***In vitro* inoculation effects and colonization pattern of *Leohumicola verrucosa*, *Oidiodendron maius*, and *Leptobacillium leptobactrum* on fibrous and pioneer roots of *Vaccinium oldhamii* hypocotyl cuttings**

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Abstract: The fine root systems of ericaceous plants possess both absorptive fibrous roots and skeletal pioneer roots. This functional differentiation (heterorhizy) can be coupled with a specific mycorrhization pattern, in which fibrous roots form more ericoid mycorrhizas than pioneer roots. However, the behaviors of root-associated fungi on the different individual roots remain largely unknown. We investigated the colonization patterns and root modifications of *Leohumicola verrucosa*, *Oidiodendron maius*, and *Leptobacillium leptobactrum* strains on *Vaccinium oldhamii* hypocotyl cuttings *in vitro*. Three putative and true mycorrhizal fungal strains (one *L. verrucosa* and two *O. maius*) exhibited typical heterorhizic colonization patterns and tended to increase pioneer root branching. However, a root endophytic fungus (*L. leptobactrum*) uniformly exhibited a lower colonization across the different roots and did not increase the number of pioneer root branches. The colonization patterns and root modification ability of mycorrhizal fungi may be linked, thereby ensuring sufficient colonization sites.

Keywords: ericoid mycorrhiza, heterorhizy, *in vitro* resynthesis, lateral root formation, mycorrhization pattern, root endophytic fungi

Introduction

Most plants belonging to core Ericaceae have extremely fine absorptive roots, known as hair roots, where ericoid mycorrhizas are formed (Read 1996, Freudenstain et al. 2016). These plants receive benefits from their association with ericoid mycorrhizal fungi, including enhanced nutrient supply and stress tolerance (Smith and Read 2008). Hyphal coils within host rhizodermal cells are a plant–fungal interface and key structure that differentiates this symbiosis (Read 1996, Peterson and Massicotte 2004). Despite the importance of mycorrhizas for plant growth, the functional and phylogenetic characteristics of the diverse fungi and their association with ericaceous fine roots remain largely unknown (Leopold 2016). There is a lack of data from *in vitro* resynthesis experiments for many fungal taxa (Vohník 2020). There are few reported examples of symbiotic functions of ericoid mycorrhizal fungi that are related to root morphogenesis (Villarreal-Ruiz et al. 2004, Scagel 2005, Vohník et al. 2007, Wei et al. 2020, Baba et al. 2021). Therefore, additional studies are necessary to understand the ecophysiological roles of ericaceous fine roots and their association with fungi.

Recently, we reported qualitative differences between individual fine roots originating from different apical meristems in blueberries (Baba et al. 2018, Baba et al. 2019). Because heterorhizy has been commonly regarded as a dimorphism that classifies individual fine roots as fibrous (thin and short absorptive roots) or pioneer (thick and long skeletal roots) (Sutton and Tinus 1983, Zadworny and Eissenstat 2011, Bagniewska-Zadworna et al. 2012), individual fine roots in the blueberry plant can be classified into hair root-like fibrous roots and thicker and longer pioneer roots (Baba et al. 2018). This means that heterorhizy can represent a type of functional differentiation between individual roots. There are corresponding mycorrhization patterns that support the differentiation between individual roots. In ectomycorrhizal plants, a pattern that mycorrhizas are more frequently formed in fibrous roots than in pioneer roots is common (e.g., Kubíková 1967, Zadworny and Eissenstat 2011, McCormack et al. 2015). Similar heterorhizic mycorrhization exists in ericoid mycorrhizal plants (Baba et al. 2021). However, there are only rare instances where research is focused on heterorhizy of core Ericaceae; furthermore, how root colonization patterns differ between fungal strains remain unclear.

