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Cultivation of mushrooms of edible ectomycorrhizal fungi associated with *Pinus densiflora* by in vitro mycorrhizal synthesis

I. Primordium and basidiocarp formation in open-pot culture

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Abstract Edible mushroom fungi in the genera *Lyophyllum*, *Tricholoma*, *Leucopaxillus*, *Suillus*, *Rhizopogon*, *Lactarius*, and *Morchella* were tested for mycorrhization with *Pinus densiflora* in vitro. Most of the tested fungi in the genera *Lyophyllum*, *Tricholoma*, *Suillus*, *Rhizopogon*, and *Lactarius* formed ectomycorrhizas 2–4 months after fungal inoculation. Mycorrhizal seedlings were then acclimatized in open-pot soil under growth-chamber conditions. Almost all mycorrhizal seedlings sustained their symbiont and developed new mycorrhizas for 8–9 months after transplantation. Under these conditions, more than half of the tested species formed primordia and *Tricholoma flavovirens*, *Rhizopogon rubescens*, and *Lactarius akahatsu* developed basidiocarps with young host plants.

Keywords Edible mushrooms · Ectomycorrhizas · Cultivation · In vitro synthesis · Basidiocarp

Introduction

Generally, cultivation of the mushrooms of edible ectomycorrhizal fungi has not been very successful under regulated environmental conditions. This is due to the slow growth rate of the mycelium on nutrient media, the large problem of fruit body formation without a host plant, i.e. mycorrhiza formation, the difficulty of sustaining a mycorrhizal association with a host plant, and lack of information on fruiting mechanisms (e.g. Hall and Wang 1998a). The exceptional species are *Lyophyllum shimeji* Hongo, *Cantharellus cibarius* Fr. *Lactarius deliciosus* (L. ex. Fr.) S. F. Gray, *Tuber maculatum* Vittad, and *T. melanosporum* Vittad. Although *Lyophyllum shimeji* can form true ectomycorrhizas (Kawai 1997), fruiting without a host plant was successful in conditions similar to saprobic mushroom cultivation, i.e. a sawdust-based substrate supplemented with oat grains as organic nutrient (Ohta 1994b). Conversely, *T. maculatum*, *T. melanosporum*, *C. cibarius*, and *Lactarius deliciosus* fruited in association with young host plants under greenhouse or special plantation conditions (Fassi and Fontana 1969; Shaw et al. 1996; Guerin-Laguette 1998; Hall and Wang 1998b; Danell 1999).

Considering general ectomycorrhizal associations, including fungi other than edible mushrooms, fruiting in association with young seedlings is not an unusual phenomenon. Various fungal genera used as inocula for nursery seedlings in forest stations, e.g. *Laccaria*, *Hebeloma*, *Thelephora*, *Rhizopogon*, *Pisolithus*, *Scleroderma*, often form basidiocarps under open-pot soil and nursery conditions (e.g. Brundrett et al. 1996; Castellano 1996). Environmental factors that can affect basidiocarp formation in vitro and in open-pot culture have been discussed for *Hebeloma cylindrosporum* Romagnesi, *H. sarcophyllum* (Peck) Sacc. and *Laccaria bicolor* (Maire) P. D. Orton (Debaud and Gay 1987; Godbout and Fortin 1990, 1992; Marmeisse et al. 1999). Several other *Hebeloma* species and *Chalciporus rubinellus* (Peck) Sing., all of which are putative ectomycorrhizal species, formed mature basidiocarps on nutrient media even without host plants (McLaughlin 1970; Bruchet 1973; Suzuki 1979; Ohta 1998; Marmeisse et al. 1999). Such an ability also indicates the saprobic nature of the isolated strains of these species (Giltrap 1981). These reports on basidiocarp formation may be applicable for the cultivation of edible ectomycorrhizal mushrooms under regulated environmental conditions with the formation of ectomycorrhizas, similar to natural mycorrhizal associations in forests.

