Testing the importance of a common ectomycorrhizal network for dipterocarp seedling growth and survival in tropical forests of Borneo

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Abstract

Background: Connections between mature trees and seedlings *via* ectomycorrhizal (EcM) hyphal networks existing in dipterocarp-dominated tropical rain forests of South-east Asia could have strong implications for seedling growth and survival and the maintenance of high diversity in such forests.

Aim: To test whether EcM hyphal network connections are important for the growth and survival of dipterocarp seedlings.

Methods: We conducted four independent experiments that prevented contact of experimental seedlings with an EcM network by using a series of fine meshes and/or plastic barriers. We measured the growth and survival (and foliar $\delta^{13}C$ in one experiment) of seedlings of six dipterocarp species over intervals ranging from 11 to 29 months.

Results: Seedling growth (diameter, height or leaf number) was unaffected by exclusion from the EcM network in three experiments and there were no differences in foliar $\delta^{13}C$ values in the fourth. Seedling survival was reduced following exclusion from the EcM network in one experiment. Our results give little support to the hypothesis that dipterocarp seedlings growing in the shaded forest understorey benefit from being connected, through a common EcM network, to surrounding trees.

Conclusions: We suggest that our negative results, in contrast to studies conducted in low diversity boreo-temperate or tropical forests, are due to these high diversity forests lacking host species-specific EcM fungi, and therefore providing little opportunity for adaptive support of seedlings *via* hyphal networks.

Keywords: Borneo, dipterocarps, ectomycorrhizas, mycorrhizal networks, source-sink relationships

43 **Introduction**

44 Mycorrhizas are a symbiotic association between specialised root-inhabiting fungi and
45 the roots of living plants. The plant provides the fungus with carbon derived from
46 photosynthesis, and, in return, the fungus may improve the nutrient uptake, growth,
47 water relations, pathogen and heavy metal resistance of the plant (van der Heijden and
48 Sanders 2002; Smith and Read 2008 and references therein). Although the majority of
49 tropical trees form arbuscular mycorrhizal (AM) associations, an important minority
50 form ectomycorrhizal (EcM) associations including members of the Dipterocarpaceae
51 (Brearley 2012). Dipterocarp trees dominate the forests of South-east Asia (Slik et al.
52 2003, 2009), and there are more than 250 species on the island of Borneo alone (Ashton
53 2004). Their seeds are produced every 3-8 years in mast-fruiting events (Curran et al.
54 1999; Sakai et al. 2006; Brearley et al. 2007a) after which they germinate and become
55 colonised rapidly by EcM fungi (Lee and Alexander 1996). The main method of
56 colonisation is from the hyphae of fungi already present and forming network in the soil
57 radiating out from roots of adjacent adult trees (Alexander et al. 1992) – during the
58 process of EcM colonisation seedlings become 'connected' to this network. After a
59 mast-fruiting event, dipterocarp seedlings are found at high densities close to parent
60 trees forming seedling banks where they are limited in their growth and survival in the
61 shaded forest understorey.

62 Numerous studies have shown the existence of EcM networks in various forest
63 ecosystems with shared fungal species linkages between adults and seedlings (Beiler et
64 al. 2010; Diédhiou et al. 2011; Michaëlla Ebenye et al. in press) and Connell and
65 Lowman (1989) hypothesised that the dominance of dipterocarps in South-east Asian
66 lowland evergreen rain forests was linked to the ability of newly germinated seedlings
67 to link into this EcM-mediated resource acquisition network. Studies conducted in

68 lowland tropical forests of Cameroon found that isolation of seedlings of *Paraberlinia*
69 *bifoliolata* (Leguminosae) from roots and EcM fungi reduced seedling biomass and
70 survival (Onguene and Kuyper 2002), and a similar study in Guyana showed that
71 *Dicymbe corymbosa* (Leguminosae) had reduced growth and survival when isolated
72 from an EcM hyphal network using fine meshes (McGuire 2007). Contrasting with
73 these findings, seedlings of only one of three Caesalpinioideae legume species in
74 Cameroon had a higher growth rate in the presence of adult trees and their associated
75 roots and EcM fungi (Newbery et al. 2000). The cause of this difference in outcome
76 between studies in different locations is unknown, and further research is required to
77 extend the range of environments where this is examined including both high and low
78 diversity sites. Whether the connection into an EcM hyphal network has implications
79 for the high species richness observed in dipterocarp-dominated tropical rain forests
80 remains unsolved, and clearly, then, it is important to improve our knowledge of the
81 role of EcM networks in facilitating the regeneration of tropical forest trees.

82 The benefits of being connected into this hyphal 'wood-wide web' have been
83 reported from boreo-temperate forests (Simard et al. 2012). For example, carbon has
84 been shown to move between plants or seedlings that form a hyphal network in a
85 'source-sink' fashion whereby plants that are photosynthesising at a rapid rate, such as
86 those under higher irradiance, pass carbon to those that have lower rates of
87 photosynthesis, such as those which are strongly shaded (Francis and Read 1984;
88 Simard et al. 1997; Klein et al. 2016). Support *via* an EcM hyphal network may
89 therefore be beneficial for the survival of seedlings that are growing below the light
90 compensation point in shaded understorey environments. Francis and Read (1984) were
91 the first to show that carbon could move between plants via an AM hyphal network, but
92 not until the milestone study of Simard et al. (1997) was net movement of carbon in

93 EcM systems shown: they found that 6.6% of carbon fixed in *Betula papyrifera*
94 (Betulaceae) was transferred to *Pseudotsuga menziesii* (Pinaceae) and that 45% of this
95 transferred carbon was found in the plant shoots (*i.e.* not fungal structures). Most
96 recently, Klein et al. (2016) showed transfer of carbon from *Picea abies* (Pinaceae)
97 adult trees to roots of adjacent EcM species. However, the ecological importance of
98 this network has been under considerable debate as inter-plant carbon transfer is a
99 complex and variable process. From a phytocentric view, there is a challenge in
100 explaining how this process could be adaptive as it is only likely to be selected for if
101 adults are transferring beneficial compounds, such as carbon, to kin. If considered
102 mycocentrically, however, then the fungus will simply be moving compounds to where
103 they are most required at a given point in time.
104 EcM colonisation in shade tolerant dipterocarps has been shown to improve the
105 growth of seedlings under nursery conditions although far fewer studies have shown a
106 similar benefit under natural field conditions (Brearley 2011, 2012). We report four
107 independent studies on the island of Borneo, using seedlings of six dipterocarp species
108 with contrasting ecological characteristics. We hypothesised that seedlings that were
109 experimentally excluded from an EcM network would display slower growth rates and
110 reduced survival than seedlings that were connected to the network.
111
112 **Materials and methods**
113 *Rationale*
114 In the first three experiments reported, we planted seedlings surrounded by meshes of
115 various pore size with the intention of creating a series of barriers to in-growth by plant
116 roots and fungal hyphae. Therefore, the control treatments allowed free access to fine
117 roots and fungal hyphae, a large mesh treatment had a fine pore-size mesh (35-50 μm)

