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**ESTABLISHMENT OF ARBUSCULAR MYCORRHIZAL FUNGI (AMF) ON
MICROPROPAGATED TEAK (*Tectona grandis* L. f.)**

María Isabel Ramírez Caro

Degree in Biology, District University of Bogotá

This thesis is presented in fulfilment of the requirements for the degree of
Master of Science (Biological Sciences)

Faculty of Health, Engineering and Science

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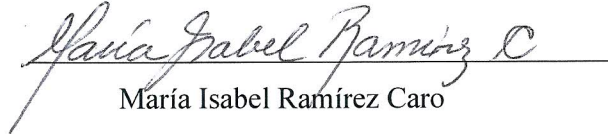
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Abstract

Improvement of root production and ability to establish mycorrhizas from different Arbuscular Mycorrhizal Fungi (AMF) inoculum sources on two micropropagated teak (*Tectona grandis* L. f.) clones was examined at the acclimatisation phase. Teak shoots were maintained on a multiplication medium containing Murashige and Skoog (MS) nutrients and organics, 30 g L⁻¹ sucrose, 0.5 µM benzyl amino purine, 0.5 µM kinetin, 2.5 g L⁻¹ agar, 2.5 g L⁻¹ gelrite and pH 5.8. After 35 days shoots were exposed to a rooting medium (RM) containing ¼ strength MS macronutrients, ½ strength MS iron, full strength MS micronutrients, and 20 g L⁻¹ sucrose, 2.5 g L⁻¹ agar, 2.5 g L⁻¹ gelrite and pH 5.5. This RM was supplemented with indole-3-butyric acid (IBA) ranging between 0 – 160 µM to evaluate the most suitable IBA concentration for producing roots without affecting survival and growth in soil. Minimum and maximum times of exposure to IBA were also examined for periods from 4 to 28 days. Rooting *in vitro*, using soils made by combining sand:perlite (1:1, v/v), sand:peat (1:1, v/v), sand:peat:perlite (1:1:1, v/v/v) and agar, were also investigated as mycorrhization *in vitro* was pursued. Rooted teak plants were inoculated with isolated AMF spores, or inoculum product, or soil-based inoculum.

It was determined that exposure to IBA between 40 and 80 µM for 8 days, followed by transfer to RM without IBA for another 14 to 28 days, induced the most roots. Higher auxin concentrations (160 µM) did not produce more roots but decreased survival after transfer to soil. Rooting *in vitro* using pasteurised soils produced rooted plantlets with shorter roots than those produced on a solidified agar medium, but the plants were small and the roots did not develop so rooting *ex vitro* was adopted. Plants grown in inoculum comprising isolated spores showed significant differences in growth, although there were no visible features of AMF establishment. Similarly, plants grown with an unprocessed inoculum product showed hyphal development, but there were no arbuscules or vesicles evident. In contrast, 100% of the plants grown in an Australian organic farming soil treatment were colonized with AMF, and arbuscules, vesicles, spores and intra and extraradical hyphae formed. The latter plants were taller and had larger root surface areas than the other treatments after 20 weeks of acclimatisation. Surprisingly, mycorrhization was achieved despite a reasonable level of phosphorus in

the soil. Variation in clone responses was also recorded. One of the clones, T201, showed a low multiplication rate and was less responsive to all treatments.

Early mycorrhization of teak plantlets might provide advantages when transferring the plantlets to the field; for example early mycorrhization might reduce transplant shock, reduce the need for inorganic fertilizers, and the extraradical mycelium could help populate the soil with AMF. Increased growth and root area found in this research cannot be solely explained by mycorrhizal establishment; it could also be a result of nutrients in the soil. Standardised rooting and mycorrhization protocols were developed for teak, but these could also be applied or adapted for other forest trees.

Key words

Teak mycorrhization, AMF, *in vitro* rooting, *in vitro* mycorrhization, teak clones.

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Abbreviations

| | |
|-------------|--|
| AMF | Arbuscular Mycorrhizal Fungi |
| BAP | 6-Benzylaminopurine |
| BEG | Banque Europeen of Glomales |
| DI | Deionised Water |
| ERM | Extraradical Mycelium |
| IAA | Indole Acetic Acid |
| IBA | Indole 3-Butyric Acid |
| ICP-OES | Inductively Coupled Plasma Optical Emission Spectrometry |
| IP | Processed Inoculum Product |
| IRM | Intra Radical Mycelium |
| MS | Murashige and Skoog's medium |
| mMS | modified Murashige and Skoog's medium |
| MY4 | a single teak clone used in the present research |
| NAA | 1-Napthaleneacetic Acid |
| rh. | relative humidity |
| ROC | Root organ culture |
| Tukey's HSD | Tukey's honestly significance difference test |
| T201 | a single teak clone used in the present research |
| UIP | Unprocessed Inoculum Product |
| VG soil | inoculum made from organic farming soil |

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Chapter 1: Micropropagation and mycorrhization

1.1. Introduction

This chapter provides background information for this research project. It details general aspects of teak, micropropagation and mycorrhization. Specific information about micropropagation on teak and mycorrhization on teak will be provided in later chapters.

1.2 Teak

Teak grows naturally in: India, Thailand, Lao People's Democratic Republic (Lao PDR) and Myanmar. Teak is a high quality tropical timber and as a result has high commercial value. Teak wood is used in transport (sea, rail, road), housing, wood carving, furniture, as poles and plywood. It is easy to work with, does not lose its shape, is resistant to termite attack, does not break easily and is durable. It is considered the most promising hardwood for plantation establishment in at least 43 tropical countries on four continents (Kollert & Cherubini, 2012). There are now 29 million ha of natural teak forest throughout the world and only 4.3 million ha of teak plantations; however, natural stands are becoming less and less available for harvesting (Goh & Monteuis, 2012; Kollert & Cherubini, 2012).

Teak requires specific physical conditions to grow well. For instance, in India growth occurs at altitudes under 1000 m with annual rainfall ranging from 900 to 2,500 mm and has maximum growth from March to October (Tiwari et al., 2002). In Thailand, teak grows best where the annual rainfall is between 1,200 and 1,500 mm with a dry season of three – five months. It seems that areas with high rainfall all year round (2,000 mm/year or more) promote teak growth (Goh & Monteuis, 2012; Midgley, Blyth, Mounlamai, Midgley, & Brown, 2007). Similar rainfall (2,500 mm/year) has been acknowledged in India (Palanisamy, Gireesan & Hedge, 2009) as producing the best growth rates. Similarly, Krishnapillay (2000) reported that between 1,250 and 3,750 mm per year is the optimum rainfall in Malaysia; however, the author stressed the importance of having a dry season (less than 60 mm) annually for a minimum of

four months. Teak has also been introduced to regions with similar climatic conditions, for example, Matto Grosso state in Brazil, which receives 1,400 mm per year of rainfall, and Ecuador, which receives from 1,400 to 2,500 mm per year rainfall, and at altitudes from 50 to 250 m above sea level (Goh & Monteuis, 2012). With regard to soil type, teak has been reported to grow well in a variety of soil types (Goh & Monteuis, 2012; Krishnapillay, 2000). For example, it has been reported that teak was successfully grown in North Eastern India where the soils are loamy sands (Singh, Tiwari & Dkhar, 2003), and also in Brazil where the soils are similar to North Eastern India (Goh & Monteuis, 2012).

Despite its use in large scale plantations, teak is also considered an important option for small landholders. Teak plantations produce returns after 15 years; however, small-scale plantings in Laos from the 1960s to the 1990s (Roder, Keoboualapha, & Manivanh, 1995) now total over 40,000 ha (Mohns, Bianchi & Noeske, 2013) and have become a valuable asset to small landholders. Short-term returns for the first three years have been obtained from rice, sesame or pineapples that have been inter-planted with teak while the trees are maturing. It has also been suggested that teak can be inter-planted with paper mulberry (*Broussonetia papyrifera*, which can be harvested after 18 to 24 months) greengram, groundnut and fingermille (Midgley et al., 2007; Roder et al., 1995; Venkateswarlu & Korwar, 2005).

1.2.1 Teak sexual propagation

Teak trees grown from seeds show a wide variety of characteristics due to the substantial genetic variation from the sexual processes (Krishnapillay, 2000; Lopez, 2013; Venkateswarlu & Korwar, 2005). Seed-grown teak trees can also introduce undesirable variation under plantation circumstances. In addition, there is inadequate supply of teak seed (particularly good quality seed) to meet the demand of expanding plantations (Midgley et al., 2007). There are seed orchards in countries such as Thailand (Dr S. Wattanasuksakul, personal communication, March 29, 2013) and Bangladesh (Al Mahmud & Hossain, 2013), but these have not been capable of producing a sufficient quantity of seeds to satisfy the demand.

1.2.2 Teak asexual propagation

Due to the lack of suitable seed, and the advantages that can be achieved through clonal propagation, techniques such as budding, cutting and grafting have gained importance in providing teak planting material (Aminah, Fadhilah & Nor Hasnida, 2013; Gavinlertvatana, 1998). Grafting has been used successfully by several workers in Thailand (Gavinlertvatana, 1998) and in India (Shirin, Rana & Mandal, 2005). Shoots from plus teak trees as old as 60 years have been grafted onto root stocks and, after five years, new shoots from the grafts have been used as a source of nodal segments for micropropagation (Shirin et al., 2005).

Cuttings have also been used to propagate teak in various ways. For example, two-month-old shoot cuttings from cloned teak have been taken from plants of different ages: 2 months (seedlings) and trees 15 to 30 years old (Husen & Pal, 2006). Husen and Pal applied auxins were applied to the cuttings once, using powder comprising auxin and 0.05% Bavastin® fungicide, at concentrations of 2000 ppm of 1-naphthalene acetic acid (NAA) or IBA (0.01 μ M NAA or 0.01 μ M IBA) or 4000 ppm NAA or IBA (0.02 μ M NAA or 0.02 μ M IBA) to induce adventitious roots. The substrate they used was vermiculite soaked in water for 24 hr and sterilized. The cuttings were maintained for 30 days in a mist chamber at 85% humidity with day temperatures of 32 °C and night temperatures of 26 °C. The authors concluded that formation of adventitious roots was markedly promoted when the plant donor was young and the auxin concentration was low. In addition, the Maegar Silvicultural Research Station (2013) is currently using cuttings from grafted buds to propagate teak, and to achieve between 25% and 100% rooting, depending on clonal variation. This procedure has been adapted for several teak nurseries in Thailand (World Teak Conference 2013: Excursion II: Teak Improvement in Thailand, n.d). Therefore, grafting is still being used to produce nodal segments for subsequent micropropagation, but this technique does not produce enough plants to supply large-scale plantations.

Teak micropropagation using mature selected trees is reported as being a feasible method to produce vigorous plants; however, there is not enough production of plant material from tissue culture, which has superior genetic quality (Callister, 2013; Nor Aini, Goh, & Ridzuan, 2009). Gavinlertvatana (1998) reported the successful

establishment of teak plantations from tissue cultured plants in 1994 – 1995 with explants obtained from plus teak trees aged 100 to 180 years old. In India, Venkateswarlu and Korwar (2005) reported successful establishment of micropropagated teak in field trials from 1997 to 2003. They concluded that plants originating from tissue culture performed better than stump coppice cuttings or seedlings and that the plantations were more homogenous. Recently, Goh and Monteuis (2012) reported the successful establishment of millions of micropropagated plants in East Malaysia, Indonesia, Tanzania, Brazil, Mexico and Ecuador. All these plantlets originated from eight plus trees and overall the cloned trees performed better than the trees produced from seeds. Both Goh and Monteuis' (2012) and Venkateswarlu and Korwar's (2005) have reported that environmental conditions, such as all-year-round high rainfall, promote high yield and increased girth. Therefore, teak grown from vegetative procedures, such as micropropagation, can provide more homogeneous plant material that can perform better than those plants originating from seedlings or stumps (Goh & Monteuis. 2012; Venkateswarlu & Korwar, 2005).

1.2.3 Teak in Australia

Teak plantations have emerged worldwide as an option to the limited number of logs that can be harvested from the native forests and as a new source of income (Kollert & Cherubibi, 2012). Reid and Stephen (2001) reported that the oldest known plantation of teak in Australia was a small 12-year-old plantation in the Northern Territory near Darwin. Reilly, Robertson, Neitzel, Clark, and Hearnden (2005) reported that teak was found to be one of the best performing exotic hardwoods in the Top End Regional Tropical Hardwood Forestry Project. More recently, Elders Forestry (2011) reported that there were 805 plantation units (161 hectares) planted in far north Queensland (Cooktown) on the Mount Ray Tree Farm. However, due to the global financial crisis in 2007, Elders Forestry has reduced investment in this area.

1.3 Micropropagation

Plant tissue culture was developed in the early 20th century and was initially used to examine different aspects of plant development. Plant tissue culture has been developed to such an extent that it now has standard applications in molecular biology, plant breeding and plant propagation. Plant propagation through tissue culture is usually

referred to as micropropagation whereas the more traditional methods of asexual propagation are called cutting, grafting and layering. Micropropagation rapidly reproduces a selected genotype using isolated parts of plants (explants) such as apical or axillary buds or pieces of leaves or flowers (Neuman, Kumar & Jafaegholi, 2009; Werbrouck & Debergh, 1994).

1.3.1 Micropropagation stages

Micropropagation is generally divided into four or five stages (George, Hall & De Klerk, 2008; Werbrouck & Debergh, 1994). Stage 0, or the preparative stage, is where plants are grown under protected conditions with environmental controls (e.g. in greenhouses) to obtain hygienic parent material at the appropriate growth stage. These conditions reduce potential microbial contamination and provide tissue that is more responsive once introduced into culture. Stage 1 involves the establishment of axenic cultures from suitable explants. The explants may include structures such as pre-existing meristems (e.g. axillary and apical buds) or the use of different tissues such as shoots, leaves and flowers to form adventitious meristems for direct organogenesis or callus for indirect organogenesis. This stage aims to establish axenic cultures (only the plant species of interest) in a controlled environment. Stage 2, shoot multiplication, involves the production of a large number of adventitious shoots or buds from the material obtained in stage 1. Stage 3 aims to allow shoot elongation and root induction. The production of adventitious and axillary shoots during stage 2 usually results in a lack of roots, so stage 3 is used to improve root induction. Disadvantages of rooting *in vitro* have also been identified and these include: increasing cost per plant and poor root development (Werbrouck & Debergh, 1994). Stage 4, or acclimatisation, is when rooted plantlets gradually adapt to external conditions. It involves plant transfer to a suitable substrate in conditions usually of high humidity and controlled light and temperature.

In the current research modifications to micropropagation on teak were made in stage 3 (see Chapter 3) and stage 4 (see Chapter 4).

1.3.2 Micropropagation conditions

Conditions for micropropagation vary between species and even genotypes and modifications in protocols for each of the above stages need development prior to

commercialisation. In general, the development of commercial protocols was initially restricted to herbaceous plants such as ferns and African violets, but woody species (McCown, 2000; Nas & Read, 2004) particularly important timber species have required considerably more development.

For the development of micropropagation protocols for woody species, several problems were initially reported. The early stages (0 and 1) caused problems such as high contamination and initial slow growth. These were overcome by applying specific sterilisation conditions such as collecting material at appropriate times of the year, maintaining plants under protected conditions, and frequent subcultures after initiation. The stage 2 components relied upon the long-term establishment of cultures before growth and multiplication occurred (referred to as stabilisation (McCown, 1988)). Stabilisation has been associated with tissue rejuvenation, which has been identified as a key factor in adventitious root induction (Binet, Lemoine, Martin, Chambon & Gianinazzi, 2007; Feijó & Pais, 1990; Hackett, Murray, Woo, Stapfer, & Geneve, 1990). Grafting, partial etiolating and successive horizontal re-culturing have been reported as techniques that assist in achieving tissue rejuvenation on *Quercus robur*, *Castanea sativa* and *Camellia* spp. (Ballester, Sánchez, San-José, Vieitez, & Vietez, 1990). Finally, once root induction occurs, the conditions under which plantlets survive after transfer to soil were seen to be very specific. Despite these problems, some economically important tree species have been now commercially micropropagated such as *Castanea sativa*, *Hevea brasiliensis*, *Olea europea*, *Eucalyptus grandis* and *Quercus robur*.

Micropropagation is one of the vegetative propagation techniques that can be used for clonal propagation of economically important tree species and can fulfil the needs of large-scale plantation establishments. Generally, once shoot cultures of these species have been obtained stabilised the most frequently reported difficulty is the induction of roots. Nevertheless, root induction can be achieved by the use of an auxin such as indole-3-butyric acid (IBA). IBA can be used to induce rooting on shoots at low concentrations (1 – 50 μM) for long periods of time (four weeks) or at high concentrations (100 – 250 μM) for short periods (two days) and then the rooted shoots can be transferred to a medium without hormones (Bennett, McDavid, & McComb,

2003; Binet et al., 2007; Padilla, Carmona, Westendorp, & Encina, 2006; Pérez-Tornero, Tallón, & Porras, 2010). Therefore, auxin (particularly IBA) at low concentrations is used to induce adventitious roots on micropropagated plants regardless of the species.

One species that has received particular attention and successful commercial production through micropropagation is *Tectona grandis* (teak). This species displays some of the difficulties listed above, such as: difficult initiation (Castro, Díaz & Linero, 2002; Kozgar & Shahzad, 2012; Tiwari, Tiwari & Siril, 2002), stabilisation as illustrated by increased root production after long periods in culture (Gupta, Nadgir, Mascarenhas, & Jagannathan, 1980; Husen & Pal, 2006; Mendoza De Gyves, Royani, & Rugini, 2007), and low survival at the acclimatisation stage (Quiala et al., 2012). However, under certain conditions these difficulties can be resolved.

1.4 Mycorrhization

Micropropagation combined with mycorrhization has been recognised as an option to provide high numbers of quality forest or crop trees for plantations or reforestation (Kapoor, Sharma & Bhatnagar, 2008; Rai, 2001). This combination has already been applied to a number of associations between ectomycorrhiza and forest trees (Brundrett et al., 2005), but due mainly to the difficulty of growing pure fungal cultures, the combination has been very limited in the development of arbuscular mycorrhizal symbiosis.

Mycorrhizal symbiosis is found on nearly 86 % of flowering plants (Brundrett, 2009) and is essentially a partnership between roots of the host plants and members of the fungal phylum Glomeromycota. As a result, the fungal partner forms organs of nutrient exchange and extraradical mycelium (ERM) from the mycorrhizal roots to the rhizosphere, thus increasing the root surface area (Marsh & Schultze, 2001).

The main function of these external threads is the absorption and translocation of nutrients (particularly phosphorus) from the rhizosphere to the root cortex (Harrison, 1997). Mycorrhizas are considered a biological tool because they can:

- act as biofertilizers and bioprotectors (Linderman, 2010; Varma & Schuepp, 1995) to enhance plant growth (Duponnois et al., 2007; Vidal, Azcón Aguilar & Barea, 1992; Wang, Xia, Wu, Liu & Hu, 2007)
- impact on plant reproduction (Koide, 2010)
- increase nutrient concentrations (particularly nitrogen (N), phosphorus (P), and potassium (K)) (George, 2010)
- improve soil aggregation and produce glomalin (Millar & Jastrow, 2010)
- reduce environmental stress when transferring plants to soil (Desjardins, Hernández-Sebastià, & Piché, 2005; Vidal et al., 1992)
- improve tolerance to low-temperature stress (Zhou, Ma, Liang, Huang, & Pinyopusarerk, 2012)
- act as a regulator for the amount of P that can be transferred to the roots of the plants (Nazeri, Lambers, Tibbett & Ryan, 2014).

Arbuscular mycorrhizal fungi (AMF) are normally present in soils and found in the presence of more than one species (Abbott & Robson, 1991; Liu & Yang, 2008). It has been suggested that the presence of specific mycorrhizal fungi may influence the occurrence of particular host species and specific types of vegetation (Janos, 1980; Van Der Heijden, Boller, Wiemken & Sanders, 1998). Therefore, a considerable amount of literature has been published on mycorrhiza and their ecosystem functions. In addition, it has been clearly identified that the successful establishment of introduced trees in a region may depend upon being able to find mycorrhizal partners in the local soil: either the host being planted with its own fungal partners (non-native), or related host plants being present that may share symbionts (Kalucka et al., 2011). It is considered that these mycorrhizal partnerships are not always specific and a single fungal species may establish symbioses with many different plant species (Dhar & Mridha, 2012; Harrison, 1997).

Nowak and Shulaev (2003) remarked on the importance of enhancing micropropagated plantlets' resistance to the factors that can affect successful establishment by using microorganisms, such as AMF, and by changing environmental conditions from heterotrophic to autotrophic growth, and that the enhancing should happen prior to the acclimatisation stage. The authors coined the term 'priming' to describe plantlets that have been exposed to these factors and have developed a type of resistance. In this context, 'biopriming' is defined as the adaptation to beneficial microorganisms and can be caused by secondary metabolic responses (Nowak & Shulaev, 2003). The research

presented here aimed to enhance micropropagated teak plantlets by adding AMF prior to the acclimatisation stage (*in vitro* mycorrhization).

1.4.1 AMF isolation

Walker (1999) explained several methods to isolate AMF, namely: soil trap cultures, plant trap cultures, pot substrate cultures, root fragment cultures, multi-spore cultures and single spore cultures. All these methods start with AMF that can be obtained from soil as mycorrhizal root pieces or spores (Brundrett, Abbot, Jasper & Ashwath, 1995; Oehl et al., 2004) and the methods differ in the quality and composition of the inoculum produced. Difficulties can occur when the fungal material is spores, including: identification of species based solely on morphological characteristics, mixtures of species due to hyphal fragments attached to dead spores, or spores being at different stages of development (Redecker et al., 2013; Schwartz et al., 2006; Walker, 1999). Calvente, Cano, Ferrol, Azcón-Aguilar and Barea's (2004) study produced two-stage plant trap inoculum and then used the inoculum with micropropagated olive trees. In the first stage, extracted spores were collected from rhizospheres of mature field-grown olives and were multiplied using alfalfa (*Medicago sativa*) (*M. sativa*) as a plant trap and sterilised sand:soil (1:1, v/v) as medium. These cultures were subcultured until evidence of pure cultures was achieved. A second trap pot culture was set using these mono-specific cultures and multiplying them in a medium composed of vermiculite:sepiolite (1:1, v/v) and clover as a host plant. Isolated spores were registered at the Banque Europeen of Glomales (BEG) culture collection. Stockinger, Walker and Schüßler (2009) used pot cultures and single-spore isolates to carry out research about identification and to distinguish between a few *Rhizophagus intraradices* (*R. intraradices*) (formerly *Glomus intraradices*; Schüßler & Walker, 2010) vouchers/cultures using molecular analyses.

Glomales inoculum production

Pot culture and plant trap culture are the most widely used techniques to multiply Glomalean fungi to produce inoculum (Calvente et al., 2004; Jin, Germida & Walley, 2013; Ramanwong & Sangwanit, 1999). To form a pot culture, spores, inert materials (e.g. sandy soils), nutrient solution and the trap plants are needed. According to Brundrett, Bougher, Dell, Grove, and Malajczuck (1996), the spores can be obtained

from field soil samples or an existing inoculum. The sandy soil should be sterilised twice in a steamer at 90 °C for at least 1 hr. The nutrient solution can be one of several common formulae used, for instance, Sorghum (Brundrett et al., 1996) or Long Ashton (Calvente et al., 2004; Nazeri et al., 2014). Finally, the trap plant has to be compatible with the fungus species and tolerant of the environmental conditions in which the pot culture will be established. Ijdo, Cranenbrouck and Declerck (2011) reported that plant species such as onion and leek (*Allium cepa*), Bahia grass (*Paspalum notatum*) and maize (*Zea mays*) (*Z. mays*) are considered as ideal hosts to AMF. The number of trap plants in each pot can affect the mycorrhization percentage per plant. In Wang et al.'s (2007) work, 50 g of inoculum was placed 5 cm below 20, 30, 40, 50 or 60 red clover seedlings. The highest rate of colonization was obtained with 20 or 30 seedlings per pot. In addition, Wang et al. suggested that pots with a high density of seedlings, such as 60, showed lower colonization with AMF.

St-Arnaud, Hamel, Vimard, Caron, and Fortin (1996) and Liu and Yang (2008) emphasised that pot culture is not a clean inoculum production technique because the inoculum may be contaminated. As an alternative to this complication, St-Arnaud et al. (1996) proposed using an *in vitro* method to produce high numbers of spores without host roots. To do so, spores of the AM fungus *R. intraradices* were induced to grow in a Petri dish using transformed *Daucus carota* roots in modified minimal medium. The spores were originally obtained from a clay soil where leeks had been growing and 10 – 15 spores were placed near the transformed, actively growing root segments in Petri dishes. These plates were incubated in the dark at 27 °C. Next, the mycorrhizal roots were placed in another Petri dish half of which contained White medium (W. medium). The hyphae from the mycorrhizal roots colonised the W. medium and produced a second generation of spores. It took from 6 to 8 weeks for the fungus to grow out of the mycorrhizal roots. St-Arnaud et al. also described how the spores growing *in vitro* turned different colours, perhaps as the maturation process occurred, from translucent to whitish then to yellow and finally to brown after a few months.

More recently, Adholeya, Tiwari, and Singh (2005) contrasted the advantages of monoxenic cultivation with pot culture techniques as a tool to produce large amounts of inoculum. When inoculum was produced using monoxenic cultivation, neither the

inoculum nor the active propagules were contaminated and large amounts of inoculum were produced quickly using a small space. In addition, Adholeya et al. emphasised the achievability of commercial inocula. Ijdo et al. (2011, p.10) reported *R. intraradices* “clade/species complex...are among the most productive so far”. This work prompted the researcher to ask three questions: Firstly, if *R. intraradices* develops symbiosis with almost all plants, as suggested, then should it be used as the main or one of the main components of all commercial inocula? Secondly, if ‘pure’ means just one species, then can *R. intraradices* be considered the most convenient ingredient for a commercial inoculum? And thirdly, does *R. intraradices* need to be transported or can it be found universally in soils? In summary, pot cultures can be easily established and consist of a mix of medium, spores, root pieces and hyphal fragments that are used as inoculum.

The thesis presented here contains information from papers that used taxonomic names valid at the time of their publication; however, wherever possible the AMF names used in this thesis have been updated to the ones proposed by both Schüßler and Walker (2010) who have published a reviewed *Glomeromycota* species list that includes an update from May 2012, and by Redecker et al. (2013). These papers were based on molecular analysis and a compilation of several papers.

1.4.2 Inoculum products

Inoculum products (IPs) have been used as a source of inoculum for several plant species (Faye et al., 2013; Herrera-Peraza, Hamel, Fernández, Ferrer, & Furrázola, 2011; Jin et al., 2013; Rai, 2001). Herrera-Peraza et al. (2011) reported results of inoculation with MicoFert^R, which is a mixture of soil and mycorrhizal root pieces of sorghum (*Sorghum bicolor*). The mycorrhizal sorghum roots were obtained by inoculating sorghum plants with a certified AM fungal strain and growing them for three months in a medium consisting of soil:sugar cane residues (3:1, v/v). Jin et al. (2013) concluded that an inoculum made with mixed fungal species is more efficient than the ones made with a fungal isolate for promoting mycorrhizal colonisation. However, some difficulties have been reported when working with IPs (Nagahashi, 2010). For instance, Tarbell and Koske (2007) evaluated eight different IPs using a sand:peat (v/v) medium as medium. The dose of inoculum applied to *Z. mays* grown for six weeks, was the same as suggested by the producer, or five or ten times this amount.

It was found that just four out of eight inocula formed mycorrhiza and the percentage of root colonisation ranged from 0.4% to a maximum of 8.0%. Similarly, Faye et al. (2013) evaluated 12 AMF IPs using twice the recommended doses and compared their efficiency with indigenous soil. It was found that only three out of twelve IPs achieved higher root colonisation than what was achieved with indigenous soil, and inconsistency on the AMF species present in the IPs was reported. Spores present in IPs are usually isolated from several sources using mechanical methods. In contrast, Rai (2001) when considering feasibility of mycorrhization *in vitro* specifically about spores, suggested that sorting spores by hand, double disinfection and changes in nutrient media were ways to assure spore germination.

There has been a discussion about the positive and negative consequences of introducing AMF into ecosystems; for example, Schwartz et al. (2006) proposed guidelines for mycorrhization. They acknowledged that there can be competition and that the introduced AMF can, after a while, be dominant over the indigenous AM fungal species. The authors also suggested evaluating the need for mycorrhizas, selecting and using the local AMF as the first option rather than non-native species, and finally, that if the local species are not compatible with the target plants there must be careful evaluation of the possible outcomes.

