EFFECTS OF INDIGENOUS BENEFICIAL MICROBES FORMULATED AS BIO-FERTILIZER ON THE GROWTH ENHANCEMENT OF TROPICAL TREE SPECIES, *Neolamarckia cadamba*

by

WENDY ONG HAN SZE

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Abstract

*Neolamarckia cadamba* is a fast-growing timber tree in Sarawak known to be growing well in its natural moist and highly fertile alluvial habitat. Its growth potential would be compromised if planted on lands which are less fertile and dry. Chemical fertilizer is therefore commonly applied in plantations of *N. cadamba* in Sarawak to supply major nutrient such as nitrogen, phosphate and potassium to the trees. However, chemical fertilizer is not user friendly to the surrounding environment, thus leading to water, soil and air pollution. As an attempt to reduce the application of chemical fertilizer, carrier-based bio-fertilizer is introduced as an alternative fertilizer in this study. Bio-fertilizer is a substance containing growth prompting living microbes that have activities in nutrient cycles of nitrogen, phosphate and potassium, which eventually enabling plants to absorb these available nutrients. It does not contain heavy chemicals and would less likely harm the environment as the microbes in bio-fertilizer originate from soil. The aim this study was to formulate a carrier-based bio-fertilizer containing high population of beneficial microbes [*Streptomyces gramineus* (nitrogen fixing microbe), *Serratia nematodiphila* (phosphate solubilizing microbe), *Bacillus cereus* Strain I (potassium solubilizing microbe) and *Bacillus cereus* Strain II (indole acetic acid producing microbe)]. Studies had been conducted on the growth media and media additive. Results showed that growth of *Serratia nematodiphila*, *Bacillus cereus* Strain I and *Bacillus cereus* Strain II were best in nutrient broth added with 0.3 % yeast while growth of *Streptomyces gramineus* bacterial strain was best in tryptic soy broth added with 0.3 % yeast. After the optimization of these parameters, high population of $10^8$ to $10^{10}$ CFU mL$^{-1}$ beneficial microbes could be obtained from shake flask fermentation. The beneficial microbes were then assimilated into suitable carrier material to form bio-fertilizer. Several carrier materials viz. cocopeat, vermiculite, sawdust, compost and charcoal were evaluated for their physical and chemical properties as well as their ability to sustain the growth of beneficial microbes. Results showed that cocopeat was a suitable carrier material that could support the growth of beneficial microbes at a high population of $10^8$ to $10^9$ CFU mL$^{-1}$. The effectiveness of the newly formulated bio-fertilizer in enhancing the root elongation of *N. cadamba* seedlings was evaluated by conducting a rhizopod assay. Rhizopod assay is a simulation of seedlings growing in a water limitation environment.
Rhizopod assay results showed that *N. cadamba* seedlings treated with dual combination of bio-fertilizer with half regime chemical fertilizer had the highest rate of root elongation, which indicating that both bio-fertilizer which containing the beneficial microorganisms, and the chemical fertilizer would be essential for root elongation. The effectiveness of bio-fertilizer in enhancing the growth of *N. cadamba* seedlings were evaluated by conducting pot and field trials. The growth of *N. cadamba* seedlings were monitored in terms of their shoot height, root collar diameter, dry shoot and root weights. Both pot and field trial results showed dual combination of bio-fertilizer with half regime chemical fertilizer treatment had the effectiveness comparable to full regime chemical fertilizer treatment in enhancing the growth of seedlings. The results from the studies conducted indicated that microbes in bio-fertilizer could recycle nutrients from the environment and thus provide seedlings with available nutrients for their intake.
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Declaration

I, Wendy Ong Han Sze, student 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 “Effects of indigenous beneficial microbes formulated as bio-fertilizer on the growth enhancement of tropical tree species, Neolamarckia cadamba” is solely original and contains no information which had been accepted for the award to candidate of any other diploma or degree, except where due references have been made in the text of the examinable outcomes; and where the work is based on collaborative research or publications, any relative contributions from respective workers or authors have been disclosed.

Wendy Ong Han Sze

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

Dr Tan Lee Tung
Table of Contents

<table>
<thead>
<tr>
<th>Content</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>I</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>III</td>
</tr>
<tr>
<td>Declaration</td>
<td>IV</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>V</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>XI</td>
</tr>
<tr>
<td>List of Figures</td>
<td>XIV</td>
</tr>
<tr>
<td>List of Tables</td>
<td>XIX</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>2</td>
</tr>
<tr>
<td>1.2 Literature Review</td>
<td>3</td>
</tr>
<tr>
<td>1.2.1 Sustaining Sarawak’s timber industry</td>
<td>3</td>
</tr>
<tr>
<td>1.2.2 <em>Neolamarckia cadamba</em> as a tropical species that is suitable reforestation in timber industry</td>
<td>5</td>
</tr>
<tr>
<td>1.2.2.1 History of <em>Neolamarckia cadamba</em></td>
<td>5</td>
</tr>
<tr>
<td>1.2.2.2 Characteristics of <em>Neolamarckia cadamba</em></td>
<td>5</td>
</tr>
<tr>
<td>1.2.3 Importance of <em>Neolamarckia cadamba</em> in timber industry</td>
<td>6</td>
</tr>
<tr>
<td>1.2.4 Evaluating the planted forest areas in Sarawak</td>
<td>7</td>
</tr>
<tr>
<td>1.2.5 Application of chemical fertilizer to timber plantations</td>
<td>9</td>
</tr>
</tbody>
</table>
1.2.6 Disadvantages of excessive application of chemical fertilizer

1.2.6.1 Water pollution

1.2.6.2 Soil pollution

1.2.6.3 Air pollution

1.2.7 Bio-fertilizer as an alternative to reduce excessive application of chemical fertilizer

1.2.8 The mechanisms of beneficial microbes in bio-fertilizer to supply plants with nutrients

1.2.8.1 Mechanisms of nitrogen fixing microbe

1.2.8.2 Mechanisms of phosphate solubilizing microbe

1.2.8.3 Mechanisms of potassium solubilizing microbe

1.2.8.4 Mechanisms of indole acetic acid producing microbe

1.2.9 Manufacturing of bio-fertilizer

1.2.9.1 Roles of carrier materials in the formulation of bio-fertilizers

1.3 Research Aim and Objectives

1.4 Thesis Outline

CHAPTER 2: FORMULATION OF BIO-FERTILIZER FOR *Neolamarckia cadamba*

2.1 Introduction

2.2 Materials and Methodology

2.2.1 Preparing microbial culture from glycerol stock solution

2.2.2 Microbial growth curves of selected bio-fertilizer strains
2.2.3 Parameters that affect microbial growth ........................................................... 22

2.2.3.1 Growth media.............................................................................................. 22

2.2.3.2 Medium additives........................................................................................ 24

2.2.4 Carrier materials analysis .................................................................................. 24

2.2.4.1 pH................................................................................................................ 24

2.2.4.2 Nitrogen, phosphorus and potassium contents............................................ 25

2.2.4.3 Water holding capacity ............................................................................... 25

2.2.4.4 Assimilation of microbes in carrier materials ............................................. 26

2.2.5 Bio-fertilizer formulation .................................................................................. 26

2.2.5.1 Formulation of carrier-based or solid bio-fertilizer .................................... 26

2.2.5.2 Comparing viabilities of selected microbial strains in liquid and carrier-
    based or solid bio-fertilizer ........................................................................ 27

2.3 Result and Discussion ............................................................................................... 28

2.3.1 Microbial growth curves of selected bio-fertilizer strains ......................... 28

2.3.2 Parameters that affect microbial growth ........................................................... 30

2.3.2.1 Growth media.............................................................................................. 30

2.3.2.2 Medium additives........................................................................................ 31

2.3.3 Analysis of carrier materials ............................................................................. 33

2.3.4 Viabilities of selected microbial strains in liquid and carrier-based bio-fertilizer
    .......................................................................................................................... 36

2.4 Summary ................................................................................................................... 39
CHAPTER 3: RHIZOPOD ASSAY TO STUDY THE EFFECTIVENESS OF BIO-
FERTILIZER ON THE RATE OF ROOT ELONGATION OF Neolamarckia cadamba

3.1 Introduction ............................................................................................................... 42
3.2 Materials and Methodology ...................................................................................... 43
    3.2.1 Germination of Neolamarckia cadamba seedlings ........................................... 43
    3.2.2 Design and preparation of rhizopod assay ....................................................... 43
    3.2.3 Data analysis ..................................................................................................... 46
3.3 Result and Discussion ............................................................................................... 46
    3.3.1 Rate of root elongation of Neolamarckia cadamba seedlings between treatment
groups ......................................................................................................................... 46
        i)  30th day of treatment ..................................................................................... 47
        ii) 60th day of treatment ..................................................................................... 48
        iii) 90th day of treatment .................................................................................. 50
3.4 Summary ................................................................................................................... 52

CHAPTER 4: SMALL SCALE POT TRIAL TO STUDY THE EFFECTIVENESS OF
BIO-FERTILIZER ON Neolamarckia cadamba ............................................................ 54
4.1 Introduction ............................................................................................................... 55
4.2 Materials and Methodology ...................................................................................... 55
    4.2.1 Preparation of seedlings and potting medium ............................................... 55
    4.2.2 Small scale pot trial design ............................................................................. 56
    4.2.3 Data analysis ..................................................................................................... 59
4.3 Result and Discussion

4.3.1 Determining the effect of different treatments on *Neolamarckia cadamba* seedlings in pot trial

4.3.1.1 The mean shoot height increment of *N. cadamba* seedlings

4.3.1.2 The mean root collar diameter increment of *N. cadamba* seedlings

4.3.1.3 The dry weights of *N. cadamba* seedlings

i) After 90 days of treatment

ii) After 180 days of treatment

CHAPTER 5: FIELD TRIAL TO FURTHER EVALUATE THE EFFECTIVENESS OF BIO-FERTILIZER ON *Neolamarckia cadamba*

5.1 Introduction

5.2 Materials and Methodology

5.2.1 Preparation of seedlings, potting medium and bio-fertilizer for field trial

5.2.2 Field trial design

5.2.3 Data analysis

5.3 Result and Discussion

5.3.1 Determining the effect of different treatments on *Neolamarckia cadamba* seedlings in field trial

5.3.1.1 The mean shoot height of *N. cadamba* seedlings

5.3.1.2 The mean root collar diameter of *N. cadamba* seedlings

5.4 Summary

CHAPTER 6: CONCLUSIONS AND FURTHER RECOMMENDATIONS
6.1 Aim of the thesis ....................................................................................................... 95

6.2 Conclusions ............................................................................................................... 95

6.3 Further Recommendations ........................................................................................ 97

6.4 Concluding statements .............................................................................................. 99

References ..................................................................................................................... 100

Appendix ....................................................................................................................... 115
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
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<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>Corp</td>
<td>Corporation</td>
</tr>
<tr>
<td>DAT</td>
<td>Days After Treatment</td>
</tr>
<tr>
<td>DCM</td>
<td>Dried Carrier Material</td>
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<tr>
<td>hr</td>
<td>Hour</td>
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<tr>
<td>HSD</td>
<td>Honestly Significant Difference</td>
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<tr>
<td>IAA</td>
<td>Indole-3-Acetic Acid</td>
</tr>
<tr>
<td>IAM</td>
<td>Indole-3-Acetamide</td>
</tr>
<tr>
<td>IBM</td>
<td>International Business Machines</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
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<tr>
<td>LPF</td>
<td>Licence for Planted Forests</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>MgO</td>
<td>Magnesium Oxide</td>
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<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>P</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PFE</td>
<td>Permanent Forest Estate</td>
</tr>
<tr>
<td>PGPR</td>
<td>Plant Growth-Promoting Rhizobacteria</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>PZSE</td>
<td>Point of Zero Salt Effect</td>
</tr>
<tr>
<td>RER</td>
<td>Root Elongation Rate</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>SFC</td>
<td>Sarawak Forestry Corporation</td>
</tr>
<tr>
<td>spp</td>
<td>Species</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>w</td>
<td>Weight</td>
</tr>
<tr>
<td>WCM</td>
<td>Wet Carrier Material</td>
</tr>
<tr>
<td>WHC</td>
<td>Water Holding Capacity</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1 The plot locations of forests in Sarawak (Forest Department Sarawak 2018).</td>
<td>7</td>
</tr>
<tr>
<td>Figure 1.2 Licence for Planted Forest (LPF) areas in Sarawak (Liew 2007).</td>
<td>8</td>
</tr>
<tr>
<td>Figure 2.1 Growth curves of selected strains used in the formulation of bio-fertilizer for <em>N. cadamba</em>.</td>
<td>28</td>
</tr>
<tr>
<td>Figure 3.1 Rhizopods in water table.</td>
<td>45</td>
</tr>
<tr>
<td>Figure 3.2 The trend of rate of root elongation of <em>N. cadamba</em> seedlings throughout the 90 days rhizopod assay.</td>
<td>47</td>
</tr>
<tr>
<td>Figure 3.3 The mean rate of root elongation of <em>N. cadamba</em> seedlings on 30(^{th}) day of treatment. Each bar in the graph represents the mean rate of elongation of <em>N. cadamba</em> seedlings ± SE (n = 3) under respective treatment group. Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).</td>
<td>48</td>
</tr>
<tr>
<td>Figure 3.4 The mean rate of root elongation of <em>N. cadamba</em> seedlings on 60(^{th}) day of treatment. Each bar in the graph represents the mean rate of elongation of <em>N. cadamba</em> seedlings ± SE (n = 3) under respective treatment group. Means with same</td>
<td>49</td>
</tr>
</tbody>
</table>
letter(s) are not significantly different according to Tukey’s HSD test ($p \leq 0.05$).

**Figure 3.5** The mean rate of root elongation of \textit{N. cadamba} seedlings on 90th day of treatment. Each bar in the graph represents the mean rate of elongation of \textit{N. cadamba} seedlings ± SE ($n = 3$) under respective treatment group. Means with same letter(s) are not significantly different according to Tukey’s HSD test ($p \leq 0.05$).

**Figure 4.1** The trend of mean shoot height increment of \textit{N. cadamba} seedlings throughout the 180 days’ pot trial.

**Figure 4.2** The mean shoot height increment of \textit{N. cadamba} seedlings after 90 days of treatment. The bars in the graph represented the mean shoot height increment of each treatment group ± SE ($n = 20$). Means with same letter(s) are not significantly different according to Tukey’s HSD test ($p \leq 0.05$).

**Figure 4.3** The mean shoot height increment of \textit{N. cadamba} seedlings after 180 days of treatment. The bars in the graph represented the mean shoot height increment of each treatment group ± SE ($n = 10$). Means with same letter(s) are not significantly different according to Tukey’s HSD test ($p \leq 0.05$).

**Figure 4.4** The trend of mean root collar diameter increment of \textit{N. cadamba} seedlings throughout the 180 days’ pot trial.
Figure 4.5  The mean root collar diameter increment of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean root collar diameter increment of each treatment group ± SE (n = 20). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Figure 4.6  The mean root collar diameter increment of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean root collar diameter increment of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Figure 4.7  The mean shoot dry weight of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean shoot dry weight of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Figure 4.8  The mean root dry weight of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean root dry weight of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).
Figure 4.9  The mean shoot dry weight of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean shoot dry weight of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Figure 4.10  The mean root dry weight of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean root dry weight of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Figure 5.1  Field trial plot design at Sabal Forest Reserve, Simunjan, Sarawak.

Figure 5.2  The trend of mean shoot height increment of *N. cadamba* seedlings throughout the 180 days’ field trial.

Figure 5.3  The mean shoot height increment of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean shoot height of each treatment group ± SE (n = 30). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).
Figure 5.4  The mean shoot height increment of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean shoot height of each treatment group ± SE (n = 30). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Figure 5.5  The trend of mean root collar diameter increment of *N. cadamba* seedlings throughout the 180 days’ field trial.

Figure 5.6  The mean root collar diameter increment of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean root collar diameter of each treatment group ± SE (n = 30). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Figure 5.7  The mean root collar diameter increment of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean root collar diameter of each treatment group ± SE (n = 30). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).
List of Tables

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Culturing of microbes in different growth media.</td>
<td>23</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Culturing of microbes in growth media added with different additives.</td>
<td>24</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>Growth rate constant and generation/doubling time of selected bacterial strains.</td>
<td>29</td>
</tr>
<tr>
<td>Table 2.4</td>
<td>Population of bacteria grown in different growth media.</td>
<td>31</td>
</tr>
<tr>
<td>Table 2.5</td>
<td>Population of bacteria grown in growth media added with different additives.</td>
<td>32</td>
</tr>
<tr>
<td>Table 2.6</td>
<td>The physical and chemical properties of carrier materials.</td>
<td>33</td>
</tr>
<tr>
<td>Table 2.7</td>
<td>Population of microbes in different carrier materials after 15 and 30 days.</td>
<td>34</td>
</tr>
<tr>
<td>Table 2.8</td>
<td>Population of viable microbes in liquid bio-fertilizer.</td>
<td>37</td>
</tr>
<tr>
<td>Table 2.9</td>
<td>Population of viable microbes in carrier-based bio-fertilizer.</td>
<td>38</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Treatment groups for rhizopod assay.</td>
<td>43</td>
</tr>
</tbody>
</table>
Table 4.1  Treatment groups for *N. cadamba* seedlings in 180 days of pot trial.

Table 5.1  Treatment groups for *N. cadamba* 180 days of field trial.
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW
1.1 Introduction

In Sarawak, the estimated timber production capacity of 8.5 million m³ per year from the natural forests is not considered sufficient for the industry (Forest Department Sarawak 2017). As such, tree species that can yield good quality timber need to be planted. In fact, Sarawak government had issued forest planting areas for timber plantations. Nonetheless, most forest planting areas are considered “unplantable” as they are located in areas which necessitate protective functions such as reducing surface erosion and controlling floods (Forest Department Sarawak 2017).

For long term sustainable supplement of timber, the Sarawak government have attempted to develop forest plantation to increase the productivity and economic returns for some of the degraded or under-utilized lands that had been left idle due to shifting cultivation (Forest Department Sarawak 2017). Timber plantation companies are also collaborating with Sarawak Forestry Corporation (SFC) in research and development to establish forest plantations with high productivity (Moh 2016).

There are many timber species that could be selected for forest plantations. These timber species include *Eusideroxylon zwageri*, *Shorea* spp., *Gmelina arborea*, *Dryobalanops*, *Rhizophora* spp., *Paraserianthes falcataria*, *Tectona grandis*, *Eucalyptus* spp. and *Neolambarckia cadamba* (Forest Department Sarawak 2017). Among these timber species, *Neolamackria cadamba* is often flavourable for reforestation as it is a native fast growing timber species with short rotation cycle of 5 to 10 years (Joker 2000). It is a light demanding species and can grow on a variety soils. However, the optimum soil for *N. cadamba* is deep, moist alluvial soil (Indian Council of Forestry Research and Education 2014). This species can grow up to 45 m tall, with bole diameter approximately 100 to 160 cm in length (Joker 2000).

