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SHORT NOTE

Maria Catarina Megumi Kasuya · Tsuneo Igarashi

In vitro ectomycorrhizal formation in *Picea glehnii* seedlings

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Abstract Twenty isolates of ectomycorrhizal fungi – 3 from *Picea glehnii*, 12 from other coniferous trees, and 5 from decidous trees – were tested for the ability to form mycorrhizae with *P. glehnii*, using an in vitro synthesis technique. Macroscopically, mycorrhizal formation was observed 3 months after inoculation, when the lateral roots began to grow. Mycelial growth was observed in all inoculated treatments, generally around and along the roots. Six months after inoculation, seedlings were harvested and the mycorrhizae were observed microscopically. Fourteen of the 20 isolates formed ectomycorrhizae with a dense sheath and a deep Hartig net; 1 formed ectendomycorrhizae with a rudimentary mantle, a well-developed Hartig net and intracellular hyphae; 3 formed pseudomycorrhizae with a mantle but without the Hartig net; and only 2 of the fungi tested, *Chalciporus pipeparatus* 5/92 and *Lyophyllum* sp. 61/92, did not form mycorrhizae at all. *P. glehnii* was a good host species since it had low specificity to ectomycorrhizal fungi isolated from trees other than *P. glehnii*.

Key words Ectendomycorrhizae · Ectomycorrhizae · Pseudomycorrhizae · In vitro synthesis · *Picea glehnii*

Introduction

Picea glehnii is one of the most important conifers of Hokkaido (Tatewaki 1958). It can be found occurring naturally in the east and north of the island growing mainly on serpentine soils, in volcanic sand and gravel areas, and in bogs. These areas are edaphically unfavorable to the growth of other tree species (Tatewaki 1958; Tatewaki and Igarashi 1971). Planting of *P. glehnii* was very sparse at first, but it has increased annually, and in 1993 the species comprised 28.3% of the planting area of Hokkaido, followed by *Abies sachalinensis* (28.1%) and *Larix kaempferii* (26.2%), two other tree species that were widely planted until 1992 (Anonymous 1994).

Under field conditions, Ogawa (1976) reported that *Tricholoma matsutake* (S. Ito and Imai) Singer was a mycorrhizal fungus of *P.glehnii* at Mt. Meakan, Hokkaido. Fruiting bodies of potential ectomycorrhizal fungi, not only of *Tricholoma* species, but also of *Amanita*, *Boletus*, *Cortinarius*, *Lepista*, *Russula*, and *Suillus*, have been observed in *P. glehnii* forests (Igarashi 1990, 1993; Takahashi 1991), but the mycorrhizal associations with these fungal species have not been verified. Kasuya and Igarashi (1994) observed five different ectomycorrhizae in seedlings of *P. glehnii* in a nursery. Subsequently, Kasuya et al. (1995) observed 34 types of ectomycorrhizae in seedlings of *P. glehnii* growing in a natural *P. glehnii* and *Abies sachalinensis* (Fr. Schm.) Masters mixed forest. Although these associations had been observed, the exact fungal species associated with this tree were not precisely determined. Compared with other forest species, the occurrence of basidiocarps of ectomycorrhizal fungi under *P. glehnii* trees is noticeably low (personal observations).

Therefore, considering the economical and ecological importance of *P. glehnii* for Hokkaido and since there is a scarcity of information on the ectomycorrhizae of *P. glehnii*, the ability of fungi collected from under different Hokkaido forest species to form ectomycorrhizae was tested with *P. glehnii* in pure culture.

Materials and methods

Twenty fungi were tested for their ability to form mycorrhizae with *P. glehnii*. Five of these fungi were isolated from basidiocarps (or mycorrhiza) collected from under deciduous trees, 14

M. C. M. Kasuya (✉)[1] · T. Igarashi
Faculty of Agriculture, Department of Forest Science,
Hokkaido University, Sapporo, 060, Japan

Present address:
[1] Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais, 36570–000, Brazil
fax: +55–31–899–2573

Table 1 Origin of the fungi used in this study. Fungi were isolated from basidiocarps collected in the forests of Hokkaido except where noted

