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Alessandra Zambonelli
Gregory M Bonito *Editors*

Edible Ectomycorrhizal Mushrooms

Current Knowledge and Future Prospects

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Editors

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Preface

If you were to stroll through a marketplace in Kunming, Kyoto, or Krakow in autumn, like thousands of other cities and towns around the world, you would be sure to see wild mushrooms on display—chanterelles, porcini, milk caps, russulas, and maybe even truffles, if you are lucky. Many of these will be the fruiting bodies of fungi that live in a close relationship with the roots of trees. These are the edible ectomycorrhizal mushrooms or EEMMs for short.

Some of the EEMMs do not look very much like supermarket button mushrooms, but in this book, we have adopted Chang and Miles's broad definition of mushrooms as "any fungus with a distinctive fruiting body that is large enough to be. . . picked by hand." Thus, we include hypogeous fungi like truffles and shoro in this wide and commercially important group of fungi.

EEMMs comprise of more than 1,000 species and represent an important food and economic resource that until now has remained only partially explored. EEMMs are an important source of protein in developing countries. The exploitation of this resource also provides a source of income for local populations. On the other hand, some EEMMs, including truffles, porcini, chanterelles, and matsutake, are considered as food delicacies and are sold at prices higher than other food products in established and flourishing international markets. Many EEMMs also have beneficial nutraceutical and medicinal proprieties.

EEMMs live in the soil as mutualistic symbionts, nourished by roots of trees and shrubs, and, as do other ectomycorrhizal fungi, play important roles in maintaining forest ecosystem health and diversity. Moreover, EEMMs interact in the soil with other biota and microbes contributing to soil formation and nitrogen fixation. Many EEMM species also function in bioprotection and soil detoxification by sequestering heavy metals.

EEMM cultivation offers new sustainable agricultural possibilities for farmers struggling to increase their agricultural income. EEMM cultivation provides not only the general benefits of forestation with mycorrhized plants but also economical and social benefits through the production and harvesting of mushrooms and truffles with high market values. Success in these ventures, however, can only be achieved by taking into consideration the biology of these fungi and their complex ecological

interactions with other soil organisms. Modern tools including comparative genomics and high-throughput sequencing are providing new biological insights relevant to EEMM cultivation and with possibilities for genetic selection of fungal and plant strains used in inoculations.

This book is subdivided in four sections pertaining to the most important aspects of EEMMs the first section covers their systematics, biology, and ecology; the second focuses on their cultivation; and the third section covers economic and social aspects of wild collected EEMMs. In the final section, Francis Martin, who has led numerous EEMM genome sequencing projects, provides an epilogue on the EEMM industry in the age of genomics.

Each chapter of this volume has been written by internationally recognized scientists who have established research programs focused on EEMMs. We are particularly grateful to these authors for the high quality of their contributions. With their effort, we have produced the most complete and up-to-date treatment of EEMMs available. Moreover, most chapters include novel research findings that have not been previously published.

We are also grateful to the many scientists who generously assisted us in reviewing the content of this book. Peer review by contributors to this volume and external internationally recognized scientists helped to maintain the rigor and high quality of material presented. In particular we owe a debt of gratitude to Ian Hall, Matthew Smith, Mirco Iotti, Antonella Amicucci, Rino Ghelfi, Andrii Gryganskyi, Khalid Hameed, Jessy Labbé María-Soledad Benítez, Martin Ryberg, Nicola Sitta, and Rytas Vilgalys.

Finally, we thank Ajit Varma, series editor, for providing us with this great opportunity and allowing us to include color figures, and Jutta Lindenborn, life sciences editor at Springer, for the help, patience, and guidance regarding the preparation of this book.

We hope that this volume will serve as a useful guide for all the students and scientists interested in soil ecology, genetics, and the cultivation of EEMMs. The contents within contain the most complete scientific treatise on EEMMs and can be used as a starting point for initiating new studies on EEMMs or for deepening one's knowledge of these fungi. It is our goal that this book provides an impetus for new research and developments with EEMMs.

Bologna, Italy — Alessandra Zambonelli
Durham, NC — Gregory M Bonito

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Abbreviations

ACP	Acid phosphatase
AFLPs	Amplified fragment length polymorphisms
ALP	Alkaline phosphatase
AQP	Aquaporin
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethylsulphoxide
EEM	Edible ectomycorrhizal
EEMF	Edible ectomycorrhizal fungi
EEMM	Edible ectomycorrhizal mushroom(s)
EM	Ectomycorrhizal (fungi)
eMyCo	ectoMycorrhizal Community database
F1000	1000 Fungal Genome Project
FGP	Fungal Genomics Program
GSH	Glutathione
GST	Glutathione S-transferases
IGS	Intergenic spacer region
ITS	Internal transcribed spacer region
J.A.AD	J (Jeune = young in French), A (Adolescent = immature in French), AD (Adulte = adult in French)
JGI	Joint Genome Institute
M.R.T.	Reasoned methods of trufficulture
MAPK	Mitogen-activated protein kinase
MAT	Mating type
MGI	Mycorrhizal Genome Initiative
MHB	Mycorrhizal helper bacteria
MiSSPs	Mycorrhiza-induced small secreted proteins
MTs	Metallothioneins
MVOC	Microbial organic compound
NGS	Next generation sequencing
OUT	Operational taxonomic unit
PC	Phytochelatins

PCR	Polymerase chain reaction
PCW	Plant cell wall
PEG	Polyethylene glycol
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
ROS	Reactive-oxygen species
s.l.	sensu lato
SMC	Saffron milk cap
SNP	Single nucleotide polymorphisms
SOM	Soil organic matter
SSRs	Simple sequence repeats
TEs	Transposable elements
TGGE	Temperature gradient gel electrophoresis
TTSS	Bacterial type III secretion system
UHTS	Ultra-high-throughput DNA sequencing
VOCs	Volatile organic compounds

Part I
Biology and Ecology of Edible Ectomycorrhizal Mushrooms

Chapter 1
Laying the Foundations

Ian R. Hall and Alessandra Zambonelli

1.1 Introduction to Mycorrhizal Mushrooms

As children we were taught that trees have leaves to collect the sunlight and roots that take up water and nutrients from the soil. For many adults, this myth perpetuates. Indeed in the not too distant past, books and scientific papers dealing with soil science gave only scant attention to the fungal bridge between soil and roots that the majority of plants depend on (Smith and Read 2008). Partly in response to this, research on these combined structures formed by mycorrhizal fungi and plant roots has blossomed so the mycorrhizal literature now runs into tens of thousands of scientific papers and books, and entering "mycorrhiza" into Google will return about half a million hits.

There are several groups of mycorrhizal fungi of which the main types are the endomycorrhizal (includes the arbuscular and orchid groups) and ectomycorrhizal (EM) (Smith and Read 2008). The EM fungi form a combined structure with root tips, which resemble the finger of a glove—the finger being the root tip and the glove a layer of fungus that can make the root tips club shaped. It has been estimated that there are more than 20,000 species of EM fungi (Rinaldi et al. 2008). Some of the above-ground mushrooms are toxic with a few, like the death cap (*Amanita phalloides* (Vaill. ex Fr.) Link), deadly (Hall 2012; Hall et al. 2003a). However, more than 1,000 species produce edible above-ground mushrooms or belowground truffles with some having superb flavors and aromas (Hall et al. 2011; see Chap. 17). Almost all of the true truffles are edible, although a few have foul

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aromas and are unlikely to finish up on a plate, while a few others are mildly toxic. In this book, we have adopted the definition of mushroom as "any fungus with a distinctive fruiting body that is large enough to be . . . picked by hand" (Hall et al. 2003a after Chang and Miles 1992).

The vast majority of the 100 odd commercial species of edible mushrooms are cultivated on animal and/or plant waste in what are essentially large factories. These are the saprobic mushrooms (Chang 2008; Stamets 2000; Vedder 1978). In contrast, the edible mycorrhizal mushrooms can only be grown in specialised plantations on the roots of suitable trees and shrubs and then only with difficulty. Examples include *Tuber melanosporum* Vittad. (Périgord black truffle), *Tuber aestivum* Vittad. (burgundy truffle), *Tuber borchii* Vittad. (bianchetto truffle) and *Lactarius deliciosus* (L.) Gray (saffron milk cap). Supplies of the rest, such as *Tuber magnatum* Pico (Italian white truffle), *Boletus edulis* sensu lato (porcini), *Cantharellus cibarius* Fr. (chanterelle) and the birch boletes (*Leccinum* spp.), can only be collected from the wild. Despite this, the world markets for many of the edible mycorrhizal mushrooms are measured in billions of euros (Hall et al. 2003a, 2007; see Chap. 20).

This volume on the edible ectomycorrhizal mushrooms has an unashamedly applied focus, but with fewer than a dozen species of these obligate mycorrhizal fungi cultivated, the complex problems outlined in the chapters that follow are anything but prosaic. They explore the early attempts at cultivation, the relationships these fungi have with other soil microorganisms, the problems we still face and new technologies that might lay the foundations for more successful methods for cultivation.

1.2 Cultivation of Truffles

Chinese records show that *Auricularia auricula-judae* (Bull.) Quél. (wood ear) was the first mushroom to be cultivated around 600 AD (Miles and Chang 1997) some 1,000 years before the button mushroom was first cultivated in France around 1650 (Spencer 1985; de Tournefort 1707 in Ainsworth 1976). But it was 150 years later still before methods were devised for the cultivation of a mycorrhizal mushroom. The first method is usually attributed to Joseph Talon (Hall et al. 2007) from Saint Saturnin-lès-Apt, France. The story goes that it was Talon who discovered that if self-sown seedlings found under truffle trees were transplanted to a new area, eventually they too would produce truffles. However, in the small village of Beuxes near Loudun, in the Poitou-Charentes region of France, Pierre Mauléon (1744–1831), a miller, is credited with the same discovery in 1790—20 years before Talon (de Laplane 2000). Another method was described in 1807 in a report by Giovio to Canonico Giacomo Sacchetti, the secretary of the Siena Academy, in which seedlings were inoculated with pieces of truffle (Biblioteca di Campagna 10: 209; see Bruni 1891).

At the times when Mauléon, Giovio and Talon lived, there is little doubt that nothing of use could have been gained from scientific texts such as they were (Hall et al. 2007, 2009), and another half century was to pass before Gibelli (1883) was to observe mycorrhizas and Frank was to coin the term "Mykorrhizen" (Trappe 2004). None would have known anything of the scientific method of integrating previous knowledge using a pathway involving observation, hypothesis, predictions and experimentation (Carey 2011) and the rigors of experimental design and the statistical analysis of scientific data (Ramsey 2011; Ruxton and Colegrave 2006). As with many good ideas, it seems likely that their flashes of brilliance were anything other than just that.

Fortunately, the importance of Talon's technique, as it has become known, was recognised by Auguste Rousseau of Carpentras, Vaucluse, when he planted 7 ha of *T. melanosporum* truffière (a truffle plantation) in 1847 (Truffes.org 2011). Production began 12–15 years after planting and returned 500 French francs per hectare (Valserres 1867). Instead of keeping his adopted technique secret, as his predecessors had done, Rousseau publicised his success and received a *médaille de première classe* at the 1855 World Fair in Paris. Planting truffle trees rapidly spread through France and Italy as a source of income particularly for those with vineyards that had been affected by *Phylloxera*, first seen in the Rhône region in 1863 (Gale 2003), or silkworm diseases such as pebrine (*Nosema bombycis* Naeg.) that began destroying the silkworm industry in France about 1845 (Datta and Nanavaty 2005; Hume 1996).

Truffle trees were also used by Jean-Charles Eyraud, a Vaucluse mayor and François Tichadou, the inspector of the National Forestry Commission, to reforest the slopes of Mont Ventoux, Vaucluse. These slopes had successively been denuded first by the thirteenth-century shipbuilders and charcoal burners from prehistoric times through to the eighteenth century when coke became available for making iron. Then, once much of the forest had been removed, sheep farmers moved in and pastoral farming dominated (Rittersma 2010; Wikipedia 2011). By 1890, the heyday of the truffle industry, there were 750 km^2 of truffières in France (EDinformatics 2005), and Gaspard Adolphe Chatin (1892) gauged production to be 1,500 tonnes in 1868 and 2,000 tonnes by 1890. Annual production of *T. melanosporum* truffles in the Vaucluse alone was 380 tonnes in 1868, 450 tonnes in 1875 and more than 700 tonnes by the end of the century (Rittersma 2010; Truffes.org 2011). That "Talon's" technique is still being used with success by some in New Zealand is also testimony to its importance (Hulley 2011).

Dramatic falls in truffle production in the post WWI and WWII years (Hall et al. 2007) were taken very seriously in France and Italy. This resulted in an injection of research funds in an attempt to solve the problem. Because a single mature tree can have several mycorrhizal fungi on its root system, in addition to potentially beneficial organisms (Frey-Klett et al. 2010; Hall et al. 2003b; Rigamonte et al. 2010), Talon's technique can result in the production of plants that are infected with a mixture of fungi and other soil microorganisms, of which truffle *might* be one.

There is also the possibility that the transfer of pests, pathogenic fungi or other disease organisms from mother plant to seedling may occur. In order to establish more controlled and predictable methods for producing truffle infected trees, in the late 1960s and early 1970s, French and Italian researchers began establishing mycorrhizas with truffle spores, akin to the method described in the 1807 letter sent to Canonico Giacomo Sacchetti, although other methods were also tried (Chevalier and Grente 1973).

An early report of the success of the spore inoculation technique for *T. melanosporum*, *T. aestivum* and *Tuber brumale* Vittad. was published by Palenzona (1969). This method is now almost universally used for the commercial production of *T. aestivum*, *T. borchii* and *T. melanosporum* truffle mycorrhized plants. Briefly, clean seedlings or cuttings are inoculated with about 10^7 spores per plant. The plants are then raised in a greenhouse or shade house and then hardened off before transplanting into truffières (Hall et al. 2007, 2009). However, the spore inoculation technique is only rarely an effective method for *T. magnatum*, an issue discussed elsewhere in this book (Chap. 9). There are also some inherent problems with the spore inoculation technique as discussed by Hall and Haslam (Chap. 11) and by Hall and Zambonelli (2012).

1.3 Cultivation of the Above-Ground Mycorrhizal Mushrooms

At the same time the French and Italian scientists were developing methods for producing truffle mycorrhized plants, others were addressing mycorrhizal problems in forestry. We must first start with a description of these because their research and findings went hand in hand with the subsequent work on the cultivation of the above-ground edible mycorrhizal mushrooms.

Early physiological experiments between the 1930s and 1960s by Baylis, Bjorkman, Burjeff, Harley, Hatch, Melin, Mikola and others showed that many plant species could not grow unless they were mycorrhizal (see Smith and Read 2008 for references). The earliest demonstration that we can find of the economic importance of mycorrhizas was by R. E. Lawrence in 1939 (Gilmore 1958). He recognised that chlorosis of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) in Golden Downs forest in Nelson, New Zealand, was probably caused by a lack of suitable mycorrhizas and cured the problem by applying duff collected from an established Douglas fir stand at Hanmer Springs 300 km to the south. In 1958, John Gilmore repeated this work and concluded that poor growth of Douglas fir in a nursery and subsequently in the field "appeared to be due to the absence of proper (mycorrhizal) inoculation, certain unfavorable nursery practices and possibly periodic anaerobic soil conditions". Eventually it was also recognised that other nursery practices, such as the use of soilless or heated potting mixes devoid of mycorrhizal fungi and used to avoid weeds and pathogen problems, were not conducive to mycorrhizal formation (Mexal 1980; Hall and Perley 2008). It was also recognised that spores of mycorrhizal fungi that might blow into a greenhouse through vents

and doors or might be resident in bare root nurseries cannot be relied on to ensure adequate mycorrhizal formation. However, much information remained unpublished, and solutions to problems had to be reinvented as they arose (Hall and Perley 2008; Swale 1998).

Mycorrhizal inocula available to foresters are soil or duff from old bare root nurseries, positioning nurseries adjacent to well-infected "mother" trees, basidiospores and pure cultures (Mexal 1980). The first is now generally rejected as too risky because the inocula could also introduce pests and pathogens, and the second because it is impractical. Spores of *Pisolithus arhizus* (Scop.) Rauschert (= *Pisolithus tinctorius*) were widely researched in the 1970s and 1980s in the USA (Marx 1976, 1991; Marx and Barnett 1974; Marx et al. 1979), and *Rhizopogon parksii* A.H. Sm. and *Rhizopogon roseolus* (Corda) Th. Fr. (=*Rhizopogon rubescens*) spores are now almost universally used in New Zealand forestry nurseries to ensure mycorrhizal formation on Douglas fir and radiata pine (*Pinus radiata* Don), respectively. In the 1970s, spores were also used to inoculate seeds (Theodorou and Bowen 1973) although we are not aware if this is now used commercially.

Unfortunately, the spores of many mycorrhizal species do not germinate readily. Further, because basidiospores are meiotic products, their effects on the plant are less predictable. With the exception of sequestrate fungi it would seem that spores are unlikely to be an efficient method for infecting plants with the majority of the edible ectomycorrhizal mushrooms. Consequently, many of the researchers in the 1970s and 1980s used cultures produced from dikaryotic sporocarps of various easily manipulable fungi to establish EMs (Giomaro et al. 2005; Molina 1979a, b; Molina and Palmer 1982; Riffle and Tinus 1982). Mason (1980) first isolated the cultures onto Hagem's or a modified Melin-Norkran's medium with glucose rather than sucrose and then maintained them on agar slopes (Mason 1980). Alternatively, the fungi were grown on solid substrate such as a peat-vermiculite mixture moistened with Ingestad's medium (Mason 1980).

In the pioneering work on above-ground edible mycorrhizal mushrooms by Nicole Poitou and colleagues at the Institut National de la Recherche Agronomique (INRA) near Bordeaux, techniques similar to Mason's (1980) and Molina and Palmer's (1982) were used to cultivate *Suillus granulatus* (L.) Roussel and *Lactarius deliciosus* (saffron milk cap) (Guinberteau et al. 1990; Poitou et al. 1983, 1984, 1989). Nicole Poitou removed small portions of very young sporocarps, placed these on nutrient agar and then incubated. Contaminated cultures were either cleaned up or rejected before permanent cultures were maintained on nutrient agar. These cultures were then used to inoculate sterile seedlings, and, once mycorrhized, field trials were established. The first success was with *S. granulatus* in 1982, 2 years after planting 2-year-old trees (Poitou et al. 1984). By 1985, a third of the inoculated trees were productive and yielded 2.5–3 kg of sporocarps each. In 1983, the INRA group also successfully produced *L. deliciosus*. Half of the trees were productive with each yielding 0.5 kg (Poitou et al. 1984, 1989). For both species irrigation increased production. The methods employed a decade later in New Zealand by Wang Yun and Ian Hall (Wang et al. 2002; Hall et al. 2003b) followed a similar route and were also successful (see Chap. 16).

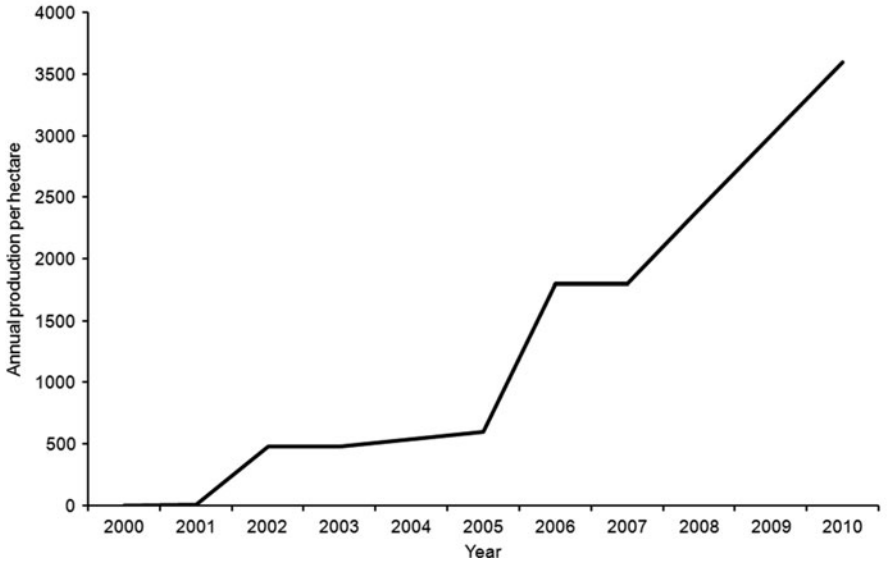

Fig. 1.1 Annual fresh weight production of saffron milk cap mushrooms (*Lactarius deliciosus*) per hectare (not cumulative) on inoculated *Pinus radiata* near Nelson, New Zealand, from planting in 2000 to 2010

1.4 Bringing Forestry and Mycorrhizal Mushroom Production Together

Growing edible mycorrhizal mushrooms in specialised plantations makes economic sense with a crop as expensive as truffles but perhaps not with mushrooms that sell for a few tens of euros per kilogram. It was because of this that attempts have been made to grow the above-ground edible mycorrhizal mushrooms as secondary crops in forestry plantations. This has been met with some reluctance by foresters who naturally are focussed on tree growth, wood and economic returns measured over decades. However, the demonstration that a relatively cheap mushroom like the saffron milk cap is capable of producing more income in just the 10th year after planting than the value of the timber at maturity (Fig. 1.1; Hall and Zambonelli 2012) could see plantation forests as a potential source of food as well as simply a source of wood or carbon credits. Similarly, co-cropping truffles with timber and nut and timber crops also has potential (see Chap. 15).

1.5 Life Cycle of the Truffles

As early as the 1950s, croziers had been observed by Lillian Hawker and Dave Minter in truffles (Fig. 1.2; Hall et al. 2010; Hawker 1954; Minter 1985), and in some species of truffle, asci containing eight spores were known (Montecchi and

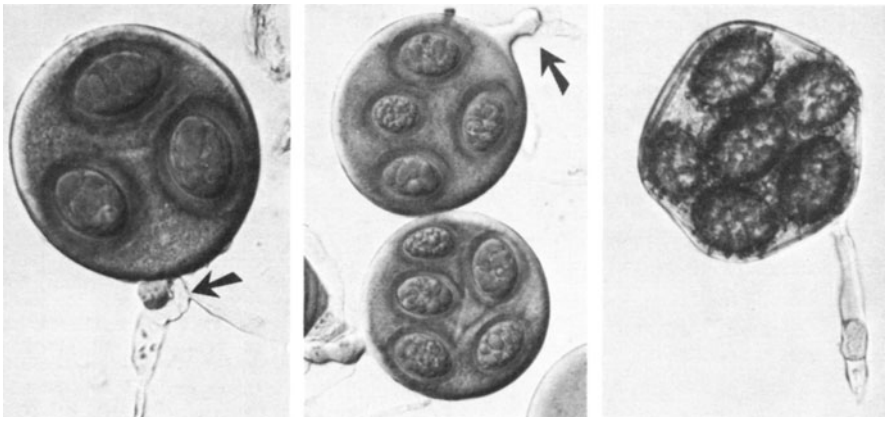

Fig. 1.2 Truffle croziers, evidence of sexual reproduction, photographed by Dave Minter in 1985 (also see Hall et al. 2010)

Lazzari 1993). From this it should have been apparent that truffle spores were almost certainly produced sexually like other genera in the Pezizales, and the vast majority of ascomycetes for which the full sexual life cycle has been observed (Alexopoulos et al. 1996). Strangely, this escaped the attention of many who expressed doubt about whether truffles outcross. Only recently did molecular biologists surprise the world by finding evidence in support of outcrossing in truffle fungi (Rubini et al. 2011a, b; see Chap. 4).

1.6 Direction of Research Over the Past 20 Years

During the 1990s and 2000s, research on the cultivation of the above-ground edible mycorrhizal mushrooms has been anything but spectacular. Research tended to be either taxonomic, peripheral and not directed at their cultivation or simply a quest for new knowledge and understanding. To illustrate this point, we searched the Mycorrhiza Literature Exchange Web site (http://mycorrhiza.ag.utk.edu/) for "*Boletus*" between 1991 and 2009. In 1994, there was one hit for a taxonomic paper (Nagasawa 1994), one in 1995 for a new find of *B. edulis* in New Zealand (Wang et al. 1995) and a review of *B. edulis* in 1998 (Hall et al. 1998). It was not until 2004 that a paper was published about trying to increase the productivity of *B. edulis* (Salerni and Perini 2004). This was followed by two taxonomic papers using molecular methods (Leonardi et al. 2005; Mello et al. 2006), one characterising the mycorrhizas of *B. edulis* (Águeda et al. 2006) and another on the effects of mycorrhizas on a damping off fungus (Martin-Pinto et al. 2006). The following year yielded a paper on phytochelatins (Collin-Hansen et al. 2007) and one on salt shock (Liang et al. 2007) in *B. edulis*. Finally, in 2007, our attention was

drawn to the puzzling absence of *B. edulis* mycelia in a forest producing porcini (Peintner et al. 2007), two papers on the synthesis of mycorrhizas of *B. edulis* (Águeda et al. 2008; Fu et al. 2009) and one on the anatomy of tuberculate mycorrhizas of *Boletus rubropunctus* Peck (Smith and Pfister 2009). Of the abstracts from the prestigious International Conference on Mycorrhiza at Berkeley in 1996, Uppsala in 1998 and Adelaide in 2001, which are included on the Mycorrhiza Literature Exchange Web site, only two papers on *Boletus* were presented.

Considering the genus *Boletus* contains *B. edulis*, one of the most commercially important edible ectomycorrhizal mushrooms (EEMMs), with an annual market almost certainly in excess of one billion euros, one can hardly conclude that it received adequate scientific attention between 1990 and 2009. Similarly, over the same period, there were only seven applied papers on *Cantharellus* and ten papers related to the cultivation of *Lactarius* spp. Admittedly there were other forums where papers on edible mycorrhizal mushrooms were presented which were not included on the Mycorrhiza Literature Exchange Web site, for example, the North American Conference on Mycorrhizae (e.g. Fort Collins 1979, Quebec 1981, Bend 1984, Gainesville 1987, Jackson 1990), European Symposium on Mycorrhizae (Dijon 1985, Prague 1988, Sheffield 1991, Granada 1994), the Spoleto conferences on truffles (1968, 1988, 2008) and the International Workshop on Edible Mycorrhizal Mushrooms (IWEMM—Uppsala 1998, Christchurch 2001, Vancouver 2003, Murcia 2005, Chuxiong 2008, Rabat 2011). However, significant papers presented at these meetings, and at many other conferences, were very likely published in scientific journals as well to gain maximum exposure. Undoubtedly, there is also considerable work that remains unpublished, perhaps for commercial reasons (e.g. Hall and Zambonelli 2012; Guerin-Laguette et al. 2011). However, to have so few published papers on the cultivation of EM mushrooms in the past 20 years, out of a total of more than 11,000 publications on mycorrhizas in the Mycorrhiza Literature Exchange database, shows that mycorrhizal research is heading in other directions.

In contrast to the above-ground mycorrhizal mushrooms, between 1991 and 2009, there were nearly 200 papers on the true truffles (*Tuber* spp.), the most expensive of the edible mycorrhizal mushrooms, with a third of these on applied areas of research. Perhaps it is also not surprising that the authors of some of the more noetic papers on *Tuber* did not let the reader go past their introductions without explaining that truffles can fetch obscene prices as justification for the research conducted.

As with much agricultural science, research on the cultivation of edible mycorrhizal mushrooms can be a slow process. For example, in New Zealand, it took a decade to produce the first Périgord black truffle and another decade to produce significant harvests in Australia. In New Zealand, it was made all the more difficult with a requirement to maintain confidentiality so that the maximum return on the investment might be achieved, whereas funding was based not on the final outcome—the establishment of a new crop—but on numbers of scientific papers,

a requirement introduced halfway through the long-term project. This is not the type of topic and timescale that a university supervisor is likely to hand to a PhD student. Instead, academia, scholarship, short-term achievable projects and the chase for impact factors are more likely to be research drivers in a university setting. This might explain the paucity of publications describing long-term research on mycorrhizal mushrooms on the Mycorrhiza Literature Exchange Web site.

1.7 Unlocking the Secrets of Edible Mycorrhizal Mushrooms

No matter how applied we might wish to be in search of the holy grail—the cultivation of the edible mycorrhizal mushrooms—it became clear in the 1980s that some issues would not be resolved unless there was a method to study how these fungi interacted with their host plants and other soil microorganisms under controlled conditions. Ideally, this had to be an easily repeatable, cheap and simple laboratory system where eventually other organisms could be introduced into the microcosm. Then, bit by bit, the immensely complex interacting web of EEM fungi, other fungi, bacteria, insects, microfauna, the roots of the host plant and those of adjacent plants could be unravelled (Kuhad et al. 2004; Griffiths et al. 2007; Bonfante and Anca 2009).

The system developed in the late 1980s and 1990s by Alessandra Zambonelli and colleagues involved growing micropropagated plants together with the mycorrhizal fungus in pure culture (Zambonelli et al. 1989). This was similar to a method developed by Randy Molina (1979a, b) but incorporated features that ensured the survival of the very slow growth characteristic of the majority of edible mycorrhizal mushrooms. *Populus alba* L. was chosen as the host plant and *T. borchii* the EEM fungus. Later Davide Sisti and colleagues (1998) replaced poplar with *Tilia platyphyllos* Scop. to achieve a better understanding of the molecular mechanisms in mycorrhizal development and regulation (Polidori et al. 2002; Guescini et al. 2003; Menotta et al. 2004). At the same time, numerous Italian researchers were developing new molecular tools for tracking mycorrhizas in soil ecological studies in the laboratory, greenhouse and natural environments, to supplement classical morphological methods (Amicucci et al. 1998, 2000; Rubini et al. 1998; Mello et al. 1999).

A newer approach, which is already showing great potential in the study of complex ecosystems, is metagenomics, which is based on the extraction of nucleic acids (DNA or RNA) directly from an environment. Although still in its infancy, it has already given us some insights into how, for example, *T. magnatum*, *T. melanosporum* and *Boletus* spp. interact with specific sets of soil organisms, as is described in more detail in the following chapters (Mello et al. 2010; Napoli et al. 2008, 2010; Peintner et al. 2007).

1.8 Conclusions

The cultivation of EEMMs has made considerable progress over the past 200 years, yet, like much of agricultural research, progress has been pedestrian. This research has concentrated on the truffles, the most expensive of the EEMMs, while the cultivation of the vast majority of above-ground edible mycorrhizal mushrooms has been completely neglected.

Some useful findings have come from recent molecular studies of EM fungi, including sequencing the *T. melanosporum* genome. This genome sequence will serve as a valuable resource for future studies regarding the biology and ecology of *Tuber*. It is anticipated that metagenomics and comparative genomics of other edible EEMMs will stimulate new frontiers of activity. We urge that this is done in concert with more long-term research projects focused on the cultivation of EEMMs.

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Chapter 2
Systematics and Ecology of Edible Ectomycorrhizal Mushrooms

Matthew E. Smith and Gregory M. Bonito

2.1 Introduction

2.1.1 Humans and Edible Fungi

Humans have consumed fungi for sustenance, medicine, and culinary delight since ancient times. Some fungi are purposely cultivated, but most edible fungi are gathered from the wild. The Romans and Greeks treated mushrooms as a special kind of food (Miles and Chang 2004), and there is historical evidence of mushroom consumption in ancient India (Chopra 1933) and Mesoamerica (Ruan-Soto et al. 2006). There are also reports of the use of fungi by the indigenous cultures of South America (Henkel et al. 2004), Africa (van Dijk et al. 2003), Australia (Trappe et al. 2008), and Asia (Yun et al. 1997). The rapid emergence of mushrooms and other fungal fruiting structures has long been shrouded in mysticism, as suggested by common names for fungi such as "toadstool," "Elvin saddle," "witches' butter," and "fairy ring."

There are deeply held superstitions and misunderstandings regarding mushrooms and truffles. For example, the Romans believed that truffles were the result of lightning strikes or thunderclaps; truffles were collected both because of their strong flavors but also because of their status as "mysterious products of the earth" (Hall et al. 2007). Similarly, mushrooms that fruit in circular patches or "fairy rings" were said to be the paths left by fairies that danced in the night (Morgan 1995). Nonetheless, through keen observation of fungi in their natural habitats, humans have observed that some fungi depend directly on the dung, wood, insects, or grains where they are found. However, the fruiting of mushrooms and truffles

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directly on soil and not associated with particular substrates has long remained a perplexing mystery. Part of the problem is that most fungi spend the majority of their life cycle growing in opaque substrates such as soil or wood, and this makes them particularly challenging to study. For many fungi, their sporocarps are the only visual cue to alert humans to their presence. However, fungal hyphal networks abound underfoot whenever we walk through forests and other natural habitats.

2.1.2 Discovering the Ectomycorrhizal Symbiosis

A major breakthrough in understanding the biology of soil- and root-dwelling fungi came in 1885 when German biologist A.B. Frank published an important paper on the association between truffles and trees (Frank 2005). He suggested that many soil-dwelling, plant-associated fungi were actually living in a mutualistic symbiosis with trees. He coined the term "mycorrhiza" (from the Greek linguistic roots "myko" meaning fungus and "rhiz" meaning root) to explain this close relationship. Although the debate about the validity and significance of mycorrhizas continued over the ensuing decades, we now understand the key ecological roles that mycorrhizas play in most ecosystems on earth (Smith and Read 2008). Biologists currently recognize at least four distinct types of mycorrhizal associations (e.g., ectomycorrhizas [EM], arbuscular mycorrhizas, ericoid mycorrhizas, and orchid mycorrhizas), but only EM fungi routinely produce large, fleshy fruiting bodies that are consumed by humans. Recent estimates suggest that at least 6,000 plant species and 20,000 fungal species are involved in this ecologically and economically important symbiosis (Rinaldi et al. 2008; Brundrett 2009).

A.B. Frank's original research focused on EM associations of truffles (*Tuber*, *Ascomycota*), but subsequent work has shown that many different groups of fungi form ectomycorrhizas, including species of Ascomycota, Basidiomycota, and at least one order in the Zygomycota (Endogonales, Mucoromycotina) (Tedersoo et al. 2010). Because EM fungi are often difficult to isolate in pure culture (Melin 1954) and challenging to work with under laboratory conditions (Palmer 1969), much of the early EM research was observational in nature or necessarily focused on a few fungi that could be readily manipulated [e.g., *Pisolithus arhizus* (Scop.) Rauschert (= *Pisolithus tinctorius*), *Rhizopogon* spp.] (Melin and Krupa 1971; Marx 1977). Studies from fumigated nursery soils demonstrated early on that at least some plants depend on EM fungi for normal growth and reproduction (e.g., *Pseudotsuga menziesii* (Mirb.) Franco) (Trappe and Strand 1969). EM fungi have subsequently been found to be integral for plant nutrition (particularly the acquisition of N and P) as well as in the mitigation of drought stress and root pathogens (Marx 1970; Parke et al. 1983; Smith and Read 2008).

2.1.3 *Edible Plant-Associated Fungi that Do Not Form Ectomycorrhizas*

Although EM fungi fruit from soil in association with plant roots, the simple occurrence of a fungal fruiting body with tree roots does not prove that the fungus is an EM symbiont of plants. There are several well-known cases where edible mushrooms consistently fruit near particular plants but are not EM symbionts. One example is the distinctive "Ash Bolete," *Boletinellus merulioides* (Schwein.) Murrill, which only fruits near trees in the genus *Fraxinus* (Ash). Despite belonging to the Boletineae (Boletales, Basidiomycota), a phylogenetic group with many EM species, this fungus does not form ectomycorrhizas but instead forms a commensalism with insects that feed on *Fraxinus* roots (Brundrett and Kendrick 1987). Morels (*Morchella* spp., Morchellaceae, Pezizales, Ascomycota) are another example. Morels are often found in association with plants that do not form ectomycorrhizas (e.g., *Malus* [apple], *Ulmus* [elm], and *Liriodendron* [tulip poplar]) or in coniferous forests that have been recently burned (Winder 2006). Although morels have been shown to associate biotrophically with roots under some conditions (Dahlstrom et al. 2000), they are not found in EM community studies, they can survive as sclerotia, and they do not need a host plant to complete their life cycle (Ower 1982; Miller et al. 1994).

2.2 Ectomycorrhizal Fungi and the Molecular Revolution

Since the time of A.B. Frank, the challenges in determining the ecology and trophic mode of fungi based on observational data were a significant obstacle. However, PCR-based molecular approaches for studying EM fungi have clarified our understanding of these important microbial plant symbionts (Horton and Bruns 2001). Since pioneering molecular studies of EM fungi in the 1990s (Bruns et al. 1989; Gardes and Bruns 1996), DNA-based tools have figuratively "peeled back the soil" to reveal important insights about three key aspects of EM fungi: (1) community ecology, (2) population biology, and (3) molecular systematics.

2.2.1 *Community Ecology*

The first molecular tools that helped elucidate the biology of EM fungi were basic "DNA fingerprinting" techniques such as RFLP (restriction fragment length polymorphism). These techniques are relatively inexpensive and can be used to identify EM fungi from sporocarps or directly on EM roots (Gardes and Bruns 1996). For species-level identification, basic DNA fingerprinting approaches have now been largely supplanted by DNA sequence-based methods that typically offer

higher accuracy and resolution (e.g., DNA barcoding). DNA barcoding refers to the sequencing of a particular locus that is considered to be species specific and can thus be used to identify fungi at the species level based on the DNA sequence. The internal transcribed spacer region (ITS) of the ribosomal DNA (rDNA) has long been the marker of choice for ecological studies of fungi, and this DNA region has recently been recognized as the "official barcode" for the kingdom (Seifert 2009; Schoch et al. 2012). ITS rDNA barcodes provide direct identification of morphological EM types (e.g., "morphotypes") when they can be directly matched to reference sequences from well-identified specimens. When direct matches are not made, ITS rDNA sequences provide at least some phylogenetic signal to help identify the fungal lineage to which the EM species belongs. In cases where ITS sequences are not sufficient for delimiting species, other informative loci may be targeted [e.g., ribosomal large subunit (LSU or 28S), elongation factor (EF1α), second subunit of RNA polymerase (RPB2)].

Molecular fungal community studies based primarily on ITS rDNA have provided several important insights about the biology of EM fungi: (1) the diversity of EM fungi is higher than expected (both at species and lineage levels); (2) species common as sporocarps are not always abundant on roots; (3) species that are frequently found on roots are not always encountered as sporocarps; (4) many abundant fungi as EM on root tips make inconspicuous sporocarps that are resupinate or sequestrate (truffle-like); (5) it is common to encounter cryptic species within fungal community studies such that two or more distinct, divergent species exist within one morphological species; (6) biogeographic patterns of EM fungi are more similar to those of plants and animals than those of *Bacteria* and *Archaea*; (7) local EM community structure is strongly influenced by dispersal, host plant species, and resource availability; and (8) different phylogenetic groups respond differently to resource fluctuations, disturbance, and management (Horton 2002; Lilleskov et al. 2002; Peay et al. 2007; Smith et al. 2007a, b; Diaz et al. 2009) (see also Chap. 7).

2.2.2 Population Genetics

One big mystery for biologists interested in the ecology of EM fungi has been to discern "what is an individual EM fungus and what does it do?" For example, does an individual EM fungus produce many sporocarps or just a few? Do they encompass large areas of forest or do they exist in small patches? Do they fruit each year or is fruiting reserved for optimal conditions? Throughout the last several decades, while molecular studies were elucidating the community structure of belowground EM fungi, population genetic studies were clarifying the size of fungal genets (i.e., fungal individuals) and dispersal patterns of many key EM fungi. A recent paper on population genetics of EM fungi exhaustively reviews the subject (Douhan et al. 2011), so we will address the topic only briefly here.

Population genetics studies of EM fungi first used culture-based somatic incompatibility tests to examine genet size and distribution on the landscape. However,

because this approach depends on living axenic cultures, culture-based studies were limited to only those fungal species with significant saprotrophic abilities. Furthermore, variable molecular markers such as RAPDs (randomly amplified polymorphic DNA) have been shown to be superior to culture-based approaches for delimiting fungal genets (Jacobson et al. 1993). More recent studies have utilized microsatellites, which have the advantage that they are specific, nondominant markers that can be used to study EM fungi directly on roots, as sporocarps or as pure culture isolates (Kretzer et al. 2005).

Although most EM fungi population studies have focused on a few EM lineages (e.g., /hebeloma, /boletus, /laccaria, /tricholoma, /russula-lactarius—see below), they have revealed great variations in population structure and in life-history strategy between members of different lineages. For example, species of *Amanita*, *Laccaria*, *Hebeloma*, and Russulaceae tend to have small genets (usually less than 5 m across), whereas species of *Boletus*, *Suillus*, and other Boletales tend to have large genets (often 10–20 m across but sometimes up to 100 m in diameter). Similarly, members of the EM lineages differ in their ability to persist in the environment. Some groups appear to survive only 1–3 years and are continually establishing new individuals from spores (e.g., Russulaceae, *Laccaria*), whereas other groups have genets that are perennial and may persist up to 35 years in some cases (e.g., many Agaricales and Boletales) (Baar et al. 1994). These patterns are interesting, especially when considered along with other phylogenetically linked traits (e.g., EM exploration strategies—Table 2.1).

Population genetic studies have definitely provided important insights into the diversity of life-history strategies among EM fungi, but there are still many aspects that have not been adequately addressed. In particular, unexplained variation within lineages and even genera suggests that additional studies are needed. In addition to the problem of variation within and among groups, more than two-thirds of the population studies on EM fungi have been conducted in the USA or Europe, and only a few tropical EM fungi have been studied from a population biology perspective (Douhan et al. 2011). Furthermore, many fungal lineages and genera that produce economically important sporocarps have yet to be studied (e.g., *Terfezia* and *Tirmania* spp. in the /terfezia-peziza depressa lineage, *Craterellus* and *Hydnum* spp. in the /cantharellus lineage).

2.2.3 Molecular Systematics

While molecular markers were being used to explore the community ecology and population biology of EM fungi, similar molecular tools were being paired with phylogenetic methods and advances in computing power to map the evolutionary tree of life (Bruns et al. 1989). Although PCR and DNA sequencing have fundamentally changed our understanding of the entire evolutionary tree of life over the last 20 years, no branch of the tree has been reorganized more thoroughly than the kingdom Fungi (James et al. 2006). Once thought to have evolved only a handful of

Table 2.1 Genera and lineages of ectomycorrhizal fungi with edible fruiting bodies

Genus	EM lineage	Order (Phylum)	Edibility	Ectomycorrhizal exploration type	References
Afroboletus[2]	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Boa (2004)
Albatrellus	**/albatrellus**	Russulales (Basidiomycota)	E	Ms	Arnolds (1995)
Alpova[3]	**/paxillus-gyrodon**	Boletales (Basidiomycota)	E	L[5]	Trappe et al. (2007)
Amanita[1,3,4]	**/amanita**	Agaricales (Basidiomycota)	$	Ms	Arnolds (1995)
Arcangeliella[3]	**/russula-lactarius**	Russulales (Basidiomycota)	E	C, S, Ms[5]	Trappe et al. (2007)
Aureoboletus	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Arora (1986)
Austroboletus[2]	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Bessette et al. (2000)
Balsamia[3]	**/tuber-helvella**	Pezizales (Ascomycota)	E	C	Trappe et al. (2007)
Bankera	**/tomentellopsis-bankera**	Thelephorales (Basidiomycota)	E	L	Boa (2004)
Barssia[3]	**/tuber-helvella**	Pezizales (Ascomycota)	E	C[5]	Trappe et al. (2007)
Boletellus	**/boletus**	Boletales (Basidiomycota)	E	L	Bessette et al. (1997)
Boletopsis	**/incertae sedis**	Thelephorales (Basidiomycota)	E	Mm	Arora (1986)
Boletus	**/boletus**	Boletales (Basidiomycota)	$	L	Arora (2008)
Cantharellus	**/cantharellus**	Cantharellales (Basidiomycota)	$	Ms	Arnolds (1995)
Catathelasma[2]	**(/catathelasma)**	Agaricales (Basidiomycota)	E	No data	Arnolds (1995)
Choiromyces[3]	**/tuber-helvella**	Pezizales (Ascomycota)	E	C[5]	Arnolds (1995)
Chroogomphus[2]	**/suillus-rhizopogon**	Boletales (Basidiomycota)	E	C, S	Arnolds (1995)
Clavariadelphus[4]	**/clavariadelphus**	Gomphales (Basidiomycota)	E	No data	Arora (1986)
Clavulina	**/clavulina**	Cantharellales (Basidiomycota)	E	S or Mf	Arora (1986), Ma et al. (2010)
Cortinarius (incl. *Rozites* and *Dermocybe*)[1,3]	**/cortinarius**	Agaricales (Basidiomycota)	E	S, Mf, Ms	Arora (1986)
Craterellus	**/cantharellus**	Cantharellales (Basidiomycota)	$	Ms	Arnolds (1995)
Cystangium[2,3]	**/russula-lactarius**	Russulales (Basidiomycota)	E	C, S, Ms[5]	Trappe personal communication
Entoloma s. str.[3,4]	**/entoloma**	Agaricales (Basidiomycota)	E	Mf	Co-david et al. (2009)
Fistulinella[2]	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Bessette et al. (2000)
Gastroboletus[3]	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Trappe et al. (2007)

Gautieria[3]	**/ramaria-gautieria**	Gomphales (Basidiomycota)	E	Mm	Trappe et al. (2007)
Genea[3]	**/genea-humaria**	Pezizales (Ascomycota)	E	S	Trappe et al. (2007)
Geopora[3]	**/geopora**	Pezizales (Ascomycota)	E	S	Trappe et al. (2007)
Gomphidius	**/suillus-rhizopogon**	Boletales (Basidiomycota)	E	Ms	Arnolds (1995)
Gomphus	**/ramaria-gautieria**	Gomphales (Basidiomycota)	E	Mm	Arora Perez-Moreno et al. (2008)
Gymnomyces[3]	**/russula-lactarius**	Russulales (Basidiomycota)	E	C, S, Ms[5]	Trappe personal communication
Gyrodon s. stricto	**/paxillus-gyrodon**	Boletales (Basidiomycota)	E	L[5]	Bessette et al. (2000)
Gyroporus	**/pisolithus-scleroderma**	Boletales (Basidiomycota)	E	L[5]	Arora (1986)
Hebeloma	**/hebeloma-alnicola**	Agaricales (Basidiomycota)	E	Mf	Perez-Moreno et al. (2008)
Heimioporus (syn. *Heimiella*)	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Bessette et al. (2000)
Helvella	**/tuber-helvella**	Pezizales (Ascomycota)	E	C	Arora (1986), Perez-Moreno et al. (2008)
Hydnotrya[3]	**/hydnotrya**	Pezizales (Ascomycota)	E	C	Trappe et al. (2007)
Hydnum	**/cantharellus**	Cantharellales (Basidiomycota)	E	Mf	Arnolds (1995)
Hygrophorus	**/hygrophorus**	Agaricales (Basidiomycota)	E	C	Arnolds (1995), Perez-Moreno et al. (2008)
Hysterangium	**/hysterangium**	Hysterangiales (Basidiomycota)	E	Mm	Trappe et al. (2007)
Imaia[2,3]	**/leucangium**	Pezizales (Ascomycota)	$	No data	Trappe et al. (2007)
Inocybe[1,3]	**/inocybe**	Agaricales (Basidiomycota)	E	S	Kuyper (1986)
Kalapuya[2,3]	**/leucangium**	Pezizales (Ascomycota)	E	No data	Trappe et al. (2010)
Laccaria[3]	**/laccaria**	Agaricales (Basidiomycota)	E	Mf	Bessette et al. (1997), Arora (1986)
Lactarius	**/russula-lactarius**	Russulales (Basidiomycota)	$	C, S, Ms	Arora (1986)
Leccinellum[2]	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Boa (2004)
Leccinum	**/boletus**	Boletales (Basidiomycota)	$	L	Arnolds (1995)
Leucangium[3]	**/leucangium**	Pezizales (Ascomycota)	$	C	Trappe et al. (2007)

(continued)

Table 2.1 (continued)

Genus	EM lineage	Order (Phylum)	Edibility	Ectomycorrhizal exploration type	References
Lyophyllum[4]	**/paralyophyllum**	Agaricales (Basidiomycota)	E	Mf	Perez-Moreno et al. (2008), Yamada et al. (2001)
Macowanites[3]	**/russula-lactarius**	Russulales (Basidiomycota)	E	C, S, Ms[5]	Trappe personal communication
Melanogaster[3]	**/paxillus-gyrodon**	Boletales (Basidiomycota)	E	L	Trappe et al. (2007), Pena et al. (2010)
Mycoclelandia[2,3]	**/terfezia-peziza depressa**	Pezizales (Ascomycota)	E	C[5]	Trappe et al. (2008)
Otidea[3]	**/otidea**	Pezizales (Ascomycota)	E	S	Arnolds (1995);
Phylloporus	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Arora (1986)
Picoa[3]	**/geopora**	Pezizales (Ascomycota)	E	S	Arora (1986), Alsheikh and Trappe (1983)
Polyozellus[2]	**/pseudotomentella**	Thelephorales (Basidiomycota)	E	No data	Pilz et al. (2003)
Porphyrellus	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Boa (2004)
Pseudoboletus	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Bessette et al. (2000)
Pulveroboletus[2]	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Boa (2004)
Ramaria[4]	**/ramaria-gautieria**	Gomphales (Basidiomycota)	E	Mm	Perez-Moreno et al. (2008)
Reddellomyces[3]	**/tuber-helvella**	Pezizales (Ascomycota)	E	C[5]	Trappe et al. (2008)
Retiboletus[2]	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Bessette et al. (2000)
Rhizopogon[3]	**/suillus-rhizopogon**	Boletales (Basidiomycota)	E	L	Visnovsky et al. (2010)
Rubinoboletus[2]	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Boa (2004)
Russula	**/russula-lactarius**	Russulales (Basidiomycota)	$	C, S, Ms	Arnolds (1995)
Sarcodon	**/hydnellum-sarcodon**	Thelephorales (Basidiomycota)	E	Mm	Arnolds (1995), Barros et al. (2007)
Scleroderma[3]	**/pisolithus-scleroderma**	Boletales (Basidiomycota)	E	L	Boa (2004)
Strobilomyces	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Arora (1986)
Suillus[3]	**/suillus-rhizopogon**	Boletales (Basidiomycota)	E	L	Arnolds (1995)
Terfezia s. stricto[3]	**/terfezia-peziza depressa**	Pezizales (Ascomycota)	$	C[5]	Díez et al. (2002)
Thelephora	**/tomentella-thelephora**	Thelephorales (Basidiomycota)	$	Ms	Sha et al. (2008)
Tirmania[3]	**/terfezia-peziza depressa**	Pezizales (Ascomycota)	$	C[5]	Díez et al. (2002)

Tremellodendron	**/sebacina**	Sebacinales (Basidiomycota)	E	S[5]	Bessette et al. (1997), Pena et al. (2010)
Tricholoma	**/tricholoma**	Agaricales (Basidiomycota)	$	Mf, Ms, L	Arnolds (1995)
Truncocolumella[3]	**/suillus-rhizopogon**	Boletales (Basidiomycota)	E	L	Trappe et al. (2007)
Tuber[3]	**/tuber-helvella**	Pezizales (Ascomycota)	$	C	Arora (1986), Trappe et al. (2007)
Turbinellus	**/ramaria-gautieria**	Gomphales (Basidiomycota)	E	Mm[5]	Arora (1986) [as *Gomphus* spp.]
Tylopilus	**/boletus**	Boletales (Basidiomycota)	E	L	Arora (1986)
Ulurua[2,3]	**/terfezia-peziza depressa**	Pezizales (Ascomycota)	E	C[5]	Trappe et al. (2008)
Xanthoconium	**/boletus**	Boletales (Basidiomycota)	E	L[5]	Bessette et al. (2000)
Xerocomus	**/boletus**	Boletales (Basidiomycota)	E	L	Arora (1986)
Zelleromyces[3]	**/russula-lactarius**	Russulales (Basidiomycota)	E	C, S, Ms[5]	Trappe et al. (2007)

Genera with edible species are indicated by an E, whereas those with species considered choice edibles are indicated by a $. Genera known to contain both edible species and deadly poisonous species are indicated by a 1, genera suspected but not proven to be ectomycorrhizal are indicated by a 2, genera with some or all sequestrate species are indicated by a 3, and genera known to be polyphyletic with only some ectomycorrhizal species are indicated by a 4. Ectomycorrhizal exploration strategies are designated according to the classifications of Agerer (2001) as follows: contact (C), short distance (S), medium-distance smooth (Ms), medium-distance fringe (Mf), medium-distance mat (Mm), and long distance (L). Genera where the ectomycorrhizal exploration strategy was inferred based on the phylogenetic relationships in addition to data from related species are indicated by a 5

times, the EM habit is now known to have arisen independently in at least 66 different fungal lineages (Tedersoo et al. 2010). Given the multiple independent origins of the EM habit, it is likely that selection pressures and adaptations leading to the symbiosis were different for the various phylogenetic lineages. We expect that the similar EM structures that are common across the different lineages (e.g., the hyphal sheath and Hartig net) probably mask large functional differences between different EM fungal groups.

Fungal molecular systematics studies have also repeatedly shown that the morphology of fruiting structures is a highly plastic trait and that similar fruiting body plans have evolved many times in different EM fungal groups. For example, "agaricoid" fruiting bodies with a stipe and gills have arisen independently in the Agaricales (e.g., *Amanita*), Russulales (e.g., *Lactarius*), Boletales (e.g., *Phylloporus*), Gomphales (e.g., *Gomphus*), and Cantharellale*s* (e.g., *Cantharellus*), whereas "coralloid" forms can be found in Gomphales (e.g., *Ramaria*) and Cantharellales (e.g., *Clavulina*). Similarly, the sequestrate, truffle-like fruiting body plan has arisen independently many different times and is present in at least 94 different genera of EM fungi (Tedersoo et al. 2010). These exciting discoveries have been paramount for understanding fungal evolution but have caused massive and rapid reorganizations in fungal taxonomy (Hibbett 2007).

2.3 The Lineage Concept

In one of the first large-scale assessments of evolutionary relationships of euagarics (mushrooms), Moncalvo et al. (2002) established an informal, rank-free nomenclature for discussing monophyletic groups of any taxonomic level. In their notation system, the monophyletic lineage name is written in non-italicized lowercase letters and preceded with the symbol "/" (e.g., /amanita). The lineage-based concept is informal and therefore mediates some of the problems that arise due to polyphyly (e.g., multiple taxa with the same genus name are dispersed in multiple different lineages) and due to phylogenetic uncertainty (e.g., we sometimes know which genera belong to a particular lineage, but we do not always know how they are related to each other within that lineage).

2.3.1 Lineages of EM Fungi

A similar lineage-based concept was adopted by Tedersoo et al. (2010) in a recent review of global patterns of biogeography and evolution of EM fungi. Tedersoo et al. (2010) identified 66 monophyletic groups of EM fungi in the Ascomycota, Basidiomycota, and Zygomycota with different ages, diversity levels, and global distributions.

In this chapter, we use the same organizational concept of the rank-free EM lineages as Tedersoo et al. (2010) to examine the biology and ecology of the edible EM fungi within this framework. Specifically, we examine which EM lineages contain edible fungi and how many genera within each lineage are considered to have edible species. We also explore what is known about the systematics, ecology, and life-history traits of EM lineages that have edible species. Based on our findings, we suggest ways in which phylogenetic knowledge of the EM lineages can inform future decisions on how to best manage EM ecosystems for edible fungi.

Throughout this chapter, we assume that species traits are phylogenetically conserved, meaning that closely related species are more likely to be similar in terms of their biology and ecology than are more distantly related species (Felsenstein 1985). This is known to be true of EM fungi for many ecological, physiological, and morphological traits (Agerer 2006; Smith and Read 2008).

2.3.2 Lineages of EEMM

Currently, 66 unique lineages of EM fungi that belong to 15 orders of fungi are recognized (Tedersoo et al. 2010), but only 34 of these lineages contain species that produce edible sporocarps (Table 2.1). We define edible fungi as those species that are regularly consumed in at least one part of the world. Some EM fungi may be considered a delicacy in one country, but not consumed elsewhere (e.g., some species of *Thelephora* are a regularly eaten specialty in China but only rarely eaten in other parts of the world). Edible EM fungi are currently placed in eight orders: Agaricales, Boletales, Cantharellales, Gomphales, Hysterangiales, Pezizales, Russulales, and Thelephorales. Some of these orders contain a large number of edible species (e.g., Agaricales, Boletales, Pezizales), but taxonomic knowledge gaps prevent us from making reliable estimates of the true number of edible species at this time. We have relatively good information for economically important genera that have been recently studied (e.g., *Tuber*—see Jeandroz et al. 2008, Bonito et al. 2012), but many genera and lineages remain poorly known (e.g., *Clavulina*—see Henkel et al. 2011).

Although it is difficult to make definitive statements about the number of edible species within all EM fungi lineages, we are better able to assess the distribution of edibles at the genus level. By our estimation, there are 82 genera of EM fungi that contain at least one edible species. The majority of these genera belong to the *Basidiomycota* (64 genera), whereas fewer belong to Ascomycota (18 genera) (Table 2.1). Many of the 66 lineages contain edible species in only one genus (e.g., *Amanita*, *Genea*, *Hygrophorus*, *Hysterangium*), but others, such as the /boletus, /tuber-helvella, /russula-lactarius, /suillus-rhizopogon lineages, contain many genera with edible species. Enumerating edible EM fungi at the genus level is insufficient to give us a complete understanding of the diversity of edibles within each lineage, but it is a useful starting point that highlights the serious need for more taxonomic work in many groups.

The phylogeny in Fig. 2.1 depicts our current understanding of how the different EM fungal lineages that contain edible species are related to one another. This figure visually highlights some key relationships: (1) a high number of lineages with edible species are *not* closely related to one another; (2) Basidiomycota constitute the majority of the lineages having edible fruiting bodies; (3) edible EM taxa are particularly concentrated within the Agaricales, Boletales, and Pezizales; (4) the Agaricales includes the largest number of distinct EM lineages with edible species; (5) the /boletus lineage has the greatest number of genera with edible species; and (6) the relationships among some key groups are not yet well resolved (e.g., Thelephorales, Cantharellales).

2.4 Biology of EM Fungi

Given the estimated 400 million years of divergence between the Ascomycota and Basidiomycota (Taylor and Berbee 2006), we expect members of these two phyla to differ significantly in terms of their biology and life cycle.

2.4.1 Biology of the Ascomycota

Although the EM Ascomycota belong to many orders (including Eurotiales, Sordariales, and Helotiales), all of the edible EM Ascomycota belong to the Pezizales (Table 2.1). Many of the seven different EM Pezizales lineages with edible sporocarps appear to have evolved from pyrophilic ancestors or humus saprobes. Most are adapted to disturbed habitats, drought-prone environments, and fire. Biotrophic Pezizales are not thought to produce lignin-degrading enzymes, and most EM Pezizales appear poorly adapted for growth on wood or other heavily lignified substrates (Egger 1986). EM Pezizales do, however, grow well in mineral soils with low organic content and with a pH $>$ 7.0 (Bonito et al. 2012).

Ascomycota spend most of their lives as haploids (1 n); they can simply germinate from spores and begin active growth and EM formation as functional individuals (Bonito et al. 2012). Many of the EM Pezizales also produce conidia, although the dispersal and/or mating functions of these asexual propagules have not yet been shown (Urban et al. 2004; Healy, Smith and Bonito unpublished). Despite the fact that they evolved separately from one another, the edible EM Ascomycota appear to share a similar, yet not completely understood, mating biology (Varda Kagan-Zur and Roth-Bejerano 2008). They are characterized by the maintenance of two mating idiomorphs (1-1-1 and 1-2-1) and are thought to reproduce through heterokaryotic mating as detailed by Rubini et al. in Chap. 4. However, the number of species and lineages that have been studied is still small, and recent studies of species in the /terfezia-peziza depressa lineage indicate that there may be exceptions to these patterns (Varda Kagan-Zur and Roth-Bejerano 2008).

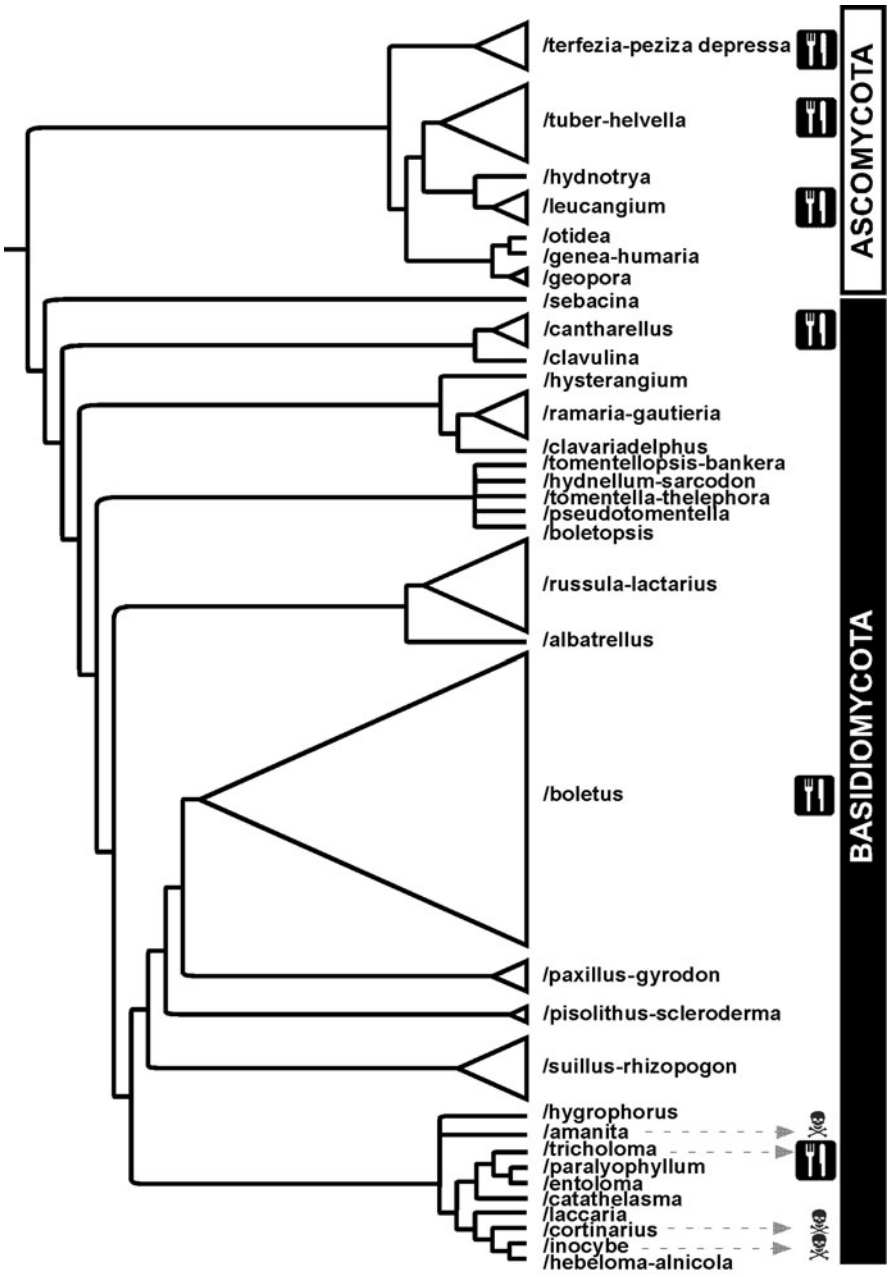

Fig. 2.1 Cladogram depicting the relationships between lineages of ectomycorrhizal (EM) fungi containing edible species. The size of the *triangle* scales to the number of genera in each lineage that contains edible species. Those lineages that also contain deadly species are depicted by the *skull* and *crossbones* symbol (e.g., *Amanita phalloides* (Vaill. Ex Fr.) Link; *Inocybe erubescens* Blytt; *Cortinarius orellanus* Fr.). The *dinner plate* symbol marks the three lineages of Ascomycota and Basidiomycota that contain the most sought-after and valuable species

All of the known pezizalean EM fungi that have been studied to date produce short-distance or contact-type ectomycorrhizas where their hyphae and cystidia grow close to plant roots. No Pezizales EM fungi are known to produce rhizomorphs, hyphal cords, or hyphal mats (Table 2.1). We hypothesize that the haploid life cycles of these EM Pezizales and their postfire evolutionary history preadapt these fungi for colonizing via spores. These traits also help to explain their success in tolerating, and at times flourishing, in disturbed habitats.

2.4.2 *Biology of the Basidiomycota*

In sharp contrast to Ascomycota, Basidiomycota EM are highly diverse in their mating biology, habitat preferences, and evolutionary histories. Most Basidiomycota produce haploid spores that must germinate and then presumably fuse with another haploid to form a dikaryon (prior to initiating ectomycorrhizas) (Horton 2006). Most EM basidiomycetes germinate in the presence of host plant roots, although germination rates are usually low and only select species are efficient at colonizing roots via spores (Ishida et al. 2008). These traits effectively limit the dispersal abilities of many EM Basidiomycota to within a few meters of a parental sporocarp (Li 2005). Exceptional basidiomycete species that establish well from spores have one or a few traits in common: they routinely produce multinucleate spores (e.g., *Laccaria* spp.), they have spores that are deposited *en mass* via animal mycophagy (e.g., *Rhizopogon* spp.), and/or they have good saprotrophic abilities (e.g., *Hebeloma* spp.) (Horton 2006; Ishida et al. 2008; Kawai et al. 2008).

The Basidiomycota EM lineages also share the important common trait that they appear to have evolved from wood or humus saprobes (Tedersoo et al. 2010). Unlike the pezizalean EM fungi, many (but certainly not all) EM basidiomycetes grow well in acidic soils with high organic matter content. Many EM basidiomycetes (including species in the /cortinarius, /hygrophorus, /ramaria-gautieria, and /russula-lactarius lineages) have been shown to produce lignin-degrading peroxidases (Bodeker et al. 2009) and also appear capable of degrading woody substrates. Although most basidiomycetes probably retain at least some wood-degrading capabilities, they exhibit a wide degree of variation in their dependence on host plants. Members of some genera are readily cultured on artificial media away from their host plants (e.g., species of *Laccaria*, *Hebeloma*, and *Suillus*), whereas members of other genera either have never been successfully propagated or die in pure culture or when transferred (e.g., species of *Tremellodendron* and *Inocybe*) (Iotti et al. 2005). Certain taxa may grow better on particular types of culture media (see Chap. 9).

As with Basidiomycota in general, the distantly related EM lineages likely exhibit wide diversity in their mating biology (Kües et al. 2011). However, because of the difficulties in germinating EM basidiospores and with culturing EM fungi in general (Ali and Jackson 1988), relatively few species have been studied in detail. The genus *Laccaria* has served as a model for studies of mating in EM fungi. Thus, we know that *Laccaria bicolor* (Maire) PD Orton has a tetrapolar mating system

with ca. 45 different mating types (Raffle et al. 1995; Niculita-Hirzel et al. 2008). In contrast, *Rhizopogon rubescens* Tul. and C. Tul. and several *Suillus* species have been shown to have bipolar mating systems (Fries and Sun 1992; Kawai et al. 2008). Unlike members of the Ascomycota, it is assumed that EM Basidiomycota generally do not produce asexual spores (e.g., anamorphs), based on evidence from in vitro culture studies (Hutchison 1989).

In contrast to the uniformly short-distance EM exploration types of the *Ascomycota*, the EM *Basidiomycota* exhibit a very wide variation in the morphology of the hyphae that emerge from ectomycorrhizas to explore for nutrients and roots to colonize. These exploration types range from contact or short-distance types for many species in the /hygrophorus, /inocybe, /clavulina, /sebacina, and /russula-lactarius lineages to the long-distance and/or tuberculate EM types in /bankera and in *Boletales* lineages (e.g., the /boletus, /pisolithus-scleroderma, /suillus-rhizopogon, and /paxillus-gyrodon lineages) (Agerer 2006). Several groups within Boletales have also been shown to regularly form sclerotia, although the biological function of these structures in relation to EM ecology is not known (Smith and Pfister 2009). Several basidiomycete EM lineages form extensive hyphal mats that are accompanied by mat-type EM formations (see Table 2.1; Dunham et al. 2007). Many of these extensively ramified EM systems are associated with highly organized rhizomorphs to less extensive hyphal cords.

2.5 Future Research and Management

2.5.1 Taxonomic Uncertainties

The exact number of EEMM species is not known at this time. This is due partly to the lack of taxonomic studies from certain parts of the world (e.g., West Africa, Southeast Asia) and also due to the large number of species complexes that still are in need of further study (e.g., *Boletus edulis* Bull.) (Dentinger et al. 2010). Basic taxonomic studies, coupled with complementary molecular data, will help to determine a realistic estimate of the number of edible EM fungal species. It is worthy to note how in even the most economically important fungal groups and in those with significant cultural value, true species diversity is unknown and underestimated. Prominent examples of this include the recent descriptions of several new choice edible species from well-studied regions of the Western USA (e.g., *Boletus rex-veris* D. Arora and Simonini, *B. regineus* D. Arora and Simonini, and *Cantharellus californicus* Arora and Dunham and *Cantharellus cascadensis* Dunham, O'Dell, and R. Molina) (Dunham et al. 2003; Arora 2008; Arora and Dunham 2008). In some cases, taxonomic uncertainties extend to generic level or higher. Genera such as *Entoloma*, *Amanita*, and *Lyophyllum* currently include both EM and saprotrophic edible mushroom species, making it difficult to enumerate the number of species with each of the different trophic modes (Tedersoo et al. 2010).

2.5.2 *Management of EEM Fungi*

Management decisions regarding EEMM should be influenced by knowledge of both the natural and evolutionary history of the particular EM species. Because lineages of EEMM are the result of evolutionary changes through an act of independent sequential events, these lineages are shaped by a range of varying selective factors. Coincidently, management strategies are expected to differ significantly between taxa and lineages. For example, the management of boletes belonging to the Basidiomycota may promote organic matter and soil acidity, while management of truffles belonging to the Ascomycota would generally avoid such conditions. Likewise, pioneer species that depend heavily on spores for dispersal require different management regimes than fungi that are sensitive to disturbance or better adapted for root colonization via mycelial spread (Peay et al. 2011).

2.5.2.1 Cultivated EM Fungi

It is interesting to note that species from only seven of the 34 EM fungal lineages with edible species have been cultivated (in some cases, by accident). These lineages include /tuber-helvella (see Chaps. 10, 11, 12 and 13), /terfezia (see Chap. 14), /cantharellus (Danell and Camacho 1997), /russula-lactarius (see Chap. 16), /suillus-rhizopogon (see Chap. 16), /tricholoma (see Chap. 16), and /laccaria (DiBattista et al. 1996). By far, the greatest successes in the cultivation of edible EM fungi have been with truffle fungi and *Tuber* in particular. Truffle fungi generally grow slowly in culture, but their spores can readily infect plant roots (see Chap. 9). Current cultivation practices start with the use of spore inoculum on appropriate host plant seedling roots, followed by outplanting, proper irrigation, and appropriate soil amendments (Le Tacon et al. 1982). Adapting similar approaches for basidiomycete mushrooms has proved extremely challenging for most edible EM. Despite the fact that many of these basidiomycete mushrooms grow readily in pure culture, their spores are generally not as effective in directly establishing ectomycorrhizas. Consequently, mycelium-based techniques for inoculating trees continue to be developed and improved for many edible EM basidiomycete mushrooms but also ascomycetes (Rossi et al. 2007). When inoculating seedlings with basidiomycete mycelium, it is important to use a mated, dikaryotic strain (such as that which arises from the vegetative tissue of a fruiting body) to ensure that fruiting is possible. Once introduced, specific genotypes can persist for years to decades (Henrion et al. 1994), depending on site conditions and the competitiveness of the given species on EM roots. Strategies for limiting competitive exclusion are also important. This can be done by introducing species where they are most competitive (e.g., within their natural range), by introducing them in areas with limited competition (e.g., enemy-release hypothesis) or by soil modification processes that reduce propagules and competitiveness of non-target taxa.

