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Ectomycorrhizae and ectomycorrhizal fungal fruit bodies in pine stands differentially damaged by pine wilt disease

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Abstract We surveyed ectomycorrhizae, ectomycorrhizal fungal fruit bodies, and soil physical properties in one heavily damaged and two lightly damaged pine stands on Mt. Tsukuba, central Japan. The rate of ectomycorrhizal root tips was not different between heavily and lightly damaged pine stands. For ectomycorrhizae, *Cenococcum geophilum* had high relative abundance in the heavily damaged pine stand. The number of ectomycorrhizal fungal fruit bodies in the heavily damaged pine stand was much lower than that in the lightly damaged pine stands.

Key words Active root tips · Ectomycorrhizal fungal fruit body · Ectomycorrhizal type · Mt. Tsukuba · Soil physical property

In Japan, pine wilt disease has severely damaged two native pine species, *Pinus densiflora* Sieb. & Zucc. and *Pinus thunbergii* Parl. This disease is caused by the pinewood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (Kiyohara and Tokushige 1971), which is mainly vectored by the cerambycid beetle *Monochamus alternatus* Hope (Mamiya and Enda 1972). The nematode infects pine trees during maturation feeding by beetles and causes cavitation in the tracheids of pine trees, which results in wilting of the pine trees (Kishi 1995). The susceptibility of pine stands is affected not only by abiotic environmental factors such as desiccation stress (Suzuki and Kiyohara 1978), but also by biotic factors such as ectomycorrhizal association (Kikuchi et al. 1991).

Ectomycorrhizae are a symbiotic association in which the host tree is associated with fungi. The host tree provides carbohydrates to the fungi via ectomycorrhizae, and, inversely, the fungi enhance host tree absorption of soil minerals and water (Finlay and Read 1986), improve host resistance to root disease (Marx 1969; Buscot et al. 1992), and ameliorate soil desiccation stress (Mexal and Reid 1973).

Regarding the relationship between ectomycorrhizal abundance and pine wilt damage, Akema and Futai (2005) found that pine wilt damage was lighter and the rate of ectomycorrhizal root tips was higher on upper slopes than lower slopes. Thus, ectomycorrhizal abundance is thought to be related to pine wilt damage, but the causal association is not clear. Kikuchi et al. (1991) reported that mortality caused by inoculation by the pathogenic nematode was lower in *P. densiflora* seedlings with ectomycorrhizae than without ectomycorrhizae. After inoculation of pathogenic nematodes on *P. thunbergii* seedlings, ectomycorrhizae did not disappear; however, ectomycorrhizal development ceased (Ichihara et al. 2001). To clarify this relationship, more field observations and experiments are needed.

Previous studies have not considered two potentially important factors: soil conditions and the ectomycorrhizal community structure. Akema and Futai (2005) reported a difference in soil moisture between the upper and lower slopes. Soil conditions such as soil moisture can influence ectomycorrhizal association (Slankis 1974). Thus, the effects of soil conditions should be considered to understand the relationship between ectomycorrhizal association and pine wilt damage. Neither Akema and Futai (2005) nor Ichihara et al. (2001) reported the ectomycorrhizal community structure on pine roots. Kikuchi et al. (1991) compared the different effects of ectomycorrhizal species on pine resistance, but they observed only two fungal species. The symbiotic function of ectomycorrhizae differs among fungal species (Nara 2006). Thus, not only the amount of ectomycorrhizae,

