

# Chapter 16

## Cultivation of Basidiomycete Edible Ectomycorrhizal Mushrooms: *Tricholoma*, *Lactarius*, and *Rhizopogon*

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### 16.1 Introduction

Edible mushrooms are produced by many species of ectomycorrhizal (EM) basidiomycetes spread across a range of genera. Some of the most highly valued species include *Tricholoma matsutake* (S. Ito and S. Imai) Singer (matsutake), *Boletus edulis* Bull. (porcini), *Cantharellus cibarius* Fr. (golden chanterelle), and *Amanita caesarea* (Scop.) Pers. (Caesar's mushroom).

Edible EM basidiomycetes have generally been far more difficult to cultivate than ascomycete truffles (Savoie and Largeteau 2011). In the last few decades, however, significant progress has been made in the cultivation of these fungi. Currently the following species can be cultivated reliably and are associated with varying degrees of commercial development: *Lactarius deliciosus* (L.) Gray (saffron milk cap) in France and in New Zealand (Poitou et al. 1984; Wang and Hall 2004; Wang et al. 2011), *Lactarius hatsudake* Tanaka (hatsutake) in China (Tan et al. 2008), *Suillus granulatus* (L.) Roussel (granulated bolete) in France (Poitou et al. 1984), *Rhizopogon roseolus* (Corda) Th. Fr. (shoro) in New Zealand (Wang

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and Hall 2004; Visnovsky et al. 2010), and *Lyophyllum shimeji* (Kawam.) Hongo (honshimeji) in Japan (Yamanaka 2008).

Improvement of techniques for the cultivation of ectomycorrhizal basidiomycetes has been the subject of a large number of studies that have mainly focused on optimizing mycorrhization methods. Several species have successfully produced mushrooms under controlled laboratory conditions, including *L. shimeji* (Ohta 1994; Kawai 1997), *C. cibarius* (Danell and Camacho 1997), *L. deliciosus* (Guerin-Laguette et al. 2000a), *Lactarius akahatsu* Tanaka (Yamada et al. 2001), *Tricholoma terreum* (Schaeff.) P. Kumm., *Tricholoma portentosum* (Fr.) Quél., and *Tricholoma saponaceum* (Fr.) P. Kumm. (Yamada et al. 2007). However, many of the most highly prized mycorrhizal mushrooms, such as *T. matsutake*, *B. edulis*, and *A. caesarea*, have, so far, defied cultivation. The conservation and management of natural populations of these fungi have therefore become urgent issues, particularly in developing countries.

Two approaches are generally used to cultivate EM basidiomycete fungi:

1. Management of natural populations: development of techniques for sustainable harvesting, habitat improvement, and optimization of yields
2. A proactive approach to “tame” these organisms: from artificial production in the laboratory to their production on large-scale plantations

This review outlines current progress in the cultivation of edible ectomycorrhizal mushrooms (EEMM) in the genera *Tricholoma*, *Lactarius*, and *Rhizopogon*.

## 16.2 *Tricholoma*: Matsutake (*Tricholoma matsutake*) and Other Species

The genus *Tricholoma* includes a large number of edible species, among which matsutake (comprising *T. matsutake* and several closely related species) are by far the most valued and well known (Ogawa 1978; Wang 1995; Hosford et al. 1997; Koo and Milek 1998; Berch and Wiensczyk 2001; Danell 2002; Chapela and Garbelotto 2004). *T. portentosum* and *T. terreum* are also popular EEMM species. Other edible *Tricholoma* species are listed in Boa (2004), Yamada (2002), and Hall et al. (2007b). Many *Tricholoma* species including edible ones (e.g., *Tricholoma giganteum* Masee) are saprophytic, while a number of the EM species (including *T. matsutake*) also have saprophytic abilities (Vaario et al. 2011a, b). Here we focus on the cultivation of *T. matsutake*, as few other members of this genus have been the subject of cultivation attempts.

### 16.2.1 *Tricholoma matsutake*

Matsutake is a highly esteemed and commercially valuable traditional autumn delicacy in Japan (Fig. 16.1). The common name derives from the words pine (“matsu”) and mushroom (“take”), as the principal host plant for this fungus in

**Fig. 16.1** Matsutake (*Tricholoma matsutake*) and bakamatsutake (*T. bakamatsutake*, arrowhead) mushrooms from natural forest in Ina, Nagano, Japan



Japan is the Japanese red pine (*Pinus densiflora* Sieb. & Zucc.). Matsutake holds a special place in traditional Japanese culture and has long been the subject of poems, drawings, and paintings (Kobayashi 1983; Ogawa 1978). The mushroom is also considered a delicacy in Korea and China. In Korea the common name is songyi (pine mushroom) (Lee 1988a; Koo and Milek 1998), while in China it is known as song-koumo, song-rong, song-jun, or qing-gang-jun (Wang and Xie 1982; Zang 1990; Wang 1995; Gong et al. 1999). In addition to their culinary attributes, matsutake have also been shown to have important medicinal properties (Ying et al. 1987; Gong et al. 1999).

