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Effects of Ectomycorrhizal Fungi on Growth of Seedlings of *Pinus densiflora*

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This study was conducted to investigate the different effects of ectomycorrhizal fungal (ECMF) species on the growth of seedlings of *Pinus densiflora*, and the effects of ECMF diversity on plant productivity. A total of five species of ECMF were isolated from root tips of pine seedlings collected from Mt. Songni and used as inocula. Pots containing pine seedlings were inoculated with either a single ECMF species or a mixture of five ECMF species. All of the seedlings formed ECM on their roots except for the control plants. The pine seedlings' growth responses varied by the different ECMF species. Also, pine seedlings inoculated with a mixture of five ECMF species showed the highest growth response. The results of the study suggest that the colonization of diverse species of ECMF will increase plant productivity, and the selection of suitable ECMF species could be an important factor for plant growth.

KEYWORDS: Ectomycorrhiza, *Pinus densiflora*, Productivity, Species diversity

Biodiversity of ecosystems is important, especially in relation to the productivity of the ecosystem. Most studies that have been conducted to evaluate productivity responses to biodiversity have involved manipulation of plant diversity (Naeem *et al.*, 1994, 1996; Tilman *et al.*, 1996; Hooper and Vitousek, 1997; Hector *et al.*, 1999). Therefore, the interpretations of many of these results has been debated (Tilman *et al.*, 1996; Grime, 1997; Huston, 1997). However, other biotic components of ecosystems are also important determinants of plant productivity. For example, herbivores, micro-organisms forming symbiotic relationships with plants, pathogens and decomposers have an effect on productivity of ecosystems. Few studies have attempted to determine the effects of consumer diversity on plant productivity.

Mycorrhizal fungi play an important role in maintaining the structure and function of ecosystems by increasing host uptake of nutrients and water and providing protection from pathogens to host plants (Smith and Read, 1997). Furthermore, different species of mycorrhizal fungi have different effects on plant growth, and the community structure of mycorrhizal fungi induces different effects on the host plant (van der Heijden *et al.*, 1998). Studies have demonstrated that increasing numbers of mycorrhizal fungal species will increase plant productivity, because each single mycorrhizal fungal species provides an added beneficial effect (van der Heijden *et al.*, 1998; Klironomos *et al.*, 2000). These results suggest that mycorrhizal fungi have the potential to control plant community structures and productivity.

Although the importance of mycorrhizal fungi in the relationship between biodiversity and ecosystem functioning is now being recognized, the effect of species diversity of ectomycorrhizal fungi (ECM the dominant mycorrhizal fungi in temperate forest ecosystems) on host plants remains unknown. To determine whether the relationship between diversity and productivity is dependent upon characteristics of ECMF species on Japanese red pine (*Pinus densiflora*) seedlings, we conducted an experiment to determine the different effects of different ECMF species on the growth of pine seedlings, which are common to the temperate zone in East Asia. In addition, the effects of ECMF species diversity on plant productivity were investigated in a greenhouse.

Materials and Methods

Isolation of ECMF. ECM roots of pine trees were collected at a pine forest around Mt. Songni in Gyeongbuk, Korea (36° 35' 43" N, 127° 49' 35" E). ECM tips were detached from collected root samples, surface-sterilized and ECMF were isolated on a modified Melin-Norkans (MMN) medium (Marx, 1969). Cultures of each ECMF strains were stored at 30°C on MMN medium until used as inocula. Isolated ECMF strains were identified by their molecular features (Table 1). These ECMF strains are available from the author by request.

Sequence analysis of ECMF. The fungal mycelium was homogenized in a PCR tube using micropestle, and DNA was extracted from ECM root tips using DNeasy Plant mini kit (Qiagen Science, USA). The internal transcribed spacer (ITS) region of rDNA was amplified using the fungal specific primer pair ITS1F and ITS4 (Gardes

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Table 1. Sequence analysis of fungal strains used as ECMF inocula in this study, using sequences of internal transcribed spacer (ITS) regions[1)]

Fungal strains	Closest sequences[2)]		
	Accession No.	Similarity (%)	Fungal species
A5	AY394921	547/553 (98%)	*Phialocephala fortinii*
A6	DQ474368	496/521 (95%)	*Cenococcum geophilum*
A8	AY599237	766/849 (90%)	*Mollisia cinerea.*
A12	AB089660	516/521 (99%)	*Leptodontidium elatius*
A18	DQ420919	429/439 (97%)	*Lachnum pygmaeum*

[1)]The isolates were isolated from ECM root tips of *Pinus densiflora* collected from Mt. Songni.
[2)]The best BLAST matches to known sequences in GenBank (NCBI).

and Bruns, 1993). Thermocycling for PCR was conducted as follows: 94°C for 3 min for 1 cycle, 94°C for 1min, 55°C for 1 min, 72°C for 1 min for 35 cycles, 72°C for 7 min for 1 cycle, for a total of 30 cycles. A sequence similarity search of the National Center for Biotechnology Information database was conducted using a Basic Local Alignment Search Tool algorithm.