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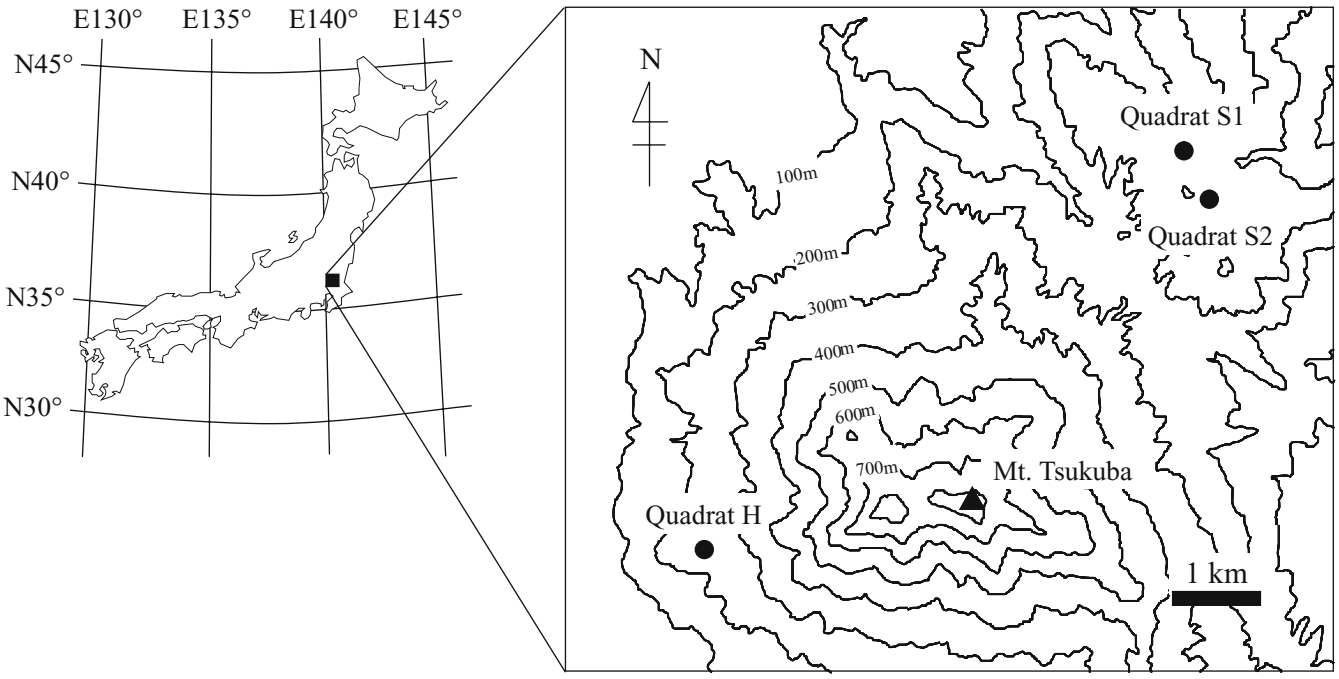

Fig. 1. Location of the study site and the three quadrats (*S1*, *S2*, *H*) in pine forests around Mt. Tsukuba, central Japan. Topographical data were provided by the Geographical Survey Institute

but also the community structure, should be clarified in relationship to pine wilt disease.

In this study, we examined whether ectomycorrhizal abundance differs between heavily and lightly damaged pine forests. In addition, we observed the relationship between ectomycorrhizal abundance and soil conditions. A survey of ectomycorrhizae, ectomycorrhizal fungal fruit bodies, and soil physical properties was conducted in one heavily and two lightly damaged pine stands on Mt. Tsukuba, central Japan.

The study site was situated on Mt. Tsukuba (36°13′31″ N, 140°06′24″ E) in central Japan (Fig. 1). Mt. Tsukuba is 877 m in elevation and is covered with weathered granite and other sediments (Takahashi 1980, 1982). The mountainside is vegetated by artificial pine forests composed of *P. densiflora* and *P. thunbergii* that are located discontinuously below 450 m elevation. Above 450 m elevation, natural forests of *Abies firma* Sieb. & Zucc, *Quercus acuta* Thunb. ex Murray, and *Fagus crenata* Blume occur. In these forests, pine wilt disease has spread since the early 1990s. The annual mean temperature at the nearest automated meteorological data acquisition site (36°03′25″ N, 140°07′30″ E; 25 m elevation) was 14.3°C and the annual precipitation was 1522 mm in 1998 (Japan Meteorological Agency 2007).

