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# Defoliation negatively affects plant growth and the ectomycorrhizal community of *Pinus pinaster* in Spain

**Montserrat Pestaña · Serena Santolamazza-Carbone**

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**Abstract** In this work, by artificially reproducing severe (75%) and moderate (25%) defoliation on maritime pines *Pinus pinaster* in NW Spain, we investigated, under natural conditions, the consequences of foliage loss on reproduction, abundance, diversity and richness of the fungal symbionts growing belowground and aboveground. The effect of defoliation on tree growth was also assessed. Mature needles were clipped during April 2007 and 2008. Root samples were collected in June–July 2007 and 2008. Collection of sporocarps was performed weekly from April 2007 to April 2009. Taxonomic identity of ectomycorrhizal fungi was assessed by using the internal transcribed spacer (ITS) regions of rDNA through the polymerase chain reaction (PCR) method, subsequent direct sequencing and BLAST search. Ectomycorrhizal colonization was significantly reduced (from 54 to 42%) in 2008 by 75% defoliation, accompanied with a decline in species richness and diversity. On the other hand, sporocarp abundance, richness and diversity were not affected by foliage loss. Some ECM fungal symbionts, which are assumed to have a higher carbon cost according to the morphotypes structure, were reduced due to severe (75%) defoliation. Furthermore, 75% foliage loss consistently depressed tree growth, which in turn affected the ectomycorrhizal growth pattern. Defoliation impact on ECM symbionts largely depends on the percentage of foliage removal and on the number of defoliation bouts. Severe defoliation (75%) in the short term (2 years) changed the composition of the ECM community likely because root biomass would be adjusted to lower levels in parallel with the depletion of the aboveground plant biomass, which probably promoted the competition among mycorrhizal types for host resources. The persistence of fungal biomass in mycorrhizal roots would be crucial for nutrient up-take and recovery from defoliation stress of the host plants.

**Keywords** Simulated herbivory · ITS · Fungal species diversity · Plant growth

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## Introduction

To understand the dynamics of terrestrial ecosystems a combined aboveground–belowground approach is required, because many important feedbacks occur between the producer and other trophic levels (Price et al. 1980; Gange and Brown 1997; Van der Putten et al. 2001; Tscharntke and Hawkins 2002; Bardgett and Wardle 2003; Hartley and Gange 2009). Nonetheless, multitrophic interaction theory has focused mainly on aboveground species assemblages, because these are easier to observe, sample and manipulate experimentally than soil communities (Gange and Brown 1997; Van der Putten et al. 2001; Tscharntke and Hawkins 2002).

Mycorrhizae are mutually beneficial symbiotic structures formed by fungi and fine roots of terrestrial plants, participating in maintaining plant biodiversity and ecosystem variability, stability and productivity (Molina et al.

1992; Brundrett 2002;Whitfield 2007; Smith and Read 2008). Approximately 7,000–10,000 fungal species form ectomycorrhizae (ECM) (Taylor and Alexander 2005), the predominant mycorrhizae of temperate forest ecosystems, that play a complex role in aboveground–belowground multitrophic interactions (Molina et al. 1992; Van der Putten et al. 2001; Bennett et al. 2006). Herbivores and mycorrhizal fungi usually coexist on the same host plant and thus may interact affecting each other's performance (Smith and Read 1997; Gange and Brown 1997; Gehring and Whitham 2002; Hartley and Gange 2009). Although great attention has been paid to the independent effects that herbivores and mycorrhizal fungi have on their host plants, comparatively few studies have examined their interactions (Hartley and Gange 2009).

Generally, the growth of forest trees decreases after insect herbivory, and the response to damage is often proportional to the amount of foliage lost (May and Carlyle 2003). Because nearly 20% of the carbon produced by woody plants is allocated belowground (Hobbie and Hobbie 2006), loss of photosynthetic area may reduce carbon flux to roots, negatively affecting mycorrhizal fungal symbionts (Gehring and Whitham 2002). On the other hand, there is evidence that herbivory may favor carbon inputs to the soil, through fine root turnover or an increase of root exudation, but at present it has been shown only in fast-growing plant species (Bardgett et al. 1998). However, the studies conducted on the effects of herbivory on the ECM fungi associated with conifers such as *Pinus* spp. have provided variable results. For example, reduced carbon assimilation in *P. sylvestris* due to artificial defoliation has been found to negatively affect ECM fungi by reducing abundance and diversity of sporocarps (Kuikka et al. 2003). Furthermore, ECM colonization of fine root tips of *P. edulis* was significantly reduced by herbivory caused by the pine tip moth *Dioryctria albovittella* (Lepidoptera, Pyralidae) (Gehring and Whitham 1991), and by the pinyon-feeding scale *Matsucoccus acalyptus* (Hemiptera, Margarodidae) (Gehring and Whitham 1991; Del Vecchio et al. 1993). However, in other studies on *P. sylvestris*, it has been shown that artificial defoliation induced a photosynthate limitation that did not alter percent ECM colonization (Markkola et al. 1995; Saikkonen et al. 1999) but produced a qualitative shift in the ECM community, because relative abundance of ECM morphotypes with high fungal biomass and high carbon demand was reduced (Saikkonen et al. 1999). Similarly, artificial defoliation on *P. contorta* did not alter ECM colonization and species richness but caused changes in species composition, relative abundance and frequency of certain morphotypes (Cullings et al. 2001, 2005). On the other hand, Gehring and Whitham (1995) found a negative impact of simulated herbivory on ECM colonization in *P. edulis*. Furthermore, it has been reported that ECM community associated with *P. edulis* may shift from Basidiomycetes to Ascomycetes, which typically have a lower biomass production and less nutritional requirements (Gehring and Whitham 2002; Mueller and Gehring 2006). Differences in the environmental variables, such as climatic and edaphic factors (Erland and Taylor 2002), defoliation type and intensity (Gehring and Whitham 2002), time of defoliation (Saravesi et al. 2008), plant susceptibility (Gehring and Whitham 1995) and duration of damage (Gehring and Whitham 2002), could account for the different fungal responses to herbivory.