Greenhouse experiment. An experiment with treatments of single species of five fungal species and a mixture of the five species was set up in the greenhouse. Five strains of ECMF isolated from root tips were used as inocula (Table 1). Vermiculites and pearlites (2 : 1, v/v) were used as the potting medium. The experiment was set up with three replicate experimental units (pots) for each possible treatment; all data analyses were done on the basis of three independent replicate blocks. Fifty seeds of *Pinus densiflora* were sown per pot (50 × 38 × 8 cm). To prevent seedling loss caused by damping off fungi, seeds were surface-sterilized. Heavier pine seeds of roughly the same size were selected, and their surfaces were sterilized by shaking them in 70% ethanol solution, using 5% NaClO for 30 min, 30% H_2O_2 for 10min, and then thoroughly rinsing them with dH_2O. The agar blocks (1 cm × 1 cm) with fungal mycelium were grinded in dH_2O and sprayed into each pot. The pots were maintained in the greenhouse and watered as needed.

Harvest and measurements. The seedlings were harvested after 16 weeks of growth. Care was taken to ensure that the root systems of individual plants in the same pot were separated and kept as intact as possible. The shoots were measured for height, and dried at 60°C for 48 hours to measure their dry weights to determine their plant biomass. Five seedlings from each pot were randomly selected for assessment of ECM colonization. Root systems were maintained in moist conditions at 4°C for mycorrhizal assessment. For each treatment, lateral roots were examined under a stereoscopic microscope to determine the percentage ECM colonization rate as the number of mycorrhizal root tips per total number of root tips in an individual plant.

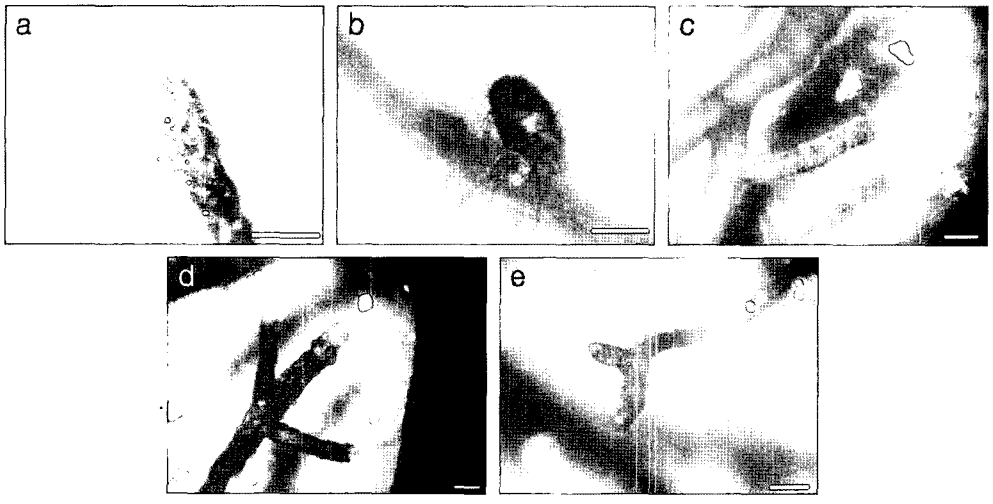

Fig. 1. ECM of five different ECMF species formed on seedlings of *Pinus densiflora* after 16 weeks of growth: a. A5, reddish brown, red spotted, thick tips, orange rhizomorph, b. A6, black tips with hairy and dark brown hyphae, c. A8, root tips with dark brown, spiny, brown hyphae, d.A12, dark brown, translucency, thick and dichotomous root tips, e. A18, light brown, smooth, long and dichotomous root tips. Scale bars represent 1 mm.

Statistical analysis: All data were subjected to one-way analysis of variance (ANOVA), and mean values were compared by Fisher's least significant difference test (LSD, $P < 0.05$) using the statistical package SPSS-WIN.

