SOIL MYCORRHIZAL INOCULUM OF NEOLAMARCKIA CADAMBA

by

JOHN SABANG

A thesis

presented in fulfilment of the requirement for the degree

Doctor of Philosophy (Science)

Swinburne University of Technology

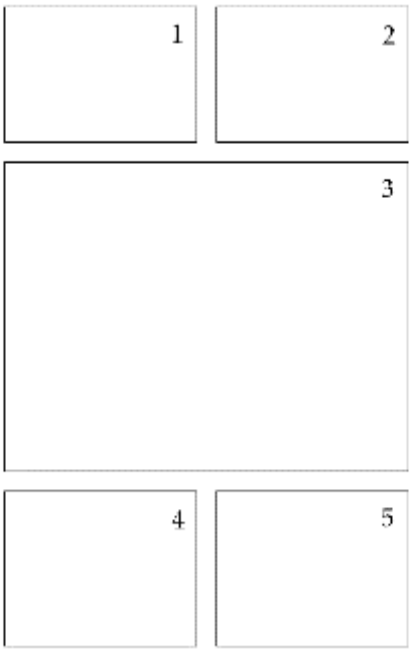
2019

SOIL MYCORRHIZAL INOCULUM OF NEOLAMARCKIA CADAMBA





JOHN SABANG



Inside front cover.
1. *Neolamarckia cadamba* seeds
2. Plantlets from seeds sown
3. Soil trap culture
4. Cross-section of soil mycorrhizal inoculum in polybag
5. Inoculated seedlings

Without Him nothing was made that has been made.

SOIL MYCORRHIZAL INOCULUM OF NEOLAMARCKIA CADAMBA

by

John Sabang
B Sc. Agriculture Science (Universiti Pertanian Malaysia)
PG Dip. Environmental Impact Assessment (Murdoch Univ.)
M Sc. Environmental Science (Murdoch Univ.)

Supervisors:

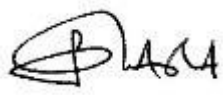
Associate Professor Dr. Peter Morin Nissom
Principal Coordinating Supervisor
Faculty of Engineering, Computing and Science
Swinburne University of Technology Sarawak

Dr. Daniel Tan Lee Tung
Coordinating Supervisor
Faculty of Engineering, Computing and Science
Swinburne University of Technology Sarawak

Dr. Mark Bloomberg
Associate External Supervisor
Faculty of Agribusiness and Commerce
Lincoln University, New Zealand

Declaration

I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution.



_____

John Sabang

Abstract

The development of forest plantations in Sarawak started at a much later date as compared to the neighboring State of Sabah. Since 1997, 42 forest plantation concessions have been issued in Sarawak covering 2.57 million hectares with a net presumably suitable plantation area of 1 million hectares. Areas planted with *Neolamarckia cadamba* made up the fourth largest area, making it the most planted indigenous tree species.

The traditional soil resource evaluation generally measures physical and chemical properties of the soil, but lacks good quality data on rhizosphere microorganisms which could be utilized in managing the issue of poor tree growth in forest plantations in Sarawak. This thesis envisages that inexpensive and simple soil bio-enhancement practices could be considered to be the way forward to increase tree growth performance and ensure the viability and profitability of timber production from forest plantations in Sarawak. The thesis encompasses a baseline study, a field trial and several ancillary nursery experiments.

The soils of the three sites, *Neolamarckia cadamba* phenology plot in Kubah N.P., forest nursery in Semengoh N.R., and the field trial plot in Sabal F.R. were generally acidic and the soil nutrient contents were rated low except for the calcium content of the phenology plot soil. The need for the use of fertilizers at the field planting site was presumably necessary for favorable tree growth. The soil procured by the forest nursery for planting media preparation was extremely low in spore count as compared to the other two sites. Coupled with poor soil at the field planting site, the need to test the soil "bio-fertilizer" produced on planting media used in raising seedlings for planting is coherent.

Soil mycorrhizal inoculum produced has about 10 times more spores than the forest nursery soil. However, the mean percentage of root colonization of inoculated seedlings was only 10%. Root colonization was also observed on non-inoculated seedlings as well, which was just slightly less, at 8%. The just slightly higher root colonization of inoculated seedlings might possibly indicate the presence of arbuscular mycorrhizal fungi specific

to *Neolamarckia cadamba* in the starter soil collected from the *Neolamarckia cadamba* phenology plot. However, arbuscular mycorrhizal fungi propagules specific to *Neolamarckia cadamba* were probably found in soils from all three sites.

The use of soil mycorrhizal inoculum to enhance the growth of seedlings planted out to the field was dismayingly not effective. The use of chemical fertilizer with an efficacy period of six months enhanced tree growth in the field. However, its use together with soil mycorrhizal inoculums, did not show any difference beyond the period of six months after planting out. To sustain the good growth of *Neolamarckia cadamba* in the field, a proper fertilizer regime was needed as part of the silviculture practices. Nitrogen, phosphorus and zinc were found to be soil nutrients that influence growth of *Neolamarckia cadamba*. *Neolamarckia cadamba* requires a proper fertilizer regime, and as observed during the field trial, regular weed control, systematic pest control, and maybe pruning of branches and multiple tree leaders, were also necessary.

The soil trap culture trial indicated that the use of a local lemongrass variety was able to enhance sporulation of the fungal spores. Spore count of soil with the nurse plant was more than double that of starter soil collected from the base of natural stands of *Neolamarckia cadamba*. Starter soil planted with nurse plants and treated without fertilizer showed better sporulation. The simple and low cost protocol for soil mycorrhizal inoculum production using soil trap culture in this trial could be used by the forest nursery in Semengoh N.R. and forest nurseries across Sarawak, if the need arises.

The pot substrate culture trial was able to produce distinguishable and definite spore characteristics, namely by spore shape and color. Five spore morphotype specimens coded D1, D2, D3, D4 and D5 were segregated based on composition of spore counts. The D1 spore specimen was the most abundant as compared to the other spore specimens. The D1 specimen spore count was significantly and positively correlated with the percentage root colonization of *Neolarmackia cadamba*. This probably indicates that the D1 spore specimen may have influenced the root colonization of *Neolamarckia cadamba*. Spores of the D1 specimen were characterized as globose shaped, a pale yellow

color and with sizes from 100-200 μm. However, root colonization by the D1 spore specimen was very low. The sterilization of planting media used in the root infectivity experiment seemed to eliminate completely all inherent fungal propagules and other soil microorganisms. However, *Neolamarckia cadamba* roots were also colonized by other non-arbuscular mycorrhizal fungi and this could be due to contamination of spore inoculum of the D1 specimen used in the single spore culture trial.

The phylogenetic tree derived for the DNA sequence of the D1 spore specimen indicated that it was clustered together with the family of Glomeraceae namely *Glomus mosseae*, and *Glomus etunicatum*. The re-propagation culture trial yielded a similar spore morphotype with simple three-layer spore walls that extended to the subtending hyphal wall. A search for similarity in spore cellular characteristics to an on-line voucher specimen database was traced to *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe. Future work could refocus on further ascertaining the nomenclature of the D1 spore specimen and its symbiotic relationship of the arbuscular mycorrhizal fungi species with *Neolamarckia cadamba*. Helper soil microorganisms should also be studied to determine their roles on the root colonization of *Neolamarckia cadamba*.

List of acronyms

AMF	Arbuscular Mycorrhizal Fungi/Arbuscular Mycorrhiza Fungal
ANOVA	Analysis of Variance
B	Boron
BLAST	Basic Local Alignment Search Tool
BN	Billion
C	Carbon
Ca	Calcium
CBE	Chloral Black E
CEC	Cation Exchange Capacity
CN	Carbon Nitrogen
CNP	Carbon Nitrogen Phosphorus
Co	Cobalt
Cu	Copper
DBH	Diameter at Breast Height
dd	Double Distilled
DFH	Dot Filter Hybridization
F.R.	Forest Reserve
Fe	Iron
FN	Forest Nursery
FTP	Field Trial Plot
ITS	Internal Transcriber Spacer
LOI	Lost on Ignition
LSD	Least Significant Difference
LSU	Large Sub-Unit
MAI	Mean Annual Increment
MM	Million
MMI	Monthly Mean Increment
MPN	Most Probable Number
MS	Malaysian Standard
MTT	Tetrazolium Bromide
N	Nitrogen
N.P.	National Park
N.R.	Nature Reserve
NC	*Neolamarckia cadamba*
NJ	Neighbouring Joining
NP	Nurse Plant
OUT	Operational Taxonomical Unit
P	Phosphorus
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promoting Rhizobacteria
pH	Potential of Hydrogen
PP	Phenology Plot
ppm	Parts Per Million
PSC	Pot Substrate Culture

PVLG	PolyVynl-Lacto Glycerol
QMI	Quarterly Mean Increment
RM	Malaysian Ringgit
RPC	Re-propagation Culture
rpm	Revolution Per Minute
SE	Standard Error
SMI	Soil Mycorrhizal Inoculum
SOM	Soil Organic Matter
SPA	Seed Production Area
SSC	Single Spore Culture
SSU	Small Sub-Unit
STC	Soil Trap Culture
TE	Tris-EDTA Buffer

Table of contents

Abstract vii
List of acronyms x
List of figures xix
List of tables xxii
Acknowledgements xxiii
1 Introduction 1
1.1 Forest plantation, bio-fertilizer and arbuscular mycorrhizal fungi 2
1.1.1 Forest plantation in Sarawak 2
1.1.2 Bio-fertilizer 5
1.1.3 Arbuscular mycorrhizal fungi 8
1.2 Aim and objectives of the study 10
1.2.1 Aim 10
1.2.2 Objectives 11
1.3 Justification 12
2 Literature Review 13
2.1 Neolamarckia cadamba 14
2.1.1 The Sarawak experience 14
2.1.2 *Neolamarckia cadamba* ecology 15
2.1.3 *Neolamarckia cadamba* growth outside Sarawak 16
2.1.4 *Neolamarckia cadamba* silviculture 17
2.1.5 *Neolamarckia cadamba* utilization 21
2.2 Mycobiont 22
2.2.1 Taxonomy of the mycobiont 22
2.2.2 Importance of the mycobiont 26
2.2.3 Utilization of the mycobiont 29
1.2.4 Inoculum of the Mycobiont 33
3 General materials and methods 36
3.1 Field trial 36
3.2 Nursery trials 38
3.3 Work flow 39
3.4 Location of the study sites 40
4 Baseline information of the study sites 43
4.1 Aims 43
4.2 Materials and methods 44
4.2.1 Sourcing of arbuscular mycorrhizal fungi propagules 44
4.2.2 Soil type classification 45
4.2.3 Topography survey 45
4.2.4 Soil disturbance survey 46
4.2.5 Rainfall measurement 47
4.2.6 Soil Analyses 47
4.2.6.1 Soil sampling 47
4.2.6.2 Soil physical properties 48
4.2.6.3 Soil chemical properties 48
4.2.7 Assessment of soil spores 49
4.2.7.1 Extraction of spores 49

4.2.7.2 Segregation of spores....50
4.2.7.3 Enumeration of spores....51
4.3 Results....52
4.3.1 Phenology plot, Kubah N.P....52
4.3.1.1 Natural stands of *Neolamarckia cadamba*....52
4.3.1.2 Soil type....52
4.3.1.3 Soil texture of pit profile....54
4.3.1.4 Properties of composited soil sample....55
4.3.1.5 Soil spores....56
4.3.2 Forest nursery, Semengoh N.R....57
4.3.2.1 Properties of composited soil sample....58
4.3.2.2 Soil spores....58
4.3.3 Field trial plot, Sabal F.R....60
4.3.3.1 Soil type....60
4.3.3.2 Soil texture of pit profile....61
4.3.3.3 Properties of composited soil samples....63
4.3.3.4 Micro-physiography....64
4.3.3.5 Soil disturbance....65
4.3.3.6 Rainfall pattern....67
4.3.3.7 Soil spores....68
4.4 Discussion....69
4.5 Conclusion....74
5 Soil mycorrhizal inoculum nursery and field trials....75
5.1 Introduction....75
5.2 Aim....76
5.2.1 Objectives and hypotheses....76
5.3 Materials and methods....76
5.3.1 Production of soil mycorrhizal inoculum....76
5.3.1.1 Collection of starter soil....77
5.3.1.2 Preparation of starter soil....78
5.3.1.3 Planting of nurse plant....78
5.3.1.4 Upkeep of nurse plants....78
5.3.1.5 Processing of soil mycorrhizal inoculum....79
5.3.2 Assessment of soil spores....79
5.3.2.1 Extraction of spores....79
5.3.2.2 Segregation of spores....79
5.3.2.3 Enumeration of spores....80
5.3.3 Preparation of NC seedlings....80
5.3.3.1 Sowing of seeds....80
5.3.4 Inoculation of seedlings....81
5.3.4.1 Preparation of planting media mix....81
5.3.4.2 Application of soil mycorrhizal inoculum....82
5.3.4.3 Transplanting of plantlets....82
5.3.4.4 Hardening and upkeep of seedlings....82
5.3.5 Assessment of seedling morphology at the forest nursery....83
5.3.5.1 Seedling height and root collar diameter....84
5.3.5.2 Seedling dry-biomass....84

5.3.6 Assessment of seedling mycorrhizal roots 85
5.3.6.1 Preparation of root staining and mounting solutions 85
5.3.6.2 Sampling roots for staining 86
5.3.6.3 Clearing and staining roots 86
5.3.6.4 Preparing diagnostic slides....87
5.3.6.5 Quantifying root colonization....87
5.3.6.6 Taking photographs of root fungal structures....88
5.3.7 Field trial plot....89
5.3.7.1 Trial design 89
5.3.8 Field planting....91
5.3.8.1 Preparation of planting site 91
5.3.8.2 Preparation planting holes and planting....91
5.3.8.3 Application of fertilizer....92
5.3.8.4 Up-keeping of trees....92
5.3.9 Assessment of trees at field trial plot 93
5.3.9.1 Tree height and root collar diameter....93
5.3.9.2 Leaf nutrient concentration 94
5.3.9.3 Tree dry-biomass....95
5.3.10 Assessment of tree mycorrizal roots 95
5.3.10.1 Clearing and staining roots....95
5.3.10.2 Preparing diagnostic slides 96
5.3.10.3 Quantifying root colonization....96
5.3.10.4 Taking photographs of root fungal structures 96
5.4 Results....96
5.4.1 Assessment spores of soil mycorrhizal inoculum and forest nursery soil..96
5.4.2 Assessment of seedlings at the forest nursery 97
5.4.2.1 Seedling height and root collar diameter 97
5.4.2.2 Seedling dry-biomass 99
5.4.2.3 Seedling mycorrhizal root 101
5.4.3 Assessment of trees at the field trial plot....103
5.4.3.1 Tree height 103
5.4.3.2 Tree root collar diameter....106
5.4.3.3 Tree diameter at breast height....108
5.4.3.4 Tree branches and internode length....110
5.4.3.5 Mean monthly increment of tree height 111
5.4.3.6 Tree dry-biomass....113
5.4.4 Leaf nutrient concentration....115
5.4.4.1 Macro-nutrients....115
5.4.4.2 Micro-nutrients....119
5.4.5 Tree mycorrhizal root 122
5.5 Discussion 123
5.6 Conclusion 131
6 Soil trap culture trial....133
6.1 Introduction 133
6.2 Objectives 134
6.3 Materials and methods....134
6.3.1 Trial design....134

6.3.2 Establishment of nurse plant 135
6.3.2.1 Collection of starter soil 135
6.3.2.2 Preparation of starter soil 135
6.3.2.3 Planting of nurse plant 135
6.3.2.4 Upkeep of nurse plant 136
6.3.3 Assessment of nurse plant morphology 136
6.3.3.1 Nurse plant dry-biomass 136
6.3.4 Assessment of soil spores 137
6.3.4.1 Extraction of spores 137
6.3.4.2 Segregation of spores 137
6.3.4.3 Enumeration of spores 137
6.4 Results 138
6.4.1 Nurse plant dry-biomass 138
6.4.2 Spore counts 140
6.4.3 Correlation of total spore counts and nurse plant dry-biomass 141
6.5 Discussion 142
6.6 Conclusion 144
7 Pot substrate culture trial and isolation of spore morphotype specimens 146
7.1 Introduction 146
7.2 Aim 147
7.3 Materials and methods 147
7.3.1 Trial design 147
7.3.2 Production of soil mycorrhizal inoculum 148
7.3.2.1 Collection of starter soil 149
7.3.2.2 Preparation of starter soil 149
7.3.2.3 Planting of nurse plant 149
7.3.2.4 Upkeep of nurse plant 149
7.3.2.5 Processing of soil mycorrhizal inoculum 149
7.3.3 Establishment of Neolamarckia cadamba seedlings 149
7.3.3.1 Planting media mix 150
7.3.3.2 Liming requirement 150
7.3.3.3 Sowing of seeds 151
7.3.3.4 Application of soil mycorrhizal inoculum 151
7.3.3.5 Transplanting of plantlets 151
7.3.3.6 Hardening and upkeep of seedlings 151
7.3.4 Assessment of seedling morphology 151
7.3.4.1 Seedling height and root collar diameter 151
7.3.5 Assessment of soil spores 151
7.3.5.1 Extraction of spores 152
7.3.5.2 Segregation of spores 152
7.3.5.3 Enumeration of spores 152
7.3.6 Assessment of seedling mycorrhizal roots 153
7.3.6.1 Preparation of root staining and mounting solutions 154
7.3.6.2 Sampling roots for staining 154
7.3.6.3 Clearing and staining roots 154
7.3.6.4 Preparing diagnostic slides 154
7.3.6.5 Quantifying root colonization 154

7.3.6.6 Taking photographs of root fungal structures....154
7.4 Results....154
7.4.1 Growth of seedlings....155
7.4.2 Segregation of spore to morphotypes....156
7.4.3 Spore count....157
7.4.4 Seedling mycorrhizal root....157
7.5 Discussion....160
7.6 Conclusion....162
8 Single spore culture trial and characterization of D1 spore specimen....163
8.1 Introduction....163
8.2 Aim....164
8.3 Materials and methods....165
8.3.1 Trial design....165
8.3.2 Preparation of spore inoculum....165
8.3.2.1 Extraction of spores....166
8.3.2.2 Segregation of D1 spore specimen....167
8.3.3 Assessment of D1 spore specimen....167
8.3.3.1 Spore count and characterization....167
8.3.3.2 Storage of spores....168
8.3.4 Establishment of Neolamarckia cadamba seedlings....169
8.3.4.1 Preparation of grower pots and liming....169
8.3.4.2 Sterilization of planting media mix....170
8.3.4.3 Transplanting of plantlets....170
8.3.4.4 Inoculation of spores....171
8.3.4.5 Upkeep of seedlings....171
8.3.5 Assessment of seedling morphology....171
8.3.5.1 Seedling height and root collar diameter....171
8.3.5.2 Seedling dry-biomass....171
8.3.6 Assessment of mycorrhizal roots....172
8.3.6.1 Preparation of root staining and mounting solutions....172
8.3.6.2 Sampling roots for staining....172
8.3.6.3 Clearing and staining roots....172
8.3.6.4 Preparing diagnostic slides....172
8.3.6.5 Quantifying root colonization....172
8.3.6.6 Taking photographs of root fungal structures....173
8.3.7 Assessment of D1 spore specimen phylogeny....173
8.3.7.1 Preparation of spores....174
8.3.7.2 Spore DNA extraction....174
8.3.7.3 DNA amplification....175
8.3.7.4 Electrophoresis of second PCR product....176
8.3.7.5 PCR product purification....176
8.3.7.6 Spore DNA sequencing....177
8.3.7.7 Spore DNA sequence processing....177
8.3.7.8 Basic Local Alignment Search Tool (BLAST) search....177
8.3.7.9 Phylogenetic analysis....178
8.3.8 Viability test of D1 spore specimen....178
8.4 Results....179

8.4.1 Seedling morphology....179
8.4.2 Characterization of spore shape, size and color....182
8.4.3 Gel electrophoresis....183
8.4.4 Phylogenetic tree....185
8.4.5 Spore viability....186
8.4.6 Seedling mycorrhizal roots....188
8.5 Discussion....189
8.6 Conclusion....193
9 Re-propagation culture trial and cellular assessment of D1 spore specimen....194
9.1 Introduction....194
9.2 Aim....195
9.3 Materials and methods....195
9.3.1 Trial design....195
9.3.2 Establishment of nurse plant....196
9.3.2.1 Preparation of planting substrate....196
9.3.2.2 Planting of nurse plant....196
9.3.2.3 Upkeep of nurse plant....197
9.3.3 Assessment of nurse plant morphology....197
9.3.3.1 Nurse plant dry-biomass....197
9.3.4 Assessment of soil spores....198
9.3.4.1 Extraction of spores....198
9.3.4.2 Segregation of spores....198
9.3.4.3 Enumeration of spores....198
9.3.5 Assessment of cellular structure of D1 spore specimen....198
9.3.5.1 Preparing diagnostic slides....199
9.3.5.2 Microscopic examination of spores....199
9.3.5.3 Taking photographs of spore structure....200
9.4 Results....200
9.4.1 Nurse plant dry-biomass....200
9.4.2 Spore counts....202
9.4.3 Spore cellular structure....204
9.5 Discussion....206
9.6 Conclusion....209
10 Summary....210
10.1 Background of the thesis....210
10.2 Outcomes of trials....210
10.2.1 Soil mycorrhizal inoculum field trial....212
10.2.2 Soil trap culture trial....214
10.2.3 Pot substrate culture trial and isolation of spore morphotype specimens 215
10.2.4 Single spore culture trial and characterization of D1 spore specimen ..216
10.2.5 Re-propagatn culture trial and cellular assessment of D1 spore specimen 217
10.3 What has the thesis established?....217
10.3.1 Soil trap culture technique....218
10.3.2 *Neolamarckia cadamba* soil mycorrhizal inoculum....218
10.3.3 *Neolamarckia cadamba* fertilizer regime....219

10.4 Future research....219
10.4.1 D1 spore specimen....220
10.4.2 Helper soil microorganisms....221
11 References....224
Appendix....236

List of figures

Figure 1. Planted areas according to tree species (Forest Department Sarawak 2014). 5
Figure 2. Work flow chart of methods and approximated duration. 40
Figure 3. Location perspective of the three study sites....41
Figure 4. Spore suspension. 1: Before agitation, 2: Agitated for easy spores picking, and 3: Spores pipetted into clean petri dish. 51
Figure 5. Soil pit profile at the phenology plot. 54
Figure 6. Percentage sand, silt and clay of soil pit profile at phenology plot. 55
Figure 7. View of spores from phenology plot soil (35X magnification). 57
Figure 8. View of spores from forest nursery soil (35X magnification). 59
Figure 9. Soil pit profile at the field trial plot. 62
Figure 10. Percentage sand, silt and clay of soil pit profile at the field trial plot. 62
Figure 11. Topography of the field trial plot. 65
Figure 12. Spatial distribution of soil disturbance in the field trial plot. 66
Figure 13. Monthly rainfall of areas within close proximity of the field trial plot. 68
Figure 14. View of spores from field trial plot soil (35X magnification). 69
Figure 15. Percentage sand, silt and clay of composited soil samples from the phenology plot, forest nursery, and field trial plot. 70
Figure 16. Spore counts of the phenology plot, forest nursery, and field trial plot soils…. 73
Figure 17. Position of blocks, treatments, and replications of soil mycorrhizal inoculum field trial. 90
Figure 18. Spore counts of soil mycorrhizal inoculum and forest nursery soil. 97
Figure 19. The maximum, minimum, mean, median, upper and lower percentiles of inoculated and non-inoculated seedlings. 1: Seedling height, and 2: Root collar diameter……..98
Figure 20. Scatter plot of seedling height and root collar diameter. 1: Inoculated, and 2: Non-inoculated. 98
Figure 21. The maximum, minimum, mean, median, upper and lower percentiles of shoot and root dry-biomass of inoculated and non-inoculated seedlings. 100
Figure 22. Scatter plot of shoot and root dry-biomass. 1: Inoculated seedlings, and 2: Non-inoculated seedlings. 100
Figure 23. Fungal structures in roots. 1: arbuscules and vesicules, 2: arbuscules and hyphae, and 3: non-mycorrhizal vesicles. 102
Figure 24. The maximum, mean, median, and upper percentiles of percentage root colonization of inoculated and non-inoculated seedlings. 102
Figure 25. Scatter plot of percentage root colonization and root dry-biomass. 1: Inoculated seedlings, and 2: Non-inoculated seedlings. 103
Figure 26. The maximum, minimum, mean, median, upper and lower percentiles of tree heights at 12 months. 104
Figure 27. Least Significant Difference test on mean tree height with one Standard Error bars (different letters indicate significant difference). 105
Figure 28. Monthly means of tree heights with one Standard Error bars. 106
Figure 29. The maximum, minimum, mean, median, upper and lower percentiles of tree root collar diameters - 5 cm above ground level at 12 months. 107

Figure 30. Least Significant Difference test on means of root collar diameter - 5 cm above ground level (different letters indicate significant difference). 108
Figure 31. The maximum, minimum, mean, median, upper and lower percentiles of tree diameter at breast height, at 12 months. 109
Figure 32. Monthly means of root collar diameters with one Standard Error bars.....109
Figure 33. The maximum, minimum, mean, median, upper and lower percentiles of trees at 12 months. 1: Number of branches, and 2: Tree internode length. 111
Figure 34. Mean monthly increment of tree height with two moving average trend lines……. 112
Figure 35. Mean monthly increment of tree height and monthly rainfall. 113
Figure 36. Scatter plot of dry-biomass and tree heights. 1: SMI above ground, 2: Control above ground, 3: SMI root, and 4: Control root. 114
Figure 37. Scatter plot of dry-biomass and tree root collar diameters. 1: SMI above ground, 2: Control above ground, 3: SMI root, and 4: Control root. 114
Figure 38. Leaf primary and secondary macro-nutrient concentrations at planting out……. 116
Figure 39. Leaf nutrient concentrations at 0 month and every quarterly. 1: Nitrogen, 2: Phosphorus, 3: Potassium, 4: Calcium, and 5: Magnesium. 118
Figure 40. Scatter plot of mean quarterly increment of tree heights and percentage leaf dry-biomass. 1: Nitrogen, 2: Phosphorus, 3: Potassium, 4: Calcium, and 5. Magnesium…… 119
Figure 41. Leaf micro-nutrient concentrations at planting out. 119
Figure 42. Leaf nutrient concentrations at 0 month and every quarterly. 1: Boron, 2: Copper, 3: Zinc, 4: Iron, and 5: Manganese. 121
Figure 43. Scatter plot of mean quarterly increment of tree heights and percentage leaf dry-biomass. 1: Boron, 2: Copper, 3: Zinc, 4: Iron, and 5. Manganese. 122
Figure 44. Fungal structures in roots. 1: and 2: Inoculated tree (Tree no. 84), 3: Non-inoculated tree (Tree no. 188), and 4: Non-inoculated tree (Tree no. 50). 123
Figure 45. The postulated monthly means of tree height of inoculated trees with fertilizer. 127
Figure 46. Position of treatments and replicates in soil trap culture trial. 135
Figure 47. View of spores under stereo microscope superimposed with vertical lines (35X magnification). 138
Figure 48. The maximum, minimum, mean, median, upper and lower percentiles of shoot and root dry-biomass of nurse plants with and without fertilizer. 139
Figure 49. The maximum, minimum, mean, median, upper and lower percentiles of sporulation by nurse plants with and without fertilizer, and starter soil (Control).....140
Figure 50. Spore composition of nurse plants with and without fertilizer, and starter soil (Control). 141
Figure 51. Scatter plot of total spore counts and dry-biomass of nurse plants. 1: NP shoot, 2: NP+Fertilizer shoot, NP root, and NP+Fertilizer root.....142
Figure 52. Position of treatments and replicates in the pot substrate culture trial. 148
Figure 53. Selected spore morphotype specimens. 1: D1, 2: D2, 3: D3, 4: D4, and 5: D5……. 153
Figure 54. The maximum, minimum, mean, median, upper and lower percentiles of inoculated seedlings without and with lime, and non-inoculated seedlings (Control). 1: Seedling heights, and 2: Seedling root collar diameters. 155

Figure 55. Total spore counts and composition of spore specimens. 156
Figure 56. Fungal structures in roots. 1: Inoculated seedling without lime, 2: Inoculated seedling with lime, and 3: Non-inoculated seedling. 158
Figure 57. Composition of root fungal structures of inoculated seedlings without lime, inoculated seedlings with lime, and non-inoculated seedlings (Control). 159
Figure 58. Scatter plot of percentage root colonization and spore counts of spore specimens. 1: D1, 2: D2, 3: D3, 4. D4, 5: D5, and 6: Others. 160
Figure 59. Position of treatments and replicates in single spore culture trial. 165
Figure 60. Enumeration of spore characteristics of D1 spore specimen (Shapes: G-globose: S/G-sub-globose, I-irregular, Size: 100μm, and Color: W-white, P/Y-pale yellow). 168
Figure 61. Means and one Standard Error bars. 1: Seedling heights, and 2: Seedling root collar diameters. 180
Figure 62. Means and one Standard Error bars of seedling shoot and root dry-biomass……..181
Figure 63. The maximum, minimum, mean, median, upper and lower percentiles of percentage spore count of D1 spore specimen's shape, size, and color. 183
Figure 64. Gel electrophoresis results of amplified D1 spore specimen's DNA. 1: First PCR, and 2: Second PCR. 184
Figure 65. Phylogenetic tree of D1 spore specimen. 186
Figure 66. Condition D1 spore specimen and at different incubation periods. 1: Autoclaved spores, 2: 40 hours, 3: 120 hours, and 4: > 30 days. 187
Figure 67. Stained and dead spores after 40 hours and 120 hours of incubation. 187
Figure 68. Hyphal structures in roots. 1: Uneven/bumpy edges, 2: Thin and septate, and 3: Smooth edges and septate. 188
Figure 69. Position of treatments and replicates in re-propagation culture trial. 196
Figure 70. Means and one Standard Error bars of dry-biomass of nurse plant shoot and root…….201
Figure 71. View of D1 spore specimen among other globose and ovoid shapes spores (35X magnification).....202
Figure 72. Spore morphotype composition of limed and non-limed substrates, and spore inoculum used in single spore culture trial.....203
Figure 73. Structure of D1 spore specimen isolated. 1: Pot substrate culture trial, and 2: Re-propagation culture trial. 204
Figure 74. Cellular spore wall details of D1 specimen.....205
Figure 75. Cellular structure of *Glomus mosseae* spore's wall (Blaszkowski et al. 2005) ……...206
Figure 76. Combo pictures of materials and methods. 1: Natural stand of *Neolamarckia cadamba*, 2: Lemongrass nurse plant, 3: Application of soil mycorrhizal inoculum, 4: Preparation of field trial plot, 5: Installation of Symon rain gauge, 6: Soil auger profile, 7: Application of fertilizer nugget, 8: Measurement of root collar diameter, 9: Measurement of tree height, 10: Tree defoliation, 11: Stem canker, 12: Leaf sampling for nutrient analysis, 13: Soil mycorrhizal inoculum field trial, 14: Soil trap culture trial, 15: Pot substrate culture trial, 16:) Root staining to determine mycorrhizal colonization, 17: Single spore culture trial, 18: Spore inoculation, 19: Crushing spore using micro-pestle for DNA extraction, and 20: Re-propagation culture trial. 238
Figure 77. Mean Isohyetal map of Sarawak 2014. 239

List of tables

Table 1. Spore characteristics of Glomeromycota from class to genus level (Oehl et al.2011, pp. 194-195) 24
Table 2. Arbuscular mycorrhizal fungi taxonomical contention (Berruti et al. 2014, pp. 161) 26
Table 3. Seed production areas and phenology plots of Sarawak Forest Tree Seed Bank….. 44
Table 4. Soil parameters and laboratory analytical methods. 48
Table 5. Description of soil pit profile at the phenology plot. 53
Table 6. Rating and general interpretation of phenology plot soil properties (Deloitte 1990, pp.7, 9-10). 56
Table 7. Spore counts of phenology plot soil (40 g) 57
Table 8. Rating and general interpretation of forest nursery soil properties (Deloitte 1990, pp. 7, 9-10). 58
Table 9. Spore counts of forest nursery soil (40 g). 59
Table 10. Description of soil pit profile in the field trial plot. 60
Table 11. Field trial plot soil properties (Upper Slope, Middle Slope, and Lower Slope) ……..... 63
Table 12. Rating and general interpretation field trial plot soil properties (Deloitte 1990, pp. 7, 9-10) ……... 64
Table 13. Composition of field trial plot based on areas of slope positions. 65
Table 14. Composition of field trial plot based on areas of disturbance severity. 67
Table 15. Spore counts of field trial plot soils (40 g) (Upper Slope, Middle Slope, and Lower Slope) ……. 69
Table 16. Soil chemical properties of the three sites. 71
Table 17. Leaf nutrient laboratory analytical methods. 94
Table 18. Sturdiness quotients of inoculated and non-inoculated seedlings 99
Table 19. Root-shoot ratios of inoculated and non-inoculated seedlings. 104
Table 20. Root-above ground ratios of trees. 115
Table 21. Tree root colonization at the field trial plot. 123
Table 22. Root-shoot ratio of nurse plants. 139
Table 23. Sturdiness quotients of inoculated seedlings with and without lime and non-inoculated seedlings (Control). 156
Table 24. Main morphological characteristics of spore morphotype specimens. 156
Table 25. Sturdiness quotients of spore inoculated seedlings without and with lime and non-inoculated seedlings (Control). 180
Table 26. Root-shoot ratios of spore inoculated seedlings without and with lime and non-inoculated seedlings (Control). 182
Table 27. Percentage sequence similarities of D1 spore specimen with the arbsuscular mycorrhizal fungi species in Sarawak. 185
Table 28. Root colonization of spore inoculated seedlings without and with lime, and non-inoculated seedlings (Control). 189
Table 29. Nurse plant root-shoot ratio grown on non-limed substrate and limed substrate 202

Acknowledgements

I would like to thank Tunku Abdul Rahman Sarawak Scholarship Foundation for awarding me the scholarship (2/TAR-S1863) to pursue this course. I also would like to thank:

Sarawak Biodiversity Centre for granting "Agreement Pertaining to Access to Collection of and Research on Biological Resources (SBC-RA-0107-JS);"

Assoc. Prof. Dr. Peter Morin Nissom, Dr. Daniel Tan Lee Tung, Ms. Cindy Wee of Swinburne University of Technology Sarawak, and Dr. Mark Bloomberg of Lincoln University, New Zealand for their guidance and moral support rendered throughout this project;

Ms. Hajarin Nyodi, Dr. Pang Shek Ling, Ms. Bibian Micheal Diway, Mr. Andy Samuel, Mr. Guru@Arai Nyam, of Sarawak Forestry Corporation Sdn Bhd, Mr. Holed Jubol of Sarawak Biodiversity Center who have kindly provided technical assistance and materials in kind for laboratory and field works needed for the thesis, *mon ami* Pastor Mark Goodey who graciously proofread draft of thesis, and

Dayang Matu @ Sina Paren Lemunad my wife, for her encouragement and enthusiasm, my three children; Priscilla, Marilyn and Kevin, immediate family members, relatives, and members of Semaba Home Fellowship Group, BEM Olive Garden Church, and friends for their prayers, moral support and well wishes.

1 Introduction

Sustainable forest management and organic agriculture are two notions that have recurrent interests as people at large are becoming more informed about the environment, conservation and a healthy living lifestyle. Sustainable forest management entails conserving protected forest areas and also relying on forest plantations rather than natural forest as a source of timber. The policy to embark on forest plantations in Sarawak for example has multi-pronged long-term strategies. Oscar Johin Ngayop (2013), reiterated that the establishment of forest plantations was geared towards providing an alternative source of wood material for the timber industries as ever increasing demand of raw materials cannot depend solely on one main source. Establishment of forest plantations was envisaged to not only relieve the harvesting on the natural forests but also off-setting the greenhouse effect by conserving and increasing forest cover. Forest plantation also entails rehabilitation and amelioration of degraded areas including abandoned shifting agriculture areas within the Production Forests in Sarawak.

Food products derived from organic agriculture fetch premium prices at supermarkets. This method of agriculture is devoid of any use of chemical fertilizers and pesticides through such management of farms to prevent soil infertility and pest problems. In organic farming, the overall health of soil-microorganism-plant-animal system is maintained and improved (FAO 1998). The interest for more environmental friendly and healthy agriculture is a strong reason to find alternatives to reliance on mineral fertilizer and pesticide (Sasvari et al. 2012, p.1796). Most bio-enhancement products have been designed for annual crops but its application was also much needed by other agricultural sectors such as for fruit and vegetable production, where synthetic agronomical inputs are not allowed or their use is limited by legal restrictions (Malusa, Sas-Paszt & Ciesielska 2012, p. 7).

Bio-fertilizers containing artificially multiplied cultures of efficient selected microorganisms were reputed to help crop plants in uptake of nutrients through

rhizospheric interactions when applied prior, on seed or directly to soil. The use of bio-fertilizers to complement chemical fertilizers is an important component of integrated nutrient management for sustainable agriculture, as they are cost effective and a renewable source of plant nutrients. One such group of microorganism that mobilize P in the soil are the mycorrhizae (Tamil Nadu Agricultural University 2014). Sasvari et al. (2012, p. 1796), reiterated that arbuscular mycorrhizal fungi (AMF) a main component of beneficial soil microorganisms, represent a promising providers of key ecological services. New findings about dominant AMF in different crop systems could be a starting point for the development of well performing and adapted inoculum suitable for field application.

One of the possible field applications of bio-fertilizer is in the establishment of forest plantations. As pointed out by Garcia-Fraile, Menendez and Rivas (2015, p. 183), several plant growth promoting rhizobacteria (PGPR) have been used worldwide for many years as bio-fertilizers, and were observed to have increased crop yields and soil fertility. Hence, they have the potential to contribute towards a more sustainable agriculture and forestry activities.

1.1 Forest plantation, bio-fertilizer and arbuscular mycorrhizal fungi

1.1.1 Forest plantation in Sarawak

The State Government of Sarawak has been committed towards the establishment of one million hectares of commercial forest plantations by 2020 (STIDC 2009, p. 6). The purpose is to cater for the timber processing industries which have been relying on the natural forests for availability of timbers. The highest log production was 19 MM m^3 recorded in 1991 but a declining trend ensued thereafter. The downstream processing industry needed to diversify based on an alternative or new source of raw material to supplement the deficit in timber supply from the natural forests. Thus, the government initiative to embark on a commercial scale of forest plantations by timber companies (Oscar Johin Ngayop 2013). To meet the future needs of timber industry and at the same time, conserve the natural forests, the State Government of Sarawak have been

encouraging and providing incentives to the private sector to establish forest plantations. As expected certain wood properties of planted materials would not necessarily match wood from natural forests due to more juvenile wood. However, this shortfall will be compensated by a relatively high wood biomass generated in manner that is sustainable from forest plantations planted with fast growing tree species (Ling & Wong 2007, p. 266).

The development of the forest plantation industry in Sarawak started at a much later date as compared to the neighboring State of Sabah. The development of large scale commercial forest plantations in Sabah started in 1974 and were largely government driven and ran by government linked companies. The endeavor continued with the participation of other privately owned companies and individuals in forest plantation development towards the late 1980's and 1990's (Annuar Mohammad 2008, p. 4).

Since 1997, 42 forest plantation concessions have been issued in Sarawak covering 2.57 MM hectares with a net presumably suitable plantation area of 1.0 MM hectares (Oscar Johin Ngayop 2013). However, the slow rate of tree planting after almost two decades of issuing the concessions was blamed by the holders as due to the lack of adequate suitable and available land to achieve the 1.0 MM hectares' target set by the State Government of Sarawak. The ambiguous definition of suitable areas for planting included poor or unsuitable soil, and excessive broken terrain (Bong 2018, p. 5). Skeletal soil, usually shallow, stony and even swampy, contains very little nutrients, and typical pest, diseases or poor soil foundation could also be found in the areas assigned. Selection and testing the correct tree species from proven seed sources, provenances and even clonal materials with the ability to grow in such areas were desperately needed.

Ismail Jusoh and Nur Syazni Adam (2012, p. 3), reported that the establishment of forest plantations was rather slow. Incidentally, the rate of forest plantation establishment was also on a declining trend since 2007 and *Acacia mangium*, the major planted species comprising 74% of the total planted area, was susceptible to heart rot disease. The major source of wood for pulp mills in Sumatra, Indonesia was from *Acacia mangium*

plantations. However, during the first and second rotations of tree planting, the plantations suffered from incidences of *Ganoderma* root rot disease which were compounded by the arrival and rapid spread of *Ceratocystis* wilt disease. Tree mortality became so high that the planting of *Acacia mangium* was no longer viable. From the setback caused by the diseases, *Eucalyptus pellita* emerged as the next best candidate species to replace *Acacia mangium* (Hardiyanto, Nambiar & Inail 2015, p. 9). Similarly, besides *Eucalyptus pellita* the contributions of other tree species such as *Falcataria moluccana, Eucalptus deglupta*, and *Neolamarckia cadamba,* locally known as Kelampayan will become important for forest plantations in Sarawak when such scenario occurs.

The status of planted areas areas at the end of 2013, according to tree species is as shown in Figure 1. *Acacia mangium* was the most planted tree species with more than 230,000 hectares followed by *Falcataria moluccana* about 43,000 hectares, and *Eucalyptus spp.* about 25,000 hectares. Planted areas with *Neolamarckia cadamba* were about 20,000 hectares, making it the most planted indigenous tree species. Other tree species planted were about 11,000 hectares which included *Gmelinia arborea*, *Sweitenia macrophylla*, *Havea brasiliensis*, *Octomeles sumatrana*, *Azadirachta excels*a, *Shorea macrophylla*, *Dyera costulata* and *Duabanga moluccana*.

The areas planted with *Acacia mangium* incidentally dwarfed the areas planted with other tree species as they were part of the 6.4 MM hectares of Production Forest land declassified for forest plantations. Establishment of forest plantation in 1999 was the initiative of the State Government of Sarawak. Planting of fast-growing tree species was meant to supply the planned pulp mill situated near Bintulu with the initial production capacity of 750,000 MT of cellulose. The project was a joint-venture between Sarawak Timber Industry Development Corporation, a state agency and the Asia Pulp & Paper Company Ltd was provided with 373,700 hectares of land for planting of fast-growing tree species such as *Acacia mangium*, *Eucalyptus* and others (World Rainforest Movement 1998).

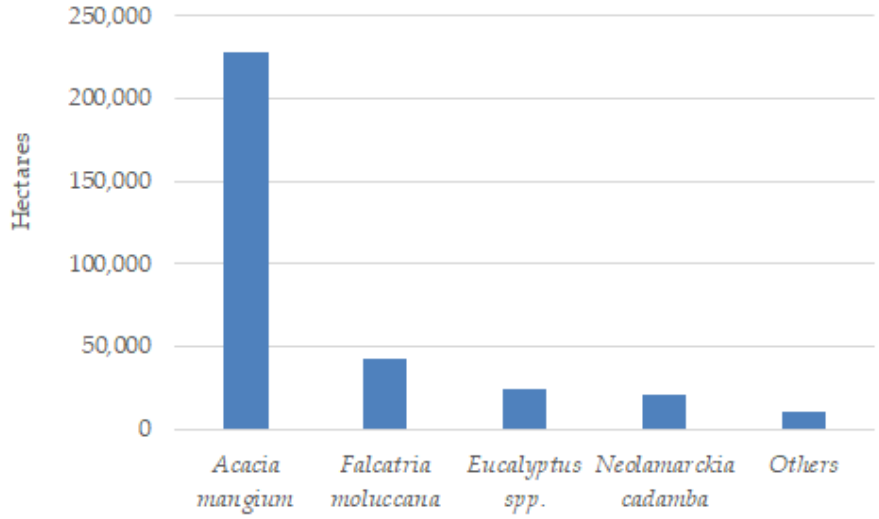


Figure 1. Planted areas according to tree species (Forest Department Sarawak 2014).

As of December 2017, total tree-planted areas amounted to only 408,000 hectares, making the set target of 1.0 MM hectares a daunting task. Nearly RM 2.0 BN has already been invested by planters, and at least another RM 3.0 BN will be needed to meet the targeted one MM hectares (Bong 2018, p. 1). Besides being plagued by a slow planting progress, the other set back is that trees planted could take years to reach maturity. The predicament necessitates time-honed experience and the required level of expertise. These may include the use of effective soil amelioration practices and bio-enhancement technology to increase productivity in such ambiguously suitable forest plantation areas. The application of the right silviculture input including the use of bio-fertilizer should also be considered to mitigate the situation.

1.1.2 Bio-fertilizer

Bio-formulation in agriculture offers an environmentally sustainable approach to increase crop yield and health product as chemical formulation used to kill pathogens, pests and weeds, have a harmful impact on the ecosystem (Arora, Khare & Maheshwari 2011, pp. 97 & 98). Bio-fertilizer is a formulation containing soil microorganisms that are able to facilitate nutrient uptake, increase nutrient availability or stimulate plant growth. As such, it could thus be considered as an alternative or a complement the chemical

fertilization used to increase the production of crops in low input agricultural systems (Martinez-Viveros et al. 2010, p. 296). The term bio-fertilizer should not be used interchangeably with organic fertilizer as the latter contains organic compounds which could increase soil fertility by their decomposition. Additionally, not all formulation PGPR can be termed bio-fertilizers. Bacteria formulation that promote plant growth by control of deleterious organisms are called as bio-pesticides. Similarly, bacteria that can enhance plant growth by producing phytohormones are regarded as bio-enhancers (Ohyama 2006, p. 1).

Bio-fertilization accounts for approximately 65% of the N supply to crops worldwide such as by planting of legumes. Legumes area plants with root nodules capable of fixing atmospheric N. The most efficient N-fixers are bacterial strains belonging to the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Allorhizobium*, which have been studied in most detail (Bloemberg & Lugtenberg 2001, p. 343).

The three major groups of microorganisms considered beneficial to plant nutrition are PGPR, AMF, and N-fixing rhizobia (Malusa, Sas-Paszt & Ciesielska 2012, p. 1). PGPR are soil bacteria that aggressively colonize plant roots and benefit plants by promoting their growth. Crop plants inoculated at an early stage of development with certain strains of PGPR improved their biomass production through a direct effect on root and shoot growth (Saharan & Nehra 2011, p. 1). Saharan and Nehra (2011, p. 1) further added that PGPR influenced the growth, yield of crop plants by an array of mechanisms in their nutrients uptake. The PGPR help in increasing such as soil N by bacteria of legume root nodules, and free-living N-fixing bacteria, other nutrients, such as P, S, Fe and Cu. They produce plant hormones that enable other beneficial bacteria or fungi, which contribute to the control of fungal and bacterial diseases, and also insect pests. As there has been much interest in PGPR research an increasing number of PGPR bio-fertilizer formulation has being commercialized for various crops.

Several PGPR have been used as bio-fertilizers for many years worldwide. Such bio-fertilizers were shown to increase soil fertility and in turn crop yields and hence they have the potential to ensure more sustainability in agriculture and forestry undertakings. However, the technologies for the production and application of bacterial-based inoculums are under constant development and improvement. Nevertheless, bacterial-based bio-fertilizer market has been steadily growing (Garcia-Fraile, Menendez & Rivas 2015, p. 183).

Bio-fertilizer using rhizobia, AMF, and other microorganisms are able to increase the availability of plant nutrients in the soils and thus, improve the yield of crops (Ohyama 2006, p. 1). Maize treated with bio-inoculants such as *Azospirillum*, *Azotobacter*, *Rhizobium*, *Pseudomonas* and AMF showed significant diverse effects compared to non-treated crop (Kannan et al. 2011, p. 48). These microorganisms selected using pot trials and field experiments are cultured and packed with carrier materials. The sterilization of carrier material can be augmented by ionizing radiation as it is one of the best ways to keep bio-fertilizers in storage for a long period and thus making it commercially viable for agriculture, forestry, and reclamation of degraded lands (Ohyama 2006, p. 1).

It is now generally recognized that AMF improve not only the P nutrition of the host plant and hence its growth by increasing resistance to drought stress and some diseases. Consequently, the use of AMF have great potential for sustainable agriculture, and their application in the form AMF inoculation for agricultural crops has been developed commercially (Nopamornbodi & Thamsurakul 2006, p. 90). The combination AMF and other bio-inoculants was proven to be significant in increasing shoot and root length of cotton plants (Sultana & Pindi 2012, p. 123). A bio-fertilizer containing a mixture of PGPR; *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus mucilaginous,* and AMF; *Glomus mosseae or Glomus intraradices* increased growth and nutrient uptake of maize (Wu et al. 2004, p. 155). It also improved soil properties, such as soil organic matter content and total N. PGPR; *Bacillus amyloliquefaciens* and *Bacillus pumilus* formulated with AMF; *Glomus intraradices* enhanced plant growth and yield, N and P nutrient

uptake of tomatoes that were equivalent to the full fertilizer rate without the inoculants (Adesemoye, Torbert & Kloepper 2009, p. 921).

AMF can interact synergistically with a various soil microorganisms such as PGPR to stimulate plant growth (Miransari 2011, p. 917). Future research should focus on functional mechanisms of soil microorganism combinations as effective inoculums (Artursson, Finlay & Jansson 2006, p. 1). The functional mechanisms of particular importance to the functioning of the symbiosis are the role of bacteria in promoting mycorrhizae formation and the AMF that determine the interaction of plant with root and N-fixing bacteria (Fitter & Garbaye 1994, p, 123).

1.1.3 Arbuscular mycorrhizal fungi

Any soil where plants have grown, will have a good chance of containing the spores of AMF. The other less conspicuous parts of the fungi are the webs of hyphae spreading through the soil, and fungal structures within plant roots that are only visible under the view of a microscope. The study and identification of AMF though perplexing, is important because AMF form symbioses with many of terrestrial plants (Young 2012, p, 823). Most tropical trees are habitually mycorrizal in natural soils. Most tropical tree taxa have AMF that penetrate the roots inter-cellularly and intra-cellularly and give rise to characteristic hyphal coils, vesicles and arbuscules. Fungal hyphae extend out from the root into the soil substrate. These loose web of hyphae may bear spores. The spores produced form the basis of the taxonomy of AMF (Alexander, Norani Ahmad & Lee 1992, p. 379).

AMF get their name from formation of branching structures characteristically called arbuscules within the cortical cells of roots. The fungal structures increase the contact area between plant and fungus, and they are the sites of exchange of the C produced by the plant and P absorbed by the fungus. Some of these fungi, also form vesicles, or sack-like reservoirs and therefore, AMF are also known as vesicular arbuscular mycorrhizal fungi (VAM). The fungi grow within plant roots through infective hyphae from hyphal fragments, or asexual spore colonization that eventually developed fungal structure

such as arbuscules within the root. Root colonization arises from extension of hyphae from one infected root to another, or from an infected root to the root of another plant (Bever et al. 2001, p. 924). Some AMF do not produce vesicules under all conditions, and so should be called just as arbuscular mycorrhizal fungi (AM) and not vesicular-arbuscular mycorrhizal fungi (VAM) (Kheyrodin 2014, p. 42). The acronyms AM has become more common than VAM, though the latter is still frequently used as there is no consensus about which name for these associations is most correct. As suggested by Kheyrodin (2014, p. 42), all papers should include VAM or AM in their title if the these associations are of interest.

AMF are mainly thought to facilitate P uptake in plants, but they can provide equally beneficial functions such as providing protection from root pathogens. Infection of *Setaria glauca* root by the fungal pathogen *Fusarium oxysporum* was suppressed by its AMF partner (Sikes 2010, p. 763). AMF in the family Glomeraceae limited the pathogen abundance of heavily infected plant. AMF in the family Gigasporaceae though greatly enhanced the growth of the simple rooted plant, was poor at protection against the pathogen.

AMF inoculum can be applied as spores, fragments of roots that were colonized by AMF, or a combination of the two, and incorporated soil mycelium. According to Gentili and Jumpponen (2006, pp. 11 & 12) the inoculation has been limited to the production of high value nursery stocks. Such nursery applications were often highly advantageous, resulting in an improved crop growth, more expedient development, and a homogeneous end product. However, they reiterated that due to the absence of practical applications for production of AMF inoculum for agricultural practices, the importance of management for maintenance of soil borne fungi must be emphasized. Indigenous AMF isolates were reported to be able to colonize the plant roots in polluted soil contaminated with various trace metals and higher concentrations of chemical constituents of the soil. The AMF isolates adapted to higher concentrations of trace metal and can be used for inoculation of the plant species used in bio-remediation programs on paper mill polluted soil (Chanda, Sharma & Jha 2014, pp. 537-539).

AMF use the C received from the plant to grow and to make glomalin which accounts for 27% of the C in soil (Whitman 2009a, pp. 1&2). It is a major component of soil organic matter (SOM) and it can last seven to 42 years depending on the soil conditions. The SOM forms stable and porous soil structure that can harbor beneficial microorganisms, retain soil moisture water, and prevent soil surface from crusting.

The study on plant roots and rhizosphere soils of forest plantation and nursery in Chittagong, Bangladesh showed the diversity of root colonization and AMF were involved in soil nutrient cycling, and enhancement of seedling survival and growth (Dhar & Mridha 2012, p. 115). The AMF colonization studied varied significantly from 10%−73% in the forest plantations studied. Maximum colonization was observed in *Acacia mangium* (73%). Vesicular colonization was recorded 15%−67% in five plantation tree species of which *Neolamarckia cadamba* as the highest at 67%. Arbuscular colonization was recorded at 12%−60% by four plantation tree species. The highest was in *Acacia mangium* (60%) and the lowest was in *Neolamarckia cadamba*. *Glomus* and *Acaulospora* were the dominant genera among the six AMF species recorded.

1.2 Aim and objectives of the study

The main focus of the thesis was on the utilization of soil mycorrhizal inoculum derived from soil trap culture technique. The utilization main objective was to enhance growth of *Neolamarckia cadamba*, one of the key forest plantation tree species that have been planted in Sarawak. Thus, the plain title of the thesis "Soil Mycorrhizal Inoculum of *Neolamarckia cadamba*." Throughout the thesis, soil mycorrhizal inoculum and *Neolamarckia cadamba* will be referred by the acronym SMI and NC respectively.

1.2.1 Aim

The aim set in the thesis was expected to be determined, fulfilled, answered by several objectives and hypotheses set.

1.2.2 Objectives

The thesis has four main objectives. The first objective was to determine potential sources of AMF propagules for use as inoculum suitable for NC.

The second objective was to test the effectiveness of SMI produced from soil trap culture. The third objective was to establish a soil trap culture technique for production of SMI which was tailored for the inoculation of NC seedlings and the fourth objective was to quantify, characterize and identify the associated AMF species.

The bottom line of the thesis is to test the effectiveness of the SMI as bio-fertilizer for NC by gauging the adaptation and performance of treated seedlings after planting out in comparison to non-treated seedlings as aforementioned in the second objective. To affirm the objective of testing the effectiveness of the SMI, three hypotheses were concocted to that effect.

1.2.3 Hypotheses

The first hypothesis was that the growth performance of inoculated NC seedlings is better than that of non-inoculated NC seedlings. The second hypothesis was that the growth performance of NC seedlings with fertilizer treatment is better than that of NC seedlings with no fertilizer treatment and the third hypothesis is that the inoculated NC seedlings with fertilizer application perform better than non-inoculated NC seedlings applied with the same type and amount of fertilizer.

Soil management strategies have been dependent mainly on chemical-based fertilizers which have cause a threat to human health and serious consequences to the environment. The exploitation of beneficial microorganisms as a bio-fertilizer has paramount importance to the agriculture sector to enhance food safety and sustainable crop production(Bhardwaj et al. 2014, p. 1). The justification to undertake the thesis was largely based on the assumption that there was a lack of data on mycorrhizae of NC in Sarawak. Thus, the situation warranted the use of the potential soil improvement technology for NC forest plantation.

1.3 Justification

The thesis also envisaged making a significant contribution in providing baseline data on low tech and low cost tree nutrition improvement technology for a key forest plantation tree species in Sarawak. Successful outcomes could lead to further work on the isolation, identification, culture and development of operational scale protocols for bio-formulation of AMF and PGPR for use of forest plantations in Sarawak in future.

In-expensive and simple soil bio-enhancement practices were envisioned as being the way forward to increase tree growth performance and thus ensure viability and profitability of timber production from forest plantations in Sarawak. This could be possible by being able to predict growth and not just survival of planted trees.

2 Literature Review

The term symbiont refers to an organism living in a symbiosis. Symbiosis is a close and prolonged interaction between organisms of different species whereby both participating symbionts benefit from each other in a relationship called mutualism. Symbionts in mutualism are often interdependent (Biology Online 2018). The term mycorrhiza covers the entire mechanism of a working relationship between a plant root and fungal hyphae. It refers to the structure, the function, and the members of this symbiotic relationship. There are seven or eight types of mycorrhizae. They differ structurally and functionally arising at different times in evolutionary history (Whitman 2009b, p. 2). According to (Brundrett 2004, p. 473), most mycorrhizae are 'balanced' mutualistic associations in which the fungus and plant exchange commodities required for their growth and survival. In contrast to 'balanced' mutualistic associations, the 'exploitative' mycorrhizae transfer processes apparently benefit only host plant as exhibited by myco-heterotrophic plants.

Trees in the families of PINACEAE, FAGACEAE, DIPTROCARPACEAE and CAESALPINODACEAE found in tropical rain forests interact with ectomycorrhizae from the phylum of Ascomycota and Basidiomycota. As suggested by Bonfante and Genre (2010, p. 2), these fungi could be said to have shaped the present forests though they may also live independently of plant roots, as demonstrated by their growth capabilities in petri dishes.. The fungi colonize the lateral roots of these trees with sheathing hyphae called Hartig net, a fungal mantle that covers the root tip. Though ectomycorrhizae are free-living organisms, the fungi are always associated with the roots of higher plants, including forest trees, wild grasses and many agriculture crops whereby the host plants are necessary for their growth and reproduction.

AMF are the most widespread fungal symbionts of plants, being associated with more than 80% of current land plants. They all belong to the phylum Glomeromycota, a monophyletic group that diverged from the same common ancestor as that of Ascomycota and Basidiomycota (Bonfante & Genre 2010, p. 4). Bonfante and Genre

(2010, pp. 2&4), reiterated that AMF host plants can survive even without their fungal symbionts, but this condition is virtually unknown in natural ecosystems, in which AMF function as true helper microorganisms, improving overall plant fitness. Plants grown in artificial non-symbiotic conditions indicated that AMF significantly contribute to the uptake of soil nutrients, increase plant biomass and enable plant resistance to physiological stress and diseases. AMF have shown to be unculturable in the absence of a plant host as they are unable to absorb carbohydrates except from inside a plant cell. These fungi depend strictly on their plant hosts for growth and reproduction, and thus they are considered as obligate biotrophs (Bonfante & Genre 2010, pp. 2&4)

The symbionts of interest in thesis is the tree NC called Kelampayan in Sarawak and its presumed mycobiont, AMF.

2.1 Neolamarckia cadamba

2.1.1 The Sarawak experience

NC is a very promising tree species for forest plantation as it was reputed to be fast growing and its seeds are in ample supply for planting material. However, attempts to plant it on a large scale have met with many obstacles and failure especially due to the unavailability of suitable planting sites. A high incidence of shoot abnormality in the form of necrotic, stunted, deformed apical shoot leading to premature death of trees was related to low tree density as observed in a forest plantation at Kanowit, Sarawak (John Sabang & Jessica Meredi 2008, pp. 39&40). Trees with deformed terminal buds exhibited a short inter-nodal length leading to early maturing and senescence of this pioneer tree species. The soils of the area studied were generally of a loamy texture and suitable for cultivation of most agricultural crops. However, nutrient levels were low as indicated by two important soil properties, Total N and Available P (Deloiite, Talisman & The DPA Group 1990, p. 7, 9&10). As reiterated by Maas, Tie & Lim (1986, p. 16), the majority of soils in Sarawak are seriously leached and low in nutrients and nutrient-retaining capacity though their physical properties are generally quite good.

NC exhibited poor performance on ridges with shallow soils, infertile soils and on warm sunny sites where the soil was too dry (Bloomberg & John Sabang 2009, p. 57). Field assessment of NC forest plantation, on steep hill country at Kanowit, Sarawak indicated that the survival rate of trees planted at 400 trees/ha was down to about 250 trees/ha, 20 months after planting. The study of showed a strong link between stem malformations depicted by stunted apical shoots, and a shortening of internodes and soil CN ratio. Percentage stem abnormality increases rapidly when CN ratio exceeded the desirable value of 10. Thus, it was assumed that NC could survive only for about three to five years after planting, requiring specific site and soil condition including sufficient moisture and availability of P, and special silvicultural practices such as fertilizer inputs, weed control, pest and disease management.

First-hand observation of planted NC trees that survived in a forest plantation in Sarawak were those that clustered along skid trails especially on sites where new soil was deposited. Forest plantation areas were mainly remnants of logged-over forest that have been clear felled. The ground skidding method of timber harvesting resulted in substantial soil disturbance. The practice of land preparation by spreading of plant debris and top soil was also done mechanically. Areas with good growth were at the lower end of the skid trail where bulldozed soil was deposited. Few trees seemed to survive beyond a few meters of skid trails (John Sabang 2008, p. 5). Site preparation practices adopted by forest plantation operators somehow, included construction of terraces using excavator akin to roadside fill conditions. This was seen as consistent with NC status as a pioneer tree species, growing rapidly on sites where disturbance has resulted in a temporary "flush" of soil nutrients (Bloomberg & John Sabang 2009, p. 57).

2.1.2 *Neolamarckia cadamba* ecology

NC is of the family RUBIACEAE and it has generic names, *Anthocepalus chinensis* and *Anthocephalus cadamba*. It is widely distributed and grows naturally in primary and secondary forests of Nepal, India, Sri Langka, Vietnam, Myanmar, Indonesia, Australia, New Guinea, Malaysia and the Philippines. The species is a large deciduous tree and has a straight cylindrical stem with or without a small buttress. The crown is typically

umbrella-shaped and small, with its branches spreading. Propagation is mainly by seeds (Kimjus et al. 2008, p. 123).

The pioneer tree species is often found in logged areas, produces small seeds that are animal-dispersed. According to Pinard, Howlett and Davidson (1996, pp. 2, 4&9), it is more abundant on skid trails than in forest gaps since skid trails are routes for animals carrying seeds. Flowering normally begins when the tree is 4-5 years old and the fruits are in the form of small capsules packed closely together to form a fleshy, yellow or orange colour infructescence containing approximately 8,000 seeds. At maturity, the small capsules split into four parts releasing the seeds (Sabrina Aslan Joe 2007, pp. 237&240). Thus, seeds could also be naturally dispersed by wind, rain, floods and rivers.

NC is an early-succession tree species found on deep, moist, alluvial soils, such as secondary forests along riverbanks and in the transitional zone between swampy, permanently flooded and periodically flooded areas. Though the tree is a light demander; the young saplings require protection from the hot sun. It is however, sensitive to drought, and excessive soil moisture (World Agroforestry Center 2015).

2.1.3 *Neolamarckia cadamba* growth outside Sarawak

Laran which is the local name of NC in Sabah is categorized as fast growing native species group. The other species in this category were *Octomeles sumatrana*, *Duabanga moluccana* and *Endospermun spp.* (Kimjus et al. 2008, p. 122). NC trial plots in Segaliud Lokan and Brumas in Sabah indicated that the Taliwas seedlot out-performed the other seedlots in terms of both tree height and DBH. The mean tree heights recorded from two trial plots at seven years after planting were 19.55 m and 28.15 cm respectively (Sabah Forestry Department 2008, p. 294).

Krisnawati, Kallio and Kanninen (2011, pp. 7&8), reported that in a 30-year rotation forest plantation in Indonesia, the NC stand attained a mean tree height of 38 m and mean DBH of 65 cm, producing 350 m^3/ha in the final harvest. Total wood production including thinning amounted to 23 m^3/ha per year. In the same report, NC forest

plantations in Java generally reached a maximum volume with MAI of 20 m^3/ha/year by nine years. On good-quality sites, it produced up to 183 m^3/ha of timber and on medium-quality sites, up to 145 m^3/ha timber with MAI of 16 m^3/ha/year producing. In poor-quality sites, MAI at 24 years of age could only reach 13 m^3/ha/year.

2.1.4 *Neolamarckia cadamba* silviculture

Production of NC seedlings in forest nurseries has been relatively easy as ample supply of seeds are obtainable on a regular basis. A gram of seed from the processed fruits contains approximately 20,000 in numbers (Sabrina Aslan Joe 2007, pp. 237&240). The seed usefulness can be maintained by proper storage. The optimum storage temperature for NC was reported to be 8^o C. Seed storage beyond three months must be under a reduced temperature in order to retain their high viability. According to Krisnawati, Kallio and Kanninen (2011, p. 4), viability of NC seeds ranged from 32-63%. The seed germination rate varied, but was generally low at about 25%. When seeds were stored in cool and air-tight boxes for about 2 ½ months, a much higher germination rate, up to 95% was obtained.

As reiterated by Budiman et al. (2015, p. 206), forest tree planting programs should be supported by suitable forest nursery techniques. The production of quality of seedlings for planting out will determine the success of reforestation endeavors. As they pointed out, management strategies starting from the forest nursery stage are crucial to ensure productive tree stands of forest plantation. Seedlings that are raised well will be able to sustain their growth once planted out by adapting well to adverse field environments. Example of a good nursery practice is the incorporation of fertilizer with arbuscular mycorrhizal fungal inoculation on the hardy and robust growing *Acacia mangium* in several forest tree nurseries, showed a significant increase in plant growth and leaf nutrient contents as reported by Jeyanny, Lee and K Wan Rasidah (2011, p. 404). Earlier work by Alexander, Norani Ahmad and Lee (1992, p. 379), showed that *Falcataria moluccana* and *Parkia speciosa* responded well to an introduced cocktail of arbuscular mycorrhizal fungi compared to indigenous spore inoculum propagated in pot culture from roots and soil collected in undisturbed forests. Plants perform better if inoculation

are done early while they are still in the nursery. As reiterated, the real payoff will often show up after planting out especially on restoration sites where soil conditions are less than optimal. Arbuscular mycorrhizal fungi produce a substance in the soil called glomalin which can make up one-third of all carbon in the soil and can persist for 40 years according to Landis and Amaranthus (2009, pp. 13&14). The sticky glycoprotein which makes the soil easy to work with, to hold water, to crumble easily into large aggregates, and thus increasing soil resistance against wind and water erosion.

Sites peculiar to NC are summarized as ecologically those of riverbanks, with possible adaptations to a variety of soil types but dominant on well accelerated fertile soils, and less suited to non-conducive soil properties such being highly leached and poorly aerated (John Sabang 2015, pp. 23&24). The use fertilizers are obviously required in infertile sites to attain desired growth of planted trees. Single application of fertilizers at the planting time of NC was the most widely used practice according to Krisnawati, Kallio and Kanninen (2011, p. 5). However as reported, some of the smallholders in villages South Kalimantan, Indonesia applied fertilizer to NC plantation trees at least twice during the first two years of the growth. Urea and Triple Super Phosphate fertilizers about 15 g per plant in a ring around the seedlings resulted in much faster tree growth. In a forest plantation in Sabah, the density of planting was kept between 278-833 trees per hectare. An extensive fertilizer regime has been adopted for NC in Sabah that includes the use of Urea, compound N, P and K fertilizer (15:15:15) and Christmas Island Rock Phosphate at planting, two rounds of the same compound fertilizer in the first year to third year and one round annually from the fourth year onwards until the seventh year (Lapongan 2008, pp. 69&73). Combined application of organic and inorganic fertilizers had significant promoting effects on NC growth, with height and ground diameter growth of fertilized treatments being 100% and 150% respectively greater than those of the control treatment. Combined application of organic and inorganic fertilizers significantly promoted NC growth. 14 kg of pig manure and 1.5 kg of Calcium super-phosphate per tree had the best promoting effect (Chu et al. 2017, p. 92).

