Mycoscience (2011) 52:59–64
DOI 10.1007/s10267-010-0069-0

SHORT COMMUNICATION

Difference of pine ectomycorrhizal biomass in relation to forest conditions

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Received: 18 March 2010 / Accepted: 17 August 2010 / Published online: 11 September 2010
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Abstract In this study, two plots in a secondary and another two in planted *Pinus densiflora* stands were exposed to different forest treatments, and the ectomycorrhizal (EM) biomass and its ergosterol content was measured for a year. The unmanaged plot in the secondary stand had greater EM biomass than those in any other plots. Whereas understory cutting had less effect on EM biomass, litter and humus removal decreased pine EM biomass and its ergosterol content, suggesting that such forest treatment alters EM biomass and its structure.

Keywords Ergosterol content · *Pinus densiflora* · Quantitative dynamics of ectomycorrhizae · Removal of litter and humus

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Ectomycorrhizal (EM) symbiosis plays an important role in matter cycles of forest ecosystems. In coniferous forests, tree fine-root systems, including EM, account for half of the soil respiration (Andrews et al. 1999; Högberg et al. 2001). As the turnover of EM is the most rapid in tree-root systems (Helmisaari et al. 2002), EM tips and the extra-radical mycelium are key factors in forest carbon cycle. Direct carbon allocation from host-plant photosynthates (sugars) to EM fungal (EMF) carbohydrates is estimated to be approximately 20% under experimental conditions (Hobbie 2006). Despite the importance of direct measurements of EMF biomass for validating carbon cycle in forests, only limited data are available. In a Japanese red pine (*Pinus densiflora*) forest, fine-root biomass and its fungal biomass were estimated to be 91.0 and 0.5–9.6 g/m^2, respectively (Satomura et al. 2003). In boreal Norway spruce (*Picea abies*) and Scots pine (*P. sylvestris*) forests, the soil EM biomass was estimated at 56.9–107.8 and 30.2–132.0 g/m^2, respectively (Dahlberg et al. 1997; Helmisaari et al. 2009). In addition, EM biomass was estimated to be 10–100 times greater than the mass of epigeous EMF sporocarps (Dahlberg et al. 1997). However, basic issues such as EM biomass in relation to stem density, seasonal changes in EM biomass, and the effects of forest management processes and sampling methods on EM biomass estimates have not been fully considered.

P. densiflora is obligately EM and is widely distributed in Japan, the Korean Peninsula, and northeastern China, mainly in mountainous regions that are characterized by nutrient-poor soil conditions (Richardson 1998). This pine was commonly used for firewood and timber until the 1960s in Japan, and this use has been continued in other countries. The pine forests in these areas have also been managed for the production of matsutake (*Tricholoma matsutake*), a gourmet wild mushroom highly valued in

Japan (Yamada et al. 1999; Murata et al. 2008). However, Japanese red pine forests as well as Japanese black pine (*P. thunbergii*) forests have been declining severely for more than 50 years due to pine wilt disease caused by the pinewood nematode, *Bursaphelenchus xylophilus* (Mamiya 1983; Kikuchi et al. 2009). In addition, these pine forests have been abandoned recently in many regions of Japan, thus accelerating the mortality of the pine trees.

Soil nutrition for EM trees is exclusively adsorbed through the symbiotic EMF (Smith and Read 2008). Therefore, pine forest establishment and maintenance are strongly affected by the symbiotic EMF. It is generally known in *P. densiflora* forests that combination practice such as removal of the understory, thinning, and removal of litter and humus, is necessary to sustain productions of edible EM mushrooms, especially *T. matsutake* (Ito and Ogawa 1979). Although such forest management is suggested to be suitable for EMF growth due to the maintenance of the oligotrophic condition of soil in the forests, scientific background data is poor, especially in the EM biomass (Kake et al. 2000; Lian et al. 2006). Removal of litter and humus layers increases abundance of EMF sporocarps (Baar and Kuyper 1993, 1998; Baar and Ter Braak 1996; Sayer 2006) in forests of other pine species.