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In this present study, we focused on diverse edible ectomycorrhizal fungi associated with *Pinus densiflora* Sieb. et Zucc., the primary host plant of *Tricholoma matsutake* (S. Ito et Imai), one of the best-known mushrooms in the world (Hosford et al. 1997). Targeted fungal genera were *Lyophyllum*, *Tricholoma*, *Suillus*, *Rhizopogon*, and *Lactarius*, all of which are popular edible mushrooms in Japan (Imazeki and Hong 1987, 1989). Our main objectives were: (1) to establish ectomycorrhizas under in vitro conditions, (2) to grow the mycorrhizal seedlings in open-pot soil under regulated environmental conditions, and (3) to induce fruiting structures, i.e. basidiocarps. This study thus concerns the methodology for producing mycorrhizal seedlings which may lead to industrial mushroom cultivation of various edible ectomycorrhizal fungi in the future.

Materials and methods

Fungal isolates

The fungi were isolated from sporocarps collected in Ibaraki Prefecture, Japan (Table 1) and included 12 putative mycorrhizal species and three putative saprobic species. The latter fungi were included in the experiments as negative controls for the experimental conditions of mycorrhizal synthesis, but also because of indications of the possibility for mycorrhization (Buscot 1992; Agerer and Beenken 1998). Most of the sporocarps were lyophilized, heated for 1 day at 60°C and stored in the Forest Center as proof specimens in an air-conditioned room at 18°C. Isolates were sustained on slants of MNC and Hamadaís media (Yamada and Katsuya 1995).

In vitro mycorrhizal synthesis

An open system of mycorrhizal synthesis was used in which the host plant root system was aseptically incubated with a fungus in vitro through the external plant shoot (Yamada et al. 1999). Axenically germinated *P. densiflora* seedlings were incubated with different fungal isolates in wide-mouth polymethylpentene plastic bottles (ca. 120 ml in volume) filled with a vermiculite/sphagnum moss mixture moistened with MNC liquid medium without glucose. Two replicate seedlings were set up for each fungal isolate. Inoculated seedlings were incubated in a growth chamber at 20°C and 5,000–7,000 lx continuous light. Sterilized, distilled water (20–30 ml) was added once to the substrate at the mid-point of the incubation period to maintain the growth of plant roots and fungal mycelium. After 4 months incubation, seedlings were removed from the bottles and gently washed with tap water; the root system was observed under dissecting and *compound* or *binocular* light microscopes (Yamada et al. 2001).

Acclimatization of mycorrhizal seedlings in open-pot soil

Soil collected from a *P. densiflora* plantation at our institute in Ibaraki, Japan consisted of brown forest soil with an organic

Table 1 Fungi tested for in vitro mycorrhiza synthesis

Species	Isolate number	Origin of the isolate (sporocarp)	
		Year	Vegetation; site
Lyophyllum semitale (Fr.) Kuhn.	AT586	1997	*Quercus serrata* forest; Yamagata, Ibaraki
Lyophyllum shimeji (Kawam.) Hong	AT608	1997	No record (commercially harvested); Daigo, Ibaraki
Lyophyllum fumosum (Pers.:Fr.) P.D. Orton	LF-1	1991	*Q. serrata* forest; Daigo, Ibaraki
	LF-2	1991	*Q. serrata* forest; Daigo, Ibaraki
Lyophyllum decastes (Fr.:Fr) Sing	AT506	1992	Grassland; Kukizaki, Ibaraki
Tricholoma flavovirens (Pers.:Fr.) Lund.	AT613	1997	*Pinus densiflora* forest; Yamagata, Ibaraki
	AT614	1997	*P. densiflora* forest; Yamagata, Ibaraki
T. portensosum (Fr.) Quel.	AT615	1997	*P. densiflora* forest; Yamagata, Ibaraki
T. saponaceum (Fr.) Kummer	AT616	1997	*P. densiflora* forest; Yamagata, Ibaraki
Leucopaxillus giganteus (Sow.:Fr.) Sing.	Lg-1	1992	No record (commercially harvested); Daigo, Ibaraki
	Lg-2	1992	*Q. serrata* forest; Suifu, Ibaraki
	AT571	1997	No record (commercially harvested); Satomi, Ibaraki
Suillus granulatus (L.:Fr.) O. Kunze	AT108	1992	*P. densiflora* forest; Tsukuba, Ibaraki
	AT577	1997	*P. densiflora* forest; Yamagata, Ibaraki
S. luteus (L.:Fr.) S. F. Gray	AT524	1992	*P. densiflora* forest; Kukizaki, Ibaraki
S. bovinus (L.:Fr.) O. Kunze	AT605	1997	*P. densiflora* forest; Yamagata, Ibaraki
	AT606	1997	*P. densiflora* forest; Yamagata, Ibaraki
Rhizopogon rubescens Tul.	AT517	1992	*P. tunbergii* forest; Kashima, Ibaraki
	RU-1	1995	*P. tunbergii* forest; Ooarai, Ibaraki
	AT630	1998	*P. tunbergii* forest; Kashima, Ibaraki
	AT632	1998	*P. densiflora* forest; Naka, Ibaraki
Lactarius hatsudake Tanaka	AT584	1997	*P. densiflora* forest; Naka, Ibaraki
	AT121	1992	*P. densiflora* forest; Tsukuba, Ibaraki
	AT124	1992	*P. densiflora* forest; Tsukuba, Ibaraki
Lactarius akahatsu Tanaka	AT561	1997	*P. tunbergii* forest; Asahi, Ibaraki
	AT583	1997	*P. densiflora* forest; Naka, Ibaraki
Morchella esculenta (L.:Fr.) Pers.	AT102	1992	*Q. myrsinaefolia* forest; Tsukuba, Ibaraki
	AT551	1997	*Prunsu mume* forest; Naka, Ibaraki