Though *N. cadamba* is fast growing, it requires a natural alluvial habitat that is moist and highly fertile (Indian Council of Forestry Research and Education 2014) to grow well. The growth potential of *N. cadamba* will be compromised if this species is planted on lands which are less fertile and dry. Investigations showed that the nutrient contents of soil obtained from lands allocated for timber plantation development in Sarawak were
low in the subsurface soil (Ishizuka et al. 2000). These lands are not constantly moist as Sarawak experiences dry season in June and July with a rainfall less than 200 mm per month (Ministry of Resource Planning and Environment 2012). Lands for timber plantation development will not be able to be situated near water intake points (less than 8 km) as this is the enforcement law set by Sarawak government to enhance the environmental performance in Sarawak (Sarawak Integrated Water Resources 2008).

Soil nutrient deficiency and dry lands are major issues faced when planting *N. cadamba*. In order to supply the soil with nutrients, fertilizers have to be supplied regularly but heavy usage of chemical fertilizers would further deteriorate the soil fertility and also lead to water, soil and air pollution (Savci 2012) as some fertilizers may also contain heavy metals and radionuclides (Savci 2012). In order to reduce the excessive usage of chemical fertilizer, bio-fertilizer is encouraged to be used, normally in combination with lesser amount of chemical fertilizer to increase the availability of nutrients in the soil and also to improve the soil condition (Bato 2009). Bio-fertilizer is a substance containing growth prompting living microbes that recycle nutrients (nitrogen, phosphate and potassium) the environment, enabling plants to absorb these available nutrients (Ajmal et al. 2018). As these microbes used in bio-fertilizer are often obtained from the soil or roots of plants, the bio-fertilizer formulated would less likely be harmful to the plant, the soil and also the environment (Saeed et al. 2015).

1.2 Literature Review

1.2.1 Sustaining Sarawak’s timber industry

Malaysia is a developing country whereby its overall economy is still depending on timber industry. Timber products, the third largest export of the country after palm oil products (Bernama 2017), is crucial in contribution to the country’s export revenue. In 2012, 36 percent of Malaysia’s total timber export earnings worth RM 20 billion were accounted by Sarawak (Tuah 2013). The major products being exported were plywood, sawn timber and logs with total export values of RM4 billion, RM758 million and RM1.8 billion respectively (Tuah 2013). As Sarawak’s timber has high demand both locally and
internationally, Sarawak is expected to eventually developed into the region’s largest tropical plywood producer (Tuah 2013). Sarawak’s timber industry will not be able to sustain such demand if the harvesting of raw timber relies solely on timbers from natural forests.

There are 7.89 million hectares of natural forest in Sarawak known as permanent forest estate (PFE). About 5 million hectares out of these were allocated as production forests for timber (Forest Department Sarawak 2017; Forest Governance Integrity Transparency International Malaysia 2013), while only 1.3 million hectares were allocated for planted forests due to some areas being high terrain areas and buffer zones allocated for protection functions (Forest Department Sarawak 2017). Planted forests are managed by holders of Licence for Planted Forests (LPF) and will be planted with timber species approved by the Director of Forest Department. Logging in PFE is rather restrictive as most natural forests are located at hilly areas which are extremely environmentally sensitive (Forest Department Sarawak 2017). Based on recommendation of The International Tropical Timber Organization (ITTO), logging in PFE is limited to 170,000 hectares per year, resulting to only 8.5 million m³ of timber that could be produced annually (Forest Department Sarawak 2017). As the rotation period for timber trees is at an average of 35 years (Forest Department Sarawak 2017), the current annual timber production within PFE is insufficient to cater for the growing demand for raw timber. More lands are required for the establishment of planted forests.

In 1960s, Sarawak had 2.25 million hectares of degraded or under-utilized land resulting from shifting cultivation (Forest Department Sarawak 2017). The State Government of Sarawak intends to utilize these lands for reforestation to provide economic returns. The Reforestation Research Programme was initiated in Sarawak to experiment whether exotic fast growing timber species such as conifers are suitable for reforesting lands which have been subjected to numerous cycles of shifting cultivation. However, the growing performance of conifers was shown to be poor due to the land condition and it was concluded that conifers would not be suitable for reforestation in Sarawak (Forest Department Sarawak 2017). Thus, Sarawak foresters are looking into other fast growing tree species for reforestation. In the early 80’s, late 1995, Acacia mangium was one of the major species selected to be planted on shifting areas.
Currently, there are many other timber species such as *Eusideroxylon zwageri, Shorea* species, *Gmelina arborea, Dryobalanops, Rhizophora* species, *Paraserianthes falcataria, Tectona grandis, Eucalyptus* species and *Neolambarckia cadamba* (Forest Department Sarawak 2017) that could be selected for planting. *Neolamarckia cadamba*, a potential fast growing timber species, is selected for reforestation as it is Sarawak’s native species with short rotation cycle of 5 to 10 years (Joker 2000). With the establishment of reforestation on these idle lands, the annual timber production will be increased and the logging in natural forests can be lessen, thus reducing the negative impact and biodiversity loss in the environment.

1.2.2 *Neolamarckia cadamba* as a tropical species that is suitable reforestation in timber industry

1.2.2.1 History of *Neolamarckia cadamba*

*Neolamarckia cadamba* is a species commonly found in Sabah and Sarawak belonging to the Rubiaceae family and is commonly known as ‘kelampayan’ or ‘laran’ in Malaysia (Ho et al. 2014). Though *N. cadamba* is a native species in Sarawak, it can also be found planted in countries such as China, India, Nepal and Thailand (Chen et al. 2011). It is a pioneer in the forest and is commonly found in secondary forest.

1.2.2.2 Characteristics of *Neolamarckia cadamba*

*Neolamacrkia cadamba* has an umbrella-shaped crown, tiered branches and cylindrical bole. It has glossy green broad leaves that are 13 to 32 cm long. At young, its bark is smooth and grey in color. As it matures, its bark is rough and longitudinally fissured (Maharashtra Forest Department 2018). It only begins flowering at a maturity age of 4 to 5 years and the orange flowers are small, globose heads shaped. The flowers are fragrant to attract pollinators (Maharashtra Forest Department 2018). Fruiting normally occurs after flowering and each gram of fruit produced will release approximately 20,000 seeds (Joker 2000).
It is commonly found growing in damp places, such as along large streams (Dubey et al. 2011). It is a light demanding species that can grow on a variety soils but it prefers deep, moist alluvial soil (Indian Council of Forestry Research and Education 2014). It is a light demander tree but extreme heat may harm the tree. It can also grow in both dry and wet conditions. With minimum 200 mm and maximum 1500 mm rainfall per year, it can still grow in such conditions (Joker 2000).

Although it is a species that could be planted on a variety of soils and is tolerate to dry and wet conditions, it could only achieve its optimum growth when planted in favorable conditions - deep, moist alluvial soil in the presence of light. When planted in favorable conditions, the height of *N. cadamba* could reach up to 17 m and would have a diameter of 25 cm at breast height (Ho et al. 2014) within a short time frame of 9 years.

### 1.2.3 Importance of *Neolamarckia cadamba* in timber industry

The sapwood and heartwood of *N. cadamba* are lightweight and hardly distinguishable as both are creamy yellow in color. The wood has fine to medium texture with no characteristics smell or taste. Its tree at a height of 13 m could produce 250 to 300 kg of wood (TNN 2017). Its wood can be easily preserved using open tank or pressure-vacuum systems and easy to work on either hand or machine tools. The density of its wood is 290 to 560 kg/cu. m. at 15 % moisture content (Krisnawati et al. 2011). For greater density and compressive strength, its wood is normally impregnated with synthetic resins. The wood produced by *N. cadamba* is non-durable but is commonly used in light construction, manufacture of plywood, dugout canoes, paper, crates, boxes, pencils and components for furniture (Maharashtra Forest Department 2018). The pulp can also be mixed with long-fibred material to produce paper of medium quality (Krisnawati et al. 2011).

Wood of *N. cadamba* is important in the plywood and furniture sectors. Both these sectors are Malaysia’s outstanding export earners. Initially, Malaysia was the main exporter of plywood, however in year 2015, China outstripped Malaysia (Ministry of Plantation Industries and Commodities Malaysia 2009). *N. cadamba* also contributes to the overall export of timber products, positioning Malaysia as the world’s largest exporter of tropical
timber in year 2005 with free on board value of RM 21.5 billion (Ministry of Plantation Industries and Commodities Malaysia 2009).

### 1.2.4 Evaluating the planted forest areas in Sarawak

Sarawak is made out of four different types of forests, namely the hill mixed dipterocarp forests, mangrove forests, peat swamp forests and shifting cultivation forests. The plots for these forests are shown in Figure 1.1.

![Figure 1.1: The plot locations of forests in Sarawak (Forest Department Sarawak 2018).](image)

In year 2009, 2.8 million hectares of PFE and roughly more than 3.33 million hectares of damaged forest due to shifting cultivation are issued as Sarawak’s LPF areas (Forest Department Sarawak 2017). Figure 1.2 summarizes the LPF areas in Sarawak.
By comparing Figure 1.1 and 1.2, it can be seen that most LPF areas are a mixture of hill mixed dipterocarp and shifting cultivation forests. Not all LPF areas in PFE are suitable for timber plantation as the terrain is hilly and rugged. Thus, only 50% of the LPF areas being issued are plantable. The soil of LPF areas are classified into sandy loams, clay, peat, grey-white podzolic, red-yellow podzolic and gley. Peat soil has low mineral contents (Andriesse 1974), sandy loam soil has low nutrient contents (Applied Agricultural Resources Sdn. Bhd. 2014), clay soil has high nutrient contents but sometimes may restrict root growth (Reid 2006), podzolic soil has low nutrient contents due to continuous leaching (Buurman & Jongmans 2005) and gley soil has poor drainage system (The James Hutton Institute 2018). High compactness of soil and its low nutrient and mineral contents may affect the growth of timber species. In order to increase economic returns, Sarawak government had issued shifting cultivation forests as LPF too. Shifting cultivation forests are natural forests which have been logged for raw materials. The nutrients in the soil of these forests are severely exhausted and their soils are harder and compact (Ishizuka et al. 2000). The growth of timber species will not be at their optimum growth rate in a nutrient deficiency environment.
Timber species at plantation areas will need to endure the hot and humid climate with temperature ranging from 22 to 33°C (Ministry of Resource Planning and Environment 2012). Annual rainfall in Sarawak ranges from 2,400 mm to 5,000 mm (Ministry of Resource Planning and Environment 2012). It will receive heavy rain during end of the year (December to March) and less rain during mid-year (June to August) (Ministry of Resource Planning and Environment 2012). Plantations will also be far from water source as it will be separated from permanent water source by a 20 m or more than 100 m wide riparian buffer zone if the plantation is located at sensitive areas (Sarawak Integrated Water Resources 2008).

1.2.5 Application of chemical fertilizer to timber plantations

Nutrient contents of soil obtained from lands allocated for timber plantation development in Sarawak were low in the subsurface soil (Ishizuka et al. 2000). Study conducted by Ishizuka et. al (2000) on Bakam Forest Reserve, Miri, showed that the soil had low nutrient and oxide content, low point of zero salt effect (PZSE) value and other strongly weathered characteristics. Study conducted by Ishizuka (1998) on Lambir Hills National Park, Miri, showed that the soil had relatively lower content of phosphorus compared to nitrogen and potassium.

To overcome timber shortfalls, excessive application of fertilizers on forest plantations is often required. Fertilization will accelerate the growth of timber species and thus shorten their rotation time (Sophie et al. 2011). The application of nitrogen and phosphate fertilizers increase rate of photosynthesis and this leads to the increment of tree productivity (Gough et al. 2004). Meanwhile, potassium fertilization will increase carbon dioxide diffusion across stomata and mesophyll (Battie-Laclau et al. 2014).

Fertilizers being commonly used to provide nutrients (nitrogen, phosphorus or potassium) to timber species at plantations are chemical fertilizer (N:P:K = 15:15:15) on Dryobalanops lanceolata (Irino et al. 2004), NPK Blue (N:P:K = 12:12:17 + 2MgO) on Acacia mangium (Vijayanathan et al. 2011), Urea and Triple Super Phosphate (TSP) on Antocephalus chinensis (Hoque et al. 2004).
1.2.6 Disadvantages of excessive application of chemical fertilizer

Currently, forest plantations are using chemical fertilizer as the nutrients are in soluble form and directly available for plants’ intake (Guo et al. 2016). However, chemical fertilizers not user friendly to the surrounding environment as they contain heavy metals and radionuclides (Savci 2012). Excessive application of chemical fertilizers would lead to water, soil and air pollution (Savci 2012).

1.2.6.1 Water pollution

Water pollution occurs due to remaining nitrogen in fertilizers not absorbed by plants. Plants only use 50% of nitrogen in fertilizers that have been applied to soil (Savci 2012). The remaining nitrogen supply will either be lost through evaporation, react with soil organic compounds or leached into water source which will be converted into nitrates by nitrifying microorganisms. These converted negatively charged nitrates will contaminate water source and thus causes harmful effects to biological organisms, especially causing toxicity in human beings when consumed. Remaining nitrogen and phosphorus compounds leached into water source will also cause eutrophication when the amount of aquatic plants and algae increase (Rathore 2016; Savci 2012).

1.2.6.2 Soil pollution

Soil pollution occurs over time after being applied with chemical fertilizer. Sodium and potassium in fertilizers deteriorate soil structure and fertility. Nitrogen in fertilizers will decrease pH of soil to acidic level and thus affecting the activity of nitrogen fixing microorganisms (Savci 2012). There are also acids from chemical fertilizer such as hydrochloric and sulphuric acid which will dissolve soil crumbs used to hold rock particles together. Without soil crumbs, soil drainage and air circulation will be reduced and plants will face difficulty obtaining oxygen and nutrients as the roots are unable to penetrate the soil (Savci 2012).
1.2.6.3 Air pollution

Air pollution is caused by excessive application of chemical fertilizer which contributes to nitrogen oxides emissions. Fertilizer containing ammonia when applied to soil will cause evaporation of ammonia too. Ammonia will be oxidized into nitric acid. Nitric acid when combined with sulphuric acid will cause acid rain after chemical transformations. Acid rain can damage vegetation and organisms living in lakes and reservoirs (United States Environmental Protection Agency 2017). Acid rain itself is not harmful to humans but when the pollutants that form acid rain - nitrogen oxide and sulphur oxide react in the atmosphere, sulphate and nitrate particles are formed. These particles can trigger heart and lungs diseases in human beings (United States Environmental Protection Agency 2017). Besides emitting nitrogen oxides, excessive application of chemical fertilizer would also contribute to the release of nitrous oxide and carbon dioxide into the atmosphere. These are greenhouse gases which get trapped in the atmosphere and might be contributing to the increment of land and water surface temperatures (Climate Monitoring & Diagnostics Laboratory 2002).

1.2.7 Bio-fertilizer as an alternative to reduce excessive application of chemical fertilizer

To reduce the application of chemical fertilizer to meet the demand of timber industry, bio-fertilizer would be a better alternative. Bio-fertilizer is a substance containing growth prompting living microbes that may enhance the growth of a plant (Ajmal et al. 2018). Bio-fertilizer provides soil with beneficial microbes which provides plants with nutrients through different mechanisms. Microbes in the bio-fertilizer have the capability either to fix nitrogen, solubilize phosphate, solubilize potassium or produce indole acetic acid. One gram of solid bio-fertilizer contains at least 10 million viable soil microbe. Thus, according to a study, only a small quantity of bio-fertilizer is needed to aid in plant growth and produce desirable results (Anandaraj & Delapierre 2010).

Bio-fertilizer can either be manufactured in the form of solid or liquid. As the usage of bio-fertilizer will be at planted forests, it will be easier to handle solid bio-fertilizer. Solid phase bio-fertilizer or normally known as carrier-based inoculants which contain soil
microbes. Microbial culture is not directly given to plants as bio-fertilizer. Instead, these microbes require assimilation in carrier materials. The process of assimilation will make bio-fertilizer easy to handle, prolong its shelf-life and also increase the effectiveness of the microbes contained within the carrier materials (Muraleedharan et al. 2010).

**1.2.8 The mechanisms of beneficial microbes in bio-fertilizer to supply plants with nutrients**

It is generally known that three main elements of nitrogen, phosphorus and potassium are required by plants to sustain their health and growth (Ball 2007). Due to the fact that these elements in the environment are often not present as readily available forms whereby plants can immediately absorb, plants would require the assistances of microbes to convert these elements into available forms. For example, to absorb nitrogen, plants would need help from nitrogen fixing microbes to convert the nitrogen gas into ammonium, which eventually convert by other microorganisms into nitrate that the plant can absorb. Besides requiring the three major elements of nitrogen, phosphate and potassium, plants would sometimes require phytohormones such as indole acetic acid (IAA), gibberellin, cytokinin and ethylene within their plant zone (Gnanamanickam 2006). All these phytohormones could improve plants’ water and nutrients absorption from soil as they could increase the root surface area by regulating plants’ root hairs’ length and density (Gray & Smith 2005). When bio-fertilizer is applied as soil inoculants, bio-fertilizer containing beneficial microbes may aid in plants’ growth by participating in nutrient cycling. It had been shown in various reports that inoculants plus fertilizer is significantly comparable to chemical fertilizers (Adesemoye et al. 2009).

**1.2.8.1 Mechanisms of nitrogen fixing microbe**

One of the main element required by plants is nitrogen. Plants are not able to directly absorb atmospheric nitrogen. Instead, they need to absorb nitrogen in the form of ammonium or nitrates through their roots (Haynes & Goh 1978). In the nitrogen cycle, nitrogen in the atmosphere would first be converted into volatile ammonia by the enzyme complex- nitrogenase (PNA potassium nitrate association 2016) present in nitrogen
fixing microbe via the process of biological nitrogen fixation. Ammonia is further converted into ammonium by ammonium-oxidizing microbe (PNA potassium nitrate association 2016). In the presence of nitrifying microbe, ammonium is converted into nitrates when the soil is sufficiently available with oxygen, moist, temperature > 20°C and pH level 5.5 to 7.5 (PNA potassium nitrate association 2016). Nitrates are preferably absorbed by plants as nitrates are non-volatile and do not require further conversion prior to absorption by plants’ roots (PNA potassium nitrate association 2016). Plants can only absorb ammonium or nitrates with the presence of nitrogen fixing microbe in the nitrogen cycle. Nitrogen fixing microbe can be found naturally in soil or roots of legumes plants. However, their existences depend on the type of plants and the supply of substrates available (Wagner 2011). Nitrogen (absorbed in the form of nitrate) is needed by plants as it is an essential component of chlorophyll and plants’ genetic material- DNA and RNA (Ohyama & Sueyoshi 2010). Plants with low nitrogen intake would have stunted growth, shorter internodes and their leaves would be pale yellow in color (Uchida 2000).

