Chapter 1
The Importance of Ectomycorrhizas for the Growth of Dipterocarps and the Efficacy of Ectomycorrhizal Inoculation Schemes

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1.1 Introduction

The Dipterocarpaceae is the most important tree family in the tropical forests of South-east Asia as they are the ecosystem dominants, especially in lowland forests of this region, where they often contribute up to a quarter of all stems and half of the above-ground biomass (Proctor et al. 1983; Davies et al. 2003; Brearley et al. 2004). They also play major role in timber production from this area as they are favoured for their fast growth rates, tall cylindrical boles and high quality wood. In addition, they are a source of non-timber forest products such as oils and resins (Shiva and Jantan 1998). Given the continued exploitation and degradation of lowland tropical forest habitats worldwide, there is interest in reforestation programmes to maintain forest cover and to provide a sustained supply of wood products. Many of these programmes have focussed on fast-growing exotic tree species but, to provide the highest quality timber, reforestation efforts now need to focus on the Dipterocarpaceae.

The majority of trees in tropical forests form arbuscular mycorrhizas but it was first noted by Singh in 1966 that dipterocarps, like many temperate forest trees, formed ectomycorrhizas (EcMs). Since then, many subsequent studies have examined the roots of numerous dipterocarps and found them to be colonised by EcM fungi (Alexander and Högberg 1986; Chalermpongse 1987; Smits 1992; Lee 1998; Hoang and Tuan 2008). There are also minor reports of arbuscular mycorrhizal (Shamsuddin 1979; Ibrahim et al. 1995; Tawaraya et al. 2003) and ectendomycorrhizal (Tupas and Sajise 1976) fungi in dipterocarp roots, but more details are needed on this potential dual colonisation before anything more concrete can be written.

In this chapter, I have (1) given a brief overview of mycorrhizal symbioses in dipterocarps, I now plan to (2) note which are the important fungal species involved

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in the EcM symbiosis and then (3) focus on the role of this symbiosis in improving plant nutrition, growth and performance, and complete (4) with a special focus on how we might critically use this knowledge to determine if we need to apply EcM inoculation in reforestation programmes.

1.2 Fungal Species Associated with Dipterocarps

We know, from fruit body surveys, that the most speciose groups of fungi found in dipterocarp-dominated forests are the families of Amanitaceae, Boletaceae and Russulaceae (Watling and Lee 1995, 1998, 2007; Watling et al. 1998, 2002; Lee et al. 2002, 2003), and these appear to form a reasonable proportion of the EcM root tips belowground (Lee et al. 1997; Ingleby et al. 1998). However, it is known that above-ground and below-ground views of the EcM community rarely show close concordance (Gardes and Bruns 1996; Yamada and Katsuya 2001), and it has recently been noted that fungi with cryptic fruiting bodies such as members of the Thelephoraceae, which appear to have been overlooked in fruiting body surveys, are also important EcM formers. For example, Ingleby et al. (2000) found a Thelephoraceae species to be common on seedlings planted in soil from Vietnamese forest and I have found two Thelephoraceae species common on nursery-grown seedlings in Malaysia (Brearley et al. 2003, 2007; Brearley 2006). Furthermore, sequences from this group dominated the molecular studies on dipterocarp EcM communities conducted by Sirikantaramas et al. (2003) and Yuwa-Amornpitak et al. (2006). As an example, half of the sequences generated by Sirikantaramas et al. (2003) were from this family. In this regard, the use of molecular identification techniques has shown us that the EcM community in dipterocarp-dominated forests is more similar to many boreo-temperate EcM communities (e.g. Richard et al. 2005; Ishida et al. 2007; Morris et al. 2008) than originally thought. As molecular work continues on the EcMs communities of dipterocarps, it will reveal a much clearer below-ground picture of the dominant species involved in this symbiosis.