*Tricholoma matsutake* is considered to be the “true” matsutake and commands the highest prices in Japanese markets. The species is distributed throughout the circumboreal Northern Hemisphere including countries in Asia (Japan, China, North and South Korea, Bhutan, Turkey, and Russia), Europe (Sweden, Finland, Norway, Germany, Czechoslovakia, Austria, Switzerland, and Italy), possibly North Africa (Algeria and Morocco), and the eastern USA (Chapela and Garbelotto 2004). In Europe, *T. matsutake* was originally identified as a different species *Tricholoma nauseosum* (A. Blytt) Kytöv. However, based on morphological characters, Kytövuori (1989) considered *T. matsutake* and *T. nauseosum* to be conspecific. The same conclusion was reached by Bergius and Danell (2000) and Matsushita et al. (2005) following the analysis of sequences from the ribosomal DNA internal transcribed spacer (ITS) and intergenic spacer (IGS) regions. To avoid confusion, Ryman et al. (2000) proposed conservation of the name *T. matsutake*, based on common usage, rather than the rarely used but older name *T. nauseosum*.

## 16.2.2 *Tricholoma magnivelare* and Other “Matsutake” Species

*Tricholoma magnivelare* (Peck) Redhead or “white matsutake” is a close relative of *T. matsutake* found in northwestern North America (Hosford et al. 1997; Chapela

and Garbelotto 2004). Although *T. magnivelare*'s market value is significantly lower than that of *T. matsutake*, it is valuable enough to be airfreighted to Asian markets and has generated a multimillion dollar export industry (de Geus and Berch 1997). A morphologically similar species from Turkey, *Tricholoma anatolicum* Doğan & Intini, is also exported to Japan (Doğan and Akata 2011; Intini et al. 2003).

*Tricholoma bakamatsutake* Hongo is called the 'foolish pine mushroom' (baka = stupid in Japanese) and is very similar to the true matsutake in morphology but has a much stronger aroma and therefore no commercial value. The species is also the only member of the matsutake group recorded in the Southern Hemisphere; it has been reported in Papua New Guinea, growing in association with *Castanopsis* trees (Otani 1976). Other species, such as *Tricholoma robustum* (Alb. & Schwein.) Ricken, *Tricholoma focale* (Fr.) Ricken and *Tricholoma zelleri* (Stuntz and Smith) Ovrebo & Tylutki that cohabit with *T. matsutake* or *T. magnivelare*, and other related species *Tricholoma caligatum* (Viv.) Ricken, *Tricholoma dulciolens* Kytöv. and *T. fulvocastaneum* Hongo, similarly have no commercial value.

### 16.2.3 Artificial Cultivation of Matsutake

Harvesting and trading of matsutake has a longer history in Japan than in any other country (Kobayashi 1983). However, matsutake harvests from Japan have dramatically decreased over the past 70 years, from 12,000 tons in 1941 to 39 tons in 2006 (Saito and Mitsumata 2008). One to three thousand tons of matsutake are imported to Japan annually from countries such as China, North and South Korea, Canada, and the United States (see Wang 1995; Hosford et al. 1997; Wang et al. 1997; Saito and Mitsumata 2008). Increasing commercial harvests from natural populations worldwide, especially in developing countries, have raised major concerns over environmental consequences including sustainability issues.

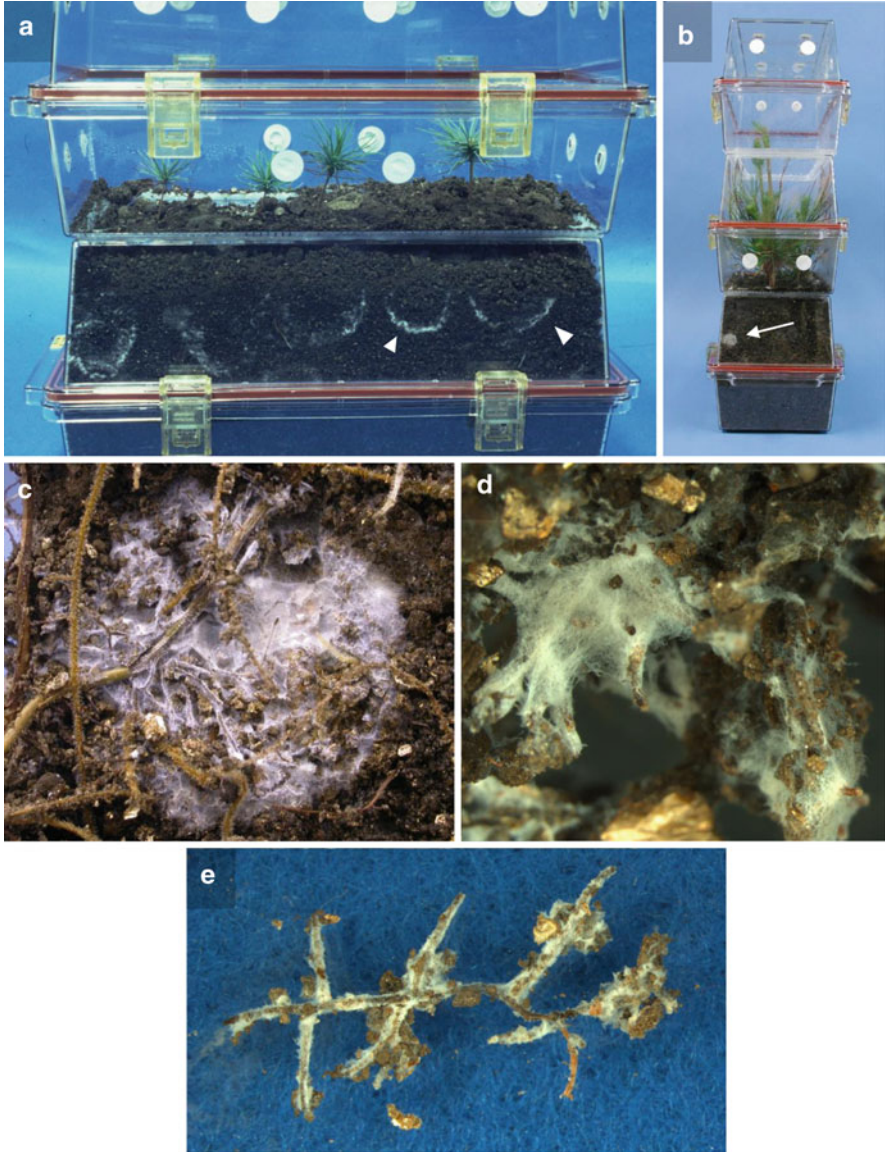
Attempts to cultivate *T. matsutake* began in Japan at the start of the twentieth century (Wang et al. 1997). Initial successes included germinating spores (Mimura 1909), obtaining pure cultures, synthesizing mycorrhizae (Masui 1927; Hiromoto 1963b), and inducing the formation of primordia in pure culture (Ogawa and Hamada 1975). Despite these achievements, however, all attempts to establish new shiros (the mycelium–soil–mycorrhizae aggregations that give rise to fruiting bodies) in pine forests using artificially mycorrhized plants have failed. This led Kawai and Ogawa (1981) to conclude that the formation of fruiting bodies under artificial conditions was 'hopelessly difficult.' Furthermore, several authors suggested that the relationship between matsutake and its hosts should be considered as parasitic or pathogenic rather than mycorrhizal (Masui 1927; Ogawa 1978; Tominaga and Komeyama 1987; Wang and Hall 2006).

