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Fine root development and mycorrhization in Norway spruce stands one year after fertilization with potassium sulphate and wood ash

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ABSTRACT: We compared the effects of forest fertilization with wood ash and potassium (K) sulphate on growth and mycorrhizal colonization of fine roots and on other soil microorganisms in Norway spruce (*Picea abies*) stands with symptoms of foliage damage. Fertilization trials with the application of 0.25 kg·m^{-2} of wood ash and similar amount of K as K sulphate were established. One year later, a total of 270 soil samples with roots were collected to determine morphological parameters of fine roots and extent of mycorrhization. Soil samples were collected to characterize soil chemical composition and number of colony forming units (CFU) of soil bacteria and fungi. The results showed that fine root biomass, length and volume, and relative abundance of living root tips were significantly higher in control sample plots than in treated plots. Abundance of bacterial CFU was higher in both wood ash and K sulphate treatments than in control plots; the Actinobacteria were more common in plots fertilized with wood ash than in other treatments. Relative abundance of several fungal species in sites fertilized with wood ash differed significantly from control sites and from sites fertilized with K sulphate.

Keywords: forest fertilization; fine root; ectomycorrhizal fungi; *Picea abies*; soil bacteria

Among the dominant forest tree species in Europe, Norway spruce (*Picea abies* /L./ Karst.) is one of the most susceptible to both abiotic and biotic stress (Modrzyński 2007) and recently, its mortality has increased in Latvia (Klavina et al. 2015a). In Latvia, 38% of the spruce forest area occurs on drained soils (Jansons 2011). In spring and early summer 2010, serious symptoms of spruce decline were noted, particularly on drained soils, and the observed foliage damage was found to be associated with soil and fine root parameters (Klavina et al. 2015a).

Potassium is often deficient for tree growth in peat soils (Kaunisto, Paavilainen 1988; Finer 1989). Analysis of soil parameters and needle chemical composition of declining spruce stands indicated a significant negative correlation of K concentration with the intensity of foliage damage (Bardule et al. 2012). This suggested that the application of K fertilizer or wood ash as a source of potassium (Pitman 2006; Augusto et al. 2008) might improve stand growth and nutrient balance. Previous studies noted the association of foliage damage of Norway spruce with belowground factors (Klavina et al. 2015a), which prompted us to compare various soil and fine root parameters in wood ash and K sulphate fertilised vs. control plots. The aim of this study was to evaluate the effects of forest fertilization with wood ash and K sulphate on morphological parameters of fine roots and mycorrhization of Norway spruce, on abundance of soil microorganisms and the effects on soil chemical properties one year after treatment.

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MATERIAL AND METHODS

Study sites and experimental design. The study sites included three middle-aged (mean age: 50 to 60 years old) *P. abies* stands on drained peat soils (56.8575N, 23.6903E; 56.8584N 23.6799E; 56.7840N, 23.4597E); forest type was *Myrtillosa turf. mel.* according to the Latvian forest classification (Zālītis, Jansons 2013). The study sites were located in the central part of Latvia in the Klīve Forest Management Unit. In all sites thinning was performed between 2007 and 2010. Typical symptoms of spruce decline were due to the spruce bud scale insect *Physokermes piceae* (Schrnk.) invasion and included discoloration and dieback of top and lateral shoots and reduction of their increment growth as well as shoot colonisation by sooty mould (*Apiosporum pinophilum* Fuckel.) at later stages; those symptoms were observed in selected sites in 2010. The initial soil chemical analysis showed that average pH ($CaCl_2$) among stands ranged from 3.0 to 3.7; total N 21… 24 g·kg^{-1} and organic C 413 … 485 g·kg^{-1}. Three different treatments (wood ash, K sulphate and control) were used with replication of three plots per treatment in each of the three *P. abies* stands (27 sample plots in total). Nine 20 × 20 m plots were established in each forest stand with a 11.3-m buffer zone between them. The fertilizer was applied in June, 2011 in the following quantities: 0.25 kg·m^{-2} wood ash corresponding to approx. 6.5 g·m^{-2} K; 14.5 g·m^{-2} K sulphate corresponding to approximately 6.1 g·m^{-2} K.