Strategies to overcome infertile soils have been relying on the usages of inorganic fertilizers. As pointed out by Bhardwaj et al. (2014, p. 1), these chemical-based fertilizers cause serious threats to human health and the environment. Thus, it was reiterated that the exploitation of beneficial microorganisms as a bio-fertilizer is the way forward for the agriculture sector to ensure food safety and sustainable crop production in the future. Landis and Amaranthus (2009, pp. 13&14), outlined several potential benefits of inoculating plants with mycorrhizae, including among others increased plant water and nutrient uptake, protection from stress and diseases, increases seedling vigor and growth, reduces transplant shock, and production of the substance glomalin. Arbuscular mycorrhizal fungi help plants by increasing the root ability to absorb soil moisture and nutrient, via the fungal hyphae to access sites beyond the normal root zone.

Numerous studies have shown that arbuscular mycorrhizal fungi has a significant influence on the growth of planted host plants but were done mainly using pot trials on the basis of the reciprocal exchange of carbon and mainly phosphorus, however solid empirical field demonstration of their use are less numerous (Fitter 1985, p. 257). The recurrent interest in developing and utilizing bio-fertilizer has been gaining ground worldwide. However, more information is needed to understand the role of various root-associated organisms in promoting plant growth and health and to make use of their potential benefits as bio-fertilizers. Mycorrhizal infection of plant roots can lead to better plant growth in somewhat unfavorable conditions. Tinker (1984, p. 77), suggested that the improved plant growth is almost always linked to the increase in phosphorus uptake, and could also be due to an improvement in plant zinc and copper nutrition. Linderman (1988, p. 366), also suggested that in soils where such soil nutrients may be deficient or otherwise less available, mycorrhizal fungi increase the efficiency of nutrient uptake, resulting in enhanced plant growth. It was reckoned that the increase in water uptake by plant was due to altering the plant's physiology by the mycorrhizae and thus reducing the effect of stress on plant during drought periods. More information is needed to understand the role of associated soil microorganisms in plant growth and health and how to utilize them to produce bio-fertilizers without losing their beneficial

features. Unless positive effects can be repeatedly shown in practical applications, the commercial of inoculums will remain uncertain (Gentili & Jumpponen 2006, p. 18).

The tree species is also very susceptible to pest infestation. There are two categories of insects that could be considered as serious pests of NC, namely defoliators and stem borers. The most severe defoliator is *Margaronia hilaralis* Walker, a PYRALIDAE; while the most sever stem borer is *Endoclita aroura* Tindale, a HEPIALIDAE (Marfaisal Marzuki 2006, pp. 236&237). Heavy defoliation causes the plant to be stunted and eventually leads to its death. The stem borer is usually most injurious at its larvae stage where it bores into the bark up to sapwood. Holes in the stem or branches provide entry points for secondary infection by fungi. The infestation seldom results in the death of a mature tree, but it does reduce the timber wood quality. The infestation on sapling size trees as observed could cause sudden tree death. Stem borer infestation was reported to be one of the many factors that leads to high mortality of NC, especially when trees are about one or two-year-old. Chai et al. (2010, p. 69), reported that intensive silvicultural practice such as monthly circle weeding was effective to control stem borer infestation in NC plantation. The stem borer could also be *Batocera numitor* found in India and Indonesia, as reported by Browne (1968, pp. 106&107). The large longhorn beetle, when at larva stage was a secondary parasite of sickly or injured dicotyledonous trees such NC. Its eggs are laid on wounds or in unhealthy bark and the larva burrows in the wood, keeping its tunnel clean by pushing out frass and sap through ejection holes made at intervals. Pupation occurs in the wood. In large trees much sapwood may be destroyed, and branches and small trees may be killed.

NC is generally considered a light-demander, requiring high light availability for seedling growth. Krisnawati, Kallio and Kanninen (2011, p. 5), reiterated that NC seedlings are thus highly susceptible to weed competition. As suggested, the area around the young seedlings need to be weeded of competing vegetation, especially of climbers and plants causing shade. They pointed out that smallholders in Indonesia practiced both manual and chemical weed controls. Weeding was done several times during the first few years after planting until the trees approached canopy closure. The

interval between two successive weeding was usually three months during the first year, and six months after the first year. The plausible mechanism for mycorrhizal plant being able to out compete weeds and earlier successional plant species was because mycorrhizal fungi pulled nutrients from the non-mycorrhizal plants as reported by Kheyrodin (2014, p. 45). However, fungi also need fungal foods like humic acid such as from mulch or litter layer on the soil surface. As suggested, the use of organic fertilizer such as mulching using compost and farmyard manure may well be the source of the needed nutrients not only for the tree but the mycobiont as well.

2.1.5 *Neolamarckia cadamba* utilization

Timber producers and users in Sarawak have been relying on a very few common timber species and as a result, many of the other timber species have been neglected, misused or under appreciated. NC was one of the 44 timber species that whas already been included in the royalty coding list of Sarawak. It is classified as; Light Hardwood (density < 720 kg/m^3), Weak (Group D wood strength), Non-durable (natural durability: < 2 years), Non-decorative feature, Uniform white to yellowish wood color (Yang 2005, pp. 123-129). Some of the recommended uses of NC were light framing, glue laminated construction, veneer and plywood, paper pulp, other panel products, light duty flooring, furniture parts, packing cases and crates

Ling and Wong (2007, pp. 262-265), reported that mean air-dry wood densities of common Sarawak timbers were highly variable. Among 100 tree species sampled, wood density was as low as 331 kg/m^3 for *Octomeles sumatrana* a DATISCEAE and 1,227 kg/m^3 for *Gymnostoma nobile* a CASAURINACEAE. NC has density of 439 kg/m^3 and was among 13% of tree species with densities of < 500 kg/m^3. However, the bending strengths of plywood samples fabricated from NC were found to be notably high and surpassed the minimum requirement by Japanese Agricultural Standards for "Structural Plywood-1999" and "Common Plywood-1973." The findings indicated that the tree species is suitable for manufacturing of both common and structural plywood (Siti Hanim Sahari 2005, p. 169).

NC is a general utility timber, suitable for joinery and light construction, particularly for interior application and can be treated with preservatives (Andrew Nyorik Nibu & Alik Duju 2005, p. 121). The wood material derived from more than 75% wood residues could be molded to form a strong high quality chair shell. The natural color gave the completed chair an attractive appearance when wood finishing was applied (Pek & Isa Kumbang 2006, p. 182).

2.2 Mycobiont

Mycobionts are a cornerstone of forest ecosystems as their vast network of mycelium, made up of hyphae carry out their essential work of adapting plants to soil condition. Trees are the obvious members the ecosystem and are intimately bound up with, and dependent on these mycobionts (Trees for Life 2018). Approximately 80-90% of all plants form symbiotic mycobiont relationships by forming hyphae networks (Hoorman 2018). As described by Hoorman (2018), the hyphae are about 1/60 the diameter of most plant root hairs and they assist the plant by foraging for soil nutrients. The mycobionts also release enzymes into the soil and break down complex molecules, reabsorbing and redistributing soil nutrients back to plant roots.

2.2.1 Taxonomy of the mycobiont

The fungi Kingdom is one of the six kingdoms of life and is currently divided into seven phyla which include the Ascomycota and Basidomycota and the defunct Zygomycota. The organisms that have traditionally been placed in the phyla Zygomycota are divided among the phylum Glomeromycota, and four subphyla as the broader relationships were still unknown or undefined to permit a clear classification (Brandt & Warnock 2011, p. 1748). The major types of mycobionts related to the fungi phyla taxa are AMF (Glomeromycota), Ecto and Ectendomycorrhizhae (Basidiomycota or Ascomycota), Arbutoid, Monotropoid and Orchidaceous (Basidomycota), and Ericoid (Ascomycota) (Barman et al. 2016, p. 1095).

The history of AMF taxonomy and the several proposed AMF classifications between 1800 and the present day can be divided in six phases. In Phase V (2001-2010), the most important event was the proposed new monophyletic phylum for AMF. All AMF species were transferred from Zygomycota to Glomeromycota (Souza 2015, p. 9). Prior to this, AMF included in the order Glomerales were placed in the phylum Zygomycota. According to Schubler, Schwarzott and Walker (2001, p. 1413), AMF could be indisputably separated from all other major fungal groups in a monophyletic clade based on molecular, morphological and ecological characteristics. Consequently, they were removed from the polyphyletic Zygomycota, and placed into the new monophyletic phylum, the Glomeromycota. The recognition of this monophyletic group, which probably diverged from the same common ancestor as the Ascomycota and Basidiomycota, gave these fungi their proper status, a new and natural systematics (Schubler, Schwarzott & Walker 2001, p. 1413). As reiterated by Berruti et al. (2014, p. 159) Glomeromycotan species, genus and families traditionally discovered and characterized based mainly on spore morphology have be replaced by molecular DNA sequencing-based analyses. It has recently contributed to a great extent light on a previously unseen and profound diversity within this phylum.

Oehl et al. (2011, p. 191) grouped the phylum Glomeromycota into three classes, five orders, 14 families and 29 genera by relating spore morphology and molecular analyses. Spore formation in ten of the arbuscular mycorrhiza-forming genera is exclusively glomoid, one is gigasporoid, seven scutellosporoid, four entrophosporoid, two acaulosporoid, and one pacisporoid. Spore bimorphism is found in three genera, and one genus is associated with cyanobacteria (Table 1). According to Souza (2015, pp. 66&67), basically, there are five different types of spore morphotypes or spore formation modes. Firstly, Acaulosporoid – spores borne laterally from the neck of a pre-diffrentiated saccule, secondly Entrophosporoid – spores borne inside the saccule neck, thirdly Gigasporoid – spores borne at the apex of bulbous hyphae, fourthly Glomoid – spores borne in the terminal or intermediate portion of reproductive hyphae, and fifthly Radial-glomoid – spores formed and organized from the central plexus of hyphae.

Table 1. Spore characteristics of Glomeromycota from class to genus level (Oehl et al. 2011, pp. 194&195).

Class	Order	Family	Genus	Spore formation
Glomeromycetes	*Glomerales*	*Glomeraceae*	*Glomus*	Glomoid (terminally on hyphae)
			Funnelifor-mis	Glomoid-Funneliformoid *sensu stricto*
			Septoglomus	Glomoid-Septoglomoid *sensu stricto*
			Simiglomus	Glomoid-Simiglomoid *sensu stricto*
		Entrophospora-ceae	*Clairodeoglo-mus*	Glomoid sensu lato-Claroideoglomoid *sensu stricto*
			Albahypha	Glomoid-Claroideoglomoid *sensu lato*
			Viscospora	Glomoid-Claroideoglomoid *sensu lato*
			Entrophospho-ra	Entrosphosporoid (in the neck of a saccule)
	Diversispora-les	*Diversispora-ceae*	*Diversispora*	Glomoid-Diversispora *sensu stricto*
			Redeckera	Glomoid (Diversisporo-) Redeckeroid *sensu stricto*
			Otospora	Acaulosporoid (on the neck of sporiferous saccule): Otosporoid *sensu stricto*
			Tricispora	Entrophosporoid-Tricisporoid *sensu stricto*
		Sacculospora-ceae	*Sacculospora*	Entrphosporoid-Sacculosporoid *sensu stricto*
		Pacisporaceae	*Pacispora*	Pacisporoid
		Acaulospora-ceae	*Kuklospora*	Entrophosporoid-Kuklosporoid *sensu stricto*

	Gigasporales	*Scutellospoceae*	*Acaulospora*	Acaulosporoid
			Orbispora	Scutellosporoid (on sporogenous cells, and forming germ shield); Orbisporoid *sensu stricto*
			Scutellospora	Scutellosporoid
		Dentiscutata-ceae	*Fuscutata*	Scutellosporoid-Fuscutatoid *sensu stricto*
			Dentiscutata	Scutellosporoid-Dentiscutatoid *sensu stricto*
			Quantunica	Scutellosporoid-Dentscutatoid *sensu stricto*
		Racocetraceae	*Cetraspora*	Scutellosporoid-Raccocetroid *sensu stricto*
			Racocetra	Sctellosporoid-Racocetroid *sensu stricto*
		Gigasporaceae	*Gigaspora*	Gigasporoid (on sporogenous cells, and forming germ warts)
Archaeospormyce-tes	*Archaeospora-les*	*Ambisporaceae*	*Ambispora*	Bimorph: Acalo- & Glomo-ambisporoid
		Archaeospora-ceae	*Archeospora*	Bimorph: Acalo- & Glomo-archaeosporoid
			Intraspora	Bimorph: Entropho- & Glomo-intrasporoid
		Geosphonaceae	*Geosiphon*	Glomoid *sensu lato* (Associated with cyanobacteria)
Paraglomeromyce-tes	*Paraglomera-les*	*Paraglomer-aceae*	*Paraglomus*	Glomoid *sensu lato*

As reiterated by Young (2012, p. 823), the identification and nomenclature of AMF is challenging, but it is important because AMF form symbioses with the majority of land plants . The phylogeny of AMF in particular concerning some taxonomical groups, was still puzzling and was an open debate (Berruti et al. 2014, p. 160). The general disagreement about the number of families and genera such as the separation of Gigasporales and Diversisporales was under contention as shown in Table 2.

Table 2. Arbuscular mycorrhizal fungi taxonomical contention (Berruti et al. 2014, p. 161)

Taxonomical status	Order	Family
Consensus	*Glomerales*	*Glomeraceae*
		Claroideoglomeraceae
	Archaeosporales	*Archaesporaceae*
		Ambisporaceae
		Geosiphonaceae
	Paraglomerales	*Paraglomeraceae*
Open debate	*Diversisporales*	*Sacculospora-ceae*
		Pacisporaceae
		Diversisporaceae
		Acaulospora-ceae
	Gigasporales	*Gigasporaceae*
		Scutellospoceae
		Dentiscutata-ceae
		Racocetraceae

Enumeration, characterization and identification of AMF involved primarily using spore specimens by phylogenetic and morphological assessment. Morphological identification of AMF spores is difficult and laborious for most researchers who lack experience. The effective way to carry out identification is to refer to experts on AMF taxonomy, and to organizations such as INVAM or the European Bank of *Glomales* (Habte & Osorio 2001, p. 23&24).

2.2.2 Importance of the mycobiont

Plant families are either predominantly ectomycorrhizal or predominantly endomycorrhizal (Linderman 1988, p. 367). The mycorrhizal fungi exist as vegetative propagules in the form of spores and hyphae structures in in soils and root fragments. The propagules of mycorrhizal fungi apparently respond to the stimulation of root exudates, and their hyphae or germ tubes grow and penetrate root epidermal cells. The colonization of host tissues progresses both internally and externally along the root surface, the latter resulting in initiation of new colonization sites. Alexander, Norani Ahmad and Lee (1992, p. 379), reported that most tropical trees are habitually mycorrhizal in natural soils. Hyphae formed penetrate the root inter-cellularly and

intra-cellularly and extend out from the root into the soil substrate. As further reiterated, these hyphae may bear spores which form the basis of the taxonomy of AMF

AMF are the most important microorganism in symbiosis with the majority of plants particularly under condition of P limitation (Jeffries et al. 2003, p. 1). The development of the symbiosis between plants and mycobionts are divided to three phases based on the AMF's life cycle according to Souza (2015, p. 54). Firstly, Asymbiotic Phase – involved the germination of quiescent spores and initial mycelium development, secondly Pre-symbiotic Phase – chemotropism activation on physical contact with root and the formation of appressorium, and thirdly Symbiotic Phase – symbioses establishment with root colonization through intra-radical and extra-radical mycelium development which further lead to sporulation. Both the plant bio-diversity, above and below ground productivity increased with increasing AMF species richness. The lowest plant bio-diversity and productivity were found at sites without or with only a few AMF species (Heijden et al. 1998, p. 70).

Out of 35 species of RUBIACEAE capable of forming mycorrhizae, 94% were the AMF type. However, generalizations should not be made for the majority of species because the exceptions are too numerous and widespread (Newman & Reddell 1987, p. 747). As pointed out, a plant belongs to a particular family, could still be mycorrhizal. AMF symbiosis in coffee a RUBIACEAE during the seedling phase was most important in enhancing its growth according to Andrade et al. (2009, p. 111). The selection of effective AMF was desired for a positive response from the association. Soil samples extracted from the rhizosphere of coffee trees in Yemen tentatively revealed the presence of five AMF species; *Glomus proliferum, Glomus etunicatum, Acaulospora sporocarpia, Archeospora* sp. and *Scutellospora nigra* (Al-Areqi et al. 2013, p. 4888).

Roots of seedlings from forest nurseries in Sarawak were examined for mycorrhizal colonization. AMF were by far the commonest type found. The prevalence of AMF in the tropics suggests that they may be of great importance for plant growth in tropical soils which are relatively poor in mineral nutrients especially P (Chong 1987, p. 11). A

pot experiment of *Falcataria mnoluccana* and *Parkia speciosa* responded well to introduce cocktail of AMF compared to with indigenous source propagated in pot cultures from roots and soil collected in undisturbed forests. *Intsia palembanica* responded better to the AMF as compared to ectomycorrhizae (Alexander, Norani Ahmad & Lee 1992, p. 379). The study on comparing abundance of AMF spores in a rehabilitated forest and a logged-over forest in Bintulu, Sarawak indicated abundance of *Glomus clarum*, *Glomus. Fasciculatum*, *Glomus macrocarpum*, *Glomus multicaule, Acaulospora laevis, Acaulospora scrobiculata, Acaulospora spinosa* at both sites, while *Gigaspora margarita* was found only at the latter site (Ong et al. 2012, p. 1). NC was found to have AMF, *Glomus* and *Acaulospora* in studies in Bangladesh (Dhar & Mridha 2012, p. 115). In the study of AMF of *Octomeles sumatrana* and NC in Niah, Sarawak, Chubo et al. (2009, p. 340) reported that AMF species from the genera *Glomus* was found to be dominant in the rhizospheres of both tree species followed by *Acaulospora* and *Gigaspora*. However, their study on sporulation based on MPN method indicated that *Octomeles sumatrana* supported better sporulation of the AMF compared to NC.

Haug, Setaro and Suarez (2013, pp. 9&10) opined that AMF communities of the pristine forest and reforestation plots share many features. As reiterated the size of the disturbed sites might influence the stability of AMF communities. Contribution of AMF inoculum from the adjacent forests to the disturbed sites is a plausible way to maintain high diversity and similar composition of AMF. The regeneration of the natural forest vegetation was regarded as not inhibited by a lack of appropriate AMF mycobionts as the highly diverse flora of the reforested sites most likely serves as host to the diverse generalist mycobionts. Planting native trees can foster the on-going process of forest rehabilitation.

The importance of the roles of soil microorganisms in sustainable low-input agriculture systems in maintaining soil fertility and bio-control of plant pathogens may increase in contrast to conventional agriculture where their significance has been disregarded by high dependent on agrochemicals (Johansson, Paul & Finlay 2004, p. 1). Better understanding of the interaction between arbuscular mycorrhizal fungi and other

microorganisms is necessary for the development of sustainable management of soil fertility and crop plants production. Artursson, Finlay and Jansson (2006, p. 1), also reiterated that arbuscular mycorrhizal fungi and bacteria can interact synergistically to stimulate plant growth through a range of mechanisms to improve soil nutrient acquisition and inhibition of fungal plant pathogens. The plant-microorganism interactions that enable sustainable, low-input agricultural cropping systems that rely on biological processes rather than chemical fertilizers to maintain soil fertility and plant health are of crucial importance.

In nutrient depleted, tropical regions with excessive rainfall, essential plant nutrients are leached from soil surfaces. AMF increase the surface area of roots for improved uptake of water and nutrients. Immobile nutrients are absorbed by the plants through diffusion as AMF can extend their external hyphae beyond the depleted zones. As a result, more volume of soil becomes accessible to plant roots. Therefore, plants with the mycorrhizal associations are more efficient in the absorption of nutrients like N, P, K, and Ca (Barman et al. 2016, pp. 1099-1100). Most soils have low supply of P, and the slow rate at which this nutrient diffuses to surrounding roots created zones of P depletion (Habte & Osorio 2001, p. 5). AMF help overcome this problem by extending their external hyphae from root surfaces to areas of soil beyond the P depletion zones. Plant roots aided by the fungal hyphae are able to explore wider soil matrix as compared to roots alone. Thus, it is imperative that the objectives and hypotheses thought of in the thesis are put under scrutiny.

2.2.3 Utilization of the mycobiont

The traditional soil resource evaluation generally measures soil physical and chemical properties but lack good quality data on rhizosphere microorganisms which could be utilized in managing the issues of poor tree growth in forest plantation in Sarawak. Soil management strategies have been dependent mainly on chemical-based fertilizers which have cause a threat to human health and serious consequences to the environment. The exploitation of beneficial microorganisms as a bio-fertilizer has paramount importance to the agriculture sector to enhance food safety and sustainable crop

production(Bhardwaj et al. 2014, p. 1). Studies in Sarawak showed that arbuscular mycorrhizal fungi in forested soils are abundance (Ong et al. 2012, p. 1). Spores extracted from soil taken from rhizosphere of natural tree stands indicated that *Neolamarckia cadamba* is a host plant to arbuscular mycorrhizal fungi (Chubo et al. 2009, p. 340). Alexander, Norani Ahmad and Lee (1992, p. 379), reiterated that they are circumstantial proof to show that plant-mycorrhizal symbiosis is important in the survival and growth of tropical tree seedlings and consequently, the regeneration and restoration of disturbed tropical rain forest.

The eco-friendly PGPR, mycorrhizal fungi, cyanobacteria and other beneficial microorganisms could enhance plant growth by improving plant nutrient uptake, and increasing plant tolerance to abiotic and biotic stresses (Bhardwaj et al. 2014, p. 1). Williams et al. (2017, p. 874), reiterated that agricultural fertilization significantly affects AMF community composition whereby crop plants can reduce C allocation to AMF in response to P fertilization. When N fertilizer is used, crop plants allocated an increasing amount of C to AMF and but received relatively less P. This suggests a shift in terms of the P–C exchange between AMF and crop plants under N fertilization regardless of availability of soil P. Any knowledge gained will help forest plantation silviculturists to understand the physiological basis of bio-fertilizers towards sustainable forestry in reducing problems associated with the use of chemical-based fertilizers.

The increasing need for environmentally friendly agricultural products has been driving the use of fertilizers based on beneficial microorganisms from array of bacteria, yeasts and fungi, each with its own mechanism to improve overall plant nutrition (Malusa, Sas-Paszt & Ciesielska 2012, p. 12). The studies done on the interactions between plant, soil, and the different microorganisms provide new possible ways to exploit them for agricultural purposes. However, as pointed out by Malusa, Sas-Paszt and Ciesielska (2012, p. 12), the inoculation of plants with these microorganisms using a formulation of inoculum with a reliable and consistent effect under field conditions is still not substantiated yet. As reiterated, the technology for inoculum production and the formulation of suitable carrier is key to their successful application.

Reforestation endeavors usually face sites where seedlings survived adequately but grew unacceptably for a number of years (Duryea 1985, p. 4). This problem of poor growth could be alleviated by producing and planting quality seedlings in the first place which include inoculation with effective beneficial microorganisms. Plant-mycorrhizal symbiosis is one of the crucial factors in ensuring healthy soil-plant relationship by enhancing nutrients uptake and the ability of plant to withstand drought periods. It also induces resistance against soil pathogens, and reduces sensitivity to toxic substances in their host plants (Barman et al. 2016, p. 1103). Activities like slash and burn cultivation, mining, waste disposal, and clear-cutting of forests are detrimental to mycorrhizae. The indiscriminate use of fertilizers and pesticides also inhibit the growth of mycorrhizae. Thus, well developed mycorrhizal bio-technologies may be better alternatives for forestry practices in the long run.

AMF inoculation has been limited to production of high value nursery stocks. In such nursery, the inoculation was often highly advantageous, resulting in improved crop growth, more expedient development, and a homogeneous end product (Gentili & Jumpponen 2006, pp. 11&12). In the absence of practical applications for the production of AMF inoculum for agricultural practices, the management for maintenance of soil borne fungi are important. Fitter (1985, p. 257) reiterated that AMF use has a significant effect on the growth performance of planted host plants but were done in pot trials on the basis of reciprocal exchange of C and mainly P, however as reckoned, unequivocal field demonstrations were less numerous.

Inoculum can be applied as spores, fragments of roots colonized by AMF or a combination of the two and incorporated into soil mycelium. The spores and hyphae can be isolated from the soil substrate and mixed with carrier substrate (Gentili & Jumpponen 2006, pp. 11&12). The use of SMI may be considered as not effective as mycorrhizal formation can be irregular and inconsistent. Although used spores have been widely used and are relatively easy to apply, however they often require large spore numbers for colonization of roots (Brundrett et al. 1996, pp. 243-245).

One of the main constraints for a large scale use of AMF in plant production is the availability of commercial inoculums (Blal 1999, p. 496). As pointed out some companies have totally or partially achieved a procedure for a commercial production of AMF inoculums. However, their utilization at a commercial level is still limited. Blal (1999, p. 496) gave example of a company called BIORIZE, which has produced a range of commercial AMF inoculums in a granular form using calcined clay as the carrier. To promote utilization of these inoculums, the company has set up a wide range of experiments with technical centers and private nurseries to determine the best conditions for their use. The utilization of their commercial inoculum "ENDORIZE" after soil disinfection was able to overcome many problems such as heterogeneity, low growth and recovery. This ensures a better rate of success in plant production, high plant quality and a guaranty, for plant recovery when planted out to the field.

Bio-technological tools employed in the production and formulation of conventional and novel inoculum products should always take into consideration the functional relationship between the wide numbers of players in bio-fertilizer technology (Vassilev et al. 2015, p. 4983). It was reiterated that apart from boosting exploitation of plant-beneficial microorganisms in playing a key role in increasing the availability of plant nutrients that otherwise are inaccessible to plants, special attention on combining the use of microorganism active natural compounds such as plant extracts and exudates and compost extracts which improve not only plant growth and development but also plant-microbial interactions.

Verbruggen et al. (2013, p. 1104) suggested three factors that determine inoculation success and AMF persistence in soils. Firstly, species compatibility under the imposed circumstances, secondly field carrying capacity - the habitat niche available to AMF, and thirdly priority effects - the influence of timing and competition on the establishment of alternative stable communities. The method use in production of SMI in the thesis was based on the simple method using soil trap culture of starter soil collected from under healthy trees of the targeted species (Sunseed Desert Technology 2012, pp. 4&5). As pointed out by Gentili and Jumpponen (2006, p. 18), more information is needed to

understand the role of various root-associated microorganisms in plant growth and health and to make use of their potential beneficial features as bio-fertilizers. Unless positive effects can be repeatedly shown in practical applications, commercial viability of inoculation programs will be uncertain. Indigenous AMF have been demonstrated to be equal to or to perform even better than commercial or culture collection isolates. Farmers including those in developing countries were encouraged to autonomously produce their AMF inoculum, starting from native soils. This makes the bio-fertilization technology more likely to be affordable to ensure the possibility of their crop system to highly sustainable according to Berruti et al. (2016, p. 9).

1.2.4 Inoculum of the Mycobiont

Husband et al. (2002, p. 2669), reiterated that the tropical arbuscular mycorrhizal fungi population are spatially heterogeneous and are not randomly associated with the different host plants. The high diversity variation across points of time, sites and hosts, implied that the arbuscular mycorrhizal fungi types are ecologically distinct from each other. Thus, they may potentially influence recruitment of host composition in tropical forests. As pointed by Burrows and Pfleger (2002, p. 120), sporulation arbuscular mycorrhizal fungi species with large spores increased significantly with increasing plant diversity, while sporulation of the arbuscular mycorrhizal species with smaller spores species varied in response to host diversity. The potential sources of AMF propagules for use as inoculum suitable for NC. could be the root zone or rhizosphere soil of a plant hosting AMF. Such soil could be used as the ingredient for the production of the inoculum (Habte & Osorio 2001, p. 5). The inoculum usually composed of soil, root fragments, and AMF spores, and fragments of hyphae. However, as reiterated by Habte and Osorio (2001, p. 5), soil may not be a reliable inoculum ingredient unless the richness of the inherent AMF are known.

Spores extracted from soil and use as inoculum tends to have very low viability or are simply already dead. Spores might be reasonably viable if collected from soil where an actively growing plant known to be infected with AMF (Habte & Osorio 2001, p. 5). Nonetheless, as pointed out by Habte and Osorio (2001, p. 5), soil together with root

tissues from the site can be taken to start a soil trap culture to increase the number of viable spore for isolation and further multiplication.

AMF inoculum are produced mainly by growing host plants in controlled conditions, whereby different fungal structures specifically spores, mycelium hyphae and mycorrhizal root residues from the plants used as the propagating host are included. For commercial large-scale applications, substrates of sand and soil with other materials are used to mass-produce arbuscular mycorrhizal fungal inoculum in pots, bags, or beds, (Malusa, Sas-Paszt & Ciesielska 2012, p. 3). Lemongrass is suitable for soil trap culture as it is easily available, easy to plant and can thrive in most soil types. As indicated by Broeckling et al. (2008, p. 739), non-resident plants root exudates influenced the fungal community by both positively and negatively impacting the relative abundance of individual species. Root exudates is the mechanism through which a plant is able to regulate soil fungal community composition. They pointed out that a net increase in fungal biomass was observed, suggesting that increases in specific carbon substrates support an increased soil fungal population (Broeckling et al. 2008, p. 739).

The soil trap culture could also be improved by using Chitin laminarin-like substance. As described by Gryndler et al. (2003, p. 283), the compounds of the substance are organics commonly present in the soils, and may be appropriate additives for soil trap culture since they are components of fungal cell walls. As reiterated, the presence of the compounds affects the abundance of mycolytic microorganisms in the soil. It was shown that its addition to sand-soil based substrates, stimulated the root colonization, growth of extra-radical mycelium and production of spores of arbuscular mycorrhizal fungi with *Allium amppelloprasum*, *Plantago lanceolata* and *Lactuca sativa* as host plants. Interestingly, stimulation of arbuscular mycorrhizal fungi sporulation was also observed when mycelium of *Fusarium oxysporum* after being autoclaved was used instead of Chitin (Gryndler et al. 2003, p. 283)).

Another suitable candidate for nurse plant besides lemongrass is *Jatropha curcas* a biofuel crop which was at one time a much hyped big cash crop in Sarawak. *Jatropha curcas* as nurse plant was able to trap the ten species of arbuscular mycorrhizal fungi of which two species produced abundant spores and the roots and the nurse plant roots were also heavily colonized (Charoenpakdee et al. 2010, p. 195). The use of organic fertilizer should be further studied in relation to the tree growth as it influenced both the community composition and the species richness of AMF and root-associated bacteria as reiterated by Toljander et al. (2008, pp. 334&335). As reported, organic fertilizers, in the form of sewage sludge, used resulted in the most distinct microorganism communities. AMF richness declined but significantly altered bacterial community in terms of a higher bacterial richness. The application of nitrogen fertilizer, Ammonium sulphate resulted in significant reductions in plant growth and richness of both arbuscular mycorrhizal fungi and bacterial species as it due decline in soil pH induced by the chemical fertilizer use (Toljander et al. 2008, pp. 334&335).

3 General materials and methods

Materials and methods presented in this chapter outline the main study, the field trial, and ancillary experiments, the nursery trials that were carried out to meet the objectives and hypothesis set in the thesis. The aim of the thesis was to determine and measure the effectiveness of soil trap culture in the production of SMI which was used to inoculated NC seedlings in a forest nursery and subsequently planted out, to simulate field establishment of a forest plantation.

Successful reforestation of NC tree species in Indonesia, was determined by morphological and physiological attributes of seedlings used in field planting (Budiman et al. 2015, pp. 206 & 207). Nursery cultural practices directly influenced the morphological and physiological attributes of seedlings. The cultural practices of high importance, were transplanting, growing density, and the pruning of both root and shoot. Forest plantation establishment requires good nursery setup and practices to produce quality planting materials. Plant nutrition management strategies are mainly focus on the use of chemical-based fertilizers. The alternative in the form beneficial microorganisms formulated as bio-fertilizer are gaining popular in the agriculture sector as they could ensure food safety and sustainable crop production (Bhardwaj et al. 2014, p. 1).

The thesis focus was on developing a low cost and low tech soil bio-enhancement technique that could complement the existing planting media used in raising NC seedlings of a forest nursery. The bio-improved planting media was postulated to enhance seedlings adaptation and growth performance once planted out to the field. To achieve the aim, the thesis comprised of a field trial and several nursery trials.

3.1 Field trial

The objectives of the field trial were to determine potential sources of AMI propagules for use as inoculum suitable for NC and test the effectiveness of SMI produced from soil

trap culture. Such sources of AMF propagules could be root zone or rhizosphere soil of a plant hosting AMF which can be used as the ingredient for the production of inoculum (Habte & Osorio 2001, p. 5). The inoculum composed of soil, dried root fragments, and AMF spores, sporocarps, and fragments of hyphae. Extraction and segregation of AMF propagules namely spores from rhizosphere soil taken from several sites of naturally growing NC were carried out.

Most soils are not adequately supplied with P, resulting in zones of P depletion surrounding roots (Habte & Osorio 2001, p. 5). AMF help overcome this problem by using their external hyphae to assist plant root to reach microsites where soil P is available. The SMI produced was expected to increase NC root colonization during seedling stage and remain effective until and after planting out of inoculated seedlings. NC seedlings treated with SMI were expected to adapt and perform under field condition. The thesis was to determine the applicability of such soil amelioration techniques for forest plantations in Sarawak. Further field trials in various forest plantations in Sarawak were envisaged if the study shows promising results. The eeffectiveness of inoculum was determined non-destructively by measuring plant height, root collar diameter, and destructively for dry- biomass accumulation (Habte & Osorio 2001, p. 16). The effectiveness of AMF development was determined by measuring AMF colonization of roots based on the presence of fungal structures; arbuscules, versicles, and hyphae through destructive sampling of roots.

The field trial shed light on the three hypothesis set. Firstly, growth performance of inoculated NC seedlings is better than non-inoculated NC seedlings. The study by Chubo et al. (2009, p. 344) at Niah, Sarawak, showed that NC has the ability to enhance the development and sporulation of mycorrhiza. As reckoned by Brundrett et al. (1996, p. 3), major research initiatives focus on manipulating mycorrhizal associations to increase tree establishment and productivity in plantation forestry and recovery of severely disturbed forest ecosystem.

Secondly, growth performance of NC seedlings with fertilizer treatment is better than NC seedlings with no fertilizer treatment. The response of NC seedlings to fertilizer treatment was highly expected as available and reserved soil nutrients of theplanting site are low. The majority of soils in Sarawak are seriously leached and low in nutrients and nutrient-retaining capacity. A study of a NC forest plantation, on steep hill country at Kanowit, Sarawak showed a strong link between stem malformations depicted by stunted apical shoots, and shortening of internodes to the soil CN ratio. The percentage stem abnormality increases rapidly when CN ratio exceeded the desirable value of 10 (Bloomberg & John Sabang 2009, p. 57). Thirdly, inoculated NC seedlings with fertilizer application perform better than non-inoculated NC seedlings applied with the same type and amount of fertilizer. NC seedlings treated with SMI and fertilizer were expected to outdo non-treated seedlings after theage of six months when the efficacy of fertilizer applied diminished. Williams et al. (2017, p. 874), reiterated that agricultural fertilization significantly affects AMF community composition. They suggested that regardless of soil P status of N fertilization caused the alteration in of P–C exchange.

The outcomes of the field trial, to determine potential sourcesof AMF propagules and test the effectiveness of SMI are outlined in Chapter 5. "Soil mycorrhizal inoculum field trial."

3.2 Nursery trials

Several nursery trials were carried out to fulfil the objectives of establishing a soil trap culture technique for the production of SMI for inoculation of NC seedlings and to quantify, characterize and identify the associated AMF species. Spores can be extracted from soil and used as inoculum but such spores are usually not viable or are mainly already dead. However, soil with root parts from a site can be taken to start a soil trap culture to boost the number of viable spore propagules (Habte & Osorio 2001, p. 5). Production of SMI in the thesis involved the collection of starter soil at a location naturally growing NC trees. SMI derived using soil trap culture was expected to contain diverse soil microorganisms including AMF from rhizosphere soil of NC natural stands.

The study on the taxonomy of the AMF was just preliminary and it could be pursued when spore isolates are more certain. Enumeration, characterization and identification involved primarily AMF spores by phylogenetic and morphologically study. Morphological identification of AMF spores can be a difficult exercise. Apart from referring to experts AMF taxonomy, online search could be done to various websites such as INVAM, European Bank of Glomales, and few others (Habte & Osorio 2001, pp. 23&24).

The outcomes of nursery trials are outlined in the following chapters of the thesis.

Chapter 6. "Soil trap culture trial"
Chapter 7. "Pot substrate culture trial and isolation of spore morphotype specimens"
Chapter 8. "Single spore culture trial and characterization of D1 spore specimen"
Chapter 9. "Re-propagation culture trial and cellular assessment of D1 spore specimen"

3.3 Work flow

The summary of the general work flow and interrelationship of the field trial study and nursery trials are as shown by the chart as shown in Figure 2. The dates stated were approximated durations taken to complete each experiment. The blue colored boxes refer to the experiment carried at the field trial plot study. The ancillary trials are denoted in respective colored boxes and by the chapter number in the thesis.

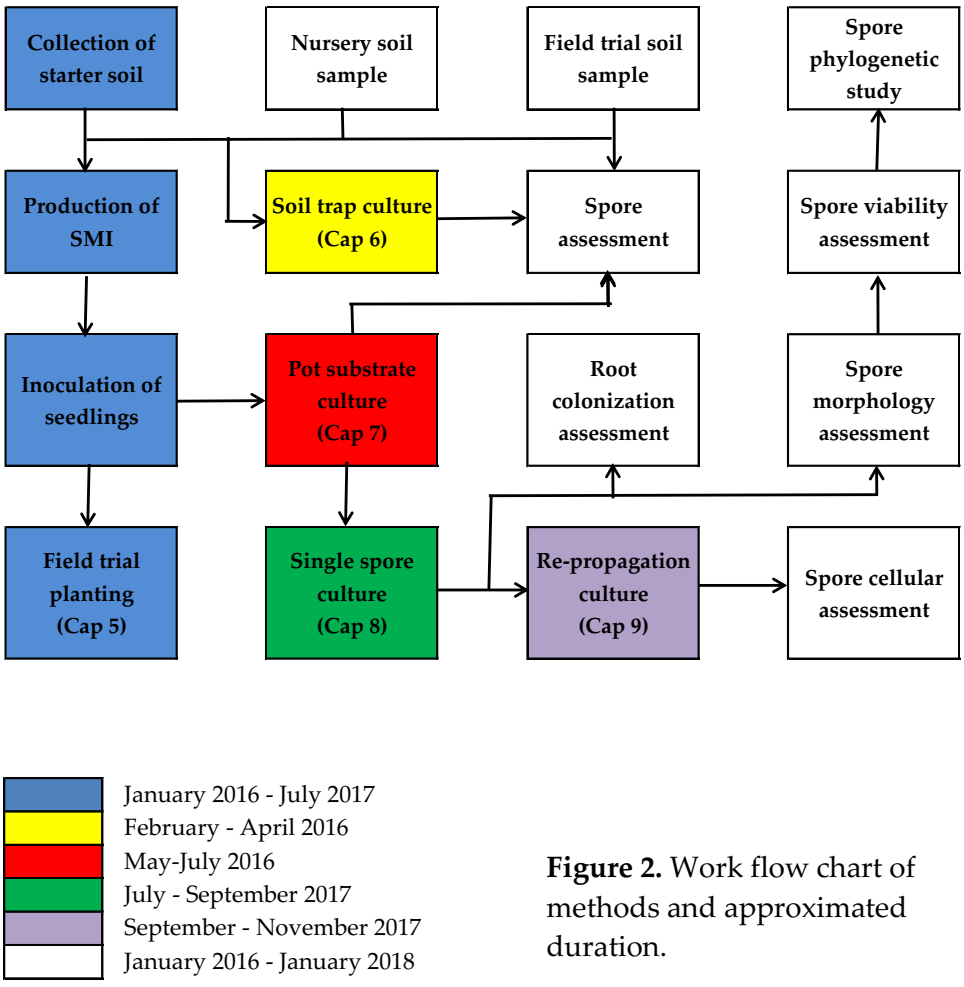


Figure 2. Work flow chart of methods and approximated duration.

3.4 Location of the study sites

The main study involves three localities namely Kubah N.P., Semengoh N.R. and Sabal F.R. as shown in Figure 3. Starter soil was collected from the NC phenology plot located within Kubah N.P. about 20 km east of Kuching. Soil trap culture trial using the starter soil was then carried out at the forest nursery in Semengoh N.R. which is about 20 km south of Kuching. NC seedlings treated with SMI were raised in the forest fursery and then brought to the field trial plot in Sabal F.R. for field planting. Sabal F.R. is about 150 km east of Kuching.

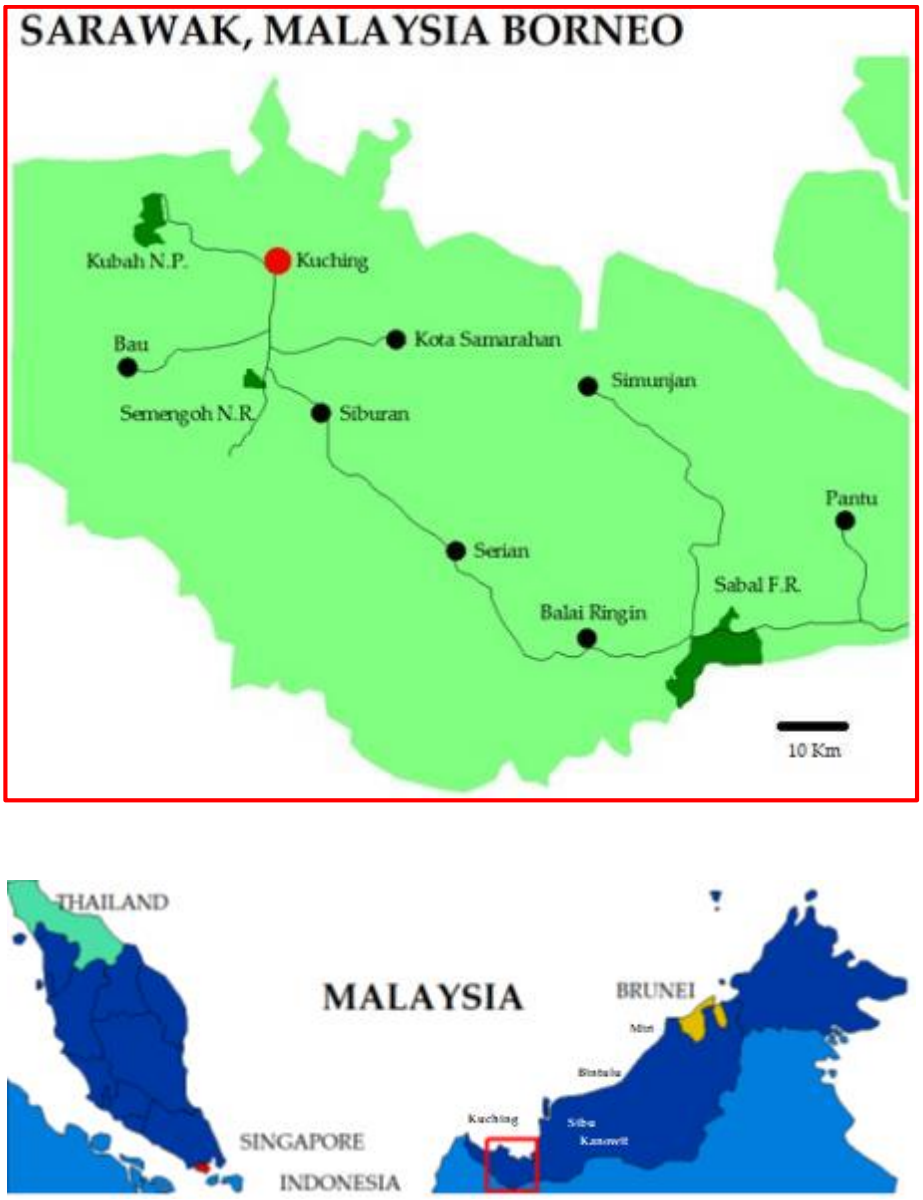


Figure 3. Location perspective of the three study sites.

The three specific localities namely the phenology plot in Kubah N.P., the forest nursery in Semengoh N.R. and the field trial plot in Sabal F.R. are referred to as PP, FN and FTP respectively in the thesis. As three specific localities were involved in the study the need to gather and know the basic information about each locality was necessary to facilitate planning and implementation of nursery trials, field trial, soil sample collection, and other logistical requirements.

The succeeding chapter outlined baseline studies carried out that were relevant in understanding the purpose, rationale, work flow, that aided in the preparation for the field study and ancillary experiments at the forest nursery as set in the thesis.

4 Baseline information of the study sites

The components in the baseline study included the vegetation, soil type classification, micro-physiography, soil disturbance, rainfall pattern, soil physical and chemical properties, and soil spore counts

4.1 Aims

The main aim of the baseline studies carried out was to derive two important pieces of information, firstly, the level of soil nutrients and the nutrient retention capacity of the three sites; FN in Semengoh N.R., PP in Kubah N.P., and FTP in Sabal F.R. Soil fertility level will determine the response to the usage of fertilizers at the planting out site and its necessity for favorable tree growth. The soil in Sabal FR was presumably typical of the majority of soils in Sarawak. According to Maas, Tie and Lim (1986, p. 16) their physical properties are generally quite good but chemically they are saddled by serious leaching, low in nutrients and nutrient-retaining capacity. Thus, the FTP site in Sabal F.R. was considerably not an ideal site for planting of NC.

Secondly, spore counts of soil samples taken from the three sites, could be used to reflect soil microorganism activity and richness which would provide the rationale to undertake the studies in the thesis. The spore counts could indicate a soil microorganism activity site as of logged-over forest or disturbed forest as mechanical clearing of vegetation and movement of earth had occurred not too long ago. The FTB could be somewhat similar to logged-over forest. The baseline study on the spore count of the soil stocked for planting media preparation at the FN in Semengoh N.R could also show its suitability as a planting media mix. The long term storage could cause the soil to be completely dry and thus affect the infective capacity of spores and thus considerably decreasing the quality of the substrate for planting media preparation.

4.2 Materials and methods

4.2.1 Sourcing of arbuscular mycorrhizal fungi propagules

The Sarawak Forest Tree Seed Bank situated in the Semengoh N.R., Jalan Puncak Borneo, Kuching has established and maintained Seed Production Areas (SPA) and PP (Seed Bank Semengoh 2013, pp. 2&3) as shown in Table 3.

Establishment of SPA and PP for priority species has been carried out since 1996. The purpose of the SPA and PP was to ensure the sustainability of seed supply from selected mother trees. SPA and PP have been established in 13 locations throughout Sarawak. SPA and PP with NC mother trees identified and selected were demarcated on the ground and protected by regular monitoring to prevent encroachment. The closest naturally growing NC trees were located in Kubah N.P. highlighted green in Table 3 below.

Table 3. Seed production areas and phenology plots of Sarawak Forest Tree Seed Bank.

Location	**Forest Type**	**No. of Trees**	
		Neolamarckia cadamba	**Other Species**
Arboretum, Semonggok Nature Reserve, Samarahan	Lowland Mixed Dipterocarp Forest	1	23
Balleh Protected Forest, Kapit	Hill Mixed Dipterocarp Forest	5	16
Baram, Miri	Secondary Forest	29	-
Kubah National Park, Kuching	Hill and Lowland Mixed Dipterocarp Forest	8	39
Lambir National Park, Miri	Lowland Mixed Dipterocarp Forest	-	38
Landeh, Samarahan	Tree Plantation	-	322
Lanjak Entimau Wildlife Sanctuary, Sarikei	Hill Mixed Dipterocarp Forest	-	23
Mukah Hill Forest Reserve, Sibu	Hill Mixed Dipterocarp Forest	18	10
Ravenscourt, Lawas	Hill Mixed Dipterocarp Forest	70	-

Selangau, Mukah	Hill Mixed Dipterocarp Forest	-	21
Similajau National Park, Bintulu	Hill Mixed Dipterocarp Forest	-	32
Sarawak Tree Improvement Centre, Sibu	Kerangas Forest	-	37
Sebangkoi, Sarikei	Hill Mixed Dipterocarp Forest	-	36

4.2.2 Soil type classification

Soil type identification was based on soil pit profile description. The soil pit was dug to about 1 m wide and 1 m deep or to limitation of any restricting layer within. Soil description procedures were based on Guidelines for a Forest Classification in Forest Management Planning (Glauner & Lagan 1995). A soil pit each was dug at PP in Kubah N.P. and the FTP in Sabal F.R.

Identification of soil types was carried out based on Keys to Soil Classification Sarawak (Teng 2004). The soils in Sarawak are currently classified into 11 soil groups (1 organic soil and 10 mineral soils), 45 families and 145 or more soil series (Teng 1993). Soil group is the highest level of classification and is identified according to its diagnostic subsurface horizon(s) or diagnostic characteristic. Many of the diagnostic parameters were adapted from USDA Soil Taxonomy and FAO Soil Map Legend of the World. Some of these parameters were adopted in total and some with minor modifications to suit the local Sarawak condition (Tie 1982, p. 2).

4.2.3 Topography survey

A topography survey was carried in FTP in Sabal F.R. to determine the micro-physiography of the site. A topography map could be used to assess the steepness of the site and determine an experimental planting layout based on the Randomized Complete Block Design (Chang 1972, pp. 33-37).

Wooden stakes were positioned in east-west and north-east directions at 3m horizontal distance intervals in the area already demarcated. Gradients of slope incline between

positions of wooden stakes were measured using a clinometer (Suunto Instrument, Finland). The vertical height of each position of wooden stake was determined using a horizontal distance and slope gradient based on the Phythogras equation. Elevation of each wooden stake position was then derived from its vertical height relatively from the starting point at left lower corner of the FTP.

The elevation of every wooden stake position were then digitized spatially using CartaLinx Spatial Data Builder software (Clark Labs, USA). A digital elevation model was then created using surface interpolation tool INTERPOL of IDRISI 32 software (Clark Labs, USA). The digital elevation model created depicts the micro-physiography of the FTP.

4.2.4 Soil disturbance survey

The FTP in Sabal F.R. and surrounding areas was once cleared of vegetation using a bulldozer and excavator crawler. Inevitably the top soil was also displaced. A 2" diameter Edelman Combination Auger (AMS, USA) was used to profile the thickness of the A Horizon at every sample point. The A Horizon is a mineral horizon formed at the surface or below a layer dominated by organic material. It composed of all or much of the original rock structure that have obliterated and intimately mixed with the accumulation of humified organic matter with the mineral fraction (Department of Agriculture Sarawak 2004, p. 28). The measurement of the A Horizon using the 2" diameter Edelman Combination Auger is illustrated by Figure 76.6 (Appendix).

Sample points were fixed using 3 x 3 m gridline in the FTP. The measurement of A-Horizon thickness at every sampling point was digitized spatially using CartaLinx Spatial Data Builder software (Clark Labs, USA). Distribution map of A-Horizon thickness was then created using surface interpolation tool INTERPOL of IDRISI 32 software (Clark Labs, USA).

4.2.5 Rainfall measurement

Collection and measurement of daily rainfall was carried out using the Symons rain gauge, a non-recording type of rain gauge. It was fabricated locally based on description by The Constructor (2017). Galvanized steel sheet is used to create a cylindrical vessel 13 (D) x 44 (L) cm. The vessel was fitted with a funnel about 13 cm below the top part. The lower part of the vessel which was detachable from the top part, held a 1 L receiving plastic bottle. The receiving bottle enveloped the funnel shank at the neck, storing any rainfall collected.

The rain gauge was installed by placing it in a hole on the ground about 12 cm deep and with the receiving end about 30 cm above ground level. The installation site was an open space of a house compound about 6 km from the FTP. Rain water collected in the receiving bottle was measured using a measuring glass with 1 mm graduation. For uniformity, rainfall was measured every morning and was recorded as rainfall of the day. Rainfall data were translated to rainfall in mm by converting amount of rainfall in ml/m^2 by factoring surface area of the Symons rain gauge which was about $0.013m^2$. The installation of the Symon rain gauge is depicted in Figure 76.5 (Appendix).

4.2.6 Soil Analyses

4.2.6.1 Soil sampling

Composited soil samples made up sub-samples were taken randomly from several points at the PP in Kubah N.P., whereas systematic grid of about 2.5 X 4.0 m spacing, sampling method was used in the FTP in Sabal F.R. The discrete sub-samples contributed an equal amount of material to the composite, taken from the same soil horizon and depth interval. Each discrete sub-sample should be thoroughly homogenized before drawing the composite (Environment Protection Authority 2005, p. 2). At each bore point, soil sub-sample was taken from a depth of 0-20cm using a 2" diameter Edelman Combination Auger (AMS, USA). As for the soil pits at PP Kubah N.P. and at FTP in Sabal F.R., soil samples were taken representing each identified soil horizon. For FN in Semengoh N.R., constituents of composited soil sample were

collected by randomly scooping at every point possible on the pile of soils which was already mechanically processed by soil shredder for planting media mixture, using a hand trowel.

Soil samples were processed at the Soil Laboratory in Sarawak Forest Tree Seed Bank Centre, Semengoh N.R. Samples were spread on plastic trays and air-dried for about a week. A steel pin roller was used to crush soil clumps before sieving them with a 2mm steel mesh.

4.2.6.2 Soil physical properties

The processed soil samples were analyzed for particle size distribution (Mechanical analysis) to determine percentage sand, silt and clay and soil texture class. Lost on Ignition (LOI) analysis was also carried out to determine the percentage of SOM. The Mechanical analysis and LOI methods was based on a laboratory manual prepared by Chin (1993, pp. 7-11, 14). The Mechanical analysis involved; pre-treatment of 10 g of soil using Hydrogen peroxide, dispersion by Calgon (mixture of Sodium hexa-meta-phosphate and Sodium carbonate), pipetting of silt and clay fractions, and finally decanting of sand fraction. LOI analysis involved incineration of oven-dry samples in a furnace at 800^{o} C for one hour.

4.2.6.3 Soil chemical properties

Analysis of soil chemical properties was done through an external party. The 2mm processed soil samples were dispatched to a private laboratory (i-TESTCHEM LABORATORY SERVICES, 1st Floor, Sub-lot 6, Contempo Commercial Centre, 94300 Kota Samarahan, Sarawak, Malaysia). Parameters and methods used to determine the soil chemical properties are as shown in Table 4.

Table 4. Soil parameters and laboratory analytical methods.

Soil parameters	Analytical methods
pH	MS 2457: 2012
Total N	MS 678: Part II: 1980-(a)
Organic C	MS 2469: 2012

Total P	In-house Method ITC/TM/S09 based on MS 678: Part VIII: 1980
Available P	In-house Method ITC/TM/S08 based on MS 678: Part VIII: 1980
Exchangeable K, Ca, Mg	In-house Method ITC/TM/S06 based on MS 678: Part IV: 1980
CEC	In-house Method ITC/TM/S07 based on MS 678: Part V: 1980

Interpretation of the soil physical and chemical properties in this baseline study was based wholly on the guideline described by Deloiite, Talisman and The DPA Group (1990, pp. 9&10).

4.2.7 Assessment of soil spores

AMF profiles of the study sites were deduced by assessing spore count. The Spore extraction method used was based on the Sucrose Extraction Method (Center for Conservation of Biology 2013). The spore segregation method described by INVAM (2017) was improvised in the thesis to enhance its efficiency.

4.2.7.1 Extraction of spores

The composited soil samples from the three sites were first air-dried for one or two days depending on wetness before sieving through a 2mm steel mesh. 10 g of soil was weighed in a 50ml centrifuge tube, and added with distilled water to its 40ml mark. The 50ml centrifuge tube was shaken vigorously using a vortex machine and left to stand for 15 minutes before centrifuging for 10 minutes at 2500 rpm using a Table Top Centrifuge (KUBOTA 2420, Japan). Water and floating organic debris were poured out gently from the 50ml centrifuge tube.

The same 50ml centrifuge tube was then refilled with 2M Sucrose-10% Calgon solution to its 40ml mark. The Sucrose-calgon solution was prepared by using 685 g of refined sugar and 100 g of Calgon flaks (Sodium hexametaphosphate) to 1 L water. The mixture was heated up and stirred rapidly until solution was clear. The tube with 2M Sucrose-

10% Calgon solution was again put through centrifuge machine for 20 minutes at 2500 rpm.

Filter paper (Whatman No. 1) was placed inside a filter funnel, and moistened with distilled water. The Sucrose-calgon solution containing spores was gently poured on the filter paper before turning on the vacuum line. Once the solution had filtered through completely, the inside of filter funnel was gently rinsed with distilled water and drained out completely by turning on the vacuum line again. The filter paper was gently removed from filter funnel and the remaining filtrate was rinsed off slowly with distilled water into a 5cm diameter plastic petri dish.

Each 5cm diameter plastic petri dish comprised of spore extracts from four tubes or 40 g of the same soil sample. Distilled water was further added to about 1/3 height of the petri dish. Spore suspension was stored in the refrigerator at 4^0C while waiting for subsequent procedure which was spores' segregation.

4.2.7.2 Segregation of spores

"Wholesome looking" spores in distilled water suspension were picked individually among roots, mass of debris, mycelium fragments and "dead" spores under view of a Leica EZ4HD stereo microscope with LED and HD Camera (Leica M, Singapore). The picking of spores was done by using a 10µl micro-pipette on to a clean petri dish filled with distilled water. To aide in pipetting the spores, the petri dish was placed in a Transsonic 310 sonicator (Elma, Germany) for about one minute. Additionally, spore suspension was also agitated using a wooden skewer before picking the spores again once the suspension had settled. The segregated spores were re-picked and transferred on to a second clean petri dish filled with distilled water. The spore picking process is illustrated in Figure 4.

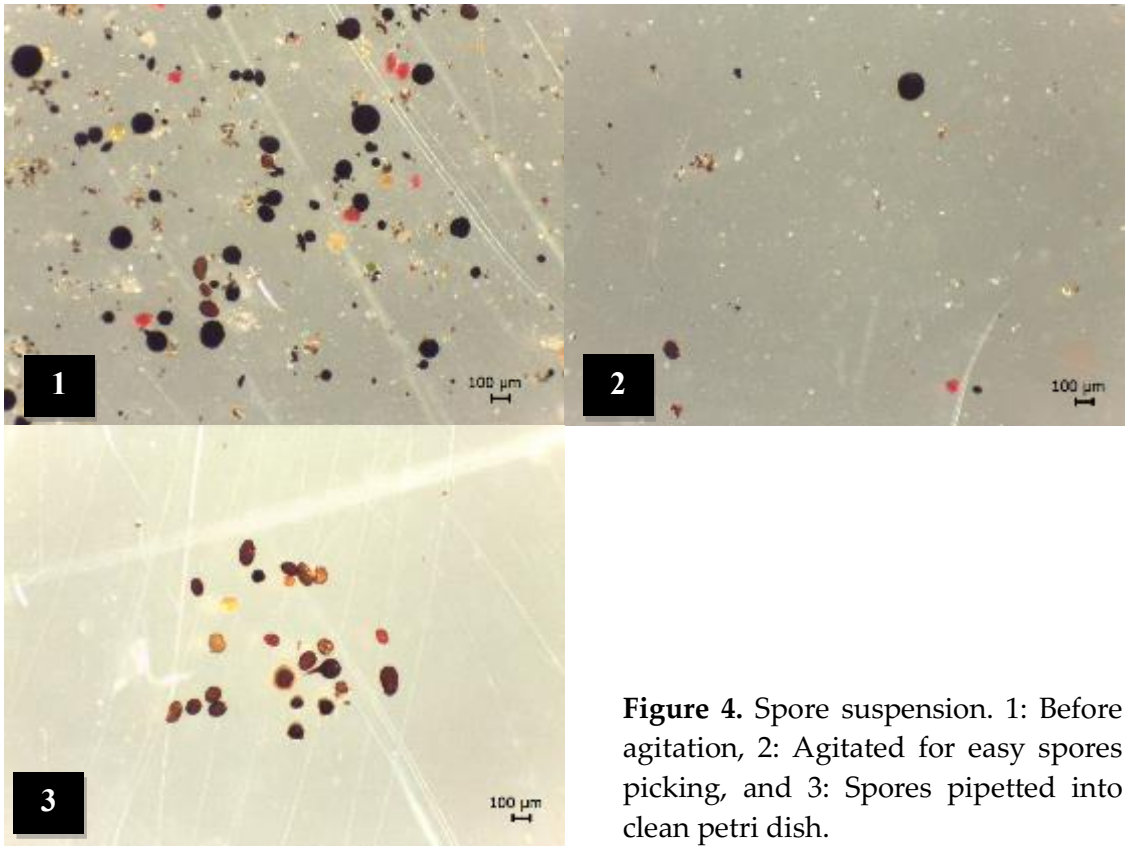


Figure 4. Spore suspension. 1: Before agitation, 2: Agitated for easy spores picking, and 3: Spores pipetted into clean petri dish.

4.2.7.3 Enumeration of spores

A direct count method was carried out for the enumeration of spores as the spore density was considered low (INVAM 2017). Spore count was done under view of a stereo microscope usually at 35X magnification. The spores were grouped based on three simple morphological groups namely globose shaped of different colors, ovoid shaped of different color, and a distinctly white to pale yellow color spores which were rather prominent. To augment the spore count, a white paper drawn with parallel lines spaced at 5 mm intervals was placed underneath the petri dish viewed. The microscopic view was moved from left to right in a zigzag manner between the lines. With the aid of mechanical tally counter almost 100% of spores were accounted for.

4.3 Results

Baseline information presented was peculiar to each study site. The information on the FN in Semengoh N.R. for example was limited to properties of the soil procured for planting media mix. The information gathered on the two others sites, were slightly more exhaustive.

4.3.1 Phenology plot, Kubah N.P.

The aim of the main study was to carry out soil trap culture using soil collected from rhizosphere of naturally growing NC trees to produce SMI for use in the thesis. The *Neolamarckia cadamba* PP in Kubah N.P. was considered an ideal site to source for AMF propagules as it was the closest and easy to access.

4.3.1.1 Natural stands of *Neolamarckia cadamba*

Five naturally growing NC trees were found at the PP (1°36′48″ N 110°11′51″ E) along the main entrance road to the Kubah N.P. headquarter office. The trees were numbered 0001-0005. The trees were about 40 cm in DBH. The PP area was once cleared and levelled for use as a camping site and since then has being left abundant and overgrown with secondary forest trees, shrubs and bushes. The depiction of one of the five trees is as shown in Figure 76.1 (Appendix). The PP sits at the edge an area that was levelled by filled up of moved soils. NC trees frequently colonized such site where the soil has been disturbed as also observed elsewhere in the N.P. such as at fringes of buildings and roads.

Based on the phenology monitoring records, the NC trees had experienced a high fruiting intensity in 2010 and followed by a medium fruiting intensity in 2011 and 2012 (Seed Bank Semengoh 2013, p. 4).

4.3.1.2 Soil type

Description of the soil pit profile is as shown in Table 5. Based on the Sarawak Soil Classification System, the soil type was originally Tutoh Soil Series, TUTOH family of

the Skeletal Soil Group based on its presence as of a buried soil at 68 cm depth. Based on definition by Galbraith (2011), buried soil shows a sequence of one or more genetic horizons covered with a surface mantle of new soil material that is at least 50 cm thick. The soil type classification of the PP in Kubah N.P, is thus based primarily on the soil formed on the surface mantle of the new soil material as the diagnostic horizons.

The clearing and levelling of the site created a mantle over the original soil. The new soil can be classified as the same type as the buried soil. Skeletal Soils are mineral soils, with a depth less 50 cm and the TUTOH soil family have developed from colluvium parent material (Teng 2004, p. 31). The presence of coarse fragments such as gravel, stones, and boulders were frequently observed as shown Figure 5. The tentative soil correlation with Soil Taxanomy (USDA, 1998) is "Clayey-/loamy-skeletal, mixed, acid, isohyperthermic, Typic Troporthents" (Teng 2004, p. 55).

Table 5. Description of soil pit profile at the phenology plot.

Soil Group	: Skeletal Soils
Soil Family	: TUTOH
Soil Series	: Tutoh
Location	: *Neolamarckia cadamba* Phenology Plot, Kubah NP, Kuching
Drainage	: Moderately well drained
Terrain	: Hill, 25^0
Vegetation	: Secondary forest (Old camping site)

Depth (cm)/Horizon	**Description**
0-10 A	Light brownish grey (10YR 6/2) Sandy clay loam, friable sub-angular blocky structure, very frequent slightly weathered gavels and stones, common fine yellow red mottles, few fine, medium and large roots, and gradual wavy boundary.
10-38 B1	Light grey (10YR 7/1) Sandy clay loam, firm sub-angular blocky structure, very frequent slightly weathered gavels and stones, common fine yellow red mottles, few fine, medium and large roots and clear wavy boundary.
38-62 B2	Light brownish grey (10YR 6/2) Sandy clay loam, firm sub-angular blocky structure, very frequent slightly weathered gavels and stones, common fine yellow red mottles, few fine, medium and large roots, and abrupt wavy boundary.

