Morphological characteristics of rhizodermal colonization by *Leohumicola* species in an ericaceous host

Takashi Baba¹ and Dai Hirose²

¹Division of Apple Research, Institute of Fruit Tree and Tea Science, NARO, 92-24 Shimokuriyagawa, Morioka, Iwate 020-0123, Japan

²School of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan
The two authors contributed equally to this work.

Corresponding author: T. Baba, E-mail: babat211@affrc.go.jp, Phone: +81-19-645-6155

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Abstract: Lifecycle and temporal functionality of ericaceous fine roots can be affected by colonization dynamics of diverse root colonizing fungi. However, states of colonization are unknown for most of the root endophytes, which also obscures their lifestyles. To recognize characteristics of colonization of *Leohumicola* fungi, we investigated (1) the vitality of rhizodermal cells by using FUN-1 stain (vital staining) and (2) morphology of colonizing hyphae by *N*-acetylglucosamine-specific staining (DAB staining). All the twelve strains, identified as *L. verrucosa*, *L. minima*, and *Leohumicola* spp., colonized vital and non-vital rhizodermal cells of *Vaccinium* seedlings. We observed at least four different states of host cells encompassing coils. DAB staining visualized intracellular hyphae that resembled coils of typical ericoid mycorrhizal fungi. Heterogeneous staining states of coils were also observed in DAB staining. The variable states of *Leohumicola* coils can be comparable with ericoid mycorrhizal fungi, and indicate various developmental and functional stages of intracellular association.

Keywords: 3,3'-diaminobenzidine tetrahydrochloride, ericoid mycorrhizal fungi, FUN 1 stain, hyphal coil, *Leohumicola*, wheat germ agglutinin-conjugated horseradish peroxidase

Abbreviations: CIVS, cylindrical intravacuolar structures; DAB, diaminobenzidine; ErM, ericoid mycorrhiza; ErMF, ericoid mycorrhizal fungi; HRP, horseradish peroxidase; PBS, phosphate buffered saline; WGA, wheat germ agglutinin

Introduction

ErM (ericoid mycorrhiza) is formed by some ascomycetous and basidiomycetous mycorrhizal fungi in association with fine roots of core Ericaceae and Diapensiaceae (Wang and Qiu 2006, Smith and Read 2008, Freudenstein et al. 2016). The ErMF (ericoid mycorrhizal fungi), harbored in host rhizodermal cells, form hyphal complexes called coils or loops, which function as the sites of nutrient exchange (Read 1996, Peterson et al. 2004, Leopold 2016). As mycorrhizal symbiosis should be vital association, both host cytoplasm and hyphal coils encased by host plant membrane are living and interacting at least for a certain period (Bonfante-Fasolo and Gianinazzi-Pearson 1979, 1982, Selosse et al. 2007, Grunewaldt-Stöcker and von Alten 2016). Assistance with nutrient absorption and conferring stress tolerance are some of the benefits of the ErMF-host association considered essential for host plant survival in their habitats especially with harsh edaphic conditions (Cairney and Meharg 2003). However, mycorrhizal status has only been confirmed for a few taxa, which makes the definitive distinction of ErMF from the other fungi that inhabit ericaceous fine roots challenging (Leopold 2016). Phylogenetic and functional diversity of ErMF remain understudied in comparing to those of arbuscular mycorrhizal and ectomycorrhizal fungi, which hamper detailed understanding of functional features of ErM and lifestyles of ericaceous fine roots.