In 1998, two 30 × 20-m quadrats (S1 and S2) were established in pine stands showing slight damage from pine wilt disease (see Fig. 1). At the same time, one quadrat (H) was established in a pine stand with heavy damage. Only *P. densiflora* was observed in S1 and H. However, nine *P. thunbergii* trees were found in S2 (Table 1). The cumulative mortality was 19.0%, 17.0%, and 68.4% in November 1997 and reached 19.0%, 18.6%, and 96.5% in December 1998 in quadrats S1, S2, and H, respectively. In quadrat S2, all *P. thunbergii* individuals were alive in 1998 and represented 18.6% of all live pine trees and 17.5% of the basal area of live pine trees. In an additional survey in November 1999, the wood of three dead pine trees was sampled in each quadrat, and the pinewood nematode was found in at least one dead tree in each quadrat.

Table 1. Characteristics of the pine population in each quadrat in 1998

Quadrat	S1	S2	H
Altitude (m)	370	370	250
Aspect[a]	NW	W	NW
Inclination (°)	18	25	10
Density[b] (number/ha)	1317	983	950
Basal area[b] (m^2/ha)	36.8	24.6	46.6
Diameter at breast height[c] (cm)	17.9 ± 0.7	17.0 ± 0.7	24.2 ± 0.8
Cumulative mortality (%)	19.0	18.6	96.5
Annual mortality (%)	0.0	1.7	28.1
Relative basal area in pine species[b]			
Pinus densiflora	1.00	0.85	1.00
P. thunbergii		0.15	

[a] NW, northwest; W, west
[b] Summed value of live and dead pine individuals
[c] Average ± standard error on all pine individuals: S1, n = 79; S2, n = 59; H, n = 57

The belowground ectomycorrhizae were surveyed in all quadrats in October 1998. A live *P. densiflora* individual was chosen randomly in each quadrat, and three soil cores were collected around the individual. Each soil core was 15 × 15 × 10 cm deep, taken from the ground surface after litter

removal. The soil cores were brought to the laboratory and washed carefully under running water on a 2-mm-mesh sieve. The plant roots remaining on the sieve were then sorted using forceps into pine roots and those of other plants based on their surface morphology.

In the pine roots, 1000 root tips were randomly chosen and observed under a dissecting microscope (maximum magnification, 40×). These root tips were divided into inactive root tips, which had a wrinkled surface or partial removal of the epidermis (Lorio et al. 1972; Ogawa 1975; Ugawa and Fukuda 2005), and active root tips, which had intact and fresh epidermis and/or ectomycorrhizal mantle (Fig. 2). The inactive and active root tips were counted, and the active root tips were divided into ectomycorrhizal and nonectomycorrhizal root tips based on the obvious presence of an ectomycorrhizal mantle (Fig. 2) and counted. Ectomycorrhizal root tips were then categorized into morphological types according to their shape and dimensions, mantle surface, and rhizomorph characters using the method of Agerer (1993); the number of root tips of each morphotype was counted. Moreover, the rate of root tips of each ectomycorrhizal type per total number of ectomycorrhizal root tips was calculated for each soil core, and the rates were averaged in each quadrat.

For all pine roots in each soil core, the pine roots connected to the 1000 observed root tips and the other roots were oven-dried at 80°C for 72 h; the dry weights were measured as "observed root weight" and "non-observed root weight," respectively.

