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Ectomycorrhizal fungi increase the vitality of Norway spruce seedlings under the pressure of *Heterobasidion* root rot *in vitro* but may increase susceptibility to foliar necrotrophs

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Abstract

We tested if root colonisation by ectomycorrhizal fungi (EMF) could alter the susceptibility of Norway spruce (*Picea abies*) seedlings to root rot infection or necrotic foliar pathogens. Firstly, spruce seedlings were inoculated by various EMF and challenged with *Heterobasidion* isolates in triaxenix tubes. The ascomycete EMF *Meliniomyces bicolor*, that had showed strong antagonistic properties towards root rot causing *Heterobasidion in vitro*, protected spruce seedlings effectively against root rot. Secondly, spruce seedlings, inoculated with *M. bicolor* or the forest humus, were subjected to necrotrophic foliar pathogens in conventional forest nursery conditions on peat substrates. *Botrytis cinerea* infection after winter was mild and the level of needle damage was independent of substrate and EMF colonisation. Needle damage severity caused by *Gremminiella abietina* was high in seedlings grown in substrates with high nutrient availability as well as in seedlings with well-established EMF communities. These results show that albeit *M. bicolor* is able to protect spruce seedlings against *Heterobasidion* root rot in axenic cultures it fails to induce systemic protection against foliar pathogens. We also point out that unsterile inoculum sources, such as the forest humus, should not be considered for use in greenhouse conditions as they might predispose seedlings to unintended needle damages.

Keywords: *Meliniomyces bicolor*, *Botrytis cinerea*, *Gremmeniella abietina*, forest nurseries, integrated pest management IPM, *Picea abies*

1 Introduction

Norway spruce (*Picea abies*) is the most widely used tree species in Northern forest nurseries; In Finland more than 100 million Norway spruce seedlings are produced annually (Finnish Food safety Authority 2010–2016). During and after winter storage in forest nurseries spruce seedlings are vulnerable to various microbial diseases. Grey mould *Botrytis cinerea* commonly infects spruce seedlings by spreading from diseased seedlings to healthy ones during winter storage or thawing resulting in mild discoloration spots or even total decay of needles (Petäistö 2006). Another disease showing belated symptoms after winter dormancy is the ascomycete *Gremmeniella abietina* that causes Brunchorstia disease in pine and spruce. The fungal hypha of *G. abietina* spreads inside the aboveground plant tissues during dormancy. The infection becomes visible gradually during the following growing season as browning of needles in the mid-section of the shoot, discolouring of foliage, presence of resin impregnated necrotic stem lesions and sometimes even dieback of the whole shoot (Petäistö 2008, Børja *et al.*2006).

During the first years after out planting, Norway spruce seedlings commonly suffer from pine weevil damage (*Hylobius abietis*) (Luoranen *et al.*2017) but in longer term, also from root rot caused by *Heterobasidion annosum sensu lato*, the group of most severe fungal coniferous tree pathogens in northern temperate regions, particularly in Europe. In Finland, two *Heterobasidion* species, *H. annosum sensu stricto* and *H. parviporum* (Niemelä and Korhonen 1998), infect spruces of all ages causing root and butt rot. *Heterobasidio*n root rot becomes a risk especially at previously infected regeneration sites, where old conifer stumps as well as decaying root pieces in soil colonised by *Heterobasidion* spp. act as infection sources for the next tree generation (Stenlid 1987, Piri 1996, Piri 2003). On infested sites, the first spruce seedlings can be infected by Heterobasidion root rot already four to five years after planting (Piri and Hamberg 2015). *H. annosum* and *H. parviporum* inhabit tree roots and spread via belowground root contacts (Garbelotto and Gonthier 2013) thus overlapping with habitats of symbiotic ectomycorrhizal fungi (EMF) in forest soils.

Formerly fungal diseases in seedling production have been tackled with chemical fungicides, but due to the restricting legislation and banning of several chemicals (European Parliament 2009) there is an urgent need of integrated pest management that can be cost efficiently utilized in forest nurseries. Mycorrhizal fungi have suggested triggering signals that move through plants causing biochemical changes affecting growth allocation, nutrition levels, inducible defence mechanisms and resistance as well as host vitality (Whipps 2004).

In arbuscular mycorrhizal angiosperm plants both positive and negative feedbacks have been observed between aboveground and belowground microbial communities; Priming and protective systemic effects against microbial and invertebrate pathogens have been shown to move to distal plant organs (Pozo and Azcón-Aguilar 2007, Pineda *et al.*2010) but the outcome seems to be context dependant (Jung *et al.*2012). Arbuscular mycorrhiza-induced jasmonic acid-regulated resistance boosts host aboveground durability towards pathogens with a necrotrophic lifestyle but in contrast may make the host more susceptible to biotrophic and viral pathogens (Pozo and Azcón-Aguilar 2007, Jung *et al.*2012).

Induced resistance has shown to move from roots to shoots also in Norway spruce, as root inoculation with the mycorrhiza helper bacterium *Streptomyces* sp. increased needle resistance against *B. cinerea* (Lehr *et al.*2007). Furthermore, Rajala *et al.*(2013) found a negative relationship between the abundance of fungal needle endophytes and the EMF richness on Norway spruce. Some earlier studies have tested the biocontrol potential of EMF against soil and air derived plant pathogens in conifers, but again the effects seem to be fungal strain specific and vary according to the physiological state of the host (Zhang *et al.*2011, Farquhar and Peterson 1991, Hwang *et al.*1995, Martín-Pinto *et al.*2006). In the case of unspecific general responses of Norway spruce to fungi, the effects of symbiotic EMF *Pisolithus tinctorus* on peroxidase activity and on the amount of free salicylic acid in roots seem to be transient in comparison to the more permanent effects caused by *H. annosum* (Likar and Regvar 2008). EMF may still permanently modify the chemical content of distal organs as the colonisation by EMF *Hebeloma bryogenes* and *Cadophora finlandica* reduced seedling growth but increased nutrient content and photosynthetic pigment levels in needles of Norway spruce (Mrnka *et al.* 2009). Thus there is an increasing interest in the long-term effects of these interactions on distant plant parts. Furthermore, it is important to address the effects of EMF on growth allocation, access to nutrients, and the formation of induced and constitutive defence (Bonello *et al.* 2006).