In our work, by artificially reproducing severe (75%) and moderate (25%) defoliation performed by the pine processionary moth *Thaumetopoea pityocampa* Schiff. (Lepidoptera: Notodontidae) on maritime pine (*Pinus pinaster* Ait.), we investigated first the consequences of foliage loss on tree growth and reproduction, and second the effect of this aboveground disturbance on reproduction, abundance, diversity, richness, and community structure of the ECM fungal symbionts. This is the first time that these interactions have been investigated in *P. pinaster* plants growing in temperate forest ecosystems of southern Europe.

We selected evergreen conifers as model species, because it is recognized that a large amount of the whole-plant carbon and nitrogen is located in the foliage (Helmisaari 1992; Millard et al. 2001). During spring and summer, mobile nutrients such as N, P, and K are retranslocated from mature needles to current shoots and needles, which require high amount of resources (Helmisaari 1992; Millard et al. 2001). While N remobilization is unaffected by foliage loss among deciduous trees, among evergreens it can be reduced by half (Millard et al. 2001), thus accentuating the importance of the presence of mycorrhizal symbionts for nutrient uptake (Kuikka et al. 2003). Moreover, some conifers have a fixed pattern of growth, which limits the capacity for compensatory development (Trumble et al.1993; Ayres et al. 2004), although a few cases have shown that defoliated trees had a higher net photosynthetic rate (Kolb et al.1999; Vanderklein and Reich 1999). Hence, we expected a strong plant response to nutritional stress, as reported previously on *Pinus* spp. after natural (Hódar et al. 2003; Kanat et al. 2005) or artificial herbivory (Millard et al. 2001). We addressed the following questions: (1) do ECM colonization, diversity and richness in maritime pine negatively correlate with defoliation degree, (2) are high-biomass ectomycorrhizal types more reduced by defoliation, and (3) is one defoliation bout enough to trigger changes in the ECM community or a second harvest (i.e. higher loss of foliage) is necessary?

We expected that simulated herbivory should force the host plant to make compensatory responses belowground, which could be translated in a reduction of ECM

colonization associated with a decline in species diversity and richness.

## Materials and methods

### Study area

The research area was located in Pontevedra (NW Spain). The study was replicated in two pine forest where *P. pinaster* was the dominant tree species. The first plot (A) was a stand of 7,000 $m^2$ located at Catoira (42°38′N, 8°41′W), at the altitude of 370 m. It was a patch of natural regeneration of a logged 60-year-old forest. The soil was an umbric regosol-type from unconsolidated materials and with an umbric A-horizon. Vegetation was composed of patches of trees (*Pyrus cordata* Desv., *Frangula alnus* Miller, *Quercus robur* L., *Pinus radiata* Don.), shrubs [*Ulex* sp., *Erica* sp., *Rubus* sp., *Calluna vulgaris* (L.) Hull and *Daboecia cantabrica* (Huds) K. Koch.], and herbaceous plants [*Agrostis curtisii* Kerguelen, *Mentha rotundifolia* L., *Pteridium aquilinum* (L.) Kunth, *Digitalis purpurea* L., *Asphodelus albus* Willd., *Narcissus bulbocodium* L., *Potentilla erecta* (L.) Rauschel, *Lithodora prostrata* (Loisel) Griseb]. The mean annual temperature was 13.4°C in 2007 and 13.3°C in 2008. The mean annual precipitation reached 1,008 mm in 2007 and 1,342 mm in 2008.

The second plot (B) was a pine cultivation of approximately 6,000 $m^2$ located at Cotobade (42°29′N, 8°29′W), at the altitude of 545 m. The soil at this location was an umbric leptosol-type, which was less than 30 cm deep, with continuous hard rock and an umbric A-horizon. Vegetation was composed of patches of shrubs [*Ulex* sp., *Erica* sp., *Rubus* sp., *Calluna vulgaris* (L.) Hull and *Daboecia cantabrica* (Huds) K. Koch.] and herbaceous plants [*Agrostis truncatula* Castrov. & Charpin, *Agrostis curtisii* Kerguelen, *Mentha rotundifolia* L., *Pteridium aquilinum* (L.) Kunth, *Digitalis purpurea* L., *Asphodelus albus* Willd., *Narcissus bulbocodium* L., *Potentilla erecta* (L.) Rauschel., *Lithodora prostrata* (Loisel) Griseb., *Hypochaeris radicata* L., *Romulea clusiana* (Lange) Nyman, *Teesdalia nudicaulis* (L.) R. Br., *Senecio lividus* L., *Aira praecox* L., *Cerastium pumilum* Curtis, *Merendera montana* (L.) Lange, *Hyacinthoides non-scripta* (L.) Chouard ex Rothm]. The mean annual temperature was 12.3°C in 2007 and 11.6°C in 2008. The annual precipitation was 1,112 mm in 2007 and 1,488 mm in 2008. Environmental parameters were obtained from two official climate stations of the regional Government (Consellería de Medio Ambiente, Xunta de Galicia). Pontevedra is characterized by an Atlantic humid climate without large frost periods and with a uniformly distributed annual precipitation over 700 mm (Martínez Cortiza and Pérez Alberti 2000). Nonetheless, autumn 2007 was considered the driest of the last 50 years in Pontevedra (Consellería de Medio Ambiente 2008).

