Original Research Article

Geomyces species, a fungal endophyte, promotes the growth of honeysuckle (*Lonicera caerulea*) through symbiosis

ABSTRACT

Honeysuckle (*Lonicera caerulea*), also known as "haskap" in Japan, is a shrub that produces edible berries and inhabits mountainous and wetland areas with harsh environments. In this study, we surveyed the relationship between *L. caerulea* and root endophytic fungi. Several endophytic fungal strains including *Geomyces* species were isolated from the roots of *L. caerulea* plants growing in wetland regions in Hokkaido, Japan. Inoculation tests revealed that *Geomyces* sp. promotes the growth of *L. caerulea* seedlings and colonizes the epidermal and cortical cells of roots, suggesting that *Geomyces* sp. acts as a mycorrhiza-like fungus for the arbuscular mycorrhizal plant. We speculate that *L. caerulea* establishes a symbiotic relationship with endophytic fungi to overcome acidic and nutrient deficient environments. This is the first report demonstrating that endophytic ascomycetes promote the growth of host plants belonging to the *Caprifoliaceae* family.

Keywords: Caprifoliaceae, endophytic fungi, ericoid mycorrhiza, PGPF, root endophyte

1. INTRODUCTION

The genus *Lonicera* (*Caprifoliaceae*) contains more than 200 species, many of which are useful; for example, *L. caerulea* produces edible fruits, and *L. confusa* and *L. japonica* are used as medicinal plants. *L. caerulea* is distributed in North America, northern Eurasia, and Japan [1], and is commonly known as "haskap" in Japan and blue honeysuckle in other countries. "Kurominouguisukagura " (*L. caerulea* subsp. *edulis* var. *emphyllocalyx*) and "keyonomi" (*L. caerulea* subsp. *edulis* var. *edulis*) grow wild in Hokkaido, Japan [2], and hereafter are referred to as haskap. Haskap is indigenous to alpine and marshy areas in Hokkaido, and forms large colonies in the Yufutsu wilderness [3,4].

Symbiosis with soil fungi has an important effect on plant growth under stress conditions. In wetlands, acidic peat soils are formed by the accumulation of undecomposed plant remains under overly humid conditions, and have adverse effects on plants because of the high concentrations of metal elements (such as aluminum and manganese) and hydrogen ions and the formation of nutrient-poor oligotrophic environment [5-7]. Plants adapt to stress conditions by employing various strategies, one of which is mycorrhizal symbiosis. For example, in acidic and oligotrophic soils, Ericaceae plants establish symbiotic relationship with ericoid mycorrhizal (ERM) fungi [8,9], such as *Rhizoscyphus ericae*, *Meliniomyces variabilis*, *Cadophora finlandia*, and other ascomycetes [10], to overcome harsh environmental conditions. Ericaceae plants have also been reported to associate with ectomycorrhizal (ECM) fungi, dark septate endophytes (DSE), and saprophytic basidiomycetes and ascomycetes; however, how these plants develop symbiotic relationships with and are affected by each of these fungal groups has not been elucidated [11-13]. Nevertheless, in recent years, it has been suggested that various endophytes improve plant stress tolerance and influence vegetation formation [14-18].

Currently, research is being actively conducted on the utilization of endophytes to promote host plant growth, enhance greening, and improve the environmental stress tolerance of crop plants. Recently, several studies reported the relationship between useful fungal strains and specific plant species [18-20]. Narisawa and colleagues showed that unlike arbuscular mycorrhizal (AM) fungi, the ascomycete endophytes are easy to isolate and cultivate and have a wide host range [19]. Since the impact of endophytes on the host varies greatly with the growth environment and host [21,22], inoculation tests are essential for elucidating the symbiotic relationship between specific plant species and endophytes. However, to elucidate the ecological function of endophytes, tests under various conditions are required, and since only a limited number of plant species have been used for inoculation tests so far, the effect of endophytes on host plant species remains largely unknown.