It is crucial to examine the universality and the mechanisms regulating heterorhizic ericoid mycorrhization to clarify the functional cooperation between fine roots and mycorrhizal fungi in ericaceous plants. Of particular interest is (1) how different root-associated fungi differentially colonize fibrous and pioneer roots and (2) how they alter root morphogenesis. We expected that true ericoid mycorrhizal fungi would show a colonization pattern that is dominant in absorptive fibrous roots, similarly to ectomycorrhizal fungi, possibly to increase the nutrient exchange efficiency.

The aim of this study was to determine (1) *in vitro* colonization patterns of different root-associated fungi on fibrous and pioneer roots, and (2) how these fungi modify the morphogenesis of the two types of roots. For this purpose, we resynthesized four root associated fungi with an ericaceous host and examined host growth as well as fungal colonization patterns.

Materials and Methods

Host plant and fungal strains

In November 2015, we collected seeds from wild *Vaccinium oldhamii* berries located in a temperate deciduous broad-leaved forest at the Field Museum Kusaki of the Tokyo University of Agriculture and

Technology, Gunma, Japan (36°32'48''N 139°24'45''E). The seeds were washed and dried before storing at 4°C. In January 2016, the seeds were again cleaned with sterile water and surface sterilized in 10% hydrogen peroxide for 10 min. The seedlings were germinated on 0.8% sterile water agar plates under 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiation and a 16 h light (23°C)/8 h dark (20°C) cycle. Because of the long dormancy, germination started after 6 months. In April 2017, uniform-sized seedlings with fully expanded cotyledons were used for subsequent resynthesis experiments.

The following four fungal strains were selected: *Leohumicola verrucosa* EF1393, a putative ericoid mycorrhizal fungus (Grunewaldt-Stöcker et al. 2013, Baba and Hirose 2020); *Oidiodendron maius* EF1409 and EF1453, a representative ericoid mycorrhizal fungus; and *Leptobacillum leptobactrum* EF1895 (basionym: *Verticillium leptobactrum*; Zare and Gams 2016), a putative root endophytic fungus belonging to the order Hypocreales. We noted that although *Leohumicola* species retrieved from ericaceous fine roots repeatedly and frequently forms hyphal coils in ericaceous rhizodermal cells, the actual mycorrhizal status of the species is yet to be resolved (Grunewaldt-Stöcker et al. 2013, Baba and Hirose 2020). Moreover, although *L. leptobactrum* has been detected in ericaceous root samples (Toju et al. 2016), the colonizing form of *L. leptobactrum* in ericaceous roots has not been elucidated. EF1393, EF1409 and EF1453 were isolated from root samples of *Rhododendron molle* subsp. *japonicum* growing at altitudes of 1900–2000 m on Mt. Nekodake, Nagano, Japan (Baba and Hirose 2020, Baba et al. 2021). EF1895 was isolated from a root sample of *Rhododendron dilatatum* var. *dilatatum* growing at an altitude of 446 m on Mt. Homan, Fukuoka, Japan. Their taxonomic status was identified based on their morphology and closest BLAST matches of rDNA internal transcribed spacer sequences (Table 1).

Resynthesis experiment

Each fungal strain was activated on potato dextrose agar (Becton, Dickinson and Co., Maryland, USA) for 21 days at 25°C in the dark. A 5 × 5 mm agar plug, taken from the edge of the cultures of each strain was placed on 10 mL of corn meal agar (Nissui, Tokyo, Japan) in a 9 × 2 cm Petri dish. These inoculated fungi were pre-cultured for 1 week at 25°C in the dark. Control plates with corn meal agar did not contain plugs. Sterile soil medium (3:1 (v/v) mixture of Kanuma soil and peat moss, with about 80% of water content) was prepared by

Table 1. Accession number and BLAST search results of each fungal strain used in this study