### Soil characterization

To analyze soil parameters, 12 samples (6 per locality) were collected by using a soil core sampler of 750 $cm^3$ (Eijkelkamp Agrisearch Equipment, The Netherlands). We assessed soil pH, organic C, total soil N, Ca, K, P and Mg. Soil organic matter was determined by the rapid method of Walkley–Black (Walkley and Black 1934) and total soil N was measured by the Kjeldahl method (Bremner and Mulvaney 1982). The relationship C/N was also estimated. The available phosphorus was determined by the Bray II method (Bray and Kurtz 1945). Potassium was determined by flame emission and Ca and Mg by absorption spectroscopy (Knudsen et al. 1982). Soil dry weights were determined after the samples had been dried overnight at 105°C. Results are summarized in Table 1.

### Experimental design

The experiment was carried out during 2007 and 2008 on the same experimental trees, to evaluate how the host plants and the fungal symbionts tolerate two consecutive defoliations during the growing season. In each plot, 45 trees (5 years old) were randomly selected and marked with plastic tags. In 2007, before the treatments, the mean tree height at site A was 2.23 ± 0.06 m, and at site B it was 2.08 ± 0.06 m. The age of the trees was estimated by counting the branch whorls. The trees were distant at least 2 m from each other to decrease the belowground hyphal connections. In each plot, the plants were randomly distributed among three blocks, and within each block they were randomly assigned to three treatments (five repetitions per treatment): a control group (unmanipulated), moderate defoliation (25%) and severe defoliation (75%). Artificial defoliation, reproducing the damage produced by

**Table 1** Soil parameters of experimental sites

| | Site A | Site B |
|---|---|---|
| pH ($H_2O$) | 4.16 ± 0.06 | 4.13 ± 0.09 |
| Organic matter (%) | 15.1 ± 2.1 | 8.43 ± 0.53 |
| Organic C (%) | 8.76 ± 1.21 | 4.89 ± 0.31 |
| N total (%) | 0.64 ± 0.09 | 0.54 ± 0.03 |
| C/N | 13.6 ± 0.8 | 9.16 ± 0.50 |
| Available P (ppm) | 16.8 ± 3.4 | 74.5 ± 11.2 |
| K (ppm) | 70.5 ± 9.6 | 36.1 ± 1.8 |
| Ca (ppm) | 30.1 ± 9.2 | 17.6 ± 2.9 |
| Mg (ppm) | 31.1 ± 5.86 | 9.2 ± 3.1 |

Mean of six samples (two per block) for each site ± SE

the pine processionary moth *Thaumetopoea pityocampa*, was applied during April 2007 and 2008. It consisted in clipping with scissors the assigned percentage (25 or 75%) of mature needles for each branch whorl, without eliminating the base of needles. During the experiment, no natural defoliation occurred in the study areas. The pine processionary moth is the most important defoliator of pines in the Mediterranean region (Devkota and Schmidt 1990). Severe outbreaks may led to strong defoliation, which imply reduced pine growth and reproduction, and eventually tree deaths (Dajoz 2000; Hódar et al. 2003; Kanat et al. 2005). In Spain, the majority of the defoliation provoked by the pine processionary caterpillar (from 20 to over 70% of the mature foliage) occurs between January and April, when larvae are in the fourth and fifth instars (Romanyk and Cadahia 1992; Hódar et al. 2003).

### Tree growth parameters

To assess the impacts of foliage loss on growth and reproduction of the selected trees, we measured on spring 2007 (before the first defoliation) and 2008 (before the second defoliation) the following parameters: tree height, stem diameter, crown height, crown diameter, number of shoots, length of the terminal leader shoot, length of the terminal shoots in the upper three branch whorls, length of four needles in the main stem and number of female cones.

### Root sampling

We collected root samples (~200 cm of fine roots) approximately 1 m around the base of each tree, by digging to a maximum depth of 20–30 cm. The main roots of each marked pine were followed to avoid including samples from other plants. Fine roots were cut with scissors, introduced in marked plastic bags and transported to the laboratory, where they were carefully washed under running tap water and stored at 4°C. Roots were collected during June–July 2007 and 2008. Following Agerer (1987–2002), mycorrhizal root tips were sorted into different morphotypes based on color, shape, texture, ramification type, mantle type and occurrence of the emanating hyphae or rhizomorphs. For each tree, subsamples of the morphotypes (depending on its abundance, it ranged from 10 to 100 mg) were placed individually in a 1.5-ml Eppendorf tube and stored at −20°C for molecular analysis, in order to provide definitive identification. To study the impact of the treatments on fungal species which are assumed to have different carbon requirements, after morphologic description and taxonomic identification (see methods below), fungal ECM were separated in two categories: smooth types with thin-mantle, without extensive extraradical mycelia, and generally with low carbon demand (low-biomass), and types with thick mantle, forming abundant hyphal bundles or rhizomorphs, and with high carbon demand (high-biomass) (Saikkonen et al. 1999; Saravesi et al. 2008) (see Electronic Supplementary Material, Fig. S1).