1.4.3 Coexisting fungi

There is usually more than one fungal species coexisting within the root of a plant (Janoušková et al., 2009; Smith & Smith, 2011; Walker, 1999). Janoušková et al. (2009) recreated this situation in coal mine sites using soil trap cultures. Three-week-old seedlings of *Tripleurospermum inodorum* (*T. inodorum*) and *Calamagrostis epigejos* (*C. epigejos*) were inoculated with *R. intraradices*, *Claroideoglomus claroideum* (*C. claroideum*) (formerly *Glomus claroideum*; Schüßler & Walker, 2010) or combinations of both fungal species were reproduced using a pot culture technique. Janoušková et al. explained that the rapid spread of fungal inoculum was due to the potential of the inoculum and it can differ between species. In this case, however, the two fungal species coexisted without evidence of spatial exclusion.

1.5 Factors influencing mycorrhization

The ubiquitous occurrence of mycorrhiza throughout the plant kingdom has led to a considerable amount of work on the factors that influence the formation of the mycorrhizal associations. These can be broadly divided into biological factors, such as inoculum source, host compatibility, and physical (environmental) factors, such as nutrient (particularly P) availability, carrier material, soil, pH, and temperature.

Soils can obtain AMF from the air, soil or fauna movements, or from inoculation by man, and the absence of AMF can be caused by erosion, fumigation or disturbance (Abbott & Robson, 1991; Tommerup, 1992). Furthermore, soils are usually populated with more than one AMF species. The mycorrhization process requires the presence of active propagules in soil, such as spores (asexual), hyphae or vesicles, living or dead roots with hyphae, infected roots with vesicles or spores, and/or ERM (Abbott & Robson, 1991; Smith & Smith, 2011; Tommerup, 1992).

1.5.1 Spores

Spores are a starting points for mycorrhization (Abbott & Robson, 1991; Bécard & Fortin, 1988; Daft, Spencer, & Thomas, 1987; Douds Jr , 1997; Gerdermann & Nicolson, 1963; Pawlowska, Douds Jr, & Charvat, 1999; Tommerup, 1992; Walker, 1999). Since the early 1960s spores have been recognised as a source of mycorrhizal establishment, but even at that time the need for a large number of spores to initiate an infection was acknowledged. Also, surface-sterilization of spores was used to verify that the infection originated from those particular spores (Becard & Fortin, 1988; Daft et al., 1987; Gerdermann & Nicolson, 1963; Pawlowska et al., 1999). Daft et al. (1987) compared four types of propagules (hyphal fragments, root segments of bluebell or *Z. mays* and spores) and evaluated the viability of spores from four different continents. Regarding spores, they concluded that recently harvested *Rhizophagus clarus* (*R. clarus*) (formerly *Glomus clarum*; Schüßler & Walker, 2010) spores were the most effective propagules to induce mycorrhization in *M. sativa*. Additionally, in terms of storage, the authors reported that *R. clarum* spores were inactive after 15 weeks at temperature of 45 °C and at 14, 45 or 75% relative humidity (rh.). They found that 5 °C was the best storage temperature for viability in combination with 45 and 75% rh. Becard and Fortin (1988) also stressed that surface sterilised spores could be used

instantly after the disinfection process or after a few weeks storage at 4 °C. It was found that spore germination was better when using environmental conditions similar to those from where the spores were collected. The time that it takes for spores to germinate ranges from two to three weeks to up to a year (Tommerup, 1992). In contrast, under *in vitro* conditions, it takes ten days (Voets et al., 2009). The most important factors that influence spore germination are: incubation conditions and extraction methods (Pawlowska et al., 2011; Tommerup, 1992).

1.5.2 Hyphae

Hyphae can occur in soil, can grow within roots, or can grow from infected roots which are also called ERM (Smith & Smith, 2011) into the rhizosphere (Abbott & Robson, 1991; Tommerup, 1992). Very early in mycorrhizal research it was determined that hyphae growing within the roots were required before spores can be formed (Bécard & Fortin, 1988). Hyphae in living roots can generate spores (Chabot, Bécard, & Piché, 1992) and free hyphal propagules after germinating (Tommerup, 1992), and therefore pieces of mycorrhizal roots can be used as inoculum (Pawlowska et al., 1999). For instance, Voets et al. (2009) exposed autotrophic four-day-old seedlings of *Medicago truncatula* (*M. truncatula*) to eight-week-old mycorrhizal roots of *M. truncatula* which had a high density of extraradical hyphae. The colonisation was assessed after 3, 6, 9, 12 or 15 days after exposure to the living, extraradical hyphae. All the four-day-old seedlings were mycorrhizal regardless of time exposure. However, the degree of infection increased with the exposure time, being the highest after 15 days. In the Voets et al. research the newest mycorrhizal plants were then placed in a fresh medium and all of them, except the ones exposed for three days, produced spores and hyphae (spore production started after a week). After four weeks the highest spore production and longest hyphae were found in plants exposed for 12 or 15 days. In other research it has been acknowledged that the primary function of mycelium, transferring P, is enhanced by links established between ERM from the same host plant species, or different host species and/or even from different spore isolates forming a wide network connecting plants (Smith & Smith, 2011).

To conclude: there are certain conditions that are important to consider when analysing fungal infectivity. Infectivity can be affected by infection stage, propagule age, ability

to survive in changing soil conditions, and presence of suitable host plants or non-mycorrhizal plants. Each condition can vary depending on the fungal species.

1.5.3 Soil cultivation

Soil cultivation practices have been shown to affect mycorrhiza in *Z. mays* and soil fertility (Borriello, Lumini, Girlanda, Bonfante, & Bianciotto, 2012; McGonigle, Evans, & Miller, 1990). McGonigle et al. (1990) noted that even though there was an increase in P shoot content and shoot dry mass of *Z. mays* grown in undisturbed soil, mycorrhizal colonisation either did not increase or was not affected by soil disturbance. In addition, it was suggested that at least 1 ml of mycorrhizal soil is needed to preserve the integrity and functionality of the mycorrhiza, particularly with regard to P uptake. Recently, Borriello et al. (2012) characterised AMF contained in soil and roots from a *Z. mays* field using molecular techniques and found that members of the *Glomeraceae* family were, among other fungal species, the most profuse.

1.5.4 Mycorrhizal root segments

Mycorrhizal root segments can also be used as a source of inoculum (Brundrett et al., 1995; Daft et al., 1987; Forbes, Ellison, & Hooker, 1996; Verma & Jamaluddin, 1995). Daft et al. (1987) used 1 cm chopped long mycorrhizal *Z. mays* root pieces, exposed for seven weeks to *R. clarus*, and mixed with pasteurised beach sand to determine the effects of various storage conditions. The authors reported that the root segments were able to induce mycorrhizal development in *M. sativa* after the first week at all temperatures tested (5, 25 and 35 °C). They found the highest level of infection in *M. sativa* at 5 °C and after four weeks of storage. In contrast, at 35 °C and at 14, 45 or 75% rh. and after four weeks of storage, the *Z. mays* root segments did not form mycorrhizas. Similarly, Vidal et al. (1992) used roots, clean of soil, which were 1 cm long and colonised with *Rhizophagus fasciculatus* (*R. fasciculatus*) (formerly *Glomus fasciculatum*; Schüßler & Walker, 2010) to inoculate micropropagated avocado plants (*Persea americana*). After 20 weeks of being exposed to the mycorrhizal roots, the avocado plants were heavily colonised (Vidal et al., 1992). Mycorrhizal roots can also be used as inoculum when produced in a pot culture (Brundrett et al., 1996).

1.5.5 Native soil

Several studies have used native soil as a source of inoculum (Faye et al., 2013; Sýkorová, Ineichen, Wiemken, & Redecker, 2007; Verma & Jamaluddin, 1995). Tarbell and Koske (2007, p. 51) used dune sand as a source of local AMF and colonisation was achieved on *Z. mays* after six weeks of exposure, calling this procedure “the standard assay for potency of inocula”. In Tarbell and Koske’s study, the percentage of root colonisation ranged from 60.5 to 73.7% and all plants were colonised. Karasawa, Kasahara and Takebe (2001) demonstrated the beneficial use of mycorrhizal host plants before planting crops. They suggested that it may be possible to determine when a soil is suitable for growing a specific crop based on the previous plant mycorrhizal colonisation level. In addition, Karasawa et al. stressed that enhancing indigenous AMF populations could act as a biofertilizer. More recently, Sýkorová et al. (2007) compared the composition of the AMF community in a non-fertilised calcareous grassland, using its soil and plants (from the site) as sources of inoculum to be used in a pot culture under greenhouse conditions, with the trap plant technique at the site and root plant samples taken from the site. AMF found in the pot culture 3 and 10 months after establishment showed a different AMF community from ones found using the other techniques. Most of the AMF species found were *Glomeromycota*.

Regarding teak, Singh et al. (2003) surveyed AMF populations in Arunachal Pradesh (North East India) on abandoned agricultural land, which had been regenerating for five years, when teak was introduced. It was found that AMF populations, most of them *Glomus*, were higher in the natural forest, which contained 42 AMF species, whereas agricultural land had only 34 species. This finding was explained as a result of the presence of permanent symbioses for long periods of time without being disturbed.

1.5.6 Environmental factors

Nutrient availability: Phosphorus (P)

Low soil P or P depletion are factors considered to promote mycorrhizal establishment (Bécard & Fortin, 1988; Lambers, Shane, Cramer, Pearse, & Veneklaas, 2006; Schroeder & Janos, 2005; Smith & Smith, 2011). As a result, mycorrhizal plants perform better than non-mycorrhizal plants in soils low in P (Shen et al., 2011). Nevertheless, colonisation has been reported under a broad range of soil P

concentrations, ranging from 1 to 25 ppm (Dickson, 2004; Herrera-Peraza et al., 2011; Janoušková et al., 2009; McGonigle et al., 1990; Singh et al., 2003).

Soil P, which can be either organic (P_o) or inorganic (P_i), is found in many different compounds and is considered to be a non-renewable resource (Shen et al., 2011). Plants mainly absorb $H_2PO_4^-$ or HPO_4^{2-} (Shen et al., 2011), but the availability of these ions depends on soil pH (Shen et al., 2011). Plants obtain limited P from soils because of its low diffusion rate and high rate of fixation (Lambers et al., 2006; Shen et al., 2011). As a result, P deficiency can restrict plant growth; however, P can be replenished using fertilizers or manure (Schroeder & Janos, 2005). In addition, Lambers et al. (2006) suggested that there could be scarcity of P in the future, so there is a need to create new crops; Lambers et al. proposed adaptations like cluster roots, similar to other plants adapted to growing in low P soils, or new cultivation systems with different plant species to be interplanted, and selected for careful crop rotations.

Soil microorganisms and plant roots producing phosphatase can release P_o from various P_o compounds in the soil (Schroeder & Janos, 2005; Shen et al. 2011). Soil moisture, temperature, chemical and physical soil characteristics are factors that affect P availability. Once the P_i is in the roots, it is translocated to the shoots (Shen et al. 2011). The levels of soil P_i on rare occasions are higher than 10 μ M (Smith & Smith, 2012), but this is still lower than the levels found in the plant where concentrations range from 5 to 20 mM (Shen et al., 2011). Schroeder and Janos (2005) studied the combined effects of P concentration, the number of plants per pot and mycorrhiza on *Capsicum annuum*, *Z. mays*, and zucchini (*Curcubita pepo*), all facultative mycotrophic species. After 10 weeks of being exposed to the inoculum, Schroeder and Janos (2005) reported that “high levels of phosphorus reduced but did not eliminate AM colonization” (p. 203). A similar conclusion was claimed by McGonigle et al. (1990) under field conditions.

In order for plants to be able to absorb P, this element has to be easily reached by the root and/or mycorrhizal mycelium as well as needing to be in an appropriate ionic form (Shen et al., 2011). Some changes in root architecture have been reported when the P content in soil is low, very low or high and these changes allow the plant to reach the

soil P more efficiently (Shen et al., 2011). For instance, Mollier and Pellerin (1999) studied P starvation effects using *Z. mays* seedlings growing in a liquid medium under greenhouse conditions for 16 days and stated that leaf area development and shoot growth were drastically reduced while root growth increased. Schroeder and Janos (2005) reported increased specific root length and root branching of *Z. mays* and *C. annuum* when planted at low and medium densities and with different P concentrations in the soil. It is generally accepted that low P promotes mycorrhization, but the absolute value of flow varies on conditions in the soil and on plant species.

Plant responses to P

The amount of P obtained by a plant through mycorrhiza can be large but it does not necessarily increase plant growth (Smith & Smith, 2011). These results imply that the amount of P absorbed via roots themselves is small. This may mean that scarcely colonised plants may have restricted growth because of low P uptake (Smith, Jakobsen, Gronlund, & Smith, 2011). Similarly, Janoušková et al. (2009) and Herrera-Peraza et al. (2011) reported that even though both *R. intraradices* and *C. claroideum* coexisted within a root or *R. intraradices* alone, P uptake was improved, yet no positive growth response was recorded for the host plants *T. inodorum* and *C. epigejos*.

Carrier materials

The importance of the carrier materials for the propagules to produce good inoculum has been highlighted. For instance, the effectiveness of three carrier materials, namely beach sand, garden soil and sphagnum peat were examined. These materials were mixed with *R. clarus* spores or a mixed isolate from India and kept at 5, 25 and 35 °C for 12 weeks. Both sand and garden soil, either wet (which was better) or dry, at 5 or 25 °C were good materials to develop mycorrhizal infection on *M. sativa*. In contrast, wet and dry peat were both ineffective carrier materials (Daft et al. 1987). Several other materials have been used, such as: expanded clay (Varma & Schuepp, 1995); steamed sand (Brundrett et al., 1996; Pereira Cavallazzi, Klauberg Filho, Luiz Stürmer, Rygielwicz, & Matos de Mendonça, 2007); sterilised sand-soil mix (1:1, v/v) (Rajan, Reddy, & Bagyaraj, 2000); Terragreen and attapulgite (Duponnois et al., 2007), and M medium solidified with 0.4% Phytigel® (Folli-Pereira, Meira-Haddad, Rasool, Otoni,

& Kasuya, 2012). There have been no comparisons of these specific carrier materials to indicate which might be more effective for mycorrhization.

1.6 Inoculation strategies

Several inoculation strategies have been used to produce mycorrhizal plants. The main objective of inoculation is to place the fungal propagules in close contact with the plant (host) roots (Adholeya et al., 2005). For instance, mixtures of hyphae, mycorrhizal root pieces, spores, AMF strains, and/or substrate can be mixed with the potting mixture (Joshee, Mentreddy, & Yadav, 2007; Rajan et al., 2000), placed in the planting holes (Schroeder & Janos, 2005), below the seeds (Janoušková et al., 2009; Wang et al., 2007), and placed close to the host roots (Duponnois et al., 2007). Therefore, inoculation strategies depend on the characteristics of the inoculum; however, there have been no thorough examinations reported to indicate which strategies may be more effective for teak.

1.7 AM Development and identification of mycorrhizal associations

Various stages in the establishment of AMF have been identified (Bécard & Fortin, 1988; Giovannetti, 2010; Marsh & Schultze, 2001; Tommerup, 1992); these include:

- 1) If the starting point is a spore, the spore germinates and produces a small hypha. Spores do not require the presence of roots to germinate.
- 2) If a root is present, the hypha branches and grows vigorously.
- 3) Once the hypha touches the root, it swells and forms appresoria; from there the hypha starts growing inside along the root.
- 4) The hypha penetrates the cortical cells and forms arbuscules.
- 5) After approximately seven days, the arbuscules degenerate and finally the hyphae grow towards the root exterior developing extraradical mycelium.

These stages can be identified microscopically and used to assess the development of mycorrhiza in roots.

Mycorrhizal associations can be misdiagnosed due to a lack of thorough microscopic observation and identification of arbuscules in the plants grown in the wild (Brundrett, 2009). Even though vesicles and/or spores and hyphae can be present, there is a need to

ensure that the plants have AMF by locating arbuscules and providing photographs if possible. It is crucial to provide additional information, such as details of the sampling method, season, vesicles, arbuscules and hyphae (Brundrett, 2009). More recently, Smith and Smith (2011) claimed that along with arbuscules, intracellular coiled hyphae also can be used as a feature to identify AMF. Therefore, a thorough inspection of inoculated roots must be done in order to identify features that indicate mycorrhizal establishment.

1.8 Staining

Adequate staining is vital for accurate identification of mycorrhiza within the root (Brundrett et al., 1996; Koske & Gemma, 1989). The staining techniques have varied over the years, and during the 1980s the trypan blue technique developed by Koske and Gemma (1989) was frequently used. Recently, attempts have been made to reduce use of this stain due to its carcinogenic characteristics, such as replacing it with ink (Vierheilig, Coughlan, Wyss, & Piché, 1998)

There are several essential steps required to stain mycorrhizal roots (Brundrett et al., 1996; Koske & Gemma, 1989): fixating, clearing, rinsing and bleaching, acidifying and staining and destaining.

1. Fixation in 50% ethanol is done to preserve the roots until they can be cleared.
2. Clearing roots is done using 2.5% (w/v) potassium hydroxide (KOH) and autoclaving the roots at 121 °C for 3 min, or alternatively the roots can be heated in a water bath at 90 °C for 10 – 30 min.
3. Rinsing consists of changing the water several times and bleaching if the roots are too dark.
4. Acidification involves soaking the roots in 1% hydrochloric acid (HCl), which allows the trypan blue dye to stain the fungal structures.
5. Staining is done by immersing the acidified roots in an acidic glycerol solution consisting of 500 mL glycerol, 450 mL of water and 50 mL 1% HCl and then adding 0.05% trypan blue. The staining process also requires heating, using an autoclave at 121 °C for 3 min or a water bath for 15 – 60 min.

5. The final step is destaining which does not need heat and can be done at room temperature by adding acidic glycerol to the roots.

Koske and Gemma emphasised that the times and volumes used for staining would depend on the root volume.

Subsequently, Vierheilig et al. (1998) suggested a modified staining method because of the health and environmental issues related to using trypan blue. The authors tested their method using seedlings of several species that were inoculated with *F. mosseae*, *R. intraradices* and *Gigaspora margarita* (*G. margarita*) and harvested seven weeks after inoculation. This method is similar to Koske and Gemma's (1989), with common steps such as clearing, staining and destaining. Clearing was done by boiling the roots in 10% KOH with the length of time depending on the root type. After rinsing then three times, the roots were boiled for 3 min in a 5% solution of ink-vinegar. Many different kinds of ink were tested. Destaining was done by rinsing with acidic tap water or with pure vinegar.

More recently, Sosa-Rodríguez et al. (2013) established mycorrhiza *in vitro* on plantlets of *Hevea brasiliensis* (*H. brasiliensis*) and used ink and vinegar to examine mycorrhizal features after 12 weeks of being exposed to the ERM. Their staining method was a simple and reproducible technique that allowed AMF assessment. This staining technique can also be adapted so that it is compatible with molecular techniques, thus allowing identification of AMF using both optical and biochemical tools (Pitet, Camprubi, Calvet, & Estaun, 2009).

1.9 Research aims

This research was establish to:

- determine the optimum conditions to induce adventitious roots/rooting on micropropagated teak
- develop an efficient rooting protocol for micropropagated teak clones
- determine how genetic variation in teak may influence root induction and production in micropropagation
- develop a mycorrhization protocol for micropropagated teak clones

- determine if there are differences in the mycorrhization of different teak clones.

Here is an overview of the remaining thesis chapters:

Chapter 2 describes general material and methods used in the practical part of this research. Chapter 3 is about improvement of teak micropropagation procedure. Chapter 4 describes several mycorrhization attempts. And Chapter 5 is about an overall discussion of mycorrhization and micropropagation.

Chapter 2: General Material and Methods

2.1 Plant material

Cultures of two teak clones (MY4 and T201) selected for rapid growth rates were available from other research projects being conducted in the Tissue Culture Laboratory at Edith Cowan University (ECU). These shoot cultures had been maintained under the standard conditions (Chapter 2 – Section 2.3.1) for two years prior to the commencement of this project. In this Chapter will be described general material and methods used in the experimental part of this research. In Chapter 3 and 4 there will be a specific material and methods section because each chapter has different experimental objectives.

2.2 Fungal material

The fungal material consisted of processed inoculum product (IP), or before being processed (unprocessed) (UIP), isolated spores or soil-based inoculum from organic farming soil from a vegetable garden (VG soil). IP was produced from a legume/grass pot culture processed into a powder with the endomycorrhizal fungi: *R. intraradices* and *Funneliformis mosseae* (*F. mosseae*) (formerly *Glomus mosseae*; Schüßler & Walker, 2010) (>100 spores g⁻¹). The UIP was a clayey soil from a pot culture, processed into a powder with the same *Glomus* species as above but mixed with dried chopped mycorrhizal grass roots. The isolated spore inoculum was a brown powder containing isolates of *Claroideoglomus etunicatum* (*C. etunicatum*) (formerly *Glomus etunicatum*; Schüßler & Walker, 2010) or an unidentified *Glomus* spp. The VG soil was obtained from a vegetable garden located in Mandurah, Western Australia (latitude 32°.50' S; longitude 115°.77' E), where *Allium* spp. had been growing. The garden was established in 1990 and had been fertilized only with compost.

2.3 Tissue culture

2.3.1 Culture room conditions

The tissue culture room conditions were: average temperature at 22 °C and a photoperiod of 16 hr light and 8 hr dark; the light was provided with fluorescent tubes (Phillips® lifemax cool day light TLD 36W/865)

2.3.2 Shoot multiplication

Shoots of teak clones MY4 and T201 were subcultured every 35 – 45 days. They were maintained on a multiplication medium modified from (Gangopadhyay et al., 2002), which contained: Murashige and Skoog (MS) nutrients and organics; 30 g L⁻¹ sucrose; 0.5 µM 6-benzylaminopurine (BAP); 0.5 µM kinetin. The pH was adjusted to 5.8 with 1M potassium hydroxide (KOH) prior to adding 2.5 g L⁻¹ agar and 2.5 g L⁻¹ gelrite. The contents were thoroughly mixed and heated in a microwave oven until the agar and gelrite had dissolved. Medium (50 mL) was dispensed into each 250 mL culture vessel, the culture vessels were sealed and then autoclaved at 120 °C for 20 min.

2.3.3 Shoot subculturing

During subculturing, individual shoots of MY4 and T201 were cut into two or three pieces, depending on the length of the shoot. Each piece had at least one node (two leaf axils; teak has opposite leaves) “nodal shoot”. Any callus that formed on the shoot base was cut off and discarded. For rooting, the terminal leaf buds plus one node were used (shoot tips). The remaining stem pieces were used to produce more shoots by transferring them to fresh multiplication medium. Four to six explants were grown per culture vessel. The culture vessels were kept at the tissue culture room conditions. All subculturing was performed in a laminar flow unit in a clean room using standard aseptic techniques.

2.3.4 Root induction

Root induction occurred on a basal rooting medium developed for other woody species (Bennett et al., 2004). This medium contained ¼ strength MS macronutrients, half strength MS iron, full strength MS micronutrients, plus 20 g L⁻¹ sucrose. IBA at various concentrations was added, depending on the concentration needed for each experiment. The pH was adjusted to 5.5 with 1M KOH prior to adding 2.5 g L⁻¹ agar and 2.5 g L⁻¹ gelrite. The contents were thoroughly mixed and heated in a microwave oven until the agar and gelrite had dissolved. Medium (50 mL) was dispensed into each 250 mL

culture vessel, the culture vessels were sealed and then autoclaved at 120 °C for 20 min. Four to six shoot tips were grown per culture vessel. The culture vessels were kept at the tissue culture room conditions. All subculturing was performed in a laminar flow unit in a clean room using standard aseptic techniques.

2.4 Greenhouse conditions for acclimatisation and plantlet growth

In this subsection three aspects of acclimatisation and plantlet growth are outlined, namely: soil preparation, greenhouse conditions, transfer to soil, and data collection.

2.4.1 Soil preparation

Three materials were used to make up the soils: white sand, peat and perlite. The white sand and the peat (Shamrock® - general purpose sphagnum peat) were sieved with a 0.5 mm sieve and the coarse particles were discarded. The perlite (The Perlite and Vermiculite Factory®), sieved white sand and peat were measured by volume in the proportions required and thoroughly mixed for all preparations using a cement mixer and adding tap water until the desired moisture content was reached. When the mixed soil was homogenous, it was placed into hessian bags and either pasteurised for four hr on two consecutive days in a vertical steam boiler (Simons Boiler Co®), (pasteurised soil) or autoclaved for 20 min at 120 °C on two consecutive days (sterilised soil). The sterilised soil was used for the *in vitro* rooting experiment. The crack pots (4 Seasons Seeds®; Figure 1) were filled with different pasteurised soil types, namely: (sand:peat) (1:1, v/v), (sand:perlite) (1:1, v/v) or (sand:peat:perlite) (1:1:1, v/v/v), depending on the experiment. For mycorrhization purposes 15 to 20 mL of half-strength sorghum nutrient solution (Brundrett et al., 1996) was added to each crack pot until the nutrient solution drained from the base, then the crack pots were left to drain freely for 24 hr (field capacity) prior to use in experiments.



Figure 1. Crack pot containing pasteurised sand:perlite (1:1, v/v) soil and 6-week-old teak plantlet. Scale bar = 10 mm.

2.4.2 Greenhouse conditions

Two greenhouses were used for plant acclimatisation and both had a light regime dependent on the seasons. The first greenhouse had benches covered with pasteurised coarse river sand and a misting system that watered for 10 s every 90 min. In addition, a bench could be covered with plastic sheeting (Figure 2) to increase the rh. to 80 – 95% or left without plastic (second bench) and thus at 50 – 65% rh. A heating mat, placed a few centimetres under the coarse sand, was set at 30 °C. The temperature

ranged in this first greenhouse from 19 °C to 30 °C .The second greenhouse had open benches and the temperatures ranged from 18 °C to 42 °C at the time of the experiments.



Figure 2. Benches covered by plastic in the first greenhouse. Scale bar = 10 cm.

2.4.3 Transfer to soils

In the current research the relative humidity was controlled carefully to avoid desiccation. Crack pots were filled with one of the three kinds of pasteurised soils and fertilised with half-strength sorghum nutrient solution until they reached field capacity. Rooted teak plantlets were removed from the culture vessels by lifting them carefully by the base with forceps. The plantlets were then rinsed individually with deionised water (DI) to remove excess of agar. At the pilot stage plantlets were acclimatised on the benches covered with plastic but without any grow top covers (Polyfoam[®]) (Figure 3).

During the experimentation period an acclimatisation procedure was established:

- 1) The plantlets were maintained immersed in water and the time taken to transplant them was kept to a minimum (one tray of 40 plantlets at a time).

- 2) As soon as each tray was placed onto the misting bench, it was watered by hand then immediately covered with grow top covers.
- 3) The sprinklers frequently sprayed water over the plantlets in order to maintain a high relative humidity (90%) in the misting bed.
- 4) The grow top covered plantlets were sprayed twice per day with the sprinklers during the 14-day period.
- 5) At the end of 14 days the grow top covers were removed and the plantlets were kept on the misting bench for the remaining 14 days.
- 6) The plantlets were given a further 28 days of hardening through gradual reduction in relative humidity (uncovered) on the second bench.



Figure 3. Grow top cover. Teak plantlets 14 days after transfer to pasteurised soil.
Scale bar = 4 cm.

2.4.4 Plantlet growth assessment

When the experiments were completed, the teak plants were harvested in the greenhouse. The number of surviving plants was counted. Each crack pot was cut with a pair of clippers and the contents placed carefully into a broad container; then the substrate was removed from the roots by gently shaking and then rinsed with filtered

water. The shoot and root system of each plantlet were separated for further measurements. Soils from the crack pots were kept for P analysis and pH measurement. The shoot length was determined using a vernier calliper. The roots were placed individually in labelled culture vessels and fixed in 50% ethanol. Roots areas were determined using digital images and calculated with the Windias program. To do this, each root was carefully cleaned and the whole root system was placed on the Windias screen and then flattened with a Perspex sheet.

2.5 Soil characterisation

2.5.1 Type of soil, pH, moisture content and phosphorus in soil.

The type of soil was determined using the hydrometer method (Bouyoucos, 1962) and field texture grade (Geeves, Craze, & Hamilton, 2007). Soil pH was determined at 25 °C using a mixture of 1:5 soil/0.01 M calcium chloride extract-direct. Moisture content was determined using the air-dry moisture content (Rayment & Higginson, 1992). Phosphorus in soil was determined as total P by inductively coupled plasma optical emission spectrometry (ICP-OES) using the method total elemental, HNO₃/HClO₄ outlined by soil analysis and methods of University of Minnesota. <http://ral.cfans.umn.edu/soil-analysis-and-methods/#27>

2.6 Mycorrhization assessment

2.6.1 Mycorrhizal infectivity

A *Z. mays* bioassay was used to determine VG soil or IP mycorrhizal infectivity (Janos, 1980; Pereira Cavallazzi et al., 2007).