Numerous studies have detected diverse fungi in ericaceous fine roots, and some of these fungi have been confirmed to form coils in ericaceous

rhizodermal cells (Perotto et al. 1996, Mclean et al. 1998, Allen et al. 2003, Palmer et al. 2007, Chambers et al. 2008, Zhang et al. 2009, Walker et al. 2011, Bizabani and Dames, 2015, Toju et al. 2016, Vohník et al. 2016, Zhang et al. 2016, Bruzone et al. 2017). However, most of their lifestyles remain unclear, hence, researchers need to accumulate a huge amount of fundamental knowledge about the association. Especially in morphology of colonization, the study by Grunewaldt-Stöcker and von Alten (2016) recently emphasized the necessity for careful observations to distinguish ErMF from other fungi; they found that *Acremonium strictum* forms hyphal coils in the rhizodermis of *Rhododendron*, but the coils are not found in living host cells unlike with the typical ErMF, *Oidiodendron maius* and *Hyaloscypha hepaticicola* (syn. *Rhizoscyphus ericae* or *Pezoloma ericae*). This observation may indicate presence of diverse lifestyles of hyphal coil-forming fungi which across saprotrophs, endophytes, and mycorrhizal fungi. These viewpoints emphasize the importance of confirming the state of the host cell encompassing hyphal coils to comprehend the lifestyles of the diverse fungi in ericaceous fine roots. Such understanding can indirectly contribute for our understanding of lifecycle and dynamics of fine roots.

Leohumicola is an asexual genus belonging to Leotiomycetes (Hambleton et al. 2005, Johnston et al. 2019). In total, seven *Leohumicola* species have been described in North America and some parts of Africa (Hambleton et al. 2005, Nguyen and Seifert 2008). However, Nguyen and Seifert (2008) speculated that *Leohumicola* is more diverse and widespread. Some ecological studies have actually detected known and unidentified species of the genus in other regions such as Asia, Europe, Australia, and South America (Hambleton et al. 2005, Chen et al. 2009, Quilliam and Jones 2012, Obase and Matsuda 2014, Takashima et al. 2014, Bonfim et al. 2016). Furthermore, Egidi et al. (2019) recently reported that *Leohumicola* is one of the genera dominating worldwide soil fungal communities. These findings indicate that taxonomy and biogeography of *Leohumicola* are understudied. *Leohumicola* possesses heat tolerance and root-endophytism; most of the isolates have been obtained from heated soils or surface-sterilized plants roots. Some species are known to associate with burnt ecosystems dominated by ErM host species (Hambleton et al. 2005, Nguyen and Seifert 2008). Some isolates of *Leohumicola* have been reported to form hyphal coils within root cells of *Rhododendron* (Grunewaldt-Stöcker et al. 2013). However, evidence of several characteristics of mycorrhizal status of *Leohumicola* are lacking; therefore, the genus is regarded as putative ErMF

(Leopold 2016). Here, we focused on the status of ericaceous host cells that encompassing coils of *Leohumicola* strains. We aimed to confirm the host cell vitality that is visualized by the method of Grunewaldt-Stöcker and von Alten (2016). Additionally, we aimed to confirm morphology of *Leohumicola* coils by the target-specific staining method of Kobae and Ohtomo (2016) which is expected to imply more detail features of intracellular hyphae than non-specific staining methods such as trypan blue staining that have generally used for observation of fungi colonizing in ericaceous fine roots.

Materials and Methods

Fungal strains

Twelve *Leohumicola* strains were investigated for their ability to colonize ericaceous fine roots. Information of these strains are shown in Table 1. Five strains, EF1393, EF1421, EF1433, EF1561, and EF1565, were isolated from hair roots of *Rhododendron molle* subsp. *japonicum* collected from Nagano prefecture, Japan. The other seven strains, NUH219, NUH500, NUH501, NUH502, NUH503, NUH504, and NUH505, were isolated from soils in the same prefecture, using dilution plates treated at 65°C for 30 min. The taxon of each strain was identified by its microscopic features and close matching of their internal transcribed spacer sequences in a BLAST search. Based on these results, EF1393, EF1433, NUH500, NUH501, NUH504 were identified as *L. verrucosa*, and EF1561, NUH219, NUH502, NUH503, NUH505 were identified as *L. minima*. EF1421 and EF1565 were treated as unidentified *Leohumicola* spp. because of non-sporulation and <98% identity with *L. minima* sequences. All fungal cultures used in this study have been deposited at the School of Pharmacy, Nihon University, and the determined sequences were deposited in the DNA Data Bank of Japan (DDBJ) (LC505462-LC505473).