For statistical analysis, the rates of active root tips per 1000 observed root tips and of ectomycorrhizal root tips per active root tips were calculated. Moreover, we determined the number of inactive root tips, active root tips, ectomycorrhizal root tips, and nonectomycorrhizal root tips per soil core volume (2250 cm^3) from those in the observed root tips using Formulas 1–4, respectively.

$$\text{[Formula 1] Number of observed inactive root tips} \times \frac{\text{observed root weight} + \text{non-observed root weight}}{\text{observed root weight}}$$

$$\text{[Formula 2] Number of observed active root tips} \times \frac{\text{observed root weight} + \text{non-observed root weight}}{\text{observed root weight}}$$

$$\text{[Formula 3] Number of observed ectomycorrhizal root} \times \frac{\text{observed root weight} + \text{non-observed root weight}}{\text{observed root weight}}$$

$$\text{[Formula 4] Number of observed non-ectomycorrhizal} \times \frac{\text{observed root weight} + \text{non-observed root weight}}{\text{observed root weight}}$$

Each root tip parameter was checked in each quadrat for goodness of fit to a normal distribution using a chi-squared test. Moreover, the variance of each parameter was compared among all quadrats using Bartlett's test. If the parameter did not show a significant difference from a normal distribution and variances were not significantly different among all quadrats, the parameter was compared among all quadrats with ANOVA. Otherwise, the parameter was compared among all quadrats using a Kruskal–Wallis test. When a significant difference among all quadrats was detected by ANOVA, the parameters were compared between pairs of quadrats with a Tukey–Kramer test. Statistical analyses were conducted in R2.4.1 (R Development Core Team 2006).

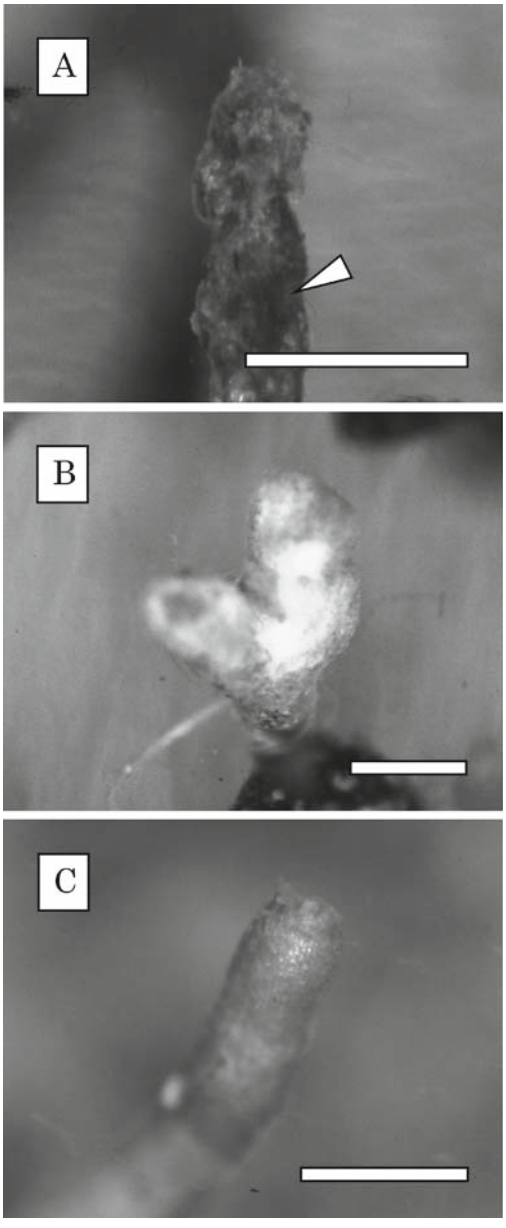

Fig. 2. Morphology of an inactive root tip (**A**), an active root tip considered ectomycorrhizal (**B**), and a nonectomycorrhizal root tip (**C**). The inactive root tip has a wrinkled surface and/or epidermis removal (*arrowhead*). The ectomycorrhizal root tip has a fungal mantle on its surface. The nonectomycorrhizal root tip has intact and fresh epidermis. *Bars* 0.5 mm

For soil conditions, an undisturbed 400-mm^3 cylindrical soil sample was taken arbitrarily at a representative point in each quadrat in October 1998. The upper soil layer is subject to the effects of material motion, which varies with

microtopography. Thus, each soil core was taken 15 cm from the ground surface. The soil cores were brought to the laboratory, and five soil physical properties were measured according to Arimitsu (1982): bulk density, maximum water-holding capacity, minimum air capacity, micropores rate, macropores rate, and saturated hydraulic conductivity.