118 to prevent the access of fine roots but allow access by fungal hyphae and a small mesh
119 treatment had a very fine pore-size mesh (0.5-1.0 μm) and/or a severing treatment to
120 prevent access to both roots and fungal hyphae. It was assumed that seedlings in which
121 fungal hyphae were allowed access through the meshes had the potential to become
122 colonised by hyphae present in the soil outside the meshes, and therefore connect into
123 the EcM hyphal network, whereas those seedlings in the treatments where hyphal access
124 was restricted would only be able to form EcMs *via* spores or hyphal fragments present
125 within their enclosed rooting volume, and would therefore not connect into the EcM
126 network outwith the meshes. This approach has been used successfully to control
127 mycorrhizal colonisation and partition of soil respiration fluxes in previous experiments
128 (Johnson et al. 2001; Heinemeyer et al. 2007; Vallack et al. 2012). A number of the
129 seedlings were raised in a nursery before being transplanted into the forest and, based
130 on prior observations (Brearley 2003), we are confident they were all colonised by EcM
131 fungi, albeit those more common of nursery conditions (*e.g.* Brearley 2006; Brearley et
132 al. 2003, 2007b; Saner et al. 2011). Whilst 'priority effects' of EcM colonisation have
133 often been found to affect subsequent competitive replacement by other EcM species
134 (Kennedy et al. 2009), replacement of nursery EcMs with those present in forest soil has
135 been seen within six months for studies in Peninsular Malaysia (Chang et al. 1994,
136 1995) and, given that the length of all our studies was over at least 11 months, we do
137 not consider this to have affected our results.

138 In one experiment we tested whether carbon was measurably transferred from
139 adult trees to seedlings through an EcM hyphal network by trenching the seedlings in
140 order to isolate them from the EcM network and then determining the $\delta^{13}C$ values of
141 newly produced leaves. This approach is based on the fact that canopy leaves have a
142 less negative $\delta^{13}C$ signature than seedlings due to differences in the atmospheric-to-

intercellular carbon dioxide ratio (O'Leary 1988; Farquhar et al. 1989) and the isotopic signature of the source carbon dioxide in the ambient air taken up for photosynthesis (Medina and Minchin 1980; Medina et al. 1986, 1991; Buchman et al. 1997). For example, if the isotopic difference between adult trees and seedlings were 5‰, using a two-source mixing model, receipt of 10% of carbon by seedlings from adult trees would result in those connected to the EcM network having a foliar $\delta^{13}C$ value 0.5 ‰ closer to adults than trenched seedlings.

Study species

Six dipterocarp species (Table 1) were selected, based on their differences in shade tolerance and maximum growth rates (Experiments 1-3), edaphic preferences (Experiment 3), and on their availability at the start of the experiments (Experiments (1-4).

Experiment 1. EcM-network exclusion and fungicide addition effects on two dipterocarp species

This experiment was carried out in the northern part of the Kabili-Sepilok Forest Reserve, on alluvial soils (5° 52' N, 117° 56' E; Fox 1973; Nilus 2004). Four plots of ca. 7 m x 7 m were cleared of the understorey vegetation and some smaller trees to reduce heterogeneity in the light environment within and between plots. Six-month-old seedlings of *Hopea nervosa* and *Parashorea tomentella* obtained from the INFAPRO nursery, Danum Valley, Sabah that had been potted in forest-derived soil (see Saner et al. 2011 and Paine et al. 2012a for nursery conditions), were planted into the four plots in March 2000. In each plot, 30 seedlings of each of the two species were randomly allocated to planting locations ca. 50 cm apart. Three treatments and two controls were

168 applied to the seedlings: (1) Control: no meshes were used, fungal hyphae and other
169 roots could fully interact with the planted seedling; (2) Sub-Control: a 1 mm pore-size
170 polyester mesh cylinder was installed around the seedling; the aim of this mesh was to
171 attempt to provide some rigidity and to protect the smaller pore-sized meshes in the
172 other treatments from larger soil invertebrates; (3) Root exclusion (-R): one layer of 35
173 µm pore-size nylon mesh (within the 1 mm pore-size polyester mesh cylinder) was
174 installed around the seedlings to allow connection to a mycorrhizal hyphal network; (4)
175 Root and mycorrhizal exclusion (-RM): two layers of 0.5 µm pore-size nylon mesh
176 (within the 1 mm pore-size polyester mesh cylinder) were installed around the
177 seedlings; the cylinders were twisted slightly every four weeks to break any hyphal
178 connections that might have occurred through the meshes; (5) Fungicide (-RM+F): as
179 the -RM treatment but with the addition of Mancozeb fungicide (Bio-Dithane 945, PBI
180 Home & Garden Ltd., Enfield, Middlesex, UK) bi-weekly at a rate of 0.08 g per
181 seedling in 50 ml of water to control the growth of EcMs on the seedling roots (Brearley
182 2003). All the mesh barriers were sewn into cylinders of 7 cm diameter with a lip of 2
183 cm above ground to prevent hyphal entry and dug into the soil to a depth of 25 cm using
184 an auger to create a hole; they remained open at the bottom. All meshes were obtained
185 from, and sewn by, Plastok Associates Ltd. (Birkenhead, Wirral, UK). Apart from the -
186 RM+F treatment all other treatments were given 50 ml of water bi-weekly to control for
187 the addition of water with the fungicide. Other than this bi-weekly fungicide solution or
188 water addition, the seedlings were given supplemental water twice weekly for the first
189 month following planting. Leaf litter and twigs lying across the meshes were removed
190 at monthly intervals to prevent fungal hyphae entering the cylinders *via* this potential
191 pathway. Other vegetation was hand-weeded from the plots throughout the experimental
192 period.

193

194 *Experiment 2. EcM-network exclusion and distance to adult tree effects on two*
195 *dipterocarp species*

196 This experiment was conducted in the Malua Forest Reserve (5º 05' N, 117º 38' E) that
197 was selectively logged for timber in the 1980s (Marsh and Greer 1992). Twenty large
198 trees (mean dbh = 69.7 ± SD 15.1 cm) of either *Dryobalanops lanceolata* or *Shorea*
199 *parvifolia* were chosen within the Sabah Biodiversity Experiment (Hector et al. 2011;
200 Saner et al. 2012). Trees were only selected if they were among the largest trees and no
201 other large dipterocarp or Fagaceae trees were within 15 m of the plots to ensure that
202 the EcM network of the focal tree was closest to the planted seedlings. At every focal
203 tree, one plot (ca. 1.5 m x 2 m) was cleared of understorey vegetation to reduce within
204 and between plot heterogeneity in the light environment under the tree canopy (2-4 m
205 away from the trunk) and one plot was established and cleared of understorey
206 vegetation outside the tree canopy (15-17 m away from the trunk), based on the
207 assumption that the tree canopy approximately reflected the extension of the rooting
208 system (Baillie and Mamit 1983; Katayama et al. 2009). One control and two treatments
209 were applied to the seedlings: (1) Control: no mesh or tube was used, fungal hyphae and
210 other roots could fully interact with the planted seedling; (2) Root exclusion (-R):
211 seedlings were planted into a PVC tube (15 cm diameter x 70 cm depth) covered at the
212 bottom with 50-µm pore-size mesh allowing fungal hyphae to grow into the tube; (3)
213 Root and mycorrhiza exclusion (-RM): seedlings were planted into a PVC tube as above
214 but with a 1-µm pore-size mesh to prevent the entry of fungal hyphae. The meshes were
215 made of monofilament PET (Sefar PETEX, Heiden, Switzerland) and were glued
216 between the bottom of the PVC tube and an additional PVC ring (15 cm diameter x 5
217 cm depth) with silica and aluminium tape. In every plot, 12 seedlings were planted at a

