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Estimation of Mycorrhizal Colonization of the Roots of Oak Seedlings Inoculated with an Ectomycorrhizal Fungus, *Laccaria amethystea*

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Six-month-old seedlings of *Quercus serrata* and *Quercus glauca* in a nursery were inoculated with the ectomycorrhizal fungus *Laccaria amethystea* encapsulated in alginate gel and grown in the nursery. The seedlings were collected at 1, 3, and 5 months after the inoculation and examined for colonization of the root system with ectomycorrhizal fungi. The roots within 5 months after the inoculation showed rudimentary ectomycorrhizal colonization. The level of colonization of the root system was estimated based on the intensity of hyphal covering on the root tips by staining with a fluorescent dye and expressed as an index of mycorrhizal colonization (IMC). IMC increased with the time after inoculation and reached values of 4 and 12% in *Q. serrata* and *Q. glauca*, respectively at 5 months after the inoculation. The determination of IMC may enable to assess the development of mycorrhizal colonization of the root system that shows rudimentary ectomycorrhizas after the inoculation.

Key Words: **colonization level, ectomycorrhizal fungi, *Laccaria amethystea*, oak seedlings, root tips.**

Ectomycorrhizal fungi can improve the nutritional conditions in the associated host plants that grow in a nutrient-poor, especially nitrogen-deficient ecosystem (Bledsoe 1992). This property has been utilized in reforestation to promote the growth of outplanted seedlings (Kropp and Langlois 1990). Such seedlings in various species have been inoculated with suitable ectomycorrhizal fungi. Prior to the outplanting performance, ectomycorrhizal formation should be assessed in the inoculated seedlings. The assessment of mycorrhizal formation is mainly based on counts of the number of fully-developed mycorrhizal root tips observed under a dissecting microscope. The fully-developed mycorrhizas have an external structure, i.e. fungal mantle. Mantle development accompanies the sequential events preceding the fully-developed morphological features. Initial mantle formation was induced 1 to 10 d after the fungal inoculation of the host-roots depending on the host-fungus combination, and then the sequence of developmental events progressed under mycorrhizal synthetic conditions *in vitro* (Massicotte et al. 1986, 1990; Malajczuk et al. 1990). However, such an early colonization can not be easily detected under a dissecting microscope. Therefore the assessment of fully-developed mycorrhizas under a dissecting microscope may underestimate the colonization of mycorrhizal fungi of the root system of seedlings after the inoculation. We should thus develop a rapid technique for estimating the development of ectomycorrhizas in nursery seedlings soon after the fungal inoculation.

Fine structure of the mycorrhizal mantle and even rudimentary colonization can be observed under a compound microscope. Especially, epifluorescence microscopy using appropriate fluorescent dyes provides high contrast images and quick analysis (Brundrett et al. 1996). A fluorescent dye, Calcofluor white M2R has been widely used for various mycological studies (Butt et al. 1989).

The objective of this study was to estimate the mycorrhizal colonization level of the root system of oak seedlings inoculated with an ectomycorrhizal fungus based

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on the intensity of hyphal covering on the root tips by staining hyphae with a fluorescent dye.

Materials and methods

Study site. The study site was a reforestation area around the Nukui dam site located in the northern part of Hiroshima Prefecture, western Japan (34°37.8′ N, 132°18.2′ E; altitude, 440 m). The soil was classified as Brown Forest Soil (Forest Soils Division 1976). At this site, reforestation had been carried out to restore the vegetation on the lateral slope around the dam site and the seedlings to be used for reforestation were prepared in the nursery. The annual mean air temperature and annual precipitation in 2001 measured at the nearest meteorological station (Kake, 34°36.5′ N, 132°19.5′ E; altitude 210 m) on 13.2°C and 1,773 mm, respectively.