Table 2 Results of mycorrhizal synthesis in vitro (*N* Number of mycorrhizal seedlings; numbers in brackets are seedlings which developed immature mycorrhizas, i.e. patched Hartig net formation)

Putative ecology	Fungal species	Isolate	Mycorrhiza formation	N	Remarks
Mycorrhizal	*Lyophyllum semitale*	586	Present	2	
	Lyophyllum shimeji	608	Present	2	
	Lyophyllum fumosum	LF1	Absent		Fungal growth incomplete
		LF2	Absent		Fungal growth incomplete
	Tricholoma flavovirens	613	Present	1 (2)	
		614	Present	1 (2)	
	T. portensosum	615	Present	2	
	T. saponaceum	616	Present	2	
	Suillus granulatus	108	Present	2	
		577	Present	2	
	S. luteus	524	Present	2	
	S. bovinus	605	Present	1	
		606	Absent		Fungal growth incomplete
	Rhizopogon rubescens	517	Present	2	
		RU-1	Present	2	
		630	Present	1 (2)	
		632	Present	1 (2)	
	Lactarius hatsudake	121	Absent		Fungal growth incomplete
		124	Absent		Fungal growth incomplete
		584	Present	2	
	Lactarius akahatsu	561	Present	2	
		583	Present	2	
Saprobic	*Lyophyllum decastes*	506	Absent		
	Leucopaxillus giganteus	Lg-1	Absent		Root growth inhibited
		Lg-2	Absent		Root growth inhibited
		571	Absent		Root growth inhibited
	Morchella esculenta	102	Absent		
		551	Absent		

layer and a pH of 5.8. Large debris (roots, twigs, stones) was removed from the soil before it was mixed and homogenized and put in transparent polycarbonate plastic pots with a lid (800 ml in volume). The filled pots were autoclaved (121°C) for 1 h and stored in a refrigerator until use. Mycorrhizal seedlings were transplanted into plastic pots with this soil following mycorrhizal examination. Each pot was amended with 50–100 ml distilled water twice a week and incubated in an air-conditioned room at (day) 23°C, RH 65% and 5,000–7,000 lx light intensity for 18 h or (night) 18°C, RH 75% and darkness for 6 h. After 9 months incubation, seedlings were removed from the pots and the root systems were observed together with those from in vitro seedlings. Shoot heights of seedlings were measured as an index of growth.

Results

In vitro mycorrhizal synthesis

Most of the putative mycorrhizal fungi formed ectomycorrhizas during the incubation period (Table 2). Seedlings inoculated with *Lyophyllum fumosum* (all four seedlings), *S. bovinus* (one of two seedlings) and *Lactarius hatsudake* (four of eight seedlings) did not form mycorrhizas and inoculated mycelia did not grow well over the substrate. The three saprobic species did not form mycorrhizas, although they produced intensive mycelia and colonized the root surface. In the case of *M. esculenta*, indefinite-shaped brown sclerotia were observed on long roots. *Lyophyllum decastes* did not form a Hartig net or other types of fungal infection in the root cortex, despite the formation of an ectomycorrhiza-like external structure.

