Root systems of declining conifer seedlings are colonised by a highly diverse fungal community

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Abstract

Fungi of roots of declining pine and spruce seedlings were assessed by pure culture isolations and direct sequencing. The isolation from 1440 roots of 480 seedlings (240 per each tree species) yielded 1110 isolates which, based on mycelial morphology and ITS rDNA sequences, were found to represent 87 distinct taxa. Direct ITS rDNA sequencing from decayed sections of 140 roots (70 per each tree species) yielded 160 sequences representing 58 taxa. In respect to the amount of examined roots, direct sequencing revealed significantly larger fungal diversity (chi-squared test; $p<0.0001$). A total of 131 taxa were found, 92 of which (70.2 %) were identified at least to a genus level. Only 14 of the total number (10.7 %) were detected by both methods, while 73 (55.7 %) were detected exclusively by isolation, and 44 (33.6 %) exclusively by sequencing. Fungi most commonly isolated were the pathogens *Fusarium oxysporum* (25.6 %) and *Nectria radicicola* (14.9 %). On the contrary, direct sequencing most frequently revealed presence of the endophyte *Phialocephala fortinii* (33.1 %) and the unidentified sp.NS234A2 (10.0 %). Our results demonstrate that a diverse fungal community inhabits roots of declining conifer seedlings, and that pure culture isolations combined with direct sequencing provides complementary data in studies of fungal communities.

Introduction

Fungi colonising roots have a significant impact on health and productivity of tree seedlings, as they are able to form beneficial, neutral or pathogenic types of associations (Wilcox 1983). In recent years, root dieback of pine and spruce was reported to be a serious problem in a number of forest nurseries over the Europe. Diseased seedlings were usually occurring in patches, exhibiting stunted growth, discoloration of needles and partial or total death of the root systems (Venn *et al.* 1986, Lilja *et al.* 1988, Unestam *et al.* 1989, Ericson *et al.* 1991, Lilja *et al.* 1992, Kacprzak 1997, Camporota & Perrin 1998, Hietala *et al.* 2001). As a rule, this led to a significant decrease in quality of plants, and in some cases resulted in loss of stock production up to 40 % (Lilja 1994). Most often, fungi from the genera *Fusarium*, *Nectria, Rhizoctonia*, and *Pythium* were reported as causal agents of the disease (Galaaen & Venn 1979, Lilja *et al.* 1992, Lilja & Rikala 2000).

Seedlings, infected with root-decay fungi, might exhibit reduced survival rates following outplanting. Consequently, the success of plantation might be also dependent on the presence of root pathogens in afforested areas, as transferred seedlings are likely more susceptible to infection due to recent replanting stress. Such risks are indeed real, as couple of studies had already shown that potential pathogens are able to persist both in forest soils on clear-cut sites and on abandoned farmland (Perry *et al.* 1987, Wilberforce *et al.* 2003). Therefore, it is important to assess root disease hazard also in different types of planting terrain.

To date, such studies are scarce, and previously fungal communities in decayed roots of conifer seedlings were mainly assessed by fungal isolations into pure culture (Lilja *et al.* 1992, Kope *et al.* 1996). However, despite the large number of isolated fungi, it was noted that this method could be biased towards fast growing species and provide only portion of total fungal community inhabiting diseased roots. More recently, it has been demonstrated that PCR based molecular methods could be a powerful tool for identification of fungi (Donaldson *et al.* 1995, Hamelin *et al.* 1996, Hantula *et al.* 2002). For example, the direct sequencing of fungal DNA from roots has proved to be a sensitive method for the detection of potentially all root-inhabiting fungi, in particular species that are usually overlooked by isolation, e.g. latent pathogens, slow-growing endophytes and unculturable species (Kernaghan *et al.* 2003). The main aim of the present work was to determine species composition and relative abundance of fungi colonising roots of decayed *P. sylvestris* and *P. abies* seedlings in three types of terrain: bare root forest nurseries, afforested clear-cuts and abandoned farmland. In order to achieve this, pure culture isolations were combined with direct sequencing of fungal DNA from decayed root tissue.

