Mycobiology 30(2): 70-75 (2002)

Mycorrhizal Formations and Seedling Growth of *Pinus desiflora* by *in vitro* Synthesis with the Inoculation of Ectomycorrhizal Fungi[1]

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(*Received February 8, 2002*)

The mycelia were directly isolated from eight species of fungal basidiocarps, confirmed to the ectomycorrhiza in the roots from the fields (forestry); *Suillus bovinus*, *Paxillus involutus*, *Lactarius hysginus*, *Russula fragilis*, *Lepista nuda*, *Lyophyllum shimeji*, *Tricholoma matsutake*, and *Russula integra*. The mycelia were pure-cultured with several transferring in various agars, and inoculated to the roots of pine (*Pinus densiflora*) seedling by *in vitro* method. After ten months growth under artificially aseptic conditions, all pine seedlings inoculated were stimulated at the growth-height, whereas those not inoculate d were nearly dead. Also, the ramifications of ectomycorrhizal pine roots formed in the synthetic *in vitro* systems and were various according to the different mycelia. Synthesis of ectomycorrhiza were clearly confirmed in ten months growth, but not distinguished at this moment. It was clearly proved that the mycelia isolated caused the ectomycorrhizae in the roots of pine seedlings.

KEYWORD: Ectomycorrhiza, Pine seedling, Synthesis

Ectomycorrhizal fungi, as an economically important group of fungi, have common symbiotic associations with trees and shrubs in forests (Harley and Smith 1983). Generally, over 5,000 species of ectomycorrhizal fungi have been described (Molina *et al.*, 1992). The symbionts span all of the phyla of true fungi (Zygomycota, Ascomycota, and Basidiomycota), and occur in at least 15 families within the Basidiomycotina and Ascomycota (Horton and Bruns 2001). Some other fungi would be involved in symbiosis with the roots of plants; various species of Tuberales with the oak trees (Isaac, 1992). The fungi that form a symbiotic relationship with a plant forming a sheath around the root tip of the host plant belong to the families of Pinaceae, Fagaceae, Betulaceae, Myrtaceae, and also include some monocotyledons and ferns (Brundrett *et al.*, 1996; Isaac, 1992; Wilcox, 1996). Those ectomycorrhizal roots, differed in morphological and anatomical characteristics, form mantle structures that morphologically changed by the outward growth of hyphae (fungal cell growth form), penetrating the plant root structure, called to Hartig nets (Martin and Tagu, 1999). Moreover, in those roots, fungal mantle with varying depth covers them, containing aggregated, branched with a characteristic fashion, and swollen hyphal cells. Fungal colonization in roots can benefit the tree through formation of a hyphal network that effectively increases the plant nutrient absorptive surface area (Rousseau *et al.*, 1994).

Although many of the ectomycorrhizal fungi involved can grow in pure culture, they are usually growing very slowly and often have quite complex nutrient requirements. They competed very poorly as saprophytes and depend on associations with higher plants in the natural situation (Danielson, 1984; Erland *et al.*, 1994). Indeed, most of forest trees are highly dependant on their fungal partners and in areas of poor soil, could possibly not even survive without them. And it is thought that high ectomycorrhizal diversity is important for the healthy functioning of woodland. Different fungi appear to have different roles. Thus, in forest management, we need to plan a strategy for protection of the ectomycorrhizal fungi (Robson *et al.*, 1994). Presently, there was found many ectomycorrhizal basidiocarps in forests, but there are few reports of morphological study of ectomycorrhizae produced by *in vitro* synthesis experiments (Yamada and Katsuya, 1995). Up to now, axenic *in vitro* synthesis of an ectomycorrhiza developed was the only way to identify the two symbiotic partners unambiguously (Wiemken, 1999). However, those different culture systems for ectomycorrhizae will be necessary for experimentally synthesized mycorrhizal formation, depending on the plant and fungal species investigated as reported by Alexander (1981), Godbout and Fortin (1985). The objective of this study was to determine the ectomycorrhizal fungi association with red pine seedling by *in vitro* synthesis experiments using fungi isolated from sporocarps of ectomy-

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[1]This work were, in parts, related to Special Awards in Korean Nationwide Exhibition Show for Science and Technology held by Administration of Education in TaeJeon were received with two students of Daejeon Science High School.

corrhizal mushroom and to investigate the formation of ectomycorrhizal roots and the growth of those seedlings.

