Effectiveness of five fungal isolates as mycorrhizal inoculants of birch seedlings

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ABSTRACT

Five fungal isolates were tested under nursery and field conditions as potential ectomycorrhizal inoculants for Downy birch (*Betula pubescens*) seedlings for a harsh site. Thirteen inoculum treatments, along with non-inoculated control seedlings, were randomly selected for the experiment. In the nursery, inoculation treatments were variably effective in enhancing mycorrhization and the inclusions of either *Laccaria laccata, Paxillus involutus* or *Thelephora terrestris* into inoculum treatments increased mycorrhization. Mycorrhization increased seedling height and reduced seedling root collar diameter relative to seedling height. In the field, mycorrhization and the inclusion of *L. laccata* into inoculation treatments in the nursery had positive effects on seedling survival while the isolate diversity and the inclusion of *Cadophora finlandica* into inoculation treatments in the nursery had negative effects. Furthermore, the isolate diversity and the inclusion of *T. terrestris* into inoculation treatments in the nursery had negative effects on shoot growth in the field. The results indicate that three of the isolates tested: *Hebeloma velutipes, L. laccata* and *P. involutus,* could potentially be used as mycorrhizal inoculants of birch seedlings for reclamation areas in Iceland, but further studies are needed.

Keywords: Container seedlings, *Betula pubescens*, ectomycorrhiza, forest nursery, reclamation.

YFIRLIT

Áhrif af smitun fimm svepprótamyndandi sveppa á birkiplöntur

Svepprótamyndun fimm sveppastofna og áhrif þeirra á vöxt og lífslíkur birkiplantna (*Betula pubescens*) var könnuð í gróðrarstöð og á erfiðu uppgræðslusvæði. Þrettán tilviljunarvaldar smitmeðferðir voru prófaðar ásamt ósmituðum samanburðarplöntum. Svepprótamyndun í gróðrarstöð var breytileg eftir smitmeðferðum. Þær meðferðir sem ollu marktækt aukinni svepprótamyndun innihéldu ýmist *Laccaria laccata*, *Paxillus involutus* eða *Thelephora terrestris*. Vöxtur plantna jókst við aukna svepprót í gróðrarstöð. Eftir gróðursetningu juku svepprótamagn og meðferðir með *L. laccata* lifun, en *Cadophora finlandica* og fjölbreytni sveppa í upphaflegum smitmeðferðum höfðu neikvæð áhrif. Fjölbreytni sveppasmits og smitmeðferðir sem innihéldu *T. terrestris* drógu úr sprotavexti plantna eftir gróðursetningu. Niðurstöðurnar sýna að þrír af þeim sveppastofnum sem prófaðir voru: *Hebeloma velutipes, L. laccata* og *P. involutus* gætu komið til greina til að smita plöntur sem ætlaðar eru í landgræðsluskógrækt, en þörf er á frekari rannsóknum.

INTRODUCTION

Mycorrhizal seedlings raised in forest nurseries generally have greater chances of becoming established in the field than those without mycorrhizae (Khasa et al. 2009). This is because the mutualistic association forms an essential root interface with the surrounding soil. Systematic mycorrhizal management should, therefore, be a key part of forest seedling production. This is possible to achieve if the chemical and biological conditions in the rooting substrate are set to promote fungal growth and mycorrhizal development in forest nurseries (Quoreshi & Timmer 1998, Óskarsson 2010). Ideally, tree seedlings leaving the nursery should be equipped with a variety of mycorrhizal fungi which offer functional benefits both in the nursery and the field (Perry et al. 1987).