Information on fungal symbiosis with Caprifoliaceae plants, especially the genus *Lonicera*, is limited to AM fungi. Inoculation of *L. confusa* and *L. japonica* with AM fungi has been attempted to promote their use as medicinal herbs and to facilitate the revegetation of contaminated soils, mainly in China [23,24]. In Japan, Ahulu et al. reported that Lonicera morrowii, native to coastal dunes, forms an AM structure, which is intermediate between the Arum- and Paris-type of arbuscular mycorrhizas [25]. To date, there has been no report on symbiosis with endophytes in *L. caerulea*, which is mainly found in alpine and wetland habitats. In this study, we isolated endophytes from *L. caerulea* growing in the Yufutsu field, the largest habitat of *L. caerulea* in Hokkaido, and found a species closely related to *Geomyces auratus*, which is ecologically and phylogenetically related to the genus *Oidiodendron*. *Geomyces* spp., commonly known as saprophytic soil fungi, have been isolated from *Ericaceae* plants in several studies but have rarely been used to perform inoculation tests [26-29]. Since Ericaceae plants also coexist with soil fungi in the native habitat and may benefit from association with similar endophytes [30,31], we conducted inoculation tests on honeysuckle and cowberry (*Vaccinium vitis-idaea*; Ericaceae). We also observed the behaviour of each host–endophyte symbiotic interaction, and investigated the contribution of endophytes to host plant growth.

2. MATERIAL AND METHODS

This study was conducted in 2017 at the Benten marsh located in Tomakomai City, Hokkaido, Japan (42°38'32.2"N, 141°45'33.9"E). Fine roots of wild haskap plants without disease symptoms as well as soil surrounding six haskap plants were sampled from the study site and brought back to the laboratory in plastic bags. The soil samples were allowed to air dry at room temperature for one week. Then, 25 ml of distilled water was added to 10 g of air-dried soil, and the sample was shaken for 1 h. The resulting soil suspension was used to measure the soil pH.

The fine root samples were washed with tap water to remove loose soil and other debris. The washed roots were transferred to a plastic bag containing a small amount of distilled water and stored in a refrigerator (4 °C). To isolate associated endophytes, the fine root samples were sterilized by soaking in 70% ethanol for 1 min 30 s and then in NaClO solution (1% effective chlorine) for 1 min 30 s on a clean bench. The sterilized root samples were rinsed with sterilized water three times for 3 min each. The rootlets were cut into 1 cm pieces on a sterilized filter paper and placed on 1.5% water-agar medium in plastic Petri dishes (9 cm diameter). The Petri dishes were incubated in the dark at 25 °C. Only mycelia that grew after 1 week were transferred to oatmeal–agar medium (18 g agar, 10 g oatmeal, 1.5 g KH_2PO_4, 1.0 g $NaNO_3$ and 1.0 g $MgSO_4 \cdot 7H2O$ in 1 L of distilled water). The isolates were used to inoculate Chinese cabbage sprouts. Briefly, 100 mL of oatmeal-agar medium was dispensed into a plastic square Petri dish, and after allowing a mycelial mat to form, a sterile sprout was placed on the medium. Non-pathogenic isolates, i.e., those that did not cause

leaf yellowing, stem browning, or reduction in the size of aboveground parts, were identified as endophytes.

Different endophyte species were identified by sequencing the nuclear rDNA internal transcribed spacer (ITS) region including ITS1-5.8S-ITS2 ITS1. DNA was isolated from mycelia growing on oatmeal agar using UniversALL^{TM} Extraction buffer II (NIPPON GENE, Tokyo, JAPAN), according to the manufacturer's instructions. PCR was performed in a 27-μL reaction volume, containing 0.5 μL of forward primer (ITS1F; [32]), 0.5 μL of reverse primer (ITS4; [33]), 12.5 μL Gene RED PCR Mix Plus (NIPPON GENE, Tokyo, JAPAN), 11.5 μL of sterilized water, and 2 μL of template DNA, under the following cycling parameters: initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 20 s, annealing at 48°C for 20 s, and extension at 72°C for 5 s, and a final extension at 72°C for 7 min. The PCR products were sequenced by MACROGEN JAPAN (Tokyo, JAPAN). Briefly, 10 μl of each sample was mixed with 5 μl of each primer, and after sequencing reaction and purification of the products, the sequences were analyzed using an Applied Biosystems 3730/xl/DNA Analyzer (Thermo Fisher Scientific K.K., Tokyo, JAPAN). The deciphered sequences were deposited to the DNA Data Bank of Japan (DDBJ) and subjected to BLAST search for molecular identification.