Future successes in EEMM domestication will likely include pioneer species adapted to disturbed habitats and EEMM species that can readily be cultured or inoculated onto roots via spores or mycelium. Pioneer EM fungi are adapted to grow well in younger aged and managed stands (e.g., tree plantations). Similar to how many animals have still not been domesticated even after many attempts certain lineages of EM fungi are probably not amenable to domestication, whereas others may be preadapted for domestication. Further studies are needed to ascertain the maturity conditions (how large an individual genet must be to initiate fruiting) and environmental cues that initiate the fruiting process in the different groups of fungi. Currently, the effect of genet size and diversity on fruiting body production and yield is unknown.

2.6 Conclusions

The majority of edible EM fungal lineages and species are currently only found in the wild. This includes many species of boletes, chanterelles, and even truffles (Wang and Hall 2004). Because nature provides the genetic resources for both cultivated and wild-collected EM species, policies that conserve natural habitats where these fungi grow and reproduce are critical. Strategies for simultaneously managing forests for both edible fungi and for timber have already been well articulated (Pilz and Molina 1996; Boa 2004; Trappe et al. 2009). Future research aimed at stimulating productivity and increasing yields from natural stands, and adaptive management approaches and refined techniques for habitat and niche modeling are needed to fully understand and conserve the diversity and productivity of EEMM (Molina et al. 2011).

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Chapter 3
Edible Ectomycorrhizal Mushroom Molecular Response to Heavy Metals

Sabrina Zeppa, Antonella Amicucci, Roberta Saltarelli, Giovanna Giomaro, and Vilberto Stocchi

3.1 Introduction

Heavy metals and metalloids are widespread in nature; they can be of natural origin, such as metalliferous rocks, or of anthropic activity origin, such as pollutions, and may be toxic for soil organisms (Zafar et al. 2007). Heavy metals are continuously being released into the environment as a result of industrial activities and technological development.

Contamination of groundwater and soil with heavy metals represents one of the major problems of pollution of natural environments and poses risks to human health. Heavy metals enter the food chain via agricultural products or contaminated drinking water (Çerbasi and Yetis 2001).

Another important consequence of heavy metal pollution is a significant decrease in saprobic and mycorrhizal fungal community diversity (Poma et al. 2006). As a consequence, this biodiversity reduction may determine a decreased litter decomposition rate and a nutrient reserve mobilization (Poma et al. 2006).

Given the dramatic consequences of soils polluted with heavy metals, for years, efforts have been made to develop methods for removing metal ions from aqueous solutions, such as chemical precipitation, ion exchange, electrochemical treatment, and membrane technologies. However, these conventional methods are ineffective and prohibitively expensive (Wang and Chen 2006). It is therefore an urgent need to develop new strategies for fast, economical, and environmentally suitable processes for removing heavy metals from the waste industry or originating from other sources.

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Recent research in this area has approached methodologies based on biosorption and bioaccumulation. The former is the use of non-living microorganisms to detoxify and control environmental contaminants (Farhadian et al. 2008), while the latter approach involves the use of living organisms that can survive in the contaminated environment and are able to accumulate metals.

Among soil organisms, fungi are highly resistant to extreme conditions of pH, temperature, and nutrient availability as well as heavy metal pollution and play important roles in element cycling, mineral transformations, and fungal–plant interactions (Gadd 2007). Fungal biomass has a high percentage of cell-wall material that shows excellent metal-binding properties; thus, it may be utilized by biosorptive processes (Zafar et al. 2007).

Because of these properties, fungi have been employed in the remediation of wastes and wastewaters, and a broad range of results have been reported (Lopez and Vazquez 2003). In fermentation and bioremediation industries, filamentous fungi have been widely used (Luef et al. 1991), having the advantage to be easily removed from liquid substrates.

More recently, there has been an increasing interest in mycorrhizal fungi. Mycorrhizal fungi participate in crucial symbiotic relationships with plants that grow on contaminated sites and alleviate metal toxicity for their host plants (Jentschke and Goldbold 2000; Schützendübel and Polle 2002). The mycorrhizal fungi show unique absorption and tolerance to heavy metals and are able to colonize degraded industrial areas that have high concentrations of metal ions such as Pb^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2+}.

As reported in the literature, mycorrhizal fungi protect the host plant from toxic compounds in the soil even if there are significant interspecific and intraspecific differences (Ouziad et al. 2005). Previous reviews have summarized the available information on amelioration of metal toxicity by EM associations (Rapp and Jentschke 1994; Leyval et al. 1997; Jentschke and Goldbold 2000). Mycorrhizal fungi may thus play an important dual role in metal homeostasis: scavenging of metal micronutrients and their supply to the host plant and detoxification of excess essential metals and non-essential metal ions as well. The mechanisms underlying the detoxification processes in filamentous and EM fungal cells, and their molecular basis, are not completely known at this time.

In EM fungi, the metal tolerance cell responses were described as extracellular or intracellular detoxification mechanisms. Extracellular mechanisms are mainly implied in avoidance of metal entry: polysaccharides and metabolites are involved in chelation and cell-wall binding, modifying the metal availability and toxicity. Intracellular systems aim to reduce metal burden in the cytosol; in particular, intracellular concentration of metals is modulated by metallothioneins (metal-binding proteins), vacuolar compartmentalization and chemical modifications (Gadd 1990). Additional antioxidative detoxification systems that allow the fungus to counteract the accumulation of reactive-oxygen species directly or indirectly, initiated by metals, may be part of tolerance mechanisms.

In this review, our intent was to collect all information known to date on edible EM mushrooms (EEMMs) response to metal pollution. Despite the identification of a

number of putative metal transporters in the sequenced genome of the EM basidiomycete *Laccaria bicolor* (Maire) P. D. Orton (Martin et al. 2008), a global view of metal homeostasis-related genes and pathways, and of their expression profiles in different life cycle stages (especially mycorrhizae) of plant-symbiotic fungi, was clearly elucidated only in the EEMM *Tuber melanosporum* Vittad. (Bolchi et al. 2011).

Studies designed to elucidate the mechanisms responsible for metal tolerance processes could improve the knowledge regarding the beneficial effects on the plant partner. Furthermore, it is a specific point of interest for the food safety of the valuable fruiting bodies produced by the EEMs. This topic will be discussed by Sitta and Davoli (Chap. 20).

3.2 Extracellular Metal Immobilization

Fungi can effectively bind metals to cell walls or perform chelation mechanisms by different organic molecules (Gadd 1993). Binding to the wall, also called biosorption, is a mechanism not depending on the metabolic activity of the fungus but on the composition of cell wall itself (Fig. 3.1). Its components of glucan-, chitin-, and galactosamine-containing polymers and, in minor extent, proteins have a large number of potential binding sites consisting of free carboxyl, amino, hydroxyl, phosphate, and mercapto groups (Gadd 1993). The metal biosorption capacity and strength are increased by the presence of melanins among the cell wall (Fogarty and Tobin 1996).

Many studies concerning the heavy metal biosorption by mushrooms have been performed, and the concentration of heavy metals in some fungal fruit bodies has been evaluated (Nilanjana 2005). Different EEMMs, such as *Agaricus bisporus* Imbach, *Boletus edulis* Bull., *Russula delica* Fr., and *Pleurotus ostreatus P. Kumm.*, absorb heavy metal like copper, cadmium, lead, zinc, iron, and manganese (Tüzen et al. 1998; Demirbas 2001; Yilmaz et al. 2003). Poitou and Oliver (1990) observed that the mycelia of *Suillus granulates* (L.) Roussel, *Lactarius deliciosus* (L.) Gray, *T. melanosporum* and *Tuber brumale* Vittad. rapidly accumulated copper ions, and the uptake of copper ions affected the absorption of potassium and magnesium ions, which were essential for the growth of fungi.

Although there is no doubt that metal sorption on fungal structures does happen, the extent to which it occurs and its significance to the mycorrhizal symbiosis are unclear. Most evidences for metal binding by fungal cell walls derive from biotechnological studies aiming at wastewater management (Jentschke and Goldbold 2000). These investigations show that fungal cells possess a high metal adsorption capacity. However, most studies have been conducted with species of the genera *Aspergillus*, *Penicillium*, *Rhizopus*, or other fungi, but data on metal uptake by EM fungi are scarce. Marschner et al. (1998) measured Pb sorption on the mycelium of the EM fungi *Paxillus involutus* Fr. and *Laccaria bicolor* P. D. Orton. They found that Pb sorption after exposure to 48 mM Pb was as high as

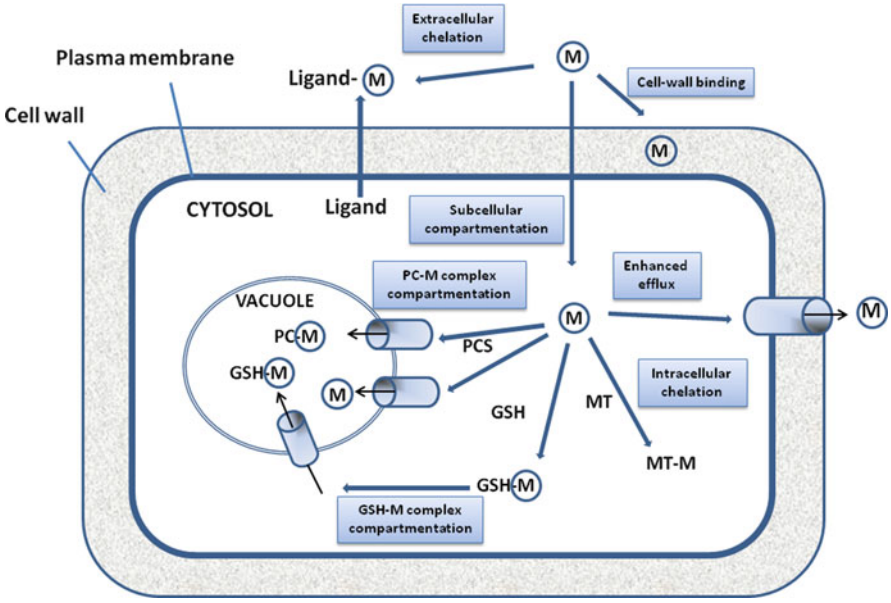

Fig. 3.1 Hypothesized schematic representation of cellular mechanisms responsible in metal tolerance in edible EM mushrooms. *M* metal ion, *MT* metallothionein, *GSH* glutathione, *PC* phytochelatin, *PCS* phytochelatin synthase

0.2–1.3 mmol (g mycelial dry weight). As this amount was similar to the cation exchange capacity of these fungi, most of the Pb was probably bound to cation exchange sites (Marschner et al. 1998).

Mycorrhizal fungi, like certain plant hosts, excrete organic acids into the rhizosphere (Landeweert et al. 2001). Organic acid exudation may either mobilize metal ions from insoluble minerals or immobilize them throughout acidification, precipitation, or chelation mechanisms (Landeweert et al. 2001); thus, they can facilitate fungal metal uptake or play a specific role in the detoxification strategy.

Organic acids excreted by fungal cells are di- and tricarboxylic acids such as citric, malic, and oxalic acid. In particular, oxalic acid is a well-known chelating agent that has been widely studied because of its ability to dissolve different minerals. In fact, in contrast to other low-molecular carboxylic acids, the oxalic acid is also able to immobilize metals very efficiently at neutral pH and even in basic solutions (Burgstaller and Schinner 1993). Sayer et al. (1999) demonstrated that pyromorphite, a stable lead mineral, can be solubilized by organic-acid-producing fungi, such as *Aspergillus niger*. The production of lead oxalate dehydrate by this fungus during pyromorphite transformation has been observed. The mechanisms of lead solubilization, or its immobilization as novel lead oxalate, have significant implications for metal mobility and transfer to other environmental compartments and organisms.

An overexcretion of organic acids with strong metal-chelating properties (oxalic and citric acids) by the soil (and insect parasitic) fungus *Beauveria caledonica*

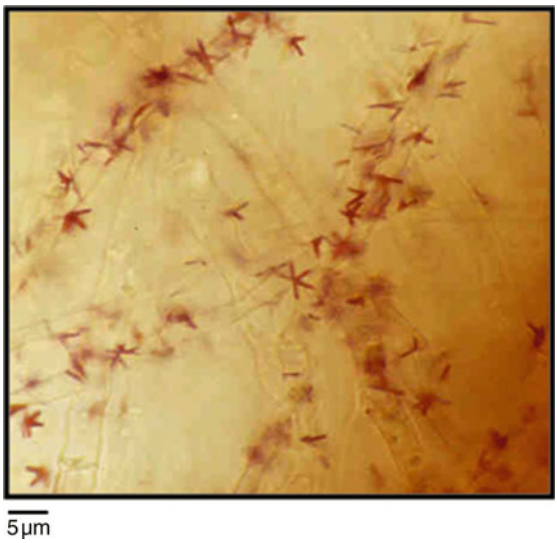

Fig. 3.2 *T. borchii* mycelia grown at high concentration of Pb^{2+}. The presence of Pb^{2+} directly on the external cell wall is visible in *red*. The analysis of content of lead in *T. borchii* mycelium was performed by rhodizonate staining technique and observed by light microscopy using 100× objective in oil immersion

Bissett and Widden has been reported by Fomina et al. (2005). Cadmium, copper, lead, and zinc oxalates were precipitated by this fungus in the local environment and also associated with the mycelium. All the processes of metal diffusion and precipitation of metal oxalates occurred in the fungal mucilaginous sheaths around the hyphae. The nature and amount of organic acids excreted by fungi are mainly influenced by the pH and buffering capacities of the environment depending on the presence and concentration of the carbon, phosphorus, and nitrogen sources; and the presence of certain metals.

T. borchii Vittad. mycelia grown in a medium with Pb $(NO_3)_2$ (5.00 mM) showed the presence of a large number of crystals of Pb^{2+} (needles and starred in red) in direct contact with the fungal wall (Fig. 3.2). This behavior would allow the fungus to perform its vital functions without suffering from this toxic element (Zeppa unpublished results).

However, organic-acid exudation should not be regarded as a general tolerance mechanism since it is both metal and species dependent (Meharg 2003). Many fungal species, such as *Rhizopus arrhizus* sensu Cunningham, *Penicillium spinulosum* Thom, and *A. niger*, have been extensively studied for heavy metal biosorption and possess species-dependent mechanisms. For example, citrate and oxalic acid contribute to the metal tolerance exhibited by *B. caledonica* and *P. involutus* (Fomina et al. 2005; Bellion et al. 2006), whereas three isolates of the EM fungi, *Pisolithus arhizus* (Scop.) Rauschert (= *Pisolithus tinctorius*), *Scleroderma verrucosum* (Bull.)

Pers. and *Scleroderma cepa* Pers. *a*, grown on pond ash, release formic acid, malic acid, and succinic acid (Ray and Adholeya 2009).

EM fungi frequently show tolerance to high aluminum concentrations (Marschner et al. 1998). Studies by Ahonen-Jonnarth et al. (2008) have demonstrated that elevated concentrations of this metal ion stimulate the production of Al-chelating oxalic acid by EM fungi colonizing plant roots, and this process may play a role in improved Al tolerance (Ahonen-Jonnarth et al. 2008). Secretion of special proteins able to sequester metal ions, like glomalin from arbuscular endomycorrhiza (González-Chávez et al. 2004), has not been identified in EM fungi.

The responses of mycorrhizal fungi to toxic metal cations are diverse, and this diversity, which is normally high, even on highly contaminated sites, could reflect species-specific genetic adaptations to cope with metal contamination.

3.3 Transport Mechanisms Involved in Metal Tolerance

Non-essential and toxic metals and metalloids enter into cells through plasma membrane permeases and channels evolved for the uptake of essential metals and other nutrients, such as Fe, Mn, Zn, phosphate, sulfate and glycerol. However, all organisms have developed mechanisms that can reduce such influx by down regulating the expression of relevant transporters at the transcriptional and post-transcriptional levels and/or by inhibiting their transport activities. Metal transport proteins may be involved in metal tolerance either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into intracellular compartments (Hall 2002) (Fig. 3.1). Most of our knowledge, concerning metal-uptake and compartmentation mechanisms, is based on studies on yeasts and filamentous fungi (Wysocki and Tamás 2010) other than EM species (Bellion et al. 2006). Although metal transporters are the most populated class of metal homeostasis-related gene products in *T. melanosporum*, the total number of predicted metal transporters in this fungus is significantly lower than in the five non-mycorrhizal ascomycetes *Saccharomyces cerevisiae* Meyen, *Neurospora crassa* Shear and B. O. Dodge, *Magnaporthe grisea* (T. T. Hebert) M. E. Barr, *Botrytis cinerea* Pers., and *Aspergillus nidulans* G. Winter (Bolchi et al. 2011).

Among the plasma membrane transporters, ZIP proteins, which take their name from the first members (Zrt- and Irt-like protein) to be identified, are key uptake transporters in eukaryotic cells, and they belong to the most populated family of *Tuber* metal transportome. In yeast and plant, different ZIP transporters are implicated in Fe, Zn, Mn, or Cd uptake. For example, in yeast, Zrt1 (that belongs to the ZIP family) mediates high-affinity Zn transport (Eng et al. 1998), and it is also implicated in Cd uptake (Gitan et al. 2003). In fact, Zn-limited cells that upregulate *ZRT1* transcription are more sensitive to Cd, and consistently, in the presence of high concentrations of Zn and Cd, Zrt1 is removed from the cell surface to prevent the uptake of toxic Cd and excess Zn (Gitan et al. 1998, 2003). Bolchi and colleagues report that *Tuber* metal transporter genes, especially those related to Cu and Zn

trafficking (e.g., *TmelZrt1*, *TmelZrc1*, *TmelCtr2*, *TmelCcc2*), display the highest expression levels in mycorrhizae. *TmelZrc1* is a zinc carrier belonging to cation diffusion facilitator (CDF) family, which, due to homology with corresponding yeast protein, is believed to transport zinc from the cytosol to the vacuole. This transporter seems to have a function in zinc storage in symbiotic hyphae and a role in resistance to zinc shock resulting from a sudden influx of this metal into the cytoplasm. The presence of *TmelZrc1* argues in favor of a sustained zinc traffic in mycorrhizae and the concomitant need to limit the potential cellular toxicity of free zinc ions in this tissue. The overexpression of *TmelCcc2*, a Cu^{2+-}transporting P-type ATPase, and its metallochaperone partner *TmelAtx1* could indicate the export of copper from the cytosol to the extracytosolic domain of the Cu-dependent oxidase Fet3, which is required for high-affinity iron uptake, as reported in yeast. Since *Tmel*Fet3 does not show an upregulation during symbiotic phase in *T. melanosporum*, *TmelCcc2* can have as its main targets other copper-containing proteins such as laccase and tyrosinase, some of which, for example, *TmelLcc1* and *TmelTyr4*, are among the top upregulated genes in *T. melanosporum* mycorrhizae (Martin et al. 2010). The multicopper oxidase system (laccase, tyrosinase)/Ccc2–Atx1 may contribute not only to cell-wall modification per se but also to the development of symbiotic multihyphal structures and to the remodeling of host plant cell walls.

Among plasma membrane metal exporters, Acr3 and Pca1 transporters constitute the main route of metal detoxification systems in *S. cerevisiae*. Acr3 is a prototype member of the arsenical resistance-3 (Acr3) family of transporters, which belongs to the bile/arsenite/riboflavin transporter (BART) super family and likely performs both As and Sb export (Maciaszczyk-Dziubinska et al. 2010). Pca1 plasma membrane P-type ATPase plays a crucial role in Cd tolerance in yeast (Adle et al. 2007). The upregulation of *Tuber* Acr3 and Pca1 orthologs in symbiotic hyphae, in the absence of any exogenously applied metal stress, indicates that they might have also more physiological roles (Bolchi et al. 2011). It is known, for example, that Pca1 is a rather promiscuous transporter capable of mobilizing various essential metals (e.g., Cu and Zn) in addition to cadmium.

Transport of metals into vacuoles is a common detoxification mechanism in eukaryotes (Fig. 3.1). In *S. cerevisiae*, the ABC transporter Ycf1 represents a major pathway for vacuolar sequestration of GSH-conjugated metals and xenobiotics (Paumi et al. 2009). Ycf1p was identified in a screen for genes conferring increased tolerance to Cd when overexpressed (Szczypka et al. 1994). The presence of this specific permease in the tonoplast of *Paxillus involutus* could explain the high Cd content in the vacuole (Blaudez et al. 2000). This hypothesis was further supported by X-ray microanalysis, which revealed that the accumulation of Cd correlated tightly with the accumulation of sulfur in electron-dense bodies in the vacuolar compartment (Ott et al. 2002). With a similar approach, it was found that an enhanced Zn efflux may act as a potential tolerance mechanism in the EM fungus *Suillus bovinus* (Pers.) Roussel (Adriaensen et al. 2006). This detoxification system, involving vacuolar sequestration, seems to be employed by *T. melanosporum*; in fact the homologs of ABC transporters (*TmelYcf1*, *TmelHmt1.1*, *TmelHmt1.2*) are encoded by the genome of this fungus.

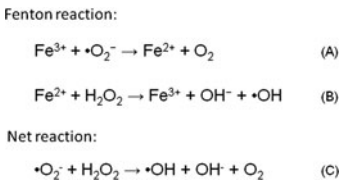

Fig. 3.3 The Fenton net reaction produces the hydroxyl radical (Eq. C) and can be broken down into two chemical reactions (Eqs. A and B)

Finally, a homolog of yeast transcription factor Zap1, involved in the regulation of numerous metal transporters in yeast and which plays a direct role in controlling Zn-responsive gene expression, was recently identified also in *T. melanosporum* (Montanini et al. 2011).

3.4 Metal and Oxidative Stress

The formation of free radical species can be initiated directly or indirectly by metals and can cause severe oxidative stress in the cell. Formation of metal-induced reactive-oxygen species (ROS) may occur via several mechanisms. The Fenton or Haber–Weiss reactions are catalyzed by redox-active metals (e.g., Cu, Fe, Cr, V) and are supposed to be a major source of hydroxyl and superoxide radicals (Halliwell and Gutteridge 1984) (Fig. 3.3).

Nevertheless, redox-inactive metals, such as Cd, Ni, Hg, and Zn, may also produce indirectly oxidative stress by different mechanism like the inhibition of specific enzymes or the depletion of antioxidant pools (Stohs and Bagchi 1995). They could also perturb intracellular Fe metabolism (Kitchin and Wallace 2008) and can lead to increased levels of free Fe ions in the cell and, via Fenton-type reactions, elevated ROS levels. The role of ROS in metal-induced damage to yeast is highlighted by increased metal tolerance during anaerobicity, protection carried out by certain free radical scavengers (Avery 2001). Redox-inactive metals such as Cd, Ni, Hg, and Zn deplete protein-bound sulphydryl groups and glutathione (GSH) (Avery 2001; Stohs and Bagchi 1995), which is the main antioxidant molecule in cells. *S. cerevisiae* neutralizes many metals and metalloids, for example, Cd, As (III), Hg, and antimonite [Sb(III)] through chelation to GSH; hence, it is possible that this process leads to reduced cytosolic GSH levels and causes cell stress by impairing the activities of GSH-dependent enzymes, such as glutathione peroxidases, glutathione S-transferases (GST), and glutaredoxins. Nevertheless, other authors observed an opposite behavior of yeast cells where GSH levels strongly increased in response to Cd (Lafaye et al. 2005) and As(III) (Thorsen et al. 2007) exposure.

In the arbuscular mycorrhizal fungus *Glomus intraradices* N. C. Schenck and G. S. Sm., grown under heavy metal stress, were identified several ESTs showing significant sequence homologies to GST-encoding genes from other organisms (Rhody 2002). This finding, together with observation on the transcriptional

upregulation of the glutathione S-transferase gene by either Cd, Cu, or Zn, could well indicate that GSTs, by catalyzing the conjugation of glutathione with a variety of reactive electrophilic compounds, contribute in the alleviation of heavy metal toxicity in the symbiotic mycelium. In addition to GST, supplementation of different heavy metal induced a putative 90-kDa heat shock protein, indicating that this fungus tried to counteract heavy metal-derived oxidative stress (Ouziad et al. 2005).

Jacob et al. (2001) analyzed transcriptional responses to cadmium in the EM fungus *P. involutus* and hypothesized a possible indirect contribute of Cd^{2+} to oxidative stress by affecting the cellular thiol redox balance. In this fungus, among overexpressed genes induced by Cd^{2+} and Cu^{2+}, a gene encoding a thioredoxin (PiTrx1) was identified. Thioredoxins are small proteins containing an active site with a redox-active disulfides (Holmgren 1989) involved in many cellular processes that require reduction reactions such DNA synthesis, sulfur metabolism, protein folding and oxidative damage repair. Additionally, in *S. cerevisiae*, thioredoxins have shown to be required to maintain redox homeostasis in response to both oxidative and reductive stress conditions (Trotter and Grant 2002) and were found to be induced upon exposure to Cd^{2+} (Vido et al. 2001). Courbot and Chalot postulated that PiTrx1, being a rapid response determinant in the handling of Cu^{2+} and Cd^{2+}, might function as a first line of defense against intracellular metal ions in *P. involutus* (Courbot et al. 2004).

Extensive investigation on the antioxidative systems in this EM fungus in response to Cd showed the induction of superoxide dismutase (SOD), in accordance with previous results of Jacob et al. (2001), and the accumulation of glutathione, along with the induction of glutathione-related systems (glutathione-dependent peroxidase, glutathione reductase) (Ott et al. 2002). SOD could promote Cd resistance through its capacity to bind and buffer cellular Cd, as demonstrated in yeast as a response to Cu (Culotta et al. 1995), or act against oxidative stress generated by this metal. A fast glutathione accumulation and maintenance of a relatively stable redox state have induced the authors to infer that *P. involutus* is able to detoxify high concentrations of Cd by a strong induction of glutathione synthesis which is followed by a sulfur-dependent transport of Cd into the vacuole (Ott et al. 2002).

Recently, the major enzymes involved in the redox-state controlling and ROS scavenging, probably involved in metal tolerance, have been identified in the genome of *T. melanosporum* (Bolchi et al. 2011). These include five superoxide dismutases (SOD); three catalases; different members of the thioredoxin, glutaredoxin, and peroxiredoxin systems as well as enzymes involved in glutathione (GSH) biosynthesis, peroxidation, and regeneration, and GSH-mediated detoxification processes. The expression analysis revealed that the components of the detoxification system of *T. melanosporum* are expressed at levels slightly above background in at least one phase of its life cycle, except for the putative catalase (TmelCTA1.C) and the glutathione biosynthetic enzyme (TmelGsh1).

Two enzymes involved in oxidative stress defense (the catalase isoform TmelCta1.B and the glutathione biosynthetic enzyme TmelGsh1) were also found to

be upregulated in *T. melanosporum* mycorrhizae. Catalase and GSH overproduction in symbiotic hyphae may serve to counteract ROS produced during the oxidative burst that accompanies plant–fungus interaction.

3.5 Metallochaperones, Metal-Chelating Peptides and Other Metal Detoxification Systems

Despite extracellular chelation and cell-wall binding capacities of EM fungi, a large amount of metal may enter into the cells. Due to their chemical reactivity and non-specific macromolecule binding potential, free metal ions are extremely rare (and toxic) species inside the cell. Even essential metals, especially the redox-active ones, must be sequestered as metal–peptide (or other small-molecule metal binding) complexes delivered to their target (apo)metalloproteins by specialized carriers, known as "metallochaperones". The orthologs of five known metallochaperones-three cytosolic (Atx1, Ccs1, Cox17) (Glerum et al. 1996; Lin et al. 1997; Wong et al. 2000) and two mitochondrial (Sco1, Yfh1) (Babcock et al. 1997; Horng et al. 2004)—were detected in the *T. melanosporum* genome (Bolchi et al. 2011).

Stable complexation and organelle (especially vacuole) trafficking/sequestration are another key mechanism for counteracting the toxicity of both essential and non-essential metal ions. In addition to its role as a redox-state regulator, glutathione is a known metal-chelating compound (especially for thiophilic metals such as Cu, Zn, and Cd) in fungi (Pocsi et al. 2004). As reported by Pocsi et al. (2004), GSH metal conjugates can be transferred into vacuoles where they can be accumulated (Fig. 3.1).

Additional metal-chelating, cysteine-rich peptides, much more efficient than GSH in complexation of thiophilic metal ions, are phytochelatins (PCs). PCs, whose consensus sequence is (c-Glu–Cys)*n*-*X*, where *X* is usually glycine and *n* ranges from 2 to 5, are synthesized from GSH by phytochelatin synthase (PCS). This enzyme is ubiquitous in plants (Cobbett and Goldsbrough 2002), but it also occurs in various metazoan organisms (Vatamaniuk et al. 2001) and very rarely in fungi.

The first fungal PCS has been characterized in Schizosaccharomyces was the fission yeast *Schizosaccharomyces pombe* Lindner, in which, just as in higher plants, it has been observed as accumulation of Cd-conjugated PCs in the vacuole mediated by specific transporters (Ortiz et al. 1992). In some fungi (e.g., *Candida glabrata* (H. W. Anderson) S. A. Mey. and Yarrow), the production of phytochelatins has been detected in response to Cd (Zhou and Goldsbrough 1995), despite the absence of genes encoding PCS orthologs into their genome. This implies the presence of a PC synthesis mechanism different from that catalyzed by PCS.

No PC has been identified in filamentous ascomycetes, except *T. melanosporum* (Bolchi et al. 2011), in which a gene (*TmelPCS*) homolog of different characterized PCSs has been characterized. TmelPCS (superfamily domain pfam05023) has all the features of a genuine PCS enzyme (Rea et al. 2004; Romanyuk et al. 2006;

Ruotolo et al. 2004). Recombinant TmelPCS was shown to support GSH-dependent metal-activated phytochelatin synthesis in vitro and to afford increased Cd/Cu tolerance to metal-hypersensitive yeast strains.

The PCS, by using GSH as substrate to synthesize PCs, is a high sulfur (Cys/GSH) demanding enzyme. Therefore, its occurrence in *Tuber* may be related to the sustained sulfur metabolism operating in this fungus (Martin et al. 2010; Zeppa et al. 2010). Sulfur is contained in a variety of cellular components and plays critical roles in a number of cellular processes, such as redox cycle, heavy metals, xenobiotics detoxification, and metabolism of secondary products (Zeppa et al. 2010). Given the ability of PCs to stably sequester various thiophilic metal ions (both essential metals such as Zn and non-essential metals such as Cd) and the metal-sensitivity phenotypes displayed by various PCS mutants, a general role in metal detoxification has been attributed to this enzyme (Cobbett and Goldsbrough 2002). *TmelPCS* is expressed at relatively low levels in all life cycle stages, with a small preference for free-living mycelia. This is consistent with the fact that in most PC-producing organisms, PCS genes are expressed constitutively even in the presence of heavy metals.

Metallothioneins (MTs) are small cysteine-rich peptides involved in metal homeostasis and detoxification. In fact they have antioxidant activity in vivo, which could be involved in the cellular response to oxidative stress (Tamai et al. 1993). A wide range of organisms formed these metal-binding proteins upon their exposure to toxic concentrations of metals such as Cu, Zn, or Cd. In non-AM fungi, MTs are predominantly induced by Cu. The distinct upregulation of the metallothionein gene (BI451899) in ericoid mycorrhizae (ERM) of *G. intraradices* by Cu stress, to some extent by Zn but not by Cd, fully agrees with the proposed primary function of fungal MTs in the detoxification of Cu. This view is corroborated by the Cu-specific upregulation of an MT from the AMF *Gigaspora margarita* W. N. Becker and I. R. Hall (Lanfranco et al. 2002).

Ramesh et al. (2009) have characterized two MT genes, *HcMT1* and *HcMT2*, from the EM fungus *Hebeloma cylindrosporum* Romagn., and the expression of these genes under metal stress conditions was studied. As revealed by heterologous complementation assays of these cDNAs in yeast, *HcMT1* and *HcMT2* encode a functional polypeptide able to confer increased tolerance against Cd and Cu, respectively. The expression levels of *HcMT1* was observed maximum at 24 h and increased as a function of Cu concentration. *HcMT2* was also induced by Cu, but the expression levels were less compared to *HcMT1*. The mRNA accumulation of *HcMT1* was not influenced by Cd, whereas Cd induced the transcription of *HcMT2*. Zinc, Pb, and Ni did not affect the transcription of both *HcMT1* and *HcMT2*. These results show that EM fungi encode different MTs and each of them has a particular pattern of expression, suggesting that they play critical specific roles in improving the survival and growth of EM colonized trees in ecosystems contaminated by heavy metals (Ramesh et al. 2009). Brennan and Schiestl (1996) showed that complexation of Cd by MTs is a key mechanism for Cd tolerance in the EM fungus *P. involutus*, while a Cu-binding MT was purified from the EM fungus *L. bicolor* (Brennan and Schiestl 1996; Howe et al. 1997).

A small-sized putative metallothionein (TmelMT; 26 amino acids) was also identified in *T. melanosporum*. TmelMT enhanced in vivo tolerance of heavy metal-hypersensitive yeast mutant to both cadmium and copper (Bolchi et al. 2011). Similarly to plants and fission yeast, *T. melanosporum* thus appears to produce both MTs and PCs.

Nicotianamine is a nonprotein amino acid derivative synthesized from *S*-adenosyl L-methionine able to bind several metal ions such as iron, copper, manganese, zinc, or nickel. No enzyme supporting the synthesis of nicotianamine, a broad-range but iron-preferential chelator present in Neurospora and other fungi (Trampczynska et al. 2006), was identified in the *T. melanosporum* genome. Under the metal-unstressed conditions, metal homeostasis/detoxification genes were found to be expressed at varying levels in each of the three life cycle stages. Further investigations are needed to identify different members of the MT family in EM fungi and to study their regulation of expression in an attempt to determine their respective functional role in heavy metal detoxification and/or tolerance.

3.6 Conclusions

Many studies have been conducted in recent years about how several organisms, among which fungi, cope with toxic metals and metalloids. Nevertheless, molecular insights into many aspects of metal biology are still lacking. In particular detailed studies on metal distribution among cytosolic compounds are scarce in edible mycorrhizal species, especially when exposed to high metal concentrations. For EEMM species, an important contribution to the global knowledge was obtained through the genome-wide screening of *T. melanosporum* (Bolchi et al. 2011), which allowed the construction of a metal homeostasis inventory for this truffle species. Despite the wide genome network depicted, the exact roles have not been assigned to the numerous proteins detected.

Furthermore, very limited is our knowledge about how metals can regulate the expression of genes participating to metal homeostasis and tolerance by activating transcription factors and signaling proteins. Importantly, the way in which this network of metal-related proteins is coordinated to determine the cellular response to metals on cellular and subcellular levels has remained largely unexplored.

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Chapter 4
Genomics of *Tuber melanosporum*: New Knowledge Concerning Reproductive Biology, Symbiosis, and Aroma Production

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4.1 Introduction

The symbiotic ascomycetes belonging to the genus *Tuber* produce hypogeous fruit bodies, known as truffles, highly priced and praised for their distinctive aroma and taste by gourmets. Although the life cycle of symbiotic ascomycetes remains to be fully elucidated, the establishment of methodologies to inoculate host plants and the growing market for these fungi have contributed to the growth of large-scale truffle cultivation programs over the last several decades. These programs were first developed in Europe, where highly valued *Tuber* spp. are endemic, then later established in other countries worldwide (Hall et al. 2007).

Molecular investigations of *Tuber* spp. were initiated more than a decade ago, with the primary goals of reliably identifying morphologically similar truffle species throughout their entire life cycle and preventing economic fraud (Henrion et al. 1994; Paolocci et al. 1995). The wealth of molecular-based knowledge amassed, especially in the last few years, has made it possible to tackle key biological questions regarding truffle reproductive biology and ecology. This research mainly yielded the following developments: recent progress on methodologies for dissecting and genotyping each of the structures that these fungi develop throughout their complex life cycle (i.e., single spore and single mycorrhizal root tip), high-throughput technologies for transcriptomic and ecological studies, and, most importantly, the recent release of the *Tuber melanosporum* Vittad. genome. This species produces the most highly valued black truffle, known as the Périgord black truffle. The availability of the *T. melanosporum* genome has made this species the model fungus not only among *Tuber* spp. but among all symbiotic ascomycetes. Thus, the characterization of the genomic organization and genes of this species that are committed to symbiosis

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provides mycologists a more comprehensive understanding of the similarities and differences between symbiotic ascomycetes and basidiomycetes. Moving from the comparison of these two fungal types, this chapter will highlight the genetic and environmental determinants of the aroma of this truffle species. Finally, recent findings on the genetic diversity and reproductive biology of *T. melanosporum* will be discussed, along with aspects relevant to the cultivation and marketing of this fungus and its importance for understanding the biology of other *Tuber* spp. of economic interest.

4.2 Basic Features of the *T. melanosporum* Genome

With 125 megabases, the *T. melanosporum* genome is the largest fungal genome sequenced thus far and is approximately four times greater in size than the genomes of other ascomycetes (Martin et al. 2010a; Galagan et al. 2005). Its unusually large size has not been attributed to genome duplications, which characterize the genomes of other ascomycetes, such as *Saccharomyces cerevisiae* Meyen ex E. C. Hansen and other yeast species (Wolfe and Shields 1997), and fungi belonging to other lineages, such as *Rhizopus oryzae* Went & Prins. Geerl. (Ma et al. 2009). Evidence indicating large-scale dispersed segmental duplications was also not observed (Martin et al. 2010a). Instead, the large genome size likely resulted from the proliferation of transposable elements (TEs), which account for about 58 % of the genome. Automatic annotation of the TEs in the *T. melanosporum* genome indicated that these elements are primarily retrotransposons, with an abundance of Gypsy/Ty3-like elements (29.5 % of the assembled genome) followed by long interspersed elements (5.6 % of the genome). The major wave of TE expansion likely occurred approximately 2–3 million years ago. In turn, TE proliferation may have facilitated deep rearrangements of the genome, resulting in a low level of gene synteny with other ascomycetes (Martin et al. 2010a).

Despite the large genome size, the number of predicted genes is surprisingly small (about 7,500) and falls within the lower range of the gene numbers observed among sequenced filamentous fungi (Martin et al. 2010a; Martin 2011). Moreover, protein-coding genes and TEs are not randomly distributed, with blocks of relatively high gene density and a scarcity of TEs separated by large genomic regions with low gene content and a high abundance of TEs. Compared to other filamentous ascomycetes, analyses of the *T. melanosporum* genome revealed a high number of simple sequence repeats (SSRs) in addition to TEs. In fact, searching for mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeats led to the identification of more than 22,000 SSRs (Murat et al. 2011). The majority of these SSRs are located in noncoding DNA, although a small number of SSRs are located within coding sequences and UTR regions. Interestingly, the gene ontology classification revealed that some of the genes carrying SSR motifs in their sequences are genes involved in regulatory networks, nucleotide binding, signaling pathways, symbiosis, and host interactions. One interesting challenge will be to determine whether changes in the SSR length and position affect the role and function of genes important for the fungal life cycle, such as those controlling symbiosis.

Another distinctive trait of the *T. melanosporum* genome is the small number of multigene families and the low level of sequence similarity of paralogous genes compared to other ascomycetes. Furthermore, the level of similarity between *T. melanosporum* predicted proteins and those of other ascomycetes is not very high (approximately 45 %), which is in full agreement with the phylogenetic reconstructions placing Pezizomycetes as the earliest diverging lineage within the Pezizomycotina (James et al. 2006).

4.3 Analysis of the *T. melanosporum* Genome Provides New Insight into the Mechanisms of Mycorrhizal Symbiosis

The availability of the complete genomes of the basidiomycete *Laccaria bicolor* (Maire) P. D. Orton (Martin et al. 2008) and *T. melanosporum* (Martin et al. 2010a) gives mycologists the opportunity to compare the genetic networks of two mycorrhizal fungi belonging to different phyla and to compare these fungi to others characterized by different lifestyles, such as saprotrophs and plant pathogens.

The *T. melanosporum* and *L. bicolor* genomes share some common features. For example, the large reduction in the number of genes encoding plant cell wall (PCW) degradation enzymes, compared to saprophytic and pathogenic fungi, appears to be specific to the mycorrhizal fungi. This significant reduction in the number of PCW-degrading enzymes suggests that mycorrhizal fungi have developed a host colonization strategy that prevents the elicitation of the pathogen-triggered response in their hosts (Martin et al. 2010a; Plett and Martin 2011). As a result, however, mycorrhizal fungi rely almost entirely on their host plants for carbon.

Of the few upregulated genes in the ectomycorrhizas of these two symbiotic fungi, the majority of them encode membrane transporter proteins essential for nutrient exchange between the fungus and the host plant. The genomes of these two mycorrhizal fungi also show the expansion of particular gene families, including the tyrosine kinase family. Kinases are proteins involved in many cellular processes, such as cellular differentiation and proliferation, and their expansion in the genomes of both mycorrhizal species suggests that kinases may have a key role in mycorrhiza development (Plett and Martin 2011).

In addition to these common features, there are also many differences in the expression patterns of symbiosis-related genes in *L. bicolor* and *T. melanosporum*, suggesting a divergent evolution of the symbiotic lifestyles of symbiotic basidiomycetes and ascomycetes. First, transcriptomic analyses have shown that only a small number of orthologous genes are upregulated in both *T. melanosporum* and *L. bicolor*. Moreover, no PCW-degrading enzymes are expressed during the symbiotic stage in *L. bicolor*, although some of these enzymes are produced in the hyphae growing in the soil (Martin et al. 2008). In *T. melanosporum*, on the other hand, some PCW-degrading enzymes are expressed during the colonization of the host roots (Martin et al. 2010a; Plett and Martin 2011). Overall, these data

suggest that the mycelia of *L. bicolor* have a higher saprotrophic capability than those of *T. melanosporum*. Along these same lines, a distinguishing feature of *T. melanosporum*, compared to other mycorrhizal fungi, is the presence of a gene that encodes invertase, an enzyme that hydrolyzes sucrose (Ceccaroli et al. 2011). In contrast to the saprotrophic and pathogenic fungi, almost all mycorrhizal species lack invertase genes and are thus unable to hydrolyze the sucrose synthesized by their hosts. Instead, mycorrhizal fungi depend on the activity of the host-derived invertase to utilize their major carbon source. This appears to be a mechanism by which host plants control the spread of their symbiotic partners but one that *T. melanosporum* may be able to avoid.

Another striking difference between the sequenced symbiotic fungi involves the presence and expression of mycorrhiza-induced small secreted proteins (MiSSPs). In *L. bicolor*, many MiSSPs have been identified, and some of these (10) resemble the effector proteins of pathogenic fungi (Martin et al. 2010a; Plett and Martin 2011; Veneault-Fourrey and Martin 2011). Recently, Plett et al. (2011) demonstrated that one of these *L. bicolor* proteins, MiSSP7, is an indispensable effector for the establishment of symbiosis. An effector protein, named SP7, has also been reported in the mycorrhizal fungus *Glomus intraradices* N. C. Schenck & G. S. Sm. (Kloppholz et al. 2011), suggesting that mutualistic and pathogenic fungi may share common host manipulation mechanisms (Plett et al. 2011; Koeck et al. 2011). Although the *T. melanosporum* genome contains several genes encoding small secreted proteins, none of these genes appear to be upregulated in the ectomycorrhizas. Whether *T. melanosporum* establishes symbiosis with its hosts using an effector-mediated mechanism is therefore a crucial question with both basic and applied relevance that will need to be addressed in the near future.

Overall, whole genome and high-throughput transcriptome analyses depict a scenario in which the mechanisms for the establishment and maintenance of symbiotic mutualism have diverged evolutionarily between basidiomycetes and ascomycetes.

4.4 Genes for the Production of Truffle Aroma

The widespread appreciation and gastronomic status of truffles reside in their distinctive aroma. The chemical compounds responsible for the truffle aroma vary among the different *Tuber* spp. both in quality and quantity. Among the volatile organic compounds (VOCs) identified in *Tuber* fruiting bodies, those derived from sulfur metabolism (S-VOCs) are the most important. Many studies have demonstrated that sulfur compounds such as bis(methylthio)methane are characteristic of the white truffle *T. magnatum* Pico, whereas dimethylsulfide and dimethyldisulfide are the main components of the black truffle aroma (for a recent review, see Splivallo et al. 2011). The release of chemicals by these species allows signaling information to be exchanged with the surrounding environment to ensure the completion of the fungal life cycle (Pacioni et al. 2007). For example, spore dispersal in these hypogeous fungi depends on mycophagists that are attracted by the volatiles emitted from

fruit bodies. Dimethylsulfide from *T. melanosporum* is regarded as the main attractant of pigs (Splivallo et al. 2011), and notably, truffle hunters originally used pigs to locate truffles.

Although many VOCs have been identified in the fruiting bodies of different *Tuber* spp., the ability of truffles to produce all of the volatile components responsible for their aroma has been questioned. Indeed, a variety of microorganisms, such as bacteria, yeasts, and filamentous fungi, have been reported to grow within truffle fruiting bodies (Barbieri et al. 2007; Buzzini et al. 2005; Pacioni et al. 2007). Pure cultures of the yeast strains isolated from *T. magnatum* and *T. melanosporum* fruiting bodies produce the VOCs characteristic of their hosts (Buzzini et al. 2005). Furthermore, chemical analyses on the mycelia of different truffle species grown in vitro found that some of the compounds present in the corresponding fruiting bodies were absent (Tirillini et al. 2000; Splivallo et al. 2007), suggesting either an exogenous origin of the truffle VOCs or a tightly controlled, stage-specific emission of these compounds.

The release of the *T. melanosporum* genome has allowed mycologists to address this particular question. Enzymes and putative metabolic pathways involved in the biosynthesis of volatile compounds have been identified. *In silico* analyses have shown that the genes required to synthesize most of the key components of the black truffle aroma are present in the *T. melanosporum* genome. More specifically, genome analysis has revealed the presence of 126 genes related to sulfur assimilation and S-amino acids interconversion and metabolism such as the genes coding for cystathionine β- and γ-lyases, thought to be involved in the production of S-VOCs (Martin et al. 2010a).

Furthermore, the genes for the enzymes of the Ehrlich pathway, likely responsible for the synthesis of other components of truffle aroma (i.e., fusel alcohols 2-methylbutanal and 3-methylbutanal) and a complete set of genes involved in the biosynthesis of isoprenoids, have been identified (for more details, see Martin et al. 2010a; Splivallo et al. 2011). Significantly, the expression of most of these genes is upregulated in the fruiting body compared to the mycorrhizas and free-living mycelium; thus, the emission of the most important components of the truffle aroma appears to depend on stage-specific gene regulation (Martin et al. 2010a; Splivallo et al. 2011). Notwithstanding, a synergic action between truffle species and their associated microorganisms that qualitatively and quantitatively shapes the arrays of chemicals that give rise to particular truffle bouquets cannot be ruled out. More chemical and genetic studies are thus required to determine the relative contributions of each partner to the aroma of truffles. A similarly intriguing and challenging question is one of the most vexing in *T. melanosporum* research: whether the changes in the aromatic properties of truffles of different geographical origin are solely due to the different pedoclimatic conditions (Bertault et al. 1998). Significant differences in the proportion of VOCs among *T. magnatum* truffles of different origin have been reported (Gioacchini et al. 2008). Chemical and population genetics analyses along with studies aimed at investigating genetic polymorphisms and the expression profiles of candidate genes for the production of the aromatic compounds in *T. melanosporum* specimens of different origin should provide meaningful insights to address this question.

4.5 *T. melanosporum* Population Genetics: Approaches, Results, and Implications

The assessment of the extent and distribution of the intraspecific genetic variability among and within natural truffle populations helps determine the history, evolution, and the present status of the population genetic structure of *Tuber* spp. Population genetics studies on *T. magnatum* and *T. melanosporum* have provided preliminary evidence on the reproductive biology of *Tuber* spp. (see below). Furthermore, population genetics analyses are of interest for tracing the geographical origins of truffles. This is particularly relevant because, historically, the market value of truffles has depended on both the species and the geographical origin of the fruit bodies. Thus, the ability to identify the geographic origin of truffles is important for associations of truffle harvesters and local governments, which aim to promote the economic and social development of rural and marginal areas. It may also prevent the erosion of local biodiversity by encouraging the use of autochthonous fungal genotypes for the artificial inoculation of host plants to be transplanted to naturally producing truffle areas.

Early studies using molecular markers suggested a very limited genetic diversity in *T. melanosporum* populations and led to the conclusion that *T. melanosporum* experienced a population bottleneck during the last glaciation (Bertault et al. 1998, 2001). Later studies, however, revealed significant genetic differences among *T. melanosporum* populations in France using polymorphisms within the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) (Murat et al. 2004). Similarly, based on amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSR), and ITS markers, *T. melanosporum* populations have been found to exhibit extensive genetic variability, with the southernmost populations showing the highest levels of genetic diversity (Riccioni et al. 2008). These findings support the hypothesis that the postglacial *T. melanosporum* expansion followed a northward pattern from refugia located in the Italian and, possibly, the Iberian Peninsula.

The successful discrimination of truffle populations according to their origin depends heavily on sampling strategies and on the number of polymorphic markers used to screen the specimens (Rubini et al. 2007). The use of a few polymorphic SSRs was sufficient to identify a phylogeographic structure in natural populations of *T. magnatum* (Rubini et al. 2004, 2005). Beyond the importance of SSRs for studying the organization of the truffle genome and their putative role in modulating gene expression, SSRs are one of the most suitable markers for population genetic analyses. In this regard, 135 SSRs mined from the *T. melanosporum* genome were recently used to evaluate the degree of polymorphism among fruiting bodies of different geographical origins; approximately 44 % of the SSRs were polymorphic, with up to 10 or more alleles in some cases, a number much higher than those found for previously characterized SSR loci (Murat et al. 2011). Thus, these new SSRs may be highly informative for identifying the provenance of *T. melanosporum* individuals.

4.6 Information from Genomic Analyses on the Reproductive Biology of *T. melanosporum*

4.6.1 *The Life Cycle and Reproductive Biology of* T. melanosporum

Karyological studies have suggested the prevalence of a dikaryotic phase in the truffle life cycle (Lanfranco et al. 1995). As a result, when DNA isolated from *T. melanosporum* fruiting bodies was amplified using codominant markers (SSR), the lack of heterozygosis in this supposedly dikaryotic structure was interpreted as a clear indicator of selfing (Bertault et al. 1998, 2001). However, a study that employed SSR markers in *T. magnatum* provided the first evidence that outcrossing can occur in *Tuber* spp. (Rubini et al. 2005). The absence of significant linkage disequilibrium among the SSR loci of truffles from the same or proximal populations indicated the occurrence of extensive gene flow between individuals, a finding in conflict with the thesis that truffles strictly self-fertilize. Definitive evidence of outcrossing in both species was later provided by the use of codominant markers to genotype the DNA isolated from the gleba and pooled spores of single *T. magnatum* and *T. melanosporum* fruiting bodies (Paolocci et al. 2006; Riccioni et al. 2008). More specifically, the spores of a few truffles exhibited two alleles at some SSR loci, while the corresponding gleba always had a single allele per locus. In light of these genetic data, it has been postulated that the gleba of truffles is made up of haploid hyphae of uniparental (maternal) origin and that the occurrence of outcrossing is either possible or mandatory (Rubini et al. 2007; Riccioni et al. 2008). Not all of the truffles analyzed displayed additional alleles of paternal origin in the pool of spores, and it has been reported that homothallic (self-fertile) ascomycetes may also have some rate of outcrossing (Kronstad 2007). The dilemma regarding *T. melanosporum*'s reproductive mode has been only recently solved by studying the structure and organization of the mating type genes in its genome. On the basis of all this information, it was possible to define a reliable model of the *Tuber* spp. life cycle (Fig. 4.1).

4.6.2 *The Mating Type Locus of* T. melanosporum

Sexual reproduction in fungi is controlled by small regions of the genome known as the mating type (*MAT*) loci (for recent reviews, see Debuchy et al. 2010; Casselton and Feldbrügge 2010). Molecular analyses have revealed that filamentous ascomycetes (Pezizomycotina) have a single *MAT* locus with two master regulators of sexual reproduction: the *MAT* gene *MAT1-1-1*, which encodes an α-box domain protein, and the *MAT1-2-1* gene, which encodes a high-mobility group (HMG) domain protein (Butler 2007; Debuchy et al. 2010).

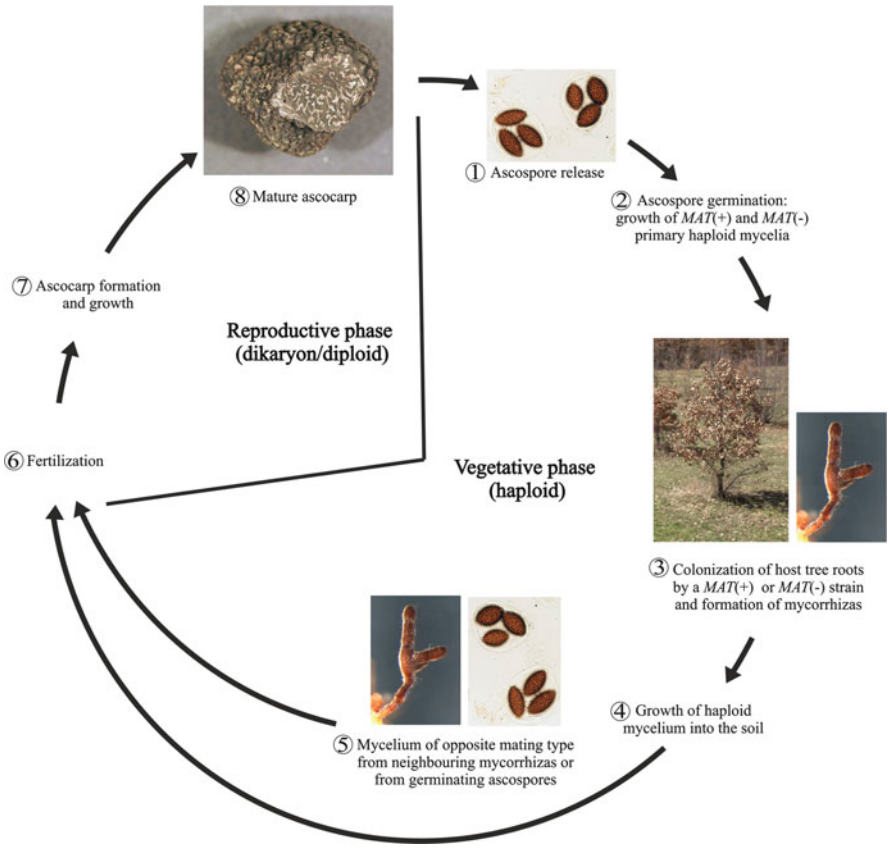

Fig. 4.1 Schematic representation of *Tuber* spp. life cycle. The ascospores released in the soil from mature ascocarps (1) germinate and produce *MAT*(+) or *MAT*(−) primary haploid mycelia (2). The primary mycelia colonize host tree roots and the ectomycorrhizas are formed (3); under a given tree, competition among different strains may result in the formation of mycorrhizas that share the same mating type. In presence of favorable climatic conditions, *MAT*(+) and/or *MAT*(−) mycelia originating from mycorrhizas grow in the soil (4); contact with mycelia of opposite mating type (5), which may originate from either ascospores or mycorrhizas from neighboring trees, is needed for fertilization to occur (6). The fertilization process gives rise to the ascocarp (fruit body, truffle) (7) which is made of the dikaryotic hyphae and the gleba, a sterile haploid mycelium of uniparental origin. Inside the mature ascocarp (8), the dikaryotic hyphae generate the asci where karyogamy takes place to form the zygotes; these diploid nuclei undergo meiosis to produce the haploid ascospores

In ascomycetes, there are two primary sexual reproductive modes: heterothallism and homothallism. In heterothallic ascomycetes, the two *MAT* genes occur in different strains; thus, heterothallic ascomycetes are self-sterile, and crossing between strains of opposite mating types is required. Incidentally, the two alternative forms of the *MAT* locus in these fungi are not two allelic versions *sensu stricto* and are instead referred to as idiomorphs (Metzenberg and Glass 1990).

In homothallic ascomycetes, a single strain harbors both *MAT* genes. Typically, the two genes are closely linked or fused within the same locus, but in a few species, such as *Aspergillus nidulans* (Eidam) G. Winter, they may reside at different loci in a single strain (Galagan et al. 2005). Therefore, homothallic ascomycetes do not have distinct sexes and are capable of selfing or crossing with any other individuals of the same species.

An *in silico* analysis of the *T. melanosporum* genome for orthologs of the *MAT1-2-1* and *MAT1-1-1* genes present in other ascomycetes revealed that the sequenced strain (Mel28) contains only the *MAT1-2-1* gene, indicating a heterothallic organization. In line with this hypothesis, only a subset of truffles produced the expected amplicon when their gleba was amplified with primers specific for *MAT1-2-1* gene. Using other PCR-based strategies, the *MAT* locus from a gleba (me206) that lacked the *MAT1-2-1* gene was cloned, and the second *T. melanosporum* mating type gene (*MAT1-1-1*) was identified therein (Rubini et al. 2011b).

The sequence comparison of the complete *MAT* regions from the me206 and Mel28 strains showed that the length of the *MAT1-2* and *MAT1-1* idiomorphic regions were approximately 7,430 bp and 5,550 bp, respectively. Although these regions are considerable in length, each *T. melanosporum* idiomorph contains only a single *MAT* gene, a situation that typifies most but not all ascomycetes (Debuchy et al. 2010). The genomic regions flanking the *MAT* locus are conserved among ascomycetes (Butler et al. 2004), and the presence of genes, such as *APN2* and *SLA2*, linked to the *MAT* locus in Pezizomycotina and some Saccharomycotina species (Butler 2007; Martin et al. 2010c) is regarded as a mark of a conserved evolutionary origin of the *MAT* locus. Interestingly, these two genes are not linked to the *MAT* locus in *T. melanosporum*. Among the genes that are linked to the *MAT* locus in *T. melanosporum*, only *COX13*, encoding for cytochrome c oxidase, resides near the *MAT* locus in other ascomycetes such as *Neurospora crassa* Shear & B. O. Dodge and *Gibberella zeae* (Schwein.) Petch (Butler 2007). This low level of synteny around the *MAT* locus likely reflects the extensive TE-driven rearrangements that the *T. melanosporum* genome has undergone (Martin et al. 2010a).

In conclusion, the analysis of the organization of the *MAT* locus in the *T. melanosporum* genome has provided mycologists some definitive evidence concerning the reproductive biology of this fungal species. The information garnered from *T. melanosporum* has also provided some clues for the characterization of the reproductive strategies employed by other *Tuber* spp. (Martin et al. 2010b).

4.6.3 *The Pheromone Receptor System in* T. melanosporum

The mating type genes encode regulatory proteins responsible for the determination of cell specificity. The mechanism of mating type determination of sexual specificity is particularly well studied in *S. cerevisiae*. In budding yeast, the recognition between cells of opposite mating types is mediated by diffusible a-factor and α-factor peptide pheromones that are produced in a mating type-specific manner. The pheromones in this yeast species are sensed by the specific G protein-coupled

receptors STE2 and STE3 that, through a mitogen-activated protein kinase (MAPK) cascade, trigger the expression of the homeodomain transcription factor *STE12*, which, in turn, activates the mating response (Leberer et al. 1997; Elion 2000).

A pheromone receptor system similar to that of *S. cerevisiae* has been described in many filamentous ascomycetes (for a recent review, see Pöggeler 2011). In heterothallic species, such as *N. crassa*, fertilization is preceded by the development of specialized male and female structures called antheridia and ascogonia, respectively. In ascogonia, a specialized hypha, the trichogyne, grows toward the hyphae of the opposite mating type attracted to the pheromone that the sexual partner emits (Bistis 1981; Kim and Borkovich 2006). Pheromones and receptors have also been identified in homothallic fungi, but in some of these fungi, the pheromone receptor system is not required for fertilization and is instead involved in later stages of sexual development (Mayrhofer et al. 2006).

Although genetic analyses have shown that *T. melanosporum* is heterothallic, the sexual fertilization step in this and other *Tuber* spp. remains elusive (Rubini et al. 2007). Indeed, fertilization structures such as antheridia have never been observed in any truffle species, and the presence of ascogonia has been reported only once (Callot 1999). Nevertheless, the majority of the genes controlling sexual reproduction in yeasts and other filamentous ascomycetes are conserved in *T. melanosporum*. In particular, its genome contains genes with sequences and structural features similar to the α-factor pheromone and the *STE2* and *STE3* receptors of *S. cerevisiae* (Martin et al. 2010a; Rubini et al. 2011b). Sequence similarity-based searches failed to identify any putative a-factor pheromone precursor genes, but this is consistent with the short length of this gene and the low level of conservation among a-factor pheromone precursor genes of different ascomycetes (Pöggeler 2011). The two-pheromone peptides are produced from larger protein precursors following different maturation pathways, and genes involved in both pathways are present in *T. melanosporum*. More specifically, the following genes have been identified: homologs of *KEX1* and *KEX2*, which encode putative carboxypeptidases; a homolog of *STE13*, which encodes a diaminopeptidase responsible for α-pheromone processing; and components of the a-factor pheromone-processing pathway (Martin et al. 2010a). Similarly, homologs of the following are present: G protein α, β, and γ subunits; components of the MAPK cascade, *STE20*, *STE11*, *STE7*, and *FUS3*; and the genes encoding the transcription factors STE11-HMG and STE12, which are associated with the pheromone pathways of *Schizosaccharomyces pombe* and *S. cerevisiae*, respectively.

The *in silico* identification of all of these genes suggests that a pheromone pathway operates in *T. melanosporum*. What remains to be identified are the specific biotic and abiotic factors that trigger the sexual pathway in this species. The availability of powerful tools, such as whole-genome custom oligoarrays (Martin et al. 2010a) and RNA-seq-based methodology (Tisserant et al. 2011), will allow for the investigation of the effects of diverse factors on the induction of sexual reproduction. Using a whole-genome oligoarray, Zampieri et al. (2010) showed that the cold stress response in *T. melanosporum* mycelia involves extensive transcriptomic changes and suggested that a cold period is necessary to initiate the development of truffle fruiting bodies.

4.6.4 *From the Genome to the Field: The Tracing of* T. melanosporum *Strains of Opposite Mating Type Reveals an Unexpected Biased Distribution*

Truffle production in both natural and cultivated truffle grounds is highly unpredictable. Until very recently, environmental determinants were the only factors considered to affect truffle fruiting. The finding that *T. melanosporum* is a heterothallic fungus has raised the question of whether the spatiotemporal distribution of strains of opposite mating types might, in addition to pedoclimatic conditions, represent a limiting factor for the occurrence of fruiting.

The availability of commercial kits for the isolation of soil DNA, the development of quick procedures for typing single truffle ectomycorrhizas (Paolocci et al. 1999), and the use of mating type-specific primers (Rubini et al. 2011b; Martin et al. 2010b) have made it possible to monitor the sexual identity of *T. melanosporum* strains at sites where truffles are naturally produced (Rubini et al. 2011a). In this study, single mycorrhizas and fruiting bodies collected beneath twelve productive sites within the same truffle ground were genotyped using *MAT*-specific primers and SSR markers (Rubini et al. 2011a). It was observed that strains with opposite mating type were never present on the same root apparatus and that a given fungal strain might spread to nearby plants by vegetative propagation to give rise to a genet. A balanced presence of mycelia of the two opposite mating types was the expected distribution for a heterothallic fungus, and this biased distribution of strains on productive host plants was a surprising result.

The same study revealed that the gleba of the harvested fruiting bodies had the same genotype as the ectomycorrhizas collected from the same site. This finding suggested that the root resident strain, regardless of its mating type, always acts as the female partner in the cross. However, the pool of spores for most of the truffles analyzed had alleles that were different from those exhibited by the mycorrhizas collected at all of the productive sites, which suggested that the male partner might not necessarily be represented by a root resident strain. Indeed, both mating types of *T. melanosporum* were detected in soil samples around a productive tree. In accordance with these findings, recent studies investigating *T. melanosporum* strain biodiversity using the polymorphism within the ITS region revealed the presence of different haplotypes in the soils of productive truffle grounds and a higher level of strain biodiversity in the soil than those displayed by the fruit bodies (Napoli et al. 2010; Mello et al. 2011). Because the saprotrophic activity of *T. melanosporum* is limited (Martin et al. 2010a; Iotti et al. 2002), the persistence and spread of its free-living mycelia in the soil is expected to be more limited, spatially and temporally, compared to strains that reside in roots, and this may negatively interfere with truffle production.

As the "ideal situation" of the co-occurrence of both *MAT1-2-1* and *MAT1-1-1* strains is not met on naturally colonized host plants, could it be achieved by artificially inoculating host plants? To address this question, host plants were inoculated under controlled conditions with single *T. melanosporum* fruiting bodies as spore donors and 6 and 19 months postinoculation (pi), the screening for

the presence of both mating types performed (Rubini et al. 2011a). Mycorrhizas of both mating types were formed and, at 6 months pi, the ratio of the two types was approximately similar in all inoculated plants. The screening performed at 19 months pi, however, indicated a drastically different situation: the majority of the plants had mycorrhizas that were all of the same mating type; of the few plants that displayed both mating types, there was a marked prevalence of one of the two types. Thus, under forced conditions, *T. melanosporum* strains seem to compete with each other for the colonization of host plant roots, and as a result, only a single strain persists on a given plant. Remarkably, the distribution pattern observed on artificially inoculated host plants strongly resembled the distribution patterns found on plants from a site where truffles are naturally produced. Taken together, these data indicate that a competition occurs between *T. melanosporum* strains for the colonization of their host plants and corroborate the view that an unbalanced representation of both mating types may be the main factor limiting *T. melanosporum* production in both artificial and natural sites (Rubini et al. 2011a).

The genetic basis underlying competition phenomena between truffle mycelia of different mating types has yet to be elucidated. It might be somehow related to self/nonself recognition phenomena. At this regard, Iotti and coworkers (2012) showed that orthologs of the genes known to control heterokaryon incompatibility (HI) in other fungal species are present in the *T. melanosporum* genome, although they lack the key functional domains involved in the HI process. Moreover, as in vitro dual culture experiments between pairs of genetically different *T. melanosporum* strains revealed neither the existence of any HI reaction nor the formation of anastomoses, it has been argued that in this truffle species any vegetative incompatibility-related phenomena might depend on mechanisms that act before hyphal contact (Iotti et al. 2012).

If the results concerning the biased distribution of the two fungal sexes on host plants are confirmed by additional experiments, then many practices in the cultivation of this fungus will need to be carefully reconsidered. For example, *T. melanosporum* orchards should be set taking into consideration the mating type of the fungal strain(s) on the host roots to maximize the chances of the appropriate strains mating with each other. Additionally, the screening of artificially inoculated host plants would be recommended to certify the *Tuber* spp. on their roots along with the mating types present.

Finally, because of the possibility that other *Tuber* spp. of economic interest are also heterothallic (Martin et al. 2010b), parallel studies should be undertaken to determine whether an unbalanced distribution of strains with opposite mating type is a common feature among other *Tuber* spp.

4.7 Conclusions

The last few years have seen a tremendous increase in our knowledge on *T. melanosporum* biology, much of which can be attributed to the sequencing of its genome. *Tuber* spp. are obligate symbiotic fungi, whose life cycles cannot be entirely

reproduced under controlled conditions. In light of this fact, our improved understanding of *T. melanosporum* population genetics, the progress made in characterizing the genes and pathways committed to symbiosis and reproduction and the wealth of recently developed genetic tools and screening methodologies will allow mycologists to address relevant issues concerning this and other *Tuber* spp. along with other symbiotic ascomycetes. Indeed, *T. melanosporum* is now a reference species among ascomycetes that can be used to understand how the ectosymbiotic lifestyle has been acquired by these fungi and for understanding the propagation strategies used by heterothallic species. In summary, *T. melanosporum* genomics is crucial for addressing both fundamental and applied questions concerning ascomycete fungi that are of outstanding ecological and economical relevance.

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Chapter 5
State of the Art of the Research on *Boletus edulis*

Antonietta Mello

5.1 Introduction

The genus *Boletus* belongs to the family Boletaceae, order Boletales, and consists of a complex of ectomycorrhizal fungal species. A large number of plants are suitable hosts: Fagales, Fagaceae (*Castanea*, *Castanopsis*, *Fagus*, *Lithocarpus*, *Quercus*) and Betulaceae (*Carpinus*, *Corylus*, *Betula*, *Ostrya*, *Populus*); Malvales, Malvaceae (*Tilia*) and Cistaceae (*Cistus*); Malpighiales, Salicaceae (*Salix*); Ericales, Ericaceae (*Arctostaphylos*); and Pinales, Pinaceae (*Abies*, *Keteleeria*, *Picea*, *Pinus*, *Tsuga*) (Olivier et al. 1997; Águeda et al. 2006; Mello et al. 2006). Among many sections, the section *Boletus* Singer (=*Edules* Fr. 1838) known as "porcini" is a conspicuous group of wild, edible mushrooms (Singer 1986; Murat et al. 2008; Sitta and Floriani 2008). Their reported distribution throughout the Northern Hemisphere was mostly limited to the sampling of European species. Nevertheless, they are popular in Europe and are becoming an economic resource North America also (Arora 2008). A global geographic sampling together with the selection of four loci—the fast-evolving in nuclear internal transcribed spacer (Fig. 5.1), the nuclear large subunit of the ribosome, the largest subunit of the nuclear gene encoding RNA polymerase II, and the mitochondrial ATPase subunit 6—has allowed Dentinger et al. (2010) to trace the molecular phylogenetics of these mushrooms. On this basis authors could recognize 18 reciprocally monophyletic species, show the monophyly of porcini, and expand their known distribution to Australia and Thailand. However, this global analysis did not consider *Boletus pinetorum*, a new species described using both morphological and molecular analysis by Korhonen et al. (2009) who focused on section *Boletus* in Fennoscandia.