Synthesis experiment

Sterile blueberry seedlings were obtained from seeds of open-pollinated rabbiteye blueberry (*Vaccinium virgatum* Ait.) according to Grelet et al. (2009) with slight modification. Seeds were surface sterilized for 15 min in 10% hydrogen peroxide and germinated on 0.8% sterile water agar under irradiation with 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16 h light (20°C)/8 h dark (20°C) cycle. Any contaminated agar plates were discarded. Approximately one month after germination, seedlings with fully expanded

Table 1. Detail information of *Leohumicola* strains used in this study

Strain	Taxon	Source	Locality	DDBJ no.	bp	Description	Total score	Query cover (%)	E value	Identity (%)	Accession
EF1393	<i>L. verrucosa</i>	<i>Rhododendron molle</i> subsp. <i>japonicum</i> root	Mt. Nekodake, 2000m, timberline	LC505462	927	<i>L. verrucosa</i> ITS region; from TYPE material	933	56	0	98.85	NR_121306.1
EF1421	<i>L. sp. 1</i>	<i>Rhododendron molle</i> subsp. <i>japonicum</i> root	Mt. Nekodake, 2000m, timberline	LC505463	498	<i>L. minima</i> ITS region; from TYPE material	789	93	0	97.02	NR_121307.1
EF1433	<i>L. verrucosa</i>	<i>Rhododendron molle</i> subsp. <i>japonicum</i> root	Mt. Nekodake, 1900m, <i>Betula ermanii</i> forest	LC505464	918	<i>L. verrucosa</i> ITS region; from TYPE material	915	56	0	98.28	NR_121306.1
EF1561	<i>L. minima</i>	<i>Rhododendron molle</i> subsp. <i>japonicum</i> root	Mt. Nekodake, 1500m, grass pasture	LC505465	932	<i>L. minima</i> ITS region; from TYPE material	920	55	0	98.47	NR_121307.1
EF1565	<i>L. sp. 2</i>	<i>Rhododendron molle</i> subsp. <i>japonicum</i> root	Mt. Nekodake, 1500m, grass pasture	LC505466	917	<i>L. minima</i> ITS region; from TYPE material	824	56	0	95.23	NR_121307.1
NUH219	<i>L. minima</i>	A horizon soil	Sugadaira, 1351m, <i>Miscanthus sinensis</i> grassland	LC505470	532	<i>L. minima</i> ITS region; from TYPE material	937	95	0	99.8	NR_121307.1
NUH500	<i>L. verrucosa</i>	A horizon soil	Mt. Nekodake, 1667m, <i>Betula platyphylla</i> forest	LC505469	529	<i>L. verrucosa</i> ITS region; from TYPE material	891	96	0	98.24	NR_121306.1
NUH501	<i>L. verrucosa</i>	A horizon soil	Mt. Nekodake, 1667m, <i>Betula platyphylla</i> forest	LC505467	921	<i>L. verrucosa</i> ITS region; from TYPE material	933	56	0	98.85	NR_121306.1
NUH502	<i>L. minima</i>	A horizon soil	Mt. Nekodake, 1667m, <i>Betula platyphylla</i> forest	LC505468	926	<i>L. minima</i> ITS region; from TYPE material	909	56	0	98.09	NR_121307.1
NUH503	<i>L. minima</i>	A horizon soil	Sugadaira, 1351m, <i>Miscanthus sinensis</i> grassland	LC505472	529	<i>L. minima</i> ITS region; from TYPE material	933	96	0	99.8	NR_121307.1
NUH504	<i>L. verrucosa</i>	A horizon soil	Mt. Nekodake, 1667m, <i>Betula platyphylla</i> forest	LC505473	922	<i>L. verrucosa</i> ITS region; from TYPE material	941	56	0	99.05	NR_121306.1
NUH505	<i>L. minima</i>	A horizon soil	Sugadaira, 1351m, <i>Miscanthus sinensis</i> grassland	LC505471	529	<i>L. minima</i> ITS region; from TYPE material	933	96	0	99.8	NR_121307.1

cotyledons possessing one or two true leaves, were transferred to flasks containing each fungal strain pre-cultured as described below.

