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Occurrence and types of ectomycorrhizae present in seedlings of *Picea glehnii* in a natural forest in Hokkaido

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***Picea glehnii* seedlings whose ages ranged from 2 to 5 years and which were growing in a *P. glehnii-Abies sachalinensis* natural forest where the surface had been scarified 6 years before, were assessed for the occurrence and types of ectomycorrhizae. Based on the macro- and microscopic characteristics, 34 types of ectomycorrhizae were classified. The basidiomycetes were clearly dominant and it was common to observe more than one type of mycorrhiza occurring in the same root tip. The diversity of mycorrhizal types and the presence of at least one type of ectomycorrhiza in a single seedling, observed in this work, indicate a very dynamic system of mycorrhizal formation in *P. glehnii* seedlings, which allows them to survive and grow in a forest surface subjected to soil scarification.**

Key Words——ectomycorrhizal types; forest surface scarification; macroscopic and microscopic characteristics; *Picea glehnii*; seedling age.

Results of many investigations on functioning of mycorrhiza have demonstrated its important bearing on forestry practices. Among well-kown benefits of mycorrhizae to the tree can be listed the improvement of seedling survival and growth by enhancing uptake of nutrients and water, lengthening root life, and protection against pathogens (Mikola, 1973).

Picea glehnii (Fr. Schm.) Masters is one of the most important "needle-leaf" trees of Hokkaido, especially in its east and north (Tatewaki, 1958), but mycorrhizae of this tree species have been little investigated. Natural occurrence of ectomycorrhizae in nursery beds was observed by Kasuya and Igarashi (1994). Under field conditions, Ogawa (1976) reported the presence of *Tricholoma matsutake* (S.Ito et Imai) Sing. as a mycorrhizal fungus of *P. glehnii* at Mt. Meakan in Hokkaido. Fruitbodies of potentially ectomycorrhizal fungi, not only *Tricholoma* species but also *Amanita, Boletus, Cortinarius, Geastrum, Lepista, Russula*, and *Suillus*, have been observed in *P. glehnii* forests (Igarashi, 1990, 1993; Takahashi, 1991), but the mycorrhizal associations of the fungal species have not been verified. Furthermore, all ectomycorrhizal fungi do not necessarily produce fruitbodies, and even among them that produce fruitbodies, they may not be observed depending on the environmental conditions, period of observation, and/or host plant (Trappe, 1977).

One characteristic of *P. glehnii* forests is the presence of *Sasa* spp. covering the forest surface and reaching until 2 to 4 m in height. Surface scarification has been used as a forestry practice to remove *Sasa* spp. and to stimulate seed germination and tree growth (Watanabe and Sasaki, 1993). Unfortunately, this process removes not only the *Sasa* species, but also all of the organic layer and sometimes the upper part of horizon A, including natural mycorrhizal inocula.

Considering that ectomycorrhizal fungi may play an important role in the seed germination and establishment of trees after surface scarification, this work was carried out to investigate the occurrence and characterize the types of ectomycorrhizae associated with *P. glehnii* seedlings in a natural forest composed of *P. glehnii* and *Abies sachalinensis* (Fr. Schm.) Masters.

Materials and Methods

Sampling On 16th September 1993, 15 samples of *P. glehnii* seedlings, each of age 2, 3, 4 and 5 years, i.e., a total of 60 seedlings, were collected in a *P. glehnii-A. sachalinensis* mixed natural forest located at the Uryu Experimental Forest, Northern Hokkaido. The forest stand (stand 320) had been scarified six years earlier (in October 1987), for removal of *Sasa* sp. which covered the forest surface. The samples were taken in an area of 10 m in width and 1.5 km in length parallel to a forest road. In this area, besides *P. glehnii* seedlings, *A. sachalinensis* (smaller than 50 cm) and *Betula ermanii* Cham. (smaller than 80 cm) and the shrub *Rubus idaeus* Linn. var. *aculeatissimus* Regel et Tiling were dominant.