### 1.2.8.2 Mechanisms of phosphate solubilizing microbe

The other important element required by plants is phosphorus. Phosphorus exists as insoluble and soluble forms in the soil. Plants are not able to absorb insoluble phosphorus and thus, only 0.1% of the total phosphorus in the soil is available for plant intake (Illmer & Schinner 1995). With the presence of phosphate solubilizing microbe, insoluble forms of phosphorus such as aluminum phosphate, tri-calcium phosphate and iron phosphate are solubilized to soluble phosphorus through mineralization process (Illmer & Schinner 1995). During the mineralization process, phosphate solubilizing microbe secretes low molecular mass organic acids. These acids chelate mineral ions, at the same time decrease pH (Pradhan & Shukla 2005), causing the release of phosphate ions from phosphate minerals by hydrogen ion substitution for calcium ion (Goldstein 1994; Mullen 2005; Trivedi & Sa 2008) to attract phosphorus into the solution. More than one type of organic acid may be secreted by one microbe. The simultaneous secretion of organic acids increases the insoluble phosphate solubilizing reaction (Khan, Zaidi & Ahmad 2014). Besides transforming insoluble phosphorus into soluble phosphorus via the production of organic acid, processes involving chelation and to reduction performed by microbes may
also contribute to the solubilizing of insoluble phosphorus. Phosphorus is needed by plants to increase root ramification and strength. Plants’ gain vitality and are resistance to diseases. Besides that, phosphorus also helps in seed formation. Low intake of phosphorus in plants reduces plants’ growth (Sharma et al. 2013), produces dark leaves, unable to flower and most importantly the inhibition of root development (Khan, Zaidi & Ahmad 2014).

1.2.8.3 Mechanisms of potassium solubilizing microbe

Potassium is another important element required by plants. The total potassium content in soil may exceed 20,000 ppm (University of Minnesota 2016). However, only a certain amount of potassium can be readily absorbed by plants. Potassium exists in the soils in three forms (slowly available, readily available and unavailable) (Plant and Soil Sciences eLibrary 2018). Slowly available potassium is fixed between layers of clay minerals. This source of potassium will only be slowly released and plants cannot absorb much of this potassium during a single growing season. Meanwhile, readily available potassium can be found in soil water. Plants can readily absorb this form of potassium. However, the uptake is usually minimal as the uptake is affected by moisture, aeration and temperature of soil (University of Minnesota 2016). Unavailable potassium is in the form of insoluble crystals and these crystals require mineral weathering in order to release potassium. Although this weathering process happens naturally, it takes a long time for the weathering process to take place. Thus, another alternative way to solubilize insoluble potassium into soluble potassium for plants’ intake is with the help of potassium solubilizing microbe which produces organic acids that can solubilize insoluble crystals of potassium (University of Minnesota 2016). Organic acids such as tartaric acid and citric acid are generated by potassium solubilizing microbes - *Bacillus mucilaginosus* and *Bacillus edaphicus*, to solubilize potassium compounds (Richards & Bates 1989). Other organic acids include oxalic acid, gluconic acid, 2-ketogluconic acid, malic acid, lactic acid, succinic acid, glycolic acid, propionic acid, fumaric acid and malonic acid (Etesami, Emami & Alikhani 2017). These organic aids trigger the release of potassium ion from potassium-bearing minerals by lowering the soil pH. In addition, organic acids can also trigger the release of potassium ions from potassium mineral via chelating (Etesami,
Emami & Alikhani 2017). Potassium is needed by plants during their early growth as it increases plants’ protein production. Potassium also increases plants’ protein production, improves the effectiveness of water, nutrients and carbohydrates movement in plant tissue. It also improves plants’ resistance towards diseases and pests, promoting vitality (University of Minnesota 2016).

1.2.8.4 Mechanisms of indole acetic acid producing microbe

Microbes colonizing the rhizosphere region of plants are known as plant growth-promoting rhizobacteria (PGPR) which produce phytohormones such as indole acetic acid (IAA), gibberellin, cytokinin and ethylene (Gnanamanickam 2006) stimulate plants’ growth. There are many types of PGPR, each type producing different phytohormones. Indole acetic acid producing microbe is a type of PGPR that produces phytohormone or plant growth regulator, IAA. This microbe is able to produce IAA in the presence of rich supply of substrates. IAA is the main auxin in plants which controls important physiological processes in plants namely division and enlargement of cell, tissue differentiation and light and gravity responses (Leveau & Lindow 2005). IAA producing microbes produce IAA via Trp-dependent and Trp-independent pathways. In Trp-dependent pathway, tryptophan-2-monooxigenase present in the microbes convert tryptophan to indole-3-acetamide (IAM). IAM-hydrolase is then metabolized by IAM into IAA (Mohite 2013). In Trp-independent pathway, the substrate present is ammonium instead of tryptophan. IAA is needed to increase the osmotic contents, increase the permeability of water, decrease the wall pressure, increase cell wall and protein synthesis in plant cells (Mohite 2013). The changes in osmotic content, wall pressure and protein synthesis lead to the elongation of primary root, lateral and adventitious root formation (Leveau & Lindow 2005), which increases the nutrient uptake in plants.

1.2.9 Manufacturing of bio-fertilizer

For the manufacturing of bio-fertilizer, propagation of beneficial microbes is needed. Back in 1950s, open pond production system is a common way to ferment microorganism culture, especially algae (Arora 2012). This method is cheaper, easy to construct, does not require much technological knowledge to operate and easy to clean up after
fermentation. However, this method can cause microbial culture to be easily contaminated (Arora 2012). Thus, fermentation of microbial culture using shake flask fermentation in the laboratory is more preferred as it is convenient and sterile. Larger scale fermentation could also occur in a bio-reactor. Microbial culture is allowed to ferment until the concentration of the microbes (CFU ≥ 10⁸) are great enough to be mixed with carrier materials to form bio-fertilizer (FNCA Biofertilizer Project Group 2006; Sreedhar & Mohan 2016). The bio-fertilizer mixture should be contained within two layered polythene bag to increase the shelf life of bio-fertilizer (Sivasakthivelan & Saranraj 2013). The mixture in the bag is kneaded (Kaljeet, Keyeo & Amir 2011) and left to cure prior to usage at trials.

### 1.2.9.1 Roles of carrier materials in the formulation of bio-fertilizers

The shelf-life of common carrier-based bio-fertilizer is around six months (Brar et al. 2012). Microbial inoculants need to survive during storage period and also compete for nutrients with other native microorganisms when introduced into the soil, thus, these inoculants would need to obtain nutrients from their carrier materials (Muraleedharan, Seshadri & Perumal 2010).

Good carrier materials have properties such as non-toxic to plants and microbial inoculant, high moisture absorption capacity, easy to process and sterilize, easy to obtain, cheap and good pH buffering capacity (Forum for Nuclear Cooperation in Asia 2006). Carrier materials that can sustain large quantities of microbes are peat, organic wastes, by-products of plants, perlite, mineral soils and alginate. These materials had been used as culture media and the microbial growth had been tested (Brockwell & Bottomley 1995). Other commonly used carrier materials in commercial production include vermiculite, compost, sawdust, cocopeat and charcoal. Sawdust has large surface area and thus high water holding capacity (Arora, Tiwari & Singh 2014), cocopeat acts like sponge and can absorb lots of moisture (Yuvaraj 2016), vermiculite improves aeration and retains moisture (Siddiqui 2006) while compost has microporous structure which enables high water holding capacity (Somarathne, Yapa, P & Yapa, N 2013).
Carrier materials will normally be crushed and powdered before usage (My Agriculture Information Bank 2015). Those acidic in nature, such as cocopeat, would need to be neutralized with 1% (w/w) calcium carbonate prior to usage (My Agriculture Information Bank 2015). All carrier materials need to be sterilized before being mixed with microbial inoculum to decrease the death rate of inoculum cells during storage period (My Agriculture Information Bank 2015).

1.3 Research Aim and Objectives

The aim of this study was to formulate a bio-fertilizer which could reduce excessive application of chemical fertilizer by acting as an alternative, enhancing the root elongation and growth of *N. cadamba*. To achieve the aim, the following objectives were listed:

(i) To determine the suitable growth media and additive for the fermentation of bio-fertilizer microbes.

(ii) To determine the suitable solid carrier material for the formulation of bio-fertilizer.

(iii) To determine the population of viable microbes in both liquid and carrier-based bio-fertilizers and their activities in fixing nitrogen, solubilizing phosphate, solubilizing potassium and producing IAA hormone.

(iv) To determine the effectiveness of bio-fertilizer on the rate of root elongation of *N. cadamba* via rhizopod assay.

(v) To determine the effectiveness of bio-fertilizer on the growth of *N. cadamba* via pot and field trial.
1.4 Thesis Outline

This thesis was reported in five chapters and the chapters are as listed below:

Chapter 1: Introduction and literature review

Chapter 1 introduces the problem faced and the purpose of carrying out the study. It also includes literature reviews regarding Sarawak’s timber industry, *Neolamarckia cadamba*, Sarawak’s planted forest areas, chemical fertilizer and bio-fertilizer with beneficial microbes. The aim and objectives of carrying out this study were also listed in this chapter.

Chapter 2: Formulation of bio-fertilizer for *Neolamarckia cadamba*

Chapter 2 describes the methods used to increase the cell number of beneficial microbes used in the formulation of bio-fertilizer. It also describes about the selection of carrier material for the formulation of bio-fertilizer. The population of viable microbes in the manufactured bio-fertilizer was counted and their efficiencies to fix nitrogen, solubilize phosphate, solubilize potassium and produce IAA were also tested.

Chapter 3: Rhizopod assay to study the rate of root elongation of *Neolamarckia cadamba*

Chapter 3 describes the methods used to study the rate of root elongation of *N. cadamba* in water limited environment. The rate of root elongation is affected by indole acetic acid (IAA) which is produced by IAA producing microbes present in the bio-fertilizer.

Chapter 4: Small scale pot trial to study the effectiveness of bio-fertilizer on *Neolamarckia cadamba*

Chapter 4 describes the methods used to evaluate the effectiveness of bio-fertilizer in enhancing the growth of *N. cadamba*. This chapter will focus on the change in height, diameter and dry weight of *N. cadamba* seedlings over a period of 180 days under different treatments.
Chapter 5: Field trial to further evaluate the effectiveness of bio-fertilizer on *Neolamarckia cadamba*

Chapter 5 describes the change in height and diameter of *N. cadamba* seedlings in field trial experiment conducted over 180 days. The purpose of conducting a field trial is to determine whether the bio-fertilizer bring formulated would still be able to enhance the growth of *N. cadamba* seedlings when grown in an environment with many other interacting conditions.

**Chapter 6: Conclusions and further recommendations**

Chapter 6 describes the overall findings of this study. In this chapter, the future recommendations for this study would also be discussed.
CHAPTER 2: FORMULATION OF BIO-FERTILIZER FOR

Neolamarckia cadamba
2.1 Introduction

Beneficial growth prompting living microbes that recycle major nutrients such as nitrogen, phosphorus and potassium are present naturally in soil. However, the populations of these microbes in the soil may vary, depending on the conditions of the soil (Sustainable Agriculture Research & Education 2012). Thus, bio-fertilizer application would ensure provision of beneficial microbes to the soil which in turn would provide plants with the necessary nutrients.

For the preparation of bio-fertilizer, fermentation of microbial culture via shake flask fermentation in the laboratory is usually preferred. It would be important to ensure that the microbial cultures contain high population of microbes through the usage of suitable media and medium additives suitable for the selected microbial strains. As carrier materials are often recommended for formulation of bio-fertilizers. It would be important to confirm that the microbial cultures are assimilated well and remain viable in the carrier material to form a stable bio-fertilizer.

This chapter will focus on the preparation of bio-fertilizer which includes the fermentation of bio-fertilizer beneficial microbes, determination of suitable carrier material and determination of the viability of various selected microbial strains in the carrier materials.

2.2 Materials and Methodology

2.2.1 Preparing microbial culture from glycerol stock solution

Glycerol stock cultures of nitrogen fixing microbe (*Streptomyces gramineus*), phosphate solubilizing microbe (*Serratia nematodiphila*), potassium solubilizing microbe (*Bacillus cereus* Strain I) and indole acetic acid producing microbe (*Bacillus cereus* Strain II) were obtained from a previous project conducted in Swinburne University of Technology Sarawak Campus on liquid bio-fertilizer for the same timber species, *N. cadamba* (Chua 2018). All the microbial strains selected were bacteria. Inoculating loop was inserted into
each stock culture and each loop of culture was inoculated onto nutrient agar plate. The plates were incubated at 28°C for 2 days.

2.2.2 Microbial growth curves of selected bio-fertilizer strains

The 250-mL Erlenmeyer flasks with screw caps each containing 75 mL nutrient broth were sterilized and left to cool down. The nutrient broth consisted of peptone, beef extract and sodium chloride (HiMedia Laboratories 2015). A loop of microbial colony was inoculated into the nutrient broth of these Erlenmeyer flasks. The inoculated flasks were incubated at 28 °C for 2 days on a rotary shaker at 160 rpm. In order to increase the volume of fermentation production, each of these 250-mL flasks containing the growing culture were then inoculated into a 1-L Erlenmeyer flasks containing 750 mL of fresh sterile nutrient broth. The cultures in the flasks were incubated on a rotary shaker at 160 rpm, 28 °C.

During the fermentation of microbial culture in the 1-L Erlenmeyer flask, the optical density of the culture was measured at 600 nm for the first 6 hours and thereafter once daily for 5 days, until the microbial culture’s growth reached the slow-down phase. The slow-down phase is when the microbial population growth levels off due to rate of death and rate of cell division starting to equal (Virtual Amrita Laboratories Universalizing Education 2011). During the fermentation period, viable microbial concentration will also be determined daily by performing plate count. Based on the optical densities obtained, the microbe’s growth curve was plotted.

2.2.3 Parameters that affect microbial growth

2.2.3.1 Growth media

The 250-mL Erlenmeyer flasks with screw caps each containing 75 mL of growth media were sterilized and left to cool down. A loop of microbial colony was inoculated into Erlenmeyer flasks containing growth media as stated in Table 2.1. The inoculated flasks were left on a rotary shaker at 160 rpm, 28 °C for 2 days. The cultures in the flasks were
then inoculated into 1-L Erlenmeyer flasks containing 750 mL of similar growth media as stated in Table 2.1 and left to ferment on a rotary shaker at 28 °C for 2 to 3 days. After fermentation period, the viable microbial concentrations were determined by plate count method.

**Table 2.1: Culturing of microbes in different growth media.**

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Growth Media</th>
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<tbody>
<tr>
<td><strong>Streptomyces gramineus</strong> (Nitrogen fixing microbe)</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td></td>
<td>Tryptic soy broth</td>
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<tr>
<td><strong>Serratia nematodiphila</strong> (Phosphate solubilizing microbe)</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td></td>
<td>Pikovskaya broth</td>
</tr>
<tr>
<td><strong>Bacillus cereus Strain I</strong> (Potassium solubilizing microbe)</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td></td>
<td>Aleksandrov broth</td>
</tr>
<tr>
<td><strong>Bacillus cereus Strain II</strong> (Indole acetic acid producing microbe)</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td></td>
<td>Lysogeny broth</td>
</tr>
</tbody>
</table>
2.2.3.2 Medium additives

The 250-mL Erlenmeyer flasks with screw caps each containing 75 mL growth medium were sterilized and left to cool down. A loop of microbial colony was inoculated into each of these Erlenmeyer flasks with the addition of additives as stated in Table 2.2.

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Growth Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces gramineus</em> (Nitrogen fixing microbe)</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td></td>
<td>Tryptic soy broth + 0.1 % glucose</td>
</tr>
<tr>
<td></td>
<td>Tryptic soy broth + 0.3 % yeast</td>
</tr>
<tr>
<td><em>Serratia nematodiphila</em> (Phosphate solubilizing microbe)</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.1 % glucose</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.3 % yeast</td>
</tr>
<tr>
<td><em>Bacillus cereus Strain I</em> (Potassium solubilizing microbe)</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.1 % glucose</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.3 % yeast</td>
</tr>
<tr>
<td><em>Bacillus cereus Strain II</em> (Indole acetic acid producing microbe)</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.1 % glucose</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.3 % yeast</td>
</tr>
</tbody>
</table>

The inoculated flasks were left on a rotary shaker at 160 rpm, 28 °C for 2 days. The cultures in the flasks were then inoculated into 1-L Erlenmeyer flasks containing 750 mL of the same growth media as stated in Table 2.2 and left to ferment on a rotary shaker at 28 °C for 2 to 3 days. After fermentation period, the viable microbial colonies were determined via plate count method.

2.2.4 Carrier materials analysis

2.2.4.1 pH

A ratio of 1:2.5 of carrier material to water [gram to gram] was used when measuring the pH of carrier materials (Okereke & Okeh 2007). Approximately, 2 g of cocopeat was
added into a 10-mL beaker containing 5 mL of water. The mixture was stirred with a glass rod. Electrode of a pH meter was inserted into the solution in order to determine the pH of cocopeat. Similar steps were repeated for the pH analysis of other carrier materials such as vermiculite, sawdust, compost and charcoal.

2.2.4.2 Nitrogen, phosphorus and potassium contents

The nitrogen, phosphorus and potassium content of carrier materials were tested by Sarawak Plantation Services Sdn Bhd, an outsourced accredited laboratory located in Kuching, Sarawak. The analysis method being carried out was based on Malaysian Standard Plant Chemical Analysis; MS 677: Parts III, IV, V: 1980.

2.2.4.3 Water holding capacity

Prior to the experiment, carrier materials were dried in an oven for 3 days at 100 °C to eliminate moisture content present in the carrier materials. After the removal of moisture content from carrier materials, 10 g of each dried carrier material (DCM) was weighed and placed on a folded filter paper placed in a filter funnel. The filter funnel was fitted on top of a conical flask. Maximum amount of water was added to thoroughly wet the carrier material. Excess water not absorbed by the carrier material was left to drip into the conical flask. When dripping stopped, the mass of the wet carrier material (WCM) was weighed again. The WCM is the mass of carrier material with the maximum capacity of water it can hold. Water holding capacity (WHC) of each carrier material was calculated using Equation 2.1 and Equation 2.2 (Arora, Tiwari & Singh 2014).

i) To determine the amount of water absorbed by carrier material

   Equation 2.1:
   \[ W (g) = WCM (g) – DCM (g) \]

ii) To determine the water holding capacity of carrier material

   Equation 2.2:
   \[ WHC (mL/g) = \frac{W (mL)}{10 (g)} \]
2.2.4.4 Assimilation of microbes in carrier materials

The carrier materials in this study included cocopeat, vermiculite, sawdust, compost and charcoal. All carrier materials were milled and passed through 100-mesh sieve. Carrier materials that were not neutral in nature were neutralized with calcium carbonate (1%) w/w (My Agriculture Information Bank 2015). Approximately, 1 g of each carrier material was placed into universal bottles and sterilized in an autoclave to eliminate contaminants.

After sterilization, the universal bottles containing the carrier materials were dried in an oven for 3 days at 100 °C to eliminate the moisture before being mixed with 500 µl of >10^8 CFU mL^-1 microbial inoculum. The microbial inoculums were left to assimilate in carrier materials for 30 days at 28 °C. After 15 and 30 days, the viable microbial concentration in each carrier material was determined by plate count method.

2.2.5 Bio-fertilizer formulation

2.2.5.1 Formulation of carrier-based or solid bio-fertilizer

Carrier materials were sieved and cocopeat which was acidic in nature, was neutralized by adding 1 % (w/w) of calcium carbonate. Each carrier material weighted 200 g was inserted into a 300 x 400 mm biohazard bag. The bags were tied using cable ties and sterilized. After sterilization, the tied bags of carrier materials were dried in an oven for 3 days at 100 °C to eliminate moisture with. Dried bags of carrier materials were placed in a biological cabinet and UV light was turned on for 30 minutes to further sterilize the carrier materials as this would lower down the risk of contamination. A volume of 400 mL of nitrogen fixing microbial inoculant from the shake flask fermentation was added into the 200 g of carrier materials. The mixture was mixed thoroughly before the bag was sealed using an electric sealer. Two third of the space was left in the sealed bag for aeration (Kaljeet, Keyeo & Amir 2011). The bag was left to cure at 28 °C for 15 days (My Agriculture Information Bank 2015). Curing is a process for microbial inoculants to adapt to the carrier material and increase their tolerance to drying (Albareda et al. 2008). The same steps were repeated for phosphorus solubilizing, potassium solubilizing and
IAA producing microbial inoculants. All the four bags containing carrier materials with different microbial inoculants were mixed together in a 500 x 570 mm biohazard bag prior to usage in pot and field trials.