62-68 Bb	Very pale brown (10YR 7/4) Sandy clay loam, friable, sub-angular blocky structure, very few slightly weathered grit and gravels, and abrupt wavy boundary.
68+ R	Boulders

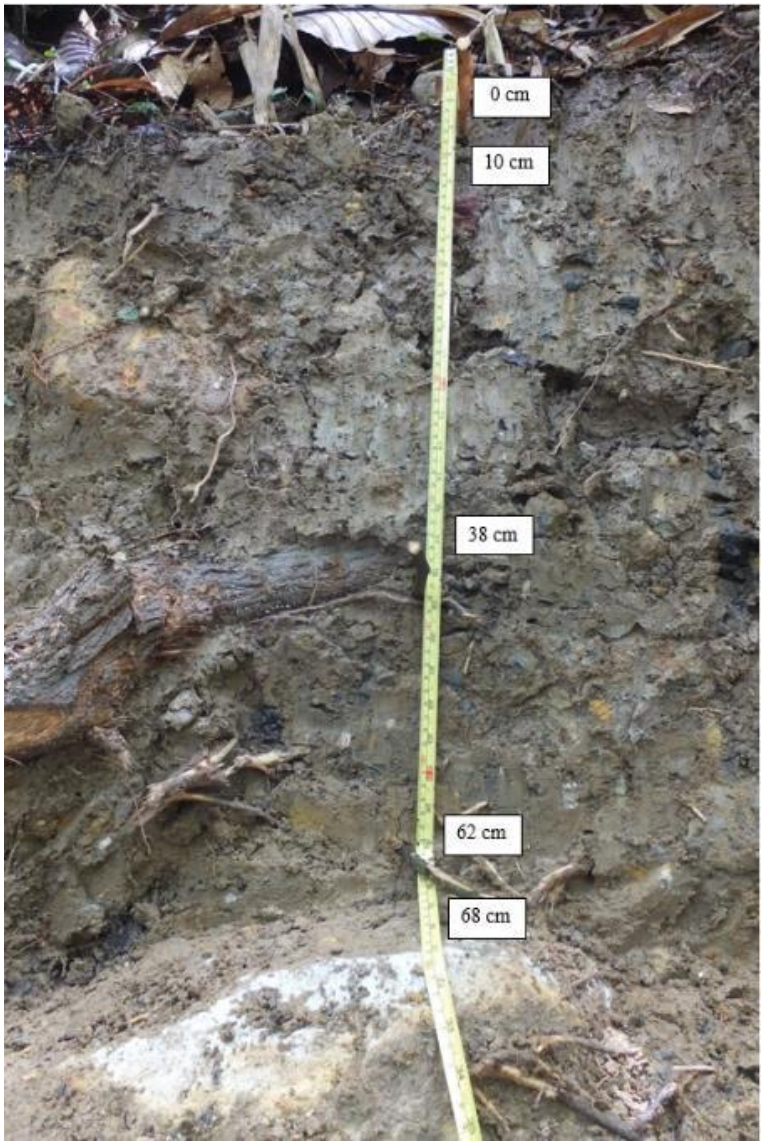


Figure 5. Soil pit profile at the phenology plot.

4.3.1.3 Soil texture of pit profile

Mechanical analysis on soil samples taken from the soil pit at the PP indicated that the original soils (62-68cm depth) had a high sand content exceeding > 80% (Figure 6). The

presence of higher percentage clay and silt above the original soil layer was the result of earth being moved mechanically to raise the level of the site. Sandy Clay Loam was the dominant soil textural class (0-62 cm depth) as seen deposited above the thin original Sandy Loam layer.

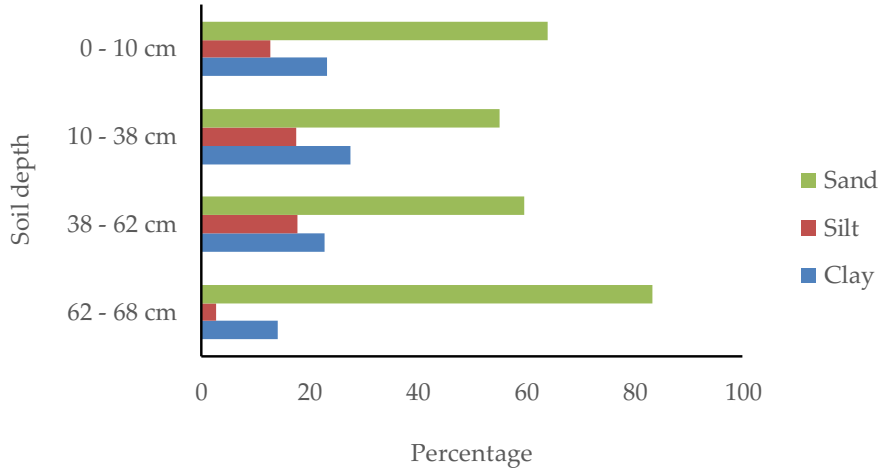


Figure 6. Percentage sand, silt and clay of soil pit profile at phenology plot.

4.3.1.4 Properties of composited soil sample

Composited soil sample taken from depth of 0-20 cm of PP in Kubah N.P. were also analyzed for particle size distribution and soil pH besides soil chemical properties. Generally the soil textural class of the site was Sandy Loam and the soil pH of 5.3 is acidic and is rated low (Deloiite, Talisman & The DPA Group 1990, p. 10). Though the soil pH is rated low, it was relatively higher than most soils in Sarawak which are about a pH of 4.

The rating of other soil parameters and its general interpretation based on (Deloiite, Talisman & The DPA Group 1990, pp. 7, 9&10) are as shown in Table 6. The soil chemical properties of the PP indicated that the site was that of poor soil in terms of soil nutrient level and retention. However, levels of Ca seemed to be exceptionally high as compared to the other elements. The low CEC and relatively medium level of Ca could have contributed to a relatively less acidic soil pH reading.

Table 6. Rating and general interpretation of phenology plot soil properties (Deloiite, Talisman & The DPA Group 1990, p. 7, 9&10).

Soil parameters	Lab Result	Rating	General interpretation
Textural class	Sandy loam	-	-
pH	5.3	Low	Acidic soils, possible Al toxicity and excess of Cu, Fe, Mn and Co; Ca, K, N, Mg, P, B, S and Mo deficiencies
Total N (%)	0.11	Low	-
Organic C (%)	1.07	Low	-
Total P (ppm)	96	Low	-
Available P (ppm)	3	Low	Fertilizer response most likely
Exchangeable K (meq)	0.12	Low	Response to K fertilizer likely
Exchangeable Ca (meq)	5.31	Medium	Generally adequate
Exchangeable Mg (meq)	0.48	Low	Response to Mg fertilizer expected
CEC (meq)	6.92	Low	Low nutrient retention/reserves

4.3.1.5 Soil spores

Segregated spores were categorized into three simple morphotypes namely ovoid shaped of different colors, globose shaped of different colors, and specifically white to pale yellow color spores which were either globose, sub-globose or irregular shapes. They are as depicted under view of stereo microscope in Figure 7.

Spore extraction, segregation and spore count was carried out in three replications (Table 7). The globose shaped of different colors spores were most dominant, followed by ovoid shaped of different colors spores and the prominently white to light yellow color spores.

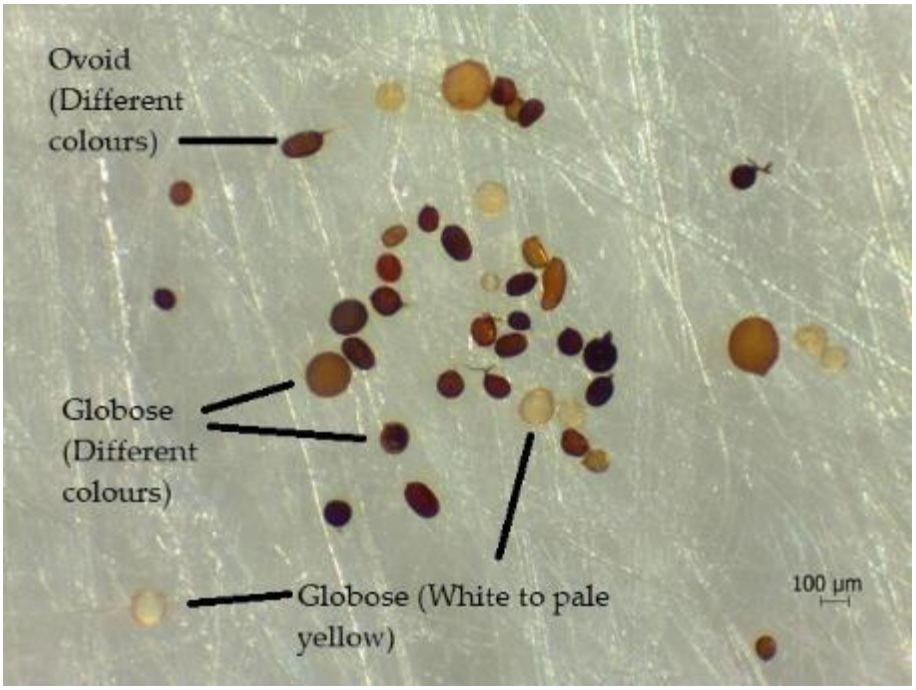


Figure 7. View of spores from phenology plot soil (35X magnification).

Table 7. Spore counts of phenology plot soil (40 g).

Replication	Ovoid (Different colors)	Globose (Different colors)	Globose, sub-globose to irregular (White to pale yellow)	Total
1	33	134	9	176
2	59	135	44	238
3	79	199	20	298
Rounded Average	57	156	24	237

Chubo et al. (2009, p. 340), reported that the mean number of spores studied in Niah, Sarawak ranged from 45–142 per 50 g dry soil. The spore count of the PP was almost double of that studied in Niah, about 296 per 50 g dry soil.

4.3.2 Forest nursery, Semengoh N.R.

Soil used in this study was the current soil stock used for preparation of planting media at the FN in Semengoh N.R. The soil stock was procured from private supplier of nursery soil costing about RM 270 per consignment of a seven MT truck.

4.3.2.1 Properties of composited soil sample

The composited soil sample from the FN was also analyzed for particle size distribution, soil pH other soil parameters. The rating of other soil parameters and their general interpretation based on (Deloiite, Talisman & The DPA Group 1990, p. 7, 9&10) are as shown in Table 8. The overall soil textural class of the soil procured for planting media preparation was classified as Clay as it constituted about 49% Clay, 14% Silt and 37% Sand (Department of Agriculture Sarawak 2004, p. 72). The soil pH of 4.3 is acidic and is typical of most soils in Sarawak. The soil properties of the soil procured at the FN is of poor soil in term of soil nutrient level and retention.

Table 8. Rating and general interpretation of forest nursery soil properties (Deloiite, Talisman & The DPA Group 1990, pp. 7, 9&10).

Soil parameters	Lab Result	Rating	General interpretation
Textural class	Clay	-	-
pH	4.3	Low	Acidic soils, possible Al toxicity and excess of Cu, Fe, Mn and Co; Ca, K, N, Mg, P, B, S and Mo deficiencies
Total N (%)	0.08	Low	-
Organic C (%)	0.87	Low	-
Total P (ppm)	135	Low	-
Available P (ppm)	7	Low	Fertilizer response most likely
Exchangeable K (meq)	0.10	Low	Response to K fertilizer likely
Exchangeable Ca (meq)	0.62	Low	Response to Ca fertilizer expected
Exchangeable Mg (meq)	0.15	Low	Response to Mg fertilizer expected
CEC (meq)	7.54	Low	Low nutrient retention/reserves

4.3.2.2 Soil spores

Segregated spores were also categorized into three simple morphotypes namely ovoid shaped of different colors, globose shaped of different colors, and specifically white to pale yellow color spores globose, sub-globose or irregular shapes. Similarly, spore

extraction and segregation was done in replication. Example of spores viewed under the stereo microscope is of that of Replicate 1 and is as shown in Figure 8.

Soil spore count of the FN was very low and was comprised mainly of globose shape of different colors (Table 9).

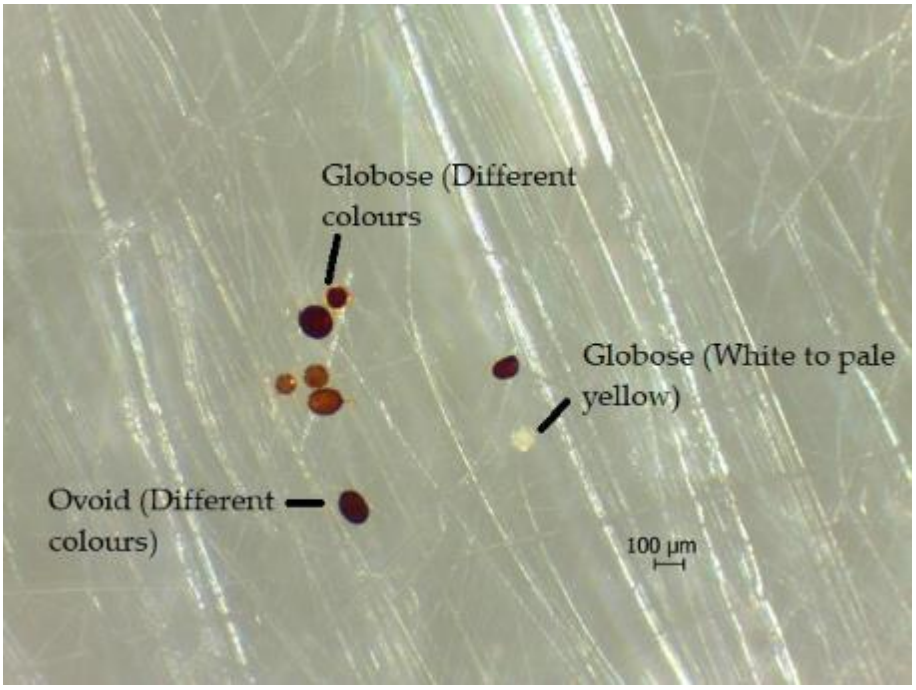


Figure 8. View of spores from forest nursery soil (35X magnification).

Table 9. Spore counts of forest nursery soil (40 g).

Replication	Ovoid (Different colors)	Globose (Different colors)	Globose, sub-globose to irregular (White to pale yellow)	Total
1	1	20	10	31
2	3	13	0	16
3	5	28	6	39
Rounded average	3	20	5	29

4.3.3 Field trial plot, Sabal F.R.

SMI treated and non-treated NC seedlings were planted out at the FTP in Sabal F.R. The FTP was considered an ideal site to test effectiveness of SMI produced as the soil and topography were quite representative of the majority of forest plantation areas in Sarawak.

4.3.3.1 Soil type

Description of soil pit profile of the FTP is as shown in Table 10. Soil type was classified as Bekenu Soil Series, BEKENU family of the Red Yellow Podzolic Soil Group based on the Sarawak Soil Classification System. Red Yellow Podzolic soils have soil colour with hues 10YR or redder; chroma of more than 4 and values of more than 6 (Teng 2004, pp. 46&48). The distinctive soil horizon is either cambic, argillic or kandic within 100cm of the soil surface. The BEKENU family soils have a Fine loamy or Fine silty particle size class developed from non-calcareous sedimentary rock parent material. The tentative soil correlation with Soil Taxonomy (USDA, 1998) according to Teng (2004, p. 65) is "Fine loamy, siliceous, isohyperthermic, Typic Kandiudults (Paleudults)."

The soil profile was generally yellowish except for the presence of a AB Horizon (10-18cm depth) which was light grey in color and having the presence of fine reddish yellow mottles within the horizon and below (Figure 9). As the soil pit was located at mid slope the sign of lateral movement of soil water was clearly seen by greying of soil matrix and emergent of reddish mottles due to the oxidation of soil Fe content.

Table 10. Description of soil pit profile in the field trial plot.

Soil Group	: Red Yellow Podzolic Soils
Soil Family	: BEKENU
Soil Series	: Bekenu
Location	: Sabal Forest Reserve, Serian-Ari Aman Road
Drainage	: Moderately well drained
Terrain	: Low hill, 10^0
Vegetation	: Shrubs
Depth (cm)/Horizon	**Description**

0-10 **A**	Yellowish brown (10YR 5/4) Sandy clay loam, friable sub-angular blocky structure, abundant fine, few medium and large roots, and clear smooth boundary.
10-18 **AB**	Light grey (5Y 7/1) Sandy clay loam, firm sub-angular blocky structure, many fine reddish yellow mottles, few fine roots, and diffuse irregular boundary.
18-57 **B1**	Yellow (10YR 7/6) Sandy clay loam, firm sub-angular blocky structure, few fine reddish yellow mottles, few fine roots, and diffuse smooth boundary.
57-100+ **B2**	Yellow (10YR 8/6) Sandy clay loam, slightly sticky and plastic sub-angular blocky structure, few fine reddish yellow mottles, and few fine roots.

4.3.3.2 Soil texture of pit profile

Particle size distribution analysis indicated that the top half of soil horizons were generally Sandy Clay Loam (0-56cm depth) whereas the bottom half was Sandy Loam (Figure 10). The soil type was classified as that of the BEKENU family as the Clay content was between 18% and 34% (Teng 2004, p. 49).

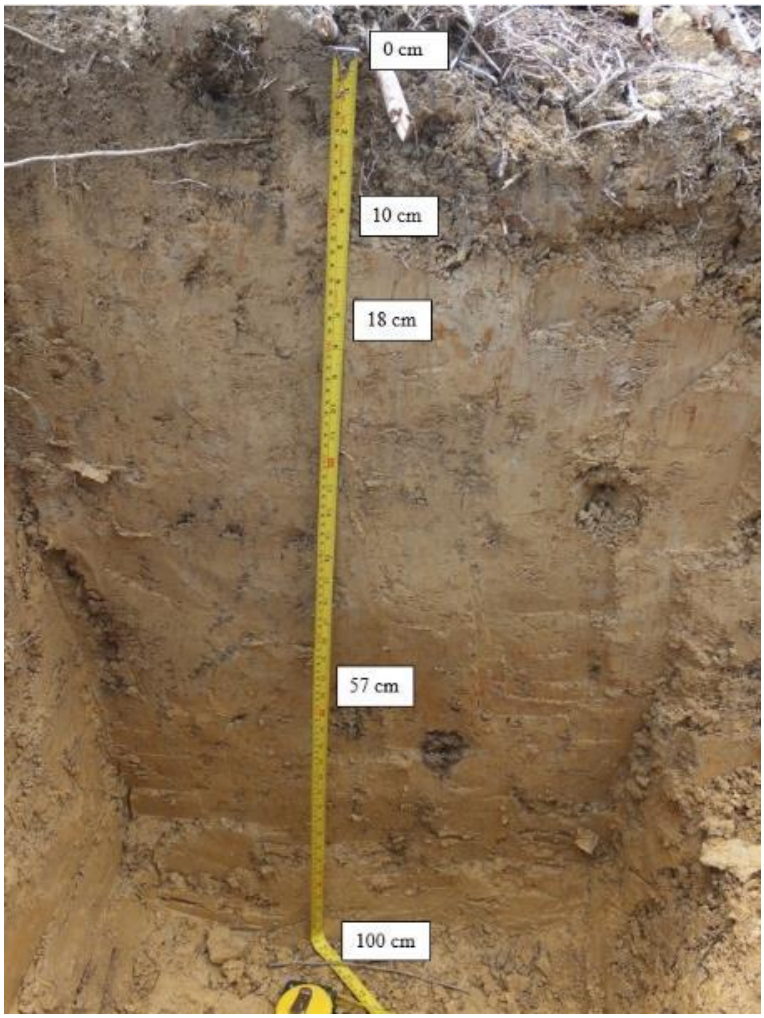


Figure 9. Soil pit profile at the field trial plot.

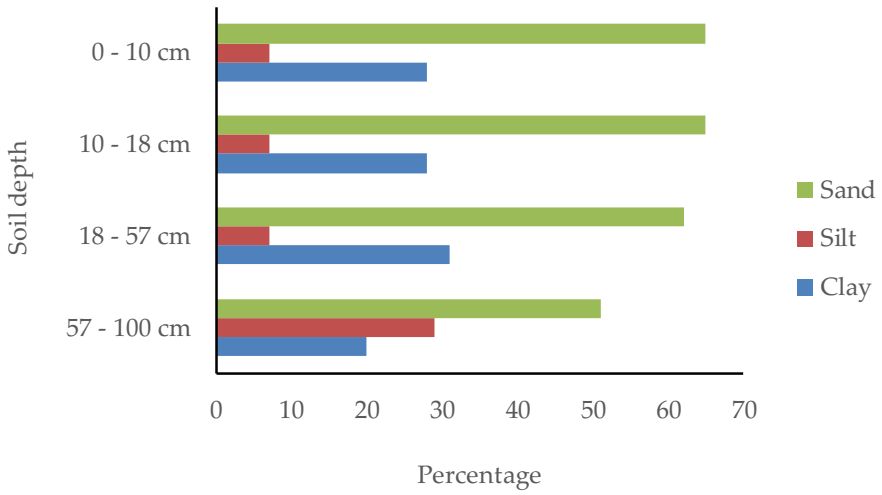


Figure 10. Percentage sand, silt and clay of soil pit profile at the field trial plot.

4.3.3.3 Properties of composited soil samples

Composited soil samples were taken separately from the three Blocks (Upper Slope, Middle Slope and Lower Slope) of FTP in Sabal F.R. and were also analyzed for particle size distribution, soil pH and other soil parameters. The obvious difference among the slope positions was that the Middle and Lower Slopes had a higher proportion of Sand content as compared to the Upper Slope (Table 11).

Table 11. Field trial plot soil properties (Upper Slope, Middle Slope, and Lower Slope).

Soil parameters	**Upper Slope**	**Middle Slope**	**Lower Slope**
Textural class	Loam	Sandy Loam	Sandy loam
(% Sand, % Silt, %Clay)	(48, 28, 23)	(55, 35, 10)	(68, 13, 18)
pH	4.2	4.2	4.5
Total N (%)	0.10	0.08	0.11
Organic C (%)	1.15	0.91	1.14
Total P (ppm)	127	129	102
Total P (%)	0.013	0.013	0.010
Available P (ppm)	3	3	3
Exchangeable K (meq)	0.09	0.06	0.09
Exchangeable Ca (meq)	0.29	0.20	0.31
Exchangeable Mg (meq)	0.11	0.06	0.15
CEC (meq)	6.80	6.13	5.57
Calculated Soil CNP ratio	91:8:1	71:6:1	112:11:1
Calculated Soil Base Saturation (%)	7	5	10

The levels of their soil parameters were generally the same for all Slope positions. Based on calculated Soil CNP ratio and Soil Base Saturation. The Middle Slope had the highest reserved P whereas the Lower Slope had the highest available base cations. The rating of soil properties and its general interpretation based on (Deloiite, Talisman & The DPA Group 1990, pp. 7, 9&10) are as shown in Table 12. The soil properties indicate that the FTP site is also that of poor soil in terms of soil nutrient level and retention.

Table 12. Rating and general interpretation field trial plot soil properties (Deloiite, Talisman & The DPA Group 1990, pp. 7, 9&10).

Soil parameters	Lab Result	Rating	General interpretation
Textural class	Sandy Loam-Loam	-	-
pH	4.2-4.5	Low	Acidic soils, possible Al toxicity and excess of Cu, Fe, Mn and Co; Ca, K, N, Mg, P, B, S and Mo deficiencies
Total N (%)	0.08-011	Low	-
Organic C (%)	0.91-1.15	Low	-
Total P (ppm)	102-129	Low	-
Available P (ppm)	3	Low	Fertilizer response most likely
Exchangeable K (meq)	0.06-0.09	Low	Response to K fertilizer likely
Exchangeable Ca (meq)	0.20-0.31	Low	Response to Ca fertilizer expected
Exchangeable Mg (meq)	0.06-0.15	Low	Response to Mg fertilizer expected
CEC (meq)	5.57-6.80	Low	Low nutrient retention/reserves

4.3.3.4 Micro-physiography

The topography of the FTP in Sabal F.R. is as shown in Figure 11. It was made up of a small valley with a long slope facing south and short slope facing north. The elevation difference between the lowest and highest spots was about 8 m and thus the general micro-physiography of FTP and its vicinity, with local relief of about < 50 m above sea level could be classified as that of Low Hills (Loi et al. 1985, p. 16). The spatial distribution based on slope positions, Upper Slope (22m-24m), Middle Slope (25m-27m), and Lower Slope (28m-30m) was fairly not equal as shown in Table 13. The Lower Slope area was also made up of sites with same elevation as the Middle Slope area. Thus, randomization of treatments and replicates in the experimental design was necessary for the FTP so as to avoid biasness in term of slope positions.

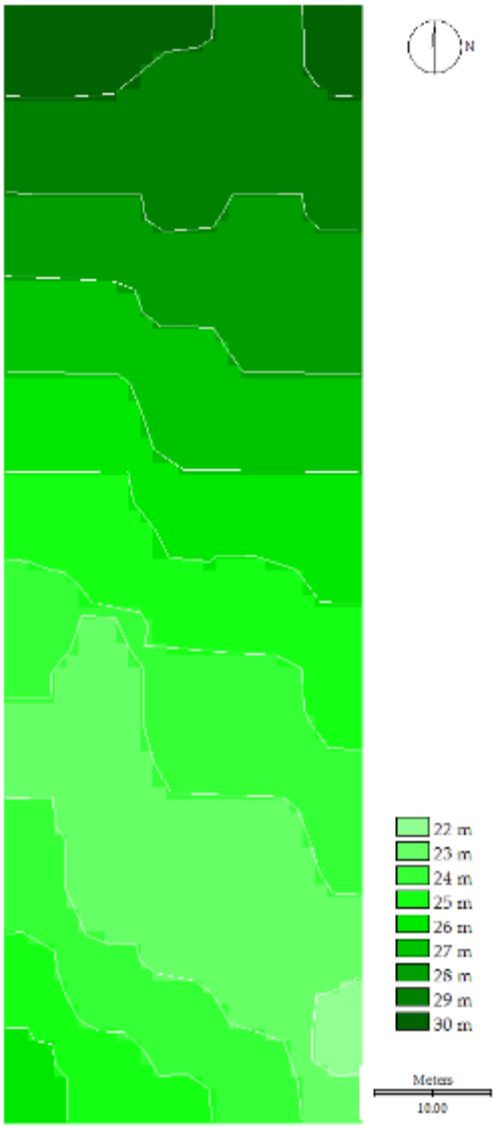


Figure 11. Topography of the field trial plot.

Table 13. Composition of field trial plot based on areas of slope positions.

Slope Position	Area (m^2)	Percentage
Upper Slope (28-30m)	820	31
Middle Slope (25-27m)	940	33
Lower Slope (22-24m)	1,060	36
Total	2,820	100

4.3.3.5 Soil disturbance

Soil disturbance was measured in terms of top soil lost. The A-Horizon identified was categorized into thickness groups of 0-5cm, 6-10cm, and 11- >20cm. The thickness was

observed using soil auger. Soil auger profile with 0-5cm A-Horizon could be considered as highly disturbed and was observed on the ground to be slightly compacted and almost void of vegetation cover. On the other hand, spots within the FTP with 11- >20 cm A-Horizon could either be undisturbed or have amassed top soil when mechanical land clearing of the area was done in the past. The spatial pattern of soil disturbance as reflected by thickness of soil A-Horizon is as shown in Figure 12.

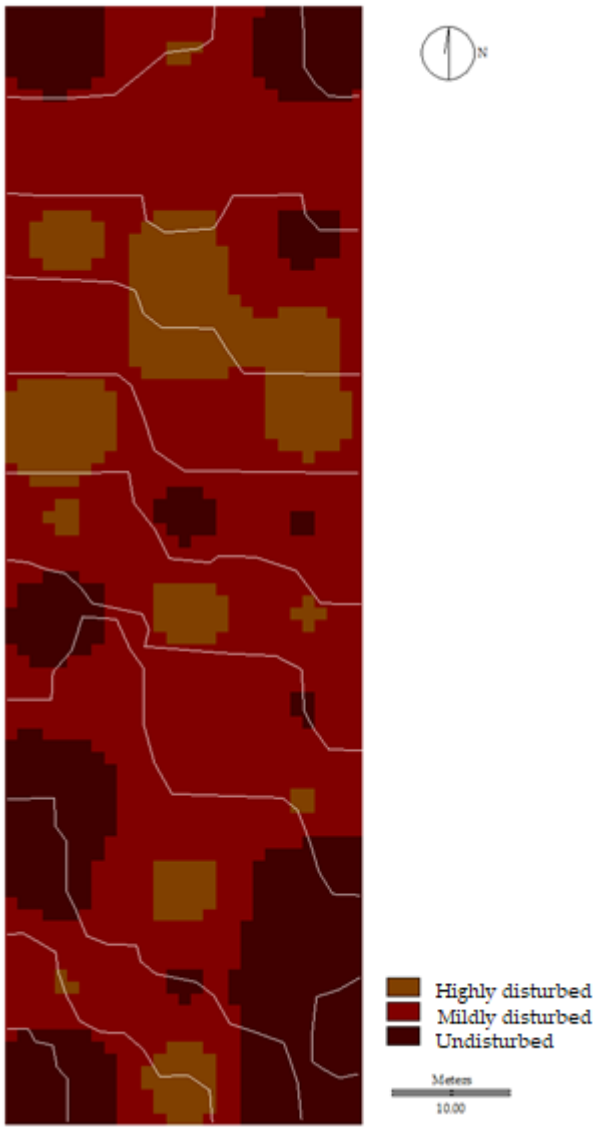


Figure 12. Spatial distribution of soil disturbance in the field trial plot.

The proportion of severity of soil disturbance is as shown in Table 14. Highly disturbed soil only made up 15% of the area whereas the undisturbed or good sites were actually larger by almost 10%. However, the disturbed spots were roughly found about the centre of the FTP running north-south, whereas the good or undisturbed patches were mainly

distributed at the corners and edges of the FTP. Similar to the variation in slope position of the FTP, randomization of treatments and replicates in the experimental design was necessary for the FTP so as to avoid biasness in terms of soil disturbance.

Table 14. Composition of field trial plot based on areas of disturbance severity.

Severity	Area (m^2)	Percentage
Highly disturbed	417	15
Mildly disturbed	1,729	61
Undisturbed	674	24
Total	2,820	100

4.3.3.6 Rainfall pattern

The daily rainfall data collated was segmented from the date of tree planting to the date of the first monthly plot assessment and every subsequent monthly plot assessment. The monthly rainfall recorded was the cumulative daily rainfall readings prior to the particular day when tree assessment was carried out. Monthly rainfall collected was calculated based on the radius of the rain gauge top part which was exactly 6.5 cm in radius.

The rainfall pattern of areas within the vicinity of the FTP was typical of the rainfall pattern in Sarawak whereby heavy rain generally occurs roughly at the end and beginning of the year from about November until March the following year (Figure 13). However, rainfall recorded for December was unusually low. It may be due to a recording lapse during the period. Camerlengo, Mohd Azmi Ambak and Mohd Nasir Saadon (2000, p. 135), reported that an important increase in rainfall intensity in the southern tip of Sarawak occurred in December. The southernmost part of Sarawak apparently registered the highest rainfall intensity and it corresponded high annual rainfall observed in that area.

Total rainfall recorded during the field trial period of 12 months was 4,018 mm. Based on the Mean Isohyetal Map of Sarawak 2014 (Figure 77, Appendix), the FTP and

surrounding areas were within the rainfall isohyetal of 3,500-3850 mm (Jabatan Pengairan dan Saliran Sarawak 2017). The increasing trend of the annual rainfall in Sarawak in recent years was also reflected by surplus rainfall recorded of about 250-500 mm during the study proper.

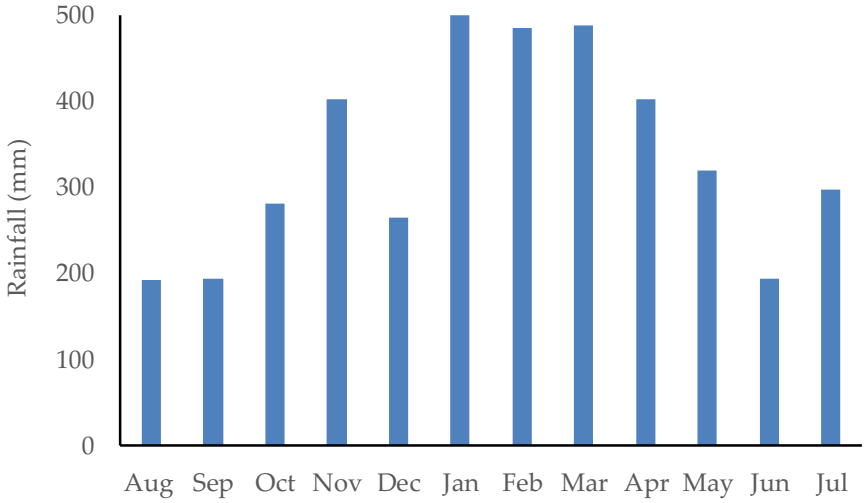


Figure 13. Monthly rainfall of areas within close proximity of the field trial plot.

4.3.3.7 Soil spores

Spore extraction, segregation and spore count was carried out on composited soil sample collected from each slope position. Segregated spores were also categorized into three simple morphotypes namely ovoid shape of different colors, globose shape of different colors, and specifically white to pale yellow color spores of globose, sub-globose to irregular shapes. The illustration of the different spore morphotypes based on composited soil sample taken from Lower Slope is as shown in Figure 14.

Globose shape spores of different colors were most dominant, followed by about equal spore count of ovoid shape of different colors spores and the conspicuous white to light yellow color spores (Table 15).

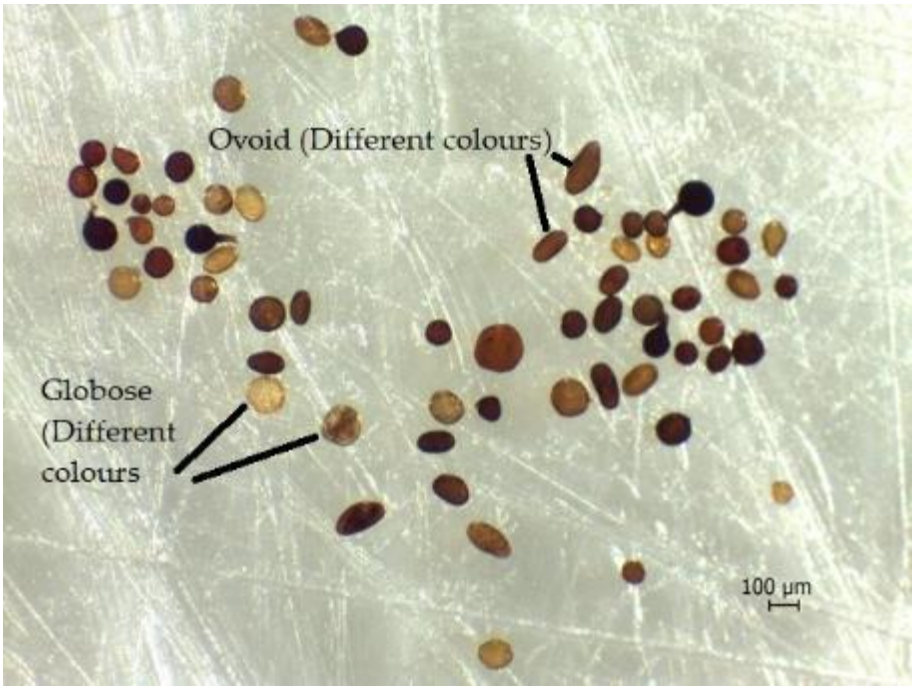


Figure 14. View of spores from field trial plot soil (35X magnification).

Table 15. Spore counts of field trial plot soils (40 g) (Upper Slope, Middle Slope, and Lower Slope).

Sample	Ovoid (Different colors)	Globose (Different colors)	Globose, sub-globose to irregular (White to pale yellow)	Total
Upper Slope	2	14	2	18
Middle Slope	18	91	15	124
Lower Slope	28	158	33	219
Rounded average	16	88	17	120

4.4 Discussion

They were some remarkable similarity between the PP site in Kubah N.P. and FTP site in Sabal F.R. though in terms of micro-physiography and vegetation cover, they were in contrast. Interestingly, both sites have gone through some kind of ground surface disturbance where vegetation was cleared using heavy machinery and resulted in

movement and deposition of soil materials. The soil disturbance in the FTP was as the consequence of site preparation activity for a tree planting program in the past. However, soil movement was probably limited to top soil and SOM as compared to the PP in Kubah N.P. McCauley, Jones and Olson-Rutz (2017, p. 2) pointed out that the SOM serves multiple functions in the soil, such as retention of nutrients, increasing water holding capacity, and soil aggregation, which are important properties of soil quality . The reduction of SOM levels decrease soil fertility, increase fertilization needs, and increase soil erosion.

Natural regeneration of vegetation has occurred at both sites with the emergence of bushes and shrubs and proliferation of pioneer tree species as in the case of the PP in Kubah N.P., NC trees, of which five of the trees were involved in the thesis as aforementioned (Table 3).

They were some differences in the properties of the composited soil samples collected. The PP soil and FTP soil were both Sandy Loam based on textural class with PP soil having slightly more sand content by 10%. The soil the FN has high clay content at almost 50% and thus its Clay textural class (Figure 15).

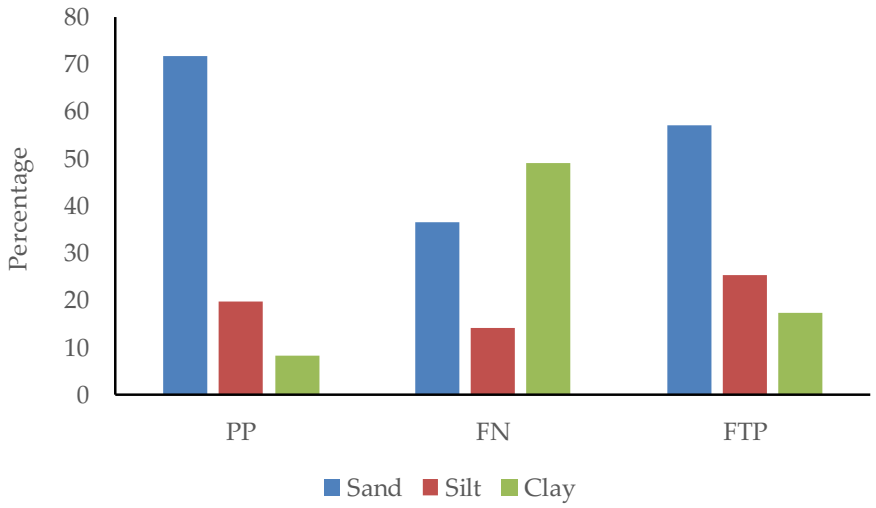


Figure 15. Percentage sand, silt and clay of composited soil samples from the phenology plot, forest nursery, and field trial plot.

The soils of the three sites were generally acidic and typical of most soils in Sarawak. However, pH of PP soil in Kubah NP was slightly higher and the Exchangeable Ca content was exceptionally higher as compared to the other two sites (Table 16). This may be due to some external factors such as due to dumping of cement and construction waste as the soil parent rock was not calcareous in nature. The lithology of Kubah N.P. was reported to be largely of Sandstone nature (Hazebroek & Morshidi 2001, p. 350).

Generally, the soil nutrient contents of all the soil samples were rated low except for Ca content in the PP soil. FN soil maybe slightly higher in P content and incidentally the CEC was also higher than the two others sites. The Clay soil texture probably contributed in relatively higher CEC and higher nutrient retention/reserves. Total P and Available P content of FN soil were also relatively higher and is probably due to the higher percentage SOM present as compared to the composited soil samples from the PP in Kubah N.P. and the FTP in Sabal F.R.

CEC is the soil's ability to hold and supply nutrients to meet plants requirement. As pointed out by McCauley, Jones and Olson-Rutz (2017, p. 3), Soils with high CEC typically have high amounts of clay and/or organic matter that is able to bind more cations such as Ca or K as compared to soils that are more silty or sandy. Higher CEC resulted in greater buffering capacity of soils

Table 16. Soil chemical properties of the three sites.

Soil parameters	Phenology Plot	Forest Nursery	Field Trial Plot
pH	5.3	4.3	4.3
Soil organic matter (%)	4	10	4
Total P (ppm)	96	135	119
Available P (ppm)	3	7	3
Exchangeable Ca (meq)	5.31	0.62	0.27
CEC (meq)	6.92	7.54	6.17

According to Maas, Tie and Lim (1986, p. 16), the physical properties of such soils are generally quite good but are saddled by serious leaching, low in nutrients and nutrient retaining capacity. Bloomberg and John Sabang (2009, p. 57), pointed out that NC needs rapid growth to beat leaf pests and herbivores. It should succeed on productive sites with low CN ratio and high levels of available water. Conversely, it will exhibit poor performance on ridges with shallow soils, infertile soils and on warm sunny sites where the soil is too dry. A study of a forest plantation at Kanowit, Sarawak showed a strong link to the stem abnormality resulting in poor growth once CN ratio exceeded the desirable value of 10. NC status as a pioneer tree species, grows rapidly only on sites where disturbance has resulted in a temporary "flush" of soil nutrients.

This scenario created the need for soil improvement technique on planting media used in raising seedlings at forest nurseries which will be used in field planting programs. The adverse field environment posed challenges to seedlings out from nursery to adapt, survive and grow well once planted out (Heijden et al. 1998, pp. 70&71). AMF species richness might increase the chance of including one very effective isolate that will be benefit the host tree, resulting in more efficient exploitation of soil P and to a better use of the resources available in the system.

They were obvious differences in spore counts of soil samples collected from the three sites as shown in Figure 16. Both PP and FTP sites were disturbed by displacement of soil materials and clearing of vegetation. The PP soil in Kubah N.P. has almost double the number of spore count compared to the FTP soil in Sabal F.R. and this could be due to it more matured regenerated secondary forest. The soil procured by the FN for planting media preparation was probably taken from good soil site with top soil intact. However, the spore count was extremely low as compared to the other two sites. This could be due to the prolonged storage period or the processing of soil by mechanical shredding that was done on the pile of soil sampled.

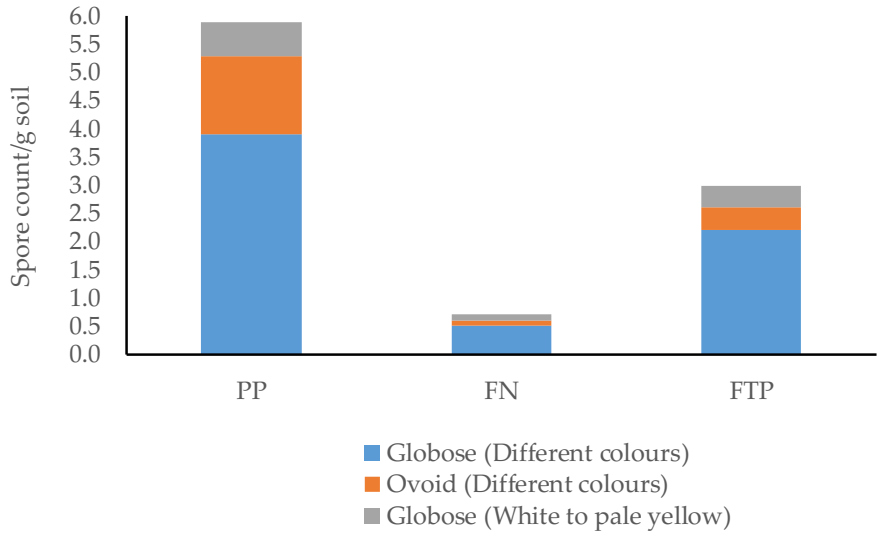


Figure 16. Spore counts of the phenology plot, forest nursery, and field trial plot soils.

Ong et al. (2012, p. 4), recorded an average of 261 spores per 20g soil isolated from rehabilitated forest, and an average of 44 spores per 20 g soil from the logged-over forest in Bintulu, Sarawak. The rounded average spore count of the FTP corresponded to the logged-over forest site as reported. The FTP area in Sabal F.R. could considered as a disturbed forest. Mechanical clearing of vegetation and movement of earth had occurred not too long ago at the area and somewhat has regenerated ecologically similar to a logged-over forest.

The rounded average count of spores from the FTP seemed to indicate the site closely mimic that of the logged-over forest in Bintulu, Sarawak as reported by Ong et al. (2012, p. 4). The FTP was considered as disturbed forest as mechanical clearing of vegetation and movement of earth had occurred not too long ago creating somewhat an environment similar to a logged-over forest. Ruiz-Lozano and Azcon (1996, p. 183), reported that spores of three *Glomus* species used to compare the effect of long term storage under different soil water conditions, had their infective capacity affected. The infective capacity of the spores decreased considerably when the substrate was completely dried. McGee et al. (1997, p. 773), also reported that the density of viable propagules of AMF over time was reduced by severe disturbance of soil substrate. They

reiterated that even the AMF that survived to 12 months formed dormant spores and were unable to initiate root colonization. Unless positive effects can be repeatedly shown in practical applications, commercial viability of inoculation programs will be uncertain (Gentili & Jumpponen 2006, p. 18).

4.5 Conclusion

The baseline study carried out indicated several important information. Firstly, the laboratory analysis on soil samples of the tree sites indicated low level of soil nutrients and further compounded by low CEC or nutrient retaining capacity. In term of fertility ranking of soils of the three sites, relatively FN in Semengoh N.R. > PP in Kubah N.P. > FTP in Sabal F.R. The response to usage of fertilizers at the planting out site was highly likely and presumably necessary for favorable tree growth. The soil in Sabal F.R. was typical of the majority of soils in many parts of Sarawak. Thus, the FTP site in Sabal F.R. was considerably not an ideal site for planting of NC if proper silviculture practices are not sufficiently carried out such as the use of bio-fertilizer.

Based on spore count of soil samples, microorganism richness at the PP site in Kubah N.P. was higher compared to the FTP in Sabal F.R. Additionally, the soil stocked for planting media preparation at the FN in Semengoh N.R. was very low in spore count.

The interest to develop and utilize bio-fertilizer has been recurring. However, more information is needed to understand role of various root-associated microorganisms in plant growth and health and make use of their would-be beneficial features as bio-fertilizers. The focus of field trial in the thesis was on utilization of SMI derived from soil trap culture with the aim to enhance the adaptability, resilient and growth of inoculated NC seedlings in the field. To complement and to shed light on the constituents and biological properties of material used several ancillary nursery trials were also carried out.

5 Soil mycorrhizal inoculum nursery and field trials

5.1 Introduction

This field trial was the mainstay of the thesis and it was prompted by the notion that AMF propagules can be sourced and culture and the effectiveness of SMI derived could be used in enhancing growth of NC. Soil from the rhizosphere of a plant hosting AMF can be used as inoculum. Such soil inoculum can be composed of soil, plant root fragments, and inherent AMF spores, sporocarps, and fragments of hyphae (Habte & Osorio 2001, p. 5).

To enrich the SMI, nurse plant (NP) are usually utilized to enhance sporulation of AMF propagules. AMF cultures may be produced by various methods, of which soil trap cultures is the simplest of procedures to produce AMF inoculums according to Walker (1999, p. 2). He suggested soil collected from the area of interest be mixed with a disinfected substrate, which could be soil, sand, or just montmorilonite clay. This mixture could be put in a suitable container and sown with either seeds or seedlings. The resulting plants are maintained for a period of usually one to six months, for the mycorrhizae to be established. As reiterated the SMI produced will be a mixed-species of AMF.

SMI produced was expected to increase root colonization of NC at seedling stage and remain after planting out. NC seedlings treated with SMI was postulated to adapt and perform better at planting out as compared non-treated seedlings. The field trial was expected to determine the applicability such soil improvement technique for forest plantations in Sarawak. Future field trials in various forest plantations in Sarawak were envisaged if the trial showed promising results. The manipulation mycorrhizal associations to increase plant productivity in plantation forestry is in need of continuous studies as reiterated by Brundrett et al. (1996, p. 3).

5.2 Aim

This trial aim to fulfil and answer two objectives and three hypotheses set in the thesis.

5.2.1 Objectives and hypotheses

The first objective was to determine potential sources of AMI propagules for use as inoculum suitable for NC and the second objective was to test the effectiveness of SMI produced from soil trap culture.

Thus, to affirm the objective of testing the effectiveness of the SMI, three hypotheses were formulated to that effect. The first hypothesis was that the growth performance of inoculated NC seedlings is better than non-inoculated NC seedlings. The second hypothesis was that the growth performance of NC seedlings with fertilizer treatment is better than NC seedlings with no fertilizer treatment, and the third hypothesis was that the inoculated NC seedlings with fertilizer application perform better than non-inoculated NC seedlings applied with the same type and amount of fertilizer.

5.3 Materials and methods

This field trial in the thesis encompassed several stages; starting from collection of started soil to be used in soil trap culture, deriving SMI from the soil trap culture, sowing of NC seeds, inoculation and raising of seedlings, and planting out seedlings to the field.

5.3.1 Production of soil mycorrhizal inoculum

SMI production was based on a simple soil trap culture method as described by Sunseed Desert Technology (2012). As reiterated, the infected plant roots and the spores and hyphae of the beneficial fungi present in the soil can colonize roots of new plants. Good starter soil can be collected from any undisturbed site with native vegetation even though it comprised of mostly tall trees. To multiply the AMF from starter soil, soil trap culture is used. Mycorrhizal dependent plants often called “bait plants” are grown.

These plants will become infected with the AMF, and in turn causing the fungal population to multiply.

Local lemongrass variety, *Cymbopogon citratus*, was used as the "bait plant" or NP to enhance the sporulation of AMF following Abdullahi, Lihan and Edward (2014, p. 118). They reported that lemongrass planted recorded the highest mean root colonization and spore counts as compared to onion. Their study indicated that AMF-plant interaction was host preference. Lemongrass planted resulted in the mass proliferation of *Glomus mosseae*, *Glomus geosporum* and *Glomus etunicatum* and thus, it was considered as more suitable NP as compared to onion for production of AMF inoculum.

5.3.1.1 Collection of starter soil

Starter soil for the soil trap culture was collected from the ground around bases of five naturally growing NC trees at the PP, 103^0 6' 48" N and 110^0 11' 51" E, located along the main entrance road to the Kubah N.P. headquarter office. Chubo et al. (2009, p. 344), reported that the rhizosphere soil of NC contained AMF with *Glomus spp.* dominating the tree species located in Niah, Sarawak. The study did also show that NC has ability to enhance the sporulation of the AMF. Such source could be root zone or rhizosphere soil of a plant hosting AMF which can be used as the ingredient for the production of inoculum (Habte & Osorio 2001, p. 5). The soil inoculum composed of soil from root zone or rhizosphere soil of a plant hosting AMF. However, soil as the source may not be a reliable inoculum ingredient unless the abundance, diversity, and activity of the indigenous AMF are known. Matured trees in its natural habitat may harbor associated AMF. As reported by Rodriguez-Morelos et al. (2014, p. 1), the diversity of AMF genera and species was found to be two times greater in matured trees of *Swietenia macrophylla* as compared to its seedlings.

Collection of starter soil carried out was using a 2" diameter Edelman Combination Auger (AMS, USA) bored to depth of 20 cm. The bore points were distributed randomly within three meters' radius at each of the tree stand. About 0.01 m^3 of soil including fine

roots were collected from each tree and brought back to the Soil Laboratory at Sarawak Forest Tree Seed Bank, Semengoh.

5.3.1.2 Preparation of starter soil

As the starter soil was wet due to rain during collection, soil collected from each tree was spread out on plastic trays and air dried overnight. The slightly moist soil then was put through 6mm plastic square mesh to remove coarse roots and rock fragments. The sieved soil from all trees were then mixed together by laying it on a tarp. Two corners of the tarp were drag over itself until the soil pile rolled to the edge of the tarp. The action and was repeated several times until the starter soil looked homogenized.

The well mixed starter soil was then put in a large plastic planter box measuring 920 (L) x 345 (W) x 275 (H) mm, to a level about 5 cm below the edge. Planter box was then place under nursery shade net with water sprinkler system.

5.3.1.3 Planting of nurse plant

Tillers from a clump of lemongrass were separated and trimmed to 30 cm length. Their roots were completely trimmed off, and the stump washed under running tap water. The lemongrass prepared for planting is as shown in Figure 76.2 (Appendix). Four healthy looking tillers were then planted in the planter box containing the tarter soil. The tillers were inserted in the soil about 5 cm deep and the soil around each tiller was made firm by applying light pressure with palm of the hand.

5.3.1.4 Upkeep of nurse plants

Fertilizer was used to nourish NP. As described by Miyasaka et al. (2003, p. 3) fertilizer should contain a low level of P as the AMF will not form association with NP. Fertilizer formulation of N P and K at the ratio of 20:5:10 was prepared by mixing proportionate amount of strait fertilizers comprising of Urea (46% N), Triple Super Phosphate (46% P) and Muriate of Potash (60% K). The formulated fertilizer, about 5 g was applied once during planting and subsequently every fortnightly or at least six times for the whole duration of the soil trap culture. The fertilizer mix was first dissolved in tap water and

applied around the NP by dripping using a pipette. The NP was water twice daily using the water sprinkler system and kept in the planter box for about 12 weeks.

5.3.1.5 Processing of soil mycorrhizal inoculum

NP were cut at soil level and discarded, about one week before processing the soil trap culture to SMI following Miyasaka et al. (2003, p. 3). Watering of the NP was also stopped simultaneously. The adverse treatment on the NP was supposed to induce the root colonizing AMF into reproducing spores(Sunseed Desert Technology 2012, p. 4).

The roots and soil in planter box were emptied and soil clumps were loosened to crumb size and NP root balls were minced using machete into small fragments. Soil and root fragments were blended together using the Tarp Method (My Square Foot Garden 2014). Two corners of the tarp were pulled over to move soil and root fragments close to one edge. The tarp was laid flat before taking the opposite two corners and pulling them to the opposite direction until all soil and root mix was running down the middle of the tarp. The action was repeated until SMI looked well mixed.

5.3.2 Assessment of soil spores

The outcome of soil trap culture was deduced by assessing the spore count. The Spore extraction method used was based on the Sucrose Extraction Method(Center for Conservation of Biology 2013). The spore segregation method described by INVAM (2017) was improvised in the thesis to enhance its efficiency.

5.3.2.1 Extraction of spores

The procedures used for extraction of spores are as described under Materials and Methods in Chapter 4.

5.3.2.2 Segregation of spores

The procedures used for segregation of spores are as described under Materials and Methods in Chapter 4.

5.3.2.3 Enumeration of spores

The procedures used for enumeration of spores are as described under Materials and Methods in Chapter 4.

5.3.3 Preparation of NC seedlings

Direct sowing of NC seeds was reported to be not very successful as the small seed size need to be protected from to dry or too wet condition and exposure to direct sunlight (Krisnawati, Kallio & Kanninen 2011, p. 4). Hence, the need to prepare seedlings adequate cares are required for the field planting. Jaenicki (1999, p. 12) indicated that they are three phases in seedling development in the nursery; establishment, production, and hardening. The establishment phase begins with seed germination and root growth. The production phase is manifested by rapid shoot growth and at the hardening phase, seedlings are gradually accustomed to field conditions.

Seed germination, preparation of potting media, and planting methods in the thesis were based on standard practices established by the FN at Sarawak Forest Tree Seed Bank, in Semengoh N.R. and also the guidelines prepared for seven nurseries previously under purview of the Forest Department of Sarawak (Phang 1991, p. 1).

5.3.3.1 Sowing of seeds

Due to the small size of NC seeds, the seeds can be mixed with fine sand at ratio of 1:10 before sowing as suggested by Krisnawati, Kallio and Kanninen (2011, p. 4). As also suggested, a salt or pepper pot can be used for sowing and the seedbeds should be protected from heavy rain and not watered too much as damping-off disease can be a problem. Consequently, seedlings were placed in well-ventilated conditions.

About 0.5 g of NC seeds registered as SFTSB (P)/01/02/0115 0005(6) with 94.5% germination rate was obtained from the Sarawak Forest Tree Seed Bank, in Semengoh F.R. Seed viability of NC influenced the field survival and growth. Budiman et al. (2015, p. 209&210), reported that the more viable seeds resulted in better seedling height and diameter growth, and higher survival of one year old seedlings after planting in the field.

Seeds were sown by gently sprinkling them on two plastic trays measuring 400 (L) x 300 (W) x 40 (H) mm. Each tray was prior filled with about 3 L of Terrahum substrate (Klasmann-Deiimann Gmbh, Germany). After seeds were sown, the germination substrate was kept moist using water mister system operated 2-3 times a day in the greenhouse. After about six weeks the plantlets from germinated seeds measuring about 1 cm tall were ready to be transferred to polybags already packed with planting media mix.

5.3.4 Inoculation of seedlings

More than 400 polybags were filled with planting media mix were prepared first while awaiting the NC plantlets to be ready for pricking. Good seedling development depends to a large part on the planting media used. Plant that develops good root system in a well-balanced substrate, will be able to adapt to the harsh environment of a field. It needs a well-developed and strong root to reduce need of additional watering and fertilizing. Good physical and chemical properties of the substrate and used of genetically superior plant to a large extent o ensure healthy root system (Jaenicki 1999, p. 30).

5.3.4.1 Preparation of planting media mix

Planting media main ingredient was the existing stock of soil at FN in Semengoh F.R. The soil before being used in the planting media mix was first processed by soil shredding machine. The planting media mix comprised of about 15 baskets of shredded soil (about 12 kg/ basket), 5 baskets of river sand (about 16 kg/ basket) and 20 kg of compost (processed oil palm fruit bunch). The ingredients were blended together with 3 kg of Controlled Release Fertilizer and instant release NPK fertilizer blend (SK COTE® PLUS 10-26-10 + 2.5 MgO + TE) and 0.5 kg of Gypsum Rock Phosphate. The blending of planting media mix was done using a motorized concrete mixer. The fertilizers used consist of a core of soluble nutrients surrounded by a polymer coating and nutrient release is controlled by the chemical composition and thickness of the polymer coating. Plant nutrients are gradually release over extended periods for three months to 18 months (Landis & Dumroese 2009, p. 5).

Perforated polybags measuring 7.5 (D) x 15 (H) cm were then filled with about 850 g of the planting media mix each. The polybags made of black polyethylene have several drainage holes at the bottom are most commonly used by forest nurseries in developing countries (Jaenicki 1999, p. 23). The polybags packed with the planting media mix were laid out on rows of 10 on gravel base under the FN shade net.

5.3.4.2 Application of soil mycorrhizal inoculum

Prior to transplanting of the NC plantlets, slightly more than 200 polybags bags packed with planting media mix were added with the SMI produced. The application of SMI to polybags is depicted in Figure 76.3 (Appendix). The remaining polybags were left untreated as they were. The amount of the inoculum suggested was on the ratio of 1:20 volume of planting media (Miyasaka et al. 2003, p. 4). The top part of planting media mix of each polybag was removed and refilled back after adding of the SMI. However, about 100 ml of SMI was used even though the volume of planting media per polybag was about 1 L, thus making the SMI usage a ratio of 1:10.

5.3.4.3 Transplanting of plantlets

Inoculated and non-inoculated planting media mix in polybag were prepared to receive the NC plantlets by making small holes using a small stick. A plantlet was gently pinched off with the sowing media to keep their root system intact and inserted to hole made in each polybag. The soil around plantlet was made firm by light pressure with the thumb and index finger.

Plantlets were watered twice daily and kept under the FN shade net (50 % shade) for about one month as they developed to seedling size.

5.3.4.4 Hardening and upkeep of seedlings

The seedlings on reaching an age about one-month old were taken out of FN shade net for hardening process by placing them on wooden slate benches in an open area. As reiterated by Jaenicki (1999, p. 16), seedlings need to get accustomed to the conditions at a planting site. Seedlings hardening process of should begin by gradually reducing

watering to once a week and simultaneously reducing shade about 4-6 weeks before planting out. Seedlings should be planted out as soon as they have reached their optimum size height of 15-30 cm depending on tree species and the condition of planting site. As suggested, some slow-growing species and strong weed competition at the planting site, much larger seedlings may be needed.

Watering continued to be carried out daily in the morning and afternoon by hosing with tap water. To keep seedlings free from pest and diseases, insecticide, SAFARI 60S (Dinotefuran 60%), 30-60 ml per 16 L water and fungicide, ANCOM THIRAM 80 (Thiram 80% w/w), 10-30 g per 16 L water were applied once a month or as necessary. N P K fertilizer with ratio of 15:15:15 were applied by slipping fertilizer granules at the side of polybag at three weeks' interval with rate of about 1 g per seedling.

Seedlings kept in the open condition were segregated for planting out to the field. Out of more than 400 seedlings prepared in the FN only 108 inoculated seedlings and 108 non-inoculated seedlings were needed for field trial planting based on the experimental design to be implemented. Krisnawati, Kallio and Kanninen (2011, p. 5), reckoned that, NC seedlings can sometimes be planted out when they are 10-15 cm tall under good care. Planting seedlings of about 1 cm in root collar diameter showed satisfactory results.

Sturdy and healthy looking trees which about two and a half months old by then were selected and transported by road from the FN in Semengoh N.R. to the FTP in Sabal F.R.

5.3.5 Assessment of seedling morphology at the forest nursery

AMF activity was determined by the presence root colonization through destructive sampling of roots. Growth of the seedlings done non-destructively by measuring plant height, root collar diameter, followed by destructive measurement of biomass accumulation (Habte & Osorio 2001, p. 16). Thirty seedlings each from the inoculated and non-inoculated batch of seedlings prepared for planting out were sampled randomly to study the effect of SMI treatment on plant biomass and the presence of mycorrhizal roots.

5.3.5.1 Seedling height and root collar diameter

Seedling height and root collar diameter measurements were taken first before seedling root balls were separated from adhering soil for assessment of spore counts and root colonization. Seedling height was measured from ground level to apical shoot using recoil measuring tape. Root collar diameter was measured using ABSOLUTE Digimatic Calipher (Mututoyo, Japan).

Seedling 'sturdiness quotient' could be used to determined seedling quality. Seedlings with small quotient are preferred as sturdy plants are expected to better chance of survival especially on marginal planting. A quotient more than six is undesirable according to Jaenicki (1999, p. 8). As reiterated, such seedlings have greater height relative to stem diameter and were considered weak and lanky. The sturdiness quotient should closely predict survival and growth in the field. Seedling sturdiness quotient was expressed by height of seedling in cm divided by root collar diameter in mm as shown in the equation below. This equation was used to study the effect of initial morphology on field performance of NC seedlings referred as White Jabon in Bogor, Indonesia (Budiman et al. 2015, p. 207).

$$\text{Sturdiness quotient} = \frac{\text{Height of seedling (cm)}}{\text{Root collar diameter (mm)}}$$

5.3.5.2 Seedling dry-biomass

Destructive sampling of seedlings to obtain dry-biomass measurement to determine such as the shoot to root ratio, a good indicator seedling quality. Jaenicki (1999, p. 8), pointed out that the shoot to root ratio is an important measure for survival seedling after planting out. As suggested, the ratio relates the transpiring area to the water absorbing area, which are basically plant shoot and plant roots usually measured by determining their dry weights. Healthy plants should have a root to shoot ratio of 1:1 to 1:2 in order to enhance the capacity of the roots to the aboveground biomass for anchorage and absorbing water and nutrients from the soil. To allow for high absorption

capacity and storage of moisture and nutrients, especially on marginal soils, seedlings with high root to shoot ratio is an added advantage. Root to shoot ratio is expressed by root dry weight divided by shoot dry weight as shown in the equation below (Budiman et al. 2015, p. 207).

$$\text{Root-shoot ratio} = \frac{\text{Root dry weight}}{\text{Shoot dry weight}}$$

Plant top, stem, branches and leaves, was cut at root collar level. Root ball was freed from soil by gentle kneading and shaking. Soil adhering to roots with was rinsed off under running tap water through 2mm sieve. Care was taken to keep root systems intact. Any detached roots caught in sieve were collected together with intact roots. Plant shoot and the wet roots were spread on cardboard boxes and left to dry at room temperature

Dry-biomass measurement of each seedling was done separately for root system and, stem and leaves for above ground plant part. The plant parts were oven-dried at 65^{o} C for three days or longer until repeat weighing at daily basis gave identical results (Bloomberg, Mason & Jarvis 2008, p. 106).

5.3.6 Assessment of seedling mycorrhizal roots

The quality of an inoculum is determined by the density of viable spores it contains and also total number of infective propagules and its effectiveness is assessed in terms of the amount and the rapidness of roots of host plant being colonized, through destructive sampling of roots (Habte & Osorio 2001, p. 16). Fungal structures in a mycorrhizal root cannot be seen without differential staining. Once root is stained and mount on slide, extent of mycorrhizal colonization can be measured (INVAM 2017).

5.3.6.1 Preparation of root staining and mounting solutions

The usage of non-vital stains of Trypan blue, cotton blue, CBE and more recently ink is which cannot distinguish between living and dead fungal tissues has been the standard

technique to visualize and quantify root colonization by AMF. For light microscopic studies, the technique that results in images of fungal structures with the highest contrast could be the most effective (Vierheilig, Schweiger & Brundrett 2005, p. 402).

Aqueous Trypan blue was used to stain roots. It was prepared to the concentration of 0.05% w/v of Trypan blue in Lactoglycerol solution (Brundrett et al. 1996, p. 181). The Lactoglycerol solution was first prepared by mixing 100 ml each of distilled water, Lactic acid and Glycerol.

Stained root segments were mounted on glass slide using a concoction of PolyVinyl-Lacto-Glycerol (PVLG) (Brundrett et al. 1996, p. 185). The mountant was prepared by mixing 16.6 g of Polyvinyl alcohol, and 100 ml each of distilled water, Lactic acid and Glycerol (INVAM 2017).

5.3.6.2 Sampling roots for staining

After washing roots from soil, wet roots were left to dry at room temperature until they were just slightly moist. Healthy looking lateral roots of about 1 mm diameter were detached from root systems and cut into 1 cm segments. Root samples were kept moist in plastic bags and refrigerated at 8^{o} C until further use (Brundrett et al. 1996, p. 174).

5.3.6.3 Clearing and staining roots

Potassium hydroxide has been the only chemical used to clear root (Brundrett et al. 1996, p. 179). The 1 cm root segments were put in a glass beaker filled with 10% w/v Potassium hydroxide with distilled water. The root segments were put in an autoclave set at 121^{o} C for 20 minutes' liquid cycle.

The cleared root segments were then rinsed by gently agitating in a glass beaker with distilled water before soaking in aqueous Trypan blue for duration of 2-3 days for staining process to take effect. Stained roots were taken out from staining solution and rinsed by gentle agitation with distilled water in glass beaker. Stained roots were finally

soak in beaker containing 50% glycerol for 2-3 days to allow the excess stain to leach out before mounting them on glass slides (Brundrett et al. 1996, p. 182).

5.3.6.4 Preparing diagnostic slides

The stained root segments soaked in glycerol were picked using forceps and placed first on paper towel to remove excess glycerol. Three root segments were arranged lengthwise on a glass slide before a small drop of PVLG mountant was added. Glass cover slip was gently placed on roots and gently tapped to flatten roots and to allow mountant to flow around freely (Brundrett et al. 1996, p. 185).

Cover slip was then fastened to the glass slide by clipping it using a cloth peg before and rinsing under running tap water to removed excess mountant and smear on cover slip. The glass slides were left to dry on a tray lined with paper towel. Root segments under cover slip were then secured using nail polish and left to dry further before they were ready for microscopic examination.

5.3.6.5 Quantifying root colonization

Microscopic examination of root segments and quantifying of root colonization was based on gridline intersect method view under compound light microscope as described by Mcgonigle et al. (1990, pp. 497&498) and Brundrett et al. (1996, pp. 185&186).

A hairline lens was inserted in one of the eyepieces of a compound light microscope LEICA ICC50W (Leica Microsystems, Switzerland) to act as line of intersection with the root segments being viewed. Slide was positioned at one end of root segments and the stage of the microscope was move to the opposite end of the root segments at every 2 mm intervals. At each root intersect, fungal structures of interest observed were recorded. According to Souza (2015, p. 2), the AMF morphological structures arbuscules, vesicles, and hyphae formed inside plant root, have huge surface areas of contact between plant root cells with AMF structures, and AMF structures with resources in soil.