The selection of fungal isolates (different species or different strains within species) for inoculation can influence growth and health of nursery seedlings and their subsequent field survival and growth (Stenström & Ek 1990, Pera et al. 1999, Brundrett et al. 2005). Certain isolates are better suited than others for hampering root diseases or are better capable of improving nutrient and water acquisition for given tree species and conditions (Thomas et al. 1983, LeTacon & Bouchard 1986, Sousa et al. 2012). The fungal isolates chosen can also be very variable in their ability to form mycorrhizae and to compete with each other under the conditions in which the seedlings are growing (Parlade et al. 1996, Jonsson et al. 2001). This ability depends, at least partly, on the fungal carbohydrate requirements. Plants can control mycorrhizal colonization by controlling carbon allocation to short roots, depending on the efficiency of the symbionts (Hoeksema & Kummel 2003). Thus, fungi with low carbohydrate requirements are often best suited for inoculation in forest nurseries.

Theoretically, the choice of potential inoculants is large for each tree species, for example, several fungi: *Amanita* spp., *Boletus* spp., *Cenococcum* spp., *Cantharellus* spp., *Cortinarius* spp., *Hebeloma* spp., *Hymenogaster tener* Berk., *Inocybe* spp., *Laccaria* spp., *Lactarius* spp., *Leccinum* spp., *Paxillus* spp., *Peziza spp*., *Ramaria* spp., *Russula* spp., *Scleroderma* spp., *Thelephora terrestris* Ehrh. and *Tricholoma* spp. form mycorrhizae with birch, (Dighton & Mason 1985, Atkinson 1992). Of these, *Hebeloma* spp., *Inocybe* spp., *Laccaria* spp., *Paxillus involutus* (Batsch) Fr. and *T. terrestris* are able to form mycorrhiza on young birch seedlings under unsterile conditions (Flemming 1985, Fox 1986). These fungi are commonly found in forest nurseries and are often used for inoculating forest seedlings. In addition, *Cadophora finlandica* (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew is among numerous less known ectomycorrhizal fungi that can be common in forest nurseries (Menkis et al. 2005).

In the present study, five fungal isolates, belonging to five fungal species that commonly form ectomycorrhizae with birch seedlings and were commercially available, were tested as potential mycorrhizal partners of birch during seedling production and after planting in the field. The object of the study was to compare the effectiveness of the fungi to increase mycorrhization in the nursery and to improve seedling survival and growth after planting at a difficult reclamation site in Iceland.

MATERIALS AND METHODS

Four week old Downy birch seedlings (seed source Næfurholt, 64°00’ N, 19°53’W; elevation 132 m), a total of 210 plants, were transplanted into 1.5 L pots, three seedlings per pot. The potting substrate was local sedge peat (source location: 64°00’N, 21°10’W) mixed with rhyolitic pumice (1 part pumice in 2 parts peat; pumice grain size 0.5-0.7 cm). The acidity of the substrate mixture was pH 5.2.

Thirteen randomly chosen inoculation treatments out of 21 possible arrangements of five fungal isolates were tested in the experiment (Table 1). During transplanting into the pots, two millilitres of diluted ectomycorrhizal inoculum (1 part vegetative mycelium [Plant-Works Ltd., Sittingbourne, UK] in 4 parts tap water; v:v) were injected into the planting

Table 1. Relative amounts of each fungal isolate and the resulting diversity of fungal isolates in each inoculation treatment.

	Inoculation treatments													
Isolates	**1***	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
C. finlandica							0.5			0.33	0.33	0.33	0.33	
H. velutipes			1			0.5			0.33			0.33		0.25
L. laccata		1			0.5				0.33	0.33	0.33			0.25
P. involutus				1		0.5		0.5	0.33	0.33		0.33	0.33	0.25
T. terrestris					0.5		0.5	0.5			0.33		0.33	0.25
Isolate diversity	0	1	1	1	2	2	2	2	3	3	3	3	3	4

* Non-inoculated control seedlings

holes, a method adapted from Boyle et al. (1987). A few seedlings, not included in the study, were also inoculated with each of the isolates separately for examination of ectomycorrhizae. Non-inoculated seedlings served as controls. The isolates tested included *Hebeloma velutipes* Bruchet (isolate PW Hv1; belonging to the *H. crustuliniforme* [Bull.] Quel.) complex, *Laccaria laccata* (Scop. ex Fr.) Berk. & Bromme (isolate PW *Lal*1), *Paxillus involutus* (isolate PW *Pax*1), *Thelephora terrestris* and *Cadophora finlandica* (syn. *Phialophora finlandia* C.J.K. Wang & H.E. Wilcox; *Mycelium radicis atrovirens* Melin). The *H. velutipes* and *C. finlandica* isolates were isolated in Iceland from a sporocarp associated with a 2 year old birch transplant and a mycorrhizal short root in natural birch woodland, respectively. Information on the origins of the other isolates was not available.