Seedlings inoculated with ectomycorrhizal fungus and their maintenance in the nursery. *Quercus serrata* Murray and *Q. glauca* Thunb. were used as the host plants. The seeds of both tree species were collected from the deciduous forests around the Nukui dam site in 2000. The seeds were washed, submerged in tap water for 1 week and then planted in paper pots (5 cm diameter, 20 cm height; FS520, Nihon Beet Sugar Manufacturing Co. Ltd., Tokyo) in November 2000. The pots were filled with unsterilized subsoils that were collected from the lateral slope at the study site and put in a large plastic container. Pot seedlings were set in the field adjacent to the reforestation area in November 2000. Germinated seedlings were inoculated with an ectomycorrhizal fungus, *Laccaria amethystea* (Bull.) Murr. FFPRI215001, in May 2001. Fungal culture was grown in Hagem medium (glucose, 5.0 g; malt extract, 5.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NH_4Cl, 0.5 g; iron (III) citrate $\cdot$ nH_2O 0.005 g; and distilled water 1,000 mL; pH 5.6) (Saito 1992) for 7 months at 25°C in darkness. Cultured mycelium was harvested, homogenized with a blender at 16,800 rpm for 30 s, and centrifuged at 3,500 rpm for 5 min. Fragmented hyphae were encapsulated in the beads of alginated gels (Mauperin et al. 1987). The maximal diameter of the gel beads was 4.6 mm. Five to ten gel beads were put into the bottom of a small hole dug along the taproot of one seedling (about 5 cm depth from the surface) and the hole was covered with soils. Two seedlings in both plant species each were arbitrarily collected from the nursery beds at 1, 3, and 5 months after the inoculation of *L. amethystea*. Collected seedlings were divided into the above- and below-ground parts. The roots were washed with tap water to remove soil particles and stored in 50% ethyl alcohol until analysis.

Observation of hyphal covering on the root tips of oak seedlings. The roots were observed under a dissecting microscope to examine the formation of ectomycorrhizas. Stored root systems in ethanol were washed with tap water, and ten lateral roots were sampled arbitrarily from the whole root system in each seedling. Sampled roots were submerged in Calcofluor white M2R (F3543, Sigma, St. Louis) solution (CW, 0.2% w / v) for a few seconds (West 1986), washed with distilled water for 30 s, and mounted on glass slides with distilled water. The mycorrhizal root tips stained with Calcofluor white M2R were observed at a magnification of 200 × under U excitation (filter block, BP330-385; dichroic mirror, DM400; barrier filter, BA420) using a microscope (BX40, OLYMPUS, Tokyo) equipped with an epifluorescent attachment BX-FLA. Rudimentary ectomycorrhizas that showed a discontinuous and thin fungal mantle were evaluated based on the intensity of hyphal covering on the root tip surface.

Table 1. Classification of mycorrhizal colonization level of root tips based on the characteristics of mantle development in the root system of *Quercus* seedlings inoculated with *Laccaria amethystea*.

Mycorrhizal colonization level of root tips	An area with hyphal covering (%)	Relative value of mantle development
I	0	0
II	0–10	0.05
III	10–50	0.3
IV	50–90	0.7
V	90–100	1

Each root tip was classified by five levels of mycorrhizal colonization estimated based on the hyphal covering, as shown in Table 1, and the number of root tips for each colonization level was counted. More than 130 root tips corresponding to 7% of the total root tips in each seedling were observed in the seedlings of both *Quercus* species at different times after the inoculation except for the *Q. glauca* seedlings at 1 month after the inoculation (58 tips). The index of mycorrhizal colonization (IMC) of the whole root system was calculated from the following equation:

$$\mathrm{IMC} = [\Sigma(m_i \times n_i) / R] \times 100$$

where m_i is the relative value of mantle development in each root tip, as shown in Table 1, n_i is the root tip observed, and R is the number of total root tips observed in each root system. IMC corresponds to the number of fully-developed mycorrhizal root tips in the 100 root tips observed. Determination of IMC was performed for duplicated samples in each collection.

Results and discussion

The length of the taproots exceeded 15 cm in all the oak seedlings at 5 months after the inoculation. Even though lateral roots were formed extensively in both

seedlings at 5 months after the inoculation and the number of first-order lateral roots reached 131 in *Q. serrata* and 154 in *Q. glauca*, respectively, very few root tips showed a mature ectomycorrhizal structure.

The mature mycorrhizas observed were classified into two types based on their morphological characteristics. One was distinctly black, and had many emanating hyphae and a star-like structure on the mantle surface, typical of *Cenococcum geophilum* Fr. (Ingleby et al. 1990; Agerer 1999). The appearance of this mycorrhiza was found only at 5 months after the inoculation in the seedlings of both oak species. This mycorrhizal fungus contaminated the pot soils and colonized the oak roots because the seedlings were cultivated in the nursery under natural conditions. The other type showed a brown monopodial structure with a thick mantle surface and in which many hyphae radiated from the mantle (Fig. 1e). The fungal septa displayed a clamp connection. The mantle surface showed a loosely organized network of hyphae forming a net prosenchymatous structure. These morphological characteristics were typical of the ectomycorrhizas formed by *Laccaria* (Brand and Agerer 1986; Ingleby et al. 1990; Agerer 1999). In this study, the latter type of mycorrhizas was focused on in the whole root system of oak seedlings as to be the inoculated *L. amethystea*.