The objectives of this study were to: (1) determine the EM biomass of Japanese red pine forests in relation to stem density and stem size of pine trees, and (2) assess the effects of forest management practices on the EM biomass. We also assessed the ergosterol content of EM because ergosterol is a unique sterol of fungi (Nylund and Wallander 1992) allowing for measurement of the fungal biomass in symbiotic EM tips.

Four experimental plots were established in two *P. densiflora* stands in the experimental forest of Shinshu University, Nagano, Japan (35°51″N, 137°56″E). Two experimental plots (20 m × 20 m each) were established in a 40- to 50-year-old secondary stand, and another two plots were established in a 50-year-old planted stand. Tree density in the planted stand had been controlled by thinning, and the latest management procedure conducted in 2003 halved the density. The secondary stand had been maintained without any forest management for at least several decades. These two stands were approximately 100 m apart and differed in tree stem size and density (Table 1). The two stands were established on flat land 770 m in elevation. The soil type was Andosol, and soil depths of the A0 and A layers in both stands were 3–5 and 30–50 cm, respectively. Annual mean temperature and precipitation in both stands were 11.1°C and 1359 mm, respectively (Japan Meteorological Agency). The two stands had common understory vegetation, such as *Rhus trichocarpa*, *Lyonia ovalifolia* var. *elliptica*, *Acanthopanax sciadophylloides*, *Ilex crenata*, *Clethra barbinervis*, and *Acer* sp. A few *Quercus serrata* trees were also present in the secondary stand.

The two plots in the secondary stand were 30 m apart and managed as follows: no management (Sn); and cutting of all shrubs and herbs (Sc). The two plots in the planted stand were 15 m apart and were managed as follows: cutting of all shrubs and herbs (Pc) and removal of the litter and humus layer as well as cutting (Pcr). In the Sc, Pc, and Pcr plots, shrubs and herbs were cut in May and August 2005 and 2006. In the Pcr plot, litter and humus (A0) layers were removed using rakes in May 2005, and fresh litter was also removed in May 2006. Management practices were conducted 5 m beyond the plot perimeters to reduce edge effects. In each plot, pine tree number, height, and diameter at breast height were measured. Based on these parameters, basal areas and stem volume (aboveground biomass) were estimated.

Seven soil blocks (5 cm × 5 cm, 10 cm deep) were collected monthly at random locations in each plot from June 2005 to August 2006. In total, 105 soil blocks were collected from each plot. A soil block sample consisted of upper (0–5 cm deep) and lower (5–10 cm deep) subsamples. Each soil subsample was kept in a polyethylene bag and stored at 4°C for up to 1 month until processed. Each soil subsample was sieved (500-μm mesh) and rinsed with

Table 1 Characteristics of the pine forests in each plot

Plot	Pine tree					
	Age (years)	Density (trees/ha)	Mean height (m)	Diameter at breast height (cm)	Basal area (m^2/ha)	Stem volume (m^3/ha)
Sn	40–50	800	20.8 (0.5)	25.7 (1.1)	47.2	525
Sc	40–50	525	19.6 (0.6)	24.4 (1.4)	28.2	308
Pc	50	314	23.7 (0.2)	39.6 (1.2)	31.7	353
Pcr	50	300	24.2 (0.2)	39.6 (0.9)	42.7	456

Numbers in parentheses indicate standard error

Sn secondary stand with no forest treatment, *Sc* secondary stand with cutting of shrubs and herbs, *Pc* planted stand with cutting of shrubs and herbs, *Pcr* planted stand with cutting of shrubs and herbs and removal of litter and humus layers