### Molecular characterization

Taxonomic identity of the morphotypes was ascertained by PCR amplification and direct sequencing of the internal transcribed spacer (ITS) (Horton and Bruns 2001). Fungal DNA was extracted from frozen ECM root tips using the EZNA Fungal DNA Kit (Omega Bio-Tek, USA) according to the manufacturer's instructions. DNA samples were run by gel electrophoresis, with 1.5% agarose gel and 1 µl of ethidium bromide, at 60 V for 15 min. Results were visualized under a UV-transilluminator. We obtained a gross estimation of DNA concentration and molecular length by using Lambda Phage (Sigma, USA) and BioMarker Low (Bioventures, USA) respectively. The PCR reaction mix consisted of 1 µl of undiluted DNA (concentration ranged between 10 and 50 ng/µl), 23.8 µl sterile ultrapure water, 3 µl of 10× PCR buffer–$MgCl_2$, 0.6 µl of 0.2 mM $dNTP_s$ 0.5 µl of each primer and 0.6 µl of 1U/µl of Taq enzyme (BioTaq; Bioline, USA). We performed PCR with the following primer combinations: ITS1/ITS4, which amplify basidiomycete and ascomycete species, or ITS1F/ITS4B, which is intended to be a basidiomycete-specific primer pair (Gardes and Bruns 1993). PCR conditions were optimized for each primer combination, but the general reaction protocol was as follows: 94°C for 3 min, 94°C for 30 s, 55°C for 30 s and 72°C for 1 min (40 cycles) and 72°C for 10 min. Successful amplifications were verified by gel electrophoresis at 60 V for 30 min. When PCR products were not successfully amplified, the eluted DNA was purified with PowerClean DNA Clean-Up Kit (MoBio Laboratories, USA) following manufacturer's instructions, and PCR reaction was repeated. PCR products were purified and sequenced by Macrogen Laboratories (http://www.macrogen.com).

Forward and reverse sequences were aligned and edited using SeqMan™ software (DNAStar, Madison, WI, USA). All sequences were identified to genus or species level by querying the GenBank database, using the nucleotide–nucleotide (blastn) BLAST search option, available through the National Center for Biotechnology Information (NCBI), and the UNITE (Koljalg et al. 2005) online database through blastn algorithm, searching sequences from INSD, the International Nucleotide Sequence Database (GenBank, EMBL, DDBJ). We considered only sequences with 90–100% similarity and *E* value (expectation value) equal to zero. Assignment to taxonomic categories was performed using the following criterion:

sequence similarity ≥98% gives a match for species identification, while sequences with similarity between 90 and 97% were identified to genus groups.

### Sporocarp sampling

To study sexual reproduction of the ECM species, all the sporocarps growing 2 m around the marked trees were collected weekly during 2 years, from April 2007 to April 2009. Sporocarps were transported to the laboratory to be identified, counted, dried (60°C, 48 h) and weighed.

### Ecological parameters

Ectomycorrhizae colonization percentage was calculated by using the gridline intersect method under a dissecting microscope (Brundrett et al. 1996). Three replicates of 60 short pieces (~1 cm) of roots were examined per tree. The fragments were randomly dispersed in a 9-cm-diameter Petri dish with grid lines.

Once obtained a taxonomic identity of ECM root tips and sporocarps, we assessed the relative abundance of ECM sporocarps (number of sporocarps produced by each taxon/total number of sporocarps per tree per year), and ECM root tips (total root tips produced by each taxon/total ECM root tips per tree per year). Finally, we evaluated species richness of ECM belowground and aboveground, and Shannon–Wiener diversity index (H′), calculated by using the following equation:

$$H' = \sum_{i=1}^{S} pi \ln pi$$

where $p_i$ is the proportion of root tips or sporocarps produced by a taxon $i$ per tree and year. The Shannon–Wiener index increases when the number of taxa increases or the distribution of the species becomes more even.

### Statistical analysis

Prior the analyses, variables were checked for normality by Kolmogorov–Smirnov test. The effect of 2007 defoliation on tree growth parameters of 2008 was assessed by analysis of variance (ANOVA), using sites and blocks as random factors. No transformations were required to satisfy ANOVA assumption. Pairwise comparisons were performed using the Fisher's least significant differences test (LSD). Female cone production was analyzed by using Kruskal–Wallis test, followed by Mann–Whitney $U$ test for pairwise comparisons.

Analysis of covariance (ANCOVA), with sites and blocks as random factors, and tree growth parameters as covariates, was employed to determine, in either year, whether ECM colonization percentage and low/high-biomass species abundance varied among the treatments. Data were previously subjected to logarithm and square-root transformation to meet the assumption of normality and homogeneity of variances. Pairwise comparisons were performed with LSD test.

Species richness per year was analyzed using a generalized linear mixed model (GLMM) with Poisson distribution and logarithm link function, using sites and blocks as random factors. The effect of defoliation on diversity was tested per year by a generalized linear mixed model (GLMM) with normal distribution and identity function, using sites and blocks as random factors. Spearman's rank correlation coefficient was used to analyze the relationship between treatments and belowground ECM taxa.

To analyze the effect of treatments on the number of sporocarps and biomass (dry weigh) in either year, we used an ANCOVA with sites and blocks as random factor and tree growth parameters as covariates. Data were previously submitted to $\log_{10}(x + 1)$ transformation.

Treatment consequences on species richness and diversity of sporocarps were analyzed per year by using a generalized linear model (GLM) with Poisson distribution and logarithm link function.

Significance was declared at $P < 0.05$. The analyses were performed with GenStat release 10.2 (VSN International, Hemel Hempstead, UK).

## Results

### Tree growth parameters

In 2007, prior to defoliation, we did not find any significant difference in tree growth parameters among the trees (data not shown). On the other hand, defoliation performed in 2007 resulted in a significant reduction of tree growth in 2008 (Table 2). Specifically, we found a negative effect of defoliation on tree height ($F_{2,79} = 3.47$, $P = 0.036$), stem diameter ($F_{2,79} = 5.11$, $P = 0.008$), number of shoots ($F_{2,79} = 3.83$, $P = 0.026$), length of terminal shoots on the first whorl ($F_{2,79} = 4.90$, $P = 0.010$), second whorl ($F_{2,79} = 3.34$, $P = 0.041$), and third whorl ($F_{2,79} = 5.33$, $P = 0.007$). Defoliation marginally affected crown height ($F_{2,79} = 2.54$, $P = 0.085$), length of terminal leader shoot ($F_{2,79} = 2.98$, $P = 0.057$), and needle length ($F_{2,79} = 2.27$, $P = 0.110$), but not crown diameter ($F_{2,79} = 1.47$, $P = 0.236$), and female cone production (Kruskal–Wallis = 0.814, $P = 0.666$).