Soil and needle chemical analysis. One set of soil samples and current year needles were collected from each sample plot in September 2012. Needles were collected from tops (upper part of the crown with optimal light conditions) of seven trees and used for analyses according to ICP Forests Manual (Rautio et al. 2010). A soil core sampler (5.3 cm in diameter) was used to obtain samples from a depth of 0–20 cm. Chemical analyses of soil and needles were conducted using established standard methods (International Organization for Standardization /ISO/ standard). Samples were prepared for analysis according to ISO 11464:2005. Total carbon was determined using a LECO CR-12 elemental analyser according to LVS ISO 10694 standard by sample ashing in oxygen flow at 900°C and measurement of the absorption of infrared spectrum. Total nitrogen (N) content was determined using the modified Kjeldahl method (ISO 11261:1995) and total phosphorus (P) was measured spectrophotometrically using the ammonium molybdate method (LVS 398:2002). Contents of K, calcium (Ca) and magnesium (Mg) were determined by an atomic absorption spectrophotometer with an acetylene-air flame in Aqua Regia extract (soil samples) and in concentrated HNO_3 (needle samples). Soil pH ($CaCl_2$) was measured potentiometrically in 0.01 M $CaCl_2$ suspension according to ISO 10390:2002.

Root sampling and analysis. Ten replicate soil samples from each sample plot were obtained for root analysis (total of 270 samples). Sampling was conducted in October 2012. Samples were collected from a depth of 0–20 cm using a core sampler (Ø 5.3 cm) and stored at 4°C for a period not longer than two weeks. All woody roots were removed from soil samples and rinsed under tap water. Coarse roots (more than 2 mm in diameter) were discarded. Non-conifer roots were also discarded. The remaining fine roots were cut into 1-cm segments and evenly spread in Petri dishes with a grid on the bottom (mesh size 7 × 7 cm). Starting from the upper left corner in 30 grid squares, age class of all single fine roots and their ectomycorrhizal morphotypes (in the case of living mycorrhizal fine roots) were determined (Agerer 1986–2006) using a stereomicroscope (Leica MZ-7.5, Solms, Germany). Age classes of roots were determined based on external features and the roots were divided into three classes: (1) fine roots with white turgid root tips; sometimes darkened but with well-formed and undamaged mycorrhizal mantle; the stele is white and elastic (Young root); (2) more suberized fine roots or fine roots with damage of the external mantle surface; the stele is still elastic and light to slightly brown (Old root); (3) fine roots which normally are referred to as dead; the stele is brownish and easily broken and no elasticity remains; root tips are blackened and fragile (Dead root) as described previously (Klavina et al. 2015a). Morphological parameters of fine roots including length, volume, surface area and number of root tips were determined using an Epson Perfection V750Pro scanner (Epson, Tokyo, Japan) (http://www.epson.com/cgi-bin/Store/support/supDetail.jsp?UseCookie=yes&oid=66134&infoType=Doc) and WinRHIZO 2005 C (Regent instruments Inc., Canada) software (http://regent.qc.ca/assets/winrhizo_software.html). After scanning, roots were dried at 60°C for 1 h and weighed.

One to five single root tips of each distinct morphotype were separately placed in 1.5-ml centrifugation tubes and stored at –20°C for the molecular identification of fungal taxa. DNA extraction and PCR using primers ITS1F (Gardes, Bruns 1993) and ITS4 (White et al. 1990) were conducted as in a

Table 1. Characteristics of soil and needle chemical composition in samples from fertilized and control *Picea abies* stands [plot design was three treatments (wood ash, K sulphate and control) × three stands × three replicate plots]; values show the mean value ± standard error

	Soil (0–20 cm)			Needles (current year)		
	stand	treatment × stand	average	stand	treatment × stand	average
pH ($CaCl_2$)	n.s.	n.s.	3.7 ± 0.1	–	–	–
C (total, g·kg^{-1})	n.s.	n.s.	357 ± 25	**	n.s.	539 ± 7.2
N (total, g·kg^{-1})	*	n.s.	16.9 ± 1.4	*	n.s.	15.0 ± 0.5
P (total, g·kg^{-1})	n.s.	n.s.	0.5 ± 0.1	n.s.	n.s.	1.2 ± 0.1
K (g·kg^{-1})	**	*	241 ± 28	n.s.	n.s.	5.6 ± 0.4
Ca (g·kg^{-1})	**	n.s.	8.5 ± 1.2	n.s.	n.s.	3.5 ± 0.3
Mg (mg·kg^{-1})	**	n.s.	0.8 ± 0.1	n.s.	n.s.	1.3 ± 0.1