Materials and Methods

Ectomycorrhizal fungi. The basidiocarps and their related ectomycorrhizal roots were collected from the forest of the Dae-Jeon in Korea during June 2000 to November 2000. The handling operations for isolating all fungi were performed in the clean bench. The ectomycorrhizal roots were first observed by microscope dissecting, and confirmed with the descriptions of the species of basidiocarps (Lee *et al.*, 2000a, b). Above all, the ectomycorrhizal mycelia obtained from the tissue of basidiocarps were cultured on potato dextrose agars (PDA; Difco); From the basidiocarps collected, the mycelia of eight different basidiocarps were selected and transferred for the *in vitro* synthesis of ectomycorrhizal roots (Table 1), being well known to those of *Suillus bovinus*, *Paxillus involutus*, *Lactarius hysginus*, *Russula fragilis*, *Lepista nuda*, *Lyophyllum shimeji*, *Tricholoma matsutake* and *Russula integra*.

Seed germination. The seeds of *Pinus densiflora* were obtained from National Forest Institute and used for this experiment. Germination of these seeds were forcibly induced for this work, while the seeds that not used stored in refrigerator at 0~4°C. The seeds were germinated with the method modified by Brundett *et al.* (1996). The heavier pine seeds were selected with the same size of seed and surface sterilized by shaking in the 70% ethanol solution including 0.1% Tween. These seeds were additionally sterilized in 5% sodium hypocholorite (NaOCl) for 20 min. Finally, the seeds were treated with 30% H_2O_2 solution for 10 min and washed three times with the sterile distilled water. The sterile seeds were placed in the sterile synthetic soil (vermiculite : peat moss = 1 : 1) (Yamada *et al.*, 1999) and watered with sterile water every day for germination. The containers containing the seeds were sealed with the wrap and kept in 23°C incubator of a dark room.

Culture of seedling. All works related to seedling growth were carefully done in the clean bench, preventing from other pathogenic fungi. The sterilized air filtered through the membrane filter (0.2 μ) was supplied to the container of seed every three days. The growth chamber were controlled at 23°C, and light eighteen hours on a day. The soil materials were made of the pure white Perlite and carbon paper in the Petri dishes (87×15 mm). The part of roots in young seedling were placed on the carbon paper (the Perlite packed in other side) in the Petri dishes and the young shoot were, throughout the hole sized with 0.5 mm, located in the out sides of Petri dishes. The young seedling were stored for 2 months for root-growth without any treatment, and then inoculated with 10 mm block of agar containing the various mycelia grown in PDA agar. The mycelial blocks were placed near the roots of young seedling and were induced to be synthesized to the ectomycorrhiza (Beckmann and Heyser; 1992; Beyeler and Heyser, 1997). The young seedlings were watered every a week by pouring about 20 m*l* of sterile water and were maintained in the rectangle of glass water tank with wrap sealing, removing the water stress for plant.

***In vitro* etomycorrhizal synthesis.** The roots of young seedling were inoculated with the mycelial block and induced for 8 months to synthesize ectomycorrhiza. The roots of seedling were observed every a month by naked eyes or microscope dissecting. The Petri dishes containing the seedling were controlled in the plant growth chambers at the same conditions with the above mentioned. For all steps above, water and air supply were done in clean bench in order to prevent the infection from

Table 1. The fungal species directly isolated from the basidiocarps collected in forest and their possible relationships of ectomycorrihizae[a]

Mycelial species	Ectomycorrizae	Host plant descriptives[e]
Suillus bovines	Dichotomous[b]	*Pinus densiflora* trees[1,2,3,4], conifers and deciduous trees[3,4]
Paxillus involutus	Irregularly pinnate[b]	Conifers & deciduous trees[3,4], especially, birch[1]
Lactarius hysginus	Monopodial-pinnate & pyramidal[b]	Conifers[4]
Russula fragilis	–[c]	Deciduous trees (birch, oak)[1,3]
Lepista nuda	–[c]	Hardwoods trees and conifers[1,3,4]
Lyophyllum shimeji	–[c]	*Quercus serrata* & pine trees[4]
Tricholoma matsutake	Dichotomous[d]	*Pinus densiflora* trees[2,3,4]
Russula integra	–[c]	deciduous trees (birch) & conifers[1]

[a]The fungal mycelia directly isolated from the basidiocarps by the special techniques.
[b]Formations of ectomycorrhizal roots in the soil, see Agerer (1991) previously worked.
[c]Formations of ectomycorrhizal roots in the soil, not observed.
[d]Formations of ectomycorrhizal roots in the soil, see Lee (2000) previously worked.
[e]The relationships of host plant roots mentioned by Laessøe and Lincoff (1998) [1], Lee (1988)[2], Park and Lee (1996, 1999)[3], and Imazeki *et al.* (1999)[4].

other pathogenic fungi. Also in a growth chamber, young pine seedlings were grown in glass water tank, being covered with wrap. The ectomycorrhizal tips formed were counted with the gridline intersect method (Brundrett *et al.*, 1996).