Macroscopic characterization The shoots were cut off and their height and butt diameter were measured before being discarded. The root systems were washed in running water to remove adhered soil, blotted with a paper towels and cut into 1- to 2-cm segments. Some of these were observed immediately under the stereomicroscope (Ingleby et al., 1990; Agerer, 1994) for mycorrhizal as-

Table 1. Types of ectomycorrhizae of *Picea glehnii* seedlings of ages ranging from 2 to 5 years growing under a *P. glehnii-A. sachalinensis* mixed forest, classified according to the macro and microscopic characteristics.

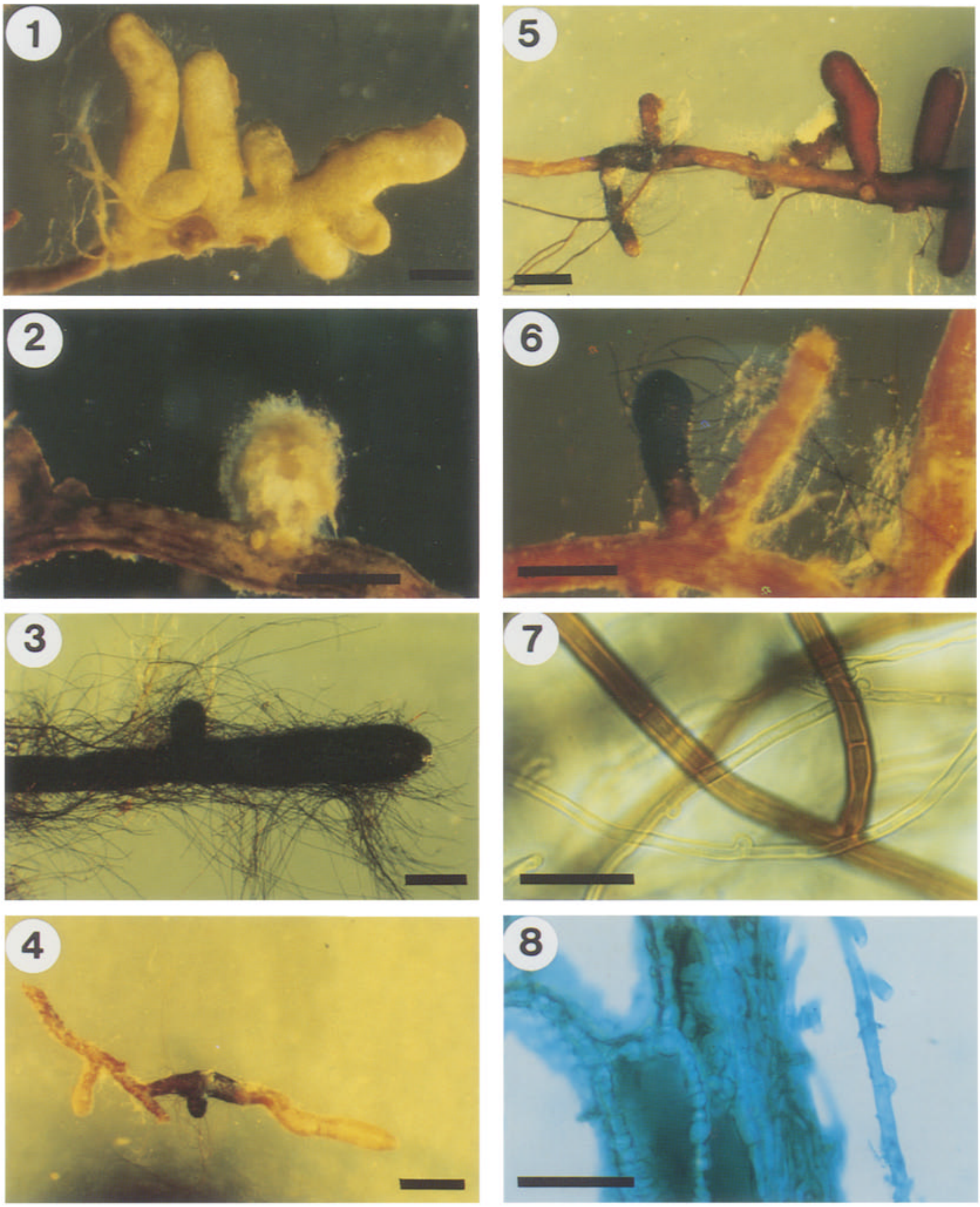
Type	Macroscopic characteristics					Microscopic characteristics								
	Ectomycorrhizae					Mantle					Emanating hypha			
	Color	Ramification	Main axis Length (μm)	Main axis Diam[2] (μm)	Riz[1]	Edge[3]	Thickness (μm)	Structure IPV[4]	Structure Diam[2] (μm)	Hartig net hypha diam[2] (μm)	Ab.[5]	Length[6]	CC[1]	Diam[2] (μm)
01	black	simple	<6.5	<0.6	−	cu	5–30	ns	1–4	1.5–4	+++	long	−	2–6
02	very dark grayish brown	simple	<5	<1	+	cs	20–30	is	1–2.5	1–2	+++	long	+	2–5
03	dark reddish brown	simple	<4	<0.4	+	cs	25–40	is	2–3	2–2.5	+	short	+	2–3
04	dark reddish brown	simple	<2	<0.3	+	cs	15–35	ns	1.5–2	<1	++	short	+	1–2
05	brown	simple	<1.5	<0.4	−	lo	5–20	ns	1.5–3	1.5–3	++	long	−	1.5–3
06	brown	branched	<8	<0.4	+	cu	10–25	is	2–3	2–3	++	short	+	3–3.5
07	reddish brown	simple	<3	<0.4	−	lo	2.5–20	ns	1–3	1–3	+++	long	+	1.5–3
08	dark yellowish brown	branched	<4	<0.5	−	cs	10–45	is	2–3	1.5–2.5	+	short	+	2–3
09	yellowish brown	simple	<3	<0.5	−	cu	15–60	is	2–3	1.5–4	++	short	−	2–3
10	yellowish brown	simple	<5	<0.3	−	cu	10–50	is	1–4	1–2	++	short	+	1.5–3
