Seed Provenance Impacts Growth and Ectomycorrhizal Colonisation of *Picea abies* Seedlings

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Abstract

We studied morphological parameters and ectomycorrhizal (ECM) fungal colonisation of Norway spruce (*Picea abies*) seedlings produced from seeds of Western (W1 and W2 provenances), Central (C1 and C2 provenances) and Eastern (E1 provenance) seed regions in Latvia. In total, 50 seedlings of each seed provenance were cultivated in a sphagnum peat substrate in the plastic pots, which were randomly arranged in an open field in order to allow natural ECM fungal colonisation. After six-year cultivation in a forest nursery, significant differences both in morphological parameters (shoot height, root collar diameter and shoot volume) and in ECM fungal colonisation were detected among seedlings of different regions and provenances. Seedlings of C2 and W2 showed significantly better growth and possessed significantly higher number of ECM morphotypes, while seedlings of W1, and in particular of C1, were of the poorest growth and possessed the lowest number of ECM morphotypes. Nine fungal taxa were revealed amplifying the internal transcribed spacer of fungal ribosomal DNA from 102 selected ECM root tips; amplicon separation on denaturing gradient gel electrophoresis (DGGE) gels and sequencing methods were used. Basidiomycete *Amphinema byssoides* (33.1%) and ascomycete *Wilcoxina* sp. (50.3%) dominated fungal communities in roots but their abundances significantly varied among seedlings of different regions and provenances. Principal component analysis showed that fungal communities in seedling roots from the Western, Central and Eastern seed regions were separated from each other thereby showing their certain specificity. In conclusion, the results demonstrated that despite *P. abies* seedlings of different seed regions and provenances were exposed to the same growth conditions and natural ECM fungal colonisation, the genetic background of the seeds has likely influenced both seedling growth and their root colonisation by ECM fungi. This can be of significant practical importance for successful establishment and growth of seedlings in the nursery and after their outplanting in the field.

Key words: *Picea abies*, forest nursery, seed provenance, ectomycorrhiza, fungal community

Introduction

Norway spruce (*Picea abies* (L.) Karst.) is a principal tree species of temporal and boreal forests and one of the most important commercially grown conifers in Europe (Larsson 2001). In Latvia, *P. abies* occupies ca. 17% (537.4 thousand hectares) of forest land and therefore is widely used in reforestation (Jansons 2011). Among other factors, seed provenance may have a significant effect on survival and growth of replanted tree seedlings (Ying 1991) and the use of local seed provenances is often recommended because they are thought to be better adapted to local site conditions (Rone 1984, Gailis 1993). For *P. abies*, the territory of Latvia is divided into three seed regions (Western, Central and Eastern) each possessing established seed orchards of local provenances. Commercial nursery production in Latvia exceeds 23 million *P. abies* seedlings yearly. For seedling production, spruce seeds are predominantly used from the Central region (53%) and to a lower extent from the Eastern (23%) and Western (24%) regions (personal communication, L. Zvejniece, Deputy Director for production at Latvia's State Forests, Seeds and Plants).

Genetic factors are directly linked to the seed provenance and may significantly affect quality and consequently the growth of the conifer trees (Eiche and Andersson 1974). Ectomycorrhizal (ECM) fungi may also significantly affect early survival and the growth of *P. abies* seedlings (Menkis et al. 2007, Menkis et al. 2011) through improved nutrition and supply of water (Smith and Read 1997). However, information on

how the genetic background of *P. abies* seedlings affects communities of the ECM fungi is scarce. A recent Finnish study, which deployed rooted *P. abies* cuttings of different clones, has shown a statistically significant clone-specific effect on the ECM fungal diversity and abundance of dominating fungal taxa (Velmala et al. 2013). Furthermore, similar effects of host genotype were also observed in mid-age clonal *P. abies* stands concomitantly showing that the ECM fungal diversity and community structure were commonly associated with the growth parameters of the hosts (Korkama et al. 2006). The latter observations may also suggest that seed provenance might not only influence growth and productivity of forest stands, but also diversity and composition of the ECM fungal communities. The ECM fungal communities could be an important factor while selecting particular seed provenance for large scale cultivation of *P. abies* seedlings (Korkama et al. 2006).

The aim of the present study was to investigate whether *P. abies* seedlings representing different seed regions and provenances in Latvia differ in growth parameters (shoot weight, volume and root collar diameter) and ECM fungal colonisation of roots (richness of morphotypes, ECM species diversity and composition).