The fungal structures were noted in preferential order of arbuscule if arbuscules were present, vesicle if vesicles and hyphae were present, and hyphae if only hyphae were present. If no mycorrhizal structures were present, the intersect line was noted as non-present (Comas, Carlisle & Patterson 2011). Colonization of root was calculated as percentage of intersection that have AMF structures present out of total root intersects as shown by the equation below.

$$\text{Percentage root colonization} = \frac{\text{Arbuscules + Vesicles + Hyphae}}{\text{Total root intersections}} \times 100$$

AMF hyphae were presumed to be those seen within the root, are aseptate, typically thicker and have uneven and bumpy edges. Hyphal coils in root cells were also be marked as asbuscules. Relatively small vesicles and occurring more than one to a cell was discounted as non-mycorrhizal vesicles (Comas, Carlisle & Patterson 2011). Souza (2015, p. 2), added that AMF usually have the mycelium absent of septum, but occasionally could be found in senescent mycelium parts, especially in the genus *Diversispora, Gigaspora, Racocetra* and *Scuttelospora.* Kheyrodin (2014, p. 42), pointed out that they are two types extra-radical hyphae morphologies. Larger and thicker hyphae are called runner hyphae, and the thinner walled, very fine, ephemeral hyphae that fan out into nutrient rich micro-sites in the soil are called the absorptive hyphae. The absorptive hyphae are decomposed in days or weeks while runner hyphae may remain for years

5.3.6.6 Taking photographs of root fungal structures

Acquisition, analysis and processing of digital images were done using computer software LAS EZ (Leica Application Suite) Version 3.3.0 (Leica Microsystems, Switzerland). The software allows the use Leica microscopes and cameras on a Windows OS computer.

Essential to all work with AMF associations, microscopic methods enable observe the presence and identification key fungal morphological criteria in plant roots, especially arbuscules (Vierheilig, Schweiger & Brundrett 2005, p.402). Therefore, microscopic method adapted in the specific study permit an easy detection of anatomical features that are peculiar to AMF.

5.3.7 Field trial plot

5.3.7.1 Trial design

Field trial planting plan was based on Randomized Complete Block Design (Chang 1972, pp. 33-37) with four treatments of nine replication each, distributed in three blocks. Random Number Tables were used as a guide in randomization of the replicates. The four treatments of the field trial were as follows:

1. Seedlings treated with soil mycorrhizal inoculum (SMI)
2. Seedlings treated with soil mycorrhizal inoculum and fertilizer (SMI+Fertilizer)
3. Seedlings treated fertilizer only (Fertilizer)
4. Seedling without treatments (Control)

The field trial planting plan is as shown in Figure 17. Each replication was represented by nine trees. Trees were planted 3 x 3 m apart and the buffer zone between replications was 1 m wide. Trees were numbered in ascending order starting from top left corner to the right in Block I and in zigzag manner until Block III. Based on the topography of the site, Block I, II and III, were roughly categorized as Upper Slope, Middle Slope and Lower Slope respectively. The FTP is as shown in Figure 76.13 (Appendix).

Chang (1972, p. 17), reiterated that the purpose of grouping trial units in blocks is to ensure that they are uniform as possible so that the observed differences among treatments are due to the actual differences between treatments. One of the greatest sources of error in field experiments is due to soil heterogeneity. Variability in plot yield was decreased as the plot size increases up to about 0.1 acre (0.04ha), but for plots larger

than 0.1 acre the decrease in variability was very small (Chang 1972, p. 19). The sub-plot of each treatment at the FTP was about 0.45 ha.

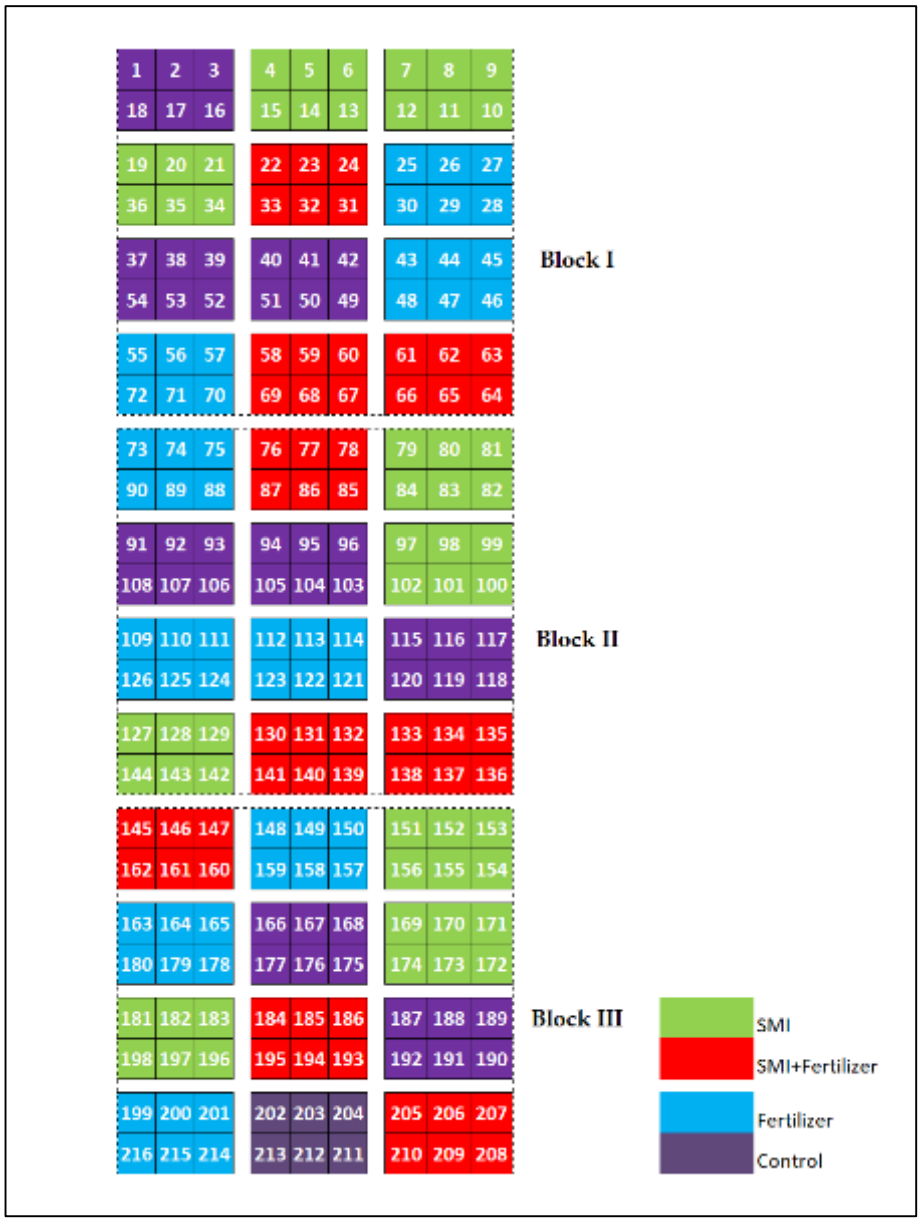


Figure 17. Position of blocks, treatments, and replications of soil mycorrhizal inoculum field trial.

5.3.8 Field planting

5.3.8.1 Preparation of planting site

The FTP was established in Sabal F.R. after soil and topography survey were carried out of the site selected to determine size and alignment of the plot. The selected site was then demarcated on the ground based on layout of the experimental design.

Undergrowth was slashed first, followed by felling of trees and trimming of branches. No burning of plant debris was carried out as all tree stems and branches were cut to short sections and left to decompose and also to provide thick ground cover. The mulch on the ground resulted in the FTP weed free for at least two months after which the decomposition of woody material set in. The preparation of site for the field trial is illustrated in Figure 76.4 (Appendix).

Alignment of planting lines was carried out in the east-west and north-south direction. Plant debris obstructing planting lines was moved to either side of planting lines. The planting distance adopted in the study was 3 x 3 m with 1 m buffer between replicates. Krisnawati, Kallio and Kanninen (2011, p. 5), reported that the planting distance of NC in the field was usually around 3-4 x 3-4 m though spacing of 3 x 2 m was also used. A wider spacing of 4-5 x 4-5 m was usually being practiced by smallholders in South Kalimantan, Indonesia.

5.3.8.2 Preparation planting holes and planting

Digging of planting holes was done a day prior to planting. Cubical holes were dug out using a hoe that could easily accommodate the root ball of NC seedlings. Polybag was first removed from root ball before putting seedling in hole dug. Backfilled soil was placed firmly around seedling by gently firming the soil around with back of the hoe or just by foot stamping. Watering was done after all the seedlings have been planted and watered again for the second time the following day.

5.3.8.3 Application of fertilizer

Gofcote N P K nugget slow release fertilizer 10-26-10+3MgO+0.2B (Xi'an Longer Business, China), imported and rebranded as SK 10, 10:26:10+4MgO+TE (Kimia Utama, East Malaysia, 6A, Lorong Sukun 28, Upper Lanang, 96007 Sibu, Sarawak, Malaysia) was used in the field trial. The SK 10 nugget fertilizer has been used extensively by the FN in Semengoh N.R. in its tree planting activities in the past. The 20 g spherical shape nugget fertilizer was specially formulated for Sarawak soil namely for *Acacia mangium* planting. It has a longevity of six months. Suggested application rate for field planting was two nuggets per planting hole (Kimia Utama, East Malaysia, 6A, Lorong Sukun 28, Upper Lanang, 96007 Sibu, Sarawak, Malaysia).

Fertilizer was applied once during planting. Four shallow holes were dug around and about 10 cm away from seedling root collar. Four SK 10 fertilizer nuggets were buried in the holes (Figure 76.7 Appendix). The fertilizer rate per seedling calculated was based on the average weight of the nugget fertilizer, and its P content equivalent to the amount of P from 50 g of Triple Superphosphate fertilizer (45% K). The use of the strait fertilizer 50 g Triple Superphosphate was observed to enhance early growth of trees in several forest plantation trials in Sabah (Mitchell 2015). The response NC seedlings to fertilizer treatment was highly expected as available and reserved soil nutrients of planting site was low as majority of soils in Sarawak are seriously leached and low in nutrients and nutrient-retaining capacity. However, their physical properties are generally quite good (Maas, Tie & Lim 1986, p. 16).

5.3.8.4 Up-keeping of trees

Grasses and shrubs started to emerge and flourish two months after planting. Circle weeding around bases of trees and blanket slashing between tree rows were carried out every quarterly with machete and motorized backpack grass cutter. The weeding work was necessary as the emerging weeds were outgrowing the planted trees. The easiest way to control weeds is by using weedicides. However, Druille et al. (2013, p. 99), reported that AMF root colonization found in plants grown in soils treated with high

dosage of herbicide decreased as compared to untreated ones. The herbicide active ingredient glyphosate remains in the soil and products its degradation affected AMF spore viability and their ability to colonize roots.

The main tree disease observed in the FTP was stem canker (Figure 76.11 Appendix). About 40% of the trees affected were observed to be at different stages of stem canker development such signs of blotches and bruises that eventually developed to crack and open wound on stem. The stem canker might have caused death of at least two the trees planted due to secondary infection. No control measure was taken as it seemed that nothing could be done about it. According to Browne (1968, p. 829), stem canker pathogen could be *Gleosporoium anthocephali* that causes lesions on the leaves, petioles, fruits, and stems, particularly on seedlings. Trees densely planted, and are under stress due to insect pest attack will be more susceptible to attack by canker fungi. Open wound was the entry point for the pathogenic fungus. These could lead to canker and dead of the infected trees as the fungal mycelia diffused into the inner bark and killing the cambial tissue and providing avenues for further infection by wood decaying fungi (Annya Ambrose & Empenit Empawie 2008, p. 18).

5.3.9 Assessment of trees at field trial plot

Tree assessment was the most important data collection for this field trial. The field trial plot is as shown in Figure 76.13 (Appendix). The assessment carried out were mainly measurement of tree height, root collar diameter, DBH and leaf nutrient concentration. The final assessment involved, destructive sampling of selected trees to measure tree dry-biomass and also to observe the presence of tree mycorrhizal roots.

5.3.9.1 Tree height and root collar diameter

Tree height and root collar diameter measurements were taken immediately after planting. Monthly measurement was done consecutively. Tree height measurement was taken from ground level to apical shoot using recoil measuring tape and an improvised wooden measuring stick especially needed for tall trees (Figure 76.9 Appendix). Root collar diameter was measured using ABSOLUTE Digimatic Caliper (Mututoyo, Japan).

Due to oversized root collar diameter of some trees at 12 months old, additional measurement of stem diameter of every tree was taken 5 cm above ground level (Figure 76.8 Appendix). Stem DBH measurement was also taken but was confined to trees that have grown to height of > 1.3 m.

5.3.9.2 Leaf nutrient concentration

Composited leaf samples were taken separately for each treatment by nipping eight spots with size of about 2.25 cm^2 from the edges of newly expanded young leaves of each tree. Leaf sampling was carried immediately after planting and consecutively at every three months' interval. The oven dried weight of each composited leaf sample were 5-10 g and it was just sufficient for carrying out leaf nutrient analysis. One whole newly expanded young leaf was taken from each tree at the final leaf sampling (Figure 76.12 Appendix).

Leaf samples were oven dried at 105^{o} C overnight, packed in zip-lock plastic bag and kept in a desiccator awaiting dispatch to a private laboratory (i-TESTCHEM LABORATORY SERVICES, 1st Floor, Sublot 6, Contempo Commercial Centre 94300 Kota Samarahan, Sarawak, Malaysia). Leaf samples were cut to very fine pieces using scissor for the chemical analysis. Parameters and methods used to determine leaf nutrient concentration are as shown in Table 17.

Table 17. Leaf nutrient laboratory analytical methods.

Leaf nutrients	**Analytical methods**
N	MS 677. Part III: 1990-Clause 2
P, K, Ca, and Mg	MS 677: Part IV, V, VI, VII: 1980
B	In-house Method ITC/TM/P12 based on MS 417: Part 7: 2001_Clause 6.2.3
Cu, Zn, Fe and Mn	In-house Method ITC/TM/P08, P09, P10, P11 based on MS 677: Part II: 1980-ASS

5.3.9.3 Tree dry-biomass

Trees were also be sampled for above and below ground dry-biomass. Sampling was confined to 10 trees each from the SMI and Control treatments. Trees were selected from the range of the tallest to smallest. Uprooting of trees was done using a hoe to loosen soil before slowly yanking root system from the ground. Trees were cut into short sections for easy transportation back to the laboratory.

Soil in root ball was separated by kneading and gentle shaking. Soil clumps adhering to roots with soil was rinsed off under running tap water through 2mm sieve. Care was taken to keep root systems intact. Any detached roots caught in sieve were collected together with intact roots. After rinsing for the second time, plant tops and the wet roots were left to dry at room temperature, on boxes made from cardboard.

Dry-biomass measurement of each tree was done separately for whole root system and, stem, leaves and shoot for above ground part of tree. Samples were oven dried at 65^{o} C for 3 days and beyond until repeat weighing at daily basis gave identical results (Bloomberg, Mason & Jarvis 2008, p. 106).

5.3.10 Assessment of tree mycorrizal roots

After washing away soil, wet roots were left to dry at room temperature until slightly moist. Before whole root system was oven dried to determine its dry-biomass, healthy looking lateral roots of about 1 mm diameter were selected to be cleared and stained by detaching from root systems and cut into 1 cm segments.

5.3.10.1 Clearing and staining roots

Clearing and staining procedure of tree root segments was similar to the procedure used on seedling roots. Roots were cleared using Potassium hydroxide and autoclaving. Aqueous Trypan blue was used to stain root segments followed by leaching of excess stain by soaking in glycerol.

5.3.10.2 Preparing diagnostic slides

Preparation of diagnostic slides was also similar to procedures for seedlings stained roots. Cloth peg was also used to fasten cover slip on roots mounted on glass slides before securing with nail polish.

5.3.10.3 Quantifying root colonization

Similarly, quantifying of tree root colonization was based on gridline intersect method using compound light microscope as described by Mcgonigle et al. (1990, pp. 497&498) and Brundrett et al. (1996, pp. 185&186). A hairline lens was inserted in one of the eyepieces of the Leica DM750 (Leica Microsystems, Switzerland) compound light microscope that provided line of intersection with the roots. At each root interception along intersect line, the fungal structures in roots was noted if present.

5.3.10.4 Taking photographs of root fungal structures

Acquisition, analysis and processing of digital images were done using computer software LAS EZ (Leica Application Suite) Version 3.3.0 (Leica Microsystems, Switzerland) and Leica DM750 compound light microscope and camera LEICA ICC50W (Leica Microsystems, Switzerland).

5.4 Results

The results of the study characterize the two stages of NC growth namely seedling stage at the FN and tree stage at the FTP. The spore counts of SMI produced and existing soil from the FN in Semengoh N.R. used for planting media mix preparation was also compared.

5.4.1 Assessment spores of soil mycorrhizal inoculum and forest nursery soil

There was apparent difference in spore count of SMI produced and spore count of soil at the FN in Semengoh N.R. as shown in Figure 18. The SMI has about 10 times more spores than the FN soil. As discussed in Chapter 4, the soil procured by the FN for plating media preparation was probably taken from good soil site with intact top soil.

However, the spore count was extremely low. This could be due to the prolonged storage period of the soil procured. In terms of spore count of the different spore morphotypes, globose shape of different colors > Ovoid shape of different colors > Globose shape with white to pale yellow colors.

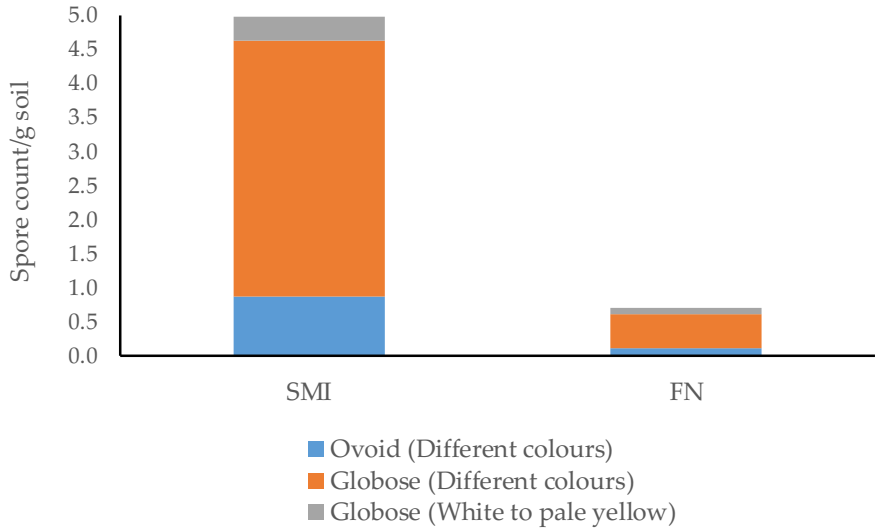


Figure 18. Spore counts of soil mycorrhizal inoculum and forest nursery soil.

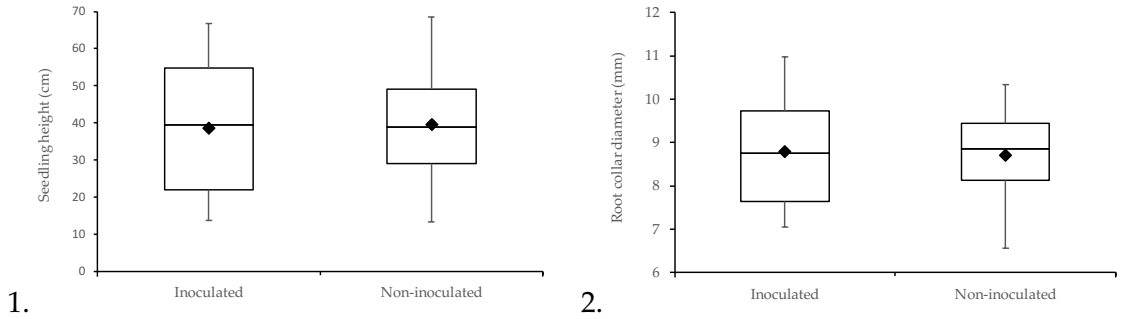
5.4.2 Assessment of seedlings at the forest nursery

Seedling height, stem diameter and dry-biomass measurements were used to estimate seedlings growth performance.

5.4.2.1 Seedling height and root collar diameter

The maximum, minimum, mean, median, upper and lower percentile of seedling height and root collar diameter are as shown in Figure 19. The mean height and root collar diameter indicated that the growth performance of inoculated seedlings was about the same as non-inoculated seedlings. The mean heights and root collar diameters were 38 cm, 39 cm, and 8.8 mm and 8.7 mm respectively.

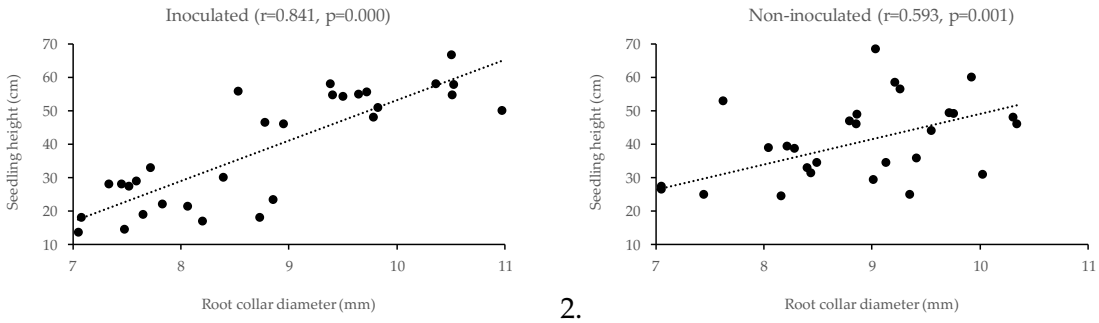
Though growth patterns were almost similar for both inoculated and non-inoculated seedlings, the upper and lower percentile results indicated that non-inoculated seedlings were more uniformed or less variation in size as compared to the inoculated seedlings.



1. 2.

Figure 19. The maximum, minimum, mean, median, upper and lower percentile of inoculated and non-inoculated seedlings. 1: Seedling height, and 2: Root collar diameter.

The correlations between root collar diameter and seedling height of inoculated and non-inoculated seedlings are as shown in Figure 20. With Pearson correlation of 0.841, the inoculated seedling heights were positively and significantly highly correlated with root collar diameter as compared to the non-inoculated seedlings. This indicates that the above ground morphology of inoculated seedlings was more symmetrical as compared to the non-inoculated seedlings.



1. 2.

Figure 20. Scatter plot of seedling height and root collar diameter. 1: Inoculated, and 2: Non-inoculated.

The sturdiness quotients based on mean height in cm and root collar diameter in mm of inoculated seedlings and non-inoculated seedlings are as shown in Table 18. A small quotient indicates a sturdy plant with a higher expected chance of survival, especially if the planting site is not conducive for non-hardy plants (Jaenicki 1999, p. 8). Inoculated seedlings seems to be slightly sturdier than non-inoculated seedlings

.

Table 18. Sturdiness quotients of inoculated and non-inoculated seedlings

Treatment	Mean height (cm)	Mean root collar diameter (mm)	Sturdiness quotient
Inoculated	38	8.8	4.32
Non-inoculated	39	8.7	4.48

5.4.2.2 Seedling dry-biomass

The maximum, minimum, mean, median, upper and lower percentile of root and shoot dry-biomass of inoculated and non-inoculated seedlings sampled from the seedling batch for planting out are as shown in Figure 21.

The seedlings with and without SMI treatment were generally of the same size based on means of shoot and root dry-biomass. However, root system of inoculated seedlings was more uniformed as compared to non-inoculated seedlings. The shoot dry-biomass of inoculated and non-inoculated trees indicated that the seedlings above ground morphology were about the same. The mean root and shoot dry-biomass of inoculated and non-inoculated seedlings were 6.5 g, 8.5 g and 6.4 g, 8.4 g respectively.

The correlation between shoot dry-biomass and root dry-biomass are as shown in Figure 22. The correlation was low for inoculated seedlings as compared non-inoculated seedlings. The Pearson correlation were 0.387 and 0.659 respectively. However, the correlation of shoot and root dry-biomass was significantly positive. Thus, the root to

above-ground allometry of non-inoculated seedlings was considered relatively more proportionate as compared to the inoculated seedlings.

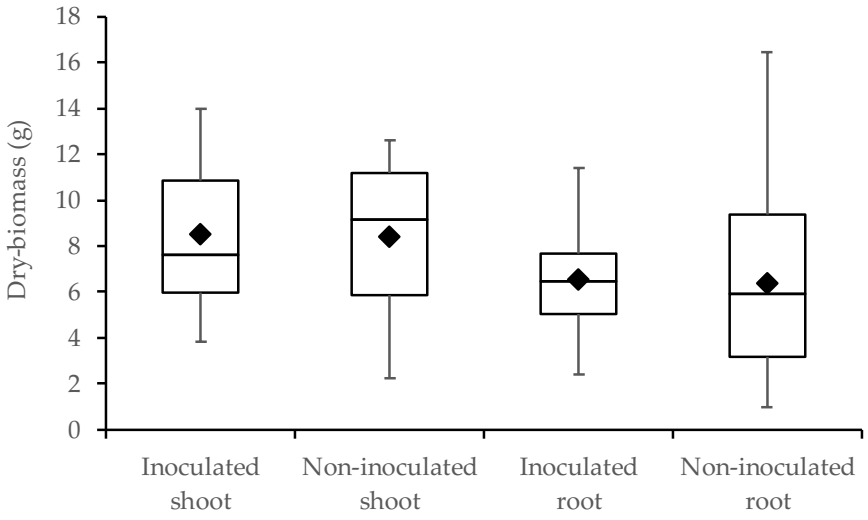
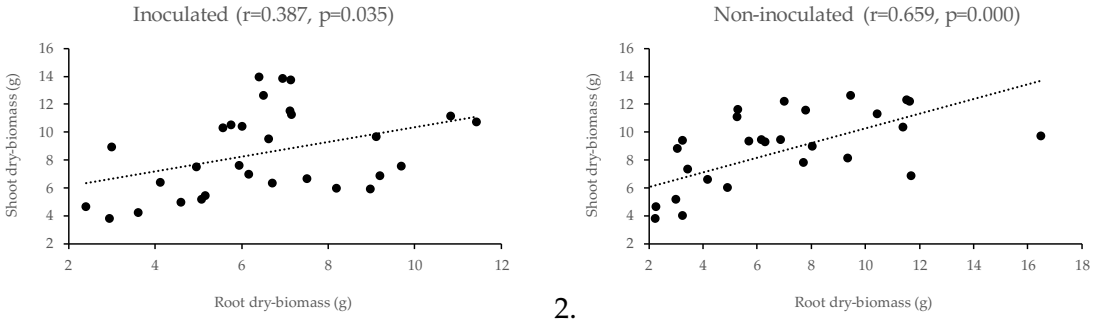


Figure 21. The maximum, minimum, mean, median, upper and lower percentile of shoot and root dry-biomass of inoculated and non-inoculated seedlings.



1. 2.

Figure 22. Scatter plot of shoot and root dry-biomass. 1: Inoculated seedlings, and 2: Non-inoculated seedlings.

The root-shoot ratios of inoculated seedlings and non-inoculated seedlings based on means of dry-biomass is as shown in Table 19. The inoculated and non-inoculated seedlings showed similar root-shoot ratios.

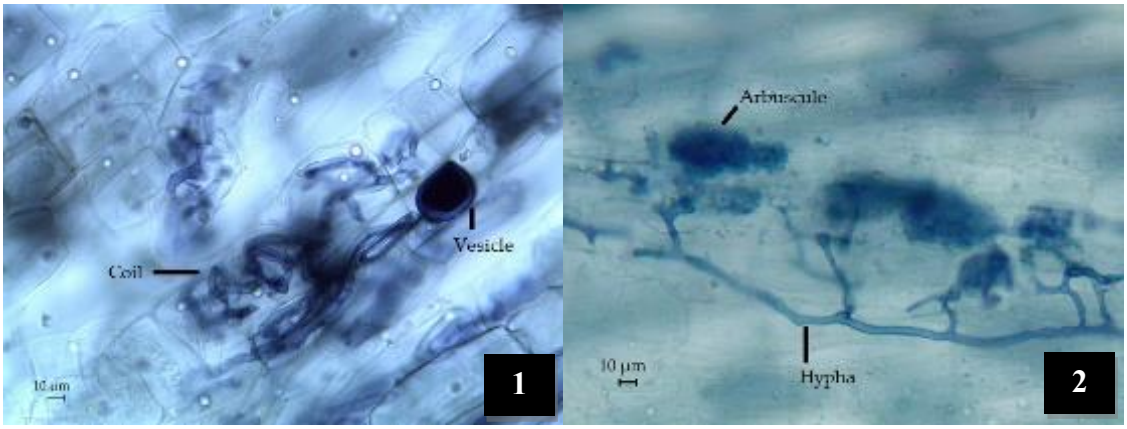
Table 19. Root-shoot ratios of inoculated and non-inoculated seedlings.

Treatment	Mean of root dry-biomass (g)	Mean of shoot dry-biomass (g)	Root-shoot ratio
Inoculated	6.5	8.5	0.76
Non-inoculated	6.4	8.4	0.76

5.4.2.3 Seedling mycorrhizal root

Some fungal structures of stained roots on glass slides prepared were clearly observed under compound light microscope. The identification and assessment of the AMF colonization, were rather fuzzy at times. No presence was recorded when identification of AMF hypha structures was thought doubtful. Observation was done as objective as possible by focusing on fungal structures of interest in order of their clarity; vesicles > arbuscules > hyphae. Quite often small vesicles and usually clumped-up more than one to a cell were observed and they were considered as non-mycorrhizal (Figure 23).

Maximum, mean, median and upper and percentile of percentages of seedling root colonization are as shown in Figure 24. The rate of root colonization was dismaying very low especially the inoculated seedlings. The difference in mean percentage root colonization between inoculated and non-inoculated seedlings was very small. Mean percentage root colonization were 10% and 8% respectively.



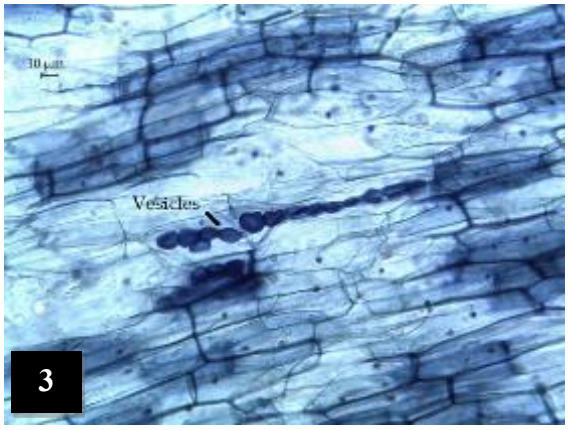


Figure 23. Fungal structures in roots. 1: arbuscules and vesicules, 2: arbuscules and hyphae, and 3: non-mycorrhizal vesicles.

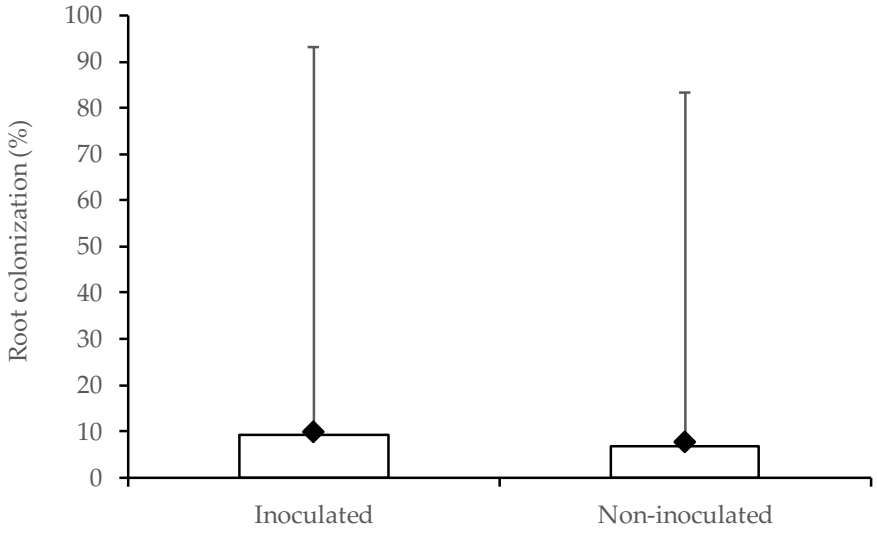
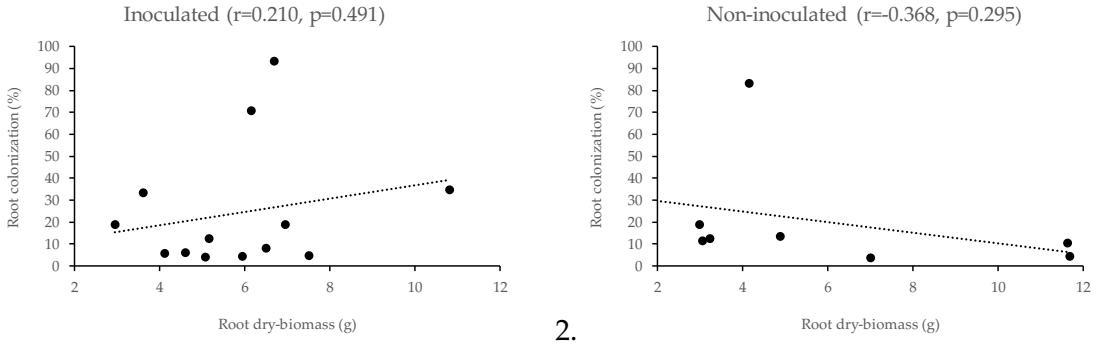


Figure 24. Maximum, mean, median and upper percentile of percentage root colonization of inoculated and non-inoculated seedlings.

Correlation between root colonization with root dry-biomass seedlings are shown in Figure 25. There was no clear correlation with the plant morphology as data for root colonization were clustered to < 10%. A very weak and rather insignificant positive correlation was seen between percentage of root colonization and root dry-biomass of inoculated seedlings. As discussed earlier, root dry-biomass of inoculated seedlings were more uniformed as compared to non-inoculated seedlings. The use of SMI probably have some influence on root development of seedlings at the nursery stage.

Seedlings with poor root system development may not be able to fare well when planted out to the field.



1. 2.

Figure 25. Scatter plot of percentage root colonization and root dry-biomass. 1: Inoculated seedlings, and 2: Non-inoculated seedlings.

5.4.3 Assessment of trees at the field trial plot

Several tree parameters were measured at 0 month until 12 months which was the duration of the study proper at the FTP in Sabal F.R. The main parameters were tree height and root collar diameter especially the measurement taken of 12 months old trees as they will reflect the adaptability of seedlings planted to the site. Good adaptation to the site will ensure their survival and steady growth within and beyond the first year of growth.

5.4.3.1 Tree height

Tree height was the most crucial parameter in determining growth performance of NC. As a pioneer tree species it need to outgrow competitors as forest gaps closes in. Fast growing individual exhibiting long internodes, will shoot high and not be suppressed by competitors such bushes, shrubs and other secondary forest tree species. Observation in some forest plantations usually showed that NC trees remained stunted by exhibiting short internodes and leading to high mortality rate.

The maximum, minimum, median, mean, upper and lower percentiles of tree height of the one-year-old trees are as shown in Figure 26. The graph depicted that the growth performance of trees was in the order of Fertilizer > SMI+ Fertilizer > SMI > Control. The

use of SMI was not effective as compared to the use of fertilizer to enhance growth performance in terms of tree height. The only noticeable effect of the application of SMI is that the tree height was more uniformed as compared to the non-inoculated trees. Apparently, the use of SMI with fertilizer also resulted in slightly shorter trees as compared to fertilizer treatment alone.

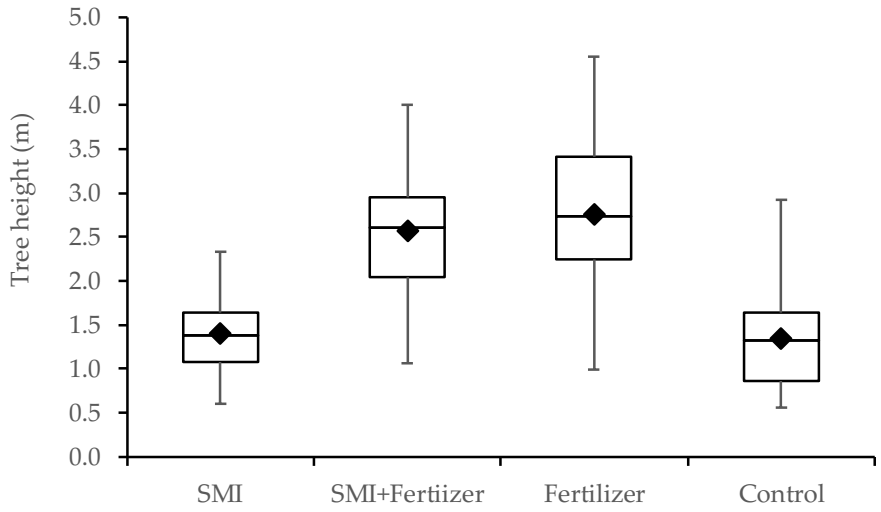


Figure 26. The maximum, minimum, median, mean, upper and lower percentiles of tree heights at 12 months.

The Least Significant Difference test (p=0.05) was carried out on mean tree height as shown in Figure 27, as the Single Factor ANOVA analysis using Excel (Microsoft Office Professional Plus 2016) indicated P value < 0.01. The means of tree height of the four treatments; SMI, SMI+Fertilizer, Fertilizer and Control were, 1.39 m, 2.66 m, 2.84 m and 1.38 m respectively. There was no significant difference between mean height of inoculated seedlings and non-inoculated trees. However, there was significant difference between mean height of trees with and without fertilizer.

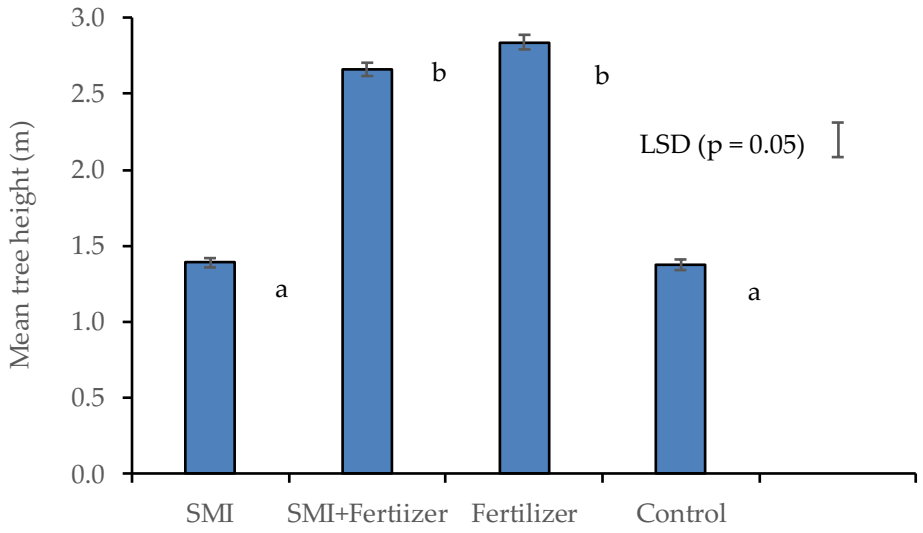


Figure 27. Least Significant Difference test on mean tree height with one Standard Error bar (different letters indicate significant difference).

The postulated growth trend of the inoculated tree with fertilizer after six months was expected to exceed the non-inoculated seedlings. However, no pattern was shown by the inoculated seedling exceeding growth of non-inoculated trees with fertilizer from six months onwards as shown in Figure 28. On the contrary, non-inoculated trees fared much better than inoculated trees even at the age of three months (October). The mean tree heights at age three months were 1.13 m and 1.23 cm respectively.

As for inoculated seedling without fertilizer and control treatment trees, the tree height was different at the date of planting. However, the growth differences tapered from eighth month onwards. The original seedling heights were 39 cm and 46 cm respectively.

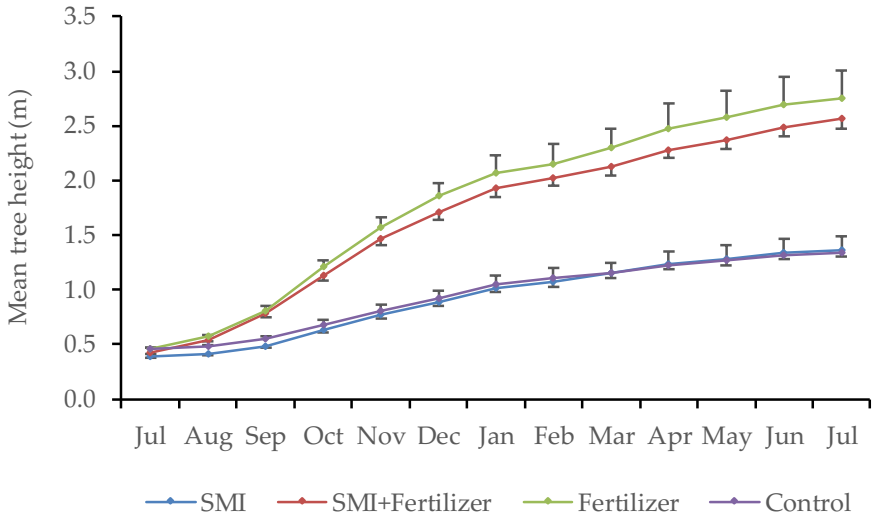


Figure 28. Monthly means of tree heights with one Standard Error bars.

5.4.3.2 Tree root collar diameter

The maximum, minimum, median, mean, upper and lower percentiles of tree root collar diameter at 5 cm above ground level of 12 months' trees are as shown in Figure 29. The graph depicted that the growth performance in terms of root collar diameter of trees were in the order of Fertilizer > SMI+ Fertilizer > SMI = Control. The means of root collar diameter were 5.4 cm, 5.3 cm, 2.4 cm, and 2.4 cm respectively. Similarly, the use of SMI was not effective as compared to fertilizer usage in enhancing tree growth. The use of SMI with fertilizer on the contrary also resulted in slightly smaller root collar diameters.

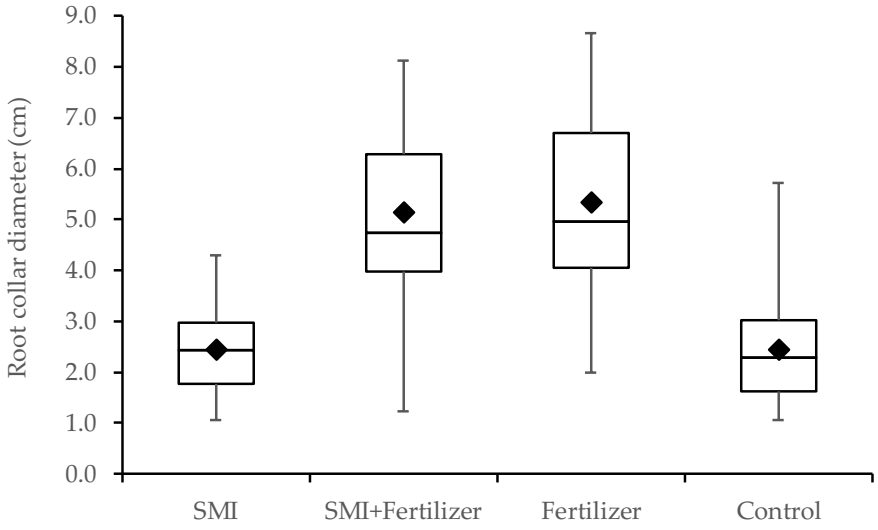


Figure 29. The maximum, minimum, median, mean, upper and lower percentiles of tree root collar diameters - 5 cm above ground level at 12 months.

The Least Significant Difference test (p=0.05) was also carried out on means of tree root collar diameter as shown in Figure 30, as the Single Factor ANOVA analysis using Excel (Microsoft Office Professional Plus 2016) indicated P value < 0.01. The means of root collar diameter of the four treatments - SMI, SMI+Fertilizer, Fertilizer and Control were, 2.4 cm, 5.1 cm, 5.3 cm and 2.4 cm respectively. There was no significant difference between mean tree root collar diameter of inoculated seedlings and non-inoculated trees. Similarly, there was no significant difference of mean root collar diameter between inoculated tree with fertilizer and non-inoculated trees with fertilizer. However, there was significant difference between mean root collar diameter of trees with fertilizer and without fertilizer.

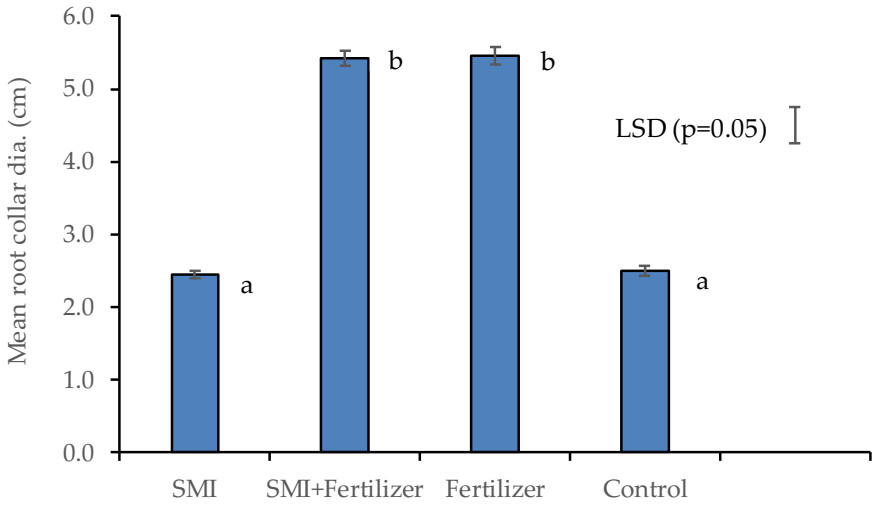


Figure 30. Least Significant Difference test on means of root collar diameter - 5 cm above ground level with one Standard Error bar (different letters indicate significant difference).

5.4.3.3 Tree diameter at breast height

The maximum, minimum, median, mean, upper and lower percentiles of tree DBH, taken at 1.3 m above ground level is as shown in Figure 31. The percentage number of tree achieving 1.3 m height or DBH measurement for the four treatments; SMI, SMI+Fertilizer, Fertilizer, and Control were 31%, 96%, 93%, 44% respectively. The number of DBH measurable trees were less than 50% for trees without fertilizer as compared to 100% (including dead trees) for trees with fertilizer. The mean DBH were 1.6 cm, 3.1 cm, 3.2 cm and 1.6 cm respectively for the four treatments. Similarly, there was no difference between mean DBH of inoculated trees and non-inoculated trees. DBH of trees with fertilizer was double than those without fertilizer. There was just a slight difference in mean DBH between inoculated trees with fertilizer and non-inoculated trees with fertilizer.

The monthly mean root collar diameters of trees are as shown in Figure 32. Similarly, root collar diameter inoculated trees did not exceed that of non-inoculated trees. In fact, mean root collar diameter of both inoculated and non-inoculated trees without fertilizer (Control) were almost the same since the date of planting. Non-inoculated trees with

fertilizer seemed to fare much better than the inoculated trees from the age of nine months (April). The mean root collar diameters were 5.5 cm and 5.2 respectively.

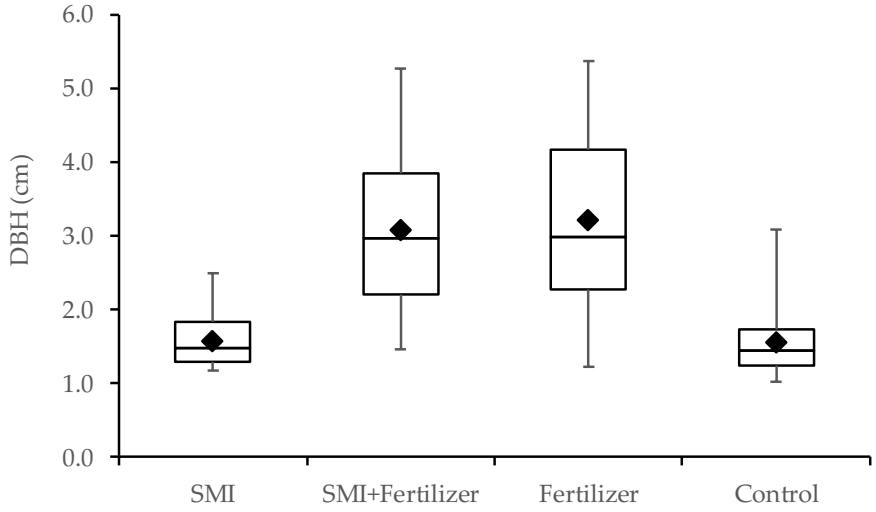


Figure 31. The maximum, minimum, median, mean, upper and lower percentiles of tree diameter at breast height, at 12 months.

.

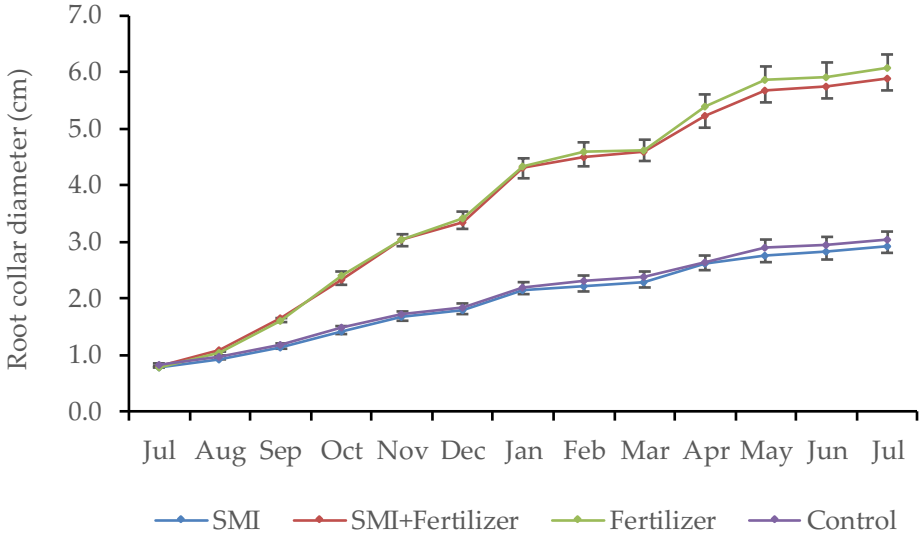


Figure 32. Monthly means of root collar diameters with one Standard Error bars.

Besides the weed problem, about 40% of the planted trees were severely attack by a defoliating insect pest (Figure 76.10 Appendix). Symptoms of defoliation indicated that

it was caused namely by the larva stage of the butterfly *Margaronia* that was reported elsewhere also by Norhayati Ahmad Sajali et al. (2010, p. 14). The spraying of pesticide, 20 ml emulsifiable concentrate of 84% w/w Malathion (Malathion 84 EC, Halex) mixed with 16 L of water, on affected trees was found to be effective in stopping further damage. However, the attack recurred few months after the pesticide spraying.

5.4.3.4 Tree branches and internode length

The maximum, minimum, median, mean, upper and lower percentiles of the number of branches exhibited by trees with the different treatments are as shown in Figure 33. At 12 months of age the trees produced about 30 branches. Great majority of trees in the Tropics branch sooner or later and can be divided into three growth types; firstly, branches of equal status, secondly branches of different orders, and thirdly the main stem consists of a succession of sympodially growing axes with terminal parts curve over and form branches. The crown shape of mature trees depend partly on which of these categories they belong to, and their adult characteristics and their growing environments (Echereme, Mbaekwe & Ekwealor 2015, pp. 3&4). In the monopodial juveniles of giants the lateral branches are short-lived and eventually fall off at maturity, and permanent lateral limbs developed (Whitmore 1990, pp. 47&48). The NC juvenile tree has a monopodial crown structure. At maturity the crown metamorphosed into a sympodial structure.

The maximum, minimum, median, mean, upper and lower percentiles of the internode length of tree stem of the different treatments are shown in Figure 33. The order of tree internode length of the FTP was; non-inoculated trees > inoculated trees, either with or without fertilizer. A study of a NC forest plantation on steep hill country at Kanowit, Sarawak showed a strong link between stem malformation in the form of stunted apical shoots, shortening of internodes and soil CN ratio in plantation. Percentage stem abnormality increases rapidly when the CN ratio exceeded the desirable value of 10 (Bloomberg & John Sabang 2009, p. 57). The internode length of inoculated trees was shorter as compared to non-inoculated trees. Though the SMI treatment seemed to have adversely affected tree height it did not however affect the growth of branches in the

inoculated trees. King et al. (1997, p. 627), reiterated that the spacing of branches along central stems was related to growth rate and light level in forest saplings and trees in tropical moist forest in Panama. All species showed increases in branch spacing with increasing light and growth rate of diameter.

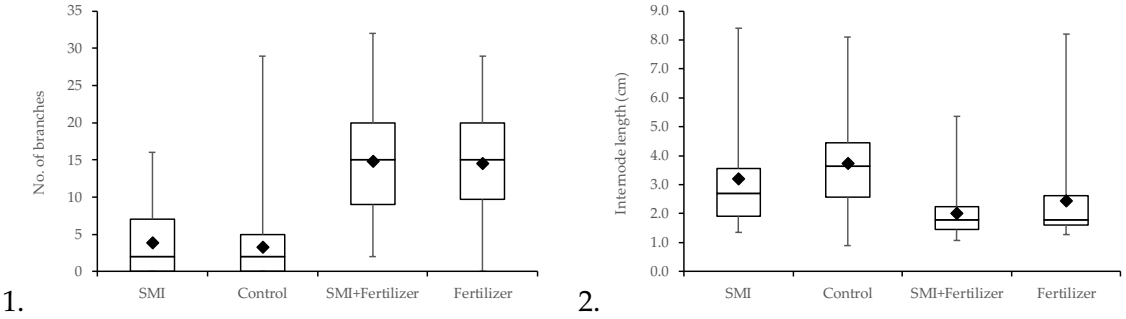


Figure 33. The maximum, minimum, median, mean, upper and lower percentiles of trees at 12 months. 1: Number of branches, and 2: Tree internode length.

5.4.3.5 Mean monthly increment of tree height

Mean Monthly Increment (MMI) of tree height was derived by subtracting mean tree height of a particular month with the mean tree height of the previous month. The array of MMI of the four treatments are as shown in Figure 34. Two moving average trend line was incorporated into the graph to illustrate clearly the monthly rate of tree growth.

The trend line showed that rapid growth was shown by trees following fertilizer application for three months (August-October). Growth slowed down for the next three months (November-January), and growth seemed to plateau from the seventh month onwards. The trend of slowing growth was expected as the fertilizer used has the efficacy period of six months. As discussed earlier the use of SMI was not able to propel the efficacy the fertilizer used.

The trend line for trees without fertilizer treatment was similar to trees with fertilizer except that the decline in growth was much gentler as compared to sharp drop of treatments with fertilizer. MMI height of tree with SMI treatment were slightly higher as compared to non-treatment (Control) except for month of February and May.

However, the small edge in MMI of tree height shown by the inoculated tree (SMI) was pale in comparison to trees with fertilizer treatment.

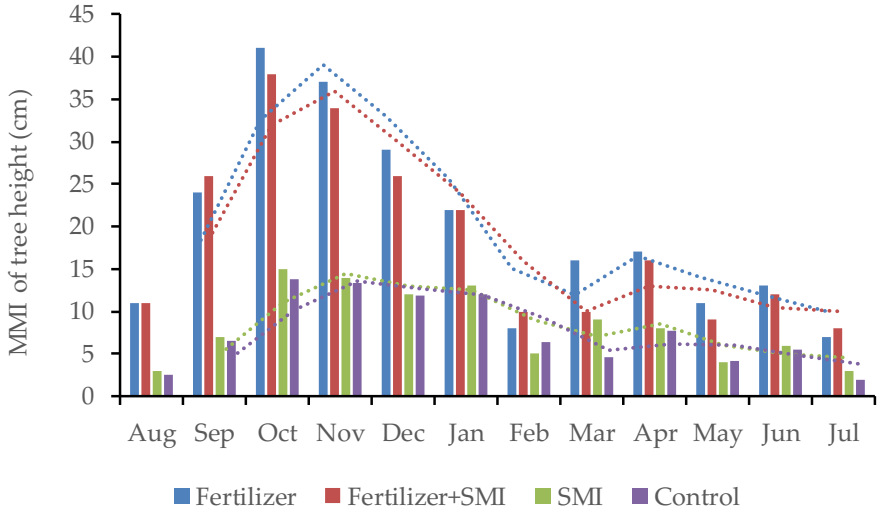


Figure 34. Mean monthly increment of tree height with two moving average trend lines.

Monthly amount of rainfall was thought as an important influencing factor to tree growth at the FTP. The MMI of tree height versus monthly rainfall is as shown in Figure 35. However, it indicated that inoculated trees showed no advantage to rainfall fluctuation such as enhanced capacity to absorb soil moisture during dry period. Though the rainfall were consistently high from January-March, MMI of tree height were declining and thus the amount of available water to tree could be considered excessive for the tree need. Monthly rainfall of 200-300 mm was probably about sufficient to meet water requirement of NC.

As discussed earlier MMI of tree height accelerated for the first three months, slowed down during the next three months and plateau off from the seventh month onwards. They were noticeable sudden drop in MMI of tree height in the month of February and May (Figure 35). The two sudden drops were observed to have coincided with heavy infestation of a defoliator or leaf eating insect that not only devoured leaves but the tree apical shoots as well. The leaf eating pest caused stunting effect on the trees. With the

use of insecticide to kill the larvae, growth of tree recovered as depicted in the month of March and June.

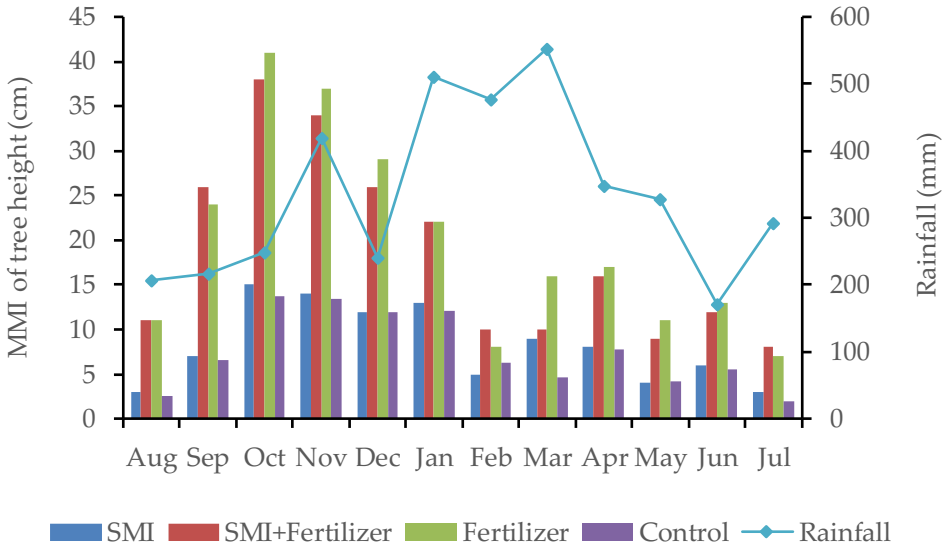


Figure 35. Mean monthly increment of tree height and monthly rainfall.

5.4.3.6 Tree dry-biomass

Measurement of tree dry-biomass was taken among the SMI treated and Control trees. The 10 trees were selected ranging from the tallest to the smallest. Tree heights were exponentially correlated with tree above ground dry-biomass and root dry-biomass especially for non-inoculated (Control) trees as shown in Figure 36. This indicated that physiologically, tree wood became denser to keep trees upright as the tree grew taller between the height of 1.2-1.5 m. Inoculated trees exhibited slightly more dry-biomass as compared to the non-inoculated trees.

Similarly, tree root collar diameter was also exponentially correlated with above ground and root dry-biomass biomass especially for non-inoculated trees (Control) as shown in Figure 37. This probably also indicated that abrupt formation of dense wood occurred by when trees grew to about 3.0 cm root collar diameter.

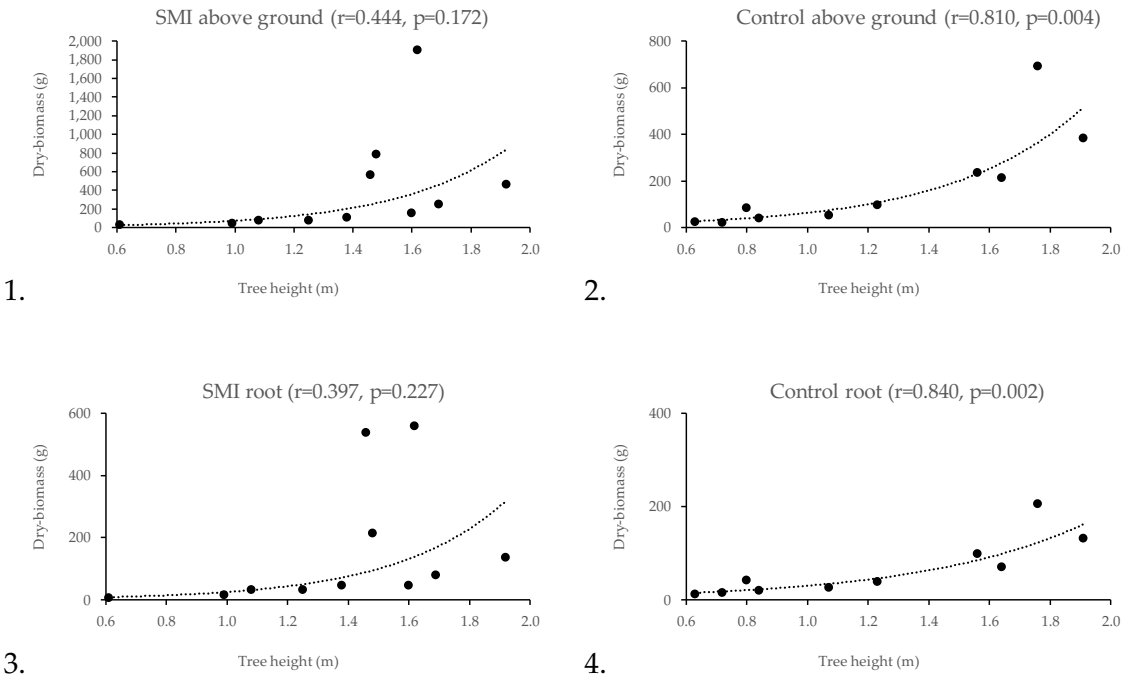


Figure 36. Scatter plot of dry-biomass and tree heights. 1: SMI above ground, 2: Control above ground, 3: SMI root, and 4: Control root.

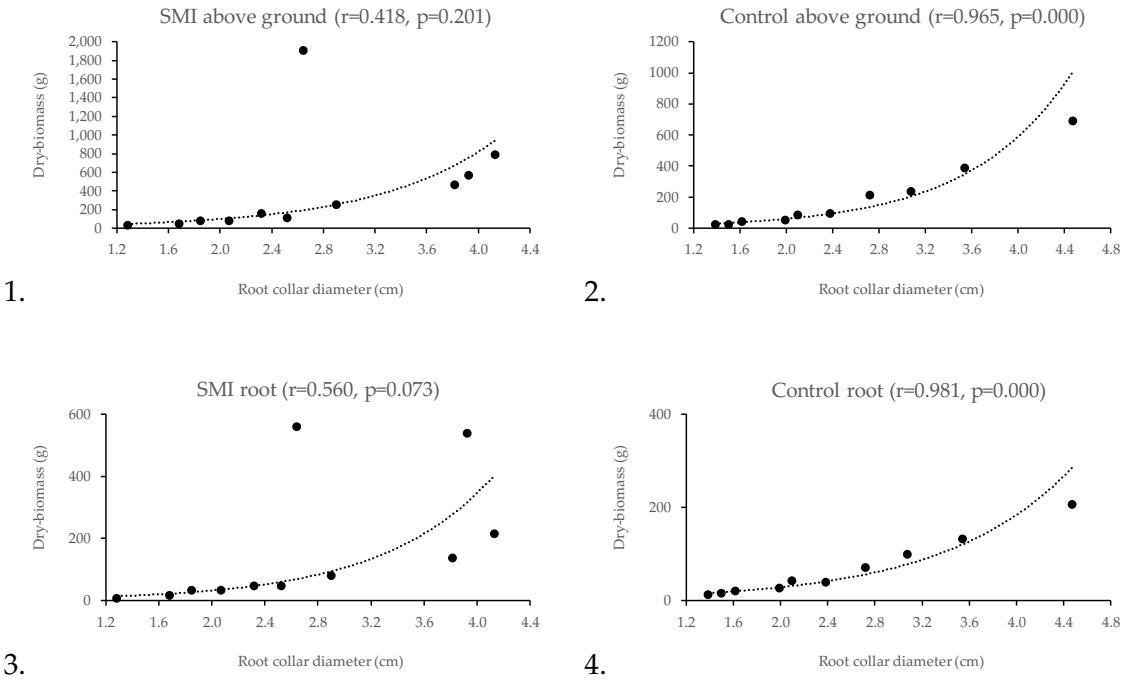


Figure 37. Scatter plot of dry-biomass and tree root collar diameters. 1: SMI above ground, 2: Control above ground, 3: SMI root, and 4: Control root.

The root-above ground ratios of inoculated trees and non-inoculated trees based on means of above ground and root dry-biomass are as shown in Table 20. The inoculated and non-inoculated seedlings showed proportionate dry-biomass of root and shoot and thus the result indicate almost similar root-above ground ratios. The root-above ground ratio of non-inoculated trees (Control) was somewhat higher and thus, presumably giving slight advantage in terms of absorption and soil moisture and nutrients (Budiman et al. 2015, p. 210).

Table 20. Root-above ground ratios of trees.

Treatment	Mean of root dry-biomass (g)	Mean of above ground dry-biomass (g)	Root-above ground ratio
SMI	156	407	0.36
Control	67	185	0.38

5.4.4 Leaf nutrient concentration

Leaf nutrient concentration "ascending" relationship with MMI of tree height was studied for macro-nutrients; N, P, K, Ca and Mg, and micro-nutrients; B, Cu, Zn, Fe and Mn.

5.4.4.1 Macro-nutrients

Laboratory results of primary (N, P, and K) and secondary (Ca and Mg) leaf macro-nutrient concentrations based on percentage of leaf dry-biomass at 0 month are as shown in Figure 38. The nutrient concentration of inoculated and non-inoculated seedlings was almost the same for all the elements except for slight difference in N at time of planting. The order of nutrient concentration was N > K > Ca > P = Mg. N, K, and Ca seems to be absorbed in larger amount as compared to P and Mg. There was no noticeable increase of uptake of the nutrients especially P by inoculated seedlings in the FN before being planted out to the FTP.

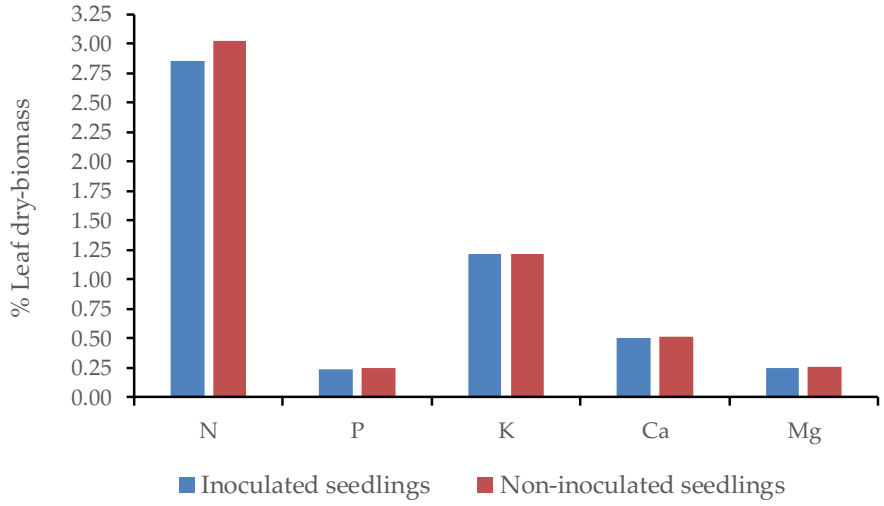


Figure 38. Leaf primary and secondary macro-nutrient concentrations at planting out.