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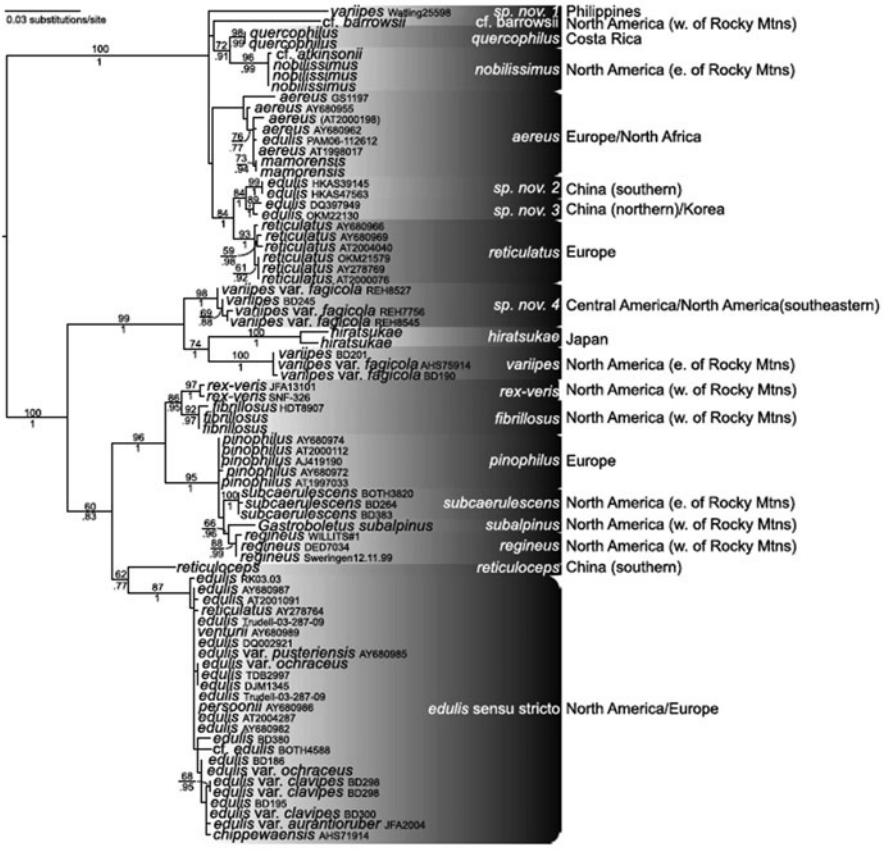

Fig. 5.1 Phylogram of the best maximum likelihood tree using *Boletus* ITS sequences. Clades that correspond to species are *shaded* and labeled with the specific epithet on the *right*. Geographic range of each species is indicated at *right*. Figure reprinted by "Molecular Phylogenetics and Evolution," 57, Dentinger et al., Molecular phylogenetics of porcini mushrooms (*Boletus* section *Boletus*), 2010, with permission from Elsevier

Many attempts to cultivate edible boletes have been made (Hall et al. 1998). This practice mostly concerned species belonging to *B. edulis* sensu lato (s.l.), such as *Boletus edulis* Bull.: Fr. sensu stricto (s.s.), *B. aereus* Bull.: Fr., *Boletus pinophilus* Pilát & Dermek, and *Boletus aestivalis* (Paulet) Fr. (Fig. 5.2). These fruiting bodies are in high demand; these mushrooms have a pleasant flavor and texture and are used in huge quantities for the production of dried mushroom soups. Preparations of these dried mushrooms sometimes include different species even some not belonging to section *Boletus*, such as *Boletus violaceofuscus*. Although Helbling et al. (2002) report that *B. edulis* induces allergic IgE-mediated symptoms, no taxonomic indication is given concerning *B. edulis* (sensu stricto or sensu lato), so the identity of the specific mushroom culprit remains unclear. Therefore, specific primers have been designed within the internal transcribed spacer rDNA region for the unambiguous

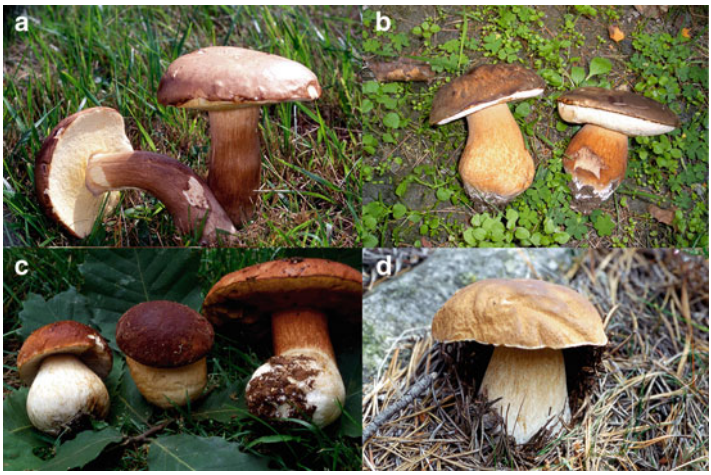

Fig. 5.2 Pictures of *Boletus aestivalis* (**a**), *Boletus aereus* (**b**), *Boletus pinophilus* (**c**), and *Boletus edulis* (**d**). Photos by Alfredo Vizzini

detection of these five species (Mello et al. 2006). The availability of these primers represents a crucial resource for detecting these fungi. Next steps are focused toward the analysis of the genetic structure of these fungi, as well as the certification of mycelia to be used as inocula in long-term reforestation programs focused on increasing bolete production. The broad host range of *B. edulis* makes it a suitable species to be introduced with various host tree species during reforestation efforts.

5.2 From *Boletus edulis* Molecular Identification to Cultivation

Many researchers have synthesized ectomycorrhizae of the *B. edulis* species complex on various hosts (Froidevaux and Amiet 1975; Tozzi et al. 1980; Molina and Trappe 1982a, b; Ceruti et al. 1983–1984, 1985; Poitou et al. 1982; Duñabeitia et al. 1996). However, the identification of the mycelia obtained by strain isolation and used as inoculum could not be confirmed by molecular analysis nor could the identification of the synthesized ectomycorrhizae. Because of this and other problems, when seedlings inoculated with *Boletus* species have been outplanted, the ectomycorrhizae of *Boletus* sp. are quickly replaced by other ectomycorrhizal fungi, and fruiting body production has never been successful (Olivier et al. 1997; Meotto et al. 1999; Wang and Hall 2004). Only more recently, Águeda et al. (2008) were able to synthesize ectomycorrhizae of *B. aereus*, *B. edulis*, and *B. reticulatus* (=*B. aestivalis*) with *Cistus* sp. and to provide molecular identification of the fungal strains isolated from fruiting bodies. Controlled mycorrhization now seems feasible opening new perspectives on the cultivation of these commercial mushrooms, which

have been collected exclusively from the wild (Cannon and Kirk 2007). In addition, *B. edulis* is regarded as a species tolerant to salinity stress, an adaptation that should be selectively advantageous in reforestation ecosystems (Liang et al. 2007). In order to understand the adaptive mechanisms of *B. edulis* to salt stress, proteomics was used to study the global expression profile of proteins in response to this stress. When the *B. edulis* was exposed to salt stress conditions (4 % NaCl, w/v), proteomic changes included proteins related to multiple cellular processes (e.g., metabolisms, energy-related processes, DNA repair, cell cycle control, and stress tolerance) (Liang et al. 2007).

5.3 In Field Characterization of *Boletus edulis* Mycelium and Mycorrhizae

Tracking the dynamics of an ectomycorrhizal fungus is a difficult task because fruiting bodies do not reflect the distribution of underground mycelial networks (Dahlberg 2001). Generally, the first analyses on the distribution of an ectomycorrhizal fungus focused on the fruiting bodies, which were followed by analyses of the mycorrhizae and extraradical mycelium in the soil. Few studies have been conducted on the ecology of the *B. edulis* complex. A first description and molecular characterization of the ectomycorrhizae of *B. edulis* on *Cistus ladanifer* L. was made by Águeda et al. (2006) by sampling field ectomycorrhizae and soil rhizomorphs under the fruiting bodies of *B. edulis*. Mycorrhizae of *B. edulis*, *B. aestivalis*, and *B. aereus* on *Castanea sativa* were detected in the field by Peintner et al. (2007). These mycorrhizae were morphologically similar to each other and showed the anatomo-morphological features previously described on *C. ladanifer*, except the color, which was darker ochre. In this investigation, ectomycorrhizae of *B. edulis* s.l. and soil fungal communities were characterized by molecular methods in a habitat with high production of *B. edulis* s.l. fruiting bodies. The first outcome of this investigation was that large amounts of basidiomes result from abundant mycorrhizae, but rare mycelia. While *Boletus* fruiting bodies dominated the aboveground fungal community, *Boletus* mycelia were rare in the soil and had a scattered distribution (Peintner et al. 2007). This finding is in agreement with a study by Zhou et al. (2001) showing that amounts of subterranean mycelia and mycorrhizae are not always correlated to the number of fruiting bodies formed by them. It is known that fructification is affected by habitat characteristics and climatic conditions (Bonet et al. 2004; Pinna et al. 2010) and depends on the presence of mycelium in the soil. Thanks to the progress of real-time PCR techniques, a quantification of *B. edulis* extraradical mycelium in a Scots pine forest soil was carried out (De la Varga et al. 2011). In this work the productivity of fruiting bodies was not correlated either with the concentration of *B. edulis* mycelia in soil samples or with the abundance of *B. edulis* mycorrhizae, whereas a statistically significant positive correlation was detected between the concentration

of mycelia of *B. edulis* in the soil samples and the presence of *B. edulis* mycorrhizae in these samples. The lack of correlation between the concentration of the mycelium in the soil and the productivity of fruiting bodies has also been shown in *T. melanosporum* orchards (Suz et al. 2008).

5.4 Applications of *B. edulis*

While ecologists are interested in deciphering the lifestyle of boletes, the common mushroom *B. edulis* is also an object of research aimed to discover important properties in edible fungi (see also Chap. 18). Since fungi are the most productive biological sources of various primary and secondary metabolites, they have long been exploited by the pharmaceutical and food industries. Heleno et al. (2011), in a targeted analysis of metabolites in wild *Boletus* species, revealed an important source of proteins, carbohydrates, fatty acids (mainly linoleic acid), sugars (mainly mannitol and trehalose), and vitamins (tocopherols and ascorbic acid) in *B. edulis*, *B. aereus*, and *B. reticulatus*, as well as phenolic acids. The finding of various macronutrients favors the consumption of these species not only for their taste and aroma but also as source of nutrients. On the other hand, the presence of phenolic acids, which has been correlated to antioxidative properties, can be exploited by food and pharmaceutical industries. In addition, polysaccharides extracted from *B. edulis* have shown important antioxidative properties and might be employed as ingredients in healthy and functional food to alleviate the oxidative stress (Zhang et al. 2011). As result of recent investigations, a lectin with antitumoral properties has been found, opening new perspectives in research aimed at developing new drugs for cancer therapy (Bovi et al. 2011). 1-Octen-3-ol is a characteristic aroma compound produced by mushrooms, including *B. edulis*, and is often added as flavoring in processed products such as dehydrated soups (probably because of huge losses of 1-octen-3-ol during the preparation of these food products) (Zawirska-Wojtasiak 2004). De Pinho et al. (2008) correlated the pattern of volatiles with the overall aroma of 11 wild edible mushrooms and discovered that only *B. edulis* presented linoleic acid that, as all a long-chain unsaturated fatty acids, shows antibacterial activity and is used as antimicrobial food additive. While metabolites are the object of interest for industrial applications, few studies have investigated their biological role in nature, such as their role in the interactions with other fungi, bacteria, and plants. In this regard, Splivallo et al. (2011) examined specific aspects of volatile ecology and biology of other ectomycorrhizal mushroom highly appreciated for their special taste and aroma, the truffles. To date, more than 200 volatile organic compounds have been described from various truffle species, and the biosynthetic pathways/genes involved in volatile biosynthesis have been traced in the recently sequenced genome of *Tuber melanosporum* Vittad. (Martin et al. 2010).

In a recent survey aimed to investigate metallic elements in wild-grown mushrooms, the use of *B. edulis* has established a baseline measure of regional mineral status and heavy metal pollution (Falandysz et al. 2011). It is known that

mushrooms are involved in the biogeochemical cycling of metallic elements and metalloids, and there is interest in understanding the mechanisms involved in bioconcentration of metallic elements by mushrooms.

5.5 Conclusions

Among the sections of the genus *Boletus*, the species belonging to the *B. edulis* complex have received more attention because of their commercial use. The application of morphological and molecular methods has allowed the production of *B. edulis* certified mycelia, and consequently inocula, and in vitro mycorrhizae, but research in field is still in its infancy. Although the detection of mycorrhizae and extraradical mycelium is feasible, no conclusions can be given on how this fungus completes its life cycle in time and space or on the population size. More long-term studies are needed to elucidate the genetic structure of *B. edulis*. Recently the ultrahigh-throughput DNA sequencing (UHTS) has dramatically changed the nature of biological research. These new sequencing technologies provide a tremendous amount of DNA sequence data with reduced costs, effort, and time as compared to Sanger sequencing (Fox et al. 2009). Among the UHTS numerous applications, genome sequencing/resequencing is opening new perspectives through understanding the biology and ecology of fungi. The sequencing of the mycorrhizal genomes has just started. The first sequenced genome is that of the basidiomycete *Laccaria bicolor* (Maire) P. D. Orton followed by the genome of the ascomycete *T. melanosporum* (Martin et al. 2008, 2010). The comparison of genomic traits in the two ectomycorrhizal fungi clearly indicated that the evolution followed along different ways in ascomycetes and basidiomycetes. Since the availability of genome sequences from ecologically and taxonomically diverse fungi would allow comparative studies to understand the evolutionary mechanisms of symbiosis, the US Department of Energy Joint Genome Institute launched the Fungal Genomics Program (FGP) (Martin et al. 2011). This program scheduled the sequencing of the genomes of 25 mycorrhizal fungi that have been chosen on the basis of their different affiliations, types of symbiosis they establish, and host specificity (http://mycor.nancy.inra.fr/blogGenomes/?p=1334). The genomes of some of them [*Amanita muscaria* (L.) Lam., *Cenococcum geophilum* Fr., *Hebeloma cylindrosporum* Romagn., *Laccaria amethystina* Cooke, *Oidiodendron maius* G. L. Barron, *Piloderma croceum* J. Erikss. & Hjortstam, *Paxillus involutus* (Batsch) Fr., *Pisolithus microcarpus* (Cooke & Massee) G. Cunn., and *Pisolithus arhizus* (Scop.) Rauschert (= *Pisolithus tinctorius*) have been just sequenced and preliminary data presented at the first Mycorrhizal Genomics Workshop held in Nancy in September 2011. *B. edulis* genome sequencing will follow soon [together with that of *Cantharellus cibarius* Fr., *Coltricia cinnamomea* (Jacq.) Murrill, *Cortinarius glaucopus* (Schaeff.) Fr., *Gymnomyces xanthosporus* (Hawker) A. H. Sm., *Lactarius quietus* (Fr.) Fr., *Meliniomyces bicolor* Hambl. & Sigler, *Paxillus rubicundulus* P. D. Orton, *Ramaria formosa* (Pers.) Quél., *Rhizoscyphus ericae* (D.

J. Read) W. Y. Zhuang & Korf, *Scleroderma citrinum* Pers., *Suillus luteus* (L.) Roussel, *Sebacina vermifera* Oberw., *Tomentella sublilacina* (Ellis & Holw.) Wakef., *Tricholoma matsutake* (S. Ito & S. Imai) Singer, *Tulasnella calospora* (Boud.) Juel, and *Terfezia boudieri* Chatin] allowing the identification of crucial genetic traits driving symbiosis and fruiting body development. This could, in the future, provide tools for the management of this precious fungus *in situ*.

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Chapter 6
Influence of Edaphic Factors on Edible Ectomycorrhizal Mushrooms: New Hypotheses on Soil Nutrition and C Sinks Associated to Ectomycorrhizae and Soil Fauna Using the *Tuber* Brûlé Model

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6.1 Introduction

6.1.1 A Global Perspective on Soil Organisms and Inorganic C Sinks

Soil is a complex system where basic processes for terrestrial communities take place (Ruf et al. 2003). Soil represents an important substrate for a large part of the earth's biodiversity. Soil properties determine ecosystem function and vegetation structure, and they serve as a medium for root development and provide moisture and nutrients for plant growth (Minnesota Forest Resources Council 1999). Food chain interactions among the soil biota (including plant roots) have major effects on the quality of crops (affecting human and animal nutrition and other aspects of life on earth), the incidence of soil-borne plant and animal pests and diseases (affecting production

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levels), and beneficial organisms such as edible ectomycorrhizal mushrooms (EEMM) (Brussaard et al. 2007).

Trueman and Gonzalez-Meler (2005) explain that the interplay between plant C intake, soil properties, community structure of the soil organisms and climate agents will determine the biosphere's organic terrestrial C sequestration strength at future atmospheric CO_2 concentrations. They studied tree growth and stabilization of SOM (soil organic matter) in an ecosystem exposed to elevated CO_2 concentrations and reviewed other works, concluding that the inability to retain C in soils exposed to elevated CO_2 may stem from the lack of stabilization of SOM, allowing its rapid decomposition by soil heterotrophs. They explain that increases in heterotrophic respiration in response to elevated CO_2 may result in soils becoming a net source of CO_2 into the atmosphere if input of new C does not keep pace and indicate that these facts make it impossible to forecast soil organic C sequestration potential.

In this scenario, it is important to have soil inorganic C sinks and biological models for the study of soil reaction and the decarbonation–recarbonation soil processes associated with fungal and soil organism communities, vegetation, and climate, in order to evaluate in-soil C cycling and C sequestration.

6.1.2 *A Global Perspective on EEMM, Soil Nutrients, and Inorganic C Sinks*

Ectomycorrhizal fungi (EM) mobilize soil nutrients, improve soil quality, and are important in the remediation of soil loss (Poma et al. 2006; Lian et al. 2007). Moreover, Wang and Hall (2004) indicate that the most highly prized edible mushrooms belong to the ectomycorrhizal group (EEMM). The total market for these fungi is measured in billions of US dollars, and the production of EEMM provides higher economic returns than any other forest product in many Mediterranean woods. In these areas, truffles (species of *Tuber* Micheli ex Wiggers), *Boletus pinophilus* Pilát and Dermek, *B. edulis* Bull.: Fr., and *Lactarius deliciosus* L.: Fr. are the fungi that yield the highest economic benefits (Moreno et al. 1986; Oria 1989, 1991; Montecchi and Sarasini 2000; Riousset et al. 2001).

The biology of EEMM has been fully described. However, there are still gaps in our knowledge of EEMM biology, and there is insufficient knowledge of the ecological relationships between EEMM and soil properties, processes and organisms. Among the numerous interactions between soils and EM-EEMM populations, there are two which may be relevant to soil processes on a significant scale: fungal metabolism could have greater impact than is generally estimated on soil reaction (as measured by pH) and on the amount of soil carbonates.

Cooke and Whipps (1993) report that fungal mycelia excrete H^+ as a result of the cation metabolism. This H^+ excretion is linked to K^+ capture, which occurs by

means of active transport, and exceeds concentration gradients of 5,000:1. Therefore, fungi could strongly acidify their immediate soil environment (hyphosphere), and their mycelia could provoke a significant change in soil properties and soil–EM–plant interactions. One of the immediate consequences on the soil acidification process is the change in the equilibrium of soil carbonates and inorganic C sinks.

6.1.3 The Tuber Brûlé: A Model to Study EM-EEMM Soil Ecology

The properties of carbonated soils limit the development of many EM-EEMM species. Truffles are nevertheless an exception to this rule, since the calcareous soils of Mediterranean forests afford the habitat required by many *Tuber* species.

Tuber melanosporum Vittad. has great economic value and grows in well-drained calcareous soils. Its phytotoxic activity provokes clearings in the vegetation (where the growth of other plants is inhibited either permanently or for part of the year), called burns or brûlés, where the mycelia bear fruit. The environmental conditions created by the brûlé are very favorable for *T. melanosporum* development, mycorrhization, and fruiting (Papa 1980; Plattner and Hall 1995; Dessolas et al. 2008; García-Montero et al. 2009a; Chevalier and Palenzona 2011).

Callot (1999), Ricard (2003), Jaillard et al. (2007), and Chevalier and Palenzona (2011) explain that *T. melanosporum* is closely linked to soil pH and to calcium carbonate availability and highlight the interesting decarbonation–recarbonation processes observed in *T. melanosporum* brûlés. García-Montero et al. (2009a) describe the *T. melanosporum* brûlé as an interesting biological model to study the soil reaction and the decarbonation–recarbonation soil processes associated with the EM-EEMM community, vegetation, and climate.

In the past 10 years we have provided new evidence of the influence of soil reactions and carbonate availability on *Tuber* ecology (in particular) and on EM-EEMM ecology (in general). The objective of this chapter is to review the results obtained during these 10 years of research into the influence of soil carbonate on truffles and soil organisms. As a result, we propose new hypotheses on soil chemical reactions, development of EM-EEMM populations and their interactions with soil inorganic C sinks.

Moreover, in this chapter, we will provide new evidence linking the presence and activity of soil fauna with *T. melanosporum*. Thus, we evaluate (1) earthworm impact on soil reaction and carbonate availability on *T. melanosporum* brûlés and (2) the impact of *T. melanosporum*'s ectomycorrhizal activity on biodiversity and soil quality.

6.2 Interactions Between Carbonates and Soil pH with EM

6.2.1 *Impact of EM Populations on the Amount of Carbonates in Soils*

The amount of soil carbonates depends on biological soil-forming factors such as relief, time, and climate (Breemen and Buurman 1998). Some important aspects include the processes of dissolution, transport, and accumulation of soil carbonates that depend on the relationships among water dissolution, gaseous CO_2, soluble H_2CO_3, HCO_3^-, and solid $CaCO_3$ phases.

An increase in H^+ in the microenvironment of the EM-EEMM hyphosphere could therefore provoke an alteration in these chemical balances and the dissolution of soil carbonates. This hypothesis is consistent with Poitou (1988), who showed that, in vitro, *T. melanosporum* mycelia acidify their environment and dissolve calcium carbonate. Callot (1999) reports on the doctoral thesis of Pottier (1986), who carried out an experiment on the growth conditions of the *T. melanosporum* mycelia with populations of associated bacteria. It was demonstrated that, regardless of the initial conditions, pH values of 5.5–6 were reached in all the cultures. The conclusion is that the *T. melanosporum* mycelium associated with bacterial populations strongly acidifies its environment. This experiment additionally confirms that Ca^{2+} favors the development of the *T. melanosporum* mycelium. Callot (1999) also reports that the roots and the microbiota associated with limestone soils create microsites of acidified soil, which in turn enables the dissolution of calcareous rock.

García-Montero et al. (2009b) studied the differences in pH and carbonate fractions in soils inside and outside the *T. melanosporum* brûlés (the study of exchangeable Ca^{2+} was difficult because the soil exchange complex is saturated in exchangeable Ca^{2+}). They indicated that there are no significant pH differences between the soils inside the brûlés and those outside brûlés. Nevertheless, the total carbonate is significantly lower inside the brûlés than outside. *T. melanosporum* mycelia may solubilize all the carbonate fractions, which would then dissolve in the water of the hyphosphere (this could buffer the increase in H^+ produced by the mycelia). The carbonate-enriched water would allow the mobilization and redistribution of carbonates throughout the brûlé soil. Part of the carbonate-enriched water would be leached out and contribute to soil carbonation losses, especially during periods of rain. The problem of carbonate leaching in *T. melanosporum* brûlés has been pointed out by Callot (1999) and Ricard (2003). Further studies are needed to confirm these experimental observations.

6.2.2 *Impact of Soil Carbonates on EM Populations*

Decarbonation–recarbonation soil processes could condition the dynamics of many EM-EEMM populations. Several experimental studies on liming and tree ectomycorrhizae reinforce this hypothesis:

1. Liming stimulates fine root development (Schneider and Zech 1990; Huettl and Zoettl 1993; Kreutzer 1995; Bakkera et al. 2000).
2. Liming modifies the EEMM community structure (Jonsson et al. 1999), increases the relative frequency of certain EM morphotypes (Erland and Soderstrom 1991; Antibus and Linkins 1992), decreases the relative proportion of some smooth-type EM in favor of hairy-type EM and increases the total number of EM tips (Bakkera et al. 2000). Even a moderate level of liming has long-lasting effects on ectomycorrhizal community composition (Kjøller and Clemmensen 2009).

The amounts of soil carbonates and the decarbonation–recarbonation processes also have an impact on truffle culture. *T. melanosporum* is present in different Mediterranean regions, and its distribution overlaps the natural regions for *Tuber aestivum* Vittad., *Tuber brumale* Vittad., *Tuber rufum* Pico and *Tuber mesentericum* Vittad. These truffle species compete in the same habitats (truffières) and frequently contaminate and cause damage to *T. melanosporum* truffières and reduce their production.

Simultaneous fruiting body production of various truffle species can be observed within the same brûlé. Ricard (2003) indicates that the development of the *T. melanosporum* brûlé triggers a process within the mycorrhizal population that favors *T. melanosporum* development over other fungi. The interactions of the species observed in time and space, associated with the processes involved in the brûlé development, generate a fungal succession (Falini and Granetti 1998; Callot 1999; Riousset et al. 2001; Ricard 2003; Granetti et al. 2005; Sourzat 2005):

1. *Tuber brumale*, *T. rufum*, *T. aestivum*, and *T. mesentericum* fruiting bodies are frequently collected outside *T. melanosporum* brûlés or just inside the edge of the brûlé.
2. Inside the brûlés, a temporal succession of the various truffle species can also be observed: *T. rufum* fruit bodies are the first to be collected, followed by *T. melanosporum*, and finally *T. brumale*. Interactions therefore take place between *T. melanosporum*, *T. aestivum*, *T. brumale*, *T. mesentericum*, and other fungi.
3. *T. rufum* may also enter natural and cultivated brûlés when the production of *T. melanosporum* begins to decline (García-Montero 2000; Riousset et al. 2001). In many areas of Spain, collectors call the fruiting bodies of *T. rufum* "bordes" (edges); this common name refers to the way in which the fruiting bodies of this species are collected on the outer edges of the *T. melanosporum* brûlés.

Sourzat and Dubiau (2001) and Sourzat et al. (2001) conclude that *T. melanosporum* brûlés could be unproductive for truffles due to different reasons. After noting that *T. aestivum* and *T. brumale* are present as mycorrhizal contaminants, they propose that environmental modifications or a loss of vigor in *T. melanosporum* might lead to its replacement by *T. aestivum* and *T. brumale*. Sourzat (2005) also indicate that natural *T. melanosporum* brûlés often contain *T. aestivum* and *T. brumale* as predominant competitor fungi but these species never fruit. The author explains that the contamination pressure of these other two *Tuber* species did not have a

negative effect on *T. melanosporum* production when grass and brush were limited on the brûlés. Nevertheless, the author was unable to say whether the aggressiveness of *T. melanosporum* and its resistance to the pressure of fungal contamination are qualities of the environment or of the soil.

García-Montero et al. (2008a) carried out a study to further explain the impact of soil on the interactions between truffle species inside the *T. melanosporum* brûlés, as compared to the *T. aestivum* and *T. mesentericum* brûlés in the same study area. These studies were done using ten conventional soil properties of surface horizons to determine how these properties affect the development and fruiting of these truffles. The results showed that the abundance of active carbonate is significantly higher in brûlés that produce only *T. melanosporum* than in those that produce *T. aestivum* and *T. mesentericum*.

Riousset et al. (2001) indicate that *T. rufum* lives in chalky soils in identical habitats to those occupied by *T. melanosporum*. García-Montero et al. (2009b) studied the relationships among pH, soil carbonates, and the presence of *T. melanosporum* versus *T. rufum* fruiting bodies outside the brûlés. The results showed that *T. rufum* fruiting bodies were collected in soils (outside the brûlés) with a greater concentration of total carbonate than *T. melanosporum* soils (inside the brûlés). The results also showed significantly higher active carbonate content inside the brûlés than in external soils (with *T. rufum*).

Therefore, the results described, in addition to Valverde-Asenjo et al. (2009), suggest that active carbonate, and its associated exchangeable Ca^{2+}, could be a major factor in the greater fruiting and aggressiveness of *T. melanosporum* and *T. brumale* when compared to *T. aestivum*, *T. mesentericum*, and *T. rufum*. These authors highlight that this similar response by *T. melanosporum* and *T. brumale* to soil carbonates may be related to their close phylogenetic relationship (Roux et al. 1999; Wang et al. 2006).

6.2.3 Impact of Calcareous Amendments on Truffle Culture

New studies are needed to understand the cultivation practices that increase total carbonate, active carbonate, and exchangeable Ca^{2+}. Particular attention should be given to the use of calcareous amendments in truffle culture, under strict ecological control, which would favor *T. melanosporum* production and its presence over other truffle species. Chevalier and Palenzona (2011) explain that the idea of using calcareous amendments in truffle culture goes back a long way. They describe the works of various authors on *T. melanosporum* and *Tuber uncinatum* Chatin (= *T. aestivum*) plantations on acid soils that had been treated with calcareous amendments in the United States, France, New Zealand, and Australia. Ricard (2003) also suggests adding fine limestone calcareous amendments in truffle culture, although he recommends using them carefully and in moderation. This assertion agrees with Callot (1999), who highlights the importance of high concentrations of surface carbonate in truffle culture. Moreover, Riousset et al.

(2001) report that calcareous amendments are being used in truffle culture to eradicate *T. brumale* from *T. melanosporum* cultivations. However, Valverde-Asenjo et al. (2009) explain that the calcareous amendments have not produced the expected effects of eradicating *T. brumale* from *T. melanosporum* cultivations.

García-Montero et al. (2009a) embarked on a succession of controlled calcareous amendments to eradicate *T. brumale* from *T. melanosporum* cultivations in Spain in order to expand the current knowledge of the effect of calcareous amendments on *T. melanosporum* plantations. These authors explain that calcareous amendments in *T. melanosporum* plantations significantly modified the soil's carbonated fraction in the brûlés one year after their application (mean content of active carbonate and total carbonate was higher than in nearby soil brûlés without calcareous amendments). These amendments will be continued in order to evaluate the development of the carbonated fraction in the monitored brûlé soils.

García-Montero et al. (2009a) also indicate that *T. melanosporum* and *T. brumale* production increased considerably in the amended plantations after 1 year. Therefore, liming does not negatively affect the *T. brumale* ectomycorrhizae in contaminated *T. melanosporum* truffières. These results and all cited observations indicate that active carbonate and its associated exchangeable Ca^{2+} could play a significant role in boosting production of both truffle species, thereby increasing the profitability of contaminated truffières.

6.3 Active Carbonate, Exchangeable Ca^{2+}, and the *Tuber* Brûlé Model: New Hypotheses on Soil Nutrition and C Sinks

There are still gaps in our understanding of *Tuber* ecology. In particular, Ricard (2003) points out that there is insufficient knowledge as to how physical–chemical soil properties influence truffle development. Most studies have failed to supply statistical data associated with the ecology and development of brûlés (García-Montero et al. 2007b). Recently, Chevalier and Palenzona (2011) have pointed out that current truffle culture techniques have not advanced much since the Second International Truffle Congress in Spoleto (1988).

However, new techniques are now being proposed thanks to fungal ecology studies: these include indirect control of the rootlet network by tree pruning and root cutting, root growth management, soil loosening, stimulation of burn formation, and organic fertilization. These authors also indicate that future research will benefit from a better knowledge of crucial points such as intraspecific variability, saprophytic nutrition habits, burn formation mechanisms, factors affecting fruiting body formation, influence of drought stress, or soil eutrophication by nitrogen. *Tuber* cultivation practices should benefit greatly from breakthroughs in understanding these key areas.

6.3.1 *Influence of Active Carbonate and Exchangeable Ca^{2+} on the Fruiting of* Tuber melanosporum

Various authors have examined soil properties to determine those with the greatest impact on *T. melanosporum* development. They indicate that *T. melanosporum* grows only in calcareous soils and prefers a C/N ratio close to 10, in which the soil texture tends to be balanced. Lulli et al. (1999), Castrignano et al. (2000), Callot et al. (2001), Ricard (2003), and Chevalier and Palenzona (2011) report that the development of *T. melanosporum* increases in deep, well-drained, and aerated soils with a reservoir of available water during dry periods. The most suitable texture for *T. melanosporum* is variable and depends on the stoniness of the soil. Both texture and stoniness should promote good aeration and water drainage. Organic matter content must be low to avoid pH modification, and considerable porosity is required, originating from biological activity.

Sourzat (2001) indicates that the soil parameters tolerated by *T. melanosporum* are highly variable. Callot (1999) and Ricard (2003) propose that the physicochemical properties of topsoil have a limited impact on truffle culture. Chevalier and Palenzona (2011) and others have emphasized the importance of conducting a thorough analysis of soil properties, including porosity, permeability and aeration, water balance, soil flora and fauna, exchangeable Ca^{2+} and recarbonation throughout the entire profile, as these factors determine the growth and production of *T. melanosporum* (Callot 1999; Bragato et al. 2001; Callot et al. 2001; Ricard 2003). Callot et al. (2001), Ricard (2003), and Granetti et al. (2005) also highlight the lack of knowledge as to how the physicochemical properties of fine soil influence truffle development.

García-Montero (2000) and García-Montero et al. (2006, 2007a) studied the relationship between *T. melanosporum* productivity and soil properties. Multivariate statistical analysis showed that the productivity of *T. melanosporum* is only slightly (and positively) influenced by the overall action of active carbonate, stoniness, organic carbon, clay content, and exchangeable cations present in the soil surface horizons. However, simple, positive, and very significant correlations were found among the percentage of active carbonate, percentage of exchangeable Ca^{2+} and the production of carpophores (Fig. 6.1). These authors explain that active carbonate is a fine fraction of calcareous rock measuring <50 μm, which is susceptible to rapid mobilization and is highly chemically active. Active carbonate indicates the extent and reactivity of carbonate surfaces and constitutes an important reserve of exchangeable Ca^{2+}. Continuous active carbonate formation preserves high levels of Ca^{2+} in the soil solution, regulates soil pH, and maintains the exchange complex, as it counteracts losses from leaching and other processes (Breemen and Buurman 1998).

The availability of active carbonate in soil depends on complex soil, climatic, and biological factors that are favored by soil stoniness. García-Montero et al. (2007a) showed that the percentage of surface stoniness is directly correlated to active carbonate concentration and *T. melanosporum* production. Surface stoniness regulates soil temperature and favors soil condensation and humidity, whereas

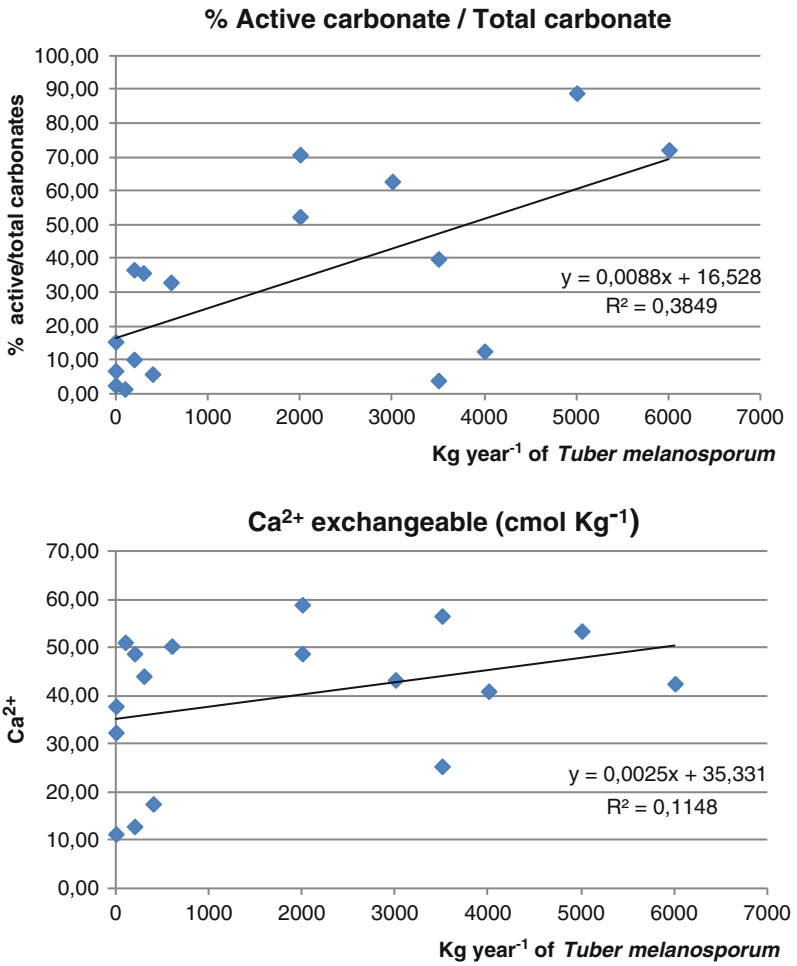

Fig. 6.1 Simple, positive and significant correlations among the percentage of active carbonate, percentage of exchangeable Ca^{2+} and the production of *Tuber melanosporum* carpophores (García-Montero 2000)

belowground stoniness lowers the fine earth volume and thereby encourages moisture. Certain types of rocky calcareous fragments are highly porous and retain and release significant amounts of water. These factors cause microenvironments to form on the lower side of rocks, which encourages the development of soil fauna, roots, microorganisms, and fungal mycelium. All of these factors modify the balance, dissolution, and precipitation of $CaCO_3$ and favor surface accumulation of secondary $CaCO_3$. Furthermore, rainwater flows over the rocks and accumulates in the lower parts, producing precipitations of carbonates in air spaces under the rocks when the water evaporates (Breemen and Buurman 1998; Callot 1999; Bragato et al. 2001; Callot et al. 2001; Ricard 2003).

Ricard (2003) suggests that the lack of studies on active carbonate in truffle culture is an oversight, and Jaillard et al. (2007) indicate that it is difficult to judge its impact. Ourzik (2001) points out that the knowledge of *T. melanosporum* biology is insufficient to establish a direct relationship between active carbonate and this truffle. These authors' objections have a rationale, since this carbonated fraction is highly variable and dynamic. In summary, Poitou (1988), García-Montero (2000), Ourzik (2001), and García-Montero et al. (2006, 2007a) emphasize the significance of active carbonate and exchangeable Ca^{2+} in the growth of *T. melanosporum* mycelium, and Chevalier and Palenzona (2011) conclude that exchangeable Ca^{2+} is the single most important element for truffle production. New studies are necessary on the interactions among active carbonate, exchangeable Ca^{2+} and *T. melanosporum*, linked to cultivation practices and soil physical and chemical properties.

6.3.2 *The* Tuber *Brûlé Model: A Feedback Process and New Hypotheses on Soil Nutrition and C Sinks*

Chevalier and Palenzona (2011) highlight the need to study the mycelium and the means by which the fruiting body acquires nutrients, as this is a key factor in its cultivation. The nutritional mechanism of the *T. melanosporum* fruiting body is still under discussion, and the theory of fruiting body independence has been called into question. The implications for truffle fertilization are important. Chevalier and Palenzona (2011) and Dessolas et al. (2008) indicate that the mechanism of brûlé formation is poorly known: they only know that truffles "prepare their nest" by emitting volatile herbicidal substances which strongly degrade minerals and soil organic matter.

Sourzat (2004), Granetti et al. (2005), and other authors have explained the production and development of brûlés. Chevalier and Palenzona (2011) suggested that truffle production may be correlated to tree vigor and brûlé growth. They explain that the idea of *T. melanosporum* aggressiveness proposed by Sourzat (2001) is a reflection of appropriate ecological conditions for the truffle and that its vigor may be assessed by measuring the ratio of the tree canopy radius to the brûlé's radius and its annual growth rate. In any case, Callot et al. (2001), Ricard (2003), Granetti et al. (2005), and Chevalier and Palenzona (2011) indicate that the biological and physiochemical conditions governing brûlé development have yet to be clarified.

García-Montero et al. (2007b) indicate that in different types of woodland, *T. melanosporum* showed a significant difference in the amount of productive carpophores. They also point out that the larger the size of the brûlés, the greater the carpophore production of *T. melanosporum*. Nevertheless, there were no significant differences in brûlé size among the different types of woodland tested. In summary, brûlé size was a significant feature in the production of *T. melanosporum*. To understand the processes related to brûlé development, these authors studied the relationship between brûlé size and soil features. The statistical

analysis revealed that *T. melanosporum* brûlé size is related to the percentage of active carbonate present in the soil surface horizons and that the collective influence of the other soil features studied is not significant to the development of *T. melanosporum* brûlés.

Soil carbonate solubilization and leaching increases with rainfall, respiration, and metabolism of roots and mycelia and other factors that increase the partial pressure of CO_2 (pCO_2) in the soil. Bicarbonate precipitates as carbonate when it is concentrated in capillary water (evaporated or absorbed by the roots). This also occurs when the pCO_2 diminishes due to aeration caused by macrofauna and the disappearance of biological respiration of the roots at depth, among other factors (Poitou 1988; Bencivenga and Granetti 1989; Coli et al. 1990; López and López 1990; Wild 1992; Marañés et al. 1994; Breemen and Buurman 1998; Callot 1999; Ourzik 2001; Ricard 2003). *T. melanosporum* eliminates plants from their brûlés, and the disappearance of their roots favors the accumulation of active carbonate. In summary, the development of the brûlé encourages the formation of large amounts of active carbonate and exchangeable Ca^{2+}. *T. melanosporum* activity and brûlé size are simultaneously favored by a high concentration of both factors, which suggests a feedback process. These conclusions agree with Chevalier and Palenzona (2011) who also conclude that the single most important element for truffle production is exchangeable Ca^{2+}.

García-Montero et al. (2009a) propose the following hypothesis as a guide for research into the role of active carbonate and exchangeable Ca^{2+} and the feedback processes associated with the brûlé. They hypothesize that *T. melanosporum* and *T. brumale* may favor or benefit from soils that are rich in active carbonate and exchangeable Ca^{2+} to promote their ectomycorrhization in plants with nutritional deficiencies due to lime-induced chlorosis.

Little is known about the physiological relationship between *Tuber* species and their symbiont plants (Ricard 2003). The proposed hypothesis on lime-induced chlorosis and *Tuber* mycorrhization is related to the experiments of Dupré et al. (1982) on the mycorrhization of *T. melanosporum* in substrates watered with nutrient solutions. These experiments showed that the lack of phosphorous favors the ectomycorrhization of *Quercus pubescens* Willd. The experiments of Gelpe and Timbal (1986) also demonstrate that a high active carbonate content induces chlorosis in *Quercus rubra* L. Moreover, Püttsepp et al. (2004) indicate that local soil nutrient status influences EM colonization, and low P and K availability favors EM colonization.

The abundance of active carbonate in soils raises the pH and increases HCO_3^- and exchangeable Ca^{2+}, which immobilizes P, B, Fe, and Mn (Gaucher 1971; Follet et al. 1981; Loué 1986; Douchafour and Souchier 1979; Wild 1992; Callot 1999). Moreover, a significant rise in pH affects the absorption of microelements by plants. An increase in pH reduces the assimilability of Al, Co, Cu, Fe, Zn, and Mn (Loué 1986). A pH of 7.9–8.4, typical of *T. melanosporum* soils, leads to increasing deficiencies of Co, Cu, Mn, and Zn in plants (Gaucher 1971; Follet et al. 1981; Wild 1992). When the pH is raised, Mg^{2+} may be transformed into a non-exchangeable cation with no satisfactory explanation (Wild 1992), leading to the formation of insoluble calcium borates (Follet et al. 1981).

Reactions between Ca^{2+} and P can occur in the soil solution, soil colloids, or precipitated calcium carbonate. The degree of P adsorption on precipitated calcium carbonate is inversely related to particle size (Follet et al. 1981). An overabundance of Ca^{2+} and HCO_3^- immobilizes Mg^{2+} or leads to its deficiency in plants (Douchafour and Souchier 1979; Follet et al. 1981).

In summary, our soil nutrition hypothesis proposes that soils with high active carbonate and exchangeable Ca^{2+} produce host plant chlorosis, which is a symptom of an underlying nutrient deficiency. This nutrient deficiency could lead to greater colonization of *T. melanosporum* and *T. brumale* ectomycorrhizae and favor the growth of mycelia and fruiting body production. The development of *T. melanosporum* brûlés could simultaneously encourage the formation of new amounts of active carbonate and exchangeable Ca^{2+} based on a feedback model proposed as the best way of explaining the cause–effect of all these observations.

This brûlé model is essential for EM-EEMM fungi species from an evolutionary and ecological viewpoint, as it assumes that *T. melanosporum* and *T. brumale* have the ability to modify soil pH, soil carbonate, and exchangeable Ca^{2+} availability and may thus have a direct impact on the structure and dynamics of the mycorrhizal community. This brûlé model also has an added value in the form of a study of the C sequestration/loss process, as inorganic and organic carbon, in relation to the soil–plant-*Tuber* system.

6.4 Soil Fauna and the *Tuber* Brûlé Model: New Hypotheses on C Sinks

The diversity of soil fauna includes a quarter of described living species, the majority of which are insects and arachnids (Decaëns et al. 2006). Soil fauna play an essential role in agroecosystems by maintaining their functionality and productivity (Wardle et al. 1995), and it is also important in decomposition, nutrient recycling and the maintenance of physical and chemical soil properties and structure (Davidson and Grieve 2006). Just like plants, most soil animal taxa are directly influenced by soil parameters, since their mobility is limited.

Various soil invertebrate taxa, including Nematoda, Collembola, and Oligochaeta, are known to graze on fungal mycelia and can substantially alter fungal morphology and physiology (Harold et al. 2005; Bobby and Jones 2008). Such effects are likely to alter fungal fitness and therefore their combativeness in interaction with other soil microorganisms, including fungi (Rotheray et al. 2011).

Since fungi are an attractive food source for soil arthropods, certain fungi may have developed strategies to avoid grazing. The deleterious effect of toxins on the development of coleopterans has been studied intensively (Mier et al. 1996). Böllmann et al. (2010) hypothesized that crystals on the hyphal surface as well as toxic or bitter-tasting secondary metabolites may serve as feeding protection and will have a strong impact on the feeding preference of Collembola.

6.4.1 *Soil Fauna and EM Populations*

There is still much to learn about ectomycorrhizal ecology, and very little in particular is known about interactions between the EM–plant complex and soil fauna. In contrast, more is known about the interactions between arbuscular mycorrhizae and soil fauna (Hoffmann 2011).

The relationship between Collembola and ectomycorrhizae is beginning to receive increased attention because Collembola can affect plant growth and mycorrhizal infection (Kaneda and Kaneko 2004). Ectomycorrhizae are capable of "connecting" with nearby trees, even if they belong to different species, thus creating networks that establish a flow of nutrient and photosynthetic products among all the interconnected plants. The ecological role of these networks is crucial and is currently the subject of study. In addition to nutrient and water transport among plants (which sometimes only favors one of them), it has been demonstrated that there are other positive effects on plants, particularly on those aspects related to the capacity of new land colonization and the degree of forest evolution and maturity (Domínguez et al. 2006). These EM-plants are conditioned by several factors inherent in their soil habitat, and Collembola populations may condition network size or even interrupt them. Therefore, the relationship between Collembola preferences and mycorrhizal activities is important for understanding the potential effects of Collembola on plant mycorrhizal symbiosis, especially since a highly active mycorrhizal mycelium may be more effective than a senescent one in plant nutrient acquisition (Kaneda and Kaneko 2004; Menta et al. 2011; Tarasconi et al. 2011).

In summary, from a global perspective, soil fauna is an important component of soil systems. For this reason, there is growing interest in studying the interactions between soil fauna and soil properties (including soil pH and carbonate balances) and in determining their impact on EM–plant systems and soil C sinks.

6.4.2 *Soil Mesoarthropods and the* Tuber *Brûlé Model: Preliminary Studies*

Callot (1999), Lulli et al. (1999), and Castrignano et al. (2000) highlight that the *T. melanosporum* brûlé requires considerable soil porosity and that much of this porosity is due to the activities of earthworms and ants. Chevalier and Palenzona (2011) propose the use of cultural practices that favor soil fauna and emphasize the importance of avoiding cultural practices that may damage soil life (such as pesticides) They also propose the experimental introduction of earthworms into truffle culture. However, there is little information about the relationships between *T. melanosporum* and soil fauna.

The mesofauna composition of the soil (from 200 μm to 2 mm) is highly influenced by humidity, pH, organic matter, and humus type (Hagvar 1982; Kuznestova 2002). It

is also necessary to determine how these factors affecting soil fauna are correlated to the type of vegetation and the existing EM–plant systems. Plants modify the physical and chemical properties of the soil (Materna 2004), which in turn indirectly influences soil fauna composition (Wild 1992). The *T. melanosporum* brûlé creates a particularly hostile environment that induces low in-soil humidity, modifies its temperature regime, and changes soil properties inside the brûlé.

Recently, Menta et al. (2011) and Tarasconi et al. (2011) have begun to study the interactions between mesoarthropods and *T. melanosporum* in a series of comparative studies between different Mediterranean areas in Italy and Spain. For this purpose, they used the QBS-ar biological quality index proposed by Parisi et al. (2005). In the Italian plots a total of 12 soil fauna taxonomic groups were found. Many of these are typical of forest environments: pseudoscorpions (Pseudoscorpionida), arachnids (Araneida), acarid (Acari), isopods (Isopoda), diplopods (Diplopoda), symphylans (Symphyla), pauropodans (Pauropoda), collembolans (Collembola), hemipterans (Hemiptera), hymenopterans (Hymenoptera), coleopterans (adults and larvae) (Coleoptera), and dipterans (adults and larvae) (Diptera). These studies found that there were distinct differences between the mesoarthropod communities inside and outside the *T. melanosporum* brûlé and that the communities outside the brûlés had a higher diversity. The Spanish plots showed a smaller number of groups (10) than the Italian plots: arachnids, acharina, diplopods, symphylans, collembolans, hemipterans, dermapterans, hymenopterans, coleopterans (adults and larvae), and dipterans (adults and larvae).

Soil mesoarthropod communities were partially different in the brûlé (1) the density of acharina and wasps and the number of biological forms outside the brûlé were higher; (2) the number of mesoarthropod taxa was lower inside the brûlés; and (3) some important groups, such as mites and ants, were negatively affected by the brûlé. However, collembolans do not seem to be affected by the brûlé. It is necessary to determine whether these soil fauna differences are a consequence of the direct action of the compounds produced by the *T. melanosporum* mycelium or are caused as an indirect effect of the truffle on vegetation and chemical and physical soil characteristics.

6.4.3 Earthworms and the Tuber *Brûlé Model: Cast Impacts on Soil Recarbonation and C Cycling*

6.4.3.1 Earthworms and Truffle Culture

Callot (1999), Ricard (2003), and Pargney et al. (2008) indicate that earthworms are often found around the truffle fruiting bodies in the brûlé soils. These soils usually have low levels of organic matter, and earthworms search near the carpophores for the bacterial populations and droppings of micro-arthropods and macrofauna. Lulli et al. (1999) and Castrignano et al. (2000) point out that the action of earthworms in

the soil benefits *T. melanosporum*. Callot (1999) stresses that the impact of cultural practices greatly reduces earthworm activity.

Callot (1999) and Ricard (2003) highlight the relationships between earthworm activity, pCO_2, and soil pH in *T. melanosporum* brûlés. They propose that in calcareous, poorly aerated, and badly drained soils, the episodes of rain and dryness increase the pH to over 8.4 (and local areas may sometimes reach pH 10), while in well-aerated and drained soils with high earthworm activity, the pH is generally maintained below 8.4. They explain that the pCO_2 increase is reflected in a lower pH that falls below 7 when pCO_2 reaches 0.1 atmospheres (often achieved in calcareous soils and rhizospheres). They conclude that pCO_2 in the soil atmosphere depends not only on the respiration of roots and microorganisms but also on soil aeration which is strongly conditioned by earthworm activity (generating up to 5,000 km of galleries per hectare in grassland soils) (Callot 1999).

6.4.3.2 Earthworm Calcite Granules

Earthworm activity around *T. melanosporum* carpophores may affect the soil carbonate dynamics associated to the brûlés. In this regard, Callot (1999) proposes that earthworms could provoke local soil decarbonation, as the calcite granules are coated with mucus (uncarbonated), consisting of clay and polysaccharide, during the intestinal transit through the earthworm.

Spherical soil concretions composed of calcite crystals have been found in soils containing earthworms. These calcite granules range from single calcite crystals to aggregations up to 2.5 mm in diameter and are produced by the calciferous glands of all species of earthworm of the family *Lumbricidae*. Wiecek and Messenger (1972) proposed a preliminary estimation on the abundance of earthworm calcite granules in some deeply leached forest soils in North America. They estimated a contribution of 1.1 kg/ha per year of calcite granules in the upper portions of the horizons (0–50 mm). However, they did not estimate the calcite granules associated with earthworm casts. Jongmans et al. (2003) found earthworm calcite granules down to a soil depth of 50 cm, and Canti and Piearce (2003) indicate that these granules are regularly found in soil profiles, although they confirm that little is known about their origins and dynamics.

Gago-Duport et al. (2008) explain that earthworm calcite granules do not appear to have any biological function. Coleman et al. (2004) and Briones et al. (2008) indicate that calciferous glands provide a mechanism for regulating CO_2 in blood and tissue. Briones et al. (2008) explain that both environmental and metabolic CO_2 can be fixed in this way and show that ^{13}C-labeled air rapidly enters the calciferous gland and can be detected in both the gland and the granules after 4 days. Based on laboratory studies, Canti (2009) indicates that the C calcite granule is extracted from the earthworm's dietary intake (litter), atmospheric CO_2, and SOM; however, very little appears to come from soil calcium carbonate. Canti's conclusions (2009) may be of great value regarding soil C cycling and carbonate dynamics.

Lee et al. (2008) report that earthworms produce calcite granules in sufficient volume to have a measurable impact on soil C cycling, although they indicate that the volume and role of this earthworm bio-mineralization process is yet unknown. Jongmans et al. (2003) found that soils without earthworm activity undergo decalcification processes that could be ascribed to the production of organic acids in the litter layer and to the absence of soil homogenization by earthworms.

6.4.3.3 Earthworm Casts and the *Tuber* Brûlé Model: A New Hypothesis on Soil C Sinks

García-Montero et al. (2011) proposed the *T. melanosporum* brûlé model to analyze the interactions between earthworm calcite granules and decarbonation–recarbonation soil processes. They conducted studies in the topsoils of brûlés to determine the balance of carbonate and organic carbon associated to earthworm casts, as compared to the soils from which the casts were produced. These studies sought to demonstrate in the field Canti's (2009) laboratory proposals regarding the sources of C for the synthesis of calcite granules by earthworms.

García-Montero et al. (2011) indicate that earthworm cast samples had a significantly higher percentage of total carbonate levels and higher pH than the surface soils from which the casts were produced; however, there were no significant differences in the percentage of SOM. They also indicate that soils from *T. melanosporum* brûlés from a truffière with an abundance of casts had a significantly higher pH and a lower percentage of total carbonate, percentage of active carbonate, and SOM levels than nearby brûlés with low cast levels. These results agree with Chan (2003), who showed that cast pH is significantly higher than that of the bulk acid soil material used in a laboratory experiment. Wiecek and Messenger (1972) also indicate that earthworm casts are less acid than the ingested food and propose that the weathering of earthworm calcite granules is partly responsible for high pH values in the top horizons of acid soils under forest cover in some North American forests.

These field results obtained in *T. melanosporum* brûlés confirm Canti's (2009) laboratory results: there is a significant increase in carbonate contents in the earthworm casts (presumably due to the calcite granules) that cannot be explained by existing levels of carbonate in the original soils from which the casts were produced. This confirms that, in the field, the C in the calcite granule is mainly extracted from the earthworm's dietary intake (litter), SOM, and atmospheric CO_2. This was demonstrated by Canti (2009) using artificial substrates with marked differences in $\delta^{13}C$ levels.

Therefore, the calciferous glands of earthworms may be of great importance in evaluating C cycling and C sequestration in the soil, as they could fix different forms of C into bio-mineral granules formed by calcite crystals, and promote long-term soil C fixing. Lee et al. (2008) indicate that these biomineral granules are stable, and Canti (2009) reports that earthworm calcite granules have been found in archaeological soils and sediments after many years.

Earthworm calciferous glands may also have a major impact on soil reaction and recarbonation processes associated to *T. melanosporum* brûlés and other EM-EEMM populations. It is therefore advisable to further our knowledge of the role played by earthworm casts and their calciferous glands on a local and global scale.

6.5 Conclusions

Fungi can strongly acidify their immediate soil environment, and their mycelia can provoke a significant change in soil properties and soil–EM–plant interactions. One of the immediate consequences of the process of soil acidification is the change in the equilibrium of soil carbonates.

The *T. melanosporum* brûlé is an interesting biological model for studying soil reaction and the decarbonation–recarbonation soil processes associated with the EM-EEMM community, soil fauna (earthworms, mesoarthropods, and others), and vegetation. Several studies have indicated that a high concentration of active carbonate and exchangeable Ca^{2+} in the soil are major factors that favor *T. melanosporum* fruiting body production and increases in brûlé size; this points to the interest of studying the use of calcareous amendments in truffle culture. The amount of active carbonate is significantly higher, and total carbonate is significantly lower inside than outside the *T. melanosporum* brûlés. These patterns and other works confirm that *T. melanosporum* mycelia acidify their hyphosphere and solubilize carbonated fractions. Subsequently, the particular environmental conditions of brûlé soils may favor a secondary carbonate precipitation with a net increase in active carbonate, which preserves high levels of exchangeable Ca^{2+}.

Furthermore, field results obtained in *T. melanosporum* brûlés and recent experimental studies show that the synthesis of calcite granules by earthworms is based on C sources that are mainly extracted from the earthworm's dietary intake, soil organic matter, and atmospheric CO_2. Therefore, the earthworm's calciferous glands could cause brûlé soil recarbonation based on long-term fixing of C in the form of calcite.

Based on these findings, we propose new hypotheses on EM-EEMM ecology, soil biology, and inorganic C soil sinks, suggesting that (1) the model which best explains the cause–effect of all brûlé observations is a feedback process; (2) this model assumes that the ability of *T. melanosporum* to modify soil properties may have a direct impact on soil-plant nutrition and the degree of plant mycorrhization, and this hypothesis could have a major impact on EM-EEMM from the evolutionary standpoint; and (3) the integrated action of *T. melanosporum* and/or other EEMM populations, in combination with earthworms, could be of great importance in the cycling and sequestration of inorganic C in the soil.

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Chapter 7
Ectomycorrhizal Fungal Communities of Edible Ectomycorrhizal Mushrooms

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7.1 Introduction

Ectomycorrhizal (EM) fungi are a wide group of fungi that include around 20,000–25,000 species (Rinaldi et al. 2008). An estimated 1,000 species of EM fungi produce edible fruiting bodies (Hall et al. 2011; Chap. 1). Edible ectomycorrhizal mushrooms (EEMMs) are a quite heterogeneous group of fungi characterized by different reproductive structures either epigeous (mushrooms, puffballs-like, etc.) or hypogeous (truffle-like) that just happen to be edible. As a consequence, they do not form a distinct taxonomic group but are instead spread across many Ascomycota and Basidiomycota genera. Hence, they cannot be expected to have similar niches in soil communities but are instead adapted to different soil conditions (Smith and Read 2008).

In natural habits EM fungi form complex communities characterized by multiple species, and coexistence is governed by competition dynamics (Kennedy 2010) and host availability (Tedersoo et al. 2012). EM fungal competition for host plant carbon resources and soil nutrients is affected by biotic and abiotic factors particularly host plant, soil chemistry, and environmental conditions (Jumpponen and

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Egerton-Warburton 2005; Kennedy 2010). The soil also includes numerous other microorganisms such as pathogenic and saprobic fungi and bacteria that are known to interact with the EM fungi (Paulitz and Linderman 1991; Cairney and Meharg 2002; Frey-Klett et al. 2007) although these interactions remain poorly investigated (Lindahl et al. 2001; Zambonelli et al. 2009).

The knowledge of EM fungal community composition and dynamics in relation to environmental conditions is an important ecological basis for understanding interactions between different EM fungi. EM competitive relationships can affect survival and spread of EM species in the soil. This is particularly important in EEMM cultivation where a valuable EM species is introduced in the field through the planting of EM infected plants (see Chap. 9). Replacement of the introduced EEMM by native EM fungi is one of the most important causes of truffle cultivation failures (Hall et al. 2007).

This chapter aims to provide a brief review of the approaches applied to the study of EM fungal communities including morphological and molecular methods, as well as resources available on the Web for the identification of EM fungi. Further, we report on recent studies specifically carried out on EEMM communities.

7.2 Methods Used in the Study of EM Fungal Communities

7.2.1 Fruiting Body Surveys

The classical approach in the study of EM fungal communities was simply through collection and identification of fruiting bodies (Arnolds 1991). Fungal species were determined as mycorrhizal through the basis of mycorrhizal synthesis or direct observation of this condition with the host plant in nature (Brundrett 2008; Chap. 1). Such approach tends to underestimate the EM fungal diversity because fruiting body production is sporadic and seasonal; many species have cryptic fruiting, while others may not fructify at all such as some species of Thelephoraceae, Sebacinaceae, and *Cenococcum geophilum* Fr. (Horton and Bruns 2001), all of which have been shown to be common in many edible ectomycorrhizal (EEM) fungal communities (see Sect. 7.3). Moreover, due to the climatic dependence of EM fruiting body production, numerous surveys over many years are necessary to get baseline estimates of fungal diversity (Dahlberg 2001). For these reasons, molecular and morphological analyses of mycorrhizas are now standard methods used to study EM fungal communities.

7.2.2 Morphological Identification of Mycorrhizas

Investigation of the EM fungal communities by morphological characterization (morphotyping) of their mycorrhizas was systematically defined by Agerer (1987–2008, 1991), who provided a large set of morphological characters to unequivocally

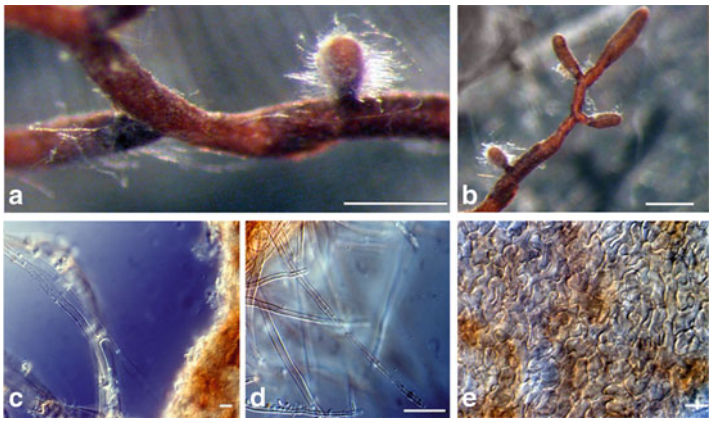

Fig. 7.1 Mycorrhizas of *T. magnatum* obtained by spore inoculation in greenhouse: infected tips (**a** and **b**, bar = 1 mm), external hyphae forming an anastomosis (**c**, bar = 10 μm), awl-shaped cystidia (**d**, bar = 10 μm), epidermoid external mantle (**e**, bar = 10 μm)

describe discrete EM morphotypes. This approach results in a panorama of underground EM fungal diversity. However, using Agerer's methods, only a small fraction of the EM diversity can be attributed to define fungal species with any certainty, and most EM morphotypes remain unidentified. In these cases, the EM species is named using a provisional binomial derived from the genus of the host tree and a characterizing epithet, for example, *Piceirhiza gelatinosa* (Agerer 1991). Studies of EM communities using only morphological methods tend to produce an erroneous estimation of the EM richness. Indeed, multiple species could be included in single morphotypes (e.g. cryptic species), but it is just as likely that a single fungal species produces different morphotypes (Burke et al. 2005). In fact, the morphology of the symbiotic structures formed by an EM fungal species can vary depending on the host plant and the fungal strain, as well as the environmental conditions, tip aging, and phenotypic expression (Martin et al. 2007). For instance, different strains of the EEMM *Tuber borchii* Vittad. were found to form slightly different morphotypes (Giomaro et al. 2000; Sisti et al. 2003), and in the same way the host plant was shown to influence the morphology of the *Tuber brumale* Vittad. mycorrhizas (Giomaro et al. 2002).

Among the EEMMs, morphotyping of mycorrhizas is a commonly used tool for identifying species of truffles in the genus *Tuber*. Truffle mycorrhizas were first described by Zambonelli et al. (1993, 1995), who provided the first key for their identification on *Quercus pubescens* Willd and *Pinus pinea* L. host plants. The most informative characters for *Tuber* mycorrhizas are color, form of cystidia, and shape and dimension of the mantle cells (Fig. 7.1). These characters are used for an online interactive key, called "TuberKey" (Zambonelli et al. 2000b). Even if these keys are useful for the identification of *Tuber* mycorrhizas in commercial seedlings, morphotyping does not always guarantee correct identification of symbiotic truffle species. For example, the mycorrhizas of *Tuber indicum* Cooke and

Massee and *Tuber melanosporum* Vittad. (Zambonelli et al. 1997) have similar cystidia and mantle cell shapes, and in some stages of development, cystidia may be lacking altogether. The same is true for those of *T. borchii* and *Tuber maculatum* Vittad., as well as some other species of *Tuber* (Zambonelli et al. 1999). Morphotyping is a useful technique for discriminating mycorrhizas formed by competitive EM species in truffle orchards and nurseries, such as the "AD" morphotype, described in French for the right angle "angles droits" cystidia cells (Giraud 1988) and more recently identified as *Trichophaea woolhopeia* (Cooke and W. Phillips) through DNA sequencing approaches (Agueda et al. 2008; Rubini et al. 2011).

7.2.3 Molecular Identification of Mycorrhizas

The recent explosion of studies applying PCR-based molecular techniques in fungal identification and genotyping has greatly advanced our understanding of fungal diversity (Peay et al. 2008). The internal transcribed spacer (ITS) rDNA region is now widely used as a DNA barcode for taxonomic identification of ectomycorrhizal fungi (see Chap. 2) and is the foremost marker used to characterize whole fungal communities. The comparison of the ITS sequences obtained from an EM community against those deposited in International Nucleotide Sequence Database (INSD: GenBank, EMBL, and DDBJ) is commonly performed by BLAST best-hit analyses because of its speed and simplicity (Porter and Golding 2011). This approach often allows for the identification of the fungal taxa of interest at genus or even species level resolution, or, at worst, to assign it to one of the main EM lineages (thelephoroid, sebacinoid, agaricoid, etc.). However, there are problems concerning the taxonomic reliability of public sequence databases (Nilsson et al. 2006), and caution is warranted in taxonomic determinations made solely through BLAST searches. For this reason, it is convenient to perform BLAST searches against multiple databases and to adopt a conservative naming approach when the more significant BLAST hits are poorly supported or contrasted. A case of inconsistent reliability of the public sequence database occurs for the genus *Tuber*. While ITS accessions of many distinct species, such as *Tuber magnatum* Pico and *Tuber aestivum* Vittad., appear to be accurately identified, those of other less studied taxa are not. For example, the 107 ITS1-5.8S-ITS2 sequences labeled as *T. borchii* deposited in GenBank (accession date 4 Apr 2012) fall into five different phylogenetic groups (similarity <90 %) that represent distinct species (see also Zambonelli et al. 2010). In another instance, four ITS sequences with a pairwise similarity exceeding 99 % (EU784422, EU784423, EU784424, EU784428) obtained from specimen vouchers of the Kew herbarium (Brock et al. 2009) have mistakenly been accessioned as three different species (*T. borchii*, *Tuber dryophilum* Tul. & C. Tul. and *T. maculatum*). This is in part due to the difficulties of discriminating species of whitish truffles from each other, given the few distinguishing microscopic characters of spores and peridium that have been found useful. Similar cases in GenBank are common for other groups of EM fungi. Effort has been made to annotate reliable sequences in public databases (Tedersoo et al. 2011).

Various criteria have been reported in literature for taxonomic affiliation of morphotypes forming an EM community based on ITS sequence similarity values (Landeweert et al. 2003; Smith et al. 2007) although it is unrealistic to establish a global cut-off value for taxon delimitation that works for all groups of fungi. In fact, the ITS region is not equally variable across the fungi and far-reaching knowledge of genetic variability within each lineage is still needed (Nilsson et al. 2008). The best way to classify species detected on the roots is through the comparison of their ITS sequences to those of the fruiting bodies collected in the same experimental area or from a curated reference databases (Dahlberg 2001; Liu et al. 2011).

The identification of fungal symbionts is routinely done these days through DNA extraction from one or a few colonized root tips, followed by PCR analyses and sequencing. In order to reduce time and costs of molecular identification, "direct PCR"-based method has been developed by Iotti and Zambonelli (2006) and can be applied to the molecular characterization of EEM fungal communities (Peintner et al. 2007; Iotti et al. 2010; Benucci et al. 2011a). This method amplifies the ITS1-5.8S-ITS2 region directly from a small fragment of fungal mantle (0.01–0.02 mm^2 wide) included in the PCR reaction mixture. The fragment of fungal mantle is preferentially excised using fine needles from the distal end of an EM root tip under a stereomicroscope and then transferred directly into the PCR tube. The addition of 20–40 μg of bovine serum albumin (BSA) to the PCR mixture is necessary to scavenge Taq DNA polymerase inhibitors such as phenolic compounds and melanins, which can strongly limit the activity of Taq DNA polymerase (Kreader 1996; Giambernardi et al. 1998; Sejalon-Delmas et al. 2000). Direct PCR techniques bypass the time and expense of DNA isolation and preserve the structure of the mycorrhized tips, which are then available for morphological characterization. This requisite has two primary advantages (1) it allows the description of rare morphotypes, and (2) it can be used to correlate erroneous molecular or morphological data when similar mycorrhizas formed by different fungal species are mixed on the same roots. The risk of amplifying DNA from mycelium or other propagules of nontarget fungal species in the rhizoplane is also drastically reduced. To date only <0.5 % of nontarget fungal species have been amplified from our research staff after analyses of about 500 EM morphotypes collected from different experimental sites. Similar direct PCR method has been also used for identification of truffles and other EEM fruiting bodies (Bonuso et al. 2006; Bonito 2010).

The great number of ITS fungal sequences deposited in GenBank during the last 10 years made possible the design of species-specific primers that are used in simple or multiplex PCR and are able to identify unequivocally mycorrhizas EEMMs (Table 7.1). The use of specific primers coupled with direct PCR approaches allows for a drastic reduction in time and cost for genotyping and routine screening. Moreover, specific primers can be used to detect mycorrhizas or mycelium of target taxa in soils and natural habitats and allow researchers to follow fungal dynamics with the aid of "real-time" PCR (Peintner et al. 2007; Hortal et al. 2008; Zampieri et al. 2010; Suz et al. 2008; de la Varga et al. 2012; Iotti et al. 2012). Specific primers could be a useful tool for assessing the geographical distribution and ecology of *T. aestivum* in countries such as the Czech Republic and Slovakia, where the collection of fruiting bodies is forbidden (Gryndler et al. 2011).