The correlations between each soil physical property and root tip parameter, including ectomycorrhizal root tips, were determined. Each soil physical property in all quadrats was checked for goodness of fit to a normal distribution using a chi-squared test. For the root tip parameters, the average and sample standard deviation were calculated in each quadrat; these values were checked for goodness of fit to a normal distribution using a chi-squared test. For normally distributed factors, correlations were analyzed using Pearson's product–moment correlation in R2.4.1 (R Development Core Team 2006).

The occurrence of fungal fruit bodies was surveyed in all quadrats from May to October 1998. Each quadrat was gridded using a width of 50 cm, and the fungal species and the location of the closest grid point to the fruit bodies were recorded once every 2 weeks. In this study, ectomycorrhizal fungi were determined to at least the genus level based on Molina et al. (1992), Smith and Read (1997), and Imazeki and Hongo (1987, 1989). The species that could not be identified to genus were not considered ectomycorrhizal fungi. In addition, the number of the closest grid points to ectomycorrhizal fungal fruit bodies was counted in each species. The rate of grid points with each species of ectomycorrhizal fungal fruit bodies per total number of grid points with all ectomycorrhizal fungal fruit bodies was calculated in each quadrat.

The rate of active root tips was normally distributed in each quadrat ($P > 0.050$), and variances were not significantly different among quadrats ($P = 0.072$). The rate of active root tips differed among quadrats (Fig. 3; $P = 0.019$). A difference was detected between S1 and H ($P = 0.018$), but not between S1 and S2 ($P = 0.605$) or between S2 and H ($P = 0.060$). The rate of ectomycorrhizal root tips per active root tip was normally distributed in each quadrat ($P > 0.050$), and the variances were not significantly different among quadrats ($P = 0.489$). The rate of ectomycorrhizal root tips was not significantly different among quadrats (Fig. 4; $P = 0.064$).

The numbers of inactive root tips, active root tips, and ectomycorrhizal root tips per volume of soil core were normally distributed in each quadrat ($P > 0.050$); however, variances differed among quadrats ($P = 0.009$, 0.031, and 0.005 in inactive, active, and ectomycorrhizal root tips, respectively). The number of inactive, active, and ectomycorrhizal root tips was not significantly different among quadrats (Table 2; $P = 0.061$, 0.066, and 0.051 in inactive, active, and ectomycorrhizal root tips, respectively). The number of nonectomycorrhizal root tips per volume of soil core was normally distributed in each quadrat ($P > 0.050$), and the variances were not different among quadrats ($P = 0.385$). The number of nonectomycorrhizal root tips was not significantly different among quadrats (Table 2; $P = 0.098$).

The soil physical properties in each quadrat are shown in Table 3. Neither soil physical properties nor root tip parameters differed significantly from a normal distribution ($P > 0.050$). Soil physical properties were correlated with some root tip parameters, although most correlations were not significant (Table 4). The soil macropores rate was significantly correlated with the average and standard

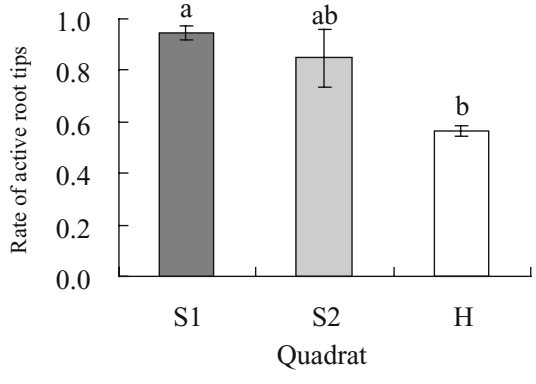

Fig. 3. Rate of active root tips per total root tips in each quadrat. Active root tips consist of ectomycorrhizal and nonectomycorrhizal root tips. *Bars* indicate the standard error. There is a difference among quadrats [analysis of variance (ANOVA), $P = 0.019$]. *Different letters* indicate differences between quadrats (Tukey–Kramer test, $P = 0.018$)