Strain	Taxon	Accession number	Best identified BLAST match taxa (Accession number)	Query cover (%)	Identity (%)
EF1393	<i>Leohumicola verrucosa</i>	LC505462	<i>L. verrucosa</i> (NR_121306)	56	98.85
EF1409	<i>Oidiodendron maius</i>	LC574987	<i>O. maius</i> (LC131008)	91	99.68
EF1453	<i>Oidiodendron maius</i>	LC574988	<i>O. maius</i> (MT321755)	90	99.56
EF1895	<i>Leptobacillum leptobactrum</i>	LC600768	<i>L. leptobactrum</i> (NR_154111)	93	97.08

autoclaving at 121°C for 30 min. Fifty milliliters of soil (about 10 mm in height) was added to each pre-cultured plate. The root systems of young *Vaccinium* seedlings initially possess only a few skeletal roots, including primary roots (Baba et al. 2019), and thinner individual roots may be initiated from such root systems. Therefore, we employed hypocotyl cuttings as plant material to induce sufficient new pioneer roots whose apical parts can accept fungal colonization. The root systems of seedlings were excised on sterile water agar plates. For each plate, three cuttings were inserted with equal spacing so that their hypocotyls were fully buried in the medium and were above the colony glowing zone. The dishes were then sealed with Parafilm, and five dishes were replicated per strain. These cuttings were cultured under 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiation and a 14.5 h light (25°C)/9.5 h dark (20°C) cycle. After 64 days, the soil and cuttings were harvested.

Contamination was checked by culturing a pinch of soil medium on potato dextrose agar for 1 week at 20°C. No contamination was observed from the fungal morphology on the agar plate. For each vessel, one seedling was randomly selected. After removing the attached debris in water, plants were separated into the leaves, stems, and roots. Thereafter, leaves and individual roots were counted. According to the method described by Baba et al. (2018), root systems were scanned using an image scanner (600–1200 dpi resolution, 8-bit grayscale, TIFF format; GT-9800F, EPSON, Nagano, Japan), and total root length was calculated with Scion Image (Scion Corporation, Maryland, USA). After drying at 60°C for 48 h, shoot and root dry weights were determined. Moreover, one additional plant was selected from each vessel for mycorrhizal observation. These plants were fixed in 70% ethanol and fungal hyphae were stained brown according to the method described by Kobae and Ohtomo (2016) with slight modifications. Fixed roots were cleaned by soaking in 10% KOH for 24 h and then in 2% HCl for several hours. After washing with phosphate buffered saline (pH 7.4), the hyphae were labeled and placed in 1.5 mL phosphate buffered saline containing 1% (w/v) bovine serum albumin (Wako, Osaka, Japan) and 0.4 $\mu\text{g mL}^{-1}$ wheat germ agglutinin-conjugated horseradish

peroxidase (Vector, California, USA) for more than 16 h. Furthermore, after washing again with phosphate buffered saline, hyphae were stained in 1.5 mL phosphate buffered saline containing 0.2 mg mL^{-1} 3,3'-diaminobenzidine tetrahydrochloride (Nacalai Tesque, Kyoto, Japan) and 0.1 $\mu\text{L mL}^{-1}$ of 30% hydrogen peroxide for more than 1 h. Subsequently, the staining reaction was completed by transferring the samples into Tris-ethylenediaminetetraacetic acid buffer (pH 8.0). Stored roots were mounted with 50% glycerol for microscopic observation.

Under bright-field microscopy (BX50, Olympus, Tokyo, Japan), individual roots were visually classified into fibrous roots that showed hair root-like morphology or pioneer roots that are approximately twice as large in diameter (Baba et al. 2018). For both types, one to four individual roots were selected per individual plant. However, no fibrous roots could be selected in four control individuals owing to insufficient development. For individual root, root diameter and hyphal coil frequency were measured in a 300 μm -length located between the 1st and 2nd root orders. This area was chosen because fungi are not found around the meristem in actively growing roots, and rhizodermal cells slough off from root surface as the root grows (Peterson et al. 1980, Berta and Bonfante-Fasolo 1983). Therefore, to observe the root parts where fungi actively colonize, the most apical parts in the 1st order and basal parts in the 2nd order were excluded from observations. Furthermore, except for the control plants, the observed root parts were limited to those that had extraradical hyphae attached. Additionally, the presence of coils was confirmed in each observable rhizodermal cell, and the coil frequency percentage was calculated. On the root images scanned as above, lateral root numbers were counted. Each root length was measured using the segmented line tool, ImageJ (National Institutes of Health, Maryland, USA).