Leaf macro-nutrient concentration N, P, K, Ca and Mg of tree at the planting month (July), third month (October), sixth months (January), ninth months (April) and twelfth months (July) are as shown in Figure 39.

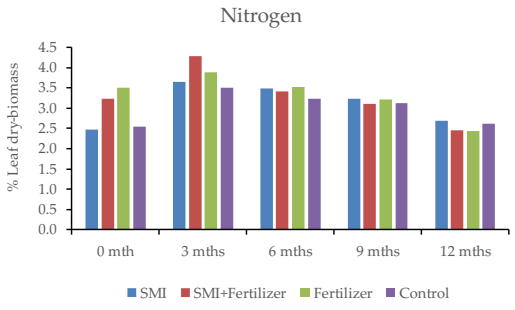
N concentration of all treatments started low and its uptake peaked at the third month, and gradually drop at the sixth month until twelfth month. As for P concentration, it was high initially for all treatments suggesting sufficient P was provided for at the FN stage but it dropped rapidly from third months onwards except for treatments with fertilizer. Ca concentration on the other hand, showed that the level was very high at the initial stage, suggesting bountiful supply of Ca in the FN but it dropped to about half at the third month onwards. In contrast to Ca concentration, K and Mg concentrations gradually built up or remained constant until the twelfth month. The Mg concentration shoot up by about 30%.

The leaf nutrient concentration dynamics of N and P was quite consistent with the MMI of tree height. Trees showed rapid growth following fertilizer application for the first three months (August-October). Growth slowed down for the next three months (November-January), plateau from the seventh month (February) onwards and dropped

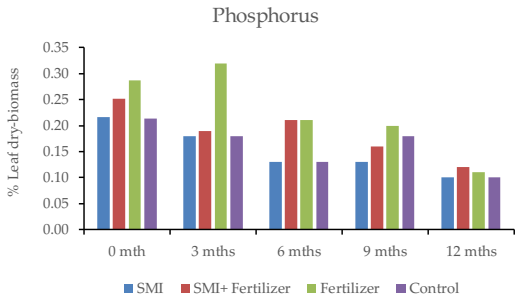
at 12 months (July). The trend of slowing growth was expected as the fertilizer use has the efficacy period of 6 months.

As for K, Ca and Mg, the trend was reversed and this could be due to their lower requirement by NC, and/or probably their supply was sufficient or excessive. The leaf critical nutrient concentration could be between for 3-4% for N and between 0.2-0.3% for P based on leaf dry-biomass to sustained desired growth. Critical nutrient concentration has been the basis to assess plant nutrient status based on plant chemical parameters. It indicates concentration just deficient or just adequate for maximum growth (Smith & Loneragan 1997, p. 10).

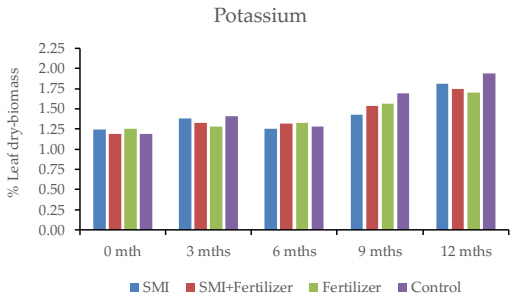
The use of SMI did not indicate any efficiency in the nutrient absorption by inoculated trees either with or without fertilizer especially for critical elements like N and P.



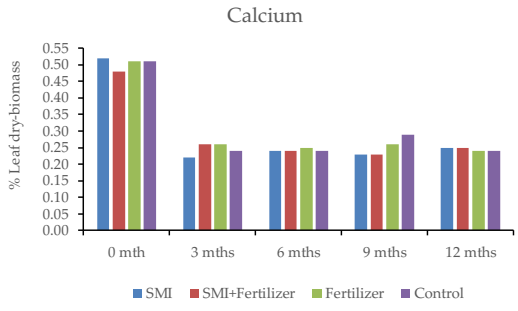
1.



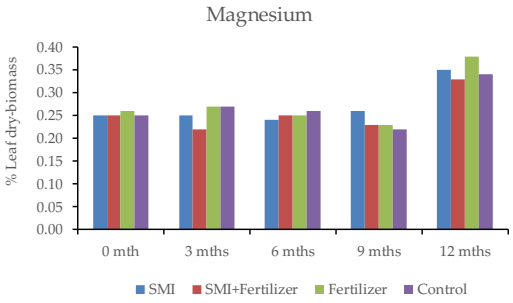
2.



3.



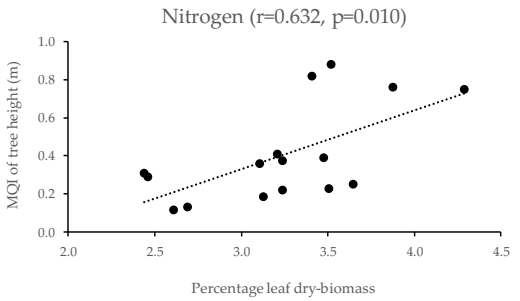
4.



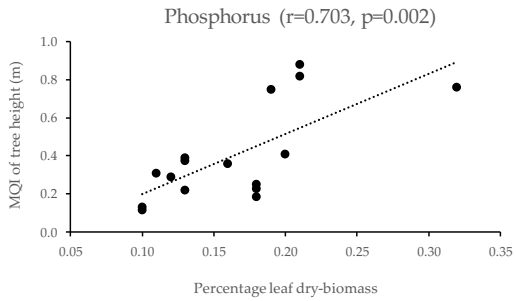
5.

Figure 39. Leaf nutrient concentrations at 0 month and every quarterly. 1: Nitrogen, 2: Phosphorus, 3: Potassium, 4: Calcium, and 5: Magnesium.

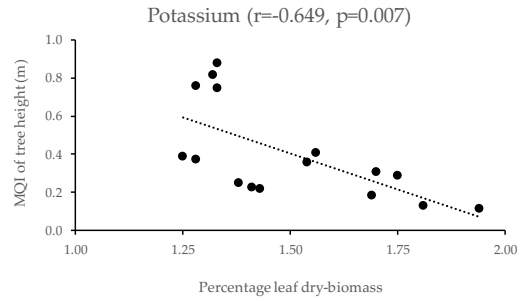
Correlation of mean quarterly increment (MQI) of tree heights and leaf nutrient concentrations based on percentage leaf dry-biomass is as shown in Figure 40. Significant positive correlation was shown for leaf N and P concentration with Pearson correlation of 0.632 and 0.703 respectively. This indicated that N and P played an important role on the growth of NC. On the other hand, leaf K concentration showed significant negative correlation with MQI of tree height with Pearson correlation of 0.649. This indicate that K concentration accumulated in leaves even when growth of trees was declining. Leaf Ca and Mg concentration showed no correlation to growth of trees though leaf Mg concentration seemed to have same pattern as leaf K concentration.



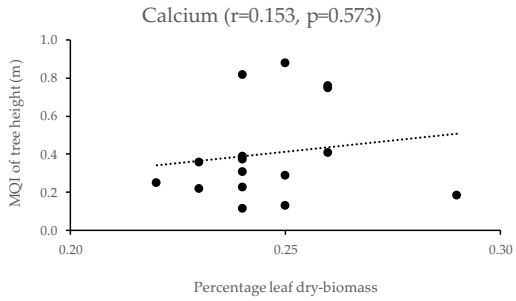
1.



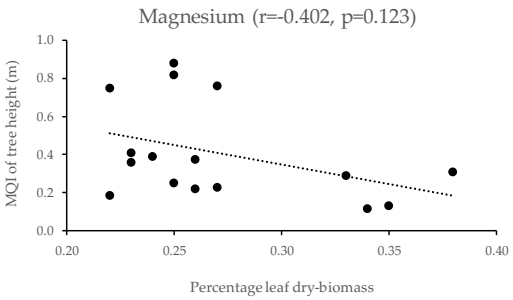
2.



3.



4.



5.

Figure 40. Scatter plot of mean quarterly increment of tree heights and percentage leaf dry-biomass. 1: Nitrogen, 2: Phosphorus, 3: Potassium, 4: Calcium, and 5. Magnesium.

5.4.4.2 Micro-nutrients

Leaf micro-nutrients concentrations of B, Cu, Fe, Zn, and Mn at 0 month are as shown in Figure 41. The leaf nutrient levels of trees from inoculated and non-inoculated tree were almost the same for all the elements except for slight difference in Zn and Fe concentrations. The order of high to low of the micro-nutrient concentrations of tree leaves was Mn > Fe > B > Cu > Zn. Mn and Fe were deemed to be required in higher amount by NC. Similarly, like the macro-nutrients, there was no noticeable increase of micro-nutrients uptake by inoculated seedlings while in the FN.

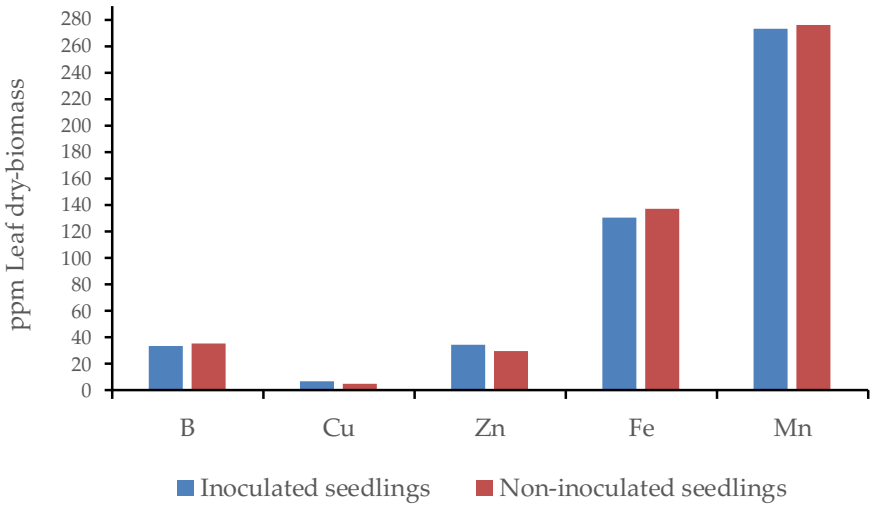


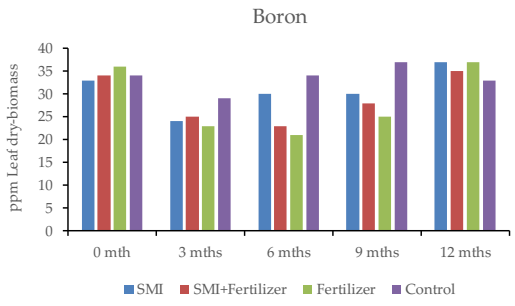
Figure 41. Leaf micro-nutrient concentrations at planting out.

Leaf micro-nutrient concentration of B, Cu, Zn, Fe and Mn for tree with ages of 0 month (July), 3 months (October), 6 months (January), 9 months (April) and 12 months (July) old in the FTP are as shown in Figure 42. The amount of micro-nutrients needed by the

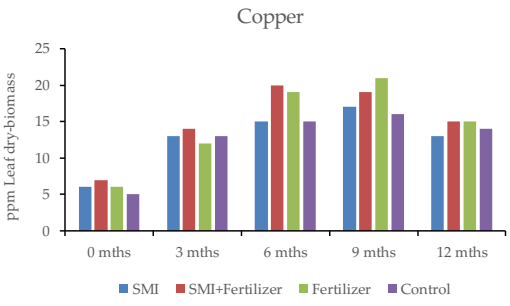
tree was generally smaller, by 1,000X as compared to the macro-nutrients. Generally, the leaf micro-nutrient concentration dynamic was quite similar to the leaf macro-nutrient concentration. As shown by Figure 42, Fe showed similar pattern with Mg, while Cu and Mn were similar with K, and Zn with N.

The uptake of leaf Zn concentration showed peaked at third and sixth month, and gradually drop at ninth until the twelfth month. The nutrient concentration dynamic of Zn was consistent with the MMI of tree height as described for leaf nutrient N and P concentrations. Trees showed rapid growth following fertilizer application for the first three months (August-October). Growth slowed down for the next three months (November-January), plateau from the seventh month (February) onwards and dropped at 12 months (July).

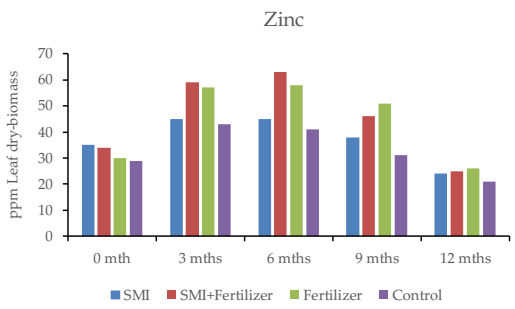
The critical nutrient concentration for Zn could be between 40-60 ppm of leaf dry-biomass for NC to sustained good growth. Interestingly Zn concentration of inoculated trees was consistently higher than non-inoculated trees as shown in Figure 42. However, the higher level of leaf Zn concentration did not seem to enhance the growth of inoculated trees as growth non-inoculated trees was much better as discussed earlier.



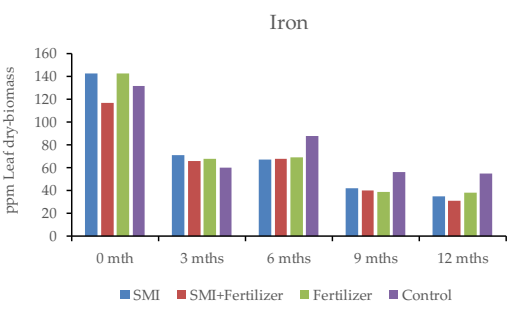
1.



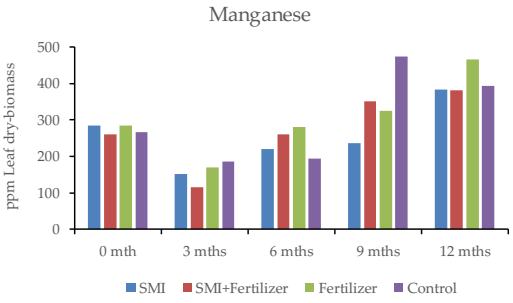
2.



3.



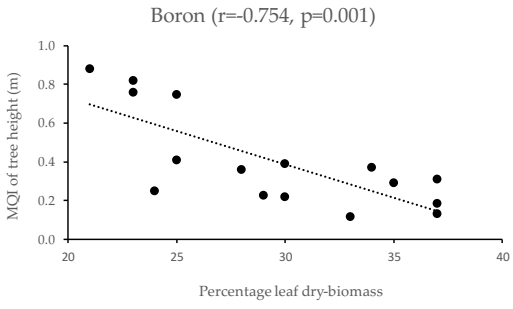
3.



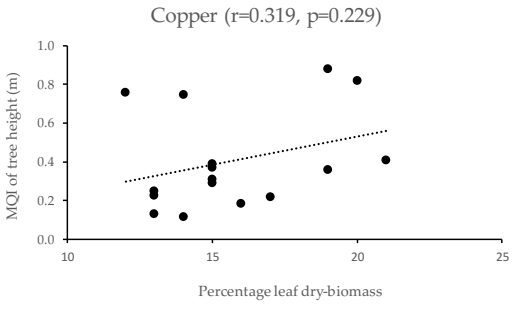
5.

Figure 42. Leaf nutrient concentrations at 0 month and every quarterly. 1: Boron, 2: Copper, 3: Zinc, 4: Iron, and 5: Manganese.

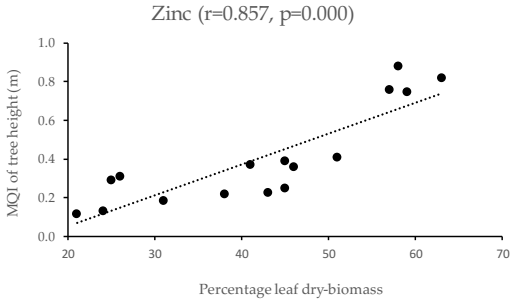
MQI of tree heights and leaf micro-nutrient concentrations based on percentage leaf dry-biomass is as shown in Figure 43. Significant positive correlation was shown for leaf Zn concentration with Pearson correlation of 0.857. This indicated that Zn could have played an important role on the growth of NC. On the other hand, leaf B concentration showed significant negative correlation with MQI of tree height with Pearson correlation of 0.754. This indicate that leaf B concentration like K, accumulated in leaves even when growth of trees was declining. Leaf Cu, Fe and Mn concentrations showed no correlation to growth of trees though leaf Mn concentration seemed to have same pattern as leaf B concentration.



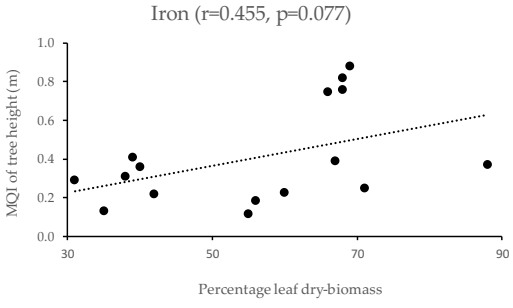
1.



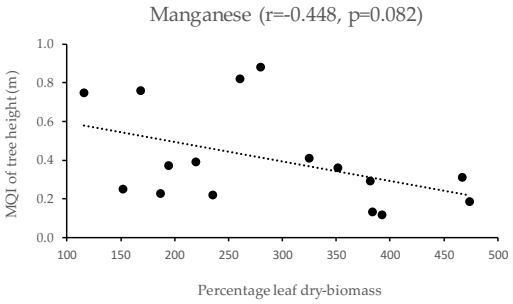
2.



3.



4.



5.

Figure 43. Scatter plot of mean quarterly increment of tree heights and percentage leaf dry-biomass. 1: Boron, 2: Copper, 3: Zinc, 4: Iron, and 5. Manganese.

5.4.5 Tree mycorrhizal root

Roots collected for dry-biomass measurements were sub-sampled to prepare diagnostic glass slides to determine mycorrizal root. The diagnostic glass slides preparation yielded low success as root samples were too woody for sufficient clearing. Only one tree from SMI treatment and fives trees from Control treatment could be diagnosed (Table 21).

Tree root colonization by AMF was found on both inoculated tree (Tree no. 84) and non-inoculated tree (Tree no. 188) at about 25%, and 30% respectively. The mycorrhizal roots were those sampled at the distal ends and were non-woody. AMF having symbiotic relationship with NC were most likely present inherently in the soil at the FTP, since roots of non-inoculated tree were also colonized. Temporal variation of AMF communities in a tropical forest reported by Husband, Herre and Young (2002, p. 131), indicated that as the forest tree seedlings matured, the fungal diversity decreased and as previously rare fungal types replaced formerly the dominant fungal ones. Different seedlings ages sampled were colonized by significantly different fungal populations, and thus indicating both time and host age influenced mycorrhizal population.

Some fungal structures of stained roots on glass slides were observed clearly under compound light microscope such as arbuscules, vesicles and hyphae, however identification the arbuscular mycorrhizal fungi was quite fuzzy. Hyphae observed as thin and septate were considered as non-mycorrhizal (Figure 44).

Table 21. Tree root colonization at the field trial plot.

Treatment	Tree no.	Fungal structure intercepted	Interception (%)
SMI	84	Arbuscule and Hypha	25
	2	None	0
	50	Hypha (Septate)	9
Control	167	None	0
	188	Arbuscule, Vesicle and Hypha	30
	211	Hyha (Thin)	19

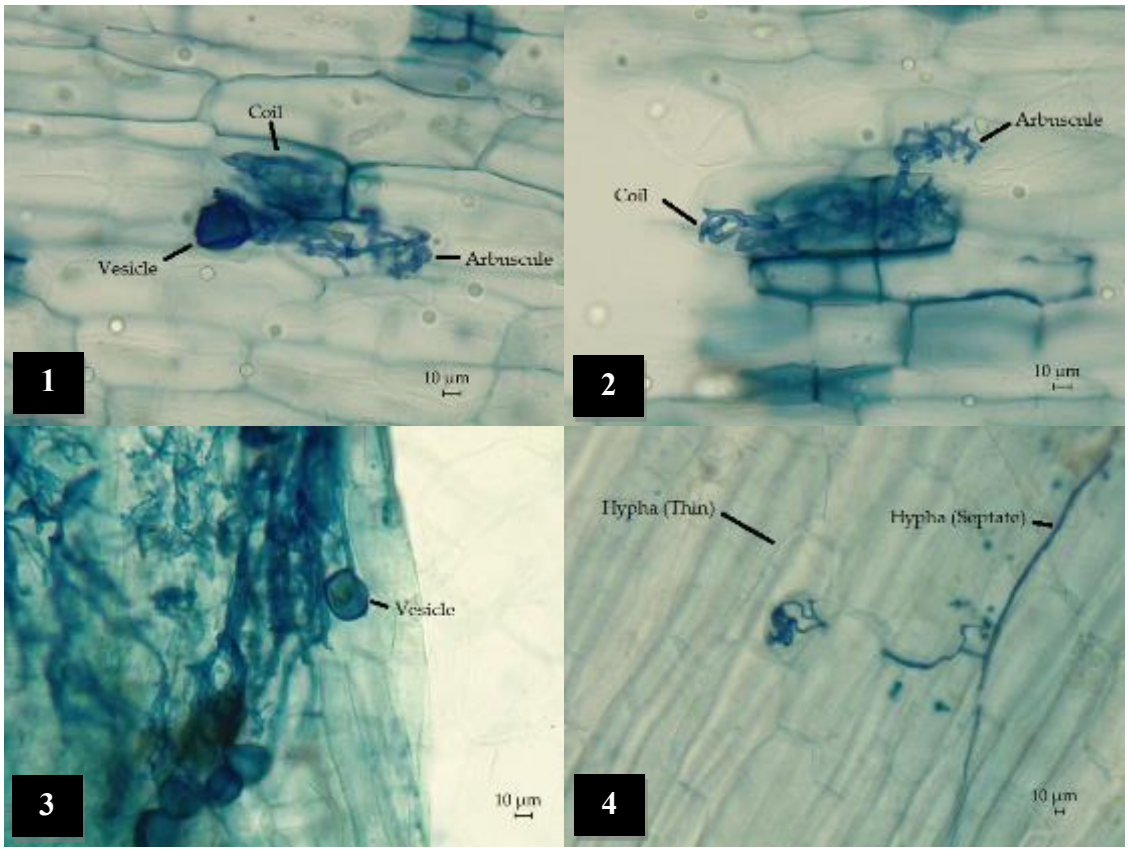


Figure 44. Fungal structures in roots. 1: and 2: Inoculated tree (Tree no. 84), 3: Non-inoculated tree (Tree no. 188), and 4: Non-inoculated tree (Tree no. 50).

5.5 Discussion

This field trial described the two growth stages of NC, namely seedlings at the FN and trees at the FTP. As a pioneer forest tree species, its role is to quickly start the regeneration of forest gaps due to soil disturbance. Where ever there is opening of forest

canopy such as road construction, naturally growing trees were found thriving along such roads. Thus, NC was sometimes coined as “Kayu kaki Lipan” meaning bulldozer track tree by the local Iban community. The ability of such pioneer forest trees species to colonize disturbed soil environment was probably due to its fecundity. However, as pointed out by Kiers et al. (2000, p. 106), small-seeded pioneer tree species were dependent on mycorrhizae for initial survival and growth. Host species affected both the richness and the community composition of AMF in roots as AMF-plant interaction network was significantly nested as neither that of geographic distance, soil type, nor distance from a creek (Phillips 2012, p. 67).

Planting of NC has been carried out extensively by some large forest plantation companies in Sarawak due to its reputation as a fast growing tree by merely observing the naturally growing ones. However, the planted trees have not been growing as fast as the wild ones and thus, it ended up being relegated to just a site demanding species with preference for nutrient rich soils and moist sites. NC is good example of a plantation tree species where such site factors are important to the success of the forest plantation. NC abnormally, were also observed at open sites where soil has been disturbed or rejuvenated by logging, earthworks or natural processes (Bloomberg & John Sabang 2009, p. 55).

A high root-shoot ratio refers to the proportion of the root dry weight to the shoot dry weight, is preferred as it suggests good roots to support to the aboveground biomass. Good root system is not only for strong anchorage for plants but also for high absorption and storage capacity of soil water planting sites have poor soil moisture retention (Budiman et al. 2015, p. 210). Jaenicki (1999, p. 13), reiterated that to enhanced growth of most agroforestry tree species, inoculation with AMF is necessary. As pointed out that the AMF inoculation can increase plant disease resistance and help alleviate plant stress by enhancing the plant's water and nutrient uptake. Early inoculation of AMF at the nursery increase the propagation success of cuttings and seedlings. Thus, it is imperative that AMF inoculation is well established when producing seedlings especially when they are destined to planting site with degraded soils.

This field trial used soil trap culture of NC to produce a crude bio-fertilizer in the form of SMI to enhance the growth of planted trees in the field. To enhance the symbiotic relationship between fungi and host tree, the use of fertilizer was also tested with the purpose of boosting health of planted out trees at the initial stage so as to facilitate root colonization and sporulation of the AMF.

Though the soil trap culture carried out was able to enrich AMF propagules as shown by the spore count whereby the SMI produced has about 10 times more spores than the FN soil. However, the mean percentage of root colonization of inoculated seedlings was only 10%. Root colonization was also observed on non-inoculated seedlings which was just slightly less at 8%. The just slightly higher root colonization of inoculated seedlings might possibly have indicated presence of AMF specific to NC in the starter soil collected from the PP in Kubah N.P. The use of SMI probably have some influence on root development of seedlings at the nursery stage. The cause of root system uniformity of inoculated seedling by AMF root colonization was however, uncertain.

Budiman et al. (2015, p. 210), reported that NC seed viability of NC influenced the field survival, height, and diameter growth of a year old seedlings after planting out. They reiterated that the height growth of seedlings in the nursery significantly influenced their height growth in the field. Seedling height class of, 20 - < 30 cm, and diameter class of > 4.5 mm gave good sturdiness quotient of 5.47. Non-inoculated seedlings in this study seemed to be sturdier than inoculated seedlings with sturdiness quotients of 4.48 and 4.32 respectively, however, their root to shoot ratios turned out to be the same. The only noticeable effect of SMI was that the tree height of inoculated trees was more uniformed as compared to the non-inoculated trees. This could be due to root system based on root dry-biomass of inoculated seedlings at the FN which was more uniformed as compared to non-inoculated seedlings.

The use of SMI did not enhance tree growth in the field. The Least Significant Difference test ($p=0.05$) was carried out on means of tree height. The means of tree height of treatment SMI, and Control were, 1.39 m, and 1.38 m respectively. There was no

significant difference between mean tree height of inoculated seedlings and non-inoculated seedlings. Similarly, there was no significant difference between mean tree root collar diameter of inoculated seedlings and non-inoculated seedlings. The means of root collar diameter were both 2.4 cm.

The result of the study indicated that the use of fertilizer enhanced tree growth in the field. The Least Significant Difference test (p=0.05) was carried out on means of tree height. The means of tree height of treatments - Fertilizer and Control were, 2.84 m and 1.38 m respectively. There was significant difference between mean tree height of seedlings with fertilizer and without fertilizer. There was also significant difference between mean root collar diameter of seedlings with of trees with and without fertilizer. The means of root collar diameter were 5.3 cm and 2.4 cm respectively.

The result of the study indicated that the use of SMI with fertilizer did not enhance tree growth in the field as compared to the use of fertilizer only based on the Least Significant Difference test (p=0.05) carried out on means of tree height. The means of tree height of the treatments, SMI+Fertilizer, and Fertilizer only were, 2.66 m, and 2.84 m respectively. There was no significant difference between mean tree height of inoculated seedlings with fertilizer and non-inoculated seedlings with fertilizer. There was also no significant difference of mean root collar diameter between inoculated seedlings with fertilizer and non-inoculated seedlings with fertilizer. The means of root collar diameter were 5.1 cm and 5.3 cm respectively. The early growth performance of NC with fertilizer in the FTP at Sabal F.R. with mean height of 2.84 m and mean root collar diameter of 5.4 cm of were better off than the reported early growth of NC in a provenance-progeny tests in West Java Province, Indonesia (Sudrajat et al. 2016, p. 16). As reported, four-month-old seedlings were planted at the two field conditions with annual rainfall and temperature of 2,580 mm and 27 °C, and 2,440 mm and 28 °C respectively. The soils at both sites had low level of N P K and Organic C with pH of 5.1 and 4.2, respectively. The first site was an open private land with slope that ranged from 5-15%, and subjected to high grazing by domestic animal, was planted with irregular agricultural crops in several parts of area. The second site was an even topography area within a state forest land covered

with dense weed that grew rapidly even after clearing. The planted NC seedlings were given 3 kg of manure each as basic fertilizer with the spacing of 3 x 3 m between planting hole. The mean heights and root collar diameters of the two sites were 2.42 m and 4.49 cm, and 2.14 m and 4.08 cm respectively.

The actual and postulated growth trend of the inoculated seedlings with fertilizer after six months is as shown in Figure 45. The use of SMI was not able to propel the efficacy of the fertilizer used and in fact slightly affected growth. Antunes et al. (2012, p. 532), reported that use of fertilizer affected AMF colonization of plant roots and consequently the growth of fungi. Their findings were consistent with the optimal foraging theory, whereby AMF from N- deficient or P-deficient soils grew larger but reduced plant growth more in those conditions relative to AMF isolated from non-deficient soils.

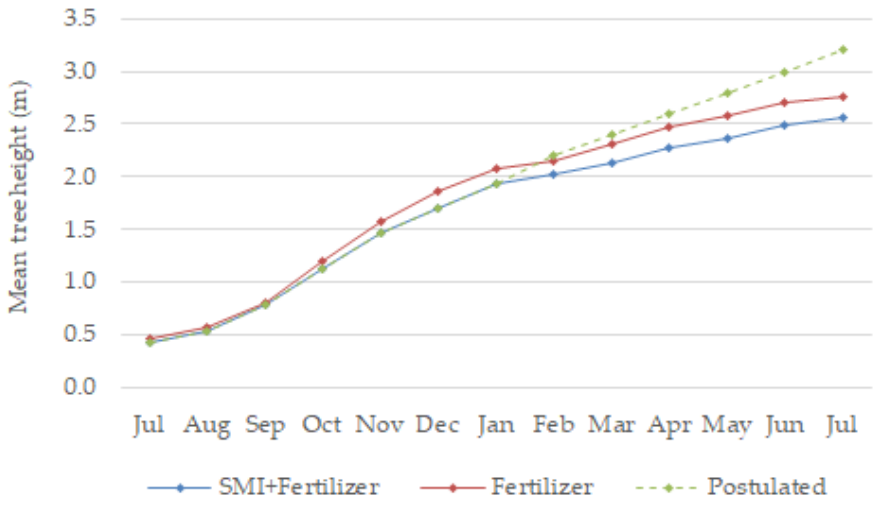


Figure 45. The postulated monthly means of tree height of inoculated trees with fertilizer.

The use of SMI as a bio-fertilizer for growth of seedlings for planting out was not effective. Even complementing SMI used with slow release chemical fertilizer did not propel further growth at the FTP. The efficacy period of the slow release fertilizer was six months, and no significant difference in growth was seen beyond the six months' period after planting. Quality seedlings were related to increased survival and

productivity at field planting as they are able to produce new roots quickly to get anchored in the ground, and start assimilating and growing (Jaenicki 1999, p. 8). As reiterated, to ensure of adequate establishment AMF infection, a well-developed root system, sun-adapted foliage, a large root collar diameter, a balanced root to shoot ratio, a good carbohydrate reserves, and an optimum mineral nutrition content are required.

Measurement of tree dry-biomass at the FTP also did not showed influence by SMI treatment. Root and above ground dry biomass were exponentially correlated with root collar diameter of non-inoculated trees (Control) as compared to inoculated trees. However, inoculated trees exhibited slightly more dry-biomass as compared to the non-inoculated trees. This abrupt formation of denser woody by inoculated trees probably occurred when root collar diameter was about 3.0 cm.

MMI of tree height was also used to study the influence on the amount of monthly rainfall received. However, inoculated trees showed no advantage to rainfall fluctuation such as enhanced capacity to absorb soil moisture during dry period. The amount of available water to tree was considered too excessive. Monthly rainfall of about 200-300 mm was probably sufficient to meet water requirement of NC. The two instances in sudden drop in MMI of tree height was rather coincidences of heavy infestation by a defoliator or leaf eating insect that not only devoured leaves but the tree apical shoots also. The leaf eating pest caused stunting effect on the trees.

MMI of tree height of tree of inoculated trees (SMI) were slightly higher as compared to non-inoculated trees (Control) except for month of February and May. However, the small edge in MMI of tree height was pale in comparison to trees with fertilizer treatment. Incidentally, leaf Zn concentration of the inoculated trees was consistently higher than non-inoculated trees. However, the role of Zn in tree growth of NC was uncertain.

To sustained good growth of NC in the field, proper fertilizer regime was needed as part of the silviculture practices. N, P, and Zn were found to be soil nutrients that influence

growth of NC at the FTP. The study by Ani Sulaiman, Wan Rasidah Abdul Kadir and Mohd. Shukari Midon (1990, p. 119), showed that morphology of six years old *Acacia* applied with different combinations of N and P fertilizers during its early growth showed differences in height and diameter growth increment. The wood basic density was found to be significantly different at all levels of treatments. The application N fertilizer alone has some effects in improving the heartwood to sapwood ratio. Combination of P and N improved the mechanical strength of timber while with N alone, the strength appeared to decrease. Miller (1981, p. 157), suggested that the patterns of nutrient uptake varies with tree age. Prior to canopy closure, tree growth was very dependent on the availability of soil nutrients and response to a number of nutrients was as expected. Responses to fertilizer were unlikely when foliage biomass have fully formed. However, on low N capital sites, immobilization of N in biomass and humus, led to the progressive development of deficiency as tree aged but may also eventually disappeared, as tree demands for soil nutrients fell with age.

Leaf nutrient concentration "ascending" relationship with MMI of tree height was studied for macro-nutrients N, P, K, Ca and Mg and micro-nutrients B, Cu, Zn, Fe and Mn. The leaf nutrient concentration dynamics of N, P and Zn were quite consistent with the pattern of MMI of tree height. Trees showed rapid growth following fertilizer application for the first three months. Growth slowed down for the next three months, plateau from the seventh month onwards and dropped at the twelfth month. The trend of slowing growth was expected as the fertilizer use has the efficacy period of six months.

The leaf critical nutrient concentrations could be between 3-4% for N, between 0.2-0.3% for P, between 40-60 ppm for Zn of leaf dry-biomass for NC to sustained good growth. They indicate concentration just deficient or just adequate for maximum growth (Smith & Loneragan 1997, p. 10). The nutrient proportions in the tissues needed for unimpaired growth, are similar among woody and herbaceous plants (Ericsson 1994, p. 159). However, the quantities required per unit of time differ significantly between species owing to different inherited potentials for growth. Generally, nutrient-use efficiency becomes higher when trees grow older and the processes associated with internal

nutrient cycling are operating fully. Nonetheless, the outcome of the thesis indicated that to sustain the growth of *Neolamarckia cadamba* in the field, repeated fertilizer application is needed every six months if a similar make and origin of chemical fertilizer is to be used. Leaf critical nutrient concentration of nitrogen, phosphorus and zinc could be further ascertained as a guide to developing the fertilizer regime of *Neolamarckia cadamba*.

There was no there is no noticeable increase of the macro-nutrients and micro-nutrients uptake by inoculated seedlings while at the FN as well as at the FTP. The use of SMI did not indicate any efficiency in the nutrient absorption by inoculated trees either with or without fertilizer especially for soil nutrients like N, and P. Agricultural practices limited to chemical fertilization have caused ecological caste. The presence in the soil of high levels of fertilizer dramatically altered the interaction between plants and microorganism communities. Generally, in a nutrient-rich environment, a plant can directly uptake enough nutrient from the soil, without the service provided by the AMF partners. As a result, the dependency of plants on their AMF partners gradually diminishes, and AMF community richness and diversity decline (Berruti et al. 2014, pp. 168&169).

The percentage root colonization by AMF was low and it occurred both in SMI treated and non-treated seedlings and trees as well. Root colonization was also probably caused by AMF propagules present in soils that the seedlings were in contact with namely, planting media at the FN in Semengoh N.R. and soil at the FTP in Sabal F.R. The increase in root colonization by the effect of SMI treatment when seedlings grew bigger once planted out to the field was also uncertain. Tree root colonization by AMF was found on individual trees sampled both inoculated and non-inoculated were about 25%, and 30% respectively. AMF having symbiotic relationship with NC was most likely influenced by inherent AMF propagules in the soil at the FTP. As aforementioned, roots of the non-inoculated tree were also colonized. Williams et al. (2017, p. 874), reiterated that agricultural fertilization significantly affects AMF community composition. They indicated that the use N fertilizer caused plants to increase the supply of C to AMF and

in return received relatively less P. The alteration in P-C exchange between plants and the mycobionts occurred regardless of soil P status under N fertilization. Open field condition may adversely affect AMF root infection. Closa and Goicoechea (2011, p. 65), demonstrated that changes in the composition of understory vegetation within beech forests strongly affected the infectivity of native AMF in clear-cut areas. The removal of over-story impairs the correct development of herbaceous species as high light intensity negatively affects the establishment of shade species.

The NC trees were also susceptible to insect pest attack especially by a leaf eating caterpillar. Tree left untreated with regular pesticide application was stunted and eventually dies off. The proliferation of leaf defoliator was probably due to high rainfall received prior to the severe pest attack in February and May. According to Huberty and Denno (2004, p. 1383), plant sap feeder pest are adversely affected by continuous water stress. They reiterated that despite enhanced foliar N during concurrent reductions in plant cells turgor and water content, pests' ability to access or utilize N was interfered. As indexed by (Browne 1968, p. 92), the larva moth of *Margaronia hilaralis* synonyms *Arthroschista hilaris*, *Diaphania hilaris* found from India to New Guinea was a defoliator of NC and *Duabanga sonneratiodes*. The larva has a grey color with numerous black spots, and when feeding, folds part of a leaf over as a shelter and skeletonizes the tissues from below. It is considered to be a pest of some importance in Sabah, as it particularly sometimes destroys the terminal buds of the host.

5.6 Conclusion

The field trial indicated that the use of SMI derived from the soil trap culture of NC did not enhance growth of NC in the field. Root colonization of inoculated seedlings at the FN was very low. The use of SMI may have some influence on root system of inoculated seedlings and may have adverse impact even on trees with fertilizer. Fast growing individual trees exhibit long internodes as NC trees observed in forest plantations exhibiting short internodes usually remained stunted and have high mortality rate. The use of fertilizer was able to increase tree growth by more than double as shown by tree

height and root collar diameter of NC in the FTP. However, tree growth plateau after six months consistent with the efficacy period of the fertilizer used.

Complementing SMI with fertilizer used did not propel growth of trees. The efficacy period of the slow release fertilizer was six months, and no significant difference in growth was seen beyond the period of six months after the planting out. The percentage root colonization by AMF was low. However, it was observed on both inoculated and non-inoculated seedlings in the FN and trees in the FTP as well. AMF specific to NC were probably found in soils from all three sites; PP in Kubah N.P., FN in Semengoh N.R. and FTP in Sabal F.R.

To reduce tree mortality and sustained growth of NC, good silvicultural management was necessary. NC requires proper fertilizer regime, regular weed control, systematic pest control and maybe as observed the rarely needed pruning of branches and multiple leaders of trees.

6 Soil trap culture trial

6.1 Introduction

Soil trap culture (STC) is one of the many methods for culturing and isolating AMF (Walker 1999, p. 2). Some AMF species were only detected when STC method was employed as compared to rhizosphere soil trees growing in its natural habitat. This information has great potential for bio-technology application when performing reintroductions or reforestation such as with tropical tree mahogany (Rodriguez-Morelos et al. 2014, p. 1).

In a STC, soil is collected from the area of interest put in a suitable container, where seeds are sown or seedlings planted. The plants are then maintained for a period of time suitable for the establishment of AMF. The usual result of STC is a mixed-species AMF (Walker 1999, p. 2). To produce SMI spores, host plant or rather the NP should be kept for 16 weeks (Miyasaka et al. 2003, p. 3). The technique can be improved from time to time such as choice of NP and location of starter soil collection.

The NP grown to host AMF in the production of SMI should be fast growing, produce a large quantity of roots within a relatively short time, hardy and of course be readily colonized by AMF. The best NP tested using a soil-sand mix were *Cynodon dactylon*, *Sesbania grandiflora*, *Zea mays* and followed by *Panicum maximum* as reported by Habte and Osorio (2001, p. 10). *Cymbopogon citratus* (lemongrass) and *Alium cepa* (onion) seedlings were reported to be also suitable candidates for mass production of AMF spores. Both crops were reported to be suitable NP for AMF due to their fast growth; and ability to produce numerous fine and hairy roots for abundant sporulation (Abdullahi, Lihan & Edward 2014, p. 118). The genus *Cymbopogon* was widely distributed in the Tropical and Sub-tropical regions of Africa, Asia and America, comprising of 144 species. *Cymbopogon* was famous for its high content of essential oils which have been used for cosmetics, pharmaceuticals, and perfumery applications. Two main species, *Cymbopogon flexuosus* and *Cymbopogon citratus* were commercially

cultivated in the Democratic Republic of Congo, Madagascar, and the Comoros Island. *Cymbopogon citratus* was most widely distributed and used in every part of the world (Avoseh et al. 2015, p. 7438).

Besides using the right NP, appropriate planting medium producing AMF inoculum condition must be regulated such as containing a low level of P. The fungi will not form associations with the host plant if there is much P available in the planting medium (Miyasaka et al. 2003, p. 3)

6.2 Objectives

The objective was to establish a STC technique for production of SMI for inoculation of NC seedlings, and that is simple and low cost for use by the FN in Semengoh N.R. and forest nurseries of forest plantation across Sarawak.

6.3 Materials and methods

The STC trial encompassed several stages; starting from collection and processing of starter soil, planting of NP, and enumerating of spores' production. It involved three treatments as follows:

1. Starter soil with nurse plant (NP)
2. Starter soil with nurse plant and low P fertilizer (NP+Fertilizer)
3. Starter soil only (Control)

6.3.1 Trial design

Each treatment comprised of ten grower pots. The 30 grower pots were then position based on the Complete Randomization Design (Chang 1972, p. 27) as shown in Figure 46. Random Number Tables were used as a guide in pot position randomization. The STC trial is as shown in Figure 76.14 (Appendix).

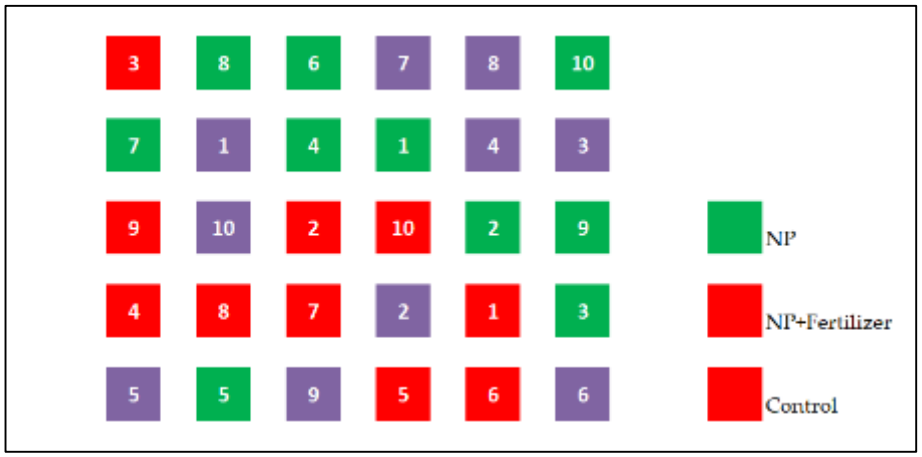


Figure 46. Position of treatments and replicates in soil trap culture trial.

6.3.2 Establishment of nurse plant

6.3.2.1 Collection of starter soil

The procedures used for the collection of starter soil are as described under Materials and Methods in Chapter 5.

6.3.2.2 Preparation of starter soil

The procedures used for the preparation of starter soil are as described under Materials and Methods in Chapter 5.

The well mixed starter soil was then put in plastic grower pots measuring 150 (D) x 120 (H) mm (L 150-S GAFRI, Malaysia) to a level about 5 cm below the edge. Grower pots were filled with the processed starter soil and place under nursery shade net with water sprinkler system at the FN in Semengoh N.R.

6.3.2.3 Planting of nurse plant

The procedures used for the planting of nurse plant are as described under Materials and Methods in Chapter 5.

6.3.2.4 Upkeep of nurse plant

The procedures used for the upkeep of nurse plants are as described under Materials and Methods in Chapter 5.

6.3.3 Assessment of nurse plant morphology

Assessment on NP morphology and extraction of spores from soil were carried out after about three months. About one week before the destructive sampling was carried out, the NP tops were cut at root collar level. Watering regime was also stopped. The adverse treatment on the NP was supposed to induce the root colonizing AMF into reproducing spores (Sunseed Desert Technology 2012, p. 4). The rate of development of AMF colonization was determined by growing the indicator plant in a media optimized for mycorrhizal activity and then monitoring AMF colonization of roots through destructive sampling of roots and/or determining biomass accumulation (Habte & Osorio 2001, p. 16).

6.3.3.1 Nurse plant dry-biomass

NP root ball was freed by kneading and gentle shaking adhering soil off. Soil clumps adhering to roots with were rinsed off under running tap water through a 2mm sieve. Care was taken to keep root systems intact. Any detached roots caught in sieve were collected together with intact roots. After washing, plant shoot and the wet roots were left to dry on boxes made from cardboard at room temperature.

Dry-biomass measurement of each NP was done separately for whole root system and plant shoot. Plant parts were oven-dried at 65^{o} C for three days and beyond until repeat weighing at daily basis gave identical results (Bloomberg, Mason & Jarvis 2008, p. 106). A high root-shoot ratio indicates high absorption and storage capacity of water, which is an advantage, especially in conditions of limited soil moisture. Root-shoot ratio was expressed by root dry-biomass divided by shoot dry-biomass (Budiman et al. 2015, p. 207). Soil loosen from roots of NP was used to assess the presence and enumeration of spores.

6.3.4 Assessment of soil spores

The effectiveness of STC technique was deduced by assessing soil spore count. The Spore extraction method used was based on the Sucrose Extraction Method (Center for Conservation of Biology 2013). The spore segregation method described by INVAM (2017) was improvised in the thesis to enhance its efficiency.

6.3.4.1 Extraction of spores

The procedures used for extraction of spores are as described under Materials and methods in Chapter 4.

6.3.4.2 Segregation of spores

The procedures used for segregation of spores are as described under Materials and methods in Chapter 4.

6.3.4.3 Enumeration of spores

The procedures used for enumeration of spores are as described under Materials and methods in Chapter 4.

Spores composition was also measured to determine percentages of different spore morphotypes. The three simple morphological segregation were globose shape of different colors, ovoid shape of different colors and a distinctly white to pale yellow color spores. Spore suspension in petri dish was swirled several time to let spores congregate and settled down. Images of spores gathered about the center of petri dish was captured using LEICA EZ4 HD stereo microscope with LED and HD Camera (Leica Microsystems, Singapore) using 35X magnification. Vertical lines (blue color) of 10 columns with 1.59 cm width were superimposed on image of spores as shown in Figure 47. Percentage spore composition was calculated by number of the different spore morphotypes crossing or touching the vertical lines.

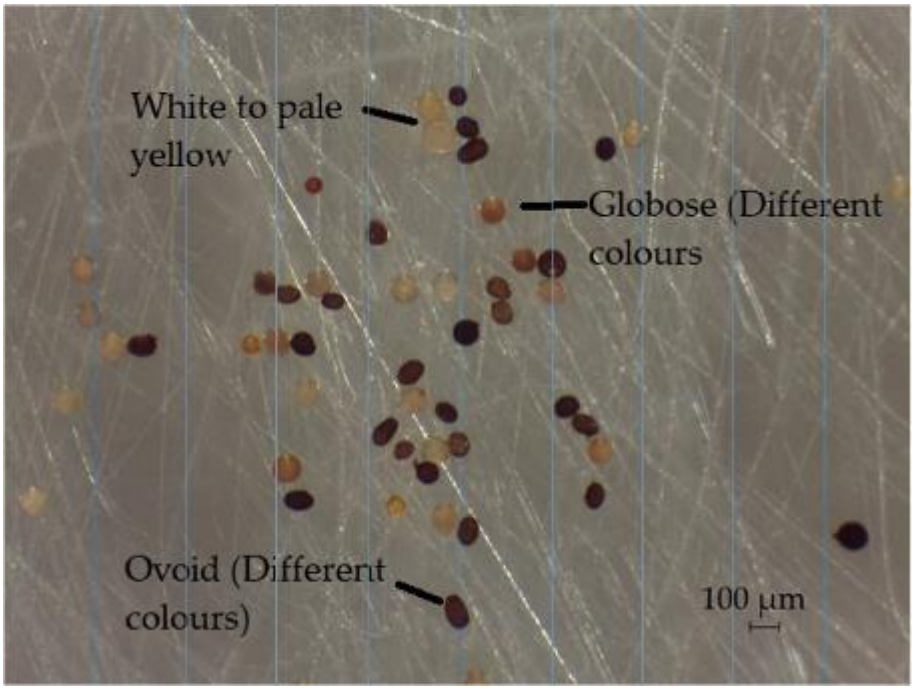


Figure 47. View of spores under stereo microscope superimposed with vertical lines (35X magnification).

6.4 Results

The results of this trial cover the morphology of NP in terms of shoot and root dry-biomass, and spore counts.

6.4.1 Nurse plant dry-biomass

The maximum, minimum, median, mean and lower and upper percentiles of dry-biomass of the NP at three months after planting are as shown in Figure 48. The means shoot dry-biomass of the of nurse plant with and without fertilizer were 3.5 g and 2.2 g respectively The above ground and root dry-biomass were inversely related with NP without fertilizer and NP with fertilizer. NP with fertilizer has more above ground dry-biomass as compared to NP without fertilizer.

However, the root dry-biomass of NP with fertilizer in this trial was lesser than NP without fertilizer. This indicated that used of fertilizer enhanced NP above ground

growth but impeded its root growth. The means of root dry-biomass of NP with and without fertilizer were 1.0 g and 1.3 g respectively.

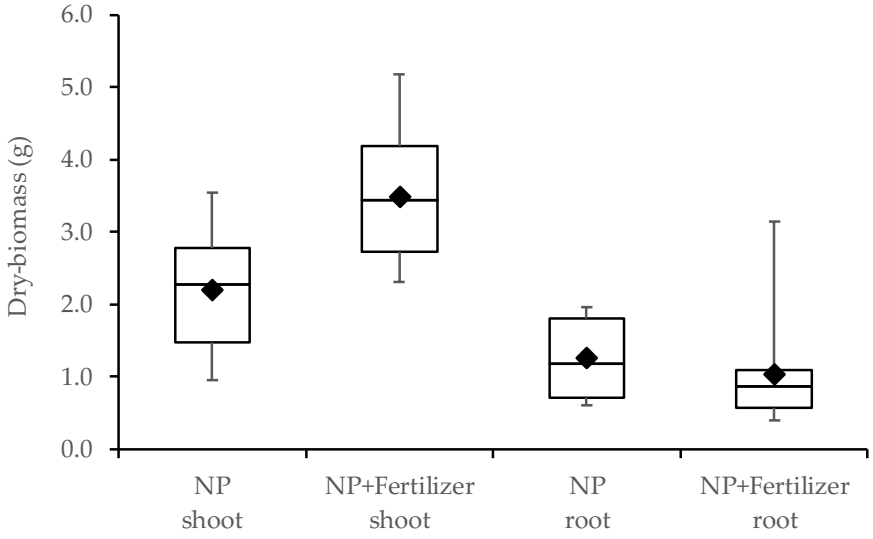


Figure 48. The maximum, minimum, median, mean and lower and upper percentiles of shoot and root dry-biomass of nurse plants with and without fertilizer.

The root-shoot ratio refers to the proportion of the root dry weight to the shoot dry weight and reflects the capacity of the roots to support the aboveground biomass not only for anchorage but also in absorbing water and nutrients from the soil (Budiman et al. 2015, p. 210). The root-shoot ratios of NP with fertilizer and without fertilizer based on means of dry-biomass of shoot and root are as shown in Table 22. The NP without fertilizer showed higher root-shoot ratios or double of NP with fertilizer and it augured well for root colonization and production of AMF spore.

Table 22. Root-shoot ratio of nurse plants.

Treatment	Mean of root dry-biomass (g)	Mean of shoot dry-biomass (g)	Root-shoot ratio
NP	1.2	2.2	0.54
NP+Fertilizer	1.0	3.5	0.29

6.4.2 Spore counts

The maximum, minimum, median, mean and upper and lower percentiles of spore counts of the three treatments is as shown in Figure 49. Spore counts based on treatments were in the order of NP > NP+Fertilizer > Control. The result indicated that the local lemongrass used was able to enhance sporulation of AMF in starter soil collected from the PP in Kubah N.P. The used of the low P fertilizer formulation had adverse effect on rate of sporulation. Spore count of NP with fertilizer was just slightly higher compared to spore count of starter soil only. The spore counts by NP with and without fertilizer, and starter soil only were 9, 19 and 7 spores/g soil respectively. Sporulation by NP without fertilizer was enhanced by two folds.

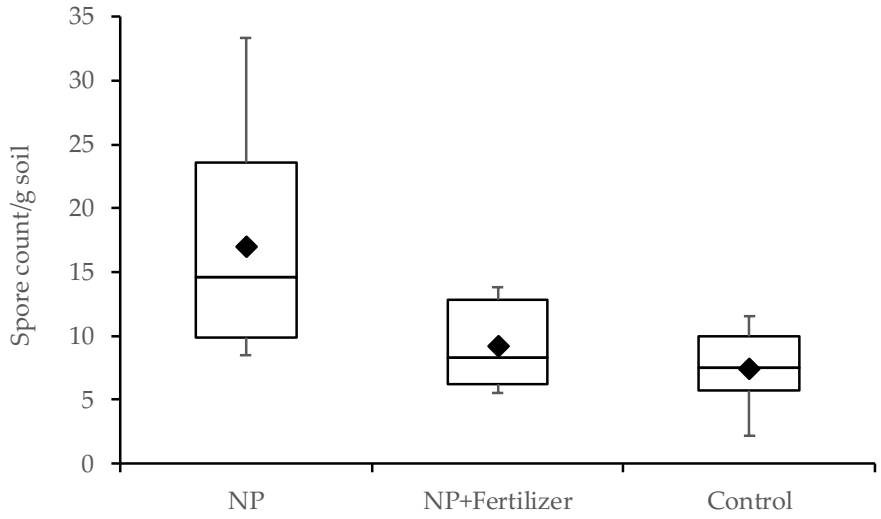


Figure 49. The maximum, minimum, mean, median, upper and lower percentiles of sporulation by nurse plants with and without fertilizer, and starter soil (Control).

The composition of spore morphotypes based on spore shapes and colors are as shown in Figure 50. The composition of spores of treatment NP only was more varied as compared to NP+Fertilizer and Control. It had more than triple the percentage of the globose shape andwhite to pale yellow color spores. As reiterated by (Walker 1999, p. 2), the usual result of STC is a mixed-species of AMF.

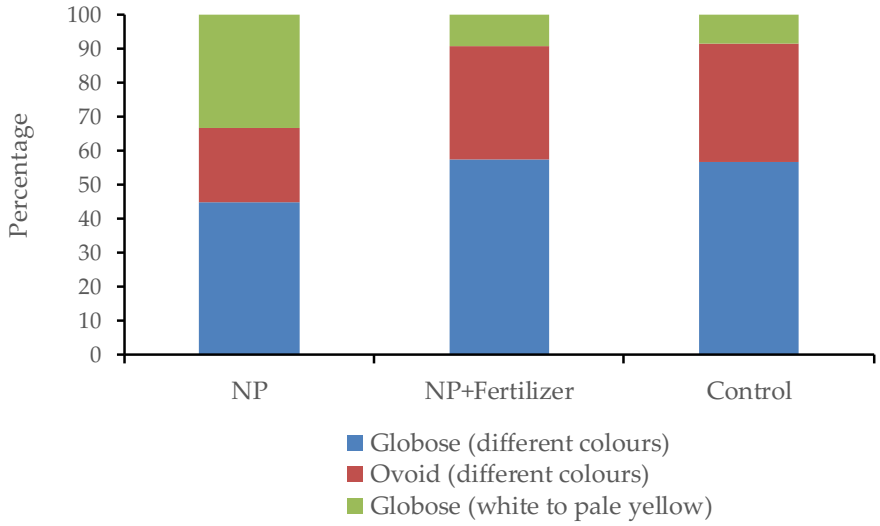
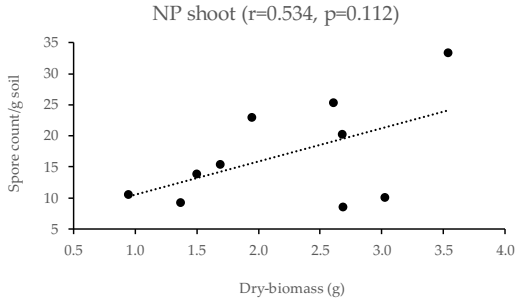


Figure 50. Spore composition of nurse plants with and without fertilizer, and starter soil (Control).

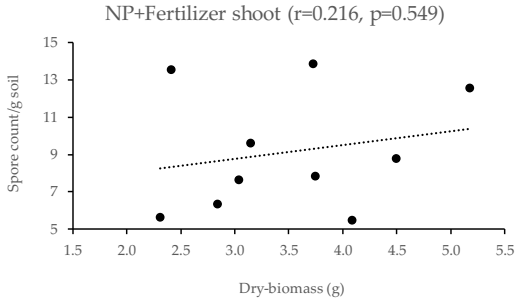
6.4.3 Correlation of total spore counts and nurse plant dry-biomass

The correlation between total spore count and shoot and root dry-biomass of NP with and without fertilizer is shown in Figure 51. The shoot dry-biomass of NP with fertilizer was higher compared to the NP without fertilizer. However, there was no correlation between the pore count and above ground dry-biomass of NP for both treatments.

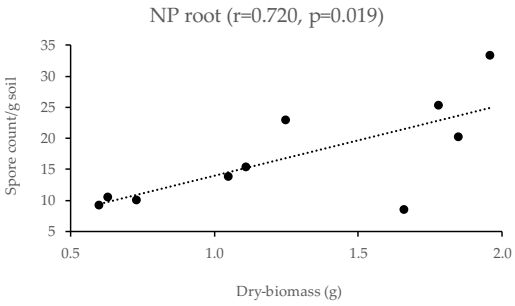
On the contrary the root dry-biomass of NP with fertilizer had less dry-biomass as compared to NP without fertilizer. NP without fertilizer has generally more root dry-biomass than NP with fertilizer except for one of the plants with a root dry-biomass of 3.2 g. Apart from having more root dry-biomass, the root dry-biomass NP without fertilizer was also significantly and positively correlated to spore count with a Pearson correlation of 0.720. The spore count increased with increased root dry-biomass.



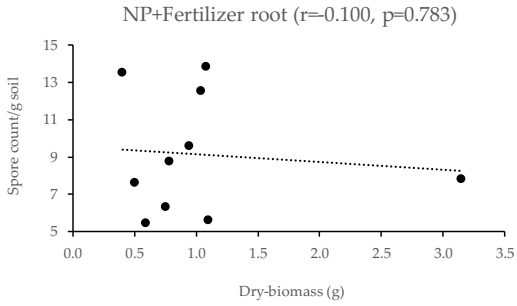
1.



2



3.



4.

Figure 51. Scatter plot of total spore counts and dry-biomass of nurse plants. 1: NP shoot, 2: NP+Fertilizer shoot, 3: NP root, and 4: NP+Fertilizer root.

6.5 Discussion

The local lemongrass variety used in the STC trial was easily available and very easy to cultivate. A lemongrass plantation was shown to have maximum height, total biomass production, and essential oil content after two years of planting, under the treatment of 50 kg N per hectare (Rashmi & Singh 2008, p. 188).

Most households in Sarawak have their own plants in their backyard planted for condiment in every day cooking.

The low P fertilizer formulation used was easy to prepare and the straight fertilizers needed were also readily and cheaply available. The effectiveness of the STC for the production of SMI was gauged by the presence and sporulation of spores presumably either AMF specific or those non-specific to NC. The functional equilibrium model applies to AMF and that fertilization should reduce allocation to arbuscules, coils, and extra-radical hyphae, the fungal structures are directly involved in nutrient acquisition and transfer to plants according to Johansson, Paul and Finlay (2004, p. 1895). In their study to compare AMF responses to N enrichment at five grasslands distributed across North America, it was demonstrated that N enrichment impacts mycorrhizal allocation across a wide range of grassland ecosystems.

The trial indicated that the STC using a local lemongrass variety was able to enhance sporulation of the fungal spores. The spore count of soil with NP was more than double

that of starter soil only (Control). The mean spore count of starter soil with NP and without NP were 19 and 7 spores/g soil respectively. Starter soil with treatment NP+Fertilizer showed less sporulation as compared to NP without fertilizer. The means of spore counts of starter soil with NP and fertilizer was only 9 spores/g soil. NP with fertilizer showed more above ground dry-biomass as compared to NP without fertilizer. However, the root dry-biomass was less than NP without fertilizer. This indicates that the use of fertilizer enhanced lemongrass above ground growth but impeded the root growth. Lemongrass and onion were both colonized by AM fungi and served as good host plants for mass multiplication of *Glomus mosseae, Glomus geosporum and Glomus etunicatum* due to environmental adaptability based on the study by Abdullahi, Lihan and Edward (2014, pp. 118&119). Significant differences in spore density and root colonization were observed between lemongrass and onion nourished weekly with Hoagland's nutrient solution without P at 100 ml/pot, for three months. Lemongrass was the most suitable host plant for mass multiplication of AMF spores as compared to onion for inoculum production. As reiterated by Miyasaka et al. (2003, p. 3), nourishment of NP should contain a low level of P as the AMF will not form associations with it.

The technique can be improved from time to time such as varying the choice of NP used and the various locations of starter soil collection. Okiobe et al. (2015, p. 7), reported that the most favorable conditions for AMF spore production was using a local variety of *Sorghum bicolor* as the host plant in the trial on the effect of Rorison's nutrient solution and the effect of a soil-sand mix variation. On the contrary, the nutrient solution significantly increased root colonization from 5 to 36%, and AMF spore production 12 to 23 spores/g of soil. Other suitable NP tested using a soil-sand mix media included *Cynodon dactylon*, *Sesbania grandiflora*, *Zea mays*, and *Panicum maximum* as reported by Habte and Osorio (2001, p. 10), could be the closest alternatives. Additionally, starter soil collected from the area of interest could be mixed with a disinfected substrate, which could be soil, sand, or expanded montmorilonite clay before planting of the NP (Walker 1999, p. 2)

Apart from having more root dry-biomass, there was positive correlation of spore counts and root dry-biomass of NP without fertilizer. Spore count increased with increase in NP root dry-biomass. The composition of spore morphotypes based on spore shapes and colors also indicated that starter soil with NP without fertilizer was more varied as compared to NP with fertilizer. The average percentage of globose shape with white to pale yellow colour spores was more than triple that of NP with fertilizer.

The alternative method of spore production is by AMF root-organ culture. The use of AMF root-organ cultures allows the aseptic production of spores of various AMF species. Despite the artificial nature of this in vitro system, the fungus forms typical colonization structures; arbuscules, and vesicles and extra-radical mycelium and spores (Fortin et al. 2002, pp. 13&14). The production of spores, morphologically and structurally was similar to those produced in pot cultures. However, empirical tests of many nutrient media and physiological conditions have failed to provide sustained hyphal growth from germinated spores in the absence of host roots. Mass production technologies for AMF were still dependent on various host plants. Kaushish, Kumar and Aggarwal (2011, pp. 2971&2976), reported that lemongrass and farmyard manure were found to be the best tools for the production of AMF in a fertilizer form as farmyard manure was the supply of amino acids needed by the fungi.

6.6 Conclusion

Sporulation of starter soil collected from natural stands of NC at the PP in Kubah N.P. was enhanced by the use of the local lemongrass variety by at least two fold. Lemongrass and onion seedlings were shown to be suitable NP for mass production of AMF spores The use of the low P fertilizer, formulation of N P and K at a ratio of 20:5:10 derived by mixing the proportionate amount of the strait fertilizer of Urea (46% N), Triple Super Phosphate (46% P) and Muriate of Potash (60% K), applied at the rate of 5 g per pot during planting and subsequently five more times fortnightly, seemed to show an adverse effect on AMF sporulation. The fertilizer used enhanced growth of above ground biomass of NP, but was shown to have the opposite effect on root development.

Incidentally, the root dry-biomass of NP without fertilizer was also significantly positively correlated with spore counts. Fertilizer formulation 20:5:10 of N, P, and K used was probably still too high in P content.

The simple and low cost protocol for SMI production using STC in this trial could be used by the FN in Semengoh N.R. and forest nurseries across Sarawak if the need arose. The NP used to host AMF in the inoculum production was fast growing, produced a large quantity of roots within a relatively short time, was hardy and of course was readily colonized by AMF. The use of lemongrass is quite suitable for SMI production as it is easily available, easy to plant and can thrive in most soil types. It is also free from pest and most plant diseases. An improved technique of soil trap culture using lemongrass may have to exclude the use of any form of fertilizer.

7 Pot substrate culture trial and isolation of spore morphotype specimens

7.1 Introduction

The usual result of STC is a mixed-species culture of AMF species. Such a culture can then be used as a base for further purification of AMF specific to NC. Consequently, a pot substrate cultures (PSC) could be carried out. PSC involved a small quantity of substrate from existing STC, either mixed through the disinfected substrate, or added in a depression into which either a mycorrhiza-free plant is placed or seeds are sown. The cultures usually established quickly, and spores were normally produced within a month or two of the sub-culturing attempt (Walker 1999, p. 2). This trial in the thesis used the PSC method to produce AMF spores specific to NC by planting the host plant itself. This trial focused on extracting spores from rhizosphere soil of NC seedlings inoculated with the SMI.