To investigate the symbiotic potential of endophytes, an inoculation test of endophytes was conducted using haskap as a host on oatmeal-agar medium. Fruits of wild haskap plants were collected, and seeds were removed from the pulp. The removed seeds were wrapped in gauze, soaked in NaClO solution (1% effective chlorine) containing 0.5% Tween-20 for 10 min on a clean bench. The sterilized seeds were rinsed with sterilized water three times for 3 min each and sown on 1.5% water-agar medium [3]. The germinated seeds were transferred to Petri dishes containing half-cut oatmeal-agar medium covered with mycelia. Each Petri dish was sealed with Parafilm, and its lower half was covered with aluminum foil to protect from light. The Petri dishes were then incubated vertically at 25 °C, 16 h light/8 h dark photoperiod, and 600 lux (180 μmol/m2/s) for 14 wk. The Petri dishes were randomly rearranged once a week to ensure uniform light exposure. The survival rate, shoot length, leaf number, and total root length of seedlings were measured, and the ratio of shoot length to the total root length was calculated.

To further explore the symbiotic potential of endophytes, an inoculation test was conducted using cowberry as a host. Fruits were collected from commercially potted plants, and seeds were surface sterilized and sown as described above. The germinated seedlings were sequentially transferred to plastic Petri dishes (9 cm diameter) containing oatmeal-agar medium covered with endophytes. The Petri dishes were sealed with Parafilm and incubated under the conditions described above for 5 wk. Only the growth of the root parts was observed, and the shoot length was not measured.

To analyze the growth of cowberry root and their association with endophytes, seedlings were removed from the medium, and roots were rinsed with distilled water to wash off the agar medium. The morphology of roots and presence of root hairs were observed under a stereomicroscope. Intraroot mycelium was observed by staining. Briefly, the roots were immersed in 10% KOH and heated at 80 C for 1 h. Subsequently, the roots were rinsed with distilled water and soaked in 1% HCl at room temperature for 15 min. The roots were rinsed again with distilled water and then soaked in Chlorasol Black E solution (50 mL each of lactic acid, glycerol, and deionized water, and 0.15 g of Chlorasol Black E) at room temperature for 7 d. The stained specimens were observed under a compound microscope and photographed.

Statistical analysis was performed using Student's t-test. Differences between *Geomyces* sp.-inoculated and un-inoculated (control) samples with P-value < 0.05 were considered significant.

3. RESULTS

A total of 47 endophytic species were isolated from 644 haskap root fragments and classified into 14 taxa (Table 1). The results of inoculation tests showed that *Geomyces* sp. significantly increased the shoot length of haskap seedlings by more than 2-fold compared with the un-inoculated control (Fig. 1) but significantly decreased the total root length and shoot length/total root length ratio (Table 2).

Table 1. Endophytic fungal species isolated from haskap roots.

Putative species	No. of isolates	Accession No.	Closest match		
			Species name	Isolation source	Accession No.
Cladophialophora sp.	1	LC374639	*Clado chaetospira*	Soil	EU035406
Cryptosporiopsis sp.	3	LC180168*	*Pezicula ericae*	Ericaceae root	KR859174
Dothideomyctes sp.	2	LC374631*	Dothideomycetes sp.	Pinaceae root	KF973193
Geomyces sp.	1	LC374638	*Geomyces auratus*	Soil	MF106206
Helotiales sp. 1	1	LC180166	Helotiales sp.	Diapensiaceae root	AB598109
Helotiales sp. 2	3	LC374632*	Helotiales sp.	Ericaceae root	KX440125
Lachnum sp.	1	LC180192	*Lachnum* sp.	Ericaceae root	KJ817272
Leptodontidium sp.	14	LC180167*	*Leptodontidium* sp.	Ericaceae root	AB846993
Leotiomycetes sp.	1	LC374643	*Dactylaria appendiculata*	Ericaceae root)	KM580040
Oidiodendron sp. 1	1	LC180165	*Oidiodendron* sp.	Ericaceae root	AB847057
Oidiodendron sp. 2	2	LC180173*	*Oidiodendron* sp.	Diapensiaceae root	AB598107
Oidiodendron sp. 3	3	LC180176*	*Oidiodendron* sp.	Ericaceae root	AB847062
Phialocephala sp.	2	LC180190*	*Phialocephala* (unculured)	Ericaceae root	HF947841
Preussia sp.	1	LC374622	*Preussia funiculata*	Salicaceae root	GU934563
Rhizoscyphus sp. 1	2	LC180180*	*Rhizoscyphus* aff. *ericae*	Ericaceae root	AB847069
Rhizoscyphus sp. 2	3	LC374629*	*Rhizoscyphus* aff. *ericae*	Ericaceae root	AB847029
Sordariales sp.	1	LC374642	*Zopfiella tabulata*	Dung	AY999132
Sordariomycetes sp.	2	LC180175*	Sordariomycetes sp.	Ericaceae root	AB846990

Fungal species	1	LC374641	*Entrophospora infrequens*	Spore		U94714
Unidentified	2	-	-	-		-

*Representatives are shown.