2.6.2 Spores in organic farming soil

The organic farming soil was collected using a soil auger at 10 and 20 cm deep. The five soil samples each of approximately 500 g, were mixed in a plastic bag and then sealed and stored at 4 °C. Spore isolation procedure was as described by Gerdermann and Nicolson (1963) and Brundrett et al. (1996) with some modifications. Soil samples of 50 g were wetted, one at the time, with 1 L of water for at least 30 min. The mixture of soil and water was agitated for 2 min and it was decanted through a series of sieves 63, 100, 250, 500 and 710 µm. The material was collected in the sieves and then rinsed

on to pre-wetted filter paper in a Buchner funnel before vacuum filtration. The spores collected in the filter papers (only from 100, 250, 500 μm sieves) were counted using a dissecting microscope.

2.6.3 Staining

Ethanol-fixed roots were taken from the culture vessels and rinsed in a Petri plate filled with tap water (using a sieve to avoid loss of the fine roots). The roots were chopped into pieces approximately 2 – 4 cm in length. The pieces were placed into the tube or culture vessel. Then, 10% KOH (w/v) was added to cover the root and the culture vessel was sealed. The tubes were placed in a metal rack and then in the oven at 60 °C for a minimum of 30 min or until the root became transparent (usually) 45 – 60 min (Figure 4 A). The clarifying time varied depending on the root volume and thickness. Ten roots were clarified at a time. There was no subsampling because the root volume was relatively small.

After clarification (Figure 4 B), and when the KOH was cold (approximately 10 min), the roots were gently rinsed with filtered water in a laminar work station using a sieve to avoid loss of the fragile and transparent roots. The roots were placed back into the tube or culture vessel and 1% HCl was added for one or two min. The acid was decanted and replaced with 0.05% (w/v) trypan blue in lactoglycerol (lactic acid:glycerol:water) (1:1:1, v/v/v) (Brundrett et al., 1996), or 5% (v/v) blue ink (Parker®) in acetic acid without the root acidification step (Vierheilig et al., 1998; Walker, 2005). The roots were stained for at least 24 hr. The stained roots were destained and preserved in 50% glycerol (v/v). The roots were then assessed individually for mycorrhizal colonisation.



Figure 4. Clearing roots by heating in 10% KOH at 60 °C for 30 – 60 min. A) Before clearing. B) After clearing.

The stained roots were assessed individually using a Leika DMLB[®] compound microscope in bright field and phase settings. Photomicrographs were taken using a

Nikon D5000[®] camera. The slides were prepared with water (C, Walker, personal communication, February 23, 2011). Mycorrhization assessment was done by identifying the presence of hyphae, vesicles, spores or arbuscules and the area occupied by the mycorrhiza.

Chapter 3: Root induction, acclimatisation and plantlet growth

3.1 Teak micropropagation

3.1.1 Shoot multiplication

Since the early 1980s several attempts have been made to propagate teak using tissue culture technologies (Gupta et al., 1980). The initial work concentrated on establishing cultures of plus genotypes that were typically grown from field growing, mature trees because the plus genotype was required for assessment of growth characteristics. This work revealed the difficulty of initiating teak cultures, which produce high levels of contamination and inhibition of growth by the production of excessive amounts of phenolics (Akram & Aftab, 2009; Shirin et al., 2005; Venkateswarlu & Korwar, 2005). However, researchers have found that these problems can be overcome through changing the sterilising agents and frequently subculturing the initiated explants (Akram & Aftab, 2009; Tiwari et al., 2002), or using apical buds from epicormic shoots grown under greenhouse conditions (Castro et al., 2002). Once aseptic cultures are obtained, the multiplication of teak shoots has occurred with fewer problems, and researchers have reported successful long-term maintenance and multiplication (Mendoza De Gyves et al., 2007). Obtaining aseptic cultures has led to the commercial micropropagation of teak in India, Thailand, Malaysia and Australia (Elders Forestry Management, ITC Teak Project 2006, 2011; Goh, Chang, Jilimin, & Japarudin, 2010; Goh & Monteuis, 2012; Venkateswarlu & Korwar, 2005).

Most of the published work on the micropropagation of teak has used basal media based on MS or modified MS (mMS) (Castro et al., 2002; Gupta et al., 1980; Mendoza De Gyves, 2007) and has required the presence of a cytokinin for shoot multiplication. The most common used cytokinins are: BAP (6-benzylaminopurine) from 0.4 to 22.2 μM (Akram & Aftab, 2009; Castro et al. 2002; Gangopadhyay et al., 2002; Gupta et al., 1980; Mendoza De Gyves, 2007; Shirin et al., 2005; Tiwari et al., 2002) and/or kinetin from 0.4 to 2.3 μM (Gangopadhyay et al. 2002; Gupta et al., 1980; Nor Aini et al., 2009). MS has also been used in a liquid form, specifically by Quiala et al. (2012), who

employed glass beads to support the plantlets and supplemented the medium with 4.4 μM BAP. However, several researchers have found that high BAP levels can induce hyperhydricity when in a liquid medium (6.66 μM BAP, Quiala et al., 2012) or a solid medium (4.44 μM BAP, Castro et al., 2002).

The basal medium for the growth of teak shoots is sometimes supplemented with an auxin, for example: 2 μM IBA (Akram & Aftab, 2009), 0.05 μM IBA (Mendoza De Gyves et al., 2007), 1.0 μM NAA (Shirin et al., 2005), 0.00 or 0.57 μM IAA (Tiwari et al., 2002). Sometimes other supplements are included, such as: myo-inositol (Shirin et al., 2005), gibberelin (GA_3) (Mendoza De Gyves et al., 2007), pectin (Mendoza De Gyves et al., 2007) or silver nitrate (AgNO_3) (Akram & Aftab, 2009). These supplements, however, are not essential.

The above approaches have been applied to explants from teak trees up to 100 years old (Gupta et al., 1980; Quiala et al., 2012; Shirin et al., 2005; Tiwari et al., 2002), that is, trees well into their mature growth phase. Generally, once teak shoot cultures have been established they can be maintained for long periods. In one case, however, this long term maintenance was found to be associated with genetic variation (Gangopadhyay et al., 2003).

3.1.2 Root induction

Rooting of some woody species has been recognised as one of the major impediments to clonal propagation. For example, root induction of teak has been reported since the early published work on this species (Gangopadhyay et al., 2002; Gupta et al., 1980) and has been performed on different explant sources, such as cuttings (Husen & Pal, 2007), nodal explants from softwood shoots (Akram & Aftab, 2009), and axillary buds from greenhouse grown epicormic shoots (Daquinta et al., 2001). One of the most important parameters determining the success of the rooting ability is the time that the shoots produced through multiplication have been in culture. As the subculture period increases, the ease of rooting increases (Gupta et al., 1980; Nowak & Shulaev, 2003). In addition, early work has shown that variation in basal medium composition is important. Where MS based media have been used, reduction in the concentration of nutrients is preferred (Akram & Aftab, 2009; Mendoza De Gyves et al., 2007).

Alternatively, media based on White's medium has been used (Gangopadhyay et al., 2002; Gupta et al., 1980; Nor Aini et al., 2009); this also has lower levels of nutrients than MS.

Spontaneous production of adventitious roots on teak shoots *in vitro* has been reported on media without plant growth regulators (Bonal & Monteuuis, 1997, Castro et al., 2002), but auxin has been found to substantially increase root production (Mendoza De Gyves et al., 2007; Nor Aini et al., 2009). Pure or combined auxins have been used, such as IBA, Indol-3-acetic acid (IAA) and NAA. The concentrations used ranged from 0.24 to 25 μM and exposure time ranged from 30 to 42 days (Akram & Aftab, 2009; Bonal & Monteuuis, 1997; Kozgar & Shahzad, 2012; Mendoza De Gyves et al., 2007; Nor Aini et al., 2009; Shirin et al., 2005). In addition, transfer to a medium without hormones after exposure to auxin has been suggested as an important step to allow root elongation (Gupta et al., 1980). *Ex vitro* rooting also has been reported by a number of workers (Castro et al., 2002, Daquinta et al., 2001, Quiala et al., 2012, Tiwari et al., 2002; Venkateswarlu & Korwar, 2005). Variations in auxin treatments using this approach have included: 0.005 M NAA and 0.005 M IBA in powder form (Daquinta et al., 2001); 0.02 M IBA for 30 sec (Castro et al., 2002); 9.8 μM IBA for two min (Tiwari et al., 2002); and 492.1 μM IBA for 2 min on shoots produced from cytokinin free multiplication medium (Quiala et al., 2012). With these treatments rooting ranged from 78% to 100%.

It is widely recognised that liquid media can reduce costs and allow the automation of the micropropagation process, but this approach still requires a solid matrix to give support to the forming roots. For example, Gangopadhyay et al. (2002) supplemented the liquid media with coir and a combination of IBA and IAA and obtained 1.2 ± 2 roots per shoot; Shirin et al. (2005) also used liquid media supplemented with 15 μM NAA and filter paper and obtained 1.6 roots per shoot; whereas Quiala et al. (2012) used glass beads.

3.1.3 Teak acclimatisation

Successful acclimatisation of teak plantlets in soil depends largely on the number of adventitious roots formed *in vitro* and the abiotic conditions (e.g. rh. and soil

composition) (Akram & Aftab, 2009; Bonal & Monteuuis, 1997; Castro et al., 2002; Mendoza De Gyves et al., 2007; Shirin et al., 2005; Tiwari et al., 2002), the medium used at the multiplication stage (Quiala et al., 2012), and being able to maintain steady growth during acclimatisation (Nowak & Shulaev, 2003). Plantlets are most frequently transferred to soil after auxin treatment is applied to the shoots (*in vitro* rooting) (Castro et al., 2002; Daquinta et al., 2001; Tiwari et al., 2002). Alternatively, a two-stage process has been used where the auxin treatment is applied *in vitro*, followed by hardening (Mendoza De Gyves et al., 2007).

Several materials have been used for acclimatisation, including: boiled sand (Bonal & Monteuuis, 1997), soil:vermiculite (1:1, v/v) (Mendoza De Gyves et al., 2007; Tiwari et al., 2002), Soilrite® (comprised of Irish peatmoss 75%: expanded perlite 25%) (Kozgar & Shahzad, 2012; Shirin et al., 2005), sand soaked with half-strength MS without organics (Shirin et al., 2005), and a combination of sterilised peat moss and coarse sand (1:1, v/v) (Akram & Aftab, 2009). For rooting *ex vitro*, Daquinta et al. (2001) used Zeolite®, Tiwari et al. (2002) used soil:vermiculite (1:1, v/v), and Venkateswarlu and Korwar (2005) used sterilised soilrite ® or cocopeat in the area near to the roots and soil in the rest of the polyethylene bags. Quiala et al. (2012) used an organic matter (humus and sugarcane mill baggase (waste): Zeolite® mixture (1:1, v/v).

Several techniques have been developed to avoid plant desiccation during the first days of hardening off after having been transferred. For instance, Bonal and Monteuuis (1997) used a misting system for 15 weeks. Tiwari et al. (2002) reported similar procedures using covering on misting benches for 15 – 20 days at 33 ± 2 °C and 90% rh. (Tiwari et al., 2002) or covered with polyethylene film for two weeks (Mendoza De Gyves et al., 2007). Shirin et al's (2005) procedure involved two steps: first, they kept the plantlets under culture room conditions in sand for 15 days, and then the bottles were placed in a misting chamber for 15 days: next, the plants were transferred to poly bags containing a mixture of sand:soil:farm manure (1:1:1, v/v/v) and were kept under greenhouse conditions. In general, regardless of the technique used, survival from 85% to 100% has been obtained once the plantlets developed roots (Castro et al., 2002; Kozgar & Shahzad 2012; Mendoza De Gyves et al., 2007; Nor Aini et al., 2009; Venkateswarlu & Korwar, 2005).

Another common practice as a measure to avoid fungal infections is the use of fungicides applied to the plantlets at the time of transferring to the soil or substrate (Akram & Aftab, 2009; Bonal & Monteuis, 1997; Shirin et al., 2005). Several fungicides have been used for this purpose, including: Thiram 80® (Bonal & Monteuis, 1997), Bavasting® (Shirin et al., 2005), Dithane® (Akram & Aftab, 2009), and Chlorothalonil® (chloronitrile) (World Teak Conference 2013: Excursion II: Teak Improvement in Thailand, n.d).

3.1.4 Survival and growth of micropropagated teak

Survival and development features have been used as criteria to compare plants produced through conventional propagation techniques (seed and/or cuttings) with those produced through tissue culture. Teak plants, obtained from the terminal buds of the upper branches of 100-year-old teak trees, were compared with teak seedlings (Gupta, Timmis, & Mascarenhas, 1991). At year three, results indicated that plants obtained from micropropagation flowered at an earlier stage, suggesting that micropropagated plants achieve maturity earlier than those in the wild (reproduced from seed), which usually flower after 7 – 10 years. However, after five years there was no difference in diameter at breast height (DBH) between the micropropagated plants and the seed-produced plants. Thus, based on flowering time, micropropagated plants seemed to mature faster (Gupta et al., 1991). Venkateswarlu and Korwar (2005) concluded that after two years of growth teak trees from micropropagated material showed greater uniformity and superior growth than trees obtained from stumps (cuttings).

3.2 Aims of this chapter

The aims of the research described in this chapter were to:

- determine the optimum conditions for root induction
- determine whether there is variation between clones in rooting ability
- determine whether auxin treatments influence growth and development of plantlets in soil and
- develop an acclimatisation protocol for successful mycorrhization.

3.2 Material and methods

3.2.1 Low auxin concentrations for root induction

To identify the most suitable auxin concentration for root induction, several auxin concentrations were examined by using a single clone (MY4). Low concentrations of IBA were tested initially. Teak shoot tips (Chapter 2 - 2.3.3) were transferred into a basal rooting medium (Chapter 2 - 2.3.4), supplemented with 0, 5, 10, 20, 40, or 80 μM IBA for 7 days. The shoot tips were then transferred into new culture vessels, with the same basal medium but without IBA, for a further 28 days. There were 30 replicates (5 shoot x 6 culture vessels) for each auxin treatment. The culture vessels were maintained under standard culture room conditions (Chapter 2 - 2.3.1) and they were placed randomly (randomised block design). At the end of five weeks, the number of roots formed was counted. The rooted plantlets were then transferred to pasteurised soil (sand:perlite:peat) (1:1:1, v/v/v) for 28 days under mist and a further 28 days of hardening on open benches in the greenhouse. Plantlet heights and survival (Chapter 2 – 2.4.4) were measured 56 days after transfer to soil.

3.2.2 High auxin concentrations for root induction

To assess the effect of high concentrations of IBA, shoot tips were transferred into basal rooting medium (Chapter 2 - 2.3.4), supplemented with 0, 80, 100, 120 or 160 μM IBA for 7 days. The shoot tips were then transferred into culture vessels, with the same basal medium but without IBA, for a further 28 days. There were 30 replicates (5 shoot x 6 culture vessels) for each auxin treatment. The culture vessels were maintained under standard culture room conditions (Chapter 2 - 2.3.1) and they were placed randomly (randomised block design). At the end of five weeks, the number of roots formed was counted. The rooted teak plantlets were then transferred to pasteurised soil (sand:perlite:peat) (1:1:1, v/v/v) for 28 days under mist and a further 28 days of hardening in the greenhouse. Plantlet heights and survival (Chapter 2 – 2.4.4) were measured 56 days after transfer to soil.

3.2.3 Suitable exposure time to IBA

Once the most appropriate auxin treatment (80 μM IBA) was determined, a suitable exposure time was investigated. Shoot tips of two clones (MY4 and T201) were

exposed to basal rooting medium (Chapter 2 - 2.3.4), supplemented with 80 μ M IBA for 4, 6, 8, 14 or 28 days. Once each exposure time was reached, the shoots were transferred to the same basal rooting medium without IBA. There were 30 replicates (5 shoot x 6 culture vessels) for each treatment. The culture vessels were maintained under standard culture room conditions (Chapter 2 - 2.3.1) and they were placed randomly (randomised block design). The number of roots formed was counted 28 days after the initial exposure to IBA.

3.2.4 *In vitro* rooting using sterilised soil

In vitro growth of the roots of two clones (MY4 and T201) in sterilised soil and liquid medium was evaluated. Shoot tips of these clones were transferred onto basal rooting medium supplemented with 80 μ M IBA for 8 days. Using aseptic techniques, 50 mL of each sterilised soil, sand:peat (1:1, v/v); sand: perlite (1:1, v/v) or sand:peat:perlite (1:1:1, v/v/v) and 50 mL of liquid basal rooting medium (without IBA, agar and gelrite) after autoclaving was transferred to sterile 250 mL culture vessels. In addition, a control of standard rooting medium (i.e. solidified with agar and gelrite) without IBA was used. The teak shoot tips were transferred into these culture vessels for a further 28 days. There were 30 replicates (5 shoot x 6 culture vessels) for each treatment. The culture vessels were maintained under standard culture room conditions (Chapter 2 - 2.3.1) and they were placed randomly (randomised block design). The number of roots formed on each plantlet was counted five weeks after initial exposure to IBA.

This experiment was conducted twice. In the first, the soils were placed in hessian bags and sterilised (twice) and then transferred to sterilised culture vessels. In the second, the soils were sterilised (once) with soil in the culture vessels (from where the data were collected). All the procedures were done using aseptic techniques.

3.2.5 Growth of teak in different pasteurised soils

The effect of different pasteurised soils on plant growth (height) was evaluated. Shoot tips of MY4 were transferred onto basal rooting medium supplemented with 80 μ M IBA for 8 days. Using aseptic techniques, shoots were transferred to a basal rooting medium without IBA for a further 28 days. The culture vessels were maintained under

standard culture room conditions (Chapter 2 - 2.3.1) and they were placed randomly (randomised block design). The rooted plantlets were transferred to three different types of pasteurised soils: sand:peat (1:1, v/v); sand:perlite (1:1, v/v) and sand:peat:perlite (1:1:1, v/v/v). There were 40 replicates for each type of soil. At the greenhouse the cell trays were placed on a covered bench with a misting system for 28 days and for a further 28 days on an uncovered bench with a misting system. Plant height and survival (Chapter 2 – 2.4.4) were scored after these 56 days of transfer to these soils.

3.3 Statistical analysis

One-way and two-way between-groups analysis of variance (ANOVA) were performed to test differences between means. Levene's test was used to determine differences of variance. Where variances were not equal, natural logarithm transformations were performed. Where significant differences were seen due to treatment, Tukey's Honestly Significant Difference Test (Tukey HSD) and Scheffe's test were used. The latter were only used when the data were unbalanced. Where only proportions were scored, X^2 analysis was used; this applied to rooting percentage and to survival after acclimatisation. SPSS 21 and 22 versions were used for all statistical analysis.

3.4 Results

3.4.1 Low auxin concentrations: 0, 5, 10, 20, 40 and 80 μM IBA

The different concentrations of IBA increased the number of adventitious roots produced per shoot, plantlet growth (height), and percentage of rooting (Table 3.1), but had no impact upon plantlet survival in pasteurised soil (Figure 5). The effect of auxin concentrations on the number of roots produced was statistically significant, $F(5, 149) = 52.44$, $p < 0.001$, with the no auxin treatment producing significantly fewer roots per shoot than all other treatments (Figures 5A & 5B). After 56 days of transfer to inert soil, there was no impact on survival (χ^2 ; $p = 0.23$) (Figure 5D); the main effect of auxin concentrations on plant height was also statistically significant, $F(5, 151) = 6.99$, $p < 0.001$ (Figure 5C). Figure 6 shows a teak plantlet that had developed adventitious roots.

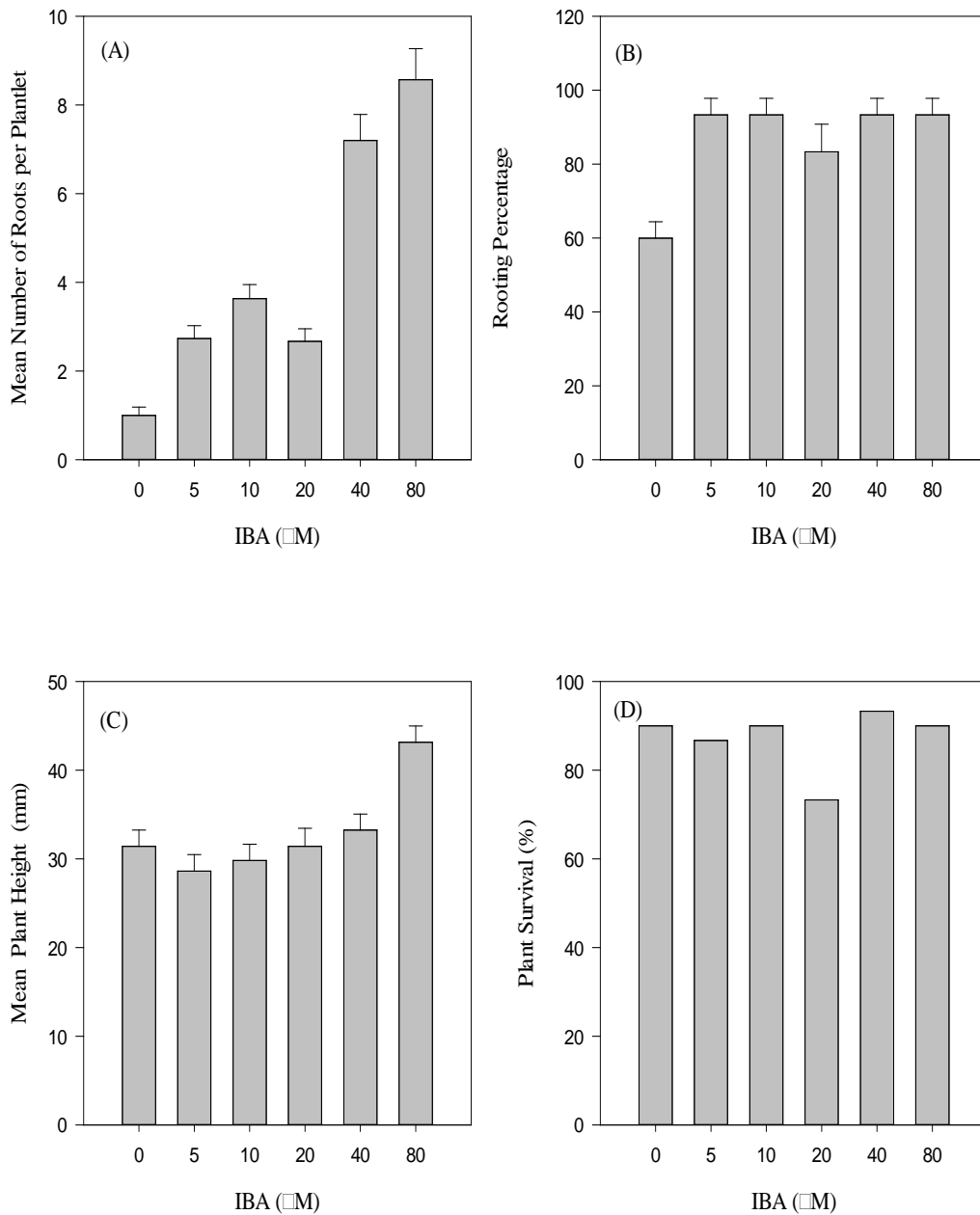


Figure 5. Response of clone MY4 exposed to 0, 5, 10, 20, 40 or 80 μM IBA for 7 days and then transferred to the same medium without auxin for a further 28 days. Means were calculated from 30 replicates per treatment and error bars represent standard error. A) Number of roots per plantlet and B) Rooting percentage. C) Plant height and D) Survival 56 days after transfer to pasteurised soil.

Table 3.1.

Response of clone MY4 exposed to low concentrations of IBA for 7 days and then transferred to the same medium without auxin for a further 28 days: number of roots and rooting percentage. Height measured after 56 days of acclimatisation.

| | | | | | | | <i>F</i> |
|-----------------|------------------|------------------|------------------|------------------|------------------|------------------|----------------------|
| [IBA μ M] | 0 | 5 | 10 | 20 | 40 | 80 | |
| Number of roots | 0.4 _a | 0.9 _a | 1.3 _a | 1.1 _a | 1.9 _b | 2.1 _b | 52.44 ^{***} |
| Height(mm) | 3.4 _a | 3.3 _a | 3.4 _a | 3.4 _a | 3.5 _a | 3.7 _b | 6.99 ^{***} |

Note = ^{***} = $P \leq 0.05$. Means with different subscripts within rows were significantly different at the $P \leq 0.05$ based on Tukey HSD post hoc paired comparisons.



Figure 6. Rooted teak plantlet. Scale bar = 1 cm.

3.4.2 High auxin concentrations: 0, 80, 100, 120 and 160 μM IBA

High IBA concentrations increased the number of roots produced per shoot, rooting percentage, and plantlet height (Table 3.2), but decreased survival 56 days after transfer to soil (Figure 7). The effect of auxin concentrations on the number of roots was statistically significant, $F(4, 125) = 75.67, p < 0.001$. Tukey's test indicated that the control treatment had significantly fewer roots than all the other treatments, and that all the auxin treatments produced an average number of roots per plantlet (Figures 7A & 7B). The main effect of auxin concentration on plantlet height was also statistically significant, $F(4, 103) = 19.62, p < 0.001$. Scheffe's test indicated that plantlets treated with 80 μM IBA were significantly higher than plantlets from all other treatments (Figure 7C). However, plants exposed to the highest concentrations had presented the lowest survival ($\chi^2; p < 0.001$) (Figure 7D).

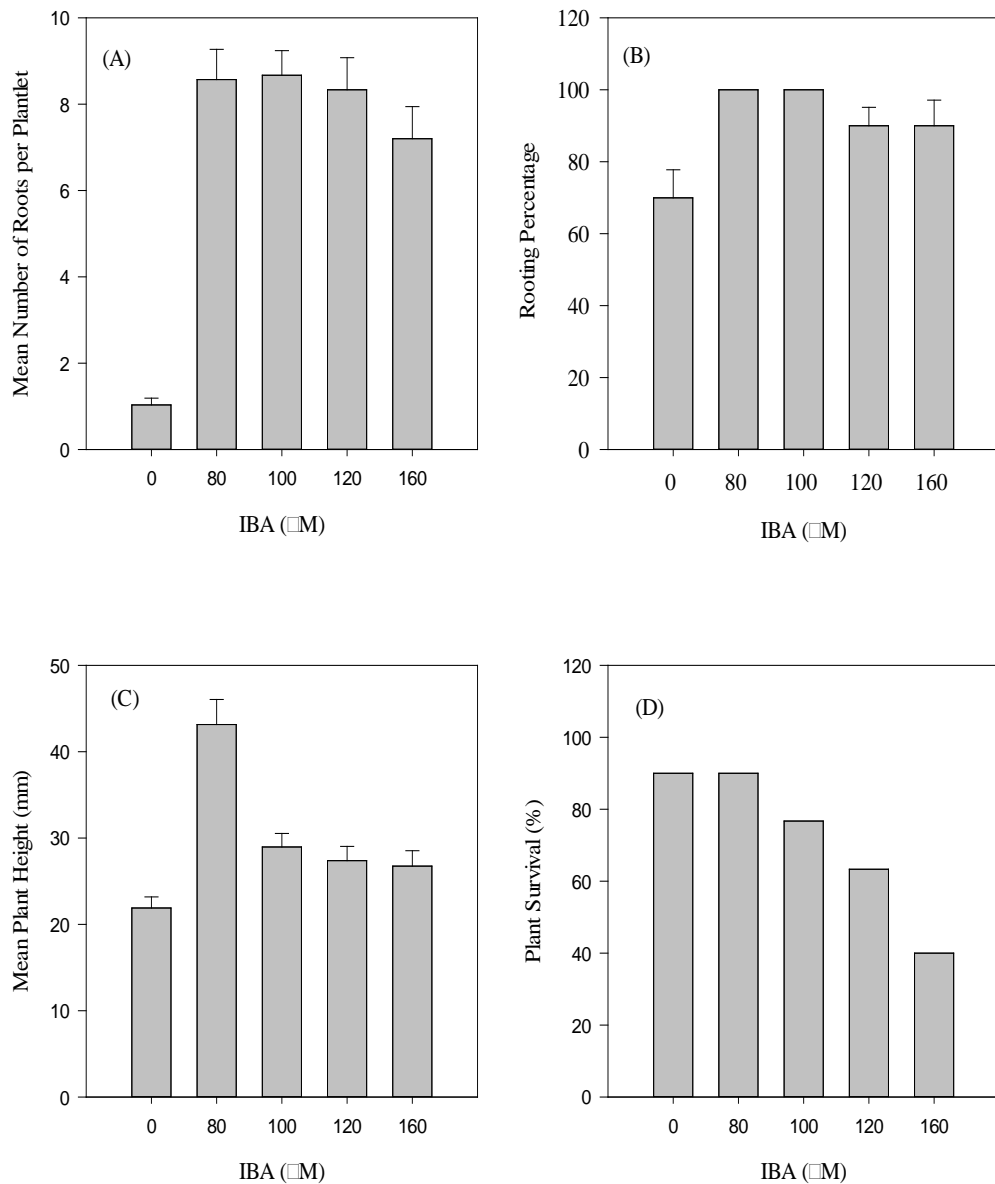


Figure 7. Response of clone MY4 shoots exposed to 0, 80, 100, 120 or 160 μM IBA for 7 days and then transferred to the same medium without auxin for further 28 days. Means were calculated from 30 replicates and error bars represent standard error. A) Number of roots per plantlet and B) Rooting percentage. C) Plant height and D) Survival 56 days after transfer to pasteurised soil ($n = 81$).