### ECM root tips and sporocarp identification

A total amount of 59,588 ECM root tips (Table 3) were counted under the dissecting microscope. The number of

**Table 2** Tree growth parameters per site measured in 2008

| | Site A | | | Site B | | |
|---|---|---|---|---|---|---|
| | 0 (%) | 25 (%) | 75 (%) | 0 (%) | 25 (%) | 75 (%) |
| Tree height (m) | 3.26 ± 0.15 a | 3.23 ± 0.14 a | 3.09 ± 0.13 a | 2.70 ± 0.19 a | 2.35 ± 0.14 b | 2.15 ± 0.15 b |
| Stem diameter (cm) | 6.74 ± 0.43 a | 6.99 ± 0.52 a | 5.74 ± 0.37 b | 6.52 ± 0.45 a | 5.47 ± 0.45 b | 4.71 ± 0.54 b |
| Crown height (m) | 2.51 ± 0.15 a | 2.63 ± 0.15 a | 2.35 ± 0.15 a | 1.76 ± 0.09 a | 1.50 ± 0.19 b | 1.37 ± 0.11 b |
| Crown diameter (m) | 1.75 ± 0.05 b | 1.85 ± 0.09 a | 1.64 ± 0.08 b | 1.59 ± 0.04 a | 1.54 ± 0.04 a | 1.54 ± 0.06 a |
| Number of shoots | 192.3 ± 18.3 b | 253.0 ± 30.3 a | 149.5 ± 15.1 c | 185.9 ± 16.1 a | 209.5 ± 12.2 a | 218.0 ± 21.4 a |
| Terminal leader shoot length (cm) | 36.0 ± 3.3 a | 34.2 ± 3.0 a | 30.3 ± 3.1 a | 18.1 ± 2.9 a | 10.8 ± 2.9 b | 10.2 ± 2.0 b |
| First whorl terminal shoot length (cm) | 26.0 ± 2.8 a | 25.0 ± 2.4 a | 18.3 ± 2.1 b | 11.7 ± 1.8 a | 9.40 ± 1.30 ab | 7.68 ± 1.00 b |
| Second whorl terminal shoot length (cm) | 23.4 ± 3.0 a | 23.4 ± 2.2 a | 16.2 ± 2.3 b | 8.37 ± 1.15 a | 8.31 ± 1.36 a | 6.99 ± 1.44 a |
| Third whorl terminal shoot length (cm) | 13.0 ± 2.3 a | 17.5 ± 2.3 a | 9.41 ± 1.27 b | 7.13 ± 1.11 a | 7.06 ± 1.60 ab | 5.03 ± 0.70 b |
| Needle length (cm) | 19.4 ± 0.6 a | 18.5 ± 0.6 a | 17.9 ± 0.4 b | 14.3 ± 0.7 a | 13.7 ± 1.0 ab | 13.1 ± 0.4 b |
| Number of female cones | 1.00 ± 0.28 b | 2.87 ± 0.77 a | 2.47 ± 0.52 a | 7.47 ± 1.49 a | 6.64 ± 1.68 a | 7.31 ± 2.28 a |

Different letters indicate significant differences at $P < 0.05$

ECM root tips per tree, ranged between a minimum of 15 and a maximum of 1,421, with a mean value of 337.83 ± 19.82. We obtained a total of 419 ECM root tip samples, sorted among 44 morphotypes. Of those 419 samples, only 323 were analyzed by direct sequencing, because in the other cases the anatomical and morphological characterization of the ECM was sufficient to establish the taxonomical identity. The analysis revealed the presence of 19 genera, 17 species and 4 uncultured ECM fungi (see Supplementary Material, Table S1). Actually, only 17 genera are documented as ECM fungi (15 Basidiomycetes and 2 Ascomycetes), while the other 2 are the soil fungus *Cryptococcus* spp. and the litter decomposer *Mycena* sp., which were excluded from the analysis. The phylum Ascomycota was represented by *Phialophora* sp., *Meliniomyces* sp., and two uncultured ECM fungus. We identified three species that have not been previously documented in symbiosis with *P. pinaster* (Pestaña Nieto and Santolamazza Carbone 2009): *Boletus aestivalis*, *Inocybe praetervisa*, and *Rhizopogon verii*. The most common species identified by molecular tools from ECM root tips at site A were *Tomentella sublilacina*, *Thelephora terrestris*, *Pseudotomentella tristis*, *Russula drimeia*, *Xerocomus badius*, *Suillus bovinus*, and *Paxillus involutus*, while at site B the most common types were *Rhizopogon luteolus, Suillus bovinus*, and *Thelephora terrestris* (see Supplementary Material, Fig. S2 a, b).

We collected 193 sporocarps in 2007 (192 at site A and 1 at site B) and 374 sporocarps in 2008 (271 at site A and 103 at site B) (see Supplementary Material, Table S2). The sporocarps belonged to 12 genera and 16 species: *Amanita gemmata*, *A. muscaria*, *A. rubescens*, *Amanita* sp., *Boletus* sp., *Cortinarius alboviolaceus*, *Hydnum repandum*, *Inocybe soluta*, *Laccaria amethystina*, *L. laccata*, *Paxillus involutus*, *Russula ochroleuca*, *R. sardonia*, *Russula* sp., *Scleroderma citrinum, S. verrucosum*, *Suillus bovinus*, *Thelephora terrestris*, and *Xerocomus badius*. This is the first time that *Cortinarius alboviolaceus* and *Inocybe soluta* are reported in association with *P. pinaster* (Pestaña Nieto and Santolamazza Carbone 2009). At site A, in 2007 the dominant ECM species in aboveground fungal community were *Paxillus involutus* (45.6%) and *Suillus bovinus* (45.6%), while in 2008 they were *Thelephora terrestris* (27.7%) and *Russula* sp. (18.8%). At site B, the most abundant species in 2008 was *Inocybe soluta* (56.3%).