Paxillus involutus (Basidiomycota) and *Meliniomyces bicolor* (Ascomycota) form ectomycorrhizal associations with Norway spruce roots in boreal forests. *M. bicolor* is part of the *Pezoloma ericae* (formely *Rhizoscyphus ericae*) aggregate including phylogenetically related fungi (*M. variabilis*, *R. ericae* and *C. finlandica*) inhabiting roots of both hardwood and herbaceous plants in various habitats, both in the Northern and Southern hemisphere (Vrålstad *et al*. 2000, Hambleton and Sigler 2005, Bruzone *et al*. 2017). Individual genets of fungal species belonging to the *P. ericae* aggregate can simultaneously form both ericoid and

ectomycorrhizal associations (Grelet *et al*. 2010). Previously it has been found that certain isolates of *P. involutus* and *M. bicolor* were able to restrict or even halt the growth of *H. parviporum* and *H. annosum* strains *in vitro* on nutrient agar plates in laboratory (Hyder *et al.*2013). Hence, we aim to test in triaxenic cultures how certain EMF isolates, that have shown to be antagonistic against *Heterobasidion* species *in vitro*, affect the viability of Norway spruce seedlings. In addition, we study the effects of the antagonistic EMF *M. bicolor* on spruce growth allocation, and susceptibility of distant organs towards necrotic foliar pathogens *B. cinerea* and *G. abietina* in conventional forest nursery conditions. As a result, we gather elementary knowledge prior to compile recommendations for the use of certain EMF strains in nursery.

2 Material and Methods

2.1 Triaxenic testing of protective effects of EMF against Heterobasidion root rot

2.1.1 Fungal strains and preparation of vegetative inoculum

The ectomycorrhizal strains that outperformed standard antagonism against *H. parviporum* and *H. annosum* (Hyder *et al.* 2013) were selected to be used in the triaxenic test (Table 1). Additionally, *Thelephora terrestris*, a common nursery EMF, and a common root endophyte *Phialocephala fortinii* were included as non-antagonistic controls. *H. parviporum* isolates were collected from Norway spruce (isolate A) thinning stump and from living trees (B,C) and *H. annosum* isolates from final cutting stumps (D, E) of Scots pine in southern Finland. All fungal isolates were collected from different stands and represented different genotypes based on pairing tests (Stenlid 1985). Vegetative mycelia of *H. parviporum* (A, B, C) and *H. annosum* (D, E) were grown on Brown & Wilkins (1985) agar on Petri dishes. Twice autoclaved wooden sticks (121 ˚C 60 min; Ø 3mm x 20mm) were placed on top of the cultures for three weeks to allow mycelia to grow into the sticks. The ectomycorrhizal, root endophytic and *Heterobasidion* fungal isolates used in this study were obtained from the culture collection of the Natural Resources Institute Finland, except *P. involutus* BOUX which originates from France, INRA-Nancy. *T. terrestris* strain was initially isolated from a mycorrhizal root tip of a Norway spruce seedling (Flykt *et al.* 2008). *M.bicolor* and *P. fortinii* were isolated similarly from spruce mycorrhizas and the Finnish *P. involutus* strains were originally isolated from fruiting bodies (details in Vuorinen *et al.* 2015).

2.1.2 Preparation of triaxenic synthesis tubes

The potential of EMF isolates to protect Norway spruce seedlings from *H. annosum* and *H. parviporum* was tested using a modification of the synthesis tube system described previously by Timonen *et al*. (1993). Briefly, surface sterilized (30% H_2O_2 15 min) seed-orchard-seeds of Norway spruce (ROI-89-1002 SV. 109) of southern Finnish origin were germinated on glucose (5%) agar plates. After germination seedlings were transferred into glass tubes (Ø 25 mm x 200 mm) on 9 ml slants of Brown & Wilkins agar covered with Leca-clay balls (acid washed and twice autoclaved for 20 min 121°C) moistened with 2,5 ml of Modified Melin-Norkrans (Marx 1969) nutrient solution with reduced sugar and malt content (½ MMN). Tubes were closed with cotton wool plugs.

2.1.3 Tube experiment

Triaxenic synthesis tubes containing spruce seedlings were placed in Conviron PGR15 growth chambers (Controlled environments Ltd., Manitoba, Canada) under 280 µM light intensity at 23 °C and 65% humidity with a 16 h day length, and 8 hours of darkness. Lights were composed of high pressure sodium (250W, Lumalux®, Sylvania) and metal halide (250W, Britelux®, Sylvania) light bulbs (Feilo Sylvania Finland Oy).