Materials and methods

Diseased *Pinus sylvestris* and *Picea abies* seedlings were collected from three bare-root forest nurseries, three replanted clear-cuts and one afforested farmland. All four plantations were established during spring of the same year. The aboveground symptoms of all sampled seedlings were needle discoloration and stunted growth. Following excavation, all of them showed root dieback and decay. From each root system, three to five core roots with decay symptoms were randomly selected, and from each selected root, a single segment about 5 mm in length was cut at the zone of advancing decay. Three of those were immediately used for isolation of fungi into pure culture. In addition, from 10 randomly selected plants from each site, one segment per root system was designated for direct sequencing.

The isolation of fungal cultures was attempted from 1440 core roots derived from 240 pine and 240 spruce seedlings. For isolation from the nursery plants we used three

different types of agar medium (one type per each root from a single plant), 2 % water agar, vegetable juice agar (Barklund & Unestam 1988) and Hagem agar (Stenlid 1985). All isolations from replanted clear-cuts and afforested farmland, as well as all subsequent subculturings of all obtained strains were done exclusively on Hagem agar. The cultures obtained were grouped into mycelial morphotypes based on mycelial morphology. For identification, one to ten representative cultures from each morphotype were ITS rDNA sequenced. Moreover, a total of 140 segments of core roots representing 70 pine and 70 spruce seedlings was selected for direct ITS fungal rDNA sequencing from root tissue. In all procedures, extraction of DNA, amplification and sequencing followed the method described by Rosling *et al.* (2003).

Databases at both GenBank (Altschul *et al.* 1997) and at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala were used to determine the identity of sequences. The criteria used for deciding on the taxon or genus for a given strain was its intra- and interspecific ITS sequence similarity to those present in the databases. Fungal community structures were compared by calculating qualitative (SS) Sorenson similarity indices (Magurran 1988). The occurrence of a given fungus in respective datasets was compared by chi-squared tests, which were calculated from actual numbers of observations (presence/absence data) (Fowler *et al.* 2001).

Results and discussion

Out of 1500 roots used for isolation, 1110 (74.0 %) gave fungal growth, and the remaining 390 (26.0 %) were either colonised by bacteria or remained sterile. As in all cases a single isolate per root was obtained, this part of work yielded a total 1110 of distinct cultures, which were found to represent 87 different taxa. Of those, 77 (88.5 %) were identified at least to genus level. The fungi most frequently isolated were ascomycetes and deuteromycetes: *Fusarium oxysporum*, *Nectria radicicola, Nectria* sp.702, *Trichoderma harzianum, Phialocephala fortinii, Penicillium spinulosum, T. viride and Zalerion varium*.

The results showed that high fungal diversity does exist in decayed roots even within a single root system. Thus, the isolations from three different roots of the same plant had resulted in three similar outcomes only in 17.0 % of seedlings from the nurseries, in 17.5 % of seedlings from the clear-cuts, and 21.7 % of seedlings from abandoned farmland. By contrast, two and three different outcomes were observed in 54.0 % and 29.0 %, 45.8 % and 36.7 %, and 61.7 % and 16.7 % of plants from respective types of terrain.

Amplification of fungal ITS rDNA from 140 root segments was successful for 123 (87.9 %), producing 1 to 4 distinct amplicons in each PCR reaction. Direct sequencing of all amplicons resulted in 160 sequences representing 58 fungal taxa. The fungi most commonly detected by direct sequencing were the ascomycetes *Phialocephala fortinii*, Unidentified sp.NS234A2, *Leptosphaeria* sp.1169, *Nectria radicicola, Nectria* sp.702, *Xenochalara juniperi*, *Fusarium oxysporum* and *Zalerion varium*.

The efficacy of direct sequencing was higher than that of isolation. For example, direct sequencing from 140 root segments yielded 58 taxa, while the isolation from the same number of root samples would count only 27 species as estimated from the species accumulation curves (data not shown). Moreover when sequenced, a single root segment delivered up to 4 sequences of different fungi, when during the isolation similar segment never yielded more than one culture. When pooled, direct sequencing and isolation detected a total of 131 fungal taxa, 92 of which (70.2 %) were identified at least to a genus level. The overlap between the two methods was very low (S_s = 0.19). Only 14 (10.7 %) of the taxa were both sequenced and isolated, 44 (33.6 %) were detected exclusively by sequencing, and 73 (55.7 %) exclusively by isolation. In conclusion, the results showed that pure culture isolations combined with direct sequencing provide complementary data in studies of fungal communities and reveal high abundance of species in roots of declining conifer seedlings.

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