Inoculation treatments were arranged randomly in a heated greenhouse and grown for 75 days (14°C night and ≥16°C day) from June to September 2005. Fertilizers were fed through the watering system. For the first 20 days, seedlings were fed with a dilute (0.2 g L^{-1}) Superex (Nursery Stock Superex [19-10-24], Kekkila Oyj, Finland) solution along with 0.08 g L^{-1} monopotassiumphosphate (0-52-34; Haifa Chemical Ltd, Israel). This corresponded to 0.04 gL^{-1} N, 0.03 g L^{-1} P and 0.06 g L^{-1} K. After this the nutrient solution was slowly strengthened, without the monopotassiumphosphate, and was eventually applied as 2.0 g L^{-1} Superex towards the end of the nursery period. This corresponded to 0.38 g L^{-1} N, 0.08 g L^{-1} P and 0.40 g L^{-1} K. Every day the pots were watered with the nutrient solution, through a drip watering system, until the field capacity of the potting mixtures was exceeded and excess water seeped through the underside drain holes of the pots.

Each pot represented one treatment unit, replicated five times in blocks. After cessation of shoot growth, the root systems of plants in each pot were carefully separated and during the process most of the potting substrate fell off the roots. Seedling height and root collar diameter were measured. Random samples (ca. 10% of root systems) were removed from each plant and then pooled per pot to estimate the amount of mycorrhizae. The number of mycorrhizal short roots was counted and expressed as a ratio of total number of short roots in each sample (Brundrett et al. 1996).

In October 2005, all bare root seedlings were planted in a reclamation area (Óskarsson 2012) near Hekla volcano in south Iceland (64°05' N, 19°41' W; elevation 260 m). The site was a pumice field that had been seeded with Bering's tufted hairgrass (*Deschampsia beringensis* Hultén) a few years earlier to stabilize the soil surface. The experiment in the field had the same layout as in the greenhouse. At planting, each seedling received 0.17 g of granular fertilizer (12-15-17; Áburdarverksmidjan, Reykjavík, Iceland) placed at the seedling base. This equalled 0.02 g N, 0.01 g P and 0.02 g K.

The field experiment was surveyed for 6 years. Surviving plants were counted and their total height, root collar diameter and the length

of current year terminal shoot were measured at the end of each growing season. The length of dieback on the previous year's terminal shoot was also measured.

The effects of inoculation treatments in the nursery and the number of isolates added to the inoculum (isolate diversity) on seedling mycorrhization and plant parameters in the nursery and the field were tested using one-way ANOVA and Tukey's test to find significant differences between means. The correlation between individual fungal isolates, mycorrhization and plant parameters was investigated using Pearson correlation method, corrected for the effect of seedling position within the experiments in the nursery and the field. Statistical analyses were done by SAS 9.2 for Windows (SAS 2002-2010).