Table 3.2.

Response of clone MY4 exposed to high concentrations of IBA for 7 days and then transferred the same medium without auxin for a further 28 days: Number of roots and rooting percentage. Height measured after 56 days of acclimatisation.

| | | | | | | <i>F</i> |
|-----------------|------------------|------------------|------------------|--------------------|--------------------|----------------------|
| [IBA μ M] | 0 | 80 | 100 | 120 | 160 | |
| Number of roots | 0.3 _a | 2.1 _b | 2.0 _b | 2.1 _b | 2.0 _b | 75.67 ^{***} |
| Plant height | 3.0 _a | 3.7 _c | 3.3 _b | 3.3 _{a,b} | 3.3 _{a,b} | 19.62 ^{***} |

Note = ^{***} = $P \leq 0.05$ Means with different subscripts within rows were significantly different at the $P \leq 0.05$ based on Tukey HSD post hoc paired comparisons.

3.4.3 Suitable exposure time to IBA

Prolonged exposure to 80 μ M IBA inhibited the number of roots formed by both clones (Figure 8). A factorial between groups ANOVA was performed and indicated that there was differences between clone MY4 produced significantly more roots than T201 $F(7, 144) = 167.09, p = \leq 0.001$ (Table 3.3),. However, there was not interaction between clones and time of exposure.

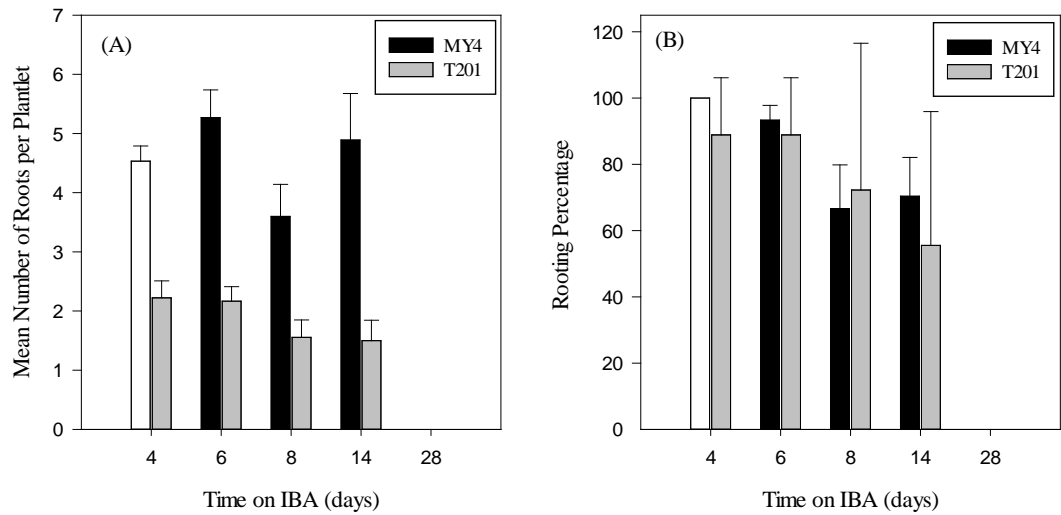


Figure 8. Comparison of the rooting response of two clones MY4 or T201 exposed to 80 μM IBA for 4, 6, 8, 14 or 28 days. Means were calculated from 30 MY4 replicates and 18 T201 replicates per treatment and error bars represent standard error. A) Root induction. B) Rooting percentage after different time exposure to 80 μM IBA.

Table 3.3.

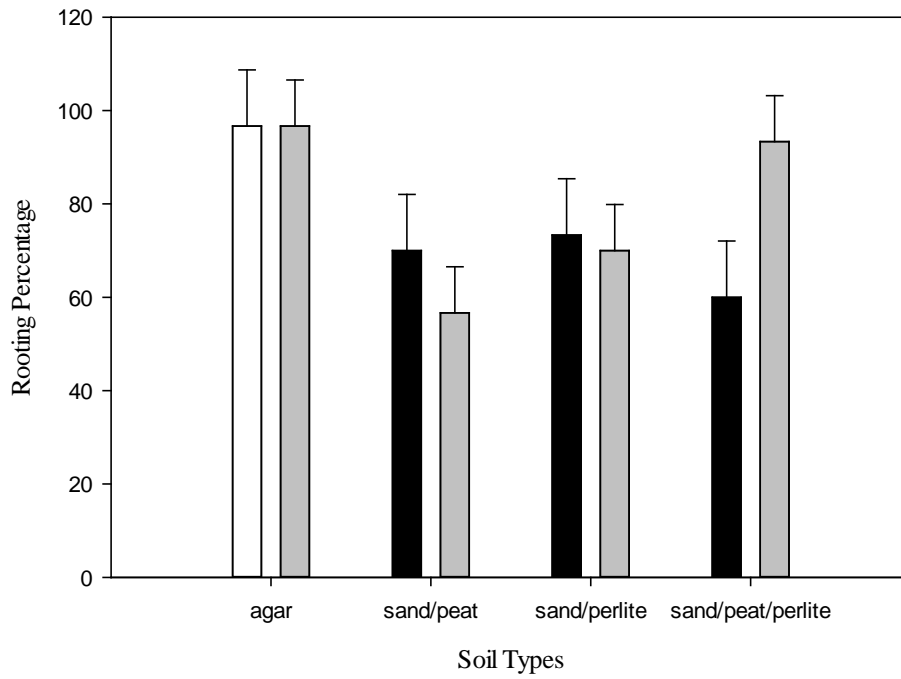
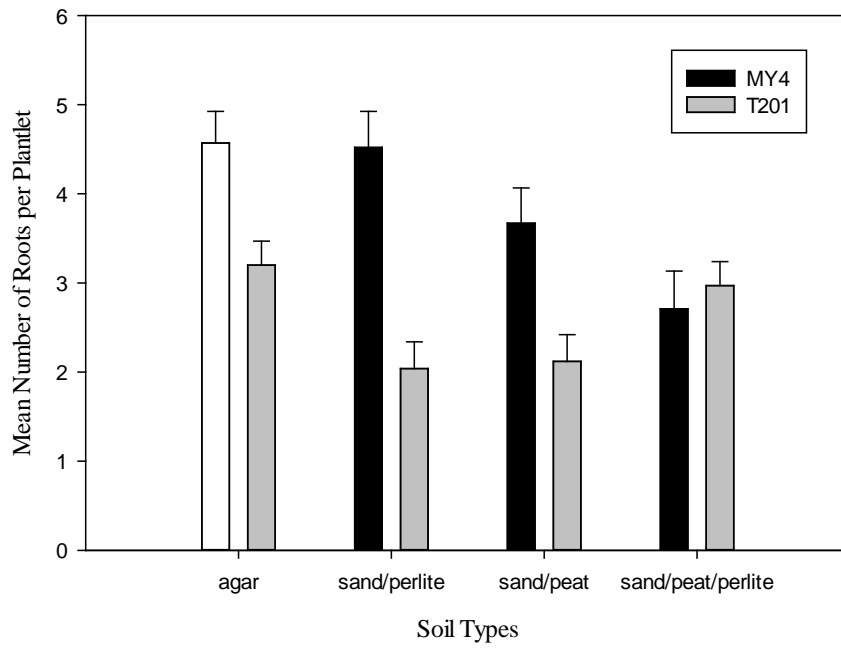
Rooting of clones MY4 and T201 developed *in vitro* after different times of exposure to IBA.

| | | | | | <i>F</i> |
|--------------------------|------------------|--------------------|------------------|------------------|------------------------|
| Days of exposure to IBA | 4 | 6 | 8 | 14 | |
| Number of roots | 1.2 _a | 1.4 _{a,b} | 1.3 _a | 1.6 _b | 3.189 ^{***} |
| Clone | | | | | 167.087 ^{***} |
| Days of exposure X clone | | | | | 1.298 ^{***} |

Note = ^{***} = $P \leq 0.05$. Means with different subscripts within rows were significantly different at the $P \leq 0.05$ based on Tukey HSD post hoc paired comparisons.

3.4.4 *In vitro* rooting using liquid medium and sterilised soils

The number of roots produced differed significantly between the two clones MY4 and T201 (Figure 9, Figure 10 and Table 3.4). The effect of soil type on number of roots produced was statistically significant $F(7, 177) = 2.76, p = 0.044$. The effect of soil types on clones was also statistically significant $F(7, 177) = 25.68, p < 0.001$. There was an interaction between soil types and clones $F(7, 177) = 4.47, p = 0.005$. However, there was a significant difference between soil types ($\chi^2; p < 0.026$).



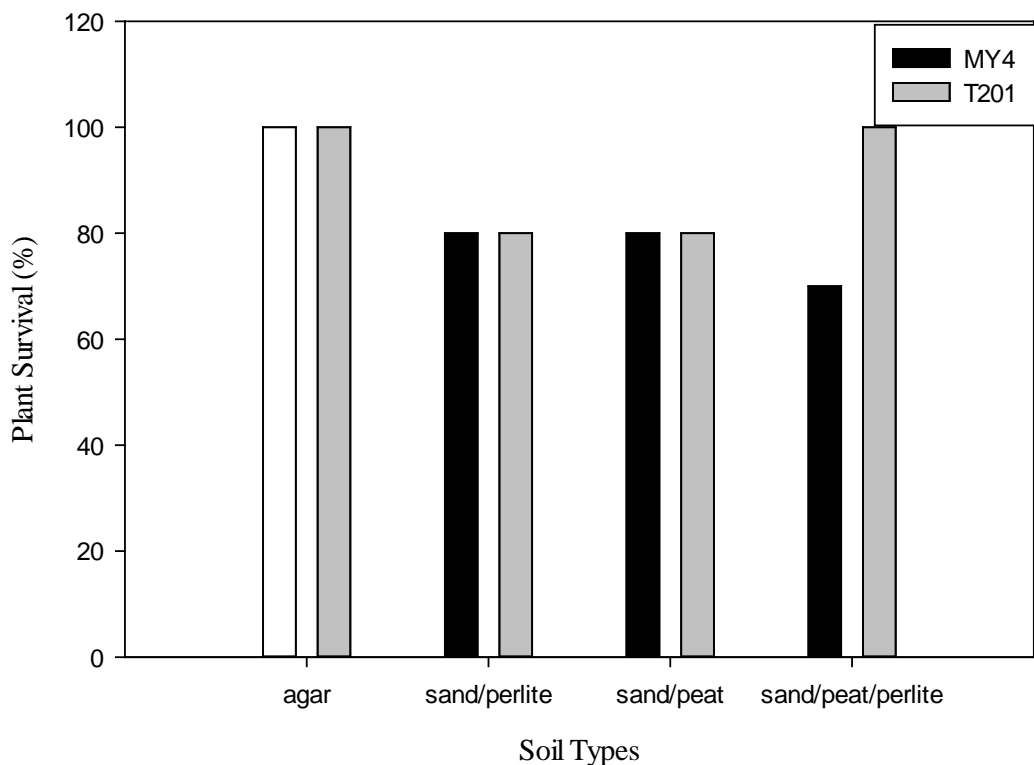


Figure 9. Response of clones MY4 and T201 to sterilised soil types. Means were calculated from 30 replicates and error bars represent standard error. A) Number of adventitious roots formed from shoots exposed to 80 μ M IBA for 8 days and development for a further 4 weeks in agar or different kinds of soil types; B) Rooting percentage. C) Plant survival.

Table 3.4.

Response of clones MY4 and T201 to sterilised soils used *in vitro*.

| | | | | | <i>F</i> |
|-----------------|------------------|------------------|------------------|-------------------|----------------------|
| Soil type | agar | sand/perlite | sand/peat | sand/peat/perlite | |
| Number of roots | 1.3 _a | 1.1 _a | 1.2 _a | 1.1 _a | 2.76 ^{***} |
| Clone | | | | | 25.68 ^{***} |
| Soil type*clone | | | | | 4.47 ^{***} |

Note = ^{***} = $P \leq 0.05$ Means with different subscripts within rows were significantly different at the $P \leq 0.05$ based on Tukey HSD post hoc paired comparisons.



Figure 10. Adventitious roots induced *in vitro* using liquid medium and sterilised soil types. Scale bar = 5 mm.

3.4.5 Teak grown on pasteurised soils

When clone MY4 was grown in different soil types there was no difference in either plant height (Figure 11A) or survival (Figure 11B) 56 days after transfer to the pasteurised soils ().

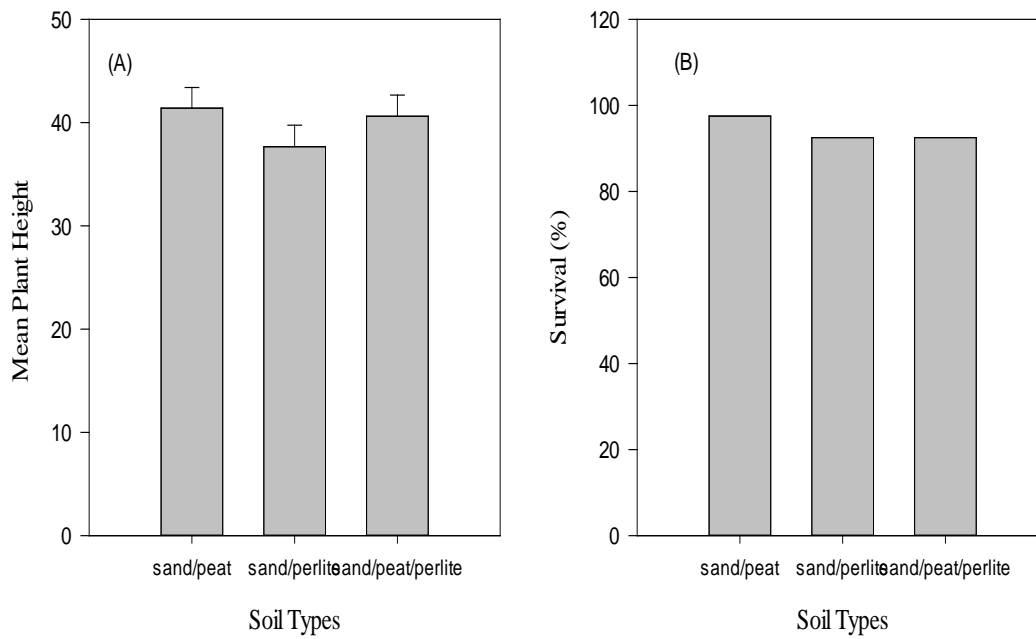


Figure 11. Clone MY4 grown on pasteurised soil types after 56 days of transfer. Means were calculated from 40 replicates and error bars represent standard error . A) Plant height. B) Percentage of survival.



Figure 12. Teak grown in pasteurised soil types (P = sand:peat (1:1, v/v);
S = sand:perlite (1:1, v/v); C = sand:peat:perlite) (1:1:1, v/v/v).

3.5 Discussion

3.5.1 Adventitious roots

IBA promoted *in vitro* adventitious root formation on micropropagated teak in the experimental conditions applied. In particular, concentrations at both 40 and 80 μM IBA added to the medium induced the most roots per shoot; 80 μM IBA (8.6 ± 0.7 roots per plantlet) and 40 μM (7.2 ± 0.6 roots per plantlet). This finding corresponds with studies of Mendoza De Gyves et al. (2007), who were able to induce maximum 2.3 roots per shoot using 2.5 μM IBA in combination with putrescine. However, Mendoza De Gyves et al. tested a narrower IBA range (0 – 10 μM). Nor Aini et al. (2009) did not obtain any roots using 0 μM IBA but obtained 8.0 roots per shoot with 24.6 μM IBA. In the research presented here a few spontaneous roots formed in the absence of IBA, but rooting of the teak plantlets was improved with the addition of IBA. These findings confirmed that low concentrations of IBA promoted the development of numerous roots that could allow good establishment when transferred to soil. Therefore, for clone MY4, IBA concentrations of 40 and 80 μM when used in combination with mMS, were sufficient to produce an equivalent number of roots per shoot to what had been previously reported.

Other kinds of auxin have been used to induce root formation on teak, such as: NAA (Akram & Aftab, 2009; Daquinta et al., 2001; Husen & Pal, 2006; Shirin et al., 2005; Kozgar & Shahzad, 2012) and IAA (Gangopadhyay et al., 2002), but the number of roots formed in these studies was similar or lower than produced in this research. Therefore, neither NAA nor IAA is likely to be suitable for adventitious root induction on teak. Akram & Aftab (2009) and Daquinta et al. (2001) worked with teak and demonstrated that high rooting percentage can be achieved with a combination of NAA and IBA at low concentration. But, it is important to realise that even though the same substances can be used to induce root formation, each clone or explant has originated from different parent material so it may respond slightly differently.

The findings also showed that IBA concentrations from 80 to 160 μM IBA produced a similar number of roots per plantlet; however, with concentrations higher than 80 μM IBA both growth and plant survival were reduced when plantlets were transferred into

soil. This has previously been demonstrated with teak (Castro et al., 2002) and other woody species, where a high survival rate and growth were achieved using low IBA concentrations (Bennett et al., 2003; Joshee et al., 2007).

Some studies have used very high IBA concentrations, but the time of exposure has been reduced. For instance, Castro et al. (2002) used 0.02M IBA for only 30 s and achieved 90% rooting under *ex vitro* conditions. Quiala et al. (2012) showed that low cytokinin enhanced rooting percentage in combination with high auxin concentration (492.1 μ M IBA) with a short exposure of 2 min. The research presented here is consistent with other studies that show strong relationships between high auxin treatment and low survival (Quiala et al., 2012).

Micropropagated plantlets generally have sparse root systems, or they have only a few root hairs (McCown, 1988); development of the root system allows the absorption of water and nutrients and thus leads to a more successful establishment (Glocke, Nowak, & Shulaev, 2003; Gribaudo, Zanetti, Morte, Previati, & Schubert, 1996). This is why it is important to induce the maximum number of roots (Bonal & Monteuis, 1997; Palanisamy et al., 2009). Gribaudo et al. (1996) reported that two woody plants, kiwifruit and apple, developed three kinds of adventitious roots: *in vitro*, *in vivo* [*ex vitro*] (newly part grew from *in vitro*), and ones produced in soil after four and five weeks of acclimatisation. *In vitro* roots formed in Bonal and Monteuis' (1997) work were replaced by thin and branched roots with white ends. It has been proposed that the roots formed *ex vitro* cannot be seen as new roots but as a regeneration of *in vitro* induced roots. Similarly, Kozgar and Shanzad (2012) described adventitious roots as "thick and well developed with secondary branching" (Kozgar & Shanzad, 2012, p. 199). The research presented here supports previous research on root formation of micropropagated plants and implies that there is a relationship between adventitious roots formed *in vitro* and high rates of successful establishment when plantlets are transferred to the soil. (Bonal & Monteuis, 1997; Glocke, Delaporte, Collins, & Sedgley, 2006).

3.5.2 Suitable exposure time to IBA

The findings show that the shortest exposure time to IBA produced the most roots and that the longest exposure time produced fewest roots and negatively affected rooting percentage with no roots being produced on either clone when exposed to IBA for 28 days. Some researchers have reported that exposure of teak and other woody plantlets to auxin and then transferring them to a medium without auxin prior to transferring to soil was useful (Azcón-Aguilar, Padilla, Encina, Azcón & Barea, 1996; Glocke et al., 2006; Joshee et al., 2007; Kozgar & Shahzad, 2012), whereas others have reported no benefit (Nas & Read, 2004). In contrast, some researchers have reported direct transfer to soil after auxin exposure (Akram & Aftab, 2009; Mendoza De Gyves et al., 2007; Nas & Read, 2004; Nor Aini et al., 2009; Shirin et al., 2005). When teak plantlets were kept in an auxin treatment for six weeks (Nor Aini et al., 2009; Shirin et al., 2005), the number of roots obtained in these studies was lower than the number produced in the research presented here. Whereas differences in number of roots produced by each of either clone in this work may be due to genetic differences (different root ability production), it appears that when the liquid rooting medium is used the number of roots tends to be lower (Gangopadhyay et al., 2002; Shirin et al., 2005). In addition, the lower number also can be explained as differences in levels of stabilization achieved for each clone, even though they have been subcultured for the same length of time (McCown, 1988). The production of adventitious roots at the longer exposure time was expected and has been explained as an 'inhibitory effect' (Nowak & Shulaev, 2003, p. 108). This inhibitory effect can be avoided by transferring the plantlets to a medium without hormone as soon as the adventitious roots start appearing.

In comparison with other studies, the auxin concentrations used in this research were generally higher; however, it was found that between six and eight days of exposure and transfer to a medium without IBA were sufficient to induce maximum root induction and growth. A similar procedure was reported by Glocke et al. (2006), who stressed that short time exposure to a low IBA concentration produced a higher number of roots and emphasised the importance of transfer to a medium without auxins to allow root development as the key to successful establishment.

The research presented here also indicates that exposure to IBA can be reduced from the minimum of 30 days reported in other studies (Akram & Aftab, 2009; Bonal & Monteuis, 1997; Kozgar & Shahzad, 2012; Mendoza De Gyves et al., 2007; Nor Aini et al., 2009; Shirin et al., 2005) to 8 days without impact on rooting and survival. It is important to note that the base of all the shoots exposed to 28 days of the 80 μM IBA treatment showed swellings (callus) but no roots emerged. Furthermore, higher auxin concentration and longer exposure time may cause stress and thereby it may affect subsequent mycorrhization. Therefore, it was concluded that an IBA treatment of 80 μM IBA for 8 days would be adequate to induce maximum rooting with high survival and growth when transferred to soil for both of the clones used in this work.

3.5.3 *In vitro* rooting using a liquid medium and sterilised soils

The current research found that pasteurised soil types did not allow root development in comparison with agar; in addition, the two clones produced fewer roots *in vitro* in comparison with agar when the MY4 clone was grown in sand, peat and perlite (1:1:1. v/v/v) and the T201 clone was grown in sand and perlite (1:1. v/v). Other kinds of substrates have also been experimented with, such as liquid rooting medium, which was used by Shirin et al. (2005) and with filter paper as root support. The number of roots formed was very low. In contrast to the previous findings such as Gangopadhyay et al. (2002) reported no root formation, even though the teak plantlets were established and they were exposed to a White medium supplemented with a combination of 9.84 μM IBA and 1.1 μM IAA for 72 hr. However, these authors reported a mean of 1.2 roots per plantlet when using the same rooting medium mix and coir as a support. Again, differences in the number of roots produced in this research and in previous studies may be due to different culture conditions and/or genetic material.

High numbers of *in vitro* roots have been acknowledged as an indication of the possible number of roots to be formed *ex vitro* (Bonal & Monteuis, 1997), but from findings presented here, the roots of the plantlets transferred to inert soils in *in vitro* conditions could not develop. This may have been due to the development of anaerobic conditions in the substrate. Nonetheless, because it was thought that this mode of plantlet production could be used to induce mycorrhization *in vitro*, the experiment was conducted twice; but there was no elongation of the roots in either case. The plantlets

were very small and the result was not encouraging. It was hoped that this attempt to select inert material/s for *in vitro* root growth might allow root elongation and mycorrhization establishment simultaneously. However, due to the poor performance of the teak plantlets under the conditions tested, this approach was abandoned and *ex vitro* mycorrhization was pursued.

3.5.4 Substrate for acclimatisation

Careful selection of substrate has been reported as important for acclimatisation of many species including teak (Venkateswarlu & Korwar, 2005). In the current research substrate was examined because there are some differences between the soil substrates that are recommended for acclimatisation and those recommended for mycorrhization. The plantlet survival in this research was higher than 90% in all soil types. The mist system was supplemented with grow top covers to prevent leaching, again relating to later attempts at mycorrhization.

Even though a variety of materials have been tested, there is still a lack of information reported by researchers about prior treatment and origin of the soil used in other work (Akram & Aftab, 2009; Mendoza De Gyves et al., 2007; Shirin et al., 2005; Tiwari et al., 2002). For the purpose of mycorrhization selection of the substrate may be very important. For instance, soil collected from regions in which teak occurs may provide undetected mycorrhizal partners. These regions include: Malaysia (Bonal & Monteuuis, 1997) and India (Shirin et al., 2005; Venkateswarlu & Korwar, 2005). In addition, sterilised soil mixtures negatively affected plantlet survival after being transferred to soil (Nor Aini et al., 2009). The pasteurised sand and perlite (1:1, v/v) used in this research allowed elongation and development of roots and provided a clean substrate to allow mycorrhization. The selection of the substrate at hardening off must be based on the objectives of the next procedure. In the research presented here, sand and perlite were selected because there were clean, easy to obtain and there was not effect of soil treatments on height of teak plantlets after 56 days of acclimatisation. The procedure could thus be standardised and reproduced, which conditions are normally recommended when examining mycorrhization (Brundrett, 2009; Smith, 2008).

In this research the acclimatisation was done using the same substrate for transfer to soil and subsequent mycorrhization (Chapter 4). Other researchers have used several materials, some of organic origin (Quiala et al., 2012; Venkateswarlu & Korwar, 2005), such as farmyard manure in combination with other materials (Kozgar & Shahzad, 2012; Shirin et al., 2005), whereas others have used inert substrates (Bonal & Monteuis, 1997) or combinations including soil (Mendoza De Gyves et al., 2007). The criteria for selection may be due to availability and cost. For the research presented here, it was assumed that acclimatisation using the same substrate for transfer to soil and mycorrhization would be more efficient and less likely to damage plantlets in early stages of development.

There have been several reports on the development of micropropagation protocol for teak. The research presented here provided reproducible conditions that allowed successful acclimatisation of teak plantlets that were ready for mycorrhization. Improvement of root production depended upon the use of auxin and developing an appropriate exposure time. The time that auxin is required did not change after 14 days; however, it appears that after transplanting the plantlets to substrates, that longer exposure to higher auxin concentrations reduces their survival and /or the growth. There were also differences in response to the treatments between the clones used in this research. Clone MY4 produced significantly more roots more quickly than T201 given the same treatment. This variation may also be reflected in shoot multiplication: T201 multiplied more slowly and therefore it was not possible to include both clones in all experiments. Variation in root number produced between clones has also been reported by others (Glocke et al., 2006) and it has been suggested that ease of rooting might be an important criterion for selecting clones to be used in large-scale plantation production (Palanisamy et al., 2009). Therefore, root production (and possibly shoot multiplication) should be evaluated for each clone because it is a key factor for later establishment.

The following chapter is about inducing mycorrhization in readily micropropagated teak plantlets.

Chapter 4: Mycorrhization

4.1 Micropropagated plantlets and AM fungal species

In other researches mycorrhization has been established in micropropagated woody plants of high economic value. The AMF inoculum used has been obtained from a variety of sources; and examples of the associations that have been reported include: *R. fasciculatus* on avocado (*Persea americana* Mill) (Vidal et al., 1992), *Glomus* spp. on pear (*Pyrus communis* L.) and peach (*Prunus persica* x *Prunus amygdalus*) (Rapparini, Baraldi, Bertazza, Brazanti, & Predieri, 1994); *R. intraradices* on *Annona cherimola* (Azcón-Aguilar et al., 1996), and *F. mosseae* on grape (*Vitis berlandieri* x *V. riparia* Kober 5 BB), kiwifruit (*Actinidia deliciosa* cv Hayward) (Gribaudo et al., 1996) and olive (*Olea europaea* L.) (Binet et al., 2007). In addition, Krishna et al. (2005) reported mycorrhizal formation on micropropagated grape with a variety of AMF, including: *Acaulospora laevis*, *A. scrobiculata*, *A. colombiana* (Formerly *Entrophospora colombiana*, Schüßler & Walker, 2010) *Gigaspora gigantea*, *Rhizophagus manihotis* (Formerly *Glomus manihotis*, Schüßler & Walker, 2010) (*R. manihotis*), and *Fuscutata heterogama* (Formerly *Scutellospora heterogama*, Schüßler & Walker, 2010) (*F. heterogama*) inoculated as individual species or as a mixture of *R. manihotis*, *F. mosseae* and *G. gigantea*. Similarly, Calvente et al., (2004) have established *R. intraradices*, *F. mosseae*, *Rhizophagus clarus* (Formerly *Glomus clarum*, Schüßler & Walker, 2010) (*R. clarus*) and *Glomus viscosum* on two varieties of olive (Arberquina and Leccino). Therefore, the *Glomeraceae* family is mainly found in mycorrhizal associations with micropropagated plants. This is not surprising due to the ubiquitous occurrence of this family with these associations in general (Brundrett, 2004).