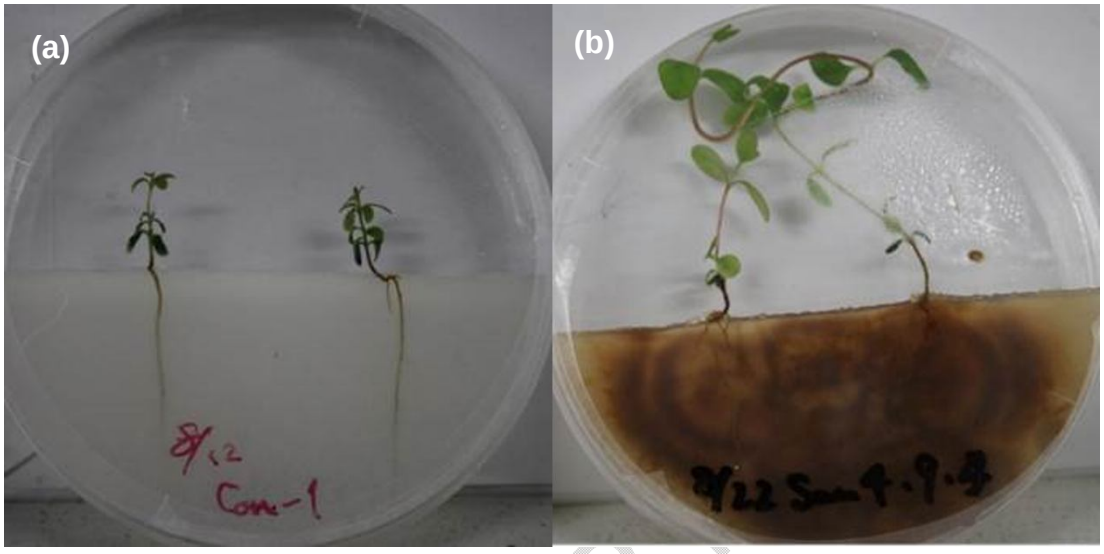


Fig. 1. Photographs of haskap seedlings grown with or without *Geomyces* sp. for 14 wk. (a) Control (un-inoculated) haskap seedlings. (b) Haskap seedlings inoculated with *Geomyces* sp.

Table 2. Measurements of *Geomyces*-inoculated and un-inoculated (control) haskap seedlings grown for 14 wk.

Treatment	Measurements (mean ± standard error)						
	Shoot length (mm)	No. of leaves	No. of root tips	Total root length (mm)	Shoot length/total root length	Survival rate (%)	No. of seedlings
Control	12.3 ±1.5 [a]	8.5 ±0.7 [a]	6.2 ±0.7 [a]	51.8 ±6.2 [a]	0.77±0.14 [a]	96.15	26
Geomyces sp.	29.3 ±6.6 [b]	7.9 ±1.3 [a]	6.3 ±1.0 [a]	38.6 ±7.0 [b]	0.28±0.03 [b]	70.00	20

The survival rate of haskap seedlings inoculated with *Geomyces* sp. was slightly lower than control seedlings (Fig. 2).