11	yellowish brown	simple	<7	<0.5	−	lo	5–10	ns	2–3	1–4	+++	long	+	1.5–4
12	yellowish brown	simple	<4.5	<0.5	−	cu	5–75	ns	2–2.5	2–3	++	short	−	2–3
13	yellowish brown	simple	<5.5	<1	−	cu	5–20	ns	1–3	1–2.5	++	short	+	2.5–3
14	brownish yellow	simple	<1.5	<0.4	+	cs	15–40	is	2–3	2.5–3	+	short	−	3–5
15	brownish yellow	branched	<4	<0.5	−	cu	10–40	ns	1–1.5	2–3	+	long	+	2–2.5
16	brownish yellow	branched	<5.5	<0.5	−	cu	5–20	ns	1–3	1.5–3	+++	long	+	1.5–3
17	brownish yellow	branched	<6.5	<0.7	+	cu	15–55	ns	1–3	2–5	+	long	+	2–5
18	brownish yellow	branched	<8	<0.5	−	cs	10–45	is	1–2	1–2	+++	long	+	1–2
19	brownish yellow	branched	<5	<0.4	−	cu	10–25	rs	1–2	1–2	+	short	+	2–2.5
20	yellow	simple/oval	<1.5	<1.3	−	cu	25–35	ns	<1	<1	+++	long	−	<1
21	yellow	simple	<1.5	<0.3	−	cu	8–15	rs	1–3	1–2	++	short	−	1–2
22	yellow	simple	<2	<0.4	−	cu	5–30	is	1–3	1–2	++	long	+	1–2
23	yellow	simple	<10	<0.5	−	lo	5–50	ns	1–5	1–3	+++	long	−	1–6
24	yellow	simple	<10	<0.8	−	cu	5–30	is	1–2	1–3	++	short	+	2–3
25	yellow	simple	<8	<0.6	−	cu	10–70	ns	1–3	1–4	++	short	+	1–3
26	yellow	branched	<4	<0.5	−	cu	10–40	is	1–2.5	1–2	+	short	−	1–3
27	yellow	branched	<3.5	<0.3	−	lo	5–30	is	1.5–4	1.5–3	++	long	−	1–2
28	yellow	branched	<9	<0.4	−	cu	5–40	ns	1–2.5	1–2	+	short	−	1–2
29	yellow	branched	<5	<0.8	+	cs	10–90	is	2–3	1–2	++	short	+	1–3
30	pale yellow	simple	<2.5	<0.4	−	cu	10–70	is	1.5–2	1.5–2	+++	long	+	2–3
31	pale yellow	simple	<1	<0.2	−	lo	10–25	ns	2–4	1–2	+++	long	+	1.5–2
32	pale yellow	simple	<2.5	<0.3	−	cu	5–30	ns	1–3	1–3	++	short	−	1.5–3
33	pale yellow	simple	<2.5	<0.3	+	cs	50–100	is	2–4	2–4	+	short	−	2–4
34	pale yellow	simple	<4.5	<0.4	+	cu	20–80	ns	1–2	1–2	++	short	+	1–2