1.3 Nutrient Relationships

There are numerous experiments showing that EcMs can improve plant nutrient uptake (Smith and Read 2008) and, in the Dipterocarpaceae, this has also been shown to be the case. For example, Lee and Lim (1989) correlated percentage EcM colonisation (% EcM) with foliar phosphorus (P) in dipterocarp seedlings they studied. Seedlings from a site with lower available soil P showed a positive correlation between foliar P and % EcM suggesting that EcMs enhanced uptake of P at this site. Hadi and Santoso (1988, 1989) presented data suggesting inoculation with EcM fungi increased foliar concentrations of nitrogen (N), P, potassium

(K), calcium (Ca) and magnesium (Mg) of five dipterocarp species (although it is not entirely clear if their data are for nutrient concentrations or total nutrient content in the seedlings). The first experiments to show an unequivocal increase in foliar nutrients in response to EcM colonisation were those of Lee and Alexander (1994) and Yazid et al. (1994) who clearly showed increased P concentrations in response to EcM colonisation in two species of *Hopea* studied. Additionally, in the experiments of Lee and Alexander (1994), whilst there were no suggestions of EcM colonisation increasing shoot N, K or Mg concentrations, there was some indication of improved Ca nutrition. These differential responses may be due to the experimental conditions used but may also represent a certain degree of inter-specific difference in fungal benefits to the host plant or may even be due to an EcM diversity effect on nutrient uptake (Baxter and Dighton 2005).

Improved mineral nutrition has also been shown under field conditions where reduction of EcM colonisation (by Mancozeb fungicide) led to reduced foliar N and P in both *Hopea nervosa* and *Parashorea tomentella* in addition to reduced Ca and Mg in *Hopea nervosa* (Brearley 2003). I have also shown, through stable isotope analysis, that EcM dipterocarps can also obtain more N from organic material (Brearley et al. 2003) with a positive correlation found between % EcM colonisation and uptake of organic N.

An inoculation experiment that purports to show increases in nutrient uptake of *Shorea seminis* when inoculated with two EcM species is that of Turjaman et al. (2006). Total shoot N and P contents were indeed greater in the EcM inoculated seedlings but, surprisingly, on a dry-weight basis, inoculation actually led to a decrease in concentrations of these elements in the shoot (up to 55% in the most extreme case). Smits (1983) also considers that dipterocarp EcMs may be providing thiamin to plants although, in the absence of a strong presentation of data, we should discount this hypothesis for now.

Increased nutrient concentrations within plants are generally likely to lead to improved growth, and the role of foliar nutrients in determining seedling performance is also important during out-planting as it has been shown that higher levels of foliar N allow dipterocarp seedlings to better avoid photodamage when transferred to high irradiance conditions and to allow more rapid acclimation to these conditions (Bungard et al. 2000).

1.4 Inoculation Experiments

Many of the inoculation experiments reported are in the grey literature and have a number of shortcomings. The most serious of these is that they are poorly reported and do not provide sufficient detail for the experiments to be evaluated fully nor repeated by other researchers. Pseudoreplication or the lack of statistical analyses in many cases also makes evaluation of the results problematic. In this chapter, I will focus on papers which, I feel, have mostly overcome these shortcomings or are otherwise noteworthy.

The first reported experiment attempting to inoculate dipterocarp seedlings with EcM fungi and determine seedling responses was that of Hadi and Santoso (1988). They inoculated species of *Boletus*, *Russula* and *Scleroderma* using pieces of chopped fruiting body on the roots of five dipterocarp seedling species. A shortcoming of this experiment was that the roots were not examined to determine the extent of EcM formation by any contaminant fungi. Nevertheless, after 6 months growth, inoculation appeared to at least double seedling height in all fungus/seedling combinations. Furthermore, their approach to inoculation is rarely used as it is difficult to control the inoculum viability and potential supplied to the roots with this method.

Initial experiments, using chopped root inoculum (Lee and Alexander 1994), found that EcM colonisation increased plant dry weights in *Hopea odorata* and *Hopea helferi*. This increase was generally greatest in the absence of additional nutrients supplied to the soil. However, the problem with experiments using root inoculum is that the species of fungi on the inoculant roots cannot be determined and therefore controlled experiments using defined EcM species are needed. In Malaysia, inoculation experiments have focussed on strains of *Pisolithus* species and a member of the Thelephorales (FP160: Lee et al. 2008). In Indonesia, the use of *Scleroderma* for inoculation appears more popular although the range of species being used has increased recently (Turjaman et al. 2007).