At the age of four weeks, 30 replicate seedlings per each root associated fungi (Table 1) were inoculated with two agar plugs taken from the peripheral growth zone of an actively growing fungal colony in ½ MMN agar. 30 seedlings were left as uninoculated controls. Tubes were incubated for six weeks and ectomycorrhiza formation was assessed by dissecting microscope prior to further inoculations with pathogens. *Heterobasidion* isolates (A–E) were introduced into the system by placing wood sticks colonised with each isolate close to the base of the seedlings. This resulted in total 48 treatments with five replicate seedlings each; seedlings pre-infected with six EMF fungi, one endophyte and no EMF-control, and all these challenged with five *Heterobasidion* isolates and the pathogen free control treatment. Seedlings were grown in growth chambers for four months and irrigated with 2 ml of sterile water once a month.

After altogether six months, as soon as the seedlings seemed to reach stationary stage, the vitality of the seedlings was assessed visually to a five-step discrete variable according to the following classification: i) dead seedlings with totally brown needles (B), ii) chlorotic needles with brown lesions (CB), iii) chlorotic needles (C), iv) light green needles (LG), and v) healthy seedlings possessing only green needles (G). Seedlings were removed from the

158 tubes, washed, dried in +70˚C and weighed for their biomass. The roots of seedlings with
159 severe *Heterobasidion* infection had already started to decay before the sampling.

160 ***2.2 Impact of antagonistic EMF on susceptibility to foliar pathogens***

161 *2.2.1 Preparation of plant and EMF material for the nursery experiment*
162 In late May seed-orchard-seeds of Norway spruce (ROI-89-1002 SV. 109) were sown in six
163 Plantek-81F containers (vol 85 cm^3, BCC) on top of unfertilized blonde sphagnum peat
164 (PP03, Kekkilä Group, Vantaa, Finland) supplemented with five different inoculums (Table
165 2) from which one contained 15% (vol:vol) vegetative fungal hypha of *M. bicolor* grown in
166 solid silica-based malt extract medium as in Vuorinen *et al.*(2015) (Table 2). The vitality of
167 EMF vegetative inoculum was confirmed on ½ MMN agar.

168 In each of the six containers (9 × 9 plants per container) two rows (9 plants in a row) were
169 randomly filled with one of the four treatment substrates (18 seedlings/substrate/container),
170 and one row of each container was filled with conventional fertilized blonde Sphagnum-peat
171 (Ctrl treatment, Table 2). The seedlings were germinated in a glasshouse, and transferred
172 outside to the nursery field in the end of June. Between June and August each seedling was
173 fertilized weekly with approximately 5 ml 0.1% w:w liquid fertilizer Turve Superex® N-P-K
174 (12–5–27) (Kekkilä Group, Vantaa, Finland) to keep the peat conductivity as low as 0.5–1
175 mS cm^{-1}. The experiment was established at the Haapastensyrjä nursery, Southern Finland
176 and plants were grown in the Suonenjoki nursery (62.625N, 27.122E) in Eastern Finland.

177 *2.2.2 Preparation of spore suspensions and foliar infection*

178 The foliar inoculations with the two fungal pathogens *Botrytis cinerea* and *Gremmeniella*
179 *abietina*, and the uninfected water only- treatment were performed in early June and August.
180 Two out of six Plantek-81F containers were randomly chosen to each foliar treatment and
181 labelled accordingly, totalling in 36 replicate seedlings per treatment and 18 replicate Ctrl
182 seedlings in fertilized peat (Table 2). *B. cinerea* was grown on potato dextrose agar (PDA) at
183 +17 °C for three days in darkness followed by three to seven days under photoperiodic
184 ambient lighting (16 hours of light:8 hours of dark). The *B. cinerea* spores from the fungal
185 cultures were liberated into 10 ml of sterile water by rubbing the plates with a sterile glass
186 rod. Hyphae from the spore suspension were removed by running the suspension through
187 sterile cotton gauze. *G. abietina* was grown on autoclaved wheat substrate (10 g wheat and 25
188 ml distilled water in 100 ml growth bottle) for one to two months at +17 °C under artificial

189 light with the photoperiod of 16 hours of light and 8 hours of dark. The spore mass on the
190 culture was collected by a sterile loop and suspended into 20 ml of sterile water. The number
191 of spores was counted by Neubauer-improved chamber (Wertheim, Germany). Before
192 inoculation, the shoots were sprayed with tap water to moisten the foliage. The seedlings
193 were spray-inoculated with 2 ml of spore suspension of either *B. cinerea* isolate BcSjk1.1
194 (Petäistö *et al.* 2004) or *G. abietina* containing ca. 300 000 spores/ml. The vitality of spore
195 suspensions was confirmed by monitoring the spore germination on PDA plates. The water
196 only-seedlings were sprayed with 2 ml of autoclaved tap water. After inoculation, the
197 seedlings were maintained in greenhouse under plastic cover for three days and the shoots
198 were repeatedly sprayed with tap water to keep the foliage humid. Thereafter the seedlings
199 were grown in a plastic greenhouse ventilated by opening side panels and doors. The
200 seedlings were monitored daily for two weeks after the inoculation. A throughout inventory
201 of needle damage was done twice, in October after the growing season and in May after
202 wintering in the open field nursery under natural snow cover.

203 *2.2.3 Sampling and determination of fungal colonisation*

204 In early June seedling height was measured and roots were carefully washed with tap water.
205 From each combination of foliar and growth substrate treatments ten replicate root systems
206 were randomly chosen for EMF colonisation assessment.