Most inoculated seedlings grew vigorously, i.e. 50% had developed two-needles by the fourth month of incubation. Root systems of the seedlings grew throughout the substrate in the bottles. Seedlings inoculated with three *Leucopaxillus giganteus* isolates were inhibited in both shoot elongation and foliage development by the second month. Their root systems were only 2–3 cm long and had few branches at the end of the incubation period.

Acclimatization of mycorrhizal seedlings in open-pot soil

Most seedlings used in the mycorrhizal synthesis were transplanted into open-pot soil conditions. Seedlings inoculated with two *Lyophyllum fumosum* isolates or *Lactarius hatsudake* isolate AT124 were not transplanted due to unsuccessful fungal infection in vitro. Seedlings inoculated with *Lyophyllum decastes* and *M. esculenta* were transplanted because they had developed infective mycelia and subsequent mycorrhization could possibly occur under different soil conditions.

All seedlings grew shoots and roots 1 month after transplanting into the open pots and these continued to

Table 3 Mycorrhizal acclimatization and formation of fruiting structures in open-pot soil (*H* Height in mm, *N* number of mycorrhizal seedlings/number of seedlings tested, *W* width in mm)

Putative field ecology	Species	Isolate	N	Distribution	Seedling height (cm)	Fruiting structures		
						Type	H	W
Mycorrhizal	*Lyophyllum semitale*	586	2/2	Upper half	7.3	Primordium	4	
	Lyophyllum shimeji	608	2/2	Overall	8.5	None		
	Tricholoma flavovirens	613	2/2	Overall	7.0	None		
		614	2/2	Overall	8.5	Primordium	2	
	T. portensosum	615	2/2	Overall	8.2	Basidiocarp	18	
	T. saponaceum	616	2/2	Overall	7.9	Basidiocarp	12	
	Suillus granulatus	108	2/2	Overall	10.2	None		
		577	1/1	Overall	12.2	Primordium	3	
	S. luteus	524	2/2	Overall	7.7	Primordium	2.5	
	S. bovinus	605	1/2	Overall	6.6	None		
	Rhizopogon rubescens	RU-1	2/2	Overall	12.1	None		
		630	1/1	Overall	10.5	Basidiocarp		10
		632	2/2	Overall	12.5	Basidiocarp		10
	Lactarius hatsudake	121	0/2	None	7.3	None		
		584	2/2	Overall	8.3	Primordium	3	
	Lactarius akahatsu	561	2/2	Overall	8.3	None		
		583	2/2	Overall	7.1	Basidiocarp		38
Saprobic	*Lyophyllum decastes*	506	0/2	None	6.7	Basidiocarp	10	
	Morchela esculenta	551	0/2	None	6.9	None		

Table 4 Comparison of mean height (cm±SE) of mycorrhizal seedlings inoculated with fungi from different genera. Different letters attached to values indicate significant differences between the means ($P<0.05$)

Genus	Number of seedlings	Height
Lyophyllum	4	7.88 (0.74) a
Tricholoma	8	7.9 (1.01) a
Suillus	5	9.7 (1.96) b
Rhizopogon	5	11.7 (1.47) c
Lactarius	6	7.85 (0.32) a

develop throughout the incubation period (Table 3). Most of the in vitro mycorrhizal seedlings developed new mycorrhizas all over the pot soil, but development was limited to the upper half of the soil column in the case of *Lyophyllum semitale*. No mycorrhizas were observed on seedlings inoculated with *Lactarius hatsudake* AT121 (two seedlings), *S. bovinus* AT605 (one seedling), or the putative saprobic fungi (*Lyophyllum decastes* and *M. esculenta*). Seedling heights varied between and within inoculated isolates (5.5–13 cm) (Table 3). The mean shoot height of mycorrhizal seedlings (8.9 cm, $n=28$) was significantly greater than that of non-mycorrhizal seedlings (6.8 cm, $n=8$) (one-way ANOVA, $P<0.05$). At the fungal genus level, mean shoot heights were also significantly different ($P<0.05$) (Table 4).