tap water to remove soil particles. The remaining pine EM tips and fine roots (diameter <2 mm; Persson 1983; Helmisaari et al. 2009) were transferred to a Petri dish for stereomicroscopic observation. In this study, numeric data of fine roots included EM tips. Active and inactive EM tips were distinguished based on morphological properties: active EM tips with turgid surfaces were collected; inactive EM tips with wrinkled surfaces were discarded as necromass. In addition, EM tips of *Quercus* (in the Sn and Sc plots), present in small amounts, were discarded. Small soil particles on the EM tips were carefully removed using a brush or by ultrasonic cleaning for 30 s. Pine EM and fine roots were separated, dried for 3 days at 60°C, and weighed. Root samples collected from November 2005 to August 2006 were freeze-dried for ergosterol analysis. EM samples with <50 tips were not weighed; rather, their weights were estimated from the mean values of other EM samples with >50 tips collected at the same sampling time in each plot (Fig. 1). These EM samples were not subjected to ergosterol analysis.

The ergosterol levels of EM samples were analyzed as described by Satomura et al. (2003): saponification at 75°C, redissolution with 2 ml dichloromethane–methanol solution (1:1/v:v), filtration by a membrane filter (0.20-μm pore size), and high-performance liquid chromatography (HPLC) analysis at a flow rate of 1.0 ml/min. The retention time of authentic ergosterol (Sigma, St. Louis, MO, USA) was 16 min. The ergosterol concentration of each EM sample was measured, and the correlation between EM dry weight and ergosterol content was calculated for each experimental plot. Mean EM and fine-root dry weight were evaluated using three different indices: (1) weight per unit area of forest floor (g/m^2), (2) weight per individual pine tree (g/tree; kg/tree), and (3) weight per stem volume (g/m^3; kg/m^3) to compare the biomass between plots in relation to stem size and tree density of the host pines. Vertical distribution of EM biomass was calculated as the EM ratio in the upper soil subsample (0–5 cm in depth) to that in the full soil sample (0–10 cm in depth). In samples with <50 EM tips, this value was not calculated. Temporal changes of EM biomass were compared using 3-month moving averages ($n = 21$) to reduce data variance that might derive from spatial heterogeneity of EM in soil. Significant differences among means for all four plots was calculated by Tukey's honestly significant difference test for multiple comparisons ($P < 0.05$) and that between two plots in the same stand was calculated by Student's t test ($P < 0.05$) (KaleidaGraph 4.0; Hulinks, Tokyo).

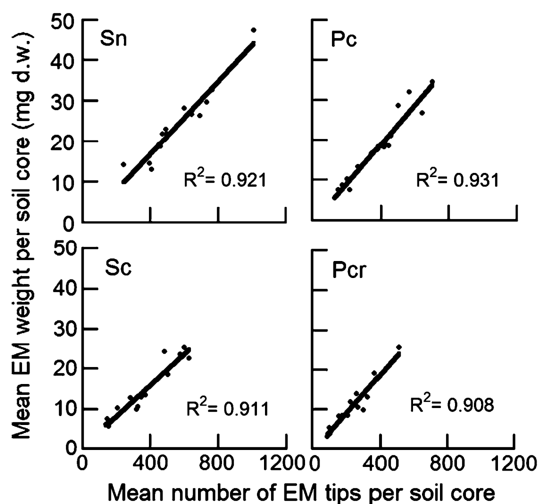

Fig. 1 Correlation between the number of ectomycorrhizal tips and weight. Each data indicates the monthly mean ectomycorrhizal (EM) tip number and weight at a given sampling time ($n = 14$–15)

In the secondary stand, EM weight was not different between Sn and Sc plots in 2005 except in July, whereas it was consistently higher in the Sn plot than in the Sc plot in 2006, with significant differences on February, May, and June (Fig. 2, $P < 0.05$). In the planted stand, EM weight was consistently higher in the Pc plot than in the Pcr plot, except early in the monitoring period, although these differences were not significant (Fig. 2). Temporal patterns of fine-root biomass in the secondary stand were generally similar between plots in 2005 (Fig. 2). In 2006, fine-root biomass in the Sn plot was higher than that in the Sc plot, with significant differences in May and June 2006 ($P < 0.05$). In the planted stand, no significant differences were found (Fig. 2). The EM ratio generally showed an increasing trend throughout the monitoring period in the Sn, Pc, and Pcr plots and was stable in the Sc plot in 2006 (Fig. 2).