Table 7.1 A set of ITS-specific primers for identification of EEM

EEM species	Specific primers[a]	Sequences	References
Tuber melanosporum	**ITSML**[b]	TGGCCATGTGTCAGATTTAGTA	Rubini et al. (1998)
	ITS1TM[c]	GTATTCCCGAACACAAACCT	Suz et al. (2006)
	ITS2TM[c]	AGACTTGTGACTGATCCAGG	
	T.mel_for	TTGCTTCCACAGGTTAAGTGA	Bonito (2010)
	T.mel_rev	TAAAGTCCGTCGTTCATGC	
T. brumale	**SYLV1**	TCGTCAGTGGTACACAATGT	Douet et al. (2004)
	SYLV2	ATGACAGAACAAATAGAATGTAA	
	ITSB[b]	CAATGTCAGAGCCAATCTAATGC	Rubini et al. (1998)
T. indicum	**IndF1**	ACCTGTGGGAGATCTCCAC	Douet et al. (2004)
	IndF2	GGCCATGTGTCAGATTTACTG	
	IndR	CATAGACTAGCAATTCACTCCTG	
	ITSCHCH[b]	AACAACAGACTTTGTAAAGGGTTG	Rubini et al. (1998)
T. aestivum	**Tu1sekvF**	AGAGCACCAAACCACAG	Gryndler et al. (2011)
	Tu2sekvR	ACCACAGCGTCTACCAA	
	UncI	TGGGCCGCCGAAAACTTG	Mello et al. (2002)
	UncII	CTGACGAGATGCCCCGGA	
T. macrosporum	**Tmacr For**	CGTCGCTCATCAAAGCAGTC	Benucci et al. (2011b)
	Tmacr Rev	CCGCCAGTACCACCAGGAG	
T. rufum	**Ru1f**[d]	TGCTTTCCCAGGTGGTTGG	Iotti et al. (2007)
	Ru2f*[d]	TTGCTTTCCCAGGGAATTGG	
T. magnatum	**Tmag1**[e]	GGATGCGTCTCCGAATCCTGAAT	Amicucci et al. (1998)
	Tmag2	TCGGGCCCTTTCTCAGACTGCTG	
	TSMAGN	GTCACTGAAAACCCACTCACG	Rubini et al. (2001)
	ITSBACK3	TGAGGTCAACCCAGTTGGACAGT	
	P7	TCCTACCAGCAGTCTGAGAAAGGGC	Mello et al. (1999)
	M3[f]	TGAGGTCTACCCAGTTGGGCAGTGG	
	TmgITS1for[c]	GCGTCTCCGAATCCTGAATA	Iotti et al. (2012)
	TmgITS1rev[c]	ACAGTAGTTTTTGGGACTGTGC	
	TmgITS1prob[c]	TGTACCATGCCATGTTGCTT	
T. maculatum	**TmacI**	GACACAGGCTCCCGATAAAACAC	Amicucci et al. (1998)
	TmacII	CAGCAGCACTGATAGCCCCG	
	rTmacII[e]	CGGGGCTATCAGTGCTGCTG	Amicucci et al. (2000)
T. borchii	**TboI**	TGTATGGGATGCCCTATCGGACT	Amicucci et al. (1998)
	TboII	CTATTACCACGGTCAACTTC	
	rTboII[e]	GAAGTTGACCGTGGTAATAG	Amicucci et al. (2000)
	TBA	TGCCCTATCGGACTCCCAAG	Mello et al. (1999)
	TBB	GCTCAGAACATGACTTGGAG	
T. puberulum	**TpuI**[e]	TCTGTTACCAGGGTCCACATT	Amicucci et al. (1998)
	TpuII	GGCTTCTGGGTTGAGGTGTTT	

(continued)

Table 7.1 (continued)

EEM species	Specific primers[a]	Sequences	References
T. dryophilum	**TdryI**	ATCGGGCTCCCAAGCAAAACA	Amicucci et al. (1998)
	TdryII	TCTACTACCATGGTTCACTTT	
Boletus edulis	**BE1**	CATTATCGAGTTAGACCGGGAAG	Lian et al. (2008)
	BE-2	CCATGCCCTCGAGATCAGATC	
	Bedu1F	ATGGAGGAGTCAAGGCTGTC	Mello et al. (2006)
	Bedu2R	TAGATTAGAAGCGATTCACT	
B. pinophilus	**Bpin1F**	GGGAAAATGGACAAGGACCC	Mello et al. (2006)
	Bpin1R	AGTCAGTCTCACGACCGACTTT	
B. aereus	**Baer2F**	GAAGCCGTTTCCTCGGACTCC	Mello et al. (2006)
	Baer1R	TGAGGCTTAGGGTTCGAGTC	
B. aestivalis	**Baes1F**	AAGGGGTTTCCTCGGACTCT	Mello et al. (2006)
	Baes2R	AATTGGAAGCGATTCACCGCW	
B. violaceofuscus	**Bvio1F**	CACACTTTGTCGCATGTCCAT	Mello et al. (2006)
	Bvio1R	CCACCATCGTGTGCTTGTGT	
Lactarius deliciosus	**FWD-Ldel**[c]	TTGACGCAAAAGTCGTGCAC	Parladé et al. (2007)
	RVS-Ldel[c]	ATCGGTTCGATCCCAAAAGG	
	STQ Ldel[c]	TCTCGCATAAAATCCA	
Rhizopogon roseolus	**FWD-Rro**[c]	TCGACTTTGCGCGACAAG	Hortal et al. (2008)
	RVS-Rro[c]	CATGCGCTTCAGCAAACG	
	STQ-Rro[c]	ATCATTATCACGCCGAAAG	

[a]Forward primer is in bold. Probes are in italic
[b]In multiplex PCR with ITS4LNG (TGATATGCTTAAGTTCAGCGGG) as reverse primer
[c]In real-time PCR
[d]ITS4 as reverse primer
[e]In multiplex PCR with ITS4 as reverse primer
[f]In multiplex PCR with ITS1 as forward primer

7.2.4 Metagenomics

Metagenomics can be defined as "the application of modern genomic techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species" (Chen and Pachter 2005). Metagenomics is based on the analysis of nucleic acids (DNA or RNA) extracted directly from the environment. Metagenomics can be used to study soil fungal communities and to this aim several approaches are suitable. Polymerase chain reaction-temperature gradient gel electrophoresis/denaturing gradient gel electrophoresis (PCR-TGGE/DGGE), cloning and sequencing of rDNA amplicons and 454 amplicon pyrosequencing have been used to elucidate the composition and diversity of EEM communities (Peintner et al. 2007; Napoli et al. 2010; Mello et al. 2011). An ever-increasing number of studies on EM communities are under way using next-generation sequencing approaches (454 and Illumina

platforms), which provide more complete community characterization and previously unimaginable amount of sequence data. However, quality control measures (e.g. denoising, chimera checking) and reference databases continue to be improved, and caution should be taken when interpreting results given inherent biases of all PCR-based approaches (Kunin et al. 2010; Tedersoo et al. 2010).

Another promising high-throughput molecular tool for studying fungal communities is the microarray technique. PhyloChip design is based on the use of several thousand oligonucleotide probes derived from DNA barcodes to allow the rapid and parallel identification of a high number of species from many processed samples. Various PhyloChips have been designed for detecting target fungal species, but phyloarrays have been poorly applied for characterization of EM communities (Reich et al. 2009), and probes had been designed only for a low number of EEMM species (Bruns and Gardes 1993; El Karkouri et al. 2007). Nonetheless, new and custom arrays and probes are available, including those targeting functional genes, and small-scale PhyloChips are a promising time-saving and cheap approach for monitoring specific fungal species over years and/or space (Reich et al. 2009). However, one major limitation of phyloarrays is that they can only detect known taxa and genes; thus, they are not suitable for discovery applications.

7.2.5 *Ectomycorrhizal Database*

Recently a number of free online databases have been created to facilitate identification of ectomycorrhizas. DEEMY (http://www.deemy.de) (Rambold and Agerer 1997) and UNITE (http://unite.ut.ee/index.php) (Kõljalg et al. 2005) are the most widely used tools for morphological and genetic data gathering, respectively. Together, UNITE and GenBank are the most important genetic database available for characterizing ectomycorrhizal fungi. Other useful Web databases for research on ectomycorrhizas have been created by (a) Canadian Forestry Service (CFS), which hosts an image database (POE) and a database containing brief descriptions of the ectomycorrhizas (EDD) (http://www.pfc.cfs.nrcan.gc.ca/biodiversity/bcern/index_e.html) and (b) University of Wisconsin (USA) that hosts the "Database of the Chestnut Mycorrhizae," which is a collection of ITS region sequences of EM fungal species in association with plants of the *Castanea* genus. However, these tools are limited and do not give information pertaining to the community to which given EM fungi belong or concerning the role of different species in a given community. Moreover, these databases do not supply exhaustive information on the ecology and the geographical distribution of the fungal species.

Another new Web tool worth mentioning is eMyCo "*ecto*-*my*corrhizal *co*mmunity database," which collects and analyzes data about EM communities (Lancellotti et al. 2012). This database has been developed specifically to assist researchers working on the ecology of the EM fungi. eMyCo is composed of independent surveys, each characterizing a single EM community at a certain time and place. For each

survey, site description (host community, soil, geographic localization), survey methodology and operational taxonomic units (OTUs) characterized by a simplified set of morphological features of their mycorrhizas and the ITS sequences of the fungal species are maintained. Abundance and frequency values of each OTU in a community are also provided. The values of OTU richness, of the Shannon (Shannon and Weaver 1949) and Pielou (Pielou 1969) indices, together with a chart representing rank-abundance curve (Whittaker 1965), are calculated automatically. In this way, eMyCo allows the use of data concerning ecological parameters in a coherent way to compare EM communities.

7.3 Studies of EEM Fungal Communities

Reported here is an overview of important studies on EEM fungal communities, with an emphasis on the most important advances concerning community composition and dynamics.

7.3.1 Ectomycorrhizal Communities of "Porcini"

Only a few studies have been conducted on *Boletus edulis sensu lato* (porcini) EM communities. Using morphological and molecular identification, Peintner et al. (2007) surveyed EM, saprobic and pathogenic fungi in an area located near Parma (Italy) that is productive with *B. edulis* s.l. Thirty-nine (39) EM fungi were identified on root tips, whereas 40 fungal species were found in the soil by cloning and sequencing. However, the overlap between above- and below-ground fungal communities was very low, and *Boletus* mycelia were rare and scattered. DNA from most species within the *B. edulis* s.l. group (*Boletus edulis* Bull., *Boletus pinophilus* Pilát and Dermek and *Boletus aereus* Bull.) appears to be poorly represented adjacent to fruiting bodies (Peintner et al. 2007). Only *Boletus aestivalis* (Paulet) Fr. (syn. *Boletus reticulatus* Schaeff.) ectomycorrhizas and soil mycelia were detected abundantly just below their fruiting bodies (Peintner et al. 2007). A recent study performed using real-time PCR confirms that *B. edulis* productivity (in terms of fruiting bodies) was not correlated either with the concentration of soil mycelium or with the presence or abundance of ectomycorrhizas (de la Varga et al. 2012).

7.3.2 EM Communities of T. melanosporum

T. melanosporum EM communities have been studied with much attention to the persistence of its mycorrhizas and its competitiveness with resident fungal species when infected plants are transplanted for establishing truffle orchards. Many of

these studies were carried out in France, in Spain, and in Italy during the last 30 years using morphological methods (Giraud 1988; Granetti and Baciarelli Falini 1997; de Miguel and Sáez 2005). These studies identified a number of EM species commonly found in truffle orchard, which appear to be competitive with *T. melanosporum*. In particular, two other *Tuber* species, *T. brumale* and *T. aestivum*, appear to be quite competitive with *T. melanosporum* in many conditions (see Chap. 10). Other EM fungi considered as highly competitive and agressive with *T. melanosporum* belong to the *T. woolhopeia* species complex (Agueda et al. 2008; Rubini et al. 2011), which have been known for decades as forming the AD morphotype.

In order to better understand the ecology of *T. melanosporum* and its relationships with other EM fungi, a number of field studies in natural truffières have been carried out in zones that produce truffles and others that do not produce truffles. Productive zones usually correspond with the brûlé (burnt area), an area of soil around the host plant where the growth of grasses is drastically reduced by substance (probably volatile organic compounds—VOCs) produced by the truffle mycelium (Pacioni 1991; Splivallo et al. 2007; Streiblová et al. 2012). Results from quantitative PCR indicates that mycorrhizas and mycelium of *T. melanosporum* are particularly abundant inside the brûlé and decrease beyond it (Garcia-Barreda and Reyna 2011; Suz et al. 2008). The high abundance of *T. melanosporum* inside the brûlé appears to lower the overall species richness of EM fungi and fungal soil communities (Pacioni 1991; Garcia-Barreda and Reyna 2011; Napoli et al. 2008; Mello et al. 2011; Belfiori et al. 2012). Also the composition of the fungal communities is affected by *T. melanosporum* inside of the brûlé. The studies of Mello et al. (2011) carried out using pyrosequencing in truffle ground located in France showed that the number of Ascomycota, which were found to be the dominant fungal phylum in the studied truffle ground, is more abundant inside the brûlé than outside. In contrast, the number of Basidiomycota increases outside the brûlé confirming the previous results obtained by Napoli et al. (2010) by DGGE technique. Recent studies on soil fungal biodiversity carried out in central Italy in *T. melanosporum* natural and cultivated truffle grounds (Belfiori et al. 2012) highlighted only few EM OTUs such as Basidiomycetes of the genus *Hymenogaster* and members of Thelephoraceae are in common with the French truffle grounds studied by Napoli et al. (2010) and Mello et al. (2011).

7.3.3 EM Communities of T. aestivum

T. aestivum EM fungal communities have mostly been investigated in truffle orchards. In one of the first studies, carried out in a 5-year-old *T. aestivum* orchard using morphological approaches, it was possible to detect 15 nontarget morphotypes, among which were *T. brumale* and *Hymenogaster citrinus* Vittad. (Zambonelli et al. 2005). In this study, the effect of different mulching materials on the EM community was compared, and it was shown that mulch type significantly influences the degree

of EM colonization in the upper 0–15 cm of soil. The type of mulching material appears to have effected ectomycorrhizal colonization through its impact on soil temperature and moisture. The decreased soil temperatures in summer and the increased soil moisture caused by straw and black mulching cloth favor contaminant EM fungi in the studied environmental conditions. Similar results were obtained in Spain by Etayo and De Miguel (2001).

In one study, *T. aestivum* EM communities were studied in a productive 24-year-old orchard in Italy using molecular methods and by sequencing the ITS regions. In this study, 29 EM taxa were identified (Benucci et al. 2011a). The most abundant EM species was *Tricholoma scalpturatum* (Fr.) Quél., a species that has also been detected in a *T. magnatum* natural truffière in northern Italy (Iotti and Zambonelli 2006). Similar to other truffle communities, multiple *Tuber* species are present (e.g. *T. rufum* Pico, *T. brumale* and *Tuber rapaeodorum* Tul. & C. Tul.), and the Thelephoraceae shows high diversity (Benucci et al. 2011a). Other EM species detected belonged to Pyronemataceae, Sebacinaceae, Inocybaceae, families showing a similar composition of truffle fungal communities in other studies (e.g. Bonito et al. 2011). *T. aestivum* mycorrhizas were abundant and showed a certain host preference for hornbeam (*Carpinus betulus*), rather than hazelnut (*Corylus avellana*) (Benucci et al. 2011a).

7.3.4 *EM Communities of* T. borchii

There are few studies on *T. borchii* EM communities in orchards compared to *T. melanosporum* because its cultivation is quite recent. In recent years, however, *T. borchii* cultivation has become popular in many countries (Hall et al. 2007). In a pioneering study conducted in a *P. pinea–T. borchii* orchard, it was shown that *T. borchii* ectomycorrhizas were widespread in the truffiére four years after planting, when the orchard began to produce *T. borchii* fruiting bodies (Zambonelli et al. 2000a). However, the number of *T. borchii* ectomycorrhizas drastically decreased 12 years after planting when the canopies closed, which suggests that *T. borchii* is an early stage fungus and that its ectomycorrhizas are distributed in the youngest part of root system (Zambonelli et al. 2000a). A study on *T. borchii* EM communities carried out in natural truffières confirmed that *T. borchii* mycorrhizas are very abundant in productive points but are present also one meter far from them (Iotti et al. 2010). Seventy ectomycorrhizal taxa were identified, many of which were rare. *T. borchii* dominated belowground, forming 20 % of ectomycorrhizas. Thelephoraceae, Inocybaceae, and Sebacinaceae were the other main EM species. Species composition was markedly affected by the host plant (oak or pine), yet community structure and composition was also influenced by the location the soil cores were collected from. The high presence of *T. borchii* in productive points affected the composition of EM community structure in a similar way as was observed in *T. melanosporum*- and *T. aestivum*-dominated communities (Benucci et al. 2011a; Napoli et al. 2010). In *T. borchii* EM communities, other *Tuber* species

were found sharing the same habitat, in particular, *Tuber dryophilum* Tul. and C. Tul, an inferior edible truffle morphologically similar to *T. borchii* but without commercial value. However, its mycorrhizas were never found together with those of *T. borchii* showing the two species differentiate in the micro-niche colonization as suggested for other EM fungi in *Pinus* forests (Iwanski and Rudawska 2007). Also, at this site multiple cryptic species of *T. borchii* were present (Bonuso et al. 2010) but their mycorrhizas were never found mixed together (Iotti, unpublished data).

7.3.5 *EM Communities of* T. magnatum

T. magnatum, the Italian white truffle, is the most expensive EEMM species. Nevertheless, with all of the attention that scientists have given this species, its cultivation is still not feasible and only a few truffle orchards are able to sporadically produce fruiting bodies (Hall et al. 2007). Even if methods have been developed to produce *T. magnatum*-infected plants (Mello et al. 2001; Rubini et al. 2001) (Fig. 7.1), mycorrhizas of this truffle seem to disappear when the plants are transplanted in the field (Hall et al. 2007). Also, in natural productive *T. magnatum* orchards, mycorrhizas appear to be absent (Di Massimo et al. 2010) or quite rare (Bertini et al. 2006; Murat et al. 2005). In contrast, other species of *Tuber* such as *T. rufum*, *T. brumale*, *T. maculatum*, and *T. borchii* (Murat et al. 2005; Bertini et al. 2006; Iotti and Zambonelli 2006) are frequent EM fungi on host roots in natural *T. magnatum* truffières. For instance, soil analyses using specific *Tuber* primers revealed a surprisingly high *Tuber* diversity in natural *T. magnatum* truffières with more than five different *Tuber* spp. (Zampieri et al. 2010). Soil analyses using *T. magnatum*-specific primers designed on β-tubulin gene by Zampieri et al. (2010) or more recent real-time PCR analysis using specific ITS *T. magnatum* primers (Iotti et al. 2012) revealed that *T. magnatum* mycelium grows extensively in the soil of the truffière, not only in the productive point but also forming large patches. Soil analysis using DGGE technique carried out in productive and in not productive points of a single *T. magnatum* natural truffière found some EM fungi such as *C. geophilum* and *Clavulina cinerea* (Bull.) J. Schröt. present only in nonproductive points that may negatively influence *T. magnatum* development (Mello et al. 2010).

Indeed, the EM communities of *T. magnatum* natural truffières located in different Italian forest environments are under investigation in order to verify common traits and differences. About 130 EM fungal OTUs have been identified molecularly on the roots collected from four experimental sites, and more than half of them belonged to thelephoroid and sebacinoid taxa. Among the truffle species, *T. rufum* mycorrhizas were the most frequent and abundant. Preliminary results obtained from the samples collected in *T. magnatum* fruiting points revealed different fungal community structures between soil and root EM communities (Fig. 7.2). Only 19 on 45 OTUs were present both as free-living mycelium and as ectomycorrhizas (particularly *Inocybe* spp. and *Sebacina* spp.). Members of

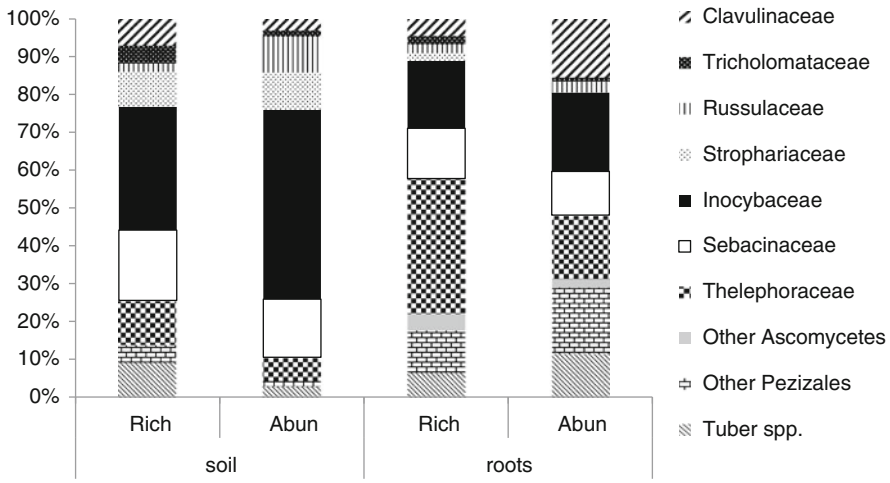

Fig. 7.2 Abundance of the fungal families of soil (**a**) and root (**b**) EM communities present in the fruiting points of two *T. magnatum* natural truffieres located in Tuscany (Montaione, FI) and Emilia Romagna (Argenta, FE)

Inocybe were relatively more abundant in soil than on the roots, whereas ascomycetes, Clavulinaceae, and thelephoroid fungi prefer the symbiotic state (Iotti, unpublished data).

7.4 Conclusions

In cultivating EEMMs, competition from resident EM fungi is one of the biggest problems, particularly in the initial few years after planting when the introduced species is vulnerable to being replaced. This emphasizes the need to better understand the drivers and dynamics of soil EM communities and the environmental factors that favor micro-niche colonization by EEMMs. The application of molecular techniques for taxonomic affiliation has dramatically changed microbial ecology so that it is now possible to investigate the composition and interactions among microbial communities in soil, on the roots, and above-ground, providing a more complete and precise scenario of their structural composition and dynamics.

Through these studies, basic characteristics of the studied EEM fungal communities have emerged:

- Most of EEMMs dominate the EM fungal communities in productive areas (like *T. borchii*, *T. melanosporum*, and *T. aestivum*). These species affect the composition of EM communities reducing richness and abundance of EM species (Iotti et al. 2010; Belfiori et al. 2012).

- Other species like *B. edulis* or *T. magnatum* have a quantity of mycorrhizas in the soil that does not appear to be correlated to the fruiting body productivity; the mycelium of these species in the soil is scattered in productive areas for porcini and widespread in large patches in *T. magnatum* truffières.
- It is common that phylogenetically related species shared the same EM communities, such as some species of *B. edulis* complex, *T. borchii* and *T. dryophilum* and *T. melanosporum* and *T. brumale*. However, most of these species tend to colonize different niches and may coexist within the same habitat. It would be useful to better understand the micro-environmental conditions (like shown in Chap. 6) in micro-niches where the most valuable species are present.

Although our knowledge of the composition EM communities and of soil microbial communities has increased in the past several years, more studies are needed if we are to better understand the ecological variables driving the dynamics of EEM fungal communities, the biotic and abiotic factors affecting synergic or competitive relationships and the fruiting body formation and development. Future studies should take into account the capacity of some EEMMs to form endomycorrhizas with different host species or particular environmental conditions. For instance, it is well known that the EEMMs of the genus *Terfezia* (the desert truffles) are able to form ectomycorrhizas or endomycorrhizas depending on the growth conditions (Fortas and Chevalier 1992; Chap. 14). Moreover, it was recently found that some species of the genus *Tuber* (*T. borchii* and *T. aestivum*) are able to thrive inside host cells of some Orchidaceae (Selosse et al. 2004; Illyés et al. 2010).

These studies, together with advances in genomics (Chap. 21), may be able to solve the mysteries of *T. magnatum* soil biology and ecology and provide the scientific principles to enable its cultivation.

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Chapter 8
Ectomycorrhizal Helper Bacteria: The Third Partner in the Symbiosis

Elena Barbieri, Paola Ceccaroli, Francesco Palma, Deborah Agostini, and Vilberto Stocchi

8.1 Introduction

Forests function in a capacity that improves environmental quality and water and carbon stocks and are critical to the conservation of biodiversity. To this end, the application of techniques for controlled mycorrhization may be important for improving reforestation strategies. Indeed, for efficient nutrient uptake, most plant species need to be associated with mycorrhizal fungi that supply minerals, increase growth, and confer stress resistance (Singh et al. 2011). The management of these symbioses in natural and agricultural environments is of significant ecological and economic importance.

The establishment of a functional symbiosis between plants and microorganisms affects the chemical–biological nature of rhizosphere. Important examples of this include nitrogen-fixing symbiosis between bacteria and leguminous plants and the symbiosis between mycorrhizal soil fungi and numerous plant species. The microbial populations that colonize roots often differ between plants involved, in a symbiotic relationship with an active molecular dialogue between partners. This crosstalk between plant and microbe can result in response mechanisms capable of attracting additional microbial species that are not directly involved in the symbiosis. Recently, some of these species, so far considered alien to the symbiosis, have been identified as important components of the ectomycorrhizal system. The establishment of mycorrhizal symbiosis alters the composition and amount of root exudates, thus changing the microbial equilibrium of the rhizosphere (Duponnois and Garbaye 1992; Barea et al. 1997; Frey-Klett and Garbaye 2005; Frey-Klett et al. 2007). This effect, known as the "mycorrhizosphere effect," seems to favor the occurrence of particular bacteria involved in the mycorrhization process, called "mycorrhizal helper bacteria" (MHB)

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(Dakora 2003; Frey-Klett et al. 2007), as well as the growth of bacteria, called "mycorrhizal associated bacteria," whose role is still unclear (Assigbetse et al. 2005; de Boer et al. 2005).

The bacterial populations present in the fruiting bodies of several species of mycorrhizal fungi exceed numerically those usually found directly in soil for the same bacterial species, whereas the diversity of species observed seems to be greatly reduced (Gazzanelli et al. 1999; Sbrana et al. 2002). These MHB populations have different roles and undoubtedly promote the establishment of mycorrhizal symbiosis and stimulate the development of mycelium resulting in a greater surface contact between the plant root and the fungus mycelium; this is easily verifiable through bacteria and fungi coculture (Founoune et al. 2002; Deveau et al. 2007). While both ectomycorrhizal (Garbaye 1994) and endomycorrhizal (Meyer and Linderman 1986) fungi can interact with different bacterial species, we will focus on the interactions relating ectomycorrhizal fungi.

In the past several years, the potential ability of bacteria associated with ectomycorrhizas to fix atmospheric nitrogen has been suggested (Frey-Klett et al. 2007). This hypothesis originates from the observation that ectomycorrhizas are particularly frequent on plants typical of land ecosystems characterized by a deficiency in combined nitrogen. Based on the hypothesis of the presence of nitrogen-fixing bacteria, previous research allowed for the detection of the occurrence of genes directly involved with nitrogen fixation process.

Several studies have found that potential nitrogen-fixing bacteria can be associated with ectomycorrhizal and arbuscular mycorrhizal fungi (Frey-Klett et al. 2007) and that the highly conserved *nifH* gene, which encodes for the nitrogenase reductase, is present in *Pinus sylvestris* and *Pinus nigra* ectomycorrhizas (Timonen and Hurek 2006; Izumi et al. 2006a).

These experiments suggest a real possibility that bacteria present in mycorrhizal tissues contribute to the nutritional needs of both fungi, and consequently plants, providing them with available nitrogen derived from atmospheric nitrogen (N_2). The recent detection of nitrogen-fixing bacteria in fruit bodies of *Tuber borchii* and *Tuber magnatum* and in association with soil and ectomycorrhizal fungi led to the hypothesis that nitrogen fixation activity may play an important role in fungal growth and in the development of the ascocarp (Rainey et al. 1990; Barbieri et al. 2005a, 2007; Frey-Klett et al. 2007). However, in order to unequivocally demonstrate this role, more targeted investigations are needed.

In this chapter, we first introduce the major biological characteristics of ectomycorrhizas followed by a description of different types of interaction taking place between bacteria and mycorrhizal fungi. Promotion of plant growth, release of active molecules, and nutritional exchange strategies are discussed within the establishment of plant–bacteria–fungus interactions. Particular attention is focused on interactions between bacteria and ectomycorrhizal fungi belonging to the *Tuber* genus with established or potentially synergistic properties important to its fruiting and life cycle.

8.2 Defining the Mycorrhizosphere

The influence of plant assimilates on microbial communities has been defined in relation to the rhizosphere, the narrow zone of soil surrounding living roots. The rhizosphere is characterized by increased microbial activity stimulated by the exudation of organic substances from the root (Grayston et al. 1997). However, since plant roots in natural and semi-natural ecosystems are commonly mycorrhized, the rhizosphere concept has been extended to include the fungal component of the symbiosis, resulting in the term "mycorrhizosphere." The mycorrhizosphere is the zone influenced by both the root and the mycorrhizal fungus and includes the more specific term "hyphosphere," which refers only to the zone surrounding individual fungal hyphae. Since mycorrhizas and fungal hyphae are more or less ubiquitous in natural soils, it could be argued that all soil could be included in the term "mycorrhizosphere" (Johansson et al. 2004).

8.2.1 Occurrence of Ectomycorrhizas

Fossil records indicate that ectomycorrhizal associations emerged at least 50 million years ago (Mya). For example, Hydnangiaceae appeared before 40 Mya according to Ryberg and Matheny (2012), although there is molecular evidence that this emergence dated to more than 180 Mya (Martin et al. 2007). In ectomycorrhizal associations, hyphae of fungal symbionts produce extensive nets of mycelium that extends beyond exploratory roots of plants. The mycelium acquires soil minerals through solubilization, particularly of phosphorus and nitrogen, and exchanges these nutrients with their host plants, which may allocate as much as 10–20 % of their photosynthate to the mycobiont. Ectomycorrhizal (EM) fungi produce a hyphal mantle during the colonization process that tightly covers the root tip, while epidermal (and in some cases also cortical) cells become separated by the development of labyrinth-like hyphae (the Hartig net), which increases the surface contact area with root cells. Thus, in ectomycorrhizas, hyphae remain extracellular, inducing important changes to root morphogenesis, whereas their presence only leads to thin modifications in epidermal or cortical cells (Bonfante 2001).

EM fungi alter the physical, chemical, and microbiological characteristics of the surrounding soil and create a special environment called mycorrhizosphere in which the microbial communities differ from those in the rhizosphere and in other portions of the soil (Izumi et al. 2006b). There is considerable evidence that EM fungi influence the taxonomic composition of communities of soil bacteria, although this effect varies among mycorrhizas of different fungi species and between different root tips colonized with the same species (Burke et al. 2008; Izumi et al. 2008; Kataoka et al. 2008). For example, Brooks and colleagues have demonstrated that EM fungal hyphae may control organic phosphorus resources in soil by selecting against soil bacteria with higher abilities to mobilize phosphorus

from organic compounds. This interaction could result in complementary and synergistic modes of action providing a sustained supply of phosphorus from organic and inorganic fonts to the EM host (Brooks et al. 2011).

8.3 Interactions Between Ectomycorrhizal Fungi and Associated Bacteria

Mycorrhizal symbiosis has long been considered as a bipartite relationship between plant roots and mycorrhizal fungi. However, in natural conditions, ectomycorrhizal fungi physically and metabolically interact with a wide community of soil bacteria with potential unfavorable, neutral, and beneficial consequences (Bowen and Theodorou 1979; Barea et al. 2002; Johansson et al. 2004; Frey-Klett et al. 2007, 2011; Bonfante and Anca 2009).

Bowen and Theodorou (1979) and then Garbaye and Bowen (1989) demonstrated that the rhizosphere microflora could have positive or negative impacts on the mycorrhizal symbiosis, depending on particular bacterial isolates involved. Garbaye and Duponnois (1992) showed that pure bacterial strains such as *Pseudomonas* spp. and *Bacillus* spp. stimulated the *Pseudotsuga menziesii–Laccaria laccata* symbiosis. This was the experimental evidence for the so-called helper effect of those bacteria named MHB. The MHB are not plant-specific, but are clearly selective about the fungal species, so they can be defined as fungus-specific (Garbaye 1994). They exist in arbuscular and ectomycorrhizal systems and now are currently the most investigated group among bacteria interacting with mycorrhizas. Some research has proposed that the ability of *Pseudomonas* spp. to utilize trehalose may be key for bacterial growth in association with the edible ectomycorrhizal fungus *Cantharellus cibarius* without causing fungal cell damage (Rangel-Castro et al. 2002). Some MHB isolated from *Lactarius deliciosus* are able to enhance establishment of mycorrhization with *Pinus pinea* and *Pinus pinaster* (Barriuso et al. 2008). More recently, Wu et al. evaluated positively the effects of the co-inoculum between the ectomycorrhizal fungus *Boletus edulis* and the MHB *Bacillus cereus* (HB12 or HB59) on the growth and nutrient uptake of *Pinus thunbergii* (Wu et al. 2011). Experimental evidence suggests that a release of active diffusible molecules occurs prior to physical contact between bacteria and/or mycorrhizal fungi, which is important for the establishment of their interactions. Recent data show the production of volatile organic compounds (VOCs) from all the members of the symbiosis, important for inter- and intra-organism communications (Splivallo et al. 2007; Tarkka and Piechulla 2007). Studies performed on the ectomycorrhizal fungus *Laccaria bicolor* S238N and the MHB strain *Pseudomonas fluorescens* BBc6R8 have indicated that both fungal trehalose and bacterial thiamine play a key role in the mutually beneficial interactions of two organisms (Deveau et al. 2010). The main significant functions of MHB are in plant protection against root pathogens, nutrient mobilization from soil

minerals and fixation of atmospheric nitrogen in forms available to plants, fungi, and microbes (Frey-Klett et al. 2007). In addition, they may have an active role in ascocarp decomposition and spore dispersal.

8.3.1 Plant Protection

Mycorrhiza-associated bacteria contribute, together with the fungal symbiont, protection against root pathogens. For example, there is now abundant evidence that some *Streptomyces* species colonize the rhizospheres of plant roots and even plant tissues (Coombs and Franco 2003), and it has been suggested that antibiotic production by the streptomycete may protect the host plants against phytopathogens (Challis and Hopwood 2003). More recently, Tarkka et al. (2008) illustrated the streptomyces mechanism that induces plant defenses and facilitates symbiosis formation. Moreover, Frey-Klett et al. (2005) revealed a significantly higher proportion of fluorescent *Pseudomonads* inhibiting the growth of root-pathogenic fungi belonging to the genera *Rhizoctonia*, *Fusarium*, *Phytophthora*, and *Heterobasidion* in the Douglas-fir *L. bicolor* ectomycorrhizas than in the surrounding bulk soil. These findings suggest that MHB could have evolved selective mechanisms of interaction with their microbial surroundings, having neutral or positive effects on their host mycorrhizal associations but negative effects on the root pathogens that might threaten their very habitat.

8.3.2 Nutrient Mobilization

Recent findings suggest that ectomycorrhiza-associated bacteria complement the roles of the external mycelium by mobilizing nutrients from minerals. In fact, these bacteria can solubilize calcium phosphate, rock phosphate, iron phosphate, and aluminium phosphate by secretion of organic acids. For example, different experiments revealed a higher proportion of culturable bacteria able to release iron from the biotite, in *Quercus sessiliflora–Scleroderma citrinum* ectomycorrhizas than in the bulk soil (Uroz et al. 2007). Other authors demonstrated that the solubilization of rock phosphate is enhanced by formation of mixed biofilms between phosphate-solubilizing saprotrophic fungi and *Bradyrhizobium elkanii* strain (Jayasinghearachchi and Seneviratne 2005).

8.3.3 Nitrogen Fixation

Nitrogen, a critical component of many biomolecules, is essential for growth and development of all organisms. Although roughly 78 % of the Earth's atmosphere is

composed by nitrogen, it is in a form (N_2) that is not biologically accessible. However, the ability to fix atmospheric nitrogen via the nitrogenase enzyme complex has evolved in some bacteria. Many eukaryotic organisms are able to obtain fixed nitrogen through their symbiotic interactions with nitrogen-fixing prokaryotes. In the last decade, special attention has been paid to potential nitrogen fixation by bacteria associated with ectomycorrhizas. Several studies have found that potential nitrogen-fixing bacteria can be associated with ectomycorrhizal and arbuscular mycorrhizal fungi (Frey-Klett et al. 2007; Kretzer et al. 2009). The presence of nitrogen-fixing bacteria in these diverse ectomycorrhizal types clearly supports their potential for improving plant nutrition. Using molecular methods, several authors found DNA sequences of the *nifH* gene (encoding nitrogenase) in *P. sylvestris–S. bovinus* and *P. nigra–Suillus variegatus* ectomycorrhizas (Timonen and Hurek 2006; Izumi et al. 2006a). Furthermore, Barbieri et al. showed the predominance of α-Proteobacteria represented by *Sinorhizobium/Ensifer* and *Rhizobium/Agrobacterium* groups as well as by nitrogen-fixing *Bradyrhizobium* spp. in *T. borchii* and *T. magnatum* ascocarps (Barbieri et al. 2005a, 2007). More recently, *Sphingobium* sp. strain TMG 022C was isolated from the ascocarp of white truffle *T. magnatum*. This strain is capable of growing in nitrogen-depleted conditions and may thus be important in mycelial nutrition and ascocarp decomposition (Pavić et al. 2011). These results suggest that diazotrophic bacteria present in ectomycorrhizal tissues contribute to nitrogen input in forest ecosystems by directly providing nitrogen of atmospheric origin to the two partners of the symbiosis, thus bypassing classic nitrogen mineralization pathways.

8.3.4 Specificity in Cell-to-Cell Communication

Mechanisms used by prokaryotes to communicate with eukaryotic cells are an important topic that has been widely investigated, particularly in regard to pathogenesis. Bacteria that cause disease may express virulence genes in order to successfully invade a host cell (Alfano and Collmer 2004). However, little information is available on fungal–bacterial signaling. The crosstalk may involve a combination of bacterial type III secretion system (TTSS) as well as host cell cytoskeleton rearrangement, quorum sensing, stress responses, competence, conjugation, motility, sporulation, biofilm formation, antibiotic, and volatile microbial organic compounds (MVOCs) production (Cotter and DiRita 2000; Miller and Bassler 2001). Many symbionts, such as nodulating rhizobia, rely on TTSS for communication (Freiberg et al. 1997; Marie et al. 2001). Moreover, a significant number of bacteria occurring in the *Laccaria proxima* mycorrhizosphere have been described for their TTSS (Warmink and van Elsas 2008). These data could help to identify in the TTSS a specific trigger in which bacteria and fungi become associated. Among soil bacteria, the quorum sensing mechanisms are again well described for *Rhizobium* sp., nodulation (Rodelas et al. 1999), nitrogen fixation, and symbiosome development (Daniels et al. 2002).

In this regard, as discussed above within *Tuber*-associated bacteria, another molecular determinant for fungal–bacterial attachment is described by a specific secreted protein, a lectin that binds *Rhizobium* sp. (Cerigini et al. 2008).

Moreover, some bacterial metabolites may favor hyphal growth. For example, during its interaction with *Amanita muscaria*, *Streptomyces* sp. AcH505 produces high levels of auxofuran, a secondary metabolite that promotes the extension of the fungal mycelium. In another case, unidentified volatile substances synthesized by some bark beetle-associated bacteria induce the growth of their symbiotic fungi (Adams et al. 2009).

In contrast, an example of specificity crosstalk between *Tuber* and associated bacteria showing a negative effect on mycelia growth is demonstrated by MVOCs produced by *Staphylococcus pasteurii*, which is strongly antagonistic toward *T. borchii* mycelium when grown together (Barbieri et al. 2005b). This bacterial strain, found on the roots of micropropagated plantlets, appears to selectively inhibit the growth of *T. borchii*, but not that of *Hebeloma radicosum* (Bull.) Ricken, supporting the specificity in cell-to-cell communication between bacteria and ectomycorrhizal fungal partners (Zambonelli et al. 2009).

8.4 Truffle Life Cycle: A New System for Studying the Interactions Between Edible Ectomycorrhizal Fungi and N_2-Fixing Bacteria

Ectomycorrhizal symbiosis is often a necessary event that precedes fruit body formation, such as that of truffles. Truffles of many *Tuber* species are edible and in great demand because of their particular organoleptic properties. The presence of nitrogen-fixing bacteria recently shown in the white truffle fruit bodies and in association with soil and other ectomycorrhizal fungi led to the hypothesis that nitrogen fixation activity may play an important role in fungal growth and in the development of the ascocarp.

Although there are many studies concerning interactions between ectomycorrhizal fungi and bacteria, the underlying mechanisms behind these associations are generally not well understood, and their functional properties still require further experimental confirmation.

8.4.1 Truffle Life-Cycle Phases and Bacterial Interactions

Truffles belonging to the Pezizales establish ectomycorrhizal symbiosis with the roots of gymnosperms and angiosperms (Pegler et al. 1993). The truffle life cycle is highly complex and includes morphogenetic changes which can be summarized in three different stages: in the first stage, spore germination generates a filamentous

mycelium that successively establishes a symbiotic association with the plant roots leading, in the second stage, to the formation of the mycorrhizas where the fungus and the plant are in an intimate symbiotic relationship. In the final stage, an hypogeous ascoma containing asci and ascospores is formed (Trappe 1979; Harley and Smith 1993). Hypogeous mycorrhizal fungi constantly interact with microorganisms present in the soil, but questions remain about when and how bacteria and mycorrhizal fungi interact and when and how bacteria become associated with truffles and mycelium.

Recent molecular approaches demonstrate that prokaryotes are associated not only with the extra radical hyphae of mycorrhizal fungi, but they also occur in their mycorrhizas and ascocarps, i.e., the *Cytophaga/Flexibacter* strain found in *T. borchii* (Barbieri et al. 2005a), suggesting that bacteria occur within the symbiotic fungi during their life cycle.

From the perspective of the bacterium, there are a number of ways in which it may establish an interaction with the ectomycorrhizal fungi, but many questions have not yet been answered (1) Is there a specific stage of the fungal life cycle that attracts bacteria? (2) How do bacteria adhere, colonize, and infect fungal tissues and remain associated with the fungus? (3) What are the advantages for the host to maintain this association?

It is important to assess whether there are potential helper bacteria for the truffle ontogenetic cycle. Some of our studies are planned to address this, and a recent molecular approach based on 16S rRNA ribosomal genes (rDNAs) has allowed us to identify a large number of microbial species associated with the most important species of white truffle *T. borchii* and *T. magnatum* at different stages of maturation, greatly expanding the scenario of the microbial ecology that characterizes truffle development and maturation. Indeed, the molecular approaches used for both *T. borchii* and *T. magnatum* fruit bodies generated a high variety of 16S rDNA sequences from six phylogenetic groups: α-, β-, γ-Proteobacteria; Bacteroidetes; and low G + C and high G + C Gram-positive bacteria. However, the largest portion of operational taxonomic units (OTUs) within these species was identified as α-Proteobacteria with close relatives among the *Sinorhizobium/Ensifer*, *Rhizobium/Agrobacterium*, and *Bradyrhizobium* groups. The prevalence of α-Proteobacteria associated with *T. magnatum* ascocarps, regardless of their stage of maturation, was confirmed by the quantitative FISH approach (85 % of α-Proteobacteria among all FISH-positive cells). This method demonstrated the occurrence of rhizobia-like bacterial cells, detected by hybridization of the specific *Rhizobium* probe RHI1247. The most representative clones among the α-Proteobacteria were closely related to *B. elkanii* (Barbieri et al. 2005a, 2007). This OTU was consistently present within each ascoma analyzed. *B. elkanii* was previously described as an inoculant for soybean, which is capable of fixing atmospheric nitrogen in symbiotic interactions with leguminous plants (Rumjanek et al. 1993). No information is currently available on the specific interaction of this species with ectomycorrhizal fungi.

Preliminary studies on the survey of α-Proteobacteria occurring in the ectomycorrhizas collected in productive experimental truffle grounds by 16S rDNA

sequencing retrieval also revealed the presence of *Bradyrhizobium* spp. within *T. borchii* ectomycorrhizas (unpublished data). These results provide the first evidence that potential nitrogen-fixing bacteria may be involved in the entire life cycle of truffle and that the nitrogenase expression may be a common feature of this symbiosis (Fig. 8.1).

8.4.2 Toward Molecular Signaling for Truffle–Bacterial Attachment

Although the interaction between fungi and bacteria has been extensively described in several different contexts, including agriculture and clinical environment to food microbiology (Frey-Klett et al. 2011), more studies are needed in these areas to acquire significant progress in the interaction between bacteria and ectomycorrhizal fungi belonging to the *Tuber* genus and in different phases of the truffle life cycle.

One of the few examples of molecular signaling for fungal–bacterial attachment in truffles is given by the TBF-1 protein secreted by *T. borchii* in the ascocarp, a species-specific lectin that binds *Rhizobium* sp. (Cerigini et al. 2008).

The participation of lectins in the *Rhizobium*–Leguminosae association has been extensively investigated, and it was proposed that the interaction occurs between lectins present in the roots of the leguminous plants and the bacterial surface polysaccharides (Hamblin and Kent 1973; Bohlool and Schidt 1974; Kijne et al. 1997; Hirsch 1999; Fraysse et al. 2003; Laus et al. 2006). The TBF-1 is expressed only during the fructification phase (De Bellis et al. 1998), when the presence of nitrogen-fixing bacteria could be required for fruiting body development, and fungus bacteria mediator molecules, such as lectins, may be involved in these mutualistic associations. *Rhizobium* spp. produces different surface polysaccharides which are either secreted or included into the capsule surrounding the cell (Laus et al. 2006; Skorupska et al. 2006). The rhizobial exopolysaccharides (EPS), species-specific complex heteropolysaccharides, are exported outside the cell, and they seemed to be involved in the attachment of some rhizobacteria to arbuscular mycorrhizal hyphal structures (Bianciotto et al. 2001).

In a recent paper (Cerigini et al. 2008), we employed some of the *Rhizobium* strains isolated from *T. borchii* and *T. magnatum* ascomata and a few reference *Rhizobium* strains, which showed a mucoid phenotype, for crude EPS extracts testing to evaluate their ability to bind TBF-1 in an hemagglutination inhibition assay. One interesting result was that TBF-1 lectin binded only to EPS extracted by the *Rhizobium* strains from *T. borchii* ascoma. The correlation between the specificity of the lectin and its ability to recognize the correspondent bacteria was very strict: it did not react either with specific *Rhizobium* symbionts of Leguminosae nor with *Rhizobia* isolated from the phylogenetically related organism *T. magnatum*.

The high binding specificity of TBF-1 towards the rhizobial surface polysaccharides led us to suppose an active role of the protein to attract and to sort

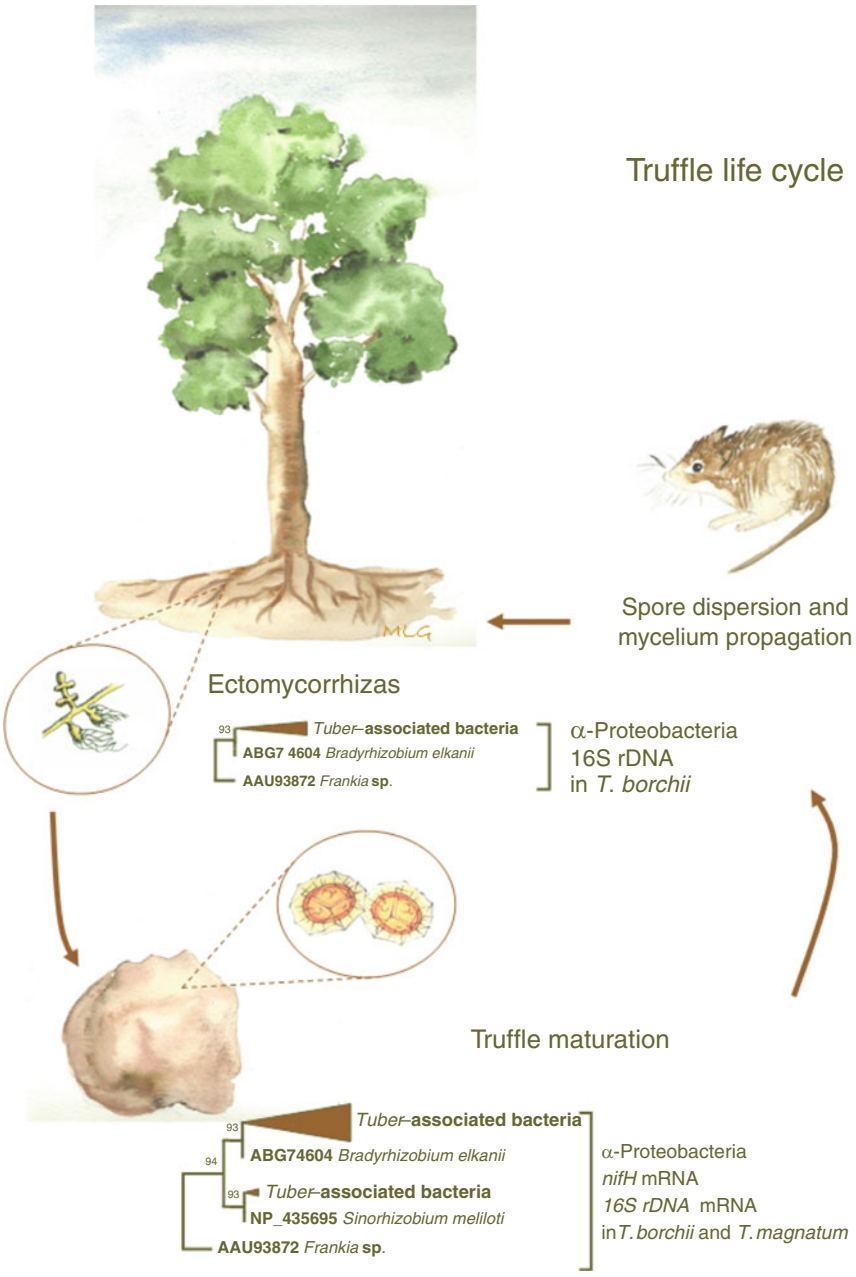

Fig. 8.1 Neighbor-joining phylogenetic analysis of the NifH amino acid-deduced sequences extracted from ascomata of both *Tuber magnatum* and *Tuber borchii* RNA overlapping with the 16S rDNA sequence analyses from the RNA extracted from the same truffle species and 16S rDNA sequences from DNA extracted from *T. borchii* ectomycorrhizas collected in productive experimental truffle grounds. Sequences obtained were compared with homologous sequences obtained by t/BLASTX algorithm. Bootstrap analyses were based on 1,000 re-samplings of the sequence alignment. The sequence of *Frankia* sp. was included as the out-group

these bacteria during the fructification phase. The ability of this protein to selectively bind the respective *T. borchii*-associated *Rhizobia* supports the hypothesis of its involvement in species-specific interaction with soil bacteria.

In which phase of the fungal biological cycle these bacteria interact, mycelium propagation, ectomycorrhizal establishment, or ascoma formation, still remains to be discovered. Since TBF-1 lectin is expressed only in the fructification phase, it could be hypothesized that rhizobia first infect the developing ascocarp and remain attached to the hypha which emerge from germinating truffle spores. Further investigation will be needed to confirm this hypothesis, and although truffle is not fully culturable in vitro, *T. borchii* is one of the ideal experimental models because the entire biological cycle is already available in vitro for this species (e.g., mycelial culture, pre-symbiosis, and ectomycorrhizal synthesis as well as experimental truffle-producing areas for ascocarp collection).

8.4.3 Can Nitrogen-Fixing Bacteria Play a Role in the Nutrition of Fruiting Bodies in Truffles?

Extending previous research from our group, we have recently addressed the problem of the molecular link between *nif* gene expression and the energetics of N_2 fixation in truffles. In our efforts to identify N_2-fixing bacteria within truffles, we also demonstrated nitrogen fixation activity throughout the study of bacterial nitrogenase gene expression and by assay of enzymatic activity in *T. magnatum*. The nitrogenase activity, evaluated by an acetylene reduction assay, was 0.5–7.5 μmol C_2H_4 h^{-1} g^{-1}, comparable with early nodules of legumes associated with specific nitrogen-fixing bacteria. The phylogenetic analysis of nitrogenase gene *nifH* from *T. magnatum* ascomata at different stages of maturation revealed the presence of α-Proteobacteria belonging to *Bradyrhizobium* spp., and expression of *Bradyrhizobia nifH* genes was also detected (Barbieri et al. 2010). Since *Bradyrhizobium* spp. is found abundantly within the mature ascoma, the conditions these bacteria encounter in the colonized tissue may approach those required for free-living nitrogen fixation (Agarwal and Keister 1983; Casella et al. 1988). Indeed, *T. magnatum* ascomata develop in soil exposed to variable temperatures during a period from late summer to autumn/winter, where drought and moisture alternate. These seasonal fluctuations affect their development.

Nitrogenase activity in the specific association of *T. magnatum* with diazotrophic bacteria has important implications for the biology of this fungus. Although the saprotrophic strategy of ascocarp development is debated (Barry et al. 1994; Zeller et al. 2008), *T. magnatum* ascomata can grow in soils that appear to have low numbers of mycorrhizas (Bertini et al. 2006). Moreover, direct linkages among belowground mycelium, mycorrhizas, and ascocarps in *T. magnatum* truffle ground are not evident (Zampieri et al. 2010). Under these conditions, enhanced nutrient

availability by nitrogen-fixing bacteria might have a beneficial effect on the development and maturation of the fruit bodies.

The Italian white truffle is highly regarded by chefs and gourmets and commands very high market prices, but repeated attempts to cultivate it have been unsuccessful (Murat et al. 2005; Hall et al. 2007). Traditional cultivation techniques, which involve planting *Tuber* colonized plants in suitable sites, do not consider the potential effects of nitrogen-fixing bacteria on truffle development (Zambonelli and Iotti 2006). The recent studies on *Tuber*-associated bacteria provide new insights into the role of the nitrogen-fixing bacteria, occurring within fungal tissue during the maturation of *T. magnatum* ascomata, and suggest new possibilities for improving the cultivation technique of this prized delicacy.

The ability of nitrogen fixation demonstrated for the white truffle *T. magnatum* has been recently analyzed in other species, and preliminary results from our laboratory show that nitrogenase expression of α-Proteobacteria is also associated with *T. borchii*, *Tuber aestivum*, and *Tuber melanosporum* (unpublished data). This evidence leads to hypothesize that a common mechanism in nitrogen utilization occurs during truffle development and maturation.

8.5 Conclusions and Perspectives

The studies described in this chapter are useful in advancing knowledge in plant–fungal–bacteria interactions and helpful in guiding future investigations on nitrogen-fixing bacteria associated with ectomycorrhizal fungi, with particular attention to the genus *Tuber*. Indeed, the expression analyses of both nitrogenase gene and enzyme activity in *T. magnatum* ascocarps and the occurrence of nitrogen-fixing bacteria in other truffle species suggest that diazotrophic bacteria may contribute to nitrogen input in forest ecosystems. These microbes can therefore be assigned to the recently revisited mycorrhizal helper bacteria group, given that they positively interact with the functioning of the mycorrhizal symbiosis. Nevertheless, direct confirmation of nitrogen fixation and quantification of the net nitrogen input remain to be performed and improved understanding of the regulation of the nitrogenase genes expression is needed.

Current evidence suggests that mutualistic fungal–bacterial interactions are more widespread than expected and that their influence may be crucial in ecosystems. The possibility that the competitiveness of ectomycorrhizal fungi in the soil and perhaps the composition of fungal communities could be influenced by nitrogen-fixing bacteria is an interesting scientific speculation, which may have serious implications for micropropagated plant production in forestry and agronomy.

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Part II
Cultivation of Edible Ectomycorrhizal Mushrooms (with Main Focus on Truffles) and Case Studies

Chapter 9
Techniques for Host Plant Inoculation with Truffles and Other Edible Ectomycorrhizal Mushrooms

Mirco Iotti, Federica Piattoni, and Alessandra Zambonelli

9.1 Introduction

Most of edible ectomycorrhizal mushrooms (EEMMs) are obligate symbionts. They cannot complete their life cycle without a host plant supplying the fungal mycelium with the photosynthates necessary to grow and develop (Karwa et al. 2011). For this reason, it is impossible to produce mature fruiting bodies of *Boletus* spp., *Tuber* spp., *Cantharellus* spp. or other EEMMs on organic substrates, as is done with saprobic edible mushrooms (Boa 2004). Currently, the production of EEMMs is almost totally via natural production. Only a few species, mainly truffles, are cultivated extensively around the world (Hall et al. 2003; Parladé 2007).

Production of well-mycorrhized plants, followed by their planting into suitable sites for soil and climate characteristics, is the crucial point in modern EEMM cultivation. The inability to cultivate most of the EEMMs is due to the difficulties in obtaining properly colonized plants and in maintaining their mycorrhizas in the field. During the past few years, the number of EEMM colonized plants available on the market has increased. This can mainly be attributed to the wide distribution and diffusion of truffle culture around the world. However, the traditional spore inoculation methodologies applied to *Tuber* spp. do not guarantee well-mycorrhized plants or consistent quality. Hence, some batches may result into being poorly colonized or not colonized at all, with the possibility of being heavily contaminated with other ectomycorrhizal (EM) fungi (Hall et al. 2003). In this context, new biotechnologies may certainly improve the efficiency of inoculation techniques for EEMMs. Moreover, recent advances in the genomics of EEMMs may reveal new insights into the biology and ecology of EEMMs, which are crucial for planning mycorrhization and cultivation programs tailored to each species

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(Martin et al. 2010). For example, the sexuality in truffles has been recently clarified (Paolocci et al. 2006; Rubini et al. 2011a) as the competitive interactions between strains of opposite mating types on root systems (Rubini et al. 2011b). Moreover, the identification of fungal genes involved in early symbiotic interactions and the characterization of enzymatic repertoires involved in fungal nutrition could improve the mycorrhization protocols for EEMMs. The aim of this chapter is to summarize the main methods currently used for inoculating EEMMs, including their preparation, application, limitations, and future perspectives.

9.2 Inoculum Types

Several types of natural and laboratory-produced EM fungal inocula have been used in the past to colonize plant roots in sterile, semi-sterile or non-sterile conditions (Repáč 2011). However, only three types of fungal material are extensively used as EEMM inoculum: spores (gamic inoculum), mycelial pure cultures (vegetative inoculum), and colonized roots (symbiotic inoculum).

9.2.1 Spore Inoculum

Spores are largely used for mycorrhizal synthesis in the greenhouse (semi-sterile conditions) but not to produce mycorrhizas in vitro. In fact, it is difficult to obtain sterile and vital spore inocula. The associated contaminants, such as bacteria or fungal parasites, may lead to unpredictable effects by favoring or selectively inhibiting root colonization (Bedini et al. 1999).

A fungal species is suitable for spore inoculation when (1) fruiting bodies are easily found and (2) its spores are produced in large amounts and can rapidly and extensively colonize the host plant root system (Lu et al. 1998). These characteristics made Gasteromycetes (*Pisolithus* spp., *Scleroderma* spp., *Rhizopogon* spp.) ideal candidates for infecting forest tree species with spores (Repáč 2011). Spore inoculum has also been successfully used for different EEMM species such as *Terfezia* spp. (see Chap. 14), *Lactarius* spp. and *Suillus* spp. (González-Ochoa et al. 2003). However, large-scale inoculation programs with spores have been applied in commercial nurseries only for *Tuber* species, and for more than 30 years, spores have been the preferred inoculum for producing infected plants with *Tuber melanosporum Vittad.*, *Tuber aestivum Vittad.*, and *Tuber borchii Vittad.* (Hall et al. 2007; Karwa et al. 2011).

There are many advantages to spore-based inoculations. Inoculum is relatively cheap, easy to prepare and less time consuming, and it does not require specialized equipment or training. Spores are obtained by crushing whole sporomata (in case of truffles) or only the fungal caps (in case of porcini or gilled mushrooms) using a

mortar and pestle or an electric mill or blender. To facilitate fungal tissue grinding, sterile water coupled with sand or other types of fine abrasive particles may be added into the crushing process. The spore suspension obtained can be injected into the planting hole or absorbed by sterile vermiculite that preserves the spores between lamellae, thus allowing inoculum placement around the root system. In the literature, there are many recommendations concerning the number of spores to provide to each plant. Although these values may vary according to species and experimental conditions, at least 10^6 spores per plant are generally recommended (Weden 2004; Hall et al. 2007).

Either fresh field-collected fruiting bodies or preserved fungal material can be used as source for spore inoculum. Preservation is often necessary because EEMM fruiting bodies are often not available at inoculation time (seasonal and/or irregular fruiting) so that short- or long-time preservation of spores is required. Moreover, preservation could be useful to reduce production costs, which are affected by the fluctuating economic value of EEMM fruiting bodies (e.g., truffles) used in spore inoculations.

Truffles are preferentially stored for short time in sterile moist sand at 4 °C. This method of preservation maintains unchanged their infective potential for up to 2 years (Zambonelli et al. 2010) and improves spore germination, perhaps due to the activity of microorganisms present in the fruiting bodies (Hall et al. 2007). Air-drying, freezing at −20 °C, and freeze-drying are simple and cheap practices commonly applied for long-time preservation of EEMMs.

A major concern with the spore inoculum technique has to do with the accidental presence of nontarget EM fungal propagules. If introduced, these undesired EM fungi may become established on the host plant root system where it may partially or completely replace the target species. This problem is relevant for truffles when large quantities of fruiting bodies are used to prepare the inoculum and, particularly, when low-grade fruiting bodies are used as inoculum (a tactic to reduce the cost of inoculum that we do not recommend). Low-grade truffles usually include immature or deteriorated fruiting bodies and nontarget taxa that may be difficult to identify. The incorporation of these less valuable and potentially more infective species of truffles can contaminate entire batches of plants (Ferrara and Palenzona 2001). In this context, *T. borchii* and *Tuber maculatum* Vittad. can contaminate *Tuber magnatum* Pico inocula, while *Tuber brumale* Vittad. or *Tuber indicum* Cooke & Massee can spoil *T. melanosporum* inocula (Tibiletti and Zambonelli 1999). The recent detection of *T. indicum* mycorrhizas in *T. melanosporum* truffle plantations in Europe and the USA (Murat et al. 2008; Bonito et al. 2011) demonstrates that lackadaisical applications of spore inoculum may lead to not only economic losses but also ecological damage through the introduction of invasive alien species. In order to avoid these problems, many countries require morphological and molecular taxonomic verification for all fruiting bodies used for inoculation. To this aim, rapid molecular identification based on direct PCR (Bonuso et al. 2006; Bonito 2009) might also be applied to reduce analytical costs and time.

9.2.2 *Inoculating with Mycelium*

Mycelium has been considered the most suitable inoculum source for infecting plants with EM fungal strains (Marx and Kenney 1982). However, the large-scale nursery application of this inoculum type strongly depends on the saprobic potential of the fungal species of interest and its ability to produce large amounts of mycelium in axenic conditions. Mycorrhizal synthesis using pure mycelial cultures has been obtained for many edible ectomycorrhizal (EEM) ascomycetes and basidiomycetes, either in greenhouse or in vitro conditions (Águeda et al. 2008; Danell and Camacho 1997; Giomaro et al. 2005; Yamada et al. 2001; Zambonelli et al. 2008). However, it is only extensively used for a few EEMM species such as *Lactarius deliciosus* (L.) Gray and *Tricholoma matsutake* (S. Ito & S. Imai) Singer (see Chap. 16). The main limitation in using EEMM mycelia as inoculum has to do with the difficulty in maintaining pure cultures and in producing adequate amounts of biomass for large-scale mycorrhization programs. This is particularly challenging for truffle species.

Mycelial inoculum has four main advantages over spore inoculum (1) lower contamination risks, (2) fewer problems with fluctuation in availability or cost, (3) higher rate of infection, and (4) better genetic tractability. The use of pure cultures can ensure that inocula are free of undesired EM fungi or contaminants that may inhibit root colonization of the target fungal species. Mycelia are also able to colonize the fine roots more rapidly than spores, thus reducing the risk of contamination during plant growth. For example, some mycelial strains of *T. borchii* are able to form mature mycorrhizas on *Quercus robur* L. in less than 1 month in semi-sterile conditions (Iotti, unpublished data). Moreover, vegetative inoculum enables the infection of plants with fungal genotypes specifically selected for infectivity, host plant affinity, ecological conditions or fruiting characteristics. This is important given that genetic differences between *Tuber* strains result in different levels of infection and in different affinities to the host plant both in vitro (Giomaro et al. 2000) and in greenhouse (Zambonelli et al. 2008) conditions. When suitable strains are available for mycelial expansion, it is possible to optimize the mycorrhization process, bypassing many constraints encountered when using spores as inoculum.

Mycelia of EEM fungi can be generated from either spores, ectomycorrhizas or fruiting bodies, but the success of this procedure depends on the quality of the source used (purity and age). In vitro germination of spores has been reported for a number of EEMM species including *T. melanosporum* (Fischer and Colinas 2005), *T. matsutake* (Murata et al. 2005), and *Cantharellus cibarius* Fr. (Danell 1994) yet is challenging for most EEMM species (Fries 1987; Nara 2009). Resulting mycelium from single spores is haploid (n), although basidiomycetes may quickly undergo plasmogamy to form dikaryons (n + n) if compatible monokaryons are available. Isolation of mycelia from ectomycorrhizas is more feasible, although the risk of contamination by other species of free-living, endophytic, or rhizospheric fungi or bacteria can be high. For this reason, ectomycorrhizas must first be surface sterilized in order to destroy propagules of contaminants but safeguard the vitality of the symbiotic fungus. To surface sterilize roots, excised root tips are washed two to

three times in sterile water by vortexing and spinning and then dipped in a calcium or sodium hypochlorite solution for 1 or a few minutes, according to the concentration of active chlorine. Alternatively, a 1–2-min bath in a 3–6 % hydrogen peroxide solution is used to sterilize root tips. After sterilization, mycorrhizal tips are rinsed three times with sterile distilled water and are placed on or submerged in Petri dishes filled with appropriate medium (see below) supplemented with antibiotics (e.g., 50–100 μg/ml of streptomycin) (Mukerji et al. 1998). Still, most pure cultures of EMM fungi have been obtained from fresh fruiting bodies, which offer lower contamination risks and easier isolation procedure. To isolate from fruiting bodies, small fragments of fungal tissues (<1 mm) are excised from the truffle gleba (preferentially from the white sterile veins) or from the inner tissues of the intersection zone between stipe and pileus (for epigeous fungi) and then transferred on agar plates. Isolations are more successful from fresh and immature fruiting bodies. With this approach, prompt growth of the hyphae from the excised fruiting body fragment and low levels of contamination are observed, even when no antibiotics are added to the culture medium (Iotti, unpublished results). Pure cultures isolated from basidiomata tissues are stable dikaryons and should be able to fruit after establishing symbiosis with the host plant. Some EEM basidiomycetes are also able to produce primordia or mature fruiting bodies in vitro, such as *Boletus reticulatus* Schaeff. (Yamanaka et al. 2000), *Lyophyllum shimeji* (Kawam.) Hongo (Ohta 1994) and *Phlebopus portentosus* (Berk. & Broome) Boedijn (Sanmee et al. 2010). In contrast, because pure cultures originated from ascomata of heterothallic ascomycetes such as *Tuber* spp. are composed only by homokaryotic maternal tissues, they are unable to fruit. For this reason, at least two compatible mycelial strains are necessary for producing fertile truffle orchards, as both mating types must be present to guarantee fruiting body production after planting (Rubini et al. 2007).

Once mycelium has been isolated on appropriated agar (more rarely Phytagel or Gelrite-based) media, isolates are incubated in the dark at 20–22 °C and maintained in active growth through repeated subculturing. The growth curve of a fungal colony developing on an agar plate proceeds in four phases (1) lag, (2) initial growth, (3) linear growth, and (4) staling (Hudson 1992). The shape of the curve is heavily dependent upon the fungal species and cultural conditions, but generally EEMM mycelia show longer phases and lower growth rates compared to saprobic fungi and other nonedible EM fungi (Iotti et al. 2002, 2005). For example, the lag phase of *Tuber* mycelia is commonly 7 days long and maximum growth rates are less than 1 mm per day. It is so for *T. magnatum* mycelia recently isolated in our laboratory, reaching the staling phase after one mm of radial growth in each subsequent culture. During the staling phase, many colonies lose their vitality and, consequently, the ability to generate new colonies. For this reason, it is necessary to cut an agar plug with hyphal tips from the edge of the fungal colony and to transfer it in a new plate when hyphae are still in active growth. Similarly, mycelial senescence is detrimental for root colonization.

Mycelial agar plugs and mycelial slurries from liquid cultures have been commonly used to synthesize ectomycorrhizas in a variety of in vitro systems (Petri plates, flasks, jars, tubes, etc.) (Repáč 2011). However, most common procedures

for preparing high amounts of mycelial inoculum come from the method proposed by Molina (1979). As a general rule, isolates are initially grown in flasks as submerged cultures (30–50 days). Actively growing mycelium is then mechanically fragmented, and the resulting mycelial slurry is used to inoculate tubes containing a mixture of sterile peat moss and vermiculite (1:29 v/v) moistened with the liquid medium (see Sect. 9.2.2.1). After a further 30–50 days of growth in the dark at 22 °C, the colonized substrate is removed from the tubes, washed with tap water and squeezed dry 5 times to remove fungal catabolites and residual nutrients. The vegetative inoculum is then mixed into an appropriate potting mixture or is localized around the plantlet root systems. This procedure enables one to obtain a rapid and uniform colonization of the substrate (Molina and Palmer 1982) but it is a time-consuming procedure. However, a number of biotechnology approaches can be applied to improve production processes of mycelial inoculum. Submerged fermentation in bioreactors is the most promising approach for efficient production of mycelia in liquid culture, and it also has recently been applied to EEMM species (Carrillo et al. 2004; Tang et al. 2008; Liu et al. 2009). Bioreactors allow production of higher amounts of fungal biomass in shorter time because fermentation parameters such as temperature, pH, oxygen, organic and inorganic nutrients, and fungal catabolites can be continuously controlled, maintaining the optimal conditions for mycelial growth.

Promising approaches for producing commercial mycorrhizal inocula also involve the use of various combinations of natural, semi-synthetic, and synthetic polymers (Siddiqui and Kataoka 2011). Entrapment of mycelial fragments in natural polysaccharide gels, such as calcium alginate, is a proven method to obtain efficient inocula for root colonization with different EM fungal species (Repáč 2011). However, this inoculant technology was unsuccessful for some EEMMs. In fact alginate-entrapped inocula of *L. deliciosus* (Parladé et al. 2004) and *Tuber* spp. (Kuek and Zambonelli, unpublished data) completely failed in forming mycorrhizas when mixed with the plant growth substrate, although viability of their mycelia was proven to be unaffected. More studies are needed to find and test alternative polymers that do not affect the colonization ability of the EEMM mycelia.

9.2.2.1 Culture Media

Many EEM fungi can be cultured in semi-synthetic and synthetic culture media, although the growth rate is extremely variable between species. Mycelia of *Boletus* spp., *Amanita* spp., *Tricholoma* spp., and *Lactarius* spp. are routinely maintained on the most common media (or their modified version) reported in the literature, such as potato dextrose agar (PDA), biotin–aneurin–folic acid (BAF) agar (Oort 1981), malt extract agar (MEA), and modified Melin-Norkrans (MMN) agar (Marx 1969). Specific media have also been developed to better support the growth of *C. cibarius* (modified Fries medium) (Danell 1994) and *Tuber* spp. (modified woody plant medium) (Iotti et al. 2002).

9.2.2.2 Preservation

Preservation of pure EEMM cultures is a priority. Beyond guaranteeing purity and viability of valuable EEMM strains, preservation has to guarantee the maintenance of their morphological, physiological and genetic integrity over time (Ryan and Smith 2004; Voyron et al. 2007). Many methods about fungal preservation are available (Nakasone et al. 2004), but only a few of them are suitable for EEMM species, in particular for *Tuber* mycelia (data not published). The choice of preservation method depends on the species of interest and project aims. Short-term preservation by repeated subculturing is simple, cheap and valid for most EEM fungi, but the risk of contamination as well as desiccation of the agar is high. Moreover, repeated transfers can lead to losses in vigor and infectivity due to mutations or modifications of gene expression (Coughlan and Piché 2005). To compensate this problem, in some cases, changes in media composition (i.e., alternation of nutrient-rich media with nutrient-poor media) can rejuvenate old cultures and minimize viability loss (Nakasone et al. 2004; Repáč 2011).

A low-cost method for long-term culture preservation is oil overlay. This consists of submerging a fungal colony grown on agar slants completely with oil. The tubes must be tightly sealed and the oil level must be checked periodically and refilled as necessary. Similarly, disks cut out of fungal growth on agar can be stored in tubes containing sterile distilled water. Cultures maintain highest vitality when stored at 4 °C (Richter 2008). Such conditions are valid only for some EEMM species. For example, long-term vitality of *Tuber* mycelia cannot be guaranteed in these conditions (personal observation).

Cryopreservation in electrical freezers at −80 °C is a technique with high survival rates (Kitamoto et al. 2002). In this method, small agar plugs sampled from actively growing fungal colonies are preserved into cryotubes containing the appropriate liquid media and dimethyl sulphoxide (DMSO) or 10 % glycerol as cryoprotectants. The cryotubes are then placed directly in a deep freezer. Some EM fungal species may remain viable under these conditions longer than others (Obase et al. 2011).