Results

Basidiocarps. The basidiocarps and their related ectomycorrhiza have been collected and classified as a basic work (Lee *et al.*, 2000a, b). Among more than 40 different species of basidiocarps, only eight basidiocarps were selected. Particularly, the basidiocarps frequently collected in the forests were identified, as shown in Table 1 and confirmed, as the species of ectomycorrhiza, with the descriptions of species (Agerer, 1991; Laessøe and Lincoff, 1998). Various types of ectomycorrhiza resulted from the interactions between the fungus and host plant were described in some species of basidiocarps (not described in detailed), but those made by four species of the others were not found in any descriptions in Table 1. The pure culture was conducted under aseptic conditions by general procedures of fungal isolation. The mycelia were directly isolated from the sterile tissues of basidiocarps, but not from ectomycorrhizal roots. The mycelia listed in Table 1 were confirmed with the formations of ectomycorrhiza at the bases of basidiocarps, being collected from the forestry. The pure culturing of mycelia isolated from ectomycorrhizal basidiocarps, being helpful to or symbiotic with the roots of plant, was focused in this work and induced to synthesize the ectomycorrhiza under artificial conditions. The mycelia obtained from several pieces of tissue in the sterile parts of basidiocarps were compared with those of the other pieces. The just simple line of mycelia, having the similar to each other at glance, were selected for this work without any other complicated methods mentioned above. Only one or two transferring of mycelia was carefully made for minimizing the loss of ectomycorrhizal abilities.

Inoculation. The germination of pine (*Pinus densiflora*) seeds was made within three or four weeks in the sterile synthetic soil, when supplied with the proper amount of sterile water. After a month's growth, the young seedlings of pine were transplanted into the Petri dishes, and their roots were carefully placed on the carbon paper inside mass of Perlite. This regarding was followed to those conducted at the laboratory of Professor Heyser in Bremen University (Beckmann and Heyser; 1992). The roots placed on the carbon paper (mentioned above) were induced to produce the root hairs in the Petri dish for 2 or 3 weeks, as gently pouring the sterile water. After then, two blocks of agar containing the mycelia grown were picked up and placed near the roots of seedling under aseptic conditions. The seedlings of pine were watered once a week under controlled rooms and supplied with small amounts of mineral nutrients. This simple method was carefully conducted under great desire or dream for the formations of ectomycorrhiza.

Young seedlings. Two or three months later after inoculation, the growth of seedlings was shown to be quite different by naked eyes, but was not significant in the measurement. The seedlings not inoculated were observed to be pale green in the leave and to be nearly died five or six months later, as compared with those inoculated with the mycelia (Fig. 1). The seedlings inoculated grown faster than those not inoculated, and produced the small pretty tips of ectomycorrhiza, ramifying or dichotomous roots with yellow or pale brown tiny root branches, after

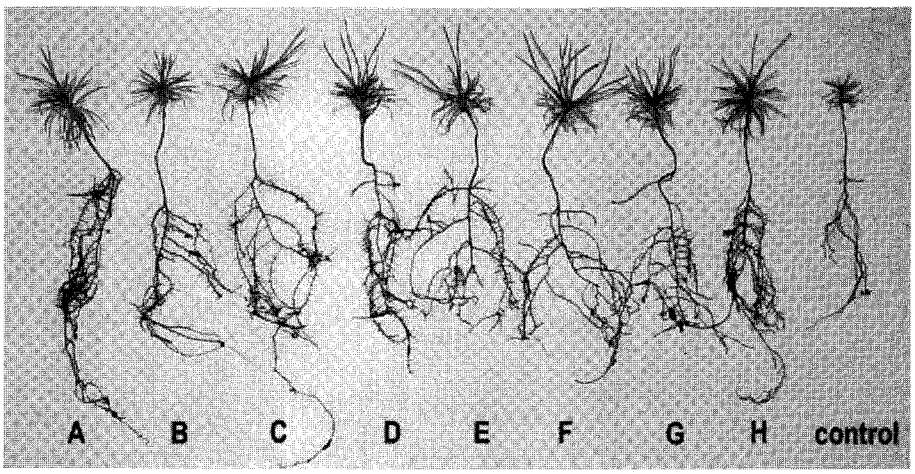

Fig. 1. The plant growths of *Pinus densiflora* stems and roots were cultured for ten months after inoculated with the eight different fungal agar blocks; the roots inoculated with the PDA cultured with A) *Suillus bovinus*, B) *Paxillus involutus*, C) *Lactarius hysginus*, D) *Russula fragilis*, E) *Lepista nuda*, F) *Lyophyllum shimeji*, G) *Tricholoma matsutake*, and H) *Russula integra*.