A 100 mL flask containing 20 mL cornmeal agar (Nissui, Tokyo, Japan) was inoculated with approximately $5 \times 5 \text{ mm}^2$ plugs cut from the edge of each fungal colony on a preincubated cornmeal agar plate. Each strain was cultured for 2 weeks in the dark at 25°C. Colonies were completely covered with approximately 20 mL an autoclaved mixtures of peatmoss and Kanuma soil (3:1, v:v). Two sterile seedlings were subsequently introduced into the medium, and culture continued under irradiation of $150 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and 14.5 h light (25°C)/9.5 h dark (20°C) condition. After two to three months, the seedlings were gently pulled off and cleaned with distilled water. Fine root systems of seedlings were subjected to further observations.

Observation of hyphal structures in roots

We observed the rabbiteye blueberry roots by using vital and DAB staining procedures recently developed by Grunewaldt-Stöcker and von Alten (2016) and Kobae and Ohtomo (2016), respectively, with slight modification.

Vital staining using FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl- (benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide] was originally developed for viability test for *Saccharomyces cerevisiae* (Millard et al. 1997), and extended its application to root cells with fungi by Grunewaldt-Stöcker and von Alten (2016). FUN-1 fluoresces when it binds with certain biomolecules such as peptides, proteins, or nucleic acid and excited with light between 470 to 590 nm (Millard et al. 1997, Molecular Probes 2001). This response itself does not require involvement of life activity, however, loading of FUN-1 into cells and vacuoles shapes distinguishable fluorescence structures such as orange-red CIVS (cylindrical intravacuolar structures) and green nuclei (Millard et al. 1997, Grunewaldt-Stöcker and von Alten 2016). In addition, formation of CIVS is known to require adenosine triphosphate (Millard et al. 1997). We employed this method as an easy screening of cells with different activity. Non-fixed fine root systems were stained with FUN 1 cell stain (F-7030, Probes, Thermo Fisher Scientific, Waltham, MA, USA; 25- μM working solution in distilled water) for at least 15 min in the dark. The stained roots were subsequently rinsed in distilled water. Small portions containing several root branches were excised, mounted with distilled water, and observed under an epifluorescence microscope (Nikon Eclipse Ni, Nikon, Tokyo, Japan) equipped with a digital

camera (DS-Fi2, Nikon) and a mercury lamp (Intensilight C-HGFI, Nikon). The fine roots were also screened under bright field. Portions with intracellular hyphae were further observed under epifluorescence through Texas Red (excitation, 560/40; dichroic mirror, 595; barrier, 630/60) and FITC (excitation, 480/30; dichroic mirror, 505; barrier, 535/45) filter cubes. The host cell vitality was confirmed by the presence of formed cylindrical intravacuolar structures (CIVS) and/or fluorescent plant nuclei. To calculate the frequency of vital host cells, several lateral roots with colonization signs per root system were randomly selected irrespective their length. Different association state of each observable coil-having cell within a root segment of a few hundred micrometers in the middle of each lateral was determined. Effect of species or sources of fungal strains on the frequency was tested by analysis of variance adopted for binomial generalized linear mixed model with a random effect of strain.