[1] Riz=rhizomorph, CC=clamp connection; + present and − absent: [2] Diam=diameter; [3] cu=compact-uneven, cs=compact-smooth, lo=loosely; [4] IPV=inner plan view: ns=net synenchyma, is=irregular synenchyma, rs=regular synenchyma; [5] Ab=abundance: +++ abundant; ++ frequent but not abundant; + sometimes present; [6] Long: majority of emanating hyphae are 100 m; short: majority of emanating hyphae <100 m. Terminology of mantle edge and inner plan view is based on Ingleby et al. (1990).

sessment with respect to colonization status, color (Munsell soil color charts, 1990) form and size of ectomycorrhizae and to verify the presence of rhizomorphs and the abundance and length of emanating hyphae. As the root hairs of non-mycorrhized roots were, generally, shorter than 100 μm, emanating hyphae were considered to be long when the majority of them were longer than 100 μm, and short when the majority of them were shorter than 100 μm.

Microscopic characterization The remaining segments were stored in 2.5% glutaraldehyde in phosphate buffer at 4°C for later evaluation of mycorrhizal microscopic characteristics (Haugh and Oberwinkler, 1987; Ingleby et al., 1990; Agerer, 1994). For this, segments with typical mycorrhizae were selected and dehydrated in a graded ethanol (EtOH) + butanol (n-BuOH) series (EtOH: H_2O

(1 : 1); EtOH: n-ButOH: H_2O (5 : 10 : 4); EtOH: n-ButOH: H_2O (50 : 19 : 31); EtOH: n-ButOH: H_2O (50 : 38 : 12); EtOH: n-ButOH: H_2O (40 : 56 : 4); EtOH: n-ButOH (24 : 76); n-ButOH 100%) for 60 min each, and infiltrated with paraffin. Transverse and longitudinal sections of 10 μm thickness were cut with the aid of a microtome and the sections were stained with 0.05% toluidine blue O in distilled water or safranin (1% in EtOH 50%) and fast green (1% in EtOH absolute + clove oil). These segments were observed under a light microscope with micrometer and photographic capabilities. The characteristics recorded were: diameter of emanating hyphae and presence of clamp connection, mantle edge, thickness and inner plan view, presence of Hartig net and di-



Figs. 1–8. Ectomycorrhizae of *Picea glehnii* seedlings, of ages ranging from 2 to 5 years growing in a *P. glehnii*-*A. sachalinensis* mixed forest.
1. Type 18 showing the branched ramification. 2. Type 20 showing the oval shape. 3. Type 1, branched mycorrhiza of *Cenococcum geophillum*. 4–6. Two types of mycorrhizae occurring in the same root: *C. geophillum* (Type 1) with Type 25 (4), with Type 4 (5) and with Type 11 (6) 6, 7. Mycelia of *C. geophillum* (brown) and Type 16 (hyaline and with clamp connection). 8. Transverse section of mycorrhiza Type 25. Scales: 1–6 = 0.5 mm and 7–8 = 20 μm.

ameter of hyphae in the mantle and Hartig net.