2.2.5.2 Comparing viabilities of selected microbial strains in liquid and carrier-based or solid bio-fertilizer

Microbial inoculants from the shake flask fermentation were distributed into two batches, one for the preparation of liquid bio-fertilizer, the other carried based or solid bio-fertilizer.

For the manufacturing of liquid bio-fertilizer, 10 mL of microbial inoculant from each 1-L Erlenmeyer flask containing nitrogen fixing microbe, phosphate solubilizing microbe, potassium solubilizing microbe and IAA producing microbe was pipetted and added into sterile universal bottles respectively. The bottles were incubated at 28°C for 180 days.

For the manufacturing of carrier-based bio-fertilizer, 400 mL of microbial inoculant from each 1-L Erlenmeyer flask containing nitrogen fixing microbe, phosphate solubilizing microbe, potassium solubilizing microbe and IAA producing microbe was added into 300 x 400 mm biohazard bags, each containing sterilized dry carrier materials respectively. The microbial inoculant and carrier material were mixed thoroughly before the bags were sealed using an electric sealer and incubated at 28°C for 180 days.

Viable microbial concentration in both liquid bio-fertilizer and carrier-based bio-fertilizer was determined by plate count at 60 days and 180 days respectively. The ability of microbes in bio-fertilizer to fix nitrogen, solubilize phosphate, solubilize potassium and produce IAA were confirmed by culturing them on Jensen agar, Pikovskaya agar, Aleksandrov agar or in Trytophan broth.
2.3 Result and Discussion

2.3.1 Microbial growth curves of selected bio-fertilizer strains

By understanding each of the microbial strain’s growth curve, the growth of each strain could be controlled. In this study, all the bio-fertilizer microbes being analysed were bacterial strains. Bacteria reproduce asexually whereby they undergo binary fission, splitting a single cell into two similar cells. In this study, the bio-fertilizer bacteria were grown in a closed system whereby no additional medium was added and no waste was removed. In this type of system, bacterial growth curve will compose of lag phase, exponential or log phase, stationary phase and death phase (Figure 2.1).

Figure 2.1: Growth curves of selected strains used in the formulation of bio-fertilizer for *N. cadamba*. 
Generation or doubling time, which is time taken for bacteria cells in the flask to double in number, was determined using equation formula (Equation 2.3) (Neidhardt, Ingraham & Schaechter 1990). The calculated generation times were tabulated in Table 2.3.

Equation 2.3:
\[ g = \frac{\ln 2}{k} = \frac{0.693}{k} \]

where \( g \) = generation/doubling time
\( k \) = growth rate constant

Table 2.3: Growth rate constant and generation/doubling time of selected bacterial strains.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Growth rate constant, K (hr(^{-1}))</th>
<th>Generation/Doubling time, g (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces gramineus</em> (Nitrogen fixing microbe)</td>
<td>0.0176</td>
<td>39.38</td>
</tr>
<tr>
<td><em>Serratia nematodiphila</em> (Phosphate solubilizing microbe)</td>
<td>0.0234</td>
<td>29.61</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> Strain I (Potassium solubilizing microbe)</td>
<td>0.0317</td>
<td>21.86</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> Strain II (Indole acetic acid producing microbe)</td>
<td>0.0247</td>
<td>28.06</td>
</tr>
</tbody>
</table>

It can be seen that bacterial strain with higher growth rate constant had shorter generation time. By judging the growth curves in Figure 2.1, *Bacillus cereus* Strain I with the shortest generation time required only 36 hours on rotary shaker till completion of exponential phase. *Serratia nematodiphila* and *Bacillus cereus* Strain II bacterial strains with similar generation times required 48 hours on rotary shaker while *Streptomyces gramineus* with the highest generation time required 60 hours on rotary shaker till completion of exponential phase.
2.3.2 Parameters that affect microbial growth

For carried based bio-fertilizer, the population of microbe was recommended to be $10^8$ cells per gram of carrier material (FNCA Biofertilizer Project Group 2006; Sreedhar & Mohan 2016). Thus, it is recommended that the bacterial cultures being fermented have a high cell count of at least $10^8$ CFU mL$^{-1}$. Parameters such as growth media, medium additives and temperature that would affect bacteria population are studied, with an aim which is to increase bacterial population.

2.3.2.1 Growth media

Bacteria would require suitable biochemical and biophysical environment to multiply. Biochemical environment or also known as nutritional environment is the growth media being supplied to bacteria for their growth, either for the purpose of isolating or growing pure batch cultures. Each growth media being designed is different in the aspect of nutrient components as each caters for bacteria with specific growth requirements.

In this study, the growth media that acted as control was nutrient broth. It is a liquid general purpose medium made of peptone and beef extract that can support the growth of most bacteria that are not nutrient demanding (Laboratorios Conda 2010). Based on literature reviews on the four bacterial strains (Streptomyces gramineus, Serratia nematodiphila, Bacillus cereus Strain I and Bacillus cereus Strain II), other recommended growth media other than nutrient broth which might be suitable for their respective growth are tryptic soy broth, pikovskaya broth and lysogeny broth. Tryptic soy broth is a nutritious medium which can support a wide range of aerobic bacteria (BD 2014), pikovskaya broth for the culturing of phosphate solubilizing bacteria (HiMedia Laboratories 2012) and lysogeny broth which is a nutrient-rich media that allows rapid bacterial growth. All the recommended media acted as the variable media in this study. Table 2.4 summarizes the result which shows the population of bacteria grown in each growth media.
Table 2.4: Population of bacteria grown in different growth media.

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Growth Media</th>
<th>Population of Bacteria (CFU mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptomyces gramineus</strong></td>
<td>Nutrient broth</td>
<td>2.50 X 10⁵</td>
</tr>
<tr>
<td>(Nitrogen fixing microbe)</td>
<td>Tryptic soy broth</td>
<td>2.00 X 10⁷</td>
</tr>
<tr>
<td><strong>Serratia nematodiphila</strong></td>
<td>Nutrient broth</td>
<td>5.24 X 10⁸</td>
</tr>
<tr>
<td>(Phosphate solubilizing microbe)</td>
<td>Pikovskaya broth</td>
<td>3.02 X 10⁶</td>
</tr>
<tr>
<td><strong>Bacillus cereus Strain I</strong></td>
<td>Nutrient broth</td>
<td>6.00 X 10⁸</td>
</tr>
<tr>
<td>(Potassium solubilizing microbe)</td>
<td>Lysogeny broth</td>
<td>5.10 X 10⁶</td>
</tr>
<tr>
<td><strong>Bacillus cereus Strain II</strong></td>
<td>Nutrient broth</td>
<td>1.95 X 10⁹</td>
</tr>
<tr>
<td>(Indole acetic acid producing microbe)</td>
<td>Lysogeny broth</td>
<td>2.73 X 10⁷</td>
</tr>
</tbody>
</table>

Based on the results tabulated in Table 2.4, growth of *Serratia nematodiphila*, *Bacillus cereus* Strain I and *Bacillus cereus* Strain II were better in Nutrient broth compared to growth in another growth media. The nutrient components in Nutrient broth such as peptone and yeast extract, provided bacteria with nitrogenous compounds. Its other components like amino acids and vitamin B complex (MP Biomedicals 2018) aided in supporting the growth of *Serratia nematodiphila*, *Bacillus cereus* Strain I and *Bacillus cereus* Strain II. Growth of *Streptomyces gramineus* was better in Tryptic soy broth compared to growth in Nutrient broth. This might be due to Tryptic soy broth containing some other vitamins and amino acid (Abd El-Salam, Abd El-Ghany & El-Tahan 2010) that supported the growth of *Streptomyces gramineus*. To further enhance the growth of bacteria and preserve their population in the selected growth media, additives were added as stated in section 2.2.3.2.

2.3.2.2 Medium additives

The growth of soil bacteria is usually influenced by the availability of nutrients which would provide soil bacteria with energy to multiply and perform cellular biosynthesis (Todar 2012). Previous studies had shown that the addition of carbon source such as glucose during the fermentation of soil microbes would be important to stimulate bacterial growth, albeit it has to be in small amount of 0.1%. High concentrations of glucose may
cause a decline in the growth of bacteria (Reischke, Kumar & Bääth 2015). Another type of medium additive that could enhance growth of soil bacteria is yeast extract. Yeast extract provides nitrogen (Costa et al. 2002), vitamin and protein sources which could provide a good growth condition for the multiplication of bacteria (Chaitanya Biologicals Private Limited n.d.). The recommended amount of yeast extract to be added to bacteria cultured in growth media is 0.3 % (Neogen Corporation 2018).

Table 2.5 summarizes the result which shows the population of bacteria grown in each growth media added with different additives.

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Growth Media</th>
<th>Population of Bacteria (CFU mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces gramineus</em> (Nitrogen fixing microbe)</td>
<td>Tryptic soy broth</td>
<td>2.00 X 10⁷</td>
</tr>
<tr>
<td></td>
<td>Tryptic soy broth + 0.1 % glucose</td>
<td>1.21 X 10⁴</td>
</tr>
<tr>
<td></td>
<td>Tryptic soy broth + 0.3 % yeast</td>
<td>1.02 X 10⁸</td>
</tr>
<tr>
<td><em>Serratia nematodiphila</em> (Phosphate solubilizing microbe)</td>
<td>Nutrient broth</td>
<td>5.24 X 10⁸</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.1 % glucose</td>
<td>2.70 X 10⁸</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.3 % yeast</td>
<td>2.10 X 10⁹</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> Strain I (Potassium solubilizing microbe)</td>
<td>Nutrient broth</td>
<td>6.00 X 10⁸</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.1 % glucose</td>
<td>2.55 X 10⁸</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.3 % yeast</td>
<td>8.00 X 10⁸</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> Strain II (Indole acetic acid producing microbe)</td>
<td>Nutrient broth</td>
<td>1.95 X 10⁹</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.1 % glucose</td>
<td>7.82 X 10⁸</td>
</tr>
<tr>
<td></td>
<td>Nutrient broth + 0.3 % yeast</td>
<td>1.00 X 10¹⁰</td>
</tr>
</tbody>
</table>

Based on the results, it can be seen that growth media added with 0.3 % yeast extract enhanced the growth of *Streptomyces gramineus, Serratia nematodiphila, Bacillus cereus* Strain I and *Bacillus cereus* Strain II. Bacterial population in nutrient broth added with 0.1 % glucose did not show growth enhancement. In fact, bacterial population in culture added with glucose showed a decline in growth when compared to the control – nutrient broth. Growth decline in bacteria was probably due to the catabolism of amino acids for...
energy after the depletion of glucose being added (Robbins & Taylor 1989). Once amino acids were catabolized, nitrogen was given off as ammonia while the separated carbon was converted into energy source. An increase in ammonia caused a rise in pH of the culture and eventually led to a decline in bacterial growth (Robbins & Taylor 1989).

2.3.3 Analysis of carrier materials

Carrier materials are important in the manufacturing of bio-fertilizers as they provide protective microenvironment or niche to the microbial inoculants. They ensure that the environment is suitable for the colonization of microbes. When choosing potential carrier materials, criteria such as pH, nutrient content and water holding capacity are examined. The pH of carrier material should be around 6.5 to 7.0. Based on Table 2.6, all the carrier materials including cocopeat which had been neutralized by the addition of calcium carbonate, had pH 7.0 to 8.0, which is slightly alkaline. Vermiculite which is slightly more alkaline compared to the other carrier materials may be suitable for microbial inoculants which require neutral to alkaline condition to grow.

Table 2.6: The physical and chemical properties of carrier materials.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Cocopeat + calcium carbonate</th>
<th>Vermiculite</th>
<th>Sawdust</th>
<th>Compost</th>
<th>Charcoal</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.00</td>
<td>7.66</td>
<td>7.12</td>
<td>7.05</td>
<td>7.11</td>
</tr>
<tr>
<td>Total nitrogen (%)</td>
<td>0.51</td>
<td>0.14</td>
<td>0.48</td>
<td>1.67</td>
<td>0.23</td>
</tr>
<tr>
<td>Total phosphorus (%)</td>
<td>0.06</td>
<td>0.09</td>
<td>0.03</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>Total potassium (%)</td>
<td>1.30</td>
<td>2.98</td>
<td>0.03</td>
<td>0.24</td>
<td>0.14</td>
</tr>
<tr>
<td>Water holding capacity (mL/g)</td>
<td>1.98</td>
<td>0.96</td>
<td>0.64</td>
<td>0.73</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Carrier materials should contain nutrients made available to both the microbial inoculants during storage. During application in soil, microbes would also require nutrients from carrier materials as nutrients in soil would be limited due to competition between bio-
fertilizer microbes and native soil microbes (FNCA Biofertilizer Project Group 2006). Laboratory results showed that all the selected carrier materials contained nitrogen, phosphorus and potassium but in different quantities. Nitrogen content was highest in compost while phosphorus and potassium contents were highest in vermiculite. Cocopeat had the second highest nitrogen and potassium contents while compost had the second highest phosphorus content.

Carrier materials should also possess high water holding capacity as they can absorb more microbial inoculant broth. High moisture level is desirable in the manufactured biofertilizer as moisture is constantly lost during storage and the survivability of microbial inoculant would be low if the moisture level is low (College of Tropical Agriculture and Human Resources 2018). In this study, results showed that cocopeat had the highest water holding capacity followed by vermiculite.

The ability of carrier materials to sustain microbial population another important criterion to study. Table 2.7 shows the viable microbial concentration of each microbe in different carrier materials at 15 and 30 days respectively.

Table 2.7: Population of microbes in different carrier materials after 15 and 30 days.

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Day</th>
<th>Cocopeat</th>
<th>Vermiculite</th>
<th>Sawdust</th>
<th>Compost</th>
<th>Charcoal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptomyces gramineus</strong> (Nitrogen fixing microbe)</td>
<td>0</td>
<td>1.30 X 10^8</td>
<td>1.30 X 10^8</td>
<td>1.30 X 10^8</td>
<td>1.30 X 10^8</td>
<td>1.30 X 10^8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.24 X 10^8</td>
<td>0</td>
<td>0</td>
<td>Contaminated</td>
<td>Contaminated</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.20 X 10^8</td>
<td>0</td>
<td>0</td>
<td>Contaminated</td>
<td>Contaminated</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>15</td>
<td>30</td>
<td>0</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Serratia nematodiphila</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Phosphate solubilizing microbe)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.38 X 10^9</td>
<td>2.38 X 10^9</td>
<td>2.38 X 10^9</td>
<td>2.38 X 10^9</td>
<td>2.38 X 10^9</td>
<td>2.38 X 10^9</td>
</tr>
<tr>
<td></td>
<td>2.20 X 10^9</td>
<td>2.90 X 10^8</td>
<td>0</td>
<td>Contaminated</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.84 X 10^9</td>
<td>0</td>
<td>0</td>
<td>Contaminated</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus Strain I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Potassium solubilizing microbe)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.81 X 10^8</td>
<td>7.81 X 10^8</td>
<td>7.81 X 10^8</td>
<td>7.81 X 10^8</td>
<td>7.81 X 10^8</td>
<td>7.81 X 10^8</td>
</tr>
<tr>
<td></td>
<td>5.50 X 10^8</td>
<td>4.20 X 10^7</td>
<td>0</td>
<td>Contaminated</td>
<td>Contaminated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.03 X 10^8</td>
<td>0</td>
<td>0</td>
<td>Contaminated</td>
<td>Contaminated</td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus Strain II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Indole acetic acid producing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>microbe)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.12 X 10^10</td>
<td>1.12 X 10^10</td>
<td>1.12 X 10^10</td>
<td>1.12 X 10^10</td>
<td>1.12 X 10^10</td>
<td>1.12 X 10^10</td>
</tr>
<tr>
<td></td>
<td>5.36 X 10^9</td>
<td>0</td>
<td>0</td>
<td>Contaminated</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.83 X 10^9</td>
<td>0</td>
<td>0</td>
<td>Contaminated</td>
<td>Contaminated</td>
<td></td>
</tr>
</tbody>
</table>

Note: Population of microbes in CFU g\(^{-1}\).
Based on the results tabulated in Table 2.7, not all the carrier materials were able to support the growth of microbes. Although sawdust had been mentioned as a suitable carrier material, it did not support the growth of *Streptomyces gramineus*, *Serratia nematodiphila*, *Bacillus cereus* Strain I and *Bacillus cereus* Strain II. Compost and charcoal could support the growth of most microbes but did not qualify as a suitable carrier material as they were unable to maintain low level of contamination during the 30 days’ study. Meanwhile, vermiculite which was able to maintain low level of contamination would only support the growth of *Serratia nematodiphila* and *Bacillus cereus* strain II. Cocopeat was the only carrier material that was able to support the growth of all the microbes (*Streptomyces gramineus*, *Serratia nematodiphila*, *Bacillus cereus* Strain I and *Bacillus cereus* Strain II), at the same time maintaining low level of contamination. When microbes assimilated in cocopeat on day 0, they started to multiply and thus, the microbial population was high on both day 15 and 30.

Cocopeat was chosen as the carrier material to be used for bio-fertilizer formulation as it supported the growth of all the beneficial microbes that would be used in the formulation.

### 2.3.4 Viabilities of selected microbial strains in liquid and carrier-based bio-fertilizer

Bio-fertilizer could be prepared either as liquid or solid form (Ngampimol & Kunathigan 2008). Liquid bio-fertilizer is inconvenient to be brought to the field as it may cause spillage during transportation. Thus, a carrier-based or solid bio-fertilizer is preferable as it is easier to be transported for usage in the field and is able to improve the vitality of microbes (van Veen, van Overbeek & van Elsas 1997). In this part of the study, the viabilities of selected microbial strains in liquid and carrier-based bio-fertilizer were studied.

The population of viable microbes in both liquid and carrier-based bio-fertilizer were determined at 90 and 180 days respectively. The results were tabulated in Table 2.8 and Table 2.9.
<table>
<thead>
<tr>
<th>Microbe</th>
<th>0 day</th>
<th>90 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptomyces gramineus</strong> (Nitrogen fixing microbe)</td>
<td>$1.50 \times 10^8$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Serratia nematodiphila</strong> (Phosphate solubilizing microbe)</td>
<td>$1.81 \times 10^9$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Bacillus cereus Strain I</strong> (Potassium solubilizing microbe)</td>
<td>$1.77 \times 10^8$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Bacillus cereus Strain II</strong> (Indole acetic acid producing microbe)</td>
<td>$1.02 \times 10^{10}$</td>
<td>$1.6 \times 10^6$</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Population of microbes in CFU g$^{-1}$. 
Table 2.9: Population of viable microbes in carrier-based bio-fertilizer.

