

Dynamics of ectomycorrhizal fungi after windthrow

Simon Egli, Martina Peter and Simone Falcato

WSL Swiss Federal Research Institute, Zürcherstrasse 111, CH-8903 Birmensdorf, Switzerland.
simon.egli@wsl.ch; martina.peter@sbiol.uhp-nancy.fr

Abstract

Ectomycorrhizal fungi are dependent on the carbohydrates produced by higher plants. Their main energy source is lost immediately if the higher plant is eliminated, e.g. as the result of windthrow. In this study we investigated how long ectomycorrhizal fungi can survive in the soil and still be capable of colonizing naturally regenerating seedlings on an uncleared windthrow site. A biotest was used for measuring the mycorrhizal inoculum potential of the soil and morphological and molecular techniques for identifying the ectomycorrhizas. Ten years after the windthrow event, the number of infective ectomycorrhizal fungi in the soil of the windthrow plot was significantly smaller than in the adjacent forest. The remaining species, including *Tylospora asterophora*, *Telephora terrestris* and *Cenococcum geophilum* were still able to fully mycorrhize the new seedlings. The results are discussed with respect to possible implications for forestry practice.

Keywords: windthrow, ectomycorrhizal fungi, inoculum potential, regeneration

1 Introduction

Mycorrhizal fungi are instrumental in the uptake of water and nutrients by plant roots. They increase the survival of seedlings, enhance their drought tolerance and protect the root system from microbial pathogens (SMITH and READ 1997). Clearcutting and wildfire reduce the number of fruiting mycorrhizal fungi (PERRY *et al.* 1987; KROPP and ALBEE 1996; VISSER and PARKINSON 1999) since they remove trees and thus the fungi's main energy source. Ectomycorrhizal fungi require carbohydrates from trees in order to complete their life cycles (SMITH and READ 1997). The stumps and roots of cut trees can act as sources of carbohydrates which can keep alive the ectomycorrhizal soil mycelium for some years at a reduced level, but which is insufficient to produce fruit bodies. Little is known about how long the mycelia of ectomycorrhizal fungi can survive in the soil without living trees. Until recently, it has only been possible to assess the below-ground ectomycorrhizal species composition by morphologically characterising ectomycorrhizal root tips. This, however, raises methodological difficulties because of the lack of resolution by morphotyping (PETER *et al.* 2002b). Nowadays molecular techniques have greatly advanced ways of studying ectomycorrhizal diversity at the root level. Such knowledge is of great importance since we know that a high ectomycorrhizal diversity may increase the stability of forest ecosystems and their resilience to disturbance (PERRY *et al.* 1989). Moreover, disturbance of the ectomycorrhizal flora may have negative effects on the regeneration success of forest trees since it can reduce or retard the formation of ectomycorrhizae on the fine roots of seedlings. In the present study we used both morphotyping and molecular techniques to determine the effects of a windthrow event on the below-ground ectomycorrhizal species composition over a period of 10 years following the event.

As one technique we used a seedling bioassay (BRUNDRETT *et al.* 1996) to examine the ectomycorrhizal inoculum potential (i.e. the ectomycorrhizal diversity in the soil and the potential of these fungal species to colonize roots) of the soil sampled in a windthrow and a forest control plot over time. In the second technique we used directly sampled naturally regenerated *Picea abies* seedlings from both the windthrow and forest control plot in the year 2000, i.e. ten years after the storm event. The aims of the study were to investigate whether the ectomycorrhizal inoculum potential decreases over time, and whether, as a consequence, the regeneration success of forest trees decreases after a windthrow event due to a lack of ectomycorrhizal inoculum in the soil.

2 Materials and methods

2.1 Study plots

Two 10 x 10 m sampling plots were defined in 1992, two years after the storm. One plot was in the uncleared, naturally regenerating area of the windthrow site Schwanden and the other in the adjacent old Norway spruce (*Picea abies* [L.] Karst.) forest. The windthrow plot contained, as a result of the storm, no living old trees. The minimum distance to the nearest living old Norway spruce tree was more than 100 metres. There were no young trees or tree seedlings from the former understory within the plot. The forest plot was situated 10 m inside the forest, about 200 m away from the windthrow plot.