Isolate	Fungal species	Forest tree species	Age of trees[b]	Soil type
Am 18/92	*Amanita porphyria* (Fr.) Secr.	*Abies sachalinensis* (Fr. Schm.) Masters.	Old	Brown forest
Ap 53/92	*Amanita pantherina* (DC.: Fr.) Krombh	*Abies sachalinensis* (Fr. Schm.) Masters.	Old	Brown forest
As 36/92	*Amanita spreta* (Peck) Sacc.	*Quercus mongolica* Fischer var. *grosseserrata* (Blume) Rehd. et Wils.	Young	Volcanic ash
Ar 13/92	*Amanita rubescens* Pers.: Fr.	*Abies sachalinensis* (Fr. Schm.) Masters.	Old	Brown forest
Bp 10/92	*Boletus pulverulentus* Opat.	*Picea abies* Karst.	Old	Brown forest
Cg 78/93[a]	*Cenococcum geophillum* Fr. (syn. *C. graniforme* (Sow) Ferd and Winge)	*Abies sachalinensis* (Fr. Schm.) Masters.	Mid	Weakly dried brown forest
Co 75/93	*Cortinarius vibratilis* (Fr.) Fr.	*Picea glehnii* (Fr. Schm.) Masters.	Mid	Weakly dried brown forest
Cp 5/92	*Chalciporus piperatus* (Bull.: Fr.) Bataile	*Abies sachalinensis* (Fr. Schm.) Masters.	Old	Brown forest
Ge 58/92	*Geastrum mirabile* (Mont.) Fisch.	*Picea glehnii* (Fr. Schm.) Masters.	Mid	Weakly dried brown forest
Le 65/92	*Lepista graveolens* (Peck) Murrill	*Quercus dentata* Thunb.	Old	Brown forest
Ln 59/92	*Lepista nuda* (Bull.: Fr.) Cooke	*Betula platyphylla* var. *japonica* Hana	Old	Brown forest
Ln 73/92	*Lepista nuda* (Bull.: Fr.) Cooke	*Pinus sylvestris* Linn. and *Pinus rigida* Mill. mixed forest	Young	Brown forest
Ls 84/93	*Lyophyllum shimeji* (Kawam.) Hongo	*Quercus mongolica* Fischer var. *grosseserrata* (Blume) Rehd. et Wils.	Mid	Weakly dried brown forest
Ly 61/92	*Lyophyllum* sp.	*Quercus mongolica* Fischer var. *grosseserrata* (Blume) Rehd. et Wils.	Old	Brown forest
Pa 60/92	*Paxillus* sp.	*Pinus strobus* Linn.	Old	Brown forest
Pt 1/92	*Pisolithus tinctorius* (Pers.)	*Pinus pumila* Regel and *Betula ermanii* Cham Coker and Couch mixed forest	Young	Volcanic ash
Sf 16/92	*Scleroderma flavidum* Ell. et Ell.	*Pinus resinosa* Ait.	Mid	Sandy
Sf 23/92	*Scleroderma flavidum* Ell. et Ell.	*Pinus resinosa* Ait.	Mid	Sandy
Sc 72/92	*Scleroderma* sp.	*Pinus sylvestris* Linn. and *Pinus rigida* Mill. mixed forest	Young	Brown forest
Su 77/93	*Suillus luteus* (L. Fr.) SF. Gay	*Picea glehnii* (Fr. Schm.) Masters.	Mid	Weakly dried brown forest

[a] Isolated from mycorrhiza
[b] *Young* 1–25 years, *Mid* 26–60 years, *Old* 60+ years

from under coniferous trees, and 1 from a stand containing both types of trees (Table 1). The dried specimens of isolated fungi basidiocarps were preserved and stored in the herbarium of the Laboratory of Sylviculture at Hokkaido University.

The in vitro synthesis technique used in this assay was that described by Kasuya et al. (1992), with minor modifications. First, 60 cm^3 of a mixture of vermiculite and peat moss (10:1 v/v) was added to the test tubes (200 × 30 mm) lined with filter paper (100 × 100 mm). The tubes were capped with a glass cup, and the space between the cup and tube wall was sealed with cotton. The tubes and their contents were autoclaved at 121 °C for 60 min. Afterwards, 40 ml of a previously autoclaved MMNb solution (Kottke et al. 1987) was added aseptically to each tube. Seeds of *P. glehnii* were surface sterilized with 30% H_2O_2 for 20 min (Molina and Palmer 1982), rinsed repeatedly with sterilized distilled water, and placed on sterile MMNb agar (Kottke et al. 1987) in Petri dishes to germinate. After 25 days, uncontaminated seedlings were aseptically transferred into test tubes, and the roots were placed between the wall and the paper inside the test tube. Inoculation with the desired ectomycorrhizal fungus (Table 1) was done 7 days after transplanting, by placing two mycelial disks (7 mm in diameter) taken from the margin of the colony, between the filter paper and the test tube wall close to the stem of the plant. Another mycelial disk, similarly prepared, was placed on the top of the vermiculite/peat moss mixture, also close to the stem. Tubes were then wrapped with aluminum foil up to the top of the substrate and placed in a growth chamber at 28 °C day and 14 °C night, under lights which provided an average of 1500 lx for 14 h/day. Five replications were used for each fungus.