Primordium and basidiocarp formation

Under in vitro conditions, the mycorrhizal fungi formed neither primordia nor basidiocarps. Under open-pot conditions, several species formed primordia and basidiocarps in association with a host plant at late stages of incubation (Table 3, Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13). Five months after transplanting into the pot soil, *R. rubescens* AT630 developed a small but mature basidiocarp, i.e. basidiospore formation, on the surface of the soil ca. 10 days after formation of a primordium (Figs. 12, 13). *Tricholoma portentosum* (AT615) (Figs. 2, 3), *T. saponaceum* (AT616) (Fig. 4), and *Lactarius akahatsu* (AT583) (Figs. 7, 8, 9, 10) also produced basidiocarps 6–9 months after transplantation. Several primordia were produced subsequently with their basidiocarps but did not develop fully into basidiocarps. Several other fungal species also formed primordia after 4–9 months incubation in the soil, but no basidiocarps developed (Figs. 1, 5, 6, 11). Production of basidiocarps and primordia differed between and within species (Table 3).

The putative saprobic *Lyophyllum decastes* formed primordia in vitro and basidiocarps in pot soil without forming a mycorrhizal association with roots of *P. densiflora*.

Fig. 1 A primordium (*P*) and a mycorrhizal tip (*M*) of *Tricholoma flavovirens*. The *arrow* indicates a rhizomorph ▶

Fig. 2 *Tricholoma portentosum* basidiocarp

Fig. 3 *Tricholoma portentosum* basidiocarp. Longitudinal section of the basidiocarp with distinct gills (*arrow*)

Fig. 4 *Tricholoma saponaceum* basidiocarp with a distinct pileus (*arrow*)