DAB staining was established for high contrast imaging of arbuscular mycorrhizal fungi under bright field condition, and is aimed at clear observation of hyphae morphology by staining *N*-acetylglucosamine within fungal cell walls. In this method, unstained roots were fixed in 70% ethanol solution. Fixed samples were cleared in 10% KOH for 24 h at 60°C, further cleared in 3% HCl for several minutes at room temperature, and rinsed twice with phosphate buffered saline (PBS; pH 7.4). The roots were soaked in 5 ml PBS containing 1% (w/v) bovine serum albumin (Wako, Osaka, Japan) and $0.4 \mu\text{g ml}^{-1}$ WGA (wheat germ agglutinin)-conjugated HRP (horseradish peroxidase) (Vector, Burlingame, CA, USA) for more than 16 h at room temperature. After rinsing twice with PBS, roots were immersed in 5 ml PBS containing 0.2 mg ml^{-1} 3,3'-DAB (diaminobenzidine) tetrahydrochloride (Nakarai Tesque, Kyoto, Japan) and $0.1 \mu\text{l ml}^{-1}$ of 30% hydrogen peroxide. The root samples were kept in DAB solution for at least 1 h at room temperature before the stain was replaced with Tris-ethylenediaminetetraacetic acid buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid; pH 8.0) to terminate HRP reaction. The stained roots were mounted with 50% glycerol and observed by bright field microscopy (the above one or BX50, Olympus, Tokyo, Japan).

Results and Discussion

All the *Leohumicola* strains formed coils and colonized in both vital and non-vital host rhizodermal cells (Table 2). Colonization of NUH502 is representatively shown in Fig. 1.

Table 2. Frequency of *Leohumicola* spp. strains within different states of association (I to IV) and vitality of colonized cells

Strain	Taxon	Source	Number of observed root segments	Count of colonized cells per association					Frequency of vital cell (%)
				I	II	III	IV	Total	
EF1393	<i>L. verrucosa</i>	Root	10	16	8	23	19	66	36.4
EF1421	<i>L. sp. 1</i>	Root	14	9	3	12	7	31	38.7
EF1433	<i>L. verrucosa</i>	Root	8	27	6	8	7	48	68.8
EF1561	<i>L. minima</i>	Root	14	7	3	40	6	56	17.9
EF1565	<i>L. sp. 2</i>	Root	14	4	8	7	40	59	20.3
NUH219	<i>L. minima</i>	Soil	7	25	12	12	21	70	52.9
NUH500	<i>L. verrucosa</i>	Soil	11	16	16	4	46	82	39.0
NUH501	<i>L. verrucosa</i>	Soil	10	31	37	3	14	85	80.0
NUH502	<i>L. minima</i>	Soil	9	14	2	7	32	55	29.1
NUH503	<i>L. minima</i>	Soil	9	22	3	1	8	34	73.5
NUH504	<i>L. verrucosa</i>	Soil	13	25	9	8	36	78	43.6
NUH505	<i>L. minima</i>	Soil	15	3	2	20	59	84	6.0

Species or source did not significantly affect the frequency of vital cell ($P < 0.05$).

Fig. 1. Representative bright field (a, d, g, j) and epifluorescence images (taken through Texas Red (excitation, 560/40; dichroic mirror, 595; barrier, 630/60. (b, e, h, k)) and FITC (excitation, 480/30; dichroic mirror, 505; barrier, 535/45 (c, f, i, l)) filter cubes) of four different states in rhizodermal cells of *Vaccinium virgatum* seedlings with NUH502 hyphal coils. a, b, c Images of state (1) both a strongly fluorescent plant CIVS and nucleus are obvious. d, e, f Images of state (2) plant CIVS are hardly observed and the plant nucleus are weakly fluorescent. g, h, i Images of state (3) no plant nucleus and CIVS are not confirmed but many fungal nuclei show fluorescence. j, k, l Images of state (4) no fluorescent structures are found. Host plant cells were considered vital in states (1) and (2) but non-vital in states (3) and (4). Each asterisk indicates one of CIVS of host plant cells. Each alphabet, n_p and n_f , indicate one of plant or fungal nuclei. White bar is 20 μ m.