The mean value of EM weight per unit area was significantly greater in the Sn plot than in the others and significantly greater in the Sc plot than in the Pcr plots (Table 2). EM weight per individual tree did not differ significantly between plots. EM weight per stem volume was greater in the Sn plot than in the Pc and Pcr plots, and was greater in the Sc plot than in the Pcr plot. Within the planted stand, EM weight per stem volume was significantly greater in the Pc plot than in the Pcr plot. EM weight per tip number exhibited a higher trend in the planted stand and was significantly higher in the Pc plot than in the plots of the secondary stand. In the vertical distribution of EM, >76% (w/w) was found in the upper soil layer in the Sn, Sc, and Pc plots, and only 67.9% was found in the upper soil layer in the Pcr plot. Ergosterol content was highly correlated with EM weight in all plots (Fig. 3). The mean value of Ergosterol per EM weight was significantly smaller in the Pcr plot than in the others (Table 2). Fine-root weight was significantly greater in the Sn plot than in the Pc and Pcr plots and in the Sn plot than in the Sc plot (Table 3). As with EM biomass, fine-root biomass per

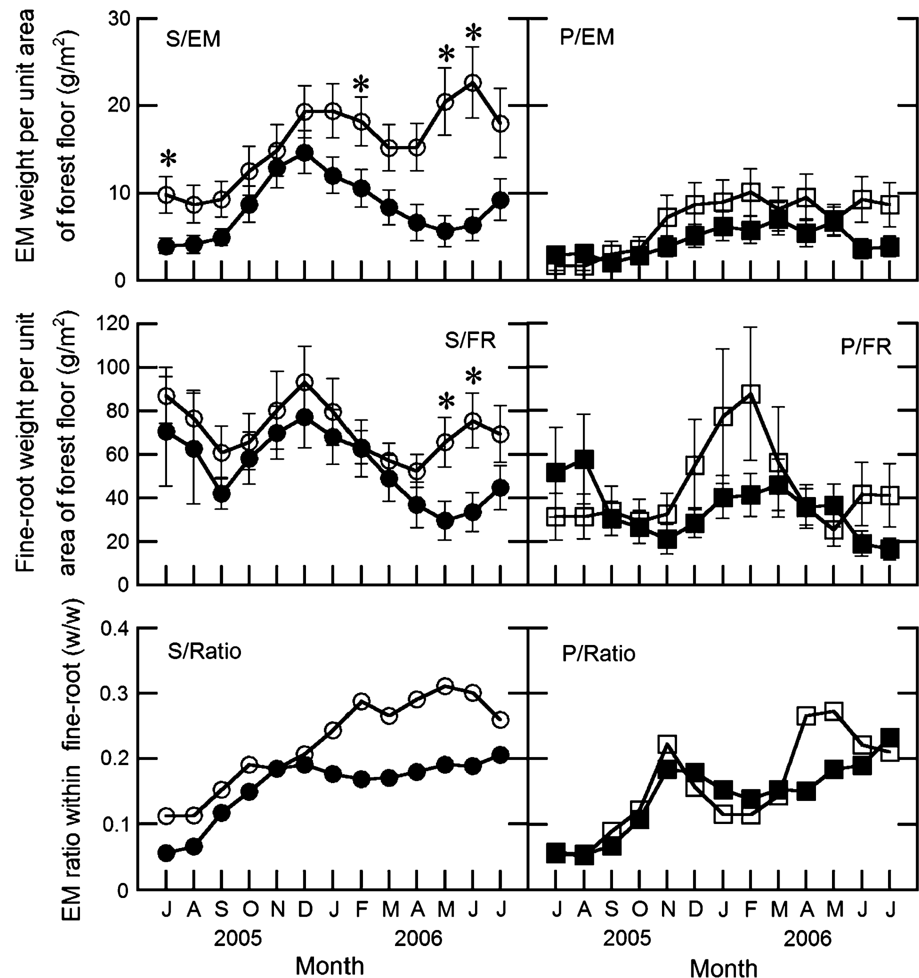

Fig. 2 Monthly changes in ectomycorrhizal and fine-root biomass. Ectomycorrhizal (EM) and fine-root (FR) biomass in each stand (S and P). Values are 3-month moving averages ± standard error ($n = 21$). *Open circles* Sn plot, *filled circles* Sc plot, *open squares* Pc plot, *filled squares* Pcr plot. *Asterisks* indicate significant differences between plots in the same stand by Student's t test ($P < 0.05$)

Table 2 Mean ectomycorrhizal (EM) weight and ergosterol concentration in each plot

Plot	Mean EM dry weight, $n = 105$				Ratio (%) in upper soil layer (w/w)	Ergosterol conc. (mg/g EM dry weight)
	(g/m^2)	(g/tree)	(g/m^3 stem vol.)	(μg/EM tip)		
Sn	15.0 (1.4) a*	188 (17) a	286 (26) a	42.0 (0.9) bc	78.4 (1.8) a	1.17 (0.39) a
Sc	8.1 (0.9) b*	154 (18) a	263 (30) ab	39.7 (1.2) c	78.4 (2.1) a	1.18 (0.62) a
Pc	6.5 (1.0) bc	234 (36) a	182 (28) bc*	47.5 (1.6) a	76.6 (3.2) ab	1.16 (0.56) a*
Pcr	4.3 (0.6) c	170 (24) a	93 (13) c*	44.6 (1.5) ab	67.9 (3.4) b	0.89 (0.61) b*

Numbers in parentheses indicate standard error. Different letters indicate significant differences between plots (Tukey's honestly significant difference test, $P < 0.05$). Asterisks indicate significant differences between plots in the same stand by Student's t test ($P < 0.05$)