Another valuable ultra low temperature technique of preservation is cryopreservation in liquid nitrogen. It was assessed for the first time in 1953 for human sperm, and it is now becoming routine in mycology. For example, microbial collections at the American Type Culture Collection (ATCC) are cryopreserved in liquid nitrogen. In this process, hyphae are preserved by cooling to low sub-zero temperatures (−196 °C), at which all biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. Thus, cellular and genetic damage is prevented. The use of cryoprotectants is indispensable in preventing cellular damage resulting from decreases and increases in temperatures. Cryoprotectants generally have two modes of action: intracellular penetrating agents, such as DMSO and glycerol, and extracellular agents such as sugars (sorbitol, sucrose, lactose, trehalose, and others). By preserving cultures through deep freezing mutations are prevented, labor is reduced, infectivity is preserved, and cultures can readily be accessed. The main disadvantages of this technique are the high costs

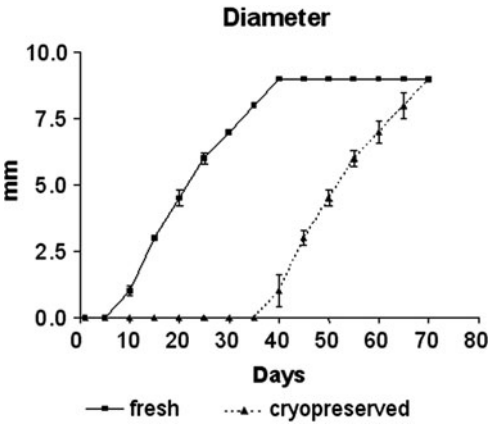

Fig. 9.1 Development of *T. borchii* mycelium before and after cryopreservation

required to purchase and maintain the deep freezing. Moreover, cryopreservation determines an increase in the lag phase of the first subculture after thawing. Cryopreservation success of pure cultures varies among species and strains, as concluded by Nagai et al. (2000).

The first successful attempts of cryopreservation with EM mushrooms were performed on *C. cibarius*, by Danell and Flygh (2002). We adopted such procedure on *Tuber* species after some small changes: replacement of DMSO by glycerol or addition of sucrose to DMSO. The first attempts of cryopreservation in liquid nitrogen of *T. borchii* were positive: after 2 months of cryopreservation, the two strains tested regenerated hyphae after thawing, although their lag phase was much longer than the one of the fresh transplanted mycelium (Fig. 9.1). However, once the lag phase was over, mycelium growth of the cryopreserved strains was similar to the one observed for the fresh strains. Similarly, initial attempts of deep freezing of *T. borchii* at −80 °C were successful too: after 2 months of cryopreservation followed by 20 days of direct storage −80 °C, the same strains developed hyphae, but in this case the lag phase was much shorter (8 days) (Piattoni et al. 2010). The same procedure of cryopreservation was applied to a single strain of *T. aestivum*. Again, tissue growth was re-established; however, the lag phase was much longer than observed for cryopreserved *T. borchii* strains (Piattoni et al. 2010).

9.2.3 Colonized Root Inoculum

Inoculum with colonized EM root tips allows one to obtain well-colonized plants regardless of the EEMM species. This approach exploits the high infectivity of hyphae emanated from active symbiotic tissues. This method was perfected for *Tuber* species to bypass the need of using spores, for instance, for species whose spores are difficult to germinate, such as *T. magnatum* (Gregori 2002) or for species which mycelial strains have low infectivity. The inoculum consists of whole

mycorrhized plants called "mother plants" (Zuccherelli 1990) or root fragments infected with the target EEMM (Chevalier and Grente 1973). In the first case, the mother plants, previously inoculated with spore or mycelium, are grown in the middle of large pots surrounded by sterile plantlets so that infected and uninfected root systems are in close contact. In the second case, mycorrhizal fragments are aseptically excised from the washed root system of the mother plant and then added to the potting mix next to the uninfected plantlet roots. Large-scale production of colonized root inoculum requires the use of numerous and well-infected mother plants initially obtained using sporal or mycelial inoculation techniques. However, the long-term maintenance of the mother plants in greenhouse conditions carries a high risk of spreading undesirable and competitive EM fungi, which can contaminate complete batches of plants. Although frequent and careful controls may be planned by growers, contaminated roots may escape detection. To reduce the risk of contaminations, colonized root inoculum may be obtained in vitro by mycelial inoculation. Over the last 50 years, a large number of in vitro techniques to grow whole plants or root organ cultures associated to EM fungi have been developed (Coughlan and Piché 2005; Giomaro et al. 2005), but they have been exploited only to improve the understanding of plant–fungus interactions. The better advances in this research sector involved the use of transformed root organs obtained by transferring of the Ri T-DNA plasmid of *Agrobacterium rhizogenes* into the host plant genome. Transformed organs show increased growth rate and branching without addition of exogenous plant growth regulators to the culture medium, and they can be used to produce mycorrhizas in axenic culture. However, transformed root clones of an EM plant able to establish EM association have only been obtained for *Cistus incanus* L. (Wenkart et al. 2001; Zaretsky et al. 2006). Mycorrhized transformed roots of *C. incanus* increased the hyphal growth of *T. melanosporum* mycelium and preserved its vitality and infectivity for months (Ventura et al. 2006). The characteristics of *C. incanus* root organs are appropriate for the long-term in vitro maintenance of EM isolates and of their infectiveness; moreover, they represent an alternative source of clonal inoculum for plant mycorrhization (Coughlan and Piché 2005). Recently, a method to obtain *T. magnatum* mycelium and mycorrhizas by using transformed roots has also been patented (Buee and Martin 2009).

9.3 Substrate

The type of soil used to grow inoculated plants affects the rate of root colonization by the target fungal species and potential contaminants. Donnini et al. (2009) and Zambonelli et al. (2009) demonstrated that the use of different soils affects the competitive abilities of truffle species. Indeed, natural soils from suitable areas have long been used as substrates to grow mycorrhized plants. However, large-scale operation for commercial purposes is not always feasible due to economic and ecological reasons. Given this, various potting mixes and soil-less plant growth

media have been developed by nurserymen. However, specific details about the composition of the potting mixes, inoculum amounts, amendments, and greenhouse conditions vary among nurserymen, and most remain trade secrets (Hall et al. 2003). Vermiculite, perlite, wood ash, bark, sand, dolomite, green compost, and peat moss are the most common ingredients used to prepare potting mixtures. In general, truffle species prefer low organic matter mixtures supplemented with calcium carbonate components (Pruett et al. 2009) to raise pH to ~8.0, whereas porcini mycorrhizas grow well in acid and peaty potting mix. Vaario et al. (2002) demonstrated that pine bark is a suitable substrate component for mycorrhization of *T. matsutake* on its host plant. Pot type, pot size, and shape used to grow inoculated plantlets are also crucial to develop appropriate highly branched root systems, optimize the effectiveness of the inoculum mass and facilitate good quality control (Palanzòn and Barriuso 2007).

9.4 Quality Control of the Mycorrhized Plants

Quality control measures are essential to successful EMM cultivation. A number of methods for the certification of truffle-in plants have been proposed by different scientists (Palanzòn and Barriuso 2007; Sisti et al. 2010). The percentage of fine roots colonized by the inoculated fungal species and the presence of undesirable EM associations are used as the main criteria for quality evaluation of mycorrhized plants. A high level of root colonization before transplanting is advisable for the development of secondary infections in the field. However, it is difficult to establish a suitable level of root colonization to guarantee the fungus persistence and spreading on roots after outplanting. The optimum level of mycorrhization mostly depends on the climatic and soil characteristics of the plantation site, which affect competitiveness, extent and rate of spreading of the EEMM inoculated species. Zambonelli et al. (2005) showed that a 30 % initial rate of root colonization with *T. aestivum* reached values of 50–70 % in mycorrhizal seedlings after 5 years when planted in a suitable soil. Hortal et al. (2009) found a higher persistence of *L. deliciosus–Pinus pinea* L. ectomycorrhizas with an initial colonization percentage over 50 %. A threshold colonization rate of about 30 % was considered acceptable by many authors (Wang and Hall 2004; Bencivenga et al. 1987; see also Chap. 14), although higher colonization levels of the target species favors the persistence of the introduced EEMM species after planting in field soils.

Although certification of commercially mycorrhizal plants is performed by some nurserymen, with the assistance of public research institutes, it is still not common for the industry and consistent standards are lacking. Also, performing large-scale quality control measures for entire plant batches is time-consuming and expensive. Its cost may represent up to 10–15 % of production costs (Bernardini, Raggi Vivai, personal communication). Moreover, previously such nursery sector has been

poorly defined and regulated by existing laws. At the assessment time, however, in few Italian regions mycorrhizal plants offered for sale are to be evaluated based on standard procedures (Bagnacavalli et al. 2012).

Root colonization by undesired fungal species may be due to negligent nursery management practices or inoculum contamination. In the first case common sources of contamination include airborne spores, insufficiently or non-sterilized potting mixtures and equipment (Brundrett et al. 2005). However, mycorrhizas of these opportunistic contaminants are relatively easy to detect with a microscope because they usually display distinctive anatomo-morphological characteristics. For example, mycorrhizas of *Pulvinula convexella* (P Karst.) Pfister (=*Pulvinula constellatio*) and *Sphaerosporella brunnea* (Alb. & Schwein.) Svrček & Kubička are common greenhouse contaminants but differ from truffle ectomycorrhizas on the basis of their mantle type and emanating hyphae (Amicucci et al. 2001). Inoculum contamination is much more difficult to detect because the contaminant species are congeneric with the target EEMM (see Sect. 15.2.1) and their mycorrhizas have often similar morphological features (Zambonelli et al. 1995; Kovács and Jakucs 2005; Águeda et al. 2008). Thus, quality control measures prior to outplanting are critical.

For these reasons, the use of molecular tools for quality control assessments is becoming more widely used across the EMM industry. This compensates for the limitations of morphological identification and helps to reduce the marketing of mycorrhized plants of poor quality. During the last 20 years, many PCR-based methods have been developed for species-specific identification of a large number of target fungi, including most of the valuable EEMM species (see Chap. 7). Most of these are based on the amplification of a small fragment of the fungal genome (usually the ITS regions of rDNA) with specific primers in single or multiplex PCR reactions (see Table 7.1). This approach is also amenable to direct PCR, thus saving labor and time costs (Iotti and Zambonelli 2006; Bonuso et al. 2006; Bonito 2009; Bonito et al. 2011).

It is undeniable that molecular analyses increase the cost of quality controls and, consequently, the price of mycorrhized plants. Yet, these costs are minimal compared to the cost of properly establishing an EMM orchard and are a wise safeguard against failure. Quality control measures will vary depending on inoculation procedure used. Generally, when using pure culture-based inocula, the molecular confirmation of the isolated strains coupled with morphological check for post-inoculation contaminants is sufficient. Similarly, when spore inoculation methods are applied, analyses should be carried out on inoculum prior to inoculating plants to guarantee that the correct species is being inoculated. Root systems and ectomycorrhizal colonization levels of inoculated plants ought to be checked prior to being sold or planted to confirm that roots are healthy and well colonized by the target species. If poorly mycorrhized plants are introduced in the field, nontarget fungal species may take over the root system resulting in orchard failure. A current challenge for the EEMM industry is the lack of quality control standards (that can be applied internationally) and the lack of independent labs available for testing inoculated plants using molecular and morphological approaches.

9.5 Conclusion

Unfortunately, recent advances in biotechnologies have been poorly utilized in large-scale production of mycorrhizal plants. Truffle-infected plants are still produced using the traditional spore inoculum techniques which, although efficient, do not offer the opportunity to use select fungal genotypes in producing mycorrhizal plants. In contrast, mycorrhizal plants from other EEMMs are routinely produced with mycelia, but we are not aware of any genetic screening programs in place for these isolates. Mycelium-based inoculation procedures offer the possibility for selecting fungal strains according to the different ecological conditions. This could be important for widely distributed species, such as *T. aestivum* and *T. borchii* in Europe, which exhibit great genetic diversity and perhaps contain cryptic species with different ecological requirements (Weden et al. 2005; Bonuso et al. 2010). Moreover, mycelium-based inoculations offer other advantages including lower risks of contamination and a more standardized level of colonization than is obtained with the more heterogeneous spore inoculum. Still, more research is needed on the physiology and nutritional requirements of each EEMM in order for large-scale culturing of their mycelia and reliable inoculum production. New insights into mycelial biomass production and symbiotic establishment are expected to result from recent mycorrhizal genome sequencing programs. Moreover, the development of mycelium-based technologies must be in step with the improvement and application of cryopreservation to avoid loss of valuable fungal genotypes and preserve EEMM biodiversity over time. To this end, a cryobank for EEMM species is advised.

In summary, modern biotechnologies developed at the end of the last century brought the first great opportunity for quality control of commercial EM plants inoculated with EEMMs. Molecular characterization of EEMMs minimizes the risk of misidentification and enables processing of a high number of samples resulting in a parallel saving of labor and expense. However, molecular identification of fungal symbionts is still not routine, even though costs of reagents and equipment continue to decrease and molecular tools continue to be developed for diagnostics. In order to further reduce costs and time involved in root-tip morphological typing and tip counting, quality control measures of commercially infected plants could be nearly entirely performed by molecular tools. In this way, the quantitative assays specifically developed to estimate the mean amount of biomass of an EEMM species on the roots of a plant batch as well as the level of contaminants can be optimized and may represent one of the main priorities of future research programs.

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Chapter 10
Soils and Techniques for Cultivating *Tuber melanosporum* and *Tuber aestivum* in Europe

Gérard Chevalier and Pierre Sourzat

10.1 Introduction

Scientific truffle cultivation began in France in 1973, with the large-scale production and planting of truffle-mycorrhized plants (Chevalier 1983). To optimise this development, J. Grente first proposed a rational method of trufficulture in 1972 outlining the details in his text "Perspectives pour une trufficulture moderne" at the same time as J. Delmas, at INRA in Bordeaux, established the first detailed studies of truffle soils (Grente and Delmas 1972–1974; Delmas 1976). Grente's method was founded on four principles:

1. The choice, and if necessary, the improvement of the *environment*
2. The choice of *tree species* best adapted to the environment at hand
3. The *inoculation* of host trees
4. The *maintenance of environmental conditions* favorable to mycorrhization and then to fruiting

This chapter focuses on the analysis of environmental conditions for truffle cultivation with particular attention to soil characteristics and cultural methods to improve the suitability of soils for truffle production of *Tuber melanosporum* Vittad. (Fig. 10.1a) and *Tuber aestivum* Vittad. (Fig. 10.1b, c). While genetic studies suggest that *T. aestivum* and *T. uncinatum* Chatin are synonyms (with *T. aestivum* having taxonomic priority) (Wedén et al. 2005; Paolocci et al. 2004), they are still marketed as different species. Most researchers consider them to be ecological forms of the same species having different maturation times (autumn for *T. uncinatum*,

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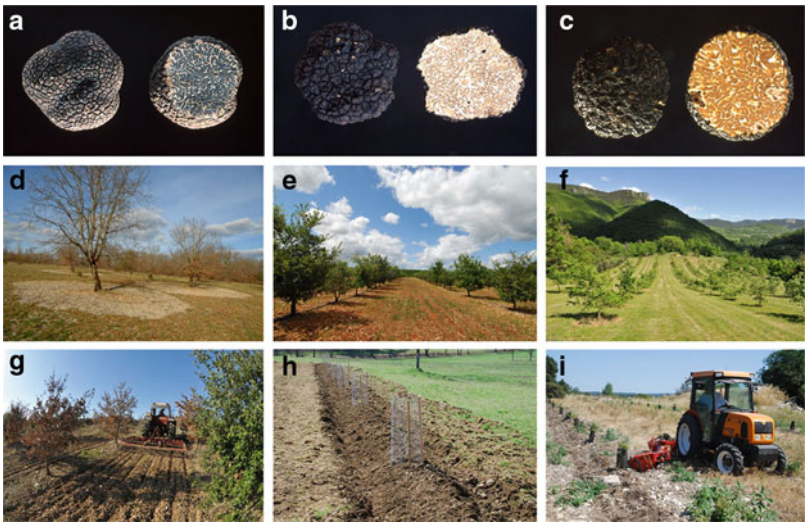

Fig. 10.1 *Tuber melanosporum* ascoma (**a**), *Tuber aestivum* f. *aestivum* ascoma (**b**), *Tuber aestivum* f. *uncinatum* ascoma (**c**), Different methods of *T. melanosporum* cultivation traditional method (**d**), truffle arboriculture (or Pallier) method (**e**), chalk grassland (or lawn ecosystem of Tanguy) method (**f**),classical method of tilling the soil (**g**), young plantation established with the J.A.AD. method (**h**), tilling the soil with the J.A.AD method (**i**)

summer for *T. aestivum*) and ecological preferences (Chevalier 1979; Chevalier et al. 1979, 1994; Chevalier 2010a; Gregori 1991, 2010; Mello et al. 2002). The form of *T. aestivum* marketed as *T. uncinatum* has a greater value in the marketplace. It matures later in the season and is characterised by a darker gleba and smaller peridium warts (Chevalier et al. 1979) and has a stronger and more pleasant aroma (Fig. 10.1b). While there is little interest in cultivating the less valuable summer form of *T. aestivum* (*T. aestivum* f. *aestivum*) (Fig. 10.1c), techniques for cultivating the autumn form of *T. aestivum* (*T. aestivum* f. *uncinatum*) have been specifically developed in Europe. Thus, the ecological requirements and cultivation techniques described in this chapter apply to *T. aestivum* f. *uncinatum* (in the text above it is simply called *T. aestivum*).

10.2 Truffle-Producing Soils

Soil physico-chemical characteristics are a determining factor in truffle production. Even when a plant is well-mycorrhized and the climate is favorable, fruiting cannot take place if certain soil characteristics are inappropriate. Delmas provided a thorough study of truffle soils in the early 1970s, and similar studies were made in Italy in the 1980s by Bencivenga and Granetti, and Lulli and Bragato

(Bencivenga et al. 1990; Bragato et al. 1992, 2001; Lulli et al. 1991, 1992, 1999). Soil conditions favorable to truffle production vary by species, and those favorable for *T. melanosporum* are not necessarily the same as for *T. aestivum*.

10.2.1 Geologic Substrate and Soil Types

10.2.1.1 Sedimentary Bedrock

French soils producing *T. melanosporum* have been characterised by Delmas (Grente and Delmas 1972–1974; Delmas et al. 1981), Callot (1999) and Sourzat (Sourzat 1989–1995–2002, 2008, 2011); those in Italy by Bencivenga et al. (1990), Lulli et al. (1991, 1992, 1995, 1999), Bragato et al. (2001) and Raglione et al. (1992, 2001, 2011); French soils producing *T. aestivum* have been characterised by Le Tacon (1997); those in Italy by Granetti et al. (2005) and Gregori (2010); those in Hungary by Bratek et al. (2010) and those in Sweden by Wedén et al. (2004). The following outline is drawn from these authors.

Delmas (Grente and Delmas 1972–1974) defines precisely the physico-chemical characteristics of French truffle soils. They extend very specifically over the extensive Jurassic, Cretaceous or Tertiary sedimentary rock deposits from the Aquitaine Basin to the Mediterranean zone. In Périgord, most truffle soils are situated on limestone of the middle or upper Jurassic period that contains bands of hard limestone. On the other hand, in Provence the banding is more varied, and in this very rugged region, the peculiar features of the climate, there is no neat relationship between the type or layer of limestone and productivity.

Truffle soils are often not very deep, and, at the extreme, truffle cultivation occurs on lithosols, represented mainly by rendzina profiles or brown calcareous soils. Briefly, they may be nearly devoid of limestone, but the complex is saturated with calcium and the pH is neutral. Nevertheless, in these calcic brown soils, the soil structure remains granular or finely polyhedral. Carbonates can lead to very different levels of truffle production depending on their abundance but also depending on their nature (hardness, solubility, particle size, composition). Other soil types can be encountered, but they are always relatively shallow and overlay fissured limestone bedrock. Besides the essential presence of limestone (or calcium), the nature of the substratum (fissuring, position of sediments, thickness and structure) has an important influence on the growth of the host tree and the ensuing mycorrhizal symbiosis.

The principal agronomic features of successful truffle production are good crumb structure, good circulation of water and gases throughout the profile (absence of hydromorphic layers engendering asphyxia), sufficient organic matter content of appropriate quality characterised by a calcic mull in equilibrium with a C/N ratio of approximately 10, sufficient resistance to surface erosion, a balanced texture (clay–silt–sand) and a mineral composition in which no essential element is markedly deficient or in excess.

Callot (1999) confirmed Delmas' work (Grente and Delmas 1972–1974), particularly the varied geologic age of sedimentary formations, and added to the list of truffle-producing regions by including the Devonian limestone of the Eastern Pyrenees to the stoney Quaternary limestone alluvia of the Rhone and Durance River valleys. He also emphasised the importance of the nature of the geologic substrate for the production of truffles and their variability.

In Italy, *T. melanosporum* truffières are essentially situated on sediments dating mainly from the Mesozoic Era and, more specifically, of Jurassic and Cretaceous periods (Mannozzi-Torini 1970; Pacioni 1986). The range of substrata seems more restricted than in France. The soils are calcareous rendzinas rich in clay with a light and friable texture that favors water penetration. They are generally superficial, well aerated and permeable. Their texture is essentially sandy, sandy–silty or sandy–clayey, whereas in central Italy, there are brown soils developed from red clays by decarbonisation of the subjacent limestone.

In contrast to *T. melanosporum*, *T. aestivum* develops in sedimentary terrain of very different geologic age, from the Paleozoic Era to Quaternary and recent alluvia. This diversity of substrata (and the fact that *T. aestivum* is less thermophilic than *T. melanosporum*) explains the vast geographic distribution of this species, from Morocco to Sweden and from Ireland to Azerbaijan (Bagi and Fekete 2010; Chevalier 2010a). In France, *T. aestivum* soils are rendzinas or brown calcareous soils.

In Italy, the celebrated "truffe de Fragno" (*T. aestivum* f. *uncinatum*) develops, in the province of Parma, on terrain derived from sedimentary rock (marly limestone) from the Mesozoic (Cretaceous) and Cenozoic Eras (Palaeocene, Eocene and Oligocene Epochs) called "flyschs".

In Hungary, *T. aestivum* soils belong to several types. More than one-third of those tested soils are brown forest soils with clay illuviations, 25 % are Ramann's brown forest soils, 17 % are meadow alluvial soils, while meadow soils and meadow chernozems each make up almost 10 % of the soils (Bratek et al. 2010).

In Sweden, where the soils date from the Paleozoic Era, the soil sites are characterised by either moraine marlstone or different kinds of gravel and sand mixtures, or a mix of the two; the bedrock can be stratified, predominantly crystalline, partly fine oolitic limestone, reef-shaped limestone, reef limestone, marly limestone, marlstone, sand limestone and lime sandstone.

10.2.1.2 Volcanic and Metamorphic Bedrock

Although the majority of favorable substrata are sedimentary, exceptions exist. Callot (1999) points out the atypical case in which productive truffières develop on acid recarbonated soils derived from crystalline rock, in the eastern Pyrenees. These truffières are, in fact, situated in colluvial zones where calcareous gravel provided the available calcium needed for truffles. An even more unusual example of very productive truffières has been recently identified on granitic sand resulting from the decomposition of alkaline granite in the eastern Pyrenees [Chevalier G, Urban A, Pla T (2009) Truffières à *Tuber melanosporum* atypiques sur granites

alcalins dans le Vallespir, Pyrénées-orientales (unpublished)]. This phenomenon raises the question of the role of limestone in the fructification of *T. melanosporum*. In fact, these soils do not contain limestone, but they do contain calcium. This calcium does not come from an exogenous contribution as in the case of the recarbonated truffière soils, but rather from an endogenous contribution resulting from decomposition of the calcic feldspar (anorthite) component of the bedrock.

Callot's work (1999) has confirmed the importance of a highly fractured substrate, which facilitates development of a deep root system, and of the soil's capacity to store water reserves. However, even in a limestone region, not all situations favor truffles. In fact, the permeability of the subsoil is essential. The structure of the subsoil, which determines drainage, conditions the speed at which a truffière enters into production and the intensity of truffle production.

Callot (1999) has also brought out the importance of having a horizon in contact with the bedrock, which demonstrates a high level of fauna-induced porosity. This horizon, called "Sbio", is an excellent indicator of good drainage and of soils favorable to truffles. Soil fauna, a major factor determining the structure and aeration of the soil surrounding the ascomata, regulates the microbial activity in truffière soils (see Chap. 6). Soils with high potential as truffières are always well drained and have high levels of soil fauna activity. The most productive truffières are most often located in well-drained deep soils. Compact clayey subsoils are always unfavorable.

This information acquired during the 1990s jostles traditional dogmas, which state that "productive truffle soils are most often relatively shallow, and, at the limit, truffles can be cultivated on some lithosols" or "most of the best truffières are situated in a textural region that excludes the extremes". Excessively sandy soils can support excellent truffières, if they are sufficiently rich in calcium, just as a very stony site can produce truffles.

The quality of the subsoil is fundamental for truffle production. In truffle cultivation, analysis of the subsoil has often been neglected, in favor of physico-chemical analysis of the surface fine earth. Before installing a truffle plantation, one is well advised to excavate a soil trench (preferably down to the rock) and examine the profile, thus avoiding gross errors in planting on unsuitable terrain.

10.2.2 Physical Characteristics of "Truffle Soils"

A wide range of soil factors interplay which makes it difficult to define the ideal truffle soil. Principle component analysis of soil physico-chemical data has permitted only partial discrimination of productive truffle soils from those that do not produce brûlés or truffles. Productive soils are characterised by simultaneously relatively elevated contents of fine sand and total limestone, associated with relatively small amount of fine silt, nitrogen and organic matter (Delmas et al. 1981). Statistical analysis has, however, permitted Raglione et al. (1992) to significantly distinguish natural sites producing *T. melanosporum* from those producing

T. aestivum and *T. brumale*, which are often located in close proximity. A similar methodology was later applied by Lorenzoni et al. (1995) in studying natural *T. melanosporum*, *T. aestivum* and *T. brumale* sites. Some of the variables studied (pH, carbonates, silt and assimilable manganese) also permitted distinction of *T. melanosporum* soils from those of *T. aestivum* and of *T. brumale*.

The outcome is that analyses made of the surface soil alone are insufficient to judge the truffle-producing capacity of a soil. What is more, it is not useful to analyse all the elements. From the soil physical point of view, evaluation of stoniness and granulometric analysis of the fine soil is indispensable. From the chemical characteristics point of view, the key elements to analyse are pH (water), total limestone, exchangeable calcium, organic matter, and the C/N ratio, realising that the optimum values depend on the truffle species to be cultivated.

10.2.2.1 Soil Structure

The soil structure in *T. melanosporum* truffières is always granular. In central Italy, despite severe soil erosion, which prevents the regular development of horizons, natural truffières have similar morphological characteristics. The structure of the horizons where truffles develop is characterised predominantly by fine to medium granular and coagulated aggregates, rather than fine subangular polyhedric aggregates. The fine earth, even in the presence of abundant skeletal material (over 80 %), is never compact, but is rather well organised, supple and friable. As a result, the soil has good permeability and drainage. In addition, the soil does not develop surface cracks, even in periods of severe drought (Raglione et al. 1992).

The brûlé, which is formed by the truffle mycelium, causes a structural modification of the surface soil. It is well known that a heavy soil outside the brûlé can become light and ashy within the brûlé. The effect of calcium on soil structure is equally well known. This is not the same process as the mineral degradation by the truffle fungus that contributes to the loosening of the soil (Neel et al. 2007).

The soil structure of *T. aestivum* truffières in northeastern France consists of good to excellent aggregation, on account of the nearly constant presence of limestone and of the high organic matter content. The individual aggregates are angular and very distinctive. This excellent structure assures roots and their fungal associates' suitable aeration and compensates for clayey or clayey–silty characteristics of many of these soils. Good soil structure permits water circulation and prevents development of hydromorphic phenomena in the upper soil profile, despite a heavy texture. The constant presence of stones or blocks of limestone and fissures in the bedrock assures deep drainage.

In Italy, in the region of Parma, the structure is comparable with an aggregation moderately developed and fine. The particles are of medium size, polyhedral and angular. The macroporosity is good, comprising abundant very fine pores. Drainage is excellent, on account of the soil structure, which permits water and gas circulation that prevents hydromorphic phenomena developing in the upper profile.

10.2.2.2 Soil Texture

Truffles display a remarkable adaptability because they can fruit in a range of soil types, from sandy to stoney and with varying proportions of clay and silt. Among the most important conditions required by truffles are good aeration and water circulation. The nutrition of the fungus is assured by the mineral macro-elements and organic matter derived from the soil and roots.

The texture of soils producing *T. melanosporum* is extremely variable. Taking into consideration truffières covering nearly all of the French truffle regions, the granulometric analyses made by Delmas et al. (1981) provide the following results: 25–99 % fine soil; 7.2–45.8 % clay; 3.5–53.3 % fine silt; 2.6–36.2 % coarse silt; 4.3–63.2 % fine sand and 0.5–70 % coarse sand.

Sourzat (2008) advises against planting in soils of greater than 40 % clay. Nevertheless, he indicates that in Quercy, France, some very productive *T. melanosporum* truffières occur on soils containing up to 49 % clay, in a shallow stony rendosol. In fact, biological activity that brings about a change in the characteristics of the organic matter in the brûlé can compensate for high clay content. Likewise, significant stoniness can greatly compensate for elevated clay content (37–42 %) in certain truffle-producing soils of the southwest on hard Jurassic limestone. Truffles are capable of developing in stony environments, taking advantage of the little fine earth accumulated between the stones. In fact, the habitat is not really truly unfavorable except for its sensitivity to drought and the small proportion of fine earth with respect to the mass of constantly ventilated substrate (Sourzat 1989, 1995, 2002, 2011). On the other hand, sandy soils (up to 85 %) can support excellent truffières, contrary to the opinion that in situating truffières "marginal textures must be excluded". Silty soils can also produce truffles. The excess silt, in limestone terrain, can be rebalanced with sufficient organic matter content. The importance of stoniness needs to be taken into account in soil analysis, though this is rarely done for practical reasons. Analysis exclusively of the fine earth can lead to avoidance of truffle cultivation in soils with elevated levels of clay, while significant stoniness can compensate for this imperfection (Ricard 2003).

For *T. aestivum*, in France, the soil texture in natural truffières is also extremely variable, from silty–clay to clayey–silt, and more rarely silty, silty–sandy or clayey. Textures are therefore generally "heavy" (up to 52.8 % clay). These heavy textures permit the tree and the *Tuber* mycelium to obtain sufficient water. Very silty textures are rare, probably due to the poor structure induced by this granulation. Sandy textures are nearly absent due to the soil types, generally acidic, that one encounters with this texture, and the poor reserves of available water associated with this texture. In Hungary, more than 65 % of the habitats in the Carpathian Basin have a heavy clay texture, 20 % are clayey soils, while the remainder are either clay loam or loam. In Italy, in the region of Parma, the stoniness of the soil is light to moderate, with very small (2–6 mm) and small (6–20 mm) angular limestone gravel. The texture is variable, ranging from the balanced type ("terre franche") to the "franco-argileux" type. On the contrary, in Sweden, the soils are generally light, even tending to sandy with 10.4–32.6 % clay (averaging 19.3 %),

9.8–64.7 % silt (averaging 25.1 %) and 12.9–79.8 % sand (averaging 55.6 %). In the soil texture triangle, Italian soils are centrally situated between the more clayey French soils and the more sandy Swedish soils. These differences are explained by the diversity of bedrocks between southern Europe and Sweden, the Swedish soils having undergone the effects of the last Ice Age. The same is true for the soils of northern Europe–Denmark, northern Germany, Poland, the Baltic republics and Byelorussia. In comparison with *T. melanosporum*, *T. aestivum* is capable of fruiting in "heavy" soils.

10.2.3 Soil Chemical Characteristics

Soil physical analysis is important to clarify the functionality of the soil in terms of drainage, aeration and biological activity, but this alone is not sufficient to assess a soil for truffle production. In France, reference values for truffière production were defined in the 1970s by Delmas and Poitou at INRA's Mushroom Research Station in Bordeaux. Since then, these values are always taken into consideration by specialised laboratories when assessing the suitability of soil analyses. However, there is a need for the 1970s standards to be updated. The elements classically taken into account are pH (water), richness in total and active limestone (Drouineau Galet method), total organic matter, carbon (generally the method of Anne using sulfochromic oxidation), total nitrogen (Kjeldahl method), exchangeable calcium, potassium and magnesium (extraction by neutral ammonium acetate) and plant available phosphorus (Joret Hébert method). Sometimes extractable trace element concentrations are also taken into account.

10.2.3.1 Organic Matter

Delmas et al. (1981) highlighted the highly variable level of organic matter in French truffières that range from 0.8 % to 8.3 %. Sourzat (2008) also notes organic matter levels of between 4 % and 8 % in the southwest of France (maximum 9.7 %) and 1.5–4 % in the southeast occasionally with as low as 0.54 % in sandy soils. However, generally productive truffle soils contain a low organic matter content in the surface A horizons (Callot 1999). Truffles are therefore capable of fruiting in both soils with very low organic matter content as well as those with elevated levels.

The quality of organic matter in the surface layer of soil is also important. Before truffle production begins, the soil of the brûlé becomes clear of vegetation, and organic matter levels fall. Consequently, truffles are often harvested in soils with little organic matter, with mull humus C/N ratio of approximately 10. The quantity and the nature of the soil organic matter evolve over the course of time. The speed with which organic matter is transformed depends on microbial activity and the soil fauna, in relationship with the temperature, humidity and aeration of the

soil habitat. The action of the truffle mycelium, which breaks down the organic material, must not be forgotten (Chevalier 2010b). The brûlé contains less free fresh organic matter (of rapid evolution), than of stable, highly evolved, greatly polymerized structural organic matter. In central Italy, organic matter content varies from 1.13 % to 17.4 % (averaging 4.58 %). A level of 17.4 % is abnormally high. Italian truffle-producing soils are therefore, on average, higher in organic matter than those of southeastern France. Increases in organic matter content, for example, caused by the build-up of debris from grass and weeds can lead to the appearance of non-target ectomycorrhizal species.

In French soils producing *T. aestivum*, levels of organic matter content are generally between 4.4 % and 21.0 %, and occasionally may even go over 21 %. Limestone coats the non-transformed organic matter. This protective layer prevents further breakdown of the organic matter which then accumulates in the profile and gives the calcareous mull its characteristic black color. This high level of organic matter is in part responsible for the excellent structure of these soils. In Hungary, the organic matter is almost always high ranging between 2.76 % and 10.80 % and averaging 6.45% indicating a balanced supply of humus. In Italy, the soils of zones producing *T. aestivum* are characterised by quite high levels of organic matter, both in truffle-producing locations (11–14 %) and in neighbouring nonproducing locations (8–12 %). Nevertheless, organic matter levels can also be low, as in the province of Parma where it is only 1.6 %. In Swedish truffières, organic matter content ranges from 5.96 % to 21.22 %. In summary, soil organic matter levels are elevated in French, Italian and Swedish truffle soils and are higher in *T. aestivum* than in *T. melanosporum* soils.

10.2.3.2 Carbon

In *T. melanosporum* truffières, levels of carbon range from 0.47 % to 5.0 % in France and from 0.55 % to 3.25 % (averaging 1.55 %) in Italy. In *T. aestivum* truffières, carbon levels range from 2.6 % to 12.4 % (averaging 5.6 %) in France and from 3.47 % to 12.33 % in Sweden. Thus, *T. aestivum* appears to be found in soils with higher soil C levels than *T. melanosporum*.

10.2.3.3 Total Nitrogen

In *T. melanosporum* truffières, levels of total nitrogen vary from 0.46 ‰[1] to 5.22 ‰ (averaging 2.37 ‰) in France, compared to 0.80 ‰ to 2.78 ‰ (averaging 1.5 ‰) in Italy (Bencivenga et al. 1990; Bragato et al. 1992, 2001; Lulli et al. 1991, 1992; Lulli and Primavera 1995). In *T. aestivum* truffières in northeastern France, total nitrogen levels range from 2.6 ‰ to 7.6 ‰ and in the region of Parma from 1.1 ‰

[1] % refers to parts per hundred (percent), whereas ‰ refers to parts per thousand.

to 6.1 ‰ (averaging 4.2 ‰). Thus, *T. aestivum* also appears to occur in soils with higher total nitrogen concentrations than *T. melanosporum*.

10.2.3.4 Carbon to Nitrogen Ratio

The C/N ratio provides information on the evolution of the organic matter. In France, the C/N ratio in truffières varies from 8.57 to 13.7. Sourzat (2008) points out the very low C/N ratios (e.g. 5.9) in some soils with very little organic matter but rich in limestone. In such situations, the low level of organic matter is a function of the high level of limestone which stimulates soil microbial activity and a breakdown of the organic matter. Low C/N ratios often correspond to very sandy soils; however, sandy soils can also have higher C/N ratios in areas where ligneous organic matter accumulates. High C/N ratios can also be found in natural truffières located in very stony soils with 48 % to 56 % total limestone where a highly calcareous fine black earth develops between the stones.

In *T. aestivum* truffières of northeastern France, the C/N ratio is generally between 8.9 and 12, indicative of an excellent capacity for mineralization of organic matter and a good availability of mineral nitrogen, principally in nitric form. Nevertheless, C/N can reach much higher levels (up to 20.4), well above the upper limit for *T. melanosporum*. In the region of Parma, C/N varies from 8.2 to 16.9 (averaging 10.8). Swedish soils are similar to those in France with C/N levels as high as 18.2 although the minimum is 9.7 and the average 13.

10.2.3.5 pH

French soils producing *T. melanosporum* are generally alkaline, with pH ranging between 7.7 and 8.35. However, exceptions exist with a pH 7.0 in a truffière in southwestern France on a sandy soil with 0 % total limestone but with 3.8 ‰ exchangeable calcium (CaO) (Sourzat 2008) points to. In Italy, productive truffières are also found on soils with pH as low as 7.05 although normally the pH is up to 8.25 with an average of 8.00 so that in central Italy pH is the most uniform chemical parameter.

For *T. aestivum* in France, due to the constant presence of limestone from the surface down, the pH is always above 7 and ranges between 7.1 and 8 in the A1 horizon as a function of the amount and nature of organic matter. In central Italy, owing to the limestone presence, the pH is likewise elevated (despite the high percentage of organic matter) giving values near neutrality or slightly alkaline. In the region of Parma, values range from 7.1 to 8.1 (averaging 7.5). In Hungary, the pH is generally weakly alkaline or neutral, or possibly mildly acidic (6.7–7.94; averaging 7.17). In Sweden, the pH is quite variable. It can reach as high as 7.9 or as low as 6.8 (averaging 7.5).

10.2.3.6 Total Limestone

In French *T. melanosporum* truffières, the level of total limestone can be highly variable and go from just a trace to as high as 74 % in some soils. Similarly, in central Italy, the level of total limestone can vary from just a trace to 83.9 % averaging 29.6 %. Although the majority of Italian truffle soils are calcareous, some are simply calcic.

Detailed studies of productive and unproductive sites led by Callot (1999) showed that the most productive soils generally have surface A horizons with, in addition to low organic matter content, a clear recarbonation and a strong macro porosity caused by the soil fauna (Callot 1999). Deeper, these soils exhibit a level of unconsolidated calcium carbonate accumulation (K), a horizon of strong faunal macroporosity (Sbio) and a well-drained calcareous subsoil (C) with water storage potential.

Using calcareous gravel on paths and roads can help render adjacent acidic soils suited to truffle production (Callot 1999; Sourzat 2008). Similarly, the incorporation of large amounts of limestone into acidic to very acidic soils has been practiced in the USA (Garland 2001), Australia (Malajczuk and Amaranthus 2008) and New Zealand (Hall et al., 2007). Similar techniques have also been used in France to produce *T. aestivum* and more recently *T. melanosporum* in Limousin (central France) on soils derived from metamorphic bedrock (schist) with initial pH 5.2 [Chevalier 2003; Chevalier G, Urban A, Pla T (2009) Truffières à *Tuber melanosporum* atypiques sur granites alcalins dans le Vallespir, Pyrénées-orientales (unpublished)].

Generally *T. aestivum* soils are less rich in limestone than those of *T. melanosporum*. In France, the majority of natural *T. aestivum* truffières are characterised by the presence of limestone from the surface down. The level of limestone is highly variable and ranges from 0.4 % to 52 %. The amount of total limestone has no effect on the quality of truffière soils. Only its presence at the surface is important. In Italy, the level of calcium carbonate in *T. aestivum* soils is just as variable, although generally it is relatively low and between 0.9 % and 12 %. However, in the region of Parma, it can be as high as 52.9 % with an average of 21.9 %. In Hungary most of the soils contain little (5–8 %) or only traces of lime (0.1–5 %), while in Sweden, total limestone can be from just a trace to 10.5 %.

10.2.3.7 Exchangeable Calcium

In France, reference values for exchangeable calcium are generally from 4 % to 16 ‰. But in the French eastern Pyrenees, values of 2.31 ‰ are recommended on recarbonated schist and 2.13 ‰ on alkaline granite.

In the calcareous truffières of central Spain, truffle development and ecological conditions of brûlés favor the formation of significant quantities of active limestone and exchangeable calcium (Garcia-Montero et al. 2007a, b; Chap. 6).

The quantity of active limestone is significantly higher and that of total limestone is lower within the brûlés than outside of the brûlés. But then the activity of *T. melanosporum*, its fructification and the size of the brûlés are simultaneously advantaged by high concentrations of active limestone and exchangeable calcium. This results in a feedback process, which in return favors development of truffle mycelium. This feedback explains why the development of *T. melanosporum* and the production of truffles increase with the size of the brûlé, which is itself a consequence of a successful symbiosis. This feedback loop also provides the truffle with an advantage over its competitors, especially *T. aestivum* and *T. mesentericum*, when the active limestone reaches a high concentration in *T. melanosporum* brûlés. Garcia-Montero (2007a, b; Chap. 6) deduced that the active limestone is an essential factor for fruiting, development of brûlés and the aggressiveness of the black truffle when in competition with other truffle species. These observations are also valid for central Spain, where the soils are very calcareous. However, the occurrence of truffières on crystalline bedrock in France requires a rethink of the roles of total limestone, active limestone and exchangeable calcium in truffle production.

In wild *T. aestivum* truffières of northeastern France, the level of exchangeable calcium ranges from 2.8 ‰ to 7.9 ‰. The absorbent complex is always saturated, mainly with exchangeable calcium, because limestone is always present (Chevalier G, Urban A, Pla T (2009) Truffières à *Tuber melanosporum* atypiques sur granites alcalins dans le Vallespir, Pyrénées-orientales (unpublished)). In Sweden, levels of exchangeable calcium are on average slightly higher at 3.6–10.7 ‰ (averaging 6.7 ‰). It is surprising that the minimum of 2.8 ‰ is a little higher than the value of 2.13 ‰ recently determined for *T. melanosporum* on alkaline granite.

10.2.3.8 Exchangeable Magnesium

In French *T. melanosporum* truffières, exchangeable magnesium levels are from 0.05 ‰ to 0.52 ‰, and although normal levels of exchangeable MgO considered normal is from 0.10 ‰ to 0.30 ‰, truffières on sandy soil can have much lower or higher values (as low as 0.08 ‰ or as high as 0.75 ‰) (Sourzat 2008). Sourzat has concluded that lower or higher than normal levels of magnesium do not seem to affect truffière production. In natural *T. aestivum* truffières of northeastern France, levels of exchangeable magnesium are slightly elevated but sufficient (0.05–0.41 ‰). Italian values from 0.12 ‰ to 0.59 ‰ are close to those in France. In the region of Parma, values vary from 0.07 ‰ to 0.30 ‰. In Sweden, the values are generally good, similar to those in France: 0.09–0.45 ‰ (averaging 0.19 ‰).

10.2.3.9 Exchangeable Potassium

In French *T. melanosporum* truffières, exchangeable potassium content varies from 0.04 ‰ to 0.50 ‰. Levels from 0.1 ‰ to 0.3 ‰ are considered normal. Truffle soils are not deficient in this element. In Italy, values are higher: 0.1–1.17 ‰.

In the wild *T. aestivum* truffières of northeastern France, the levels of exchangeable potassium are medium: 0.25 ‰ to 1.04 ‰ (averaging 0.59 ‰). In Italy (in the region of Parma), they range from 0.089 ‰ to 0.520 ‰ (averaging 0.306). In Hungary the majority of the studied soils have high potassium content (0.139–1.200 ‰; averaging 0.498 ‰).

10.2.3.10 Available Phosphorus

For *T. melanosporum* in France, levels of available phosphorus run from 0.006 ‰ to 0.980 ‰ with upper figure being an abnormal exception. In Italy, levels are between 0.006 ‰ and 0.025 ‰.

In soils producing *T. aestivum*, as is the case with most calcareous soils, phosphorus content is low, between 0.02 ‰ and 0.08 ‰. In Italy (in the region of Parma), values range from 0.001 ‰ to 0.018 ‰ (averaging 0.008 ‰). In Hungary, the phosphate content of the tested soils exhibits great variability, with a very high content in a third of the studied soils (0.013–2.534 ‰; averaging 0.423 ‰). The values in Sweden (0.02–0.12 ‰) are consistent with the French values. Low levels of active phosphorus in soils do not seem to affect truffle production.

10.3 Climate

The climate is also a decisive factor for truffle production that needs to be evaluated according the distribution of its rainfall and temperature variations during the truffle's growth cycle. *T. melanosporum's* climatic requirements can be summarised by relatively damp and warm springs with no late frosts, hot summers punctuated with rainstorms, relatively damp autumns without early frosts and winters without heavy frosts (which might destroy the truffles) and moderate rainfall. The elevation of southwest France's truffle grounds is generally between 100 and 400 m. In the southeast, the Mediterranean climate allows the establishment of truffle grounds above this elevation. In the Hautes-Alpes, natural truffle grounds are present up to 1,500 m on well-oriented sites and at the lower limit of the larch tree-line, while on the Larzac plateau, in the Aveyron, some exist at 800 m on sites with a sunny aspect.

In very favorable climatic conditions, the range of soils that support truffle production is wide and, for example, includes soils with trace levels of limestone or with organic matter imbalances. However, when climatic conditions are marginal, only exceptionally favorable soils permit mycorrhization and fructification.

10.4 Cultivating *Tuber melanosporum* in Europe

Over the past 40 years, considerable progress has been made in the cultivation of truffles with millions of truffle-mycorrhized trees planted in France, Italy and Spain.

10.4.1 Modern Approaches for Truffle Cultivation

10.4.1.1 Three Methods Used in France in the Recent Past

When we speak of truffle cultivation in France, we mean principally growing the Périgord black truffle, *T. melanosporum*. The method was first devised at the start of the nineteenth century when observant country dwellers in the southwest and southeast decided to sow acorns or plant oaks with this in mind. The cultivation of Burgundy truffle, *T. aestivum* f. *uncinatum*, has recently taken off in the northeast of the country, since the eighties. Among other species which are cultivated more or less privately, there is the white summer truffle, *T. aestivum* (*T. aestivum* f. *aestivum*), the winter truffle (*Tuber brumale* Vittad.) and the Bagnoli truffle (*Tuber mesentericum* Vittad.). Numerous truffle species are also found in truffle-producing areas and can cause problems. The species the most feared in truffières are *T. brumale* and *T. aestivum* (*T. aestivum* f. *aestivum*)—even if some growers tend to put them forwards as worthwhile species.

Three techniques for cultivating *Tuber melanosporum* in Europe can be distinguished, each with their variations, which have different results depending on the environment and changing times. Truffle growers do not all agree on the best method to use; and additionally each one introduces his own nuances to his preferred approach according to his soil, the climate and, above all, whether or not there is an existing family tradition of truffle cultivation.

The main methods are:

1. *Traditional method*: practised before the introduction of mycorrhized plants—whose followers are becoming fewer (Fig. 10.1d)
2. *Arboriculture* (known as the "*Pallier*" *method*): conceived from simple modern principles since the marketing of mycorrhized plants started in 1974 (Fig. 10.1e)
3. *Chalk grassland* or "*Tanguy*" *method*: from the 1990s onwards, based on the functioning of natural truffle grounds (Fig. 10.1f)

Other methods of truffle cultivation have been proposed (Rebière 1974; Fioc 1987; Ricard 2003); however, here we focus on the three main methods of truffle cultivation stated above.

10.4.1.2 Choosing a Modern Truffle Cultivation Method

The results obtained in France and Europe—and above all in countries where there are no species of truffle naturally present in the environment—lead us to consider the justification for using each method, in particular those of truffle arboriculture and chalk grassland. In Australia, where *T. brumale* as well as all other species of *Tuber* other than *melanosporum* are absent, the common hazelnut seems to maintain a perpetual liaison with *T. melanosporum*, for example, in Manjimup, Western Australia. In France, the same hazelnut tree has a strong affinity with *T. brumale*,

which contaminates it in numerous regions despite a high level of mycorrhization of plants checked by the INRA and the CTIFL (Centre Technique Interprofessionnel des Fruits et Légumes—Inter-professional Technical Centre for Fruits and Vegetables). On the other hand, the Downy oak (*Quercus pubescens* Willd) and Green oak (*Quercus ilex* L.) are species perfectly adapted to *T. melanosporum* as there is strong affinity between these species. This relationship is confirmed in Australia and New Zealand.

The parallel between the "Tanguy" method of truffle cultivation in chalk grassland and the workings of natural truffle grounds in southern France is easy to demonstrate. Natural truffle grounds develop in open spaces or clearings, especially in environments resulting from the neglect of cultivated land, when these spaces become wasteland reach 10–15 years of age. Such natural truffle grounds correspond to a particular state or type of vegetation (Mesobromium, Xerobromium, etc.) during the evolution of the wasteland towards its final form (woodland) in calcareous zones. They display the characteristics of biodiversity that we attempt to develop in the most efficient grassy plantations.

Truffle arboriculture appears to give noteworthy results in the absence of pressure due to contamination; whereas truffle cultivation in chalk grassland allows us to limit such contamination. Everything seems to show that in France, the choice of a method should be governed by the principle of precaution. It is a question of choosing the route which is likely to present the fewest risks, even if one can assume that these risks are very low in certain open or lightly wooded landscapes.

Precautionary technical procedures, faced with contamination by mycorrhizal fungi present in the environment, include three stages:

Stage 1: Assure the best possible re-establishment of the plant mycorrhized with *T. melanosporum* during the first year following planting, and even the second

Stage 2: Discourage the growth of the mycorrhized plant, in order to avoid contamination by various fungi: creation of the natural environment preferred by the truffle is sought after during the formation of burnt areas

Stage 3: Improve the quantity and quality of the production while maintaining its durability, once truffle fructification has been triggered

It is possible to foresee two further stages:

Stage 4: Renovate the old plantation so as to give a second life to the production by opening up the environment

Stage 5: Rip up the old truffle wood to provide a fresh base to the truffle plantation

The method of cultivation in three stages was defined in the "Guide Pratique de Trufficulture" (Sourzat 1989–1995–2002, 2011). In this 3rd edition (2002) of the "Guide Pratique de Trufficulture", the three steps were summarised by "Planter" (Planting for the 1st step), "Entretenir" (Maintaining for the 2nd step) and "Produire" (Producing for the 3rd step). This truffle cultivation method was perfected for European countries where there are climate and soil conditions favorable for truffle growth but where there are often risks of other EM fungi contaminations. However, this method can also be adapted to other conditions.

The new methods J.A.AD J (Jeune = young in French), A (Adolescent = immature in French), AD (Adulte = adult in French) (Dessolas et al. 2007–2008, Pargney et al. 2011) and M.R.T. (the reasoned methods of trufficulture) (Chevalier 2010b) comprise also three equivalent steps (Fig. 10.1h, i). All these methods are described below.

10.4.2 Establishing a Truffle Plantation (First Step)

The objective is a perfect planting of mycorrhized trees by the end of the second year of vegetation so that the trees are then able to withstand the demands of the truffle ecosystem and of the natural conditions preferred by the truffle.

10.4.2.1 Terrain and Soil

In order to restrict the damage caused by a prolonged dry period, the chosen site should ideally have easy accessibility and preferably proximity to an existing (or created) water source to enable better water management while the trees are getting established and during the production period. The site should not be on too steep a slope or without services or access for modern machinery. Cold aspects (north, northeast, northwest) in an enclosed valley are not suitable because of the possibility of immature truffles freezing in November and December. Whenever possible, the rows of trees should be aligned north–south in order to allow the sun to warm up the soil in the afternoons during the cold winter period.

Soil must be limestone with a water pH of around 8 and have physico-chemical characteristics suited to truffle production. These characteristics should be ascertained from the start by means of a complete soil analysis, if there are no other indications as to the potential of local truffle production. Lime can be added if the content of this element is low. When soil has water pH neutral or acid, it is necessary to add lime in order to increase pH at a good level (at less 7.8), which is the case in many new countries that are cultivating black truffles, including Australia, New Zealand, USA, Chile and Argentina. Doses can be varied from 10 to 100 tonnes per ha. Soil analysis should be carried out from the start with a sample of at least 500–800 g for a full analysis. Several samples of soil should be taken from different locations at depths of between 5 and 10 cm. These extractions should be mixed up together in a bucket in order to get a representative sample. If the soil on the site is homogenous, a single sample can be sent to the laboratory for analysis. Subsoil must be permeable and allow run off of excess water. If the drainage characteristics of the subsoil are unsure, a trench can be dug with a mechanical digger and analysed by a skilled technician. When there is superficial topsoil on broken bedrock, it is not recommended to make a passage of a ripper. The use of it can transform the land into a field of big stones. In this case, a stone crusher will have to be used as far as possible before launching into tree planting.

The plantation should avoid sites of recent oak deforestation without at least 2 or 3 years of wheat or barley cultivation in between, during which time all the roots of the previous trees are removed. The purpose of carrying out this work is to limit the problem of contamination from other mycorrhizal fungi species present on the roots of the felled oaks. If an oak hedge or woodland is situated around the edge of the planting site, a gap of at least 8 m should be left between their border and the first planting. Big trees, which are already mycorrhized by other kind of fungi (especially basidiomycetes), can contaminate the young plants infected with *T. melanosporum*. Creating a trench between the bordering trees and the first row of newly planted oaks will protect the small ones from contamination of the big trees via belowground roots. Size of the field can be taken in consideration in wooded environment with oaks. Big fields (at least 1 ha) are preferred over smaller fields because they offer a larger buffer against contaminating fungi.

10.4.2.2 Preparation of the Terrain

If planting is to take place on recently cultivated land, the soil should be worked in the autumn then prepared with the aid of a plough or *vibroculteur* in the same way as for planting a fruit orchard. Once the planted area is again covered with grass or grazing, only the plant rows or even just the immediate area surrounding each tree, should be carefully worked by hand (with a fork or hoe), motorised plough or weed cutter. Working within a defined space helps to preserve the ecosystem of the limestone orchard, which is a very favorable environment for the formation of natural truffières and truffle plantations. The size of the hole to be dug should be in the order of 20–30 cm in depth and 50–80 cm in diameter. Its size depends on the suitability of the topsoil (fine soil). If the soil is loose, a hole 50–60 cm in diameter is sufficient; if it is very stony and shallow, it can be done with a mini digger and can be 60 cm–1 m in diameter. The big stones will have to be removed and the topsoil replaced around the plant. The best time to dig the hole for planting is before winter, in order to benefit from the effect of the frost on the soil, especially if plantation is done on the spring. Cut the grass very short with a weed cutter before digging the hole when the plantation is in grass, fallow land, sheep pasture or juniper. It is not necessary to remove all small shrubs (juniper, wild roses, sloe, brambles). If there are not too many, it is enough to reduce their size by appropriate pruning. Do not leave a large oak tree in the middle of the plantation in the hope that it will become truffle producing. To keep one beautiful oak tree, it is necessary to do a sanitary perimeter (8–10 m) around it in order to prevent contamination.

10.4.2.3 Tree Spacing

The trees should be planted in straight lines 4–6 m apart; the rows should be 6 or 8 m apart (400–200 plants per hectare). Spacing of 4 m on the row allows for more easy irrigation. The spacing can be decided upon according to the nature of the soil

(deep topsoil: wide spacing) or according to availability of irrigation (trees with rapid growth: wider spacing more important). For example, in the case of deep topsoil with irrigation, one could plant at 6 m × 10 m (166 plants per hectare). Dense planting demands important interventions which take time and a commitment to pruning, trimming and thinning the trees. The goal of these interventions is to save *space of conquest* (available space) between the brûlés. Irrigation is necessary because the dryness of the soil is increased as the tree grows.

10.4.2.4 Recommended Tree Species

Young plants should be infected by the black truffle, *T. melanosporum* to the exclusion of all other contaminating species. These infected plants should be able to be monitored beforehand (batch sampling) by official organisations (CTIFL or INRA in France). Downy oaks (*Q. pubescens*) and holm oaks (*Q. ilex*) can be planted in the southwest of France. In the southeast, one can plant pedunculate oaks (*Quercus robur* L.) or kermes oaks (*Quercus coccifera* L.) which are adapted to dry and stony terrain. *Q. pubescens* takes longer to fruit (8–10 years) than *Q. ilex* (5–7 years) but production is generally more sustained. Hazelnut trees (*Corylus avellana* L.) can be planted by way of experiment (not more than 10 % in the plantation) in order to observe the infection potential of the planting area (presence of *T. brumale* spores). The use of *C. avellana* is always a delicate thing, although interesting results can often be observed in some parts of France and in the countries where it is not native. Root system of this species grows faster than those of *Q. pubescens* and *Q. ilex*, and may be contaminated easily. The different species can be positioned in separate rows or intermixed within the row.

10.4.2.5 Staking

In many cases trees need to be staked when planted or to help support their growth. Choose a staking method suited to protection of the plant (netting or shrub shelter). For maximum sun between rows in winter, staking along the north–south axis is preferable. Avoid staking in the direction of the slope in the case of erosion prone land.

10.4.2.6 Planting

Planting should take place in winter. *Q. ilex* is preferably planted at the end of the winter so as not to run the risk of frost damage to the young trees coming out of incubation (greenhouse). Plant in a well-drained soil, not too dry and not too sticky. Remove the plants from their packaging as soon as they are received. Place them upright in netting or plastic buckets. Take care to store the plants where they will not be liable to freezing, drying out or blanching (from lack of light).

Ensure that the soil at the bottom of the container or root ball is not too dry. If the surrounding soil is dry and powdery, immerse it in a bucket of water for one to two minutes until the bubbles stop rising to the surface (soaking the plant). After digging out the hole, open or unclip the container and delicately position the root ball in the hole and cover it with fine soil. Avoid large stones or soil containing grass or large roots coming into contact with the root ball. Do not allow the root ball to touch the wooden stake (leave 5 cm between the root ball and the stake). Fill in the hole and pack down the soil around the plant with the toe of shoe or boot. Put two or three centimetres of soil above ground level. Protect the tree with wire netting or possibly a shrub-shelter (Tubex). The use of a 60 cm high shrub-shelter (Tubex) allows mounding of earth around it which is preferable to mulching. The soil forming a mound up against the tube of the shrub-shelter increases the water retention of the soil and aids maintenance. What is more, the mound of soil, which will naturally dry out towards the top, encourages the development of the *brûlé*. When the planting is carried out with soil mounding in limestone pasture, the mound must be at least 15–20 cm high to be truly effective against drying out. It is essential that the young plants are watered during the week after planting in order to limit the stress of transplanting.

10.4.2.7 Maintenance of the Young Plantation

It is important that the saplings are protected against rabbits and roe deer by means of wire netting (or shrub-shelter). These animals eat leaves of the truffle trees and delay truffle fruiting. Where wild boars might be present in the area, an electric fence should be installed and checked at least once a fortnight. A chemical weed killer used twice a year under the fenceline increases its effectiveness. Competing weeds that appear during the summer months should be removed. Where there is little grass growth (shallow soil), removal of grass by weedeater is preferable to the use of glyphosate. Chemical foliar weedkillers (glyphosate, gluphosinate) should be used as a last resort and with great caution. Caterpillar of the butterfly bombyx *Lymantria dispar* L. damages the foliage. If need be, treat with an insecticide which does not kill bees. With powdery mildew, which provokes white spots on leaves, use liquid sulphur.

10.4.2.8 Irrigation

In the first 2 years, be sure that the plants do not suffer from water shortage. Watering can be done with containers of water or from a big tank or can be automated with drippers attached to a polyethylene pipe. Watering should commence around mid-May. The most important watering months for oak tree growth are May and June. Watering every 8–10 days is recommended during the hot months of June, July and August. Trees planted using the mounded earth method can be watered directly into the shrub-shelter (Tubex) every 10–12 days.

10.4.3 Maintenance Between Planting and Fruiting (Second Step)

The object is to provide *T. melanosporum* with the optimum conditions for production, particularly once the natural truffle has begun to form. In traditional truffle areas in an oak wooded environment, it appears that the cultivation conditions, similar to those necessary for fruit tree cultivation, present the risk of a drift in the status of the mycorrhization, and of fruiting, towards *T. brumale*. Authors of some of the first manuals on truffle cultivation in France (De Bosredon 1887; Pradel 1914) recommended to "*leave it to nature once the brûlés begin to form*".

10.4.3.1 Soil Maintenance

There are two possible options: to till the soil with manual or mechanic tools or control the growth of the native flora which form the basis of the truffle ecosystem. Both options are recommended even if the second solution seems preferable in the long run, considering the results of scientific experiments and observing the evolution of the environment once the formation of the natural or wild "truffière" has started. The first option can be used without inconvenience when there are no risks of fungi contaminations.

Work the soil as little as possible so as not to encourage the propagation of the competing fungi (*T. brumale* and *T. aestivum*), that is, a very superficial working of the soil (5 cm) once or twice a year with machinery, moving further away from the row of trees as they grow so as not to disturb the progression of the brûlé (Fig. 10.1g). The presence of some hardy plants (brambles, grass, etc.) can be favorable to the truffle in the same way as vine stock or lavender stems. It is not necessary to suppress everything. Clear between the planting rows at less once or twice a year and clear around the trees within the row with a weedeater as often as necessary to restrict the growth of the grass. Do not use chemical weedkillers other than on vigorous plants which are difficult to control. The presence of some competing young plants (to the growth of the tree) is not prejudicial to the truffle if the tree's growth is already well established. A balance has to be found between grass growth and tree growth. The growth of the grass should not compromise the growth of the tree and slow down truffle production if the tree lacks vigour. The ideal would be to work around each tree by hand with a hoe, a practice that may seem ill suited to modern agricultural concepts. Manual working of the soil around the surface of the brûlé will not upset the equilibrium between *T. melanosporum* and its companion fungi. In deep soil, mechanical tilling is less dangerous than in shallow one as there can appear fungi equilibrium under the tilled layer.

10.4.3.2 Irrigation

In wooded oak environments, do not irrigate unless there has been no rain for 3–4 weeks. It is important to control the growth of the trees as their lateral roots are susceptible to contamination. If the trees have taken well in the first year or the

first 2 years, the truffle trees will easily tolerate a long period without water (one month and plus). In open areas without oak trees frequent irrigation can be done: trees grow faster and can produce earlier with the risk of a shorter duration of truffle production.

10.4.3.3 Pruning

Prune lightly to achieve a spread out shape or stake upright if the tree has a creeping or rambling tendency. Do not cut a whole stem from the base of the tree. Go rather for gradual pinching, that is, cutting one stem or branch on several different occasions. A tree gains nourishment from the leaves as much as from the roots.

10.4.3.4 Protection Methods

Look out for powdery mildew on *Q. pubescens* and treat with liquid sulphur. Treat for leaf-eating caterpillars (*L. dispar*) in May and June with ecological or chemical insecticide and look out for moths (*Acrocercops brongniardella* Fabricius) on the *Q. ilex*. The larvae of *A. brongniardella* unstick the cuticle of leaves and protect the planted area against wild boar with electric fencing.

Remove the shrub-shelters (or Tubex) once the tree is more than 50 or 60 cm high. Install individual tree protection against deer instead of the shrub-shelters, or rabbit proof fencing in addition. To protect against deer, cut 1.5 m of wire netting (Ursus) 95 cm in height to place around the tree (cost of this protection around 1 €). This protection is held in place in the soil with stones or with the aid of one or two metal spikes made from concrete reinforcing iron rods.

10.4.3.5 Fertilisation and Feeding

Once soil analysis has been performed, correct any possible deficiencies. An improvement of crushed lime is advised in soils with low levels of this element (1–2 %). A simple soil analysis (water pH and limestone level) can indicate what is needed.

10.4.4 Improving and Maintaining Truffle Production (Third Step)

Once production has started, the aim is to ensure optimum production for as long as possible.

10.4.4.1 Soil Maintenance

In working the soil, the aim is to obtain larger truffles in soil that is aerated, vital and equipped with good water reserves. With mowing or slashing of the grass, a part of the water reserve of the soil is saved. The soil must be turned at the point when the vegetation of the tree and the growth of the mycelium of *T. melanosporum* begin in the soil with the spring warmth. It used to be done by hand with the aid of a one or multi-pronged implement. It is generally done up to the end of harvesting up to mid-May when the soil is fairly dry, to a depth of 5–8 cm. When the work is done by tractor, the raking implements used are the plough or the *vibroculteur* (sometimes *Actisol* or rotary hoe); some people prefer the disc harrow (*cover crop*) which does not pull out or break the roots. Manual working with a "bigos" (a 3- or 4-pronged hand tool) is preferable on very good producing trees taking care not to cut the roots. If it is carried out without turning the soil, simply lifting it to increase the aeration, the work can be done much later, up to the end of June. When the work is done manually or lightly by machinery, it should be restricted to the area covered by the brûlé.

10.4.4.2 Irrigation

Watering should be carried out according to the life cycle of the truffle given that the most crucial period is in August and that the truffle can withstand a certain amount of dryness (20–25 days on average without climatic heat wave). The month of June is generally the time when the truffle primordia are formed. For good management of the truffle's water requirements, rain gauges must be used and rainfalls recorded.

Agricultural irrigation systems are recommended. Use of micro-sprinklers at a low rate of flow (40–80 l per hour) and low pressure (1–2 bars) is a practical and economical solution. It requires good filtration at the water source. It is pointless watering non-productive brûlés. With a tank of water, it is possible to irrigate the best brûlés in the truffle plantation limiting the supply to 20 l per square metre, or 20 mm. In the interests of water economy, irrigate only the parts of the brûlé which are most likely to yield truffles. In July, watering can take place every 15–20 days (20–25 mm); in August, every 10–12 days (20–25 mm) and in September, every 15–20 days (20–25 mm). Covering the ground with branches prolongs the time the truffle can withstand the dry period and counteracts a weak or non-existent water supply. The soil type (sandy or clay) and the climactic conditions will determine the moisture content of the soil and the method of irrigation

10.4.4.3 Pruning and Thinning

Pruning of the truffle trees is carried out differently according to the regional conditions. A new approach has been identified in France by the Station Trufficole du Montat. It consists of maintaining *T. melanosporum*-infected tree in a state in

which the truffle is stronger than the tree. In this approach, the trees that are not producing truffles are removed to allow more room for the ones that are productive. Pruning should be done gradually on the producing trees. In order to minimise the possibility of failure, it is best to prune the trees in a plantation in stages: a third in the first year and so on each year. Pruning is usually done in spring during the months of March or April. Pruning in August can help to reduce vigorous tree growth.

As truffle trees mature, orchards may need to be thinned. The aim is to keep a *space of conquest* for the brûlé which in principle grows at the rate of 10–25 cm a year in the case of a tree with good *T. melanosporum* production. Where the *space of conquest* is inclined to diminish in size, some trees should be cut down. Such thinning encourages new growth and preserves the environmental conditions favorable to *T. melanosporum*.

10.4.4.4 Complementary Inoculation

Some new experiences in France and Spain confirm old practices for improving production. They consist of bringing truffles under trees that are not yet producing. Truffles harvested at the beginning of the season are preserved in a deep freezer. In the spring, they are crushed and mixed up with vermiculite to create the inoculum. This inoculum is added in a small trench under each tree at the limit of the canopy. Doses of truffle can be between 10 and 30 g per tree. Nevertheless, with smaller dose, good results were observed. Benefits on truffle production were obtained on trees with brûlés where the fructification of the fungus has not already triggered.

10.4.4.5 Fertilisation and Additives

The mode of feeding the developing truffle fruiting body is not well understood. Once the soil is tested, adjust any possible deficiencies. Organic mineral fertilisation to improve truffle production can be experimented. Only treat one part of the brûlé, about a quarter of the whole area at most, so that a negative outcome will not affect the whole. Improving the biological activity of the soil (earthworms and insects) by adding small branches/twigs is good for truffle nutrition. The addition of topsoil on the surface of the productive brûlés can be beneficial.

10.4.4.6 Protection

Treat leaf-eating caterpillars (*L. dispar*) if there is a significant presence. Use insecticide appropriate to the extent of the infestation. Protect the site against wild boar with an electric fence. Put reinforced wire netting (a 2.4 × 3.6 m sheet with 20 cm squares) on the best brûlés when, despite electric fencing, wild boars are likely to come and root around, attracted by the irrigation during a dry summer.

10.4.4.7 Harvesting and Grading

Harvesting takes place from the end of November up to mid-March and is accomplished with the aid of a trained dog or pig. The truffle fly can be a useful indicator of truffles in Europe. Harvesting should be done in drained soils, which are neither frozen nor too wet.

Harvested truffles are sorted and the surrounding earth is removed. The truffles should be wrapped in a piece of cotton cloth and kept in a cool place until market day. They can be stored in the fridge, taking care to put them in an airtight container. In south of France, in winter, only high quality *T. melanosporum* is sold at the market. Black truffles that are damaged or rotten should be broken up and buried in the soil under the young trees, which are not yet producing or used as inoculum as described.

10.4.5 Improved Techniques for Truffle Cultivation: Deep Cultivation and Severe Limb Pruning with J.A.AD. and M.R.T. Methods

Many authors recognise the interest in cultivating the soil, to decompact it and to aerate it in order to obtain larger truffles in a living environment provided with an ample reserve of water. Although earlier techniques recommend shallow cultivation, so as not to damage the root system, recent methods of truffle cultivation recommend much deeper cultivation (15–20 cm) of the soil. These include the J.A.AD. (Dessolas et al. 2007–2008; Pargney et al. 2011) and M.R.T. (Chevalier 2010b), which have been described in detail in recently published works. These methods give a special attention to regenerating root systems and take into account particularly the biology of the truffle and its host, especially the different phases of the truffle cycle. Compared with earlier methods, the originality of the new methods rests on several points: different management of the zones in production (soil cultivation) and the inter-rows (revegetation), deep cultivation of the soil from the outset and severe pruning from the outset (Fig. 10.1h, i).

Deep cultivation leads to the deep formation of truffles, of better quality because they are less exposed to drought, to freezing and to parasites. What is more, evaporation from the soil is reduced. Finally, whether it is done manually or with adapted tools, it results in some (but not excessive) root pruning, in a manner that promotes root regeneration in order to feed the truffle mycelium.

Judicious cultivation of the soil, through limiting evaporation and pruning of roots, and a severe limb pruning regimen, by reducing transpiration of the tree during the summer, can contribute to an improved economy of water in truffle orchards (Chevalier and Wehrlen 2008). These practices, coupled with an increased depth of soil achieved by building raised beds (J.A.AD.), in some cases, have permitted the harvest of truffles without irrigation in 2003, a year characterised by a heat wave and extreme drought. Well-adapted cultivation methods can permit the harvest of some truffles 3–4 years after planting (Chevalier 1983).

10.5 Conclusion

A greater familiarity with the physico-chemical characteristics of soils favorable to development of *T. melanosporum* and *T. aestivum* has permitted improved techniques for working the soil that better address the needs of the host plant and the truffle fungus. It should be emphasised that the soil requirements of *T. aestivum* are less strict than those of *T. melanosporum*, but *T. aestivum* is also more demanding of water.