207 EMF colonisation and number of fungal morphotypes were determined under dissecting
208 microscope. Representative root tips from each morphotype were amplified with the Thermo
209 Scientific Phire Plant Direct PCR Kit® as described in detail in Velmala *et al.*2013. Root tips
210 were crushed in 20 µl Dilution Buffer and 1 µl was used as template in a 25 µl reaction
211 volume, and amplified with primers ITS1F (Gardes and Burns 1993) and ITS4 (White *et*
212 *al.*1990). PCR products were separated in 1% Synergel (Diversified Biotech, MA, USA) for
213 3h 120V. Distinguishable bands were cut and dissolved in water, and used as template for re-
214 amplification with DreamTaq DNA Polymerase (Thermo Scientific, Waltham, MA, USA)
215 with the same primers for 25 cycles. End products were sequenced by the Macrogen
216 Sequencing Service (Macrogen Europe, The Netherlands) using the ITS4-primer. The
217 sequences were revised in Geneious R6.1.8 (Biomatters, Auckland, New Zealand, available
218 from http://www.geneious.com/). The OTUs were identified by comparing the sequences
219 against sequences in UNITE (http://unite.ut.ee/) and the INSD database. Sequences are
220 deposited in GenBank under the accession numbers MF947534–MF947542.

221 *2.2.4 Determination of biomass and nitrogen content*

222 Roots and shoots were separately oven dried for 48h in 50°C and weighted for biomass
223 determination. Needle nitrogen concentration was determined with a CHN-100 analyser
224 (Leco, analysis based on ISO 10694 and ISO 13878 standards) in Natural Resources Institute
225 Finland. Before analysis 200 mg powdery needle sample (from 0.5 g of dry needles
226 homogenised in 2 ml screw cap tubes with FastPrep® (MP Biomedicals, CA, USA) for 2 x
227 30s with 4.5 m/s speed) from three replicate seedlings from each row of the water only-
228 treatment were pooled resulting in altogether four samples per growth substrate.

229 ***2.3 Statistical analyses***

230 The data from both experiments was analysed as linear models with R software version 3.2.3.
231 (R Core Team 2015). We used ***glht*** function from 'multcomp' (Hothorn *et al*. 2008) package
232 to make Dunnett's multiple comparison of means and to calculate the confidence intervals for
233 general linear hypotheses. Model assumptions for homogeneity of variances, and normality
234 of residuals were checked by plotting residuals against fitted values, and creating a Q-Q plot
235 of residuals. Bar plot figures were plotted with 'ggplot2' (Wickham 2009) and arranged in
236 grids with 'gridExtra' (Auguie 2016) packages.

237 *2.3.1 Triaxenic tube experiment*

238 As the explanatory variable we used a 24 level variable that was coined to cover all eight
239 combinations of root associated fungi with either no pathogen (=control), *H. parviporum* (A-
240 C) or *H. annosum* (D, E). All treatments were compared to the reference, 0-level, seedlings;
241 No inoculation with root associated fungi and no pathogens. Shoot and root biomass were
242 fitted as linear models with ***lm*** function. For needle vitality class response variable we fitted
243 a generalised linear model ***glm*** assuming Poisson error distribution and log link function (no
244 over-dispersion was observed). The relationship between vitality and root and shoot growth
245 were calculated as Pearson's product-moment correlations.

246 *2.3.2 Foliar pathogen experiment in the nursery*

247 As the explanatory variable we used a 15 level variable that was coined to cover all foliar and
248 growth substrate combinations. All treatments were compared to the reference level
249 seedlings; water only- foliar treatment grown in Ctrl fertilized peat (Table 2). Root biomass,
250 shoot:root- ratio, and shoot height were fitted as linear models with ***lm*** function. For EMF

colonisation and needle damage percentage response variables we fitted a generalised linear model ***glm*** with binomial error distribution and logit link function. EMF richness count was analysed as a generalized model assuming Poisson error distribution and log link function (no over-dispersion was observed). N content and concentration was analysed as linear models with growth substrate as the explanatory variable, and all substrates were compared to Ctrl fertilized peat substrate. The relationship between EMF, *Meliniomyces* sp., *T. terrestris*, *C. geophilum* and *Amphinema* sp. colonisation, and species richness with needle damage %, N content, shoot and root growth were calculated as Pearson's product-moment correlations.

3 Results

3.1 Triaxenic testing of spruce, EMF and root rot pathogen Heterobasidion spp.

The vitality of seedlings varied between green healthy seedlings and slightly chlorotic yellow phenotypes when no pathogens were present (Fig. 1a). When seedlings without any root associated fungi were subjected to either *H. parviporum* or *H. annosum* isolates they turned brown and died. In general, it seemed that all EMF provided some protection against root pathogens, and seedling needle vitality was only slightly lowered in most treatments (Fig. 1a). *H. parviporum* or *H. annosum* isolates did not show within species differences and thus the results are bulked together within *Heterobasidion* species.

Through the treatments, shoot biomass was the highest in *T. terrestis* and *P. involutus 2* inoculated seedlings. *M. bicolor* inoculated seedlings had the most even shoot growth when infected with *Heterobasidion* isolates (Fig. 1b), and showed least disease symptoms under *H. annosum* infection (Fig. 1a). The correlation between vitality and shoot biomass was moderate (0.53, $P<0.001$).

Root growth responded differently to various root inoculations; when *Heterobasidion* was present root growth was significantly reduced in seedlings inoculated with *P. fortinii* and *P.involutus 1*. *P. fortinii* and *P.involutus 1* also resulted in the lowest vitality classes even without pathogens present (Fig. 1a). *P. involutus* strains 2, 3, and 4 were able to efficiently protect seedlings against *Heterobasidion* infection, even though some discoloration and redness of needles was more common than with other EMF. Roots inoculated with *M. bicolor* and *T. terrestris* were least affected by *Heterobasidion* isolates (Fig. 1c). Root biomass had a strong positive correlation with vitality class ($r=0.7$, $P<0.001$) indicating that root growth in *in vitro* set ups can be used to extrapolate the vitality of seedlings.