Results and Discussion

ECM colonization rates. In the single fungal species and mixture of five ECMF species treatments, all fungal species were successfully established on *P. densiflora* seedlings (Fig. 1). It was possible to distinguish ECM morphotypes based on the morphological features formed on roots inoculated with a mixture of five ECMF species. The percentage colonization rates differed in roots colonizing different fungal species, ranging between 7% and 22% (Fig. 2). Seedlings with fungal isolates, A12 and A18, had significantly higher colonization rates than seedlings with other ECMF species.

In the mixture of five ECMF species treatments, the total colonization rate of ECMF was significantly higher than in single species treatments (Fig. 2). Three species treated in the mixture of five ECMF species (i.e., A5, A12 and A18) had dominantly colonized roots (Fig. 3). Two ECMF species treated in the mixture of five ECMF species, A12 and A18, showed significantly higher colonization rates and were also dominant species. However, two species, A6 and A8, showed low colonization rates when treated in the single fungal species, and almost disappeared when treated in the mixture of five ECMF species. The result suggests that significant competitive reduction of fungal species colonization occurred in the mixture of five ECMF species treatments.

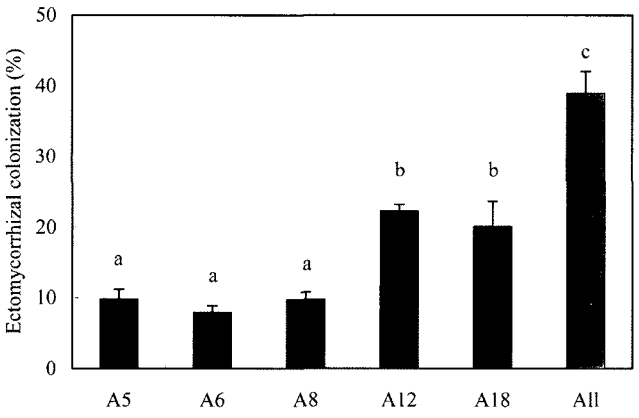

Fig. 2. Colonization rates of the treated ECMF on roots of seedlings of *P. densiflora*. All: mixture of all of the species. Different letter on each bar indicates significantly different mean at $P < 0.05$.

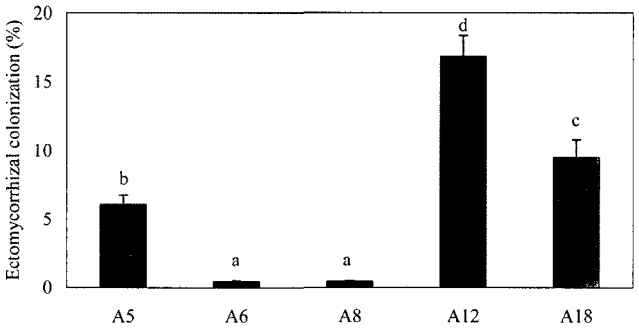

Fig. 3. Percent ECM colonization for each fungal species in the treatment inoculated mixture of five fungal species on seedling of *P. densiflora*. Different letter on each bar indicates significantly different mean at $P < 0.05$.

Plant growth responses. The results of ANOVA showed that mycorrhizal fungal treatments had a significant effect on the height of shoots and the dry weight of shoots and roots of *P. densiflora* seedlings (Figs. 4~6). The seedlings showed no significant difference in height among different single fungal species treatments, except for A5 which showed significantly low height (Fig. 4). Analyses of the mean dry weight of pine seedlings showed the same result among treatments (Fig. 5). It has been proven that height is a reliable predictor of pine seedling growth (Newton *et al.*, 1993; Randall and Johnson, 1998). All the pine seedlings inoculated with ECMF produced trees with significantly higher heights and dry weights than seedlings without fungal inoculation. The growth of the pine seedlings varied by their

Table 2. Result of ANOVA of root colonization rates, heights of shoots and biomass of seedlings of *Pinus densiflora* colonized with different ECMF species

	F	d.f.	P
Root colonization rate	52.321	5, 90	<.001
Height	10.237	6, 1050	<.001
Biomass	13.762	6, 1050	<.001
Root/shoot ratio	27.185	6, 1050	<.001