This trial also attempted to test the use of lime to ameliorate the acidic soil by thorough mixing with the planting media. Both soil type and habitat determined the distribution of AMF communities (Alguacil et al. 2016, p. 3354). Below surface lime placement was reported to be effective for correcting soil acidity and enhancing soil microorganism activity (Fuentes et al. 2006, p. 123). The relative abundance and diversity of bacteria were positively related to pH, while fungi were only weakly related with pH. The apparent direct influence of pH on bacterial community composition was probably due to the narrow pH ranges for optimal growth of bacteria as compared to fungal community (Rousk et al. 2010, p. 1). However, below pH 4.5 there was universal inhibition of all microbial variables, probably derived from increased inhibitory effects due to release of free aluminum or decreasing plant productivity (Rousk et al. 2010, p. 12). Faster turnover rates and increased mineralization of organic matter were found in lime-treated than in non-limed soils (Fuentes et al. 2006, p. 123).

Mineral fertilizers significantly influenced the community composition of AMF and ascomycetes in maize roots, and bacteria in root associated soil aggregates. Reductions

in plant growth and richness of both AMF and bacterial taxa, arose from Ammonium sulphate treatment largely appear to be linked to a decline in soil pH (Toljander et al. 2008, p. 335). The liming compound increases soil pH by combining with hydrogen ions in the soil solution. One of the commonly used liming materials is Calcitic lime, a ground limestone composed of mostly Calcium carbonate (Peters, Kelling & Schulte 1996).

7.2 Aim

This trial aim was to determine whether AMF form mycorrhizal roots with NC and that the sporulation through PSC was able to provide distinguishable and definite AMF spore morphotypes. Based on root colonization, AMF spore morphotype specimens were screened and selected for use as spore inoculum. Potential isolates suitable for NC could be identified and characterized.

This trial was also carried out to test whether an improved soil condition from acidic to a neutral state had any effect on AMF root colonization and sporulation. Soil acidification was one of the several proposed causal stresses underlying forest decline due to the effects on AMF and need for remedial liming. Liming stimulated sporulation of several taxa initially apparently absent from the declining site. The quantity of colonization generally increased with pH for both sites (Coughlan et al. 2000, pp. 1543&1546). Root samples from the soil treatments revealed the presence of AMF and root colonization increased at pH 7. Soil pH and the levels of two micronutrients, Mn and Zn, play significant roles in triggering AMF populations.

7.3 Materials and methods

7.3.1 Trial design

This trial encompassed several stages; including collection of starter soil, STC, SMI production, preparation of planting media, and PSC. Root colonization of planted NC was assessed for mycorrhizal colonization which were then correlated with different spore morphotypes isolated. The treatments in this nursery trial were:

1. Inoculated seedling (SMI)
2. Inoculated seedling with liming (SMI+Lime)
3. Non-inoculated seedling (Control)

Each treatment comprised of 10 seedlings. The seedlings were position based on Completely Randomized Experiment arrangement (Chang 1972, p. 27) as shown in Figure 52 below. A table of random digits was used as a guide in the seedling position randomization. The PSC trial is as shown in Figure 76.15 (Appendix).

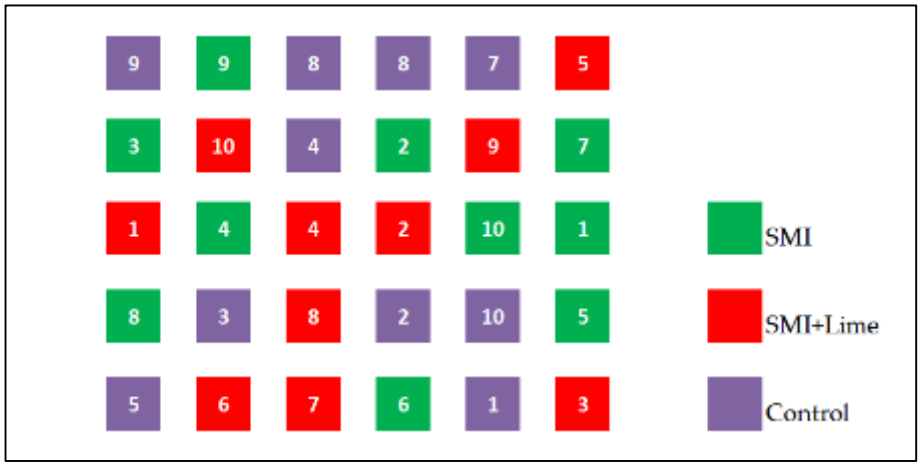


Figure 52. Position of treatments and replicates in the pot substrate culture trial.

7.3.2 Production of soil mycorrhizal inoculum

SMI production was based on a simple soil trap culture method as described by (Sunseed Desert Technology 2012). As reiterated, the infected plant roots and the spores and hyphae of the beneficial fungi in the soil can colonize new plants. Good starter soil can be collected from any undisturbed area containing native vegetation including most grown trees. To multiply the AMF from starter soil, soil trap culture was used. Mycorrhizal dependent plants often called "bait plants" are grown. These plants will become infected with the AMF, and in turn caused the fungal population to multiply.

A local lemongrass variety, *Cymbopogon citratus*, was used as the "bait plant" or NP to enhance the sporulation of AMF following Abdullahi, Lihan and Edward (2014, p. 118).

They reported that lemongrass recorded the highest average mean of root colonization and spore count as compared to onion. Their study showed that AMF-plant interaction was host preference. Lemongrass favored the mass multiplication of *Glomus mosseae*, *Glomus geosporum* and *Glomus etunicatum* and thus, it was considered as more suitable NP as compared to onion for AMF inoculum production.

7.3.2.1 Collection of starter soil

The procedures used for collection of starter soil are as described under Materials and Methods in Chapter 5.

7.3.2.2 Preparation of starter soil

The procedures used for collection of starter soil are as described under Materials and Methods in Chapter 5.

7.3.2.3 Planting of nurse plant

The procedures used for planting of nurse plant are as described under Materials and Methods in Chapter 5.

7.3.2.4 Upkeep of nurse plant

The procedures used for upkeep of nurse plant are as described under Materials and Methods in Chapter 5.

7.3.2.5 Processing of soil mycorrhizal inoculum

The procedures used for processing of soil mycorrhizal inoculum are as described under Materials and Methods in Chapter 5.

7.3.3 Establishment of *Neolamarckia cadamba* seedlings

Preparation of planting media mix, seedlings establishment, and maintenance was based on standard practices adopted by the FN at Sarawak Forest Tree Seed Bank in Semengoh NR. There are three phases in seedling development: establishment, production, and hardening. The establishment phase includes seed germination and first root growth.

The production phase is manifested by rapid shoot growth. During the hardening phase, seedlings are gradually accustomed to field conditions (Jaenicki 1999, p. 12). Seed germination, preparation of planting media, and planting methods were based on standard practices established by the FN at Sarawak Forest Tree Seed Bank Centre, in Semengoh NR and the guidelines prepared for seven nurseries previously under purview of the Sarawak Forest Department (Phang 1991, p. 1).

7.3.3.1 Planting media mix

The procedures used for planting media mix are as described under Materials and methods in Chapter 5.

30 polybags packed with the planting media mix were laid out on gravel base of the FN shade net. Preparation of the planting media also involved testing for soil acidity and the requirement for liming. Most soils in Sarawak are low in pH and liming is necessary to improve the acidic condition to about neutral (pH 7) by using Calcium carbonate ($CaCO_3$).

7.3.3.2 Liming requirement

10 g soil was added to each of the six plastic bottles containing; 0, 5, 10, 15, 20 and 25 ml distilled water. To each bottle, the reversed amounts; 25, 20, 15, 10, 5, and 0 ml of 0.04N Calcium hydroxide ($Ca\ [OH]_2$) were added to a total volume of 25 ml each. The bottles were shaken for 40 minutes before taking readings of the soil suspension by pH 700 pH/mV/^oC/^oF meter (Eutech Instruments, Singapore).

An Excel 2013 Scatter (x, y) graph was used to correlate pH readings and the concentration of 0.04N Calcium hydroxide. The linear regression equation $y = 0.0828x + 6.3567$ (Y=pH reading, x= concentration level of 0.04N Calcium hydroxide) was used to determine the concentration of Calcium hydroxide needed to neutralize soil pH. Liming needed was 0.003 g of Calcium carbonate per g soil of planting media mix.

7.3.3.3 Sowing of seeds

The procedures used for sowing of seeds are as described under Materials and methods in Chapter 5.

7.3.3.4 Application of soil mycorrhizal inoculum

30 polybags filled with planting media mix were prepared for the PSC trial. Prior to transplanting of the NC plantlets, planting media in 20 out of 30 polybags was inoculated with the SMI produced. The amount of the inoculum used was at the rate of 1 to 20 volumes of planting media (Miyasaka et al. 2003, p. 4). The top part of planting media in each polybag was removed and refilled back after applying about 100 ml of the SMI.

7.3.3.5 Transplanting of plantlets

The procedures used for planting of plantlets are as described under Materials and Methods in Chapter 5.

7.3.3.6 Hardening and upkeep of seedlings

The procedures used for hardening and upkeep of seedlings are as described under Materials and Methods in Chapter 5.

7.3.4 Assessment of seedling morphology

Growth of the seedlings was assessed non-destructively by measuring plant height, and root collar diameter. AMF activity was determined by the presence of root colonization through destructive sampling of the roots (Habte & Osorio 2001, p. 16).

7.3.4.1 Seedling height and root collar diameter

The procedures used for assessment of seedling height and root collar diameter are as described under Materials and methods in Chapter 5.

7.3.5 Assessment of soil spores

The main outcome of the PSC trial was derived from the isolation and segregation of dominant spore morphotypes from rhizosphere soil of NC seedlings. The spore

extraction method used was based on the Sucrose Extraction Method (Center for Conservation of Biology 2013). The spore segregation method described by INVAM (2017), was improvised in the thesis to enhance its efficiency.

7.3.5.1 Extraction of spores

The procedures used for the extraction of spores are as described under Materials and Methods in Chapter 4.

7.3.5.2 Segregation of spores

The procedures used for the segregation of spores are as described under Materials and Methods in Chapter 4.

7.3.5.3 Enumeration of spores

The procedures used for the enumeration of spores are as described under Materials and Methods in Chapter 4.

Several attempts were made to group and re-group extracted spores to several morphotypes based on guidelines based on important spore features used to identify Glomalean fungi as described by Brundrett et al. (1996, pp. 144-151). However, due to lack of knowledge and experience, five spore morphotypes were identified primarily because of relative spore abundance and distinguishable morphological features. The five spore morphotype specimens were coded as D1, D2, D3, D4, and D5.

Spores with definite shapes and colors, globose, sub-globose to irregular, and white to pale yellow are referred to as the D1 specimen in the thesis. Henceforth, ovoid reddish brown spores as the D2 specimen, ellipsoid orange spores as the D3 specimen, globose reddish brown spores as the D4 specimen, and globose amber spores as the D5 specimen. Spores with non-matching shapes and colors were grouped under other spore specimens. The pictorial illustration of shapes and colors of the spore morphotypes specimens are as depicted in Figure 53.

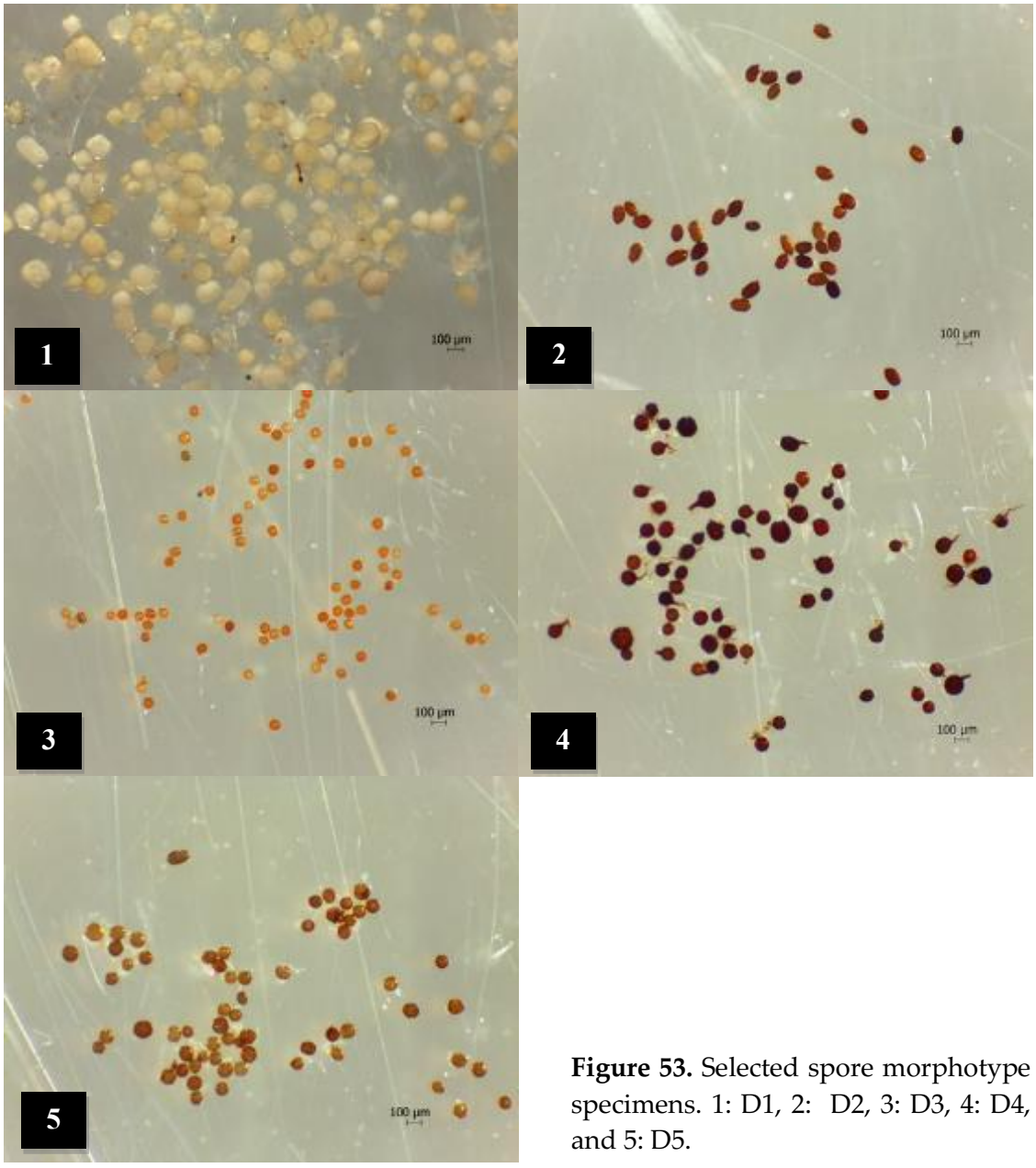


Figure 53. Selected spore morphotype specimens. 1: D1, 2: D2, 3: D3, 4: D4, and 5: D5.

7.3.6 Assessment of seedling mycorrhizal roots

One way to assess the quality of an inoculum is to determine the density of viable spores it contains and also the total number of infective propagules in the inoculum. The quality of inoculum can also be assessed in terms of the degree and the speed with which the inoculum colonizes roots of the host plant, through destructive sampling of roots (Habte & Osorio 2001, p. 16). Fungal structures in a mycorrhizal root cannot be seen without differential staining. Once the root is stained and mounted on a slide, the extent of mycorrhizal colonization can be measured (INVAM 2017).

7.3.6.1 Preparation of root staining and mounting solutions

The procedures used for preparation of root staining and mounting solutions are as described under Materials and Methods in Chapter 5.

7.3.6.2 Sampling roots for staining

The procedures used for sampling roots for staining and mounting solutions are as described under Materials and Methods in Chapter 5.

7.3.6.3 Clearing and staining roots

The procedures used for clearing and staining roots are as described under Materials and Methods in Chapter 5.

7.3.6.4 Preparing diagnostic slides

The procedures used for preparing diagnostic slides are as described under Materials and Methods in Chapter 5.

7.3.6.5 Quantifying root colonization

The procedures used for quantifying root colonization are as described under Materials and Methods in Chapter 5.

7.3.6.6 Taking photographs of root fungal structures

The procedures used for taking photographs of root fungal structures are as described under Materials and Methods in Chapter 5.

7.4 Results

Results of the trial comprised of seedling growth, spore count and the differentiation of spores segregated into morphotypes, assessment of root fungal structures, and correlation of root colonization and spore morphotypes.

7.4.1 Growth of seedlings

The maximum, minimum, mean, median, upper and lower percentile of seedling height are as shown in Figure 54. The growth performance of seedlings was in the order of inoculated seedlings with lime > inoculated seedling without lime > non-inoculated seedlings. Their mean heights were 37 cm, 34 cm, and 32 cm respectively. Inoculated seedlings were the most uniform as compared to the two other treatments.

A similar pattern was observed for seedling root collar diameter maximum, minimum, mean, median, upper and lower percentile as shown in Figure 54. The use of lime seemed to enhance the growth of seedlings in terms of height and root collar diameter. The mean root collar diameters of inoculated seedlings with lime, inoculated seedling without lime, and non-inoculated seedlings were 9.5 mm, 8.8 mm and 8.6 mm respectively. Seedling heights for inoculated seedlings were less variable as compared to the two other treatments.

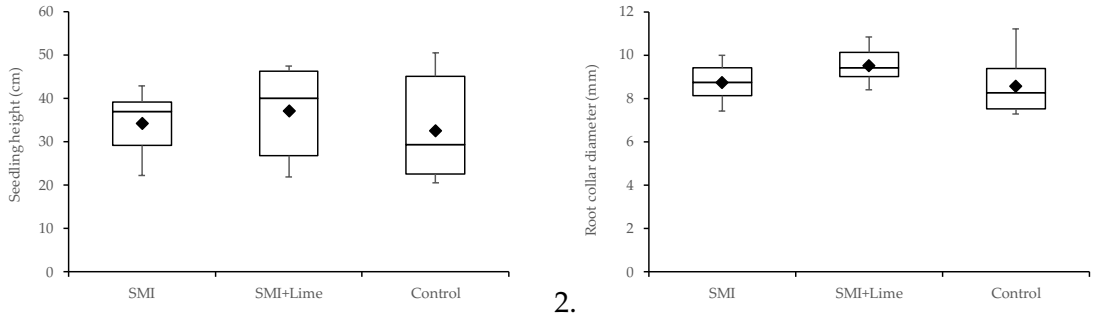


Figure 54. The maximum, minimum, mean, median, upper and lower percentile of inoculated seedlings without and with lime, and non-inoculated seedlings (Control). 1: Seedling heights, and 2: Seedling root collar diameters.

The sturdiness quotients based on mean height in cm and root collar diameter in mm of inoculated seedlings with and without lime and non-inoculated seedlings are as shown in Table 23 below.

Table 23. Sturdiness quotients of inoculated seedlings with and without lime and non-inoculated seedlings (Control).

Treatment	Mean height (cm)	Mean root collar diameter (mm)	Sturdiness quotient
SMI	34	8.8	3.86
SMI+Lime	37	9.5	3.89
Control	32	8.6	3.72

7.4.2 Segregation of spore to morphotypes

Spores were segregated into different morphotype first before doing the spore count. The main morphological characteristics of the spores segregated are as shown in Table 24. Of the five spore morphotypes, spores of the D3 specimen and the D5 specimens were the easiest to distinguish. The next easiest to distinguish were spores of the D1 specimen, followed by the D2 specimen and the D4 specimen.

The spore size was quite variable especially the D4 spore specimen and the D1 spore specimen. The latter was also variable in spore shapes which consisted of globose, sub-globose and presumably irregular ones as well.

Table 24. Main morphological characteristics of spore morphotype specimens.

Specimen	Color	Shape	Size (μm)
D1	White to pale yellow	Globose, sub-globose to irregular	50-120
D2	Reddish brown	Ovoid	50-120
D3	Orange	Ellipsoid	40-60
D4	Reddish brown	Globose	50-150
D5	Amber	Globose	50-80

7.4.3 Spore count

The composition and total spore count per g soil of the different spore specimens are as shown in Figure 55. Total spore count per g soil indicated that inoculated seedlings without lime > inoculated seedlings with lime > non-inoculated seedlings. The spore counts were 2.9, 2.0, and 1.5 spore/ g soil respectively.

Based on composition of spore count, the D1 specimen seemed to be the most abundant of all the spore morphotypes specimens. The D1 spore specimen made up more than 50% of inoculated seedling without lime. The next most abundant spores were those categorized as other spore specimens. The spore count of the other spore morphotypes specimens indicate their small presence as compared to the spores of the D1 specimen and others specimens.

The use of lime was shown to enhance seedling growth but it did not have an effect on sporulation by the inoculated seedlings.

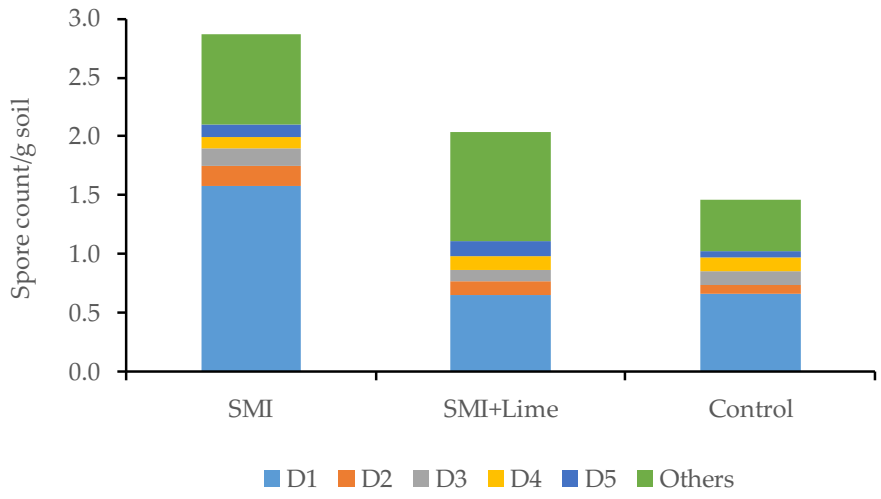


Figure 55. Total spore counts and composition of spore specimens.

7.4.4 Seedling mycorrhizal root

Some fungal structures of stained roots on slides prepared were observed clearly under the compound light microscope but due to limited experience and knowledge the identification of AMF intra-radical and or extra-radical structures was quite fuzzy. A

negative observation was recorded especially when attempts to distinguish root fungal structures namely hyphae were doubtful. Anyhow, observation was done as objectively as possible on fungal structures based on the order of clarity arbuscules > vesicles > hyphae as illustrated in Figure 56. The depiction of the fungal structure in roots of non-inoculated seedlings and the composition of spores of all morphotypes in the spore count indicated that AMF propagules were also present in planting media mix used to raise the NC seedlings at the FN.

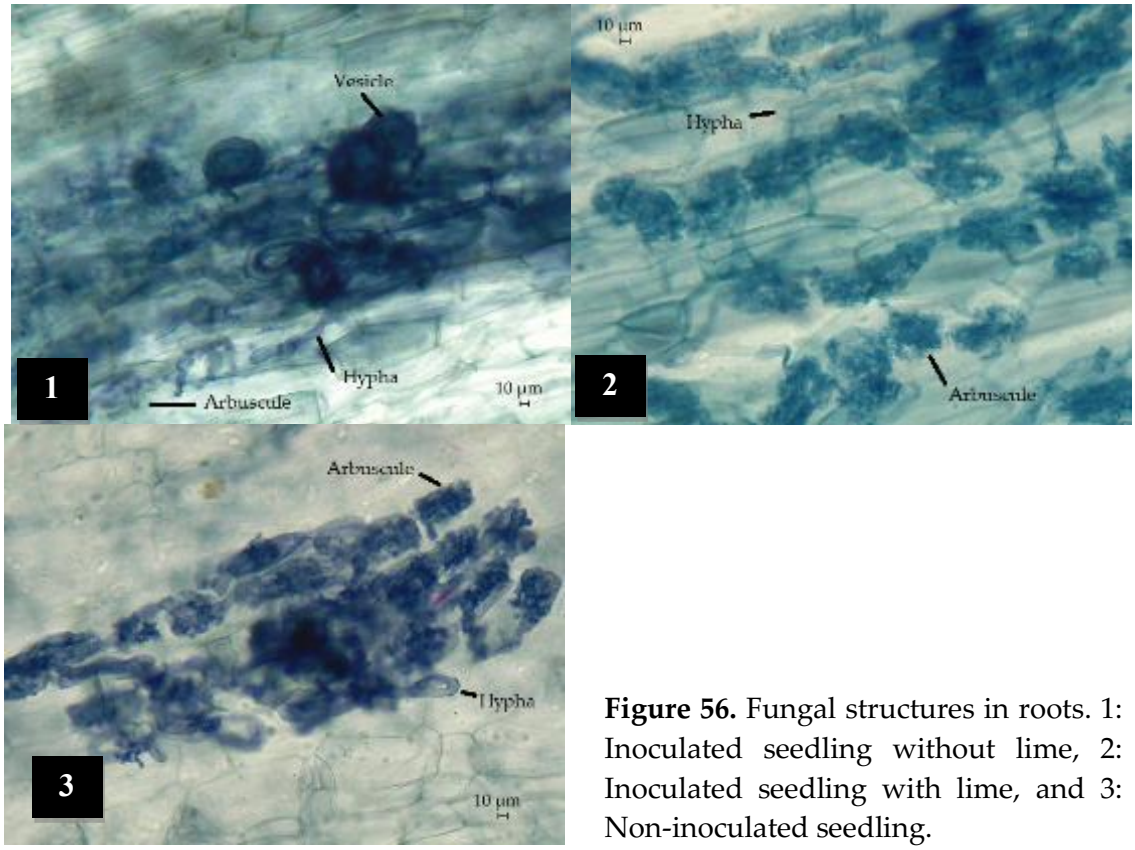


Figure 56. Fungal structures in roots. 1: Inoculated seedling without lime, 2: Inoculated seedling with lime, and 3: Non-inoculated seedling.

The proportion and average percentage of root fungal structures is as shown in Figure 57. The average percentage of root fungal structures was in the order of inoculated seedlings with lime > inoculated seedlings without lime > non-inoculated seedlings. The order of root colonization seemed to be reversed of the spore count of inoculated seedlings with and without lime. However, the average percentage of root fungal structures of inoculated seedlings with lime was largely of the hypha type. Inoculated seedlings without lime had a higher incidence of the more conspicuous vesicles and

arbuscules fungal structures as compared to seedlings with lime. The incidences of arbuscules and vesicles for the former and later were 2% and 1%, 3% and 4% respectively.

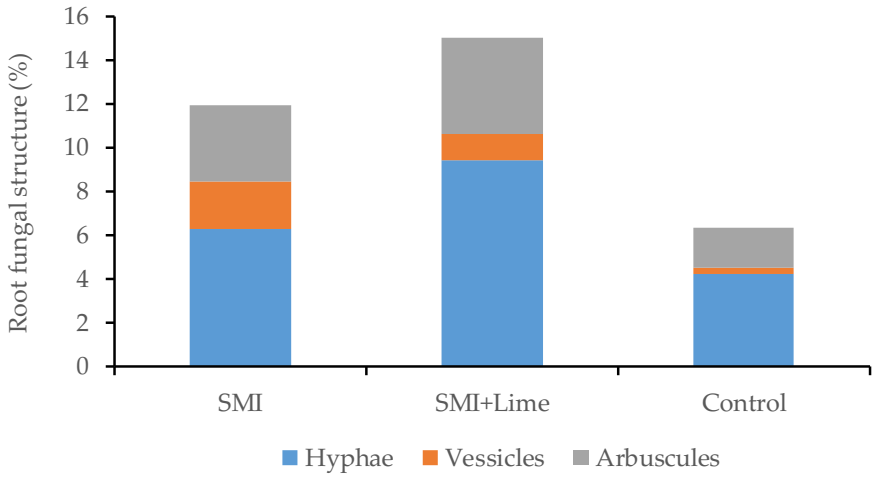
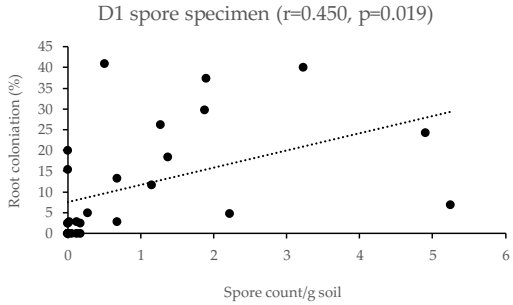
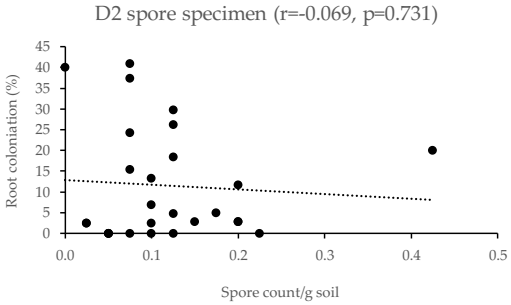


Figure 57. Composition of root fungal structures of inoculated seedlings without lime, inoculated seedlings with lime, and non-inoculated seedlings (Control).

The correlation of the spore counts with percentage root colonization for D1, D2, D3, D4, and D5 specimens, and theother specimens are as shown in Figure 58. The correlation test indicated that there was no definite correlation between spore count and root colonization for all specimens except the D1 spore specimen. Though the correlation coefficient was quite low, with a Pearson correlation of 0.450, the positive correlation was significant (p=0.019). The positive trend probably indicated that the D1 spore specimen may have contributed to the root colonization of NC seedlings.



1.



2.

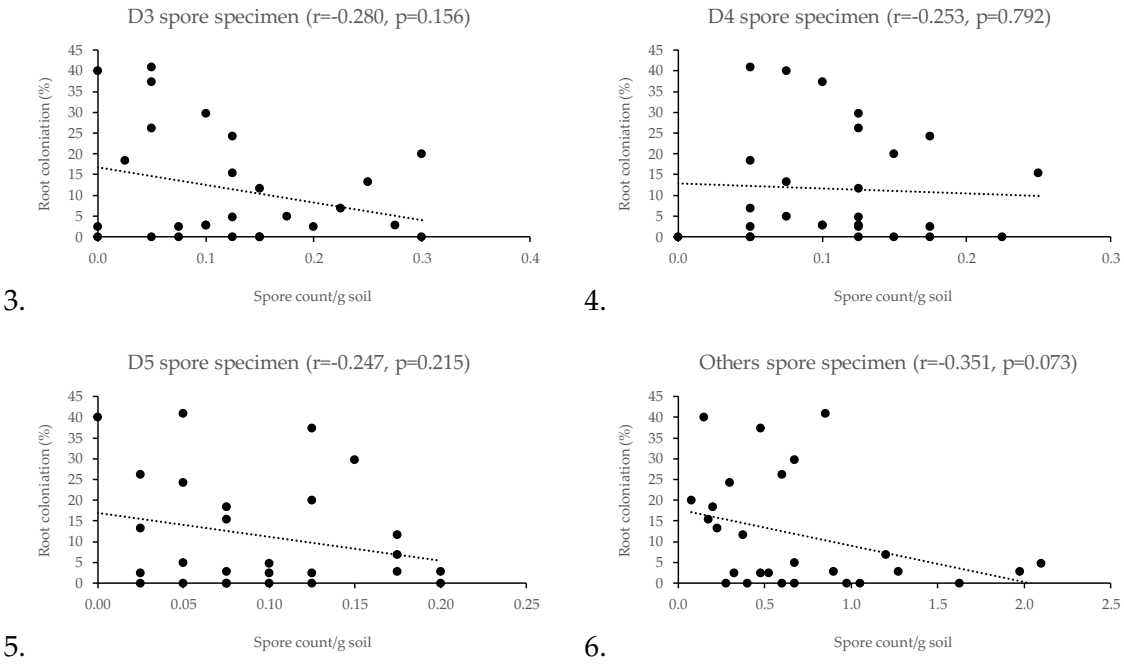


Figure 58. Scatter plot of percentage root colonization and spore counts of spore specimens. 1: D1, 2: D2, 3: D3, 4. D4, 5: D5, and 6: Others.

7.5 Discussion

Potential of AMF spores for use as spore inoculum suitable for NC isolated were screened and selected based on root colonization. The PSC was presumably able to produce distinguishable and definite spore characteristics namely by spore shapes and color. Five spore morphotype specimens coded D1, D2, D3, D4 and D5 were segregated based on the composition of spore counts. The D1 spore specimen was the most abundant whereas the spore counts of other spore specimens were very small.

Though the Pearson correlation of the D1 spore specimen spore count and seedling root colonization was small, r=0.450, the relationship was significant and positively correlated, p=0.019. The relationship pattern, though small might probably indicate that the D1 spore specimen had an influence on the root colonization of NC as all the other spore morphotype specimens including the other specimens showed no correlation pattern at all. Spores of the D1 specimen could be characterized as globose, sub-globose to irregular shape, white to pale yellow color and with size from 50-120 μm.

The trial also carried out a test to determine whether improved soil condition from acidic to neutral state had any effect on AMF root colonization and sporulation. Liming shown to have stimulated sporulation of several AMF taxa which initially were apparently absent from the declining site (Coughlan et al. 2000, pp. 1543&1546). The quantity of colonization generally increased with soil pH for both sites. Root samples from the soil treatments revealed that the presence of AMF and root colonization increased at pH 7.

The depiction of the fungal structures in roots of non-inoculated seedlings and the presence of spore morphotype specimens in the spore counts indicated that AMF propagules were also present in planting media mix at the FN in Semengoh N.R. Spore production is highly dependent on physiological parameters of the AMF and on environmental conditions (Redecker, Hijri & Wiemken 2003, p. 113). Under certain conditions or during certain seasons of the year, some AMF may produce many spores and therefore appear to be dominant root colonizers, whereas under different conditions, they may not sporulate at all. The dynamics of spore production versus root colonization may differ among species. Non-sporulating species may not be detected at all whereas prolific spore-producers dominate the views of AMF ecology.

The use of Calcium carbonate in neutralizing soil pH was beneficial in enhancing growth of NC seedlings in terms of height and root collar diameter. However, liming did not show a great effect on sporulation by AMF. Though overall root colonization of inoculated seedlings with lime was the highest, however, the average root fungal structures were largely of the hypha type and the combination of the conspicuous vesicles and arbuscules structures was about the same as that of the inoculated seedling without lime. Acidic soil initially has larger spore population but lower taxonomic diversity than the healthy site based on a study by Coughlan et al. (2000, p. 1543). Their study showed that liming stimulated sporulation of several taxa initially apparently absent from the declining site spore population. The quantity of colonization generally increased with soil pH. Liming of acidic forest soils, could also possibly further reduce AMF populations due to the development of an AMF community lacking tolerance to a more elevated soil pH.

Al toxicity, along with other pH-driven changes to soil chemistry and biology have a major influence on the microorganism community structure and function, consequently altering nutrient cycling crop productivity and overall ecosystem services (Sullivan, Barth & Lewis 2017, p. 5). It was construed that the imbalance of microorganisms in soils leads to reduced soil microorganism health which impacts cropping system productivity. Liming was shown to have a dramatic impact on soil fertility by decreasing soil acidity that enabled beneficial organisms to flourish.

7.6 Conclusion

The use of SMI produced from soil trap culture at the FN in Semengoh NR, indicated evidence of root colonization of NC seedlings. Sporulation of the fungi propagules namely spores seemed to be related to root colonization and spore count of the D1 specimen. The spores of the D1 specimen were the most abundant and relatively easiest to distinguish. The characterization of the D1 spore specimen is elaborated further in following chapter in the thesis.

The use of lime was able to enhance NC seedling growth. However, liming was observed to limit sporulation of AMF and had a relatively small impact on the root colonization of NC seedlings.

8 Single spore culture trial and characterization of D1 spore specimen

8.1 Introduction

Spores are the most important propagules for most AMF and their impact produced on their hosts will depend on the ability for fast spore germination and colonization (Maia & Yano-Melo 2001, p. 281). Any type of inoculum can be placed close to the seedling roots at the time of transplanting. If a crude inoculum contains four to eight infective propagules per gram, application of 50 g/kg soil usually produces rapid initiation of AMF colonization of target plants with a minimal lag period (Habte & Osorio 2001, p. 15).

The PSC trial carried out described in the preceding Chapter was able to produce distinguishable and definite spore's characteristics namely based on spore shapes and colors. The five spore morphotype specimens coded D1, D2, D3, D4 and D5 were segregated based on the composition of spore counts. The D1 spore specimen was the most abundant compared to spores of other specimens. The D1 specimen spore count was also significantly positively correlated with root colonization though the Pearson correlation, r=0.450 was small. However, the small correlation probably indicated that the D1 spore specimen influenced the root colonization of NC as all the other spore morphotype specimens including the specimen termed others showed no correlation pattern at all. Spores of the D1 specimen were characterized as globose, sub-globose to irregular shape, white to pale yellow color and spore size ranging from 50 to120 μm.

This single spore culture trial (SSC) involved the use of the the D1 spore specimen to inoculate NC seedlings. The amount of spore inoculum applied directly to soil is dependent on the quality of the inoculum (Habte & Osorio 2001, p. 13). Spore inoculum can be pipetted directly onto roots either at the time of transplanting or to roots of an established plant after making a hole adjacent to the roots. A commercial bio-fertilizer product PalmaGro (Felda Agricultural Services, Kuala Lumpur) contained at least 200 mycorrhizal fungi spores for every 10 g of product. The recommended application rate

for oil palm seedlings was 50 g per seedling. The application rate of another AMF mycorrhizal bio-fertilizer was 10 g or 1 spoonful per plant (Nopamornbodi & Thamsurakul 2006, p. 101). AMF bio-fertilizer can be used at any stage of plant growth. However, for maximum benefit it should be applied during the seedling stage or placed at the base of the plant hole before planting.

The number of spores required for inoculation by the commercial bio-fertilizer was about 1,000 spores per seedlings. The spore inoculum used in the study were presumably spores of the D1 specimen. The outcome of the trial on effectiveness of SMI produced as described in Chapter 5 indicated that NC seedlings were able to form mycorrhizal roots namely of the AMF type. However, root colonization was very low at < 13%. Root colonization of maize separately for arbuscular colonization, vesicular colonization, and hyphal colonization were between 10-33%, 0.4-1%, and 19-49% respectively (Mcgonigle et al. 1990, p. 499). Seedlings of a small seeded pioneer species *Luchea seemannii* was also reported to be between 25-36% root colonization (Kiers et al. 2000, p. 110).

The proportion of successes with SSC may be very low depending the condition of the spores. Often field collected spores are contaminated with fragment of hyphae (Walker 1999, p. 3). A fragment of hyphae can adhere to the spore, and this may act as a propagule rather than the spore itself. Quite often a spore appears healthy, but is actually dead and contains a spore of a different species of AMF in it.

8.2 Aim

The main aim of the SSC trial was to determine the infectivity of the D1 spore specimen and its root colonization of NC seedlings. The study also characterized the D1 spore specimen that included spore morphology, viability, and phylogenicity.

8.3 Materials and methods

8.3.1 Trial design

Spores referred to as the D1 spore specimen used in this SSC trial were sourced mainly from STC trial (Chapter 5), and PSC trial (Chapter 6). The treatments in this trial were;

1. Spore inoculum (DI)
2. Spore inoculum with liming (D1+Lime)
3. Without spore inoculum (Control).

Each treatment comprised of four seedlings. The seedlings were position in groups of four according to the treatments as shown in Figure 59. The treatments were placed about 1 m apart to prevent cross contamination. The SSC trial is as depicted in Figure 76.17 (Appendix).

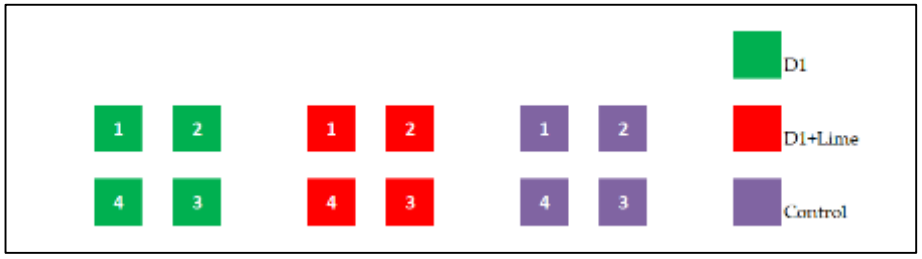


Figure 59. Position of treatments and replicates in single spore culture trial.

8.3.2 Preparation of spore inoculum

About 200 spores of the D1 specimen were needed for spore inoculation per NC seedling. Thus, more than 1,600 spores required for the two treatments of four seedlings each need to be extracted, segregated, and stored properly to be used as spore inoculum. While waiting for NC plantlets to be ready for transplanting, planting media mix was packed in plastic grower pots. The spore collection process was very tedious and probably took about 30 man hours to collate the sufficient number needed.

The spore extraction method used was based on the Sucrose Extraction Method (Center for Conservation of Biology 2013). The spore segregation method described by INVAM (2017) was improvised in the thesis to enhance its efficiency.

8.3.2.1 Extraction of spores

Soils from STC and PSC trials were spread on plastic trays and air dried first for one or two days depending on wetness before sieving through a 2mm steel mesh. 10 g of soil was weighed in a 50ml centrifuge tube, and added with distilled water to its 40ml mark. The 50ml centrifuge tube was shaken vigorously using a vortex machine and left to stand for 15 minutes before centrifuging for 10 minutes at 2,500 rpm using a Table Top Centrifuge (KUBOTA 2420, Japan). Water and floating organic debris were poured out gently from the 50ml centrifuge tube.

The same 50ml centrifuge tube was then refilled with 2M Sucrose-10% Calgon solution to its 40ml mark. The Sucrose-calgon solution was prepared by using 685 g of refined sugar and 100 g of Calgon flaks (Sodium hexametaphosphate) to 1 L water. The mixture was heated up and stirred rapidly until the solution was clear. The tube with 2MSucrose-10% Calgon solution was again put through centrifuge machine for 20 minutes at 2,500 rpm.

Filter paper (Whatman No. 1) was placed inside a filter funnel, and moistened with distilled water. The Sucrose-calgon solution containing spores was gently poured on the filter paper before turning on the vacuum line. Once the solution had filtered through completely, the inside of filter funnel was gently rinsed with distilled water and drained out completely by turning on the vacuum line again. Filter paper was gently removed from the filter funnel and the remaining filtrate was rinsed off slowly with distilled water into a 5cm diameter plastic petri dish.

Each 5cm diameter plastic petri dish comprised of spore extracts from four tubes or 40 g of the same soil sample. Distilled water was further added to about 1/3 height of the petri

dish. Spore suspension was stored in the refrigerator at 4^0C while waiting for subsequent spore segregation.

8.3.2.2 Segregation of D1 spore specimen

Definite "globose, sub-globose," and irregular shapes and white to pale yellow color spores in distilled water suspension were picked individually from root and soil debris, mycelium fragments and also "dead" spores which were black in color. The segregation process was done under a Leica EZ4 HD (Leica M, Singapore) stereo microscope with a 10µl micro-pipette. Spores picked were transferred into a fresh petri dish filled with distilled water. To make the picking of spores slightly easier, the petri dish with spore suspension was placed in a Transsonic 310 sonicator (ELMA, West Germany) for about one minute. Additionally, the spore suspension was agitated using a wooden skewer and let to settle before resuming the spores picking process. Some of the spores were so entangled with masses of mycelium that extra effort was needed to dislodge spores one at a time with an improvised prodder made from SWG 28 Nichrome wire (0.36mm diameter). The spores pipetted were then transferred on to a second clean petri dish filled with distilled water.

8.3.3 Assessment of D1 spore specimen

A direct count method was carried out for the enumeration of spores as the spore density was considered low (INVAM 2017). Spore counts were done under view of a Leica EZ4 HD (Leica M, Singapore) stereo microscope.

8.3.3.1 Spore count and characterization

The spore count was carried out to measured percentages and composition of the D1 spore specimen based on traits as observed on images captured. The spore suspension in the petri dish was swirled several time. Images of spores gathered about the center of the petri dish were captured using the stereo microscope with an LED and HD Camera (Leica M, Singapore) using 35X magnification. Vertical lines or columns with 1.59 cm width were created on the images captured. The percentage spore composition was calculated by the number of the spores crossing or touching vertical lines. The D1 spore

specimen traits were firstly shapes: globose (G), sub-globose (S/G), irregular (I), secondly color: white (W), and pale yellow (PY), and thirdly spore sizes: 50-100µm, 100-200µm as illustrated in Figure 60.

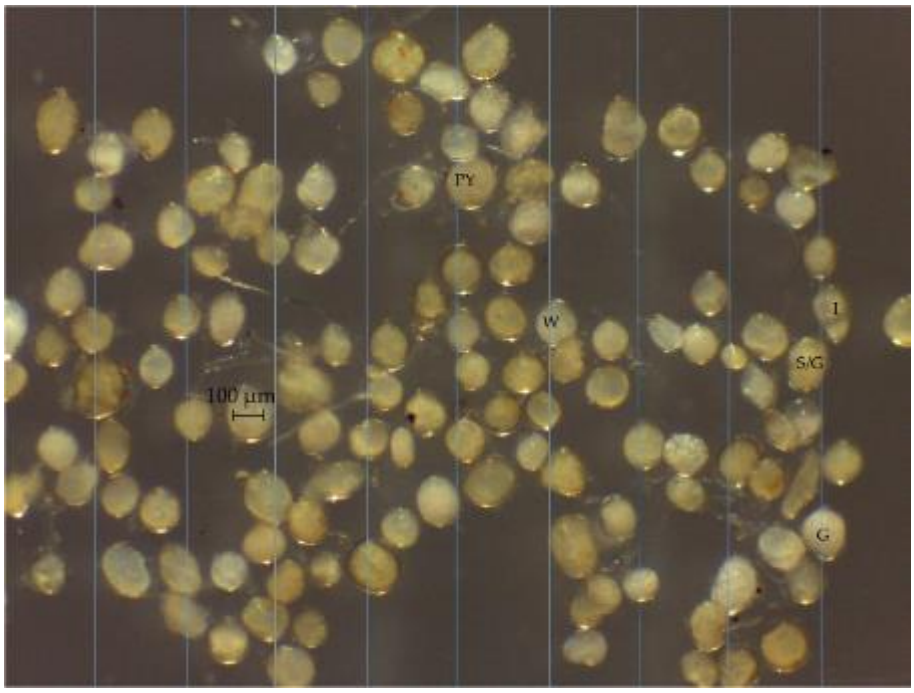


Figure 60. Enumeration of spore characteristics of D1 spore specimen (Shapes: G-globose: S/G-sub-globose, I-irregular, Size: 100µm, and Color: W-white, P/Y-pale yellow).

8.3.3.2 Storage of spores

Segregated spores of the D1 specimen for the SSC trial were sub-sampled first for DNA analysis and a spore viability test. The remaining spores were separated into eight batches of about 200 spores and kept in petri dishes filled with distilled water. The spore suspensions were kept in a refrigerator at 8^{o} C until the NC plantlets were ready for transplanting and inoculation. The D1 spore specimen was also sub-sampled for DNA extraction and a viability test.

8.3.4 Establishment of *Neolamarckia cadamba* seedlings

Before the spore inoculation of seedlings was to be carried out, planting media mix for 12 grower pots was prepared first while awaiting the NC plantlets to be ready for transplanting. Media preparation and seedlings establishment, were based on standard practices adopted by the FN at Sarawak Forest Tree Seed Bank in Semengoh N.R.

8.3.4.1 Preparation of grower pots and liming

The planting media main ingredient was soil stock that was supplied to the FN. The soil supplied before being used in the planting media mixture was first processed by a soil shredding machine. The planting media was a mix of about 15 baskets of shredded soil (about 12 kg/basket), 5 baskets of river sand (about 16 kg/basket) and 20 kg of compost (processed oil palm fruit bunch) were blended together with 3 kg of controlled release fertilizer and instant release NPK fertilizer blend (SK COTE® PLUS 10:26:10:2.5 MgO + TE) and 0.5 kg of Gypsum Rock Phosphate. The blending was done using a motorized concrete mixer. The well mixed planting media was then put in grower pots (L 150-S GAFRI, Malaysia) measuring 150 (D) x 120 (H) mm to a level of about 5 cm below the edge.

Preparation of planting media also involved testing for soil pH and requirement for liming and sterilization of the soil mixture. Soil acidity was corrected by liming or adding Ca amendments to the soil. The lime requirement was based on the amount of Calcium carbonate ($CaCO_3$) or its equivalent that had to be applied to the soil to raise its pH to a certain desired value, about pH 7.

10 g soil each was added to six plastic bottles containing; 0, 5, 10, 15, 20 and 25 ml of distilled water. To each bottles an opposite amount of 0.04NCalcium hydroxide ($Ca[OH]_2$) was added to a total of 25 ml volume. The bottles were shaken for 40 minutes before taking readings of the soil suspension using a pH 700 pH/mV/^{o}C/^{o}F meter (Eutech Instruments, Singapore). An Excel 2013 Scatter (x, y) graph was used to correlate the pH readings and concentration 0.04N Calcium hydroxide. A linear regression equation y = 0.0828x + 6.3567 (Y=pH reading, x= concentration level of 0.04N Calcium hydroxide

derived was used to determine the concentration of Calcium carbonate needed to neutralize soil pH. Liming needed was 0.003 g of Calcium carbonate per g soil of planting media.

About 2.6 g of lime was added to each grower pot meant for treatment with lime. Soil and lime were blended in a plastic bag by shoving it back and forth several times. The grower pots were refilled and kept at room temperature until the planting media mix was sterilized.

8.3.4.2 Sterilization of planting media mix

Limed and non-limed planting media mix in grower pots was emptied separately into glass beakers for sterilization. Soil sterilization was carried out by autoclaving at 120^{o} C for 15 minutes. The autoclaving was repeated twice before the grower pots were refilled back with the sterilized soil. The grower pots were covered with plastic paraffin film and kept secluded at room temperature while waiting for NC plantlets to be ready for transplanting.

8.3.4.3 Transplanting of plantlets

NC seeds registered as SFTSB (P)/01/02/0115 0005(6) from the Sarawak Forest Tree Seed Bank, in Semengoh N.R. were sown and after six weeks the plantlets from germinated seeds measuring about one cm tall were ready to be transferred to grower pots.

Prior to spore inoculation, transplanting of the NC plantlets was done first. NC plantlets of eight grower pots were treated with the spore inoculum of the D1 specimen while the remaining four were left untreated (Control). Plantlets from the germination tray were gently pinched with the germination media to keep their root systems intact. A small planting hole was made using a small stick in which the plantlet was inserted and the soil around it made firm by light pressure with the thumb and index finger.

8.3.4.4 Inoculation of spores

Grower pots with NC plantlets were brought to the laboratory for spore inoculation. Four small holes about one cm deep were made around plantlets. Spores of the D1 specimen were collected using a 100µl micro-pipette under a stereo microscope and deposited in the holes around plantlets about 50 spores per hole. The spore inoculation of NC plantlets is illustrated as shown in Figure 76.18 (Appendix). Holes around plantlets were closed by firming soil with finger pressure.

8.3.4.5 Upkeep of seedlings

The plantlets in grower pots were laid on a raised steel mesh bench with nursery shade net until they developed into seedlings about two and half months old. Watering was done in the morning and afternoon daily by gently hosing with tap water.

8.3.5 Assessment of seedling morphology

AMF activity was determined by the presence of root colonization through destructive sampling of NC roots. Determination of growth of the seedlings was done non-destructively by measuring plant height, root collar diameter, followed by destructive measurement of biomass accumulation (Habte & Osorio 2001, p. 16). Seedlings were kept under nursery shade for about eight weeks before measurement and destructive sampling was carried out.

8.3.5.1 Seedling height and root collar diameter

The procedures used in determining seedling height and root collar diameter are as described under Materials and Methods in Chapter 5.

8.3.5.2 Seedling dry-biomass

The procedures used in determining seedling dry-biomass are as described under Materials and Methods in Chapter 5.

8.3.6 Assessment of mycorrhizal roots

One way to assess the quality of an inoculum is to determine the density of viable spores it contains and also the total number of infective propagules in the inoculum. The quality of inoculum can also be assessed in terms of the degree and the speed with which the inoculum colonizes roots of the host plant, through destructive sampling of roots (Habte & Osorio 2001, p. 16). Fungal structures in roots cannot be seen without differential staining. Once root is stained and mountd on a slide, the extent of mycorrhizal colonization could be measured (INVAM 2017).

8.3.6.1 Preparation of root staining and mounting solutions

The procedures used in preparation of root staining and mounting solutions are as described under Materials and Methods in Chapter 5.

8.3.6.2 Sampling roots for staining

The procedures used in sampling roots for staining are as described under Materials and Methods in Chapter 5.

8.3.6.3 Clearing and staining roots

The procedures used in clearing and staining roots are as described under Materials and Methods in Chapter 5.

8.3.6.4 Preparing diagnostic slides

The procedures used in preparing diagnostic slides are as described under Materials and Methods in Chapter 5.

8.3.6.5 Quantifying root colonization

The procedures used in quantifying root colonization are as described under Materials and Methods in Chapter 5.

8.3.6.6 Taking photographs of root fungal structures

The procedures used in taking photographs of root fungal structures are as described under Materials and Methods in Chapter 5.

8.3.7 Assessment of D1 spore specimen phylogeny

Almost all molecular identification systems for AMF are based on the ribosomal DNA. The genes of this genome region are available in high copy number and possess highly-conserved as well as variable sectors, which allows to distinguish taxa at many different levels. Phylogenetic analyses based on DNA sequences allow direct conclusions about the evolutionary history and the relationship of the taxa in question (Redecker, Hijri & Wiemken 2003, p. 114). Among the regions of the ribosomal cistron, the ITS region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intra-specific variation (Schoch et al. 2012, p. 6241).

Procedures on the selection and cleaning of spores and DNA extraction of spores were based on INVAM (2017). Procedures on nested PCR on extracted spore DNA followed the method described by Lee, Lee and Young (2008, pp. 342&343). PCR product purification followed the instruction manual of Gel/PCR Kit Ver.03.25.14 (GenepHlow, Geneaid Biotech Limited).

A set of new primers designed by Lee, Lee and Young (2008, p. 339) new primers were shown to be much better specificity and to cover all known AMF groups. The small subunit rRNA gene was targeted of the new primers AML1 (ATC AAC TTT CGA TGG TAG GAT AGA) and AML2 (GAA CCC AAA CAC TTT GGT TTC C) as phylogenetic relationships among the Glomeromycota were well understood for this gene. The specificity of the new primers was tested and proven reliable for 23 different AMF spore morphotypes, and non-AMF DNA of plants, with Basidiomycota and Ascomycota as negative control.

8.3.7.1 Preparation of spores

Degraded, dead or parasitized spores were removed from petri a dish kept in the refrigerator for a minimum of one day. Selected spores were placed in a 5cm diameter plastic petri dish containing distilled water and placed on an ultrasonic bath (ELMA Transsonic 310, West Germany) for about 30 seconds to remove debris from the spore surface and to break up any spores that were structurally unsound.

Spores were transfered to a sterilized 5cm diameter glass petri dish. Under a view of a Leica EZ4 HD (Leica M, Singapore) stereo microscope. Debris was removed and the sterile-distilled water was replenished a few times until "immaculate looking" spores remain. Spores selected for DNA extraction were transfered to a new sterile glass petri dish containing fresh sterilized distilled water. The cleaned spores in sterile distilled water were inspected once again and stored at 4^{o}C while waiting for the DNA extraction procedure.

8.3.7.2 Spore DNA extraction

A droplet of cleaned spores was pipetted on a sterile 5cm diameter glass petri dish. To remove as much of the water as possible from around the spores, spores were dragged away from the water droplet using a sterile SWG 28 Nichrome wire (0.36 mm diameter) flattened tip. A small volume about 2.0 µl of Tris EDTA buffer (TE) was dropped on spores to enable picking up using amicro-pipette. With theaide of a stereo microscope single a spore was transferred to a 1.5ml micro-centrifuge. The micro-centrifuge tube with spore seen attached to the bottom was immediately placed in ice before pressing to crush spore against the inside of the tube with a micro-pestle. The crushing of a spore in the 1.5ml micro-centrifuge is as illustrated in Figure 76.19 (Appendix).

Once the release of spore contents has been verified under the stereo microscope, 14 µl of ice cold TE was quickly added. The micro-centrifuge tube contents were shaken and allowed to rest at the bottom of the tube on ice. The tube was immediately immersed in the water bath for four minutes at 94^{o} C and immediately placed on ice once again. The DNA extracted was stored at -20^{o} C or could be amplified straightaway.

8.3.7.3 DNA amplification

100pmol primer stocks of NS1 - GTA GTC ATA TGC TTG TCT C, NS4 - CTT CCG TCA ATT CCT TTA AG, AML1 - ATC AAC TTT CGA TGG TAG GAT AGA, and AML2 - GAA CCC AAA CAC TTT GGT TTC C (First Base Laboratories, Malaysia) were prepared separately by adding sterile dd water to a holding tube containing primer pellets based on the molecular weight of primers. The molecular weight of NS1, NS4, AML1 and AML2, were 5.784 g/mol, 6.018 g/mol, 7.415 g/mol, and 6.654 g/mol respectively. 10pmol primer working stocks were then prepared by adding 10 µl of primer stock with 90 µl of sterile dd water.

For the first amplification, sets of master mix cocktail made up 2 µl DNA extract, 1 µl each of 10pmol NS1 and 10pmol NS4 primers, 10 µl PCR Master Mix REDiant 2X (First Base Laboratories, Malaysia) to a total volume of 20 µl with 6 µl sterile dd water was prepared. A PCR reaction profile of a Gradient Thermal Cycler (Corbett Research, Australia) was set at 94^{o} C for 3 minutes for initial denaturation, followed by 30 cycles of denaturation at 94^{o} C for 30 seconds, annealing at 40^{o} C for 1 minute, elongation at 72^{o} C for 1 minute and final extension period at 72^{o} C for 10 minutes. The first PCR product was then diluted to 1/100 with TE 1X and used as DNA template in the second PCR.

Second sets of master mix cocktail to total volume of 20µl for the second PCR were prepared. The cocktail comprised of 2 µl diluted first PCR product, 1µl each of 10 pmol AML1 and 10pmol AML2 primers, 10 µl PCR Master Mix REDiant 2X (First Base Laboratories, Malaysia), and 6 µl sterile dd water. The Gradient Thermal Cycler (Corbett Research, Australia) was set for the second PCR reaction profile at 94^{o} C for 3 minutes for initial denaturation, followed by 30 cycles of denaturation at 94^{o} C for 1 minute, annealing at 50^{o} C for 1 minute, elongation at 72^{o} C for 1 minute and a final extension period at 72^{o} C for 10 minutes. The second PCR product was stored at -20°C or immediately tested for its quality using gel electrophoresis.

8.3.7.4 Electrophoresis of second PCR product

1.5% agarose gel (1.5 g + 98.5 ml LB solution 20X) was cast in an electrophoresis tray to a depth of about one-third the height of the comb teeth. Gel solidified approximately in 20 minutes. The gel tray was placed in the electrophoresis chamber and sufficient LB solution was added to cover the surface of the gel. The comb was carefully removed and additional buffer was added to fill in the wells created. Each well was loaded with 5 µl of PCR product, leaving a single lane for a 100-bp marker (0.1µg/ µl). The gel electrophoresis was set to run at 90 V for 30-45 minutes. Gel was removed from the tray and stained by soaking in 5µg/ml Ethidium bromide solution for about 10 seconds before soaking in distilled water for about 15 minutes. The mage of the gel stain was recorded using Polaroid film from Bench Top UV Trans-illuminator (UVP BioDoc-IT Imaging System, USA). The result of the electrophoresis was analyzed to determine whether DNA extraction and amplification product was reliable and suitable for DNA purification.

8.3.7.5 PCR product purification

50 µl of PCR buffer (GenepHlow, Geneaid Biotech Limited) with 10 µl of PCR product in 1.5ml micro-centrifuge tube was first vortex. Since the mixture did not turn yellow to purple, there was no need to add 1 µl of 3M Sodium acetate (pH 5.0) as in specified in the instruction manual. Sample mixture was then transferred to DFH Column place in a 2ml collection tube (GenepHlow, Geneaid Biotech Limited). Sample mixture was spun in a centrifuge at 14,000 x g for 30 seconds. The flow-through was discarded, and the DFH Column was placed back in the 2ml collection tube. 60 µl of wash buffer added with absolute ethanol (GenepHlow, Geneaid Biotech Limited) was pipetted into DFH Column and left to stand for 1 minute. The DFH Column and collection tube was spun again in a centrifuge at 14,000 x g for 30 seconds to discard the flow-through. The DFH Column was put back in the 2ml collection tube, spun in a ccentrifuge at 14,000 x g for 3 minutes to dry the column matrix.

The dried DFH Column was placed in a new 1.5ml micro-centrifuge tube and about 5 µl of Elution Buffer and TE (GenepHlow, Geneaid Biotech Limited) added in to the center

of the column matrix. The DFH Column was left to stand for 2 minutes to allow complete absorption and was spun again in a centrifuge at 14,000 x g for 2 minutes at room temperature to elute purified DNA. The purified DNA was stored at -20°C and ready to be dispatched for the sequencing procedure.

8.3.7.6 Spore DNA sequencing

The purified spore DNA was dispatched to a private facility at First Base Laboratories Sdn Bhd, Lot 7-1, Jalan S P 2/7, Taman Serdang Perdana, Seksyen 2, 43300 Seri Kembangan, Selangor, Malaysia. Accompanying the purified DNA were primer AML1 and AML2 which were used in the direct genomic sequencing for both forward and reverse reactions.

8.3.7.7 Spore DNA sequence processing

The spore DNA sequences received from First BASE Laboratories Sdn Bhd were first processed using Sequence Scanner 2 software (Aplied Biosystems 2012). Lower quality data was found at the beginning and end of a sequence. Trimming was done to eliminate these potentially misrepresentative regions from the data needed for subsequent processing. Each sequence trimmed was saved as a FASTA file format.

The trimmed sequences of reverse primer and forward primer were further processed pairwise using a BioEdit Sequence Alignment Editor Software (Hall 1999). The pair sequences were merged as a single FASTA file using an accessory application, "Cap Contig Assembly". The sequences perfectly aligned somewhere in the middle area where both the forward and reverse sequences overlapped. The original forward and reverse sequences were deleted and the 'contig-0' file containing the merged sequences was saved as a FASTA file using the same filename.

8.3.7.8 Basic Local Alignment Search Tool (BLAST) search

To find the most complete set of homologous sequences, a search by similarity of a selected query sequence against a sequence database can be performed using tools like BLAST or FASTA (Bottu 2009, p. 33). The newly saved merged sequences were then

matched to the DNA Sequence Databases for similarity using the Nucleotide BLAST search application at the Internet site of the International Center for Biotechnology Information (NCBI, USA). The BLAST search was set to organism name or identification option before commencing the search.

Organism options in the BLAST search were confined to previous studied AMF species in Sarawak namely *Glomus clarum*, *Glomus fasciculatum*, *Glomus macrocarpum*, *Glomus microcarpum*, *Glomus multicaule*, *Acaulospora laevis*, *Acaulospora scrobiculata*, *Acaulospora spinosa* and *Gigaspora margarita* (Ong et al. 2012, p. 4) and *Glomus mosseae, Glomus geosporum and Glomus etunicatum(*Abdullahi, Lihan & Edward 2014, p. 116).

8.3.7.9 Phylogenetic analysis

Phylogenetic analysis was conducted using MEGA version 6 software (Tamura et al. 2013). The DNA sequences to be analyzed i.e. the 10 AMF species in Sarawak which were obtained from the BLAST search and the D1 spore specimen fragment sequence were first aligned by ClustaW data application. Trimming was done at the beginning and end of all sequences to same length with the D1 spore specimen fragment. A consensus phylogenetic tree was computed using a Neighbouring-joining (NJ) model with bootstrap of 1,000 replicates. *Mortierella polycephala* (Accession no. X89436) and *Endogone pisiformis* (Accession no. X58724) sequences were used as the out group. The NJ method constructs a tree by sequentially finding pairs of neighbors, which are the pairs of Operational Taxonomic Units (OTU) connected by a single interior node. The clustering method minimizes the length of all internal branches and thus the length of the entire tree. The algorithm sequentially connects every possible OTU pair and finally joins the OTU pair that yields the shortest tree (Vandamme 2009, p. 26).

8.3.8 Viability test of D1 spore specimen

Viability determination of AMF spores is of great importance as it concerns SSC, mono-axenic culture, germplasm collection, inoculum production and dormancy studies. Methods for viability determination included MPN estimates, spore germination tests, and spore staining methods (Bansal & Mukerji 2002, p. 217). The viability test of spores

used in the study was developed by (An & Hendrix 1988, pp. 259&260), for determining the viability of endogonaceous spores with a vital stain, -3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT). A stock solution containing 0.5 mg of 98% Thiazolyl Blue Tetrazolium (Sigma, USA) per ml of distilled water was prepared.

To make comparison between dead and viable spores, a 1 ml suspension of 10 spores of the D1 specimen was pipetted into a screw cap glass bottle and killed in an autoclave at 120^{o} C for 15 minutes. Three other bottles were pipetted with 1 a ml suspension of 30 spores each. 1 ml of MTT stock solution was added to the bottles and mixed by Vortex mixer. The screw cap bottles with spore suspension were closed tightly, and incubated at room temperature for 40 hours, 72 hours and about 30 days. The spores were observed with a Leica EZ4 HD (Leica M, Singapore) stereo microscope at 35X magnification. Living spores were stained by MTT whereas spores killed by autoclaving turned blue.

8.4 Results

Spores of the D1 specimen were characterized in terms of morphological attributes, gel electrophoresis of extracted DNA, similarity with sequence databases, phylogenetic tree, spore viability, and its infectivity on NC seedling roots. Seedling morphology in terms of height, root collar diameter and dry-biomass is also described.

8.4.1 Seedling morphology

The mean and standard error of seedling heights and root collar diameters are as shown in Figure 61. The growth performance seedlings were in the order of non-inoculated seedling > spore inoculated seedling without lime > spore inoculated with lime seedlings. Their mean heights were 53 cm, 43 cm, and 23 cm respectively.

An almost similar pattern was observed for seedling root collar diameter except that spore inoculated seedlings without lime were considerably the same as non-inoculated seedlings as shown in Figure 61. The use of lime with the D1 specimen spore inoculation seemed to hamper the growth of the seedlings in terms of height and root collar

diameter. The mean of root collar diameters of the spore inoculated seedlings with and without lime, and non-inoculated seedlings were 7.0 mm, 9.1 mm and 9.0 mm respectively. The result was in contrast to the growth of SMI inoculated seedlings with lime as discussed in Chapter 7 of the thesis. The mean seedling height and root collar diameter of SMI treated with lime seedlings was greater than seedlings without lime treatment.

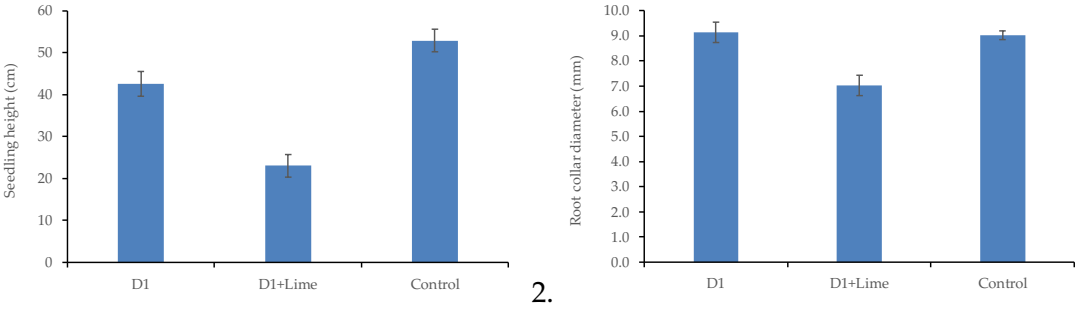


Figure 61. Means and one Standard Error bars. 1: Seedling heights, and 2: Seedling root collar diameters.