<table>
<thead>
<tr>
<th>Microbe</th>
<th>0 day</th>
<th>90 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces gramineus</em></td>
<td>$1.50 \times 10^8$</td>
<td>$1.04 \times 10^8$</td>
<td>$1.96 \times 10^7$</td>
</tr>
<tr>
<td>(Nitrogen fixing microbe)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Serratia nematodiphila</em></td>
<td>$1.81 \times 10^9$</td>
<td>$3.38 \times 10^8$</td>
<td>$1.08 \times 10^7$</td>
</tr>
<tr>
<td>(Phosphate solubilizing microbe)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus Strain I</em></td>
<td>$1.77 \times 10^8$</td>
<td>$1.00 \times 10^8$</td>
<td>$1.50 \times 10^7$</td>
</tr>
<tr>
<td>(Potassium solubilizing microbe)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus Strain II</em></td>
<td>$1.02 \times 10^{10}$</td>
<td>$2.00 \times 10^9$</td>
<td>$1.40 \times 10^8$</td>
</tr>
<tr>
<td>(Indole acetic acid producing microbe)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Population of microbes in CFU g$^{-1}$.

By comparing the microbe populations in both Table 2.8 and 2.9, it could be observed that the viabilities of all microbial strains were supported by carrier material. Although the microbe population in carrier-based bio-fertilizer reduced every 90 days, the reduction was not drastic. Meanwhile, the microbe population in liquid bio-fertilizer reduced drastically during the 180 days’ study. Liquid bio-fertilizer containing microbes (*Streptomyces gramineus, Serratia nematodiphila, Bacillus cereus* Strain I and *Bacillus cereus* Strain II) respectively did not show any growth signs at 180 days. At 90 days, only *Bacillus cereus* Strain II still had viable microbial cells. All microbes which showed growth signs were cultured on Jensen agar, Pikovskaya agar, Aleksandrov agar or in Nutrient broth added with tryptophan respectively to determine whether these microbes still possess their ability to fix nitrogen, solubilize phosphate, solubilize potassium or produce IAA. Results showed that all microbes being cultured still retained their ability.
From the results, it can be shown that all of the selected beneficial strains (*Streptomyces gramineus*, *Serratia nematodiphila*, *Bacillus cereus* Strain I and *Bacillus cereus* Strain II) used in the formulation of bio-fertilizer assimilated well in cocopeat. This study proved that carrier-based bio-fertilizer supported the viabilities of all microbial strains better compared to liquid bio-fertilizer. Thus, the formulated bio-fertilizer used in this study will be carrier-based.

### 2.4 Summary

This chapter reported how the selected beneficial microbial strains isolated from previous study were fermented and formulated as solid carrier-based bio-fertilizer. The selected strains were *Streptomyces gramineus*, *Serratia nematodiphila*, *Bacillus cereus* Strain I and *Bacillus cereus* Strain II. The growth of *Serratia nematodiphila*, *Bacillus cereus* Strain I and *Bacillus cereus* Strain II were best in nutrient broth added with 0.3 % yeast extract, while growth of *Streptomyces gramineus* was best in tryptic soy broth added with 0.3 % yeast. Nutrient broth supports the growth of less fastidious bacteria (HiMedia Laboratories 2015) while tryptic soy broth supports the growth of bacteria that are not extremely fastidious (BD 2014). All the beneficial microbes multiplied via shake flask fermentation in their respective growth media added with additives. The optimum duration for the fermentation was 48 to 72 hours varied among the selected bacterial strains, as analysed in their respective exponential growth curves.

As liquid bio-fertilizer is inconvenient to be transported, carrier-based bio-fertilizer will be preferable. Among the five carrier materials being studied, the suitable carrier material for the beneficial microbes was cocopeat. Cocopeat was able to support the growth of microbes assimilated in it due to its suitable physical and chemical properties. It had neutral pH after being neutralized by calcium carbonate, average levels of NPK and high water holding capacity (Yuvaraj 2016). Prior to usage, cocopeat was neutralized with calcium carbonate. Inoculants of beneficial microbes from shake flask fermentation were mixed with cocopeat according to its water holding capacity, which was 400 mL of inoculant with 200 g of cocopeat.
The viabilities of carrier-based bio-fertilizer being formulated and liquid bio-fertilizer was tested. The microbial population of each bacteria was determined at 90 days and 180 days respectively. Results showed that population of viability microbes in carrier-based bio-fertilizer was higher compared to the population of viable microbes in liquid bio-fertilizer. This results indicate that carrier-based bio-fertilizer was able to maintain the vitalities of microbes better than liquid bio-fertilizer as carrier materials could protect microbes from biotic and abiotic stresses (van Veen, van Overbeek & van Elsas 1997).

Thus, carrier-based bio-fertilizer will be formulated according to the methods stated above in section 2.2.5.1. After the bio-fertilizer had been cured for at least 15 days, the bio-fertilizer being prepared would be brought to experimental trial locations for further studies.
CHAPTER 3: RHIZOPOD ASSAY TO STUDY THE EFFECTIVENESS OF BIO-FERTILIZER ON THE RATE OF ROOT ELONGATION OF *Neolamarckia cadamba*
3.1 Introduction

The optimum growing conditions for *Neolamarckia cadamba* are soil which is moist and also rich with nutrients. Nutrients can be provided in the form of fertilizers, however, finding lands near water sources will be challenging. The growth potential of *N. cadamba* will be reduced if this species is planted in soils that are low in moisture.

For *N. cadamba* growing naturally in the forest, the main source of water would be from the rainfall as it would be unlikely to have nearby permanent water intake points. Forest plantation would receive rain water but during mid-year, from June to August, plantation soil would be dry as there will be less or no rain (Ministry of Resource Planning and Environment 2012).

Similar to any other plants, *N. cadamba* would need to adapt to water-limited environments, such as by growing their roots to reach out to search for water. Based on Charles Darwin’s hypothesis, the tip of a plant’ root is reckoned to be like the brain of the plant (Baluška et al. 2009). The root would be able to sense for water and move towards it (Bao et al. 2014). The rate of root elongation is known to be influenced by an important auxin known as indole acetic acid (IAA) (Pacheco-Villalobos et al. 2016).

In this study, the bio-fertilizer which was formulated to include the IAA producing microbes apart from nitrogen-fixing, phosphorus solubilizing and potassium solubilizing microbes. Indole acetic acid stimulates cell elongation of roots by decreasing cell wall pressure and increasing osmotic contents, permeability of water and protein synthesis (Mohite 2013). The above mentioned modifications made to root cells would stimulate root elongation of *N. cadamba*. Currently, there is only one study conducted by Chua (2018) on the effect of IAA on *N. cadamba* and only some on timber species such as *Eucalyptus camaldulensis* (Karthikeyan & Sakthivel 2011) and *Dalbergia sissoo* (Singh, Yadav & Bhatt 2012).

This chapter will focus on studying whether IAA producing microbes will be able to enhance the root elongation of *N. cadamba* using rhizopod assay. In rhizopod assay, *N. cadamba* seedlings growing in rhizopods were placed in a water table filled with water. The rhizopod assay design was based on the design by Mahoney & Rood (1990). With
modifications, the rhizopod assay design initially used for Banksia species (Canham, Froend & Stock 2015) was adopted for Neolamarckia cadamba. ‘Rhizopod’ was the name given to the apparatus used to grow seedlings as ‘rhizo’ represented root while ‘pods’ represented growth cylinders (Mahoney & Rood 1990).

3.2 Materials and Methodology

3.2.1 Germination of Neolamarckia cadamba seedlings

Neolamarckia cadamba seeds originated from Kubah and Lambir, Sarawak were provided in kind by Sarawak Forestry Corporation (SFC). The seeds were sown in sowing medium containing compost and compound coated chemical fertilizer added with MgO (NPK 15:15:15) in a ratio of 12:1 based on SFC’s nursery practice. The rectangular tray containing seeds and sowing medium was placed in greenhouse and watered twice a day by using the water sprinkler system. Neolamarckia cadamba seeds were left to germinate for 120 days. Once the height of seedlings in the tray were around 5 cm tall, they were pricked and transplanted into rhizopod tubes containing potting media.

3.2.2 Design and preparation of rhizopod assay

Based on SFC’s nursery practice, potting medium was prepared based on ratio of 12 parts forest topsoil, 6 parts sand, 6 parts compost and 3 parts compound coated chemical fertilizer added with MgO (NPK 15:15:15). All these components were mixed in a concrete mixer before being used for rhizopods.

The 120-days old N. cadamba seedlings were pricked sowing medium and transplanted into rhizopods. The rhizopod assay design was modified based on the design by Mahoney & Rood (1990). Each rhizopod was made of PVC tube with diameter of 10 cm and height of 0.5 m. Prior to the transplant, the initial root length of each sampling was measured and recorded. A total of 45 rhizopods were filled with potting medium and the bottom ends were covered with gravel before being sealed using cotton fabric and placed inside a water table. Seedlings growing in rhizopods placed in water table is a simulation of
seedlings growing in a water limitation environment as water source is further away from the roots of seedlings. Seedlings in the rhizopods were treated under different treatments as shown in Table 3.1. The treatments listed in Table 3.1 are similar to the treatments studied in pot trial.

Figure 3.1: Rhizopods in water table.
Table 3.1: Treatment groups for rhizopod assay.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
<th>Sample Size (seedlings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td><em>Neolamarckia cadamba</em> seedlings were not treated with fertilizers, carrier material and bio-fertilizer microbes (acting as control)</td>
<td>9</td>
</tr>
<tr>
<td>T2</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with 5.0 g chemical fertilizer</td>
<td>9</td>
</tr>
<tr>
<td>T3</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with 2.5 g chemical fertilizer</td>
<td>9</td>
</tr>
<tr>
<td>T4</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with dual combination of 2.5 g chemical fertilizer with 20.0 g bio-fertilizer</td>
<td>9</td>
</tr>
<tr>
<td>T5</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with 20.0 g bio-fertilizer</td>
<td>9</td>
</tr>
</tbody>
</table>

Note: Bio-fertilizer consists of a mixture of carrier material and bio-fertilizer microbes (nitrogen fixing microbe, phosphate solubilizing microbe, potassium solubilizing microbe and IAA producing microbe).

Fertilizers were placed on the potting soil in each rhizopod, circling the seedling. *N. cadamba* seedlings were watered fortnightly (only once in the morning) using a watering can for a duration of 90 days. The water table maintained at 0.25 m would be the only water source for the seedlings during the period between watering. The seedlings were placed in a shaded area. During the 90 days of study, three random seedlings from each treatment were harvested as samples at intervals of 30 days at 30th, 60th and 90th day. Potting media with the seedling samples were taken out of the rhizopods. Potting media
were carefully removed from the roots of seedlings to avoid damaging the roots. Each of these seedling samples were removed from the potting medium together with the roots of seedlings. The root lengths were measured from the base of shoot till the tip of roots using measuring tape. The measurements were recorded in centimetre (cm) and the rate of root elongation of each seedling was calculated based on the following elongation formula (Equation 3.1) (Canham, Froend & Stock 2015).

Equation 3.1:
\[
\text{Root Elongation Rate; } \text{RER} = \frac{(L_2-L_1) \text{cm}}{(T) \text{day}}
\]

where
- \( L_2 \) = Final length of root;
- \( L_1 \) = Initial length of root;
- \( T \) = Number of days between root measurement

### 3.2.3 Data analysis

The data obtained from rhizopod assay were subjected to analysis of significance difference among the means of treatment groups by ANOVA at \( p \leq 0.05 \) using SPSS statistical software (version 23, IBM Corp. USA) to compare the effects due to treatment groups.

### 3.3 Result and Discussion

#### 3.3.1 Rate of root elongation of *Neolamarckia cadamba* seedlings between treatment groups

As shown in Figure 3.2, during the entire 90 days of experiment, treatments with bio-fertilizer, either entirely bio-fertilizer of 20.0 g, or in combination with 2.5 g of chemical fertilizer, had both shown to be superior in enhancing root elongation starting from 30\(^{th}\) day until 90\(^{th}\) day compared to treatments with chemical fertilizers and control.
Figure 3.2: The trend of rate of root elongation of *N. cadamba* seedlings throughout the 90 days rhizopod assay.

i) 30\textsuperscript{th} day of treatment

Based on statistical analysis using SPSS statistical software (version 23, IBM Corp. USA), at 30\textsuperscript{th} day of treatment, the dual combination of 2.5 g of chemical fertilizer with 20.0 g bio-fertilizer (T4) treatment had the highest mean rate of root elongation at 0.143 cm/day ($p \leq 0.05$), followed by seedlings treated with 20.0 g of bio-fertilizer treatment (T5) at 0.137 cm/day ($p \leq 0.05$). Seedlings treated with treatments consisting of bio-fertilizer showed significant difference in mean rate of root elongation, with p value $\leq 0.05$ when compared to control group.

Interestingly, as shown in Figure 3.3, seedlings treated with 5.0 g of chemical fertilizer (T2) had the lowest root elongation mean rate of 0.04 cm/day. The seedlings without any treatment (T1) having the second lowest rate at 0.06 cm/day, while the seedlings treated with 2.5 g of chemical fertilizer (T3) having the third lowest rate at 0.073 cm/day. All
there three treatments were also not significant from each other, thus suggesting that chemical fertilizers had no effect on root elongation after 30 days of treatment.

![Bar chart showing the mean rate of root elongation of N. cadamba seedlings on 30th day of treatment. Each bar in the graph represents the mean rate of elongation of N. cadamba seedlings ± SE (n = 3) under respective treatment group. Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).](image)

**Figure 3.3:** The mean rate of root elongation of *N. cadamba* seedlings on 30th day of treatment. Each bar in the graph represents the mean rate of elongation of *N. cadamba* seedlings ± SE (n = 3) under respective treatment group. Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

ii) **60th day of treatment**

At day 60, all *N. cadamba* seedlings treated with dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer (T4) had continue to demonstrate the highest mean rate of root elongation on day 60, 0.167 cm/day (p ≤ 0.05). Unlike 30th day, this mean was now more significant than the 20.0 g bio-fertilizer treatment (T5), which had the second highest mean rate of root elongation, 0.146 cm/day. The effects of root elongation noted since 30th day of treatment may have generated more roots, render the absorption of the chemical fertilizer to be more efficient. This absorption of fertilizer would in turn
supported higher root growth. This explain why T4 treatment with the mixed bio-fertilizer and chemical fertilizer, was more superior than T5 treatment, which had only bio-fertilizer.

As shown in Figure 3.4, mean root elongation rate of *N. cadamba* seedlings treated with 2.5 g of chemical fertilizer (T3) was 0.067 cm/day (p ≤ 0.05). This was significantly higher if compared to seedlings treated with 5.0 g of chemical fertilizer (T2) which showed mean root elongation rate of 0.046 cm/day. At this point, it showed that higher chemical fertilizer application of 5.0 g had no significant effect on root elongation if compared to lower application of 2.5 g.

![Figure 3.4: The mean rate of root elongation of *N. cadamba* seedlings on 60th day of treatment. Each bar in the graph represents the mean rate of elongation of *N. cadamba* seedlings ± SE (n = 3) under respective treatment group. Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).](image-url)
iii) 90th day of treatment

The root elongation effects of various treatments on day 90th day were the same as 60th day except that by 90th day, there was no significant difference (p ≤ 0.05) between T4 treatment (20.0 g of bio-fertilizer with 2.5 g of chemical fertilizer) and T5 treatment (20.0 g of bio-fertilizer). Based on Figure 3.5, seedlings treated with T4 treatment had the highest mean rate of root elongation at 0.207 cm/day, while seedlings under T5 treatment had the second highest mean rate of root elongation at 0.188 cm/day. By 90th day, the root growth and elongation may have reached the optimum level for both treatments.

Following the trend as observed in 60th day, upon 90th day, the mean rate of root elongation of N. cadamba seedlings treated with 2.5 g chemical fertilizer (T3), 0.076 cm/day (p ≤ 0.05) was still significantly higher than those treated with 5.0 g chemical fertilizer (T2), which was 0.037 cm/day.
Figure 3.5: The mean rate of root elongation of *N. cadamba* seedlings on 90<sup>th</sup> day of treatment. Each bar in the graph represents the mean rate of elongation of *N. cadamba* seedlings ± SE (n = 3) under respective treatment group. Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

The enhancement of root growth could be attributed to the IAA producing bacteria, *Bacillus cereus* Strain II, which present in the bio-fertilizer formulation. *B. cereus* Strain II used in this experiment was shown to produce IAA activities at a value of 0.92 µg/mL in previous study (Chua 2018). As such, root elongation effect of this bio-fertilizer formulation was as anticipated. Another study by Ghost et al. (2003) had also shown that *Bacillus* spp was able to enhance root growth in *Brassica campestris* seedlings ten days after germination by synthesizing IAA.

The slower root elongation due to higher chemical fertilizer was out of our expectation. The rhizopod assay was designed for *N. cadamba* seedlings to grow in a water-limited environment. Higher application of 5.0 g chemical fertilizer to seedlings in the rhizopod
assay might have caused higher osmotic pressure in rhizopod soil rather than in seedlings’ roots (Hammer 2000). This reverse osmotic pressure occurred once water in soil started to dry out, causing the concentration of nutrients in soil to rise as nutrients could not evaporate like water (Hammer 2000). The higher osmotic pressure in soil than the inside of *N. cadamba* seedlings’ root cells would cause root cells to start losing water (Hammer 2000). Dehydration of root system would slow down rate of root elongation as roots could not elongate when their elongation zones lose turgescence (Lawlor 1969).

The results from this rhizopod assay showed that dual combination of 20.0 g of bio-fertilizer with 2.5 g of chemical fertilizer treatment could increase the root growth of *N. cadamba* seedlings as seedlings treated with this treatment had the highest mean rate of root elongation from 30th to 90th day of the rhizopod assay. Previous study conducted on *Paulownia kawakamii* also showed that the application of dual combination of chemical fertilizer with bio-fertilizer increased the root growth of *Paulownia kawakamii* seedlings (Farahat et al. 2014). *Neolamarckia cadamba* and *P. kawakamii* are both fast growing timber tree species which could be found in Asia.

### 3.4 Summary

This chapter describes the study used to determine whether the bio-fertilizer, which contains indole acetic acid producing bacteria, would enhance the root elongation of *N. cadamba* seedlings. Rhizopod assay was used where the *N. cadamba* seedlings were grown inside rhizopods made of PVC tubes with a water table of 0.5 m, which is generally used as a simulation of limited water condition. The seedlings were watered fortnightly to minimize the amount of water being directly supplied to soil of seedlings under water-limited environment. In normal nursery operation, seedlings would be watered thrice daily using a water sprinkle system.

The 90-day rhizopod assay had achieved the objective to show that *N. cadamba* seedlings treated with bio-fertilizer, or combination of bio-fertilizer and half regime chemical fertilizer, had shown significant higher mean root elongation rate if compared to treatment with full or half regime chemical fertilizer as well as negative control. Bio-fertilizer containing IAA producing microbe (*Bacillus cereus* Strain II) had aided in the root
elongation of *N. cadamba* seedlings. *B. cereus* Strain II could help elongate root length and increase surface area of roots, thus leading to an increment in dry weight of roots (Adachi et al. 2013). The potential of *B. cereus* Strain II in the root elongation of *N. cadamba* seedlings was further supported by the study conducted by Chua (2018). In the study conducted by Chua (2018), *N. cadamba* seedlings treated with dual combination of chemical fertilizer and bio-fertilizer containing IAA producing microbe (*B. cereus* Strain II) had shown significantly higher mean root dry weight if compared to seedlings treated with dual combination of chemical fertilizer and bio-fertilizer without IAA producing microbe (*B. cereus* Strain II).