Formation of mycorrhiza was evaluated 6 months after the inoculation. Intact seedlings were removed from the test tubes, and their roots were examined under a dissecting microscope. Selected root pieces, suspected to be mycorrhiza, were examined microscopically after to be freeze cutting (Agerer 1991) and when necessary stained using toluidine blue O. Sections were examined to ascertain whether the fungal sheath, Hartig net and intracellular hyphal penetration were present.

Results

Seedlings, including those from the non-inoculated control, grew at the same rate. Three months after inoculation, lateral roots had begun to grow out and mycorrhizal formation could be observed in some treatments. Six months after inoculation, mycelial growth was verified in all inoculated seedlings, generally around and along the root. Fourteen isolates of mycorrhizal fungi (70%), out of the 20 isolates collected and purified, formed ectomycorrhizae with 90–100% of colonization, while 3 (15%) formed pseudomycorrhizae, 1 (5%) formed ectendomycorrhizae and 2 (10%) did not form mycorrhizae (Table 2). Furthermore, many fungi origi-

Table 2 Characteristics of *Picea glehnii* ectomycorrhizae or root systems 6 months after inoculation with potentially ectomycorrhizal fungi in vitro (*Ih* intracellular hypha, *NM* nonmycorrhizal, *PM* pseudomycorrhizal, *EEM* ectendomycorrhizal, *EM* ectomycorrhizal)

Fungus code[a]	Characteristics[b]					Type
	Color	Ramification	Mantle	Hartig net	Ih	
Am 18/92	White/pale yellow	Simple	+	+	−	EM
Ap 53/92	White/pale yellow	Simple	+	+	−	EM
Ar 13/92	White	Simple	+	+	−	EM
As 36/92	White	Simple	+	+	−	EM
Bp 10/92	Pale yellow	Simple	+	−	−	PM
Cg 78/93	Black	Simple	+	+	−	EM
Co 75/93	Yellowish-brown	Simple	+	+	−	EM
Cp 5/92	Brownish-yellow	Simple	−	−	−	NM
Ge 58/92	Yellowish-brown	Simple	+	−	−	PM
Le 65/92	Yellowish-brown	Branched	+	+	−	EM
Ln 59/92	Brownish-yellow	Simple	+	+	−	EM
Ln 73/92	Pale yellow	Simple	+	+	+	EEM
Ls 84/93	Pale yellow	Simple	+	+	−	EM
Ly 61/92	Yellowish-brown	Simple	−	−	−	NM
Pa 60/92	Brown	Branched	+	+	−	EM
Pt 1/92	Brownish-yellow	Branched	+	+	−	EM
Sc 72/92	Yellow	Simple	+	+	−	EM
Sf 16/92	Pale yellow	Simple	+	+	−	EM
Sf 23/92	Pale yellow	Simple	+	+	−	EM
Su 77/93	Pale yellow	Simple	+	−	−	PM

[a] See Table 1 for scientific names of ectomycorrhizal fungi
[b] + Present, − absent

nally isolated from hosts other than *P. glehnii* (Tables 1, 2) successfully colonized this tree species.

Among the three fungi isolated from basidiocarps collected in *P. glehnii* forest (Table 1), only *Cortinarius vibratilis* (Co 75/93) formed ectomycorrhizae, while *Geastrum mirabile* (Ge 58/92) presented a rudimentary mantle and no Hartig net, and *Suillus* sp. (Su 77/93) presented a well-developed sheath but no Hartig net (Table 2). Therefore, *G. mirabile* (Ge 58/92) and *Suillus* sp. (Su 77/93) comprised the so-called pseudomycorrhizae as described by Molina (1981). Two isolates of *Lepista nuda* were collected, and the isolate from a mixed conifer forest (Ln 73/92) was capable of forming ectendomycorrhizae, while the isolate from a deciduous host (Ln 59/92) formed typical ectomycorrhizae (Table 1).