The sturdiness quotients based on mean height in cm and root collar diameter in mm of seedlings inoculated with the D1 specimen spore inoculum with and without lime and non-inoculated seedlings are as shown in Table 25 below. A sturdiness quotient higher than 6 is undesirable (Jaenicki 1999, p. 8). Inoculated seedlings especially with lime seem to be slightly sturdier than non-inoculated seedlings.

Table 25. Sturdiness quotients of spore inoculated seedlings without and with lime and non-inoculated seedlings (Control).

Treatment	Mean height (cm)	Mean root collar diameter (mm)	Sturdiness quotient
D1	43	9.1	4.72
D1+Lime	23	7.0	3.29
Control	53	9.0	5.89

The means and standard errors of the shoot and root dry-biomass of spore inoculated seedlings with and without lime, and non-inoculated seedlings are as shown in Figure 62. The mean of the shoot and root dry-biomass of spore inoculated seedlings was generally thesame as non-inoculated seedlings, and was especially much higher in shoot dry-biomass as compared to spore inoculated seedlings with lime. The pattern indicated that liming adversely influenced plant biomass formation. The mean shoot dry-biomass and root dry-biomass were 7.8 g, 8.0 g, 3.6 g, and 2.8 g, 2.7 g, 2.3 g respectively. The result was in contrast to the growth of SMI inoculated seedlings with lime as shown in Chapter 7. The mean seedling shoot and root dry-biomass of SMI treated seedlings with lime was greater than seedlings without lime treatment.

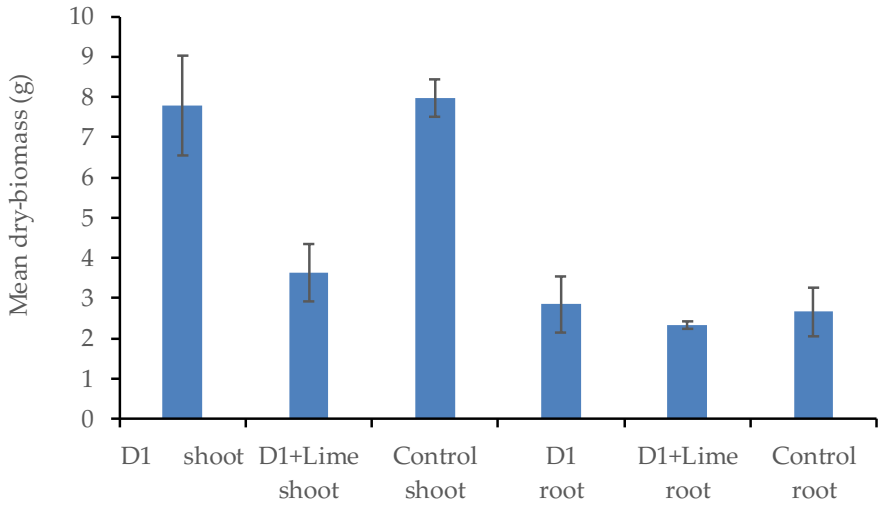


Figure 62. Means and one Standard Error bars of seedling shoot and root dry-biomass.

The root-shoot ratio refers to the proportion of the root dry weight to the shoot dry weight, and reflects the capacity of the roots to support the aboveground biomass not only for anchorage but also in absorbing water and nutrients from the soil. A high root-shoot ratio indicates high a absorption and storage capacity of water, which is an advantage, especially in conditions of limited soil moisture (Budiman et al. 2015, p. 210). The root-shoot ratio of the D1 specimen spore inoculated seedlings with lime was double

that of the spore inoculated seedlings without lime and non-inoculated seedlings based on the means of the dry-biomass of shoot and root is as shown in Table 26.

Table 26. Root-shoot ratios of spore inoculated seedlings without and with lime and non-inoculated seedlings (Control).

.Treatment	Mean of root dry-biomass (g)	Mean of shoot dry-biomass (g)	Root-shoot ratio
D1	2.8	7.8	0.36
D1+Lime	2.3	3.6	0.64
Control	2.7	8.0	0.33

8.4.2 Characterization of spore shape, size and color

The maximum, minimum, mean, median, upper and lower percentile of the morphological characteristics namely shape, size and color of the D1 spore specimens are shown in Figure 63. The spore morphology of the D1 specimen could be generally described as typically of a globose shape, 100-200μm in size and pale yellow in color. The percentage spore counts of the morphological characteristics were 80% globose shape, 84% 100-200μm in size, and 69% pale yellow in color.

The D1 spore specimen was relatively easy to segregate but because of the slight variation in spore color, the collation could be quite confusing. According to Souza (2015, pp. 67-69), AMF spore size can vary from 22-1,050 μm and some genus can be classified based on this characteristic. The nine described spore shapes were; globose, sub-globose, elliptical, oblong, ovoid, irregular, triangular, knobby and pulvinate. Other unofficial denominations were funnel-shaped, rounded dome-shaped or tear-drop shaped. Spore shape may vary among species, and within species such as the globose, sub-globose and irregular spores of *Funneliformis mosseae.* As for color, there were many variations between AMF spores, varying from white to black, with variants like bright, light, pale dark. There were variations in colour even in spores of the same species such as *Acaulospora capsicula* which could be dark red-brown and orange brown depending on spore maturity and integrity.

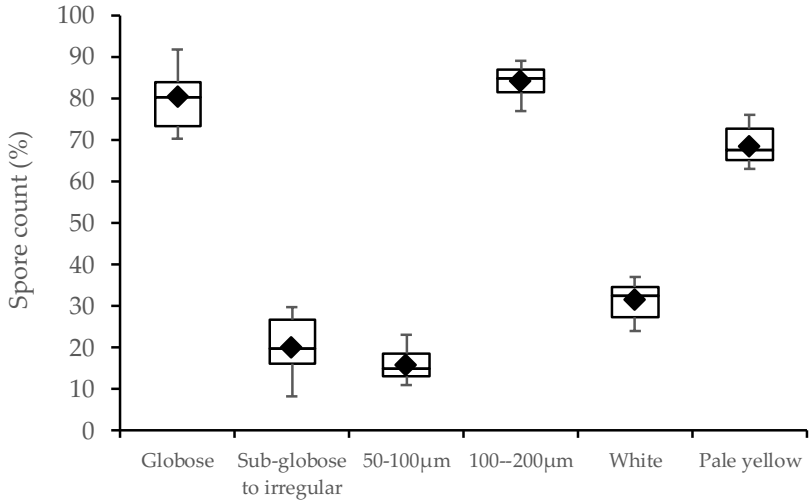


Figure 63. The maximum, minimum, mean, median, upper and lower percentile of percentage spore count of D1 spore specimen's shape, size, and color.

8.4.3 Gel electrophoresis

The agarose gel electrophoresis separates DNA fragments according to their sizes. The two best specific priming sites for AMF were found near 300 and 1100bp on the SSU rRNA gene and named AML1 and AML2, respectively (Lee, Lee & Young 2008, pp. 241&242). Amplified sequence length with primer pairs NS1–NS4, AML1–AML2 and NS31–AM1, were about 1,100bp, 795bp and 550bp respectively.

The gel electrophoresis results of the amplified D1 spore specimen sequence from first PCR using a primer pair of NS1-NS4 and second PCR using a primer pair of AML1-AML2 are as shown in Figure 64. The primers used were able to amplify the appropriate sequence lengths of the D1 spore specimen. Molecular research on exploration of genetic variation in AMF varies in systematics and taxonomy though tools for field sample identification are continually being developed (Reddy, Pindi & Reddy 2005, p. 1701). This broad research area almost entirely relies on PCR amplification of the rDNA sequences, with nuclear encoded small subunit, 17S/18S rDNA being the most common.

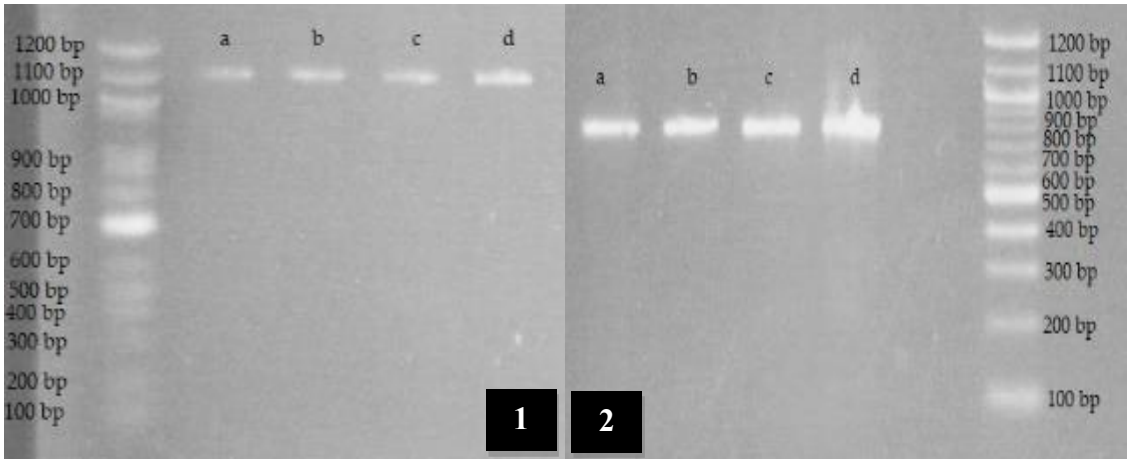


Figure 64. Gel electrophoresis results of amplified D1 spore specimen's DNA replicates. 1: First PCR, and 2: Second PCR.

7.4.1 BLAST search

Sequence similarity searching, typically with BLAST is the most widely used, and most reliable, strategy for characterizing newly determined sequences. Sequence similarity searches can identify "homologous" proteins or genes by detecting statistically significant similarity that reflect common ancestry (Pearson 2013, p. 1).

A sequence similarity search was made for the D1 spore specimen sequence and sequence databases of AMF species in Sarawak as reported by Ong et al. (2012, p. 4) and Abdullahi, Lihan and Edward (2014, p. 116). The result is as shown in Table 27. Out of the 12 AMF species involved in the BLAST search, only 10 were found at the NCBI database. The percentage similarity was in the order of *Acaulospora scrobiculata* > *Glomus fasciculatum/Acaulospora spinosa* > *Glomus macrocarpum/Acaulospora laevis/Gigaspora margarita/Glomus geosporum/Glomus etunicatum* > *Glomus clarum/ Glomus mosseae.*

Table 27. Percentage sequence similarities of D1 spore specimen with the arbsuscular mycorrhizal fungi species in Sarawak.

Species	Accession number	Sequence similarity (%)
Glomus clarum,	AJ852597.1	614/720 (85%)
Glomus fasciculatum	Y17640.2	586/677 (87%)
Glomus macrocarpum	FR772325.1	585/682 (86%)
Glomus microcarpum	Not available	Not available
Glomus multicaule	Not available	Not available
Acaulospora laevis	FR750214.1	607/690 (86%)
Acaulospora scrobiculata	KP144305.1	602/687 (88%)
Acaulospora spinosa	KU900348.1	634/729 (87%)
Gigaspora margarita	HF968773.1	626/725 (86%)
Glomus mosseae	U96144.1	622/731 (85%)
Glomus geosporum	AJ245637.1	589/682 (86%)
Glomus etunicatum	AJ852598.1	631/730 (86%)

8.4.4 Phylogenetic tree

A phylogenetic tree, also known as a phylogeny, is a diagram that depicts the lines of evolutionary descent of different species, organisms, or genes from a common ancestor. Phylogenies are useful for organizing knowledge of biological diversity, for structuring classifications, and for providing insight into events that occurred during evolution (Baum 2008).

The phylogenetic tree of the D1 spore specimen is as shown in Figure 65. It seems that the spore of D1 specimen was clustered together with the family of Glomeraceae *Glomus fasciculatum* (Y 17640.2), *Glomus clarum* (AJ852597.1), *Glomus geosporum* (AJ245637.2), *Glomus macrocarpum* (FR772325.1), *Glomus mosseae* (U96144.1), and *Glomus etunicatum* (AJ852598.1). There are two families of AMF under the order Glomerales, Glomeraceae and Entrophosporaceae. The current family of Glomeraceae has a type AMF species which is *Glomus macrocarpum* (Souza 2015, p. 117). Based on branch length, the D1 spore specimen is closest related to *Glomus mosseae* (U96144.1), and *Glomus etunicatum* (AJ852598.1).

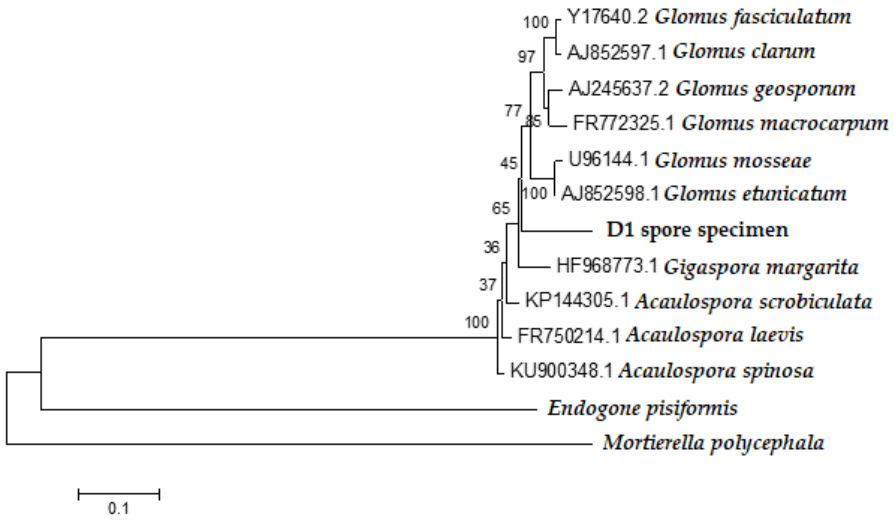
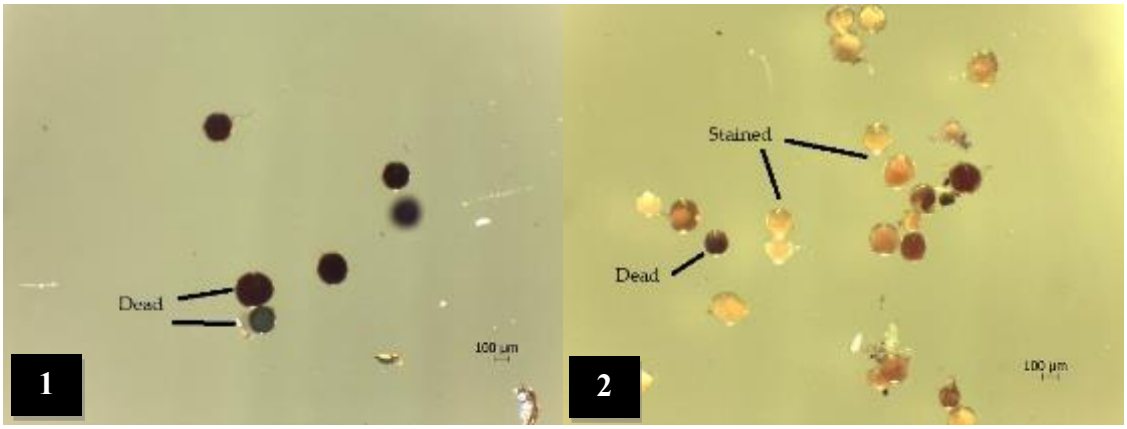


Figure 65. Phylogenetic tree of D1 spore specimen.

8.4.5 Spore viability

The outcome of the viability test of the D1 specimen with MTT is as illustrated in Figure 66. The figure shows spore condition after 40 hours of incubation of spores killed by autoclave, and other spores after 40 hours, 120 hours and > 30 days of incubation. Dead spores such as those that were killed by autoclave were stained black or dark blue while those presumably alive were stained reddish.



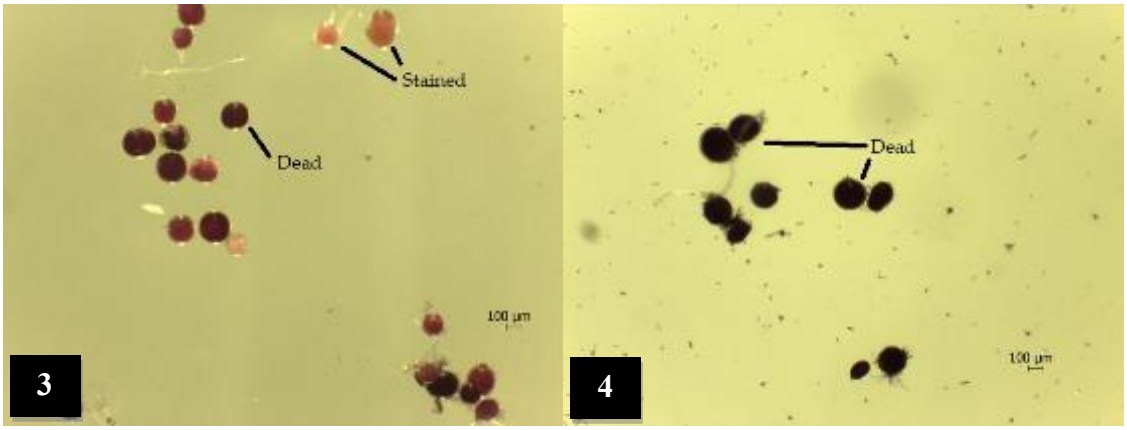


Figure 66. Condition D1 spore specimen and at different incubation periods. 1: Autoclaved spores, 2: 40 hours, 3: 120 hours, and 4: > 30 days.

Viability of spores stored in refrigerator at 8° C and incubated over time at room temperature is as shown in Figure 67. The viability test indicated that average percentage spore count of viable spores was about 95% after incubation at room temperature for 40 hours. Prolonged condition of spores under room temperature resulted in a deterioration of spore viability with the reduction of viable spore count. All of the spores incubated at room temperature perished after about one month.

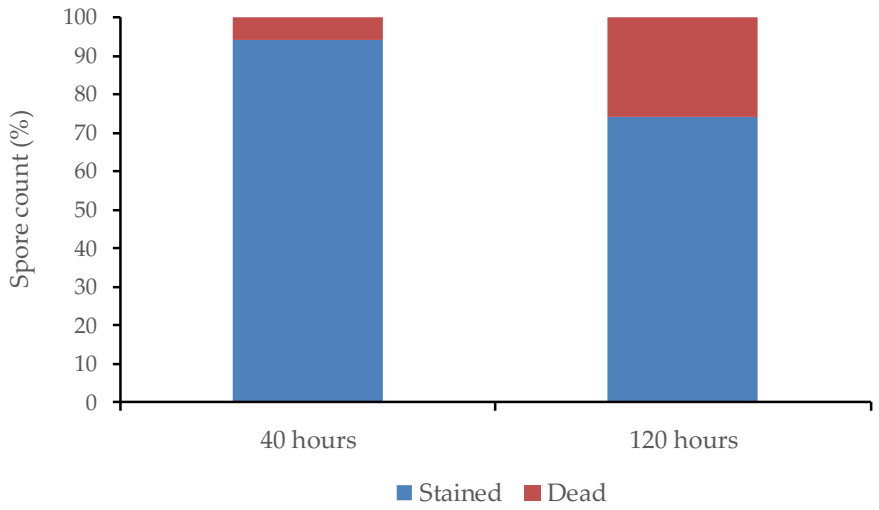


Figure 67. Stained and dead spores after 40 hours and 120 hours of incubation.

8.4.6 Seedling mycorrhizal roots

The sterilization of planting medium seemed to eliminate completely all inherent fungal propagules as fungal structures of stained roots mounted on glass slides were hardly seen. However, careful observation under the compound light microscope indicated some form but a quite fuzzy observation of root colonization. Observation was done as objectively as possible and fungal structures of interest in order of clarity were arbuscules, vesicles and hyphae. However, the D1 spore specimen presumably yielded only hyphal structure. Hyphae that were thin, smooth edged and septate were considered as non-AMF (Figure 68).

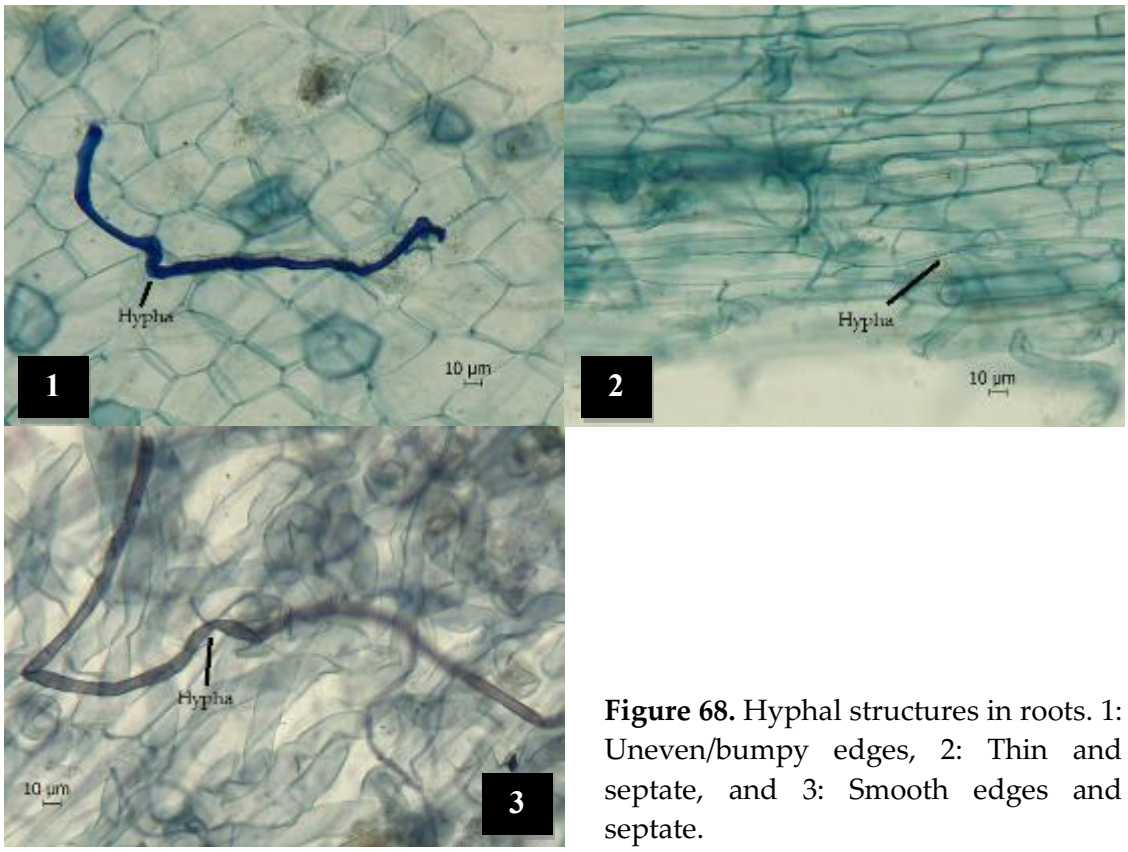


Figure 68. Hyphal structures in roots. 1: Uneven/bumpy edges, 2: Thin and septate, and 3: Smooth edges and septate.

The percentages of root colonization by spores of the D1 specimen NC roots in planting media mix with and without liming was very low (Table 28). Even though, planting medium was sterilized, seedling roots were also colonized by other non-AMF. Segregated spores were probably mixed with spores and mycelium of fungi not related to the D1 spore specimen. The variations in spore shape, size, and color, of the D1

specimen probably indicates the presence of other fungal entities. The variation in spore morphology of the D1 specimen could also result in the inclusion of spores from some other endophyptic fungi besides Glomeromycota during the spore segregation procedure.

Table 28. Root colonization of spore inoculated seedlings without and with lime, and non-inoculated seedlings (Control).

Treatment	Replicate	Fungal structure	Root colonization (%)
D1	1	Hypha	1.7
	2	Hypha	1.7
	3	None	0
	4	None	0
D1+Lime	1	Hypha (Thin and septate)	21.7
	2	Hypha	1.8
	3	Hypha (Smooth edges and septate)	1.8
	4	Hypha (Septate)	5.5
Control	1	None	0
	2	None	0
	3	None	0
	4	None	0

8.5 Discussion

The main aim of this trial in the thesis was to determine the infectivity of the D1 spore specimen by its root colonization of NC seedlings. The trial also included a study to characterize the spores of the D1specimen based on its spore morphology, viability, and phylogeny analyses. Enumeration and identification of AMF spores is a difficult and time consuming exercise for most researchers.

The NC seedling morphology in terms of mean of heights and root collar diameters showed similar pattern whereby inoculated seedlings with lime was the shortest and smallest as compared to inoculated seedlings without lime and non-inoculated seedlings. However, the dry-biomass showed differences. The mean of root dry-biomass

of spore inoculated seedlings without lime was very low as compared to seedlings with lime and seedlings which were non-inoculated. Spore inoculum of the D1 specimen may have an influence on the physiology of NC seedlings and their root formation. The fungal community composition was less strongly affected by soil pH, which was consistent with pure culture studies, demonstrating that fungi generally exhibit a wider pH range for optimal growth (Rousk et al. 2010, p. 1).

Spores of the D1 specimen were sourced from STC and PSC trials. Due to the spore abundance and distinguishable morphology, with an unclear hyaline edge, white to pale color, the D1 spore specimen was comparatively easy to segregate and collate. The D1 spore specimen could be generally describes as typically of globose shape, 100-200µm in size and pale yellow in color. AMF have traditionally been identified by the morphology of their spores. The limitation of morphological identification is that field-collected spores are often parasitized or degraded and therefore unidentifiable. This problem can be circumvented by setting up STC where soil samples from the field site are brought into contact with suitable plant hosts of AMF under controlled conditions in order to propagate the species occurring at the field site and to obtain fresh spores of all developmental stages (Redecker, Hijri & Wiemken 2003, pp. 113&114).

The only way to identify AMF before molecular techniques was by a careful microscopic examination of the spores. Unfortunately, spores are relatively simple structures that offer only a limited number of potential discriminating features. A single AMF culture can sometimes produce spores that differ substantially in morphology and color, and spores found in soil are often not in pristine condition so they may not match fresh reference material produced under controlled conditions. Reliable identification requires a good deal of patience and experience, and only a handful of experts worldwide can confidently identify the whole range of AMF by examining spores (Young 2012, p. 823). PCR-based techniques have become mandatory to obtain sufficient quantities of DNA, as these microorganisms are non-culturable and thus only a small quantity of DNA can be isolated from spores and infected roots.

The BLAST search results indicated that the D1 spore specimen DNA sequence similarity to selected AMF species found in the NCBI database was not very high. This could be due to the effect on the re-suspending procedure of the freeze-dried oligonucleotide primers during DNA amplification. Primers are usually shipped in dry form and the dried DNA pellet could become dislodged from the bottom of the tube during shipping and it could also easily fly out of the tube when first opened as electrostatic attraction is present. Brief centrifuging should be done first before opening the shipped tube for the first time (Binder & Hibbett 2003). Nevertheless, the phylogenetic tree derived for the DNA sequence of the D1 spore specimen and DNA database in the NCBI of the selected AMF species in Sarawak indicated that the D1 spore specimen was clustered together with the family of Glomeraceae namely the *Glomus fasciculatum*, *Glomusclarum*, *Glomus geosporum*, *Glomus macrocarpum*, *Glomus mosseae*, and *Glomus etunicatum*, and the closest being the last two AMF species.

Spores of the D1 specimen and those incubated with MTT stain over time at room temperature, were differentiated into black or dark blue stain by dead spores and those presumably still alive which were shown by a reddish stain. The viability test indicated that spores kept under prolonged time at room temperature resulted in a deterioration of the spore viability and completely diminished after about one month. Thus, spores extracted need to be kept refrigerated at a maximum temperature of 8^{o} C to maintain their viability. After spores of AMF have been isolated from soil or inoculum, their germination should be assessed. Commonly, not all the spores of AMF are ready to germinate and infect host plants. This is because spores exhibited a stage of dormancy in which they do not germinate until conditions for growth and development are favorable (Habte & Osorio 2001, p. 25). However, some spores were unable to germinate even under favorable conditions, a phenomenon known as innate dormancy. It can persist for a few days to months. Innate dormancy can be overcome by treatments such as slow drying, cold treatment at 4^{o}C, or soaking in water.

An alternative method to determine spore viability is the spore germination test by using Sodium hypochlorite placed on a millipore filter over a 1% water-agar medium in a petri

dish, and evaluated every seven days. Spores maintained in a water-agar medium were observed to have presented longer germ tubes than spores in the other treatments (Maia & Yano-Melo 2001, p. 281). The staining method using MTT works with uniformly stored spores using specific incubation times, but not with spores of mixed ages, which could include young spores. Thus, MTT may not be useful as a viability stain for field isolated spores (Meier & Charvat 1993, p. 1007).

The viability of the D1 spore specimen used in the NC root infectivity test or SSC trial was presumed to be at 80% based on spore incubated with MTT stain for 40 hours at room temperature. Juge et al. (2002, p. 37), indicated that cold stratification for 14 days at 4^{o} C significantly increased spore germination. Longer cold storage periods reduced spore mortality from 90% to 50% but considerably altered the hyphal growth pattern. The cold environmental factors affect the physiology of AMF spores. McGee et al. (1997, p. 773), reported that, 16-21% of spores from field soils were found to be viable in fresh soil and 6-7% after 24 months of storage by using tetrazolium red as the vital stain. The density of viable propagules of AMF in soil declined over time and was reduced by severe disturbance.

Root colonization by spores of the D1 specimen was very low. The sterilization of planting media seemed to eliminate completely all inherent fungal propagules as fungal structures of stained roots mounted on glass slides were hardly observed. The percentage root colonization of NC by the D1 spore specimen with and without liming was very low and seedling roots were also colonized by other non-AMF spores. Segregated spores were probably mixed with some other endophyptic fungi besides the Glomeromycota. The observation of fungal root structures could probably be improved by using proper root staining techniques. Vierheilig, Schweiger and Brundrett (2005, p. 402), opined that for light microscopic studies with a focus on the morphology of the intra-radical structures of the AMF, the technique that results in images with the highest contrast was the CBE stain method in combination with interference contrast microscopy. Some aspects of the beneficial role of AMF in overall plant growth under diverse edaphic conditions, have been thoroughly investigated.

The study on the taxonomy of the AMF in this thesis was just preliminary and it could be pursued further when purified isolates are more certain. The need to do so was also dependent on the bottom line of the thesis which was to test the effectiveness of SMI on NC seedlings at planting out as compared to non-treated seedlings. Some aspects like their beneficial role in overall plant growth under diverse edaphic conditions, have been thoroughly investigated. However, the biosystematics, culturability and field performance evaluation of this group of fungi are still in some areas lagging behind for want of suitable techniques, which have become hurdles fto the further progress of research (Reddy, Pindi & Reddy 2005, p. 1699).

8.6 Conclusion

The spores of the D1 specimen could be generally describes as typically of a globose shape, 100-200µm in size and pale yellow in color. The phylogenetic tree indicated that the D1 spore specimen was clustered together with the family of Glomeraceae and the closest being *Glomus mosseae* and *Glomus etunicatum*.

Extracted spores need to be kept in refrigerator at a temperature of at least 8^{o} C to avoid spore viability to deteriorate and completely diminish if kept too long in a non-refrigerated condition. The viability of the D1 spore specimen used in the NC root infectivity test was presumed to be at 80% based on spore incubated with MTT stain for 40 hours at room temperature. However, root colonization was very low. The sterilization of planting medium seems to eliminate completely all inherent fungal propagules and other soil microorganisms. The percentage root colonization of NC by the spore inoculum with and without liming was very low. Seedling roots were also colonized by other non-AMF presumably mixed with propagules of some other endophyptic fungi besides Glomeromycota.

Lastly, a variety of AMF spores are commonly encountered in the vicinity of the host roots. It is possible that more than one AMF may colonize the roots simultaneously.

9 Re-propagation culture trial and cellular assessment of D1 spore specimen

9.1 Introduction

AMF are usually propagated by growing them with a living host plant in PSC (Brundrett et al. 1996, p. 162). Large populations of homogeneous spores are frequently necessary to conduct research with AMF, such as spore germination and storage, seedling inoculation and field application. Thus, a broadly applicable method to enhance sporulation of AMF in PSC was needed (Douds & Schenck 1990, p. 413)

A re-propagation culture (RPC) or additional propagation cycle was carried out for young PSC where spore inoculum used showed poor infectiveness based on a root colonization assessment. In the RPC, the pot content of SSC was not disturbed to maintain hyphal networks and spore-hyphal aggregates, and seeds of the same host were re-planted. The rate of 50-60% of first parent culture seems to suffice for the additional propagation cycle (INVAM 2017).

This RPC trial in the thesis used soil substrate from SSC of NC using spore inoculum of the D1 specimen as described in Chapter 9. The RPC trial re-utilized lemongrass as NP as it was used before in STC for the production of SMI as described in Chapter 5. The best NP tested using a soil-sand matrix were *Cynodon dactylon*, *Sesbania grandiflora*, *Zea mays* followed by *Panicum maximum* as reported by Habte and Osorio (2001, p. 10). However, lemongrass was easily available, fast growing, produces a large quantity of roots within a relatively short time, was hardy and of course, could be readily colonized by AMF. According to Abdullahi, Lihan and Edward (2014, p. 118), lemongrass (*Cymbopogon citratus*) and onion (*Alium cepa*) seedlings were also suitable candidates for mass production of AMF spores. Both crops were reported to be suitable NP for AMF due to their fast growth; and ability to produce numerous fine and hairy roots for abundant sporulation.

The study on the taxonomy of AMF based on spore morphology of the D1 specimen was also carried out on spores produced from the RPC trial. However, the taxonomy study was just preliminary and it can be pursued further when purified isolates are more certain. The need to do so is dependent on the effectiveness of the SMI and spore inoculum of the D1 specimen to not only colonize roots of NC seedlings treated, but also with the SMI treatment, whereby inoculated seedling adapted and performed better at planting out as compared to non-treated seedlings.

9.2 Aim

The aim of this trial in the thesis is to determine whether the re-propagation culture substrate from SSC trial as described in Chapter 8, on inoculated NC seedlings will boost sporulation of the D1 spore specimen.

The second aim was also to characterize spore morphotype D1 specimen at a cellular level and to determine its similarity to an on-line database of spore specimen voucher collection. Enumeration and identification of the D1 spore specimen was done morphologically.

9.3 Materials and methods

Substrates from the SSC trial using spore inoculum of the D1 specimen were used in the RPC trial. Local lemongrass, *Cymbopogon citratus* variety was re-used as NP to enhance the sporulation of the AMF following Abdullahi, Lihan and Edward (2014, p. 118). However, no fertilizer was used in this RPC trial in contrast to the STC trial described in Chapter 6. The STC trial indicated that the use of low P fertilizer formulation did not enhance sporulation.

9.3.1 Trial design

The re-propagation culture involved two treatments as follows:

1. Limed substrate of single spore culture (Limed substrate of SSC)
2. Non-limed substrate of single spore culture (Non-limed substrate of SSC)

Each treatment comprised of four replicates of grower pots planted with the NP. The grower pots were positioned in groups of four according to the treatments as shown in Figure 69. Illustration of RPC trial is as shown in Figure 76.20 (Appendix).

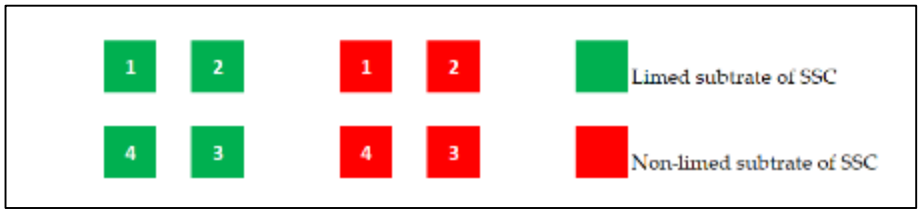


Figure 69. Position of treatments and replicates in re-propagation culture trial.

9.3.2 Establishment of nurse plant

9.3.2.1 Preparation of planting substrate

The root ball of NC seedlings from SSC trial as described in Chapter 8, was kneaded and gently shaken to loosen adhering soil. The loose substrate was put on a plastic trays and air dried overnight. The slightly moist soil then was put through a 6mm plastic square mesh to break up soil clumps. The loose substrate was then placed in grower pots (LS 150-S GAFRI, Malaysia) measuring 150 (D) x 120 (H) mm.

9.3.2.2 Planting of nurse plant

Tillers from a clump of lemongrass were separated and trimmed to 30 cm length. Their roots were completely trimmed off, and the stump washed under running tap water. Healthy looking tillers were then planted one each in the grower pots already filled with limed and non-limed substrates from the SSC trial. A tiller was placed in a 5 cm deep hole and the planting substrate around it made firm by applying light pressure with the palm.

9.3.2.3 Upkeep of nurse plant

Fertilizer application to nourish NP as described in Chapter 6 was not done in this RPC trial. The NP were left on a steel mesh bench under the nursery shade net for about three months before destructive sampling was carried out. Watering was done in the morning and afternoon daily by hosing gently with tap water. About one week before the destructive sampling of the NP, watering of NP was completely stopped.

9.3.3 Assessment of nurse plant morphology

Growth of the indicator plant can be monitored over time non-destructively by measuring leaf number, plant height, stem diameter, and leaf-area index, or by destructively determining biomass accumulation (Habte & Osorio 2001, p. 16). Assessment of NP morphology and extraction of spores from the grower pot substrate was carried out about three months after planting. About one week before the destructive sampling was carried out, the nurse plant tops were cut at root collar level. The watering regime was also stopped. The adverse treatment on the NP was supposed to induce the root colonizing AMF into reproducing spores (Sunseed Desert Technology 2012, p. 4).

9.3.3.1 Nurse plant dry-biomass

The nurse plant root ball was freed by kneading and gentle shaking adhering substrate off. Soil clumps adhering to roots were rinsed off under running tap water through a 2mm sieve. Care was taken to keep root systems intact. Any detached roots caught in the sieve were collected together with intact roots. After washing, the plant shoot and the wet roots were left to dry on boxes made from cardboard at room temperature.

Dry-biomass measurement of each NP was done separately for the whole root system and plant shoot. Plant parts were oven-dried at 65^{o} C for three days and beyond until repeat weighing on a daily basis gave identical results (Bloomberg, Mason & Jarvis 2008, p. 106). Root-shoot ratio was determined by root dry-biomass divided by shoot dry-biomass of NP (Budiman et al. 2015, p. 207). The root-shoot ratio reflects the capacity of

the roots to support the aboveground biomass not only for anchorage but also in absorbing water and nutrients from the soil.

9.3.4 Assessment of soil spores

The root ball of NP was separated from planting substrate by kneading and gentle shaking. Substrates loosened from the root ball of NP were used to carry out extraction of spores. The Spore extraction method used was based on the Sucrose Extraction Method (Center for Conservation of Biology 2013). The spore segregation method described by INVAM (2017) was improvised in the thesis to enhance its efficiency.

9.3.4.1 Extraction of spores

The procedures used in extraction of spores are as described under Methods and Materials in Chapter 4.

9.3.4.2 Segregation of spores

The procedures used in segregation of spores are as described under Methods and Materials in Chapter 4.

9.3.4.3 Enumeration of spores

The procedures used in enumeration of spores are as described under Methods and Materials in Chapter 4.

Spore composition was also measured to determine percentages of different spore morphotypes. The three simple morphological segregations were globose shaped of different colors, ovoid shaped of different colors and a distinctly white to pale yellow color spores of the D1 specimen.

9.3.5 Assessment of cellular structure of D1 spore specimen

Spores were characterized following species descriptions and illustrations of voucher specimens at Saprotrophic, Pathogenic and Symbiotic Fungi of Poland (http://www.zor.zut.edu.pl) under the phylum Glomeromycota and order Glomerales.

9.3.5.1 Preparing diagnostic slides

Permanent glass slides of the D1 spore specimen were prepared by mounting spores using a mixture of PVLG and Melzer's reagent (1:1 v/v) as described by Blaszkowski et al. (2005). PVLG mountant was prepared by mixing 16.6 g of Polyvinyl alcohol, and 100 ml each of distilled water, Lactic acid and Glycerol. Melzer's reagent was prepared by mixing 100 g of Chloral hydrate, 1.5 g of Iodine and 5 g of Potassium iodide in 100 ml distilled water (INVAM 2017).

A small drop of PVLG + Melzer's mountant was placed on the surface of a clean glass slide. About 10 cleaned spores were pipetted in a small droplet away from the mountant. Spores were dragged away from the water droplet using the flattened tip of an improvised prodder made from SWG 28 Nichrome wire (0.36mm diameter). The mountant was allowed to set for 3-5 minutes to make it become more viscous before adding a cover slip. A clean cover slip was placed on the spores. Light to moderate pressure was applied on the cover slip to crush spores by applying with the end of a needle done under the view of a stereo microscope. Slides were placed in an incubator at 65^{o} C for 2-3 hours to clean the spores and the mounting medium from oil droplets and air bubbles. The spore slides were ready for microscopic examination once the under cover slips were secured using nail polish.

9.3.5.2 Microscopic examination of spores

The measurement of fungal spores, spore diameter, wall thickness, hyphal thickness and thickness at the attachment point was carried with a LEICA DM750 compound light microscope (Leica Microsystems, China).

Although the morphology and architecture of external hyphae and internal mycorrhizal structures can differ between families of AMF, there are few differences between species within each genus(Bever et al. 2001, pp. 924&925). Therefore, taxonomy of these fungi is based on the discrete characters of the spore cellular structure, which can vary from simple to very complex for a single multi-nucleate cell. On the basis of spore wall

characters and spore ontogeny, AMF are grouped into genera and species. However, the majority of the AMF species remain undescribed based on spore characteristics.

Spores were characterized following species descriptions and illustrations of voucher specimens at the Saprotrophic, Pathogenic and Symbiotic Fungi of Poland (http://www.zor.zut.edu.pl/Mycota/Species%20descriptions.html) under the phylum Glomeromycota and order Glomerales. Fungi of the order Glomerales were described as usually hypogeous, requiring soil for spore germination and producing mycorrhizae with arbuscules and a vesicular fungal structure in the root. Spores formed either blastically at the tip of a sporogenous hypha or intercalary inside them. Spores occurred singly, in clusters or sporocarps having peridium. The order Glomerales included one of the genus, *Glomus* (Blaszkowski et al. 2005).

9.3.5.3 Taking photographs of spore structure

Acquisition, analysis and processing of digital images was done using computer software Leica Application Suite (LAS EZ) Version 3.3.0 (Leica Microsystems, Switzerland). The software allows the use of a LEICA DM750 compound light microscope (Leica Microsystems, China) and a LEICA ICC50 high definition microscope camera (Leica Microsystems, China) which was attached to an MS Windows workstation.

9.4 Results

The results of the trial cover the morphology of NP in terms of shoot and root dry-biomass, spore count, cellular characterization of the D1 spore specimen.

9.4.1 Nurse plant dry-biomass

The means and standard errors of dry-biomass of the NP, at three months after planting are as shown in Figure 70. The shoot and root dry-biomass of the NP grown in non-limed substrate were slightly higher than limed substrate of SSC trial as described in Chapter

8. The mean shoot and root dry-biomass of non-limed and limed soil substrates were 4.5 g, 4.4 g and 2.6 g, 2.5 g respectively.

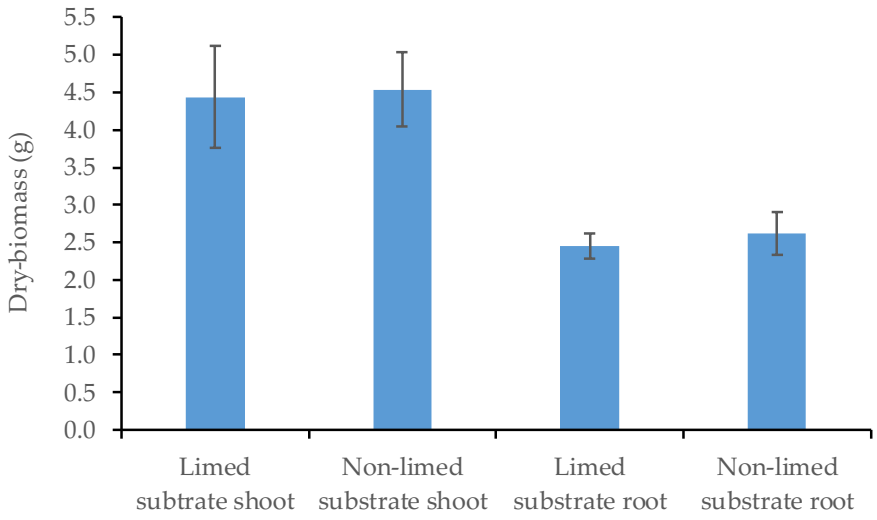


Figure 70. Means and one Standard Error bars of dry-biomass of nurse plant shoots and roots.

The root-shoot ratio refers to the proportion of the root dry weight to the shoot dry weight, and reflects the capacity of the roots to support the aboveground biomass not only for anchorage but also in absorbing water and nutrients from the soil. A high root-shoot ratio indicates a high absorption and storage capacity of water (Budiman et al. 2015, p. 210). The root-shoot ratios of NP grown on non-limed substrate and NP grown on limed substrate of SSC carried out earlier based on means of dry-biomass of shoot and root were 0.58 and 0.57 respectively as shown in Table 29. The root-shoot ratios of NP grown on non-limed soil substrate and limed soil substrate were generally the same and thus the capacity for root colonization and production of AMF propagules could also be considered equal as neither had any individual advantage in the absorption of soil nutrients and moisture over.

Table 29. Nurse plant root-shoot ratio grown on non-limed substrate and limed substrate.

Treatment	Mean of root dry-biomass (g)	Mean of shoot dry-biomass (g)	Root-shoot ratio
Non-limed substrate	2.6	4.5	0.58
Limed substrate	2.5	4.4	0.57

9.4.2 Spore counts

The RPC trial using non-limed and limed substrates with local lemongrass as NP yielded spores with a variety of morphological characteristics (Figure 71). The spores were distinguished into those that were similar to spores of the D1 specimen, and others that were grouped as simply of globose, and ovoid shaped.

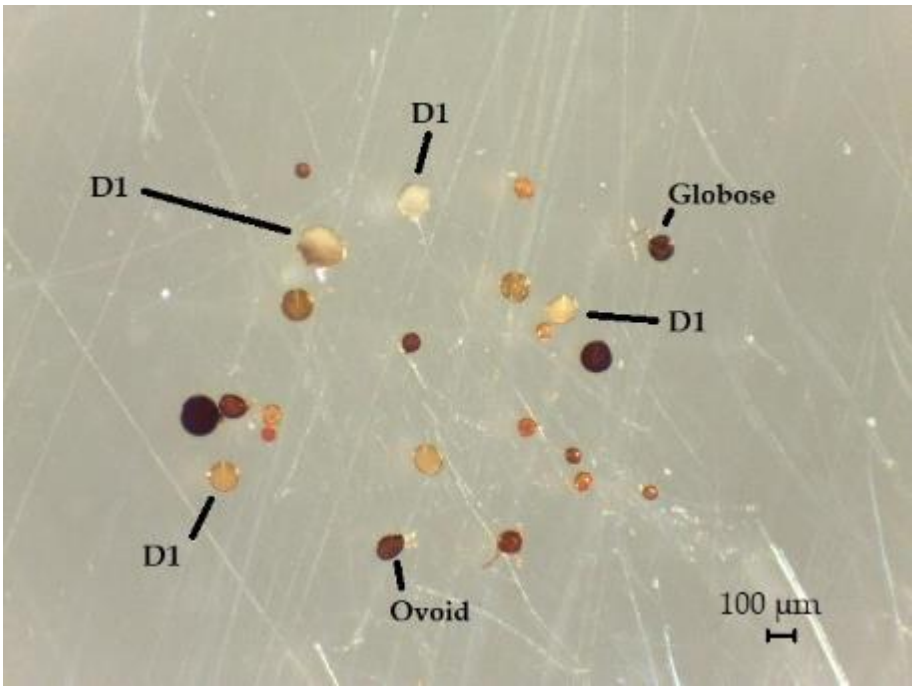


Figure 71. View of D1 spore specimen among other globose and ovoid shapes spores (35X magnification)

The RPC trial yielded about four fold more spores of globose and ovoid shapes of different colors as compared to spores of the D1 specimen as shown in Figure 73. The

spore count indicated that the sporulation of theD1 spore specimen was higher in non-limed substrate as compared to limed substrate. However, NP yielded low spore counts of the D1 specimen which were 0.4 and 0.1 spores per g substrate respectively. However, based on the presumed 0.2 spore/g of spore inoculum used to inoculate 850 g of planting media in the SSC trial (Chapter 7), it seemed that the RPC trial probably doubled the spores of the D1 specimen. The slightly higher dry-biomass of NP grown in non-limed substrate probably contributed to a slightly higher spore count.

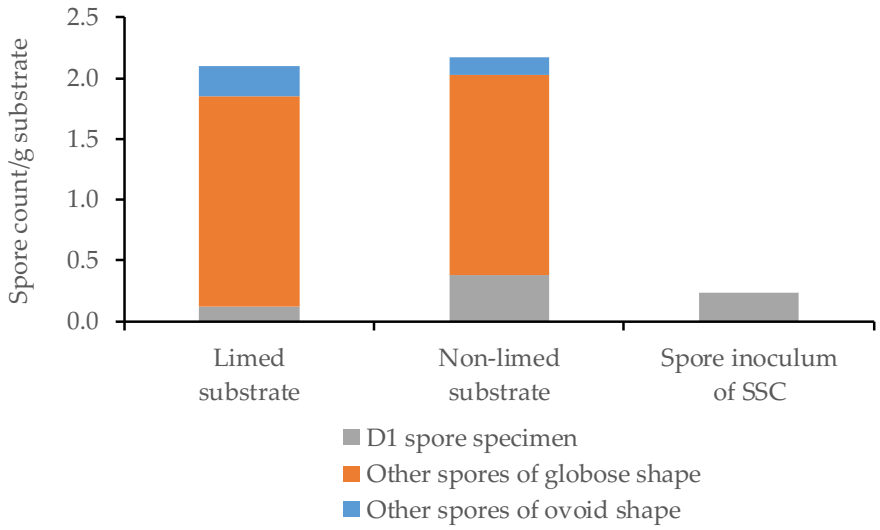


Figure 72. Spore morphotype composition of limed and non-limed substrates, and spore inoculum used in single spore culture trial.

The RPC trial indicated that the local lemongrass variety was able to enhance sporulation of the D1 specimen. The Sporulation induced by NP grown on non-limed substrate was enhanced by almost two fold. The presence of other spore morphotypes beside the D1 specimen was unexpected as planting media used was sterilized by autoclaved before spore inoculation was carried out as described in Chapter 8. Spore morphotypes composition was more varied from NP grown on non-limed substrate as compared to limed substrate. The outcome of the trial was similar to the result shown by the STC trial as described in Chapter 6 which was an AMF mixed-species.

9.4.3 Spore cellular structure

Meltzer+PVLG stained spores on a diagnostic slide as observed under the compound light microscope of the D1 spore specimen from the RPC trial, and a similar spore of the D1 specimen from the PSC trial as described in Chapter 7 are as shown in Figure 73. The spore morphology indicated similar simple spore wall and the presence of sub-tending hypha structure.

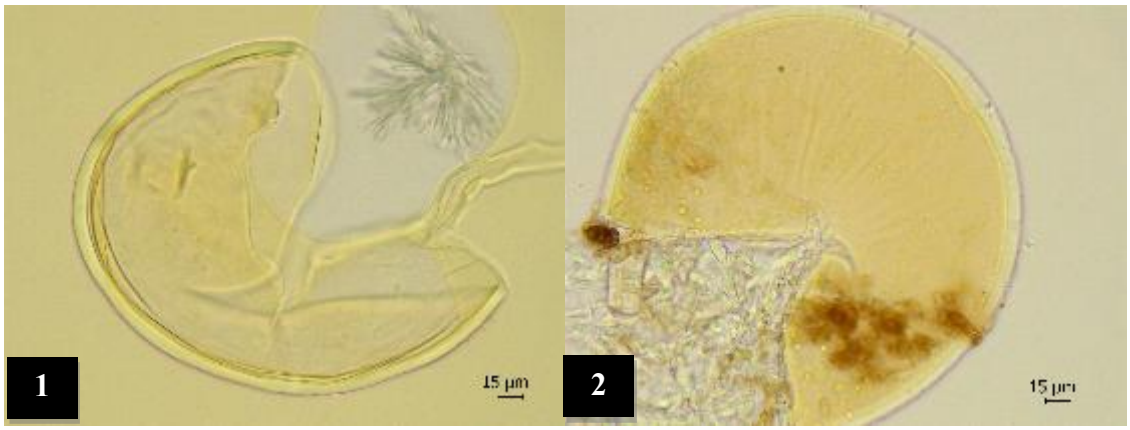


Figure 73. Structure of D1 spore specimen isolated. 1: Pot substrate culture trial, and 2: Re-propagation culture trial.

The details of spore cellular structures as seen under the compound light microscope at 400X magnification are as shown in Figure 74. Basically the spore of the D1 specimen had spore walls that consisted of 3 layers that extended to the sub-tending hyphal wall.

A search for similarity in the spore's sub-cellular characters following species descriptions and illustrations at Saprotrophic, Pathogenic and Symbiotic Fungi of Poland website (http://www.zor.zut.edu.pl/Mycota/Species%20descriptions.html) indicated that thevoucher specimen as shown in Figure 75 had the closest features to that of the D1 spore specimen isolated in the thesis. The specimen voucher was traced to that of *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe spore. Spores were described as usually single in the soil; in loose aggregates or compact sporocarps, pale yellow to golden yellow, globose to sub-globose, 80-280µm diameter, sometimes irregular with a

dimension of 80-140 x 195-280 µm, and with one subtending hypha (Blaszkowski et al. 2005).

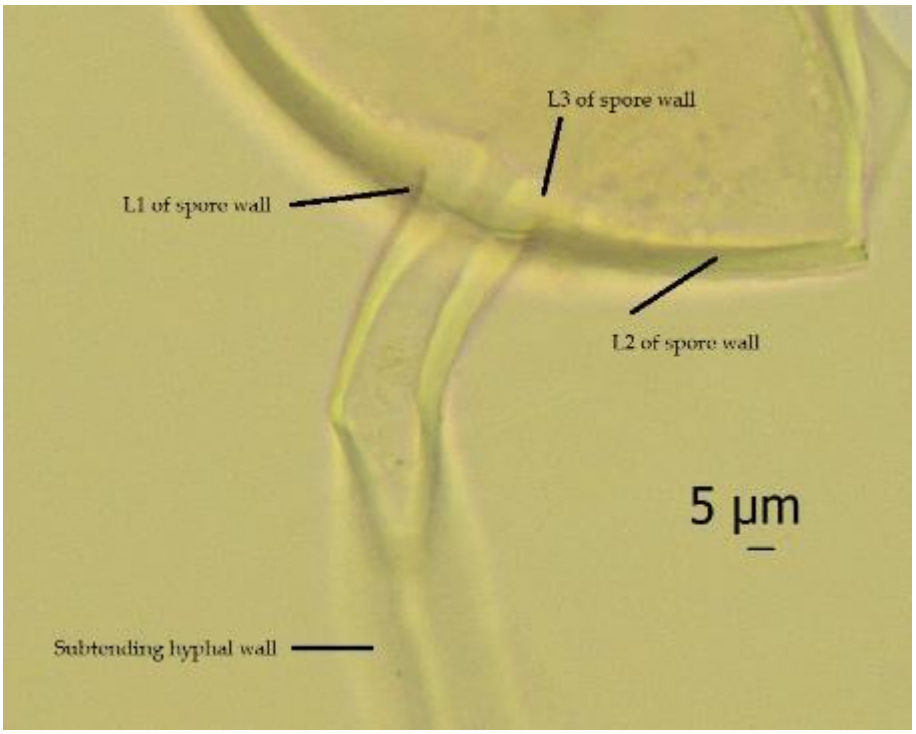


Figure 74. Cellular spore wall details of D1 specimen.

The cellular structure of spores as described by Blaszkowski et al. (2005), was first and foremost one wall with three layers (swl 1-3). Spore wall layer 1 consisted of mucilaginous; hyaline, 1.1µm thick, stained reddish white in Melzer's reagent, and usually present only in most juvenile spores. Spore wall layer 2 was semi-flexible; smooth, hyaline, 1.2µm thick, rarely present in mature spores, and usually visible in the form of highly decomposed fragments. Spore wall layer 3 was laminated, pale yellow to golden yellow, and 4.5µm thick. The sub-tending hypha was depicted as pale yellow to golden yellow; funnel shaped, and 25µm wide at the spore base. The wall of the sub-tending hypha was pale yellow to golden yellow; 2.2-4.3µm thick, composed of three layers (shwl 1-3), continuous with spore wall layers 1-3 in most mature spores, and the wall is 1-layered or consists of two layers continuous with spore wall layers 2 and 3.

Pores close by a curved septum, and are continuous with the inner-most sub-layers of the laminate spore wall layer 3.

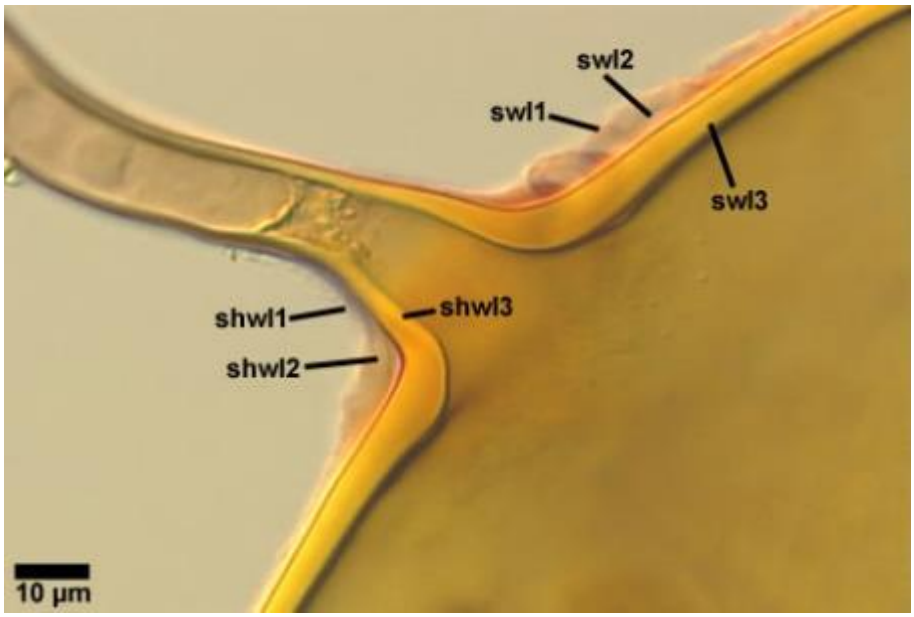


Figure 75. Cellular structure of *Glomus mosseae* spore's wall (Blaszkowski et al. 2005).

9.5 Discussion

The shoot and root dry-biomass of the NP grown in a non-limed substrate were slightly higher than limed substrate. The mean shoot and root dry-biomass were 4.5 g, 4.4 g and 2.6 g, 2.5 g respectively. The root-shoot ratio refers to the proportion of the root dry weight to the shoot dry weight, and reflects the capacity of the roots to support the aboveground biomass not only for anchorage but also in absorbing water and nutrients from the soil. A high root-shoot ratio indicates high absorption and storage capacity of water, which is an advantage, especially in conditions of limited soil moisture (Budiman et al. 2015, p. 210). The root-shoot ratios of NP grown on non-limed substrate and NP grown on limed substrate based on the means of dry-biomass of shoot and root were were generally the same and thus the capacity for root colonization and production of AMF propagules could be considered identical.

Species compatibility, carrying capacity, and priority effects are all processes that directly or indirectly affect competition for root or soil space, and therefore are important in determining the persistence and success of AMF species (Verbruggen et al. 2013, pp. 1107&1108). How these processes operate in plant-AMF interactions, can be optimized to stimulate the establishment of introduced strains. Choosing a highly mycorrhizal dependent crop and optimizing the nutrient environment are two management factors that are very likely to increase the establishment success of new strains.

Not much research work appears to deal with the culturability, phylogeny, taxonomic aspects and nature of symbiosis of AMF (Reddy, Pindi & Reddy 2005, pp. 1699&1700) This could be due to three reasons. Firstly, AMF are obligate symbionts; hence they cannot be cultured in pure form in the laboratory and large-scale multiplication is not possible. Second, is the non-availability of reliable characteristics for phylogeny and taxonomy as these fungi do not produce sexual states and exist only in an imperfect form. Establishment of phylogenetic relations, identifications and classifications are based on morphological features of the asexually produced propagules. In the absence of spores, the intra-radical structures at best allow identification to the family level.

Identification of AMF spores is a difficult and time consuming exercise for most researchers in the field (Habte & Osorio 2001pp. 23&24). The limitation of morphological identification is the fact that field-collected spores are often parasitized or degraded and therefore unidentifiable. Soil samples from the field site were brought into contact with suitable plant hosts of AMF under controlled conditions in order to propagate the species occurring at the field site and to obtain fresh spores of all developmental stages (Redecker, Hijri & Wiemken 2003, pp. 113&114). A single AMF culture can sometimes produce spores that differ substantially in morphology and color, and spores found in soil are often not in pristine condition so they may not match fresh reference material produced under controlled conditions. Reliable identification requires a good deal of patience and experience, and only a handful of experts worldwide can confidently identify the whole range of AMF by examining spores (Young 2012, p. 823).

The RPC of limed and non-limed substrates with local lemon grass as NP yielded spores with a variety of morphological characteristics. The spores of globose and ovoid shapes of different colors were about four fold more compared to those of the D1 spore specimen. The result indicated that soil substrates from the SSC trial with spore inoculum of the D1 specimen were contaminated with other fungi propagules. Some fungal phylotypes, can appear predominant in the greenhouse experiment or in bait plants. Thus, these phylotypes can be considered r strategists, rapidly colonizing non-colonized ruderal habitats in early successional stages of the fungal community. In contrast, other phylotypes were detected almost exclusively in roots sampled from plants naturally growing in the grassland or from bait plants exposed in the field, indicating that they preferentially occur in late successional stages of fungal communities and thus represent the K strategy (Sykorova et al. 2007, p. 1).

The proportion of successes with SSC may be very low depending on the species of fungus and the condition of the spores. Often, only one or two per cent of attempts will be successful, especially from field collected spores. Propagules of other fungi, which are usually spores or possibly hyphal fragments or roots particles, can be transferred in many ways. Culture trials in the open are inevitably prone to contamination (Walker 1999, p. 3). The NP yielded low spore counts, however it seems that the RPC has doubled the spores of the D1 specimen. The result is consistent with the STC as described in Chapter 5. NP without fertilizer has more than tripled the percentage of the globose shape with white to pale yellow color spores which were eventually referred to as spores of the the D1 specimen (Chapter 8). Density of the media had the greatest effect on spore formation (Ridgway, Kandula and Stewart (2004, p. 338). Silica sand/pumice medium with bulk density of 1 produced the greatest number of spores as compared to other combination of three different media (silica sand, pumice and potting mix). The use of river sand was deemed inappropriate as it was denser than soils which were also prone to water logging.

The RPC trial yielded a similar spore morphotpe as the D1 specimen. The cellular morphology indicated a similar simple spore wall and the presence of sub-tending

hypha. The spore wall consisted of three layers that extended to the subtending hyphal wall. A search for the similarity of spore's cellular characters to an on-line voucher specimen database was traced to *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe spore. *Glomus mosseae* spores were described as usually single in the soil; in loose aggregates or compact sporocarps, pale yellow to golden yellow, globose to sub-globose, 80-280µm in diameter, sometimes irregular, 80-140 x 195-280µm, and with one subtending hypha (Blaszkowski et al. 2005). Abdullahi, Lihan and Edward (2014, pp. 118&119), indicated that the use of lemongrass as NP was able to enhance sporulation. and served as a good host plant for mass multiplication of *Glomus mosseae*, *Glomus geosporum* and *Glomus etunicatum* due to environmental adaptability.

The study on the taxonomy of the AMF was just preliminary and could be pursued further when purified isolates are more certain. However, the crux of the thesis was to test the effectiveness of SMI on NC seedlings in enhancing their adaptation and performance once planted out to the field. Thus, the further taxonomy study of AMF isolates may not be really necessary as the effectiveness of SMI used was not encouraging as described in Chapter 5.

9.6 Conclusion

The RPC of non-limed and limed substrates from the SSC trial on NC yielded a low spore count of the D1 specimen as compared to spores of other morphoplogical characteristics. To enhance sporulation activities, properly some amendment to soil substrate could be used. The sporulation rate of the D1 spore specimen was observed as doubling the number of spore inoculum used in the initial SSC trial. However, spore inoculum of the D1 specimen used in the SSC trial was presumably mixed with propagules of other fungi. The RPC also yielded a similar spore morphotpe as the D1 specimen with a simple three layered spore wall that extended to the subtending hyphal wall. A search for similarity of spore cellular characters to an on-line voucher specimen database was traced to *Glomus mosseae*.

10 Summary

The summary covers background and objectives of the thesis, outcome of trials, what the thesis has established, recommendation, and proposed future research.

10.1 Background of the thesis

Thus far, the use of arbuscular mycorrhizal fungi particularly for growing *Neolamarckia cadamba* in Sarawak has not been properly researched. Information on the influence of fertilizer on *Neolamarckia cadamba* is still scarce let alone information on the combined effects with the use of bio-fertilizer. Therefore, the field trial was carried out to investigate the effectiveness of a combination of soil mycorrhizal inoculum and chemical fertilizer on the growth performance of *Neolamarckia cadamba* seedlings in the nursery and at planting out site. It was envisaged that forest plantations may consider including this technique in their goal of achieving maximum production of viable planting stocks.

Thus, the main focus of the thesis was on the utilization of soil mycorrhizal inoculum derived from soil trap culture method. The justification to undertake the thesis was largely based on the assumption that there was a lack of data on mycorrhizae of *Neolamarckia cadamba* in Sarawak. The situation limits the effective use of the potential soil bio-enhancement technology in *Neolamarckia cadamba* forest plantation. The utilization main objective was to enhance the growth of *Neolamarckia cadamba*, one of the key forest plantation tree species that have been planted in Sarawak. Thus, the title of the thesis "Soil Mycorrhizal Inoculum of *Neolamarckia cadamba*."

10.2 Outcomes of trials

There were some remarkable contrasts between the phenology plot site in Kubah N.P. and the field trial plot site in Sabal F.R. in terms of micro-physiography and vegetation cover. Interestingly, both sites have gone through some kind of ground surface disturbance where vegetation was cleared using heavy machinery and has resulted in

the movement and deposition of soil materials. Soil movement in the field trial plot site was probably limited to top soil and soil organic matters as compared to the phenology plot site in Kubah N.P.

Natural regeneration of vegetation has occurred at both sites with the emergence of bushes and shrubs and proliferation of pioneer tree species as in the case of the phenology plot in Kubah N.P., *Neolamarckia cadamba* trees, of which five of the trees were used in the thesis.