3.2 Impact of M. bicolor on susceptibility of spruce seedlings against foliar pathogens

3.2.1 Needle damage became visible after winter dormancy

Needle damage in autumn after the first growing season in nursery was low (1.35±0.1%) and no statistically significant differences could be observed between the treatments. After winter dormancy, needle damage caused by necrotrophic pathogens became visible, and in May clear symptoms with almost half of the needles injured could be seen in *G. abietina* infected seedlings (Fig. 2a). Needle damage in spring was statistically significantly higher in *G. abietina* infected seedlings than in the Ctrl seedlings treated with water only. The foliage of water only- treated seedlings suffered some damage especially when growing in Humus substrate, which possessed even higher amount of needle damage than *B. cinerea* infected seedlings (Fig. 2a).

Mortality of the nursery experiment seedlings after winter was low, less than 0.7%. The highest mortality rates were observed when seedlings were exposed to *G. abietina* and *B. cinerea* foliar treatments; 15 out of 162 seedlings died in both *G. abietina*- (evenly in all growth substrates) and in *B. cinerea*- treatments (half growing in sieved humus and the rest in Me, Ctrl-M, and Ctrl-H substrates). Only four seedlings died during the experiment from the water only- treatment and again mostly from the humus substrate.

3.2.2 B. cinerea and G. abietina caused needle damage despite EMF colonization

EMF colonisation in either *M. bicolor* or *Humus* treatments did not protect seedlings against foliar pathogens: *B. cinerea* infection severity seemed to be independent of growth substrate and EMF status. On the contrary, the severity of needle damage caused by *G. abietina* was high in growth substrates containing EMF inoculum (Me, Humus) or well-balanced nutrients (Ctrl) (Fig. 2a, Table 2).

3.2.3Needle N content was lowest in seedling growing in EMF inoculated substrates

Shoot height and biomass as well as root biomass were affected by both foliar pathogen treatments and growing substrate (Figs 2b and 2c). Fertilized peat (Ctrl) and sterile humus (Ctrl-H) inoculations ensured the best shoot growth. These seedlings were on average 20% taller than in other treatments (Fig. 2b). Furthermore, the good nutrient status in Ctrl and Ctrl-H treatments was also reflected as the highest total N contents of needles (3.40±0.11 mg and 3.65±0.30 mg, ns, respectively). The lowest N content was found in *M. bicolor* inoculated seedlings (2.09±0.35 mg, $P<0.001$), and Ctrl-M (2.48±0.13 mg, $P<0.01$) and Humus

313 treatments (2.55±0.07 mg, P<0.05) when compared to the reference level (Ctrl). Needle N
314 content correlated negatively with *Meliniomyces* sp. colonisation (r=-0.49, P<0.01), and
315 positively with colonisation percent of *T. terrestris* (r=0.45, P<0.01) and *Amphinema* sp.
316 (0.36, P<0.05) and EMF species richness (r=0.40, P<0.02). Needle N concentration was on
317 average 1.14±0.03% in all growth substrates.

318 Roots had significantly lower biomass in Ctrl-M and *M. bicolor* inoculated substrates
319 compared to uninoculated Ctrl substrate, especially when exposed to *B. cinerea*. The
320 infection by *G. abietina* reduced root growth statistically significantly in all substrates except
321 Ctrl-H (Fig. 2d), and shoot growth was restricted even more than root growth as the
322 shoot:root- ratios were statistically significantly lower after *G. abietina* infection than in the
323 water only- foliar treatment (Fig. 2c). EMF colonisation % had a weak positive relationship
324 with root growth (r=0.29, P<0.001). Furthermore, EMF species richness had a weak positive
325 relationship with both shoot and root biomass (r=0.24and r=0.26, P<0.001).

326 *3.2.4 Roots were well colonized with M. bicolor*

327 As expected EMF colonisation was highest in *M. bicolor* inoculated substrate, almost 87%,
328 but there was a lot of variation in colonisation levels between seedlings in different
329 treatments (Table 3). In all the other substrates, roots were abundantly colonised (from one
330 fifth to almost 60%) with *Thelephora terrestris.* Also other root associated fungi were found
331 in lower quantities on the roots; forest humus inoculated seedlings, Humus, hosted the most
332 diverse fungal community associated to their roots comprising *Amphinema*, *Tylospora,*
333 *Cenococcum*, *Meliniomyces*, *Varicosporium, Phialocephala, Psilocybe* and
334 *Archaeorhizomyces* fungal genera (Table 3).

335 **4 Discussion and conclusions**

336 In the triaxenic tube experiment EMF colonisation of spruce roots by *M. bicolor*, *T.terrestris*
337 and three out of four *P. involutus* isolates provided protection against *Heterobasidion* root
338 rot, and as in the study by Hyder *et al.* (2013), the strongest protective effect was provided by
339 the ascomycetous EMF *M. bicolor*. In general the same strains, that were found to be
340 antagonistic to each other when subjected to mycelial confrontation *in vitro* (Hyder *et*
341 *al.*2013), had positive impacts on the viability of the seedlings also in present triaxenic
342 system which contained a tree seedling as the tested subject. However, a few exceptions were
343 found, most probably due to differences in experimental systems; the variable protective