Material and Methods

Seed material and production of seedlings

Seeds of five different provenances representing the Western (W1 and W2 provenances), Central (C1 and C2) and Eastern (E1) seed regions of *P. abies* in Latvia (Figure 1, Table 1) were obtained from the seed storage centre of JSC Latvian State Forests. Seeds were collected in commercial seed stands (provenances W1, C1, C2 and E1) from at least 50 trees in each or in a seed orchard (Remte seed orchard – W2). Remte seed orchard represents first generation clonal material of 54 clones. In total, 50 seedlings of *P. abies* were produced from seeds of each provenance adding up to 250 seedlings altogether. In April 2006, seeds were sowed and seedlings were cultivated for three years bare-rooted in sandy soil at the Forest Research station in Kalsnava (N 56º68', E 25º96') (Figure 1). Before the study, conifer seedlings were regularly cultivated in this soil and no substrate sterilisation was carried out. Seedling cultivation included fertilization twice a year using NPK fertilizer (Bayer CropScience, Monheim, Germany), and at initial stage of cultivation application of pesticide Previcur 607 (Bayer CropScience, Monheim, Germany) and twice a year application of pesticide Actara (Syngenta, Basel, Switzerland). No artificial ECM fungal inoculation was carried out. In April 2009, three-years-old seedlings of *P. abies* were separately replanted in 2 L plastic pots (MCI 17, East Riding Horticulture Ltd., York, UK) filled with a sphagnum peat substrate KKS-M1, pH 4.5 (SIA Laflora, Jelgava, Latvia). By this, root systems of the seedlings were separated from each other. Each plastic pot with established seedlings of different seed provenances was labelled and randomly arranged in an open field, where they were grown for next three years. At this stage of seedling cultivation NKP fertilizer was applied once in a season (approx. 6 g per seedling) and extra fertilization with Vito-Silva mineral fertilizer (AS Spodriba, Dobele, Latvia) in spring 2010. No pesticides were applied to the seedlings after transplantation in pots.

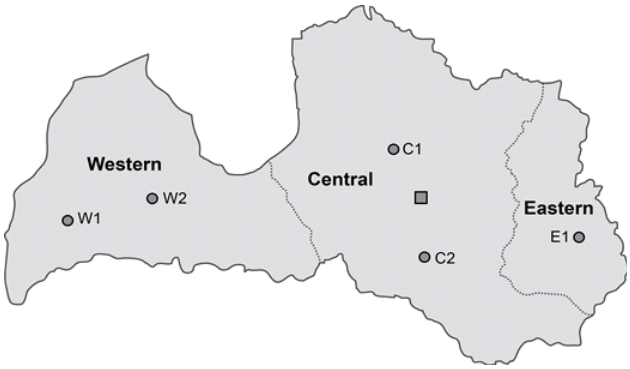

Figure 1. Map of Latvia showing the Western, Central and Eastern seed regions **(**separated by a dashed line**)** of *Picea abies*. Provenances W1, W2, C1, C2 and E1, from which seeds were used, are indicated by the circles and the nursery, where seedlings were cultivated, is indicated by a square

Seedling measurements and assessment of ectomycorrhizal roots

Measurement of seedlings and root sampling were done six years after seed sowing in January, 2012, i.e. at the time of dormancy. Seedling height and root collar diameter were measured. Root samples were collected taking all root system out of the pots and cutting one part, ca. 10% of all root volume, with the substrate using secateurs. Root samples were placed in plastic bags, transported to the laboratory and stored at 4 ºC until further processing. Roots were carefully washed in tap water, cut in 1 cm-long sections and evenly spread in water in Petri dishes with a grid (mesh size 7 × 7 mm) on the bottom and examined under stereomicroscope Leica MZ-7.5 (Leica, Solms, Germany). For each seedling, 30 randomly selected squares of the grid were used to estimate the number of fine living roots, their mycorrhization and ECM fungal morphotypes (Agerer 2001). ECM roots were identified by the presence of mantle, external hyphae

or rhizomorphs and the absence of root hairs (Smith and Read 1997). The ECM roots were divided into different morphotypes based on their colour, form and texture of the mantle and pattern of rhizomorphs and/or external mycelia (Agerer 1986-2006). One to five root tips of each ECM morphotype per seedling were collected, placed individually in 1.5 ml centrifugation tubes. Root tips were stored in 70% ethanol at -20ºC. Ethanol was removed and roots dried on sterile filter paper just before molecular identification procedure.