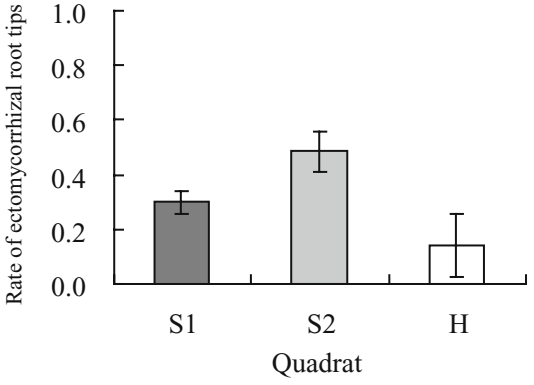

Fig. 4. Rate of ectomycorrhizal root tips per active root tip in each quadrat. *Bars* indicate standard error. No difference was observed among quadrats (ANOVA, $P = 0.064$)

Table 2. Number of inactive and active root tips per soil core of 2250 cm^3 in each quadrat

Category of root tips[a]	Quadrat		
	S1	S2	H
Inactive root tip	141 ± 70	1455 ± 1192	1645 ± 237
Active root tips			
Ectomycorrhizal	587 ± 109	3957 ± 1787	262 ± 216
Nonectomycorrhizal	1419 ± 282	3762 ± 916	1942 ± 611
Total	2006 ± 333	7719 ± 2545	2204 ± 488

Each number indicates the average ± standard error
[a] For the data analysis, please see the methods in the text

Table 3. Soil physical properties in each quadrat

Quadrat	S1	S2	H
Bulk density (Mg/m^3)	0.568	0.653	1.126
Maximum water-holding capacity (m^3/m^3)	0.667	0.494	0.422
Minimum air capacity (m^3/m^3)	0.097	0.243	0.146
Porosity (m^3/m^3)			
Micropores	0.394	0.300	0.194
Macropores	0.371	0.437	0.374
Saturated hydraulic conductivity (m/s)	5.85×10^{-5}	17.15×10^{-5}	13.80×10^{-5}

Table 4. Correlation coefficients between soil physical properties and parameters of root tip containing ectomycorrhiza

	Bulk density (mg/m^3)	Maximum water-holding capacity (m^3/m^3)	Minimum air capacity (m^3/m^3)	Porosity (m^3/m^3)		Saturated hydraulic conductivity (m/s)
				Micropores	Macropores	
Rate of active root tips per total root tips[a]						
Average	–0.994	0.873	–0.060	0.971	0.233	–0.461
SD	–0.436	–0.163	0.919	0.104	0.994	0.679
Rate of ectomycorrhizal root tips per active root tips						
Average	–0.756	0.239	0.689	0.488	0.870	0.334
SD	0.949	–0.956	0.270	–1.000*	–0.022	0.638
Number of inactive root tips per soil core						
Average	0.705	–0.985	0.677	–0.904	0.433	0.917
SD	–0.241	–0.364	0.981	–0.104	0.995	0.816
Number of active root tips per soil core[a]						
Average	–0.344	–0.261	0.954	0.004	1.000**	0.749
SD	–0.314	–0.292	0.963	–0.028	1.000*	0.770
Number of ectomycorrhizal root tips per soil core						
Average	–0.445	–0.154	0.915	0.114	0.993	0.672
SD	–0.319	–0.287	0.961	–0.022	1.000*	0.766
Number of nonectomycorrhizal root tips per soil core						
Average	–0.167	–0.433	0.993	–0.178	0.985	0.857
SD	0.163	–0.702	0.978	–0.489	0.875	0.978

