. 1474; Farahat et al. 2014, p. 855). Additionally, the study of *Paulownia kawakamii* also showed seedlings treated with integrated treatments gained similar root dry weight readings to those treated with full rate chemical fertiliser (Farahat et al. 2014, p. 855).

In short, Consortium B containing IAA producing bacteria, *Bacillus* I6 strain, had shown in this study to significantly promote the root growth of *N. cadamba* seedlings in both consortial and integrated treatments if compared to negative control and positive control respectively. In addition, Consortium A also showed significant plant growth enhancing effect on the root dry weight. Its integrated formulation could enhance root growth at the same rate as positive control by reducing chemical fertiliser application rate at 50%. Nonetheless, Consortium B and its integrated treatments performed better in promoting root growth by showing significantly heavier root dry weight than Consortium A and Consortium A-chemical fertiliser respectively.

4.4 Summary

Six bacterial strains with high levels of biological activities were selected for compatibility test. These strains were *Pseudomonas* N8, *Streptomyces* N11, *Serratia* P8, *Bacillus* K5, *Bacillus* I6 and *Arthrobacter* I7. The compatibility test showed *Streptomyces* N11, *Serratia* P8, *Bacillus* K5 and *Bacillus* I6 strains were compatible to each other therefore suitable to be incorporated as consortial formulations. These four strains were also evaluated for their growth characteristics in order to ascertain required incubation time of each strain to reach the recommended concentration range of 10^7-10^8 cfu/mL for microbial consortial formulation. *Serratia* P8, *Bacillus* K5 and *Bacillus* I6 strains could reach the concentrations of 2.80×10^{10} cfu/mL, 4.67×10^9 cfu/mL and 2.23×10^{10} cfu/mL respectively after 24-hour incubation. *Streptomyces* N11 strain required 72-hour incubation to reach 4.67×10^9 cfu/mL because it is an Actinobacteria strain which normally consumed more time to reach over 10^8 cfu/mL. When the incubated strains were diluted and mixed into microbial consortia formulations, each of them could meet the recommended concentration range of 10^7-10^8 cfu/mL.

The four selected strains formulated into Consortium A were *Streptomyces* N11, *Serratia* P8 and *Bacillus* K5 strains. Consortium B would contain *Streptomyces* N11, *Serratia* P8, *Bacillus* K5 and *Bacillus* I6 strains. In addition, these consortia were also integrated with half regime chemical fertiliser to form Consortium A-chemical fertiliser and Consortium B-chemical fertiliser treatments respectively. These consortial and consortium-chemical fertiliser treatments were applied to six-month-old *N. cadamba* seedlings for the evaluation of plant growth enhancing effect and investigation of the possibility to reduce the usage of chemical fertiliser. The effects of applied treatments were determined by stem height increment, root collar diameter increment, leaf area, shoot dry weight and root dry weight. For consortial treatments, both microbial consortia showed significant plant growth enhancing effects on all the stated plant growth parameters to negative control. However, Consortium B which contained IAA producing *Bacillus* I6 strain showed significant effects on root growth to Consortium A. Since the shoot growth could be enhanced by root growth as the nutrients absorbed by the root would enhance the shoot growth, the seedlings treated with Consortium B also gained significantly higher stem height increments, root collar diameter increments, leaf area readings and shoot dry

weight readings than Consortium A. When the consortia were integrated with half regime chemical fertiliser, both Consortium A-chemical fertiliser and Consortium B-chemical fertiliser treatments showed on par stem height increments, leaf area readings and shoot dry weight readings to full regime chemical fertiliser positive control. Besides that, the integrated treatments could also significantly enhance the root collar diameter growths of *N. cadamba* seedlings. For root growth, Consortium B-chemical fertiliser showed significant results to both Consortium A-chemical fertiliser and positive control whereas Consortium A-chemical fertiliser gave similar root dry weight readings to positive control. It would be interesting to study if Consortium B-chemical fertiliser would show significant plant growth enhancing effects on stem height, root collar diameter, leaf area and shoot dry weight to Consortium A-chemical fertiliser if an extended pot experiment period was provided.

In conclusion, the microbial consortia containing nitrogen fixing strain, phosphate solubilising strain, potassium solubilising strain and IAA producing strain significantly improved the growth of six-month-old *N. cadamba* seedlings. In addition, these microbial consortia could also support the growth of seedlings at half regime chemical fertiliser. Thus, the integrated treatments could effectively reduce the chemical fertiliser usage by 50%. The aim of pot experiment was achieved.