Fig. 5 Primordium of *Suillus granulatus*

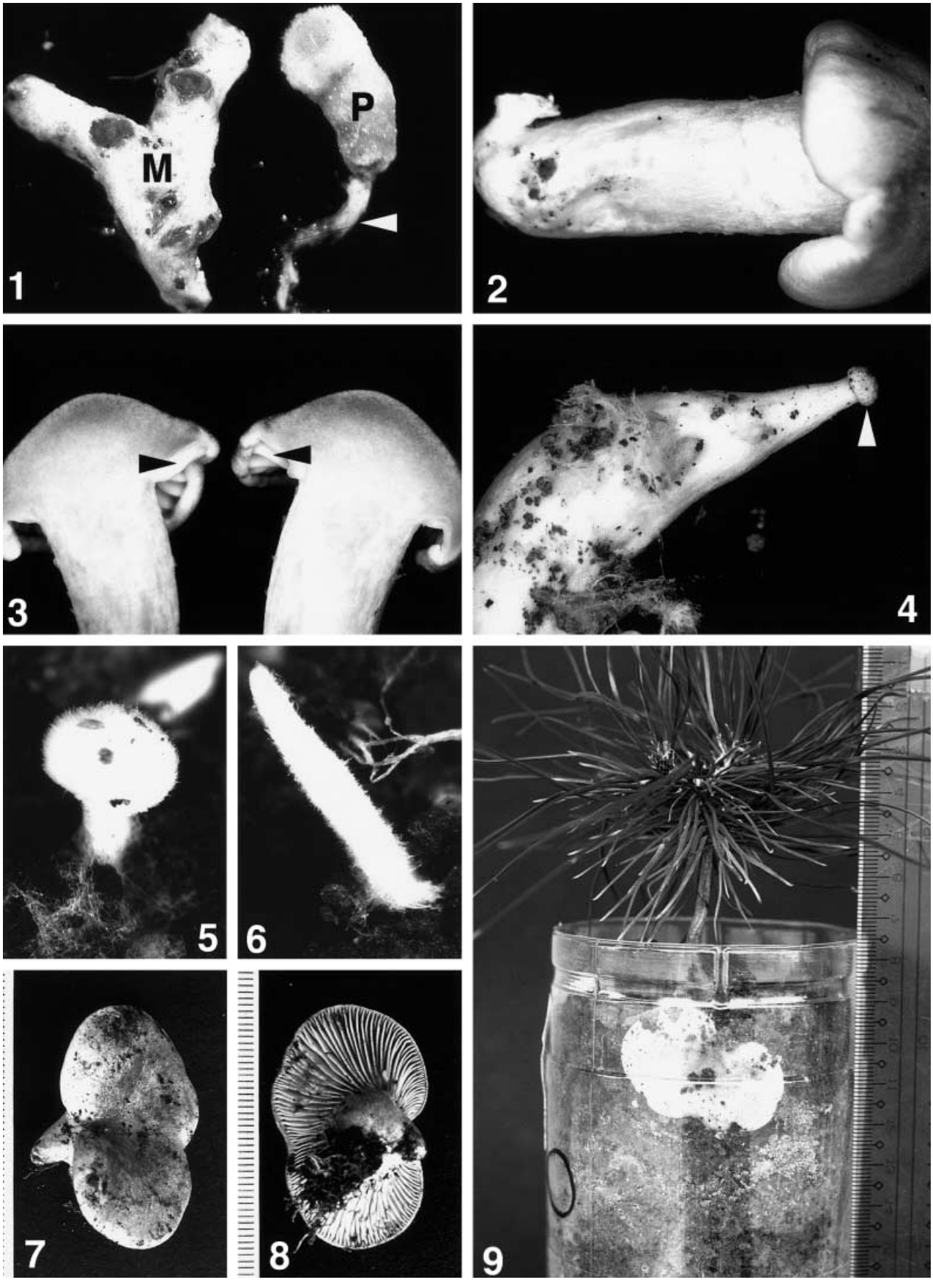
Fig. 6 Primordium of *Suillus luteus*

Figs. 7–10 *Lactarius akahatsu*

Fig. 7 Surface of the pileus of the basidiocarp

Fig. 8 Reverse side of the pileus

Fig. 9 Growing basidiocarp in association with a host plant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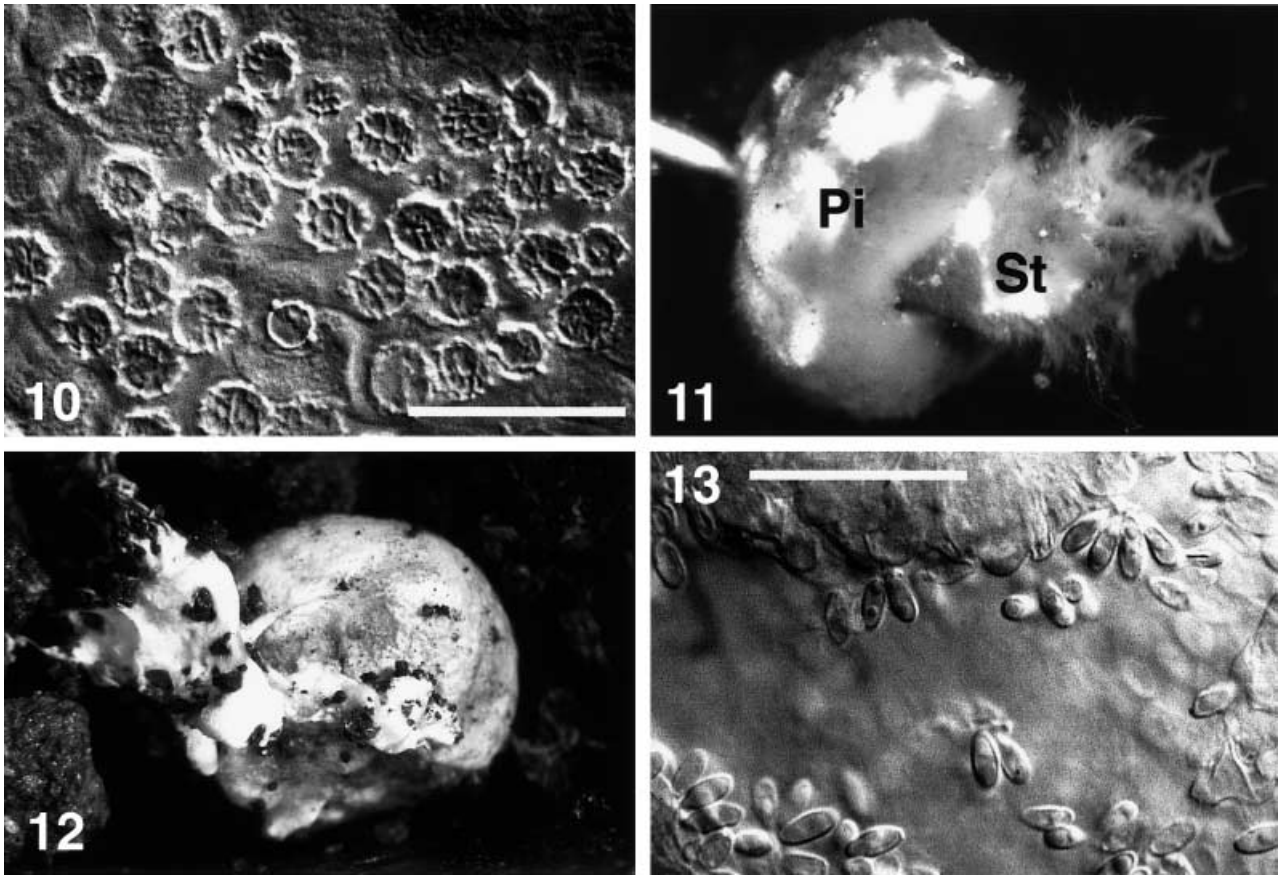