Although Masui (1927) described 'mycorrhizae' formed in nature by matsutake on *P. densiflora* roots, convincing photographs of Hartig nets were lacking in literature reporting successful mycorrhizal synthesis with matsutake

(e.g., Tominaga 1963; Hiromoto 1963b; Ogawa 1978; Yokoyama and Yamada 1987; Eto 1990; Hu 1994). These were subsequently provided by Wang (1995). The development of improved microscopy and staining techniques has allowed more detailed analysis of the ectomycorrhizae formed by matsutake in natural shiros of *P. densiflora* forests in Japan by Yamada et al. (1999a) and Gill et al. (1999, 2000). Both groups concluded that matsutake formed structures consistent with typical ectomycorrhizae including fungal sheaths and well-developed Hartig nets. Gill et al. (2000) used molecular markers to further demonstrate that the EM structures observed in situ were due to matsutake. However, these authors also stressed that the commonly observed presence of black, necrotic, root cortical tissues in aging mycorrhizae may reflect a unique behavior of *T. matsutake* that is not typical of other EM fungi.

These field observations were soon substantiated by the in vitro synthesis of ectomycorrhizae between *T. matsutake* and *P. densiflora* seedlings and the demonstration of the rapid formation of well-developed Hartig nets (Yamada et al. 1999b; Guerin-Laguette et al. 2000b; Vaario et al. 2000). The clarification of the mycorrhizal status of *T. matsutake* and the improvement of methods for mycorrhization of *P. densiflora* seedlings indicated the feasibility of cultivating the fungus in association with host plants under controlled conditions. However, all attempts to transfer plants mycorrhized in vitro by *T. matsutake* to open pots in nurseries led to regression of the mycorrhization (Wang 1995; Yamada et al. 2002). The ‘acclimatization’ of mycorrhized plants to non-sterile environments appears to be a major obstacle to the successful cultivation of matsutake. Ogawa (1978) suggested that a critical mass of mycelium on host roots may be required to sustain further development of the fungal colonies. Guerin-Laguette et al. (2003b) and Yamada et al. (2006) used large-scale in vitro cultivation systems to obtain well-developed mycorrhizae and shiro-like aggregates. It was also shown that the incorporation of surfactants (e.g., Tween 80) or olive oil into soil-containing substrates stimulated the formation of a shiro-like mycelium in pure culture (Guerin-Laguette et al. 2003b). While mycorrhization was still achieved under these conditions, the presence of these additives impacted negatively on seedling growth, therefore limiting their use in matsutake cultivation. Guerin-Laguette and Matsushita (unpublished, see also Fig. 16.2) developed large culture systems (ca. 5–15 L vessels) using low amounts of such adjuvants. Although mycorrhizae and large shiro-like aggregates were formed in culture vessels for over 3 years (Fig. 16.2), no further development was observed following the transplantation of seedlings under non-sterile conditions (Guerin-Laguette et al. unpublished). In South Korea, a method for producing matsutake mycorrhized pine seedlings in vitro was developed and patented (Park et al. 2007). Mycorrhized seedlings prepared using this technique have been out-planted into the field, but the persistence of mycorrhizae has not been monitored (Koo, personal communication). More recently, Kobayashi et al. (2009) were successful in retaining matsutake mycorrhizae for 2 years in the field on a small percentage of out-planted *P. densiflora* seedlings following in vitro mycorrhizal synthesis. This significant result was supported by positive DNA typing and re-isolation of matsutake mycelium from mycorrhizae. However, all studies





**Fig. 16.2** Long-term in vitro mycorrhization of *Pinus densiflora* by *Tricholoma matsutake* (Guerin-Laguette and Matsushita, unpublished). (a) Matsutake mycelium (arrowheads) growing from the inoculum 5 months after inoculation. (b) Host growth and shiro-like development (arrow) 20 months following inoculation. (c, d, and e) Close-up of shiro-like aggregates and mycorrhizae 3 years after inoculation

observed a progressive decline in matsutake mycorrhizae in out-planted seedlings. Such limited persistence therefore remains the most important issue to overcome for successful cultivation of *T. matsutake*.