Data analysis

Statistical analyses were performed using R (version 3.6.3; R Core Team, 2020). Leaf number, leaf dry weight, root number, root dry weight, and total root

Table 2. Growth of *Vaccinium oldhamii* hypocotyl cuttings inoculated different root endophytic fungi

Strain ^a	Leaf number				Shoot dry weight (g)				Root number				Root dry weight (g)				Total root length (cm)			
Control	6.2	±	0.5 ^b	a ^c	2.3	±	0.4	a	17.2	±	5.1	a	0.28	±	0.13	a	2.2	±	1.0	a
EF1393	9.0	±	0.3	b	3.8	±	0.7	a	16.0	±	2.0	a	0.43	±	0.11	a	7.2	±	1.1	b
EF1409	10.0	±	0.7	b	3.5	±	0.7	a	19.6	±	6.1	a	0.71	±	0.23	a	11.9	±	3.1	b
EF1453	9.8	±	1.0	b	3.2	±	0.6	a	35.2	±	6.3	a	0.82	±	0.25	a	16.5	±	3.3	b
EF1895	9.6	±	0.7	b	4.6	±	0.8	a	29.2	±	8.7	a	0.63	±	0.16	a	16.1	±	5.8	b

^a EF1393, *Leohumicola verrucosa*; EF1409 and EF1453, *Oidiodendron maius*; EF1895, *Leptobacillium leptobactrum*.

^b Values are mean ± SE ($n = 5$).

^c Different lower-case letters indicate significant differences between strains (Tukey's HSD test with natural log transformation, $P < 0.05$).

length were log-transformed and compared between different strains using Tukey's HSD test. The diameter, length, and lateral root number of individual roots were compared between different strains or root types by analysis of variance with Tukey's post-hoc test using the "car" and "emmeans" packages. For this analysis, we constructed generalized linear mixed models with Gaussian distribution, using the "lmerTest" package, to incorporate the two fixed effects with individual plants as a random intercept. Hyphal coil frequency was similarly analyzed using a statistical model with binomial distribution, but data from the control treatment were removed to eliminate excess zeros.

Results and Discussion

During the incubation period, all plants survived, and only one cutting in the control group failed to form roots. Leaf number and total root length significantly increased in the presence of the tested fungi without inter-strain differences (Table 2). Shoot and root dry weights and root number did not significantly differ among the treatments. Although we did not investigate the environment inside the dishes, a possible cause of the beneficial effects was CO₂ production by the inoculated fungi (Lukešová et al. 2015, Cassarrubia et al. 2016). However, as Isutsa et al. (1994) reported, elevated CO₂ levels do not necessarily improve plant growth when the irradiance level is low or when stomata are not fully functional. Plant-growth is promoted by related fungal strains in *ex vitro* environments (Bizabani et al. 2016, Schmidt et al. 2018, Baba et al. 2021). For example, Schmidt et al. (2018) reported that *L. leptobactrum* produced siderophores *in vitro* and promoted the growth of *Miscanthus × giganteus* in a greenhouse experiment. Considering our results about root morphology, it was possible that factors other than CO₂ concentration had, at least partly, affected root growth (see below).