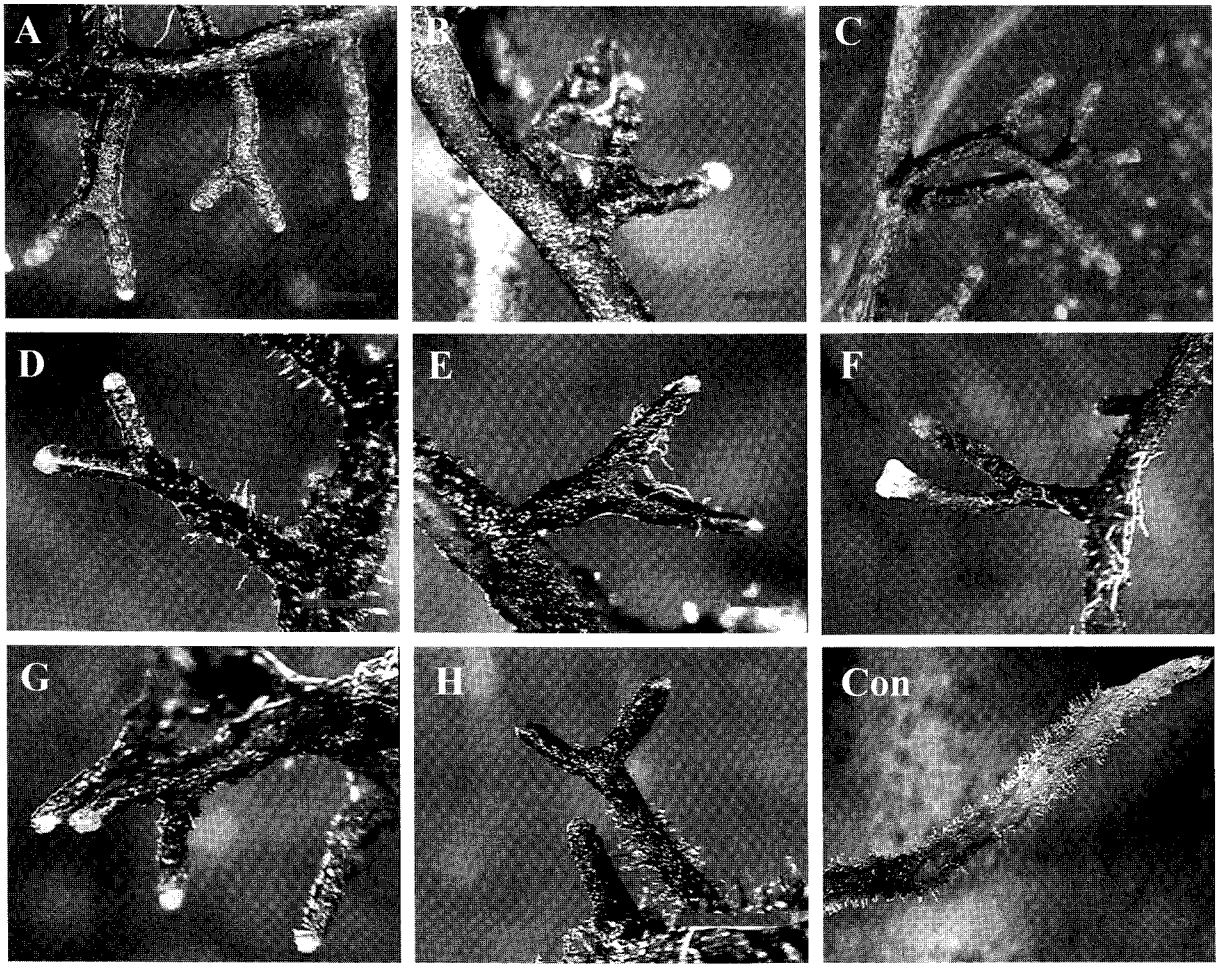

Fig. 2. Formations of the ectomycorrhizal roots (mostly, dichotomous) of *Pinus densiflora* after 12 monthscultures under artificial conditions; The ectomycorrhizal roots interacted with the fungal species of A) *Suillus bovinus*, B) *Paxillus involutus*, C) *Lactarius hysginus*, D) *Russula fragilis*, E) *Lepista nuda*, F) *Lyophyllum shimeji*, G) *Tricholoma matsutake*, and H) *Russula integra*. No formation of ectomycorrhizal roots in the plants of control (Con) and the bright brown colors in the dichotomous roots were considered to be newly formed in the old black roots. The scale bar indicated 0.2 mm.

six or seven months cultures (Fig. 2). The different growth of seedling was observed, but the formations of ectomycorrhiza not found in five or six months seedlings. When pine seedlings were inoculated with the isolated ectomycorrhizal fungi and grown *in vitro* synthesis system during ten months, remarkable growth effect was shown when compared to the controlled (Table 2, Fig. 1). These seedlings were so stimulated to grow, having more two-needles and being differentiated many small roots than those controlled in the pine seedlings. The growth of these seedlings roots was stimulated 40~60% more when compared to the controlled (Table 2). Also, the beginning states and the yellow tiny tips of ectomycorrhiza also found in the roots of these pine tree seedlings inoculated (Fig. 2). Conclusively, the growth of pine seedling was clearly related to the inoculation of the fungal mycelia (Table 2), but was not consistent with the formations of ectomycorrhizal roots in the numerical correlation.

The formations of ectomycorrhiza were formed in six or seven months after inoculation, and were similar in the roots of young seedlings. Therefore, any formation of ectomycorrhiza was never found in the roots of seedling not inoculated, whereas the several found in those inoculated. Much root hairs were covered around the feeder roots of seedling not inoculated, whereas the black sheath supposed to the mantles of fungal