218 spacing of ca. 50 cm and dug into the soil to a depth of 70 cm. Six seedlings were the
219 same species as the focal tree and six seedlings were of the other tree species. All
220 seedlings where raised in a local nursery at the Malua Field Station, Malua Forest
221 Reserve, Sabah, with conditions similar to those at the INFAPRO nursery noted earlier,
222 and ca. 6 months old and 0.5 m tall when planted into the field. Seedlings were
223 randomly allocated and planted in September 2006. Seedlings were watered once at the
224 beginning of the experiment. Leaf litter and twigs lying across the meshes were
225 removed at monthly intervals to prevent fungal hyphae entering the cylinders. Other
226 vegetation was hand-weeded from the plots throughout the experimental period. An
227 index of light interception (% of canopy openness at the plot level) was measured at the
228 beginning, middle (6 months) and end (11 months) of the experiment, using a Spherical
229 Densiometer Model A.
230
231 *Experiment 3. EcM-network exclusion and soil type effects on four dipterocarp species*
232 This experiment was carried out in the northern and central parts of Kabili-Sepilok
233 Forest Reserve on two contrasting soil types (Nilus 2004; Dent et al. 2006). Ten
234 understorey plots of ca. 5 m x 5 m were chosen within both the sandstone and the
235 alluvial soil types respectively, and understorey vegetation cleared to reduce
236 heterogeneity in the light environment within and between plots. Within each plot,
237 seedlings of *Shorea beccariana*, *S. multiflora* (both sandstone soil specialists),
238 *Dryobalanops lanceolata* and *Parashorea tomentella* (both alluvial soil specialists)
239 were planted in April 2003 at an equal spacing of ca. 1 m (seedlings were grown from
240 seeds collected within the Kabili-Sepilok Forest Reserve during the 2002 mast-fruiting
241 event and were ca. 6 months old when transplanted). They were subjected to three
242 treatments and one control: (1) Control: no tube or mesh was used, fungal hyphae and

other roots could fully interact with the planted seedling; (2) Sub-Control: seedlings were planted in PVC tubes of 15 cm in diameter and 35 cm in depth that were open at the bottom (with 5 cm above the soil surface). Three rectangular windows of 7 cm width x 20 cm depth were made in the tube, allowing both mycorrhizal hyphae and plant roots to penetrate. Six small holes (of 5 mm diameter) were cut in the tubes at the level of the soil surface to aid in drainage. (3) Root exclusion (-R): seedlings were planted in PVC tubes as above and the windows were covered in 35 μm pore-size mesh (Plastok Associates Ltd., Birkenhead, Wirral, UK), allowing only mycorrhizal hyphae to penetrate. (4) Root and mycorrhizal exclusion (-RM): Seedlings were planted in PVC tubes but there were no rectangular windows in the tubes and a knife was used to cut around the edges of the tubes once per week to sever any fungal hyphae that might have entered through the small drainage holes. Once planted, seedlings were not given additional water and there were no on-going manipulations (such as removal of leaf litter and twigs lying across the piping or weeding of vegetation). The two sandstone species (*Shorea beccariana* and *S. multiflora*) grown in the alluvial plots were harvested in July 2004 (after 15 months) due to high mortality rates; all other seedling/soil type combinations were followed for 29 months. An index of light interception (% of canopy openness) was measured at the beginning of the experiment with hemispherical photography using a Minolta X-700 camera with a Rokkor 7.5 mm fisheye lens; images were subsequently analysed using Gap Light Analyser (Frazer et al. 1999).

Experiment 4. EcM-network effects on carbon isotope ratios on one dipterocarp species

Twenty areas with seedling banks of *Shorea multiflora* were selected in March 2000 in two separate areas of Kabili-Sepilok Forest Reserve. Ten areas were in the vicinity of research plots in the northern part of the Reserve and another ten were along a trail

268 running north-south through the Reserve. In each area, a circular plot of 68.5 cm
269 diameter was trenched to a depth of 5-10 cm (varying with the local microtopography)
270 and a plastic barrier was placed in the trench. An equally-sized and shaped plot
271 (situated between 0.45-3.2 m from the trenched plot; mean: 1.25 m) was marked out
272 using a circle of plastic, lain on the forest floor but remained otherwise unaltered in
273 order to act as a control. Each plot contained a mean of 13.5 (± 4.9 SD) seedlings of
274 which 11.8 (± 4.7 SD) were *Shorea multiflora*. The number of leaves and height of each
275 seedling was recorded so that after 13 months, one leaf that had been produced during
276 that interval was randomly selected from one seedling within each plot. The leaves
277 were dried at 50° C for at least one week, ground in liquid nitrogen and a sample of 1
278 mg was analysed for $\delta^{13}C$ (PDZ Europa ANCA-GSL preparation module connected to a
279 20-20 isotope ratio mass spectrometer, Northwich, Cheshire, UK). Isotope ratios were
280 calculated as: $\delta^{13}C$ (‰) = $(R_{sample}/R_{standard} -1) \times 1000$ where R is the isotope ratio of
281 $^{13}C/^{12}C$ of either the sample or the standard (Pee Dee Belemnite). In addition, one leaf
282 was collected from the canopy of eight large individuals of *Shorea multiflora* (40-45 m
283 tall; C. R. Maycock pers. comm.; R. N. Thewlis pers. comm.) and analysed for $\delta^{13}C$ as
284 above.
285
286 *Seedling measurements*
287 Non-destructive measurements of seedling height (to the apical meristem), basal
288 diameter and leaf number as well as survival rate were taken periodically. In
289 Experiment 1, six measurements were taken over 24 months (March 2000-February
290 2002), in Experiment 2, three measurements were taken over 11 months (September
291 2006-August 2007), in Experiment 3, 10 measurements were taken over 29 months
292 (April 2003-September 2005). Seedlings that died or were severely damaged by

293 mammals or tree/branch falls, where the meshes were damaged or where there was poor
294 drainage and the tubes became waterlogged (Experiment 2 only) were removed from
295 the growth analyses. For the individual growth analyses a total of n=233 (Experiment 1),
296 n=267 (Experiment 2), and n=317 (Experiment 3) observations were included. Only
297 seedlings grown under dark conditions (<5% canopy openness) were included in the
298 analysis for Experiment 2.
299
300 *Statistical analyses*
301 Based on an initial screen, we assumed linear growth, as individual seedlings showed
302 relatively constant increases in diameter, height and leaf number over time. The linear
303 model was fitted for every seedling and the individual regression slope (r) extracted.
304 The slopes were then standardised by dividing by the mean height, diameter or leaf
305 number of the last measurement, termed in this paper as relative growth rate (Paine et al.
306 2012b). A linear mixed-effects model for each study was carried out in R 3.2.0 (R
307 Development Core Team 2015), using the *nlme* library (Pinheiro and Bates 2000).
308 Treatment and species (all experiments), plus planting distance (Experiment 2), or
309 habitat (soil) type (Experiment 3) were treated as fixed effects; as we were specifically
310 interested in selected species effects they were included as fixed, rather than random,
311 effects, plot was included as a random effect. Unequal variance was observed and
312 accounted for by defining a linear increase in variance with time (Experiment 1) or light
313 level by species (Experiments 2 and 3). In the case of Experiment 2, adding
314 conspecificity/heterospecificity did not significantly improve the fit of the model (in all
315 cases: $\chi^2<16.5$, $P>0.15$) so this variable was removed for ease of comparison with the
316 other studies. Analysis of survival rates was made on binomial count data of seedlings
317 that survived compared to those that died, including the same structure of fixed and