2.2 Sampling procedure

Bioassay

The bioassay was carried out according to BRUNDRETT *et al.* (1996) with minor modifications. Each year, beginning in the second year after the storm event, 10 soil samples of about 0.5 l were randomly collected from each of the two plots to a depth of 10 cm using a cylindrical soil corer (corresponding to 10 replicates per plot). The samples were taken between May 1st and May 15th, from 1992 to 2000. Each soil sample was separately stored in a plastic bag at 4 °C and within 48 hours transferred individually into a sterilized (120 °C, 20 min) clay pot. Norway spruce seeds were surface sterilized in 30% hydrogen peroxide for 30 min and 10 seeds were planted in each pot. Pots were maintained in a climatized greenhouse (temperature range: 15 to 25 °C) under natural day/night conditions, and watered twice weekly with tap water. Four months after planting, the seedlings were all harvested within one week and counted to determine the surviving percentage. The roots of each pot (corresponding to one replicate) were pooled, carefully washed under running tap water and stored in 6% formaldehyde until they could be classified according to morphotype. In the year 2000, the roots of the bioassay were not stored in formaldehyde, but investigated immediately after the washing procedure so that they could be subjected to molecular analysis (see below).

Field sampling

In spring 2000, eight naturally regenerated seedlings, at most 4 years old, were sampled from both the windthrow and the adjacent forest plot. Within the 10 m x 10 m windthrow plot only two seedlings were found, and the remaining six were taken from the vicinity around the plot. The roots of each seedling were carefully washed under running tap water and separately processed immediately after washing.

2.3 Analysis of root material

Morphotype classification

The whole root system from each bioassay sample, which corresponded to one bioassay pot, was examined in water under a dissecting microscope. The root tips were classified into ectomycorrhizal morphotypes based on distinctive macroscopic and microscopic features according to AGERER (1991). The frequency of each morphotype was estimated as a percentage (number of root tips of each morphotype divided by total number of root tips). For each pot, the morphotype diversity was calculated using Shannon's index of diversity: $H = -\sum p_i \ln p_i$, where p_i was the frequency of each morphotype i (MAGURRAN 1988) and averaged over all 10 pots.

Molecular identification (PCR-RFLP)

The ectomycorrhizal species on the roots from both the bioassay and field seedlings from the year 2000 were identified molecularly. Freshly washed roots were treated as follows: From each morphotype within one sample (corresponding to the roots of one pot or of one field seedling), one to three mycorrhizal root tips were collected and subjected to molecular analyses. The aim was to detect as many different ectomycorrhizal species present in a sample as possible. The morphologically unambiguously identifiable morphotypes, *Cenococcum geophilum* and *Russula ochroleuca*, were sampled only once. The single root tips were placed separately into 1.5 ml tubes and stored at -20°C prior to DNA extraction. Ectomycorrhizal fungi on mycorrhizal root tips were identified by comparing restriction fragment length polymorphisms (RFLP) obtained from digestions of the internal transcribed spacer (ITS) region of rDNA, which had been amplified from extracted DNA by the polymerase chain reaction (PCR) as described by PETER *et al.* (2001a). DNA extraction followed the protocol of KAREN *et al.* (1997). For DNA amplification, we used the primer pair ITS 1 and ITS 4 (WHITE *et al.* 1990), according to the PCR protocol of GARDES and BRUNS (1996) with minor modifications as described in PETER *et al.* 2001a. RFLP types were obtained using the restriction enzymes MboI, HinfI, and TaqI. Fragment sizes were determined on an ABI Prism 310 Genetic Analyser using GeneScan 3.1 Analysis and Genotyper 2.5 Software (Applied Biosystems). Taxotron software (Pasteur Institute, Paris, France) was used to analyse and interpret the RFLP types using single-linkage cluster analysis following the description of KAREN *et al.* (1997), with minor modifications as described in PETER *et al.* (2001a).

RFLP types were compared with a reference database consisting of approx. 180 DNA patterns of different ECM fungal species, most likely associated with spruce, from most major basidiomycetous ECM genera.

3 Results

From the bioassay seedlings, a total of 12 ectomycorrhizal morphotypes were classified. Two of them were identified as *Cenococcum geophilum* and *Russula ochroleuca*. In the year 2000 all the 12 morphotypes were found on the seedlings from both the windthrow and forest plots, except on the field seedlings from the windthrow plot where only eight morphotypes were present (Table 1). No tendency for there to be a change over time in morphotype composition or abundance was detected in either the windthrow or the forest plot. Shannon's index of diversity did not show any clear differences between the windthrow plot and the forest plot in the sampling period (Fig. 1). This was confirmed by a mixed model ANOVA (the plot as the fixed factor, the year as the random factor; $F_{1,8} = 1.205$, $p > 0.05$).

In the molecular analyses, a total of 17 ITS-RFLP types were distinguished; 10 of them were identified to species or genera by comparing the RFLP patterns with the reference library (Table 1). Seven RFLP types remained unidentified. 10 years after the windthrow

event eight ectomycorrhizal species or unidentified RFLP types were found on the windthrow plot, compared to 14 in the adjacent forest. Five of these 14 RFLP types, namely *Boletus edulis*, *Cortinarius* species, *Russula ochroleuca*, *R. fellea* and *Xerocomus badius*, are species that form large conspicuous fruit bodies. All of them were found only on field seedlings grown in the forest plot. Only five species or types were found on both plots: *Cenococcum geophilum*, *Telephora terrestris*, *Tylospora asterophora* and two unidentified RFLP types. Quantitatively *Tylospora asterophora* was the most important mycorrhiza-forming fungus on the windthrow plot: more than half of all the root tips molecularly analyzed were derived from this fungus.