4.1.2 AM fungal species

AMF have been usually multiplied using pot cultures (Brundrett et al., 1996, Smith, 2008). Examples of pot cultures have been reported by Bécard and Fortin (1988), who reproduced *Gigaspora margarita* on leek (*Allium porrum*) (*A. porrum*) and Varma and Schüepp (1994), who reproduced *R. intraradices* after three to four months of growing *Z. mays* as a trap plant with expanded clay as medium. Verma and Jamaluddin (1995)

used a soil pot culture to multiply fungal species found in the rhizosphere of teak. Rajan et al. (2000) described the multiplication of *A. laevis*, *G. margarita*, *Funneliformis caledonius* (Formerly *Glomus caledonium*, Schüßler & Walker, 2010;), *R. fasciculatus*, *R. intraradices*, *Ambispora leptoticha* (Formerly *Glomus leptotichum*, Schüßler & Walker, 2010) (*A. leptoticha*), *Funneliformis geosporus* (Formerly *Glomus macrocarpum*, Schüßler & Walker, 2010 ;), *F. mosseae*, and *Scutellospora calospora* from India, Western Australia and the USA, with Rhodes grass as a trap plant with sterilised sand and soil (1:1, v/v) as a medium. Similarly, Dupponois et al. (2007) used attapulgite (calcined clay) as medium and *R. intraradices* with *A. porrum* as the plant trap, whereas Pereira Cavallazzi et al. (2007) used soil from apple orchards to produce pure strains using three kinds of trap plants. Janoušková et al. (2009) used sand and zeolite to reproduce *R. intraradices* and *C. claroideum* over a period of four months. Pot cultures are usually set up under glasshouse conditions and the resulting inoculum consists of a mixture of chopped mycorrhizal root pieces from the trap plant, plus spores, hyphae and substrate (Gianinazzi & Vosátka, 2004). This kind of inoculum can be used to inoculate seedlings, plants, or micropropagated plantlets, such as Australian *Acacia* (Dupponois et al., 2007); *Tripleurospermum inodorum* L. and *Calamagrostis epigejos* L. (Janoušková et al., 2009), *Malus prunifolia* (Pereira Cavallazzi et al., 2007) and teak stumps (Rajan et al., 2000).

4.1.3 Teak and AM fungal species

Surveys of teak rhizospheres suggest that there are several AMF species that can form associations with teak roots. For example, Jamuluddin, Chandram and Malakar (2002) identified spores of AMF present in soil from a teak forest which was used as a source of inoculum in a plantation. The species included: *R. intraradices*, *Glomus aggregatum*, *F. mosseae*, *Acaulospora cavernata* (Formerly *Acaulospora scrobiculata*, Schüßler & Walker, 2010;), *Sclerocystis* sp., *Scutellospora* sp., and *Gigaspora* sp. On the other hand, Alvarado, Chavarría, Guerrero, Boniche and Navarro (2004) surveyed for spores of fungal species in the rhizosphere of 41 teak plantations in Costa Rica and found that nearly half of the teak trees showed low colonisation. In addition, it was found by the latter that soil pH negatively affected both root development and fungal growth. Prasetyo, Dewi, Utomo and Anderson (2010) reported the presence of *A. leptoticha*, *F. mosseae* and *R. fasciculatus* in the rhizosphere of teak grown in natural soils and in

revegetated waste rock. And Irianto and Santoso (2005) reported an increase in height and diameter of 6-month-old teak seedlings as a consequence of inoculation with *G. aggregatum* or the inoculum product Mycofer® (a combination of the AMF species: *C. etunicatum*, *G. margarita*, *R. manihotis*, and *Acaulospora spinosa*) in the field.

Micropropagated teak has also been shown to form mycorrhizas with several AM fungal species. For example, Ramanwong and Sangwanit (1999) inoculated micropropagated teak with spores of fungal species obtained from teak plantation soils or from soil in pots growing teak seedlings. The AM fungal species were cultivated through pot culture techniques with *Z. mays* or teak seedlings and the following species were used: *A. cavernata*, *G. aggregatum*, *Glomus deserticola*, *Glomus multicaule* (Formerly *Glomus multicauli*, Schüßler & Walker, 2010) *Sclerocystis coccogenum* (Formerly *Sclerocystis microcarpus*, Schüßler & Walker, 2010) as well as unidentified black spores.

Thus, *G. aggregatum*, *R. fasciculatus*, *A. leptoticha* and *F. mosseae* have been used for inoculating teak or their presence has been reported frequently associated with teak trees. Therefore, teak seedlings or plantlets seem to be able to establish symbioses with several fungal species, and the inoculum can be obtained from soil under teak plants or from a commercial inoculum product.

4.1.4 Factors affecting mycorrhization of micropropagated plants

The relationship between mycorrhizas and external factors that may affect their establishment on micropropagated plants at the acclimatisation stage has been investigated by several researchers. Factors that are important are the same as those for non-micropropagated plants and include: source of inoculum, substrate composition, inoculation technique, nutrients, acclimatisation and harvesting time.

An assortment of inocula has been used to inoculate micropropagated plants, including: defined culture collections (Calvente et al., 2004), chopped roots colonised with *R. fasciculatus* (Vidal et al., 1992), soils from plantations (Binet et al., 2007; Calvente et al., 2004; Pereira Cavallazzi et al., 2007; Ramanwong & Sangwanit, 1999; Verma & Jamaluddin, 1995) and from unspecified sources (Gribaudo et al., 1996). Substrate and

inoculum delivery techniques for micropropagated plants have not yet been standardised. For instance, in Vidal et al. (1992) avocado plantlets at initial stages of root development were transferred to sealed glass flasks containing steamed sterilised sand and soil (2:5, v/v) or to open pots with soil and sand (1:1, v/v) or peat and perlite (1:1, v/v) mixtures and inoculated with 1 g of inoculum placed close to the adventitious roots. Vidal et al. found that the best time for inoculation was when the rooted plantlets were transferred to open pots. Likewise, rooted kiwifruit or grapevine plantlets were transferred to a sterilised soil and sand mix (1:1, v/v) and inoculated with 1.5 g (20 propagules) of *F. mosseae* (Gribaudo et al., 1996); rooted olive plantlets were transferred to a soil, sand and vermiculite (1:1:1, v/v/v) mixture and supplemented with mycorrhizal soil produced by the trap plant technique (Calvente et al., 2004). The latter authors found that in colonising indigenous strains were more effective than strains from collections.

The fertilizer regime, acclimatisation conditions and harvesting time are also likely to be important factors contributing to successful mycorrhization (Binet et al., 2007; Castro et al., 2002; Joshee et al., 2007; Maherali & Klironomos, 2007; Rapparini et al., 1994). Examples of nutrient solutions that have been used include: Long Aston added weekly at rates of 10 mL of the full strength solution (Vidal et al., 1992), or 20 mL but without phosphorus (Rapparini et al., 1994), or 25% low P content (Calvente et al., 2004), 25% Hoagland's solution (Azcón-Aguilar et al., 1996) or no fertilizer at all (Pereira Cavallazzi et al., 2007).

Two different approaches have been used to decide the best time for inoculation: at the beginning of acclimatisation (Azcón-Aguilar et al., 1996), or a few days or weeks after (Binet et al., 2007). Generally acclimatisation consisted of a gradual reduction of rh. (Binet et al., 2007). Mycorrhizal plants were harvested between four weeks and six months after the inoculation, and several features were assessed as a result of the mycorrhiza establishment (Azcón-Aguilar et al., 1996; Binet et al., 2007; Calvente et al., 2004; Vidal et al., 1992)

4.1.5 Assessment of mycorrhization

Several measures have been used to assess the establishment of AMF on plants: shoot dry weight (Vidal et al., 1992), root dry weight (Vidal et al., 1992), plant height (Calvente et al., 2004; Schroeder & Janos, 2005), shoot fresh weight (Calvente et al., 2004), root fresh weight (Calvente et al., 2004), plant weight (Herrera-Peraza et al., 2011; Schroeder & Janos, 2005), and leaf area (Herrera-Peraza et al., 2011; Huat, Awang, Hashim & Majid., 2002). Other measures such as phosphorus, nitrogen, potassium, magnesium and sulphur content have also been used as an indication of mycorrhiza establishment (Huat et al., 2002; Schroeder & Janos, 2005; Vidal et al., 1992). Specifically with teak, plant height, stem girth, leaf area, dry weight and plant phosphorus content have been measured (Rajan et al., 2000).

For micropropagated plants, the features measured have been: shoot height and survival (Binet et al., 2007), shoot fresh and dry weight, leaf area and shoot height (Herrera-Peraza et al., 2011). More specifically with teak, the diameter at root collar, plant dry weight, root dry weight, plant height, shoot dry weight and shoot levels of phosphorus, nitrogen and potassium (Ramanwong & Sangwanit, 1999) have been measured. However, these features may be influenced by uncontrollable factors (e.g. nutrients availability) other than mycorrhizal establishment, particularly when soil is used as inoculum (Sykorova et al., 2007) or substrate. In most cases there is an increase in the parameter measured once mycorrhizas have developed.

4.1.6 Teak and mycorrhizas – microscopic assessment.

There are only a few reports of mycorrhizal establishment on teak plants and plantlets (Rajan et al., 2000; Ramanwong & Sangwanit, 1999) and as a result there are few photomicrographs showing mycorrhizal colonisation of teak (Alvarado et al., 2004). Photomicrographs are considered an important feature to help in an identification of mycorrhizal establishment (Brundrett, 2004; Öpik et al., 2013; Smith & Read, 2008).

4.2 Aim of this chapter

The aim of the research described in this chapter was to determine the optimum conditions to establish AMF on micropropagated teak plantlets.

4.3 Materials and methods

4.3.1 Inoculum sources

Sources of inoculum used in this research were detailed in Chapter 2 – Section 2.2.

4.3.2 Physical conditions

The mycorrhization experiments were performed from July 2010 to July 2011 and the light conditions in the greenhouses (31°57'8" S; 115°51'32" E) depended on the season.

4.3.3 Preliminary inoculation

Crack pots were filled with each of three pasteurised soil types: sand:peat (1:1, v/v), sand:perlite (1:1, v/v), and sand:peat:perlite (1:1:1, v/v/v). Prior to the teak rooted plantlets being transferred, 1.0 g of processed inoculum product (IP) was placed underneath each MY4 plantlet per crack pot and the plantlets' roots were dipped carefully into IP. There were 30 replicates for each type of soil. The crack pots were placed in cell trays randomly (randomised block design) for the acclimatisation stage, which took place under mist for 28 days, followed by a further 28 days on open benches under greenhouse conditions (Chapter 2 - Section 2.4.2).

4.3.4. Single species as inoculum

Spore inoculum consisted of two species of *C. etunicatum* (inoculum 1) or *Glomus* spp. (inoculum 2). Both samples were used at two concentrations by suspending 0.5 (A) or 2.5 g (B) of spore powder in a litre of sterilized MilliQ-plus water. The inoculum powder contained 10,000 spores g⁻¹; therefore concentration A contained 5 spores mL⁻¹ and concentration B contained 2.5 spores mL⁻¹.

Spore isolate inoculation

Crack pots were filled with pasteurised soil containing sand:perlite (1:1, v/v). Then, each crack pot was flushed with 25 mL of half strength sorghum nutrient solution (Brundrett et al., 1996) and left to drain for 24 hr. Plantlets of clone MY4 were carefully transferred using 20 – 35 replicates for each treatment in the greenhouse. Each plantlet received 10 mL of one of the following inoculum treatments: *C. etunicatum*, 5

spores mL⁻¹, or 2.5 spores mL⁻¹; *Glomus* spp., 5 spores mL⁻¹, or 2.5 spores mL⁻¹; each treatment was placed within a single cell tray. The control plantlets received 10 mL of MilliQ-plus water without spores. To each crack pot, 3 ml of half strength sorghum nutrient solution was added at week four. The five treatments were separated in the benches to avoid cross-contamination. The acclimatisation was done as outlined in chapter 2 - 2.4. The plantlets were harvested at the end of 8 weeks (Chapter 2 – Section 2.4.4) and shoot length, root surface area and mycorrhization were assessed as outlined in Chapter 2 – Section 2.6.3.

4.3.5 IP and UIP - inoculum preparation

IP inoculum was prepared by thoroughly mixing 700 g of pasteurised soil containing sand:perlite (1:1, v/v) with 10 g of IP (>500 spores, information obtained from supplier). Similarly, UIP inoculum was prepared by thoroughly mixing 1 kg of pasteurised soil containing sand:perlite (1:1, v/v) with 100 g (>10000 spores, information obtained from the supplier) UIP and 1.5 g of dried chopped mycorrhizal grass roots. Then, crack pots were filled with these inocula and placed in cell trays, one treatment per tray. The control treatment consisted of pasteurised soil containing sand:perlite (1:1, v/v). Each crack pot was flushed with 25 mL of half strength sorghum nutrient solution and allowed to drain freely for 24 hr.

4.3.6 Plant trap pre-treatment

Z. mays seeds were soaked in MilliQ-plus water for 24 hr. Seeds were surface sterilised and placed on sterile filter paper (70 mm in diameter) moistened with 10 mL of sterile MilliQ-plus water. These seeds were incubated at 26 °C for 24 hr, and after germination were used to establish pot cultures with organic farming soil or IP.

4.3.7 Soil as inoculum

Roots growing in the organic farming soil were assessed for mycorrhizal colonisation. Because these roots showed mycorrhizal colonisation this soil was used to establish a soil-based inoculum. Characteristics of this soil are reported in Table 4.7.

VG soil

The organic farming soil pot culture was established by first preparing pasteurised soil (sand:perlite (1:1, v/v)) and then mixing this inert substrate with the organic farming soil (1:1, v/v). Nursery pots (2 L) were filled with 700 g of this substrate mixture. Each of these pots was flushed with half-strength sorghum nutrient solution and allowed to drain freely for 24 hr. Then, five germinated *Z. mays* seeds were sown in each pot and the pots were placed on benches in the second greenhouse. Pot weight was determined individually and the soil was maintained at field capacity by watering daily with DI water. A sample of the *Z. mays* root system was harvested after four weeks and assessed for mycorrhizal colonisation. The establishment of mycorrhizas was obvious. The *Z. mays* plants were harvested six weeks after being established and their roots were chopped into pieces no more than 2 cm long. The VG soil inoculum was prepared by thoroughly mixing these chopped mycorrhizal roots back into the pot culture substrate.

4.2.8 IP, UIP and VG soil inoculation

MY4 and T201 rooted plantlets were transferred individually into crack pots filled with IP, UIP, VG soil or control inocula and placed in the greenhouse. There were 30 replicates per treatment. Roots of plantlets transferred to the IP treatment were first dipped into IP powder. The crack pots were placed in cell trays arranged in alternate rows of MY4 and T201 plantlets for each treatment. The acclimatisation was done as outlined in Chapter 2 - Section 2.4.3. Finally, after acclimatisation, the cell trays were placed in the second greenhouse for a further 10 weeks.

To each crack pot, 10 mL of half-strength sorghum nutrient solution was added after 5, 10 and 15 weeks. At 10 and 20 weeks, half of the plantlets were harvested. Plants inoculated with VG inoculum were harvested at 20 weeks only and the replicates were set up with MY4 clone only. For each inoculum treatment plant height, root area and mycorrhization were assessed as outlined in Chapter 2 – Sections 2.4.4 and 2.6.3.

4.2.9 IP pot culture

Nursery pots were filled with 700 g of IP inoculum and watered to field capacity with half-strength sorghum nutrient solution. Five germinated *Z. mays* seeds were sown into each pot and placed on bare benches in the greenhouse. Field capacity was maintained

by watering daily with DI water. A sample of the root system was harvested and assessed for mycorrhiza four weeks after being established. The *Z. mays* plants were harvested six weeks after being established. The substrate from these cultures with chopped mycorrhizal *Z. mays* roots was thoroughly mixed and it was used as IP pot culture inoculum.

Inoculation with VG soil, IP pot culture, IP inocula

Rooted plantlets of MY4 were transferred individually into crack pots filled with VG soil, IP, IP pot culture or control inocula and then placed in the greenhouse. Roots of plantlets transferred to the IP treatment were first dipped in processed IP powder. There were 25 replicates for each treatment. The cell trays were placed on covered benches but each treatment was placed separately into the cell trays to avoid cross-contamination. The same acclimatisation procedure was followed as in Chapter 2 – 2.4.3. Teak plants were harvested 20 weeks after being established. Plant height, root area and mycorrhization were assessed as outlined in Chapter 2 – Sections 2.4.4 and 2.6.3.

4.3 Results

4.3.1 Preliminary inoculation

Teak plants were assessed 56 days after acclimatisation. Their survival was affected by the acclimatisation procedure (data not shown). The observed roots grew in combinations of sand and peat (1:1, v/v) and sand, peat and perlite (1:1:1, v/v/v) and showed large amounts of debris on roots. Similar problem was acknowledged by Douds Jr. (1997). Microscopic observation of roots did not show any sign of mycorrhization.

4.3.2 Control roots

There were several features found in both the control treatment and in inoculated plants; the features consisted of small bodies within the cytoplasm (Illustrated in figures 14, 15 and 16).



Figure 13. Teak root from control treatment after 20 weeks of acclimatisation. Cells show small cytoplasm's bodies stained with acetic acid and ink 5%. Scale bar = 0.01mm.

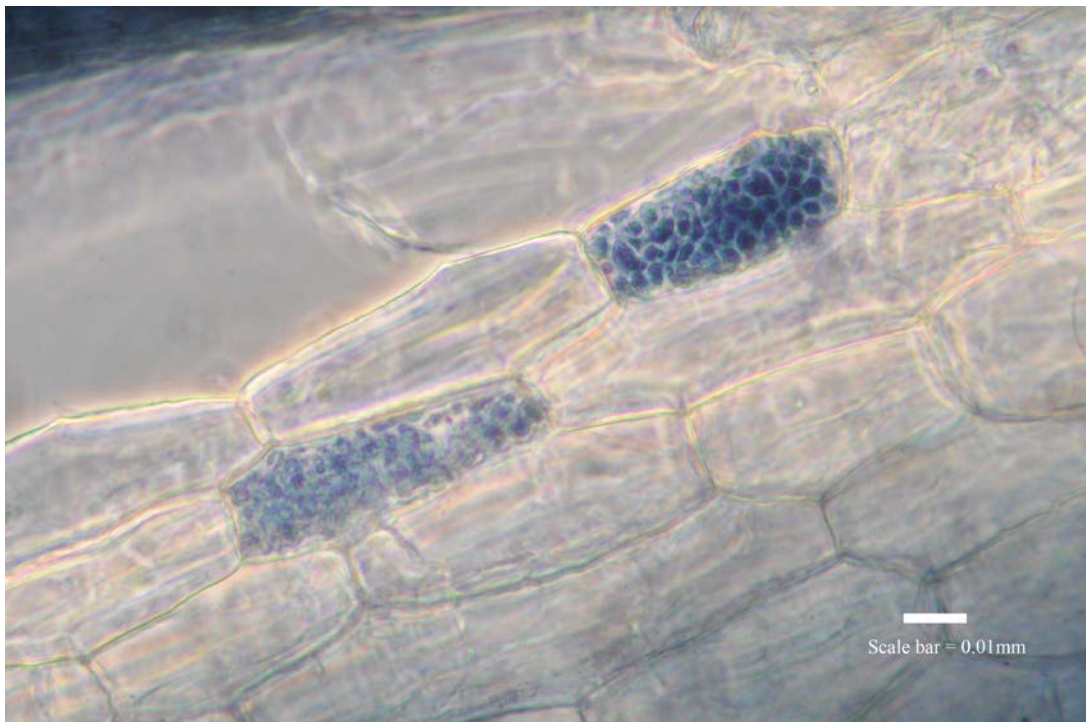


Figure 14. Teak root from control treatment after 20 weeks acclimatisation. Cells show small cytoplasm bodies stained with acetic acid and ink 5%. Scale bar = 0.01mm.

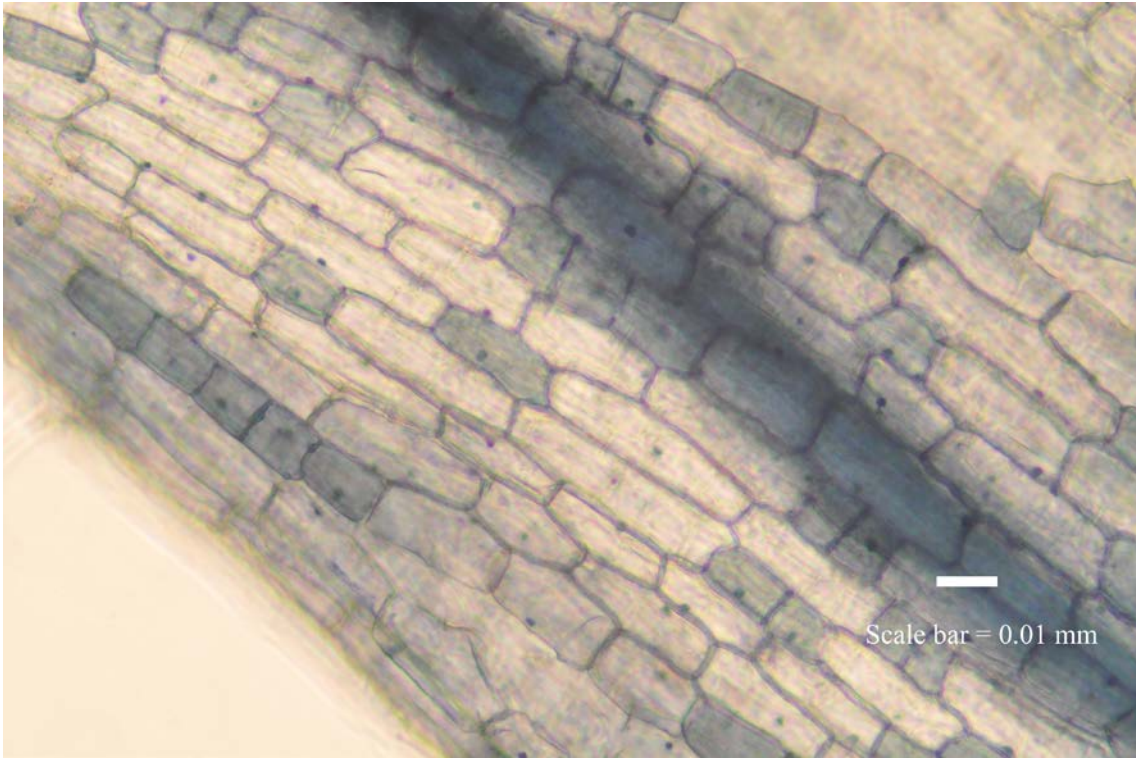


Figure 15. Teak root from control treatment after 20 weeks of acclimatisation. Cells show their nucleus and cytoplasm stained with acetic acid and ink 5%. Scale bar = 0.01 mm.

4.3.3 Single species inoculation

Claroideoglossum etunicatum.

The effect of different concentrations of the spores [*C. etunicatum*] were not related to MY4 plantlet height $F(2, 57) = 0.383, p = 0.683$, but seemed to be associated with increased root surface area $F(2, 57) = 4.12, p = 0.021$ after 8 weeks of acclimatisation (Figures 17A and 17B and Table 4.1). There was no microscopic evidence of mycorrhizal development.

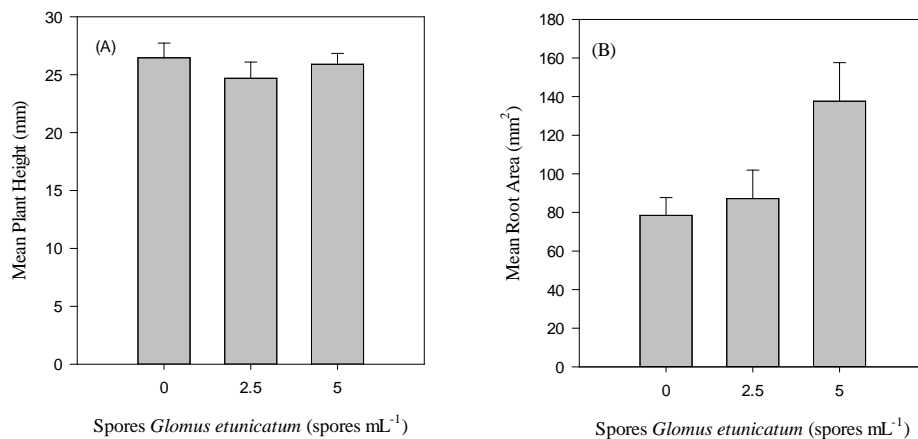


Figure 16. Response of clone MY4 to inoculation with *C. etunicatum* at 0, 2,5 or 5,0 spores mL⁻¹ after 8 weeks of acclimatisation. Means were calculated from 20 replicates per treatment and error bars represent the standard error. A) Plant height. B) Root surface area.

Table 4.1.

Response of clone MY4 to inoculation with isolated spores of *Claroideoglomus etunicatum*.

| | | | | <i>F</i> |
|--------------------------------------|------------------|--------------------|------------------|---------------------|
| Spores mL ⁻¹ | 0 | 2.5 | 5.0 | |
| Height (mm) | 26.1 | 24.7 | 25.9 | 0.38 |
| Root surface area (mm ²) | 4.2 _a | 4.2 _{a,b} | 4.7 _b | 4.12 ^{***} |

Note = ^{***} = $P \leq 0.05$ Means with different subscripts within rows were significantly different at the $P \leq 0.05$ based on Tukey HSD post hoc paired comparisons.

Glomus spp.

The effect of different concentrations of *Glomus* spp. increased height and root surface area of clone MY4 after 8 weeks of acclimatisation (Figures 18A & 18B and Table 4.2). Plant height differed significantly among the treatments $F(2, 97) = 4.88, p = 0.010$. Mean root surface areas also differed significantly among the treatments $F(2, 97) = 6.04, p = 0.003$. There was no microscopic evidence of mycorrhizal development.

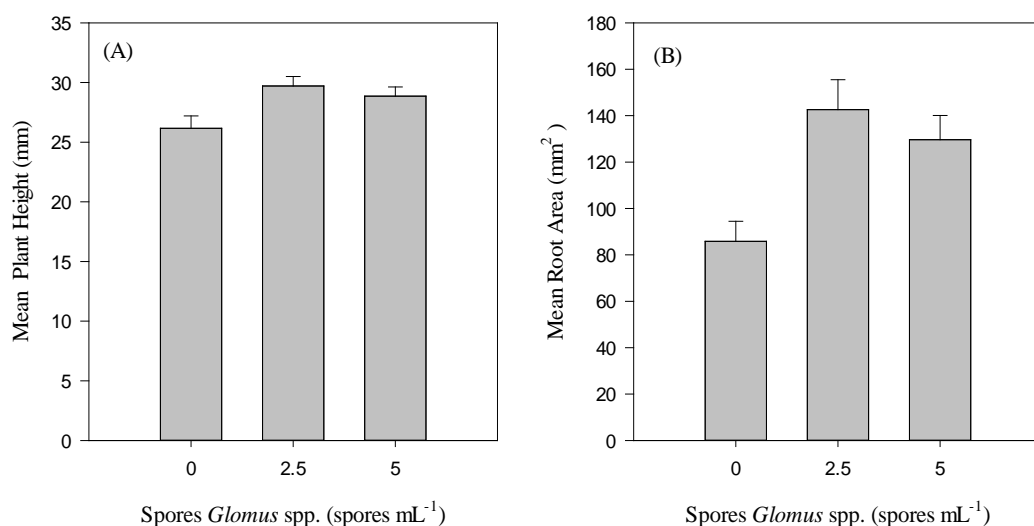


Figure 17. Response of clone MY4 to inoculation with *Glomus* spp. at 0 or, 2.5 or 5.0 spores mL⁻¹ after 8 weeks of acclimatisation. Means were calculated from 35 replicates per treatment and error bars represent the standard error. A) Plant height B) Root surface area.

Table 4.2.

Response of clone MY4 to inoculation with isolated spores of of *Glomus* spp.

| | | | | <i>F</i> |
|--------------------------------------|-------------------|-------------------|-------------------|---------------------|
| Spores mL ⁻¹ | 0 | 2.5 | 5.0 | |
| Height (mm) | 26.4 _a | 29.9 _b | 29.8 _b | 4.88 ^{***} |
| Root surface area (mm ²) | 4.3 _a | 4.8 _b | 4.7 _b | 6.04 ^{***} |

Note = ^{***} = $P \leq 0.05$. Means with different subscripts within rows were significantly different at the $P \leq 0.05$ based on Tukey HSD post hoc pared comparisons.

4.3.4 UIP, IP, VG soil inocula

MY4 clone – 10 weeks

The main effect of UIP treatment on plant height was not statistically significant $F(2, 43) = 3.22, p = 0.050$, but it increased root surface area $F(2, 43) = 4.99, p = 0.011$ of clone MY4 after 10 weeks of acclimatisation (Figures 18A & 18B and Table 4.3).

Microscopic examination revealed hyphae growing in 60% of the assessed roots from UIP treatment; however, arbuscules or vesicles were not evident.