The coefficients were calculated for the average and the sample standard deviation of each parameter in all quadrats
Asterisks on the coefficients indicate significant correlations (Pearson's product-moment correlation: * $P < 0.05$, ** $P < 0.01$)
[a]The active root tips consist of ectomycorrhizal and nonectomycorrhizal root tips

Table 5. Morphological characteristics of each ectomycorrhizal type

Type no.	Mantle surface				Rhizomorphs		Type of ramification[c,d]	Additional remarks
	Color[a]	Features[b,c]	Weave[c]	Thickness[c]	Color[a]	Connection with mantle[c]		
1	BB	W	Tight	Thick			U	*Cenococcum geophilum*
2	O	Sm	Tight	Thick			D, M	
3	BB	W	Tight	Thick	BB	Restricted point	U, D	
4	W	Sl	Tight	Thin	W	In flat angles	D, C	Mantle is thin on the ochroid ground
5	O	C, Sm	Loose	Thick			D, I	Mantle looks like thin films on brown ground
6	OW	Sm	Tight	Thick			D, C	
7	BB	Sm	Tight	Thin			D	
8	W	Sl	Tight	Thin	W	Restricted point	D	Mantle is thin on the brown ground
9	W	Sm	Loose	Thick			I	
10	BB	Sm	Tight	Thin			U, I	
11	W	Sl	Tight	Thin	W	Restricted point	D	
12	W	Sl	Tight	Thin	BB	Restricted point	I	Silvery mantle on brown-black ground
13	W	Sm	Tight	Thick			U	

[a]BB, black to brown; O, ochre; OW, ochre to white; W, white
[b]W, wooly; Sm, smooth; Sl, silvery; C, cottony
[c]Terms are according to Agerer (1993)
[d]U, unramified; D, dichotomous; M, monopodial-pyramidal; C, coralloid; I, irregularly pinnate

deviation of the number of active root tips per soil core ($P = 0.006$ and $P = 0.014$ with the average and standard deviation, respectively).

Ectomycorrhizal root tips were categorized into 13 ectomycorrhizal types based on surface character (Table 5). Ectomycorrhizal type 1 was identical to ectomycorrhizae formed by *Cenococcum geophilum* Fr. based on the blackish mantle surface with woolly emanating hyphae. Ectomycorrhizal type 1 represented 58.9% of all ectomycorrhizal root tips in H; however, in S1 and S2, type 1 represented

only 5.2% and 10.5% of ectomycorrhizal root tips, respectively (Table 6).

Eighteen species and a total of 57 grid points of ectomycorrhizal fungal fruit bodies occurred across all quadrats (Table 7). Except for the ectomycorrhizal fungal fruit bodies, the fungal genera *Collybia*, *Oudemansiella*, *Marasmius*, *Mycena*, *Leucocoprinus*, *Agaricus*, *Entoloma*, *Coltricia*, *Geastrum*, and *Lycoperdon* were observed in all quadrats. In H, only one ectomycorrhizal fungal fruit body, *Russula cyanoxantha* (Shaeff.) Fr., was confirmed. The number of species of ectomycorrhizal fungal fruit bodies was higher in quadrats S1 and S2 than in quadrat H, and the number of grid points with ectomycorrhizal fungal fruit bodies showed the same trend.

Table 6. Percentage of root tips of each ectomycorrhizal type relative to the total number of ectomycorrhizal root tips in each quadrat

Type no.	Quadrat		
	S1	S2	H
1	5.2	10.5	58.9
2	12.7	26.8	
3	3.0	23.6	
4	5.6	5.3	
5	34.4	10.5	
6	4.3	5.8	
7	30.0		
8	2.5		
9	2.4		
10		10.9	13.4
11		6.7	
12			26.4
13			1.3
Total relative frequency	100	100	100
Total number of types	9	8	4

The rate of ectomycorrhizal root tips was not significantly different among quadrats (Fig. 4). No significant difference was observed in the number of ectomycorrhizal root tips per soil core (Table 2). These results suggest that ectomycorrhizal root tips are abundant in live pine trees, even in heavily damaged pine stands. The rate of active root tips in quadrat H was significantly lower than in quadrat S1 (Fig. 3). However, in quadrat S2, the rate of active root tips varied among soil cores, but the difference was not significant. Thus, differences in the rate of active root tips between heavily and lightly damaged pine stands might be related to environmental factors, including soil conditions.