mechanisms of EMF against root pathogens may include production of antifungal substances, induction of inhibitory compounds and root exudates from the host plant, and even microbial competitive potential amongst other things (Marx 1969, Marx 1973, Duchesne *et al.*1989, Chakravarty and Hwang 1991, Buscot *et al.*1992). For example the *T. terrestris* strain, that did not show any antagonism against *Heterobasidion* isolates *in vitro* plate tests (Hyder *et al.*2013), provided protection against the less aggressive root rot species *H. parviporum* (Swedjemark and Stenlid 1995) in the present study. *T. terrestris* grew thick external mycelia in the tubes (data not shown) and thus most likely formed also a physical barrier around the roots. Furthermore, *T. terrestris* seemed to provide efficient nutrient allocation to support good growth. Similarly Buscot *et al*. (1992) reported greater vigour of mycorrhizal Norway spruce seedlings when inoculated with *P. involutus* and *Laccaria laccata* since EMF could have increased host protection trough reinforcement of plant resistance and production of antifungal phenolics even though no direct antagonism was present. Likar and Regvar (2008) noted that induction of defence cascade after inoculation with the EMF *P. tinctorius* was only transient, and thus they claimed that Norway spruce could recognise the infecting fungus and activate and adjust appropriate defence mechanisms. Then again EMF colonisation have shown explicit protective effects against *Heterobasidion* spp., and also other root pathogenic fungi (*Rhizoctonia solani*, *Fusarium* damping-off, *Ilyonectria destructans* (in both pine and spruce (Farquhar and Peterson 1991, Hwang *et al.*1995, Buscot *et al.*1992, Zhang *et al.*2011, Martín-Pinto *et al*. 2006). Thus also in root tips that were highly covered with dense *M. bicolor* hypha, fungal colonization might have provided a physical protective barrier around roots in addition to direct antagonism of *M. bicolor* against *Heterobasidion*.

The most evident exceptions in the tube experiment were *P.involutus* isolate 1 and the root endophyte *P. fortinii*, which lacked all antagonism, and tended to reduce the fitness of the seedlings even in the absence of root pathogens. Similar within species variability of antagonistic abilities of certain EMF (*P. involutus*) against *Heterobasidion* isolates has previously been reported amongst others by Červinková (1989). The root endophyte *Phialocephala sphareoides* was shown to protect spruce seedlings from *H. parviporum* root infections *in vitro* by means of antifungal metabolites (Terhonen et al. 2016). Yet the antagonistic abilities of root endophytes are variable; Tellenbach *et al*. (2013) found only one antagonistic root endophytic fungal strain from over 80 tested isolates. Furthermore, also growth reduction in spruce, caused by the root endophyte *P. fortinii*, has been previously reported by Reininger *et al.* (2012), although they emphasize that plant growth responses are

both fungal strain and host species dependent. Despite possible negative correlations between host biomass and endophytic biomass it has also been speculated that spruce might even actively attract root endophytic fungi to provide protection against more serious pathogens (Tellenbach *et al.* 2011). Nevertheless, the present results suggest that this hypothesis may not hold with the necrotrophic *Heterobasidion* sp. pathogens.

M. bicolor was selected from the tube experiment for further testing in nursery conditions as it showed good antagonistic and protective properties *in vitro*. It has also shown to be a suitable EMF species for large scale inoculum production (Vuorinen *et al.*2015), and to abundantly colonize the roots of spruce. However typically the exploration of the extraradical mycelium of ericoid mycorrhizal fungal species of the *P. ericae* aggregate is narrow (Read 1984) and there is no evidence for the formation of large mycelial networks (Grelet *et al.* 2010).

In the nursery experiment, the infection of foliar pathogen *G. abietina* caused severe damage after winter in particular in seedlings growing in conventional fertilized peat (Ctrl), in contact with *M. bicolor* or in forest humus containing natural microbial fauna and flora. Hence, on the contrary to its effectiveness towards root pathogens *M. bicolor* was not able to provide protection from the foliar *G. abietina* infection. *G. abietina* changed resource allocation towards roots, as the decrease in growth was stronger in shoots than in roots. The addition of forest soil microbes along with humus increased the EMF diversity, N contents and biomass of the seedlings but did not provide benefits against foliar pathogens. Instead, the addition of fresh unheated humus seemed to be a slight risk factor probably due to the exposure of seedlings to wild needle damage caused by the microbes of the soil.

The only soil treatment where shoot and especially root growth seemed to be unaffected by *G. abietina* was the heat-treated humus. These seedlings also had the highest N storage in their needles and the best root and shoot growth within all the substrates. This was probably due to the autoclaving of humus that released microbe-bound nutrients or other beneficial compounds into the growing media without exposing seedlings to potential pathogens. Heating of humus up to less than 200 degrees Celsius has shown to increase the quantity of water soluble potassium (K) and phosphorus (P) without yet reducing the levels of N (White *et al.* 1973). *S*eedlings growing in the heat-treated humus were also the ones most abundantly colonised by EMF *T. terrestris*. *T. terrestris* has been found to secrete both N and P solubilizing exoenzymes in moderate quantities (Velmala *et al.*2014a), and the seedlings with high *T. terrestris* colonisation had high N content in our study.

Good nutritional status increases seedling survival (van der Driessche 1992). Seedlings grown in conventional fertilized peat invested more resources belowground when exposed to foliar stress by *B. cinerea* compared to the situation with no foliar stress, as has been reported previously (Velmala *et al.*2014b). Lack of nutrients seemed to reduce shoot and root growth significantly in Ctrl-M and *M. bicolor* inoculated treatments. Reduced growth was especially clear under foliar stress caused by *B. cinerea* and *G. abietina* when compared to the conventional (Ctrl) substrate. The frequently reported short term slowdown effects of EMF on shoot growth in early developmental stages of seedlings (Corrêa *et al.*2006, Vaario *et al.* 2009) could clearly be seen also in the present study, even though the amplitude seems to be milder under high N availability.