Fig. 10 Basidiospores; *bar* 20 µm

Fig. 11 *Lactarius hatsudake* primordium with a differentiated pileus (*Pi*)and stipe (*St*)

Fig. 12 Basidiocarp of *Rhizopogon rubescens*

Fig. 13 Basidiospores of *Rhizopogon rubescens*; *bar* 20 µm

Discussion

Eleven edible mushroom species formed ectomycorrhizas in vitro with *Pinus densiflora* in the present study. The mycorrhizal status of *Lyophyllum semitale*, *T. portentosum*, *T. saponaceum*, *Lactarius hatsudake*, and *Lactarius akahatsu* was demonstrated experimentally for the first time. Mycorrhiza synthesis with other species and various host plants has been reported elsewhere (Lamb and Richards 1971; Chu-Chou 1979; Molina and Trappe 1982, 1994; Malajczuk et al. 1982; Danielson 1984; Ingestad et al. 1986; Yamada and Katsuya 1995; Parladé et al. 1996; Kawai 1997; Hall and Wang 1998a). Although Nezzar-Hocine et al. (1998) suggested that in vitro mycorrhizal synthesis is relatively difficult in the genus *Tricholoma*, all three *Tricholoma* species formed well-developed mycorrhizas in this study. Yamada and Katsuya (1995) also reported mycorrhizal synthesis between *P. densiflora* and 21 fungal species, including two species of *Russula*, which are also difficult to manipulate in vitro (Taylor and Alexander 1989). These results highlight the suitability of *P. densiflora* for mycorrhiza synthesis with diverse fungal species in vitro.

With *Lyophyllum shimeji* and *Lyophyllum decastes*, which are taxonomically allied and are both root-colonizing fungi, this study yielded contrasting results. The results support the ecological characteristics of these species, i.e. the former as putative ectomycorrhizal and the latter as saprobic in Japan (Imazeki and Hong 1987). However, *L. decastes* forms ectomycorrhizas in Europe (Parladé et al. 1996; Agerer and Beenken 1998). Japanese populations of this species may be quite different from those in Europe. Furthermore, the root epiparasitic fungus *Morchella esculenta* (Buscot 1992) formed no ectomycorrhizas, despite colonization of pine roots and formation of sclerotia in vitro. This suggests that the ecologically non-mycorrhizal fungi tested here served successfully as negative controls. The growth inhibitory effect of *Leucopaxillus giganteus* probably derived to some extent from the nutrient deficiency of the pine seedlings, because this fungus grew aggressively all over the substrate in vitro but not on the surface of roots.

Several putative mycorrhizal isolates did not form ectomycorrhizas in vitro. This was most likely due to insufficient growth of the mycelium on the substrate, especially in the case of *Lactarius hatsudake* AT121 and AT124. The vermiculite/sphagnum moss substrate used in the present study may be unsuitable for mycorrhization with some fungus/plant combinations (Peterson and Chakravarty 1991; Nezzar-Hocine et al. 1998). The fail-

ure of mycorrhization may also be explained by host specificity in the case of *Lyophyllum fumosum*, which was collected in hardwood forests. It is, however, difficult to evaluate the effect of host specificity in a simple experiment (Molina et al. 1992; Massicotte et al. 1994) and further studies are necessary.