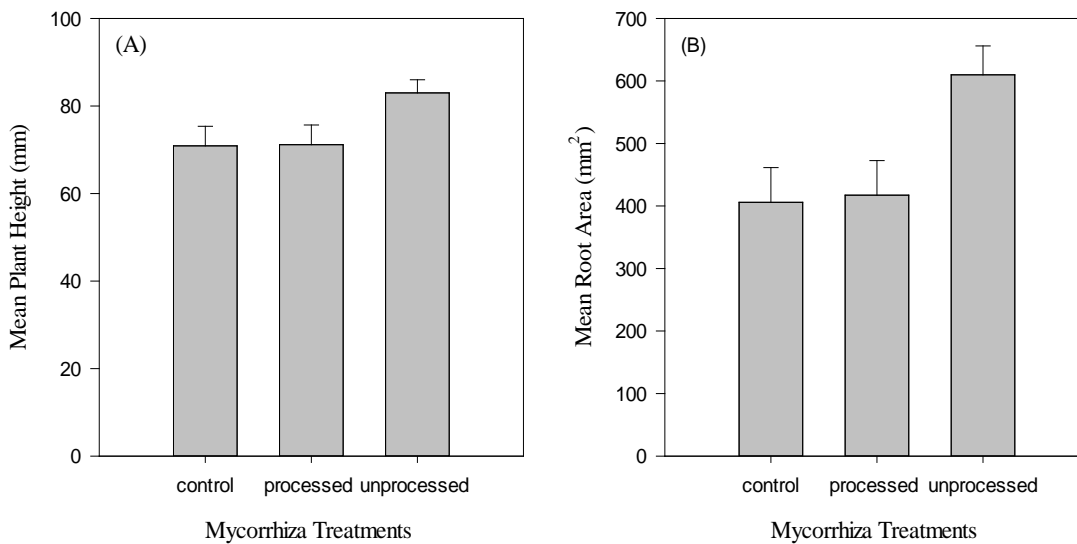


Figure 18. Response of teak clone MY4 to inoculation with processed (IP) or unprocessed (UIP) inoculum product after 10 weeks of acclimatisation. Means were calculated from 15 replicates per treatment and error bars represent the standard error. A) Plant height. B) Root surface area.

Table 4.3.

Response of clone MY4 to inoculation with inoculum product (IP), and unprocessed inoculum product (UIP) after 10 weeks of acclimatisation.

| | | | | <i>F</i> |
|--------------------------------------|--------------------|--------------------|--------------------|---------------------|
| Treatments | control | IP | UIP | |
| Height (mm) | 70.9 | 71.1 | 83.0 | 3.22 |
| Root surface area (mm ²) | 405.9 _a | 417.4 _a | 609.9 _b | 4.99 ^{***} |

Note = ^{***} = $P \leq 0.05$. Means with different subscripts within rows were significantly different at the $P \leq 0.05$ based on Tukey HSD post hoc paired comparisons

T201 clone – 10 weeks

The effect of mycorrhiza treatments was not statistically significant on plant height $F = (2, 42) = 1.66, p = 0.202$ (Figure 19A) or root surface area $F = (2, 43) = 0.468, p = 0.630$ (Figure 19B) of clone T201 after 10 weeks of acclimatisation. There was no evidence of mycorrhizal establishment (Figure 20).

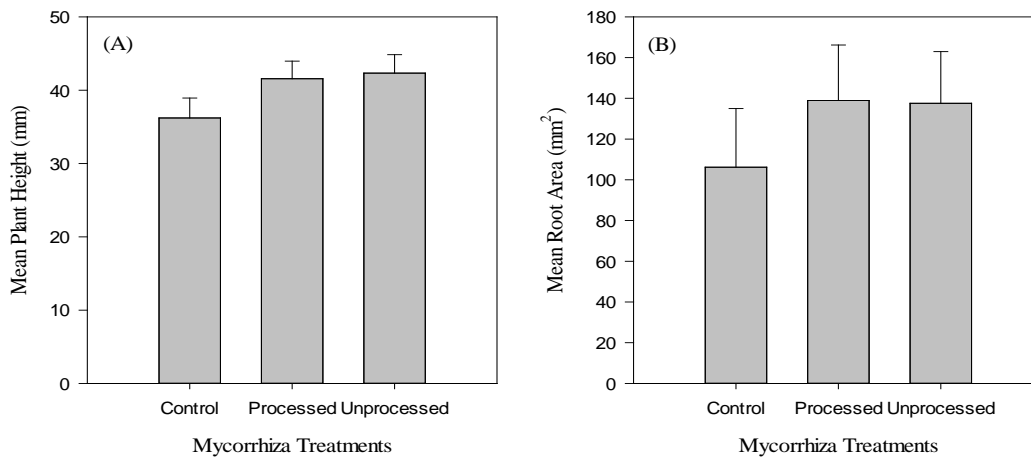


Figure 19. Response of teak clone T201 to inoculation with processed (IP) or unprocessed (UIP) inoculum product at 10 weeks of acclimatisation. Means were calculated from 15 replicates per treatment and error bars represent the standard error. A) Plant height. B) Root surface area.

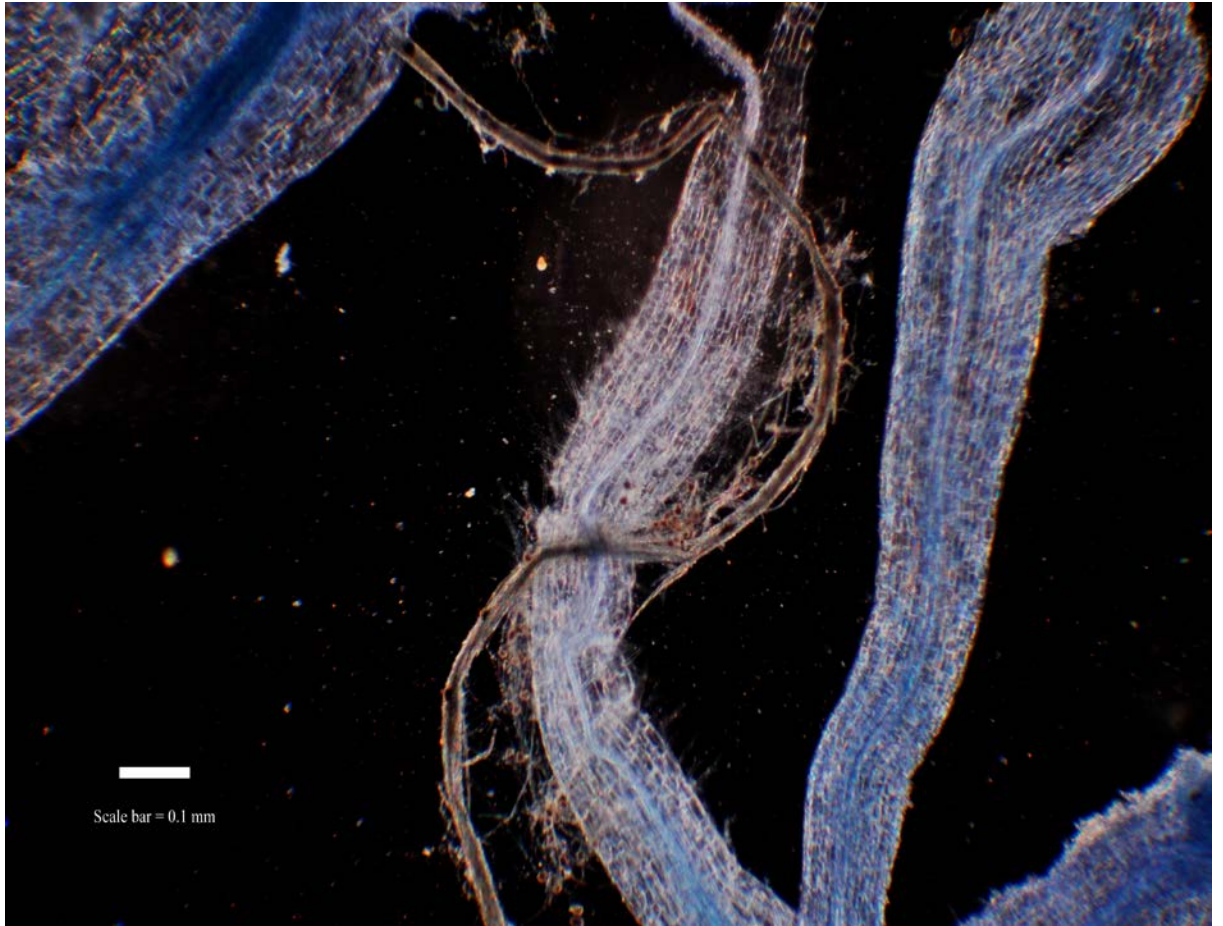


Figure 20. Unprocessed inoculum product (UIP) treatment. Mycorrhizal dry grass root acting as propagules and T201 clone teak root stained with acetic acid and ink 5% and observed using phase contrast. Scale bar = 0.1 mm.

MY4 clone – 20 weeks

The main effect of mycorrhizas treatments on plant height was statistically significant, $F(3, 51) = 16.91$, $p < 0.001$ (Figure 21A). Similarly, the main effect of mycorrhiza treatments on root surface area was statistically significant, $F(3, 50) = 16.94$, $p < 0.001$, (Figure 21B and Table 4.4) after 20 weeks of acclimatisation. There was a 100% mycorrhizal establishment on plants from the VG soil treatment, 60% of hyphae growing from UIP treatment, and 0% of mycorrhiza establishment from IP and control treatments (Table 4.6). However, arbuscules, vesicles were only evident on teak plants from VG soil treatment. Figure 22 shows what the hyphae found in IP treatment looked like.

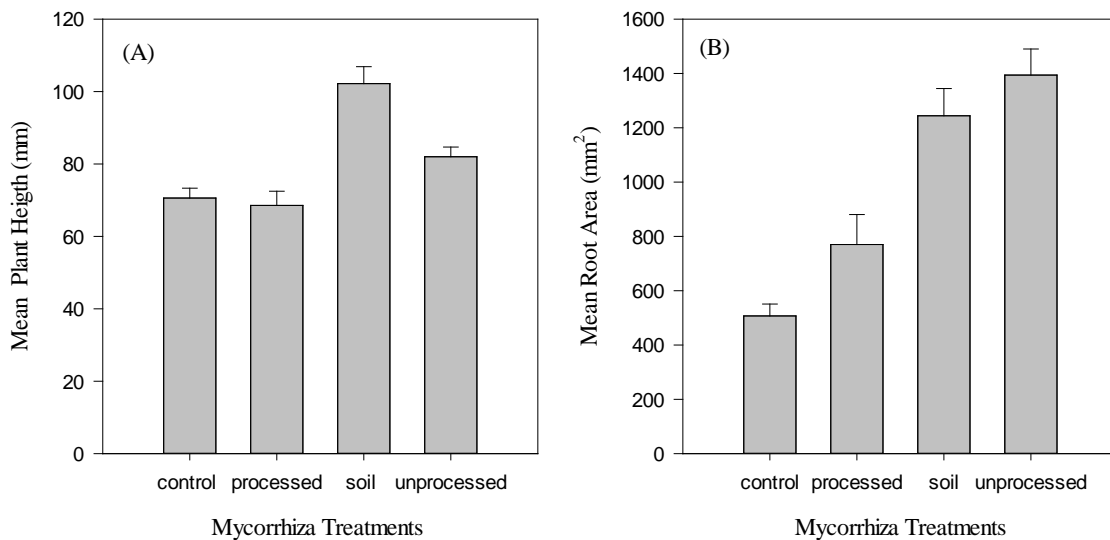


Figure 21. Response of clone MY4 to inoculation with processed (IP), unprocessed (UIP) inoculum product and soil from organic farming (VG soil) after 20 weeks of acclimatisation. The means were calculated from 15 replicates per treatment and error bars represent the standard error. A) Plant height. B) Root surface area

Table 4.4.

Response of clone MY4 to inoculation with inoculum product (IP), unprocessed inoculum product (UIP) and VG soil after 20 weeks of acclimatisation.

| Treatment | Control | IP | VG soil | UIP | <i>F</i> |
|--------------------------------------|---------------------|-------------------|--------------------|-------------------|----------------------|
| Height (mm) | 70.6 _{a,b} | 68.5 _a | 102.2 _c | 82.0 _b | 16.91 ^{***} |
| Root surface area (mm ²) | 6.2 _a | 6.5 _a | 7.1 _b | 7.2 _b | 16.95 ^{***} |

Note = ^{***} = $P \leq 0.05$. Means with different subscripts within rows were significantly different at the $P \leq 0.05$ based on Tukey HSD post hoc paired comparisons

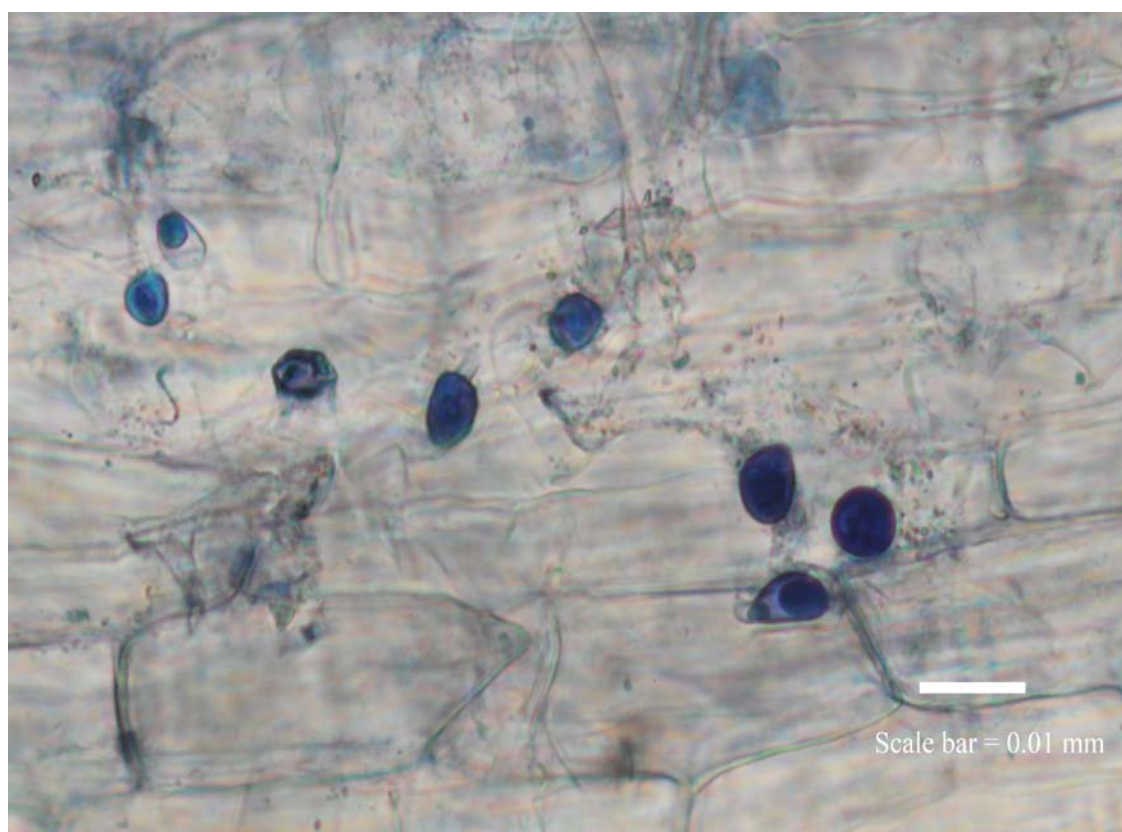


Figure 22. MY4 teak roots exposed to inoculum product (IP) after 20 weeks of acclimatisation showing growing hyphae stained with acetic acid and ink 5%. Scale bar = 0.01mm.

T201 clone – 20 weeks

Mycorrhizal treatments neither affect the height $F(2, 38) = 2.87, p < 0.069$ (Figure 23A) nor the root area $F(2, 37) = 0.91, p < 0.410$ (Figure 23B) of T201 plants after 20 weeks of acclimatisation. There was no microscopic evidence of mycorrhizal development.

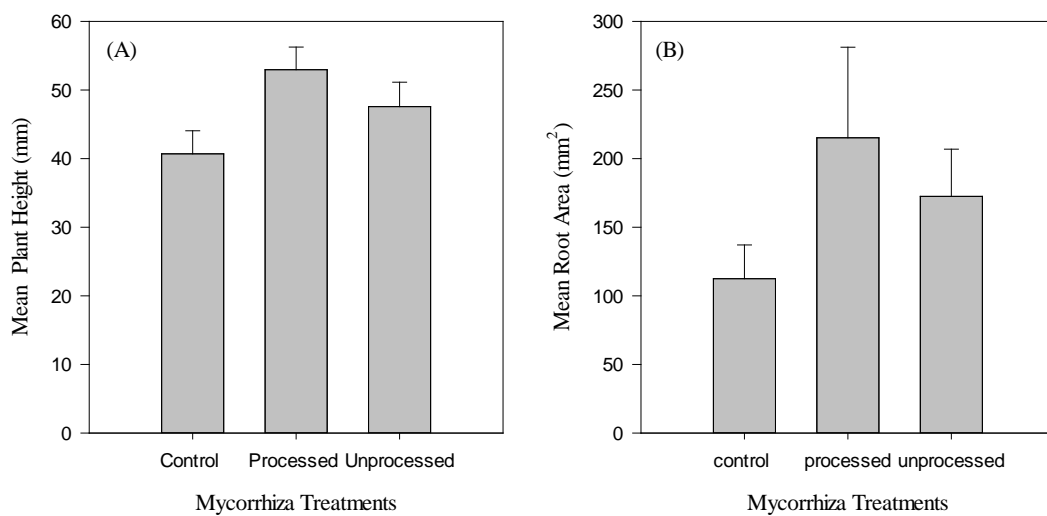


Figure 23. Response of clone T201 to inoculation with processed (IP) or unprocessed (UIP) inoculum product after 20 weeks of acclimatisation. The means were calculated from 15 replicates per treatment and error bars represent the standard error. A) Plant height; B) Root surface area

4.3.5 Inoculation with IP pot culture, VG soil and IP inocula

The effect of mycorrhizal treatments on the height of the MY4 plants was statistically significant $F(3, 95) = 39.43, p < 0.001$ (Figure 24A). Similarly, mycorrhizal treatments was also statistically significant on root surface area $F(3, 95) = 54.64, p < 0.001$, after 20 weeks of acclimatisation (Figure 24B) (Table 4.5). Figure 25 shows the appearance of hyphae found in IP treatment. The VG soil treatment resulted in the largest root area ($1017.3 \text{ mm}^2 \pm 36.4$). Figures 26 to 31 show features of AMF once the mycorrhizas were established within the teak roots. There was a 100% mycorrhizal establishment on plants from the VG soil treatment and 0% on plants from IP pot culture or IP inocula treatments (Table 4.6).

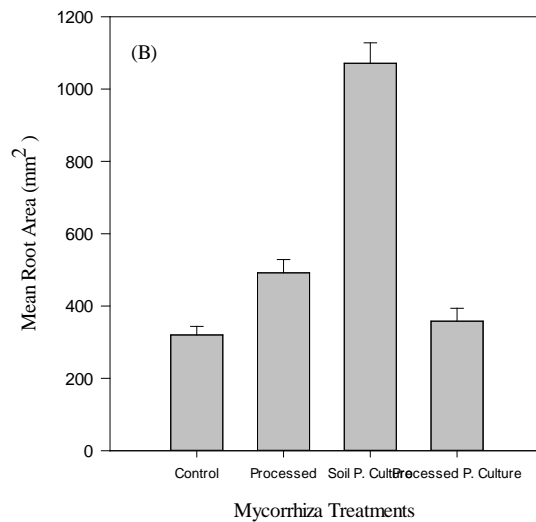
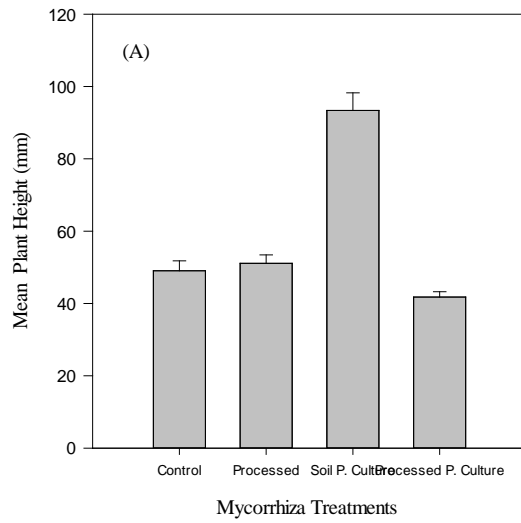


Figure 24. Response of clone MY4 to inoculation with inoculum product pot culture (IP pot culture), inoculum product (IP) or organic farming soil (VG soil) after 20 weeks of acclimatisation. The means were calculated from 25 replicates per treatment and error bars represent the standard error. A) Plant height. B) Root surface area.

Table 4.5.

Response of clone MY4 to inoculation with inoculum product (IP), organic farming soil (VG soil), or inoculum product pot culture (IP pot culture) after 20 weeks of acclimatisation.

| | <i>F</i> | | | | |
|--------------------------------------|-------------------|-------------------|-------------------|-------------------|----------------------|
| Treatment | control | IP | VG soil | IP pot culture | |
| Height (mm) | 3.85 _a | 3.91 _a | 4.49 _b | 3.72 _a | 39.43 ^{***} |
| Root surface area (mm ²) | 5.71 _a | 6.1 _b | 6.9 _c | 5.8 _a | 54.64 ^{***} |

Note = ^{***} = $P \leq 0.05$. Means with different subscripts within rows were significantly different at the $P \leq 0.05$ based on Tukey HSD post hoc paired comparisons

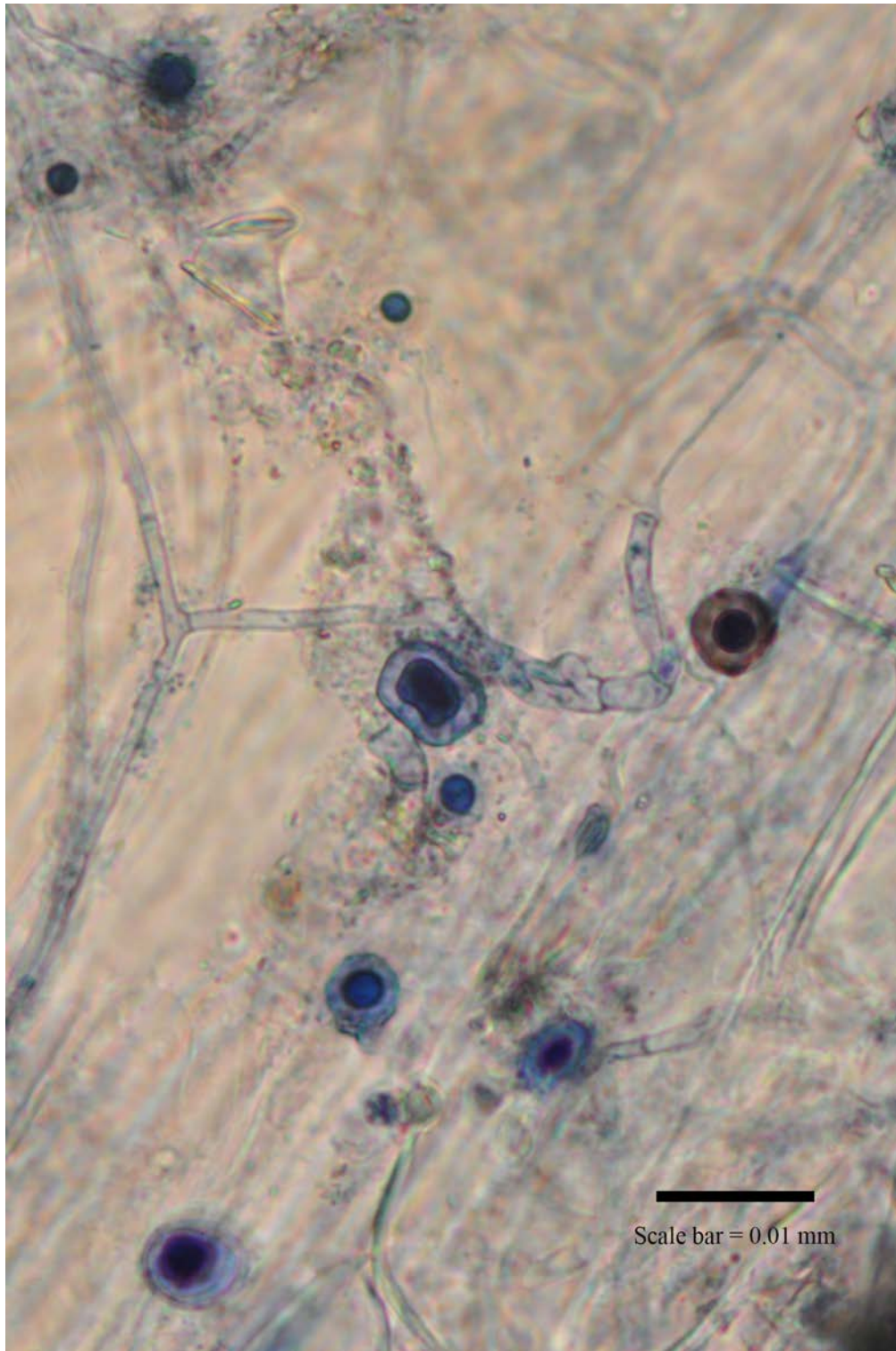


Figure 25. Teak roots grown exposed to inoculum product pot culture (IP pot culture) after 20 weeks of acclimatisation stained with acetic acid and ink 5%.
Scale bar = 0.01mm.

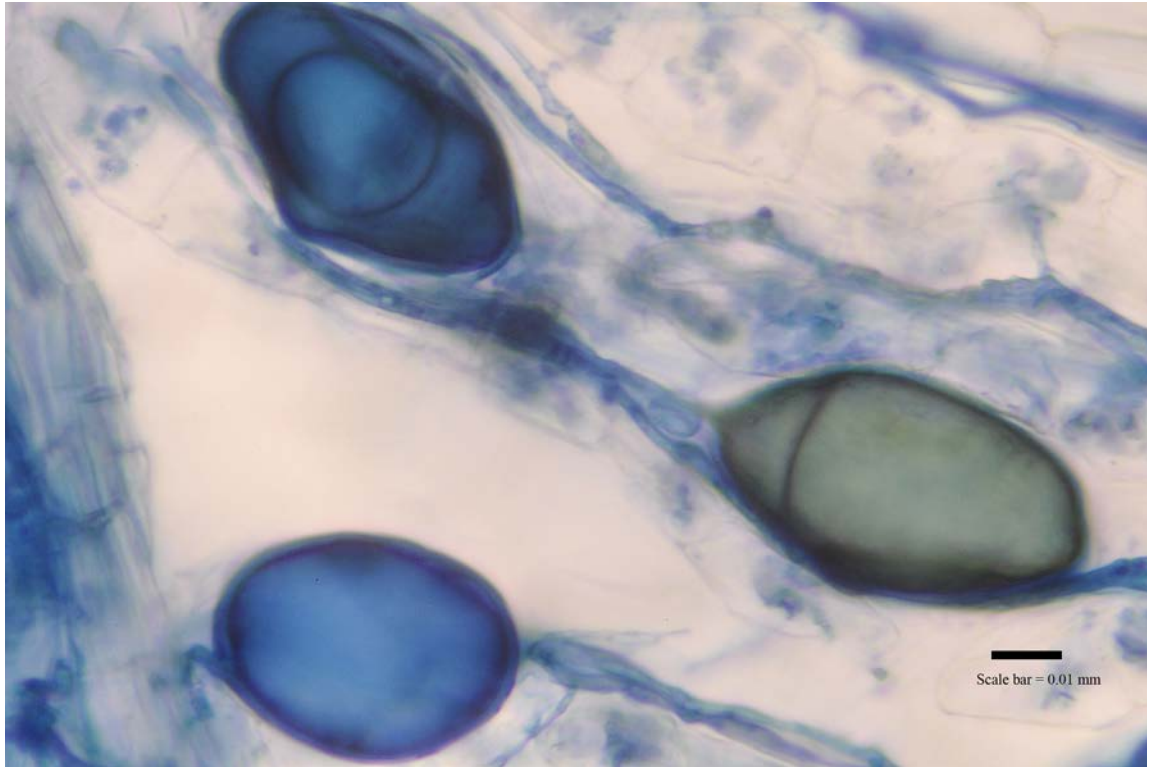


Figure 26. Glomoid mycorrhiza on teak roots from VG soil after 20 weeks of acclimatisation stained with acetic acid and ink 5%. Scale bar = 0.01mm

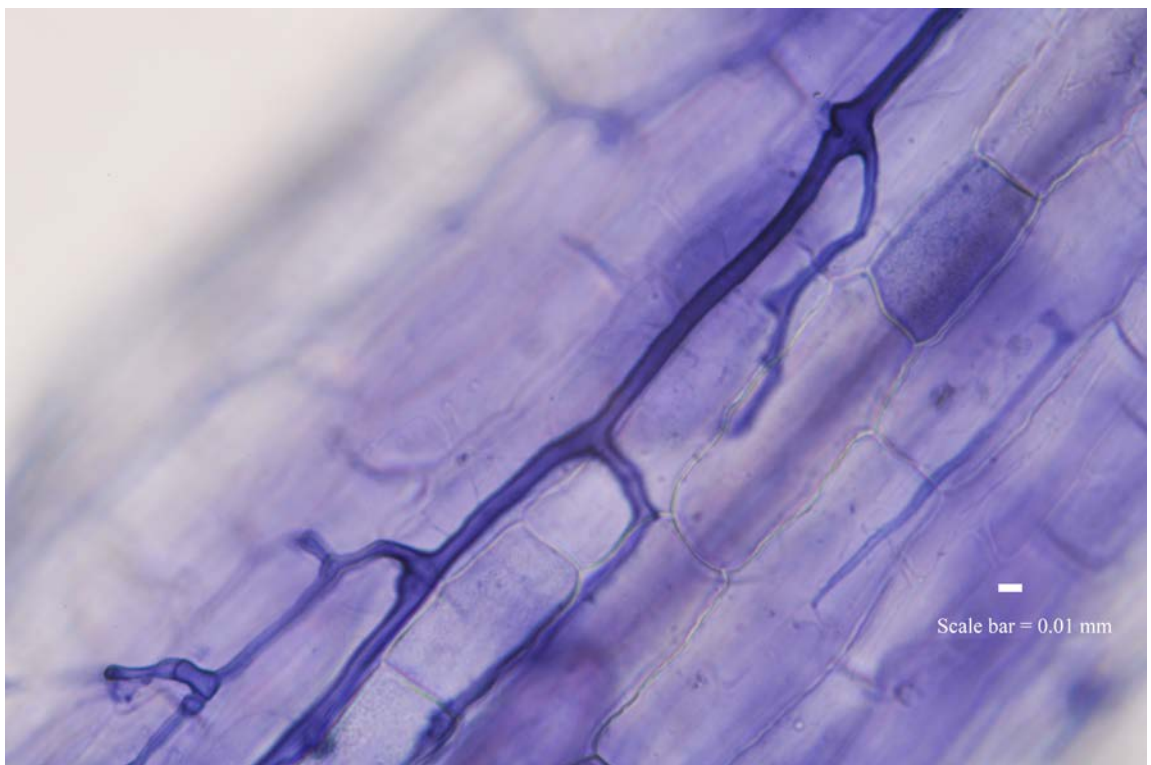


Figure 27. Glomoid intraradical mycelium on teak roots from VG soil after 20 weeks of acclimatisation. Stained with acetic acid and ink 5%. Scale bar = 0.01mm.