Results and Discussion

Averages of heights of shoots were 6.85, 8.46, 17.25 and 26.34 cm and diameter were 0.94, 1.43, 2.75 and 5.30 mm, respectively for seedlings aged 2, 3, 4 and 5 years. Growth of these seedlings, both shoot and root systems, was about 2 to 4 times less than that seedlings in the nursery (unpublished data, 1993), although the latter showed many old and dead roots, especially among the 4- and 6-year-old seedlings (Kasuya and Igarashi, 1994). Both samplings were conducted in the same period and in the same experimental forest, but five types of ectomycorrhizae observed in the nursery seedlings (Kasuya and Igarashi, 1994) differed from those observed under natural conditions.

A total of 34 types of ectomycorrhizae were classified after macro- and microscopic characterization (Table 1). Egli et al. (1993) observed 29 types in *Picea abies* (L.) Karst. in adult stands, whereas Yamada and Katsuya (1994) found 34 types in *Pinus densiflora* Sieb. et Zucc. seedlings and a total of 46 types upon consideration of the mycorrhizae associated with adult trees. Although the numbers of mycorrhizal types are close, it is not possible to compare the data, since this method of evaluation is subjective and there are differences due to fungus-host combination (Egli et al., 1993). Nevertheless, according Ingleby et al. (1990), the microscopic characteristics of mycorrhizae seem to be largely host-independent, i.e., mycorrhizae formed by the same fungus with different tree species were found to be similar.

The colors varied from black to pale yellow and within the same color more than one type of mycorrhizae could be seen (Table 1). The color was very useful for the first screening, but a more definite classification of type was only possible after microscopic observation, as stated by Haugh and Oberwinkler (1987), Ingleby et al. (1990), Egli et al. (1993), and Agerer (1994).

Mycorrhizal ramification was useful primarily to screen the branched (Fig. 1) versus oval (Fig. 2) mycorrhizae, but not for the simple one, which sometimes exhibited branching, such as *Cenoccocum geophillum* Fr. (syn. *C. graniforme* (Sow) Ferd et Winge)(Figs. 3-6). The oval form (Type 20) was not found frequently, being associated only with a 3-year-old seedling, and thus it was one of the more unusual mycorrhizal types (Fig. 2).

The presence of rhizomorphs is useful to describe mycorrhizae, as well as the presence or absence of clamp connections (Haugh and Oberwinkler, 1987). The high number of mycorrhizal types presenting hypha with clamp connections indicates that basidiomycetes were dominant, but we are unable to identify the fungi beyond the group (Figs. 7, 8). The ascomycete *C. geophillum* (Type 1), however, was easily identified by the presence of thick and black hyphae (Figs. 3, 7).

Some fungi placed into the same class type presented different abundances and lengths of emanating hyphae (Table 1). Since these characteristics can be modified with mycorrhizal age (Ingleby et al., 1990), they