Few researchers have attempted the synthesis of *T. matsutake* mycorrhizae under non-sterile conditions using pure mycelial inoculum. Guerin-Laguette et al. (2005) inoculated mature red pine trees in situ in a Japanese forest near Hiroshima and reported the successful formation of ectomycorrhizae. Results were confirmed by characterization of Hartig nets and PCR detection of matsutake in the inoculated roots. However, the development of mycorrhizae beyond the initialization of the Hartig net was not observed, suggesting that the quantity of inoculum may need to be increased. Similarly, Shindo and Matsushita (2009) succeeded in establishing mycorrhizae of *T. matsutake* under non-sterile conditions on *P. densiflora* seedlings and saplings. Although mycorrhiza formation was confirmed by microscopy and PCR analyses, details of any further development were not reported.

An alternative approach to cultivation of EM fungi relies on the natural mycorrhization of seedlings. In the case of *T. matsutake*, seedlings have been successfully mycorrhized following their planting in naturally established shiros, a method similar to the traditional Talon's technique used for truffle cultivation (see Hall et al. 2007a). Many researchers in Asia have used this technique with success to obtain mycorrhizal seedlings (e.g., Tominaga 1973; Ogawa 1978; Kareki 1980; Lee et al. 1984; Lee 1988b; Ogawa and Ito 1989; Masuhara 1992). Although previous attempts to establish new shiros with plants mycorrhized using this method have failed (e.g., Ogawa and Ito 1989; Lee 1988b), recent results in South Korea have shown promise (Ka, personal communication).

#### **16.2.4 Ecological Management of Natural Populations of *T. matsutake* and *T. magnivelare***

Despite the general lack of success in using artificially mycorrhized plants for establishing *T. matsutake* in plantations, considerable progress has been made by Japanese researchers in developing methods to maximize production in forests where the species occurs naturally. Such methods include reducing the litter layer to 30-mm depth by raking and removal of shrubs and large trees to allow adequate aeration and sunlight to reach the forest floor (Ogawa and Ito 1989). Koo and Milek (1998) suggested that an economic and efficient way to manage matsutake forests was to locate shiros and manage the local environment around the shiros every year rather than the whole forest. Modifying the soil humidity and temperature by erecting irrigated plastic tunnels over shiros (the so-called Hiroshima method) stimulated fruiting (Tominaga 1975; Tominaga and Komeyama 1987). In South Korea, plastic tunnels have been replaced with small plastic hoods or caps to cover shiros or individual fruiting bodies (Lee 1988b). Park et al. (1995) reported that weather conditions had a significant impact on the annual production of matsutake in South Korea, especially rainfall and temperature. Vegetation management was also shown to increase matsutake production although the effects only lasted for 3 years (Park et al. 1997). Research on *Tricholoma matsutake* in China (e.g., Wang

and Xie 1982; Pu et al. 1982; Chen 1983; Wei et al. 1985; Liao et al. 1991; Gao and Dai 1996; Fu et al. 1996, 1999; Gong et al. 1999; Zhou 2002) mainly concentrated on the ecology of the species and the identification of methods for stimulating fruiting in natural forests. Gong et al. (1999) reported that the number of shiros and fruiting bodies increased by 9.8 % and 44.7 %, respectively, in experimental plots in Yunnan following 3 years of management of matsutake forests.

Economic management factors can also affect matsutake production in forests. In Japan it was shown that habitat improvement was most effective and matsutake production greatest on community-owned lands managed under a traditional bidding system (“iriai”) of land use. Habitat deterioration and poor yields occurred on private land where harvesting and selling rights were controlled by owners (Saito and Mitsumata 2008). Similarly, recent studies in China and Bhutan suggest that community-based management of natural matsutake areas offers the best potential to ensure sustainable exploitation of the fungus, which in turn can contribute to the development of rural communities whose livelihood is extremely dependent upon this resource (Amend et al. 2010a; Brooks and Tshering 2010). However, Faier (2011) points out that the many obstacles arising from social, cultural, and ecological factors render the improvement of a commodity exchange such as matsutake extremely difficult.

White matsutake (*T. magnivelare*) are produced abundantly in the forests of the Pacific Northwest coast regions of the USA, British Columbia, and Eastern Canada. Commercial harvesting of white matsutake started 30 years ago and has increased significantly in the last 20 years (Hosford et al. 1997; Berch and Wiensczyk 2001). Environmental consequences of commercial harvesting of the species have aroused public concern and provoked ecological studies aimed at providing information on how to manage matsutake forests and encouraging local bodies to enforce regulations to manage harvesting (Berch and Wiensczyk 2001; Pilz and Molina 2002). Results from such studies suggest that careful hand-picking of *T. magnivelare*, as opposed to more destructive raking techniques, is less likely to jeopardize future resources of the fungus (Luoma et al. 2006).