Although our study is based on few plants per strain with relatively small number of root segments, this result implies the genus-level characteristics of *Leohumicola* to colonize vital ericaceous rhizodermal cells. This vital association is also a clear evidence for endophytism and should support mycorrhizal status in *Leohumicola* spp. The mycorrhizal status of *Leohumicola* may be further indicated by the similarities in colonization dynamics with other ErMF (Bonfante-Fasolo et al. 1981, Grunewaldt-Stöcker and von Alten 2016). Intracellular colonization of our *Leohumicola* strains had at least four different states (Fig. 1): (1) presence of strongly fluorescent plant nuclei and CIVS (Fig. 1a, b, c), (2) hardly observed plant CIVS and weakly fluorescent plant nuclei (Fig. 1d, e, f), (3) no plant nucleus or CIVS confirmed, but many fungal nuclei fluorescent (Fig. 1g, h, i), and (4) no fluorescent structures found (Fig. 1j, k, l). Host plant cells were considered vital in states (1) and (2) but non-vital in states (3) and (4). There was variation of the vitality of host cells with coils among strains, which was not affected significantly by the species and sources of strains. In (1) and (2), the coils were often comparatively loose and sometimes a few fluorescent fungal nuclei were detected. Compared to (1) and (2), hyphal coils were denser and dark-colored in (3) and (4). Most of plant CIVS were spherical rather than cylindrical, which perhaps related to time course of observation (Millard et al. 1997, Grunewaldt-Stöcker and von Alten 2016). No fungal CIVS could be found possibly because of the FUN 1 cell stain concentration. These four states were summarized as a schematic diagram with their possible temporal relation (Fig. 2). The four states are analogous to observations for *Hyaloscypha hepaticicola* and *Oidiodendron maius* studied by Grunewaldt-Stöcker and von Alten (2016). The authors illustrated possible continuous changes in

which fluorescent plant nuclei and CIVS were present in vital host cells with incomplete to normal fungal coils. However, plant CIVS were often absent in intensely colonized host cells, and host cells without vital signs were marked by green fungal nuclei. Using transmission electron micrographs, Bonfante-Fasolo et al. (1981) also showed corresponding changes during hyphal colonization by unidentified mycorrhizal fungi with wild *V. myrtillus* L. hair roots. In that study, at least three different states of host cells were distinguished a plant nucleus surrounded by highly vacuolated fungal hyphae, living hyphae with nuclei and vacuoles containing electron-dense granules accompanied by disorganized host cytoplasm, and an empty host cell containing empty and collapsed hyphae. Although comparisons of cell states between these studies need to be made carefully due to differences of the methods and samples, the affinity of colonization dynamics may indicate similarity in the interaction process throughout the lifespan of each coil between *Leohumicola* spp. and other ErMF.

DAB staining highlighted extraradical, intercellular, and intracellular hyphae of all the *Leohumicola* strains (Fig. 3, 4). As seen in the vital staining samples, the coils of all strains studied resembled those of typical ErMF, *H. hepaticicola* and *O. maius* (e.g. Martino et al. 2007, Vohník et al. 2016, Wei et al. 2016, Fehrer et al. 2019). In general, EF1393, EF1561, and NUH502 frequently formed typical dense coils that often completely filled the host cells (Fig. 3a, d, i). Compared to these three strains, EF1433, NUH501, and NUH504 formed relatively sparse and more amorphous coils (Fig. 3c, h, k). Coils of EF1421, EF1565, NUH219, NUH500, NUH503, and NUH505 showed an intermediate morphology, resembling the former three strains more than the latter three strains with

Fig. 2. Schematic diagram of different states of host rhizodermal cells colonized by *Leohumicola* hyphal coils corresponding Fig. 1. Vitality of host cells or hyphal coils is indicated by + (vital) or - (non-vital).