CHAPTER 5:

CONCLUSION AND FURTHER RECOMMENDATIONS

5.1 Conclusion

This study aimed for the isolation and characterisation of Sarawak indigenous plant growth enhancing microorganisms as environmental-friendly alternatives for chemical fertilisers which have been widely used in the silviculture of *Neolamarckia cadamba*. The isolated microorganisms were screened through the selective media and characterised into four growth enhancing traits which included nitrogen fixation, phosphate solubilisation, potassium solubilisation and IAA production. Their efficiencies in nitrogen, phosphate, potassium and IAA production were evaluated. The four best isolated strains which were compatible to each other, and showing good growth characteristics, were used for microbial consortia formulations. As expected, the microbial consortia showed significant plant growth enhancing effects on *N. cadamba* seedlings and were able to reduce the application rate of chemical fertiliser up to 50%. Hence, the targeted aims and objectives have been achieved successfully. According to the findings in this study, the isolated strains had the potential to be formulated further as commercial biofertiliser for application in *Neolamarckia cadamba* plantations.

5.2 Further Recommendations

This study focused on the isolation of nitrogen fixing microorganisms, phosphate solubilising microorganisms, potassium solubilising microorganisms and IAA producing microorganisms from rhizospheric soils collected from *N. cadamba* trees in several locations in Sarawak. Semi-solid medium could be considered for isolation of nitrogen fixing microorganisms in future works because nitrogen fixation was reported to be more efficient in low oxygen environment (Stewart & Pearson 1970, p. 293). Although the biological nitrogen fixation activities have been proved in this study, nitrogen fixing microorganisms could be further tested with acetylene reduction assay which was widely reported in studies of biological nitrogen fixation activities (Gothwal et al., p. 103; Rice & Paul 1971, p. 1049). The best isolate from each growth enhancing trait was selected for microbial consortia formulations. These isolates namely *Streptomyces* N11, *Serratia* P8, *Bacillus* K5 and *Bacillus* I6 strains had demonstrated superior activities in nitrogen fixation, phosphate and potassium solubilisation and IAA production respectively. As expected, the microbial Consortium B, which contained all the isolates, either used per se or integrated with half regime of chemical fertiliser showed overall effective plant growth enhancing effects on *N. cadamba* seedlings in six-month pot experiment. By using the integrated treatments, reduction of 50% on chemical fertilisers usage was possible without compromising the growth of *N. cadamba* seedlings as demonstrated in this six-month pot experiment. As mentioned earlier in Chapter 4, Consortium B-chemical fertiliser treatment may require longer pot experiment period to show significant plant growth enhancing effects on shoot growth. According to a review for pot experiment of woody species, the pot experiment study duration could range from 0.2 months to 48 months (Kawaletz et al. 2014, p. 485). Hence, the plant growth enhancing effects of consortium-chemical fertiliser may be further evaluated in pot experiment with study duration up to 48 months. Since the plant growth enhancing effects of isolated strains were proven in the pot experiment, the isolated strains should be further applied to the *N. cadamba* seedlings or juvenile trees in field trial experiments for further evaluation of their tolerance to environmental stresses and interactions with soil indigenous microorganism community.

The current integrated formulations contain selected strains in consortium with a reduced regime of chemical fertiliser by 50%. It is possible that the portion of chemical fertiliser could be further reduced for future experiments. There were some microbial consortia studies showing 75% chemical fertiliser reduction in their pot experiments. The first study was conducted on the temperate tree species, *Paulownia kawakamii*. The study utilised the integrated application of microbial consortia and 25% chemical fertiliser on the *P. kawakamii* seedlings. The integrated treatment showed similar shoot growth and root growth to the full strength chemical fertiliser treatment (Farahat et al. 2014, p. 855). The second study presented the application of biofertiliser with 25% chemical fertiliser on the mustard seedlings. The reduced application of chemical fertiliser did not affect the shoot growth and root growth of mustard seedlings as shown in 100% chemical fertiliser treatment (Banerjee, Datta & Mondal 2010, p. 344). As a result, the plant growth enhancing effect of isolated strains could be further evaluated on *N. cadamba* seedlings with chemical fertiliser dosage lower than 50%.