### ***16.2.5 Basic Research Contributing to a Better Understanding of Matsutake Ecology, Biology, and Physiology***

The development and application of DNA-based markers to improve the understanding of the biology, ecology, and reproduction of matsutake is an area that has made considerable progress over the past 10 years. Four main types of molecular markers have been developed (1) ribosomal DNA spacers (Kikuchi et al. 2000; Guerin-Laguette et al. 2002), (2) retroelements and related markers (Murata et al. 1999), (3) microsatellites (Lian et al. 2003), and (4) single nucleotide polymorphisms (SNP) (Xu et al. 2007; Amend et al. 2009). Molecular markers have been used to identify *T. matsutake* mycorrhizae from naturally established



populations (Kikuchi et al. 2000; Gill et al. 2000) or following artificial inoculation in the laboratory (Murata and Yamada 1999) and the field (Guerin-Laguette et al. 2005). The use of molecular markers has also provided information on the genetic structure and distribution of natural populations. Polymorphisms in the IGS1 spacer region and the uneven distribution of corresponding ribotypes were revealed in Japanese populations of *T. matsutake* (Guerin-Laguette et al. 2002). Markers based on ribosomal DNA are convenient to use but have limited application due to their low variability (Chapela and Garbelotto 2004; Matsushita et al. 2005). Microsatellites, retrotransposon-based markers and SNPs have all provided overwhelming evidence that, in nature, matsutake outcrosses and establishes from basidiospores, as inferred from high genetic variability of matsutake populations observed even within individual fairy rings or shiro (Murata et al. 2005; Lian et al. 2006; Amend et al. 2009, 2010b). Although the cytological events of sexual recombination have yet to be observed, the hypothesis that sexual reproduction and recombination play an important role in natural populations of *T. matsutake* is now widely accepted. Furthermore, an analysis of microsatellite data showed, for the first time, that a single matsutake genet could colonize multiple *P. densiflora* trees (Lian et al. 2006). SNPs have also been used to demonstrate the effect of environmental factors such as forest age (Amend et al. 2009) or landscape features (Amend et al. 2010b) on the shaping of natural populations of *T. matsutake*. Another application of molecular markers was demonstrated by the development of PCR systems based on the retroelement sigmamarY1 that allowed traceability of matsutake mushrooms from the main producing areas in Asia (Murata et al. 2008; Xu et al. 2010). Use of these techniques may resolve important commercial issues such as counterfeiting and help to rationalize the international trade of *T. matsutake* and other high-value gourmet mushrooms.

The saprophytic potential of *T. matsutake* was first discovered by Hiromoto (1963a, b). Later analysis of shiro development revealed features typical of saprophytic basidiomycetes, including the formation of chlamydospores and hyphal coil apices that may trap nematodes (Wang 1995; Wang et al. 1997; Wang and Hall 2006). This fungus has also been shown to produce a range of extracellular enzymes including amylases, cellulases, and proteinases (Terashita and Kono 1987; Terashita et al. 1995; Hur et al. 2001). Vaario et al. (2002) showed that matsutake could utilize *P. densiflora* bark as the sole carbon source in various cultural conditions and produce  $\beta$ -glucosidase allowing degradation of plant cell walls. Recently, Vaario et al. (2011a, b) carried out a number of field and laboratory experiments showing that *T. matsutake* was able to synthesize hemicellulolytic enzymes and use *Pinus sylvestris* L. root bark as the sole carbon source. The pool of available hemicellulose and the activity of enzymes contributing to its degradation were significantly higher in the shiro soil than in nearby control sites. These results demonstrate that *T. matsutake* is a facultative saprobe in vitro and in situ. Despite these recent observations, all attempts to produce *T. matsutake* fruiting bodies using methods for cultivating saprotrophic fungi have failed (Kawai and Ogawa 1981; Ogawa and Ito 1989), although primordia have been produced on solid media (Kawai and Ogawa 1976; Ogawa 1978; Wang 1995).

Only a few studies have further examined the relationships between matsutake and its host plants during *in vitro* mycorrhizal synthesis. Under conditions of nil or low levels of exogenous soluble sugar, no negative effects of *T. matsutake* mycorrhization on *P. densiflora* growth have been reported (Yamada et al. 2006, 2010), and stimulation of host growth has been demonstrated (Guerin-Laguette et al. 2004). Recent studies have shown the compatibility between several conifer species and *T. matsutake* isolates from diverse geographical locations (Yamada et al. 2010; Vaario et al. 2010).

Difficulties in cultivating matsutake may be related to a range of complex biological factors, including interactions with other microorganisms in the soil. In a recent study, Vaario et al. (2011b) identified several fungi (*Tomentellopsis*, *Piloderma*) and bacteria (*Thermomonosporaceae*, *Nocardia*, *Streptomyces*) which correlated positively with the presence of matsutake in mixed forests dominated by *P. sylvestris* in Finland.