1.4.1 Inoculation with Single Species

1.4.1.1 Malaysian Inoculation Experiments

In Malaysia, exotic *Pisolithus* isolates from Brazil (Pt 441 originally from under *Eucalyptus citriodora*) and Thailand (Pt msn) were effective at forming EcMs on four dipterocarp seedling species (although there was a certain degree of host-fungal compatibility with one or other *Pisolithus* isolate having a greater % EcM on each of the seedling species; Lee et al. 1995). Using Chilvers et al.'s (1986) cardboard inoculum technique, Yazid et al. (1994) showed that Pt 441 formed a high percentage of functional EcM colonisation (c. 80%) on *Hopea odorata* and *Hopea helferi* and that this increased the growth (dry weights increased by 7.3 and 3.6 times, respectively) and foliar P concentrations (by at least 40%) after 9 months growth. Similar results in terms of the growth of *Hopea odorata* were seen (although with a lesser growth response) when coconut husk:vermiculite inoculum was added in a ratio of 1:4 to a sterilised soil:sand mix (Yazid et al. 1996). In contrast, problems were found when trying to inoculate a local species: *Pisolithus aurantioscabrosus* (Lee et al. 1995). Why this is, is not clear but may simply reflect the ability of *Pisolithus tinctorius* to form EcMs with a wide host range (Martin et al. 2002), whereas *Pisolithus aurantioscabrosus* has only been reported to be associated with *Shorea parvifolia* and *Shorea macroptera* to date (Watling et al. 1995a, b; Martin et al. 2002).

Initial tests of successful inoculation between *Hopea odorata* and *Hebeloma crustulinifome* (Lapeyrie et al. 1993) were reported, but growth responses were not shown and this exotic European strain does not appear to be used any more. Recent work has focussed on Thelephoraceae FP160 (Lee et al. 2008), which significantly increased stem height, root length and biomass of *Hopea odorata* after 6 months growth in the nursery by 30%, 62% and 40%, respectively (Lee et al. 2008). It currently appears very difficult to bring further tropical dipterocarp-associated EcM species into culture (S.S. Lee, pers. comm.), and the species that are being used form a very limited subset of those available. The best approach here would be a wide-ranging screening using a variety of fungal structures, media and growth conditions although, of course, this will be very labour intensive with potentially little reward. Perhaps, the floating culture technique of Sangtiean and Schmidt (2002) may help South-east Asian researchers to culture some of the later stage EcM species found in these forests. This technique allowed Sangtiean and Schmidt (2002) to carry out culture experiments on *Amanita*, *Lactarius* and *Russula* species, which are common in South-east Asian forests (see above).

1.4.1.2 Indonesian Inoculation Experiments

In Indonesia, the use of *Scleroderma* species (and especially *Scleroderma columnare*) appears to be favoured, probably from the initial work of Ogawa (1993, 2006) in the early 1990s. Sadly, much of this early work is difficult to evaluate as it is not clearly reported (e.g. Supriyanto et al. 1993; Kikuchi 1997) but, more recently, a number of much better controlled inoculation experiments have been conducted by Turjaman et al. (2005, 2006, 2007). They showed that the growth of *Shorea pinanga* was improved by the addition of spore tablets of *Pisolithus tinctorius* (aka *Pisolithus arhizus*) and *Scleroderma columnare* species. Both fungal species improved the growth of *Shorea pinanga* (150% increase in dry weight after 7 months) although there was some EcM colonisation of the controls. Survival rates (86–87%) were also much higher than the control (16%), which is an equally important factor to take into consideration when planning reforestation schemes. In a follow-up experiment (Turjaman et al. 2006), tabletted spore inoculum was compared with alginated bead mycelial inoculum of *Pisolithus tinctorius* and *Scleroderma columnare*. Percentage EcM colonisation was higher (61–65%) when seedlings were inoculated with spores than with mycelium (35–37%), and there was, again, at least a doubling of dry weight after 7 months growth. In the most recently reported experiment (Turjaman et al. 2007), inoculation of four fungal species on the roots of *Shorea balangeran* increased seedling growth. Whilst we might expect the use of *Boletus* sp., *Scleroderma* sp. and *Strobilomyes* sp. to increase seedling growth as these are known to be EcM forming fungi, the use of *Calvatia* sp. also increased seedling growth, which was unexpected as this is not thought to be an EcM forming genus (Rinaldi et al. 2008).