Molecular identification of fungi

Three to five root tips of each ECM morphotype from seedlings of each provenance (Table 1) were randomly selected. Isolation of DNA was done following protocol described by Vainio et al. (1998). Internal transcribed spacer of fungal ribosomal DNA (ITS rDNA) was amplified by PCR using fungal specific primer ITS1F (Gardes and Bruns 1993) with a 40 bp GC clamp (Muyzer et al. 1993) and universal primer ITS2 (White et al. 1990). The 50 µl PCR reaction was performed with Dreamtaq DNA polymerase (Fermentas, Thermo Scientific, Waltham, MA, USA) in Arktik TM Thermal Cycler (Thermo Scientific, Waltham, MA, USA). The cycling conditions were optimized according to manufacturer's instructions: 5 min initial denaturation at 95 ºC, followed by 35 amplification cycles (30 s denaturation at 95 ºC, 30 s annealing at 57 ºC and 60 s extension at 72 ºC) and 5 min final extension at 72 ºC (Thermo Scientific, Waltham, MA, USA). Success of PCR-amplification was verified on 1% agarose gels using electrophoresis. Denaturing gradient gel electrophoresis (DGGE) was performed using D-GENE gel system (Bio-Rad, Hercules, USA) at 75 V, 60 ºC for 16 h as described by Korkama et al. (2006). Based on DGGE analysis, all unique PCR amplicons, and between two and three amplicons of the same pattern were selected. Selected samples were amplified by PCR using original DNA extracts and a primer pair ITS1F-ITS4 (White et al. 1990, Gardes and Bruns 1993). Sanger sequencing of these samples was performed by Macrogen Europe (Macrogen Inc., Amsterdam, The Netherlands). The resulting sequences were analysed in BioEdit v. 7.0.9.0 (Hall 1999). To identify the fungal taxa, sequences were compared with reference sequences at GenBank (Altschul et al. 1997) and UNITE http://unite.ut.ee/ (Kõljalg et al. 2005) using blastn. The criteria used for identification were: sequence coverage > 80%; similarity to taxon level 97–100 %, similarity to genus level 94–96%. Sequences generated in the present study are available from GenBank under accession numbers KP172303-KP172311.

Table 1. *Picea abies* seed provenances used in the present study

Seed region [a]	Provenance	Position of forest stand or seed orchard	Year of seed collection
Western	W1	Saldus forest district, Sesile forestry (N56°36', E21°35')	1992
	W2	Seed orchard Remte, Saldus forest district, Remte forestry (N56°44', E22°47')	1993
Central	C1	Cesis forest district, Zaube forestry (N57°02', E25°45')	1990
	C2	Jekabpils forest district, Briezi forestry (N56°23', E25°47')	1998
Eastern	E1	Ludza forest district, Ludza forestry (N56°30', E27°45')	1990

[a] The territory of each seed region of *P. abies* in Latvia is shown in Figure 1.

Statistical analyses

Direct shoot measurements (shoot height and root collar diameter) were used to calculate shoot volume for each seedling. Shoot volume was calculated using a cylinder volume formula ($V=\pi \times r^2 \times h$, where 'r' is a radius of root collar and 'h' is a height of seedling shoot) modified from Stenström and Ek (1990).

Relative abundance of the ECM morphotypes was calculated as a proportion of living roots of each morphotype from a total number of living fine roots observed in analysed grid squares. Based on molecular species identification data, relative abundance of the ECM morphotypes was compared with fungal species data. If molecular data revealed that two species formed one morphotype, its relative abundance was divided between species.

Differences in seedlings morphological parameters (height, root collar diameter and stem volume), the number of ECM morphotypes and relative abundance of ECM fungal taxa among seedlings produced from seeds of different provenances were analysed by one-way analysis of variance (ANOVA) and Tukey's test, which provides confidence intervals for all pairwise differences between means (Chalmers and Parker 1989, Fowler et al. 1998). Each parameter was tested for normal distribution using Shapiro and Wilk normality test (Royston 1982). If data were not normally distributed, Wilcoxon test was used instead (Hollander and Wolfe 1999). Statistical analyses were performed in R 2.15.3 software (Vienna, Austria) (R Development Core Team 2008). Shannon diversity indices and quantitative Sørensen similarity indices were used to characterise diversity and composition of fungal communities among seedlings from different provenances (Shannon 1948, Magurran 1988). These indices were calculated in ComEcoPaC (Drozd 2010). Comparisons among fungal communities were made using principal component analysis (PCA) in Canoco 4.5 (ter Braak and

Smilauer 1998). Spearman rank correlation coefficients (Hollander and Wolfe 1999) were calculated to evaluate correlation between relative abundance of ECM taxa and seedling morphological parameters.