Seedlings inoculated with forest humus had the highest EMF richness, and established widely associations with both basidio- and ascomycoteus fungi that are recognized as EMF and endophytic fungi commonly found on spruce (Vohnik *et al.* 2013, Rosling *et al.* 2011). However proportions of other EMF than *T. terrestris* were minor, underlining the strong competitive strength of *T. terrestris* in nursery conditions. Yet, foliar infection of *G. abietina* seemed to reflect in ectomycorrhizal formation as the colonisation degree of these seedlings was the lowest in all substrates except for the heat-treated humus substrate. Similarly, we have found that severe exposure to the needle pathogen *Gibberella avenacea* has a slight negative effect on the EMF richness on Norway spruce seedlings showing slow long-term growth performance (Velmala *et al.* 2014b). Regardless of these indications of basipetal, top-down, movement of pathogen induced systemic signals we found no signs of EMF induced acropetal systemic effects. Moreover, in the present study the positive effect of high EMF colonization and diversity seems to be due to either direct antagonism or root mediated improved nutritional status. Thus, it seems that the host-microbe interactions are highly species and even strain-specific, and no general responses can be expected without throughout knowledge on the identity of the organisms. It appears very characteristic to EMF-host interactions that the within-species variations of effects on host performance are as high as the variation among different EMF species (Pennanen *et al*., unpublished). Our results also contradict the findings on the effects of *C. finlandica*, the close relative of *M. bicolor*, on spruce needle chemistry (Mrnka et al. 2009) which further supports our claim that even phylogenetically closely related EMF species may induce very different effects in trees.

In conclusion, our study showed that there are several potential EMF isolates that protect Norway spruce seedlings towards *Heterobasidion* root rot *in vitro*. Nevertheless, seedlings

that were inoculated with the antagonistic EMF isolate *M. bicolor* or were naturally colonised by *T. terrestris* in the nursery showed variable aboveground susceptibility towards foliar pathogens in nursery. Before introducing EMF inoculations into forest nursery practice further studies of possible effects of the tripartite interactions should be considered.

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6 Figure captions

Figure 1 Seedling vitality classes and growth of six month-old Norway spruce seedlings grown in glass tubes on Brown and Wilkins -media and inoculated with seven root associated fungi and subjected to *Heterobasidion* spp. pathogens. Seedling vitality is scored based on visual symptoms: the highest class includes only green needles (G), the middle classes light green (LG) and chlorotic needles (C), and the lowest classes either chlorotic needles with brown lesions or totally brown dead needles (B). Figure panels group *H. parviporum* and *H. annosum* infected seedlings visually together. The mycorrhizal inoculum is visible on the X-axis; (details in Table 1). Grey bars indicate that there is no statistically significant difference between the treatments and white bars show that the treatment in question differ statistically significantly ($P<0.05$) from the leftmost base line 0 treatment (no EMF and no *Heterobasidion*). The black error bars show the 95% confidence interval of the mean (n=5).

Figure 2 Needle damage %, and shoot and root growth of 1-yr-old Norway spruce seedlings in spring after wintering under natural snow cover grown in different substrates and subjected to two foliar fungal diseases *Botrytis cinerea* and *Gremmeniella abietina*. Figure panels group seedling subjected to same foliar treatments visually together (Water only, *B. cinerea* and *G. abietina*). The growth substrate is visible on the X-axis: **Ctrl** conventional fertilized peat, **Me** inoculated with EMF *M. bicolor*, **Humus** inoculated with natural forest humus layer and their sterilized controls **Ctrl-M** (no EMF) and **Ctrl-H** (sterilized humus), respectively (details in Table 2). All treatments are compared to the leftmost reference level **Ctrl-**

treatment that is fertilized peat and **water only**- foliar treatment; Grey bars indicate that there is no statistically significant difference, and white ($P<0.05$) and light grey ($0.05<P<0.1$) bars show that the treatment in question differ statistically significantly from the **Ctrl**-reference treatment. The black error bars show the 95% confidence interval of the mean.

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628

Tables

Table 1 The root associated fungal strains used in the triaxenic tube experiment

Name	Fungi	Antagonism against *Heterobasidion* spp.
0 (reference level)	No EMF	-
Pfor	*Phialocephala fortiini* R-RS07	No antagonism[a, b]
Mbic	*Meliniomyces bicolor* R-MF01	Large inhibition[b]
Tter	*Thelephora terrestris* R-NC10[c]	No antagonism[a]
Pinv1	*Paxillus involutus* F-CY01	Small inhibition[a]
Pinv2	*Paxillus involutus* BOUX	Small inhibition[a,b]
Pinv3	*Paxillus involutus* F-SS02	Medium inhibition[b]
Pinv4	*Paxillus involutus* F-YF05	Medium inhibition[b]

a) *H. annosum* b) *H. parviporum* c) isolate MT43205. Inhibition zone data is derived from Hyder *et al.* 2013.

