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# Contrasting responses of the bacterial communities in ectomycorrhizal roots and rhizosphere soils to defoliation or winter hardening

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ABSTRACT

We examined the effects of photosynthetic activity of the host tree on the bacterial communities in ectomycorrhizal (ECM) roots and in rhizosphere soil. The seedlings of two host tree species, birch *(Betula pendula)* and pine (*Pinus sylvestris*), were subjected to winter hardening treatments for inducing total (birch) or partial (pine) restriction of the photosynthesis. Additionally, physical defoliation was performed to prevent both tree species from photosynthetic activity entirely. The bacterial communities were analyzed by RT-PCR of 16S rRNA followed by DGGE. After winter hardening, the bacterial communities in ECM roots of both host species shifted considerably while no consistent change was observed after physical defoliation. In contrast, no significant separation of the communities in rhizosphere soil was observed after winter hardening in either of the host species although the communities were significantly altered after physical defoliation compared to those of non-defoliated control in both tree species. It is therefore interesting and curious that the bacteria communities of ectomycorrhizal roots respond differently than the rhizosphere soil, to both defoliation and winter-hardening

In temperate and boreal forest ecosystems, ECM roots harbor distinctive bacterial diversity. The bacterial communities associated with ECM roots, for instance, are different from those in bulk soils (Vik et al., 2013) and in surrounding rhizosphere soils (Nguyen and Bruns, 2015). Additionally, it has been reported that different bacterial communities occurred in the roots with ECM fungi and those without (Izumi et al., 2008; Nguyen and Bruns, 2015). While the unique bacterial diversity in ECM roots is well known, the factors influencing the diversity are poorly understood.

Bacterial communities associated with host tree roots are known to respond to the variations in photosynthetic activity of the host. The seasonal shifts of rhizosphere bacterial communities have been observed depending on changes of host tree phenology (Mocali et al., 2004). Additionally, abrupt termination of host photosynthetic activity by removing leaves in growing season has been reported to induce changes of the associated ECM fungal community (Cullings et al., 2001). Therefore, it is very likely that the bacterial communities in ECM roots respond to the treatments that manipulate photosynthetic activity of the host tree although such possibility has not yet been explored.

The aim of the present study was to examine possible shifts in the bacterial communities in ECM roots and rhizosphere soil found in two different host tree species, birch (*Betula pendula*) and Scots pine (*Pinus sylvestris*), when the host trees were subjected to the treatments inducing the reduction in their the photosynthetic activity by winter hardening treatment or by physical defoliation. The bacterial communities were analyzed based on rRNA molecules that reflect metabolically active populations of the bacteria to maximize the chance of detecting functionally important groups.

Forest soil was collected from birch/pine stands in a mixed forest near Uppsala, Sweden (59° 51′ 0″ N, 17° 38′ 0″ E) in June and passed through a 5 mm mesh sieve. The soil had a pH of 6.5 and total carbon and nitrogen contents of 128 g $kg^{-1}$ and 6 g $kg^{-1}$ respectively. Fig. 1 presents the experimental treatments and timing of sample collection. One month old birch and Scots pine seedlings, aseptically germinated, were transferred to pots (10 × 10 × 15cm) containing the forest soil and grown in a climate chamber (I-36LLVL, Percival Scientific Inc., Perry, USA) with a day/night cycle of 14/10 h and air temperature cycle of 18/14°C. One seedling was planted per pot. The both tree

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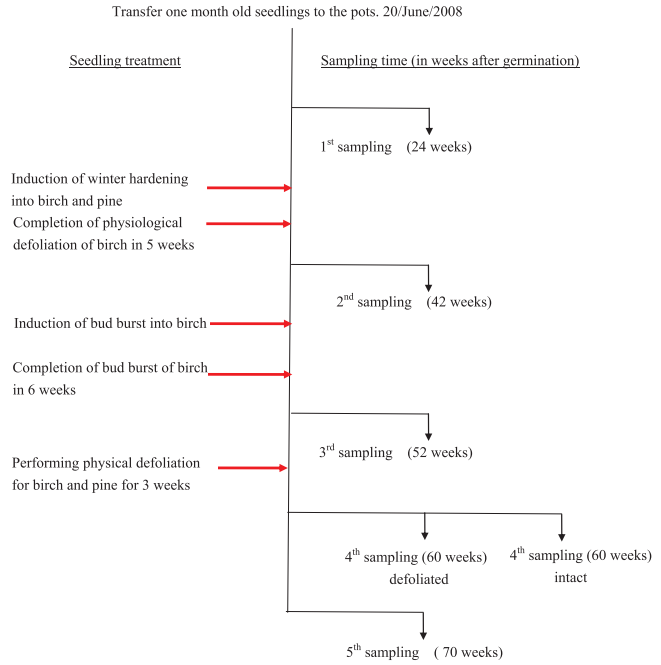


**Fig. 1.** Schematic diagram of sampling schedule.

species were subjected to the winter hardening that was attained by stepwise reduction of day length and air temperature until to reach at 4/20 h day/night cycle with 8/4 °C air temperature cycle (Ekblad et al., 1995). For induction of bud burst in birch, the both species were subjected to stepwise increasing of day length and air temperature until to reach 13/11 h day/night cycle with a air temperature of 20/16 °C (Ekblad et al., 1995). A sub-set of the seedlings of both tree species was subjected to 100% physical defoliation by clipping leaves. The defoliated seedlings were grown another three weeks without leaves by continuous cutting of re-emerging leaflets before the final harvest of the defoliated seedlings (Fig. 1). The remaining seedlings not subjected to physical defoliation were grown under constant conditions for the final harvest.