Results

After six-year cultivation in a forest nursery significant differences in morphological parameters were detected among seedlings produced from seeds of different regions and provenances (Table 2). Shoot height was highest in seedlings of C2, and then was followed by seedlings of E1, W2, W1, and it was the lowest in seedlings of C1. Root collar diameter was the highest in seedlings of C2 and W2, which were followed by seedlings of W1, E1, and it was the lowest in seedlings of C1 (Table 2). As a result, stem volume was the highest in seedlings of C2, and then was followed by seedlings of W2, E1, W1, and it was the lowest in seedlings of C1. Number of ECM morphotypes was the highest in seedling of W2, which were followed by seedlings of E1, C2, W1, and it was the lowest in seedlings of C1 (Table 2). Consequently, seedlings of C2 and W2 showed significantly better growth and possessed significantly higher number of the ECM morphotypes, while seedlings of W1, and in particular of C1, were of the poorest growth and possessed the lowest number of ECM morphotypes (Table 2).

The molecular analysis of 102 selected ECM root tips revealed the presence of nine distinct fungal taxa, including five basidiomycetes and four ascomycetes (Table 3). Despite the observed richness of taxa in each phylum, 60.5% of roots were colonised by ascomycetes and 39.5% were colonised by basidiomycetes (Table 3). Basidiomycete *Amphinema byssoides* (33.1%) and ascomycete *Wilcoxina* sp. (50.3%) dominated fungal communities in roots but their abundances significantly varied among seedlings from different regions and provenances (Table 3). Abundance of *A. byssoides* was significantly higher in seedlings of either E1 (41.5%), W1 (37.0%) or W2 (34.9%) than in those of C1 (24.5) or C2 (26.7%). Abundance of *Wilcoxina* sp., by contrast, was the highest on seedlings of C1 (63.4%), then it was followed by seedlings of C2 (53.5%), W1 (49.0%) and W2 (46.5%), and it was the lowest in seedlings of E1 (40.0%). The remaining fungal taxa were relatively rare (0.2% – 4.9%). *Tomentella* sp. was observed exclusively on seedlings of the

Table 2. Morphological parameters of the seedlings from different seed regions (Western, Central, Eastern ones) and provenances (W1, W2, C1, C2, E1)

Seedling morphological parameter	Western		Central		Eastern
	W1*	W2	C1	C2	E1
Shoot height, cm	66.8±1.1ab**	70.5±1.1bc	63.2±1.0a	80.9±1.1d	71.7±1.1c
Root collar diameter, cm	1.42±0.03ab	1.45±0.02b	1.33±0.02a	1.45±0.03b	1.37±0.03ab
Stem volume, cm^3	109.1±5.4ab	117.6±4.1bc	90.6±4.1a	136.6±6.5c	109.6±5.6ab
No. of ECM morphotypes	3.9±0.18ab	4.9±0.18c	3.6±0.16a	4.1±0.17ab	4.4±0.12bc

* There were 50 plants assessed from each provenance.
** Within the same row, values followed by different letters are significantly different ($p < 0.05$). Data variation within each provenance is represented by standard error.

Table 3. Relative abundance of fungal taxa detected by morphotyping and Sanger sequencing of ectomycorrhizal root tips of *Picea abies* seedlings representing different seed regions (Western, Central, Eastern) and provenances (W1, W2, C1, C2, E1)

Fungal taxa	GenBank accession number	Reference sequence*	Western		Central		Eastern	All
			W1 (15836/21)**	W2 (16717/21)	C1 (15258/18)	C2 (14928/21)	E1 (15699/21)	(78438/102)
Ascomycota								
Meliniomyces bicolor	KP172305	UDB017336	3.8a***	3.6a	3.9a	4.5a	2.2a	3.6
Tuber sp.	KP172308	AJ534705	0.1a	0.4a	0.7a	3.9b	3.7b	1.7
Wilcoxina sp.	KP172310	DQ150131	49.0bc	46.5bc	63.4a	53.5ab	40.0c	50.3
Wilcoxina mikolae	KP172311	AY880942	5.1a	4.4a	5.0a	4.2a	5.6a	4.9
All Ascomycota			58.1	54.9	73.0	66.2	51.5	60.5
Basidiomycota								
Amphinema byssoides	KP172303	KM504494	37.0a	34.9a	24.5b	26.7b	41.5a	33.1
Amphinema sp.	KP172304	JN943926	2.3a	4.0a	2.2a	6.6a	6.5a	4.3
Thelephora terrestris	KP172306	HM189966	0.2a	-	0.1a	-	0.5a	0.2
Tomentella sp.	KP172307	UDB008872	2.2a	4.7b	-	-	-	1.4
Tylospora asterophora	KP172309	JF300573	0.3a	1.5a	0.2a	0.5a	-	0.5
All Basidiomycota			41.9	45.1	27.0	33.8	48.5	39.5
Shannon diversity index			1.77	1.90	1.56	1.88	1.89	