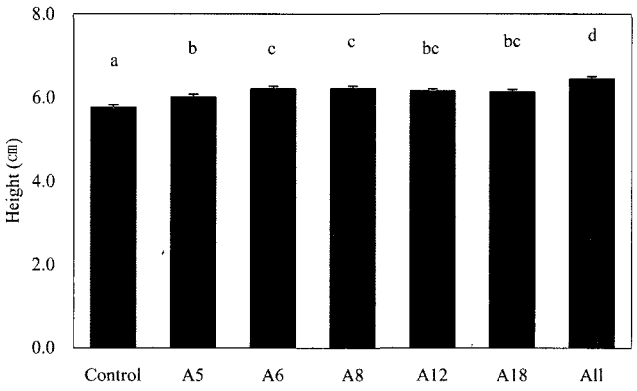

Fig. 4. Mean heights of seedling of *P. densiflora* after 16 weeks of growth with inoculation of ECMF. All: all species treatment. Different letter on each bar indicates significantly different mean at $P < 0.05$.

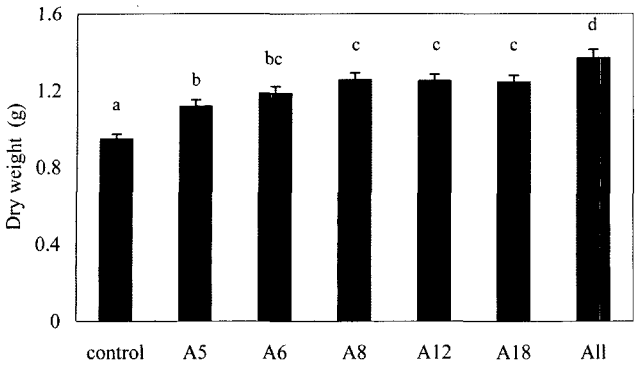

Fig. 5. Mean dry weight of seedling of *P. densiflora* after 16 weeks of growth with inoculation of ECMF. All: all species treatment. Different letter on each bar indicates significantly different mean at $P < 0.05$.

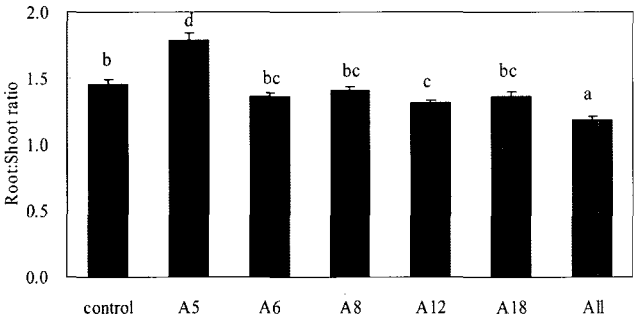

Fig. 6. Root: shoot ratio of *P. densiflora* after 16 weeks of growth with inoculation of ECMF. All: all species treatment. Different letter on each bar indicates significantly different mean at $P < 0.05$.

tent with earlier studies suggesting that ECMF influenced on plant growth (Jones *et al.*, 1998; van der Heijden *et al.*, 1998; Kiers *et al.*, 2000). However, the variation in growth of seedlings among different ECMF species was lower than expected. The two species, A12 and A18, had the highest root colonization rates but seedling growth was not significantly different from seedlings with other fungal species. Root colonization rates showed high variations among fungal species treatments, compared to growth responses to different fungal species. The results in this study did not show a significant correlation between ECM colonization rate and plant growth (correlation coefficient, 0.200, $P = 0.704$).

The pine seedlings inoculated with a mixture of five ECMF species grew better than seedlings inoculated with a single fungal species (Fig. 4). The plant biomass was also significantly higher in those treated with mixture of five ECMF species than those treated with a single fungal species (Fig. 5). These results suggest that increasing the number of ECMF will increase seedling growth. Inoculation of different fungal species significantly affects the root-to-shoot ratio of pine seedlings (Fig. 6). The root-to-shoot ratio of seedlings treated with a mixture of five ECMF species was significantly lower than those treated with a single fungal species, but this was largely due to higher shoot growth than root growth. Treating seedlings with a combination of ECMF species might produce better results with seedling roots than other treatments. In the single species treatment, the root-to-shoot ratio of pine seedlings was different, likely because of the different effects of ECMF on water and nutrient absorption.

This study showed how inoculating different ECMF produces different growth responses in pine seedlings, and how inoculating seedlings with multiple (rather than single) species of ECMF increases plant productivity. These results suggest that colonization of diverse species of ECMF will increase plant productivity, and the selection of suitable ECMF species could be an important factor for plant growth.

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