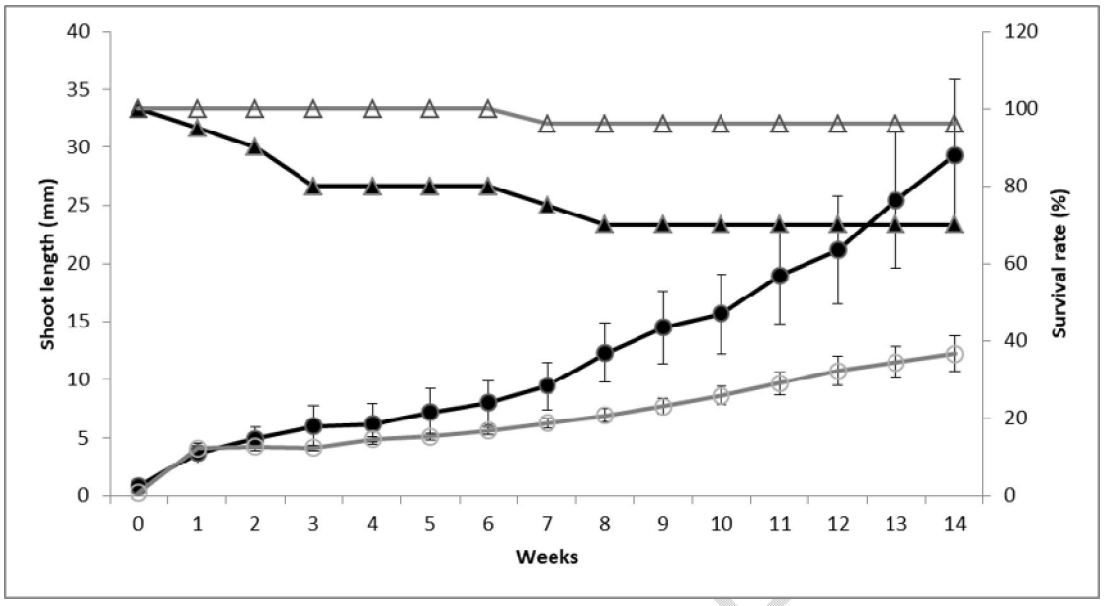
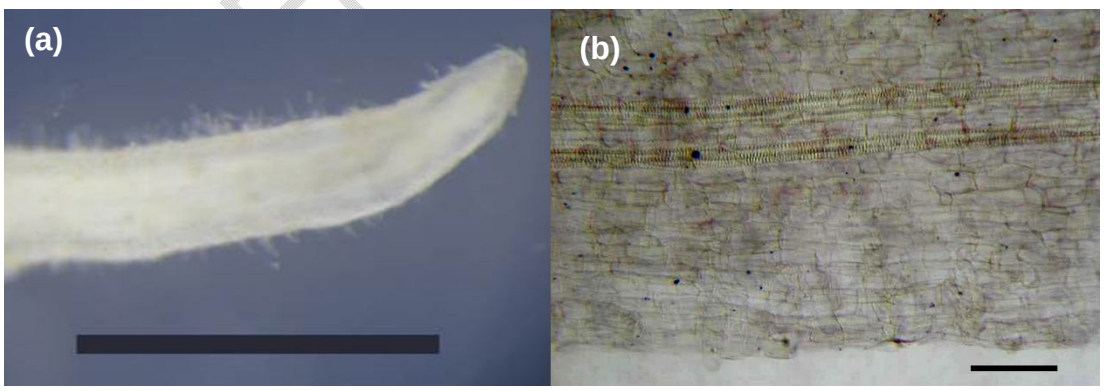


Fig. 2. Shoot growth and survival rate of haskap seedlings cultured with or without (control) *Geomyces* sp. for 14 wk. Open and filled circles represent the shoot length of control (un-inoculated) and *Geomyces*-inoculated seedlings, respectively (left axis). Open and filled triangles represent the survival rate of control (un-inoculated) and *Geomyces*-inoculated seedlings, respectively (right axis). Data represent mean ± standard error (SE; n = 26 control seedlings, 20 inoculated seedlings).

The roots of control seedlings produced root hairs (Fig. 3a) and showed no fungal infection (Fig. 3b). On the other hand, the roots of *Geomyces* sp.-treated seedlings were covered with mycelium. Staining of roots showed that the mycelium penetrated the epidermal cells and extended into the cortical cells (Fig. 3d). The intracellular mycelium of *Geomyces* sp. was not as clearly coiled as that of ERM, but fine mycelium randomly filled the cells. In addition, vesicle-like structures were observed both on the entire surface and interior of roots (Fig. 3d, arrow).