Fig. 3. Coils formed by *Leohumicola* strains, EF1393 (a), EF1421 (b), EF1433 (c), EF1561 (d), EF1565 (e), NUH219 (f), NUH500 (g), NUH501 (h), NUH502 (i), NUH503 (j), NUH504 (k), and NUH505 (l) stained by WGA-HRP and DAB. Black bar is 20 μm.

Fig. 4. Characteristic structures and staining state of *Leohumicola* strains. (a) Unusual swollen intracellular hyphal complexes of NUH505 strain. Asterisk indicates one of them. (b) Normal coil (C_n) and sparse coil-like structure (C_s) of NUH502 strains. (c) Heterogeneity in staining intensity in coils of EF1421 strain. Black bar is 20 μ m.

their highly dense hyphae and full occupation of host cells (Fig. 3b, e, f, g, j, l). In addition to coil formation, Grunewaldt-Stöcker et al. (2013) reported that several *Leohumicola* isolates formed dark microsclerotia-like cells that were closely packed in the basal region of emerging lateral roots. In our strains, EF1393, NUH219, NUH500, and NUH505 occasionally formed similar structures and constructed unusually swollen hyphae, although they retained more coil like forms (Fig. 4a). Most of our strains additionally formed net or loop-like intracellular structures (Fig. 4b). These indeterminate hyphal complexes were easily found in strains forming dense and fully expanded coils (e.g. EF1393), but somewhat ambiguous in strains forming coils with many gaps (e.g. EF1433). Considering that similar sparse structures were found with vital and non-vital host cells in vital staining samples, the developmental connection of these structures with normal coils is unclear. In addition, as the above observations, we did not confirm certain morphological traits that were shared within each species (data not shown). Detail

morphological features of coil should be examined with their phylogenetic and functional differences in future studies.

Regarding to DAB staining, staining states of our *Leohumicola* strains seemed to be more heterogeneous than in arbuscular mycorrhizal fungi (Kobae and Ohtomo 2016). Intracellular coils showed various staining intensity, their color ranged from very light brown to dark brown (Fig. 4c). There were also non-uniformly stained coils, for example, the enter points of hyphae were stained intensely in many coils. Extracellular hyphae also tended to be strongly stained. Although our sample preparation may have enhanced staining heterogeneity, for instance, through deacetylation due to extended KOH incubation and additional HCl immersion, it is likely that these staining states reflected various developmental and functional states of hyphae related to *N*-acetylglucosamine distribution in the cell wall and/or presence of specific obstacles to the staining. As seen in arbuscular mycorrhizal fungi whose chitin chains are fewer and more fragmented in arbuscules than

other hyphae and spores (Bonfante-Fasolo, 1988), our *Leohumicola* strains may have a highly heterogeneous chitin and an *N*-acetylglucosamine enrichment pattern. Otherwise, weakly stained coils may have been covered by certain substances, which impaired permeability of WGA-HRP and/or DAB. For example, extracellular sugar residues produced by extraradical hyphae of the infective *H. hepaticicola* strains probably blocked WGA-fluorescein isothiocyanate access to the longitudinal cell wall (Bonfante-Fasolo and Gianinazzi-Pearson 1982, Bonfante-Fasolo and Perotto 1986, Bonfante-Fasolo et al. 1987). As the referenced studies carefully confirmed the stained states, the mechanism by which the stained state of *Leohumicola* fungi appears needs to be carefully examined. Because such a staining pattern in target-specific staining can provide further insights into functional dynamics in the lifecycle of individual coils, as well as the possibility of continuous states observation in vital staining, effectiveness of DAB staining should be compared among ErMF and relative endophytes in the future. Finally, despite such heterogeneous staining pattern and its disadvantage of hiding melanized cells, the DAB staining provided a clear image of the hyphae of *Leohumicola* (Fig. 3, 4). Therefore, this technique is expected to be an effective observation method for at least *in vitro* sample of ErMF and other endophytes.

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