318 random effects as outlined above, with the function *glmer()* and a binomial distribution
319 in the *lme4* library (Bates et al. 2015). The statistical tests are reported based on the
320 analysis of relative growth rates for all three non-destructive measurements (height,
321 diameter and leaf number) and for survival, but for simplicity only the increase in
322 diameter is shown graphically (for additional graphical representation of all non-
323 destructive measurements see supplementary material). We present the *F*-test or Chi-
324 square (survival analysis) statistic with associated *P*-values obtained through the
325 *anova()* command and *t*-test statistic with associated *P*-values obtained through the
326 *summary()* command for main effects and their interactions as outlined in Tables 2, 4
327 and 5. Note that with non-orthogonal designs in complex models the outcome from the
328 *anova()* command and the *summary()* command may differ slightly (Hector et al. 2010;
329 Hector 2015). Experiment 4 was analysed using a straightforward one-way ANOVA to
330 compare foliar $\delta^{13}C$ values between large trees and trenched and untrenched seedlings.
331
332 **Results**
333 *Experiment 1*
334 *Diameter growth.* For relative diameter growth rate, significant main effects of
335 treatment and species were observed (treatment: $F_{4,220}$=12.8, P<0.0001; species:
336 $F_{1,220}$=19.2, P<0.001) and there was also a significant interaction between treatment and
337 species ($F_{4,220}$=2.7, P<0.05). For *Hopea nervosa*, fungicide addition (-RM+F)
338 significantly reduced growth by 40% (mean ± 95% CI: 5-75%) compared to the
339 seedlings of the root exclusion treatment (-R) ($t_{4,220}$=2.2, P<0.05), however for
340 *Parashorea tomentella*, fungicide addition (-RM+F) did not affect diameter growth rate.
341 In contrast to our hypothesis, seedlings of *Parashorea tomentella* in the root and
342 mycorrhizal exclusion treatment (-RM) grew significantly ($t_{4,220}$=3.1, P<0.01) faster

than seedlings of the root exclusion treatment (-R) (mean ± 95% CI: 38% ± 19-50%) (Figure 1 and Table 2, supplementary material Figure S1).

Height growth. There was no effect of the treatments on height growth rates but *Parashorea tomentella* showed a significantly faster relative height growth rate than *Hopea nervosa* ($F_{1,220}$=4.1, P<0.05) (Table 2, Figure S2 a and b).

Leaf growth. Relative growth rate in leaf number showed significant main effects of the treatment ($F_{4,220}$=5.0, P<0.001) and species ($F_{1,220}$=116.3, P<0.0001). *Hopea nervosa* seedlings grew significantly faster those of *Parashorea tomentella* ($t_{1,220}$=6.0, P<0.0001). No significant treatment effects were observed for *Parashorea tomentella,* however for *Hopea nervosa*, control seedlings grew significantly faster than both seedlings of the root (-R) and mycorrhizal exclusion (-RM) treatment ($t_{4,220}$=2.2, P<0.05 and $t_{4,220}$=2.9, P<0.01 respectively). Fungicide addition significantly reduced growth compared to control seedlings ($t_{4,220}$=4.2, P<0.0001) (Table 2, Figure S3 a and b).

Survival. No effects of the treatments were observed for seedling survival but seedlings of *Hopea nervosa* showed a significantly greater survival rate compared to *Parashorea tomentella* (χ^2=6.4, P=0.01) (Tables 2 and 3).

Experiment 2

Diameter growth. There was no effect with respect to either the treatment or the planting distance from the large trees but *Dryobalanops lanceolata* seedlings showed

367 significantly greater relative diameter growth rates than *Shorea parvifolia* seedlings
368 ($F_{1,238}$=10.2, P<0.01; Figure 2 and Table 4, Figure S4).
369
370 *Height growth.* There was no effect of connection to an EcM network or species on
371 height growth. However, the root exclusion (-R) treatment of *Dryobalanops lanceolata*
372 showed a 73% increase in height growth rate when planted close to a large tree
373 compared to those that were planted away from the tree ($t_{1,238}$=2.5, P<0.05) (Table 4,
374 Figure S5 a and b).
375
376 *Leaf growth.* Leaf growth in *Shorea parvifolia* was significantly reduced in the root and
377 mycorrhizal exclusion treatment (-RM) compared to the root exclusion (-R) treatment
378 ($t_{2,238}$=2.4, P<0.05) (Table 4, Figure S6 a and b).
379
380 *Survival.* A significant treatment effect (χ^2=13.3, P=0.001) was found, as seedlings with
381 the root and mycorrhizal exclusion treatment (-RM) showed a lower survival rate than
382 the root exclusion (-R) treatment and the control seedlings. Seedlings of *Dryobalanops*
383 *lanceolata* showed a significantly higher survival rate compared to *Shorea parvifolia*
384 (χ^2=4.4, P<0.05) (Tables 3 and 4).
385
386 *Experiment 3*
387 *Diameter growth.* A significant interaction between treatment and soil type ($F_{3,276}$=2.7,
388 P<0.05) and between species and soil type ($F_{3,276}$=5.4, P<0.01) was found. Seedlings
389 with the root and mycorrhizal exclusion treatment (-RM) of three species (*Parashorea*
390 *tomentella*, *Shorea beccariana* and *S. multiflora)* grew faster in the sandstone soil type
391 compared to seedlings with only the root exclusion treatment (-R). Seedlings of

392 *Dryobalanops lanceolata* with the root exclusion treatment (-R) grew marginally faster
393 on the alluvial soil type ($t_{1,285}$=1.7, P<0.10) and also showed more rapid growth
394 compared to seedlings with the root and mycorrhizal exclusion treatment (-RM)
395 ($t_{1,285}$=2.2, P<0.05) (Figure 3 and Table 5, Figure S7).
396
397 *Height growth.* Seedlings of all four dipterocarp species showed significantly different
398 height growth rates ($F_{3,276}$ = 3.6, P<0.05). *Parashorea tomentella* seedlings with the
399 root exclusion treatment (-R) grew faster on the sandstone soil type ($t_{1,276}$=2.1, P<0.05)
400 (Table 5, Figure S8 a and b).
401
402 *Leaf growth.* A significant interaction between species and soil type was observed for
403 relative leaf growth rates ($F_{3,276}$ = 4.7, P<0.01). *Dryobalanops lanceolata* seedlings
404 grew significantly faster on the alluvial compared to the sandstone soil type ($t_{1,276}$=2.0,
405 P<0.05); for all other species there were no differences between the soil types (Table 5,
406 Figure S9 a and b).
407
408 *Survival.* A marginal species effect (χ^2=7.2, P<0.10) and a significant soil type effect
409 (χ^2=6.0, P=0.01) were found, however no effect of the treatments was observed after 15
410 months (Tables 3 and 5). Notably, the sandstone specialists *Shorea beccariana* and *S.*
411 *multiflora* showed lower survival rates on alluvial soil but the species by soil type
412 interaction was not significant.
413
414 *Experiment 4*
415 There was no difference between the foliar $\delta^{13}C$ values of seedlings grown in trenched
416 (-35.05‰ ± 0.22 SE) or untrenched (-35.00‰ ± 0.22 SE) plots but both were