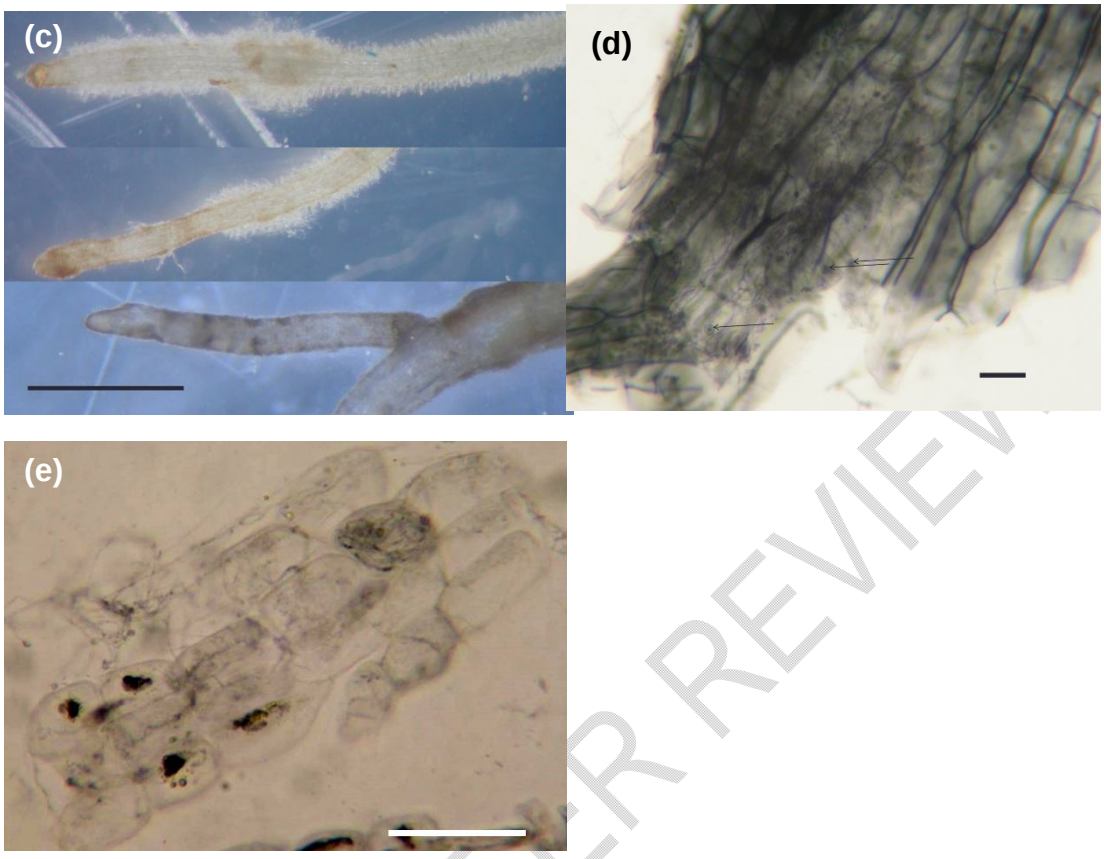


Fig. 3. Microscopic visualization of the roots of *Geomyces*-inoculated or un-inoculated (control) haskap and cowberry seedlings. (a, b) Root tip (a) and non-colonized root of control haskap seedlings grown aseptically for 14 weeks. (c, d) Root tips (c) and root (d) of haskap seedlings inoculated with *Geomyces* sp. and grown for 14 weeks. The image in (d) shows intracellular colonization by *Geomyces* sp., and the arrow indicates a small vesicle in the haskap root. (e) Intracellular colonization by *Geomyces* sp. in cowberry (Vaccinium vitis-idaea) root. Scale bars: 1 cm (a); 50 µm (b, e); 1 mm (c); 20 µm (d).

The results of inoculation tests performed using cowberry seedlings were similar to those obtained with haskap. *Geomyces* sp. formed intracellular mycelium in the roots (Fig. 3e). The typical intracellular hyphal coil of ERM was not observed, and fine mycelium randomly filled the cells. In addition, vesicular structures were observed both on the entire surface and interior of roots.

4. DISCUSSION

In this study, the plant growth-promoting ability of *Geomyces* sp. was demonstrated for the first time using haskap as a host. *Caprifoliaceae* plants are known to associate with AM fungi, and the genus *Lonicera* has been reported to develop a structure, which is intermediate between the Arum- and Paris-type of arbuscular mycorrhizas. Additionally, symbiosis with AM fungi has been shown to alleviate cadmium (Cd) toxicity and improve nutrient uptake in acidic soil [23-25]. Except for AM fungi, no endophytes have been reported to contribute to the growth of *Caprifoliaceae* hosts. Although Dalpe (1989) [26] (as *Pseudogymnoascus roseus*) and Vohnik et al. (2007) [12] inoculated *Ericaceae* plants with

Geomyces spp., to the best of our knowledge, no growth promoting effects were reported in these studies.