All seedlings that formed mycorrhizas in vitro developed new mycorrhizas in open-pot soil; non-mycorrhizal seedlings did not form new mycorrhizas. This suggests that the environmental setting was suitable for acclimatization of the in vitro mycorrhizas and that the mycorrhizal status is stable under both in vitro and open-pot soil growth conditions (Tables 2, 3). Mycorrhiza disappearance and succession (contamination) to another symbiont is problematic in the cultivation of mycorrhizal mushrooms. In this study, the tested mycorrhizal fungi competed with air-borne microorganisms such as sporulating microfungi, bacteria, and mites, as well as algae on the soil surface, all of which were observed microscopically. An important aspect of the practice of acclimatization is to protect the inoculated fungus from competitive air-borne mycorrhizal fungi in greenhouse conditions (Mamoun and Olivier 1996; Guerin-Laguette 1998; Selosse et al. 1998).

Mycorrhizal associations in pot soil were regarded as symbiotic in view of the significantly better growth of mycorrhizal relative to non-mycorrhizal seedlings. Of the fungi tested, *S. granulatus* and *R. rubescens* were associated with the highest shoot growth. *Suillus* spp., *R. rubescens*, and *Lactarius* spp. are ecologically specific to pine forests, while *Lyophyllum* spp. and *Tricholoma* spp. fruit in pine, mixed pine-*Fagaceae*, and *Fagaceae* forests in Japan (Imazeki and Hongo 1987). Although mycorrhization effects on plant growth vary with the experimental growing conditions, it is interesting to note that pine-specific fungi led to better host growth in open-pot cultures.

In this study, half of the tested mycorrhizal fungi formed basidiocarps and some of the others formed primordia in association with a host plant (Table 3). Thus, aside from mycorrhizal acclimatization in open-pot soil, the environmental conditions were suitable for the formation of fruiting structures. Godbout and Fortin (1990) reported that a shift to a shorter photoperiod from 18 to 12 h per day strongly favored basidiocarp formation in *Laccaria bicolor* (Maire) Orton. Since we used an 18-h photoperiod, a reduction might induce efficient fruiting of our test fungi. With regard to temperature, Debaud and Gay (1987) reported a significant effect of thermal shock (±6°C as against the 18°C control) on the rate of fruiting body production in *Hebeloma cylindrosporum* Romagnesi associated with *Pinus pinaster* (Sol.) in vitro. *Tricholoma matsutake* fruits in the autumn when the soil temperature is below 19°C in natural pine forests in Japan (Kinugawa 1963). This supports the results obtained here with the three *Tricholoma* spp. and other fungi tested. Kawai (1997) suggested the importance of host plant aging in relation to the fruiting of *Lyophyllum shimeji* and other mycorrhizal fungi. However, most reports of fruiting under various experimental conditions, and also the results of the present study, suggest that carbon availability, i.e. efficient CO_2 fixation, allows development of mycorrhizas on host-plant roots and fruiting structure development irrespective of host age (Lamhamedi et al. 1994). In fact, provision of exogenous organic nutrients in the substrate can support the fruiting of ectomycorrhizal fungi even in the absence of a host plant (Ohta 1994a, b).

To characterize the biochemistry and molecular biology of primordium formation and subsequent basidiocarp formation, it is necessary to identify specific signals inducing these developmental switches under simple experimental conditions (Moore 1998). There have been few such studies to date. Of 35 *Lyophyllum shimeji* strains screened, three formed primordia when incubated at 15°C in an axenic culture without a host plant (Ohta 1994a); they also developed mature basidiocarps under the same conditions (Ohta 1994b). Thus temperature is an important factor in the fruiting response of mycorrhizal as well as saprobic fungi. In *Hebeloma vinosophyllum*, formation of primordia and basidiocarps was induced in response to a nutrient deficiency in the growth medium in the absence of a host plant (Suzuki 1979). Under field conditions, various factors related to meteorology and biological community can affect fruiting. Fluctuation in rainfall has a marked effect on the production of sporocarps of various fungi in forests (Fogel 1976; Selosse et al. 1998; Agerer 1985; Selosse et al. 1998). Despite the difficulty of controlling fruiting under natural meteorological conditions, Guerin-Laguette (1998) succeeded in inducing basidiocarp formation of *Lactarius deliciosus* in association with container-grown European red pine seedlings. Thus the two *Lactarius* species we tested may be able to acclimatize in semi-natural conditions and form fruiting structures. This could lead to production of their mushrooms under greenhouse or semi-natural plantation conditions as reported for *Cantharellus cibarius* and *Tuber* spp. (Shaw et al. 1996; Hall and Wang 1998a; Danell 1999).

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