417 significantly more negative than the value of -30.31‰ ± 0.34 SE obtained from the
418 canopy leaves of large trees ($F_{2,45}$=79.06, P<0.001). No effect of the treatment (trenched
419 vs. untrenched) on seedling survival rate was observed (Table 3).
420
421 **Discussion**
422 Several studies have addressed the benefits to seedlings of tropical forest trees of being
423 in contact with EcM hyphae radiating out from tree roots (Alexander et al. 1992;
424 Yasman 1995; Newbery et al. 2000), but few have tested the importance of
425 incorporation into a common EcM network under field conditions. Two independent
426 prior studies by Onguene and Kuyper (2002) and McGuire (2007) reported significant
427 increases in seedling mass (35%) and height growth (73%) respectively, that they
428 related to incorporation into the EcM networks of Caesalpinioideae trees in studies in
429 Cameroon and Guyana, respectively. In contrast, the key result from our analysis
430 across four complementary experiments with dipterocarps in South-east Asia is that
431 there are minimal effects of experimentally imposed treatments that alter seedling
432 incorporation into an EcM hyphal network on measures of dipterocarp seedling growth
433 in understorey conditions. Only two growth measures (the number of leaves of *Shorea*
434 *parvifolia* in Experiment 2 and the diameter of *Dryobalanops lanceolata* in Experiment
435 3) suggested any importance of an EcM hyphal network. There was some evidence that
436 exclusion from the EcM network reduced seedling survival, as, in Experiment 2,
437 seedling survival was lower in the –RM treatment compared to the –R treatment and the
438 control, although there is the possibility that this was due to waterlogging. In our
439 combined studies we thus did not detect any benefit to seedlings from being connected,
440 through a common EcM network, to surrounding mature trees.
441

442 We suggest that the lack of any effect on seedling growth of being connected to
443 an EcM network, in contrast to boreo-temperate forests (Simard et al. 2012) and low
444 diversity tropical forest (McGuire 2007) is because our lowland dipterocarp forest study
445 sites have high tree diversity and low species preference of EcM fungi. Peay et al.
446 (2015) showed 'extreme host generalism' of EcM fungi in similar tropical forests in
447 northern Borneo and it has been found that there is little evidence for host preference by
448 EcM fungal species in other tropical forests with high diversity of trees and a substantial
449 proportion of EcM trees (Tedersoo et al. 2010; Diédhiou et al. 2010; Smith et al. 2011).
450 If considered from a phytocentric perspective, an absence of host-specific EcM
451 associations removes the selective advantage of supporting seedlings *via* an EcM hyphal
452 network because there can be no guarantee that the supported seedling would be
453 conspecific kin.
454
455 Overall, the majority of measurements showed no effect (positive or negative) of
456 inclusion into an EcM network on seedling growth. However, in some cases,
457 experiment-specific findings argue for species-specific growth patterns, sometimes even
458 across the experiments. *Parashorea tomentella* seedlings in Experiments 1 and 3
459 showed increased growth rates when isolated from a common EcM network, suggesting
460 that EcM networks could even have detrimental effects on seedling growth and survival.
461 Two additional species (*Shorea beccariana* and *S. multiflora*) showed this effect in
462 Experiment 3 but only on the sandstone soil type. This result may not be entirely related
463 to an EcM network but in this case we hypothesise that providing exclusive access to
464 EcM hyphae associated with the seedlings to the rooting space inside the mesh tubes
465 prevented competition with hyphae from outside. It could also indicate that the
466 artificially induced limitation of root competition over scarce resources could be

467 directly beneficial for seedling growth (Coomes and Grubb 2000). Furthermore, there
468 was some evidence in Experiment 1 that fungicide addition limited diameter and leaf
469 growth in *Hopea nervosa*, but not in *Parashorea tomentella*. Fungicide addition
470 reduced the growth rate of this one species even though there was no significant
471 reduction in EcM colonisation (Brearley 2003). Clearly, the application of fungicide
472 will have additional effects other than simply reducing EcM colonisation such as effects
473 on soil nutrient status and impacts on pathogenic fungal populations (Newsham et al.
474 1994; Brearley 2003; Teste et al. 2006). In a similar experiment under high light
475 conditions (gaps), Brearley (2003) found that fungicide addition did reduce EcM
476 colonisation but this had a greater impact on seedling nutrient status than on seedling
477 growth. Other aspects of our experimental manipulations that may not have created
478 seedlings that were entirely disconnected from an EcM network include the depth of
479 barriers that were variable among experiment designs (*i.e.* possibly too shallow in
480 Experiment 4) and their open-bottomed nature in some experiments that might have
481 allowed colonisation by EcM hyphae from deeper soil layers (Pickles and Pither 2014).
482 In addition, there is the possibility of confounding the experimental treatments with
483 colonisation by different EcM fungal species; seedling roots isolated from the EcM
484 network would be more likely to be colonised by spore-forming fungi (and perhaps
485 retain initial greenhouse colonising fungi for longer) whereas those connected to the
486 EcM network would be more likely to become colonised via hyphal connections.
487 However, despite the potential for priority effects (Kennedy et al. 2009), there is a rapid
488 turnover of the EcM community on dipterocarp seedlings (Chang et al. 1994,1995; Lee
489 and Alexander 1996). Indeed, it would have been highly beneficial to have determined
490 the EcM fungi present on the seedlings' roots in each of the treatments (both at the
491 beginning and end of the experiments), in addition to those on adult trees, to provide

492 additional support for the efficacy of our experimental manipulations, as well as
493 comparing our different experimental designs. Importantly, it would also provide
494 support for our hypothesis of low EcM host specificity and this should be the key target
495 of future research.
496
497 Whilst we do not question the benefit to seedlings coming into contact with
498 EcM hyphae already present in the soil allowing them to rapidly form EcM associations
499 (Alexander et al. 1992), we did not find any importance of the EcM network for growth
500 of seedlings although survival was affected in one experiment. Whilst the main
501 mechanism through which connections to an EcM network have been hypothesised to
502 benefit seedlings is the provisioning of carbon for seedling growth in low light
503 environments, it could be questioned whether incorporation into an EcM network
504 provides other benefits that we have not measured. These could include improved
505 resistance to herbivores (Booth 2004), drought tolerance through hydraulic uplift
506 (Egerton-Warburton et al. 2007; Bingham and Simard 2011), or access to nutrients
507 being taken up from a larger volume of soil - possibly being more important where light
508 is less limiting. Bingham and Simard (2011) found a greater importance of an EcM
509 network under drought conditions; our sites rarely experience drought but it could be
510 informative to test the effect of EcM networks under an experimentally induced drought
511 or along a climatic gradient. Under very low light conditions, such that light was highly
512 limiting to growth (i.e. below the light compensation point), seedling survival is
513 arguably more important than seedling growth in determining future community
514 composition. In our experiment, light levels were above the light compensation point
515 for seedling growth (Eschenbach et al. 1998) such that growth was a more relevant
516 measure than survival although we did see some suggestions that the EcM network was

important for seedling survival. We altered light conditions by removal of some vegetation - this might have influenced our results but as the majority of these would have been AM species the impact of this is considered minor. An isotope labelling study (^{13}C) would be the next step to truly confirm if this lack of importance of an EcM hyphal network is indeed the case although, clearly, this is logistically challenging (Philip and Simard 2008, but see Klein et al. 2016).