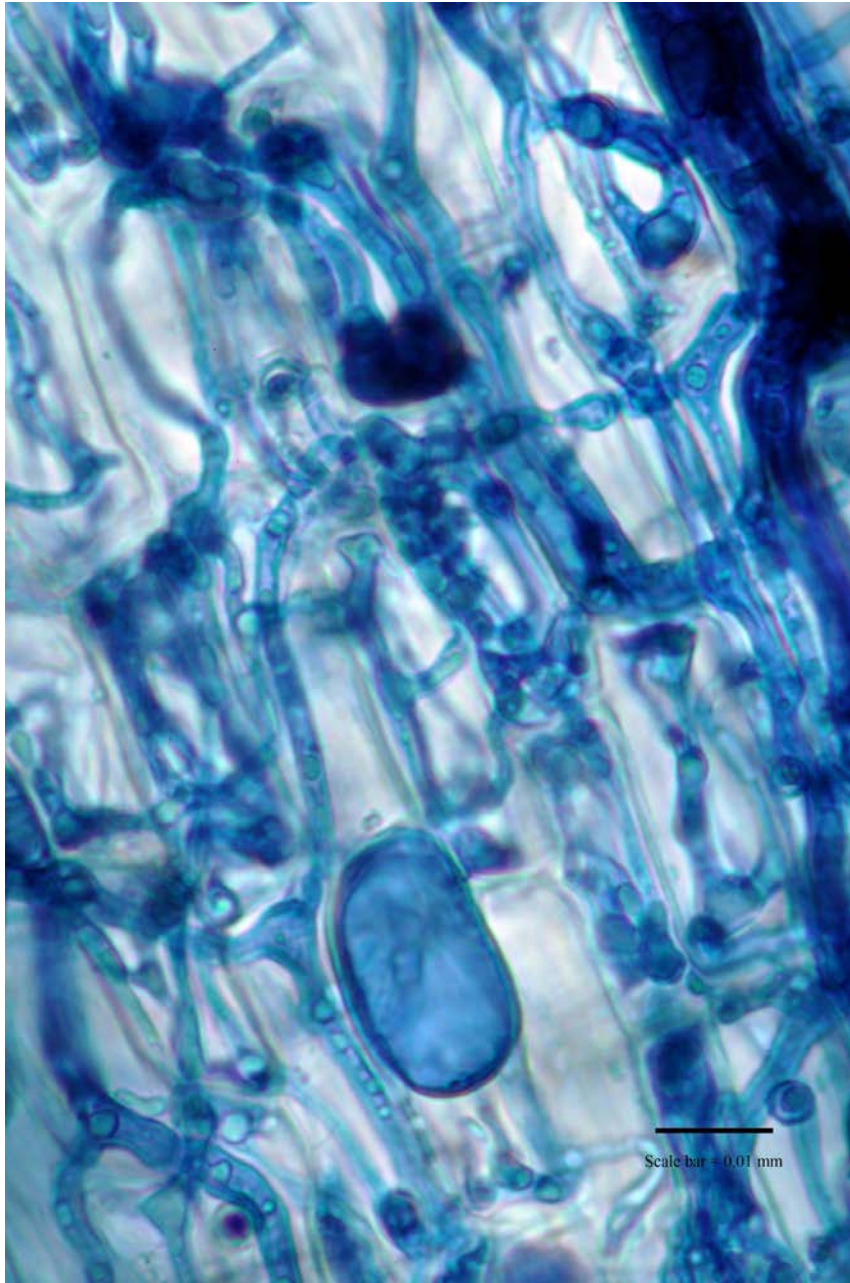


Figure 28. Colonisation by glomoid mycorrhiza on teak roots after 20 weeks of acclimatisation stained with acetic acid and ink 5%. Scale bar = 0.01mm.

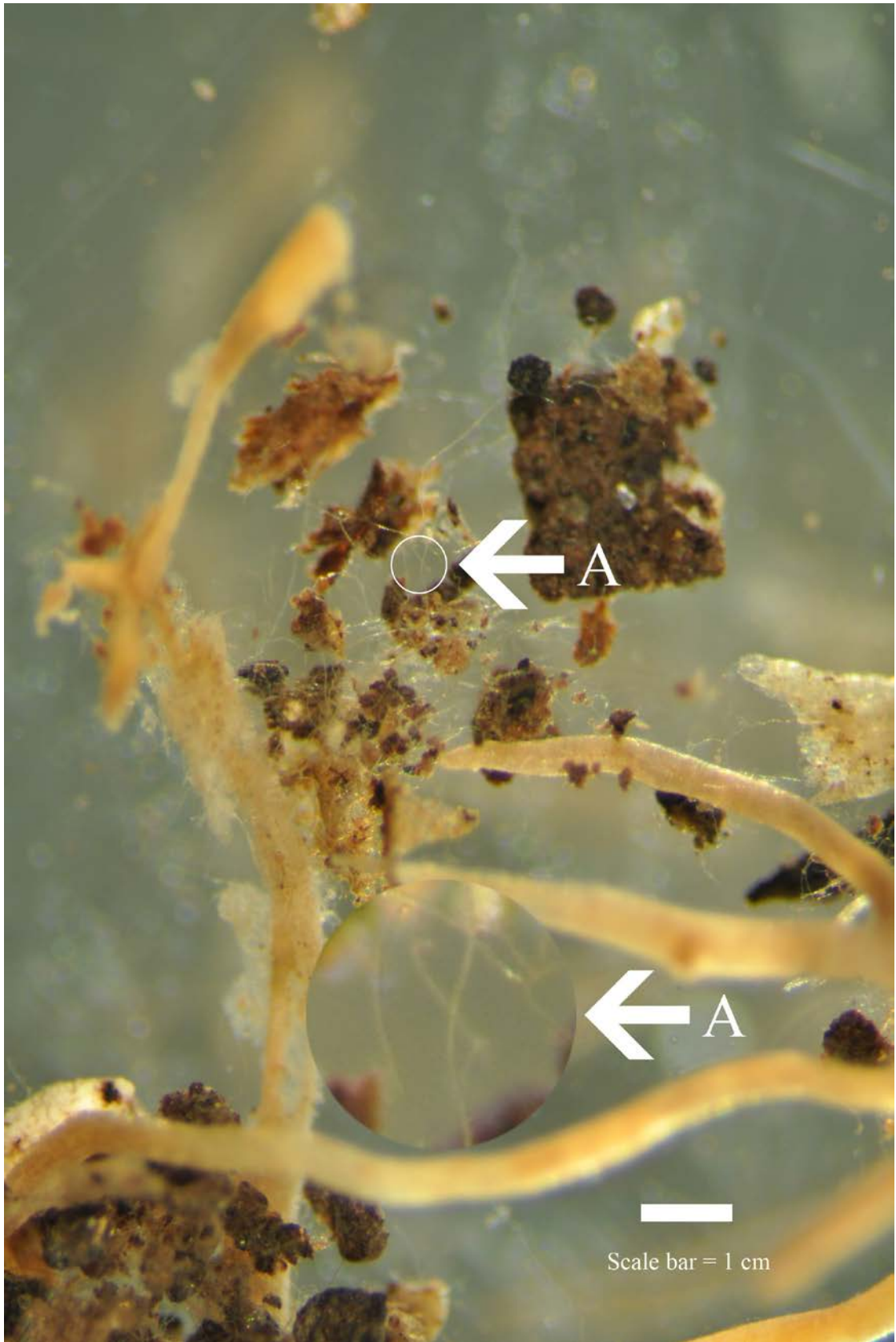


Figure 29. A) Extraradical mycelium growing from teak roots from VG soil treatment after 20 weeks of acclimatisation. Seen with stereo microscope. Scale bar = 1 cm.

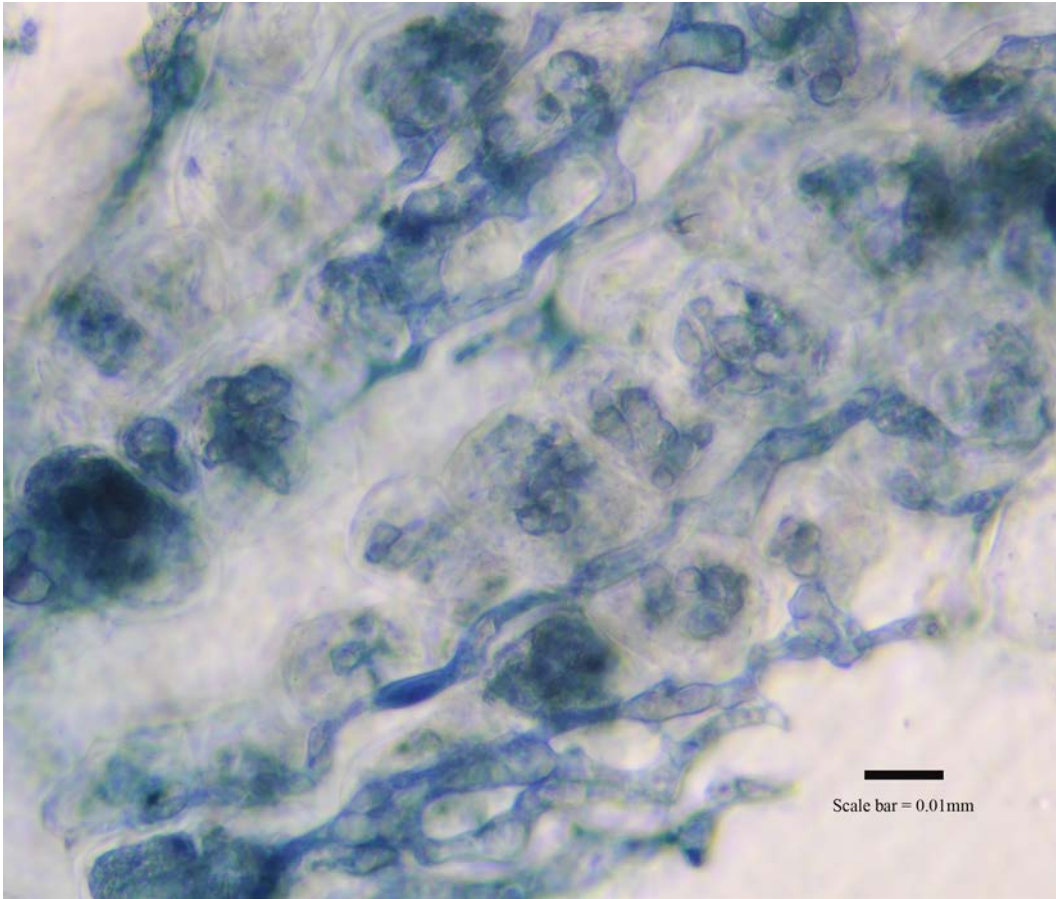


Figure 30. Arbuscules *Paris*-type on teak roots stained with acetic acid and ink 5%.
Scale bar = 0.01mm.

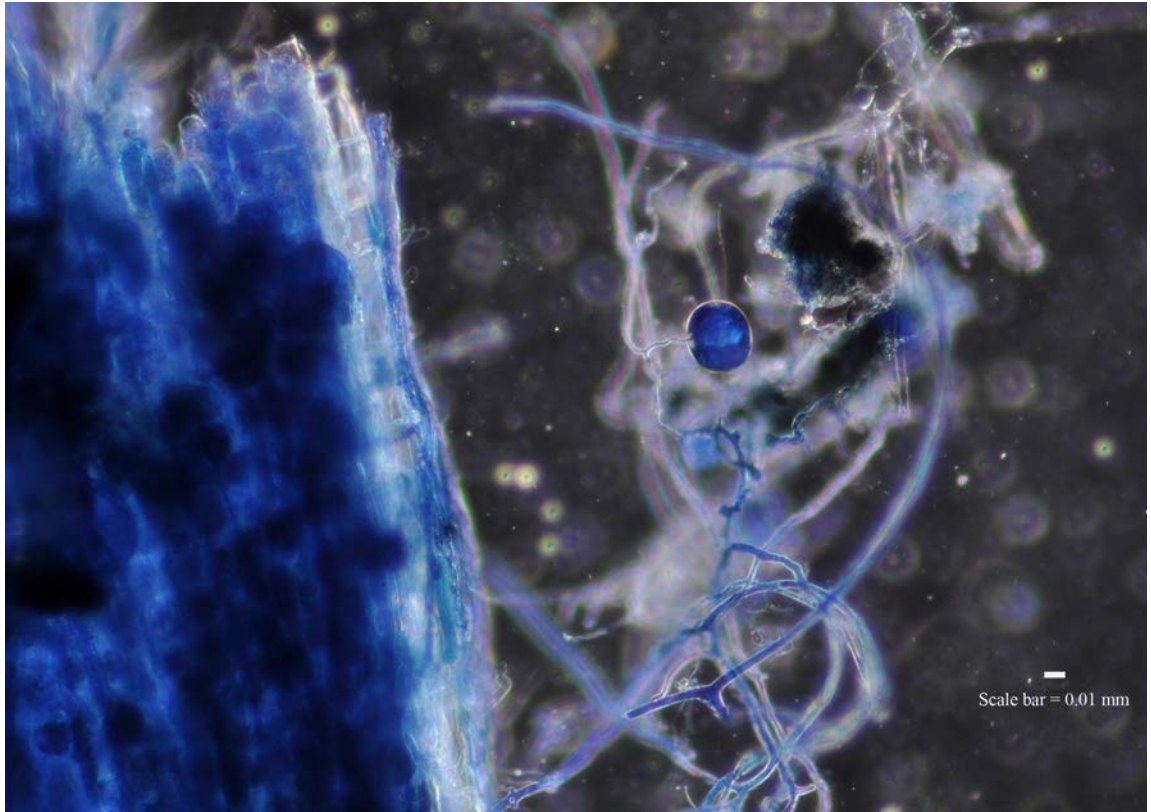


Figure 31. Soil borne spore from VG soil inoculum stained with acetic acid and ink 5% and observed using phase contrast. Scale bar = 0.01mm.

Table 4.6.

pH and P concentration of inert soils and mycorrhization percentages.

| | pH | Concentration of phosphorus (ppm) | Mycorrhization assessment % |
|---|-----|-----------------------------------|---|
| sand:peat (1:1, v/v) | 6.4 | | |
| sand:perlite (1:1, v/v) | 7.2 | | |
| sand:peat:perlite (1:1:1, v/v/v) | 7.2 | | |
| perlite (prior to experimentation) | | 46 | |
| sand/perlite (1:1, v/v) or control, after experimentation | | 26 | 0 |
| (sand/perlite(1:1, v/v)) + unprocessed inoculum (UIP) after experimentation | 6.9 | 33 | 60% of the roots plants showed some kind of hypha formation. No vesicles or arbuscules present (n = 52) |
| (sand/perlite(1:1, v/v)) + processed inoculum (IP) after experimentation | 6.9 | 22 | 30% of the roots plants showed some kind of hypha formation. No vesicles or arbuscules present (n = 53) |
| (sand/perlite (1:1, v/v)) + VG soil (1:1, v/v) after experimentation | 7.0 | 1361 | 70% of the roots occupied with intraradical mycelium, vesicles, spores and arbuscules. 100% Mycorrhizal establishment identified in all the plants of this treatment (n = 40) |

Table 4.7. Physical properties of organic farming soil

| Property | |
|---|--------------------------|
| Classification | sandy loam - Anthroposol |
| pH | 7.1 |
| conductivity | 0.77 mS/cm |
| soil moisture | 20% |
| spores per 100 g (before experimentation) | 85 |
| Previous crops | <i>Allium</i> spp. |

4.4 Discussion

4.4.1 Preliminary inoculation

The findings indicated that no mycorrhization was found within teak roots in the preliminary inoculation, so it was decided to add more inoculum and to mix it with the substrate rather than placing a small amount under each rooted plant. In addition, substrate containing peat was not used because Daft et al. (1987) reported peat as not a very efficient mycorrhizal carrier. In addition, an expert in the field suggested that after autoclaving, peat produces phenolics that may be toxic to the plants (C. Walker, personal communication, February 23, 2011).

4.4.2 Single species inoculation

After eight weeks of growth there was an increase in root surface area when plantlets of MY4 clone were exposed to the highest concentration of *C. etunicatum* or to various concentrations of *Glomus* spp., but there were no signs of mycorrhizal establishment. An increase in root area without evident mycorrhizal establishment might be explained by the very early mycorrhization stages not being visible rather than being absent. A molecular approach could perhaps have detected mycorrhizas at the very early stages of establishment (Borriello et al., 2012; Calvente et al., 2004, Öpik et al., 2013), but this procedure was beyond the scope of this project. *C. etunicatum* could be considered as one of teak's ideal fungal partners because it has been found to be the most frequent partner in a comprehensive teak rhizosphere survey in India (Verma & Jamaluddin, 1995). It is also possible that the spore isolate used in this research presented here was not ready to initiate colonisation before the plantlets were harvested.

In addition, an increase in root area can often be explained as a response to low levels of soil P (Lambers et al., 2006; Mollier & Pellerin, 1999; Schroeder & Janos, 2005; Shen et al., 2011). Shen et al. (2011) found that mycorrhization in low P soils can stimulate increases in root/shoot ratios, root branching, increases in the number of root hairs, and the formation of cluster roots.

There was also an increase in plant height, despite there being no evidence of mycorrhizal establishment when *Glomus* spp. was used as inoculum on the MY4 clone. Such increases have also been reported, but in these studies researchers used different kinds of spore isolates. For instance, in one study six months after micropropagated teak was planted it was assessed for mycorrhizal establishment (Ramanwong & Sangwanti, 1999). These authors reported that *G. aggregatum*, *A. scrobiculata* and *G. multicaule* increased plant height and *G. aggregatum*, *G. deserticola*, and *A. scrobiculata* increased root collar diameter, but they did not report seeing arbuscules. In addition, they did not find any relationship between the percentage of colonisation and increase in the growth parameter analysed. However, a strong relationship between increases in the parameter measured (for example, plant height and root fresh weight) and duration of exposure to the inoculum has been reported by Binet et al. (2007).

The importance of being able to recognise and report the anatomical features of the mycorrhizal roots (Brundrett, 2009; Smith & Read, 2008) and to compare these with roots, of non-mycorrhizal roots has also been stressed by Smith and Read (2008). In the current research, an increase in root surface area was recorded as the result of exposure to isolate spores used as inoculum, but AMF features such as vesicles, arbuscules and hyphae were not observed. Due to the complexity of these relationships, there are many possible assumptions for these findings:

- the spores may have been dormant (Giovannetti, 2010; Tommerup, 1992)
- the spores may be quiescent (Giovannetti, 2010; Tommerup, 1992)
- spore isolates storage conditions were inadequate
- dormancy of spores was induced by the isolation methods (Pawlowska et al, 2011)
- the growth rate of the hyphae from spores could be low in comparison with the growth rate of the roots (M. Brundrett, personal communication, November 5, 2010)
- unknown viability of spores
- death of spores
- not enough time to allow mycorrhizal establishment (Douds Jr, 1997; Oehl et al., 2004)
- harvesting dates of spores unknown

- inoculum doses may have been too low (researcher assumption)
- environmental conditions were not appropriate for germination of these particular isolates such as: inappropriate temperature (Giovannetti, 2010; Vierheilig & Bago, 2005), moisture (Giovannetti, 2010), pH (Douds Jr, 1997; Vierheilig & Bago, 2005)
- lack of “helper” microorganisms in the soil (Giovannetti, 2010; St-Arnaud & Elsen, 2005).

All of these may have prevented mycorrhization. In addition, because the number of spores provided was small for the current research, it was not possible to determine the spore germination ability prior to screening them. Therefore, for some or all of the above reasons, the spore isolate could not form any mycorrhiza within the teak roots within the time frame of the experiment even though it seemed to be the appropriate fungal partner.

In Ramanwong and Sangwanit’s (1999) work, and in techniques such as ROC (Chabot et al., 1992; Fortin et al., (2002); Pawlowska et al., 1999; St-Arnaud et al., 1996), were used spores as a source of inoculum but the spores were kept under specific conditions such as optimum temperature, rh., pH and they were used not long after harvesting. Other authors have emphasised the fact that spores used as inoculum take longer to establish mycorrhizas (Walker, 1999) unless the inoculum levels are high (M. Brundrett, personal communication, November 5, 2010). Chabot et al. (1992) and Pawlowska et al. (1999) stressed that spore germination time in ROC settings varied from 4 days to 8 weeks depending on the production method, namely pot culture or whether the spores produced using ROC were first or second generation, and/or the conditions under which the spores were stored. Jin et al. (2013) inoculated pea seeds with 60 spores each, and harvested the plants after 42 days, and reported that inoculation with mixed IP containing more than one *Glomus* species increased root colonisation regardless of type of mycorrhizal species. In the research presented here there were no specific storage conditions or time frame suggested for using the spores. Therefore, if a spore strain/isolate is to be used as a source of inoculum, it would be important to consider its ‘used-by-date’ and the optimum storage conditions required (Gianinazzi & Vosátka, 2004).

Several researchers have acknowledged that root exudates promote mycorrhizal establishment (Chabot et al, 1992; Giovannetti, 2010; St-Arnaud et al, 1996, Vierheilig & Bago, 2005). Specifically, Chabot et al. (1992) found that growth of the germ tube of *R. intraradices* was encouraged by the presence of roots. In addition, even though high numbers of *in vitro* roots are considered to produce an overall better survival of micropropagated plants (Bennett et al., 2003, Gribaudo et al., 1996) and the number of roots formed *in vitro* was above five in this research, it could be that at eight weeks, roots that developed *ex vitro* were not yet mature enough (Bécard & Fortin, 1988) to produce substances that stimulated further hyphal development. Consequently, in the current research it was decided to allow more time for the inoculum to establish in the subsequent experiments and for the plant to develop secondary, tertiary or even quaternary roots, which have been reported as the most likely roots to be colonised (Chabot et al, 1992, M. Brundrett, personal communication, November 5, 2012).

At various places several AMF species has been found to establish symbiotic relationships with teak (Alvarado et al., 2004; Irianto & Santoso, 2005; Rajan et al., 2000; Ramanwong & Sangwanit, 1999; Verma & Jamaluddin, 1995); however, what triggers or prevents this specific symbiosis from flourishing is still unknown. It is also possible that teak-host specificity exists or that “fungal partner specificity in AM fungi is not very high, if any exists” (Gianinazzi & Vosátka, 2004, p.9), or if the symbiosis can be established between a plant species with several AM fungal species (Janoušková et al., 2009). There are some AMF species that are not limited geographically and could be used effectively by numerous plant species (Opik et al., 2013). Whereas differences in growth between inoculated plants and the controls were seen in all the experiments in this research, these differences cannot be solely attributed to mycorrhization.

When pH is examined, it affects spore germination in most studies (Giovannetti, 2010), and the optimum pH for spore germination has been reported as being between 5.5 and 6.5 (Chabot et al., 1992; Pawlowska et al., 1999; Rajan et al., 2000; Smith & Smith, 2011). Therefore, it is preferable for the substrate to have a similar pH to that of the soils from which the spores originate. For that reason from findings presented here the

concentration of the sorghum nutrient solution was diluted and peat was removed in an attempt to maintain the pH within an acceptable range.

Two kinds of spores: *C. etunicatum* and *Glomus* spp. were used for our experiments in different concentrations (i.e. 5 spores mL⁻¹ and 2.5 spores mL⁻¹). The number of spores used by other researchers has varied from 5 to 15 (Chabot et al., 1992; St-Arnaud et al., 1996), with 1 mL of culture medium (monoxenic spores and root segments) from a six-month-old culture of *C. etunicatum* (Pawlowska et al., 1999). With teak, Ramanwong and Sangwanit (1999) used 50 spores placed beside the roots. In some cases the spore isolate used also depended on the inoculum technique (liquid, solid, gel or powder) and the number of spores was often not recorded. Thus, the optimum quantity of spore isolate to be used per teak plant has not yet been established. In the research presented here, regardless of the amount added, there was no visible mycorrhization. Given that a small number of spore isolates was reported (N. Malajczuck, personal communication, 2010) as very expensive, this suggests that it would be uneconomical to use this material as a source of inoculum for a large number of plants. The doses used in the research presented here can be regarded as a benchmark for further studies. Therefore, increased root area and height using spores as a source of inoculum in this research could not be attributed to mycorrhizal establishment because it was not possible to see with a microscope any arbuscules or/and vesicles, both of which are features indicating AMF inhabited roots.

4.4.3 Inoculum product

In this research after 10 weeks of growth there was an increase in root area but not in height when clone MY4 plantlets were exposed to UIP. The mycorrhization assessment showed signs of the very early stages of mycorrhization with the UIP inoculum. Processed inoculum products have been used by other researchers (Faye et al., 2013; Tarbell & Koske, 2007) and specifically on teak (Irianto & Santoso, 2005). The increase in root area can be explained by the reasons mentioned above for spore isolates, because the IP and UIP included spores, but differences in root area may also be attributed to other factors such as: the presence of active propagules like hyphae growing from the chopped grass, which had more time to allow mycorrhizal

establishment. IP production should include knowledge of the fungal component included (Gianinazzi & Vosátka, 2004), plus the likelihood of propagules forming before and after colonisation, the inoculum potential, and the effect of seasons on propagule production and establishment (Abbott & Robson, 1991). Hyphae growing from mycorrhizal roots require less time than spores to initiate mycorrhization (Smith & Read, 2008). The UIP used in the research presented here was enriched with mycorrhizal roots from dry grass so it is likely that this material contained more active propagules per unit volume than the IP. In addition, the UIP was in its raw form, not processed (dried and powder form), so perhaps this helped to keep the propagules intact.

Using an IP made with only two fungal species could restrict mycorrhizal establishment (Alvarado et al., 2004; Dhar & Mridha, 2006; Gianinazzi & Vosátka, 2004; Verma & Jamaluddin, 1995), and it has been suggested that fungi used in teak inocula should be species identified as fungal partners in samples obtained from roots in plantations (Calvente et al., 2004; Ramanwong & Sangwanit, 1999), or from natural ecosystems (Sýkorová et al., 2007; Tarbell & Koske, 2007), or from mixed fungi (Jin et al., 2013). Smith and Read (2008) stressed that at one site the number of AMF species identified varied depending on time of harvest (season and year). From findings presented here teak plants from UIP and IP treatments were assessed and it was not possible to see AM features such as arbuscules or vesicles, only hyphae in plants from UIP treatment. Therefore, for this particular IP perhaps more time was needed for the mycorrhizas to develop. The species in the IP were *R. intraradices* and *F. mosseae*, which are both known teak partners (Verma & Jamaluddin, 1995), but perhaps those particular isolated spores were not ready to colonise. Finally, because the IP was produced for other species and Australian conditions, it is possible that it was not well suited to the specific teak clones and the conditions used in this research.