*P < 0.05; **P < 0.01; n.s. – not significant

previous study (Klavina et. al. 2015b). Sequencing was performed by Macrogen Europe Inc. (Amsterdam, The Netherlands). Raw sequence data were analysed using the SeqMan version 5.07 software from the DNASTAR package (DNASTAR, Madison, USA). Databases at GenBank (Altschul et al. 1997) and UNITE (http://unite.ut.ee/) (Kõljalg et al. 2013) were used to determine the identity of sequences. The sequences are available from GenBank under accession numbers KT692920-KT692933.

Soil microbiological analysis. Soil samples used for microbiological analysis were also collected in October 2012 to a depth of 20 cm using a core (Ø 3.6 cm). One soil core was obtained from each plot and the three replicates were pooled together and analysed as bulk samples (nine samples in total). The samples were stored at 4°C. For microbiological analysis, 10 g of each soil sample was suspended in 90 ml of sterile water in a 500-ml Erlenmeyer flask and shaken at room temperature at 150 rpm for 30 min (Alef, Nannipieri 1998). 0.1 ml aliquots from serial dilutions of soil suspensions were plated on peptone yeast extract media for bacteria and malt media for fungi. The assay was conducted in three replicates in Petri dishes incubated at room temperature (20 ± 2°C) for three days. After incubation, the numbers of colony forming units (CFU) were counted (Vanderzant, Splittstoesser 1992) and their abundance per one gram of soil was calculated (Alef, Nannipiri 1988).

Data analysis. Multivariate Analysis of Variance (MANOVA, SPSS, Tulsa, USA) was used to test the effect of stand and fertilization treatment on morphological parameters of roots (length, volume, surface area, dry mass and number of root tips), soil and needle chemical properties and numbers of CFUs of soil microorganisms. If a significant treatment effect was observed, post-hoc Tukey's test (Fowler et al. 1998) at a confidence level α = 0.05 was performed to make comparisons among treatments. Chi-squared (χ^2) analysis calculated from the actual number of observations (Mead, Curnow 1983) was used to test for significant differences in the relative abundance of fine roots among root age classes, and in fine root mycorrhization and ECM fungal species among treatments. As the total number of root tips analysed was large, the χ^2 test was performed at a confidence level α = 0.0001. Statistical analysis was conducted using the R program (Vienna, Austria) (R Development Core Team 2011). Shannon diversity indices (Shannon 1948) were calculated for each treatment pooling the species abundance data of all samples within a plot.

RESULTS

Chemical composition of soil and current year needles was largely similar among different treatments, but total concentrations of some nutrients (K, N, Ca and Mg) differed among sampled stands (Table 1). None of the parameters showed significant *P*-values for treatment exclusively as a factor. Significant treatment interaction with stand effect was observed for soil K concentration, which was higher (P < 0.10) in sites fertilized with K sulphate than in control ones (309 ± 75 and 200 ± 13 mg·kg^{-1}, respectively) (Table 1).

There was a strong effect of stand and treatment on morphological parameters of fine roots (biomass, length, volume, and surface area), with higher values in control plots than in both fertilized treatments (Table 2). The number of root tips was lower in plots fertilized with wood ash than in the

Table 2. Morphological parameters of fine roots and abundance of soil microorganisms in fertilized and control *Picea abies* stands. Plot design was three treatments (wood ash, K sulphate and control) × three stands × three replicate plots. Values show the mean ± standard error. Different letters next to mean values indicate significant differences between treatments ($P < 0.05$)