Before planting, unfavorable factors can be corrected using appropriate cultural practices. This may include planting on raised beds to increase the thickness of useful soil, incorporating coarse material to lighten the soil, mechanical fracturing of the subsoil, amendments of limestone and calcium, eliminating weeds and ectomycorrhizal hosts and establishing irrigation. Such physical and chemical amelioration of soils could lead to a considerable growth in the zones in Europe available for truffle cultivation.

Techniques for cultivating *T. melanosporum*, as described above, have been successfully used in France for many years (Sourzat 1989–1995–2002, 2011). These methods were perfected taking into account the pressure of contamination by ectomycorrhizal fungi in wooded environments, which are a major factor limiting the success of truffle cultivation in Europe. More recent approaches for truffle cultivation including the J.A.AD. (Dessolas et al. 2007–2008; Pargney et al. 2011) and M.R.T. (Chevalier 2010b) methods are aimed to improve truffle production. Implementation of field studies on truffle cultivation together with research on the biology, ecology and genetic of truffles should continue to contribute to improved truffle cultivation techniques.

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Chapter 11
Truffle Cultivation in the Southern Hemisphere

Ian R. Hall and Wayne Haslam

11.1 Beginnings

In 1979 at a conference in Fort Collins, Colorado, Ian Hall was sitting with Jim Gerdemann and fellow students from Illinois University reminiscing about the time he had spent there as a postdoc. As happens, after years have gone by, there was a pause in the conversation, and Ian overheard a conversation in French at a neighbouring table. To describe Ian's French as schoolboy French would be a gross exaggeration, but he understood sufficient—the first truffles had been produced in a French truffière (a truffle plantation). The information lodged somewhere in his subconscious only to be resurrected some months later with the idea, "If they can do it in France, then we can do it in New Zealand to meet the out-of-season market". And so began the quest to cultivate mycorrhizal mushrooms in the Southern Hemisphere.

However, that was in 1979, and soon after, all mycorrhizal research in New Zealand was stopped, "mycorrhiza" became a dirty word, applications for funding that contained it were doomed to failure and Ian's energies were diverted into "improving" New Zealand's indigenous grasslands to carry more sheep. Fortunately, 6 years after the first "artificial" European truffles had been harvested in 1979, Jock Alison was appointed Director of the New Zealand Ministry of Agriculture and Fisheries' Invermay Agricultural Research Centre, of which Ian was part. Ian outlined the opportunities to Jock for growing truffles in New Zealand, carefully avoiding the use of the "m" word, and Jock gave the go ahead. This was also supported by a grant of $1,500 from the Miss E.L. Hellaby Trust.

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By the early 1980s, a little information on the techniques that had been used in Europe to produce truffle-infected plants had been published. Armed with this, basic principles based on previous work with arbuscular mycorrhizas, and Fred Hoyle's comment: "*If you know something to be possible, it is much easier to find it yourself*", Ian Hall and Sharon Roberts, his part-time technician, set to work. Within 12 months, they had devised a method for producing *Tuber melanosporum* Vittad. mycorrhized plants by inoculating hazel and oak seedlings with mashed-up truffle. In doing so, the ground had been prepared for new industries in the Southern Hemisphere, based on the cultivation of edible mycorrhizal mushrooms.

11.2 The First Steps in New Zealand

The first three New Zealand truffières were established in 1987 in North Otago (45°S), which was within easy reach of the New Zealand Ministry of Agriculture and Fisheries regional headquarters at Invermay near Dunedin. Two of the truffières were established on soils with a suitably high pH, whereas the soil on the third had to be modified by the application of large quantities of lime—about 4.5 kg/m^2/0.1 pH mixed into the top 0.3 m of soil. The fungus thrived on the roots of the oaks and hazels, and the following year, it was decided to sell 3,000 experimental Périgord black truffle mycorrhized trees. These were offered to anyone, provided they agreed to follow a strict set of instructions regarding soil modification and planting. A small book, "The Black Truffle" was also prepared to provide growers with basic agronomic procedures and the soil and climatic requirements of *T. melanosporum* (Hall and Brown 1989). Field information was also made freely available to visitors from Australia and elsewhere. Also in true ANZAC *esprit de corps*, the New Zealand Foundation for Research Science and Technology sent Ian Hall's proposal for further funding to Australia for assessment.

The first truffle was harvested in New Zealand on 29 July 1993 (Ian Hall's birthday; Hall et al. 2007) on Alan Hall's truffière at Waerenga-a-hika, near Gisborne (38° 40′ S), with the aid of Boss, a truffle dog trained at the New Zealand Police Dog Training Centre. Following the find of the first truffles and after the initial fanfare of media attention, no further truffles were found on Alan Hall's property for the next few years. The lack of a truffle dog did not help! Nor did the invasion of *Tuber maculatum* Vittad., probably originating from a willow that once grew adjacent to the truffière. This lack of a harvest did not escape the attention of those in charge of research funding in Crop and Food Research and the New Zealand Foundation for Research Science and Technology. Although they both were aware from the outset that harvests might take 10 years or more to begin, funding for truffle research began to be reduced in 1993 and ceased completely after 1995. Fortunately, research on other mycorrhizal mushrooms was supported, albeit at a much level lower than before. This together with income from the sale of truffle-infected trees and other commercial activities, there was almost sufficient funding to hold the edible mycorrhizal mushroom team together.

T. melanosporum truffles have now been harvested in New Zealand truffières in the Bay of Plenty (38° 00′ S), Poverty Bay, just south of Taumarunui, Hawkes Bay, Waipukurau, Paraparaumu, Nelson, Blenheim, North Canterbury and Ashburton (43° 53′ S). Bianchetto truffles (*Tuber borchii* Vittad.) have also been harvested at Te Puke (37° 45′ S), Waipukurau, greater Christchurch, Lake Hayes and near Queenstown (45° 02′ S), while Burgundy truffles have been harvested just south of Oamaru (45° 08′ S). These truffières were established both on soils with a suitably high pH (>7.2, ideally 7.9) as well as on naturally acidic soils (pH 5.3–6.9) that had been heavily limed. In 2011 the total harvest for NZ was estimated at less than 100 kg from a few ha of mature truffières. Yields range from the equivalent of >300 kg/ha in the north of the country to just a few kg/ha in the south.

11.3 Commercialisation of Truffles in Australasia

In New Zealand the primary aim of the first experimental truffières established in 1988 was to determine if large applications of lime to a variety of soils would successfully modify their pH, whether the fungus would survive in such soils in various parts of the country and whether truffles would be produced in them. The industry was essentially a government-supported initiative, and the first to produce commercial quantities was Alan Hall's truffière near Gisborne when in March 1997 large numbers of large, but very immature, *T. melanosporum* truffles were discovered by accident. Subsequently, all but one of the truffières established in 1988 on warm sites produced.

The latitudes, elevation and some climatic data for the productive truffières are provided in Table 11.1 and Figs. 11.1, 11.2 and 11.3. In Fig. 11.1 the climatic data are compared with those in the *T. melanosporum* areas of Mende, Lozere, France, and Perugia, Italy, which are arguably the coolest and warmest areas of Europe to produce this truffle. Comparative soil temperature data are not available for many of the truffle-producing areas of Europe, Australia and New Zealand, although rough comparisons can be drawn from air temperatures combined with sunshine hours, both of which impact on soil temperature. Wind run might also affect soil temperature, but again, long-term data is not always available.

In Australia in the early 1990s, the industry began as a purely commercial activity. There was no necessity to repeat the New Zealand experiments, and significant plantings, some large, were planned from the outset. The first were contract grower plantings in Tasmania established by Périgord Truffles of Tasmania. In these, Périgord Truffles of Tasmania undertook to harvest, grade and market all truffles produced for a 50 % share of proceeds with the grower carrying the risk. The first black truffles in Australia were harvested in Tasmania in 1999 on a property owned by Truffles Australis at Deloraine (approx 41° 32′ S). Later developments in Tasmania were largely managed investment schemes, for example, two offered by the Tasmanian Truffle Enterprises Group in 2000 and 2002, each about 40 ha. Another managed investment scheme near Deloraine established by Agri-Truffle Pty Ltd involved some 90 investors with 20,000 trees planted over 60 ha between

Table 11.1 A rough climatic comparison of centres adjacent to or with similar climates to Périgord black truffle (*Tuber melanosporum*), Italian white truffle (*T. magnatum*), Burgundy truffle (*T. aestivum*) and bianchetto truffle-producing areas (From: Hall et al. 2008)

	Périgord black	Italian white	Burgundy	Bianchetto	Latitude (°:′)	Elevation (m)	Annual rainfall (mm)	Accumulated degree days (>10 °C)	Mean daily air temperature in summer July/Jan (°C)	Mean daily air temperature in winter Jan/July (°C)	Annual sunshine hours	"Summer" sunshine hours (Apr–Sep/Sep–Apr)
France												
Caen					49°:18′	67	711	852	17.0	4.5	1,762	1,191
Bourges					47°:06′	166	723	1,115	19.2	3.3	1,827	1,279
Dijon					47°:18′	227	732	1,137	19.7	1.6	1,831	1,307
Clermont Ferrand					45°:50′	329	563	1,102	19.2	2.7	1,990	1,317
Lyon					45°:42′	201	825	1,312	20.7	2.6	1,975	1,411
Orange (Marseille*)					44°:08′	60	810	1,562	22.0	4.0	2,837	1,814
Toulouse					43°:36′	153	655	1,513	21.3	5.4	2,051	1,350
Switzerland												
Lugano, Ticino					46°:00′	273	1,545	1,421	21.3	3.1	2,028	1,245
Croatia												
Istria (Rijeka, *Trieste)					45°:13′	85	1,045*	1,837	23.0	5.0	2,388	1,405*
Italy												
Cuneo (*Torino)					44°:55′	384	948	1,379	22.0	3.5	1,989*	1,246*
Bologna					44°:30′	84	589	2,009	24.6	2.4	2,064	1,458
Rimini					44°:00′	13	702	1,746	22.6	3.3	–	-
Arezzo					43°:28′	249	755	1,349	21.0	4.0	-	-
Perugia					43°:06′	493	816	1,622	22.4	4.4	2,061	1,457
Campobasso					41°:36′	807	628	1,436	21.5	3.8	2,113	1,365
Spain												
Pamplona, Navarra					43°:48′	449	963	1,342	20.0	4.5	-	-
Perarrua, Huesca (*Zaragoza)					42°:16′	517	644	1,479	21.7	2.9	2,638*	1,689*
Arroyo Cerezo, Valencia					40°:07′	1,344	552	749	18.9	3.1	-	-

Cuenca, Cuenca (*Madrid)	40°:05′	1,001	569	1,352	21.7	3.1	2,678*	1,714*
Santiago de la Espada, (*Albacete)	38°:20′	1,328	673	1,547	23.2	3.3	2,705*	1,713*
North America								
Chapel Hill, N.C. (Washington*)	36°:10′	190	1,054	2,341	25.5	4.0	2,173*	1,545*
Ukiah, California	39°:20′	189	943	2,341	23.0	8.5	≈3,000	-
Placerville, California	38°:43′	570	979	1,832	23.5	6.7	≈3,400	-
Greeneville, Tennessee (Knoxville*)	36°:06′	402	1,125	1,657	23.1	0.8	2,603*	-
Australia								
Pallamana, SA	35°:03′	76	345	1,909	21.0	9.5	≈2,920	1,703
Goulbourn, NSW	34°:75′	670	642	1,546	20.6	6.5	≈2,555	1,490
Canberra, ACT	41°:54′	578	617	1,476	20.5	5.6	2,811	1,623
Armidale, NSW	30°:52′	980	792	1,612	20.3	6.3	≈2,920	1,582
Manjimup, WA	34°:25′	287	1,011	1,811	20.0	10.4	≈2,555	1,582
Cooma, NSW	36°:23′	812	502	1,292	19.3	4.5	≈2,555	1,582
Orange, NSW	33°:38′	948	886	1,168	19.0	5.0	≈2,920	1,764
Healesville, VIC	37°:68′	131	1,021	1,438	18.6	8.2	≈2,190	1,490
Colac, VIC	38°:34′	134	731	1,293	18.2	8.2	≈2,190	1,369
Launceston, TAS	41°:54′	166	677	891	16.7	6.6	≈2,555	1,521
Deloraine, TAS	41°:52′	237	950	568	14.5	5.7	≈2,555	1,460
Chile								
Talca	35°:26′	102	749	1,496	20.0	6.0	2,518	1,725
Temuco	38°:15′	120	1,179	784	16.0	7.5	1,728	1,454
Panguipulli	39°:39′	287	2,430	921	17.0	7.5	2,032	1,454
New Zealand								
Te Puke (*Tauranga)	38°:00′	91	1,754	≈1,400	18.7	9.3	2,277*	1,320*

(continued)

Table 11.1 (continued)

	Périgord black	Italian white	Burgundy	Bianchetto	Latitude (°:′)	Elevation (m)	Annual rainfall (mm)	Accumulated degree days (>10 °C)	Mean daily air temperature in summer July/ Jan (°C)	Mean daily air temperature in winter Jan/ July (°C)	Annual sunshine hours	"Summer" sunshine hours (Apr–Sep/ Sep–Apr)
Opotiki					38°:00′	6	1,400	1,493	18.5	9.2	2,169	1,227
Gisborne					38°:40′	9	1,058	1,430	18.3	9.0	2,172	1,283
Taumarunui					38°:55′	171	1,443	1,292	18.3	7.9	1,704	1,079
Waipukurau					40°:00′	137	847	1,089	17.6	7.1	1,992	1,166
Paraparaumu					40°:55′	7	1,054	1,167	17.1	8.3	2,043	1,227
Nelson					41°:15′	10	986	1,038	17.2	6.5	2,397	1,377
Blenheim Airport (* Blenheim)					41°:31′	27	738	1,179	17.7	7.0	2,447*	1,413*
Waipara (*Christchurch)					43°:05′	64	729	1,049	17.5	6.5	1,999*	1,175*
West Melton (*Christchurch Airport)					43°:29′	30	648	974	17.0	5.6	1,999*	1,175*
Ashburton					43°:54′	101	757	896	16.5	5.2	1,892	1,092
Lake Hawea (*Queenstown)					44°:50′	350	765	860	16.9	3.7	1,921*	1,260*
Shotover River (*Cromwell, **Alexandra)					45°:00′	805	661*	926*	17.3*	3.8*	2,064**	1,267**
Oamaru					44°:58′	30	537	800	15.1	5.5	1,792	1,037

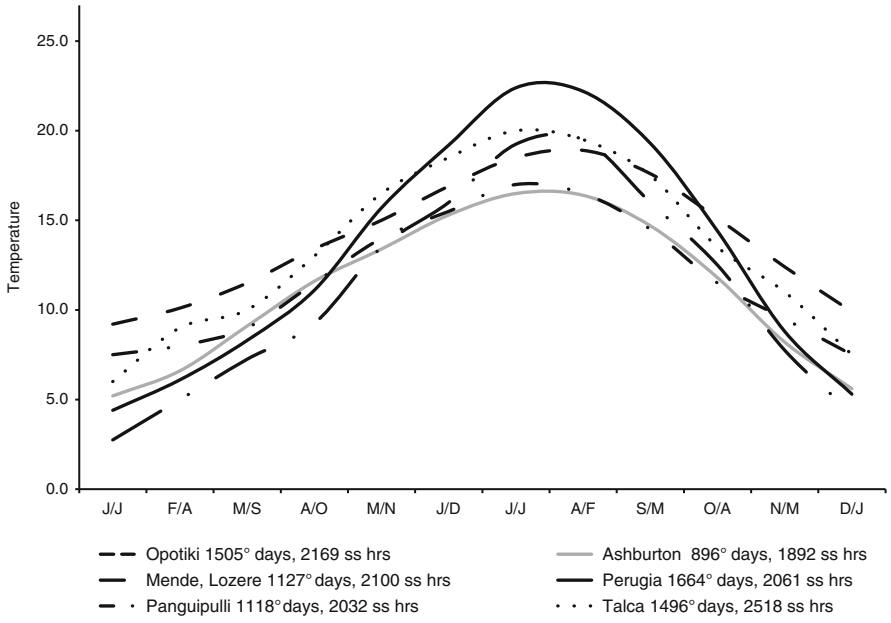

Fig. 11.1 Mean monthly temperature curves, degree days (base 10 °C) and sunshine hours (ss hrs) for truffle-producing areas near Mende, Lozere, France; Perugia, Italy; Opotiki and Ashburton, New Zealand; and Panguipulli and Talca, Chile

2001 and 2005, and the first harvest was in 2008. Initially the managed investment schemes attracted a 150 % tax write-off. This tax write-off persuaded companies and the Collins Street cockies (intelligent, professional people with money— or investors wanting to be farmers without getting their hands dirty) to invest in truffle cultivation Table 11.2.

In essence, the tax write-off helped fill what has been termed the "funding gap"—the financial bridge between the proof of an idea by science and its commercialisation (Ministry of Research, Science and Technology 2007). The tax benefit was discontinued in 2007, causing a large MIS to be cancelled in Tasmania (Australian Securities and Investments Commission 2010; Truffles Three Landco Limited 2007). Individual growers who could establish themselves as primary producers were entitled to deduct their losses from any source of taxable income or carry their tax losses forwards until production increased to a stage where a taxable profit was being made. These factors alone were sufficient to encourage the rapid establishment of a large truffle industry in Australia which is now perhaps 30 times the size of New Zealand's and set to get even larger. At the same time, New Zealand was removing all tariffs and support for agriculture that made the cultivation of truffles, which was risky to start with, even more unattractive to the entrepreneur and investor. In addition to early tax write-offs, several Australian companies also obtained significant grants from the Commonwealth Government such as $250,000 worth of RIRDC (Rural Industries Research and Development Corporation) grants to Perigord Truffles of Tasmania (2010).

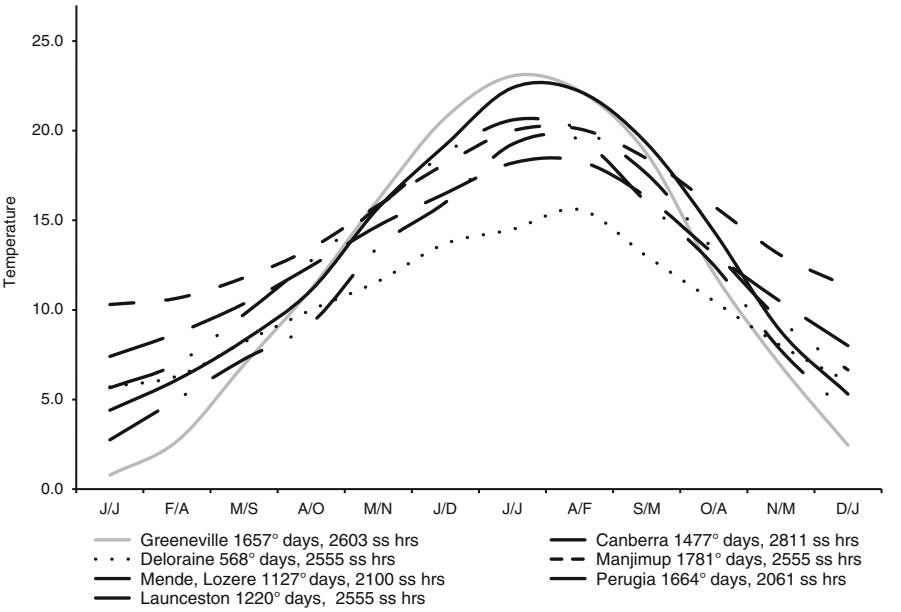

Fig. 11.2 Mean monthly temperature curves, degree days (base 10 °C) and sunshine hours (ss hrs) for truffle-producing areas near Mende, Lozere, France; Perugia, Italy; Canberra, Deloraine and Manjimup, Australia; and Greeneville, Tennessee

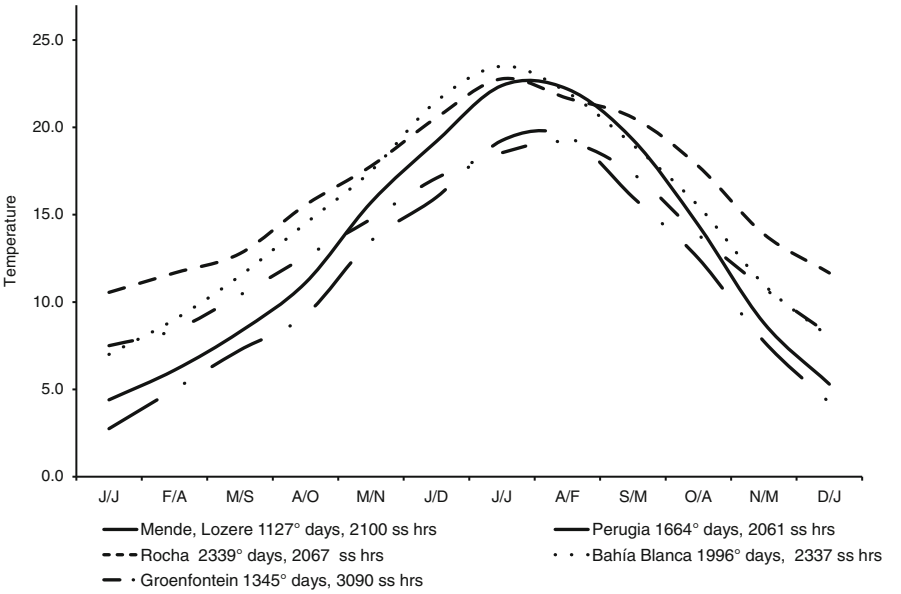

Fig. 11.3 Mean monthly temperature curves, degree days (base 10 °C) and sunshine hours (ss hrs) for truffle-producing areas near Mende, Lozere, France; Perugia, Italy; and potential truffle-growing areas near Bahía Blanca, Argentina; Groenfontein, Western Cape, South Africa; and Rocha, Uruguay

Although New Zealand had investigation grants in the late 1980s for those establishing the first truffière in a region (Cook 2010), relative to the Australian tax subsidy, these grants were tiny. In 2001 an attempt to accelerate the establishment of a larger industry in New Zealand, Crop and Food Research, one of the government-owned Crown Research Institutes, helped established TRINZ International Limited (later renamed Truffle Investment New Zealand Limited), a joint venture partnership, with an entrepreneur (New Zealand Companies Office 2011). Regrettably, before this joint venture could properly get underway, the entrepreneur was declared insolvent (New Zealand Herald 2004).

In the late 1990s, interest turned to Western Australia where in 1997 a large corporate planting of about 21 ha (13,000 trees) was established at Manjimup (34°S) to produce both wine and truffles. The Wine and Truffle Company harvested their first truffle in 2004, and by 2008, the harvest had grown to 600 kg. Manjimup Truffles planted 7 ha between 1997 and 2001 and have been producing truffle since 2007. A 70 ha planting was established at Manjimup in 2006 as a managed investment scheme, close to the existing plantings of the Wine and Truffle Company and Manjimup Truffles. They were successful in harvesting truffle in 2010 after 4 years. Significant corporate and small holder plantings have continued in the Manjimup area, and by 2011, this area was producing in excess of 2 tonnes of saleable truffle.

Significant small holder plantings have continued in all states and territories of Australia with the exception of the Northern Territory. There are a small number of growers in Queensland (29°S) and in South Australia (35°S). Plantings in Victoria (37° to 38°S), as of 2011, are probably in excess of 50 ha with about 35 growers and a mix of small holders and contract growers. New South Wales (30° to 37°S) also has a mix of small holders and contract growers numbering about 70. The total area in NSW is probably in excess of 100 ha growing at various elevations. Across Australia, it is estimated that current plantings are approaching 700 ha. Most of the Eastern State plantings have been since 2000, and many have yet to come into significant production. In 2011 the estimated number of growers and areas planted is shown in Table 11.2, and production of truffle in Australia was in excess of 3 tonnes. Of the localities listed in Table 11.2, all have been producing truffles with the exception of South Australia, Cooma and Armidale in NSW and Colac in Victoria. The left-hand column of Table 11.2 shows the various business models adopted for production in Australia.

Despite the rapid growth of the industry, there are questions on the productivity of established plantings. Although there are about 150 growers across Australia, there are probably only 10–12 plantations producing at a commercial level [in excess of 30 kg of truffle (Duell 2011)]. It is further estimated that there are another 30 or 40 plantations producing less than 30 kg, which leaves about 100 plantations with no production. The industry is still mired in secrecy, but the threat of significant investment with little or no return is pushing growers to seek answers and to work with the industry to address issues. Recent work by the Australian National University has shown that, generally, the quality of inoculated trees on the market is poor with tested samples showing root-bound and J-rooted specimens with highly variable

Table 11.2 Estimated numbers of truffle growers and planted areas in Australia in 2011

Grower type	Description	Western Australia	South Australia	Tasmania	Victoria	Australian Central Territory	New South Wales	Queensland	Total growers	Area totals (ha)
1	Larger scale corporate truffières	2	0	2	0	0	0	0	4	100
2	Larger scale managed investment scheme truffières	1	0	3	0	0	0	0	4	200
3	Smaller scale contracted grower truffières	0	0	>10	>20	0	>30	0	>60	100
4	Smaller scale independent grower truffières	>10	>3	>10	>25	2	>50	>2	>100	200
	Total numbers	>15	>3	>25	>50	2	>80	>2	>170	>600
	Estimate of total area (ha)	>150	>5	>165	>110	>5	>160	>5		>600

rates of infection and contamination with the inferior *Tuber brumale* Vittad. (Australian Truffle Growers' Association 2011). This work clearly demonstrates the poor scientific base for the industry in Australia and the work that needs to be done with truffle-grading standards, certification of infected trees and production issues to correct the uninformed errors of the past.

11.4 Chile

The demonstration that truffles could be cultivated in New Zealand generated interest, not just in Australia but also in other Southern Hemisphere countries where climatic conditions appeared suitable for truffles. Chile was at the forefront, and researchers made fact-finding visits to New Zealand, Australia and Europe. The outcome was the establishment of a number of truffières with the first established in 2003 (Ramírez et al. 2007). By the end of 2007, more than 20 ha had been planted in Metropolitan, O'Higgins, del Maule, Bío-Bío, Araucanía y Los Ríos, Los Ríos y del Maule (Duao) and Panguipulli (Región de Los Ríos). By 2010, an additional 70 ha had been planted with the final aim of expanding to 150 ha (Fundación para la Innovación Agraria 2010). The total cost was 462 million Pesos (almost $1 million US dollars) of which 52 % was contributed by the Fundación para la Innovación Agraria, a section of the Chilean Ministry of Agriculture (Fundación para la Innovación Agraria 2010). The remainder was funded by the Maule Catholic University (Fotoquinta 2009) and Agro Biotruf (2010). Clearly, there does not appear to be a funding gap in Chile.

The first Chilean *T. melanosporum* truffles were harvested in 2009 in Panguipulli, Región de Los Ríos (39° 39′ S, elevation 268 m) (Fundación para la Innovación Agraria 2010). The following year, truffles were found under *Quercus ilex* at Duao (35° 43′ S, 163 m elevation), about 12 km southwest of Talca (35° 26′ S) (Andean Truffles 2010). In 2011 truffles were also harvested near Quepe and Chufquen, Traiguén (38° 15′, elevation 120 m), 4–5 years after planting (Andean Truffles 2011). Some climatic data for these areas are shown in Fig. 11.1.

11.5 Argentina, South Africa and Uruguay

Argentina spans similar latitudes to Chile, and there are certainly areas with winter and summer temperatures that should suit the cultivation of all four species of commercialised truffles. For example, the climate in Bahía Blanca appears about right for *T. melanosporum*. Similarly, *a priori* at an elevation of 250 m on the Cerro Catedral, Uruguay (about 2°C cooler throughout the year than Rocha) seems another possibility (Fig. 11.3). Establishing truffières at high elevations has also been used by Woodford Truffles SA (2011) on its Groenfontein property and its partners' properties in South Africa (Fig. 11.3).

11.6 Quality Control of Plants

The chief method for producing *T. melanosporum*-infected plants in the Southern Hemisphere has been by inoculating clean seedlings or occasionally cuttings with a suspension of spores (Chap. 1; Hall et al. 2007, 2009). The method is capable of producing well-infected and uncontaminated plants providing certain precautions are taken: only clean, uncontaminated truffles of the desired species are used, no ascomycete or basidiomycete EM fungi contaminate the plants in the nursery and possibly, truffles are sourced from ideal locations (Hall et al. 2010). Because spores were the inoculum, it is most unlikely that there would have been a preponderance of either of the two mating types among mycorrhizas at the time the plants left the nurseries (Riccioni et al. 2010a, b).

One way of helping limit the chances of contamination is to take small samples from each truffle to be used in an inoculum and then confirm their identity using morphological and molecular tools (Ministry of Agriculture and Forestry 2008). Of course, there is always the possibility that small fragments of another species might be secreted in a crevice on the much larger piece of truffle that is not sampled. This applies whether morphological or molecular techniques are used, a factor often misunderstood by the ill-informed blinded by high-tech methodologies. It is possible that this is how some plants became contaminated with *Tuber brumale* in New Zealand (Guerin-Laguette et al. 2011). However, there are examples in Australasia of truffières with significant numbers of plants contaminated with *T. brumale*, AD-like fungi or basidiomycetes such as *Hebeloma*. Possibilities range from the accidental incorporation of previously rejected truffles in inoculum, absence of quality control during the production of inocula or prior-to-sale inspections of mycorrhizas, no after-sales follow-up surveys of mycorrhizas in established truffières, and hopefully not, callous disregard or sabotage.

The potential contamination problem on truffle plants was identified in Australia in a RIRDC report (Stahle and War 1996), but regrettably the recommendations were not followed up at the time due to the lack of a representative growers' association or any other suitable mechanism of control. The report did, however, prompt tree producers to target growing areas on the Mainland, particularly in Victoria and NSW. An Australian Truffle Growers' Association was established by Wayne Haslam in late 2006 and is presently concerned with a number of industry development issues, including the significant percentage of nonproducing truffières and reported contamination of established truffières by other species as referred to in earlier paragraphs. The Association is seeking to establish a tree certification process during 2012. This would be based on the process established in Spain for the government-subsidised truffle industry. The Australian scheme will be self-funded, and inoculated tree producers that accept the certification process will be acknowledged by the Association as being a reliable source of inoculated trees.

11.7 Quality Control of Truffles

A grading standard needs to be agreed between truffle producers in Australia and New Zealand. An attempt was made to reach agreement on a grading standard in 2009, based on the European Union grading standard, and a considerable amount of work was done towards reaching agreement between the New Zealand and the Australian Associations. However, this was rejected at a meeting of the major producers in Australia in early 2011, resulting in a rethink of a constructive approach to developing a workable standard that incorporates the European standard requirements but also meets the needs of Australian producers. Part of the problem is the difficulty of incorporating the aromatic attributes, covering the truffles allowable as imports into Australia (currently only *T. melanosporum*, *Tuber magnatum* Pico, *Tuber aestivum* Vittad./*Tuber uncinatum* Chatin and *Tuber borchii* Vittad.) and allowing cut pieces as an acceptable product. This is an ongoing activity and includes input from the electronic nose project being undertaken at the University of Western Australia (see below). Once drafted, it will need to be tested on export market clients to ensure their needs are also met.

11.8 Marketing

When Ian Hall conceived the production of truffles in the Southern Hemisphere for off-season Northern Hemisphere markets, French *T. melanosporum* production had slumped from maybe 2,000 tonnes in the 1880s (Chap. 1) to 1,000 tonnes in 1904 to an average of around 100 tonnes in the 1970s (Hall et al. 2007). Over the same time, the world's population had risen from 1.5 billion to more than 6 billion (Wikipedia 2011), and disposable income in developed countries, and in some sections of the population in developing countries, had soared (e.g. The Telegraph 2011). Australian production in 2011 exceeded 3 tonnes, but wholesale prices in the international markets appear to have been lower than those charged in the northern truffle season 6 months before. The market for Australian truffles offshore in 2011 suffered the impact of the global financial crisis and the high value of the Australian dollar. There was also significant competition between Australian producers for a share of the market. If growers do not cooperate to export the Australian product collectively, the rapid increase in production in Australia (predicted to be at least 30 % per annum, initially), then the price will continue to struggle to match the northern season price.

Another concern in Australia is that some people are calling *T. melanosporum* the "black winter truffle". This is a quality control issue and will need to be addressed for the export market. In Latin the word "brumale" is the word for "the winter solstice" and the Latin *brumalis* is "connected with winter". This is causing some concern in the industry, given the recognised contamination of some truffières with *T. brumale* and the fact that "*T. brumale*" is literally the winter truffle and worth a fraction of the price of *T. melanosporum*.

11.9 Research Funding

The truffle industry in New Zealand cannot be considered a success when compared to Australia and faces many challenges, including nonproducing truffières and inadequate government support for research and development. One reason why this new opportunity of considerable potential had to struggle to find funding in New Zealand lies with a change to the New Zealand system of funding science. In 1989 New Zealand research on truffles was aimed at establishing a new crop, its findings were confidential and publications were discouraged. In contrast, half a decade later, the system had turned turtle, and the mark of success of a science project was measured in numbers and quality of scientific papers (Hall 2008).

In New Zealand after 1995, small grants from AGMARDT and Technology New Zealand (Technology for Business Growth) were the only support for research on *T. melanosporum* in New Zealand. So in 1999, at the instigation of Ian Hall, the then Secretary of the New Zealand Truffle Association, Crop and Food Research agreed to collect a $5 research levy on each *T. melanosporum*-infected plant it sold on behalf of the New Zealand Truffle Association. This money attracted a 50/50 subsidy from Technology New Zealand, and together with a grant from AGMARDT, a modest but very worthwhile study was carried out over 3 years on factors that might affect fruiting of *T. melanosporum* (Hall et al. 2002). That the soil and temperature data accumulated over many years from productive and nonproductive New Zealand truffières is still being used is testimony to its potential value. Levies and money from New Zealand Truffle Association subscriptions were also used to carry out a further study between 2006 and 2010.

In Australia the truffle industry is rapidly maturing, and the Association is seeking to fund research and development, including production issues and marketing research. Funding sources that are being considered include higher association fees and levies on existing trees, new trees and production. The Association is able to attract Commonwealth Government support based on funds generated by the industry and is seeking an annual revenue of some A$100,000 to meet the association's operational needs and address the current list of research and development priorities: truffle rot, genetic diversity of truffle in Australia and electronic methods for determining quality.

11.10 Truffle Rot

A major problem of truffle production in parts of Australia is that a large proportion of truffles are on or close to the surface rendering them susceptible to predators and physical and weather damage. This is particularly the situation in Western Australia and parts of Tasmania. Work by Harry Eslick of the Wine and Truffle Company in Western Australia has looked at management practices to address these issues,

including the levels of canopy cover and irrigation practices (Eslick 2011a, b). While irrigation was considered one of the main areas of investigation during 2011, results have been inconclusive. However, there are indications that lower levels of irrigation may reduce rot. Bacterial pathogens have not been ruled out either, although inoculation of healthy truffles with bacteria from rotten truffles has failed to produce symptoms.

As with any crop, pests and pathogens such as nematodes, bacteria, fungi and insect larvae can affect the quality of truffles (Hall et al. 2007). With truffles, the problems are compounded because the health of the host tree also has to be considered. The solutions to these problems for other crops might be the application of fertilisers, nematocides, antibiotics, fungicides or insecticides. But a plant pathologist will always first consider if the growing conditions have been optimal and determine whether the rainfall/irrigation has been excessive or insufficient, the weather has been unusually warm or cold, etc. Although the temperature curves for Manjimup, Western Australia, suggest that winter temperatures there are well above the range for truffle-producing areas of France and Italy, it has not yet been determined if this has a bearing on truffle rot (Fig. 11.2; Table 11.1).

11.11 Genetic Diversity of *T. melanosporum* in Australia

Research by Dr. Celeste Linde of the Australian National University on microsatellite analyses of 210 truffle samples collected from various truffle-producing regions in Australia showed that there was considerable genetic diversity with 51 genotypes identified. In comparison, a similar European study found only 22 genotypes out of 206 samples. Also, more alleles per locus were identified in Australia than in the European study (Riccioni et al. 2008). For all loci, the most common allele found in Europe was also present in Australia. For one locus, the 2 alleles found previously in Europe, as well as an additional 7 alleles were found in Australia. Alleles and genotypes present in Australia match genotypes previously reported from Spain, France and Italy and therefore represent a wide geographic gene pool.

The genetic pool of truffles in Australia is sufficiently large to be an unlikely barrier to truffle production. However, an unknown at this stage is whether truffles possess vegetative incompatibility groups (VCGs) that facilitate the disappearance of one mating type after inoculation. Fungal isolates that belong to different VCGs cannot interact genetically, even if they belong to different mating types. However, VCGs have not been identified in truffles but are speculated to be present (Riccioni et al. 2010b). An alternative, and undoubtedly controversial explanation, might be that if one of the mating types is not as well adapted to the climatic or edaphic conditions, it may simply be outcompeted for sites on the roots by the other.

11.12 Electronic Nose Project

Past research on truffle aromas and possible methods to detect truffles using them has been conducted in the past but without great success (Hall et al. 2007). The incentives for more work were developments in nanotechnology in substance detection. A project under the direction of Professor Garry Lee at the University of Western Australia is primarily aimed at establishing a basis for the grading of truffles, based on aromatics, and developing a prototype tool that would assist growers in product grading, ensure consistency and retain a market premium for the highest quality truffle. The project will investigate the qualitative change in truffle volatiles at different stages of fruiting using chemical instrumentation and organoleptic techniques. The essential truffle aromas will be characterised with chemistry, using gas chromatography and mass spectrometry and correlated to the sensory analysis. Work up to 2011 is showing promising results with truffle from various parts of Australia being assessed and with subtle regional differences in aromas being detected.

11.13 The Future

Clearly, there is great potential for the cultivation of truffles and other edible mycorrhizal mushrooms in the Southern Hemisphere both for home and Northern Hemisphere off-season markets. However, the lesson learned from the Australian *T. melanosporum* programme is that cultivation practices cannot simply be transferred with an expectation of immediate success. Also, for those who are successful, marketing simply cannot be left to chance and the assumption that Northern Hemisphere clients will be pounding on your door when a new mushroom becomes available out of season. Similarly, home markets will have to be skilfully created where perhaps none existed before. The lessons show that emerging industries should:

- Do the market research first to ensure that there is a viable market for the proposed new product and there is a workable supply chain to get the product to the market.
- Do the sums thoroughly, using conservative estimates of yields and prices, to ensure a viable prospect of getting a return on capital and labour in the medium term.
- Establish an industry-wide association as early as possible, with good leadership to set industry-wide goals and have buy-in of all stakeholders.
- Only continue to invest in solving production issues when the market prospects are shown to be viable.

For the successful ventures, there is nothing the media like more than a happy story amidst the depressing miasma afflicting the world. They are often delighted to help. The media can also play a big role in ensuring the new product is introduced to and

understood by the local consumers, and have shown to be a significant market resource for local growers. Finally and above all, the new products have to be carefully introduced to the chefs, so that they can be prepared with new dishes to suit the new products. The last thing the industry needs is a chef who presents a magnificent dish and with much aplomb shaves slices of a superb truffle over the surface of the food before adding a dash of (synthetic) truffle oil (Parker Bowles 2009)!

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Chapter 12
Native and Cultivated Truffles of North America

Charles Lefevre

12.1 Introduction

Of the many truffles indigenous to North America, seven species are noted for their culinary value. Four species known collectively as the Oregon truffles are found on the west coast, while three others are found in the southern and eastern portions of the continent. North America also has European truffle species planted in orchards of inoculated trees established across much of the continent. Both the harvest of indigenous truffles and cultivation of European species are largely undeveloped industries with great cultural and commercial potential, although both face significant challenges (Pilz et al. 2009).

12.2 Oregon Truffles

At a conference entitled *Mushrooms and Man*, held in Corvallis, Oregon, on November 6–8, 1977 (Walters 1977), the recently named Oregon white truffle (James Trappe, personal communication) was proclaimed by Chef James Beard to be as good as the Italian white truffle. As one of the most influential chefs in US history, Chef Beard's statement contributed to the development of a commercial market for Oregon truffles over the ensuing decades. His praise is echoed by other prominent chefs, some of whom have expressed a preference for Oregon truffles over the celebrated European species (e.g., Czarnecki 1995). Oregon truffles have also received higher scores than their more famous counterparts in taste and aroma tests where panelists were asked to state which truffle they preferred without knowing in advance what species were presented, their relative prices, or where

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they originated (Charles Lefevre, unpublished data). These results (and accolades by renowned chefs) do not establish that Oregon truffles are "better" than the more expensive European truffles, but they do suggest that Oregon truffles merit serious attention and further study. Whether Oregon truffles are capable of favorable comparison with the European species is often irrelevant, however, given current harvest methods and their negative impact on overall quality of truffles available on the market.

12.2.1 Harvest Methods

The harvest of truffles employs flies, pigs, and trained dogs, but before a truffle industry existed in North America, there was no need for these animals. Study and surveys of the many indigenous hypogeous fungi were conducted with rakes, which was appropriate for research purposes, and had little impact considering the relatively small areas searched. Raking continued to be employed during the early development of the commercial industry in Oregon, but the habitat impact of raking quickly became extensive, and the sale of immature and unripe truffles adversely impacted their culinary reputation (Trappe 1989, 1990).

Evidence for growing disappointment with Oregon truffles was reflected in their market value, which declined precipitously in the 1980s (Stan Patterson, personal communication; Dennis Morgan, personal communication) and continued to decline between 1992 and 1998 (Lefevre et al. 2001; Schlosser and Blatner 1995). By 1998 prices had become unresponsive to supply, even in low-productivity years (Lefevre et al. 2001). Trappe (1989, 1990) called for a transition from raking to the exclusive use of truffle dogs both as a way to prevent widespread disruption of the forest floor and to selectively and reliably harvest ripe truffles. Others have reiterated the problem and repeated the call for a transition to truffle dogs (e.g., Bunyard 2008; NATS 2010; Renowden 2005; Trappe et al. 2007; Work 2008), but raking continues to be the predominant harvest method for Oregon truffles (Bauer 2012; Lefevre et al. 2001; Lefevre 2010; Pilz et al. 2009; Terry 2010).

As a natural consequence of raking, truffles are collected at all stages of maturity. Because truffles only ripen at the end of their development and tend to be consumed quickly by mycophagous animals, the proportion of truffles ripe at any given time tends to be low. Many unripe truffles are too immature to develop aroma, and the remainder must be ripened artificially to produce their aroma. The process of identifying and discarding those with no potential to ripen and ripening those that are sufficiently mature requires some education and experience (Czarnecki 1995; NATS 2010; Pilz et al. 2009) and largely explains the potential for disappointment among chefs who lack this understanding. One advantage of ripening truffles artificially is the ability to serve them 2 or 3 weeks earlier than they would ripen naturally, effectively extending the season. However, as Marin (1985) demonstrates, the intensity and complexity of artificially ripened truffles' aroma diminish as a function of the proportion of spores that have reached maturity,

suggesting that truffles harvested early and ripened artificially will, on the whole, fail to reach their culinary potential. Nevertheless, that period of time early in each season when few truffles ripen naturally often coincides with the late fall and early winter holiday season when demand for truffles is high, and raking may often be the only way to satisfy this seasonal demand. High demand early in the season and the fact that some harvesters are resistant to arguments in favor of dogs (e.g., Bauer 2012) represent a form of inertia that may hinder a transition in harvest methods from rakes to trained truffle dogs.

Growing disparagement of Oregon truffles and prices effectively "hitting bottom" by 1998 created a bleak outlook for the Oregon truffle industry. More recently, however, three separate efforts appear to have contributed to signs of recovery. The first was a conscientious effort by one company to avoid purchase of immature truffles and to ripen the truffles they purchase prior to sale. This company charges prices approximately twice those of established purveyors (Bryan McCormick, personal communication). The second effort, started in 2005, was a concerted drive to recruit dog trainers to specialize in training truffle dogs for demonstrations and seminars held at the Oregon Truffle Festival and to promote the use of truffle dogs through the festival. After several years, a limited supply of truffles harvested exclusively with the assistance of trained dogs became available locally at prices somewhat higher than those ripened prior to sale (Toby Esthay, personal communication; Eric Lyon, personal communication). The third was the Oregon Truffle Festival itself, founded in 2006, with its visible effort to promote Oregon truffles and the extensive media coverage produced by that promotion. The combined effect of these efforts is reflected in prices for both white and black truffles that have either been on an upward trend [compare Lefevre et al. (2001) and Schlosser and Blatner (1995) with Terry (2010) and Czap (2012)] or may be bifurcating, with prices for raked truffles without prior ripening increasing at a slower pace, while truffles either ripened prior to sale or harvested by dogs command substantially higher prices. At the very least, prices for all Oregon truffles, including those harvested with rakes, have once again become responsive to changes in supply (Scott Cossairt, personal communication; Owen Rice, personal communication). In some cases, prices for Oregon truffles have reached $1,100.00/kg, substantially exceeding those of the summer and autumn variants of *Tuber aestivum* Vittad. within the same markets (Ian Purkayastha, personal communication; Toby Esthay, personal communication). Improvement in the reputation of Oregon truffles and maintenance of the upward trend in prices may continue with increased truffle dog use and with proposed certification standards to ensure that truffles are harvested by dogs (Pilz et al. 2009).

In Oregon, using truffle dogs rather than rakes is likely to generate additional benefits to the industry beyond their contribution to higher quality, improved reputation, and higher prices. These include more efficient harvest of truffles that are widely dispersed and prevention of damage to subsequent crops, thereby increasing harvester yields. For example, dogs may lead to increased yields from a particular site by preventing the premature harvest of later-maturing species during the harvests earlier in the season (see *Tuber oregonense* Trappe, Bonito & Rawlinson and *T. gibbosum*

Harkn. below). Similarly, dogs permit harvest of multiple flushes, where raking brings about a premature end to production on a particular site for the remainder of the season (see *Leucangium carthusianum* (Tul. & C. Tul.) Paol. and *Kalapuya brunnea* Trappe, Trappe & Bonito below). Dogs also reduce the time and effort required to locate widely dispersed truffles (e.g., Smith et al. 2012 found harvest rates for *T. lyonii* Butters increased by approximately a factor of five using a trained dog over harvesters using rakes) and may increase the rate at which truffles are harvested on marginally productive sites. Use of dogs may thereby allow productive harvest over greatly expanded areas of forestland in western Oregon and Washington. Similarly, dogs are more efficient during low-productivity years when truffles tend to be widely dispersed in otherwise productive patches. Harvesters using dogs will thus achieve higher yields when prices are higher. The greater efficiency of dogs during periods of low productivity also effectively extends the harvest season past the point when it would ordinarily end for all Oregon truffle species. Dogs may even allow productive harvests of two species year-round (see *L. carthusianum* and *K. brunnea* below). Other benefits associated with the use of dogs include reduced ecological and aesthetic disruption of the forest litter layer and upper soil horizons and the negative public reaction that raking for truffles generates. Finally, the use of dogs to locate truffles is uniquely appealing to food, travel, and news media, attracting positive attention to the truffle industry and to the region.

12.2.2 Tuber oregonense, *Oregon Winter White Truffle*

The common name "Oregon white truffle" was originally associated with the Latin binomial *Tuber gibbosum* that was later found to be a species complex and was split into four species (Bonito et al. 2010): *T. oregonense* (Fig. 12.2e), *T. gibbosum* (Fig. 12.2d), *T. bellisporum* Bonito & Trappe, and *T. castellanoi* Bonito & Trappe. Although their geographic ranges largely overlap, the latter two are rare, and little is known of their habitat or seasonality. *Tuber gibbosum* and *T. oregonense* are both common and abundant in western Oregon and Washington. The commercial harvest of Oregon white truffles is concentrated during late fall and winter when *T. oregonense* tends to reach maturity while *T. gibbosum* is typically only beginning to develop. Thus, in early November, it was more likely to have been *T. oregonense* rather than *T. gibbosum* that James Beard actually praised.

Like other *Tuber* species, *T. oregonense* appears to produce a single annual crop of sporocarps that require several months to reach maturity and to produce the aroma that is the source of their culinary value. The first immature sporocarps are often observed September through early October and reach full size between mid-October and early November. Undisturbed sporocarps in the soil do not produce noticeable aroma until sometime after they mature, indicated by darkening of the gleba from white to brown or dark brown. The aroma production lasts for a period of days prior to spoiling. The onset of natural ripening varies annually, from late November to late January. The conclusion of the season beyond which few

T. oregonense sporocarps are found varies from mid-February to mid-March. The seasonality of *T. oregonense* ripening also varies geographically, with its onset and conclusion varying by as much as a month from one locality to the next within a season.

As with other truffles, production varies annually with weather conditions affecting both number and size of sporocarps. Conditions within the geographic range of all Oregon truffles are consistently dry during midsummer. Precipitation and temperatures become more variable from late summer to early autumn, which appears to be the critical period when weather conditions affect truffle yields in western Oregon (Luoma 1991). Although no formal study of the relationship between weather conditions and production of any Oregon truffle species has been conducted, it is understood among harvesters that late summer rains and cool conditions tend to produce higher yields, while dry weather and unusually high temperatures extending into autumn tend to produce lower yields.

Tuber oregonense is found under a wide range of conditions in natural *Pseudotsuga menziesii* var. *menziesii* (Mirb.) Franco (Douglas fir) forests throughout its range in western Oregon and southwest Washington (Fig. 12.1c), but it appears to reach its greatest abundance under a relatively narrow range of conditions that are often easily and accurately recognized from great distances, including from aircraft and satellite imagery. Like many European truffle species that thrive on fallow agricultural land that has become overgrown with suitable host trees (Hall et al. 2007), Oregon truffles thrive in similarly anthropogenic habitats with a history of use as farm or pastureland that have been planted with Douglas fir. The most productive habitats tend to be created when Douglas fir is either planted with the intention of producing Christmas trees that are subsequently neglected or to convert abandoned farm or pasture into timberland. Less frequently, Oregon truffle habitat is created when Douglas fir is planted as an ornamental in residential or semirural settings or during restoration of riparian vegetation to enhance spawning habitat for anadromous fish. These afforested stands of Douglas fir develop a distinctive appearance and are frequently adjacent to open farmland making them conspicuous.

Because truffle spores are naturally transported by animals, dispersal in the short term is limited to the territorial reach of the various mycophagous animals that eat them (Jacobs and Luoma 2008). Perhaps as a result, Oregon truffles do not tend to be found in isolated stands of Douglas fir surrounded for some distance by open farmland or pasture and are more likely to be found in stands established near older existing Douglas fir that can provide a source for spore inoculum and small mammal vectors.

Tuber oregonense is found beneath trees of various ages, from 6 years or possibly younger in some managed Christmas tree orchards (Charles Lefevre, unpublished observation) to as old as 60 years (Chris Melotti, personal communication). They often fruit most prolifically in stands between the ages of 15 and 30 years, although there are notable exceptions. In one former Christmas tree plantation visited annually by the North American Truffling Society, production was first observed when the trees were 6 years old and reached significant yields

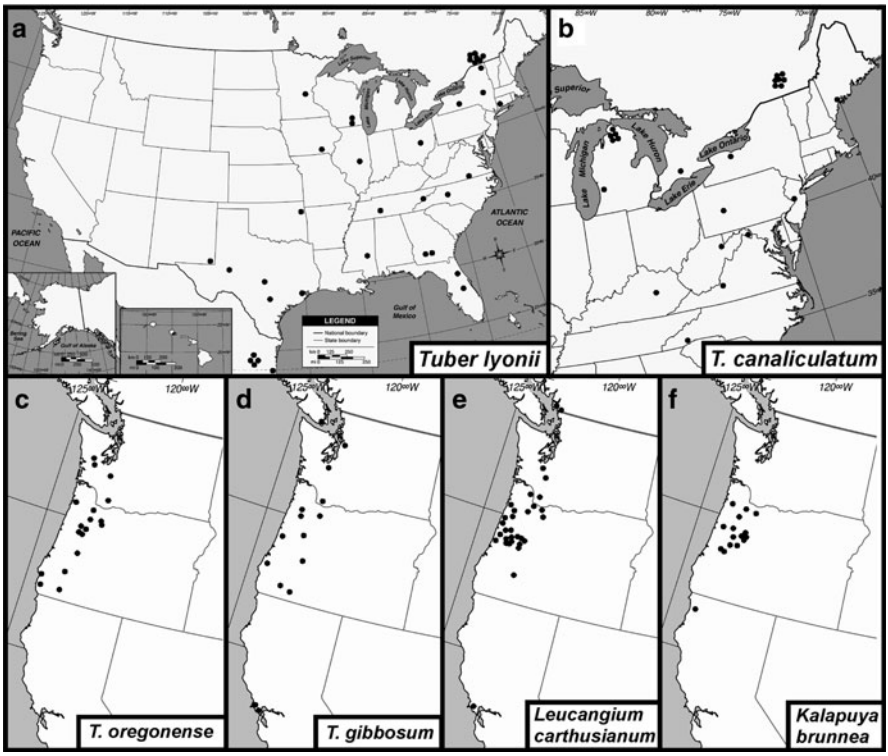

Fig. 12.1 Collection locations for (**a**) *Tuber lyonii* (modified from Trappe et al. 1996), (**b**) *T. canaliculatum*, (**c**) *T. oregonense*, and (**d**) *T. gibbosum* specimens examined in Bonito et al. (2010) and collection locations for (**e**) *Leucangium carthusianum* and (**f**) *Kalapuya brunnea* specimens held in the OSC herbarium

when the stand reached 8 years old (Paul Bishop, personal communication). The trees had been planted approximately 1.5-m apart and quickly become overcrowded. Truffle production also declined precipitously within 10 years of its onset in this closely planted stand, where stands planted at approximately 3-m spacing tend to both begin production later after the canopy closes and to produce truffles significantly longer (Charles Lefevre, unpublished observation). Examples like this suggest that stand densities may influence both the onset and duration of fruiting.

Oregon white truffles were long thought to associate exclusively with the coastal variety of Douglas fir, *P. menziesii* var. *menziesii*. However, *T. oregonense* has been observed fruiting beneath pure stands of both *Abies procera* Rehder (Margie Millard, personal communication) and *A. grandis* Lindl. (Ken Austin, personal communication) in overgrown Christmas tree plantations containing no *P. menziesii* within 100 m or more.

12.2.3 Tuber gibbosum, *Oregon Spring White Truffle*

The first immature *T. gibbosum* typically reach a size sufficient to easily locate them during the month of January, although some may be observed as early as October. They typically complete their growth within a month of becoming visible and ripen naturally over an extended period between January and mid-July with the majority ripening during the months of June and July.

Despite their development several months later than *T. oregonense*, their productivity follows a pattern similar to that of *T. oregonense* with highly productive years for one likely to also be highly productive for the other. This suggests that the productivity of both species is influenced by similar seasonal factors.

Tuber gibbosum reaches its greatest production in habitats similar to that of *T. oregonense*, and they are often found intermixed within the same stands of trees. As a result, immature *T. gibbosum* is often harvested with *T. oregonense*, which both contaminates the *T. oregonense* crop with immature *T. gibbosum* that have no capacity to ripen while simultaneously destroying the *T. gibbosum* truffle crop on that site. Use of trained dogs obviates this problem.

The principal host tree of *T. gibbosum* is *P. menziesii* var. *menziesii*, but they are known to occur in at least one pure stand of approximately 20-year-old *A. procera* planted as a Christmas tree orchard. The geographic range of *T. gibbosum* (Fig. 12.1d) differs from that of *T. oregonense* with collections from further north in NW Washington and British Columbia and further south in Northern California than any known collections of *T. oregonense* (Bonito et al. 2010).

Despite the fact that *T. gibbosum* is the name that has long been associated with Oregon white truffles, its harvest volumes is significantly lower than the more recently described *Tuber oregonense*. The relative disregard of *T. gibbosum* by harvesters may be explained by the fact that its harvest season coincides with the much larger morel and spring *Boletus* harvest in the Pacific Northwest. Some chefs who use *T. gibbosum* feel that it has the same appeal as *T. oregonense* (Jack Czarnecki, personal communication), although their aromas are not identical (Trappe et al. 2007).

12.2.4 Leucangium carthusianum, *the Oregon Black Truffle*

The Oregon black truffle, *Leucangium carthusianum* (Fig. 12.2a), received its culinary debut in the 1980s early in the development of the Oregon truffle industry (Lefevre et al. 2001). *L. carthusianum* was included in the study of hedonic response to Oregon truffles by Marin (1985) as a species with culinary appeal that was not harvested for culinary use at the time. In that study, panelists were significantly more likely to prefer the aroma of *L. carthusianum* over that of *T. gibbosum*, which may have contributed to its subsequent commercial exploitation. Despite their later introduction to culinary use, prices and harvest volumes of

Fig. 12.2 Truffle species with recognized culinary and commercial value indigenous to North America: (**a**) *Leucangium carthusianum*, (**b**) *Imaia gigantea* (photo courtesy of Todd Elliott), (**c**) *Kalapuya brunnea*, (**d**) *Tuber gibbosum*, (**e**) *T. oregonense*, (**f**) *T. canaliculatum* (photo courtesy of Gregory Bonito), and (**g**) *T. lyonii*

Oregon black truffles quickly surpassed those of Oregon white truffles (Lefevre et al. 2001; Schlosser and Blatner 1995).

The commercial raking of *L. carthusianum* takes place over a long season typically starting in October and concluding in May. In contrast to the commercial-raking season, collections from the Oregon State University herbarium (OSC) indicate that *L. carthusianum* fruits year round, and when dogs are used as the harvest method, ripe sporocarps are observed year round. Despite ripening throughout the year, the culinary quality of *L. carthusianum* appears to vary seasonally. Some harvesters, buyers, and chefs indicate that truffles producing the most appealing aromas tend to be more abundant during the spring.

Annual variation in *L. carthusianum* productivity follows patterns similar to white truffles, with cool and moist conditions in late summer and early autumn producing the highest yields and dry and/or hot conditions in late summer or early autumn producing poor yields. Black and white truffles do not respond identically to weather conditions, however, and there are occasional seasons when white truffles are plentiful while black truffles are scarce. Oregon black truffle yields generally appear to be more variable, from a near absence of sporocarps in some seasons to abundance in others. Oregon white truffle yields, in contrast, appear to be somewhat less variable and more reliable from the harvester's perspective.

Unlike *Tuber* species, *L. carthusianum* production appears to take place in a succession of flushes several weeks apart over the course of the season. The different flushes might represent wholly separate crops, but that seems unlikely given that yield trends established early in the season appear to characterize the entire season. Wholly separate crops, in contrast, might produce greater variation in yields from one crop to the next. This pattern of multiple flushes suggests that, like many other mushrooms, the crop develops in a series of cohorts arising from a single population of primordia (Stamets 2000). A transition from raking to using dogs will thus permit harvest of multiple flushes from the same site where raking early in the season tends to cause production to cease in that location for the remainder of the season presumably by disrupting resting primordia.

L. carthusianum was originally described from collections located beneath *Pinus sp.* in Chartreuse, France, but in North America, it appears to be exclusively associated with *P. menziesii* var. *menziesii* and is found west of the Cascade Mountains from northern California to southern British Columbia (Fig. 12.1e). Like *T. oregonense* and *T. gibbosum*, *L. carthusianum* thrives in anthropogenic habitats, particularly Douglas fir forests planted on former pasture or farmland. The most productive stands tend to be older than those producing the largest crops of white truffle species, and harvesters frequently observe that stands producing white truffles can undergo a transition with black truffles displacing white truffles over a period of years. Some harvesters speculate that black truffle inoculum may be introduced inadvertently or, in some cases, intentionally by the harvesters. Whether or not that is the case, *L. carthusianum* does appear to occupy a later successional niche in these afforested plantations and can occasionally be found in abundance within stands substantially older than 30 years (Dennis Morgan, personal communication).

L. carthusianum can also become highly productive in other habitat types, including areas that have never undergone a conversion to pasture or farmland (Dennis Morgan, personal communication; Stan Patterson, personal communication). Due to the secretive nature of truffle harvesting, the characteristics of other productive stand types are not well known. However, with the introduction of truffle dogs to forests of western Oregon and Washington, it is now apparent that *L. carthusianum* is more widespread than those areas where commercial harvests have taken place historically. Dogs are easily able to locate truffles too widely dispersed to be worth the indiscriminate effort of raking for them and may effectively enable profitable harvest over very large areas of forest that are not currently considered productive.

12.2.5 Kalapuya brunnea*, the Oregon Brown Truffle*

The Oregon brown truffle, *K. brunnea* (Fig. 12.2c), is the most recent addition to Oregon's truffle harvest industry. Like *T. oregonense*, it was harvested and sold for culinary use for a number of years prior to publication of its description and Latin binomial (Trappe et al. 2007, 2010). *K. brunnea* is less common and abundant than the other commercially important Oregon truffles, and its harvest is almost entirely a desirable form of "bycatch" by harvesters seeking *L. carthusianum* under young Douglas fir in western Oregon (Fig. 12.1f). Like all Oregon truffles, its habitat is not limited to the afforested plantations where most commercial harvesting tends to take place. Unlike the other Oregon truffle species, it is not known to associate with hosts other than *P. menziesii*. While relatively uncommon, it is highly regarded and receives prices comparable to, or slightly higher than, Oregon black truffles (Bryan McCormick, personal communication; Toby Esthay, personal communication).

The seasonality of *K. brunnea* follows a pattern similar to that of *L. carthusianum* with a long harvest season during which truffles ripen naturally in flushes from early autumn to late spring and possibly year round. Annual productivity also appears to be correlated with that of *L. carthusianum*, with relatively high production of both species some years contrasting with near absence in others. *K. brunnea* however is less abundant than *L. carthusianum* under all circumstances. Because *K. brunnea* tends to fruit in lower densities, the use of dogs greatly facilitates its harvest, and like *L. carthusianum*, the use of dogs will enable harvest of subsequent flushes from a particular site, where raking does not. The increased use of trained truffle dogs in Oregon may lead both to greater volumes of *K. brunnea* becoming available on the market and to a better understanding of its distribution and habitat.

12.3 Prospects for Cultivation of Native Oregon Truffles

As successful cultivation of European truffles has spread across the globe, interest in cultivation of native Oregon truffles has grown as well. Farmers are already establishing productive "orchards" without intending to do so suggesting that a crude form of cultivation is already possible simply by planting Douglas fir in the vicinity of existing stands and in the same widespread soil types (Pilz et al. 2009) where Oregon truffles already occur. Considering the predictability with which harvesters recognize and locate stands producing Oregon truffles, this simple emulation of the habitat may be a reliable way to achieve truffle production. However, the experience of harvesters also suggests that yields may remain highly variable both among stands and spatially within productive stands.

Several Oregon farmers have attempted to either introduce or enhance truffle production beneath established stands of young Douglas fir by broadcasting spores in a water suspension. Several widely publicized claims of success have gained

attention (e.g., Dubarry and Bucquet-Grenet 2001). Unfortunately, there are no reported attempts to conduct this sort of field inoculation using the controls necessary to measure a treatment effect.

Inoculation of Douglas fir roots with *T. oregonense*, *T. gibbosum*, and *L. carthusianum* has been conducted in the laboratory producing seedlings well colonized by ectomycorrhizae of the various truffle species (Charles Lefevre, unpublished data). Because the methods are not yet cost effective, no significant attempts have been made to establish orchards using seedlings inoculated under controlled conditions. Numerous growers have attempted a crude form of inoculation by dipping the roots of commercially available Douglas fir seedlings into truffle spore slurries, but no verifiable claims of success either in producing truffle ectomycorrhizae or in producing truffles within orchards established using this inoculation method currently exist.

The principal impediment to progress with cultivating Oregon truffles is their current relatively low commercial value and the relatively small economic impact of truffle production in the Pacific Northwest (Pilz et al. 2009). Cultivation of Oregon truffles may nevertheless be worth further investigation. Given low historic prices paid to harvesters on the order of USD $220/kg (Lefevre et al. 2001; Schlosser and Blatner 1995) and yields in naturally producing stands reaching 5–30 kg/ha (Lefevre et al. 2001), gross returns from naturally occurring, unmanaged *L. carthusianum* patches exceed those of many agricultural crops.

The natural production of Oregon truffles beneath Douglas fir trees also appears to be compatible with other simultaneous land uses with little or no additional establishment or management costs. Most sites where truffles are currently harvested are principally used for timber production, but there is also an example of commercial truffle harvests beneath Douglas fir planted in a riparian zone that serves to enhance salmon spawning habitat (Maxwell 2005a). Establishment of the afforested plantations where Oregon truffle production is greatest is precisely the kind of project that the Oregon Department of Forestry supports through the Forest Resource Trust as a way to offset CO_2 emissions (Cathcart 2000). In addition, restoration plantings designed to enhance salmon spawning habitat are currently funded by the Conservation Reserve Enhancement Program administered by the U.S. Department of Agriculture (USDA 2011).

The economics of farming Oregon truffles will naturally improve if the current trend toward higher prices paid to harvesters using trained dogs and employing more stringent grading standards continues. The transition to exclusive use of dogs may also increase efficiency of harvest and increase yields of black and brown truffles by protecting the primordia that comprise subsequent flushes. Similarly, the outlook for cultivation of Oregon truffles will improve if methods can be developed to consistently produce the 20–50 kg/ha yields observed in European truffle orchards (Bonet and Colinas 2001).

Management methods with potential to influence production of Oregon truffles include irrigation to emulate beneficial weather conditions in late summer, soil amendments to optimize nutrients and pH for truffle production, and stand density management. Planting density and thinning treatments both affect the speed of

canopy closure, the rate of litter layer buildup, and the crown ratio of the trees, among other factors that may influence the timing, abundance, and yield of truffle harvests. Apart from anecdotal reports of truffles reaching exceptional sizes on sites subjected to fertilization, lime application, and chemical weed control (Trappe 1990; Bonito et al. 2010), no other published reports discuss effects of forest management interventions on production of Oregon truffles.

12.4 Other North American Truffle Species with Culinary and Commercial Potential

At least three indigenous truffle species with notable culinary value are found in southern and eastern North America. These include *Tuber lyonii*, *Tuber canaliculatum* Gilkey, and *Imaia gigantea* (Imai) Trappe & Kovács. Though relatively unknown, their occurrence near many of North America's largest population centers and in the southern states has potential to generate significant culinary and cultural interest among local chefs and media. These species are harvested and sold for culinary use, although their annual harvest is currently insignificant.

Of these species, the best known is *Tuber lyonii*, the pecan or Texas truffle (Fig. 12.2g), which is found from central Mexico to southeastern Canada, roughly throughout the eastern third of the continent (Fig. 12.1a).

Tuber lyonii is associated with several host tree genera, including members of *Quercus*, *Crataegus*, *Tilia* (Trappe et al. 1996), *Corylus* (Bruhn 2007), and the pecan tree, *Carya illinoinensis* (Hanlin et al. 1989). Its fruiting season extends from March to February throughout its geographic range (Trappe et al. 1996), but it appears to vary in different regions. For example, the season for *T. lyonii* in south Georgia where it is currently collected for culinary and commercial purposes is limited to August through October (Smith et al. 2012). Like Oregon truffles, *T. lyonii* is frequently collected in natural forests but is also observed fruiting abundantly in anthropogenic habitats, most notably managed pecan orchards (Bonito et al. 2011; Hanlin et al. 1989), as well as ornamental plantings (Taber 1990), and as a contaminant in orchards of *Corylus avellana* L. inoculated with European truffle species (Bruhn 2007; Tom Michaels, personal communication). Many of these settings are routinely subjected to irrigation, fertilization, and chemical weed control. In orchards established for cultivation of the European truffles, *T. lyonii* is found in spite of heavy applications of calcium carbonate lime used to effect radical increases in soil pH. Its natural affinity for environments characterized by various horticultural interventions and broad climatic and edaphic latitude suggests that *T. lyonii* may be an excellent candidate for cultivation and potentially co-cropping with pecans, which has been discussed by Bonito et al. (2012) at greater length.

Like *T. lyonii*, the culinary value of *T. canaliculatum* (Fig. 12.2f) has been recognized for decades (e.g., Trappe 1990), although there are few reports of its

harvest by amateur mycologists or commercial harvesters. The geographic range of *T. canaliculatum* extends over most of the eastern United States (Fig. 12.1b), where it is associated with a broad range of host species (Trappe 1990), particularly members of the *Pinaceae* and *Fagaceae*. It is likely, given the scant records of its occurrence, that it is seldom prolific. However, as at least one harvester in Maryland has found, the use of trained truffle dogs may enable productive harvest of *T. canaliculatum* sporocarps otherwise too widely dispersed to be effectively located by other means (Jeffrey Long, personal communication). Seedlings of *Pinus taeda* have been successfully inoculated with *T. canaliculatum* under laboratory conditions (Gregory Bonito, personal communication), and similar to other commercially important truffle species, *T. canaliculatum* sporocarps have been found in the anthropogenic environment of a backyard garden (Donna Mitchell, personal communication).

The truffle species most recently introduced to culinary use in North America is *Imaia gigantea* (=*Terfezia gigantea* Imai) (Fig. 12.2b), a close relative of *L. carthusianum* (Kovacs et al. 2008). Its distribution is limited to disjunct populations in Japan and in the eastern United States (Kovacs et al. 2008; Trappe and Sundberg 1977). The principal habitat in the United States is an uncommon and ecologically sensitive environment in which commercial harvest, particularly using rakes, may be undesirable (Alan Muskat, personal communication). It is harvested for culinary use on a small scale in western North Carolina. Efforts to inoculate *Pinus* spp. seedlings L. with *I. gigantea* under laboratory conditions are underway (Alan Muskat, person communication), although experimental orchards remain to be established.

12.4.1 Cultivation of European Truffles in North America

As the Oregon truffle industry began to develop, farmers around the country began to plant orchards of *Corylus* and *Quercus* inoculated with *Tuber melanosporum* Vittad. The first fruiting of *T. melanosporum* outside of its natural habitat in southern Europe took place in 1987 (Bruce Hatch, personal communication) beneath 5-year-old inoculated hazelnut trees planted in Mendocino County, California (Bland 2010; Bruce Hatch, personal communication; Olivier et al. 1996; Don Reading, personal communication; Rigdon 1994). The production of truffles from the orchard was significant given its size (Rigdon 1994), and the orchard continued to produce truffles through 2007 (Bland 2010). Since that initial success, several other orchards in the United States have produced *T. melanosporum* sporocarps (e.g., O'Neill 2007), and as of March 2012, one orchard in central Idaho had produced the first *T. borchii* Vittad. sporocarps (Paul Beckman, personal communication). Orchards of trees inoculated with *T. aestivum* (both summer and autumn variants) and *T. magnatum* Pico are also established in the United States and Canada, but have not yet born fruit. The inoculated seedlings used in these orchards are produced by a growing number of nurseries in the United States and Canada, and new orchards are established at a rate of several hundred per

year. Most North American truffle orchards established to date are small with few exceeding 5 ha.

North American regions with winter and summer temperatures potentially suitable for cultivation of *T. melanosporum* occupy two large areas of the continent based loosely on AHS (1997) and climate maps included in Stamper and Koral (1979). One lies in a strip of relatively mild climate along the west coast extending from southernmost Canada to northernmost Mexico encompassing most of the area west of the Cascade mountain range and most of northern California but narrowing to the coast in central and southern California. This region also includes an extension inland to central Idaho along the Snake River Valley and a strip in California along the foothills of the Sierra Nevada range. The other major region forms a wedge with its point in New Mexico and extends to the east through the panhandle of Texas to encompass most of Oklahoma, Tennessee, and North Carolina. The northern boundary of this region includes southern Missouri, southernmost Illinois, much of Kentucky, southern West Virginia, and the non-mountainous areas of Virginia. The southern boundary of this region includes northern Arkansas, northern Mississippi, northern Alabama, north Georgia, and much of South Carolina. These boundaries are tenuous considering the imprecise knowledge of the truffles' climatic tolerances, as well as variation in meso- and microclimatic influences that may permit or prevent successful production of *T. melanosporum*. The northern boundary is also determined to some extent by the grower's tolerance for risk of frost damage to the truffle crop, which, for example, might extend the region into warmer sites in southern Indiana and southern Ohio for those growers who either take measures to mitigate winter temperature extremes or are comfortable with routine partial crop losses, and occasional total losses due to freezing conditions during the winter harvest season. Precipitation during the summer is insufficient to support reliable production of *T. melanosporum* throughout much of both major regions, and irrigation is generally advised by nurseries producing inoculated trees.