The soils of the three sites were generally acidic and typical of most soils in Sarawak. However, soil pH of the phenology plot soil in Kubah N.P. was slightly higher and also show exceptionally high exchangeable calcium content as compared to the soils from the other two sites. This may be due to some external factor such as dumping of cement and construction wastes as the soil parent rock was not calcareous in nature.

Generally, the soil nutrient content of all the soil samples from the three sites was rated low except for the calcium content of the phenology plot soil. The forest nursery soil was slightly higher in phosphorus content and incidentally the cation exchange capacity was also higher than the two others sites. The clay soil texture probably contributed in a relatively higher cation exchange capacity and higher nutrient retention and reserve. Total and available phosphorus content of forest nursery soil were also relatively higher and this was probably due to the presence of a higher percentage of soil organic matter as compared to the soil samples from the phenology plot in Kubah N.P. and the field trial plot in Sabal F.R. In terms of fertility ranking of soils of the three sites, relatively the forest nursery Semengoh N.R. > phenology plot in Kubah N.P. > field trial plot in Sabal F.R. The response to the usage of fertilizers at the planting site was highly likely and presumably necessary for favorable tree growth.

Obvious differences in the spore counts were observed on soil samples collected from the three sites. Both the phenology plot and the field trial plot sites were disturbed by displacement of soil materials and clearing of vegetation. The phenology plot soil in

Kubah N.P. had almost double the number of spore count compared to the field trial plot soil in Sabal F.R. and this could be due to its more mature secondary forest. The soil procured by the forest nursery for planting media preparation was extremely low in spore count as compared to the other two sites. Based on spore count of soil samples, arbuscular mycorrhizal fungi richness at the phenology plot in Kubah N.P. was higher compared to the field trial plot in Sabal F.R. Incidentally, the soil stocked for planting media preparation at the forest nursery in Semengoh N.R. was very low in spore count. Coupled with poor soil at the field planting site scenario, created the need for a soil bio-enhancement technique on planting media used in raising seedlings at the forest nursery which will be used in the field planting trials.

More information is needed to understand role of various soil microorganisms associated with plant roots in helping plant growth and health and to develop their beneficial properties into bio-fertilizers. Unless positive effects can be repeatedly shown in practical applications, the commercial viability of inoculation programs will be uncertain (Gentili & Jumpponen 2006, p. 18).

10.2.1 Soil mycorrhizal inoculum field trial

Soil mycorrhizal inoculum produced based on spore count had about 10 times more spores than the forest nursery soil. However, the mean percentage of root colonization of inoculated seedlings was only 10%. Root colonization was also observed on non-inoculated seedlings which was just slightly less at 8%. The just slightly higher root colonization of inoculated seedlings might possibly indicate the presence of arbuscular mycorrhizal fungi specific to *Neolamarckia cadamba* in the starter soil collected from the phenology plot in Kubah N.P. Nonetheless, arbuscular mycorrhizal fungi specific to *Neolamarckia cadamba* were probably found in soils from all three sites; phenology site in Kubah N.P., forest nursery in Semengoh N.R. and field trial plot in Sabal F.R.

The presences of arbuscular mycorrhizal fungi in roots of *Neolamarckia cadamba* were most likely due to the inherent existence of arbuscular mycorrhizal fungal propagules in the soil at the field trial plot, since roots of the non-inoculated tree were also colonized.

Root of trees colonized by arbuscular mycorrhizal fungi was found on individuals of both inoculated and non-inoculated trees at about 25%, and 30% respectively.

Non-inoculated seedlings in this study seemed to be sturdier than inoculated seedlings, however their root to shoot ratios were the same. The use of soil mycorrhizal inoculum did not enhance tree growth in the field. There was no significant difference between mean tree height and root collar diameter of inoculated seedlings and non-inoculated seedlings.

Fertilizer usage as expected enhanced tree growth in the field trial plot. There was significant difference between the mean tree height and root collar diameter of trees with fertilizer and those without fertilizer. The result of the study also indicated that the use of soil mycorrhizal inoculum with fertilizer did not enhance tree growth in the field as compared to the use of fertilizer only. There was no significant difference between the mean tree height and root collar diameter of inoculated seedlings with fertilizer and non-inoculated seedlings with fertilizer. The efficacy period of the slow release fertilizer was six months, and no significant difference in growth was seen beyond the period of six months after seedlings were planting out.

The usage of soil mycorrhizal inoculum as a bio-fertilizer for growth seedlings for planting out was not effective in complementing chemical fertilizer applied, Soil mycorrhizal inoculum combination with slow release chemical fertilizer and did not propel further growth at the field trial plot. The efficacy period of the slow release fertilizer was six months, and no significant difference in growth was seen beyond the six-month period after planting.

The monthly mean increment of tree height was also used to study the influence on the amount of monthly rainfall received. Inoculated trees showed no advantage to rainfall fluctuation such as enhanced capacity to absorb soil moisture during dry periods. The two instances in sudden drop in mean monthly increment of tree height coincided with

a heavy infestation of a defoliator or leaf eating insect that not only devoured leaves but damaging tree apical shoots also. The leaf eating pest caused stunting effect on the trees.

To sustain good growth of *Neolamarckia cadamba* in the field, a proper fertilizer regime was needed as part of the silviculture practices. Nitrogen, phosphorus and zinc were found to be soil nutrients that influenced the growth of *Neolamarckia cadamba* at the field trial plot. The leaf nutrient concentration dynamics of nitrogen, phosphorus and zinc were quite consistent with the pattern of mean monthly increments of tree height. Trees showed rapid growth following fertilizer application during the first three months. Growth slowed down for the next three months, level off from the seventh month onwards and dropped at the twelfth month. The trend of slowing growth was expected as the fertilizer used had an efficacy period of six months. The leaf critical nutrient concentration could be between 3-4% for nitrogen, 0.2-0.3% for phosphorus, and 40-60 ppm for zinc of leaf dry-biomass for *Neolamarckia cadamba* to sustain good growth.

There was no noticeable increase of the macro-nutrients and micro-nutrients uptake by inoculated seedlings while at the forest nursery as well as at the field trial plot. The use of soil mycorrhizal inoculum did not indicate any efficiency in the nutrient absorption by inoculated trees either with or without fertilizer especially of the critical nutrients.

Trees were also susceptible to insect pest attack especially by a leaf eating caterpillar. Trees left untreated with regular pesticide application were stunted and eventually died off. To reduce tree mortality and sustained growth of *Neolamarckia cadamba*, good silvicultural management was necessary. *Neolamarckia cadamba* requires a proper fertilizer regime, regular weed control, systematic pest control and maybe as observed the rarely needed pruning of branches and multiple tree leaders.

10.2.2 Soil trap culture trial

The soil trap culture trial indicated that the use of a local lemongrass variety was able to enhance sporulation of the fungal spores. The spore count of soil with nurse plants was more than double that of starter soil collected from the base of natural stands of

Neolamarckia cadamba. Starter soil planted with nurse plants and treated with fertilizer showed less sporulation as compared to nurse plants without fertilizer. The use of fertilizer enhanced shoot growth of lemongrass but impeded its root growth.

Apart from having more root dry-biomass, there was positive correlation of spore count and root dry-biomass of the nurse plants without fertilizer. Spore count increased with an increased in nurse plant root dry-biomass. The composition of spore morphotypes based on spore shapes and colors also indicated that starter soil with nurse plants without fertilizer was more varied as compared to nurse plants with fertilizer. Sporulation of starter soil collected from natural stands of *Neolamarckia cadamba* at the phenology plot in Kubah N.P. was enhanced by the use of the local lemongrass variety by at least two fold. Incidentally, the root dry-biomass of nurse plants without fertilizer was also significantly and positively correlated with spore count.

The simple and low cost protocol for soil mycorrhizal inoculum production using soil trap culture in this trial could be used by the forest nursery in Semengoh N.R. and forest nurseries across Sarawak if the need arises.

10.2.3 Pot substrate culture trial and isolation of spore morphotype specimens

The pot substrate culture was presumably able to produce distinguishable and definite spore characteristics namely by spore shapes and color. Five spore morphotype specimens coded D1, D2, D3, D4 and D5 were segregated based on the composition of the spore count. The D1 spore specimen was the most abundant whereas the spore counts of other specimens were very small.

The D1 specimen spore count was also significantly and positively correlated with root colonization. Though the Pearson correlation, r=0.450 was small it might probably indicate the D1 spore specimens influenced the root colonization of *Neolamarckia cadamba* as all the other spore morphotype specimens including spore specimen grouped as others showed no correlation pattern at all. Spores morphotype of the D1 specimen can be characterized as globose shaped, pale yellow color and with a size from 100-200 μm.

The use of soil mycorrhizal inoculum produced from soil trap culture at the forest nursery in Semengoh N.R., indicated evidence of root colonization of *Neolamarckia cadamba* seedlings. Sporulation of the fungi propagules namely spores seems to be related to root colonization.

The use of lime was able to enhance *Neolamarckia cadamba* seedling growth. However, liming was observed to limit sporulation of arbuscular mycorrhizal fungi and has relatively small impact on root colonization of *Neolamarckia cadamba* seedlings.

10.2.4 Single spore culture trial and characterization of D1 spore specimen

Root colonization by the D1 spore specimen was very low. The sterilization of the planting media seemed to eliminate completely all inherent fungal propagules as fungal structures of stained roots mounted on glass slides were hardly observed. *Neolamarckia cadamba* root colonization was also colonized by other non-arbuscular mycorrhizal fungi. Segregated spores were probably mixed with some other endophyptic fungi besides the Glomeromycota.

The BLAST search results indicated that the D1 spore specimen DNA sequence's similarity to the selected arbuscular mycorrhizal fungi species found on the NCBI database was not very high, about 85-88%. Nevertheless, the phylogenetic tree derived for the DNA sequence of the D1 spore specimen indicated that it was clustered together with the family of Glomeraceae namely *Glomus mosseae*, and *Glomus etunicatum*.

The viability test indicated that spores kept under prolonged conditions at room temperature resulted in a deterioration of spore viability and it completely diminished after about one month. Thus, spores extracted need to be kept refrigerated at temperature of about 8^{o} C to maintain their viability.

The study on the taxonomy of the arbuscular mycorrhizal fungi namely the spores of the D1 specimen in the thesis was just preliminary and it could be pursued further when purified isolates are more certain. However, the need to do so is dependent on the crux

of the thesis which was to test the effectiveness of soil mycorrhizal inoculum and the root colonization ability on *Neolamarckia cadamba*.

10.2.5 Re-propagation culture trial and cellular assessment of D1 spore specimen

The re-propagation culture trial of non-limed and limed substrates from the single spore culture trial on *Neolamarckia cadamba* yielded a low spore count of the D1 specimen as compared to spores of other morphoplogical characteristics. However, the sporulation rate of the D1 spore specimen was observed to double the number of spores used in the initial inoculation in the single spore culture trial. The spore inoculum used in the single spore culture trial was presumably mixed with propagules of other fungal types.

The re-propagation culture trial yielded similar spore morphotpes of the D1 specimen with a simple three layered spore wall that extended to the subtending hyphal wall. The search for similarity in spore cellular characters to an on-line voucher specimen database was traced to *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe.

10.3 What has the thesis established?

The thesis aim was to make a significant contribution to the use of soil bio-enhancement techniques which could sustain growth and adaptation of seedlings to the field environment of forest plantations in Sarawak, namely for the planting of *Neolamarckia cadamba*. The potential contribution of arbuscular mycorrhizal fungi for that purpose could be further explored. Unfortunately, the thesis showed that the soil mycorrhizal inoculum produced showed no positive effect on the growth performance of *Neolamarckia cadamba* planted out to the field

However, some general practical guides such as on the use of soil trap culture and the soil mycorrhizal inoculum derived from it, and the fertilizer regime needed for *Neolamarckia cadamba* could be considered for further study and refinement.

10.3.1 Soil trap culture technique

Plants are easily sampled and measured, but evaluation of the fungal community are rather intangible. However, as reiterated by Bever et al. (2001, p. 390), comprehension on the ecology and site requirement of individual fungal species is necessary as simple assumptions about the influence of arbuscular mycorrhizal fungi on plant communities need to be re-evaluated

The use of lemongrass was suitable for soil mycorrhizal inoculum production in the forest nursery as it is easily available, easy to plant and can thrive in most soil types. It is also free from pests and most plant diseases. An improved technique of soil trap culture using lemongrass may have to exclude the use of any form of fertilizer including low phosphorus formulation. Use of lemongrass as the nurse plant was able to enhance sporulation of the D1 spore specimen by doubling the spore count of the initial spore inoculum. The re-propagation culture trial was also able to yield similar spore morphotype of the D1 spore specimen.

10.3.2 *Neolamarckia cadamba* soil mycorrhizal inoculum

The use of soil mycorrhizal inoculum produced from the soil trap culture trial at the forest nursery in Semengoh N.R., indicated evidence of root colonization of *Neolamarckia cadamba* seedlings. The use of lime was able to enhance *Neolamarckia cadamba* seedling growth. However, liming was observed to limit sporulation of and had a relatively small impact on root colonization of *Neolamarckia cadamba* seedlings.

As pointed out by Landis and Amaranthus (2009, p. 15), many studies have shown that inoculating nursery stock with mycorrhizal fungi was beneficial. The benefits could be well explained by the particularly interesting newly discovered relationship between arbuscular mycorrhizal fungi and glomalin. As implied, arbuscular mycorrzal fungi associated with wide variety of plants from around the world, produce glomalin on plant roots. The substance which is a sticky protein makes soils more friable, making it useful in forest nurseries, and improving soils condition at physically poor sites meant for reforestation, and restoration activities.

10.3.3 *Neolamarckia cadamba* fertilizer regime

Growth was more than double for trees with fertilizer as compared to trees without fertilizer. *Neolamarckia cadamba* growth plateau after six months consistent with the efficacy period of the fertilizer used. Applying high rates of fertilizer among other silvicultural treatments showed long-term growth of trees and utilization potential of site. As indicated by Mead (2005, p. 249), the largest gains in forest plantations apart from site selection, species and provenance choice, draining wet sites and, tree breeding and irrigation, came from correcting soil nutrient deficiencies.

The use of slow release fertilizers is the newest and most technically advanced way of supplying mineral nutrients (Landis & Dumroese 2009, p. 10). Slow release fertilizers have gradual pattern of nutrient release that meets plant needs better, and thus minimizes leaching and improving fertilizer use efficiency as compared to conventional fertilizers. The polymer coated fertilizer products are most commonly used in forest nurseries forest, and forest conservation work. As indicated by Landis and Dumroese (2009, p. 10), these fertilizers release their nutrients over periods from 3 to 18 months depending on the type of fertilizer coating and temperature of the planting media.

Apart from meeting nutrients need of tree planted, rapid growth of mycorrhizal fungi can be assisted by the addition of humic acids. As suggested by Kheyrodin (2014, p. 45), if plant root colonization is below 40%, plants are actually not getting the help they need from the mycobionts. As pointed out, the situation can be remedied, by providing additional fungal foods to help the mycorrhizal fungi play their roles in improving plant growth, reducing plant stress, and providing root protection against diseases. Fungal foods, namely humic acid can be derived by putting on a mulch or litter layer on the soil surface.

10.4 Future research

Future research could refocus on ascertaining the nomenclature of the D1 specimen spore morphotype and its symbiotic relationship with *Neolamarckia cadamba*. Helper soil

microorganisms could also be studied to determine their roles in root colonization of *Neolamarckia cadamba*.

10.4.1 D1 spore specimen

Several studies conducted locally and in several countries of arbuscular mycorrhizal fungi population from environmental samples indicated that the genera *Glomus* was the most common or dominant. The genera *Glomus* occurred most frequently, constituting 89.1% of the total species in the study of diversity and habitat relationships, based on spore counts of soil samples from 55 fields of wheat from 11 agro-climatic regions of India (Singh & Adholeya 2013, p. 1). *Glomus* was the most dominant isolated mycorrhizal genus of which three dominant species *Glomus fasciculatum*, *Glomus macrocarpum*, and *Glomus mosseae* have been identified in a study undertaken to access the influence of paper mill effluents (Chanda, Sharma & Jha 2014, p. 527). Isolates of the species of *Glomus* and *Acaulospora* appeared to be the most abundant in the rhizosphere of vine roots in the vineyards of a commercial farms in the SteUenbosch Region of South Africa. The arbusclar mycorrhizal fungal species occurred at a soil P concentration of up to 80 ppm and a soil pH that ranged between 5.63 and 6.10 according to Meyer et al. (2005, p. 90).

The study to assess and compare the abundance of arbuscular mycorrhizal fungi in a rehabilitated forest and a logged-over forest soil using the most probable number and spore number methods showed *Glomus* (72-82%) and *Acaulospora* (17%-20%) were found to be abundant in both sites (Ong et al. 2012, p. 1). *Glomus* was also found to be dominant in the rhizospheres of *Octomeles sumatrana* and *Neolamarckia cadamba* at Niah N.P. (Chubo et al. 2009, p. 340). *Glomus* was found to be dominant out of the five arburcular mycorrhiza fungal genera recorded in roots and rhizosphere soils of *Acacia auriculiformis*, *Acacia mangium*, *Artocarpus heterophyllus*, *Dalbergia sissoo*, *Eucalyptus camaldulensis*, *Hevea brasiliensis*, *Swietenia macrophylla*, and *Tectona grandis*, collected from different locations of Madhupur forest area, Bangladesh (Dhar & Mridha 2012, p. 201).

The spores of the D1 specimen could be generally describes as typically globose shaped, 100-200µm in size and pale yellow in color. The phylogenetic tree indicated that the D1

spore specimen was clustered together with the family of Glomeraceae and the closest being with *Glomus mosseae* and *Glomus etunicatum*. Search for similarity of spore's cellular characters to an on-line voucher specimen database was traced to *Glomus mosseae*. Further work could be done to enhance identification of the spores of the D1 specimen and its infectiveness on *Neolamarckia cadamba* roots. Consistent DNA sequences of the D1 spore specimen obtained could also be registered in NCBI databases in future.

A simple and inexpensive method for producing and maintaining pot cultures of the D1 spore specimen could be carried out using transparent bags, incorporating a micro-filter to allow gaseous exchange as described by Walker and Vestberg (1994, p. 233). The method as suggested, was able to maintain fungal purity, and has the advantage of requiring less watering and maintenance than open pot cultures. The other option for propagation of the D1 spore specimen is by root-organ culture. The use of arbuscular mycorrhizal fungi root-organ cultures allows the aseptic production of spores of various arbuscular mycorrhizal fungi species (Fortin et al. 2002, p. 6). The contaminant-free cultures as suggested could enable the constant supply of clean fungal propagules, which can be observed and harvested at any stage during fungal development.

Mycorrhizal roots could also be analyzed for the taxonomy of arbuscular mycorrhizal fungi in the thesis. As suggested by Ishii and Loynachan (2004, p. 271), two improved DNA extraction techniques from Trypan blue stained root fragments developed could be used for rapid and reliable analyses. Both techniques as reiterated are useful for PCR based applications to identify species and estimate species composition after measuring the arbuscular mycorrhizal fungi colonization rate with Trypan blue staining of mycorrhizal roots as what was carried out in the thesis.

10.4.2 Helper soil microorganisms

The sterilization of planting media in the single spore culture trial seemed to eliminate completely all inherent fungal propagules and other soil microorganisms. Thus, this could probably explain the resulted of the very low percentage of root colonization of *Neolamarckia cadamba* by the spore inoculum of the D1 specimen.

Bonfante and Anca (2009, pp. 363&377), reiterated that the constant discovery of bacteria new roles attributed to a paradigm shift in viewing mycorrhizal symbioses. A as pointed out, analysis of the multiple interactions among mycorrhizal fungi, plants and bacterial cells offer new understanding of the complexity of arbuscular mycorrhizal fungi activities. The exact nature of the interactions remains unclear, much evidence supports the view that mycorrhizae can be defined as tripartite associations of plants, mycorrhizal fungi, and bacteria. The interactive mechanisms among the association members promote plant growth. Fitter and Garbaye (1994, p. 123), suggested two areas that are of particular importance to the functioning of the symbiosis; the role of bacteria in promoting mycorrhizae formation and arbuscular mycorrhiza fungi modifying the interaction of plant root and N-fixing bacteria.

The arbuscular mycorrhizal fungi extra-radical hyphae are an important interface for interactions with other soil microorganisms. Toljander et al. (2006, p. 39), reckoned that carbon that flow through this interface provides a significant source of energy that can be exploited by opportunistic or mutualistic soil organisms.

Arbuscular mycorrhizal fungi associations have been shown to reduce damage caused by soil-borne plant pathogens (Azcon-Aguilar & Barea 1996, p. 457). This ability of arbuscular mycorrhizal fungi is due to the co-existence of other rhizospheric pathogen angatonists that enable improved plant growth and health. As pointed out by Azcon-Aguilar and Barea (1996, p. 457), the level protection given varies with the pathogen involved and subjected to soil and other environmental conditions. However, as reiterated by Fortin et al. (2002, p. 11), arbuscular fungi root colonization may be adversely affected by certain bio-control microorganisms. The possibility for such interactions should be considered when developing bio-control strategies especially with the co-existence of *Trichoderma* species.

The research on other helper soil microorganisms may lead to further work on isolation, identification, culture and development of operational scale protocols for bio-formulation of arbuscular mycorrhizal fungi and other soil microorganisms suited to the

forest plantations in Sarawak. Zhang et al. (2016, p. 1022), suggested that there is a link between carbon release from an arbuscular mycorrhizal fungi and phosphorus availability via a phosphate solubilizing bacteria. The arbuscular mycorrhizal fungi released substantial carbon to the environment, triggering phosphate solubilizing bacteria growth and activity. Phosphate solubilizing bacteria enhanced mineralization of organic phosphorus, increasing phosphorus availability for the arbuscular mycorrhizal fungi. Apart from phosphate solubilizing bacteria, usage of actinomycetes could also be considered as they were also shown to have improved arbuscular mycorrhizal fungi mycelial growth, stimulated spore germination, and enhanced plant growth and nitrogen acquisition as reported by Franco-Correa et al. (2010, p. 209). Co-inoculation of actinomycetes strains, MCR9 and MCR24 with *Glomus mosseae* resulted in synergic benefits in growth and acquisition of phosphorus by the clover host plants studied.

11 References

Abdullahi, R, Lihan, S & Edward, R 2014, 'Isolation of indigenous arbuscular mycorrhizal fungi and selection of host plant for inoculum production', *International Journal of Biosciences*, vol. 5, no. 12, pp. 116-122.

Adesemoye, AO, Torbert, HA & Kloepper, JW 2009, 'Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertlizers', *Microb Ecol*,no. 58, pp. 921-929.

Al-Areqi, AHNA, Chliyeh, M, Sghir, F, Touhami, AO, Benkirane, R & Douira, A 2013, 'Diversity of arbuscular mycorrhizal fungi in rhizosphere of Coffea arabica in the Republic of Yemen', *Journal of Applied Biosciences*,no. 64, pp. 4888-4901.

Alexander, I, Norani Ahmad & Lee, SS 1992, 'The role of mycorrhizas in the regeneration of some Malaysian forest trees', *Phil. Trans. R. Soc. Lond.*,no. 335, pp. 379-388.

Alguacil, MdM, Torres, MP, Montesinos-Navarro, A & Roldan, A 2016, 'Soil characteristics driving arbuscular mycorrhizal fungal communities in semiarid Mediterranean soils', *Applied and Environmental Microbiology*, vol. 82, no. 11, pp. 3348-3356.

An, ZQ & Hendrix, JW 1988, 'Determining viability of endogonaceous spores with vital stain', *Mycologia*, vol. 80, no. 2, pp. 259-261.

Andrade, SAL, Mazzafera, P, Schiavinato, MA & Silveira, APD 2009, 'Arbuscular mycorrhizal association in coffee', *Journal of Agricultural Science*,no. 147, pp. 105-115.

Andrew Nyorik Nibu & Alik Duju 2005, 'Machining properties of *Acacia mangium* and Kelampayan,' *Applied Forest Science Reserach Seminar 2005*, Sarawak Forestry, 161-166.

Ani Sulaiman, Wan Rasidah Abdul Kadir & Mohd. Shukari Midon 1990, 'Effects of fertilizer on wood properties of plantation grown *Acacia mangium*', *Journal of Tropical Forest Science*, vol. 4, no. 2, pp. 119-126.

Annuar Mohammad 2008, *An overview of forest plantation history, development and policy in Sabah,* A guide to plantation forestry in Sabah, Sabah Forestry Department, Sabah Forest Record No. 16.

Annya Ambrose & Empenit Empawie 2008, 'Disease surveillance', *Forest Health Consultancy and Field Report 2008*, Forest plantation disease monitoring, Applied Forest Science and Industry Development Division, Sarawak Forestry Corporation Sdn. Bhd.,

Antunes, PM, Lehmann, A, Hart, MM, Baumecker, M & Rillig, MC 2012, 'Long-term effects of soil nutrient deficiency on arbuscular mycorrhizal communities', *Functional Ecology*,no. 26, pp. 532-540.

Applied Biosystems 2012, 'Sequence Scanner Software 2,' Life Technologies Corporation.

Arora, NK, Khare, E & Maheshwari, DK 2011, 'Plant growth promoting rhizobacteria: constraints in bioformulation, commercialization, and future strategies', in DK Maheshwari (ed.) *Plant growth and health promoting bacteria*, Springer, Berlin Heidelberg, pp. 97-116.

Artursson, V, Finlay, RD & Jansson, JK 2006, 'Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth', *Environmental Microbiology*, vol. 8, no. 1, pp. 1-10.

Avoseh, O, Oyedeji, O, Rungqu, P, Nkeh-Chungag, B & Oyedeji, A 2015, '*Cymbopogon* species; ethnopharmacology, phytochemistry and pharmacological importance', *Molecules*,no. 20, pp. 7438-7453.

Azcon-Aguilar, C & Barea, JM 1996, 'Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - an overview of the mechanisms involved', *Mycorrhiza*,no. 6, pp. 457-464.

Bansal, M & Mukerji, KG 2002, 'Methods in study of viability of VAM fungal spores', in KG Mukerji, C Manoharachary and BP Chamola (eds), *Techniques in Mycorrhizal Studies*, Springer, Dordrecht, pp. 217-229.

Barman, J, Samanta, A, Saha, b & Datta, S 2016, 'Mycorrhiza the oldest association between plant and fungi', *Resonanve*,no. December, pp. 1093-1104.

Baum, D 2008, 'Reading a phylogenetic tree: the meaning of monophyletic groups', *Nature Education 1(1):190*, vol. 1(1), no. 190

Berruti, A, Borriello, R, Orgiazzi, A, Barbera, AC, Lumini, E & Bianciotto, V 2014, 'Arbuscular mycorrhizal fungi and their value for ecosystem management', in O Grillo (ed.) *Biodiversity - The Dynamic Balance of the Planet*, IN TECH.

Berruti, A, Lumini, E, Balestrini, R & Bianciotto, V 2016, 'Arbuscular mycorrhizal fungi as natural biofertilizers: Let's benefit from past successes', *Frontiers in Microbiology*, vol. 6, no. 1559

Bever, JD, Schultz, PA, Pringle, A & Morton, JB 2001, 'Arbuscular mycorrhizal fungi: More diverse than meets the eye, and the ecological tale of why', *BioScience*, vol. 51, no. 11, pp. 923-931.

Bhardwaj, D, Ansari, MW, Sahoo, RK & Titeja, N 2014, 'Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity', *Microbial Cell Factories*, vol. 13, no. 66, pp. 1-10.

Binder, M & Hibbett, D 2003, *Oligonucleotides*, Clark University, viewed 2 April <http://www.clarku.edu/search-clark-university?as_q=faculty%2Fdhibbett% 2F protocols_folder%2Fprimers%2Fprimers>.

Biology Online 2018, *Symbioses*, Vicki Mozo, viewed 2 January 2018, <https://www. biology-online.org/dictionary/Symbiosis>.

Blal, B 1999, 'Utilization of commercial arbuscular mycorrhizal fungal inoculants in ornamental and woody plants production in nursery', *Acta Hortic.*,no. 496, pp. 461-470.

Blaszkowski, J, Adamska, I, Czerniawska, B, Madej, T & Ziolo, E 2005, *Making dianostic slides*, Department of Plant Pathology, University of Agriculture, Szczecin, Poland, viewed 1 July 2017, <http://www.zor.zut.edu.pl/Mycota/ Making%20 diagnostic% 20slides.html>.

Bloemberg, GV & Lugtenberg, BJJ 2001, 'Molecular basis of plant growth promotion and biocontrol by rhizobacteria', *Current Opinion in Plant Biology*,no. 4, pp. 343-350.

Bloomberg, M & John Sabang 2009, 'How site factors can influence plantation forest health,' *Pest and Disease Perspective in Planted Forests*, Sarawak Forestry, Forest Department Sarawak, and Sarawak Planted Forest Sdn Bhd, 51-58.

Bloomberg, M, Mason, EG & Jarvis, P 2008, 'Predicting seedling biomass of radiata pine from allometric variables', *New Forests*,no. 36, pp. 103-114.

Bonfante, P & Anca, I-A 2009, 'Plants, mycorrhizal fungi, and bacteria: A network of interactions', *Annu. Rev. Microbiol.*,no. 63, pp. 363-383.

Bonfante, P & Genre, A 2010, 'Mechanisms underlying beneficial plant interactions in mycorrhizal symbiosis', *Nature Communications*, vol. 1, no. 48, pp. 1-11.

Bong, K 2018, 'Road to sustanable planted forest industry', *The Borneo Post*, 25 March 2018,

Bottu, G 2009, 'Data preparation - sequence databases and database searching', in P Lemey, M Salemi and A-M Vandamme (eds), *The phylogenetic handbook*, Cambridge University Press, New York, pp. 31-54.

Brandt, M & Warnock, D 2011, 'Taxonomy and classification of fungi', in J Versalovic, K Caroll, G Funke, J Jorgensen, M Landry and D Warnock (eds), *Manual of Clinical Microbiology*, ASM Press, Washinton DC, pp. 1747-1755.

Broeckling, CD, Broz, AK, Bergelson, J, Manter, DK & Vivanco, JM 2008, 'Root exudates regulate soil fungal community composition and diversity', *Applied and Environmental Microbiology*, vol. 74, no. 3, pp. 738-744.

Browne, FG 1968, *Pests and diseases of forest plantation trees,* Clarendon Press, Oxford.

Brundrett, M 2004, 'Diversity and classification of mycorrhizal associations', *Biol, Rev.*,no. 79, pp. 473-495.

Brundrett, M, Bougher, N, Dell, B, Grove, T & Malajczuk, N 1996, *Working with mycorrhizas in forestry and agriculture,* Australian Centre for International Agricultural Research, Canberra.

Budiman, B, Sudrajat, DJ, Lee, DK & Kim, YS 2015, 'Effect of initial morphology on field performance in white jabon seedlings at Bogor, Indonesia', *Forest Science and Technology*, vol. 11, no. 4, pp. 206-211.

Burrows, RL & Pfleger, FL 2002, 'Arbuscular mycorrhizal fungi respond to increasing plant diversity', *Can. J. Bot.*,no. 80, pp. 120-130.

Camerlengo, AL, Mohd Azmi Ambak & Mohd Nasir Saadon 2000, 'Rainfall in Sarawak', *Pertanika J. Sc. & Technical*, vol. 8, no. 2, pp. 125-135.

Center for Conservation of Biology 2013, *Spore extraction: The sucrose method*, University of California, Riverside, viewed <http://ccb.ucr.edu/lab protocols.html>.

Chai, D, Empenit, Empawi, Encahrang, L & Meredi, J 2010, 'Insect pest investigation on Kelampayan at LPF0032 WTK Leseh Plantation, Kanowit', *Forest Health Consultancy and Field Report 2010*, Sarawak Forestry Corporation Sdn. Bhd.,

Chanda, D, Sharma, GD & Jha, DK 2014, 'Isolation and identification of some arbuscular mycorrhiza (AM) fungi for phtoremeidation in soil contaminated with paper mill effluent', *International Journal of Current Microbiology and Applied Sciences*, vol. 3, no. 6, pp. 537-539.

Chang, L-C 1972, *The concept of statistics in connection with experimentation,* Food and Fertilizer Technology Center, Taipei.

Charoenpakdee, S, Phosri, C, Dell, B, Choonluechanon, S & Lumyong, S 2010, 'Compatible arbuscular mycorrhizal fungi of *Jatropha curcas* and spore multiplication using cereal crops', *Mycosphere*, vol. 1, no. 3, pp. 195-204.

Chin, SP 1993, *A laboratory manual of methods of soil analysis,* Research Branch, Agriculture Department Sarawak.

Chong, L 1987, *Occurrence of mycorrhizae in seedlings of some tree species in Sarawak,* Forest Research Report, FP5.

Chu, SS, Wang, J, Tong, X, Chen, XY, Deng, XM, Lin, JY & Zeng, SC 2017, 'Effects of combined application of organic and chemical fertilizers on growth of young

Neolamackia cadamba plantation', *Journal of South China Agricultural University*, vol. 38, no. 2, pp. 92-98.

Chubo, JK, Ong, KH, Hasnah Md Jais, Mardatin, NF & Nik Muhamad Nik Abdul Majid 2009, 'Genera of arbuscular mycorrhiza occuring within the rhizospheres of Octomeles sumatrana and Anthocephalus chinensis in Niah, Sarawak, Malaysia', *ScienceAsia*,no. 35, pp. 340-345.

Closa, I & Goicoechea, N 2011, 'Infectivity of arbuscular mycorrhizal fungi in naturally regenerating, unmanaged and clear-cut beech forests', *Pedosphere*, vol. 21, no. 1, pp. 65-74.

Comas, L, Carlisle, B & Patterson, A 2011, *Arbuscular mycorrhizal (AM) staining and quantification*, PrometheusWiki, viewed 30 March 2017, <http://prometheuswiki.org/tiki-index.php?page=Arbuscular+mycorrhizal+%28AM%29+staining+and + quantification>.

Coughlan, A, Dalpe, Y, Lapointe, L & Piche, Y 2000, 'Soil pH-induced changes in root colonization, diversity, and reproduction of symbiotic arbuscular mycorrhizal fungi from healthy and declining maple forests', *Can. J. For. Res.*,no. 30, pp. 1543-1554.

Deloiite, T, Talisman, LRC & The DPA Group 1990, 'An evaluation of the soil physical and chemical properties of the major representative soil types in Sarawak', in *Integrated land-use planning for Sarawak*, vol. III, Sarawak Agricultural Development Project, State Planning Unit, Department of Development Sarawak, pp. 50.

Department of Agriculture Sarawak 2004, *Guidelines for soil description in Sarawak,* Department of Agriculture, Soil Management Branch, Kuching, Sarawak, Kuching.

Dhar, PP & Mridha, MAU 2012, 'Arbuscular mycorrhizal associations in different forest tree species of Hazarikhil forest of Chittagong, Bangladesh', *Journal of Forestry Research*,no. 23(1), pp. 115-122.

Douds, DDJ & Schenck, NC 1990, 'Increased sporulation of Vesicular_Arbuscular Mycorrhizal Fungi by manipulation of nutrient regimens', *Applied and Environmental Microbiology*, vol. 56, no. 2, pp. 413-418.

Druille, M, Cabello, MN, Omacini, M & Golluscio, RA 2013, 'Glphosate reduces spore viability and root colonization of arbuscular mycorrhizal fungi', *Applied Soil Ecology*,no. 64, pp. 99-103.

Duryea, ML 1985, 'Evaluating seedlings quality: Importance to reforestation,' *Evaluating seedling quality: principles, procedures, and predictive abilities of major tests*, Forest Research Laboratory, Oregon State University, 1-6.

Echereme, CB, Mbaekwe, EI & Ekwealor, KU 2015, 'Tree crown architecture: Approach to tree form, structure and performance: A review', *International Journal of Scientific and Research Publications*, vol. 5, no. 9, pp. 1-6.

Environment Protection Authority 2005, (ed Environment) Government of South Australia, Adelaide, p. 5.

Ericsson, T 1994, 'Nutrient dynamics and requirements of forest crops', *New Zealand Journal of Forestry Science*, vol. 24, no. 2/3

FAO 1998, *Evaluating the potential contribution of organic agriculture to sustainability goals*, Environment and Natural Resources Service; Research, Extension and Training Division, Sustainable Development Department, Food and Agriculture

Organization of the United Nations, viewed 14 April 2018, <http://www.fao.org/docrep/003/ac116e/ac116e00.htm#Toc>.

Fitter, AH 1985, 'Functioning of vesicular arbuscular mycorrhizas under field conditons', *New Phytologist*,no. 99, pp. 257-265.

Fitter, AH & Garbaye, J 1994, 'Interactions between mycorrhizal fungi and other soil organisms', *Plant and Soil*, vol. 159, no. 1, pp. 123-132.

Forest Department Sarawak 2014, 2014, Wisma Sumber Alam, 93702 Kuching, Sarawak, Malaysia.

Fortin, JA, Becard, G, Declerck, S, Dalpe, Y, St-Arnaud, M, Coughlan, AP & Piche, Y 2002, 'Arbuscular mycorrhiza on root-organ cultures', *Can. J. Bot.*,no. 80, pp. 1-20.

Franco-Correa, M, Quintana, A, Duque, C, Suarez, C, Rodriquez, MX & Barea, J-M 2010, 'Evaluation of actinomycetes strains for key traits related with plant growth promotion and mycorrhiza helping activities', *Applied Soil Ecology*,no. 45, pp. 209-217.

Fuentes, JP, Bezdicek, DF, Flury, M, Albrecht, S & Smith, JL 2006, 'Microbial activity affected by lime in a long-term no-til soil', *Soil & Tillage Research*,no. 88, pp. 123-131.

Galbraith, JM 2011, *Buried soils and their effect on taxonomic classification*, USDA Natural Resources Conservation Service, viewed 21 May 2018, <www.nrcs.usda.gov/ wps/ portal/nrcs/detail/soils/ref/?cid+nrcs142p2_053568>.

Garcia-Fraile, P, Menendez, E & Rivas, R 2015, 'Role of bacterial biofertilizers in agriculture and forestry', *Bioengineering*, vol. 2, no. 3, pp. 183-205.

Gentili, F & Jumpponen, A 2006, 'Potential and possible uses of bacterial and fungal biofertilizers', in MK Rai (ed.) *Handbook of Microbial Biofertilizers*, The Haworth Press Inc., NY, pp. 1-28.

Glauner, R & Lagan, P 1995, *Forest Site Classification in Forest Management Planning,* Short Term Consultancy Report,

Gryndler, M, Jansa, J, Hrselova, H, Chvatalova, I & Vosatka, M 2003, 'Chitin stimulates development and sporulation of arbuscular mycorrhizal fungi', *Applied Soil Ecology*,no. 22, pp. 283-287.

Habte, M & Osorio, NW 2001, *Arbuscular mycorrhizas: Producing and applying arbuscular mycorrhizal inoculum,* College of Tropical Agriculture and Human Resources, University of Hawai, Manoa Hawai.

Hall, TA 1999, 'BioEdit a user-friendly biological sequence alignment editor and analysis program for Window 95/98/NT,' *Nucl. Acids Symp.*, 95-98.

Hardiyanto, EB, Nambiar, EKS & Inail, MA 2015, 'Challenges to site management during large-scal transition from Acacia mangium to Eucalyptus pellita in short rotation forestry on mineral soils in Sumatra, Indonesia,' *IUFRO Eucalypt Conference 2015*, IUFRO, 1-120.

Haug, I, Setaro, S & Suarez, JP 2013, 'Reforestation sites show similar and nested AMF communities to an adjacent pristine forest in a tropical mountain area of South Ecuador', *PLOS ONE*, vol. 8, no. 5, pp. 1-11.

Hazebroek, HP & Morshidi, AKbA 2001, *National Parks of Sarawak,* Natural History Publications (Borneo), Kota Kinabalu.

Heijden, MGAvd, Klironomos, JN, Ursic, M, Moutoglis, P, Streitwolf-Engel, R, Boller, T, Wiemken, A & Sanders, IR 1998, 'Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity', *Nature*, vol. 386, pp. 69-72.

Hoorman, JJ 2018, *Role of soil fungus*, Ohio State University Extension, viewed 18 April 2018, <https://ohioline.osu.edu/factsheet/anr-37>.

Huberty, AF & Denno, RF 2004, 'Plant water stress and its consequences for herbovotous insects: A new synthesis', *Ecology*, vol. 85, no. 5, pp. 1383-1398.

Husband, R, Herre, EA, Turner, SL, Gallery, R & Young, JPW 2002, 'Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest', *Molecular Ecology*,no. 11, pp. 2669-2678.

Husband, R, Herre, EA & Young, JPW 2002, 'Temporal variation in the arbuscular mycorrhizal communities colonising seedlings in a tropical forest', *FEMS Microbiology Ecology*,no. 42, pp. 131-136.

INVAM 2017, *International Culture of Collection of (Vesicular) Arbuscular Mycorrhizal Fungi*, West Virginia University, viewed 5 January 2017, <https://invam.wvu.edu>.

Ishii, S & Loynachan, TE 2004, 'Rapid and reliable DNA extraction techniques from trypan-blue-stained mycorrhizal roots: comparison of two methods', *Mycorrhiza*,no. 14, pp. 271-275.

Ismail Jusoh & Nur Syazni Adam 2012, *Acacia mangium plantation in Sarawak and its wood quality*, International Institute of Plantation Management, viewed 25 March 2014, <http://www.ipicex.com/docs/2012/posters/Acacia%20mangium%20Plantation%20in%20Sarawak%20and%20its%20Wood%20Quality.pdf>.

Jabatan Pengairan dan Saliran Sarawak 2017.

Jaenicki, H 1999, *Good tree nursery practices - practical guidelines for research nurseries,* International Centre for Research in Agroforestry, Nairobi Kenya.

Jeffries, P, Gianinazzi, S, Perotto, S, Turnau, K & Barea, J-M 2003, 'The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility', *Biology and Fertility of Soils*, vol. 37, no. 1, pp. 1-16.

Jeyanny, V, Lee, SS & K Wan Rasidah 2011, 'Effects of arbuscular mycorrhizal inoculation and fertilisation on the growth of Acacia mangium seedlings', *Journal of Agricultural Science*, vol. 23, no. 4, pp. 404-409.

Johansson, JF, Paul, LR & Finlay, RD 2004, 'Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculuture', *FEMS Microbiol Ecology*,no. 48, pp. 1-13.

John Sabang 2008, 'Multi-disciplinary studies on issues faced by Kelampayan plantations', *Forest Health Consultancy and Field Reports*, Investigation, Sarawak Forestry Corporation Sdn Bhd, 24 March-8 April 2008, viewed 17 July 2014.

John Sabang 2015, 'A manual on site requirement of four forest plantation species in Sarawak', Sarawak Forestry Corporation Sdn Bhd,

John Sabang & Jessica Meredi 2008, 'Growth performance of kelampayan at Leseh Plantation, Kanowt (LPF0032)', *Forest Health Consultancy and Field Report 2008*, Applied Forest Science Industry Development Division, Sarawak Forestry Corporation Sdn Bhd,

Juge, C, Samson, J, Basten, C, Vierheilig, H, Coughlan, A & Piche, Y 2002, 'Breaking dormancy in spores of the arbuscular mycorrhizal fungus *Glomus intradices*: a critical cold-storage period', *Mycorrhiza*,no. 12, pp. 37-42.

Kannan, VR, Suganya, S, Solomon, EK, Balasubramanian, V, Ramesh, N & Rajesh, P 2011, 'Analysis of interaction between arbuscular mycorrhizal fungi and their helper bacteria by MILPA model', *Research in Plant Biology*,no. 1(5), pp. 48-62.

Kaushish, S, Kumar, A & Aggarwal, A 2011, 'Influence of hosts and substrates on mass multiplication of *Glomus mosseae*', *African Journal of Agricultural Research*, vol. 6, no. 13, pp. 2971-2977.

Kheyrodin, H 2014, 'Plant and soil relationship with fungi', *International Journal of Research Studies in Biosciences*, vol. 2, no. 9, pp. 42-49.

Kiers, ET, Lovelock, CE, Krueger, EL & Herre, EA 2000, 'Differential effects of tropical arbuscular mycorrhizal fungal inocula on root colonization and tree seedling growth: implications for tropical forest diversity', *Ecology Letters*,no. 3, pp. 106-113.

Kimjus, K, Ajik, M, Pang, KKN, Lapongan, J, Kodoh, J & Mariyani, AMA 2008, *Annex: Descriptions of species,* A guide to plantation forestry in Sabah, Sabah Forestry Department, Sabah Forest Record No. 6.

King, DA, JR, EGL, Condit, R, Foster, RB & Hubbell, SP 1997, 'Relationship between branch spacing, growth rate and light in tropical forest saplings', *Functional Ecology*,no. 11, pp. 627-635.

Krisnawati, H, Kallio, M & Kanninen, M 2011, *Anthocephalus cadamba Miq.: ecology, silviculture and productivity,* Center for International Forestry Research, Bogor.

Landis, TD & Amaranthus, MA 2009, Vol. 29 USDA, Lincoln, Nebraska, pp. 13-16.

Landis, TD & Dumroese, RK 2009, Vol. 29 USDA, Lincoln, Nebraska, pp. 5-12.

Lapongan, J 2008, *Plantation establishment and maintenance,* A guide to plantation forestry in Sabah, Sabah Forestry Department, Sabah Forest Record No. 16.

Lee, J, Lee, S & Young, JPW 2008, 'Improved PCR primers for the detection and identification of arbuscular mycorrhizl fungi', *FEMS Microbiol Ecol*, vol. 65, pp. 339-349.

Linderman, RG 1988, 'Mycorrhizal interactions with the rhizosphere microflora: The mycorrhizasphere effect', *Phytopathology*, vol. 78, no. 3, pp. 366-371.

Ling, WC & Wong, AHH 2007, 'An overview of the raw material quality, supply and demand for the wood products industry in Sarawak, with insights for future planted forests,' *Conference on natural resources in the Tropics: Development and commercialization of Tropical Natural Resources*, University Malaysia Sarawak, 259-268.

Loi, KS, Lah, J, Eilers, RG & Louie, RH 1985, 'Terrain classification in Sarawak', *Malaysian Journal of Tropical Geography*, vol. 11, pp. 12-31.

Maas, EF, Tie, YL & Lim, CP 1986, *Sarawak land capability classification and evaluation for agricultural crops,* Technical Paper No. 5, Department of Agriculture Sarawak,

Maia, LC & Yano-Melo, AM 2001, 'Germination and germ tube growth of the arbuscular mycorrhizal fungi Gigaspora albida in different substrates', *Brazilian Journal of Microbiology*,no. 32, pp. 281-285.

Malusa, E, Sas-Paszt, L & Ciesielska, J 2012, 'Technology for beneficial microorganisms inocula used as biofertilizers', *The Scientific World Journal*, vol. 2012, no. 491206, pp. 1-12.

Marfaisal Marzuki 2006, 'Common insect pests of Kelampayan,' *Applied Forest Science Research Seminar 2006*, Sarawak Forestry, 235-237.

Marfaisal Marzuki & Tracy Chendan 2008, 'Pests in Pasin and Ng Dap plantations', *Forest Health Consultancy and Field Report*, Applied Forest Science Industry Develpment Division, Sarawak Forestry Corporation Sdn. Bhd.,

Martinez-Viveros, O, Jorquera, MA, Crowley, DE, Gajardo, G & Mora, ML 2010, 'Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria', *J. Soil Sci. Plant Nutr.*, vol. 10, no. 3, pp. 293-319.

McCauley, A, Jones, C & Olson-Rutz, K 2017, Motana State University Extension, pp. 1-16.

McGee, PA, Pattinson, GS, Heath, RA, Newman, CA & Allen, SJ 1997, 'Survival of propagules of arbuscular mycorrhizal fungi in soils in eastern Australia used to grow cotton', *New Phytol.*, vol. 135, pp. 773-780.

Mcgonigle, TP, Miller, MH, Evans, DG, Fairchild, GL & Swan, JA 1990, 'A new method which gives an objectives measur of colonization roots by vesicular-arbuscular mycorrhizal fungi', *New Phytologist*,no. 115, pp. 495-501.

Mead, DJ 2005, 'Opportunities for improving plantation productivity. How much? How quickly? How realistic?', *Biomass and Bioenergy*,no. 28, pp. 249-266.

Meier, R & Charvat, I 1993, 'Reassessment of Tetrazolium bromide as a viability stain for spores of versicular-arbuscular mycorrhizal fungi', *American Journal of Botany*, vol. 80, no. 9, pp. 1007-1015.

Meyer, AH, Botha, A, Valentine, AJ, Archer, E & Louw, PJE 2005, 'The occurrence and infectivity of arbuscular mycorrhizal fungi in inoculated and uninoculated rhizosphere soils of two-year-old commercial grapevines', *S. Afr. J. Enol. Victic*, vol. 26, no. 2, pp. 90-94.

Miller, HG 1981, 'Forest fertilization: Some guiding concepts', *Forestry*, vol. 54, no. 2, pp. 157-167.

Miransari, M 2011, 'Interactions between arbuscular mycorrhizal fungi and soil bacteria', *Applied Microbiology and Biotechnology*, vol. 89, no. 4, pp. 917-930.

Mitchell, G 2015, Personal communication, 13 July 2015, Sarawak Forest Tree Seed Bank, Semengoh, Sarawak, Malaysia.

Miyasaka, SC, Habte, M, Friday, JB & Johnson, EV 2003, *Manual of arbuscular mycorrhizal fungul production and inoculation techniques,* Cooperative Extension Service, CTAHR, University of Hawai'i at Manoa, SCM-5.

My Square Foot Garden 2014, *Mixing it up-tarp method*, viewed 20 Jan 2016, <http://www.mysquarefootgarden.net/mels-mix/ >.

Newman, EI & Reddell, P 1987, 'The distribution of mycorrhizas among families of vascular plants', *New Phytologist*,no. 106, pp. 745-751.

Nopamornbodi, O & Thamsurakul, S 2006, 'Benefits of mycorrhizal biofertilizer', in *Biofertilizer manual*, Japan Atomic Industrial Forum (JAIF), Tokyo, pp. 90-91.

Norhayati Ahmad Sajali, Chai, D, Empenit Empawie & Guru Arai 2010, 'Investigation of insect pest and disease susceptibiity in different Kelampyan provenances at Kuala Baram research plot, 12-16 April 2010', *Forest Health Consultancy and Field Reports 2010*, Applied Forest Science Industry Development Division, Sarawak Forestry Corporation Sdn. Bhd.,

Oehl, F, Sieverding, E, Palenzuela, J, Ineichen, K & Silva, GAd 2011, 'Advances in *Glomeromycota* taxonomy and classification', *IMA Fungus*, vol. 2, no. 2, pp. 191-199.

Ohyama, T 2006, 'Introduction', in *Biofertilizer manual*, Japan Atomic Industrial Forum (JAIF), Tokyo, pp. 1-2.

Okiobe, ST, Angue, MA, Bougnom, BP, Onana, B & Nwaga, D 2015, 'Improvement of arbuscular mycorrhizal fungi inoculum production by nutrient solution concentration and soil texture variation', *International Journal of Agronomy and Agricultural Research*, vol. 6, no. 5, pp. 7-20.

Ong, KH, Chubo, JK, Jie, HK, Chia, SL, Shan, DAS & Philip Sipen 2012, 'Influence of soil chemical properties on relative abundance of arbuscular mycorrhiza in forested soils in Malaysia', *Turk J Agric For*,no. 36, pp. 1-8.

Oscar Johin Ngayop 2013, International Conference Forest Plantation in Sarawak - The Way Forward to Sustainable Development of Timber Industry 27 May 2013, Forest Department Sarawak.

Pearson, WR 2013, 'An introduction to sequence similarity ("Homology") searching', *Curr Protoc Bioimformatics*, vol. June, no. 03, pp. 1-9.

Pek, YK & Isa Kumbang 2006, 'Molded chair shell fabrication of Kelampayan,' *Applied Forest Science Research Seminar 2006*, Sarawak Forestry, 180-183.

Peters, JB, Kelling, KA & Schulte, EE 1996, University of Wisconsin-System Board of Regents and University of Wisconsin-Extension, Cooperative Extension.

Phang, TJ 1991, *Seed technology and nursery practice,* Forest Research Report - RR 2, Reforestation Unit, Forest Research Branch, Forestry Department Sarawak,

Phillips, WS 2012, *Drivers of arbuscular mycorrhizal fungal community composition in roots: Hosts, Neighbors, and environment*, thesis, Oregon State University, pp. 161.

Pinard, M, Howlett, B & Davidson, D 1996, 'Site conditions limit pioneer tree recruitment after logging of dipterocarp forests in Sabah, Malaysia', *Biotropica*,no. 28 (1), pp. 2-12.

Rashmi & Singh, SB 2008, 'Studying effect of nitrogen and potassium fertilizer on growth and essential oil content of *Cymbopogon citratus* and *Vetiveria zizaniodes*', *Journal of Essential Oil Bearing Plants*, vol. 11, no. 2, pp. 188-193.

Reddy, SR, Pindi, PK & Reddy, SM 2005, 'Molecular methods for research on arbuscular mycorrhizal fungi in India: problems and prospects', *Current Science*, vol. 89, no. 10, pp. 1699-1709.

Redecker, D, Hijri, I & Wiemken, A 2003, 'Molecular identification of arbuscular mycorrhizal fungi in roots: Perspective and problems', *Folio Geobotanica*,no. 38, pp. 113-124.

Ridgway, HJ, Kandula, J & Stewart, A 2004, 'Optimising production of carrot hairy roots', *New Zealand Plant Protection*,no. 57, pp. 77-80.

Rodriguez-Morelos, VH, Soto-Estrada, A, Perez-Moreno, J, Franco-Ramirez, A & Diaz-Rivera, P 2014, 'Arbuscular mycorrhizal fungi associated with the rhizosphere of seedlings and mature trees of *Swietenia macrophylla* (Magnoliophyta: Meliaceae in Los Tuxtlas, Veracruz, Mexico', *Revista Chilena de Historia Natural*, vol. 87, no. 9, pp. 1-10.

Rousk, J, Baath, E, brookes, PC, Lauber, CL, Lozupone, C, Caporaso, JG, Knight, R & Fierer, N 2010, 'Soil bacterial and fungal communities across a pH gradient in an arable soil', *International Society for Microbial Ecology*, pp. 1-12.

Ruiz-Lozano, JM & Azcon, R 1996, 'Viability and infectivity of mycorrhizal spores after long term storage in soils with different water potentials', *Applied Soil Ecology*,no. 3, pp. 183-186.

Sabah Forestry Department 2008, *Research and development,* Annual Report 2008, Sandakan Sabah, Sabah Forestry Department,

Sabrina Aslan Joe 2007, 'Determination of optimu storage temperature for *Neolamarckia cadamba* (Kelampayan) seed,' *Conference on natural resources in the Tropics: Development and commercialization of Tropical Natural Resources*, Universiti Malaysia Sarawak, 237-241.

Saharan, BS & Nehra, V 2011, 'Plant growth promoting rhizobacteria: A critical review', *Life Sciences and Medicine Research*, vol. 2011, no. 21, pp. 1-30.

Sasvari, Z, Margurno, F, Galanics, D, Tran, TNH, Tran, THH, Nguyen, DL, Le, MH & Posta, K 2012, 'Isolation and identification of arbuscular mycorrhizal fungi from agricultural fields of Vietnam', *American Journal of Plant Sciences*,no. 3, pp. 1796-1801.

Schoch, CL, seifert, KA, huhndorf, S, Robert, V, Spouge, JL, Levesque, CA, Chen, W & Fungal Barcoding Consortium 2012, 'Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi', *PNAS*, vol. 109, no. 16, pp. 6241-6246.

Schubler, A, Schwarzott, D & Walker, C 2001, 'A new fungal phylum, the *Glomeromycota*: phylogeny and evolution', *Mycological Research*, vol. 105, no. 12, pp. 1413-1421.

Seed Bank Semengoh 2013, *Activity Report,* Planting Materials Unit, AFSID, Sarawak Forestry,

Sikes, BA 2010, 'When do arbuscular mycorrhizal fungi protect plant roots from pathogens?', *Plant Signaling & Behavior*, vol. 5, no. 6, pp. 763-765.

Singh, R & Adholeya, A 2013, 'Diversity of AM (Arbusclar mycorrhizal) fungi in wheat agro-climatic regions of India', *Virol Mycol*, vol. 2, no. 2, pp. 1-9.

Siti Hanim Sahari 2005, 'Suitability of three potential plantation species for plywood production' *Applied Forest Science and Industry Development (AFSID) Research Seminar 2005*, Sarawak Forestry, 169-173.

Smith, FW & Loneragan, JF 1997, 'Interprtation of plant analysis: concepts and principles', in D Reuter and JB Robinson (eds), *Plant analysis: an interpretation manual*, CSIRO Publishing, Collingwood, pp. 1-34.

Souza, T 2015, *Handbook of arbuscular mycorrhizal fungi,* Springer International Publishing, Switzerland.

STIDC 2009, 'Seminar on viability assessment of indigenous tree species and propogation techniques for planted forest development in Sarawak', *Perkasa*, vol. 5/6, May-June 2009, pp. 1-7.

Sudrajat, DJ, Nurhasybi, Siregar, IZ, Siregar, UJ, Mansur, I & Khumaida, N 2016, 'Intraspecific variation on early growth of *Neolamarckia cadamba* MIQ. in provenance-progeny tests in West Java Province, Indonesia', *Biotropica*, vol. 23, no. 1, pp. 10-20.

Sullivan, TS, Barth, V & Lewis, RW 2017, Washington State University Extension, pp. 1-6.

Sultana, T & Pindi, PK 2012, 'Role of arbuscular mycorrhizae (AM) fungi and multi bioinoculants in cotton plant growth', *British Microbiology Research Journal*,no. 2(3), pp. 123-130.

Sunseed Desert Technology 2012, *A simple method for making your own mycorrhizal inoculum*, Sunseed Desert Technology, viewed 25 April 2014, <http://www. sunseed.org.uk/wp-content/uploads/2012/06/A-SIMPLE-METHOD-FOR-MAK I N G -YOUR-OWN-MYCORRHIZAL-INOCULUM.pdf>.

Sykorova, Z, Ineichen, K, Wiemken, A & Redecker, D 2007, 'The cultivation bias: different communities of arbscular mycorrhizal fungi detected in roots from field, from bait plants transplanted to the field, and from a greenhouse trap experiment', *Mycorrhiza*,no. 18, pp. 1-14.

Tamil Nadu Agricultural University 2014, *Biofertilizers*, viewed 14 April 2014, <http://agritech.tnau.ac.in/org_farm/orgfarm_biofertilizers.html>.

Tamura, K, Stecher, G, Peterson, D, Filipski, A & Kumar, S 2013, 'MEGA6: Molecular Evolutionary Genetics Analysis version 6.0', *Molecular Biology and Evolution*,no. 30, pp. 2725-2729.

Teng, CS 1993, *Key to soil classification in Sarawak,* Department of Agriculture Sarawak, Kuching.

Teng, CS 2004, *Keys to Soil Classification Sarawak,* Cawangan Pengurusan Tanah, Jabatan Pertanian Sarawak.

The Constructor 2017, *Types of rain gauges for measuring rainfall*, viewed 19 February, <https://the constructor.org/water-resources/types-of-rain-gauges/120801/>

Tie, YL 1982, *Soi classification in Sarawak,* Technical paper No. 6, Department of Agriculture Sarawak.

Tinker, PB 1984, 'The role of microorganisms in mediating and facilitating the uptake of plant nutrients from soil', *Plant and Soil*,no. 76, pp. 77-91.

Toljander, JF, Artursson, V, Paul, LR, Jansson, JK & Finlay, RD 2006, 'Attachment of different soil bacteria to arbuscular mycorrhizal fungal extraradical hyphae is determined by hyphal vitality and fungal species', *FEMS Microbiol Lett*,no. 254, pp. 34-40.

Toljander, JF, Santos-Gonzalez, JC, Tehler, A & Finlay, RD 2008, 'Community analysis of arbuscular mycorrhizal fungi and bacteria in the maize mycrrhizosphere in a long term-fertilization trial', *FEMS Microbiol Ecol*, p. 65.

Trees for Life 2018, *Mycorrhizas*, viewed 17 April 2018, <https://treesforlife.org.uk/forest/ecology/mycorrhizas/>

Vandamme, A-M 2009, 'Introduction - basic concepts of molecular evolution', in P Lemey, M Salemi and A-M Vandamme (eds), *The phlogenetic handbook*, Cambridge University Press, New York, pp. 1-30.

Vassilev, N, Vassileva, M, Lopez, A, Martos, V, Reyes, A, Maksimovic, I, Eichler-Lobermann, B & Malusa, E 2015, 'Unexploited potential of some biotechnological techniques for biofertilizer production and formulation', *Appl Microbiol Biotechnol*,no. 99, pp. 4983-4996.

Verbruggen, E, Heijden, MGAvd, Rillig, MC & Kiers, ET 2013, 'Mycorrhizal fungal establishment in agricultural soils: factors determining inoculation success', *New Phytologist*,no. 197, pp. 1104-1109.

Vierheilig, H, Schweiger, P & Brundrett, M 2005, 'An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots', *Physiologia Plantarum*,no. 125, pp. 393-404.

Walker, C 1999, 'Methods for culturing and isolating arbuscular mycorrhizal fungi', *Mycorrhiza News*, vol. 11, no. 2, pp. 2-4.

Walker, C & Vestberg, M 1994, 'A simple and inexpensive method for producing and maintaining pot culture of arbuscular mycorrhizal fungi', *Agricultural Science in Finland*,no. 3, pp. 233-240.

Whitman, M 2009a, 'Glomalin: Hiding place for a third of world's stored soil carbon', *Wild Ones*,no. May/June, pp. 1-2.

Whitman, M 2009b, 'Thinking about mycorrhizae and roots, and fungi, and hyphae, and how plant really get their nutrients', *Wild Ones*,no. January/February 2009, pp. 1-3.

Whitmore, TC 1990, *An introduction to tropical rain forests,* Clarendon Press, Oxford.

Williams, A, Manoharan, L, Rosenstock, NP, Olsson, PA & Hedlund, K 2017, 'Long-term agricultural fertilization alters arbuscular mycorrhizal fungal community composition and barley (*Hordeum vulgare*) mycorrhizal carbon and phosphorus exchange', *New Phytologist*,no. 213, pp. 874-885.

World Agroforestry Center 2015, *Anthocephalus cadamba*, World Agroforestry Center, viewed 21 September 2015, <http://www.worldagroforestry.org/treedb2/ species profile.php?Spid=17933>.

World Rainforest Movement 1998, *"A fortune for the people" of Sarawak*, viewed 15 April 2018, <http://wrm.org.uy/oldsite/bulletin/8/Malaysia3.html>.

Wu, SC, Cao, ZH, Li, ZG, Cheung, KC & Wong, MH 2004, 'Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial', *Geoderma*, vol. 125, no. 1-2, pp. 155-166.

Yang, MC 2005, 'Lesser-known timbers of Sarawak,' *Applied Forest Science and Industry Development (AFSID) Research Seminar 2005*, Sarawak Forestry, 122-130.

Young, JPW 2012, 'A molecular guide to the taxonomy of arbuscular fungi', *New Phytologist*,no. 193, pp. 823-826.

Zhang, L, Xu, M, Liu, Y, Zhang, F, Hodge, A & Feng, G 2016, 'Carbon and phosphorus exchange may enable cooperation between an arbuscular mycorrhizal fungus and a phosphate-solubilizing bacterium', *New Phytologist*,no. 210, pp. 1022-1032.

Appendix


9


10


11


12


13


14


15


16

Figure 76. Combo pictures of materials and methods. 1: Natural stand of *Neolamarckia cadamba*, 2: Lemongrass nurse plant, 3: Application of soil mycorrhizal inoculum, 4: Preparation of field trial plot, 5: Installation of Symon rain gauge, 6: Soil auger profile, 7: Application of fertilizer nugget, 8: Measurement of root collar diameter, 9: Measurement of tree height, 10: Tree defoliation, 11: Stem canker, 12: Leaf sampling for nutrient analysis, 13: Soil mycorrhizal inoculum field trial, 14: Soil trap culture trial, 15: Pot substrate culture trial, 16:) Root staining to determine mycorrhizal colonization, 17: Single spore culture trial, 18: Spore inoculation, 19: Crushing spore using micro-pestle for DNA extraction, and 20: Re-propagation culture trial.

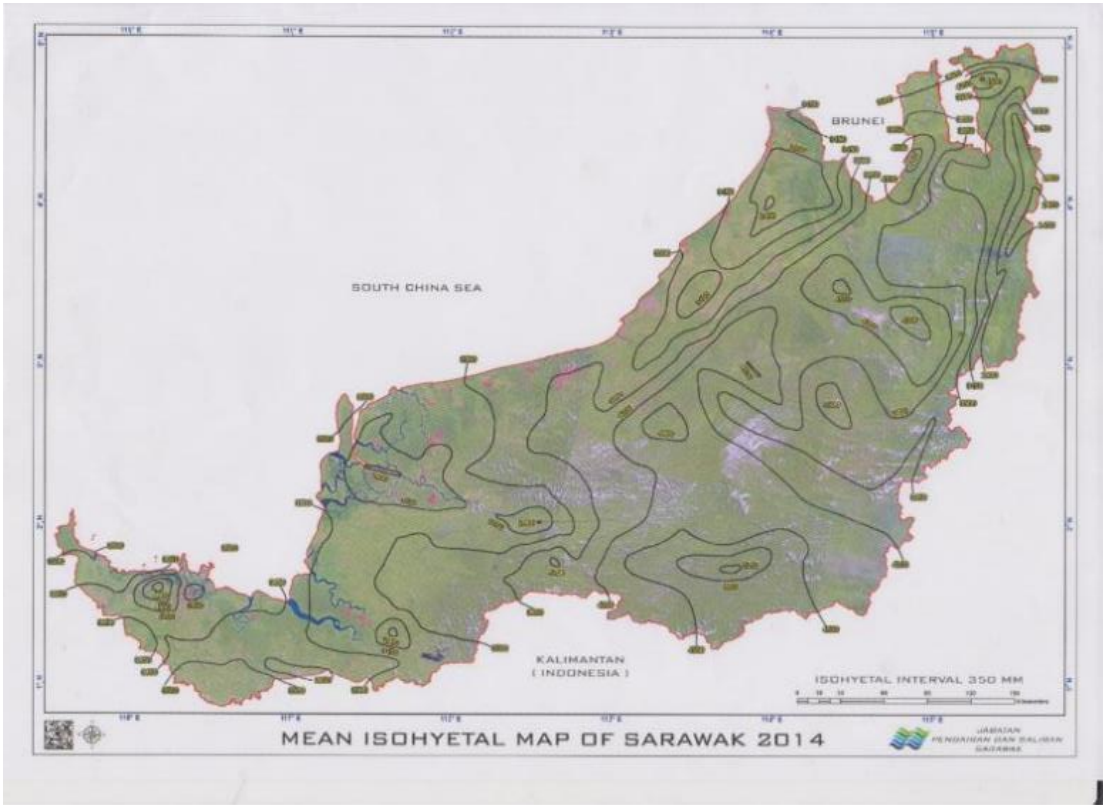


Figure 77. Mean Isohyetal map of Sarawak 2014.