In conclusion, we found that incorporation into a common EcM network has few measurable beneficial effects on dipterocarp seedling growth. That is not to say that the EcM network is unimportant, but, that within the constraints of short-term experiments (< 2 ½ years), we could not detect a signal of its influence on seedling growth. We did determine suggestions of an effect on seedling survival but this was only in one experiment and may have been an experimental artefact. We recommend that further studies should focus on the role that EcM networks play in resilience to drought periods or nutrient limitation of dipterocarp seedlings. In addition, we propose a working hypothesis, that needs further experimental testing, that the high tree species diversity and lack of benefit to trees of supporting heterospecific seedlings through a generalist EcM network is the reason for the minimal effects seen here. We welcome additional experiments and note that they need to be supported by identification of EcM fungi on seedling roots to aid interpretation. Currently, incorporation into an EcM network cannot categorically be invoked as affecting dipterocarp seedling growth or determining patterns of community diversity in dipterocarp-dominated tropical forests of Borneo.

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Disclosure statement

The authors declare that they have no conflicts of interest. The authors acknowledge that they have no financial interest or benefit arising from the direct applications of this research.

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580

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Figure legends

Figure 1: Effect of fungicide addition, but no effect of exclusion from an ectomycorrhizal hyphal network on the relative diameter growth rate (RGR) of two species of dipterocarp seedlings (top: *Hopea nervosa* and bottom: *Parashorea tomentella*) over a 24-month period at Kabili-Sepilok Forest Reserve in Sabah (Malaysian Borneo). The box indicates the data range from the lower quartile (25%) to the upper quartile (75%) and covers 50% of the data with the solid horizontal line within the box indicating the median. Whiskers indicate the data range from the lower 10% to the upper 90% (1.5 times the lower or upper quartile); outliers are indicated separately with a dot. See text for full details of experimental treatments.

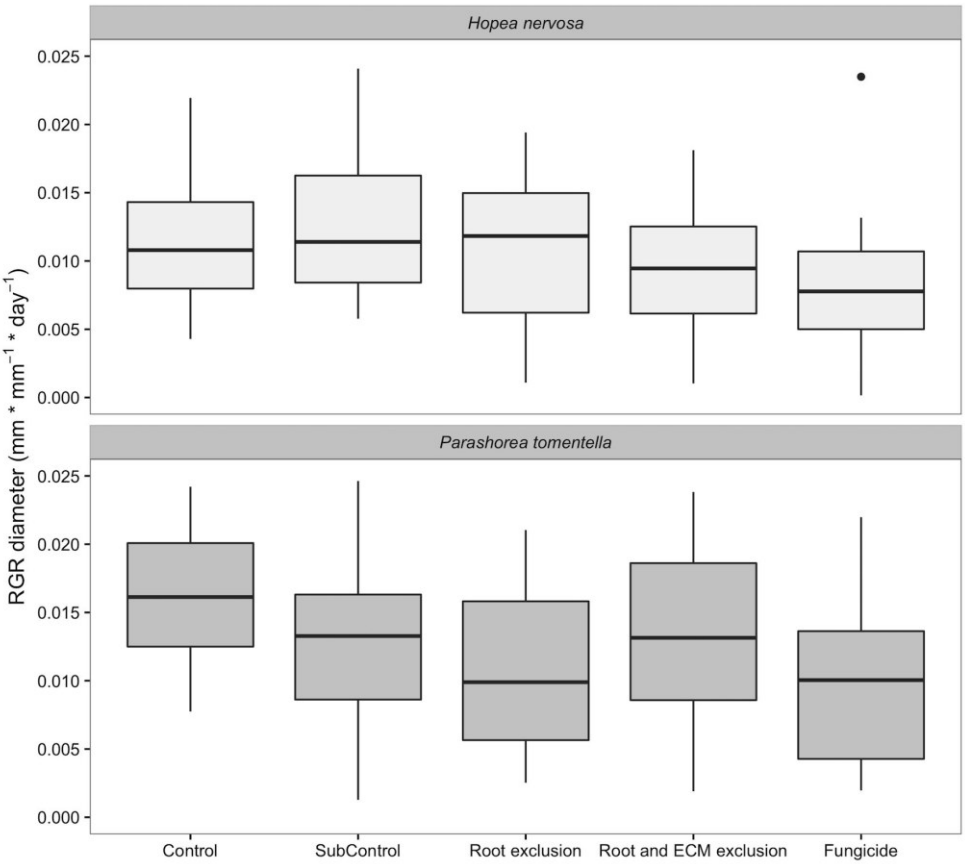


Figure 2: No effect of exclusion from an ectomycorrhizal hyphal network on the relative diameter growth rate (RGR) of two species of dipterocarp seedlings by distance from adult tree (top: *Dryobalanops lanceolata* and bottom: *Shorea parvifolia*) over an 11-month period at Malua Forest Reserve in Sabah (Malaysian Borneo). The box indicates the data range from the lower quartile (25%) to the upper quartile (75%) and covers 50% of the data with the solid horizontal line within the box indicating the median. Whiskers indicate the data range from the lower 10% to the upper 90% (1.5 times the lower or upper quartile); outliers are indicated separately with a dot. See text for full details of experimental treatments.