Soil physical properties, especially the soil macropores rate, were correlated with the number of active root tips per soil core (Table 4). Soil conditions (e.g., low soil moisture and low water potential) affect the amount of fine roots in some tree species, including *Pinus* spp. (Deans 1979; Torreano and Morris 1998; Konôpka et al. 2005, 2007). Thus, soil conditions might influence the amount of active root tips. However, variance in the rate of active root tips did not correlate with the soil macropores rate (Table 4). We therefore cannot explain the inconsistent results in the rate of active root tips described here.

The ectomycorrhizal type, which was categorized under a dissecting microscope, did not necessarily correspond to species level (Yamada and Katsuya 1995, 1996, 2001; Taniguchi et al. 2007). Our results therefore might not reflect ectomycorrhizal fungal species. However, ectomycorrhizal type 1 was identified as *Cenococcum geophilum* because this species has distinguishing surface characteristics (Table 5; Yamada and Katsuya 1995, 1996; Wu et al. 2005). In quadrat H, the percentage of ectomycorrhizal type 1 per total number of ectomycorrhizal root tips was 58.9%, which was much higher than in quadrats S1 and S2 (Table

Table 7. Epigeous ectomycorrhizal fungal fruit bodies recorded in each quadrat

Species	S1		S2		H	
	Number of grid	Relative abundance (%)	Number of grid	Relative abundance (%)	Number of grid	Relative abundance (%)
Amanita citrina var. *grisea*			1	3.6		
Amanita pseudoporphyria	1	3.4				
Amanita spissacea	2	6.9				
Amanita vaginata var. *alba*	3	10.3				
Astraeus hygrometricus			1	3.6		
Boletellus chrysenteroides			1	3.6		
Cortinarius tenuipes			3	10.7		
Inocybe maculata			2	7.1		
Inocybe sp.			1	3.6		
Leccinum extremiorientale			2	7.1		
Russula cyanoxantha					1	100
Russula rosacea	1	3.4				
Russula vesca	4	13.8				
Russula sp.	1	3.4				
Suillus bovinus	3	10.3	1	3.6		
Thelephora palmata			13	46.4		
Tylopilus castaneiceps	1	3.4	3	10.7		
Tylopilus neofelleus	12	41.4				
Xerocomus chrysenteron	1	3.4				
Total	29	100	28	100	1	100
Total number of species	10		10		1	

6). This result indicates the probability that the dominance of *C. geophilum* is different between heavily and slightly damaged pine stands.

Compared to quadrats S1 and S2, very low numbers of ectomycorrhizal fungal fruit bodies occurred in quadrat H (Table 7). This result suggests that the number of ectomycorrhizal fungal fruit bodies is different between heavily and slightly damaged pine stands.

Kikuchi et al. (1991) showed that ectomycorrhizal association decreases the mortality of *P. densiflora* seedlings caused by pathogenic nematodes. Similarly, the possibility exists that ectomycorrhizal community structure (e.g., particular ectomycorrhizal species) makes host pine trees resistant to pine wilt disease. In this study, we did not find a causal association between ectomycorrhizal association and pine wilt damage. However, our observations suggest that differences in active root tip abundance between heavily and lightly damaged pine stands vary among sites. Moreover, our results indicate that the dominance of *Cenococcum geophilum* and the occurrence of ectomycorrhizal fungal fruit bodies differ in stands with pine wilt damage. To clarify the relationships between ectomycorrhizal association and pine wilt damage, more field observations and experiments focusing on ectomycorrhizal community structure are needed.

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