1.4.2 Other Inoculation Methods

1.4.2.1 Mycorrhizal Tablets

Where sterile facilities are not available to cultivate species aseptically, researchers have used "mycorrhizal tablets". In this case, spores or mycelium are mixed with a carrier (clay or alginate beads) and applied to seedlings' rooting zones to allow hyphal contact and subsequent EcM formation. The first record of this in the South-east Asia region appears to be that of Ogawa (1993). Species used for this method are often those such as *Scleroderma* species, which have the advantage that their spores can be collected much more easily from their enclosed fruit bodies than many other gilled or pored fruit bodied species. Clay tablets at 1:100 (crushed fruit bodies:clay) were used in the experiments by Turjaman et al. (2005, 2006), and these showed increased growth of *Shorea pinanga* and *Shorea selanica* when inoculated as compared with uninoculated controls. However, in such experiments, there is a need to confirm that the tablets do not contain additional nutrients that might improve seedling growth in the absence of a mycorrhizal effect.

1.4.2.2 Mother Tree Inoculation

Other inoculation methods include inoculation from a colonised mother tree in the nursery – in other words, simply letting newly germinated seedlings' roots contact hyphae already radiating out in the soil around established, colonised trees. This method was first used by Roeleffs (1930, in Nara et al. 1999) to inoculate seedlings of *Pinus* species. The technique, also known as inoculation beds, is a low-tech method that allows EcM inoculation before planting-out but it can be rather haphazard in terms of the speed and reliability of inoculation (see Kikuchi 1997). For example, Ogawa (2006) shows a diagram of the spread of *Scleroderma columnare* fruiting bodies through a nursery containing seedlings of *Shorea leprosula* and *Shorea academica* to be between 1 and 2 m per year.

1.4.3 Production of Inoculum

In order to produce inoculum rapidly, conditions for the optimum growth of fungi in culture should be evaluated. For example, Patahayah et al. (2003a) showed that the most rapid growth of *Pisolithus albus* (aka Pt Gemas) was obtained at 25°C when grown on Oddoux medium but at 30°C when grown on MMN or Pachlewski's medium (Patahayah et al. 2003b). We have also shown that this species grows best when N is supplied in an organic form (BSA in the experiments conducted; Brearley et al. 2005). Thelephoreaceae FP160 shows best growth at 25°C (Patahayah et al. 2003b) but has minimal preferences for N source (Brearley

et al. 2005). In terms of efficient spread of EcM inoculum in the nursery, Nara et al. (1999) considered that seedlings are often maintained under sub-optimal conditions in potted soil with a high clay content and hence poor aeration, thereby slowing growth of fungal hyphae. They found that, by using a growth medium with particles of 2–4 mm diameter, the optimum growth of EcM mycelium (Th1 on *Shorea roxburghii*) was obtained. It is also important to consider the longevity of the different forms of inoculum. For example, Fakuara and Wilarso (1993) showed that mycorrhizal tablets remained effective up to 4 months in storage (this was the longest period tested). More experiments are clearly needed in this area, with longer test periods, to gain a better idea of spore longevity.

1.4.4 Field Experiments

There is now a need to determine how well inoculated seedlings and their symbiotic EcM species survive in the wild when seedlings are out-planted. This is important as, if considerable effort is being put into inoculation programmes, this is simply being wasted if seedlings or their inoculant fungal species are dying unnecessarily. Furthermore, in terms of reforestation schemes, growth is not necessarily the most important parameter, seedling survival is arguably equally as important.