Five levels of hyphal covering on the root tips, as previously indicated, were observed in the oak seedlings. In level I, hyphal covering was not observed but the root tips showed a large number of root hairs (Fig. 1a). In level II, hyphal covering on the root tips was sparse and the root tips showed few root hairs (Fig. 1b). Although in levels III (Fig. 1c) and IV (Fig. 1d) the hyphal covering on the root tips did not correspond to fully-developed ectomycorrhizas when observed under a dissecting microscope, in level V, the hyphal covering on the root tips (Fig. 1e) corresponded to fully-developed ectomycorrhizas, when observed under a dissecting microscope. Root tips with level V of hyphal covering were observed only in the seedlings at 5 months after the inoculation.

The distribution pattern of each colonization level depending on the mantle development was estimated for the seedlings of *Q. serrata* and *Q. glauca* at various months after the inoculation (Fig. 2). More than 79% of the root tips in the *Q. serrata* seedlings failed to display mycorrhizal colonization until 1 month after the inoculation. The percentage of the root tips with levels II and III of hyphal covering increased in the seedlings at 5 months after the inoculation but few root tips showed levels IV and V of covering. In contrast, the seedlings of *Q. glauca* had a thin hyphal covering on the root tips at 1 month after the inoculation. However the sum of the percentages of the root tips with levels II, III, IV, and V of hyphal covering reached 56.7% at 5 months after the