4.4.4 Optimum quantity of inoculum

While doses of inoculum have been recommended by the manufacturer, in the current research the amount used per plant needed to be higher, because it was found that mycorrhization was not achieved in the time frame of the experiments. Similar problem occurred for Tarbell and Koske (2007) who used from 5 to 10 times more than

recommended, but still only three of eight different IPs achieved very low colonisation levels at recommended doses. Faye et al. (2013) used twice the dose suggested by the producer and reported that only three IPs showed higher root colonisation than obtained using local soil where *Z. mays* was grown. Possible explanations for non mycorrhization are inappropriate storage conditions, or that the IP had been evaluated under different conditions and for a longer time of exposure and was tested with different plant species. Therefore, this particular IP may have required enrichment with a varied source of propagules, allowing more time to develop and/or for changes in environmental conditions in order to achieve similar results to those obtained by the IP producer on other species. These current research findings can be used as a benchmark for future research work.

4.4.5 Timing for mycorrhization

Mycorrhization assessment, in this work, revealed that the times taken for mycorrhizal partnerships to establish was 4 weeks on *Z. mays* as a trap plant, and 10 or 20 weeks (or earlier) on micropropagated teak using VG soil. Mycorrhiza development took from 4 to 8 weeks in ROC settings (Pawlowska et al., 1999), or with teak from 6 (Ramanwong & Sangwanit, 1999) to 7 months (Verma & Jamaluddin, 1995). These differences can be explained partly by the inoculum potential of the propagules and their stage of development (spores, germinated spores or extraradical hyphae), the physical distance between mycorrhizal propagules and the host root, or the amount of inoculum added. Colonisation after four weeks on *Z. mays* or other readily mycorrhizal plants gives an idea of the activity of the inoculum (Pereira Cavallazzi et al., 2007). Mixing fungal species in an IP might give greater mycorrhizal potential because fungal species have different germination times and thereby the mixing could avoid dormancy or quiescence of specific propagules. In this research 4, 10 and 20 weeks after inoculation were used as mycorrhization assessment times in different experiments. When isolated spores were used, no mycorrhization was observed after 8 weeks; therefore, for subsequent experiments and with different source of inoculum, the time of harvesting was 10 and/or 20 weeks that showed be sufficient to achieve mycorrhization.

4.4.6 Soil as a substrate

In the current research pasteurised sand and perlite (1:1, v/v) was used as a substrate. Several studies have reported using soil as a substrate (Binet et al., 2007; Huat et al., 2002; Janoušková et al., 2009; Jin et al., 2013; Pawlowska et al., 1999; Rajan et al., 2000; Ramanwong & Sangwanit, 1999). The soil was usually sterilised using techniques such as irradiation (Janoušková et al., 2009) or sterilisation was not reported. For example, Huat et al. (2002) used soil as a substrate and claimed that adding inoculum did not enhance plant growth. Nonetheless, it is possible that because the seeds were planted and grown for six months in a forest soil, the number of active propagules around the roots was already high. Both species (*Azadirachta* from Malaysia and teak from India) used in their research were indigenous to where the soil was collected, so there was a chance that there was no conflict between host and fungal partners. Furthermore, Huat et al. did not mention whether roots were assessed for mycorrhization before inoculation or if the soil was pasteurised or sterilised. So it is possible that the “uninoculated” seedlings used to induce mycorrhizas were already mycorrhizal. The authors also reported that the control treatment grew better than the inoculated seedlings; this rather unexpected result might be because the seedlings were already mycorrhizal and possibly the inoculum applied was incompatible with the host seedlings. In addition, the fungal species were introduced together with the commercially available inoculum, but it was not clear whether the fungi had been identified as the correct partners for the tree species. These findings could also be seen as an unintentional validation of the use of native soil as a source of inoculum. Similarly, Rajan et al. (2000) reported mycorrhization in their uninoculated treatment, but they also reported increased growth parameters due to the added inoculum. In contrast, Jin et al. (2013) reported that pea plants grown in soil achieved higher biomass than their counterparts grown in sterilised soil, regardless of the inoculum added. Pawlowska et al. (1999) reported using soil and sand (1:1, v/v), but did not mention whether the soil was treated beforehand. Therefore, findings from Huat et al. (2002), Jin et al. (2013), and Rajan et al. (2000) should be interpreted with caution because the use of soil as a substrate could act as a possible but unintentional early inoculation. Once again the selection of a generic or specific mycorrhizal fungus strain should be considered.

4.4.7 Soil at the acclimatisation stage

Using soil at the acclimatisation stage is common (Shirin et al., 2005; Venkateswarlu & Korwar, 2005). For instance, Gavilertvatana (1998) reported using soil mix combination consisting of compost and top soil. The author reported successful establishment of more than 500,000 teak plants over two or three months. However, once again there was not information about soil origin, treatment prior use, or physical characteristics. Some studies provide information, such as where the soil was collected, its composition or plant species that were growing in that particular soil; other studies do not. Also, it could be that teak found fungal partners in the soil at the potting stage or later in the field, so there may have been unintentional mycorrhization. However, when using soil it is a common practice to dip the plant or cutting in fungicide, such as Bavistin® solution (Shirin et al., 2005) or Chlorothalonil® (World Teak Conference 2013: Excursion II: Teak Improvement in Thailand, n.d) before planting. It has been demonstrated that these products help in controlling pathogens, but they may also prevent mycorrhizal establishment.

4.4.8 Soil as a source of inoculum

The findings indicate that at harvest after 20 weeks of growth there were increases in height and root area on MY4 teak plants grown in VG soil and arbuscules (*Arum-Paris* type: Dickson, 2004) and vesicles were visible within their roots. Mycorrhization was achieved on all plants of this treatment. These findings are consistent with works by Alvarado et al. (2004), Dhar & Mridha (2012), and Jamuluddin et al. (2002) who all reported teak as a mycorrhizal plant. It is understood that land that has been used for agricultural purposes has a reduced number of AMF fungal species in comparison with forest soil (Dhar & Mridha, 2012; Jin et al., 2013; Singh et al., 2003). In the research presented here the soil used as a source of inoculum was an anthroposol (McKenzie, Jacquier, Isbell, & Brown, 2004) from an organic farming vegetable garden in Western Australia and contained a high number of fungal spores per gram of soil. The high number could be due to the particular management practices during its 20 years of use as a vegetable garden in which the soil was not turned often (Oehl et al., 2004). Furthermore, the inoculum potential was high, as demonstrated when after only four weeks *Z. mays* roots were highly colonised by mycorrhizal fungi.

Teak roots from the research presented here showed patched colonisation, which included arbuscules thus indicating functional mycorrhiza, vesicles and spores (C. Walker, personal communication, April 12, 2013). AMF from several collections (Rajan et al., 2000) or soil from teak trees and nursery beds (Verma & Jamaluddin, 1995) has been used as a source of inoculum, but this research presented here is the first attempt to induce mycorrhizal teak in a different geographic zone to where is originated. This research used a source of inoculum that did not originate from teak plantation soil. It is possible that there is a gradient in host-fungal partner specificity. The fact that the same genotype of teak is cultivated widely and naturalised outside its native range (Goh, & Monteuis, 2012) shows that teak might 'find' mycorrhizal partners from different ecosystems. In studies where inoculation has been performed, and rhizosphere surveys have been done, *Glomus* species were the commonest fungal partner reported or used. *Glomus* spp. can be found worldwide (Öpik et al., 2013) or in Australian soils (Brundrett et al., 1995) and this genus has been reported as being generalist (Sykorova et al., 2007).

4.4.9 Teak clones

Significant differences were found in responses between clones MY4 and T201, with MY4 being more receptive to the treatments. Generally, clone T201 was not affected in any of the inoculum treatments. Response differences in survival rate, growth and percentage of colonisation at harvest were recorded by Rapparini et al. (1994) on pear and peach using the same inoculum, even though these two species are closely related. The authors explained that the magnitude of the response differences was dependent upon plants harvesting time. However, with regard to teak, Verma and Jamaluddin (1995) suggested that genetic variability affected the percentage of mycorrhization, with superior trees having greater colonisation. The differences seen here could be explained by the differences in the genetic makeup of the two clones. Therefore, plant genetics is likely to play an important role in responses to mycorrhization treatments.

4.4.10 Trap plants

In this work at harvest after six weeks of growth, *Z. mays* roots grown in IP inoculum did not show any arbuscules or vesicles, but hyphae were visible. In contrast, after four weeks of growth *Z. mays* roots in VG soil showed hyphae, vesicles and spores.

Mycorrhizal roots of *Z. mays* and *Allium* spp. were used as propagules. Pot cultures are widely used as a technique to multiply AM fungal species (Brundrett et al., 1995; Calvente et al., 2004; Duponnois et al., 2007; Jin et al., 2013; Ramanwong & Sangwanit, 1999), and have also been used to identify the correct fungal partners for several woody tree species (Pereira Cavallazzi et al., 2007). However, the pot culture technique has not been seen as reliable by some authors because it may become contaminated (St-Arnaud et al., 1996; Liu & Yang, 2008). *Z. mays* (Tarbell & Koske, 2007) and *Allium* spp. (Bécard & Fortin, 1988; Dupponois et al., 2007) have been reportedly used to establish a pot culture. In contrast in this work pot culture was a valuable technique that allowed assessment of the inoculum potential.

4.4.11 Hyphae as propagules

VG soil and UIP treatment were more effective in initiating the colonisation process. Hyphae are known to be efficient propagules in establishing mycorrhizal colonisation, taking 6 to 15 days for active and growing hyphae to reach young roots (Voets et al., 2009). VG soil provided teak plants with active hyphae growing from propagules, such as mycorrhizal *Allium* spp. and/or *Z. mays* roots, which contained ERM, hyphae in the soil (detached from the roots) and germinated spores. This inoculation technique using VG soil, which contained hyphae as propagules mixed with the substrate, perhaps allowed the micropropagated teak plants achieve mycorrhization establishment in shorter period than when using isolated spores as inoculum.

4.4.12 Teak – mycorrhiza assessment

The findings indicate that mycorrhizal features, such as arbuscules, vesicles, spores and/or intraradical mycelium (IRM) were found mainly within the secondary and tertiary teak roots exposed to VG soil, confirming mycorrhizal establishment. Mycorrhizal assessment on teak roots has been done by several researchers, such as Rajan et al. (2000) and Verma and Jamaluddin (1995), and specifically on micropropagated teak (Ramanwong & Sangwanit, 1999). They identified that mycorrhization occurred within newly-formed roots. In contrast, Sosa-Rodriguez et al. (2013) reported no mycorrhization on roots that were treated with IBA. Even though in this research the adventitious roots were induced with IBA, the IBA inducement did not appear to hamper the mycorrhizal establishment.

The mycorrhizas established on teak roots in this work were most probably colonised by some of the *Glomeraceae* family (C. Walker, personal communication, April 12, 2013) because arbuscules were observed scattered in the cortex: a normal feature of this family (C. Walker, personal communication, April 12, 2013). Colonisation was also found at the root apices, places that have been reported as highly likely to be colonised (Bago & Cano, 2005). In addition, hyphal growth patterns within the root were also used to identify different kinds of colonisation (Abbott & Robson, 1991). Furthermore, it was possible to observe vesicles that were reportedly formed by *Glomus* spp. (Dickson, 2004). Therefore, microscopic mycorrhizal assessment confirmed the establishment of AMF within teak roots of micropropagated teak because it was possible to identify arbuscules, vesicles and/or intraradical mycelium.

4.4.13 Soil phosphorus levels

It has been frequently reported that low levels of soil P promote the establishment of mycorrhiza (Bécard & Fortin, 1988; Ryan & Graham, 2002; Schroeder & Janos, 2005; Smith & Smith, 2011) on plants, including micropropagated plantlets. However, due to the nature of the source of inoculum of the VG soil, it was not possible to control this variable in the current experimental design. Despite this, the highest level of mycorrhization occurred in the soil treatment with a P concentration of 64 times the average soil P used in other studies. It is possible that mycorrhizal development may occur in teak at levels of soil P that were previously thought to be too high. It seems that there are also some other factors that may impact upon P soil levels. For instance, Herrera-Peraza et al. (2011) reported that inoculation responses are based on the soil's chemical and physical characteristics and there was no significant relationship between soils that have been exposed to high fertilizer input and plant responses to inoculation. In addition, although the plant responses seen within the VG soil were similar in some aspects to that of the other inoculated treatments, the growth cannot be solely attributed to mycorrhization and it is likely that some of the differences in growth are due to high nutrient levels of soil. There are two aspects to discuss here: the first refers to the plant stage when the inoculation occurs; the second is the P requirements at that plant stage. It is possible that during the earlier stage of mycorrhization, the level of soil P may not affect the establishment, but at a later stage it may. Therefore, the findings of this

research do not support the previous suggestions that P needs to be low (1.08 ppm to 35.2 ppm: Alvarado et al., 2004; Becard & Fortin, 1988; Jin et al., 2013; McGonigle et al., 1990; Rajan et al., 2000; Schroeder & Janos, 2005) to stimulate mycorrhizal establishment and development, or that there is a need to develop a new kind of crop species (Lambers et al., 2006). In this research P levels at harvest in the soil were still relatively high, yet they did not hamper mycorrhiza formation.

Therefore, there is a need for further knowledge about the minimum P soil concentration that allows mycorrhizal establishment without impacting negatively on plant development. It is necessary to know the physical and chemical properties of the soil because the presence of mycorrhizas *per se* does not guarantee P absorption and other indirect, factors such as physical and chemical characteristics of the roots, may also play a role and need to be considered.

Thus the aim of mycorrhizal research has been met. Establishment of mycorrhiza on micropropagated teak was achieved. Improvement of teak micropropagation provided readily available plantlets to allow mycorrhization. Clonal differences were found because clone MY4 multiplied at a higher rate than clone T201. In addition clone MY4 was more responsive to treatments than clone T201. The ubiquity of the *Glomeraceae* family (Öpik et al., 2013) has been recognised and that species' composition of AM communities can determine the composition of the plant community structure (Van Der Heijden et al., 1998), so micropropagated plantlets of teak adapted to Australian conditions were able to find fungal partners in an organic farming soil. These findings, obtained under experimental conditions, are expected to be replicated when the teak is transplanted to the field environment.

This early mycorrhization might help teak plants manage the stress of the new environments once out of the greenhouse. The extraradical mycelium might provide the host plant with nutrients, but at the same time could populate the soil, producing soil-borne spores. Mycorrhization of micropropagated plants during the acclimatisation stage may also then provide the field soil with mycorrhiza, which in time might reduce the use of inputs, such as inorganic fertilizers, and could improve soil quality due to the interaction within the plant rhizosphere (Barea, Azcón & Azcón-Aguilar, 2002).

Mycorrhization has been considered as an alternative to irreversible changes caused by modern agriculture by establishing mycorrhizas using mixed AMF inocula (Gianinazzi et al. 2010). These rooting and mycorrhization protocols could also be applied to different forest trees.

Chapter 5: General discussion

5.1 Micropropagation and mycorrhization

Mycorrhization and micropropagation are processes that, when combined, could provide quality forest tree material for multiple uses (Azcón-Aguilar et al., 1996; Binnet et al., 2007; Gribaudo et al., 1996; Huat et al., 2002; Kapoor et al., 2008; Rai, 2001; Varma & Schuepp., 1995; Vidal et al., 1992). In addition, it has been suggested that early mycorrhizal development could help teak plants to establish in the field (Rajan et al., 2000). According to Kollert and Cherubini (2012), there is a need to improve the quality of teak planting material in order to achieve good growth rates; however, little attention has been paid to ensure that teak, as a mycorrhizal plant, develops mycorrhiza in the early stages of micropropagation (Elders Forestry Management Limited, 2011; Monteuis & Goh, 2012; Venkateswarlu & Korwar, 2005). Monteuis and Goh (2012) did not mention any kind of mycorrhization treatment in their report, and they did not assess any of the trees for mycorrhizal establishment (O. Monteuis, personal communication, May 06, 2013). Monteuis and Goh reported successful establishment of the same true-to-type genotypes on different continents, which have similar climatic conditions; but they provided incomplete information about survival of the micropropagated teak plants after transferring to the field. Venkateswarlu and Korwar (2005) reported 95% survival at acclimatisation and 100% in the field in India. As yet, however, there is no evidence that micropropagated, mycorrhizal teak plants perform better than the non-mycorrhizal ones (O. Monteuis, personal communication, May 06, 2013). Moreover, Verma & Jamaluddin (1995) reported in a rhizosphere survey that plus trees had a high level of mycorrhization and that trees originating from other Asian countries showed lower root colonisation than teak from India.

In the current research, teak plants became mycorrhizal even though the soil used as a source of inoculum was not specifically designed for teak. There was no need to use any kind of mycorrhizal fungi selected for teak, and this confirms the promiscuous ubiquity of the globally distributed *Glomeromycota* (Brundrett et al., 1995; Brundrett, 2009; Öpik et al., 2013).

5.2 Inoculum product

The difficulties of cultivating AMF (Smith & Read, 2008) and the production of optimum inoculum for mycorrhization *in vitro* have been acknowledged (Ehinger, Croll, Koch, & Sanders, 2012; Rai, 2001). The inoculum can be produced from soil that contains pieces of mycorrhizal roots and/or spores, or from a combination of these and hyphae, or from strains selected and maintained in a pot culture (Janouška et al., 2009). Regarding spore production, Bécard and Fortin (1988) indicated that the period of spore production ranged from one to seven months in ROC settings. ROC also could be appropriate for a large-scale production of inoculum (Chabot et al., 1992), but the new spores formed were smaller and had thicker walls than the ones that were soil-borne, and many of the spores were not viable (Pawlowska et al., 1999). A further step regarding spore production was used by St-Arnaud et al. (1996), who produced 15,000 viable spores from a total of 34,000 in four to six weeks. These findings have important implications for developing an inoculum for teak. Questions that still need to be researched include:

- Should the inoculum be made of spore isolates only, or from a mixture of spores, a carrier and microorganisms to help germination?
- If other species are included in the inoculum, which species and which carriers are most beneficial?
- Should the inoculum be generic for several species or only specific for teak?
- Would the spores produced by ROC be efficient? (Ehinger et al., 2012)
- What particle sizes should be in the inoculum product?
- Would the germinability of the new spores be affected?
- Which would be the optimum storage conditions for IP?
- How long will the spore isolate last?
- How many spores and what amount of inoculum, would be needed to use for each teak plant?
- How much would the spore isolate product cost per plant?

None of these questions have been answered yet.

In addition, when using isolated spores obtained by AM monoxenic cultures, or dual cultures using transformed root organ culture as a source of inoculum, there can be

some difficulties. Firstly, even though this procedure has been reported as being feasible (Sosa-Rodriguez et al., 2013), it has only been tested at an experimental level so it is not known whether it can be applied to large-scale plantation production. Secondly, by definition, this technique requires several reagents, the maintenance of strict aseptic conditions, and wide knowledge and expertise (Bago & Cano, 2005; Ijdo et al., 2011); therefore, so the added production cost could be a disadvantage (George et al., 2008). So if ROC is also used to produce inoculum it will raise the cost even further. Thirdly, in order to have an axenic culture, the spores need to be to surface sterilized (Pawlowska et al., 1999), again adding to the cost. Some research has shown the need for other microorganisms that are associated with spores to allow germination (Bago & Cano, 2005; Giovanetti, 2010), whereas Chabot et al. (1992) suggest that high nutrient concentrations fulfil this need. There is still no precise information about which bacterial species or kinds of microorganism are needed for each AMF species to trigger spore germination. Fourthly, it has been suggested by Giovannetti (2010) that spore germination depends on the regional zone temperature from which the spores were harvested, so the spores may be restricted in effectiveness in different temperatures. Fifthly, there has also been dissension about transporting mycorrhiza from one geographical place to another because irreversible damage in the new ecosystem has been implied (Jin et al., 2013; Schwartz et al., 2006). The sixth drawback is the need to provide the right fungal species to the host plant or to produce a product that could contain ubiquitous fungal species in order to guarantee mycorrhization. Finally, the size of other particles in the inoculum product should be larger than the spores themselves. For instance, *C. etunicatum* spore sizes range from 48 to 132 μm diameter (Pawlowska et al., 1999), and *R. intraradices* spores are usually 85 μm in diameter (Chabot et al., 1992). The size of the spores and the other propagules are important features to consider for large-scale inoculum production.

Results from this research are in accordance with findings of previous work in this field. However, some researchers have questioned whether the inoculum needs to be produced in a “uniform and artificial culture environment”? (Bago & Cano, 2005; Rai, 2001; Smith & Read, 2008). It is widely known that soil pot cultures are used to multiply fungal propagules. In some cases one spore has been used to initiate an isolate (Redecker et al., 2013). Those spores can be used in studies about fungal life cycles but

ROC studies do not necessarily represent the processes in the field or in the wild (Borriello et al., 2012; Smith & Read, 2008). In contrast, it has been suggested that mixed fungal species inocula are more effective (Jin et al., 2013; Verma & Jamaluddin, 1995), and it has been suggested that there are some fungal species that do not produce spores but can still live in the soil (Jin et al., 2013; Öpik et al., 2013; Smith & Read, 2008). An organic farming soil can provide a mixture of fungal species that can be used for a pot culture, and then the substrate can be used to induce mycorrhization, as was achieved in this research.

5.3 Live inoculum

There are many reasons for using actively growing inoculum: first, in as little as a month, roots of a trap plant can be assessed for mycorrhizas; thus the inoculum potential of the soil-based inoculum can be easily determined. This assessment can be done easily with standard equipment like a compound microscope. Second, soil in which mycorrhizal fungi are actively growing (i.e. farming organic soil) in which a trap plant had been growing (in this research work, *Allium* spp.) could provide a high number of live and active propagules (Ryan & Graham, 2002). Early mycorrhization can be achieved because there is minimal disruption of the hyphal networks (Smith & Read, 2008); and since this soil-based inoculum is native or non-introduced, the inocula/fungi/propagules are already adapted to the soil's chemical and physical conditions. Fourth, the exposure of readily growing hyphae and germinating spores to uninoculated root systems is a more natural approach. Finally, the richness of this soil-inoculum base, which may contain more than one fungal species, allows the possibility of one or more fungal species colonising the root system (Janouška et al., 2009). Meanwhile, although techniques such as ROC and monoxenic pot cultures are good enough for understanding basic aspects of AMF biology, there is an urgent need to expand towards inocula that can be accessible, and are easy to obtain by both farmers and nurserymen because it is vital to preserve productive soils (Parke & Kaeppler, 2010).

5.4 Soil as a source of inoculum

There are some requirements for a soil in the field to be considered as a source of inoculum: confinement and isolation from external changes, active propagules, presence of trap plants and incubation. The soil should be isolated and protected from the changing environmental conditions *in situ*, as much as possible. The longer the soil is “incubated” in this way, the better because it will allow development of a variety of mycorrhizal species (Sýkorová et al., 2007). At least a year may be needed for establishment, to allow passage through all the seasons at least once (Abbott & Robson, 1991; Oehl et al., 2004). Ideally, the number of active propagules should be high (Nazeri et al., 2014). High number of active propagules can be obtained by allowing mycorrhizal trap plants, such as *Allium* spp. or *Z. mays* to grow without disturbing the soil, because the main objective is to harvest the soil later. The soil also should be fertilised with compost. Then to assess the soil inoculum potency small soil samples can be taken and use them in a soil trap pot culture. Once the mycorrhizas have been assessed, the trap plants could be harvested and the soil and the mycorrhizal roots mixed with an inert soil then could be used at acclimatisation. This soil could then be used with micropropagated plants. This procedure could be applied both in developed and developing countries, so the countries can be “independent and self-sufficient” (Rai, 2001, p. 164) and the procedure is efficient for a large number of plants.

There is a need to understand that mycorrhizas, as living organisms, have to be treated as such. There must be up-to-date information about the date/age of establishment of the soil, trap plants used and date when the plants were harvested and any special conditions of storage. Information about the environmental conditions where the soil is located (temperature, rainfall and soil pH) is also crucial. Further research using other micropropagated plants and their performance after transfer to the field is needed; in particular the appearance of mycorrhizal roots compared with non-mycorrhizal roots should be monitored (Abbott & Robson, 1991).

It has been stressed that the inoculum should be pure (Chabot et al., 1992). There is often apprehension about using soil due to the contamination risk associated with viruses, bacteria or pests already present in the soil. At the acclimatisation stage, mixes have been used that have not compromised the survival of teak plants; for instance

soil:sand:compost (2:1:0.5, v/v/v) have been used by Rajan et al. (2000). However, there was no effect on survival reported. In addition there are studies that have reported using soil but there was a lack of details about the location, when the soil was collected, and the plant species present in that soil, which may or may not have impacted on the fungal species present (Abbott & Robson, 1991). Therefore, it can be assumed that the species living in that soil were unlikely to affect the roots. In this research presented here, this was confirmed because 100% of the teak plantlets survived. The risk, if any, was minimized by using a pot culture grown for eight weeks under controlled conditions. In addition, the farming organic soil sample contained spores, mycorrhizal roots and extraradical hyphae and was used to establish the pot culture using *Z. mays* seedlings. A sample of root system was assessed for mycorrhizas after four weeks of establishment. The presence of mycorrhiza within the roots at this early stage indicated that the propagules were alive and active. This highlights the importance of keeping the propagules alive until use.

5.5 Survey of mycorrhizal species in the plant rhizosphere

Surveying the plant rhizosphere for mycorrhizal species may or may not give an overview of the possible fungal species that can inhabit the roots of a particular host species (Brundrett et al., 1995; Verma & Jamaluddin, 1995). However, it does not necessarily mean that all the fungi present will develop a mycorrhizal symbiosis. Some of the fungi can be dormant and their presence is due to the previous host plants. Comprehensive procedures for establishing mycorrhization were developed by Calvente et al. (2004) with olive and Verma and Jamaluddin (1995) with teak. Further research should be done to identify AMF species using reliable tools, such as molecular biology (Pitet et al., 2009; Redecker et al., 2013) and to test the effect these AMF have on teak plants, specifically in relation to the geographic origin of the inoculum. This would make fungal isolates known to improve establishment of growth of teak in particular environments. These could be incorporated into a program that looks at developing a mycorrhization process for teak.

5.6 Practical applications and further research

This research has practical applications because the demand for teak wood in the world is increasing. In addition, these rooting and mycorrhization protocols could be applied to other micropropagated forest trees. However, further research should be done on the mycorrhization protocol to be used in nurseries, or during acclimatisation, for tissue-cultured plants (including teak) for plantations, recognising that differences between clones that can be present. One of the most important aspects to be researched further will be establishing a sustainable source of inoculum. Sources of local soil-based inocula should be explored. Within a teak nursery a reliable source of actively growing inoculum could be established. Then, when the level of readily available propagules has been enhanced, mycorrhizal plants could also provide mycorrhiza to impoverished soils (Barea et al., 2002; Sanon et al., 2010). Information of teak in tropical Australia is scarce for commercial reasons, but mycorrhizal micropropagated teak can be a success just as it has been in several countries (Goh & Monteuuis, 2012). Further studies, which take into account variables, such as shelf life, minimum particle size, propagules to be formed, and their life span, native mycorrhizal fungi surveys and IPs storage conditions, need to be undertaken so that incorporation of mycorrhization into teak/forest trees is more acceptable to nursery practices.

5.7 Limitations

In the research presented here there were several limitations:

- The rate of multiplication between clones was different. Clone T201 took longer to obtain the number of plantlets required, so some of the experiments were only performed with clone MY4.
- The number of zeros inflated some of the data sets, which made them difficult to analyse. For instance, 28 days of exposure to IBA treatment produced no roots: therefore, it was decided not to include these zero values in the statistical analysis, although the meaning of those zeros was considered in the analysis of the results.
- Mycorrhizal establishment took longer than expected. It was possible that in some mycorrhization treatments, the plants were harvested too early and therefore it was not possible to detect visible signs of mycorrhizas establishment, such as arbuscules, vesicles, spores and/or hyphae.
- The mycorrhization assessment method, which included staining and microscopic observation, might not have allowed detection of the very early stages of mycorrhizal establishment.
- Another limitation could be the number of soils tested to be used as a substrate. The main idea was to work with an inert material, free of organic matter that could be ideal to promote mycorrhization. In this research sand, peat, and perlite were tested because they were easy to find.
- The number of inocula tested was limited to three sources of mycorrhiza and two genotypes (clones MY4 and T201). This was due to availability and to keep the size of the experiments under control.

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