Table 2 Content and labelling of the growth substrates used in the foliar pathogen experiment in nursery

Name	Growth media	Inoculum	V:V	Replicates
Ctrl	Fertilized blonde Sphagnum-peat[a]	None	1:0	18
Ctrl-M	Unfertilized blonde Sphagnum-peat[b]	Sipernat® growing matrix [c]	9:1	36
Me	Unfertilized blonde Sphagnum-peat[b]	*Meliniomyces bicolor* R-MF01 in Sipernat® growing matrix [c]	85:15	36
Ctrl-H	Unfertilized blonde Sphagnum-peat[b]	Sterilized forest humus [d]	1:1	36
Humus	Unfertilized blonde Sphagnum-peat[b]	Forest humus layer[d]	1:1	36

a) White 420 F6 is a fertilized, and **b)** PP03 is unfertilized, and pH adjusted (pH 4-5) blonde Sphagnum peat (Kekkilä Group, Vantaa, Finland). c) Solid silica-based cultivation matrix according to Vuorinen *et al.*2015 containing Sipernat® (Evonik Resource Efficiency GmbH, Essen, Germany), 2,5% Maltax 10 brewery malt extract (Senson Oy, Lahti, Finland) and 0.5 g/l Lignohumate AM (Amagro, Prague, Czech Republic). d) The humus was excavated from the uppermost layer of fine sandy till of a Norway spruce stand out planted in 1993 at the nearby Ruotsinkylä research area located in southern Finland (60.359012; 25.004210). Sterilization was performed twice, 20 min 121°C with a two days interval.

Table 3 EMF colonisation % and species richness of roots in the foliar pathogen experiment in nursery

1-yr-old Norway spruce seedlings were grown in different substrates and subjected to two foliar fungal diseases *Botrytis cinerea* and *Gremmeniella abietina*, and the **water only**-control**.** The growth substrates were fertilized peat **Ctrl**, unfertilized peat inoculated with *M. bicolor* **Me**, unfertilized peat inoculated with natural forest humus layer **Humus** and their sterilized controls **Ctrl-M** and **Ctrl-H***,* respectively (details in Table 2). Mean ± 1SE for each treatment are presented separately. Largest mean values are highlighted with grey fill colour, nd not detected.

		Water only	***B. cinerea***	***G. abietina***
Root tips with no EMF	***Ctrl***	55.1±13.4	47.5±9.61	78.0±9.49
	Ctrl-M	60.6±14.4	38.8±13.4	67.0±9.61
	Me	13.3±3.20	11.3±1.89	14.6±4.26
	Ctrl-H	56.8±14.0	50.4±14.3	37.4±8.82
	Humus	47.7±14.3	45.9±14.4	63.6±11.1
***Amphinema* sp.[a]**	***Ctrl***	0.10±0.10^{Ab}	0.10±0.10	nd
	Ctrl-M	nd	0.10±0.10^{Ab}	1.60±1.49^{Ab}
	Me	nd	nd	nd
	Ctrl-H	0.10±0.10	nd	nd
	Humus	1.00±1.00	1.50±1.50	nd
***Meliniomyces* sp.[a]**	***Ctrl***	0.50±0.22^{Mb}	2.20±1.08	0.40±0.22^{Mb}
	Ctrl-M	0.20±0.13	3.60±1.97	2.20±1.00^{Mv}
	Me	86.7±3.20^{Mb}	88.7±1.89^{Mb}	85.4±4.26^{Mb}
	Ctrl-H	2.90±2.47	4.30±3.98	3.70±2.08
	Humus	7.4±3.96	2.60±1.26^{Mb}	7.70±4.83
Thelephora terrestris	***Ctrl***	43.4±13.7	50.1±9.72	21.5±9.39
	Ctrl-M	38.9±14.4	57.3±13.9	29.2±10.2
	Me	nd	nd	nd
	Ctrl-H	39.8±13.2	43.6±14.5	58.3±8.71
	Humus	42.2±11.8	44.4±12.0	24.8±8.11
Cenococcum geophilum	***Ctrl***	nd	0.10±0.10	0.10±0.10
	Ctrl-M	0.30±0.15	0.10±0.10	nd
	Me	nd	nd	nd
	Ctrl-H	0.20±0.13	nd	nd
	Humus	1.40±0.98	3.90±2.08	3.20±1.97
Other[b]	***Ctrl***	0.90±0.90^{Ve}	nd	nd
	Ctrl-M	nd	0.10±0.10^{Pf}	nd
	Me	nd	nd	nd
	Ctrl-H	0.20±0.13^{Pf}	1.70±1.16$^{Pf, Ta}$	0.60±0.50^{Pf}
	Humus	0.30±0.21$^{Pf, Ve}$	1.70±1.06$^{Pf, Tf, A}$	0.70±0.52$^{Pf, Ta}$
Total root colonisation[c]	***Ctrl***	44.9±13.4	52.5±9.61	22.0±9.49
	Ctrl-M	39.4±14.4	61.2±13.40	33.0±9.61
	Me	***86.7±3.20***	***88.7±1.89***	*85.4±4.26*
	Ctrl-H	43.2±14.0	49.6±14.3	62.6±8.82
	Humus	52.3±14.3	54.1±14.4	36.4±11.1
Richness[c]	***Ctrl***	1.60±0.22	1.60±0.31	1.20±0.29
	Ctrl-M	1.20±0.25	1.80±0.20	1.70±0.21
	Me	1.0±0.0	1.0±0.0	1.0±0.0
	Ctrl-H	1.70±0.40	1.40±0.31	1.70±0.15
	Humus	2.20±0.33	2.30±0.47	2.10±0.31

a) Some samples identified even to species level: *Amphinema byssoides*, *Meliniomyces bicolor* and *M. variabilis*. *b)* Other includes infrequent root associated fungi: ***Varicosporium elodeae***, ***Phialocephala fortinii***, ***Archaeorhizomyces*** sp., ***Tylospora fibrillosa***, ***Tylospora asterophora***. *c)* significant differences in EMF total colonisation between the reference level *F6* fertilized sphagnum peat substrate in water only- foliar treatment (underlined) and *Me* treatments are emphasized with strengthen italics (P<0.05) and italics (P<0.1). No statistically significant differences in EMF richness between treatments.