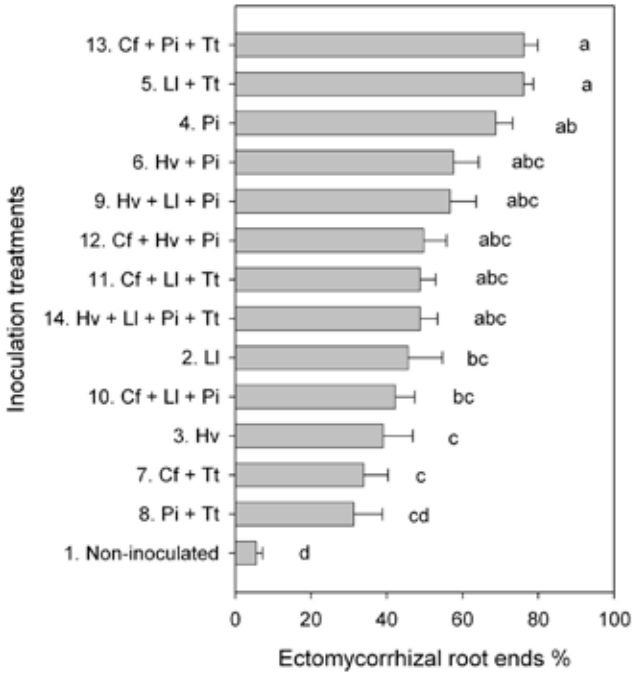

Figure 1. Ectomycorrhizal colonization of seedlings at the end of the nursery period by inoculation treatments. Each column represents treatment means (n= 15), error bars indicate standard deviations of means, and different letters to the right of the bars denote significant difference of means (Tukey's test P< 0.05). Fungal isolates in inoculation treatments are abbreviated to the left of the columns; Cf= *C. finlandica*, Hv= *H. velutipes*, Ll= *L. laccata*, Pi= *P. involutus*, Tt= *T. terrestris*.

RESULTS

All fungal isolates tested were able to form abundant ectomycorrhizae on birch seedlings under the conditions provided in the nursery. Inoculation treatments significantly influenced seedling mycorrhization (F value= 10.29, P <0.001, DF= 13). Individual treatments produced ectomycorrhizae on 34% to 76% of seedling root ends in the nursery. At the same time, 6% of short roots became ectomycorrhizal on the control seedlings. Two treatments, a mixture of *L. laccata* and *T. terrestris* and a mixture of *P. involutus*, *C. finlandica* and *T. terrestris*, produced significantly more mycorrhiza than five other inoculum treatments and control seedlings (Figure 1). Furthermore, a single isolate inoculation of P. *involutus* produced significantly more mycorrhiza than three inoculum treatments (Figure 1). Control seedlings had significantly lower mycorrhizal colonization than all but the lowest inoculum treatment (Figure 1).

The inclusion of any of three fungal isolates in the original inoculum was positively correlated with mycorrhizal colonization in the nursery. These were: *L. laccata* (r=0.14, P=0.044, n= 208), *P. involutus* (r=0.23, P=0.001, n= 208) and *T. terrestris* (r=0.15, P=0.035, n= 208). Isolate diversity in the original inoculum >0 did not influence mycorrhizal colonization in the nursery.

In the nursery, inoculation treatments significantly influenced the ratio between seedling root collar diameter (RCD) and seedling height (relative RCD; F value= 2.14, P= 0.014, DF= 13). Furthermore, mycorrhizal colonization was positively correlated with seedling height (r= 0.27, P< 0.001, n= 208) and seedling RCD (r= 0.18, P>0.001, n= 208), but negatively with relative RCD

(r= 0.29, P< 0.001, n= 208). The relationship between mycorrhizal colonization, height and relative RCD is presented in Figure 2.

After planting in the field, only one third of all seedlings survived the first winter. Seedling survival in the field was only slightly positively correlated with mycorrhizal colonization in the nursery (r= 0.14, P=0.037, n=208) and also with the inclusion of *L. laccata* in the inoculum treatments in the nursery (r= 0.15, P= 0.027, n= 208). However, the isolate diversity in the inoculum was negatively correlated with seedling survival in the field (r=-0.14, P=0.032, n=208) and the inclusion of *C. finlandica* in the inoculum treatments also reduced seedling survival (r= -0.28, P<0.001, n= 208).

About half of the surviving seedlings suffered shoot dieback during the first winter, but neither plant parameters, treatments nor isolates correlated with the degree of shoot dieback.