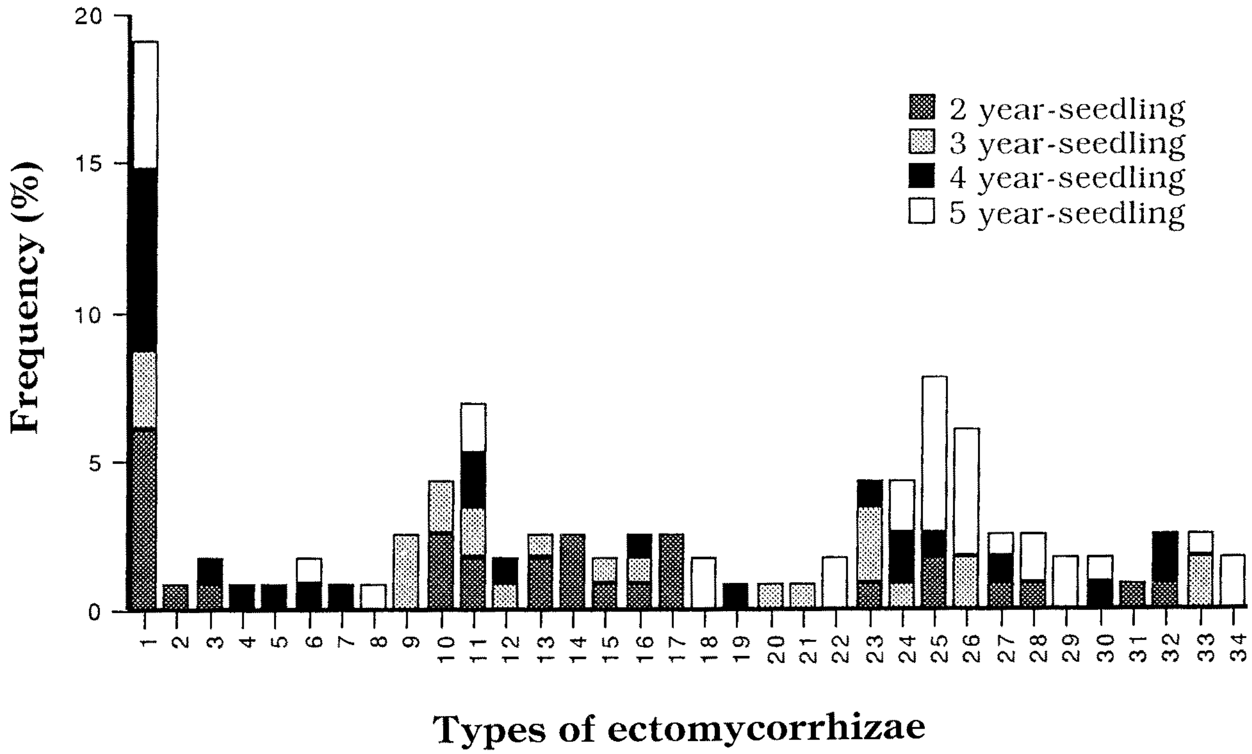


Fig. 9. Frequency of ectomycorrhizal types as a function of the *Picea glehnii* age. See Table 1 for description of ectomycorrhizal types.

Table 2. Frequency of number of ectomycorrhizal types per seedling.

Seedlings age (years)	Number of ectomycorrhizal types/Seedling		
	1	2	3
2	5	8	2
3	9	4	2
4	5	7	3
5	4	8	3

were not used for the classification.

Sporocarps of *Thelephora terrestris* Fr. could be observed in some 4- and 5-year-old seedlings, but neither hypha linked to the mycorrhizae nor typical ectomycorrhizae of this fungus could be verified when compared with the characteristics described by Ingleby et al. (1990). The seedlings presenting *T. terrestris* sporocarps rarely presented common types of mycorrhizae. No other fungal sporocarp could be observed.

The types of mycorrhizae and the frequency of these types in seedlings of varying ages are presented in Fig. 9. Some types were present only in seedlings of a specific age and others in seedlings of various ages. *Cenoccocum geophillum* (Type 1) was observe in seedlings of all age and with high frequency (Fig. 9), but it was always present in low proportion, never dominating the root system. Mycorrhiza of Type 11 was also observed in seedlings of all ages.