The carrier formulation of microbial consortia may need to be optimised for large scale biofertiliser production and application in field trial experiments. There are four basic dispersal formulations available in the market including powder, granules, slurry and liquid. The current study used liquid formulation because the microbial consortia could be diluted and applied directly on the plants. The powder formulation is suitable for spore-producing microorganisms which tolerate to heat and desiccation. The formulation is formed by ground peat and soil carriers with microorganisms at a size range of 0.075 to 0.25 mm for better adhesion to the plant seeds (Zayed 2016, p. 226). Another solid formulation is granule which can be made of peat, prill, small marble, calcite or silica grains. The microorganisms can be impregnated into the granular materials and get protected from soil stress conditions like low pH, desiccation, low temperature and waterlogging. The granule formulation can be applied to the furrow directly and thus facilitate the lateral root interactions (Zayed 2016, p. 226). The slurry form is derived from powder formulation by suspending the powder in liquid like water. Then, the slurry formulation can be directly applied to the plant seeds or furrow (Zayed 2016, p. 227). The last formulation is liquid formulation which is formed by dissolving microbial consortia in the water, mineral or organic oils. Liquid formulations can be added with some additives to improve the viabilities of microbial cells. In addition, they are easy to handle

and can be sprayed on the plant seeds evenly (Zayed 2016, p. 227). As a result, the four stated carrier formulations could be considered for further larger scale production and field trial experiments.

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Appendix

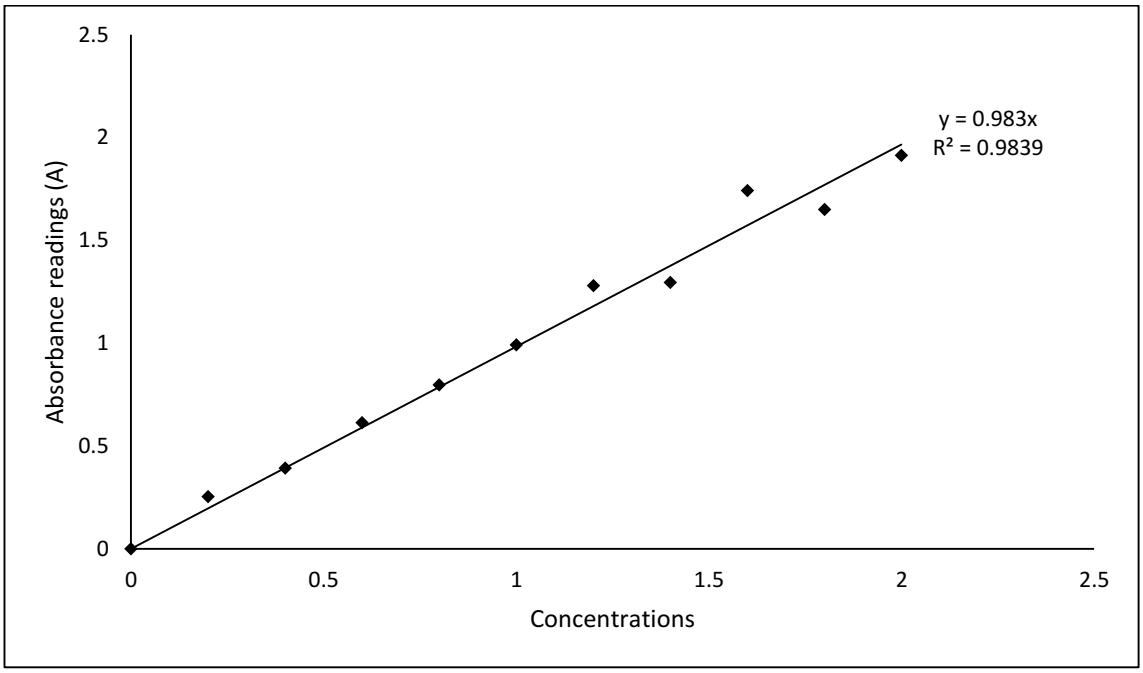

Figure 35 The standard curve of ammonia standard solutions ranging from 0.2 to 2.0 mg/L NH_3.