Shoot growth in the field generally dwindled with time (Figure 3). Seedlings inoculated with one, two or three fungal isolates, which had grown better than seedlings in other treatments in the nursery, grew successively slower with time, and so did seedling receiving four fungal isolates (Figure 3). Non-inoculated seedlings, however, grew at a similar rate for the first two years in the field as in the nursery (Figure 3). In the 6th summer in the field, a negative correlation appeared between shoot growth and isolate diversity in the nursery treatments (r= -0.27, P= 0,025, n=70; Figure 3). At the same time, the inclusion of *T. terrestris* in the inoculum treatments in the nursery correlated negatively with shoot growth (r= -0.26, P= 0.035, n= 70).

DISCUSSION

The present study agrees with other studies showing that inoculation with mycorrhizal fungi is necessary in order to increase mycorrhization of young forest seedlings in container

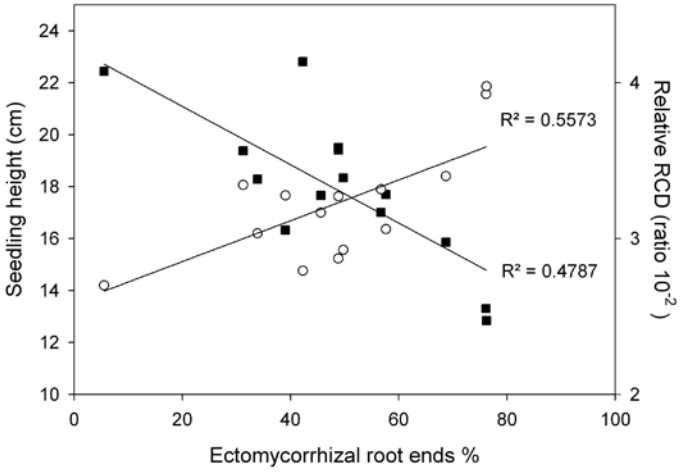

Figure 2. The relationship between ectomycorrhizal colonization and seedling height (left hand axis, o) and relative RCD (right hand axis, (■) at the end of the nursery period.

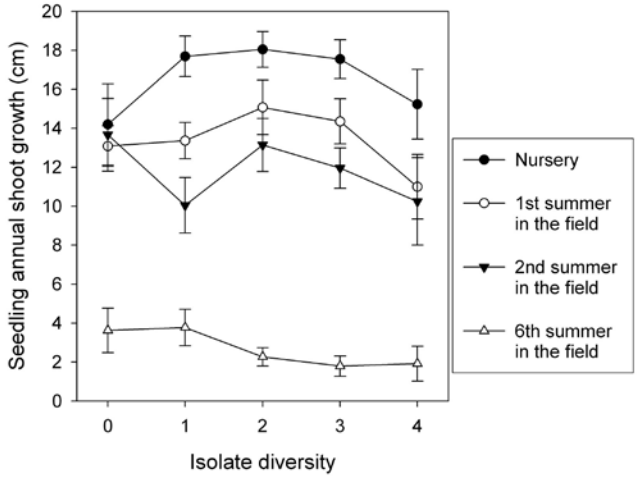

Figure 3. Seedling annual shoot increment in the nursery and the field by isolate diversity (number of fungal isolates included in treatments in the nursery). Vertical bars denote standard deviation of the means.

nurseries. Peat deposits that are commonly used as rooting substrates for containers usually lack mycorrhizal propagules (Tammi et al. 2001) whereas soils in nursery beds, where bare root seedlings are repeatedly raised, can contain numerous mycorrhizal fungi (Menkis et al. 2005). Although the sedge peat used in the present study is deficient in mycorrhizal propagules, this substrate along with the fertilization regime employed, provides excellent conditions for mycorrhization of forest seedlings (Óskarsson 2010).