**Table 3** Overall analyzed ECM root tips sorted by year, site and treatment

| Year | Site | Treatment | ECM root tips |
|---|---|---|---|
| 2007 | Site A | Control | 5,893 |
| | | Moderate defoliation | 7,032 |
| | | Severe defoliation | 5,538 |
| | Site B | Control | 2,718 |
| | | Moderate defoliation | 2,604 |
| | | Severe defoliation | 2,737 |
| 2008 | Site A | Control | 7,919 |
| | | Moderate defoliation | 7,232 |
| | | Severe defoliation | 5,457 |
| | Site B | Control | 4,886 |
| | | Moderate defoliation | 3,456 |
| | | Severe defoliation | 4,116 |
| Total | | | 59,588 |

## ECM colonization, richness, and diversity

Fungal colonization, richness and diversity consistently grew in 2008 across the treatment groups in both localities (Fig. 1). ECM colonization percentage was not affected by

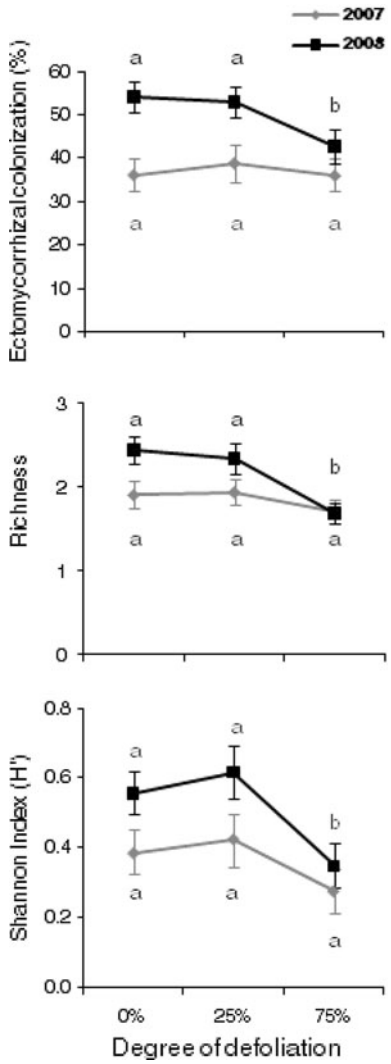


**Fig. 1** Relationship between the treatments and the percentage of ECM colonization, genera richness (mean number of taxa per tree) and Shannon–Wiener diversity index per year. *0%* Control group (undefoliated), *25%* moderate defoliation, *75%* severe defoliation. Data from both localities have been pooled. *Bars* mean ± SE; those labeled with *different letters* show significant difference at $P < 0.05$

treatments in 2007; however, in 2008 we detected a significant reduction of fungal presence (Table 4), which shifted from 54% (control group) to 42% among severely defoliated trees (Fig. 1).Tree growth parameters before defoliation, in 2007, did not have any impact on fungal colonization (data not shown), although after defoliation, in 2008, crown height ($F_{1,73} = 5.91$, $P = 0.017$) and terminal leader shoot length ($F_{1,73} = 11.53$, $P = 0.001$) had a significant positive effect on mycorrhization percentage.

The treatments did not significantly affect the abundance of high-biomass types in 2007 (Table 4), which was not influenced by tree growth parameters (data not shown). In 2008, high-biomass types were just marginally affected by treatments (Table 4) and there was a significant difference between control group and severe defoliate trees, with a reduction of 38% of high-biomass ECM among defoliated pines (Fig. 2). Tree crown diameter was significantly related with the response variate ($F_{1,68} = 7.72$, $P = 0.007$). On the other hand, low-biomass types were not affected by treatments neither in 2007, nor in 2008 (Table 4; Fig. 2). Tree growth parameters did not alter low-biomass ECM abundance (data not shown).

Considering the correlation between treatments and fungal taxa, we detected the reduction of two high-biomass types when applying a severe defoliation: *Suillus* ($r = -0.346$, $P = 0.020$) in 2007, and *Tomentellopsis* ($r = -0.352$, $P = 0.018$) in 2008. However, in 2008, the genus *Pseudotomentella*, with low-biomass type, also decreased significantly ($r = -0.327$, $P = 0.028$). Furthermore, we observed that the uncultured ECM 1 (Ascomycete) appeared associated only with defoliated trees ($r = -0.324$, $P = 0.028$).

Richness was not affected by treatments in 2007; however, a significant difference among groups was found in 2008 (Table 4), when richness was reduced by severe defoliation. The same trend was found for diversity, which was not affected by defoliation in 2007, but was significantly reduced in 2008 (Table 4).

Total sporocarp biomass, total number of sporocarps, species richness and diversity were not different between control and defoliated trees neither in 2007 nor in 2008 (Table 4). The response variates were not influenced by tree growth parameters (data not shown).

## Discussion

Defoliation has been reported to reduce photosynthetic ability and to increase the carbon cost of mycorrhizal symbiosis, leading to a reduced investment by the host plant in ECM symbionts, which are known to consume between 10 and 50% of photosynthate production (Gehring and Whitham 2002). Actually, loss of foliage may increase or decrease the relative advantage of symbiosis depending on the cost efficiency (i.e. the ratio between carbon expended belowground/nutrient acquired) of nutrient acquisition and the value of the acquired nutrients for the net primary production of the tree (Tuomi et al. 2001). Defoliation of mature needles during the growing season usually strongly affects conifer growth, because it involves the loss of a great part of carbon and nitrogen resources (Saravesi et al. 2008). In our study, growth loss in host trees was proportional to the severity of the defoliation, and it was negatively reflected on fungal symbionts colonization belowground, likely because root biomass would be adjusted to lower level in parallel with the depletion of plant biomass (Markkola et al. 1995; Saravesi et al. 2008).