## 16.3 *Lactarius*: Saffron Milk Cap (*Lactarius deliciosus*) and Other Species

### 16.3.1 Edible Mycorrhizal Mushroom Species in *Lactarius*

The ectomycorrhizal genus *Lactarius* includes a large number of edible species, amongst which *L. deliciosus* (saffron milk cap) is probably the most widely known. *Lactarius deliciosus* is a member of *Lactarius* section *Deliciosi* (Fr.:Fr.) Redeuilh, Verbeke & Walley. Members of this section share the typical *Lactarius* characteristics of flesh with brittle consistency and the presence of milky latex. Species are primarily characterized by the color of their latex which ranges from dingy yellow to bright orange (e.g., in the case of *L. deliciosus*), vinaceous red, brown, and indigo blue (Romagnesi 1958; Hesler and Smith 1960; Nuytinck and Verbeke 2007). Species of this section are found naturally throughout the Northern Hemisphere. Although many species are morphologically similar, molecular analyses suggest that intercontinental conspecificity is generally low and that there may be as many as 38 taxa worldwide within sect. *Deliciosi* (Nuytinck et al. 2007). In Europe, nine species are currently accepted (Nuytinck and Verbeke 2007). Although *L. deliciosus* is widely considered an excellent EEMM species (De Román and Boa 2006; Ortega-Martínez et al. 2011), the primarily Mediterranean *Lactarius sanguifluus* (Paulet: Fr.) Fr., and its close relatives are often more sought after by European connoisseurs (Borgarino and Hurtado 2001). Other popular edible mushrooms in this section are the vinaceous milk species *Lactarius vinosus* (Quél.) Bat. and *Lactarius semisanguifluus* R. Heim & Leclair in Europe (Borgarino and Hurtado 2001); *Lactarius hatsudake* Tanaka and *L. akahatsu* in Asia (Imazeki et al. 1988); *Lactarius rubrilacteus* Hesler & A. H. Smith in North America (Arora 1986; Wang 1993); and the two unusual blue species, *Lactarius*

*indigo* (Schwein.) Fr. and *Lactarius subindigo* Verbeken & E. Horak (Flores et al. 2005; Nuytinck et al. 2007) in America and Asia, respectively.

In the Southern Hemisphere, *L. deliciosus* has been reported in Chile (Valenzuela 2003) and in Australia (Dunstan et al. 1998; Wang et al. 2002), where its presence is the result of the introduction of exotic conifer species from the Northern Hemisphere. In New Zealand, *L. deliciosus* is only present as a result of the application of cultivation technologies (Wang et al. 2002, 2011).

All European species of *Lactarius* sect. *Deliciosi* form mycorrhizae with coniferous hosts. These are mainly represented by various *Pinus* spp. but also include *Picea*, *Abies*, and *Larix* spp. with several well-documented cases of host specificity: *L. deterrimus* Gröger with *Picea* spp., *Lactarius salmonicolor* R. Heim & Leclair with *Abies* spp. and *Lactarius porninsis* Rolland with *Larix* spp. (Courtecuisse and Duhem 1994). Worldwide, *Pinus* species are the most common hosts although these are also found in other coniferous genera including *Pseudotsuga* and *Tsuga* (Nuytinck et al. 2007). *Lactarius indigo* and *L. subindigo* are associated with both Pinaceae and hardwood species (Flores et al. 2005).

Although sect. *Deliciosi* is probably the richest section of *Lactarius* in terms of gourmet species, there are other noteworthy edible species in the genus such as *Lactarius volemus* (Fr.) Fr. and the related species *Lactarius hygrophoroides* Berk. & M. A. Curtis in Asia and America (Arora 1986; Imazeki et al. 1988; Hall et al. 2007b; Sicard and Lamoureux 2001). *Lactarius lignyotus* Fr. is an attractive species with a velvety appearance that has been considered as one of the best mushrooms in Jura, France, and Quebec (Chaumeton et al. 2000; Sicard and Lamoureux 2001). Other *Lactarius* species are renowned for their fragrance and used as flavoring, e.g., the candy cap or *Lactarius camphoratus* (Bull.) Fr. and related species (Arora 1986; Boa 2004). In total, Boa (2004) mentions 59 taxa in *Lactarius* that are known to be edible or have medicinal properties.

Research on the cultivation of *Lactarius* species has focused on *L. deliciosus*, mainly due to its popularity in Europe where the mushroom is widely collected and traded. It is particularly valued in Catalonia (de Román and Boa 2006), but is also popular in Southern France and in Eastern European countries. Another reason for ongoing research and commercial interest in the species is the considerable success obtained so far in cultivation attempts.

### 16.3.2 Mycorrhizal Synthesis with *Lactarius deliciosus*

Most methods used for the production of *L. deliciosus* mycorrhizal seedlings involve bringing actively growing vegetative inoculum into contact with receptive short roots of the host. *Lactarius deliciosus* grows slowly in pure culture (Melin and Norkrans 1948; Poitou 1978; Torres and Honrubia 1994), which led early workers to attempt mycorrhizal synthesis under aseptic conditions using nutrient-rich media (Riffle 1973; Poitou et al. 1984; Parladé et al. 1996). However, these methods only produced a limited number of mycorrhizae, and the process took at least 4 months

following inoculation (Guerin-Laguette et al. 2000a). Guerin-Laguette et al. (2000a) improved techniques for the growth of *L. deliciosus* vegetative inoculum and also showed that low-nutrient conditions without exogenous glucose were crucial for the rapid and extensive development of mycorrhizae under non-aseptic conditions in the laboratory. They further demonstrated that different nutrient conditions led to opposite effects of mycorrhization on growth of pine hosts. Host growth was shown to be stimulated by *L. deliciosus* mycorrhization under nutrient-depleted conditions and depressed under nutrient-rich conditions. Researchers in Spain and New Zealand have subsequently refined mycorrhization methods based on vegetative inoculum and developed practical, cost-effective techniques for nursery production of large numbers of mycorrhized seedlings (Wang et al. 2002; Carillo et al. 2004; Parladé et al. 2004; Díaz et al. 2009).