The fungal isolates in the present study were variably effective as mycorrhizal colonizers. While isolate numbers >0 in the inoculum didn't cause differences in the total mycorrhizal colonization, the results showed that one isolate, *L. laccata* was less effective in a single isolate treatment than in dual combination with *T. terrestris*. Interestingly, Sudhakara Reddy & Natarajan (1997) obtained similar results; *L. laccata* and *T. terrestris* were more effective together than separately in increasing the rate of mycorrhizal colonization and growth of pine seedlings. Conversely, in the present study, however, *P. involutus* was more effective alone than in combination with *T. terrestris.*

Variable outcomes in such studies are common and depend on numerous factors. For example, in studying pine and birch seedlings and several mycorrhizal isolates, the only positive effects of isolate diversity that Jonsson et al. (2001) found was on the growth of birch seedling in a low fertility substrate, whereas the effects on pine growth were negative in high fertility substrate. Kennedy et al. (2007) also found that increased diversity of *Rhizopogon* spp. isolates negatively affected mycorrhizal biomass, shoot growth and leaf N of pine seedlings. Similarly, even though Baxter and Dighton (2001) found that higher isolate diversity increased total mycorrhizal colonization and uptake of P and N of birch seedlings, shoot biomass was negatively affected.

In multi-isolate combinations, the substrate condition can determine the outcome of competition between isolates for root ends, nutrients and carbohydrates. One of the factors that can influence the competitive abilities of a particular fungal isolate is its inoculum potential (Kennedy & Burns, 2005). In some cases, where a fungal isolate has a slight advantage in competition, it can eliminate other isolates and colonize whole root systems (Hönig et al. 2000, Jonsson et al. 2001, Kennedy & Burns 2005). In other cases, the mycorrhizal colonization of each isolate decreases relative to the number of isolates (Baxter & Dighton 2001, Jonsson et al. 2001).

In the present study, inoculation treatments only had an effect on seedlings in the nursery and isolates that were synergistic became neutral or even antagonistic in the field. Similar observations were made by Jackson et al. (1995), where differences in mycorrhization and growth in the nursery due to fungal isolates did not translate into differences in the field.

With time, new roots become colonized with mycorrhizal fungi in the field, which are often better adapted to the field conditions, and the mycorrhiza from the nursery may give way to a greater variety of fungi (Dighton & Mason 1985). Nonetheless, already established mycorrhizal isolates can be very persistent. For example, isolates of *Laccaria* spp. that were used for inoculation in a nursery persisted for many years in the field on root systems without preventing the colonization of other mycorrhizal species (Selosse et al. 2000). *T. terrestris* has also been reported to be persistent on plants, perhaps mostly in environments poor in mycorrhizal competitors (Hilszczanska & Zbigniew 2006).

In the present study, the antagonistic effects in the field associated with the inoculation of *T. terrestris* in the nursery awaits further study, but could possibly be related to its persistence on roots and the investment of the mutualistic system in root mass and explorative mycelium which *T. terrestris* is known to develop (Agerer 2001). The slower shoot growth of isolate diversity > 1 in the 6th summer can also be attributed to T. terrestris, since this isolate was not part of the single isolate treatments. It is

also possible that more beneficial mycorrhizal fungi for seedling shoot growth were better able to colonize non-inoculated and single isolate treated seedlings but not seedlings with a greater variety of isolates. The overall slow growth in the field, however, reflects the harsh conditions at the site and a lack of nutrients. The poorly developed tephra-derived soil at the site is almost void of nitrogen and seedlings need repeated fertilizer additions to grow, as shown for similar situations in Iceland (Óskarsson et al. 2006).

In conclusions, three of the isolates tested: *H. velutipes*, *L. laccata* and *P. involutus*, could potentially, based on the present study, be used as mycorrhizal inoculants of birch seedlings for reclamation areas in Iceland. *L. laccata* was the only isolate in the study that appeared synergistic both in the nursery and the field. *H. velutipes* appeared neutral throughout the study, but this fungus is sometimes found fruiting beside planted birch seedlings under similar conditions. In fact the isolate used in the present study was originally isolated in the reclamation area where the study was conducted. *P. involutus*, apparently the most effective isolate in the nursery based on mycorrhization efficiency, also appeared neutral in the field. However, clearly further studies are needed on inoculum development.

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