mats was in the surfaces of feeder roots in the seedling inoculated (Fig. 2). The ramified roots attached to the feeder root, especially dichotomous branches with long or short arms, were observed on the roots in the seedlings inoculated. The yellow tips on the ramified and branched roots, indicating the new formation of ectomycorrhiza, were frequently consistent with the observations in the fields (Fig. 2A, B, D, E and G). The blooming colors of yellow and brown at the dichotomous branches (Fig. 2C) were also observed to be different from those mentioned above. Surprisingly, the marvelous pan-type tip at the yellow tip of dichotomous branches (Fig. 2F) were observed to be the secondary tips

Table 2. Numbers of ectomycorrhizal roots (dichotomous) formed and root growth (cm) of *Pinus densiflora* seedling grown for 10 months under artificial conditions, after inoculating their roots with the following fungal mycelial agar blocks[a]

Mycelial species	Formations of ectomycorrizal roots[b]	Growth of roots (cm)[c]
Suillus bovines	5	16±1.5
Paxillus involutus	1	13±1.0
Lactarius hysginus	6	19±1.5
Russula fragilis	2	13±2.0
Lepista nuda	5	13±1.0
Lyophyllum shimeji	2	13±1.5
Tricholoma matsutake	1	13±1.0
Russula integra	6	15±1.2
Control	0	8±0.5 (nearly dead)

[a]The fungal mycelia were cultured for 2 or 3 weeks on PDA, after pure culture and the mycelial mat block cut by the sized 1 cm×1 cm retingular shape.

[b]Formations of ectomycorrhizal roots were counted in the plant roots with naked eyes.

[c]Average values of root growth of ectomycorrhizal roots measured. The plants treated with no ectomycorrihizae were dead, so not presented.

of dichotomous branch, being regarded as the coral type of ectomycorrhiza in the interactions between the species of *Russula* and roots of pine (Fig. 1C in Lee *et al.*, 2000b). In conclusion, the formation of ectomycorrhiza in the roots of pine seedling were synthesized with the inoculum of mycelia isolated from basidiocarps under artificial conditions and observed to be various morphologies according to the different mycelia.