The results of the molecular analysis of mycorrhizal root tips of the bioassay 2000 revealed a relatively low number of species or RFLP types on seedlings from both the windthrow (7) and forest plots (6), but there were some differences in species composition between them. Three species were detected on both plots (*Cenococcum geophilum*, *Telephora terrestris* and *Tylospora asterophora*), whereas a *Tuber* species and three unidentified RFLP types were found exclusively on the windthrow plot, and *Tylospora fibrillosa* and two other unidentified RFLP types were found only on the forest plot (Table 1).

The percentages of the surviving bioassay seedlings showed no significant differences between the windthrow and the forest plot in any sampling year (Fig. 2). There was, however, a constant decrease over the nine years of assessment in both treatments, which was probably due to the seed material losing its germination capacity over time.

Table 1. Ectomycorrhizal species/ RFLP types and number of morphotypes of field- and bioassay-seedlings from the windthrow and the forest plots, 10 years after the windthrow.

	windthrow plot		forest plot	
	bioassay	field	bioassay	field
species:				
<i>Boletus edulis</i>				x
<i>Cenococcum geophilum</i>	x		x	x
<i>Cortinarius</i> group *)				x
<i>Russula ochroleuca</i>				x
<i>Russula fellea</i>				x
<i>Telephora terrestris</i>	x	x	x	
<i>Tuber</i> sp.	x			
<i>Tylospora asterophora</i>	x	x	x	x
<i>Tylospora fibrillosa</i>			x	x
<i>Xerocomus badius</i>				x
RFLP types:				
RFLP02-Par,Bea (Hygrophoraceae)	x			x
RFLP59-Vor		x		x
RFLPneu (Thelephoraceae)				x
RFLPSchwBiot1			x	
RFLPSchwBiot2			x	
RFLPGew97-3-BiotestPar	x	x		
RFLP11-Par	x	x		
number of species/RFLP types	7	5	6	11
	8		14	
number of morphotypes	12	8	12	12

*) *Cortinarius sanguineus/olivaceofuscus/sommerfeltii*

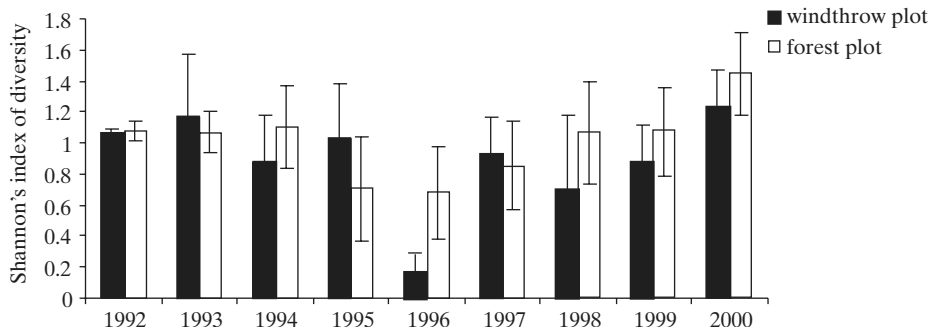


Fig. 1. Morphotype diversity (Shannon's index of diversity) of the the bioassay seedlings from 1992 to 2000 (n = 10).

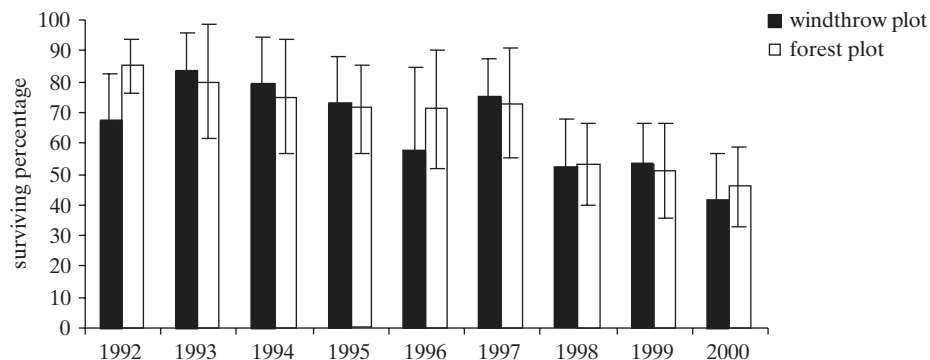


Fig. 2. Surviving percentage of the bioassay seedlings from 1992 to 2000 (n = 10).