	Treatment	Stand	Treatment × stand	Control	Fertilizer	Wood ash
Morphological parameters of fine roots						
Number of replicates				90	90	90
Volume (cm^3)	*	**	**	1.33 ± 0.15[a]	0.90 ± 0.05[b]	0.85 ± 0.05[b]
Length (cm)	**	**	**	442 ± 30[a]	366 ± 20[b]	313 ± 16[b]
Surface area (cm^2)	**	**	**	84 ± 7[a]	63 ± 3[b]	57 ± 3[b]
Number of root tips	**	**	**	2,515 ± 216[a]	2,168 ± 206[a]	1,258 ± 79[b]
Biomass (g)	**	**	*	0.35 ± 0.03[a]	0.27 ± 0.02[b]	0.26 ± 0.02[b]
Soil microbial analysis						
Number of replicates				6	6	6
Bacteria (CFU per g) × 10^6	**	n.s.	**	4.0 ± 1.3[a]	9.9 ± 2.8[b]	9.5 ± 1.6[b]
Actinobacteria (CFU per g) × 10^5	**	*	n.s.	5.5 ± 1.7[a]	4.7 ± 0.4[a]	11.5 ± 1.5[b]
Filamentous fungi (CFU per g) × 10^5	n.s.	n.s.	n.s.	1.5 ± 0.3	1.7 ± 0.3	1.6 ± 0.3

*$P < 0.05$; **$P < 0.01$; n.s. – not significant

other treatments indicating lower fine root density or branching in sites fertilized with wood ash.

The number of bacterial CFU was significantly higher in fertilized plots than in control ones (Table 2). The number of the Actinobacteria CFU was significantly higher in plots fertilized with wood ash than in the other treatments. Abundance of filamentous fungi CFU in soil samples was similar among all treatments.

Relative abundance of dead fine roots was significantly lower in control plots than in both types of fertilized sites (Table 3). However, both control plots and those fertilized with wood ash had higher abundance of young living fine roots and lower abundance of older living fine roots than the plots fertilized with K sulphate (Table 3).

Mycorrhizal colonization of fine roots was higher in plots fertilized with both wood ash and K sulphate than in control plots (Table 3). Twelve ECM morphotypes were detected (Table 3). Fungal amplification and sequencing from 60 mycorrhizal root tips resulted in 22 high-quality sequences which were found to represent 14 fungal species. Among those, 12 belonged to basidiomycetes and 2 to ascomycetes. The most common species were *Piloderma* sp., *Tylospora asterophora* and *Tomentella* sp. 3 (Table 3).

The Shannon diversity index among sites was similar, with slightly higher values in control plots (2.74) than in plots fertilized with wood ash (2.64) and K sulphate (2.67). Relative abundance of all ECM morphotypes differed significantly among different treatments (Table 3). In sites fertilized with wood ash, *Piloderma* sp. and *Tomentella* sp. 3 were more abundant and *Cortinarius* cf. *casimiri* and *Tylospora asterophora* were less abundant than in control sites and in those fertilized with K sulphate.

DISCUSSION AND CONCLUSIONS

The soil and needle chemical analysis did not indicate any strong effect of the application of wood ash and K sulphate fertilizer, but perhaps a longer period is needed to observe changes in these parameters after fertilization (Genenger et al. 2003). On the other hand, the low effect might be due to the high buffering capacity of drained peat soils. The absence of significant differences in soil pH between the wood ash treatment and the control might also be due to the relatively low amount (0.25 $kg \cdot m^{-2}$) of wood ash applied in this study, in comparison with other studies (e.g. Augusto et al. 2008; Klavina et al. 2015b).

Morphological parameters of fine roots indicated a strong fertilization effect via a reduction of fine root biomass and root length in both types of fertilized sites, which is in accordance with other studies (Clemensson-Lindell, Persson 1995). The decreased growth of fine roots in sites treated with wood ash and K sulphate might be due to tree-root system stress (Clemensson-Lindell, Persson 1995). However, the age structure of living fine roots (young

Table 3. Relative abundance of fine root age classes, mycorrhizal root tips and ECM morphotypes among control plots and plots fertilized with wood ash and K sulphate (different letters next to relative abundance values indicate significant differences between treatments $P < 0.0001$)