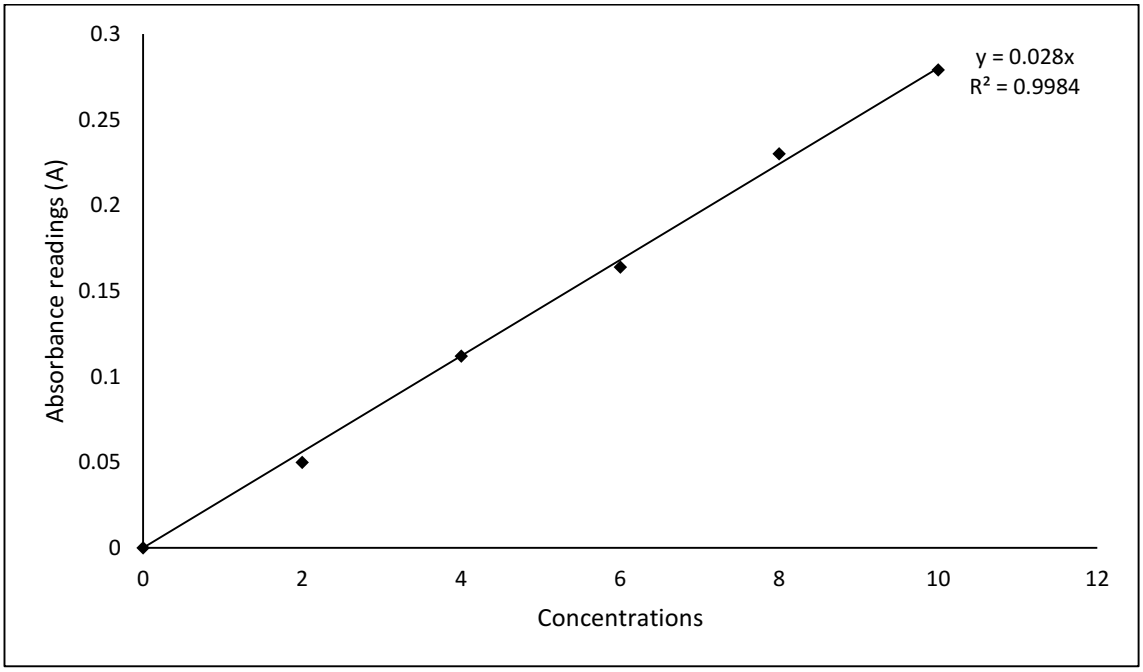

Figure 36 The standard curve of phosphate standard solutions ranging from 2.0 to 10.0 mg/L PO_4^{3-}.

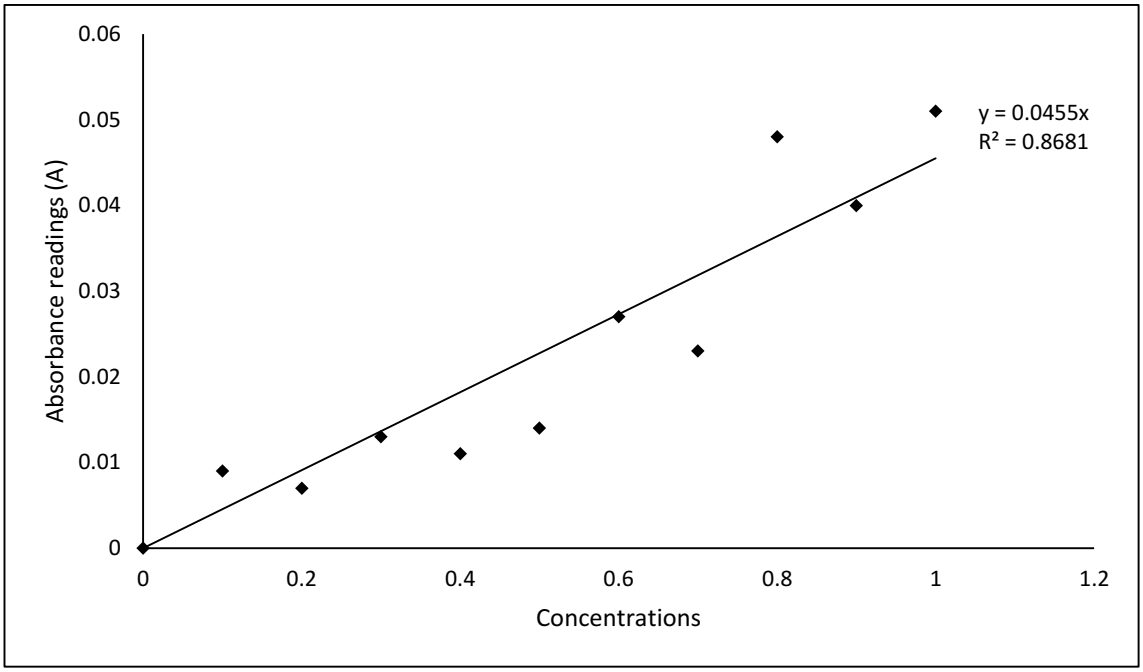

Figure 37 The standard curve of IAA standard solutions ranging from 0.1 to 1.0 µg/mL IAA.