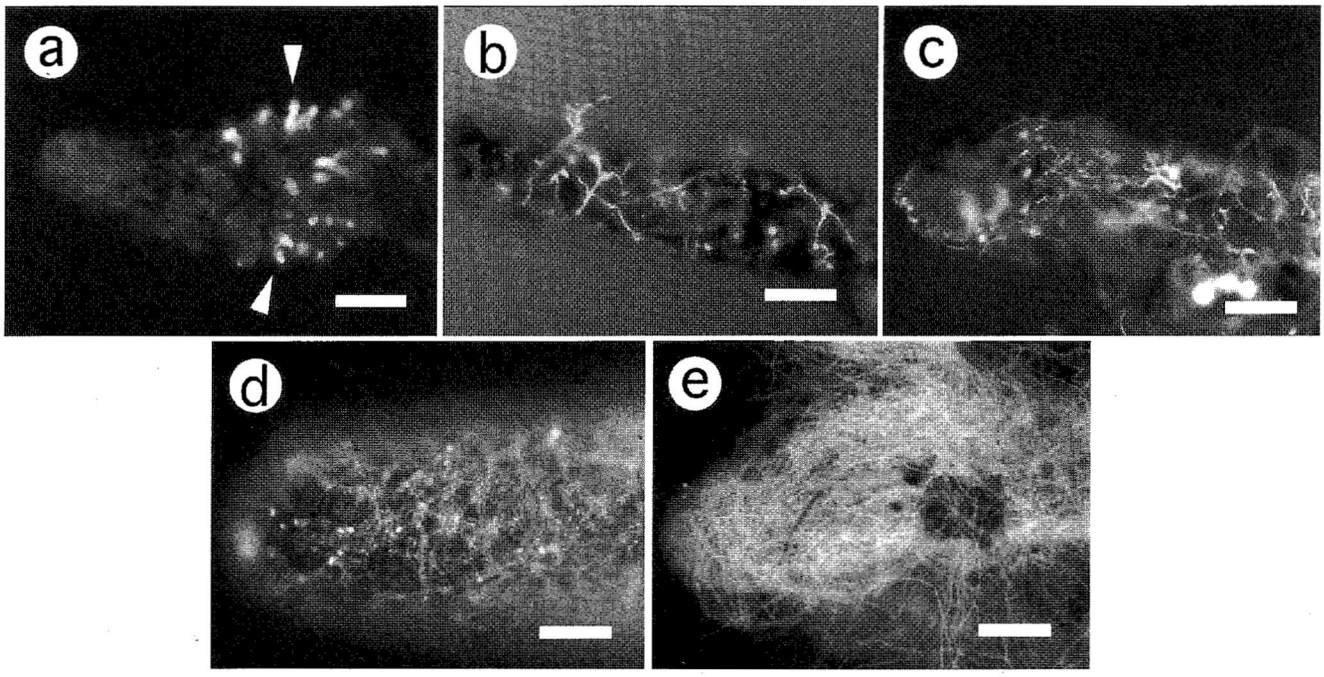

Fig. 1. Development of ectomycorrhizas of *Quercus* seedlings inoculated with *Laccaria amethystea*. a, Root tip of *Q. serrata* showing level I of hyphal covering with many root hairs on it (arrowheads); b, root tip of *Q. glauca* showing level II of hyphal covering; c, root tip of *Q. serrata* showing level III of hyphal covering; d, root tip of *Q. glauca* showing level IV of hyphal covering; e, root tip of *Q. serrata* showing level V of hyphal covering. The root tip showed a fully-developed ectomycorrhizal structure and many hyphae that radiated from the mantle. Bars = 100 μm.

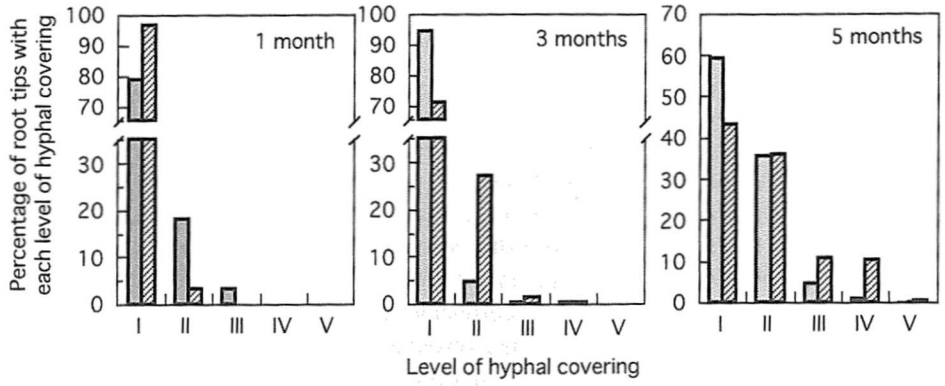

Fig. 2. Relative value of the amount of root tips for each level of hyphal covering after the fungal inoculation. ▩, *Q. serrata*; ▨, *Q. glauca*.

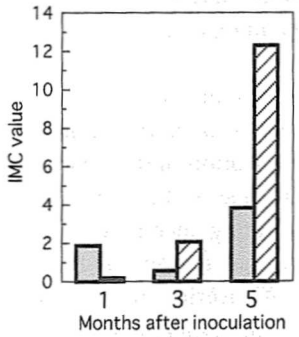

Fig. 3. IMC of the *Quercus* seedlings after inoculation with *L. amethystea*. ▩, *Q. serrata*; ▨, *Q. glauca*.

inoculation. Thus five types of hyphal covering on the root tips reflected the sequential events of mantle development to fully-developed mycorrhizas.

To estimate the level of mycorrhizal colonization of the whole root system, IMC was calculated. The IMC of the *Q. serrata* seedlings ranged from 0.6 to 3.8% (Fig. 3). Although the IMC of the *Q. glauca* seedlings was low until 3 months after the inoculation, it increased up to 12.3% at 5 months after the inoculation. The IMC in the seedlings at 5 months after the inoculation was different between *Q. serrata* and *Q. glauca*. These results indicate that colonization of the seedlings of both oak species with inoculated *L. amethystea* progressed in a different way, though fully-developed ectomycorrhizas in the roots were mostly not detected in the seedlings of both species within 5 months after the inoculation.

In conclusion, combination of CW-staining of hyphae and estimation of hyphal covering on the root tips revealed that the colonization of the seedlings with the inoculated ectomycorrhizal fungus progressed in the root system after the inoculation which showed rudimentary ectomycorrhizas, and that the colonization level of the root system increased with the time after the inoculation. Therefore, estimation of IMC may provide a semi-quantitative analysis of ectomycorrhizal colonization of an immature ectomycorrhizal root system. We have begun to apply this technique to examine the development of mycorrhizal colonization of the root system of nursery seedlings in various host-fungus combinations. This technique may enable to predict the appropriate time for the transfer to planting sites of the inoculated seedlings before they show fully-developed ectomycorrhizas.

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