			Control	K sulphate	Wood ash
Relative abundance of fine root age classes					
Total number of root tips analysed			29,102	29,288	27,985
Young living fine roots (%)			11.4[a]	7.7[c]	9.6[b]
Older fine roots (%)			36.7[a]	38.5[b]	36.6[a]
Dead fine roots (%)			51.9[a]	53.8[b]	53.8[b]
Relative abundance of ECM root tips					
Total number of root tips analysed			13,411	13,883	12,537
Mycorrhization (%)			97.4[a]	98.0[b]	98.0[b]
Shannon diversity			2.74	2.67	2.64
Morphotype, species and GenBank accession No.					
MT1	*Amphinema byssoides*, *Xerocomus fennicu*s	KT692928, KT692929	2.2[a]	0.5[c]	1.2[b]
MT2	*Amphinema* sp. 1, *Paxillus involutus*	KT692923, KT692922	0.2[a]	0.8[b]	2.0[c]
MT3	*Amphinema* sp. 2	KT692933	4.3[a]	9.6[b]	5.4[a]
MT4	*Cadophora finlandia*	KT692932	2.3[ab]	2.6[b]	1.9[a]
MT5	*Cortinarius* cf. *casimiri*	KT692930	5.4[a]	5.4[a]	3.4[b]
MT6	*Humaria hemisphaerica*	KT692924	7.0[a]	5.1[b]	4.5[b]
MT7	*Piloderma* sp.	KT692920	38.7[a]	40.0[a]	44.9[b]
MT8	*Pseudotomentella mucidula*	KT692921	5.9[ab]	4.6[a]	6.7[b]
MT9	*Tomentella* sp. 1, *Tomentella* sp. 2	KT692925, KT692926	3.4[a]	2.4[b]	3.0[a]
MT10	*Tomentella* sp. 3	KT692931	10.2[a]	9.6[a]	11.5[b]
MT11	*Tylospora asterophora*	KT692927	19.7[a]	19.2[a]	14.8[b]
MT12	Unidentified	–	0.6[ab]	0.3[b]	0.7[a]

living and older living fine roots) (Table 3) indicated relatively better fine root growth in plots with wood ash application than in sites fertilized with K sulphate. Therefore, wood ash treatment might be considered as a better alternative for fertilization of *P. abies* stands with reduced fine root vitality, as described previously (Klavina et al. 2015b). Other studies (Augusto et al. 2008) showed that a negative effect of wood ash on the growth of fine roots is temporal and that it becomes positive after a longer period of time. The increased abundance of Actinobacteria in sites fertilized with wood ash was probably due to a liming effect, as described previously (Frostegård et al. 1993). The Actinobacteria can also contribute to tree vitality by antifungal activity against some soil-borne pathogens (Kamara, Gangwa 2015).

The differences in fungal species abundance between both types of fertilized sites and control are consistent with previous studies that showed species-specific changes in the ECM fungal community composition after forest liming (Jonsson et al. 1999; Kjøller, Clemmensen 2009). Our data indicated a strong effect of wood ash application on the ECM species abundance, which is in accordance with previous studies (Klavina et al. 2015b). This study, however, demonstrates that even low amounts of wood ash may have an effect on the ECM species composition and that this effect can occur soon after fertilization. Differences in ECM communities between the sites fertilized with wood ash and other treatments might be due to specific properties of wood ash and amounts of nutrients such as Mg, Mn, Na, P introduced into soil (Augusto et al. 2008).

Higher relative abundance of the dominant ECM morphotype represented by *Piloderma* sp. in sites fertilized with wood ash corroborates results of other studies (Mahmood et al. 2001; Mahmood 2003). Lower abundance of the ECM morphotype represented by *Tylospora asterophora* in sites treated with wood ash was observed in our previous studies (Klavina et al. 2015b). Although the importance of ectomycorrhizas has been widely acknowledged, the factors determining ECM com-

munity structure and species diversity following wood ash fertilization are still poorly understood.

In conclusion, our data indicate that forest fertilization has specific effects on morphological parameters of fine roots, ECM fungal community composition and abundance of soil bacteria in *P. abies* stands on drained peat soils. These findings should become the basis for future studies to clarify the effects of fertilization on stand growth and recovery after recent foliage damage in the long term, as well as to elaborate recommendations for the forestry practitioners.

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