individual tree did not differ significantly between plots. Fine-root weight per stem volume was significantly greater in the Sc plot than in the Sn and Pcr plots. The EM ratio within fine roots was highest in the Sn plot (21.2%) and lowest in the Pcr plot (12.4%).

We monitored EM and fine-root biomass for over a year in two *P. densiflora* stands that were managed differently. Although experimental treatments were not replicated in our study, all experimental plots were placed in the flatlands, and the soil substance (i.e., Andosol) and depth of each soil layer were mostly the same among plots (data not shown). Therefore, the difference in EM and EMF biomass between plots in a stand could be a consequence of the different management practices or differences in pine tree size and density. Mean EM and fine-root biomass per unit area or stem volume were greater in the plots of the secondary stand, whereas EM and fine-root biomass per individual tree were the same among all four plots (Table 2). These results suggest that mean EM and fine-root biomass per unit area were related to pine-tree density.

EM and fine-root biomass per individual pine tree were not affected by pine thinning, which significantly affected the size of tree stems in each plot (Table 1). As mean fine-root biomass showed a similar trend among plots (Table 3), the growth pattern of pine-root systems was suggested to be stable even in the postthinning period. To clarify how EM and fine-root biomass are regulated in relation to pine stem volume, longer monitoring periods at different sites are necessary.

EM and fine-root biomass per unit area were significantly lower in the Sc plot than in the Sn plot. Kume et al. (2003) reported that annual clear cutting of the understory for more than a decade in *P. densiflora* stands positively affected maximum net photosynthesis and stomatal conductance of the pine canopy, and the authors suggested that this was the result of escaping below-ground interactions (root competition) between plants, allowing the pine EM biomass to increase. Our results, however, did not support this hypothesis. It is suggested that >1 year of cutting of shrubs and herbs are necessary to cause its significant effect on EM biomass in *P. densiflora* forests.