Discussion

Although in vitro synthesis studies tend to overestimate the symbiotic compatibility of a specific fungus and host combination under natural conditions, this system can demonstrate whether a given fungus is mycorrhizal or not with a well-characterized host plant (Molina and Palmer 1978; Peterson and Chakravarty 1991). Using such a technique here, more than 85% of the tested fungi were able to form mycorrhizae with *P. glehnii* alone (Table 2), regardless of the plant(s) with which the isolates were first associated (Table 1).

Fungi belonging to the genera *Lepista* and *Lyophyllum* have commonly been regarded solely as wood and litter decomposers. However, basidiocarps of these fungi are frequently found in forests, and some have speculated that they may be mycorrhizal (Hongo 1990; Ohta 1994). Basidiocarps of *Lepista nuda* have been found under *P. glehnii*, *Abies sachalinensis* or *Larix kaempferii* and sometimes under broad-leaved trees of Japan (Imazeki et al. 1988; Igarashi 1990; Takahashi 1991). Basidiocarps of *Lyophyllum shimeji* have been collected under *Quercus serrata*, in a *Pinus densiflora–Quercus serrata* mixed forest (Imazeki et al. 1988), and under *Quercus mongolica* var. *grosseserrata* (Takahashi 1991). As the mycorrhizal features of these fungi have not yet been described, it was interesting to discover that both fungi, *Lepista nuda* and *Lyophyllum shimeji*, formed ectomycorrhizae with *P. glehnii*. Furthermore, one isolate of *Lepista nuda* (Ln 73/92) formed an ectendomycorrhiza, with mantle and Hartig net, but also had intracellular hyphae. *Geastrum* is also a fungus commonly considered only to be saprophytic. In pure culture with *P. glehnii*, however, *G. mirabile* formed a pseudomycorrhiza, where hyphae of the mantle were surrounding the root, but no Hartig net was formed (Table 2). The *Suillus* isolate used in this study also formed pseudomycorrhiza. Usually, this fungal genus is associated with only one genus of host plant, so considering that it was isolated from a pure *P. glehnii* forest, a true mycorrhiza was expected in the in vitro system, but it did not occur.

According to Molina and Trappe (1982), a specific host often derives greater benefit from ectomycorrhizal fungi with high specificity than from those with low specificity, whenever this specificity is controlled more by the host plant than by the fungus. For example, *Alnus rubra* has symbiotic fungus with restricted host range (Molina 1979), whereas *Quercus* spp. are able to form mycorrhizae with many ectomycorrhizal fungi (Dixon et al. 1984). Results of this study suggest that *P. glehnii* also has a low specificity toward mycorrhizal

symbionts, since most of the fungi tested formed mycorrhizae.

Both the isolates that did not form mycorrhiza, *Chalciporus piperatus* 5/92 and *Lyophillum* sp. 61/92, were isolated from old forest tree stands (Table 1). Whether this is due to an intrinsic inability to form mycorrhiza, to stringent host specificity, or to their being late successional fungi remains to be determined.

Basidiocarps of some of the fungi isolated in this study, such as *Lepista nuda* and *Lyophyllum shimeji*, have not been reported from *P. glehnii* forests. However, they were able to form mycorrhizae under our test conditions. Mycorrhiza formation by *Amanita* spp., *Pisolithus tinctorius*,*Cenococcum geophillum*, *Boletus pulverulentus*, and *Paxillus* sp. in our in vitro system confirmed their broad host range (Molina et al. 1992), since they formed ecto-/ectendomycorrhiza with *P. glehnii*. Individual species of the genus *Scleroderma* are considered to be restricted either to gymnosperms, to angiosperms, or to a single host family (Molina et al. 1992). Since the two *Scleroderma* species collected here were under hosts belonging to the Pinaceae, the mycorrhizal formation obtained with *P. glehnii* was to be expected.

This study presents information on the affinity of some ectomycorrhizal fungi for *P. glehnii*. However, it is necessary also to verify the ability of these fungi to form mycorrhizae under natural conditions, and to demonstrate the benefits that their inoculation can bring to the host tree.