Discussion

In Europe, re-vegetation of woody plants with the ectomycorrizal fungi were intensively investigated in the disturbed or pollutant soils (Danielson, 1984; Erland *et al.*, 1994; Wilcox, 1996). Synthesis of ectomycorrhiza *in vitro* system were reported as a new advanced technique in this moments because they were recognized to be resulted from the interaction between the two organisms, for example, Lichens (Agerer, 1991). However, the line of mycelia related to the species of ectomycorrhizal basidiocarps was considered to be important for isolating from the field roots and sustaining the abilities symbiotic with the roots of plant. The techniques of molecular biology were focused for these ectomycorrhiza in the roots or the line of mycelia employed for this regarding. The questions whether the line of mycelia, isolated from the tissues of basidiocarps, is identified to be same of the mycelia of fungus collected were posed on the mycologists laboratory for every fungus collected from the fields. Particularly, the line of fungus isolated was considered to be more important in this work of ectomycorrhiza than in the others. The pure culture was believed to be carefully conducted with several sophisticated methods with the time enough to be confirmed. The line of mycelia should be, before inoculation work in ectomycorrhiza, identified as the same species with basidiocarps collected in the forest. However, confirmation of fungal line mentioned above usually required so many transferring on series of agars, which might cause the loss of abilities symbiosis with the roots. In other words, only few transferring of fungal line were conducted in our work (shown in Table 1), regardless of fungal species confirmation. This study was only focused to understand new formations of ectomycorrhiza by *in vitro* system. During the pure culturing of isolated the basidiocarps, more than five different lines of mycelia were obtained and compared with each other by various methods; microscopic observations of mycelia and polymorphic patterns of genomic DNA by PCR-RAPD. Only a line of fungal mycelia similar to each other in naked eyes were selected to be inoculated to the roots of pine seedling. A synthesis of ectomycorrhiza *in vitro* system should be, as a new advanced technique, more important than any other technique in the present work.

Seedling inoculated with the fungal line isolated above were, if possible, maintained at the constant temperature (23°C) under aseptic conditions. The growth of seedlings and formation of ectomycorrhizal roots were continuously observed once a month, when the sterile water was supplied to the perlite in Petri dishes. The contaminations by other fungi or bacteria were removed as possible as we could. Any contaminations were not found in our culturing systems, because the different growth was observed among seedlings inoculated or not (Table 2). Better growth of young seedling inoculated was consistent with other ectomycorrhizal works (Duchesne *et al.*, 1988a, b; Marx, 1973). Further, any tips of ectomycorrhiza were found in the roots not inoculated, whereas yellow or pale brown tips were in the roots inoculated (Fig. 2). Also, the secondary yellow tip of ectomycorrhiza were, surprisingly, formed in the roots covered with the black mantle in the pine seedlings inoculated with the fungus of *Lyophyllum shimeji* (Fig. 2F). The seedlings not inoculated grown not well and their ten months growth was similar to their four months. The seedlings not inoculated were almost death with little needles in the parts of shoot as well as no formations of ectomycorrhiza. This indicated that the pine seedlings required with the symbiotic helper, such as the ectomycorrhizal fungus mentioned in Table 1 (Lee *et al.*, 2001a, b; Wiemken, 1999). It could be, from the analysis of these results, concluded that ectomycorrhiza formed in roots of pine tree accelerated the growth of those plant with unknown growth factors.

The shapes of nodule root were believed to be dependent upon the species of plant within the different color and variation, but not upon the kinds of *Frankia* (Sil-

vester *et al.*, 1990). Similar phenomena were observed in the ectomycorrhizal roots of plant species collected from the fields in the previous work (Lee *et al.*, 2000a). In other words, the dichotomous branches of roots observed in our work due to plant species, but not due to the fungal inocula. However, the diversities of ectomycorrhiza synthesized *in vitro* system were shown in our culturing; the dichotomous branches with short Y or long Y-shaped tail, yellow tips and pale brown tips, or arrangements of the dichotomous branches in Fig. 2. The secondary tip was formed in the one tip of dichotomous branch, whereas not in the other (Fig. 2F). Growth of ectomycorrhiza was observed to be faster in the roots inoculated with *Lyophyllum* than with other fungus. These indicated that various types of ectomycorrhiza synthesized *in vitro* system within the shape of dichotomous branch would be originated from the different fungal species, listed in Table 1. The information related to the species of ectomycorrhizal basidiocarp would be confirmed with the fingerprint of DNA work, such as PCR-RAPD or RFLP. Simple devices for *in vitro* ectomycorrhizal synthesis would be much important for the experiments for *Tricholoma matsutake* in the pine or *Sarcordon asparatus* in the oak seedlings in Korea.

Acknowledgements

The present investigation was conducted with two students of Daejeon High School of Science. Particularly, the students, Lee, Hong Ki and Son, Min Sik, had taken care of this work in part of the growth of pine seedlings.

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