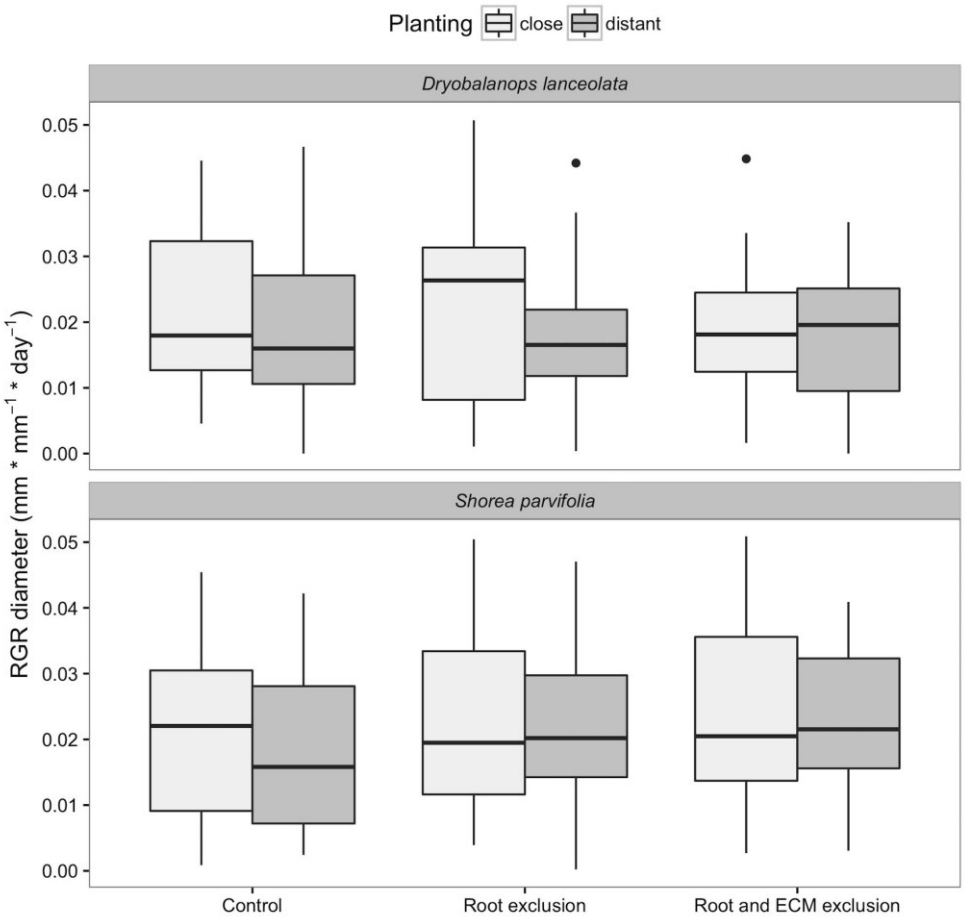
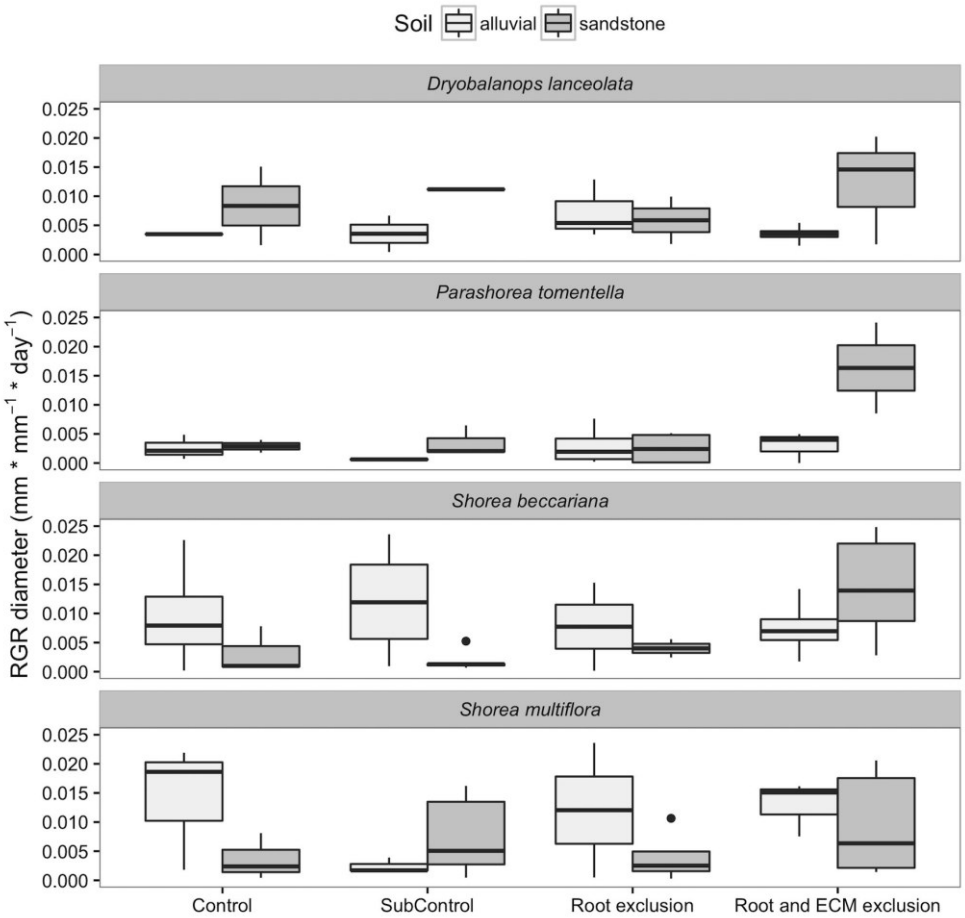


Figure 3: No effect of exclusion from an ectomycorrhizal hyphal network on the relative diameter growth rate (RGR) of four species of dipterocarp seedlings (top: *Dryobalanops lanceolata*, middle top: *Parashorea tomentella*, middle bottom: *Shorea beccariana* and bottom: *Shorea multiflora*) across soil types over a 29-month period at Kabili-Sepilok Forest Reserve in Sabah (Malaysian Borneo). Note that *S. beccariana* and *S. multiflora* growing in the alluvial soil type were harvested after 15 months due to high mortality rates. The box indicates the data range from the lower quartile (25%) to the upper quartile (75%) and covers 50% of the data with the solid horizontal line within the box indicating the median. Whiskers indicate the data range from the lower 10% to the upper 90% (1.5 times the lower or upper quartile); outliers are indicated separately with a dot. See text for full details of experimental treatments.



818 **Table 1:** Ecological information on seedlings of six dipterocarp species used to
819 experimentally assess the important of incorporation into a common EcM network on
820 seedling growth in tropical forests of Malaysian Borneo.

Name	Size	Wood density	Distribution	Experiment(s)
Dryobalanops lanceolata	Very large emergent	Medium / heavy	Common on fertile clay-rich soils in lowland northern Borneo	2,3
Hopea nervosa	Medium-sized	Heavy	Locally common in eastern Sabah	1
Parashorea tomentella	Large emergent	Light	Locally common on fertile lowland soils with occasional flooding (only on the east coast of northern Borneo)	1,3
Shorea beccariana	Medium-sized to large	Light	Common in northern Borneo on sandy soils and particularly ridge-tops associated with sandstone rocks.	3
Shorea multiflora	Small to medium-sized	Light	Common throughout Borneo on nutrient-poor or sandy soils and coastal hill slopes.	3,4
Shorea parvifolia	Large emergent	Light	Common throughout Borneo on better-drained clay soils.	2

821 Information collated from Ashton (2004), Meijer and Wood (1964), Newman *et al*. (1996, 1998) and
822 personal observations.
823

Table 2: Experiment 1: Statistical summary table and biological interpretation of exclusion from an ectomycorrhizal hyphal network on relative growth rates (diameter, height and number of leaves) and survival for two species of dipterocarp seedlings (*Hopea nervosa* and *Parashorea tomentella*) over a 24-month period at Kabili-Sepilok Forest Reserve in Sabah (Malaysian Borneo). -R = Root exclusion, -RM = Root and ectomycorrhiza exclusion, -RM+F = Root and ectomycorrhiza exclusion plus fungicide addition.

Effect	F_{df}	***P***	**Interpretation**
Diameter			
Treatment	$F_{4,220}$=12.8	<0.0001	-RM+F reduced growth of *Hopea nervosa* -RM treatment showed faster growth rate compared to -R in *Parashorea tomentella*
Species	$F_{1,220}$=19.2	<0.0001	Seedlings of *Parashorea tomentella* grew faster than *Hopea nervosa*
Treatment x Species	$F_{4,220}$=2.7	<0.05	Slower growth with =RM+F for *Hopea nervosa* but not *Parashorea tomentella*
Height			
Treatment	$F_{4,220}$=1.4	ns	No ectomycorrhizal network effect
Species	$F_{1,220}$=4.1	<0.05	*Parashorea tomentella* grew faster than *Hopea nervosa*
Treatment x Species	$F_{4,220}$=0.8	ns	No significant interaction term
Leaves			
Treatment	$F_{4,220}$=5.0	<0.001	*Hopea nervosa* control seedlings grew faster than -R and -RM -RM+F significantly reduced growth in *Hopea nervosa*
Species	$F_{1,220}$=116.3	<0.0001	*Hopea nervosa* seedlings grew faster than *Parashorea tomentella* seedlings
Treatment x Species	$F_{4,220}$=1.8	ns	No significant interaction term
Survival			
Treatment	$\chi^2_{3,7}$=4.0	ns	No effect of treatment on survival
Species	$\chi^2_{6,7}$=6.4	<0.01	Seedlings of *Hopea nervosa* showed higher survival compared to *Parashorea tomentella*
Treatment x Species	$\chi^2_{7,11}$=4.2	ns	No significant interaction term

Table 3: Survival rates (%) of dipterocarp seedlings following exclusion from an ectomycorrhizal hyphal network in four independent experiments conducted in Borneo. See text for full details of experimental set-up in each experiment. Dash (-) indicates that the treatment noted was not present in the given experiment. Asterisk (*) indicates 15 months to harvest whilst all other values are for the entire experimental period. -R = Root exclusion, -RM = Root and ectomycorrhiza exclusion, -RM+F = Root and ectomycorrhiza exclusion plus fungicide addition.