**Table 4** The impact of defoliation on ECM root tips and sporocarp variables sorted by year

| | 2007 | 2008 |
|---|---|---|
| ECM root tips variables | | |
| Percentage mycorrhization | $F_{2,84} = 0.19$, $P = 0.824$ | $F_{2,84} = 3.53$, $P = 0.034$ |
| High-biomass types | $F_{2,82} = 1.11$, $P = 0.335$ | $F_{2,82} = 2.58$, $P = 0.082$ |
| Low-biomass types | $F_{2,59} = 0.09$, $P = 0.910$ | $F_{2,59} = 0.33$, $P = 0.718$ |
| Richness | Wald statistic = 2.46, $df = 2$, $P = 0.298$ | Wald statistic = 14.24, $df = 2$, $P = 0.001$ |
| Diversity | Wald statistic = 1.29, $df = 2$, $P = 0.528$ | Wald statistic = 8.36, $df = 2$, $P = 0.015$ |
| Sporocarp variables | | |
| Total biomass | $F_{2,82} = 0.04$, $P = 0.964$ | $F_{2,82} = 0.32$, $P = 0.726$ |
| Total no. sporocarps | $F_{2,89} = 0.08$, $P = 0.923$ | $F_{2,89} = 1.58$, $P = 0.213$ |
| Richness | GLM: deviance ratio = 0.33 $P = 0.719$ | GLM: deviance ratio = 0.01 $P = 0.990$ |
| Diversity | GLM: deviance ratio = 0.40 $P = 0.673$ | GLM: deviance ratio = 0.35 $P = 0.707$ |

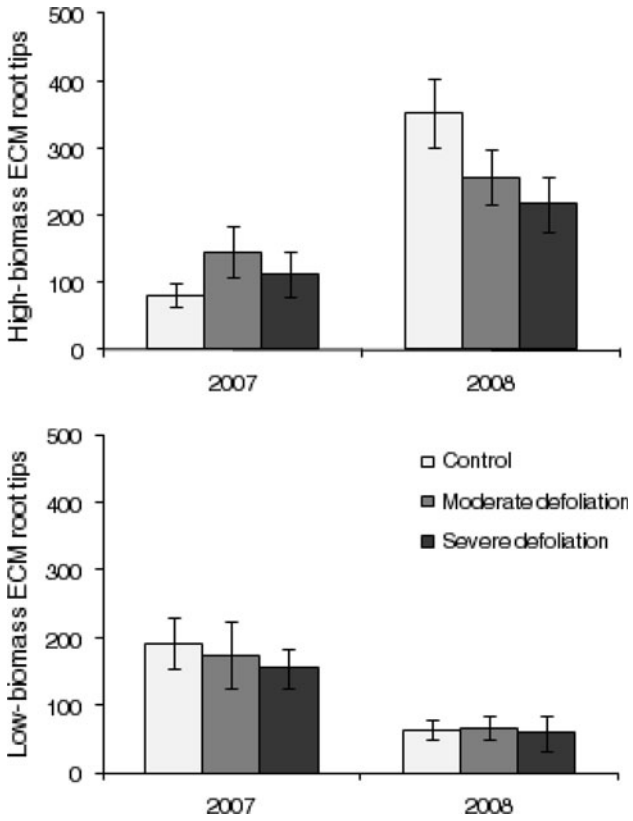


**Fig. 2** The impact of the treatments on the mean number of ECM root tips belonging to high and low biomass types. *Bars* mean ± SE; those labeled with *different letters* show significant difference at $P < 0.05$

Maritime pines defoliated in April 2007 probably suffered a consistent shortage of carbon and mineral nutrient reserves. In fact, in 2008, we registered a drastic reduction of shoot number and length, tree height and stem diameter, among severely defoliated plants, which indicates that plant experienced nutritional deficiency. On the other hand, the consequence of moderate defoliation ranged between positive effects on the number of shoot and female cones, which would suggest the existence of plant compensation capacity, to negative consequences on tree height, stem diameter and terminal leader shoot length. It is remarkable that defoliation did not depress the production of female cones. This result does not agree with other studies which reported that the production of cones was almost completely inhibited by both natural herbivory performed by pine processionary caterpillars (Hódar et al. 2003) and artificial defoliation (Kuikka et al. 2003).

Ectomycorrhiza colonization is a conservative, although important, measure, because it refers to the degree of individual nutrient transport connections between the host plant and fungal symbionts, and is closely related to the functioning of the symbiosis (Simard et al. 2002; Saravesi et al. 2008). In this study, moderate defoliation did not seriously compromise tree growth or any aspect of ECM symbiosis. On the other hand, severe defoliation significantly reduced all the studied variables of tree growth, and altered overall fungal colonization of the root tips, although sporocarp production remained unchanged. Hence, defoliation impact on ECM symbionts seems to depend on the percentage of foliage loss and on the number of defoliation bouts. In fact, severe artificial herbivory applied during 2 years reduced the fungal community belowground by 23%. This value agrees with the percentages obtained by Del Vecchio et al. (1993) (with insect defoliation) and Gehring and Whitham (1995) (with 25% of manual defoliation), who found a reduction of the ECM colonization up

to 28 and 19%, respectively, using 1-year-old defoliated *P. edulis* trees. This suggests that, in the short term, defoliated pines, even under nutritional stress, continued to invest in the maintenance of the symbiosis. In fact, those studies which showed a stronger decrease of ECM fungi (33–44%), applied a long-term (8–9 years) herbivory (Gehring and Whitham 1991; Gehring et al. 1997).