Results of rhizopod assay showed that seedlings treated with 2.5 g of chemical fertilizer (T3) had higher mean rate of root elongation compared to seedlings treated with 5.0 g of chemical fertilizer (T2). This interesting finding suggested that half regime of chemical fertilizer could actually elongate roots at a faster rate compared to full regime of chemical fertilizer when *N. cadamba* seedlings were grown in a water-limited environment. The reason behind this finding was probably due to full regime chemical fertilizer causing higher osmotic pressure in rhizopod soil rather than in the roots of the seedlings (Hammer 2000). The difference in osmotic pressure would allow water to migrate out from root cells into the soil. The root system of seedlings would eventually become dehydrated and this would affect the elongation rate of roots (Lawlor 1969).

In conclusion, the results in this study showed that bio-fertilizer containing IAA producing bacteria (*B. cereus* Strain II) could significantly enhance the root elongation of *N. cadamba* seedlings. Elongation of roots in *N. cadamba* seedlings is important as it results in efficient uptake of nutrients and water and will subsequently influence the growth of seedlings’ shoot (Hartmann, Stingh & Klingmuller 1983). When roots elongate, the dry root weight of seedlings would also increase (Adachi et al. 2013). The potential of IAA producing microbe in enhancing the root elongation of *N. cadamba* seedlings could be further supported by the study conducted by Chua (2018), whereby the mean root dry weight of *N. cadamba* seedlings treated with dual combination of chemical fertilizer with bio-fertilizer containing IAA producing microbe was significantly higher than the mean root dry weight of seedlings treated with dual combination of chemical fertilizer and bio-fertilizer without IAA producing microbe.
CHAPTER 4: SMALL SCALE POT TRIAL TO STUDY THE EFFECTIVENESS OF BIO-FERTILIZER ON *Neolamarckia cadamba*
4.1 Introduction

Bio-fertilizer contributes to sustainable forestry by increasing soil fertility (García-Fraile, Menéndez & Rivas 2015). Many countries viz. Canada, Cuba, Brazil, Europe, Russia and Asia are developing bio-fertilizers applicable for forestry (García-Fraile, Menéndez & Rivas 2015). Dual application of bio-fertilizer with chemical fertilizer is often encouraged and EVL Inc., a company in Canada, had even developed a bio-fertilizer containing several microbial strains to be used with chemical fertilizer (García-Fraile, Menéndez & Rivas 2015).

In Chapter 2, we discussed bio-fertilizer formulation in solid or carrier based form as which had shown to be stable in sustaining microbial population $10^7$ to $10^8$ CFU mL$^{-1}$, while preventing contamination for a duration of at least 180 days. In this chapter, the effectiveness of bio-fertilizer in enhancing the growth of *Neolamarckia cadamba* seedlings will be studied by conducting a small scale pot trial. The 180-day pot trial design was modified based on Sreedhar and Mohan (2016).

The purpose of conducting a pot trial is to enable the bio-fertilizer to be tested under a controlled condition in comparison with chemical fertilizer (Plant Science Consulting 2016). The results obtained from the small scale pot trial will be analyzed and discussed in this chapter to streamline field trial in the next chapter.

4.2 Materials and Methodology

4.2.1 Preparation of seedlings and potting medium

A total of 120 *N. cadamba* seedlings were used in this pot trial. Seeds originated from Kubah and Lambir were obtained from Sarawak Forestry Corporation (SFC) Seed Bank and sown in sowing media containing compost and compound coated chemical fertilizer added with MgO (NPK 15:15:15) in a ratio of 12:1 based on SFC nursery practice as described earlier. Two rectangular trays containing seeds and sowing media were placed in SFC greenhouse and watered twice daily by using the water sprinkler system. *Neolamarckia cadamba* seeds were left to germinate for 60 days. Once the height of
seedlings in the tray were around 1 cm tall, they were pricked and transplanted into black polythene potting bags (10 cm X 12 cm) containing potting media.

Potting medium was prepared by using ratio of 12 parts forest topsoil, 6 parts sand, 6 parts compost and 3 parts compound coated chemical fertilizer added with MgO (NPK 15:15:15). All the components were mixed in a concrete mixer before being shovelled into black polythene potting bags (10 cm X 12 cm), filling the potting bags up to 5 cm below mouth of the potting bags.

**4.2.2 Small scale pot trial design**

**4.2.2.1 Preparation of bio-fertilizer and pot trial**

Carrier-based bio-fertilizer was formulated based on the methods stated in section 2.2.5.1. The 60-days old *N. cadamba* seedlings that were transplanted into black polythene potting bags were left to grow in SFC nursery shed for 60 days. During this nursery stage, the seedlings were treated once with 0.3 g NPK fertilizer (NPK = 15:15:15) and watered thrice daily using the water sprinkler system. The seedlings were later raised in a shaded area for another 60 days without any fertilization before being moved out of the shed into an open area for the conduct of pot trial.

**4.2.2.2 Conducting the pot trial**

A total of 120 six-month old *N. cadamba* seedlings of similar height (± 5 cm) were placed on a bench in an open area. The seedlings were divided randomly into 6 treatment groups and each treatment had 20 seedlings. The treatment groups were as shown in Table 4.1. All the *N. cadamba* seedlings were given their respective treatments. For seedlings under test treatments, cocopeat or fertilizers were placed on the soil of the potting bag in a circular pattern surrounding the sapling. Forest topsoil was then layered on top of the cocopeat or fertilizer till the mouth of the potting bags. *Neolamarckia cadamba* seedlings under different treatments were placed randomly on the bench. The seedlings were watered daily by a water pipe and the seedlings were left to grow in the unshaded area.
The *N. cadamba* pot trial lasted for 180 days. At every 30-day intervals, the height of *N. cadamba* seedlings were measured from the top surface of soil until the shoot tip of seedlings using a measuring tape while the root collar diameter was 5 cm above surface of soil using an electronic calliper. Height and root collar diameter increment for every interval of 30 days was calculated by subtracting the new height and root collar diameter reading with the initial respective height and root collar diameter reading before application of treatment. All the height and root collar diameter increment values were exported to SPSS for means, standard errors and significant differences calculations.

At every interval of 90 days, ten seedlings from each treatment group were randomly selected and destructive sampling was carried out. Seedlings were uprooted and roots of seedlings were washed free from soil. The roots and shoots of seedlings were separated from each other by cutting near the bottom of the stem using a hedge shear. Each seedling’s root and shoot were separately wrapped using newspaper and left to dry in an oven at 60 °C for 72 hours. Once the roots and shoots were free from moisture, each of their dry weights was measured using an electronic balance. The measurements were recorded in gram (g) and these measurements were exported to SPSS for means, standard errors and significant differences calculations.
Table 4.1: Treatment groups for *N. cadamba* seedlings in 180 days of pot trial.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
<th>Sample Size (seedlings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td><em>Neolamarckia cadamba</em> seedlings were not treated with fertilizers, carrier material and bio-fertilizer microbes (acting as control)</td>
<td>20</td>
</tr>
<tr>
<td>T2</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with 20.0 g carrier material (cocopeat) without bio-fertilizer microbes</td>
<td>20</td>
</tr>
<tr>
<td>T3</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with 5.0 g chemical fertilizer</td>
<td>20</td>
</tr>
<tr>
<td>T4</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with 2.5 g chemical fertilizer</td>
<td>20</td>
</tr>
<tr>
<td>T5</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with dual combination of 2.5 g chemical fertilizer with 20.0 g bio-fertilizer</td>
<td>20</td>
</tr>
<tr>
<td>T6</td>
<td><em>Neolamarckia. cadamba</em> seedlings treated with 20.0 g bio-fertilizer</td>
<td>20</td>
</tr>
</tbody>
</table>

Note: Bio-fertilizer consists of a mixture of carrier material and bio-fertilizer microbes (nitrogen fixing microbe, phosphate solubilizing microbe, potassium solubilizing microbe and IAA producing microbe).
4.2.3 Data analysis

The data obtained from pot trial were subjected to analysis of significance difference among the means of treatment groups by ANOVA at $p \leq 0.05$ using SPSS statistical software (version 23, IBM Corp. USA) to compare the effects due to treatment groups.

4.3 Result and Discussion

4.3.1 Determining the effect of different treatments on Neolamarckia cadamba seedlings in pot trial

The efficiency of each treatment group in enhancing the growth of *N. cadamba* saplings was determined by comparing the mean shoot height increment, collar diameter increment, shoot and root dry weights of saplings in each treatment group.
4.3.1.1 The mean shoot height increment of *N. cadamba* seedlings

![Graph showing the trend of mean shoot height increment of *N. cadamba* seedlings throughout the 180 days’ pot trial.](image)

**Figure 4.1:** The trend of mean shoot height increment of *N. cadamba* seedlings throughout the 180 days’ pot trial.

Based on Figure 4.1, it could be seen that all seedlings treated with the six different treatments had shown growth in shoot heights with varying degree. The effects of each treatment was analysed based on the results obtained after 90 days and 180 days of treatment.
Figure 4.2: The mean shoot height increment of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean shoot height increment of each treatment group ± SE (n = 20). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

By interpreting Figure 4.2, it can be observed that *N. cadamba* seedlings treated with 5.0 g chemical fertilizer (T3) had the highest shoot height increment after 90 days of treatment which was significant to all the other treatment (p ≤ 0.05). *Neolamarckia cadamba* seedlings treated with dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer (T5) had the second highest shoot height increment, with a significant difference of 1.278 cm when compared to seedlings treated with full regime or 5.0 g of chemical fertilizer (T3). This difference might be due to beneficial microbes in the bio-fertilizer would still be adapting to the new environment. Bio-fertilizer microbes would often form microbial communities around the roots (Park, Cao & McSpadden Gardener 2010), and this may have happened to *N. cadamba* seedlings, thus increasing the availability of nitrogen, phosphorus, potassium and indole acetic acid for the seedlings.
*Neolamarckia cadamba* seedlings treated with 2.5 g of chemical fertilizer (T4) and 20.0 g of bio-fertilizer (T6) showed positive results when compared to seedlings under control group. The mean shoot height increment of seedlings treated with T4 and T6 were 7.039 cm ($p \leq 0.05$) and 6.717 cm ($p \leq 0.05$) respectively, showing no significant difference among each other, indicating that T6 was as effective as T4 in increasing the shoot height of seedlings. Nonetheless, T4 and T6 treatments had still shown significantly higher in height increment if compared to the control.

*Neolamarckia cadamba* seedlings treated with 20.0 g of carrier material (T2) showed minimal difference in mean shoot height increment when compared to control, which was seedlings without any treatment (T1). This indicated that carrier material alone would neither exert any significant effect in increasing the shoot height of *N. cadamba* seedlings, nor detrimental to the plant. The role of carrier material would be to protect the microbes in the bio-fertilizer formulation as discussed in Chapter 2. Van Veen, van Overbeek & van Elsas (1997) also discussed about carrier material being able to protect inoculant microbes by providing them with a protective surface, pore space or specific substrate.
Figure 4.3: The mean shoot height increment of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represent the mean shoot height increment of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

The results for mean shoot height increment of *N. cadamba* seedlings after 180 days of treatment were almost similar to the results obtained after 90 days of treatment. The only difference was that treatment T5, which is the combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer, had shown to be as good as T3, the full regime or 5.0 g of chemical fertilizer. Based on Figure 4.3, seedlings treated with T3 remained highest mean shoot height increment, followed by seedlings treated with T5 having the second highest increment, albeit the means of the two treatments were not significantly different from each other. This result indicates that the effects of the combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer was comparable to full regime or 5.0 g of chemical fertilizer in enhancing the shoot height of *N. cadamba* seedlings.
In both 90 and 180 days after treatment, T5 treatment, which is the combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer had shown significantly superior to both 2.5 g of chemical fertilizer (T4 treatment) and 20.0 g bio-fertilizer (T6 treatment). It seems indispensable for both chemical fertilizer and bio-fertilizer component to be added together in order to produce the synergistic effects that are greater than each of the components. The mean shoot height increment of seedlings treated with T4 and T6 treatments showed no significant difference among each other, albeit both treatments were significantly inferior to their combination, which was T5 treatment.

Results from 180 days of treatment indicate that beneficial microbes in bio-fertilizer were still active after being in the soil for 180 days. Previous studies had shown that bio-fertilizer could indeed increase shoot height of *N. cadamba* (Sreedhar & Mohan 2016; Chua 2018) in a 180 days of pot trial, *Dalbergia latifolia* and *Dalbergia sisso* in a 120 days’ pot trial (Nayak, Panigrahi & Gupta 2017). As discussed earlier, bio-fertilizer treatment *per se* could enhance the shoot growth as good as 2.5 g of chemical fertilizer.

Based on the overall results, it can be summarized that dual combination of 2.5 g chemical fertilizer with 20.0 g bio-fertilizer treatment showed the comparable effectiveness as 5.0 g chemical fertilizer treatment. This result is similar to previous studies on *Paulownia kawakamii* (Farahat 2014) and *Punica granatum* L. (Amin et al. 2017) that proved that dual combination of chemical fertilizer with bio-fertilizer could significantly increase shoot height of plants.
4.3.1.2 The mean root collar diameter increment of *N. cadamba* seedlings

Based on Figure 4.4, it could be seen that the root collar diameters of all seedlings treated with the six different treatments had shown growth in root collar diameters with varying degree. The effects of each treatment was analysed based on the results obtained after 90 days and 180 days of treatment.

![Figure 4.4: The trend of mean root collar diameter increment of *N. cadamba* seedlings throughout the 180 days of pot trial.](image)

**Figure 4.4:** The trend of mean root collar diameter increment of *N. cadamba* seedlings throughout the 180 days of pot trial.
Figure 4.5: The mean root collar diameter increment of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean root collar diameter increment of each treatment group ± SE (n = 20). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

The results for mean root collar diameter increment of *N. cadamba* seedlings after 90 days of treatment were almost similar to the results for mean shoot height increment obtained after 90 days of treatment. By interpreting Figure 4.5, the only difference was that the mean root collar diameter increment of seedlings treated with 5.0 g of chemical fertilizer (T3) and dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer treatment (T5) showed no significance among each other. Seedlings treated with T3 and T5 treatments concentrated on the growth of their stems as bigger stems would be required to support the weight of their shoots (Givnish 1982) which were growing significantly better compared to seedlings treated without any treatment (T1).
Figure 4.6: The mean root collar diameter increment of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean root collar diameter increment of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

The mean root collar diameter increment results observed after 90 and 180 days of treatment were similar when compared. The results were also similar to the results for the shoot height increment after 180 days of treatment. Based on Figure 4.6, throughout the 180 days of treatment, the mean root collar diameter increment of *N. cadamba* seedlings treated with 5.0 g of chemical fertilizer (T3) and dual combination of 2.5 g chemical fertilizer with 20.0 g of bio-fertilizer (T5) showed no significant difference among each other. This result indicates that both treatments have comparable effectiveness in enhancing the root collar diameter growth of *N. cadamba* seedlings.

The mean of root collar diameter increment of seedlings treated with 2.5 g of chemical fertilizer (T4) and 20.0 g of bio-fertilizer (T6) which showed no significant difference
among each other were significantly more effective than T1 treatment (not treatment) and T2 treatment (carrier material) in enhancing the root collar diameter growth of \textit{N. cadamba} seedlings. As discussed in section 4.3.1.1, when both the components from T4 and T6 were mixed together to form T5 treatment (2.5 g of chemical fertilizer with 20.0 g bio-fertilizer), the synergistic interaction of chemical fertilizer and bio-fertilizer could significantly increase the root collar diameter of \textit{N. cadamba} seedlings. Thus, in both 90 and 180 days after treatment, the mean root collar diameter increment of T5 treatment was significantly superior to both T4 and T6 treatments.

The results from 90 days to 180 days after treatment further indicate that carrier material treatment (T2) had totally no effect in enhancing the root collar diameter growth of \textit{N. cadamba} seedlings as the mean root collar diameter increment seedlings treated with T2 showed no significant difference when compared with seedlings without any treatment (T1). Carrier material is just a protective delivery vehicle for the transportation of live bio-fertilizer beneficial microbes cultured in the laboratory to the rhizophere of \textit{N. cadamba} seedlings (Brahmaprakash & Sahu 2012). It is non-toxic to \textit{N. cadamba} seedlings and does not exert any effect in the root collar diameter growth of seedlings.

Based on the overall results, it can be concluded that dual combination of 2.5 g chemical fertilizer with 20.0 g bio-fertilizer treatment had effectiveness comparable to 5.0 g chemical fertilizer treatment in enhancing both the shoot height and root collar diameter growth of \textit{N. cadamba} seedlings. This results were in harmony with the results obtained in a 180 days of pot trial conducted by Chua (2018) on \textit{N. cadamba} seedlings. Similar results were also obtained from seedlings of other tree species, such as in a 180 days of pot trial conducted by Chan (2018) on \textit{Eucalyptus pellita} seedlings and a 13 months of field trial conducted by Farahat (2014) on \textit{Paulownia kawakamii} seedlings.
4.3.1.3 The dry weights of *N. cadamba* seedlings

i) After 90 days of treatment

a) Mean shoot dry weight of *N. cadamba* seedlings

![Graph showing mean shoot dry weight of *N. cadamba* seedlings after 90 days of treatment.](image)

**Figure 4.7:** The mean shoot dry weight of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean shoot dry weight of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Based on Figure 4.7, it can be seen that *N. cadamba* seedlings treated with dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer (T5) had the highest mean shoot dry weight, 22.686 g followed by seedlings treated with 5.0 g of chemical fertilizer (T3) having the second highest mean weight, 21.034 g. The mean shoot dry weight of seedlings treated with T5 and T3 showed no significant difference among
each other, indicating that both treatments could provide seedlings with nutrients for their shoot growth.

Seedlings treated with 20.0 g of bio-fertilizer (T6) had the third highest mean shoot dry weight of 18.462 g. The mean shoot dry weight of seedlings treated with T6 and T3 showed no significant difference among each other, indicating that bio-fertilizer alone treatment was as effective as full regime chemical fertilizer treatment in enhancing the shoot growth of seedlings. Albeit T6 treatment has comparable effectiveness as T3 treatment in enhancing the shoot growth of *N. cadamba* seedlings, T6 treatment was still significantly inferior to the dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer treatment (T5).

The mean shoot dry weight of seedlings treated with 2.5 g chemical fertilizer (T4) and 20.0 g of bio-fertilizer (T6) showed no significant difference when compared. Meanwhile, when the mean shoot dry weight of seedlings treated with T4 (2.5 g of chemical fertilizer) and T2 (carrier material) treatments were compared, results also showed no significant difference. These findings show that 2.5 g of chemical fertilizer treatment could enhance the shoot growth of seedlings well but 20.0 g of bio-fertilizer treatment could enhance better the growth of seedlings’ shoot.