Chang et al. (1994, 1995) showed that the species of *Pisolithus* in the Malaysian inoculation experiments noted above did not remain competitive when colonised seedlings of *Shorea glauca* were planted into the field; indeed *Pisolithus* had mostly disappeared from the roots after 6 months suggesting that they are either early stage fungi, or are poorly adapted to the biotic or abiotic environments of the Malaysian forest soils. Using Thelephoraceae FP160, Lee et al. (2008) found it to remain competitive on the roots of seedlings (*Hopea odorata* and *Shorea leprosula*) for up to 23 months after out-planting in a sandy tin mine tailings site (after this time contaminant EcM fungi had only colonised up to 15% of the root tips of less than half the seedlings). However, the improved growth of *Hopea odorata* seen in the nursery due to inoculation with this fungus (see above) was not seen in the field (by measurement of root collar diameter) and improved growth of *Shorea leprosula* was only seen for up to 3 months following out-planting. Under field conditions, I found that the reduction in EcM colonisation by fungicide addition to the roots of two species (*Hopea nervosa* and *Parashorea tomentella*) did not lead to changes in seedling growth but that foliar nutrient concentrations were reduced (Brearley 2003). In field experiments in a degraded peat swamp forest in Kalimantan, Turjaman et al. (2007) showed that a spore suspension of *Boletus* sp. and *Scleroderma* sp. applied to the seedling rooting zone led to increased growth of *Shorea balangeran* but application of *Calvatia* sp. and *Strobilomyces* sp. did not. For the two fungal species that were beneficial, it took around 8 months for growth improvements to be seen, perhaps due to the very wet conditions at the start of the experiment (Turjaman et al. 2007). However, it is difficult to determine if the species applied were those that maintained improved seedling growth as the roots

at the end of this 40-month experiment were not examined to determine which EcM fungi were present – it would have been a notable improvement to the study design to do this. The study of Tata et al. (2009) did report this examination at the end of their experiments with *Shorea selanica* and *Shorea lamellata*, which were inoculated with spore tablets of *Scleroderma columnare* and planted in natural forests or rubber agroforests in Sumatra. Their results were complex but did not show consistent increases in growth, performance or survival of the two dipterocarp species over a 2-year period. It is notable that, among the 19 EcM types they identified using PCR-RFLP at the end of the experiment, none of them were *Scleroderma* species indicating that the inoculated fungus did not remain competitive on the roots for more than this length of time.

There is clearly a need to further evaluate the growth and survival of inoculated seedlings in the field as positive responses to EcM inoculation in the ecologically simplified, and somewhat benign, nursery environment are unlikely to be representative of that found at out-planting sites. There is an argument to be made to use indigenous species for inoculation schemes as they are anticipated to be the most effective, but we may also need to consider the potential impact of biological invasions if using exotic fungal species (Vellinga et al., 2009).

1.5 Under What Conditions Will EcM Inoculation Be Beneficial?

Whilst the body of this chapter thus far has outlined how inoculation with EcM fungi may improve dipterocarp seedling growth, and the various methods to do so, it is certainly worth considering whether inoculation should indeed be conducted at all. In the first paper on dipterocarp EcMs, Singh (1966) noted that "mycorrhizal infection should not be taken as the 'cure of all ills' in the establishment of trees in all sorts of habitats", and this warning still stands, more than 40 years later. I now pose three key questions for consideration before starting to plan inoculation schemes.

It is often considered that there is a need to inoculate seedlings prior to out-planting but, in fact, in most cases inoculation will occur naturally, and inoculation schemes may not yield any major benefits for seedling growth or survival (although we cannot be confident that the same species, or most beneficial species, of EcM fungi will be formed on each seedlings' roots every time). The first key question is, therefore, will inoculation be of benefit to the seedlings? The major benefits of inoculation are knowing that a seedling, at out-planting, is mycorrhizal with a known species of fungus which is functionally beneficial, and thus it will not need to wait to form EcMs with an unknown group of fungi present in the soil which may or may not promote seedling growth; this gives it something of an initial advantage over any out-planted non-mycorrhizal seedlings. However, the main benefits of inoculation are more likely to be shown under poor soil conditions, as I outline below.