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References

Agerer R (1991) Characterization of ectomycorrhiza. In: Norris JR, Read J, Varma AK (eds) Techniques for mycorrhizal research. Academic Press, San Diego, pp 25–74

Anonymous (1994) Forestry statistics of 1993 in Hokkaido (in Japanese). Hokkaido Government, Sapporo

Dixon RK, Garrett HE, Cox GS, Marx DH, Sander IL (1984) Inoculation of three *Quercus* species with eleven isolates of ectomycorrhizal fungi. I. Inoculation success and seedling growth relationships. For Sci 30:364–372

Igarashi T (1990) Fungi of Hokkaido (in Japanese). Hokkaido Shimbun-sha, Sapporo

Igarashi T (1993) Fungi of Hokkaido, vol 2 (in Japanese). Hokkaido Shimbun-sha, Sapporo

Imazeki R, Otani Y, Hongo T (1988) Mushrooms of Japan (in Japanese). Yama-kei, Tokyo

Hongo T (1990) Mycorrhizal Agaricales (in Japanese). Trans Mycol Soc Japan 31:281–286

Kasuya MCM, Igarashi T (1994) Study on ectomycorrhizae of *Picea glehnii* Masters. I. Occurrence and types present in a nursery. Trans Meet Hokkaido Br Jap For Soc 42:140–142

Kasuya MCM, Muchovej RMC, Bellei MM, Borges AC (1992) In vitro ectomycorrhizal formation in six varieties of pine. For Ecol Manag 47:127–134

Kasuya MCM, Igarashi T, Shibuya M (1995) Occurrence and types of ectomycorrhizae present in seedlings of *Picea glehnii* in a natural forest in Hokkaido. Mycoscience 36:335–339

Kottke I, Guttenberger M, Hamp R, Oberwinkler F (1987) An in vitro method for establishing mycorrhizae on coniferous tree seedlings. Trees 1:191–194

Molina R (1979) Pure culture synthesis and host specificity of red alder mycorrhizae. Can J Bot 57:1233–1238

Molina R (1981) Mycorrhizal inoculation and its potential impact on seedling survival and growth in Southwest Oregon. In: Hobbs SD, Helgerson OT (eds) Reforestation of skeletal soils. Proceedings of workshop, Corvallis, Oregon, pp 86–91

Molina R, Palmer JG (1978) Isolation, maintenance, and pure culture manipulation of ectomycorrhizal fungi. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathological Society, St Paul, Minn, pp 115–129

Molina R, Palmer JG (1982) Isolation, maintenance, and pure culture manipulation of ectomycorrhizal fungi. In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytopathological Society, St Paul, Minn, pp 115–129

Molina R, Trappe JM (1982) Applied aspects of ectomycorrhizae. In: Suba Rao NS (ed) Advances in agricultural microbiology. Oxford and IBM, New Delhi, pp 305–324

Molina R, Massicote HB, Trappe JM (1992) Specificity phenomena in mycorrhizal symbiosis: communities-ecological consequences and practical implications. In: Allen MF (ed) Mycorrhizal functioning, an integrative plant-fungal process. Chapman and Hall, London, pp 357–433

Ogawa M (1976) Microbial ecology of "shiro" in *Tricholoma matsutake*(S. Ito et Imai) Sing. and its allied species. III. *Tricholomamatsutake* in *Picea glehnii*and *Picea glehnii–Abies sachalinensis* forests. Trans Mycol Soc Japan 17:188–198

Ohta A (1994) Some cultural characteristics of mycelia of mycorrhizal fungus, *Lyophyllum shimeji*. Mycoscience 35:83–87

Peterson RL, Chakravarty P (1991) Techiniques in synthesizing ectomycorrhiza. In: Norris JR, Read J, Varma AK (eds) Techniques for mycorrhizal research. Academic Press, San Diego, pp 75–106

Takahashi I (1991) Fungi of Hokkaido (in Japanese). Alice-sha, Sapporo

Tatewaki M (1958) Forest ecology of the islands of the North Pacific ocean. J Fac Agr Hokkaido Univ L 4:1–486

Tatewaki M, Igarashi T (1971) Forest vegetation in the Teshio and Nakagawa district experiment forests of Hokkaido University, Prov. Teshio, N. Hokkaido, Japan. Res Bull Coll Exp For Hokkaido Univ 28:1–192