Similar to the results obtained for shoot height and root collar diameter increment, only the mean shoot dry weight of seedlings treated with carrier material treatment (T2) showed no significant difference when compared to the mean shoot dry weight of seedlings without any treatment (T1). Carrier material would not cause any detrimental effects to the shoot growth of seedlings.
b) Mean root dry weight of *N. cadamba* seedlings

![Bar graph showing mean root dry weight of *N. cadamba* seedlings after 90 days of treatment.](image)

**Figure 4.8:** The mean root dry weight of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean root dry weight of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Based on Figure 4.8, the mean root dry weight of seedlings treated with 5.0 g of chemical fertilizer (T3) and dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer (T5) showed no significant difference when compared. Both treatments showed comparable effectiveness in enhancing the root growth of *N. cadamba* seedlings.

The mean root dry weight of *N. cadamba* seedlings treated with 20.0 g of bio-fertilizer (T6) was significantly higher than the mean root dry weight of seedlings treated with 2.5 g of chemical fertilizer (T4). This indicated that bio-fertilizer stand-alone treatment was more effective than 2.5 g of chemical fertilizer treatment in enhancing the root growth of
seedlings. Beneficial microbes in bio-fertilizer enhances the growth of roots through biological nitrogen fixation and synthesis of phytohormones (Farahat et al. 2014).

Although *N. cadamba* seedlings treated with 2.5 g of chemical fertilizer treatment (T4) had significantly lower mean root dry weight compared to seedlings treated with bio-fertilizer (T6), T4 treatment was significantly superior than T1 (without treatment) and T2 (carrier material) treatments. The mean root dry weight of seedlings treated with T1 and T2 showed no significant difference when compared. This result again indicates that carrier material does not have any effect on enhancing the root growth of *N. cadamba* seedlings.
ii) **After 180 days of treatment**

a) **Mean shoot dry weight of *N. cadamba* seedlings**

![Graph showing mean shoot dry weight of *N. cadamba* seedlings](image)

Figure 4.9: The mean shoot dry weight of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean shoot dry weight of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Based on Figure 4.9, results obtained after 180 days of treatment showed that chemical fertilizer and bio-fertilizer treatments had enhanced the shoot growth of seedlings. Seedlings treated with dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer (T5) had the highest mean shoot dry weight which was significant to all the other treatments (p ≤ 0.05). Seedlings treated with 5.0 g of chemical fertilizer (T3) had the second highest mean shoot dry weight, with a significant difference of 3.959 cm when compared to seedlings treated with T5. This result indicates that dual combination of
chemical fertilizer with bio-fertilizer treatment has greater effect compared to full regime chemical fertilizer in enhancing the shoot growth of *N. cadamba* seedlings.

In both 90 and 180 days after treatment, seedlings treated with 20.0 g of bio-fertilizer (T6) and 2.5 g chemical fertilizer (T4) showed no significant difference when compared. Both treatments maintained to show comparable effectiveness in enhancing the shoot growth of *N. cadamba* seedlings. As discussed in section 4.3.1.1, only when components of T4 treatment (2.5 g of chemical fertilizer) and T6 treatment (20.0 g of bio-fertilizer) were added together, the components could produce synergistic effects that are greater than each of the components. T5 treatment, which is the dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer, was significantly superior to both 2.5 g of chemical fertilizer (T4 treatment) and 20.0 g of bio-fertilizer (T6 treatment). The results from 90 days and 180 days after treatment showed that the mean shoot dry weight of seedlings treated with T1 (without any treatment) and T2 (carrier material) showed no significant difference when compared. This results again indicate that carrier material has no effect in enhancing the shoot growth of *N. cadamba* seedlings.

Based on the overall results obtained on 90 and 180 days after treatment, it can be summarized that dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer treatment (T5) showed high effectiveness in enhancing the shoot growth of *N. cadamba* seedlings. Seedlings treated with T5 could have greater upper growth due to the synthesize of growth promoting substances by bio-fertilizer microbes. These substances were able to allow seedlings to efficiently absorb nutrients (Gommaa & Abou-Aly, 2001). Results obtained from this study were similar to results of previous study conducted by Chua (2018) which had proven that the shoot dry weight of *N. cadamba* seedlings treated with dual combination of bio-fertilizer with half regime chemical fertilizer treatment was on par with the shoot dry weight of seedlings treated with full regime chemical fertilizer treatment. Another previous study which also showed the effectiveness of dual combination of chemical fertilizer with bio-fertilizer was the study conducted by Farahat et al. (2014), whereby *Paulownia kawakamii* seedlings being treated with dual combination of chemical fertilizer with bio-fertilizer had high dry weights of leaves and stems.
b) Mean root dry weight of *N. cadamba* seedlings

![Figure 4.10: The mean root dry weight of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean root dry weight of each treatment group ± SE (n = 10). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).](image)

There were two differences being observed when comparing results of the mean root dry weight of *N. cadamba* seedlings obtained after 90 and 180 days after treatment. The first difference was dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer treatment (T5) had enhanced the root growth of *N. cadamba* seedlings. Based on Figure 4.10, seedlings treated with T5 treatment had the highest mean root dry weight of 16.030 g, followed by seedlings treated with 5.0 g chemical fertilizer treatment (T3) having the second highest mean root dry weight of 15.370 g. The mean root dry weight of seedlings treated with T3 and T5 showed no significant difference among each other, indicating that both treatments had comparable effectiveness in enhancing the root growth of *N.*
cadamba seedlings. Both treatments enhanced the root growth of seedlings by providing seedlings with nutrients for protein synthesis and at the same time to increase the meristematic activity (Farahat et al. 2014), thus attributing to the high means of root dry weight.

The second difference being observed was that the mean root dry weight of seedlings treated with 2.5 g of chemical fertilizer (T3) had shown to be as good as T6 treatment (20.0 g of bio-fertilizer). Both T3 and T6 treatments had comparable effectiveness in enhancing the root growth of N. cadamba seedlings after 180 days of treatment. The only similarity observed from results of the mean root dry weight of N. cadamba seedlings obtained after 90 and 180 days after treatment was that the mean root dry weight of seedlings treated with T1 (without any treatment) and T2 (carrier material) still showed no significant difference when compared.

The overall results showed that dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer treatment and 5.0 g of chemical fertilizer treatment showed comparable effectiveness in enhancing the root growth of N. cadamba seedlings. Previous study conducted by Chua (2018) also showed that dual combination of bio-fertilizer with half regime chemical fertilizer treatment could enhance the root growth of N. cadamba seedlings as seedlings treated with dual combination treatment had the highest root dry weight. Root growth enhancement by dual combination of chemical fertilizer with bio-fertilizer was also observed in another timber species, Tectona grandis. In the study conducted by Paroha et al. (2009), Tectona grandis seedlings treated with dual combination of chemical fertilizer with bio-fertilizer had high root biomass.

### 4.4 Summary

This chapter describes the 180 days of pot trial used to determine whether bio-fertilizer formulated with half regime chemical fertilizer could act as an alternative to replace the chemical fertilizer for N. cadamba seedlings.

Overall, an association could be observed between the shoot height and root collar diameter of seedlings throughout the 180 days of pot trial. As seedlings increase in shoot height, their root collar diameter would often also increase in order to support the shoot
growth of the seedlings (Nagashima & Hikosaka 2011). When the shoots and roots of seedlings grow, their dry weights would eventually increase too (Sheng & Hunt 1990).

The 180 days of pot trial results showed that dual combination of 20.0 g of bio-fertilizer with 2.5 g of chemical fertilizer treatment (T5) had comparable effectiveness in enhancing the shoot height and root collar diameter of *N. cadamba* seedlings if compared to 5.0 g of chemical fertilizer treatment (T3). Results of the pot trial were in harmony with the results obtained from the study conducted by Chua (2018) on *N. cadamba* seedlings, whereby seedlings treated with dual combination of chemical fertilizer with bio-fertilizer had high shoot length and root collar diameter. A study conducted by Farahat (2014) on *Paulownia kawakamii* seedlings showed that dual combination of chemical fertilizer with bio-fertilizer could increase both the shoot height and root collar diameter of the plant.

Interestingly, results also showed that *N. cadamba* seedlings treated with T5 had significantly higher mean shoot dry weight compared to seedlings treated with T3, albeit both T3 and T5 treatments had comparable effectiveness in enhancing the shoot height of *N. cadamba* seedlings. Seedlings treated with T5 treatment (dual combination of bio-fertilizer and chemical fertilizer) might have higher shoot dry weight due to seedlings having larger leaf area. Previous study conducted by Chua (2018) showed that *N. cadamba* seedlings treated with dual combination of chemical fertilizer with bio-fertilizer had larger leaf area compared to seedlings treated with chemical fertilizer alone. On the other hand, as predicted, the mean root dry weight of seedlings treated with T3 and T5 showed no significant difference among each other as both treatments had comparable effectiveness in enhancing the root collar diameter of seedlings. As shown in the study conducted by Farahat (2014), dual combination of 300.0 g of bio-fertilizer with 75.0 g of chemical fertilizer could increase both the root collar diameter and root dry weight of *Paulownia kawakamii* seedlings.

In conclusion, both 20.0 g of bio-fertilizer with 2.5 g of chemical fertilizer treatment and 5.0 g of chemical fertilizer treatment could significantly enhance the shoot and root growths of *N. cadamba* seedlings. Previous study on *Grevillea robusta* conducted by Gurumurthy and Sreenivasa (1999) also showed that dual combination of chemical fertilizer with bio-fertilizer could increase the plants’ height, root collar diameter, leaf
area, root and shoot dry weights. It had been shown in this study that bio-fertilizer formulated with half regime chemical fertilizer could act as an alternative to replace the chemical fertilizer for *N. cadamba* seedlings. Bio-fertilizer could enhance the growth of seedlings as beneficial microbes in bio-fertilizer were active during the 180 days of pot trial, forming microbial communities around the roots (Park, Cao & McSpadden Gardener 2010) of *N. cadamba* seedlings. The microbes encourage *N. cadamba* seedlings to absorb the nutrients being made available for them (Bio Fit Project 2018).
CHAPTER 5: FIELD TRIAL TO FURTHER EVALUATE THE EFFECTIVENESS OF BIO-FERTILIZER ON *Neolamarckia cadamba*
5.1 Introduction

The effectiveness of the formulated bio-fertilizer on the growth of *Neolamarckia cadamba* seedlings was conducted in pot trials. As discussed in the previous chapter, the effect of dual combination of 2.5 g of chemical fertilizer with 20.0 g of bio-fertilizer in enhancing the growth of *N. cadamba* seedlings during the 180 days of pot trial was comparable to the effectiveness of 2.5 g of chemical fertilizer. As pot trial might not provide the same stimulation of natural forest (Sollins 1998), the results obtained should not be assumed to be same as the results for field trial. Only during the conduct of field trial, the treatment could be tested under relevant climatic and geographic conditions (SGS 2018).

There have been many bio-fertilizer field trials being conducted on timber species. Pandove, Gangwar & Singh (2017) conducted a study on Eucalyptus clones, Rajendran and Devaraj (2004) on *Casuarina equisetifolia*, Farahat et al. (2014) on *Paulownia kawakamii* and Prakash (2014) on *Santalum album*.

Based on the results obtained from the pot trial in Chapter 4, a 180 days of field trial with only four treatment groups was conducted on six-month old *N. cadamba* seedlings. Carrier material treatment was not considered as in the previous pot trial, it was already proven that carrier material present in bio-fertilizer did not have any significant effect in enhancing the growth of *N. cadamba* seedlings. Meanwhile, bio-fertilizer stand-alone treatment was not included in the field trial as it had been proven in the pot trial that when 20.0 g of bio-fertilizer was mixed with 2.5 g of chemical fertilizer, the effectiveness of this mixed treatment showed better effectiveness in enhancing the growth of *N. cadamba* seedlings. Half regime or 7.5 g of chemical fertilizer treatment will be included in the field trial as this treatment acts as a baseline to compare the effectiveness of bio-fertilizer present in the dual combination treatment. The results obtained from the field trial will be analysed and discussed in this chapter.
5.2 Materials and Methodology

5.2.1 Preparation of seedlings, potting medium and bio-fertilizer for field trial

The methods for *N. cadamba* seeds germination and preparation of potting medium were based on the methods previously discussed in section 4.2.1. Carrier-based bio-fertilizer was formulated based on the methods stated in section 2.2.5.1.

5.2.2 Field trial design

5.2.2.1 Preparing *Neolamarckia cadamba* seedlings for field trial

The 60-days old *N. cadamba* seedlings that were transplanted into black polythene potting bags were left to grow in SFC nursery shed for 60 days. During this nursery stage, the seedlings were treated once with 0.3 g of NPK fertilizer (NPK= 15:15:15) and watered thrice daily using the water sprinkler system. When seedlings were 120 days of age, they were moved out of the shed into an open area for hardening. During the 60 days of hardening stage, the seedlings were watered daily using a water pipe and 0.3 g of NPK fertilizer (NPK= 15:15:15) was given to each seedlings once every month. After the completion of hardening stage, the seedlings were ready to be transplanted for field trial.

5.2.2.2 Conduct of *Neolamarckia cadamba* 180 days’ field trial

*Neolamarckia cadamba* seedlings were six months of age during transplanting. They were selected for similar height (± 5 cm) and transported to the site for field trial at Sabal Forest Reserve, Simunjan. Sabal Forest Reserve is a LPF area reserved for Sarawak Forest Corporation to conduct research plot, and it is mostly made out of hill mixed dipterocarp forests. Based on the soil analysis performed by Sarawak Forestry, the soil texture of the field used for field trial was clay, with a mean moisture content of 2.38% and a mean pH of 4.216. A total of 120 seedlings were planted in the field as shown in Figure 5.1. The buffer distance was 4 m while the distance between each seedling was 3 m. Treatments as stated in Table 5.1 were allocated to samplings in the field in a completely random
manner before the start of the trial. During the 180 days of field trial, the treatments were only applied once to the seedlings. Three holes were made surrounding the sampling so that chemical fertilizer and bio-fertilizer could be applied into the holes. After fertilizer application, the surrounding soil was used to cover the holes.

![Field trial plot design at Sabal Forest Reserve, Simunjan, Sarawak.](image-url)
Table 5.1: Treatment groups for *N. cadamba* 180 days of field trial.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
<th>Sample Size (seedlings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td><em>Neolamarckia cadamba</em> seedlings were not treated with fertilizers, carrier material and bio-fertilizer microbes (acting as control)</td>
<td>30</td>
</tr>
<tr>
<td>T2</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with 15.0 g chemical fertilizer</td>
<td>30</td>
</tr>
<tr>
<td>T3</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with 7.5 g chemical fertilizer</td>
<td>30</td>
</tr>
<tr>
<td>T4</td>
<td><em>Neolamarckia cadamba</em> seedlings treated with dual combination of 7.5 g chemical fertilizer with 100.0 g bio-fertilizer</td>
<td>30</td>
</tr>
</tbody>
</table>

Note: Bio-fertilizer consists of a mixture of carrier material and bio-fertilizer microbes (nitrogen fixing microbe, phosphate solubilizing microbe, potassium solubilizing microbe and IAA producing microbe).

5.2.2.3 Data collection for 180 days of field trial

Prior to application of treatments and at every interval of 30 days, the shoot height and root collar diameter of *N. cadamba* seedlings from each treatment group were measured. The shoot height was measured from the top surface of soil until the shoot tip of seedlings using a measuring tape or a long plastic pipe labelled with scale markings while the root collar diameter was measured 5 cm from the top surface of soil until using an electronic calliper. The shoot’s height readings were recorded in centimetre (cm) while the root collar diameter’s readings were recorded in millimetre (mm). Height or root collar
diameter increment for every 30 days’ interval was calculated by subtracting the new height or root collar diameter reading with the initial height or root collar diameter reading before application of treatment. All the height and root collar diameter increment values were exported to SPSS for means, standard errors and significant differences calculations.

5.2.3 Data analysis

The data obtained from field trial were subjected to analysis of significance difference among the means of treatment groups by ANOVA at $p \leq 0.05$ using SPSS statistical software (version 23, IBM Corp. USA) to compare the effects due to treatment groups.

5.3 Result and Discussion

5.3.1 Determining the effect of different treatments on *Neolamarckia cadamba* seedlings in field trial

5.3.1.1 The mean shoot height of *N. cadamba* seedlings

Based on Figure 5.2, it could be seen that all seedlings treated with the four different treatments had shown growth in shoot heights with varying degree. The shoot height growth trend for field trial is similar to the shoot height growth trend for pot trial. The effects of each treatment in field trial was also analysed based on the results obtained after 90 days and 180 days of treatment.
Figure 5.2: The trend of mean shoot height increment of *N. cadamba* seedlings throughout the 180 days’ field trial.
Figure 5.3: The mean shoot height increment of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean shoot height of each treatment group ± SE (n = 30). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

As shown in Figure 5.3, *N. cadamba* seedlings treated with 15.0 g of chemical fertilizer (T2) had the highest shoot height increment after 90 days of treatment, followed by seedlings treated with dual combination of 7.5 g of chemical fertilizer with 100.0 g of bio-fertilizer (T4) having the second highest shoot height increment. The mean shoot height increment of both treatments showed no significant difference among each other, indicating that both T2 and T4 were comparable in enhancing the shoot growth of *N. cadamba* seedlings.

The results also showed that seedlings treated with T4, which was a dual combination of 7.5 g chemical fertilizer with 100.0 g of bio-fertilizer, had significantly higher mean shoot height increment compared to seedlings treated with T3, which was 7.5 g of chemical...
fertilizer *per se*. All treatment groups were significantly better than T1, the control without treatment group during 90 days of treatment.

**Figure 5.4:** The mean shoot height increment of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean shoot height of each treatment group ± SE (n = 30). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Upon 180 days of treatment, the results for mean shoot height increment of *N. cadamba* seedlings shown in Figure 5.4 were similar to 90 days of treatment, where the seedlings treated with full-regime 15.0 g of chemical fertilizer (T2) was highest albeit not significant if compared to T4, the half-regime 7.5 g of chemical fertilizer with bio-fertilizer. Similarly, T4 treatment was significantly superior to T3 treatment, the half-regime 7.5 g of chemical fertilizer treatment.

The only difference was that the mean shoot height increment of seedlings treated with T3 (7.5 g of chemical fertilizer treatment) and T1 (without any treatment) showed no
significant difference among each other. This indicated that half regime or 7.5 g of chemical fertilizer might not be supplying sufficient or balance nutrients for the seedlings, thus limiting their growth (Burke et al. 2016). The lack in nutrients was most probably due to depletion of half regime chemical fertilizer at the end of 180 days of treatment. Bio-fertilizer could increase the availability of nutrients for seedlings as beneficial microbes in the bio-fertilizer can multiply and participate in nutrient cycling (Singh, Pandey & Singh 211).

Based on the overall results, it can be concluded that dual combination of 7.5 g of chemical fertilizer with 100.0 g bio-fertilizer treatment had the same effectiveness as 15.0 g of chemical fertilizer treatment in enhancing the shoot height of N. cadamba seedlings. This results were similar to the results obtained in the pot trial described in Chapter 4.
5.3.1.2 The mean root collar diameter of *N. cadamba* seedlings

Based on Figure 5.5, it could be seen that all seedlings treated with the four different treatments had shown varying degree of growth in root collar diameter. This observation is similar to the results obtained in the pot trial. The effects of each treatment in field trial was also analysed based on the results obtained at 90 days and 180 days of treatment.