There were significant interspecific and inter-

strain differences in the fungi's ability to modify individual root morphology. We observed morphologically different fibrous and pioneer roots, except in the control plants that mostly lacked fibrous roots (Fig. 1). Pioneer roots were approximately 100 µm larger in average diameter than fibrous roots, irrespective of the treatment ($P < 0.05$, Fig. 1A, B), but root diameter was not significantly affected by the strains. Furthermore, while the length of fibrous and pioneer roots did not differ in the control ($P > 0.05$), root length differed between the two root types irrespective of the fungal strain ($P < 0.05$). We did not observe significant strain effects on the length of fibrous roots (Fig. 1C). However, the pioneer root was significantly longer in all inoculated treatments than in the control (Fig. 1D). The lateral root numbers of fibrous and pioneer roots did not differ in the control or in the presence of EF1895 ($P > 0.05$) but they did differ in the presence of EF1393, EF1409, and EF1453 ($P < 0.05$). In addition to this, the strains did not seem to have any effect on fibrous root branching (Fig. 1E). The two *O. maius* strains significantly increased pioneer root branching when compared to that in the control (Fig. 1F). There was also a significant inter-strain difference between the two *O. maius* strains, EF1409 and EF1453. Regarding these *O. maius* strains, alteration of individual root length and branching have already been confirmed in an *ex vitro* experiment (Baba et al. 2021). Because diffusible soluble compounds produced by *O. maius* increase root formation (Casarrubia et al. 2016, Wei et al. 2020), it was possible that our *O. maius* strains affected root morphogenesis through the production of certain chemicals. Similarly, *L. verrucosa* EF1393, which had a slight but non-significant increase in average lateral root number (Fig. 1F), may have the ability to modify lateral root formation via soluble compounds. This may be hypothesized considering the improvement in root biomass in the field inoculation experiment of the *Leohumicola* species (Bizabani et al. 2016). However, EF1895

Fig. 1. Diameter (A, B), length (C, D), and lateral root number (E, F) of fibrous (red; A, C, E) and pioneer (blue; B, D, F) roots of *Vaccinium oldhamii* hypocotyl cuttings inoculated different root associating fungi. Different colored dots indicate individual root values. Thick black horizontal bars and grey error bars indicate mean and SE, respectively. Total observed root numbers are shown below: Control fibrous root, 2; Control pioneer root, 16; EF1393 fibrous root, 10; EF1393 pioneer root, 13; EF1409 fibrous root, 12; EF1409 pioneer root, 12; EF1453 fibrous root, 12; EF1453 pioneer root 12; EF1895 fibrous root, 13; EF1895 pioneer root, 13. Different lower-case letters indicate significant differences between strains ($P < 0.05$). Identity of fungal strains: EF1393, *Leohumicola verrucosa*; EF1409 and EF1453, *Oidiodendron maius*; EF1895, *Leptobacillium leptobactrum*.

Fig. 2. Frequency of intracellular coil/loop of fibrous and pioneer roots of *Vaccinium oldhamii* hypocotyl cuttings inoculated different root associating fungi. Total observed cell numbers are shown below: Control fibrous root, 66; Control pioneer root, 844; EF1393 fibrous root, 714; EF1393 pioneer root, 920; EF1409 fibrous root, 678; EF1409 pioneer root, 531; EF1453 fibrous root, 537; EF1453 pioneer root 602; EF1895 fibrous root, 780; EF1895 pioneer root, 819. Asterisks indicate significant root-type differences ($P < 0.05$). Different upper or lower-case letters indicate significant differences between strains in fibrous or pioneer roots, respectively ($P < 0.05$). Statistical analyses were conducted removing the data of control plants. Identity of fungal strains: EF1393, *Leohumicola verrucosa*; EF1409 and EF1453, *Oidiodendron maius*; EF1895, *Leptobacillum leptobactrum*.

did not affect lateral root formation of pioneer roots. This result may indicate that this species lacks the production capacity of certain chemicals such as indole acetic acid (Schmidt et al. 2018).