The theory recently proposed by Talbot et al. (2008), called "Plan B" hypothesis, suggested that mycorrhizal fungus decompose soil organic carbon as an alternate carbon source when supplies of photosynthate from the host plant are low or unavailable due to shadiness, plant dormancy, or defoliation. Several studies in temperate forests have supported this hypothesis, demonstrating that ectomycorrhizal fungi produce high extracellular enzyme activity during the winter months when photosynthetic rates decline (Buée et al. 2007; Mosca et al. 2007; Cullings and Courty 2009). Hence, under carbon deficiency, ECM fungal partners with saprotrophic abilities would be selected by the host plant. Additionally, because our plants grew in a very acid but high nutrient soil (see Table 1), it is possible that they were able to tolerate moderate defoliation maintaining the overall amount of mycorrhizal symbionts. This idea is also supported by other authors (Gehring and Whitham 1995; Saravesi et al. 2008), who showed that the degree of environmental stress (e.g., soil moisture and composition) may determine whether mycorrhizal reduction results from herbivory.

A predictable effect of herbivory on the ECM community is a shift in species composition, which may also be associated with a decline of species richness and diversity (Del Vecchio et al. 1993; Gehring et al. 1997; Saikkonen et al. 1999). In 2007, irrespective of the intensity, defoliation did not cause any consistent effect on ECM fungi from a quantitative or qualitative point of view, but in 2008 the reduction of species diversity and richness was significant for severely defoliated trees.

Gange (2007) proposed a redefinition of the Gehring and Whitham (2002) mycorrhizal response model, focusing on the impact of defoliation degree on fungal species number. The model predicted that, in absence of foliage loss or at very low level, the number of mycorrhizal species would be low due to carbon competition. As defoliation increases, a stimulation of photosynthesis would lead to a translocation of higher amount of carbon belowground, which promotes a greater richness of species. Finally, if defoliation further increases, carbon belowground would be insufficient for all the species and only a few taxa survive. Our study, based on a wide taxonomic identification of the ECM fungal community by molecular tools, provided the opportunity to test Gange's hypothesis. Actually, we did not detect a significant increase of species richness and diversity among moderate (25%) defoliated pines, thus rejecting the hypothesis, although we agree with Gange (2007) about the conceptual difficulties in establishing the amount of low, moderate or high defoliation degree for any particular mycorrhizae–host plant system.

Against the prediction, defoliation had no impact on the overall high/low biomass ECM types, although we observed a significant reduction when analyzing treatment consequence on certain ECM with high-biomass types in 2008, which agrees with another study on *P. contorta* ECM fungal community (Cullings et al. 2001). Furthermore, *Rhizopogon* spp., a high-biomass fungal type, was much more abundant among severely defoliated trees in both 2007 and 2008 (Supplementary Material, Fig. S2 a, b), although the statistical test was not significant. This fungus is known to be an early-stage, competitive ECM which easily proliferates in poor soils, also after fire, due to its basidiospores which may withstand different kind of environmental stress (Izzo et al. 2006). Possibly, its abundance suggests that this fungus may resist better carbon shortage, showing as the interaction of such fungal species, theoretically with high carbon cost, with the host plant is more complex than expected.

Under carbon deficiency, a fungal partner with a low nutritional demand, low-biomass, or that produces small or few sporocarps, may be favored (Cullings et al. 2001). Moreover, preferential allocation of photosynthate by host plant to the more beneficial fungal species has been reported, showing that this relationship is maintained only if it confers sufficient advantage to the beneficial symbiont, overcoming the cost of mutualism (Bever and Richardson 2008).

Considering sporocarp production, we found that fungal symbionts were able to proportionally allocate the same carbon resources to sexual reproduction independently of foliage depletion and host carbon limitation. Although sporocarp production of the ECM symbionts constitutes an important carbon sink for the host tree, sporocarp biomass forms only a minor proportion of the total of ECM biomass, representing, for example, up to 2% of total ECM fungal biomass in mature *P. sylvestris* and *Picea abies* forest stands (Markkola et al. 1995; Wallander et al. 2001). In addition, several sporocarps are able to mobilize carbon from soil organic matter (Chapela et al. 2001; Hobbie et al. 2002).This circumstance may contribute to explaining why, in our study, the effects of defoliation are evident belowground but not aboveground.

The mushrooms belonging to the genera *Amanita*, *Cortinarius*, *Hydnum* and *Laccaria* were not found on root tips, probably because some common fruiting species often produce few mycorrhizae. On the other hand, only 47% of the genera described from root tips were found as fruitbodies. In particular, the ECM belonging to the genera *Entoloma*, *Gomphidius*, *Lactarius*, *Meliniomyces*,

*Phialophora*, *Pseudotomentella*, *Rhizopogon*, *Tomentella* and *Tomentellopsis* were not found aboveground. This discrepancy is not surprising, because some species which do not form obvious fruiting structures (i.e. hypogeous sporocaps, resupinates) are abundantly represented belowground and vice versa, which implies that the use of different sampling methods, such as field surveys and root tip molecular analysis, need to be considered to obtain a complete scenario of the ECM community within a given ecosystem (Gardes and Bruns 1996).

In conclusion, our work showed that, despite moderate defoliation during the growing season compromising some tree growth parameters, it did not alter ECM fungal colonization and community composition. On the other hand, severe defoliation seriously affected tree performance and significantly depressed mycorrhization percentage, species diversity and richness of belowground fungal community. In a broader context, our results confirm the strong relationship between conifers and fungal partners to the extent that any disturbance inflicted on the host will be reflected on the fungal community and consequently on the whole belowground ecosystem, since mycorrhizal mycelium is considered to be the mayor route by which carbon enters the soil organic matter (Ayres et al. 2004).

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