### 16.3.3 Field Cultivation of *Lactarius deliciosus*

Successful laboratory synthesis of *L. deliciosus* mycorrhizae was followed by mushroom cultivation trials based on field plantation of mycorrhized seedlings. The field cultivation of *L. deliciosus* was pioneered by Poitou et al. (1984, 1989), who first reported the successful production of fruiting bodies from *Pinus pinaster* Aiton seedlings planted in a former vineyard near Bordeaux. Mushroom production in this plantation has continued for at least 20 years (Savoie and Largeteau 2011). In Spain, Hortal et al. (2008, 2009) demonstrated the persistence of *L. deliciosus* on out-planted mycorrhized *P. pinea* seedlings, but fruiting body formation was not reported.

Research on *L. deliciosus* cultivation started at The New Zealand Institute for Plant & Food Research Limited in the late 1990s. Pure culture isolates were obtained from wild mushrooms collected from *P. sylvestris* forest in North Wales (I. Hall, personal communication). Cultures were imported to New Zealand and used to produce mycorrhized seedlings both under laboratory and greenhouse conditions (Wang et al. 2002). Mycelia grown on a solid substrate and mycorrhizal roots were found to be the most efficient vegetative inocula for the mycorrhization of pine seedlings. *Lactarius deliciosus* mycorrhized seedlings were out-planted 1–2 years following inoculation. Wang and Hall (2004) reported the first fruiting bodies of *L. deliciosus* in a New Zealand *Pinus radiata* D. Don plantation 18 months after planting mycorrhized seedlings. The fruiting of *L. deliciosus* seems to require a longer time in Europe than in New Zealand. In France, Poitou et al. (1984) obtained the first fruiting bodies 3.5 years after plantation in a former vineyard while, in mountainous areas, it may take up to 10 years following plantation of mycorrhized seedlings (Mousain, personal communication). Plantations of varying sizes have now been established on a range of sites around New Zealand including agricultural areas, timber plantations, and coastal sand dunes. These currently cover a total of approximately 100 hectares and include ongoing experimental trials maintained by Plant & Food Research (Wang et al. 2011). Climatic conditions of



**Fig. 16.3** Saffron milk caps (*Lactarius deliciosus*) in trial *Pinus radiata* plantations at Plant & Food Research, Lincoln, New Zealand

these sites vary from cool temperate areas in the south of the South Island to subtropical areas in the North Island. All plantations older than 3 years are now producing fruiting bodies every year (Fig. 16.3), with the first mushrooms obtained on average 2 years following plantation. A 6-year-old small plantation with only 30 trees in the South Island has produced over 100 kg of *L. deliciosus* mushrooms annually since 2009 (Wang et al. 2011). In a plantation of more than 800 trees in the North Island, each tree is now producing mushrooms (Wang et al. 2011). Irrigation has proven to be crucial for the persistence and the development of the mycorrhizae and subsequent mushroom production. Under New Zealand conditions, mechanical weed control is necessary to facilitate mushroom harvesting until tree canopy closure suppresses grass growth. So far, most plantations have been established with *P. radiata*, whose growth rate is very high in New Zealand (Burdon 2002). A small number of *P. sylvestris* seedlings inoculated with *L. deliciosus* have produced mushrooms 4 years after plantation (Guerin-Laguette et al. unpublished). Cultivation of *L. deliciosus* has reached a stage at which there is considerable potential for development as a new industry in New Zealand.

#### **16.3.4 Basic Research to Advance Cultivation Techniques for *Lactarius spp.***

Worldwide, tree seedlings mycorrhized with *L. deliciosus* have become increasingly available commercially. *L. deliciosus* is now a model species demonstrating the feasibility of cultivation of basidiomycete EEMMs in plantations. However, there is a need for continuing research to examine the long-term persistence of *L. deliciosus* in pine plantations and identify correct management practices to maximize yields.



#### 16.3.4.1 Section *Deliciosi*

There is a need to improve the mycelial inoculum technologies currently available for *L. deliciosus*. For example, the selection of strains showing high colonization ability (Guerin-Laguette 1998; Guerin-Laguette et al. 2003a; Parladé et al. 2011) would be worthwhile. Mycorrhiza formation on pine seedlings using mycelial inoculum was obtained for other edible species of *Lactarius* sect. *Deliciosi*: *L. akahatsu* and *L. hatsudake* (Yamada et al. 2001) and *L. indigo* (Díaz et al. 2007; Flores et al. 2005). Recently, mycorrhization of *P. sylvestris* and *P. nigra* by *L. sanguifluus* was confirmed for the first time by microscopy and molecular evidence (Mousain et al. 2010), but the degree of mycorrhization achieved with this species remained low. Research is required to improve mycorrhization techniques for *L. sanguifluus*, which grows much more slowly in pure culture than *L. deliciosus* (Guerin-Laguette et al. 2000a).