* Similarity to the reference sequences was 99 or 100%.
** The number of ectomycorrhizal roots morphotyped/DGGE analysed.
*** Within the same row, values followed by different letters are significantly different ($p < 0.05$).

Western provenances W1 (2.2%) and W2 (4.7%). *Tuber* sp. was more abundant on seedlings of C2 (3.9%) and E1 (3.7%). Abundances of other species were similar among seedlings from different regions and provenances (Table 3). *Thelephora terrestris* was observed exclusively on seedlings with lower growth rate (W1, C1 and E1) in comparison with W2 and C2.

Shannon diversity index of fungal communities in seedlings of different regions and provenances was moderate and ranged between 1.56 and 1.90 (Table 3). Sørensen similarity index was between 0.875 and 1, showing that fungal communities in seedling roots of different provenances were very similar. Despite that PCA showed that fungal communities in seedlings from the Western, Central and Eastern seed regions were separated from each other on Axis 1, which explained most of variation (84.6%) (Figure 2). Furthermore, provenances W1 and W2 of the Western region were in close proximity thereby showing high similarity in their fungal community structure (Figure 2). By contrast, provenances C1 and C2 of the Central region were placed more distantly, showing higher differences in their fungal community structure (Figure 2). Spearman rank correlation analysis showed that there were no significant correlation between abundance of the dominant ECM fungal taxa and seedling morphological parameters.

Velmala et al. 2013). In turn, they can be of significant practical importance for establishment and growth of seedlings in the nursery and after outplanting in the field (Menkis et al. 2007, Vaario et al. 2009).

Certain differences were also detected between seedlings of different provenances within a particular region. In the Western region, seedlings of W2 showed higher shoot height and larger root collar diameter as well as higher number of the ECM morphotypes than those of W1. But in both the Western provenances the observed ECM fungal community structure was similar (Tables 2 and 3, Figure 2). Previously, *P. abies* field trials showed that progeny of W2 were among most productive yielding high biomass (Gailis 1993). Therefore this seed material has been widely used in tree breeding (Gailis 2012) and for large scale seedling production in forest nurseries (State forest service 2013). We observed even larger differences both in morphological parameters and in the ECM fungal community structure between seedlings of C2 and C1 of the Central region (Tables 2 and 3, Figure 2). Consequently, seedlings of W2 and C2 not only showed the best growth parameters within each particular region but also supported significantly higher diversity of the ECM fungi compared with seedlings of other provenances. Our data therefore suggest that there is a large variation in the growth performance of spruce

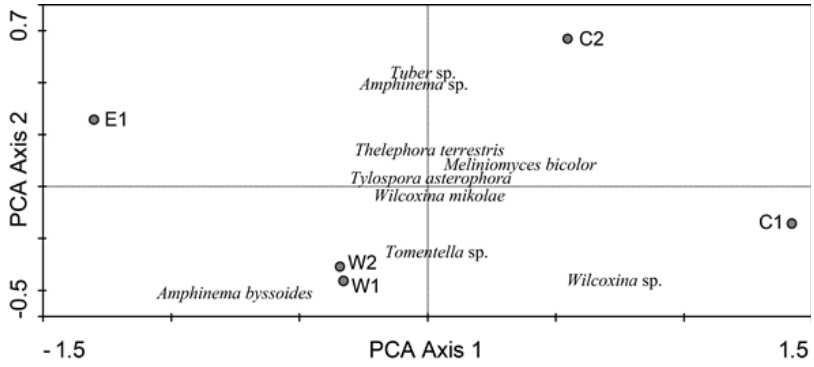

Figure 2. Ordination diagram based on principal component analysis of fungal communities in roots of *Picea abies* seedlings from different seed regions: Western (W1 and W2 provenances), Central (C1 and C2 provenances) and Eastern (E1 provenance) ones. In the diagram, 84.6% variation was explained on Axis 1 and 12.9% on Axis 2. Taxonomic names correspond to a position in the ordination (centred)