It is worth noting that climatic conditions in summer throughout much of both regions are significantly warmer than the climates in the natural habitat of *T. melanosporum* in southern Europe. Inclusion of these regions is based on successful production of truffles in several orchards in North Carolina where mean daily temperatures in summer exceed those in the producing regions of France, Italy, and Spain by 3 °C or more (Hall et al. 2007). The tolerance of *T. melanosporum* to summer heat is of less practical concern in Europe where the Mediterranean Sea interrupts the climate transect than it is in North America where large areas of the continent fall at latitudes lower than the southern limits of European *T. melanosporum* production. There is, for example, one report of modest *T. melanosporum* production at approximately 30° latitude in Dripping Springs, Texas (Price 2005), which, if borne out in additional orchards in similar summer climates, would greatly expand the area of potentially suitable climates into regions not only with warmer mean daily temperatures, but also warmer extremes and hot weather (daily high temperatures above 30 °C) exceeding 120 days over the course of the year (AHS 1997).

The regions of North America with climates potentially suitable for cultivation of *T. aestivum* and *T. borchii* cover a substantial portion of the continent, as both *T. aestivum* (Chevalier et al. 1978) and *T. borchii* (Hall et al. 2007) do in Europe. Based loosely on USDA (2012), AHS (1997), site-specific weather data available online (e.g., http://www.wunderground.com), and climate data compiled in Hall et al. (2007), the regions of North America where both species might be cultivated successfully largely encompass the climates suitable for *T. melanosporum* and extend into parts of the continent with somewhat colder winters, including parts of the Great Lakes region in the Midwest and Northeast, the southern Plains states, mountainous areas of the southwest and northern Mexico, and those regions of southern Canada with relatively mild winters on both coasts and in the Great Lakes region.

The calcareous soils required by *T. melanosporum* and *T. aestivum* are uncommon in those parts of North America with climates suitable for their cultivation, and most growers must apply lime at a rate of 60–90 tons/ha to their soils to raise the pH. While it is well documented that *T. melanosporum* and *T. aestivum* can be produced successfully in soils that have been subjected to major modification of pH (Hall et al. 2007), effects on soil biota, nutrient cycling, and truffle production resulting from lime applications on this scale are not well understood.

Other challenges facing growers of European truffles in North America include endemic fungal diseases of the principal host trees and mycophagous animals. The eastern filbert blight, *Anisogramma anomala* (Peck) E. Müll., has caused severe mortality of *Corylus avellana* inoculated with *Tuber melanosporum* in Mid-Atlantic region orchards, and it is likely to be problematic for truffle growers throughout the East Coast and Midwest regions where the disease is endemic, as well as the Pacific Northwest where it has naturalized within commercial hazelnut orchards. Sudden oak death disease, *Phytophthora ramorum* Werres & de Cock, has similarly caused severe mortality among *Q. ilex* Lour. trees that are naturalized in coastal California where truffle cultivation provides a natural complement to the wine industry. Throughout most of the Western United States, various rodent species commonly referred to as pocket gophers (family *Geomyidae*) may be uniquely problematic in truffle orchards where they not only browse the cambium layer of lateral roots and belowground portions of the stem, causing mortality among the host trees, but also consume truffles, which they are likely to encounter among the roots during the months of development and maturation prior to ripening. These as well as those challenges and uncertainties associated with truffle cultivation generally require research and subsequent modification of methods to adapt to local conditions.

12.5 Conclusion

The market potential for truffles in North America remains encouraging given growing culinary sophistication among American consumers as well as the "local" movement for sourcing food ingredients (Pilz et al. 2009). The United

States in particular has a unique opportunity to not only cultivate European truffles like other regions around the world but also to develop industries around the harvest and potential cultivation of indigenous truffles.

In Oregon and the Pacific Northwest, development of an industry based on the harvest of indigenous truffles is well underway, and with the introduction of truffle dogs, it is beginning to overcome negative perceptions resulting from sales of immature and unripe truffles. In spite of a difficult early history, the Oregon truffle industry has become a celebrated part of the larger wild mushroom industry in the Pacific Northwest and is promoted in conjunction with the wine and tourism industries to position the region as a culinary destination.

Wild production of Oregon truffles is currently abundant, due in large part to an abundance of suitable habitat. Sustaining a truffle harvest industry in the Pacific Northwest may eventually require cultivation of Oregon truffles. The habitats where Oregon truffles tend to be most productive are ephemeral and result from conversion of agricultural land to timber production; thus, trends in agricultural economics, rural land use taxation, and population demographics, among other human factors, become major influences on the long-term supply of Oregon truffles. If at some point these factors lead to a decline in the rate Douglas fir is planted on farmland, then production of Oregon truffles will also decline unless efforts are made to advance agricultural production of these species.

Culinary use of other indigenous truffle species has only just begun elsewhere on the continent. The increased availability of truffle dogs may both facilitate the development of these industries and help to prevent the kind of setback to their reputations suffered by the Oregon truffles.

Despite early success with cultivation of *T. melanosporum* in North America and promising market conditions (Pilz et al. 2009), cultivation of European truffles in orchards of inoculated trees has progressed slowly. The challenges faced by growers in North America include those of seedling quality assurance (e.g., Maxwell 2005b) and a general lack of agronomic expertise specific to truffles. The nascence of the industry is also reflected in the demographics of growers, relatively few of whom have agricultural backgrounds. This inexperience is often evident in a failure to effectively manage both competing vegetation and animal pests. Efforts to address these challenges are underway, and if the trajectory of increased professionalism in the North American wine industry can serve as a model, development of industry infrastructure, research funding, and technical expertise are likely to increase in parallel with increasing numbers of successful truffle growers.

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Chapter 13
Truffle Cultivation in China

Xianghua Wang

13.1 Current Status

Compared with Europe and North America, China has a much shorter history of scientific research on truffles. Truffles have been harvested by local people in China for many years, but the first scientific report of the genus *Tuber* in the country occurred late in 1980s. Mycologist B. Liu from Department of Biology, Shanxi University, gave the first record of truffles in China, *Tuber taiyuanense* B. Liu from Xishan, Taiyuan (the capital of Shanxi Province, after which the truffle was named) (Liu 1985). This small spiny-spored truffle, which has been placed in the Rufum clade (Wang et al. 2007), did not address commercial interests. In 1989 and 1992, respectively, *Tuber sinense* K. Tao & B. Liu was described as a new species and *Tuber indicum* Cooke & Massee was recorded from China for the first time (Tao et al. 1989; Zang et al. 1992). These two black truffles, which are highly similar to *Tuber melanosporum* Vittad. in appearance, motivated subsequent research in China on truffle ecology, taxonomy, phylogeny, and mycorrhizal relationships. Research on the cultivation of Chinese truffles followed.

García-Montero et al. (2010) gave a detailed review of research on truffles in China, which also mentioned progress on cultivation on truffles. Here we will not repeat the general content in the review, but will focus on more detailed introduction and discussion on the issues related with cultivation of truffles in China. Three parts will be included in this chapter: current status, review on relevant technology and knowledge, and advantages, challenges, and future research directions.

Truffle (*Tuber*) cultivation is a new topic in China. The earliest attempt to cultivate truffles in China started in 1987 in Taiwan (Hu et al. 2005). Six-month-old seedlings of *Cyclobalanopsis glauca* (Thunb.) Oerst. were inoculated with a

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spore suspension of *T. formosanum* nom. inval. (a small phylogenetic clade in *T. indicum* s.l.) collected in central Taiwan in 1987. One year later, the seedlings were transplanted into bigger containers and kept for another year in greenhouse before transplanting to the field. Two years before the transplantation, a site, later was used as truffle land, was limed with mixture of $CaCO_3$:$MgCO_3$ (1:1, mol/mol). In 1997, 8 years after the transplantation, the first fruiting occurred in the truffière, and the next year, ca 10-kg truffles were harvested from the 0.6 ha of truffière. This was the first successful cultivation of truffles in China.

Using similar methods and under the direction of H. T. Hu, researchers from Guizhou Academy of Forestry, Guiyang, in cooperation with a company, started cultivating *T. indicum* s.l. in 2002 in central Guizhou, a place with subtropical climate and moderately high elevation 1,000–1,500 m (Hu et al. 2010). Seedlings of two indigenous tree species (i.e., *Quercus aliena* Bl. and *C. glauca*) were inoculated by a spore suspension of *T. indicum* (1.6×10^5/ml). *Tuber* mycorrhizae formed 120 days after inoculation. One and a half years after the inoculation, the seedlings were transplanted to the field. Soil at this site is classified as yellow soil with a pH between 5.0 and 5.5. Before transplantation, the soil was limed with quicklime. In winter of 2008, five years after the transplantation, more than 1.5-kg truffles were harvested from some of seedlings of *Q. aliena* and one seedling of *C. glauca*. This is the first report of successful cultivation of truffles in mainland China.

The first report of successful cultivation of exotic truffle species in China was from Guizhou in 2008. Also cooperating with H. T. Hu from Taiwan, a company established a plantation of *T. melanosporum* near Guiyang (Longli County). At the end of 2008, the first yield, 1.0-kg ascomata of *T. melanosporum* were harvested from the truffière (Gong 2009). Although detailed information on the establishment process is lacking, it is likely similar to that of *T. indicum* and *T. formosanum*, since the truffière is at the same site as *T. indicum* s.l. mentioned above and is operated by the same research group and company.

There are several other truffle plantations established by different institutions in China, though they have not fruited yet. All of these plantations are situated in subtropical China. They include a 2-ha plantation in Cili County, northwestern Hunan, established by Hunan Academy of Forestry in 2001. This is a plantation of *T. melanosporum* established using inoculum introduced from France (Anonymous reporter 2009; Tan and Fu 2003); more than 130 ha plantations in Panzhihua, Sichuan Province, established by Panzhihua Academy of Agriculture and Forestry under the direction of researchers from IPLA (Istituto per le Piante da Legno e I'Ambiente, Italy) during 2006–2008 (Lin et al. 2008); two plantations near Chuxiong, Yunnan, established by Chuxiong Institute of Forestry and Yunnan Academy of Agriculture; five plantations near Kunming, Yunnan, established by Kunming Institute of Botany, Chinese Academy of Sciences, and Yunnan Academy of Agriculture, respectively, during 2008–2011; 2–3 plantations near Bijie, Guizhou, established by Kunming Institute of Botany, Chinese Academy of Sciences in 2008. Most of these plantations now are 3–6 years old.

To our knowledge, all of the seedlings used for these plantations were produced in China. Every institution which established the plantations above has their own

truffle nursery. The biggest nursery is owned by Panzhihua Academy of Agriculture and Forestry, Sichuan. In cooperation with IPLA, the academy produced almost 100,000 truffle seedlings in 2007, using the tree hosts *Castanea mollissima* Bl., *Pinus armandii* Franch., and *Pinus yunnanensis* Franch. (Lin et al. 2008). Hunan Academy of Agriculture owns a nursery that is able to produce 10,000 seedlings of *T. melanosporum* annually. This might be the biggest *T. melanosporum* nursery in China. Among the institutions producing truffle seedlings, two of them have registered and published the technology in State Intellectual Property Office of P.R. China. Although one of them has received an authorized patent (ZL200810058447.9), up to now there is no supply of commercial seedlings that are qualified by commercial standards in China. With the amount of truffle seedlings increasing year by year, strict quality assurance and control are becoming urgent in China.

13.2 Review of Relevant Technology and Knowledge

13.2.1 Taxonomy

Only black truffles have been cultivated in China. There are three groups of indigenous black truffles in the country: *T. indicum* group, *Tuber pseudohimalayense* G. Moreno et al. group, and *Tuber aestivum* Vittad. group, most of which are found from southwestern mountains, with elevation 1,500–2,500 m. Seven names have been used for the species of the three groups: *T. aestivum*, *T. formosanum*, *Tuber himalayense* B. C. Zhang & Minter, *T. indicum*, *T. pseudohimalayense*, *Tuber pseudoexcavatum* Y. Wang et al., and *T. sinense*. Among them, *T. aestivum*, a famous culinary truffle in Europe, was reported from the country very recently (Chen et al. 2005; Song et al. 2005). This black truffle is commercialized in southwestern China but with much less yield than *T. indicum* s.l. The Chinese material named as "*T. aestivum*" shows minor morphological differences from European material (Chen et al. 2005; Song et al. 2005; Zambonelli et al. 2012), but the close relationship between them is obvious. There is no report on the cultivation of Chinese "Burgundy truffle." Considering the high culinary value of European *T. aestivum/uncinatum* and close relationship between Chinese and European material, this Chinese counterpart is a logical candidate for cultivation.

Tuber pseudoexcavatum, a black truffle with excavated ascomata and spinoreticulate spores, is another truffle popularly commercialized and exported to Europe. It has similar commercial value as *T. indicum* group but has yet been cultivated. Mycorrhizal synthesis with this species on the European oak species *Q. ilex* L. has been reported by García-Montero et al. (2008). *Tuber pseudoexcavatum* was recently found to be conspecific with *T. pseudohimalayense* and should be treated as a synonym of the latter (Chen and Liu 2011; Manjón et al. 2009). In China, the truffle

is found under *P. yunnanensis*, which suggests a wide distribution in the Yunnan-Guizhou Plateau. There is interest in cultivating this species in China.

The truffles popularly cultivated in China belong to the *T. indicum* complex. It is this group that has stirred most debate and discussion both in China and Europe during the past two decades. Although many names have been applied to species in this complex (e.g., *T. formosanum*, *T. himalayense*, *T. indicum*, and *T. sinense*), researchers have reached a consensus that there are two separate phylogenetic species (clades) in the complex (Chen et al. 2011; Paolocci et al. 1997; Roux et al. 1999; Wang et al. 2006; Zhang et al. 2005). Since it is hard to find reliable morphological characters to define the two clades, the specific name that should be applied to each clade is still pending. The biggest barriers are the fact that morphological variations within one clade overlap those within the other clade, and up until now, sequences from type specimen are lacking (Chen et al. 2011).

Hu et al. (2005) used *T. formosanum* as the target species for cultivation. This is a black truffle which is included within the *T. indicum* B clade (Chen et al. 2011; Huang et al. 2009) and may represent a small geographical variety of the species in Taiwan. This truffle associates with *C. glauca*. It is the first truffle that was successfully cultivated in China. Chen (2003) also used ascomata of *T. formosanum* for mycorrhizal synthesis, but there is no subsequent report whether fruiting ever occurred. The Chinese truffles used by other researchers for cultivation or mycorrhizal synthesis are merely labeled as *T. indicum*, without further classification (Chen 2003; Geng et al. 2009; Hu et al. 2004, 2010; Lin et al. 2008). Since there was no detailed analysis on the identity of truffles used as inoculum, it is not clear which phylogenetic species (clade A or B) was used. According to our own experience, ascomata used for inoculations often come from markets and could have diverse origins; consequently, a mixture of the two species is most likely used for inoculations. Taking cost of identification into account, it is reasonable to believe that cultivation of *T. indicum* in China includes both species without critical sorting and may even include the unintentional inclusion of *T. pseudohimalayense*/*pseudoexcavatum*.

Besides black truffles, species of Magnatum clade, Gibbosum clade, and Puberulum clade also have high culinary value (Bonito et al. 2010; Hall et al. 2007). Although records of white truffles in China are increasing (Fan et al. 2011a, b; Garcia-Montero et al. 2010), to date there are no records of the Magnatum clade or Gibbosum clade in China. Ten white truffles were confirmed to be in China: "sp. 2," "sp. 3," and "sp. 4" in Wang et al. (2007), *Tuber excavatum* Vittad., *Tuber latisporum* Juan Chen & P. G. Liu, *Tuber lijiangense* L. Fan & J. Z. Cao (*Tuber* borchii var. *sphaerosporum* sensu Juan Chen & P. G. Liu), *Tuber liui* A. S. Xu, *Tuber polyspermum* L. Fan & C. L. Hou, *Tuber sinoexcavatum* L. Fan & Yu Li, and *Tuber zhongdianense* X. Y. He et al. (Chen and Liu 2007; Chen et al. 2008; Fan et al. 2011a, b; Wang et al. 2007). Although *Tuber borchii* Vittad. or *T. puberulum*-like species have been reported from China, the occurrence of *T. borchii* and *Tuber puberulum* Berk. & Broome in China seems doubtful, as morphology and DNA sequences generally distinguish European and Asian species (Chen and Liu 2007; Fan et al. 2011a; Wang 1988; Wang et al. 2007). In some markets in southwestern China, white truffles are sold unintentionally

mixed together with commercial black truffles. It is hard to assess the commercial value of the Chinese white truffles, for all of them are represented by very rare/small collections and there is no report on the edibility. Nevertheless, according to phylogenetic analysis, *T. zhongdianense* and *T. liui*, two truffles endemic to subalpine regions in southwestern China, could be good candidates for commercialization and cultivation due to their close relationship with *T. borchii* (Chen and Liu 2007; Wang et al. 2007).

13.2.2 Mycorrhizal Syntheses

Following methods popularized in Europe, cultivation of truffles in China began with mycorrhizal synthesis. The first mycorrhizal synthesis in China with truffles was made by H. T. Hu, National Taiwan University, Taipei, in 1987 by using *T. formosanum* (Hu et al. 2005). Spore suspension was inoculated on a Chinese fagaceous tree *C. glauca* with a dose of 1.2×10^5 spores/seedling. Hu et al. (2005) did not provide record when mycorrhizae began to form, but one year later, mycorrhizae were confirmed (Hu 1992; Hu et al. 2005), and the infected seedlings were then transplanted to establish a truffière.

After Hu's work, there were no more reports on Chinese truffle mycorrhizal synthesis until 2002. However, between 1997 and 2002, European researchers synthesized mycorrhizae by using truffles collected from China, either to obtain mycorrhizae for taxonomic comparison or identification or to test the ability of Chinese truffles to form mycorrhizae with exotic trees. Comandini and Pacioni (1997), Zambonelli et al. (1997), and Mabru et al. (2001) reported successful mycorrhizal synthesis between Chinese black truffles and European tree hosts. In total, mycorrhizae of *T. indicum* s.l. were produced on four European tree species: *Quercus pubescens* Willd., *Quercus cerris* L., *Pinus pinea* L., and *Corylus avellana* L., which indicates a broad host spectrum for the *T. indicum* complex.

During the period 2003–2009, researchers from southwestern China tried inoculating *T. indicum* s.l. on 16 tree species and successfully obtained mycorrhizae (Chen 2003; Geng et al. 2009; Hu et al. 2004, 2006; Lin et al. 2008). These tree species include the following: three pines (*P. armandii*, *Pinus massoniana* Lamb., *P. yunnanensis*), ten fagaceous trees (*C. glauca*, *C. mollissima*, *Castanopsis delavayi* Franch., *Castanopsis fargesii* Franch., *Cyclobalanopsis gilva* (Bl.) Oerst., *Cyclobalanopsis myrsinifolia* (Bl.) Orest., *Cyclobalanopsis nubium* (Hand.-Mazz.) Chun ex Q. F. Zheng, *Quercus acutissima* Carruth., *Quercus aliena* Bl., *Quercus fabri* Hance), two hazels (*Corylus* sp., *C. yunnanensis* (Franch.) A. Camus), and *Carpinus pubescens* Burk. More recently, Bonito et al. (2011) showed that *T. indicum* s.l. also established ectomycorrhizae with North American host species including pecan (*Carya illinoinensis* (Wangenh.) K. Koch) and loblolly pine (*Pinus taeda* L.). Lin et al. (2008) tried to inoculate *T. indicum* s.l. on two plants frequently occurring in the natural habitat of *T. indicum* s.l., *Coriaria nepalensis* Wall. and *Ficus tikoua* Burk., but failed to get mycorrhizae. In agreement with the European findings,

the work above confirms a very broad host spectrum for *T. indicum* s.l. Although most of these tree species have not been used for establishing truffière, the broad host spectrum suggests a feasibility of *T. indicum* cultivation in China in the future using a range of diverse host species.

Besides work on Chinese truffles, mycorrhizal synthesis between exotic truffles and Chinese trees were also tried in China. In 2002, researcher from Institute of Tropical Forestry, Chinese Academy of Forestry, Guangzhou, reported mycorrhizal synthesis between *T. melanosporum* and a Chinese tree, *Castanopsis hystrix* Miq. (Chen 2002). Mycelia of *T. melanosporum* introduced from France were used as inoculum. Inoculum was injected into the substrate 3 days after the seedlings were transplanted. Mycorrhizae formed 26 weeks after inoculation. However, according to the morphological description, abundant tapering cystidia that measured 100 μm long and had 2–3 septa grew on the surface of mantle, which did not seem to represent mycorrhizae of *T. melanosporum.* Later researchers from the same institute, Gong et al. (2003), reported successful mycorrhizal synthesis between *T. melanosporum* and five Chinese trees: *Castanopsis fissa* (Champ. ex Benth.) Rehd. & Wils., *C. delavayi*, *P. yunnanensis*, *P. massoniana*, and *Quercus variabilis* Bl. The methods are similar to that of Chen (2002). All of these trees formed mycorrhizae with *T. melanosporum*, with infection ratio of 90–100 %. Chen (2003) tried mycorrhizal synthesis by using Italian white truffle *T. magnatum* Pico in Guizhou. However, it is not clear if mycorrhizae were synthesized successfully.

By using the combination of *T. indicum* with *P. massoniana* and *P. armandii*, Hu et al. (2006) compared inoculating methods, spore density, and seedling age and evaluated different methods of inoculation on mycorrhizal formation. They found that the best way to pretreat the seedlings is to cut the main root of seedlings before new leaves emerge and then inoculate the remaining root system. They found that winter (November) is the best season for inoculation in Guizhou. Dose of inoculum is a very practical issue in producing truffle seedlings. Hu et al. (2006) also found that as the concentration of spores increased, the number of mycorrhizae is also increased. The highest spore numbers they used are 1.24×10^5/seedling for *P. massoniana* and 1.82×10^5/seedling for *P. armandii*. This is comparable with Hu et al. (2005) for *T. formosanum* (1.2×10^5/seedling) but more economical than that used by Geng et al. (2009) (5×10^7/seedling) and Hu et al. (2004) for fagaceous trees ($3.2–3.6 \times 10^6$/seedling). A detailed description of process was given by Geng et al. (2009), which can be summarized as follows: Aqueous spore suspension is prepared by blending chopped ascomata within water. Spore concentration is measured with a hemacytometer. Seeds are surface sterilized with H_2O_2 and sown in sterilized perlite/vermiculite. At the age of 3 months, seedlings are transplanted in container with substrate consisting of humus/vermiculite/peat moss or humus/soil/limestone, previously steam sterilized for 3 h. Substrates are limed before planting. Inoculum is incorporated (mixed, poured, or injected) into the soil substrates either before or after planting. Pre-inoculating in soil when sowing seeds is not recommended (Hu et al. 2006). This is the most popular procedure in producing truffle seedlings in China.

13.2.3 Ecology

13.2.3.1 Climate and Landscapes

Black truffles have been found in two regions of China: Taiwan and southwestern China. Nantou, the location in Taiwan, is characterized by a humid climate and an elevation of 1,000 m. Distribution of truffles in southwestern China is rather narrow, mainly in the adjacent localities between Sichuan and Yunnan. The most southern localities are situated south to Kunming. In southwestern China, truffle localities are characterized by rather high elevation (1,500–2,800 m) and expand across a subtropical monsoon climate zone (Liu et al. 2008; Su 2005; Tao and Liu 1990; Zhang and Wang 1990). The only fruiting plantation in mainland China is situated in central Guizhou, a mountainous place with elevation of 1,150–1,450 m, slope of 5–15 °C, and annual precipitation of 1,100 mm, essentially meeting the climatic and geographical attributes of natural truffle habitats. Most truffle localities are situated on 5–30° slopes and annual precipitation reaches ca 1,000 mm. This makes truffle hunting very hard, and truffle habitats easily undergo serious water and soil erosion when undue hunting styles are used. Actually, the situation has become quite serious during the past 15 years.

13.2.3.2 Soil

Shortly after *T. sinense* (= *T. indicum* s.l.) was described from China, researchers from China started to investigate the ecological traits of Chinese truffles, including soils. All of these investigations focused on *T. indicum* s.l. and in Sichuan and Yunnan, the only two provinces in mainland China where wild black truffles have been found. Most investigations found that the black truffles favor purple soils, which are calcareous and have a very broad pH range (5.5–8.5) (Chen et al. 1998; Su 2005; Yang et al. 2000; Zhang and Wang 1990). Tao and Liu (1990) and Lin et al. (2008) reported that black truffles also grow in yellow soils and acid red soils, respectively. Soil attributes are very rarely documented, except that Chen et al. (1998) provided some reference data: organic matter 1.43–5.32 (%), total N 0.085–0.375 (%), available P 5.8–49.6 mg/kg, and available K 149–388 mg/kg. Unfortunately, up to now, consistency of Ca^{2+} and Mg^{2+}, which is the key attributes for truffle soil, has not been well documented. However, based on the distribution of truffle and parent rock, it is reasonable to suppose high levels of Ca^{2+} in truffle soils. An interesting experience is that in our field investigations, truffles are often found near quarries, which suggests rather rich calcareous matter in the soil.

Soils in the first fruiting truffière in China were analyzed by Hu et al. (2005). Since they had been limed before transplantation, the soils were characterized by high pH values (6.3 ± 1.1) and levels of Ca^{2+} (91 ± 73 ppm). The first fruiting truffière in mainland China were in yellow soils with clay loam or sandy clay loam and an original pH of 5.5–6.0 (Longli, Guizhou) (Hu et al. 2010). Liming with

quicklime or $CaCO_3/MgCO_3$ may aid fruiting of truffles in the present, but the potential for sustainable long-term yields has not been assessed.

13.2.3.3 Host Plants

Black truffles can be found both in pure stands of *P. yunnanensis* and *P. armandii* and mixed forests with fagaceous trees. Three coniferous trees are found to be associated with *T. indicum* s.l.: *P. yunnanensis*, *P. armandii*, and *Keteleeria evelyniana* Mast. in natural habitats (Su 2005; Zhang and Wang 1990). Mycorrhizal synthesis confirmed the symbiotic relationships between *T. indicum* s.l. and the two pines (Chen 2003; Geng et al. 2009; Hu et al. 2004; Lin et al. 2008). *Pinus yunnanensis* is the most common pine in central and north Yunnan and southern Sichuan. *Pinus armandii* is a five-needled pine, distributed through north and central China to central and north Yunnan. *Keteleeria evelyniana* is a coniferous tree endemic to Yunnan. The wide distribution of these host trees implies a potential broader distribution of black truffles in China.

Besides the confirmed host trees, in natural habitats of black truffles, the following trees and shrubs also frequently occur: *Quercus acutissima* Carruth., *Quercus franchetii* Skan, *C. mollissima*, *C. delavayi*, *Coriaria sinica* Maxim., *Vaccinium bracteatum* Thunb., *Phyllanthus emblica* L., and *F. tikoua* (Chen et al. 1998; Su 2005; Tao and Liu 1990; Zhang and Wang 1990; our own investigation). Among them, some fagaceous trees, such as *Q. acutissima*, *C. delavayi*, and *C. fargesii* can form mycorrhizae with *T. indicum* s.l. in artificial conditions (Hu et al. 2004; Liu et al. 2008). Since black truffles often grow in mixed woods, these trees may also be mycorrhizal partners in the wild.

The frequent occurrence of *Alnus* spp. and *Coriaria* spp. in the natural habitats of black truffles is interesting and informative. Recent investigations on bacterial communities in ascomata of *T. magnatum* detected nitrogen-fixing bacteria and nitrogen-fixing activates within truffle (Barbieri et al. 2010; Chap. 8). As we have known, some trees of *Coriaria* and *Alnus* also have nitrogen-fixing bacteria inhabiting in their root systems. It is possible that bacteria shared by the ascomata of black truffles and trees of *Coriaria* and *Alnus* play an important role in the life cycle of both the fungi and the plants. Future research is needed on the application of helper bacteria to mycorrhization and cultivation of truffles.

13.3 Advantages, Challenges, and Future

The successful cultivation of truffles in China, although still at a limited scale, demonstrates the possibility for cultivating truffles in mountainous regions of China. During the past almost 25 years, seven institutions have conducted truffle cultivation trials and have a number of truffle plantations in the country. It is hopeful that some of these plantations will see the first fruiting in the coming 3–5 years.

Truffle seedling production is well developed, and some basic data on host trees, soil, and distribution have been obtained. The broad host spectrum of Chinese black truffles could facilitate the selection of trees for cultivation and help to establish diverse truffle plantations. In southwestern China, especially in its southern and eastern parts where typical karst landscapes are well developed (southeastern Yunnan, Guizhou, northern Guangxi), soil between rocks is poor for crops. However, these soils are rich in Ca^{2+} and may be ideal sites for truffle cultivation. The vast mountainous regions in central and southern China will make truffle cultivation prosperous. Cheaper land and labor will make Chinese truffle products more competitive in international markets if standards and quality are maintained.

At the same time, China faces big challenges in truffle cultivation. Although initial successes have been made, the sustainability of truffle harvesting practices and quantities remain open questions. There is no continuous record on the productivity of truffles for the first truffière in Taiwan. We noticed that in the first truffière in mainland China, quicklime was used to increase the pH of soil. Since efficiency of quicklime could be exhausted out in short time, the stability of annual yield may not be sustained. To our knowledge, most plantations in southwestern China are close to natural forests with ectomycorrhizal hosts or are situated on land that used to be natural forests. This does not meet the standard for truffle cultivation, which recommends that fields are free of roots from other ectomycorrhizal hosts. However, due to the large population in China and shortage of arable land, it is hard to find optimal truffle sites in mountainous regions in China (or even plains).

Most truffle plantations in China cultivate *T. indicum* s.l. at a slim profit margin. China does not have a strong truffle culture, and truffles in China have a small market. The major markets for Chinese truffles are still in Europe, supplemented by Japanese and American markets. In Europe, Asian black truffles are regarded as inferior in taste and flavor than *T. melanosporum* and even as fraud (Mabru et al. 2001; Paolocci et al. 1997). The price of Chinese truffles exported to Europe varies with season and supply but normally within the range of $50–100/kg. Compared with *T. melanosporum* and even *T. aestivum/uncinatum*, whose prices are more than ten times this, the profit of cultivating indigenous truffles in China is rather low. According to Lefevre and Hall (2001), annual yields of 15–20 kg/ha are considered good. Most Chinese plantations are still unproductive and reliable data on yields are lacking, but according to Hu et al. (2005), the yield of *T. formosanum* is 10 kg/0.6 ha. Although this yield seems reasonable, because of the lower value of this species, this yield brings 1/10 the profit that a plantation of *T. melanosporum* with the same yield.

There are two ways to improve the profitability of truffle cultivation in China. First, truffières should be established using economic species of trees. In southwestern China, two economic trees that are able to produce black truffles are *P. armandii* and *C. mollissima*. Both of these tree species are used by the local people for nut production; therefore, they offer an effective supplement for the value of truffles. A second approach for improving the profitability of truffle cultivation in China is to cultivate European truffle species such as *T. melanosporum* or *T. aestivum*. Some experiments have shown that some Chinese tree species can

Fig. 13.1 Immature *Tuber indicum* hunted by digging the soil

become well colonized by *T. melanosporum* (Gong et al. 2003). One plantation in Guizhou has already demonstrated the possibility to grow European truffles in China, though yields are still small (Gong 2009). The cultivation of European truffles in New Zealand and Australia demonstrate the possibility for developing a truffle industry with these species outside their native localities.

From a comprehensive view, truffle cultivation in China is not only a matter of money but also a matter of environmental protection. It should be considered as a huge project with multiple functions. In mountainous China, plant crops are not allowed to be planted on slopes $>30°$, but forestation is allowed. The government compensates the farmers for giving up the lands and provides seedlings to the farmers for reforestation. All truffle seedlings now produced in China use indigenous trees, well adapted to the local soil and climate. With help from mycorrhizae, these seedlings can tolerate and adapt to stressful habitats. In such cases, planting truffle-inoculated seedlings could provide multiple functions: harvesting truffles and helping to recover the vegetation. The "two-in-one" function of planting truffle seedlings will make the reforestation in mountainous regions easier to accept. The ecological significance of truffle cultivation in China provides additional value to such an effort.

Another factor, which may not be the most technical but will be the final step to guarantee success of truffle cultivation in China, is the use of truffle dogs for harvesting. Black truffles ripen from December to March in China. However, hunting truffles begins early in August. Local people usually use pickaxe to dig truffle land, and hunting truffles in China is laborious and destructive (Fig. 13.1). According to fragmentary reports of media, yield of black truffle in Panzhihua, "home of truffles in China," has decreased to 30–40 tons from 200 tons in 1990s. Even worse, since harvesting is too early to get mature products, the quality of truffles is seriously compromised. Tan and Fu (2003) investigated the quality of ascomata sold in three local markets in southern Sichuan and found that at most half

of the ascomata were mature and in good shape. Because immature truffles lack the aroma and flavor of mature specimen, the reputation of Chinese truffles suffers by such practices. Without truffle dogs, it is hard to imagine how truffle plantations can provide products of high quality, how they can be maintained, and how truffle yields can be sustained. It is reported that in Panzhihua, Sichuan, under the direction of researchers from IPLA, trials of training truffle dogs have been successful (Lin et al. 2008). Some researchers in Yunnan are also starting projects to train truffle dogs. It may take time for the local farmers to accept and adopt the use of truffle dogs, but good demonstrations on the value of truffle dogs for increasing truffle harvests, quality, truffle value, and profit are likely to facilitate their adoption.

As a country with a short history of truffle cultivation, China still has a long way to go. Truffle soil, a key factor in truffle cultivation, is poorly documented. There is still limited data available on feasibility or site selection. High-quality truffle-inoculated seedlings, a critical component in successful truffle cultivation, have not been qualified by strict commercial standards. To improve the profit of truffle cultivation in China, further mycorrhizal syntheses between European culinary truffles and Chinese trees need to be conducted. When establishing plantations, experimental designs should test the effects of limestone amount, limestone composition, fertilization, irrigation, planting density, and mulching. Experience on weed control, tilling, and pruning in truffle orchards is also lacking in China. Adopting best management practices from Europe and other regions and popularizing the use of truffle dogs in China may take time or even financial support. Nevertheless, a growing number of farmers, companies, and research institutions are showing enthusiasm for this new industry in China.

13.4 Conclusions

Although China has a much shorter history of scientific research on truffles, the country has seen the successful cultivation of *T. indicum* s.l. and *T. melanosporum*. At least ten plantations have been established in the country. Private and public enthusiasm for truffle cultivation is increasing. A series of relevant investigations on taxonomy, ecology, and mycorrhizal synthesis on black truffles have provided important data on their cultivation. Chinese black truffles *T. indicum* s.l. have a broad host spectrum and are adapted to diverse soils. Mountainous regions in southern China have suitable climate, soil, and host trees for truffle cultivation. Truffle cultivation in China should be regarded as a project with economic and ecological functions. Broad area, diverse hosts, low cost, and multiple functions are advantages for developing truffle cultivation in China. However, in order to make the industry sustainable and profitable, it is urgent to set strict standards and qualifications for seedling production, plantation establishment, and harvesting methods. Time-consuming research needs to be conducted to improve the quality of plantations in China. Truffle cultivation in China needs better integration between individual farmers, research institutions, and the government.

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Chapter 14
Terfezia Cultivation in Arid and Semiarid Soils

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14.1 Introduction

The genus *Terfezia* is formed of hypogeous fungi belonging to the family *Pezizaceae* within the order *Pezizales* (Norman and Egger 1999; Percudani et al. 1999; Laessøe and Hansen 2007). The hypogeus ascocarps of this genus are edible and are well known as desert truffles due to the nature of their distribution, which is typical of countries or territories with arid and semiarid ecosystems in the Mediterranean region, Middle East, and Southwestern Asia.

Terfezia species form mycorrhizal symbiosis with different annual and perennial rockrose species of the genus *Helianthemum*, including chamaephytes, hemicryptophytes, and therophytes (Table 14.1). Consequently, this species grows in open, sunny scrubland, or in the meadows of mountain plains, or sandy and rocky soils of arid deserts.

Since the first plantation of *Terfezia* mycorrhizal plants was established in 1999 in Murcia (Spain), most of the data related to the biotechnological aspects of the production of mycorrhizal plants and plantation management practices have been compiled in two publications of Springer (Morte et al. 2008, 2009). However, the increasing demand for this crop, not only in Spain but also in other countries, has prompted the research for new strategies to help pass from experimental scale to medium- to large-scale cultivation. One consequence of this leap, the creation of a spin-off company of the University of Murcia, called Thader Biotechnology S.L., has been necessary to satisfy the increasing demand and new data and processes, which are summarized in this chapter.

Since 1999, *Terfezia claveryi* Chatin has been cultivated throughout southeastern Spain and specific places in Israel, Abu Dhabi, and Argentina, and all plantations

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Table 14.1 Different mycorrhizal associations, obtained under controlled conditions, between species from the *Terfezia* and *Helianthemum* genus

Terfezia species	*Helianthemum* species	References
T. boudieri	*H. salicifolium*	Awameh et al. (1979)
T. claveryi		
T. nivea		
T. pinoyi		
T. leptoderma	*H. salicifolium*	Dexheimer et al. (1985)
T. claveryi		
T. leonis	*H. sessiliflorum*	Roth-Bejerano et al. (1990)
T. arenaria	*H. guttatum*	Fortas and Chevalier (1992)
T. leptoderma		
T. claveryi		
T. claveryi	*H. almeriense*	Morte et al. (1994)
T. claveryi	*H. ledifolium*	Gutiérrez (2001)
T. terfezioides	*H. ovatum*	Kovács et al. (2003)
T. claveryi	*H. violaceum*	Morte et al. (2009)
T. claveryi	*H. hirtum*	Torrente et al. (2009)
T. boudieri	*H. sessiliflorum*	Slama et al. (2010)
T. claveryi	*H. canariense*	Andrino et al. (2011)

have been established with mycorrhizal plants produced by our technology (Morte et al. 2008, 2009). More recently, experimental results have been obtained in Tunisia with *Terfezia boudieri* Chatin, using *Helianthemum sessiliflorum* (Desf.) Pers as a host plant (Slama et al. 2010). Desert truffle fructification occurs 1 or 2 years after plantation, depending on seedling quality, site suitability, and management practices, which are a critical factor for the regularity of truffle production. This chapter describes all the experiments carried out to improve desert truffle production.

14.2 Production of Mycorrhizal Plants

14.2.1 Plant Propagation

The first step in the production of mycorrhizal plants is to choose a suitable host plant species. Among the plant families cited in the literature that contain some species which form mycorrhiza with desert truffles are the following: Cistaceae (Awameh et al. 1979; Alsheikh 1984; Roth-Bejerano et al. 1990; Morte et al. 1994; Zaretsky et al. 2006), Fagaceae (Díez et al. 2002), Pinaceae (Díez et al. 2002; Honrubia et al. 2007), Fabaceae (Kovács et al. 2003), and even Cyperaceae (Ammarellou and Saremi 2008). However, only perennial and annual species from *Helianthemum* genus belonging to the Cistaceae have been reported as host plants for *Terfezia* mycorrhization (Table 14.1). For this purpose, we selected *Helianthemum almeriense* Pau, *Helianthemum violaceum* (Cav.) Pers., *Helianthemum canariense* (Jacq.)

Pers, and, more recently, *Helianthemum hirtum* (L.) Miller and *Helianthemum lippii* (L.) Dum.Cours. as host plants for large-scale production.

Most species of the host plant *Helianthemum* display erratic seed germination, and seed scarification is necessary to increase germination rates (Pérez-García and González-Benito 2006). Moreover, high mortality of the germinated seedlings has been observed during the first 2 months after germination in nursery conditions. Therefore, micropropagation techniques have been used for plant production since seed germination and plant survival with these techniques are both around 90 % (Morte et al. 2008, 2009). The in vitro micropropagation protocols of the studied *Helianthemum* species are quite rapid (about 10 weeks) because plant multiplication, elongation, and rooting occur in the same subculture. Consequently, they are also cheap because only a small amount of plant growth regulators and labor are required (Morte et al. 2009).

However, the acclimatization stage from in vitro to ex vitro conditions is the step in which many plants, above all of *H. hirtum* and *H. lippii*, do not survive. To solve this problem, we have developed a photoautotrophic *Helianthemum* micropropagation system (Andrino et al. 2011) based on the methodology described by Kozai (1991). Until now, the conventional *Helianthemum* technique has been carried out, using culture vessels with agar containing nutrients and sucrose as a carbon source for the plantlets at a low photosynthetic photon flux (PPF) (Morte and Honrubia 1992, 1997; Morte et al. 2009; Torrente et al. 2009). The in vitro environment of this micropropagation system is characterized by a high relative humidity (RH), a high ethylene concentration, still air, and a low CO_2 concentration in the vessel during the photoperiod (Fujiwara and Kozai 1995). This in vitro environment is entirely different from the ex vitro environment of the greenhouse, and it often causes the malfunction of stomata, poor epicuticular wax development, elongated shoots, low chlorophyll concentration, the hyperhydration of plantlets, low growth rates, little rooting, callus formation at the base of explants, and a low survival percentage (Kozai 1991; Majada et al. 2002; Serret et al. 1996). The photoautotrophic micropropagation technique overcomes these problems and is defined as micropropagation without sugar in the culture medium, in which the growth or accumulation of carbohydrates of cultures is fully dependent upon photosynthesis and inorganic nutrient uptake (Kozai 1991; Zobayed et al. 2004).

The photoautotrophic *Helianthemum* micropropagation system mainly consists of replacing agar by autoclave-sterilized perlite that is watered with MS medium nutrient solution (Murashige and Skoog 1962) and without a sugar source. Simply removing sugar from the culture medium without increasing PPF and CO_2 concentration inside the vessel would not promote the growth of culture or plantlets (Xiao et al. 2011). For this reason, the lids of vessels are replaced by transparent holed plastic caps (Fig. 14.1a).

Plant culture growth takes place directly in the greenhouse, with the following environmental conditions: a temperature between 16 °C and 26 °C, relative humidity (RH) of 30–65 %, 350–400 ppm of CO_2, a light intensity range of 95–295 mol m^{-2} s^{-1}, and 0.1–0.7 m s^{-1} wind speed.

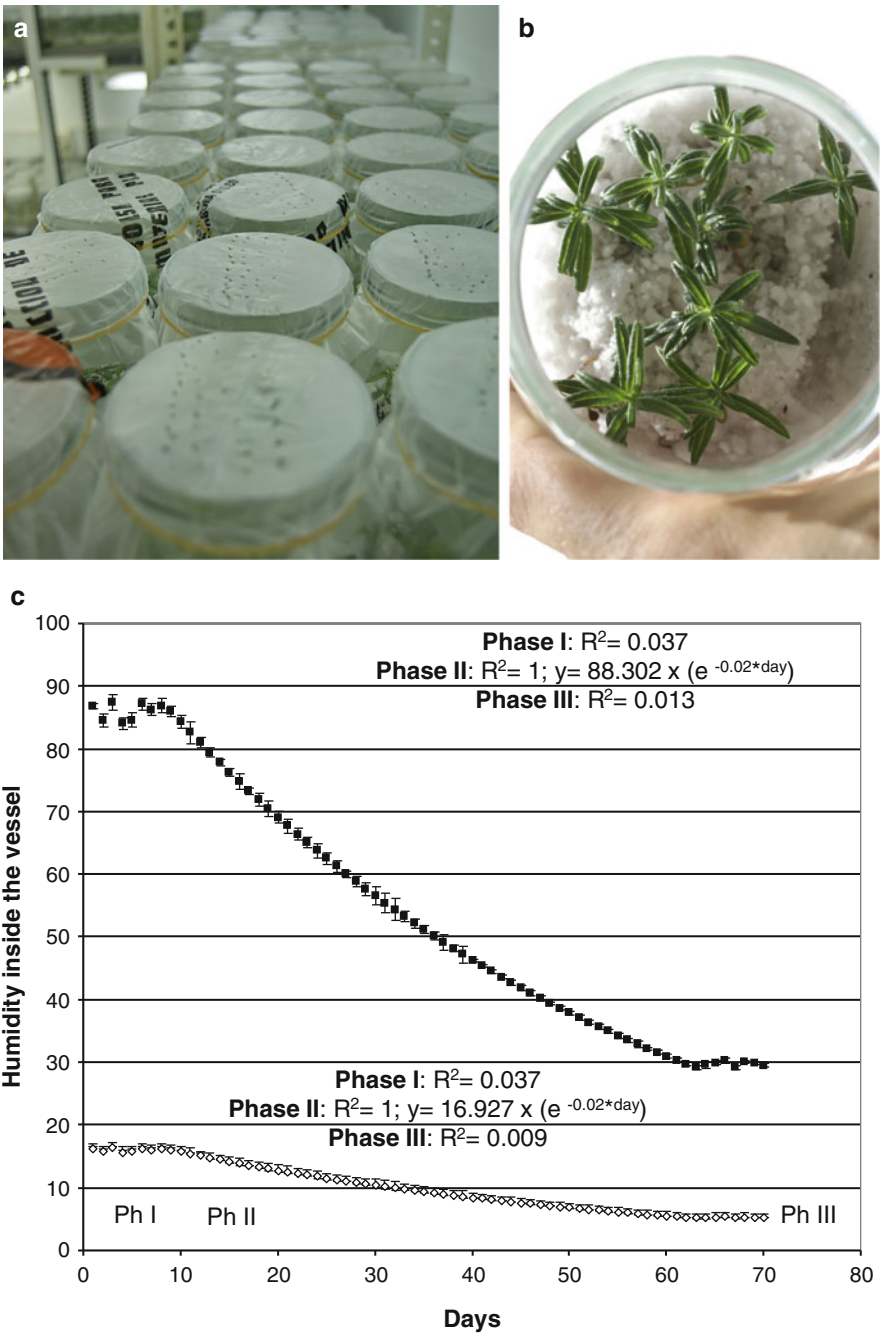

Fig. 14.1 (**a**) Photoautotrophic *Helianthemum* micropropagation system. (**b**) *H. almeriense* plants growing in perlite prior to the mycorrhization inoculation. (**c**) Phases of gradual hardening inside

After the rooting stage, the first holes are opened in the plastic cover (Fig. 14.1a). The size of the holes in the plastic ranges from 0.5 to 1 mm to facilitate the loss of RH inside the container. Their number will be determined by the vessel volume. The gas exchange balance is given by the internal vessel conditions (mainly CO_2, O_2, and RH) and external conditions (mainly CO_2, O_2, RH, and room wind speed) measured in the growing area in the greenhouse. The results show that RH loss with this method describes an exponential curve (Fig. 14.1c).

By opening holes in the plastic covers or using gas-permeable membrane disks, air diffusion or natural ventilation of the culture vessel can be improved and the CO_2 concentration inside the vessel increased during the photoperiod, resulting in enhanced photosynthesis, an increased growth rate, and hence a shorter production period (Cui et al. 2000; Kitaya et al. 2005). Meanwhile, relative humidity inside the vessel is reduced, which leads to increased transpiration and nutrient and water uptake by the plantlets. Numerous studies have shown the benefits of using gas-permeable membrane disks to enhance plantlet growth and quality as a result of increased vessel ventilation rates (Xiao et al. 2011). However, to minimize costs, we preferred to open holes in the plastic covers rather than to use gas-permeable membrane disks.

CO_2 can be a limiting factor for plant growth during the light phase. The use of perforated plastic caps versus normal in vitro culture containers avoids low CO_2 concentrations during the light phase and high concentrations during the dark one. Different gas concentrations (CO_2, O_2, and H_2O) are exchanged by passive diffusion between the perforated container and the outside atmosphere, until both are in balance. Thanks to a limited but necessary wind speed within the growing areas, renewal of the gaseous environment inside the container is favored. Due to a gradual adaptation to decreasing ambient RH, *Helianthemum* seedlings start to gradually control their stomas during the container cycle and not only during the acclimation phase. Coupled with the proper regulation of leaf transpiration, the roots are functional from the moment of emergence.

The rooting and photosynthetic ability of plantlets is usually affected by the physical and chemical nature of the supporting material (Zobayed et al. 2000). When agar was used as supporting material, the roots of *Helianthemum* species were usually thin and fragile. These roots were often damaged during transplanting, resulting in low growth or death of the plantlets. The use of porous supporting materials, such as perlite, improves the root zone environment and increases the

Fig. 14.1 (continued) the container for *H. almeriense*, *H. violaceum*, and *H. canariense* species. Humidity inside the vessel was registered as absolute humidity (*open diamonds* g/cm^3) and relative humidity (*filled square* RH %). These moisture values belong to the culture conditions 18–22 °C, 30–35 % outside RH, 360 ppm of CO_2, 150–170 µmol m^{-2} s^{-1}, and 0.3–0.5 m s^{-1} room wind speed. During phase II (when first holes have been opened), absolute humidity (g cm^{-3}) describes an exponential equation $y = 16.927 \times (e^{-0.02 * day})$. In the case of relative humidity (RH %), an exponential equation is described during phase II: $y = 88.302 \times (e^{-0.02 * day})$. Humidity values (RH %, g cm^{-3}) are the mean of 180 diary data taken every 8 min. *Bars* indicate standard error (SE)

oxygen concentration around the root system, which improves root development and enhances water and nutrient absorption by the plantlets. Moreover, the extensive root system produced in vitro appears to contribute to the higher survival percentage of plants during acclimatization to greenhouse or field conditions (Xiao et al. 2011). In addition, the use of perlite in this stage will facilitate the subsequent mycorrhizal inoculation (Fig. 14.1b).

This production system is not aseptic but fairly clean and insect-free. Moreover, photoautotrophic plantlets grown at high light intensities would be better suited to the intense irradiance found in sunlight, which also facilitates their mycorrhization.

14.2.2 Mycorrhizal Inoculation

Two types of *Terfezia* inocula have been used successfully to produce mycorrhizal plants: spores and mycelium (Morte et al. 2008). However, mature spores are used more frequently than mycelium due to the slow growth of the latter in vitro.

The best growth media for *Terfezia* mycelium growth are MMN (modified Melin-Norkrans) agar medium and PDA (potato dextrose agar) medium. The pH should be adjusted to 7.0 if the ascocarps are from alkaline calcareous soils. Desert truffle mycelium can be used directly from the plates as inoculum for in vitro mycorrhizal synthesis (Morte et al. 1994; Morte and Honrubia 1995, 1997) and from liquid fermentation for both in vitro and in vivo inoculation (Morte et al. 2008, 2009). However, only *Terfezia* strains well adapted to in vitro conditions should be used to produce mycelium by liquid fermentation in a bioreactor. A recent study on in vitro mycelium cultures of two mycorrhizal desert truffles in conditions of water stress demonstrated that *Terfezia* mycelium (strain TcS2) grows better under slight water stress (−0.45 MPa), which could improve the production of this mycelial inoculum in a bioreactor (Navarro-Ródenas et al. 2011).

Spore suspensions are made taking into account the maturation of the spores. A spore suspension from mature ascocarps consists of 6 g of dried and scratched ascocarps per liter of distilled water. This spore solution is shaken overnight (12 h). Instead of inoculating the plants directly with this spore solution, the spore solution is added to the perlite allowing spore adhesion to the pores and cavities within. Using such a mixture uses between 6 and 10 g of spores per liter of inoculum (Morte et al. 2008), which is approximately 3.5–4.5 $\times$ 10^5 mature spores per plant. The percentage of inoculum per plant is 5 % of the final container volume.

For the production of desert truffle mycorrhizal plants, five in vivo and in vitro options were designed, the time required for each of them ranging between 5 and 9 months, depending on the type of plant propagation system and inoculum source used (Morte et al. 2009) (Table 14.2). In addition, the new photoautotrophic *Helianthemum* micropropagation system proposed here (option 6, Table 14.2) allowed this time to be reduced to 3 months since fungal inoculation is carried out at the moment plants are transferred from in vitro to ex vitro conditions so that plant acclimatization and mycorrhization occur at the same time. Moreover, this

Table 14.2 Options by in vivo and in vitro methods to produce *Terfezia* mycorrhizal plants and the time required for each, depending on the type of plant propagation system and inoculum source used

Option nº	Plant material (*Helianthemum*)	Fungal material (*Terfezia*)	Time for plant production (months)	Time for plant mycorrhization (months)	Total time (months)
1	In vivo germinated seedlings	Mature spore solution	6	3	9
2	In vivo germinated seedlings	Mycelial suspension	6	1–2	7.5
3	Acclimatized micropropagated plants	Mature spore solution	4	3	7
4	Micropropagated plants	Pieces of agar with mycelium	3	2	5
5	Micropropagated plants	Mycelial suspension	3	2	5
6	Photoautotrophic micropropagated plants	Mature spores or mycelium in perlite	2	1	3

last option 6 has other advantages like (1) reduced fungal inoculum, (2) high survival percentage/smooth transition to ex vitro environment, (3) elimination of plant physiological disorders, (4) increased annual productivity per floor area, (5) reduction in labor costs, (6) simplification of the micropropagation system, and (7) no unwanted contamination due to the absence of sugar in the medium.

After the acclimation phase, seedling irrigation is established to maintain the pot water potential between −15 and −30 kPa in nursery conditions. Irrigation management is vital to prevent pathogens that could compete with the mycorrhizal symbiosis. Approximately 30–40 days after transplanting, it is necessary to make a mycorrhization quality control. With this production system, mycorrhization rates range between 75 % and 85 % after 2 months.

14.2.3 Certification of Desert Truffle Mycorrhizal Plants

Characterization of the mycorrhiza formed in the *Helianthemum* root systems by the different *Terfezia* species is extremely important to ensure the high quality of mycorrhizal plants (Morte et al. 2009). For this reason, a morphological and/or molecular analysis of the mycorrhiza should be carried out before planting. Such characterization is also important to evaluate the permanence of the mycorrhiza in field conditions.

The morphological evaluation process consists of examining the entire root system by binocular microscope, observing the abundance and condition of mycorrhizal root morphotypes (Gutiérrez et al. 2003). At this stage, the analyst should examine any root tips of doubtful identification by staining the roots (with 5 % blue ink in acetic acid or 0.01 % acid fuchsine solution). *T. claveryi* with *H. almeriense* forms an endomycorrhiza in natural field conditions, an ecto- and ectendomycorrhiza without a sheath in pot cultures, and an ectomycorrhiza with a characteristic sheath and Hartig net in vitro (Gutiérrez et al. 2003; Morte et al. 2008). Therefore, culture conditions can induce changes in mycorrhiza morphology, and there is no clear barrier between these two main types of mycorrhiza organization in *Helianthemum* species (Gutiérrez et al. 2003).

Certification of plant lots for colonization by *Terfezia* mycorrhizae is a destructive and laborious process, but it is important to sample a minimum number of plants to statistically test the percentage of mycorrhizal plants. We suggest examining 12 plants for each lot of 1,000 plants and consider as good a mycorrhization percentage of over 33 % of root system. *Terfezia* mycorrhiza has no problems with other contaminant mycorrhizal fungi due to its host specificity.

Moreover, molecular identification of the *Terfezia* mycorrhiza is very useful for evaluating the permanence of the mycorrhiza in field conditions. Due to the high number of ITS sequences from different desert truffles currently available in molecular databases, it has been possible to design specific primers for this purpose (Kovács et al. 2008).

14.3 Plantations and Desert Truffle Production

Since the establishment of the first *Terfezia* orchard in 1999 in the province of Murcia (Spain), more than 20 plantations have been established, not only in Spain but also in Israel, Argentina, and UAE (Abu Dhabi), for which around 30,000 mycorrhizal plants have been produced by our group.

In the last 10 years, carpophores have fructified yearly, and production has increased because of suitable land management techniques and irrigation (Morte et al. 2008, 2009). The application of such plantation management is necessary to maintain desert truffle production because without it, plantations could lose their productivity after 2 and 3 years (Morte et al. 2008). Even so, desert truffle production fluctuates from one year to another in the same orchard. These fluctuations could be due to other environmental or soil conditions, such as temperature and relative humidity, which influence any crop production in the field.

Among the factors that most influence desert truffle production are water availability (irrigation), weed management, season of planting, soil characteristics, and the frame of the plantation.

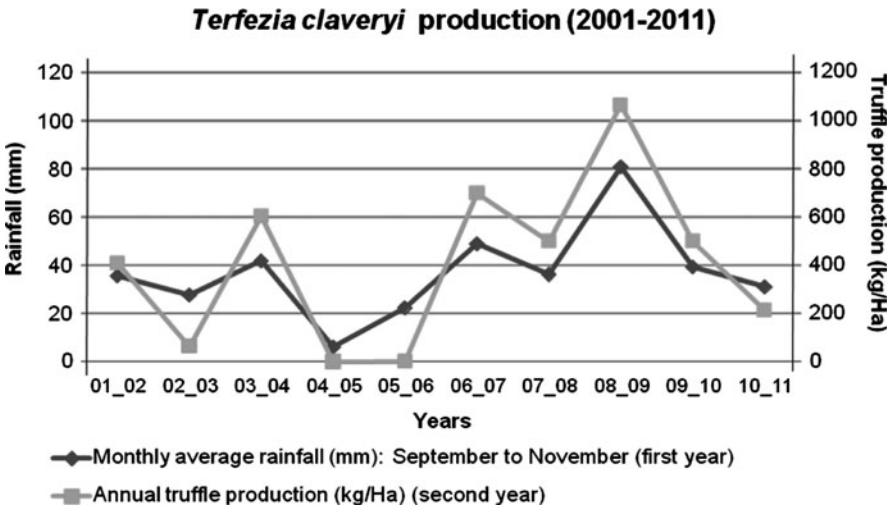

Fig. 14.2 Relation between *T. claveryi* ascocarp production and rainfall over a period of 10 years, in an orchard established in 1999 with 60 *H. almeriense* mycorrhizal plants

14.3.1 Water Availability

Irrigation is one of the most important factors for maintaining successful cultivation since desert truffle fruiting depends on rainfall (Morte et al. 2008).

The estimated desert truffle production in natural areas varies between 50 and 170 kg ha^{-1} in the province of Murcia, after years with a rainfall of between 350 and 400 mm (Honrubia et al. 2003). An irrigation system in the plantation is not necessary when the rainfall is available because the mycorrhizal association is well adapted to arid and semiarid climates (Morte et al. 2000). However, this natural desert truffle production dramatically decreases or even disappears when the rainfall is less than 150 mm, and so irrigation should be applied in dry years.

After following *T. claveryi* production for 10 years in an orchard established in 1999, we observed a statistical correlation, according to Pearson's test (coefficient value of 0.940), between the amount of precipitations during autumn (September, October, and November) of a year and the *T. claveryi* truffle production the following year (Fig. 14.2). This correlation fits a lineal equation with $R^2 = 0.87$.

This new and important finding will help maintain desert truffle production after dry years, by enabling us to adjust soil water potential to the plant physiological parameters necessary to keep the mycorrhizal symbiosis productive.

The main question is whether these fungi, known as "desert truffles," are able to resist dry conditions by themselves or due to association with the host plants. To answer this question, the effect of water deficit on the *T. claveryi* mycelium and on its symbiosis with *H. almeriense* was studied.

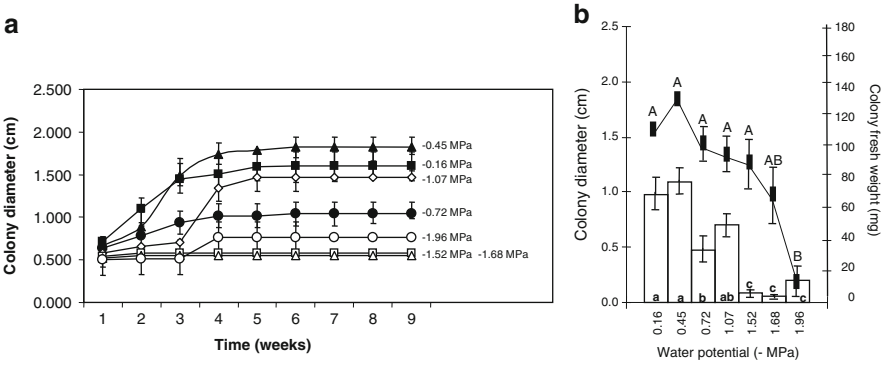

Fig. 14.3 (**a**) Mycelial growth (cm) of *T. claveryi* depending on time (weeks) at different water potentials (*filled square*–0.16, *filled triangle*–0.45, *filled circle*–0.72, *open diamond*–1.07, *open square*–1.52, *open triangle*–1.68, *open circle*–1.96 MPa). Values are the mean of six replicates. *Bars* indicate SE. (**b**) Effect of water potential on the diameter (*bars*, *lower case*) and fresh weight (*lines*, *upper case*) of *T. claveryi* colonies, grown on PEG-amended liquid MMN medium, after 9 weeks. Values followed by the same letter are not significantly different ($P \leq 0.05$) according to Tukey's test. (From Navarro-Ródenas et al. 2011, courtesy of Springer)

14.3.1.1 Effect of Water Deficit on In Vitro Mycelium Cultures of *T. claveryi*

The ability of *T. claveryi* to tolerate water stress was assessed in a pure culture (Navarro-Ródenas et al. 2011). Growth under low water potential conditions, induced using polyethylene glycol (Coleman et al. 1989; Mexal and Reid 1973), should, in theory, reflect the ability of the fungi to grow in dry soil and possibly to obtain water for the associated plant. Growth curves for the different water stress treatments showed an initial lag phase followed by an exponential growth phase and a period of maximum growth, before growth slowed and the colony finally became inactive (Fig. 14.3). The initial colony diameter of the control treatment (−0.16 MPa) exceeded that of the stress treatments; however, as time progressed, the colony diameters in the −0.45 MPa treatment (Fig. 14.3a) were greater than the control. Significant differences were found between two groups of both treatments [−0.45 > −0.16 > −1.07 MPa] and [−1.96 > −1.52 = −1.68 MPa] (Fig. 14.3a). For *T. claveryi*, growth inhibition was higher when expressed in terms of colony diameter than colony fresh weight (Fig.14.3b).

A relationship between fungal fresh weight and hyphal extension (diameter growth) could not be clearly established for *T. claveryi* (Fig. 14.3b). This observation agrees with those of Coleman et al. (1989) who reported that the relationship between fungal dry weight and hyphal extension may not be consistent for each species and can vary depending upon growth conditions. In our case, the lack of a clear correlation between the colony fresh weight and the colony diameter in

T. claveryi mycelium at −1.52, −1.68, and −1.96 MPa can be explained by the fact that, under these conditions, the mycelium grows more in thickness than in length (Fig. 14.3b). This increase in colony density with increased stress explains the type III pattern observed for *T. claveryi* (Coleman et al. 1989).

The isolate of *T. claveryi* exhibited a type III pattern characteristic of drought-tolerant species like *Cenococcum geophilum* Fr. (Coleman et al. 1989) and *Rhizopogon roseolus* (Corda) Th. Fr. (Duñabeitia et al. 2004). However, *T. claveryi* and *Picoa lefebvrei* (Pat.) Maire (Navarro-Ródenas et al. 2011) were only tolerant of moderate water stress below −1.07 MPa, similar to *Rhizopogon luteolus* (Duñabeitia et al. 2004), but they did not tolerate severe stress as other mycorrhizal ascomycete fungi could (Bois et al. 2006).

Terfezia claveryi and *P. lefebvrei* both undergo an extended lag phase before entering the exponential growth phase when grown under stress. Estimating growth rates over a shorter period (1–3 weeks) favored the control, which rapidly entered exponential growth. Estimating growth rates over longer periods (6–7 weeks) favored the stress treatments because fungi under stress have time to acclimate while growth in the control ceases (Navarro-Ródenas et al. 2011). Tolerance to water stress may result from the ability of the fungus to adjust osmotically during stress. The extension of the lag phase with increasing water stress may represent a period of osmotic adjustment (Coleman et al. 1989).

Moreover, soluble and cell wall-bound alkaline phosphatase (ALP) activities were higher when *T. claveryi* mycelium was grown under water stress (−0.45, −0.72, −1.07 MPa), and this increase was significantly different from to the control treatment (−0.16 MPa) at −1.07 MPa and −0.45 MPa for soluble and cell wall-bound ALPs, respectively (Navarro-Ródenas et al. 2011). The increased ALP activity observed in desert truffles at moderate water stress with respect to the control indicates the functional adaptation of these mycelia to drought conditions. Yet, they were able to use the phosphorus from the medium, which becomes more insoluble as water stress increases.

In arid and semiarid soils, the hydrolysis of organic phosphorus is predominantly mediated by the activity of fungal enzymes (Yadav and Tarafdar 2003). *T. claveryi* ascomata have a 2.8-times higher ALP activity than acid phosphatase (ACP) activity (Navarro-Ródenas et al. 2009). As ACP activity was not detected in *T. claveryi* mycelium (unpublished data), such activity can also be considered an indicator of the metabolic activity in these desert truffles.

It is not clear whether the drought tolerance of fungi in pure culture is transmitted to associated host plants. Parke et al. (1983) found no relationship between pure culture experiments and seedling experiments. A positive relationship between pure culture experiments and seedling experiments was observed between radiate pine seedlings and *R. roseolus* (Duñabeitia et al. 2004 and Ortega et al. 2004, respectively). The higher ALP activity in the water-stressed *T. claveryi* mycelium could be related with P accumulation in the drought-stressed mycorrhizal host plant (Morte et al. 2000).

14.3.1.2 Effect of Water Deficit on Desert Truffle Symbiosis

The effect of drought stress on the growth and water relations of the mycorrhizal association of *H. almeriense* with *T. claveryi* has been studied under nursery conditions with plants grown in pots (Morte et al. 2000). In that work, the greater tolerance of mycorrhizal plants to water stress was partially attributed to specific physiological mechanisms based on the chlorophyll content and gas exchange (Morte et al. 2000). Recently, a similar study of the association *Helianthemum sessiliflorum* (Desf.) Pers and *Terfezia boudieri* Chatin grown in pots demonstrated that mycorrhization alters plant physiology, increasing CO_2 assimilation rates and water use efficiency, which helps mycorrhizal plants to adapt to the harsh environmental conditions of deserts (Turgeman et al. 2011).

However, these studies on water deficit in mycorrhizal plants were conducted with potted plants under controlled greenhouse conditions, but recently, new work has been carried out in plots under field conditions (Morte et al. 2010). In this assay, 40 mycorrhizal plants and 40 non-mycorrhizal plants were transplanted to an experimental site in Espinardo (Murcia, Spain). Plant spacing was arranged in a 1×1-m square pattern. However, only mycorrhizal plants survived the experimental period, with a survival rate of 90 %; none of the non-inoculated plant survived to 9 months from planting. Apparently, *H. almeriense* plants are strongly dependent on the presence of a fungal symbiont in their roots for survival (Morte et al. 2010).

After acclimation, two irrigation treatments were applied to the 40 surviving mycorrhizal plants from March 2009 to May 2009. A control treatment (20 well-irrigated plants) maintained a soil matric potential (Ψ_m) of between -10 and -30 kPa (monitored with Watermark tensiometers placed at a depth of 30 cm). A water deficit irrigation condition was created (20 drought-stressed plants), in which irrigation was withheld during the study period, reaching a Ψ_m of around -120 kPa at the end of the experiment.

After the irrigation treatments, drought stress significantly affected the mycorrhizal colonization percentage, which was 70 % in nonirrigated mycorrhizal plants and 48 % in irrigated mycorrhizal plants. This effect was not observed in the potted *H. almeriense* plants where drought stress did not change the degree of root mycorrhizal colonization (Morte et al. 2000). However, no significant differences in plant growth were observed between nonirrigated and irrigated mycorrhizal plants before and after drought stress (Morte et al. 2010).

At the end of the experiment (May), most nonirrigated plants had lost all their leaves. Those watered during the dry season did not lose their leaves. Flowering for *H. almeriense* occurs in several waves (3–5) and lasts for 1 week. Flowering intensity was also similar at the end of the experimental period for both treatments (7.3 and 7.0 capsules/shoot for well-irrigated and drought-stressed mycorrhizal plants, respectively), suggesting that water deficit does not lead to more intense flowering. According to several authors, water stress in many ornamental plants may affect the flowering process, resulting in higher flowering intensity (Sánchez-Blanco et al. 1998; Nicolás et al. 2008), lower flowering intensity under severe

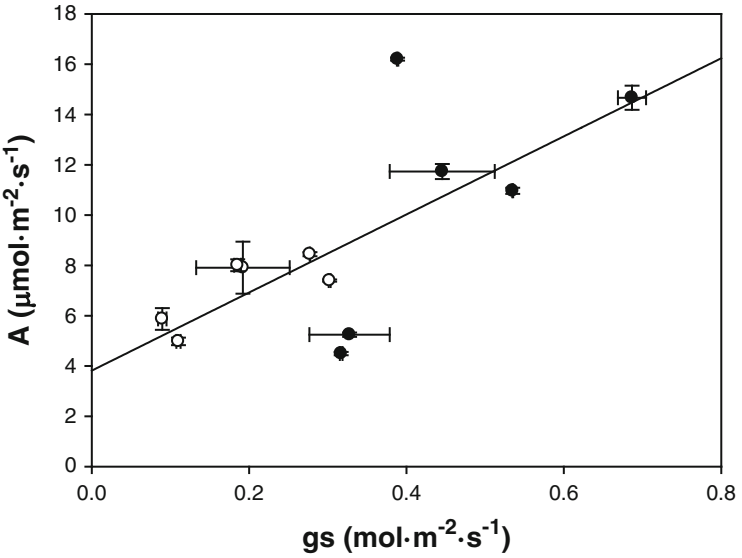

Fig. 14.4 Relationship between net photosynthesis (A) and stomatal conductance (g_s) of well-irrigated (*closed circles*) and drought-stressed (*open circles*) *H. almeriense* mycorrhizal plants at the end of the experimental period. Each point is the mean of three measurements per plant. *Bars* indicate SE. The data fitted a linear regression analysis ($A = 4 + 15g_s$, $R^2 = 0.51$, $P < 0.05$) (from Morte et al. 2010, courtesy of Springer)

drought conditions, or may be unaffected under moderate water deficit conditions (Sánchez-Blanco et al. 2009).

Stomatal conductance was more sensitive to water stress than photosynthesis. At 500 μmol m^{-2} s^{-1}, the irrigated M plants almost reached maximal stomatal conductance (0.41 mol m^{-2} s^{-1}). Nonetheless, the drought-stressed mycorrhizal plants showed a lineal response that reached less than half the maximum values (0.11 mol m^{-2} s^{-1}) (Morte et al. 2010). This stomatal conductance decreased twofold under the drought-stress conditions compared to the control mycorrhizal plants under irrigation and under light saturating conditions. What this finding indicates is the important stomatal closure that takes place under water deficit and low radiation conditions, which improved water use efficiency in the plants grown under drought conditions (Morte et al. 2010).

Moreover, a linear relationship was observed between leaf conductance and net photosynthesis, which suggests the absence of stomatal limitation in the levels of net photosynthesis under irrigated or drought conditions (Fig. 14.4).

It is well known that mycorrhizal fungi can influence water uptake ability and water use efficiency in host plants. On the one hand, the ability to maintain open stomata and photosynthesis during drought could increase the carbon supply for growth, particularly for new root growth, which requires current photosynthates (van den Driessche 1987). An extra supply of carbon is also required for truffle fruiting, and for this stage, most of the carbon derived from photosynthesis should

be allocated to mycorrhizal roots. Therefore, the inoculation of *H. almeriense* plants with *T. claveryi* provided the plant with a greater capacity to tolerate limited soil water availability.