We observed differences in colonization patterns between mycorrhizal and endophytic fungi. As with the previous *ex vitro* experiment using EF1409 and EF1453 (Baba et al. 2021), EF1393, EF1409, and EF1453 consistently showed typical heterorhizic colonization in which absorptive fibrous roots retained more mycorrhizal structures than pioneer roots (Fig. 2). For these strains, even pioneer roots with developed extraradical or intercellular hyphae often lacked intracellular hyphal coils (Fig. 3). This colonization pattern may contribute to more efficient nutrient foraging by the host plant through localizing the symbiotic organs to the absorptive individual roots. Nevertheless, the establishing mechanism of this pattern is unclear. Plants grown *in vitro* were possibly free from specific environmental stresses, such as drought, that can damage the rhizodermis. Therefore, the lower frequency of coils in pioneer roots implies

that endogenous factors decrease mycorrhization on pioneer roots. Different from fibrous roots, pioneer roots possess not only large tissues but also the ability to form secondary growths (Bagniewska-Zadworna et al. 2012), which may induce specific status in rhizodermal cells. For example, we observed rapid loss of vitality of pioneer root rhizodermis in *in vitro*-grown blueberry seedlings (Baba and Hirose unpublished data). Such specific cellular status may inhibit pioneer root mycorrhization. Compared to the typical ericoid mycorrhizal colonization of EF1393, EF1409, and EF1453, colonization by EF1895 was characterized by non-heterorhizic colonization with significant infrequency and loop- or branch-like sparse intracellular hyphae (Fig. 2, 3). As Vohník et al. (2016) stated, lower colonization frequency suggested the non-ericoid mycorrhizal status of EF1895. This non-mycorrhizal status can correspond to what EF1895 showed regarding specific colonization morphology in Fun 1 cell staining. It was observed that vital signs of colonization (cylindrical intravacuolar structures) of host cells having coils were rare (Baba and Hirose unpublished data). Considering these differences between EF1895 and the other strains, non-heterorhizic colonization of EF1895 may be associated with its endophytic lifestyle, which possibly does not require it to colonize vital host cells.

In conclusion, we revealed interspecific differences in fungal colonization patterns and inter-strain differences in fungi's ability to promote pioneer root branching. The heterorhizic colonization by putative and true ericoid mycorrhizal fungi seemed to resemble those of ectomycorrhizal fungi. Wei et al. (2020) suggested that promoted adventitious root formation by *O. maius* can contribute to increased colonization sites. The ability of this fungus to increase the laterals of pioneer roots will function to increase preferred colonization sites, namely fibrous roots. In this way, colonization patterns and the ability of mycorrhizal fungi to change root branching may be linked. Because we observed only a few fungal strains, such connections in ericaceous root associating fungi are still unclear. Considering that root endophytic or pathogenic fungi can produce indole acetic acid and modify rooting (e.g., Manici et al. 2015), the relationship between colonization patterns and root modification should be examined using more diverse ericaceous root associated fungi.

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Fig. 3. Morphology of colonization by different root associating fungi. Hyphae are stained brown. **A** Hyphal coil formation on fibrous root by EF1393, *Leohumicola verrucosa*. **B** Pioneer root without hyphal coil but having extraradical- and intercellular hyphae of EF1393. **C** Hyphal coil formation on fibrous roots by EF1409, *Oidiodendron maius*. **D** Pioneer root without hyphal coil but having a lot of extraradical- and intercellular hyphae of EF1409. **E** Hyphal coil formation and extracellular hyphal development on fibrous root by EF1453, *Oidiodendron maius*. **F** Pioneer root without hyphal coil but having a lot of extraradical- and intercellular hyphae of EF1453. **G** Hyphal loop formation on fibrous root by EF1895, *Leptobacillum leptobactrum*. **H** Pioneer root with a hyphal loop and few extraradical- and intercellular hyphae of EF1895. Arrows indicate representative coils/loops. Bar, 50 μ m.

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