	Control	**Sub-Control**	**-R**	**-RM**	**-RM+F**
Experiment 1					
Parashorea tomentella	79	83	80	71	80
Hopea nervosa	96	92	100	88	83
Experiment 2					
Near					
Dryobalanops lanceolata	98	-	97	95	-
Shorea parvifolia	98	-	97	89	-
Far					
Dryobalanops lanceolata	99	-	100	97	-
Shorea parvifolia	98	-	98	90	-
Experiment 3					
Alluvial					
Dryobalanops lanceolata	50	70	60	70	-
Shorea beccariana	30*	30*	30*	20*	-
Shorea multiflora	20*	30*	40*	10*	-
Parashorea tomentella	20	20	40	20	-
Sandstone					
Dryobalanops lanceolata	40	40	50	60	-
Shorea beccariana	60	60	40	30	-
Shorea multiflora	50	30	40	30	-
Parashorea tomentella	40	70	60	30	-
Experiment 4					
Shorea multiflora	92	-	-	88	-

Table 4: Experiment 2: Statistical summary table and biological interpretation of exclusion from an ectomycorrhizal hyphal network and distance from adult tree on relative growth rates (diameter, height and number of leaves) and survival for two species of dipterocarp seedlings (*Dryobalanops lanceolata* and *Shorea parvifolia*) over an 11-month period at the Malua Forest Reserve in Sabah (Malaysian Borneo). Interaction terms not included were not statistically significant for any of the parameters measured. -R = Root exclusion, -RM = Root and ectomycorrhiza exclusion.

Effect	F_{df}	***P***	**Interpretation**
Diameter			
Treatment	$F_{2,238}$=0.6	ns	No ectomycorrhizal network effect
Distance	$F_{1,238}$=1.1	ns	No effect of distance from adult tree
Species	$F_{1,238}$=10.2	<0.01	*Dryobalanops lanceolata* grew faster than *Shorea parvifolia*
Treatment x Species	$F_{2,238}$=0.3	ns	No significant interaction term
Height			
Treatment	$F_{2,238}$=1.2	ns	No ectomycorrhizal network effect
Distance	$F_{1,238}$=9.3	<0.01	*Dryobalanops lanceolata* grew faster closer to adult trees
Species	$F_{1,238}$=0.1	ns	No species differences
Treatment x Species	$F_{2,238}$=2.5	<0.10	*Shorea parvifolia* control seedlings grew marginally faster than -R and significantly faster than -RM but no effect on *Dryobalanops lanceolata*
Leaves			
Treatment	$F_{2,238}$=3.1	<0.05	*Shorea parvifolia* -RM seedlings grew slower than the -R treatment
Distance	$F_{1,238}$=0.9	ns	No effect of distance from adult tree
Species	$F_{1,238}$=0.1	ns	No species differences
Treatment x Species	$F_{2,238}$=3.2	<0.05	*Dryobalanops lanceolata* -RM seedlings grew slower, but no effect on *Shorea parvifolia*
Survival			
Treatment	$\chi^2_{3,5}$=13.3	<0.0001	-RM showed significantly lower survival for both species
Distance	$\chi^2_{5,6}$=1.0	ns	No effect of distance from adult tree
Species	$\chi^2_{4,5}$=4.4	<0.05	Survival rate in *Shorea parvifolia* lower than in *Dryobalanops lanceolata*
Treatment x Species	$\chi^2_{5,7}$=0.4	ns	No significant interaction term

Table 5: Experiment 3: Statistical summary table and biological interpretation of exclusion from an ectomycorrhizal hyphal network and soil type on relative growth rates (diameter, height and number of leaves) for four species of dipterocarp seedlings (*Dryobalanops lanceolata*, *Parashorea tomentella*, *Shorea beccariana* and *Shorea multiflora*) over a 29-month period at Kabili-Sepilok Forest Reserve in Sabah (Malaysian Borneo). The three-way interaction term is not included as it was not statistically significant for any of the parameters measured. -R = Root exclusion, -RM = Root and ectomycorrhiza exclusion.

Effect	F_{df}	***P***	**Interpretation**
Diameter			
Treatment	$F_{3,276}$=2.6	<0.10	See interactions below
Soil type	$F_{1,276}$=0.8	ns	No soil type effect
Species	$F_{3,276}$=0.8	ns	No species effect
Treatment x Species	$F_{9,276}$=1.3	ns	No significant interaction term
Treatment x Soil type	$F_{3,276}$=2.7	<0.05	-RM of *Parashorea tomentella*, *Shorea beccariana* and *Shorea multiflora* grew faster on sandstone soil than -R for all three species
Species x Soil type	$F_{3,276}$=5.4	<0.01	-R of *Dryobalanops lanceolata* grew faster on alluvial soil and overall faster than –RM
Height			
Treatment	$F_{3,276}$=1.0	ns	No ectomycorrhizal network effect
Soil type	$F_{1,276}$=0.9	ns	No soil type effect
Species	$F_{3,276}$=3.6	<0.05	-R of *Parashorea tomentella* grew faster on sandstone soil
Treatment x Species	$F_{9,276}$=1.0	ns	No significant interaction term
Treatment x Soil type	$F_{3,276}$=1.1	ns	No significant interaction term
Species x Soil type	$F_{3,276}$=0.4	ns	No significant interaction term
Leaves			
Treatment	$F_{3,276}$=1.1	ns	No ectomycorrhizal network effect
Soil type	$F_{1,276}$=0.1	ns	No soil type effect
Species	$F_{3,276}$<0.1	ns	No species effect
Treatment x Species	$F_{9,276}$=1.4	ns	No significant interaction term
Treatment x Soil type	$F_{3,276}$=2.0	ns	No significant interaction term
Species x Soil type	$F_{3,276}$=4.7	<0.01	*Dryobalanops lanceolata* seedlings grew faster on alluvial soil than sandstone soil
Survival			
Treatment	$\chi^2_{6,9}$=1.2	ns	No effect of treatment on survival
Soil type	$\chi^2_{8,9}$=6.0	<0.01	Survival on alluvial soil was significantly lower compared to sandstone soil
Species	$\chi^2_{6,9}$=7.2	<0.10	Seedlings of *Shorea multiflora* and *Shorea beccariana* showed lowest survival after 15 months
Treatment x Species	$\chi^2_{9,18}$=4.3	Ns	No significant interaction term
Treatment x Soil type	$\chi^2_{9,12}$=2.3	ns	No significant interaction term
Species x Soil type	$\chi^2_{9,12}$=4.6	ns	No significant interaction term