Discussion and Conclusions

The results demonstrated that despite *P. abies* seedlings of different seed regions and provenances were exposed to the same growth conditions and natural ECM fungal colonisation, they often differed in morphological parameters, and abundance and composition of the ECM fungal taxa (Tables 2 and 3, Figure 2). The results may suggest that genetic background of the seedlings has likely influenced both their growth and root colonisation by the ECM fungi as these characteristics has been similarly shown to vary within clonal families of *P. abies* (Korkama et al. 2006, seed provenances in Latvia, and that the selection of the best *P. abies* seed provenances in different seed regions is desirable to improve performance of future forest stands.

The ECM fungal taxa observed in association with roots of *P. abies* seedlings in the present study have been previously reported from forest nurseries as well as from young forest plantations (Menkis et al. 2005, Flykt et al. 2008, Klavina et al. 2013). The most dominant taxa, i.e. *A. byssoides* and *Wilcoxina* sp., colonised all seedlings of different seed regions and provenances (Table 3) at variable abundances. Similarly, predominant occurrence of certain ECM fungal taxa

was also found in different *P. abies* clones (Korkama et al. 2006). *A. byssoides* was more dominant in the Western and Eastern than in the Central provenances, and it is an efficient root coloniser of *P. abies* seedlings (Menkis et al. 2007, Vaario et al. 2009, Menkis et al. 2011). It occurs commonly in a newly established forest plantations (Kranabetter 2004, Menkis et al. 2007, Vaario et al. 2009), and when abundant it ensures better survival of seedlings after outplanting (Menkis et al. 2011). This may be of particular practical importance in terms of silviculture. *Wilcoxina* spp., the dominant ECM fungi in seedlings from the Central provenances, belongs to a group of E-strain fungi that represent early colonisers of seedling roots (Mikola 1988). *Wilcoxina* spp. are also commonly associated with the tree seedlings in soils of disturbed sites (Yu et al. 2001, Menkis et al. 2010), and in soils with naturally high organic matter content or amended with peat (Rudawska et al. 2011). Therefore, high abundance of this genus all over analysed seedlings in the present study could be substrate related. On the other hand, Velmala et al. (2014) demonstrated that *Wilcoxina* had high chitinase activity, which correlated with high N content of needles. Also, the ECM exploration type (Agerer 2001) of *Amphinema* and *Wilcoxina* differs: *Amphinema* forms the *medium distance* and *Wilcoxina – the contact type* mycorrhizas (Agerer 2001, Rudawska et al. 2011). Those differences could have an impact to seedling mineral nutrition and further nutrient supply in a field as so-called ECM exploration types are believed to differ in their efficiency of carbon (C) storage, enzymatic activities, nutrient uptake and translocation (Hobbie and Agerer 2010, Pritsch and Garbaye 2011). In the present study, the source of ECM fungal inoculum was not investigated, but likely included both ECM fungal inoculum present in the soil and air-borne ECM spores from the surrounding forests.

Above mentioned studies on ecology of the both dominant taxa indicate that the both of these taxa could have some advantages for successful seedling growth and establishment after outplantation, but they do not provide definite arguments of which taxa is more beneficial for seedling growth. Nevertheless, as in the study by Korkama et al. (2006), the seedling provenances, which showed the best growth rate (W2 and C2), were characterised as provenances with diverse ECM fungal community. Thus it appears that the diversity of the fungal community and consequently its ecological plasticity is more essential for growth of the seedlings than single dominant species.

Thelephora terrestris was observed in the provenances of the seedlings that showed lower shoot growth rate. Although it is one of the most common ECM fungi in the forest nurseries worldwide (Marx et al. 1984), this fungus often fails to support seedling establishment in the field (Ivory and Munga 1983, Lee 1992). It could result that presence of *Thelephora terrestris* and lower growth rate mark disadvantage for these seedling provenances for successful establishment in the field.

In conclusion, this study demonstrated that seed provenance may impact growth of *P. abies* seedlings and their root colonisation by the ECM fungi. In order to obtain more comprehensive picture of the processes that determine patterns of the ECM communities in tree roots, more related studies are needed, encompassing wide range of tree species and environmental conditions.

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