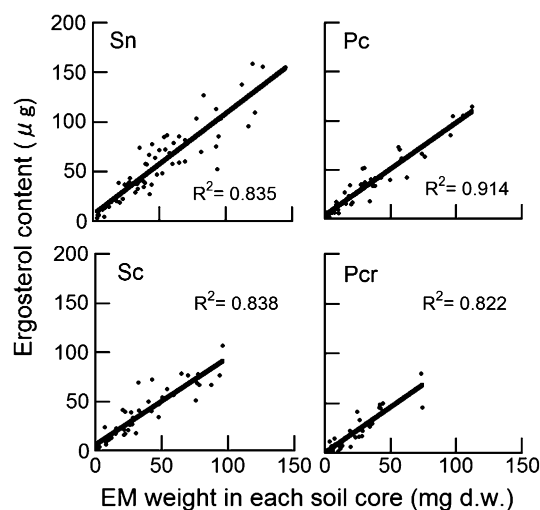

Fig. 3 Correlation between ectomycorrhizal (EM) weight and ergosterol content. Ergosterol content of soil blocks from each plot

The ergosterol content of EM obtained in this study was within the range 0.68–1.36 mg/g reported elsewhere (Salmanowicz and Nylund 1988; Antibus and Sinsabaugh 1993; Wallander et al. 1997). In addition, EM weight and ergosterol content were highly correlated, as shown in Fig. 3. Therefore, we decided that the ergosterol analysis was a valid way to estimate fungal biomass of pine EM tips. As ergosterol contents are highly different depending on fungal species, culture conditions, or life forms (Salmanowicz and Nylund 1988; Antibus and Sinsabaugh 1993; Wallander et al. 1997), further analysis are desired to estimate the accurate fungal biomass value from the data of ergosterol content.

EM biomass decreased noticeably with removal of the litter and humus layer, especially in the upper layer (Table 2; Fig. 2), and the temporal trend of EM biomass did not suggest that it would recover in a year. Kake et al. (2000) reported that the EM colonization ratio of *P. densiflora* decreased to half of the control (from 46% to 20%, calculated based on root-tip number) 1 year after completion of forest management procedures, i.e., cutting of shrubs and herbs and removal of the A0 layer. In addition, removal of litter and humus affected the EM structure as presented by ergosterol analysis, as significantly lower values were recorded in the Pcr plot (Table 2) This suggests that EM tips decreased fungal mantle thickness or hyphal density in the mantle. As fungal mantle thickness can be affected by nutrient status or other environmental factors (Smith and Read 2008), it is plausible that the increased fluctuation of water and temperature levels caused by removal of the forest floor stressed the EM fungal mantle development.

In conclusion, we demonstrated the effects of forest management on EM biomass in *P. densiflora* stands. Unmanaged secondary pine forests had more EM biomass than did planted forests. Pine EM biomass was not significantly affected by the cutting of shrubs and herbs when management was of limited duration, i.e., 1 year. Removal of litter and humus decreased pine EM biomass in the surface-soil layer and altered the structure of EM as indicated by ergosterol content.

Table 3 Mean fine-root weight in each plot

Plot	Mean fine-root dry weight, $n = 105$			EM ratio (%) within fine roots (w/w)
	(g/m^2)	(kg/tree)	(kg/m^3 stem vol.)	
Sn	70.8 (5.7) a*	0.89 (0.07) a	1.35 (0.11) ab*	21.2
Sc	55.6 (6.9) ab*	1.06 (0.13) a	1.81 (0.22) a*	14.6
Pc	43.0 (7.8) b	1.56 (0.28) a	1.22 (0.22) ab*	15.4
Pcr	34.2 (5.4) b	1.37 (0.21) a	0.75 (0.12) b*	12.4

Numbers in parentheses indicate standard error. Different letters indicate significant differences between plots (Tukey's honestly significant difference test, $P < 0.05$). Asterisks indicate significant differences between plots in the same stand by Student's *t* test ($P < 0.05$)

Acknowledgments This work was supported in part by Grant-in-Aid for Scientific Research No. 19380085 from the Ministry of Education, Culture, Sports, Science and Technology.

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