1.5.1 Successful Inoculation Schemes

For inoculation schemes to be successful, a series of well-defined and consistently repeatable techniques is needed. In other words, a pure culture of inoculum is needed to allow a regular supply, and currently there are very few fungal species being maintained in pure culture in the South-east Asian region. Access to a laboratory with sterile facilities is needed which may be problematic for a number of sites. In the absence of this, the production of mycorrhizal tablets may be useful although vagaries of fungal fruit body production and genetic variation between individual genets will remain unaccounted for. Infrequent production of dipterocarp seeds can make regular production of planting stock difficult although production of cuttings from a selected number of dipterocarp species now appears fairly routine (Moura-Costa and Lundoh 1994; Itoh et al. 2003; Haji Ahmad 2006). It must also be shown that the inoculant fungus has the ability to improve seedling growth or survival over that of non-inoculated seedlings under field conditions. It appears much easier to culture species such as *Pisolithus* or *Scleroderma* but it must be remembered that these species are not necessarily those which are most beneficial to seedling growth or, indeed, are found commonly on dipterocarp seedling roots.

1.5.2 When and Where to Inoculate?

It is often suggested that inoculation may be beneficial for seedlings planted following logging operations. However, in most cases after logging, there are still a number of smaller dipterocarp trees which will have EcM fungi associated with them and, as long as the light conditions are not detrimental to seedling growth, this should allow the rapid formation of EcMs on seedlings by hyphae, sclerotia and spores already present in the soil (Lee et al. 1996; Ingleby et al. 1998). There is little evidence so far that selective logging seriously impoverishes the fungal flora (Watling et al. 1998) although there is an indication of a loss of the some of the rarer EcM species in logged forest (Lee et al. 1996). Out-planted dipterocarp seedlings are almost certain to become colonised within a short period of time as long as they have below-ground access to roots and mycelium radiating from adult trees (Lee 1991; Alexander et al. 1992; Lee and Alexander 1996; Lee et al. 1996). The second key question is, therefore, is inoculation beneficial under all situations? If not, which situations or conditions are most likely to be improved by inoculating seedlings prior to out-planting?

Inoculation is considered more likely to be of benefit when seedlings are planted in areas where suitable EcM inoculum is not available. This may include severely degraded areas such as mine tailings (Lee et al. 2008), burnt areas (Akema et al. 2009), degraded peatlands (Turjaman et al. 2007) and areas previously used for agriculture (Ingleby et al. 2000). For example, Ingleby et al. (2000) found that the inoculation potential of soils which had been under agriculture for over 20 years

was essentially absent when compared with an undisturbed forest or plantation in Vietnam. The work of Turjaman et al. (2007) in degraded peat swamp forest is also of relevance here as they showed improved growth of inoculated dipterocarp seedlings when out-planted in a degraded area.

The final key question is, is simply adding colonised soil appropriate as inoculum? In many cases, local soil from the vicinity of dipterocarp trees may be equally as effective as any inoculation schemes although these EcMs are not necessarily the best species to promote seedling growth and there is no way to control which fungal species successfully colonise the seedlings' roots. Smits (1992) outlines a simple method by which large numbers of seedlings can be inoculated by soil colonised by EcM hyphae and spores. He advocates the use of soil collected from beneath an adult tree of the same species, but this is based on his weak assertions (Smits 1983, 1985) of a high degree of host specificity. I suggest it is equally likely that host-specific pathogens will be present (Packer and Clay 2000) and therefore suggest a general soil inoculum but ensuring that it is collected in the vicinity of dipterocarps. In the absence of any other schemes, the inclusion of forest soil should be seen as the minimum to ensure early EcM colonisation of dipterocarp seedlings.

1.6 Conclusions

Whilst EcMs are often thought to be essential for the successful growth of dipterocarp seedlings, there is surprisingly little evidence confirming this assertion under natural conditions. In nursery experiments, mycorrhizal inoculation has regularly been shown to increase seedling growth and nutrient concentrations, but when similar experiments have been conducted in the field, the results are much more equivocal with inoculation often showing minimal improvements in growth if seedlings are planted in natural forests (e.g. Tata et al. 2009). If inoculated seedlings are planted in degraded soils, the improvement in growth is often more marked although these improvements may not be maintained if the inoculated fungus does not remain competively dominant on the seedlings' roots. I therefore suggest that researchers and forest restorationists carefully consider whether EcM inoculation is of benefit in the areas they plan to re-plant.

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