In comparison with mycelial technologies, spore inoculation would allow efficient large-scale production of mycorrhizal seedlings in nurseries. However, for many species of EM fungi, spore germination has yet to be demonstrated under controlled conditions (Miller et al. 1993), and only one report details the successful formation of mycorrhizae of *L. deliciosus* following spore inoculation (González-Ochoa et al. 2003). Recently, seedlings of *Pinus massoniana* Lamb. were successfully mycorrhized from spores of *L. hatsutake* in a Chinese nursery. Fruiting bodies were produced from 3 to 4 years following plantation, and average yearly production is now over 670 kg/ha (Tan et al. 2008). More research is needed to develop effective spore inoculation technologies.

#### 16.3.4.2 Other *Lactarius* Species

Recently, in Yunnan, China, Liu et al. (2009) fermented a spawn of *L. volemus* made from triturated mature fruiting bodies, which they injected into grooves dug under established (ca. 20-year-old) *Pinus kesiya* Royle ex. Gordon trees. They reported an increase in *L. volemus* production commencing 2–3 years following the spawn injection. Similar successes have been reported for *L. deliciosus* in Spain (M. Morcillos, personal communication). However, more research is needed to validate the efficacy of these methods for the production of edible *Lactarius* spp. in established woodlands.

### 16.4 *Rhizopogon*: Shoro (*Rhizopogon roseolus*)

*Rhizopogon* is a hypogeous (truffle) genus, with more than 100 species recorded in Europe (Martín 1996) and over 150 species worldwide (Trappe et al. 2009). Among these, only *Rhizopogon roseolus* Corda (synonym *R. rubescens* Tul.) (Fig. 16.4) has



**Fig. 16.4** Fruiting bodies of Japanese shoro (*Rhizopogon roseolus*) strains produced in a trial *Pinus radiata* plantation at Plant & Food Research, Lincoln, New Zealand. Scale bar bottom right = 2 cm

commercial value. The use of the fungus has been recorded in ancient Japanese and Chinese texts (Wang and Liu 2009). In Japan, *R. roseolus* is a delicacy known as shoro (Imazeki et al. 1988), and, 200 years ago, it was the fourth most commonly eaten mushroom in Japan (Okumura 1989). Boa (2004) lists two other *Rhizopogon* species as ‘edible’ (*Rhizopogon luteolus* Krombh. and *Rhizopogon piceus* Berk. & M. A. Curtis).

Shoro is now rare in Japan (Wang et al. 2002), and attempts were made to cultivate the fungus in plantation forests from the late 1980s. Mycorrhization of pine seedlings by shoro can be achieved using either spores or mycelial inocula. The production of mycorrhized seedlings from spore inoculum is very efficient and inexpensive and is recommended when producing seedlings on large scales.

In Shimane and Kyoto Prefectures in Japan, fruiting bodies were produced from mycorrhized seedlings in 1988 and 1991, respectively (Iwase, personal communication). Yamada et al. (2001) also reported the successful mycorrhiza formation on *P. densiflora* from pure cultures of four distinct isolates of *R. rubescens*, two of which also produced basidiocarps after the successful acclimatization of the associations in open-pot soil.

In the late 1990s, plantations were established in New Zealand using *P. radiata* seedlings mycorrhized with spores from locally collected strains that were thought to have been introduced to this country with their *Pinus* hosts (Wang et al. 2002). All plantations have since produced fruiting bodies (Wang et al. unpublished).

Shoro fruiting bodies produced in New Zealand plantations were shown to be morphologically distinct from their Japanese counterparts by Visnovsky et al. (2010). These authors subsequently analyzed ITS sequence variation in collections of *Rhizopogon* subgenus *Roseoli* Grubisha & Trappe from different geographic

locations, including Japan, New Zealand, Europe, and the United States. Collections were grouped into four distinct clades. New Zealand specimens were more closely related to those from the United States, suggesting that these originated from the initial introduction of *Pinus radiata* to New Zealand from its native California. In contrast, Japanese collections of *R. roseolus* clustered closely with European representatives. The variability encountered in the ITS region was used to design multiplex clade-specific PCR primers to allow the simultaneous detection of Japanese and New Zealand shoro strains.

Visnovsky et al. (2010) also produced *P. radiata* seedlings mycorrhized in vitro from pure cultures of *R. roseolus* imported from Japan. Nursery establishment and field persistence of the Japanese shoro strains were monitored using microscopy and the multiplex specific PCR primers. The molecular diagnostic technique was also used to demonstrate the successful fruiting of Japanese shoro about 1.5 years after establishment of a trial plantation in 2007. The trial has since fruited every year with nearly 100 fruiting bodies harvested from one tree in 2011 (Fig. 16.4, Guerin-Laguette et al. unpublished). A commercial plantation of approximately 400 trees mycorrhized by Japanese shoro strains was established in the North Island of New Zealand in 2007 using seedlings produced in vitro or from excised mycorrhizae.

## 16.5 Conclusion

Over the last 15 years, research on the cultivation of *Tricholoma*, *Lactarius*, and *Rhizopogon* species has made considerable progress as a result of the development of efficient mycorrhization methods, reliable identification techniques and improved taxonomic knowledge. The cultivation of *L. deliciosus* and *R. roseolus* is now ready for development at a commercial scale. However, further research is required to address several poorly explored areas such as plantation management for optimum yields and factors influencing the longevity of mushroom production. We suggest that the current success obtained with *L. deliciosus*, in particular, will contribute to the cultivation of EEMMs in general and will encourage further research towards the understanding of “recalcitrant” but economically valuable species such as *T. matsutake*.

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