The genus *Geomyces* contains soil-dwelling saprophytic, and possibly endophytic, fungi [27,29,34], which exhibit high organic matter degradation capacity [35]. The endophytic *Geomyces* sp. has been isolated from the roots of Rhododendron spp. [36-39] as well as from the rhizosphere of plants growing in peat soils [35]. The genus *Geomyces* is an anamorph, but teleomorphs have been found in the genera *Pseudogymnoascus* and *Gymnostellatospora* (*Myxotrichaceae*) [40], and *P. roseus*, known as a teleomorph of *Gymnostellatospora auratus*, has been reported to associate with ERM fungi [26]. In addition, the genus *Geomyces* was previously considered representative of ERM fungi because *Myxotrichaceae* included the genus *Oidiodendron*. However, recent molecular phylogenetic analysis suggested that the genus *Oidiodendron* should be transferred to the *Leotiomycetes* class, and the genera *Oidiodendron*, *Pseudogymnoascus*, *Gymnostellatospora*, and *Geomyces* were shown to be phylogenetically distinct [41,42]. Thus, unlike the belief held in 1980s, a phylogenetic relationship between the genera *Oidiodendron* and *Geomyces* is highly unlikely. Nonetheless, given that *Geomyces pannorum* is a potential ERM fungus, based on multiple isolate reports, its symbiotic association with and impact on *Ericaceae* plants cannot be ignored [28,37,38]. In the current study, the growth-promoting effect of *Geomyces* sp. on haskap seedlings was probably facilitated by nutrient absorption. In the present inoculation study, control plants continued to grow throughout the incubation period, suggesting that the methodology was appropriate (Fig. 1). Intracellular mycelium, which extended from the epidermal cells to the interior of the vascular bundle in *Geomyces*-inoculated haskap seedlings, was also observed when cowberry, an ERM host, was inoculated with *Geomyces* sp. In addition, a large amount of mycelium was observed on the root surface, and vesicular structures were observed on the entire root surface and interior of roots. The intracellular mycelium formed by ERM and arbutoid mycorrhizal (ARM) fungi is considered a site of direct plant–fungus nutrient exchange [43]. The extraradical mycelium possibly expands the range of nutrient absorption available to the host. In addition, in this study, *Geomyces* sp. inhibited the growth of host roots and altered the shoot/root ratio. These results suggest that *Geomyces* sp. expands the nutrient uptake range of the host by diverting nutrients from the underground to the aboveground where they can be invested in plant growth. Vohnik et al. (2012) reported that the growth of blueberry seedlings was higher when they were co-cultured with the saprophytic fungus *Agrocybe praecox* than when they were co-cultured with the control [13]. The authors attributed this result to the rapid decomposition of organic matter in the medium by *A. praecox*. *Geomyces* spp. are known for their keratinophilic and psychrophilic properties and are capable of producing a wide variety of enzymes that perform excellent degradation in cold and polar regions [29]. In the current study, we added nutrients, including phosphate (PO_4-) and nitrate (NO_3-) to the culture medium. Therefore, it is not clear whether *Geomyces* sp. could supply nutrients not available to the host. However, in the peat soil rhizosphere, *Geomyces* sp. can decompose organic matter via its high organic matter degradation ability, and some of the released nutrients may be used by plants. Since the intracellular mycelium observed in the roots of haskap and cowberry is similar to the intracellular loop mycelium reported by Vohnik et al (2007) [28], *Geomyces* sp. may be a potential ERM fungus. ERM fungi have been reported to establish symbiotic association with and promote the growth of non-ERM hosts [12,30,31,36]. In addition, haskap has been observed to coexist with many *Ericaceae* plants in its native habitat. It is possible that haskap plants establish a symbiotic relationship with *Geomyces* sp., as shown in this study, to adapt to the harsh environment of marsh-derived oligotrophic peat soil. However, Vohnik et al. (2007) reported that the symbiotic status of *Geomyces* spp. changes between agar medium and soil, and the effect of endophytes on the host can be altered by mimicking an oligotrophic environment, i.e., by supplementing the growth medium with an organic source of nitrogen rather than with NO_3-

[28]. Therefore, soil inoculation tests are necessary to examine the symbiotic property of *Geomyces* sp.

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