Chalot et al. (1988) and Allen (1991) reported that the occurrence of more than one type of mycorrhiza in the same plant, in the same root, or even as in the same root tip is frequent. This was also veryfied in this work (Table 2) and it was easy to distinguish the presence of different types among mycorrhizae with different colors (Figs. 4–6), or when the hyphae color and diameter were distinct (Fig. 7), but care is needed when these characteristics are similar, requiring detailed microscopic observations to confirm their differences.

Results presented in this paper were obtained with seedlings collected in only one period, and the types and frequency of mycorrhizae present can vary with the period of evaluation, and the color and ramifications can be also be altered by the developmental stage (Ingleby et al., 1990; Terashima, 1992), indicating that the number of mycorrhizal types obtained here might differ if seedlings were collected in various periods of the year. However, the diversity of mycorrhizal types and the presence of at least one type of ectomycorrhiza in a single seedling, observed in this study, indicates a very dynamic system of mycorrhizal formation in *P. glehnii* seedlings, which allows them to survive and grow in a forest surface subjected to soil scarification.

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Literature cited

Agerer, R. 1994. Characterization of ectomycorrhiza. In: "Techniques for mycorrhizal research," (ed. by Norris. J. R., Read, D. J. and Varma, A. K.), pp. 25–74. Academic Press, San Diego.

Allen, M. F. 1991. "The ecology of mycorrhizae," Cambridge University Press, Cambridge. 148 p.

Chalot, M., Battut, P. M., Botton, B., Le Tacon, F. and Garbaye, J. 1988. Recent advances in physiological and practical aspects of ectomycorrhizal effects to tree development. Acta Ecologica Ecol. Applic. **9**: 333–351.

Egli, S., Amiet, R., Zollinger, M. and Schneider, B. 1993. Characterization of *Picea abies* (L.) Karst. ectomycorrhizas: Discrepancy between classification according to macroscopic versus microscopic features. Trees **7**: 123–129.

Haugh, I. and Oberwinkler, F. 1987. Some distinctive types of spruce mycorrhizae. Trees **1**: 172–188.

Igarashi, T. 1990. "Fungi of Hokkaido," Hokkaido Shimbunsha, Sapporo. 302 p. (In Japanese.)

Igarashi, T. 1993. "Continue Fungi of Hokkaido," Hokkaido Shimbunsha, Sapporo. 302 p. (In Japanese.)

Ingleby, K., Mason, P. A., Last, F. T. and Fleming, L. V. 1990. "Identification of ectomycorrhizas," HMSO, London. 112 p.

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

Mikola, P. 1973. Application of mycorrhizal symbiosis in forestry practice. In: "Ectomycorrhizae, their ecology and physiology," (ed. by Marks, G. C. and Koslowsky, T. T.), pp. 383–411. Academic Press, New York.

Munsell soil color charts. 1990. Macbeth Division of Kollmorgen Instr. Corp., Baltimore.

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

Takahashi, I. 1991. "Fungi of Hokkaido," Alice-sha, Sapporo. 366 p. (In Japanese.)

Trappe, J. M. 1977. Selection of fungi for ectomycorrhizal inoculation in nurseries. Ann. Rev. Phytopathol. 15: 203–222.

Tatewaki, M. 1958. Forest ecology of the islands of the North Pacific Ocean. J. Fac. Agr. Hokkaido Univ. **50**: 369–486.

Terashima, Y. 1993. Distribution and external morphology of mycorrhizal roots at shiros of *Tricholoma bakamatsutake* in a mixed forest of *Pasania edulis* and *Castanopsis cuspidata* var. *sieboldii*. Trans. Mycol. Soc. Japan **34**: 495–505.

Watanabe, S. and Sasaki, S. 1993. The silvicultural management system in temperate and boreal forests: a case history of Hokkaido Tokyo University Forest. Can. J. For. Res. **24**: 1176–1185.

Yamada, A. and Katsuya, K. 1994. Ectomycorrhizal distinction of Japanese red pine based on morphology. In: Proceedings of the 38th Annual Meeting of the Mycological Society of Japan. Tottori, 26–27 May 1994, p. 22. (In Japanese.)