Four replicates of the seedlings were harvested at each of five sampling times (Fig. 1). For each seedling the ECM root tips, rhizosphere soil and bulk soil were collected from both tree species. The first sampling was carried out before starting the winter hardening process. The second sampling was carried out after completion of leaf abscission in birch. The third sampling was done at the time new leaves of birch had reached the fully developed state. At the fourth sampling, both physically defoliated and intact (non-defoliated) samples were harvested. At the fifth sampling, only seedlings that were subjected to winter hardening but not physical defoliation were included.

Single ECM morphotype (Agerer, 1987), occurring in all replicates in all sampling times, were collected and pooled for each replicate. Integrity of the ECM fungal genetic identities between different replicates and sampling times were checked by comparison of the PCR RFLP patterns obtained by digesting PCR products of ITS regions with the restriction enzymes Cfo I or Taq I (Promega biotech AG, Stockholm, Sweden). Collected ECM root tips (approx. sixty) were surface-sterilized with hydrogen peroxide to remove the bacteria on the surface (Izumi et al., 2006; Nguyen and Bruns, 2015) and the sterility was confirmed by checking the last wash water of the sterilization step through plate counting (data not shown). The root tips were stored at -20 °C until further processing.

Rhizosphere soil (one to three grams of soil) was collected by shaking the root system in 10 ml double strength Phosphate Buffered Saline (PBS) after removing large, grain sized soil particles. The same amount of bulk soil was taken from the up-rooted pots and suspended in 10 ml double strength PBS. Both soil samples were stored -20 °C until further processing.

Identification of ECM fugal species and molecular analysis of the bacterial communities were carried out according to Izumi et al. (2007). The bacterial 16S RNA molecules were amplified by the primers targeting to V3 region (Muyzer et al., 1993). The sequence of the ITS 2 region of the ECM fungus in this study (EMBL accession number of the sequence: FN908811) matched to reference sequence of *Tomentella elisii* collected in Sweden (UDB 000231) with high homology (99%).

Detrended Correspondence Analysis (DCA) and Multi-Response Permutation Procedures (MRPP) was used to evaluate community composition and significance of the separations, using PC-ORD ver. 5.32 program (MJM Software Design, Grenadine Beach, USA) (McCune and Grace 2002). DCA was selected over PCA for avoiding possible distortion of ordination diagrams due to horseshoe effect (McCune and Grace, 2002). Non-metric Multidimensional Scaling (NMS) was not employed because data sets in this study did not provide reliable stress values for rigorous analysis.

In birch, the bacterial communities in ECM roots after winter hardening (2nd sampling time) were significantly ($P \leq 0.01$) distinct among all sampling times (Fig. 2A). In pine, the communities in ECM roots after winter hardening were significantly ($P \leq 0.01$) different from those collected at third, forth and fifth but not from those of the first sampling (Fig. 2B). There was no significant difference between the communities from the seedlings exposed to physical defoliation and those from non-defoliated controls in either of birch and pine (Fig. 2A and B).

In the bacterial communities of rhizosphere soil collected at the 4th sampling time, there was significant ($P \leq 0.01$) difference between physically defoliated and non-defoliated seedlings in both birch and pine (Fig. 2C and D). No statistically significant separation in the communities of rhizosphere soil after winter hardening (2nd time) from those taken in other sampling times in either birch or pine was observed (Fig. 2C and D) except that the communities collected in the last sampling time was significantly ($P < 0.01$) different from those collected after winter hardening in birch (Fig. 2C).

To our knowledge, this study is the first to show that the bacterial communities in ECM roots respond differently to the host tree experimental treatments compared to those associated with rhizosphere soil. Such unique response of the bacteria community may reflect that ECM roots harbor physiologically or functionally distinctive bacteria that were influenced by specific host tree physiology, such as winter dormancy and reduction of photosynthetic activity, and contribute to establish ECM root specific communities observed in the previous studies. Although environmental factors, such as soil temperature, may have direct impacts on the bacterial communities in ECM roots and in rhizosphere soil, the contribution of these to the observed separations of the communities are considered to be minor, because the bacterial communities in the bulk soil (without obvious association with the host) were also under the influence of the environmental conditions, but did not show any consistent shift to the experimental treatments (data not shown). Further investigation of the phenomenon is warranted, and stable isotope probing and physiological assays would lead to understanding the mechanism(s).