![Figure 5.5: The trend of mean root collar diameter increment of *N. cadamba* seedlings throughout the 180 days’ field trial.](image)

Figure 5.5: The trend of mean root collar diameter increment of *N. cadamba* seedlings throughout the 180 days’ field trial.
Figure 5.6: The mean root collar diameter increment of *N. cadamba* seedlings after 90 days of treatment. The bars in the graph represented the mean root collar diameter of each treatment group ± SE (n = 30). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

Based on Figure 5.6, after 90 days of treatment, *N. cadamba* seedlings treated with 15.0 g of chemical fertilizer treatment (T2) had the highest mean root collar diameter increment which was significant to all other treatments (p ≤ 0.05). Seedlings treated with 7.5 g of chemical fertilizer with 100.0 g bio-fertilizer treatment (T4) had the second highest root collar diameter increment. Higher effectiveness in enhancing growth of root collar diameter by full regime chemical fertilizer (T2) treatment was probably because seedlings could directly absorb the water soluble nutrients supplied by chemical fertilizer (Food and Fertilizer Technology Center 2018). T4 treatment consisting of half regime chemical fertilizer with bio-fertilizer had slightly lower effectiveness in enhancing the root collar diameter growth as the beneficial microbes in the bio-fertilizer would require
time to fix nitrogen, solubilize phosphate and potassium and produce IAA, before rendering these nutrients available for the seedlings (Vessey 2003).

As expected, T3 treatment was significantly inferior to T2 and T4 treatments in enhancing the growth of root collar diameter. T3 treatment which contains half-regime of 7.5 g chemical fertilizer (T3) was still significantly more superior than no fertilizer treatment (T1).

Figure 5.7: The mean root collar diameter increment of *N. cadamba* seedlings after 180 days of treatment. The bars in the graph represented the mean root collar diameter of each treatment group ± SE (n = 30). Means with same letter(s) are not significantly different according to Tukey’s HSD test (p ≤ 0.05).

The results for mean root collar diameter increment of *N. cadamba* seedlings after 180 days of treatment were almost similar to the results obtained after 90 days of treatment. By interpreting Figure 5.7, the only difference was that treatment T4, which is the combination of 7.5 g of chemical fertilizer with 100.0 g of bio-fertilizer, had shown to be
as good as T2, the full regime or 15.0 g of chemical fertilizer. This result indicated that both T2 and T4 treatments had comparable effectiveness in enhancing the root collar diameter of seedlings. T4 treatment could enhance good growth of root collar diameter probably because the beneficial microbes in bio-fertilizer have established growth and actively participated in nutrient cycling and provision of nutrients to the seedlings (Itelima 2018).

Interestingly, the mean root collar diameter increment of seedlings treated with 7.5 g of chemical fertilizer (T3) after 180 days of treatment remained to be significantly higher compared to the no fertilizer treatment (T1). This observation is different from the mean shoot height where the increments were no significant different between of seedlings treated with T1 and T3 after 180 days of treatment. These contrary observations may indicate that nutrients being supplied by chemical fertilizer could have been primarily used for the widening of seedlings stem instead of their height (Haase 2008). Based on Coford, Keane and Morrissey (2002), plant with larger root collar diameter tend to indicate that it also has greater root mass. It can only be assumed that nutrients had been initially used by N. cadamba seedlings to develop its root system rather than shoot so that water and nutrients could be easily absorbed by seedlings’ roots (Weaver & Himmel 1929) for survival at the field. Nutrients provided by chemical fertilizer in T3 might have been depleted after being primarily used for the development of root system, thus could not exert any significant effect in increasing the shoot height of seedlings from day 90 to day 180.

The overall results showed that dual combination of 7.5 g chemical fertilizer with 100.0 g of bio-fertilizer and 15.0 g of chemical fertilizer treatment both had comparable effectiveness in enhancing the root collar diameter of N. cadamba seedlings. This results were also similar to the results obtained in the 180 days of pot trial described in Chapter 4.
5.4 Summary

Previously in Chapter 4, the results of pot trial study had shown that bio-fertilizer formulated with half regime chemical fertilizer could act as an alternative to replace the chemical fertilizer for *N. cadamba* seedlings. The effectiveness of bio-fertilizer formulated with half regime chemical fertilizer in the growth enhancement of *N. cadamba* seedlings was further evaluated by conducting a field trial. Field trial provides the same stimulation of natural forest (Sollins 1998), thus it can further validate the results obtained from the pot trial.

This chapter describes the field trial study conducted on 120 seedlings of *N. cadamba*. Similar to pot trial, *N. cadamba* seedlings being studied in the field trial were six months old with focus only for four treatment groups. The duration of the field trial was similar to pot trial, which was 180 days. The overall field trial results showed that dual combination of 7.5 g of chemical fertilizer with 100.0 g of bio-fertilizer treatment (T4) showed effectiveness comparable to 15.0 g of chemical fertilizer treatment (T2) in enhancing the shoot height and root collar diameter of *N. cadamba* seedlings. The field trial results were in harmony with the results of previous studies conducted on *Paulownia kawakamii* and *Santalum album* whereby chemical fertilizer with bio-fertilizer treatment could significantly enhance the growth of seedlings in terms of their shoot height and root collar diameter (Farahat et al. 2014; Prakash 2014).

In a nutshell, bio-fertilizer formulated with half regime chemical fertilizer could act as an alternative to replace the chemical fertilizer for *N. cadamba* seedlings in both pot and field trials.
CHAPTER 6: CONCLUSIONS AND FURTHER RECOMMENDATIONS
6.1 Aim of the thesis

Two major issues are normally faced when planting *Neolamarckia cadamba* in Sarawak, namely the deficiencies of soil nutrient and water. Soil nutrient deficiency is normally solved by application of chemical fertilizer. However, excessive usage of chemical fertilizer would eventually lead to environmental problems. These two issues had been the inspirations where aim of this thesis was developed, which is to formulate a bio-fertilizer which can enhance the shoot and root growth for *N. cadamba*. This thesis proposed a bio-fertilizer formulation which contain four different beneficial microbes viz. nitrogen fixing microbe, phosphate solubilizing microbe, potassium solubilizing microbe and IAA producing microbe preserved in cocopeat as the carrier material.

After the assimilation of beneficial microbes in the carrier material, the bio-fertilizer created was tested using rhizopod assay for root elongation of *N. cadamba* seedlings. The bio-fertilizer was also tested in pot and field trials to study the growth enhancing effects of *N. cadamba* seedlings. This chapter would summarize the results and findings obtained in this research study that had been previously discussed in Chapter 2, 3, 4 and 5 of this thesis. Further recommendations for this research study would also be discussed in this chapter.

6.2 Conclusions

The results had shown that the high concentrations of beneficial microbial inoculants of more than $10^8$ CFU mL$^{-1}$ could be achieved in using nutrient medium with additives. Four beneficial microbial strains viz. *Streptomyces gramineus* (Nitrogen fixing microbe), *Serratia nematodiphila* (Phosphate solubilizing microbe), *Bacillus cereus* Strain I (Potassium solubilizing microbe) and *Bacillus cereus* Strain II (IAA producing microbe) were obtained from previous researcher for this study. To determine the suitable growth media for the fermentation of each microbial strain, microbial strains were cultured in different growth medias with additive. Results had shown that *Streptomyces gramineus* (Nitrogen fixing microbe) was best cultured in Tryptic soy broth with 0.3 % yeast. Meanwhile, *Serratia nematodiphila* (Phosphate solubilizing microbe), *Bacillus cereus* Strain I (Potassium solubilizing microbe) and *Bacillus cereus* Strain II (Indole acetic acid
producing microbe) were best cultured in Nutrient broth with 0.3 % yeast. The inclusion of additive to growth media could further enhance the multiplication of microbes during fermentation by supplying microbes with carbon.

Bio-fertilizer can be either in liquid or carrier-based form. Considering that the field trial location, Sabal Forest Reserve, is approximately 86 km from the laboratory, the idea of formulating a solid or carrier-based bio-fertilizer was mooted. During the study, the physical and chemical properties of five carrier materials namely cocopeat, vermiculite, sawdust, compost and charcoal were analysed, followed by each of their abilities to sustain high level of microbial population. Results showed that cocopeat was a suitable carrier material which had all the essential properties to sustain bio-fertilizer microbial population at $10^8$ CFU g$^{-1}$. There had been questionings whether liquid or carrier-based bio-fertilizer has better storage life. To answer this, a study was conducted to compare the viabilities of selected microbial strains in both liquid and carrier-based bio-fertilizer. Similar microbial inoculants were used to formulate both forms of bio-fertilizer. During the 180 days of study, both liquid and carrier-based bio-fertilizers were stored at 28 °C, away from direct sunlight. Results showed that the number of viable microbial cells in carrier-based bio-fertilizer at 90th day was conserved at $10^8$ CFU g$^{-1}$, albeit at day 180 the number of viable microbial cells started to decrease. Meanwhile, the number of viable microbial cells in liquid bio-fertilizer had started to decrease at 90th day and there was no sign of microbial growth being detected at 180th day. This results indicated that carrier-based bio-fertilizer had better storage life compared to liquid bio-fertilizer. Carrier-based bio-fertilizer has a minimum shelf life of 90 days whereby the viable microbial cells was conserved at $10^8$ CFU g$^{-1}$.

One of the objectives of this research study is to formulate a bio-fertilizer that could help in the root elongation of *N. cadamba* seedlings. As these seedlings require moist condition to grow well, the idea is to formulate a bio-fertilizer containing IAA producing microbe which could aid in the elongation of seedlings’ roots, thus helping them reach out to water sources.

*Neolamarckia cadamba* seedlings were grown inside rhizopods being placed in a water table of 0.5 m. Seedlings growing in rhizopods placed in water table is a simulation of
seedlings growing in a water limitation environment as water source is further away from the roots of seedlings. Results showed *N. cadamba* seedlings treated with treatments consisting purely of bio-fertilizer and bio-fertilizer with half regime chemical fertilizer had significantly higher mean rate of root elongation compared to seedlings treated with chemical fertilizer and no fertilizer treatments.

The effects of bio-fertilizer on the growth of *N. cadamba* seedlings was tested using a pot trial study. The pot trial was conducted in an open area at SFC. Results of the pot trial showed that dual combination of bio-fertilizer with half regime chemical fertilizer treatment and full regime chemical fertilizer treatment had comparable effectiveness in enhancing the shoot height and root collar diameter of *N. cadamba* seedlings. The results of the pot trial also showed that both dual combination of bio-fertilizer with half regime chemical fertilizer treatment and full regime chemical fertilizer treatment had comparable effectiveness in enhancing the mean root dry weight of *N. cadamba* seedlings. Interestingly, dual combination of bio-fertilizer with half regime chemical fertilizer treatment had higher effectiveness in enhancing the mean dry shoot weight of seedlings compared to full regime chemical fertilizer treatment. Dual combination of chemical fertilizer with bio-fertilizer treatment could increase seedlings’ height, root collar diameter, root and shoot dry weights.

Results from small scale pot trials had shown to be repeatable in a field trials. As in the pot trial, the bio-fertilizer formula was also shown to be among the best on par with full regime chemical fertilizer in the field trial.

### 6.3 Further Recommendations

The effectiveness of the current bio-fertilizer formulation consisting of beneficial microbes viz. nitrogen fixing microbe, phosphate solubilizing microbe, potassium solubilizing microbe and IAA producing microbe in enhancing the growth of *Neolamarckia cadamba* seedlings had been demonstrated in pot and field trials. Although this bio-fertilizer formulation had been effective, other microbes that could synthesize phytohormones other than IAA could still be considered to improve the formulation. With the addition of other microbes which could produce phytohormones viz. ethylene,
cytokinin and gibberellin (Gnanamanickam 2006), the effectiveness of the bio-fertilizer in enhancing growth of *N. cadamba* seedlings could be further increased. With controlled production of ethylene, this phytohormone has the ability to induce shoot and root to undergo differentiation and adventitious root formation (Babalola 2010. Meanwhile, production of cytokinin could induce seedling’s cell division (Schmülling 2002) and production of gibberellin could induce seedling’s stem elongation (Gupta & Chakrabarty 2013).

Bio-fertilizer is important for the sustainability of forestry as it could improve the fertility of soil. However, it would be advantageous if the bio-fertilizer being formulated could also act as bio-pesticides with the addition of PGPR that produce antibiotics, activate induced systemic resistance in *N. cadamba* or degrade xenobiotics (Srivastava & Sharma 2014). The two main pests that may harm *N. cadamba* are white grubs and *Margaronia* sp. (Nair 2000). With the development of bio-fertilizer that could also act as bio-pesticide, *N. cadamba* could better in the field.

Currently, field trial results showed that dual combination of 100.0 g of bio-fertilizer with 7.5 g of chemical fertilizer treatment that could produce effectiveness comparable to 15.0 g of chemical fertilizer treatment in enhancing the growth of *N. cadamba* seedlings. Further works need to be carried out to determine whether an increment in bio-fertilizer dosage could further reduce the dosage for chemical fertilizer in the dual combination treatment. As shown in the study conducted by Farahat et al. (2014) on *Paulownia kawakamii*, seedlings treated with 300.0 g of bio-fertilizer with 25.0 g of chemical fertilizer had higher shoot height and root collar diameter compared to seedlings treated with 100.0 g stand-alone chemical fertilizer treatment.

As the application of bio-fertilizer could also exert effect on the soil of *N. cadamba* plantations, studies regarding the chemical and biological properties of soil, soil quality index, soil pH and soil nutrient index should be conducted as these factors are also indicators to assess the effectiveness of bio-fertilizer (Mulyani et al. 2017). Research should also be conducted to study the effects of chemical fertilizer and dual combination of bio-fertilizer with chemical fertilizer on the wood density of *N. cadamba* seedlings at the field (du Toit et al. 2001).
6.4 Concluding statements

This thesis study aims to formulate a bio-fertilizer containing four species of microbial strains which could reduce excessive application of chemical fertilizer by acting as an alternative, enhancing the root elongation and growth of *N. cadamba*. Beneficial microbial inoculants assimilated well with cocopeat to form the bio-fertilizer. Cocopeat was able to maintain the population of beneficial microbes at $10^8$ to $10^9$ CFU mL$^{-1}$. When bio-fertilizer was applied together with half-regime of chemical fertilizer, the growth of *N. cadamba* was significantly enhanced. The aim and objectives of this thesis study have been achieved.
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### Appendix

1. Mean shoot height increment of *N. cadamba* seedlings in pot trial under different treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Shoot Height Increment (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 DAT</td>
</tr>
<tr>
<td>T1</td>
<td>0.639</td>
</tr>
<tr>
<td>T2</td>
<td>0.828</td>
</tr>
<tr>
<td>T3</td>
<td>5.061</td>
</tr>
<tr>
<td>T5</td>
<td>4.639</td>
</tr>
</tbody>
</table>

2. Mean root collar diameter increment of *N. cadamba* seedlings in pot trial under different treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Root Collar Diameter Increment (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 DAT</td>
</tr>
<tr>
<td>T1</td>
<td>0.229</td>
</tr>
<tr>
<td>T2</td>
<td>0.201</td>
</tr>
<tr>
<td>T3</td>
<td>2.258</td>
</tr>
<tr>
<td>T4</td>
<td>1.327</td>
</tr>
<tr>
<td>T5</td>
<td>2.484</td>
</tr>
<tr>
<td>T6</td>
<td>1.204</td>
</tr>
</tbody>
</table>
3. Mean dry weights of *N. cadamba* seedlings treated with different treatments after 90 days of treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Dry Weights (g)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shoot</td>
<td>Root</td>
</tr>
<tr>
<td>T1</td>
<td>12.988</td>
<td>5.557</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>13.321</td>
<td>5.716</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>21.034</td>
<td>11.628</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>15.958</td>
<td>8.263</td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>22.686</td>
<td>11.206</td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>18.462</td>
<td>9.698</td>
<td></td>
</tr>
</tbody>
</table>

4. Mean dry weights of *N. cadamba* seedlings under different treatment groups after 180 days of treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Dry Weights (g)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shoot</td>
<td>Root</td>
</tr>
<tr>
<td>T1</td>
<td>14.977</td>
<td>5.698</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>16.794</td>
<td>5.933</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>30.971</td>
<td>15.370</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>21.246</td>
<td>12.077</td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>34.930</td>
<td>16.030</td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>23.321</td>
<td>13.152</td>
<td></td>
</tr>
</tbody>
</table>
5. Mean shoot height increment of *N. cadamba* seedlings under different treatments.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment T1</th>
<th>Treatment T2</th>
<th>Treatment T3</th>
<th>Treatment T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2.543 cm</td>
<td>4.919 cm</td>
<td>6.162 cm</td>
<td>5.957 cm</td>
</tr>
<tr>
<td>60</td>
<td>10.010 cm</td>
<td>26.762 cm</td>
<td>15.452 cm</td>
<td>24.571 cm</td>
</tr>
<tr>
<td>90</td>
<td>25.386 cm</td>
<td>77.681 cm</td>
<td>34.510 cm</td>
<td>65.419 cm</td>
</tr>
<tr>
<td>120</td>
<td>40.100 cm</td>
<td>124.633 cm</td>
<td>54.748 cm</td>
<td>106.514 cm</td>
</tr>
<tr>
<td>150</td>
<td>51.767 cm</td>
<td>167.014 cm</td>
<td>72.986 cm</td>
<td>142.610 cm</td>
</tr>
<tr>
<td>180</td>
<td>65.719 cm</td>
<td>213.443 cm</td>
<td>96.605 cm</td>
<td>184.229 cm</td>
</tr>
</tbody>
</table>

6. Mean root collar diameter increment of *Neolamarckia cadamba* seedlings in field trial under different treatments.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment T1</th>
<th>Treatment T2</th>
<th>Treatment T3</th>
<th>Treatment T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.596 mm</td>
<td>0.713 mm</td>
<td>0.861 mm</td>
<td>0.744 mm</td>
</tr>
<tr>
<td>60</td>
<td>2.616 mm</td>
<td>6.367 mm</td>
<td>3.000 mm</td>
<td>5.637 mm</td>
</tr>
<tr>
<td>90</td>
<td>7.016 mm</td>
<td>19.672 mm</td>
<td>8.940 mm</td>
<td>16.490 mm</td>
</tr>
<tr>
<td>120</td>
<td>10.614 mm</td>
<td>30.681 mm</td>
<td>14.070 mm</td>
<td>25.446 mm</td>
</tr>
<tr>
<td>150</td>
<td>13.652 mm</td>
<td>38.117 mm</td>
<td>18.185 mm</td>
<td>33.307 mm</td>
</tr>
<tr>
<td>180</td>
<td>16.865 mm</td>
<td>47.776 mm</td>
<td>23.710 mm</td>
<td>42.229 mm</td>
</tr>
</tbody>
</table>
7. Measuring root length of *N. cadamba* seedling from rhizopod assay.

8. 180 days of pot trial conducted at SFC.
9. *Neolamarckia cadamba* seedling without any treatment at Sabal field on day 180.

10. *Neolamarckia cadamba* seedling treated with 15.0 g chemical fertilizer at Sabal field on day 180.
11. *Neolamarckia cadamba* seedling treated with 7.5 g chemical fertilizer at Sabal field on day 180.

12. *Neolamarckia cadamba* seedling treated with dual combination of 7.5 g chemical fertilizer with 100.0 g bio-fertilizer at Sabal field on day 180.