4 Discussion

The two methods applied, namely the morphological and the molecular description of ectomycorrhizal diversity, do not reveal the same results. With the morphotype survey no significant differences between the windthrow and the forest plots could be detected during the 10 years following the storm event. In contrast, the molecular approach showed clear differences between the two sites: on the windthrow plot the set of mycorrhizal fungi colonizing seedlings diminished and, 10 years after the windthrow event, consisted of just a few species. The probable reason for this divergence is that the resolution of morphotyping is considerably lower than that of the PCR-RFLP method, as has already been noted by other authors (MEHMANN *et al.* 1995; KAREN and NYLUND 1997; JONSSON *et al.* 1999; PETER *et al.* 2001b). Our results also show that the number of morphotypes normally do not correspond with the

number of RFLP types: morphotypes can comprise several RFLP types, whereas distinctive RFLP types can be assigned to more than one morphotype (PETER *et al.* 2001b). The results from other studies on the effect of clearcutting on morphotype diversity are inconsistent. MAH *et al.* (2001) found that the ectomycorrhizal diversity on seedlings regenerating in four- to five-year-old clearcut sites was lower than that on seedlings from mature forests. Similar findings are reported by REXER *et al.* (1998), but PILZ and PERRY (1984) found no significant differences in the mycorrhization two to three years after a clearcut. In another study (VISSER and PARKINSON 1999), the ectomycorrhizal community composition and structure six years after clearcutting was found to be very similar to that in mature, uncut stands. In contrast, after wildfire, the ectomycorrhizal community was severely destabilized and recovery was much slower than after clearcutting. Extensive combustion of the forest floor organic matter seems to damage the soil mycelium.

The results from our molecular analysis reveal that ten years after the storm event, seedlings that had naturally regenerated on the forest plot contained a wide range of species, similar to that found in old-growth forests, including the *Russula* and *Cortinarius* species, *Boletus edulis* and *Xerocomus badius*. Seedlings grown on the same forest soil in the bioassay and seedlings grown on the windthrow plot or on soil from the windthrow plot were not colonized by these fungi. It seems that these "old forest" species need to be linked to old forest trees and that they only colonize seedlings if connected to living trees via mycelia. If this connection is disrupted, the mycelia of these fungi are not competitive enough to form mycorrhizas on seedlings. TAYLOR and BRUNS (1999) made a similar observation when comparing the abundance and frequency of ectomycorrhizal species on roots in a mature *Pinus muricata* forest with those that colonized potted seedlings in the same soils. They ascribed this phenomenon to the species-specific colonization strategies of ectomycorrhizal fungi and the different roles of mycelial spread vs. spores. That is, species which need to be connected to old trees primarily spread and colonize seedlings through existing mycelial growth, whereas so-called pioneer species, which colonize seedlings in pot experiments and disturbed sites, are more likely to colonize from spores and resistant propagules.

On the windthrow plot eight species or RFLP types were still present 10 years after the elimination of their host trees, including *Tylospora asterophora*, *Telephora terrestris*, *Cenococcum geophilum* and a species belonging to the genus *Tuber*. *T. terrestris* is described by REXER *et al.* (1998) as the only species remaining on windthrow areas with no young trees left. These fungi, which still colonize seedlings up to 10 years after a windthrow event, seem either to be able to survive in the soil as resistant propagules or to colonize disturbed sites by spore spread.

Our study has shown that a windthrow event reduces the number of infective ectomycorrhizal fungi significantly. In particular, species which form large fruit bodies, mostly edible species of high value, are rare on windthrow sites. However, the soil of a windthrow site still contains enough mycorrhizal fungi to fully mycorrhizize ongrowing seedlings even 10 years after the event. The fact that the survival rate of seedlings growing on windthrow soils is not lower than that on forest soils suggests that the reduction in the number of ectomycorrhizal species and the changes in species composition cannot be the primary reasons for a possible impairment of the natural regeneration of windthrow sites. On the other hand, a reduction in the number of species may have negative consequences for the subsequent growth of the forest and its stability in later years, as emphasized by PERRY *et al.* (1989). BYRD *et al.* (2000) moreover conclude that a loss of species richness after a windthrow event may have profound effects on how the ecosystem functions. It could, for example, reduce forest resilience, which could, in turn, have detrimental effects on the plant-soil link, leading to less natural forest regeneration.

The number of ectomycorrhizal species should, therefore, be kept as high as possible after a windthrow event. A feasible way to achieve this goal is to protect as well as possible those young trees and seedlings that have survived a windthrow. REXER *et al.* (1998) showed that the tree seedlings that germinated on windthrow areas had obtained their mycorrhizal symbionts from the species spectrum present on the roots of the surviving young trees. Therefore, from a mycological point of view, windthrow areas should not be cleared. They should be left as untouched as possible.

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