No relationship was found between g_s and shoot water potential (ψ), which exhibited near-isohydric stomatal behavior in the face of a developing water deficit (Morte et al. 2010). The assumed primary role of this mechanism is the avoidance of a low water potential, which leads to xylem cavitation (Jones and Sutherland 1991). *H. almeriense* is a chamaephyte which loses its leaves to avoid drought and high temperatures in summer; however, if watered in the dry season, it is does not lose its leaves, which is probably due to the stomata being capable of preventing ψ from dropping below a critical threshold, such as the turgor loss point or the onset of xylem embolisms (Tyree and Sperry 1989; Franks et al. 2007).

H. almeriense, like many other Mediterranean perennials, shows a conservative water use strategy, based mainly on the avoidance of drought stress by reducing the transpiration rate, that is, by reducing stomatal conductance: the lower the soil water potential, the drier the atmospheric conditions. The results show that mycorrhizal *H. almeriense* plants are able to maintain good physiological parameters at low soil matric potentials (around −120 KPa) in field conditions, thus making them an alternative agricultural crop in arid and semiarid areas.

Two years after plantation, only the *H. almeriense* plants subjected to drought produced *T. claveryi* truffles (three in May 2011). Plants irrigated to water field capacity (−30 KPa) did not produce any ascocarps. This means that an excess of water irrigation would inhibit truffle production in the field, and a certain level of water deficit may actually stimulate the development of *T. claveryi* mycorrhizae, while elimination of the water deficit reduces that stimulus.

14.3.1.3 Molecular Base of Drought Tolerance

Finally, in an attempt to understand which molecular mechanisms are involved in the drought tolerance of desert truffles, we characterized cDNAs from *T. claveryi* and identified a sequence related to the aquaporin (AQP) gene family (Navarro-Ródenas et al. 2012). AQP are water channel proteins that facilitate and regulate the passive movement of water molecules along a water potential gradient. These proteins belong to the large major intrinsic protein family of transmembrane proteins and are represented in all kingdoms (Kruse et al. 2006; Zardoya 2005).

We cloned an AQP gene from *T. claveryi* (*TcAQP1*) (GenBank accession number JF491353) and made a functional analysis by heterologous expression in yeast, finding that this gene increases both water and CO_2 conductivity in biological membranes (Navarro-Ródenas et al. 2012).

The regulation of *TcAQP1* gene expression was tested by quantitative real-time PCR in free-living mycelium under different drought-stress conditions induced with polyethylene glycol (PEG) and in *H. almeriense* mycorrhizal roots cultivated under in vitro conditions (Navarro-Ródenas et al. 2012). When the regulation of *TcAQP1* expression in *T. claveryi* mycelium grown in in vitro culture media supplemented with PEG was evaluated, it was found that *TcAQP1* expression increased at moderate water stress (−0.45, −0.72 MPa) with respect to the control.

However, the *TcAQP1* expression decreased at higher water stress levels (−1.07, 1.52), when it showed similar expression values to the control treatment (Navarro-Ródenas et al. 2012). It seems likely that the active transporter mechanisms of *T. claveryi* mycelium contribute to maintaining cell turgor until an external water potential of −0.74 MPa is reached, at which point *TcAQP1* expression would help in turgor maintenance, facilitating water inflow. However, upon stronger water stress, ion pumping would be unable to maintain an adequate osmotic potential and *T. claveryi* mycelium would reduce its aquaporin expression to avoid loss of turgor. This bimodal behavior in *TcAQP1* expression with respect to external water stress demonstrates its role in improving the drought-stress tolerance of the mycelium.

In mycorrhizal roots of in vitro grown *H. almeriense* plants, the expression of three genes, *TcAQP1*, *EF1-alfaII* (JF491354), and *18S rRNA* (AF206926), was measured. The last two are constitutively expressed in the fungus and in the plant and can be used to normalize *TcAQP1* expression values. In this sense, the difference in the cycle threshold (Ct) values between *TcAQP1* and *EF1-alfaII* gave the *TcAQP1* expression rate, whereas the difference in the Ct values between *EF1-alfaII* and *18S rRNA* illustrated the amount of living mycelium colonizing the root and could be regarded as a measure of the degree of colonization. We observed that *TcAQP1* expression changes as a function of mycorrhizal root colonization. *TcAQP1* expression was seen to depend on the degree of colonization, with basal levels of expression when the colonization degree was low (about 16.2 %) and significantly higher levels when colonization increased (about 26.5 %). Therefore, *TcAQP1* expression was seen to be regulated to some extent during root colonization by the mycelium, which demonstrates the importance of this membrane channel in the mycorrhizal symbiosis. However, further studies are necessary to elucidate the precise role of *TcAQP1* in the symbiosis. One of the possible functions of mycorrhizal symbiosis could be related to its capacity to increase CO_2 permeability (Navarro-Ródenas et al. 2012).

It has been reported that the mycelium of many edible fungi can develop fruit bodies depending on the CO_2 concentration (Stamets 2000). Furthermore, in the cross talk between plant and fungus, the fungal responses to both root exudates and CO_2 concentration appear to define the transition from the asymbiotic to presymbiotic developmental stage (Bécard and Piché 1989). TcAQP1 is the first fungal MIP reported to have a CO_2 transport function. As in the case of other fungi, *T. claveryi* could use CO_2 as a signaling molecule in mycelium organization for ascoma formation or even for the growth and recognition of the root of its host plant, in which TcAQP1 could facilitate its passage through the membrane. This finding opens opportunities for research into the molecular mechanisms of recognition between symbionts (Bonfante and Genre 2010).

14.3.2 Weed Management

Competition from weeds has been shown to reduce desert truffle production (Morte et al. 2009). To reduce the impact of herbaceous competition, weeding is necessary,

Table 14.3 The ranges of the soil characteristics in which most of the *Terfezia* plantations have been made in Spain

Physical–chemical soil properties	
pH (1:2.5 water)	6.8–8.7
E.C. 1:5 (μS/cm)	123.1–302
Sodium (meq/100 g)	0.15–1.20
Potassium (meq/100 g)	0.28–1.5
Calcium (meq/100 g)	4.94–23.38
Magnesium (meq/100 g)	0.95–3.7
Organic matter (%)	0.58–3.92
Total organic carbon (%)	0.34–2.28
Total nitrogen (%)	0.058–0.267
C/N ratio	2.8–10.15
Total carbonate (%)	5.1–80.1
Active lime (%)	3.4–24.76
Phosphorus (ppm)	7.52–66.4
Chloride (meq/100 g)	0.05–0.09
Sulphates (meq/100 g)	0.01–0.32
Iron (ppm)	1.79–79.5
Copper (ppm)	0.31–2.73
Manganese (ppm)	3.03–57.12
Zinc (ppm)	0.3–3.12

at least once a year, in order to avoid plant competition for water and to maintain the open and sunny desert truffle ecosystem.

Two different weed control methods were used: a mechanical tilling between rows with cultivator tines set at 5–8 cm deep and the application of a commercial systemic glyphosate-based herbicide at half the recommended dose. In field conditions, the application of glyphosate has not been shown to produce any inhibition of short root formation or mycorrhizal colonization (Chakravarty and Chatarpaul 1990). However, we prefer the mechanical soil tilling to the herbicide application to keep *Terfezia* cultivation as an organic culture.

Due to the difficulty of controlling weeds in the La Garrobera plantation (Murcia, Spain), established in April 2008 with 3,000 *Helianthemum* mycorrhizal plants distributed in two hectares, the first *T. claveryi* truffles were obtained the third year after plantation (May 2011) rather than in the first or second year. A combination of the two different weed control methods, in early winter and late summer (never during or close to the fruiting season), allowed such weed control. However, it is advisable to study the long-term effects of weed control methods for desert truffle cultivation in field conditions.

14.3.3 Soil Characteristics

The ranges of the characteristics in which most of the plantations have been made in Spain are shown in Table 14.3.

In general, the soils used for plantations are poor and characterized by a clay loamy texture, basic pH (8.5), and low values of electrical conductivity (123 μs cm^{-1}), organic carbon (0.9–3.9%), and C/N ratio (7–10). These characteristics, especially pH and texture, change depending on the *Terfezia* species which grow in acid soils.

Soil texture can influence ascoma shape (Malençon 1973) but is not a determinant factor for truffle formation, since *T. claveryi* ascomata have been produced in clay loamy soils (plantations in Murcia) as well as in sandy soils (plantations in Lanzarote, Canary Islands). Similar results have been obtained for *T. boudieri* ascocarps in plantations in sand loamy and gypsy soils (Slama et al. 2010).

Soil fertilization has not been necessary until now. Fertilization has never been applied in the oldest plantation (12 years), and mycorrhizal *H. almeriense* plants are still producing *T. claveryi* truffles.

In Spain, *Terfezia* cultivation is generally associated with other crops like almond and olive trees to make the most of land use and irrigation facilities. This is possible because there is no competition for host plants between arbuscular mycorrhizal fungi from almond (Fig. 14.5a) and olive trees and the desert truffle species.

14.3.4 Season and Frame of Plantation

After testing in all seasons, spring was selected as the best time to set up a plantation owing to its moderate temperatures, the abundance of precipitations, and the long photoperiod (Morte et al. 2008). Moreover, planting in spring is essential to obtain the first ascocarps the following spring (11–12 months from plantation). When *Terfezia* orchards were established in autumn, the desert truffle production did not start in the following spring but the next one (19–20 months from plantation).

We tested different frames of plantation, 0.5 × 0.5, 1 × 1, 3 × 3, 3 × 2, and 4 × 2 m, distributed alternately in rows, ridges, and groups (9–12 plants). According to our experience, although a wide frame (4 × 2 m in rows) facilitates mechanical soil tilling and plant physiological measurements (Fig. 14.5d), a narrow frame (0.5 × 0.5 m in groups) is advisable to obtain desert truffles in the first year after plantation.

The distribution in ridges much facilitates mycorrhiza sampling (Fig. 14.5b) and ascoma collection (Fig. 14.5c, e), even for wild boars that usually eat them! In this sense, a fence surrounding the plants is necessary to avoid undesirable desert truffle hunting.

14.3.5 Conclusions

In order to obtain maximum *Terfezia* production in orchards, we recommend the following summarized management practices:

Fig. 14.5 (**a**) *T. claveryi* cultivation in *Helianthemum* species (*arrows*) associated with almond trees. (**b**) The distribution in ridges very much facilitates mycorrhiza sampling. (**c**) Ascoma of *T. claveryi* cultivated in orchard with *H. almeriense*. (**d**) Leaf gas exchange measurements (net photosynthetic rate and stomatal conductance were measured with a portable photosynthesis system LI-6400) in *H. almeriense* mycorrhizal plants in the orchard. (**e**) Ascoma of *T. claveryi* obtained from the orchard. (**f**) Desert truffles are food: a plateful of raw, sliced desert truffle as a salad, dressed in olive oil and salt

1. To use high-quality mycorrhizal plants.
2. To establish the plantation in spring.
3. To maintain a soil water potential between −50 MPa (during fruiting season) and −120 MPa (during summer) by means of an appropriate irrigation system.

This should take into account that the amount rainfall during autumn of one year directly influences *T. claveryi* truffle production the following year.
4. Weed elimination but never before and during the desert truffle fruiting season.
5. A narrow frame of plantation is advisable to obtain desert truffles the first year after plantation.
6. No fertilization.

14.4 Desert Truffle Market and Interest

Desert truffles have been known since ancient times and have been associated with Mediterranean cultures since their origins. We now know they were traded by the Greeks and Romans alike and were imported from Libya to be sold in various markets of the respective Empires (Honrubia et al. 2007). They have continued to be marketed and consumed and, even today, are linked with all three Mediterranean cultures: Christian, Jewish, and Muslim (Morte et al. 2009).

Sizable quantities of several species of wild *Terfezia* are collected and marketed in southern Europe, parts of North Africa, and other countries bordering the Mediterranean Sea. However, natural areas of desert truffles have been disappearing during the last 50 years. Large areas of the coastal desert in Egypt and Libya were mined in World War II. Certain aspects of the Gulf War seem to have ruined many truffle-gathering areas in Kuwait (Morte et al. 2008). In Spain as well as in Dubai and Abu Dhabi, this is due to the huge construction processes taking place in these "sunny" areas over the last 15 years. Moreover, climate change and global warming have reduced total rainfall and therefore the amount of naturally produced desert truffles.

The collection of desert truffles is a manual task accomplished well by those who are able to recognize the crack formed by the fungus in the soil near the host plant. Dogs or any olfactory animals are not necessary if the desert truffle hunter knows how to recognize the host plant and the crack in the soil.

Interest in desert truffles is not merely culinary and gastronomic, and not even purely commercial, since truffles also offer a high nutritional dietary value (Murcia et al. 2003). In addition, some studies have reported its antibiotic capacity (Janakat et al. 2004, 2005) and hepatoprotective activity against CCl_4 (Janakat and Nassar 2010). One of the most interesting food properties of desert truffles is their antioxidant activity with regard to their ability to inhibit lipid oxidation (Murcia et al. 2002), with higher percentages of oxidation inhibition than some common food antioxidants, even after being subjected to industrial freezing and canning processes (Murcia et al. 2002).

Moreover, they are a priori "ecological" given the ecosystem they are produced in and because of their potential for promoting rural development if we take into account the possibility of their being established as an alternative or complementary crop in a sustainable farming context (Morte et al. 2009).

The prices of the desert truffles vary between 20 € and 250 €. However, these prices are much lower than those of the *Tuber* species. One reason for this is that desert truffles are not as strongly flavored as *Tuber* species. However, marketing efforts could help promote desert truffles and increase their commercial value (Morte et al. 2008).

In Spain, *Terfezia* species are usually prepared as a plateful of raw, sliced desert truffles as a salad, dressed in olive oil and salt (Fig. 14.5f). However, the increasing interest of the general public in gastronomy has led many cooks to prepare more elaborate desert truffle dishes.

Most *Terfezia* and *Tirmania* species collected from Egypt and Morocco are sold in markets in Abu Dhabi, Doha, Kuwait, and Riyadh, which might explain why some of these countries are increasingly interested in desert truffle cultivation. Cultivation could avoid overexploitation of natural desert truffle areas that could otherwise lead to the disappearance of some desert truffle species.

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Chapter 15
Truffles, Timber, Food, and Fuel: Sustainable Approaches for Multi-cropping Truffles and Economically Important Plants

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15.1 Introduction

15.1.1 What Is a Truffle?

Truffles are a polyphyletic group of fungi that produce fruiting bodies belowground or at the soil surface with spores sequestered inside (Trappe et al. 2009), and have been derived independently numerous times across the fungal tree of life (Tedersoo et al. 2010). Truffles are an important food source for forest mammals, whereas for most truffle genera (including *Tuber* P. Micheli ex F. H. Wigg.), mycophagy acts an important mode of spore dispersal (Frank et al. 2006). Many human cultures revere truffles for their gastronomic qualities, and consequently, these fungal fruiting bodies are a high-valued commodity (Mello et al. 2006).

The majority of edible truffle species are ascomycetes, and they establish mutualistic relationships with plant roots through the formation of ectomycorrhizas (EMs), benefiting the nutrition and health of their host plant (Smith and Read 2008). This includes the most economically important edible truffle species belonging to genera *Tuber*, *Terfezia*, and *Tirmania* within the order Pezizales (Hall et al. 2007) (see Chap. 2). Other edible (and putatively ectomycorrhizal) species in this order include *Choiromyces meandriformis* Vittad. (Wedén et al. 2009); *Leucangium carthusianum* (Tul. & C. Tul.) Paol. (Li 1997); *Imaia gigantea* (S. Imai) Trappe & Kovács

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(Kovacs et al. 2008); *Kalapuya brunnea* M. J. Trappe, Trappe, & Bonito (Trappe et al. 2010); *Picoa lefebvrei* (Pat.) Maire (Sbissi et al. 2010); and *Mattirolomyces terfezioides* (Mattir.) E. Fisch. (Kovacs et al. 2007).

Of all the truffle species, the European black truffle *Tuber melanosporum* Vittad. is the best studied, both in terms of its ecology and genetics (see Chap. 4). Current data indicates that truffle fruiting bodies are the sexual products of compatible haplotypes outcrossing. Although gamete fertilization is a prerequisite to fruiting, it does not ensure truffle production (Paolocci et al. 2006; Rubini et al. 2011). Environmental factors likely act as cues in initiating the sexual cycle and can influence whether initiated truffle primordia develop to maturity. The signals, receptors, and environmental cues that are involved in truffle fertilization and maturation are still unknown. Recent studies have demonstrated that *Tuber* and other Pezizales truffle fungi produce asexual spores (mitospores) (R Healy, unpublished data). Although the ecological function of these structures is still uncertain, putatively they are involved in reproduction and/or root colonization.

15.1.2 Truffles and Sustainability

Truffles are largely collected in the wild from native and naturalized habitats. Although attempts have been made to cultivate many truffle species, the majority of truffle species have so far evaded cultivation. For instance, many *Tuber* species including the European white truffle *Tuber magnatum* Pico and the North American white truffle *Tuber gibbosum* Harkn. have yet to be cultivated in a repeatable manner.

Reductions in natural truffle habitat has been implicated in the continuous decline of truffle production in Europe over the past century, yet given global trends in increases of planted area of poplar for biofuels (*Populus* sp.), and pine (*Pinus* sp.) and Douglas fir (*Pseudotsuga* sp.) for timber (FAO 2006), potential habitat for truffle species is increasing globally.

The market price for truffles varies, depending on species, geographic origin, size, quality, and the quantity harvested during the season. The European white truffle (*T. magnatum*) is the most expensive truffle species (up the price of 5,000 €/Kg). In contrast, the Chinese truffle *Tuber indicum* Cooke & Massee is quite inexpensive (about 10–50 €/Kg). Italy currently prohibits the trade and sale of *T. indicum*, because of concerns regarding its introduction into European ecosystems and potential for genetic introgression with the native economically important sister species *Tuber melanosporum* Vittad. (Murat et al. 2008). In 2006, annual revenues based on marketed European truffle species, i.e., *T. magnatum*, *T. melanosporum*, *Tuber borchii* Vittad., *Tuber aestivum* Vittad., and *Tuber brumale* Vittad., were estimated at over ¼ billion US dollars (Splivallo 2006).

The high market value and symbiotic nature of truffles make them attractive as a centerpiece of sustainability (Fig. 15.1). Truffles grow in the companionship of living host trees, so ecosystems that are producing truffles are also sequestering

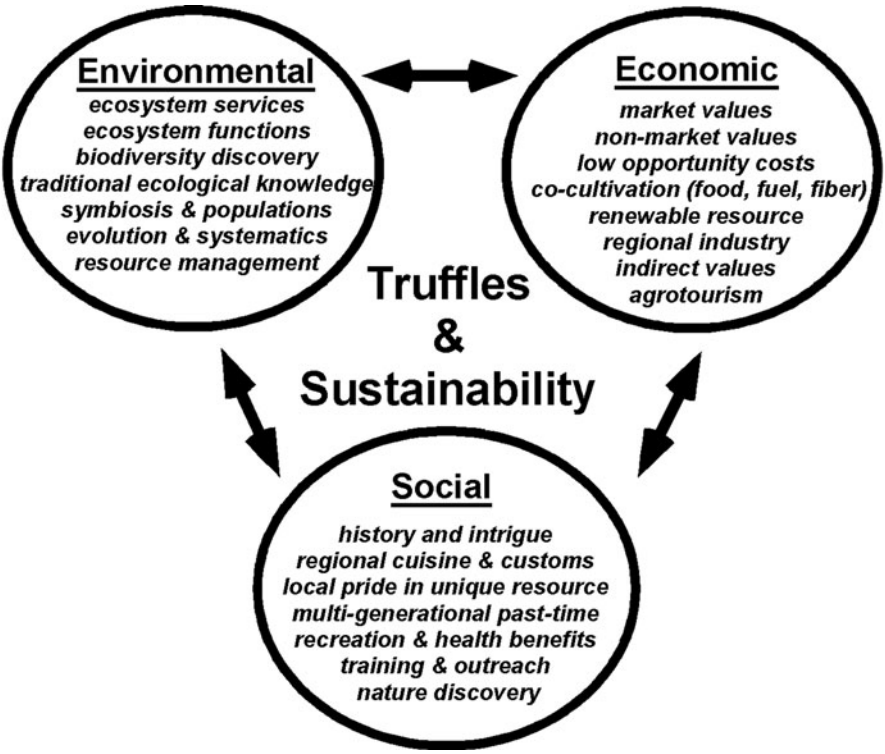

Fig. 15.1 Conceptual model of truffles as they pertain to sustainability. Truffle culture involves environmental, economic, and social components. Truffle fungi provide numerous environmental benefits, particularly to the health and function of many tree species and forest ecosystems. Economically, truffles have a high market value, but there are nonmarket and indirect values of truffles, which are harder to quantify. Socially, truffles attract interest from various social, ethnic, economic, and age groups. Truffle hunting is a healthy pastime for families and friends and can provide a sense of regional pride and uniqueness

carbon as woody biomass and in stocks of soil carbon. In many cases, economically important species of timber (Douglas-fir), nut (pecan), and biofuel (poplar and pine) trees associate with truffles, and these fungi improve in the health and growth of these plants. Truffles are also important to forest food webs and can be an important food source for diverse wildlife mammals, including flying squirrels, deer, and bear (Trappe et al. 2009). Because many truffle species are ruderal species (r-selected) and disturbance adapted, they have potential value in low-input agroforestry and reforestation applications. Truffle fungi have the ability to persist in disturbed habitats that are stressful to other fungi. Because they are directly involved in the nutrition of their host, nutrient additions are usually not needed (or conducive) for truffle production. These biological traits are adaptive to low-input agroforestry and reforestation. Finally, hunting truffles is an enjoyable pastime in many cultures, age groups, and families and is an activity that brings people to the forests. Truffles may contribute to the family diet or can be used to supplement family income. Thus, truffles have important social, ecological, and economic value.

15.2 Truffle Cultivation

Truffles are cultivated as a long-term (perennial) economic crop and in some countries as a means of land-use stability and reforestation (Bonet et al. 2006). As discussed in a previous chapter (see Chap. 9), roots of a compatible host seedling or tree must first be inoculated with truffle spores or mycelia (Hall et al. 2007; Michaels 1982) and well mycorrhized before transplanting in the field. Best practices call for simulating ecological conditions that optimize the growth of the fungus and also support growth of the host (see Chap. 10). Once truffles are produced, the truffle life cycle has been completed; however, a single genet may support annual fruiting for many years thereafter; the life span of a particular individual genet or colony is not known but could span decades given suitable edaphic and climate conditions (Garcia-Montero et al. 2006; Le Tacon et al. 1982). Most commercial truffle species benefit from climates without extreme summer heat or extreme winter cold. Primordia usually abort in conditions of low water (drought), excessive heat, and heavy frost; fruiting bodies are also vulnerable and damaged by extreme temperatures and dry conditions (Trappe et al. 2009).

Soil conditions that favor truffle production are going to vary with species. Generally, well-drained calcareous soils, with alkaline pH, commonly between 7.5 and 8.0, medium humus content, and good aeration, are desired (Granetti et al. 2005). However, soil moisture and temperature preferences vary greatly between species. Some species live in moist soil, even submerged under water for part of the year (e.g., *T. magnatum*, *Tuber macrosporum* Vittad.), and in cooler habitats almost always covered by lush vegetation. Other species (e.g., *T. melanosporum*) prefer permeable soils, never saturated with water and well heated by sunrays. Truffle species also vary in their soil preferences, and although alkaline conditions are recommended for most of the cultivated species, some species appear to tolerate soils with neutral pH values (e.g., *T. aestivum*) or lower (pH 5.0–6.0) (e.g., *T. borchii*, *T. gibbosum*) (Gardin 2005) (see Chap. 10).

Today, truffle cultivation is feasible for many species, and truffles are successfully produced in man-made truffiéres (Chevalier 1998; Hall and Yun 2002; Zambonelli et al. 2002). Most of the truffle cultivation industry is centered on the black truffle, *T. melanosporum*, but other species including *T. borchii* and *T. aestivum* are cultivated with equal and even greater success and in a broader range of hosts and habitats (Bencivenga and Baciarelli Falini 2012). Unfortunately, truffle cultivation is not yet routine for *T. magnatum* because mycorrhizas are difficult to obtain; therefore, contamination with other ectomycorrhizal species [e.g., *Tuber maculatum* Vittad., *T. borchii*, *Sphaerosporella brunnea* (Alb. & Schwein.) Svrček & Kubička, and *Pulvinula convexella* (P Karst.) Pfister (=*Pulvinula constellatio*)] is more prevalent (Bertini et al. 2006). However, in 2008 the French "Institut National de la Recherche Agronomique" requested a patent for the production of *T. magnatum* mycelium with the aim to obtain mycorrhized plants (INRA 2008). In Italy there are some old truffle orchards of *T. magnatum*, planted 20 years ago or more, that produce appreciable amounts of

truffles but only in years having suitable climatic conditions (Gregori et al. 2010; Baciarelli Falini et al. 2010). There is also recent interest in the cultivation of *T. macrosporum* (Benucci et al. 2011a, 2012b) which can grow quite large in some habitats (Glamoclija et al. 1997). Seedling roots of various hardwood species are receptive to *T. macrosporum* spores (Benucci et al. 2012a; Giovannetti and Fontana 1980–1981), and attempts to cultivate this species have been reported (Vezzola 2005).

In general, truffle cultivation needs a detailed study of the plantation site, which includes soil physical–chemical analysis, climate, topography, local vegetation, etc. Besides the choice of high-quality mycorrhized plants, pre- and post-plantation farming operations such as soil amendments, plowing, weeding, irrigation, pruning, mulching, spore-inoculum addition, and pest and disease control[1] are fundamental for successful production of fruiting bodies (Bencivenga and Baciarelli Falini 2012).

Currently, truffles are cultivated as a monocrop, but as we propose below, there is considerable potential for sustainable multi-cropping of truffles with economic host plants for meeting multiple objectives of food, fuel, and fiber (Porter et al. 2009).

15.2.1 Cases of "Spontaneous" Truffle Production

In their native habitat, truffles are perennial and fruit seasonally, some years in abundance (North et al. 1997). There are a number of instances where truffles fruit spontaneously (without human inoculation) in soils being managed for particular plant crops.

For example, in Europe both *T. melanosporum* and *T. brumale* are known to naturalize and fruit within hazelnut orchards within their native range of southern Europe (Reyna 2007). On the other hand, *T. aestivum* and *T. borchii* (as well as other nonmarketable truffle species like *Tuber rufum* Pico and *Tuber excavatum* Vittad.) sometimes fruit under planted trees belonging to *Quercus* sp., *Tilia* sp., *Ostrya* sp., and *Pinus* sp. in reforestations, city parks, and street trees (GMN Benucci, personal observation). Both *T. magnatum* and *T. borchii* are known to

[1] In Europe, truffle plantations are not so often damaged by pathogens or pests and usually do not require any particular treatment. One exception however is downy mildew [*Erysiphe alphitoides* (Griffon & Maubl.) U. Braun & S. Takam.], a common pest of oaks (e.g., *Q. pubescens*) in truffle orchards in Europe and the USA. Precautionary measure should be taken when introducing exotic hosts to a nonnative habitat (Fisher et al. 2012), and as in any nursery trade, care should be taken to avoid transporting and transplanting pests, diseases, or susceptible rootstock into one's orchard. In the USA, eastern filbert blight [*Anisogramma anomala* (Peck) E. Müll.] has become a major problem in truffle orchards using susceptible hazelnut species (*C. avellana*). Resistant hybrid cultivars have been produced, but resistance varies. Similarly, a range of diseases affect pecan. These are more of a problem in humid climates but can be managed for (Sparks 2005). Emerging diseases, such as sudden oak death (*Phytophthora ramorum* Werres, de Cock, & Man in't Veld), could be disastrous if introduced to an oak orchard (Garbelotto and Pautasso 2012).

fruit in cultivated plantations of *Populus* spp. and riparian strips along watercourses in Northern Italy (Benucci et al. 2012a, b). In the United States, young Douglas-fir timber stands in the Pacific Northwest support fruitings of *Tuber oregonense* Trappe, Bonito, & P. Rawl., *T. gibbosum*, *L. carthusianum*, and *K. brunnea*, and their natural production is the basis for a cottage truffle industry (Trappe et al. 2009). In the Southern USA, pecan orchards support large fruitings of *Tuber lyonii* Butters, a phenomenon that has been noted through anecdotes for decades and more recently through molecular analyses (Bonito et al. 2011a). In China, substantial quantities (>300 tons) of *T. indicum* are harvested from regenerated forest stands (Murat et al. 2008). In all cases mentioned, the native species of truffle appears to function as a pioneer species and is tolerant to moderate disturbances. Further investigations with these and other truffles species are surely needed, and management regimes will have to be optimized, but exciting opportunities for multi-cropping truffles and trees as a sustainable approach for aiding in land and economic stability exist.

15.2.2 Hosts Preferences of Edible Truffle Species

Some truffle species show high host specificity, they grow in association with particular host species but not others (Molina and Trappe 1982). Host specificity appears to be a gradient from high (host specific) to low (host generalist). For instance, the cultivated truffle *T. melanosporum* grows and fruits well with Northern Hemisphere angiosperm (e.g., oak, hazelnut) hosts, but poorly with gymnosperm (pines) and some Southern Hemisphere taxa (e.g., *Eucalyptus* sp.). In contrast, other truffle species including *T. borchii* and *T. aestivum* are more general in terms of host and fruit in association with many species of gymnosperms (e.g., *Pinus* spp., *Picea* spp., *Cedrus* spp.) and angiosperms (e.g., *Quercus* spp., *Populus* spp.) and even form ectomycorrhizas readily with species in genera they are not normally associated with [e.g., *Carya illinoinensis* (Wangenh.) K. Koch] (Benucci et al. 2011b, 2012b).

Currently, truffles are cultivated on a narrow set of host species. Yet many unexplored possibilities for cultivating truffles on species being used for timber, biofuels, or food (nut) production exist. Host fidelity (and lack of) for particular truffle species offers novel opportunities for their cultivation in symbiosis with economically important trees.

Many edible ectomycorrhizal Pezizales truffles associate with angiosperm hosts (e.g., *T. melanosporum*, *T. magnatum*, and *Terfezia* spp.). These fungi tend to be better adapted to soils with higher pH (see Chap. 2). In contrast, the species *T. gibbosum*, *T. oregonense*, and *K. brunnea* appear to be endemic in the Pacific Northwest, USA, growing in association with Douglas-fir, an important timber species. Often, the soils under coniferous hosts are more acidic in pH and have lower nutrient availability (Binkley and Sollins 1990). Taxonomic-level host plant preferences for edible species of truffles in the Pezizales are summarized in Table 15.1. *Terfezia* and *Tirmania* are adapted to exploit two major soil types,

Table 15.1 Phylum-level host plant preferences for common edible truffle species

Truffle species	Angiosperm	Gymnosperm
Tuber aestivum Vittad.	×	×
Tuber borchii Vittad.	×	×
Tuber brumale Vittad.	×	×
Tuber canaliculatum Gilkey		×
Tuber gibbosum Harkn.		×
Tuber indicum Cooke & Massee	×	×
Tuber lyonii Butters	×	
Tuber macrosporum Vittad.	×	
Tuber magnatum Pico	×	
Tuber melanosporum Vittad.	×	
Tuber mesentericum Vittad.	×	

acid and basic. Particular fungal species tend to show high specificity toward particular hosts (either basophilous or acidophilous species) of Cistaceae (mainly *Helianthemum* spp.), Fagaceae, and Pinaceae (Díez et al. 2002). Chapter 14 provides further details on *Terfezia* sp. cultivation.

Mattirolomyces terfezioides (Pezizaceae) is another economically interesting hypogeous ascomycete closely related to *Terfezia* genus. Kovács and colleagues (2007) reported uncertain host relationships for this species. However, they suggest that *M. terfezioides* could putatively form symbiotic relationships with woody plants such as *Celtis occidentalis* L., *Crataegus monogyna* Jacq., *Ligustrum vulgare* L., and *Robinia pseudoacacia* L. and herbaceous ones such as *Muscari racemosum* (L.) Mill., *Salvia glutinosa* L., and *Viola cyanea* Čelak; alternatively, *M. terfezioides* could be functioning a saprobe.

15.2.2.1 Truffles and Pinaceae Hosts

There are many commercialized truffle species that appear exclusively with Pinaceae hosts. As previously mentioned, in the Pacific Northwest of North America, *T. gibbosum*, *T. oregonense*, *L. carthusianum*, and *K. brunnea* are endemic to habitats with Douglas-fir. These truffle species have not yet been cultivated but appear naturally and sometimes abundantly. In Eastern North America, the endemic species *Tuber canaliculatum* Gilkey is found under conifers, particularly *Pinus strobus* L. (eastern white pine, 5-needle) and *Tsuga canadensis* (L.) Carrière (eastern hemlock) but also with *Picea* spp., and EMs have been synthesized with *Pinus taeda L.* (loblolly pine) (Fig. 15.2e, f, i). There is potential that these species could be co-cropped in timber, biomass, and Christmas tree lots across the USA or in other locations.

Some truffle species are host generalists but can fruit abundantly with coniferous host plants. *Tuber aestivum*, *T. borchii*, and *T. indicum* are three other economically important truffle species that grow and fruit well in symbiosis with a wide range of coniferous hosts (in addition to angiosperm hosts). There are a multitude of opportunities for co-cropping truffles with coniferous hosts in different geographic regions. For instance, the pine genus (*Pinus* sp.) alone accounts for over eight

Fig. 15.2 Pecan, nuts, truffles, and some mycorrhization success on pine (*Pinus taeda*) and pecan (*Carya illinoinensis*). (**a**) Adult pecan branch with nuts and *T. lyonii* fruiting bodies collected in a commercial orchard. (**b**) *T. aestivum* ectomycorrhizas (EMs) on *C. illinoinensis* seedlings (*bar* = 500 μm). (**c**) *T. borchii* EMs on *C. illinoinensis* seedlings (*bar* = 500 μm). (**d**) *T. borchii* EMs on *P. taeda* (*bar* = 500 μm). (**e**) Mycelial net *T. canaliculatum* EMs on *P. taeda* (*bar* = 500 μm). (**f**) Growing *T. canaliculatum* EMs on *P. taeda* (*bar* = 200 μm). (**g**) Outer mantle cells of *T. borchii* EMs on *C. illinoinensis* seedlings (*bar* = 30 μm). (**h**) Cystidia of *T. aestivum* EMs on *C. illinoinensis* seedlings (*bar* = 30 μm). (**i**) Cystidia of *T. canaliculatum* EMs on *P. taeda* seedlings (*bar* = 30 μm). (**j**) Cross section of *T. aestivum* EM on *C. illinoinensis* (*bar* = 30 μm). (**k**) Cystidia of *T. borchii* EMs on *C. illinoinensis* seedlings (*bar* = 30 μm). (**l**) Outer mantle type of *T. aestivum* EMs on *C. illinoinensis* seedlings (*bar* = 30 μm)

million hectares of planted forest (FAO 2006). Other genera such *Larix* sp., *Pseudotsuga* sp., *Abies* sp., and *Picea* sp. are cultivated in many countries and at many latitudes for timber, fiber, and even Christmas trees.

15.2.2.2 Truffles and Angiosperm Hosts

A number of edible truffle species tend to associate preferentially with angiosperm hosts. As mentioned previously, the black truffle *T. melanosporum* shows strong preference for angiosperm hosts including *Quercus* and *Corylus* spp. (Murat et al. 2004). Although forming EMs with some gymnosperm species, the black truffle apparently only fruits with angiosperm hosts (Garcia-Montero et al. 2007). Both fungus and plant symbionts appear well adapted to the dry climate and karst-based soils in Europe. The prized white truffle *T. magnatum* also appears to be associated only with angiosperms. It is commonly reported as a *Populus* spp. (poplar) associate but also associates with *Corylus* (hazelnut), *Quercus* (oak) and *Ostrya* (hornbeam) species (Bencivenga and Granetti 1990; Murat et al. 2005).

In the USA, *T. lyonii* fruits under oaks and pecans in residential habitats and also naturalizes in pecan orchards (Fig. 15.2a) (Bonito et al. 2011a; Hanlin et al. 1989).

In the eastern USA, many cases have occurred where large fruiting bodies of *T. lyonii* were harvested from truffle orchards established with oak and hazelnut trees pre-inoculated with *T. melanosporum* (G Bonito, personal observation). Thus, it seems that *T. lyonii* is quite compatible with the management regime and regular disturbances associated with managed orchards and lawns (Bonito et al. 2011a). Harvesting truffles in addition to pecans is a mechanism for adding value to a farm and brings in extra revenue, particularly important in non-masting years when the nut crop yield is lower. Currently, most truffle biomass produced in pecan orchards goes uncollected, due to a lack of trained truffle dogs and a strong focus on pecan production, which is one of the major economic crops of the region.

As previously mentioned, the truffle species *T. aestivum*, *T. borchii*, and *T. indicum* are host generalists and fruit with both gymnosperms and angiosperms. These species have been inoculated (and in some cases have fruited) in association with native and exotic conifer and angiosperm host plants (Benucci et al. 2011a; Bonito et al. 2011b). For example, these species can associate with economic North American tree species including loblolly pine (*P. taeda*) (Fig. 15.2d), pecan (*C. illinoinensis*) (Fig. 15.2b, c, g, h, j–l), and poplar (*Populus trichocarpa* Torr. & A. Gray).

15.3 Multi-cropping Truffles and Trees

A number of considerations need to be made for successful truffle and tree multi-cropping. Climate and soil characteristics pose the biggest constraints. In some cases, it is feasible to amend soil characteristics such as pH and nutrient level.

Truffle species each have their own habitat preferences; success in their fruiting depends on many factors including where they are being grown. When cultivating native truffle species, the cultivator may experience a "home court advantage." However, the growth of truffles as exotic species could benefit from a phenomenon known as "competition release" (Mitchell et al. 2006). If a dearth of compatible or competitive ectomycorrhizal competitors is present, then the introduced species is more likely to proliferate. Thus, host ecology and geography are important in the success (and failure) of truffle orchards, just as are soils (see Chap. 6). When experimenting with new hosts, special attention should be paid to the plant health and nutrition in order to survive at the pH required by the partner truffle species. Particular hosts, and even genotypes, may favor mycorrhization and fungal species performance (Courty et al. 2011).

15.3.1 Preliminary Steps in Multi-cropping Truffles and Economic Plants

From observations and knowledge about the ecology of plants and truffle species of interest, various host–fungus combinations can be envisioned (Table 15.2).

Preliminary steps to co-cropping European truffle species with the economically important North American nut tree *Carya illinoinensis* have recently been reported (Benucci et al. 2012b). Healthy levels of *T. borchii* (≈62 %) and *T. aestivum* (≈42 %) mycorrhizal colonization were obtained with spore-slurry inoculations (Fig. 15.2b, c, g, h, j–l). Preparations are being made to plant these seedlings as an experimental plot in Italy to ascertain whether the pecan host maintains colonized with these species in the field, and whether productive fruitings of these truffle species can be realized when associated with pecan. Such long-term research is unpredictable and is not very conducive for short-term funding cycles as has been expressed by Hall and Haslam in Chap. 11.

Outside of Europe, *T. melanosporum* has been inoculated onto native *Nothofagus* spp. in Chile (Pérez et al. 2007) and *Quercus* spp. (Michaels 1982) and pecan (G. Bonito, unpublished data) in North America. It is not clear, however, when outplanted into exotic soils whether *T. melanosporum* will be competitive against the resident ectomycorrhizal flora.

15.3.2 Possibilities for Intercropping with Economic Species and Co-inoculating with Multiple Truffle Species

Another possibility, probably little known by truffle farmers, is to cultivate a combination of truffle plants and other crops in the same field. For example, in central Italy single compatible host plants (e.g., *Quercus* spp.) naturally growing

Table 15.2 Tree species, major uses, and potential for multi-cropping with truffles

Plant species	Used for			Used in truffle cultivation	Potential multi-cropping
	Fruits	Fuel	Timber		
Quercus ilex L.		×		×	×
Quercus pubescens Willd.		×	×	×	×
Quercus cerris L.		×	×	×	×
Quercus robur L.			×	×	×
Quercus petraea (Mattuschka) Liebl.		×	×		×
Corylus avellana L.	×			×	×
Ostrya carpinifolia Scop.		×		×	×
Carpinus betulus L.			×	×	×
Populus sp.		×	×	×	×
Tilia platyphyllos Scop.			×	×	×
Tilia cordata Mill.			×	×	×
Pinus taeda L.		×	×		×
Pinus pinea L.	×		×	×	×
Pinus halepensis Mill.			×	×	×
Pinus nigra L.			×		×
Cedrus sp.			×	×	×
Carya illinoinensis (Wangenh.) K. Koch	×				×
Juglans regia L.	×		×		×
Castanea sp.	×		×		×
Larix sp.			×		×
Pseudotsuga sp.			×		×

between old olive (*Olea europaea* L.) trees have become mycorrhized with truffles and produce ascocarps. For this reason, some truffle plantation were established by alternating rows of mycorrhized plants (e.g., of *Quercus* spp.) and rows of olive trees (Granetti and Baciarelli Falini 1997).

A number of experiments have investigated the effects of co-inoculating multiple different truffle species on a single host (Mamoun and Olivier 1993; Donnini et al. 2010). Results indicate that multiple economic truffle species (e.g., *T. borchii*, *T. aestivum*, *T. melanosporum*) can be maintained on a single host and that they may in fact improve the growth of each other. Further, a greater proportion of the roots become mycorrhized, which is predicted to limit the opportunity of nontarget fungi to associate with the host (Kennedy et al. 2009).

15.3.3 Additional Considerations When Multi-cropping Economic Truffles and Trees

Co-cropping economic species of truffles alongside other crops such as nuts, timber, or pulpwoods is a reasonable improvement to the old concept of truffle cultivation aimed at solely producing truffles. With a dual cropping system, crop

failure (e.g., nuts) could be balanced by truffle production (and/or wood products). Multi-cropping of economic tree species and truffles represents a plausible alternative for boosting rural or disadvantaged economies by providing farmers with a valuable source of income. Due to the ectomycorrhizal symbiosis with forests trees, truffle cultivation does not generally require amendments, pesticides, or fertilizers and thus could be cultivated in completely organic and sustainable manner.

15.4 In Situ Inoculation of Young Forest/Timber Stands

In situ inoculation of young forest/timber stands is one approach to truffle cultivation that still needs more study. Addition of truffle spore slurry to young growing or already mature truffle orchards of forest stands could be managed to augment mycorrhization possibilities for developing roots of host trees (Bencivenga and Baciarelli Falini 2012; Baciarelli Falini et al. 2010). This is a common strategy used in truffle orchards to maintain production, although its efficacy has not been well tested. It seems unlikely that a residential community of ectomycorrhizal fungi can be replaced by spore inoculations in the field. However, if target taxa are already present, then their abundance could be augmented through in situ inoculation with spore slurries or spores could serve as propagules for sexual fertilization. A preliminary study on field inoculation with economic truffle species onto adult hazelnuts that no longer bear nuts has been carried out (Morcillo et al. 2010). This study mixed spore inocula with hydrogels, root promoting factors, and spore germination promoting factors (following the Mycoforest Technology method). An increase in amount of truffle mycorrhizas and fruiting was documented. However, because adequate controls were not included in the study, it is unclear whether the trees are responding to the disturbance, truffle propagules already in the soil, or the experimental treatment.

15.5 Future Opportunities

Considering mycorrhization successes of pecans and pines with *T. aestivum* and *T. borchii*, we foresee new mycorrhization trials with other valuable truffle species such as *T. melanosporum*, *T. brumale*, and *T. magnatum*.

Future research will also involve other potential coniferous hosts and angiosperm hosts such as the putatively ectomycorrhizal common walnut (*Juglans regia* L.) (Wang and Qiu 2006), other *Carya* species, and species of chestnut (e.g., *Castanea sativa* Mill.). Hickory and chestnut are adapted to a range of climatic and soil conditions, overlapping in cases with those suitable for truffle growth, and they may be potential symbiotic hosts for some *Tuber* spp.

Given the expansive plantations of coniferous ectomycorrhizal hosts globally, we see great opportunities for establishing these as dual truffle and timber systems.

The European species *T. borchii* and *T. aestivum* appear to be among the best suitable for such endeavors, given their broad host range and established markets. Of course, possibilities also exist for the use of North American species such as *T. canaliculatum*, *T. lyonii*, and *T. oregonense* and other tasty but less appreciated European species such as *T. macrosporum*.

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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* Massee) 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

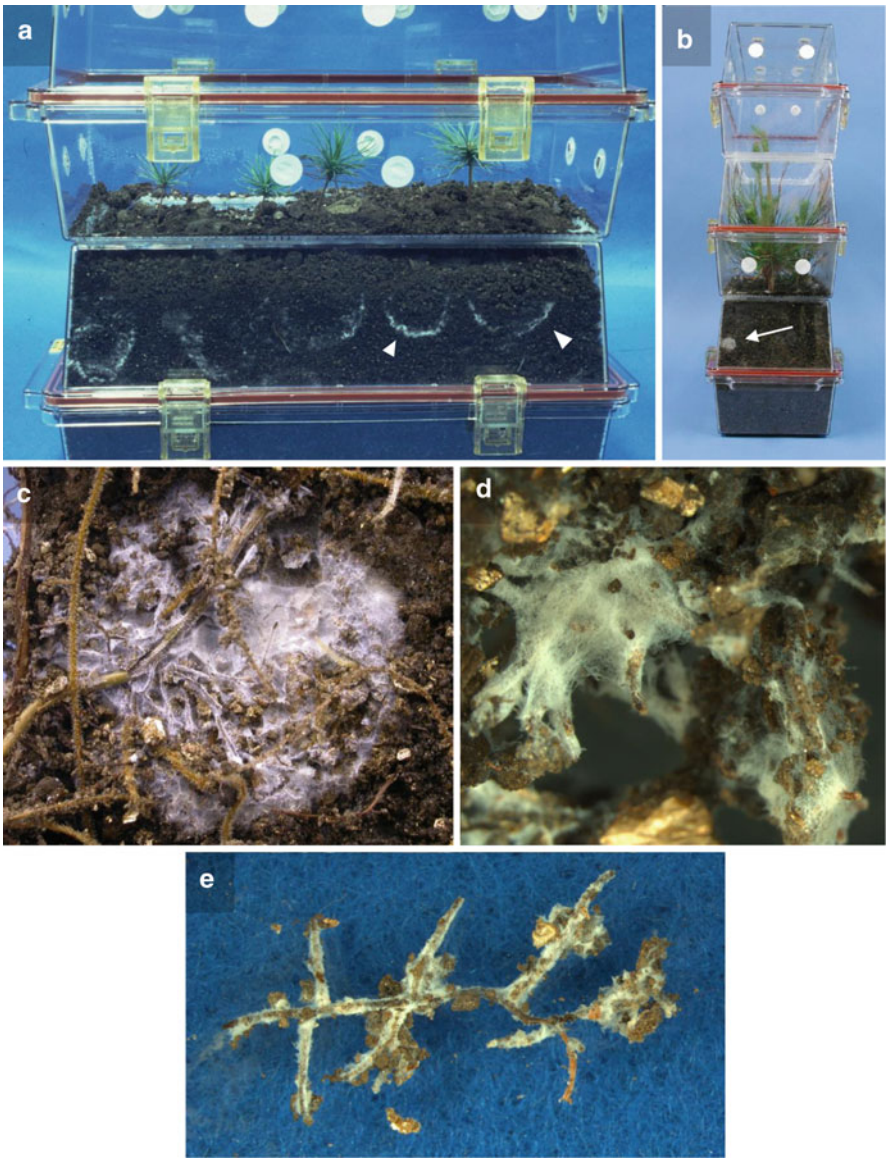

Fig. 16.2 Long-term in vitro mycorrhization of *Pinus densiflora* by *Tricholoma matsutake* (Guerin-Laguette and Matsushita, unpublished). (**a**) Matsutake mycelium (*arrowheads*) growing from the inoculum 5 months after inoculation. (**b**) Host growth and shiro-like development (*arrow*) 20 months 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 β-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, Verbeken & Walleyn. 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 Verbeken 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 Verbeken 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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Part III
Wild Collected Edible Ectomycorrhizal Mushrooms: Economics, Conservation, Management

Chapter 17
Local Communities and Edible Ectomycorrhizal Mushrooms

Eric Boa

17.1 Introduction

This chapter is about people: how they use wild edible mushrooms, the benefits they gain and the consequences of collecting and trading. It is about groups of people, often though not exclusively living in rural communities, who collect these mushrooms from the wild, usually from forests. It is a brief overview drawn from information published from many countries around the world. The majority of original sources are referenced in a review published some years ago (Boa 2004), in a publication that is freely available online in English, French and Spanish via the Food and Agriculture Organization (FAO) of the United Nations Web site.

There are two main uses for wild edible mushrooms and both are linked: you can sell them or you can eat them. Some species also have medicinal purposes, but this is an additional benefit rather than a main use. The bulk of medicinal mushrooms are cultivated, and this chapter will concentrate mostly on the potential monetary value of wild mushrooms as food. This is not an account of local markets or international trading, which is covered elsewhere in this book (see Chap. 20), but an examination of activities and events that precede the exchange of money.

There is an increasing awareness of the global diversity of edible mushrooms[1] (Boa 2004; Hall et al. 2003)—not only those that are ectomycorrhizal. Their indigenous or local uses and, more precisely, monetary or dietary value to local communities are less well known. While studies on wild edible mushrooms continue to be published, most recently in a special edition of Economic Botany

[1] Arora and Shepard (2008) argue for the term "wild edible mushroom" instead of "wild edible fungi", as used by Boa (2004). This chapter will also use "mushroom" to remain consistent with the title of the current book.

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published in 2008, there remains a poor understanding of how money earned or nutritional value to diets contributes to livelihoods and well-being.

The dietary contribution is difficult to assess because of the limited information about the relative importance of wild edible mushrooms compared to other sources and how consistent this is from one year to the next. However, there is no doubt, contrary to claims that mushrooms are "mainly water" (as are most fruits and vegetables), that mushrooms make important dietary contributions. In one of the few studies found which documents this contribution, 1.3 kg of dried leafy vegetables and/or dried wild edible mushrooms could feed a family of four for 2 weeks in Malawi (Abbott 1999). Nutritionally, they are an important source of digestible protein and fibre, especially in developing countries where options are limited by availability and cost (de Román et al. 2006).

This chapter will focus on developing countries, where our knowledge of the role played by wild edible mushrooms is weakest yet their potential economic importance is highest. This could mean a few extra dollars earned in a day, a day's wage in many places, or it could presage an economic bonanza, such as that associated with the widening discovery of *Tricholoma matsutake* (S. Ito & S. Imai) Singer throughout Asia driven by Japan's own declining national production. North Korea earned US$150 million over 5 years from its exports, a substantial sum by any measure but of national significance in an extremely poor country with restricted and limited opportunities for international trade.

There are large gaps in our knowledge of how habitat loss, particularly wooded areas and permission to harvest in existing areas affects local communities, and the degree to which they depend on collections of wild mushrooms as a source of income. Natural resource management includes studies of habitats as well as the edible mushrooms that grow there, yet there are too many studies which document long lists of species without due consideration to the ecological context. More regular consideration should be given to studies at landscape level and with the involvement of social scientists. A series of investigations in the Pacific Northwest over more than 10 years is a good example of a coordinated and rigorous attempt to explore all aspects of wild edible mushrooms in devising sensible evidence-based policy on EEMMs (Pilz and Molina 2002). The clear conclusion is that the study of human behaviour, markets and forest management is as important as the biology or ecology of mushrooms.

The collection and use of edible ectomycorrhizal mushrooms (EEMM) across the vast Miombo woodlands of southern Africa has been described previously by a number of authors (e.g., Morris 1987; Piearce 1981; Härkönen et al. 2003). These studies provide an introduction to a balanced overview of edible ectomycorrhizal mushrooms, which embraces biology, ecology and human perspectives, though funds for research were much smaller than research budgets in the Pacific Northwest. There is a useful contrast between Africa and the Pacific Northwest, where the value of the previously neglected *Tricholoma magnivelare* (Peck) Redhead also soared in response to Japan's needs, attracting migrant workers originally from Mexico and Cambodia, many with no previous experience or local knowledge of wild edible mushrooms. Mushroom collecting in Miombo has been going on for probably hundreds of years.

This is a short "balanced" introduction to the overall value of EEMM to local communities. Wherever possible, EEMMs are distinguished from wild edible mushrooms with different ecological niches, including saprobes (e.g., *Pleurotus* spp.) and pathogens (e.g., *Armillaria*). Such ecological distinctions are of little or no importance to collectors though clearly the continuing presence of host species is essential to the production of EEMM.

17.2 Local Knowledge

A knowledge of potential collecting sites and habitat preferences for various mushroom species is an advantage to a collector, as the following account by a market trader in Zomba, Malawi, suggests:

> There are lots of different *bowa* (edible mushrooms) and we have to know where to look. For example under *masuku* (*Uapaca kirkiana* Muell. Arg.) trees we find more than six different types e.g., *Nakasuku/ngundamsuku* (red or white); *Chipindi* (white and it has cracks outside and most insects and tortoises like to eat this, people know it must be edible when they eat it as it tastes like beef); *Nakatelesya*. Other *bowa* include the *utale/namichombo* which appear with the first rains. By the time farmers have started planting it is already finished. There is a certain black/brown *bowa* which white people only will buy. It grows under pines and *mkunguza* trees (mature ones). It is also found on golf ground.
>
> (Unpublished, collected by Janet Lowore, Kennedy Ndhlazi and Vicky Mzumara, March 2001)

In the above example, the person recognises an association between trees and edible mushrooms. However, collectors and traders are not always aware of the ecological interdependency. This is important because mushroom collectors compete with charcoal producers and other Miombo forest users who remove trees for fuel wood and other purposes.

Local knowledge embodies attitudes and beliefs about which species of wild edible mushrooms can be safely eaten. When local knowledge is challenged by scientific knowledge, confusion occurs. *Gyromitra esculenta* is a canned food in Sweden, yet many field guides warn severely of attempting to eat it. The mushroom is indeed poisonous when raw, but with careful cooking poses no less threat to humans than eating raw kidney beans or failing to prepare cassava so that cyanogenic compounds are removed.

Some examples of "poisonous mushrooms" will surprise those accustomed to eating boletes in Europe and North America. In certain parts of Tanzania, people will not eat mushrooms with pores because of a belief that they are unsafe (Härkönen et al. 2003). Some highly prized EEMM are simply ignored because of a lack of local knowledge. *Boletus edulis* Bull. grows freely under pine trees in Malawi, yet no one collects and eats it, though Italian traders are always on the lookout for new sources of this and allied species. These are examples where

missing or mistaken knowledge limits market opportunities and dietary options in countries where few alternatives for earning money exist.

Other valuable species are eaten and traded without receiving a price premium that new markets could offer. *T. matsutake* was traded at similar prices to other wild mushrooms in local markets in Bhutan until its value as an export species was identified by visiting Japanese (Nagoya, quoted in Boa 2004).

Local knowledge of where to collect, what to eat and how to prepare EEMM for cooking is acquired through experience and passed on through generations. However, these skills can be lost, particularly when people move to cities and lose their confidence in recognising mushroom species or acquire new prejudices. One city dweller in Malawi, and potential customers of mushrooms from a peripatetic trader, was suspicious of a commonly eaten species found in a nearby woodland. The enterprising trader would eat the mushroom in front of the potential buyer to show it was safe.

A knowledge of local markets is equally important to collectors, who often have to walk long distances to sell their produce. Success depends on the ability to deliver fresh mushrooms to market and thus favors the more robust species, such as *Cantharellus cibarius* and many African species, which can survive being carried over uneven surfaces, often in large, weighty baskets containing enough mushrooms to make the journey worthwhile.

As local knowledge of the value of *T. matsutake* to Japan became more widely shared in parts of China, communities started to compete for harvests, often with violent consequences. Fuelled by perhaps exaggerated stories of potential earnings, villagers in Sichuan engaged in sustained battles to determine local rights to matsutake sites culminating in the sabotage of water supplies—they were without water for 45 days—and destruction of a key bridge. One village threatened not only to continue their disruption of life in the rival village but to "hide the pieces of the water pipes in the forest so that they could not be repaired" (Yeh 2000).

There is no certainty that local knowledge is correct, as already noted above in the example of people not eating mushrooms with pores in Tanzania. Yet regardless of such misconceptions, the sum of what people know gives new insights and information on wild edible mushrooms that researchers may otherwise not be aware of. Such information could be used to improve and identify research gaps or plan interventions that help local communities manage their resources effectively and sustainably. Local knowledge suggests how to maximise opportunities for earning money and working with natural resource managers and local communities on sustainable harvesting.

Sound scientific knowledge is not always welcomed when it contradicts strongly held views. There is a widespread belief in some countries that EEMM can be "over-picked" and thus damage future harvests. Egli et al. (2006) have refuted this idea in Switzerland, yet there is continuing resistance to these findings by nature conservationists (Egli, personal communication). Some beliefs are difficult to change whatever the available evidence.

17.3 Diversity of Edible Species and the Relative Importance of Those that Form Ectomycorrhizae

Wasson and Wasson (1957) were the first to attempt a global overview of uses and traditions associated with wild mushrooms in *Mushrooms*, *Russia and History*, a two volume publication available only in a few specialist libraries. The book gives a broader description of the importance of mushrooms to people than the title suggests, though this early account was inevitably incomplete and soon overshadowed by the senior author's academic interest in psychoactive mushrooms.

The Wassons were the first to use the terms "mycophilic" and "mycophobic" to describe attitudes they associated culturally with different regions, though whether these remain useful descriptors today is unclear. The drift of people away from the land or local traditions within a region can rapidly erode confidence in identifying species and therefore the willingness to eat wild harvests.

With many more accounts published of wild edible mushrooms from around the world, there is now a better recognition of the incredibly rich range of species collected and eaten by humans (Fig. 17.1). During literature searches for Boa (2004), published records of edible mushroom species and "fungi with other uses" were stored in a database. Additional records were added for several years following publication, and there are now around 10,000 records from over 1,000 sources held in "WUFbase" (the wild useful fungi database). These records are not yet fully accessible on the Internet, but the intention is to do so soon. The source of each entry is recorded, and the database includes multiple records for the same species from different sources. There are around 6,500 species in the database, including poisonous relatives of edible mushrooms.

This more recent database was used by Boa (2010) to update numbers of wild edible mushrooms, and this appears in Table 17.1. There are 2,299 species of wild edible mushrooms. This includes EEMM species as well as other types. Hall et al. (2011) list 930 ectomycorrhizal species that are either edible or medicinal (or possibly both), most of which are in Boa (2004). Hall et al. do not give the properties or uses for all the species they include, and apart from those species taken from Boa (2004), all other records are taken from a 1991 Chinese publication.

An explanation is needed for the distinction made in Table 17.1 between "food", a use, and "edible", a property of a mushroom. Many fungi are edible but either insipid or worthless. It is their value as food, which is of interest. Mushroom field guides frequently fail to make the difference between a general property and confirmed use, and these publications and other published lists are the main source of records for which there is no supporting evidence that a species has actually been consumed by someone.

Because there continues to be much discussion about which species can be eaten, sources were checked for supporting evidence. There is a remarkably large number of species for which this evidence is missing. There are also many published records for which it is uncertain if they are even edible, giving a total of just over

Fig. 17.1 Selling mushrooms outside the main station in Tallinn, Estonia (**a**). Collecting niscalos (*Lactarius deliciosus*) near Palencia, Spain, growing with *Pinus nigra* (background). The mushrooms were sold to traders who would drive overnight to get to markets in Barcelona and Valencia (**b**). Mushroom collector with traditional basket from Malawi (**c**). A day's collection of matsutake is weighed and graded before being flown from Oaxaca to Japan (**d**). Mushroom collectors Malawi (**e**). *Boletus edulis* (porcini) being packed in Borgo val di Taro, Italy. A majority of porcini are imported (**f**)

1,100 species. Many authors made it clear that species were eaten and a relatively small number (62) lacking full evidence but enough to be classified as "food".

Poisonous species are included for general comparison in Table 17.1. This category includes those which are mildly toxic as well as potentially fatal. The advice in all cases is do not eat.

The database includes more than one record for the more popular species, and this is where it became difficult to decide how to arrive at a final decision. In short, a series of rules were developed which gave precedence to credible claims that

Table 17.1 Reported use and properties of 2,705 mushroom species from 90 countries

Use or property	Number of species	% of total
Food	1,118	41
Food (uncertain)	62	2
Edible	530	20
Edible (uncertain)	589	22
Poisonous	354	13
Poisonous (uncertain)	52	2

Table 17.2 Number of edible ectomycorrhizal species in the top 15 genera by abundance, based on a preliminary analysis of Hall et al. (2011) and the unpublished "WUFbase"

Genus	Number of EEMM species (Hall et al. 2011)	Number of EEMM species (WUFbase)
Russula	110	72
Lactarius	84	58
Boletus	62	32
Amanita	55	36
Tricholoma	46	27
Cantharellus	44	32
Hygrophorus	40	14
Cortinarius	39	15
Ramaria	39	22
Suillus	30	19
Tuber	28	15
Leccinium	19	10
Lycoperdon	18	13
Xerocomus	15	8
Tylopilus	13	2

a species was poisonous over claims it could be eaten, erring always on the side of safety.

The aim behind this categorization is to encourage researchers and others making inventories to check carefully on uses and not to assume that a species recorded as edible in a field guide, for example, is always eaten—if at all. Assumptions may be valid (though still presumptive) for a well-known species in a well-known region, for example, northern Italy, but this is not a good practise to follow in the Himalayan foothills, where traditions are poorly understood.

The earlier statement that there were 2,299 edible mushroom species can now be qualified: there are 1,648 unequivocal records of mushrooms that can be categorised as either food or are edible and potential food. A preliminary count says that these fall into 285 genera, of which 117 form ectomycorrhizae, based on the list published by Hall et al. (2011). These numbers are likely to change, including the total number of species, when taxonomic revisions and new genera since 2004 are taken into account and new sources of information are checked.

Analysing the species listed by Hall et al. (2011), the three genera with the highest number of edible species are *Russula*, *Lactarius* and *Boletus*. Table 17.2 lists then next 12 genera in descending order of abundance and compares the

Table 17.3 Number of species of edible mushrooms sold at local markets in Armenia, Mexico, Nepal and Tanzania combined with Zambia, adapted from Boa (2004)

Country	All species	Ectomycorrhizal
Armenia	13	4
Mexico	105	77
Nepal	20	13
Tanzania and Zambia	15	9

Original sources of information are available in Boa (2004), which includes selected information for 13 other countries. There is a major under-recording of species sold in local markets worldwide, and more information is needed on the quantities and values of this trade

number of species with those from wufbase. The order of genera stays more or less the same, but the abundance of EEMM drops significantly. The difference would appear to arise from Chinese records incorporated by Hall et al. (2011).

17.4 Local Markets and Sale of EEMM

It should be possible to demonstrate the value of EEMM to local communities through an analysis of local trade, giving a clearer indication of how many people benefit. Lists of species sold in 17 countries are given in Boa (2004), but these are clearly incomplete, and few new studies have been found. A snapshot of selected countries is shown in Table 17.3. Over half the species for sale are EEMM, though in volume and value they are likely to be a much larger part of the total trade in wild edible mushrooms.

The big markets are international, with supermarkets in better-off countries absorbing and sucking in industrial quantities of chanterelles from around Europe. There are few data on who receives money for collecting or trading on further down these long market chains.

The local selling of EEMM happens in small markets and by the roadside, the nearest opportunity for collectors to present their harvests once they have returned from their field visits. Some traders meet the collectors closer to their homes, but in Malawi there are especially women who will rise early to collect then walk up to 10 km to a market the same day, before returning home in the evening. Some sell directly but this takes time, and it is often easier to sell what you procure to a trader even though the money earned is substantially reduced compared to direct selling.

17.5 Conclusions

Edible ectomycorrhizal mushrooms account for the majority of sales and consumption of wild edible mushrooms around the world. Although their dependency on host plants marks them as biologically and ecologically different from other wild

edible species, the ultimate determinant of successful and sustainable management dependents on a thorough understanding of people. There are many gaps in our knowledge of who benefits from sales and consumption of these fungi, and future studies need to emphasise the critical role that people have in making choices and developing opportunities. Local communities benefit much from sales though uncertain demand and varying prices lead to unpredictable boom and bust cycles. EEMM and wild edible mushrooms are only one of many strategies that rural people use to earn a living. Their importance may well increase in times of financial hardship and weakened economies, putting further strains on natural resources that the rural poor increasingly fall back on when their options are limited.

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Chapter 18
Medicinal Aspects of Edible Ectomycorrhizal Mushrooms

Susanna Badalyan

"Medicines and food have a common origin" Old Chinese saying

18.1 Introduction

Mushrooms are widely appreciated all over the world for their nutritional properties and pharmaceutical value (Wasser and Weis 1999; Chang and Miles 2004). They are also used as functional food additives ("nutraceuticals" and "nutriceuticals") owing to the synergistic effects of their bioactive compounds. The early civilizations of Greeks, Egyptians and Romans, Chinese, Japanese, and Mexican people prized mushrooms for their therapeutic value and as treasures in religious rites (Hobbs 1995; Denisova 2001; Guzman 2008). Mushrooms have an established history of use in Traditional Chinese Medicine (TCM). The majority of preparations and blends possess beneficial health effects and can be used on a regular basis without harm. Medicinal mushrooms are thought to possess a total of 126 therapeutic effects (Wasser 2010).

Around 14,000 mushrooms were described among which about 7,000 species are considered edible, and more than 2,000 are regarded as highly prized edible mushrooms, including about 200 ectomycorrhizal species [*Tuber melanosporum* Vittad., *Tuber magnatum* Pico, *Tricholoma matsutake* (S. Ito & S. Imai) Sing., *Cantharellus cibarius* Fr., *Boletus edulis* Bull., etc.] (Hawksworth 2001; Wang et al. 2002b). Fruiting bodies and mycelia of edible ectomycorrhizal mushrooms

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(EEMM) are low in fat and rich in proteins, amino and fatty acids, carbohydrates, minerals, dietary fiber, and vitamins (Ulziijargal and Mau 2011). They represent a low-energy healthy nutrient used to formulate mushroom-based dietary supplements (Chung 2006; Pushpa and Purushothama 2010). EEMM are not only among the world's most expensive gourmet food but are also considered a valuable resource for pharmaceutical compounds (Ying et al. 1987). Although their nutritional value is well established and medicinal properties are gradually obtaining scientific validity, the existing problems in biotechnological cultivation of fruiting bodies and mycelia of EEMM limits the usage of their bioactive compounds (Chang 2006). Further research on EEMM biology and the development of new cultivation technologies should lead to new biotechnological and medicinal applications.

18.2 Bioactive Compounds and Medicinal Properties of EEMM

Fungi represent a seemly unlimited source of bioactive compounds, and their molecular diversity has recognized potential in drug discovery and development (Butler 2004; Štrukelj et al. 2007). Basidiomycetes mushrooms, including edible mycorrhizal species, generate the main types of bioactive compounds (polysaccharides, peptidoglucans, terpenoids, phenolic compounds, steroids, lectins, etc.) that possess a wide range of therapeutic effects (immune-modulating, antitumor, antimicrobial, antiviral, antioxidant, etc.) Stenglich 1981; Wasser and Weiss 1999; Zhang et al. 2007; Villares et al. 2012). Such bioactive compounds can be isolated from wild and cultivated fruiting bodies or from mycelial biomass. As subsidiary agents, mushroom bioactive compounds are used at certain doses for the prevention and treatment of various diseases (Borchers et al. 2004; Zaidman et al. 2005; Poucheret et al. 2006). Antimitotic polysaccharides were isolated from fruiting bodies of *Boletus* (=*Xerocomus*) *badius* Pers. (Wegiel et al. 2001). Terpenoids (sesquiterpenoids) with antimicrobial and cytotoxic effects were detected in edible *Lactarius* species, such as *Lactarius deliciosus* (L.) Gray, *Lactarius deterrimus* Gröger, and *Lactarius sanguifluus* (Paulet) Fr. (Anke et al. 1989). These compounds inhibit histamine release and reveal anti-inflammatory effects, which can be applied in the treatment of urogenital infections, cystitis, and syphilis (Tardif 2000). Many EEMM [*Boletus impolitus* Fr., *Entoloma sinuatum* (Bull.) P. Kumm., *Rhizopogon luteolus* Krombh., *Tricholoma terreum* (Schaeff.) P. Kumm.] produce alkaloids (Bastida et al. 1987a, b). The alkaloids necatorin and necotoron isolated from *Lactarius necator* (Bull.) Pers. (is mainly used as spices) possess antimicrobial activity against *Acetobacter calcoaceticus, Bacillus subtilis,* and *B. brevis* (Bross et al. 1987). A number of sterols, flavonoids, prenylphenols, and polyprenols with antimicrobial, antioxidant, and antitumor activities were reported in *Tricholoma populinum* (Fr.) Bon, *Tricholoma portentosum* (Fr.) Quél., *Suillus granulatus* (L.)

Roussel, *Suillus grevillei* (Klotzsch) Sing., and *Suillus luteus* (L.) Roussel (Tringali et al. 1989a, b; Hayashi et al. 1989; Kukina et al. 2005; Barros et al. 2007). Phenolic compounds derived from mushrooms contribute to their antioxidant properties reducing the risk of cancer, atherosclerosis, and cardiovascular and other diseases (Yang et al. 2002; Cheung et al. 2003; Elmastas et al. 2007). As major phenolic compounds, protocatechuic, *p*-hydroxybenzoic, and *p*-coumaric phenolic acids were detected in fruiting bodies of *Amanita caesarea* (Scop.) Pers., *Lactarius volemus* (Fr.) Fr., and *Suillus luteus*. However, the highest antioxidant activity was observed in *S. luteus*, in which protocatechuic acid was predominant (Barreira et al. 2011). Another study directed to the chemical composition and antioxidant activity of 12 mycorrhizal mushrooms revealed that edible species [*Amanita caesarea*, *Lactarius quietus* (Fr.) Fr., *Lactarius volemus*, *S. luteus*] can be incorporated into human diet as sources of antioxidants, whereas inedible species [*Amanita muscaria* (L.) Lam., *Amanita pantherina* (DC) Krombh., *Chroogomphus fulmineus* (R. Heim) Courtec., *Cortinarius anomalus* (Fr.) Fr., *Cortinarius collinitus* (Pers.) Fr., *Cortinarius violaceus* (L.) Gray, *Russula sardonia* Fr., *Tricholoma ustale* (Fr.) P. Kumm.] can be regarded as sources of bioactive metabolites (Reis et al. 2011a, b). Antibacterial, anti-inflammatory, hypocholesterolemic, hypotensive, antitumor, and other therapeutic effects were reported in the medicinal EEMM *Lyophyllum decastes* (Fr.) Sing. and *Cantharellus tubaeformis* Fr. (Ukawa et al. 2000, 2001a, b, 2002; Tsvetkova et al. 2006). The highly valued EEMM *Boletus edulis* (porcini) is also considered a producer of the variety of bioactive compounds with antimicrobial, antitumor, anti-inflammatory, cytotoxic, and antioxidant effects (Tang and Lu 1999; Tsai et al. 2007; Vidović et al. 2010; Palacios et al. 2011).

Biological activities (immune-modulating, mitogenic, hypotensive, nematicidal, insecticidal, antimicrobial, etc.) of fungal lectins (proteins) were described in previous studies (Wang et al. 1996b, 1998, 2002a, 2007; Konska 2006). Some lectins preferentially bind to cancer cell membranes or their receptors, causing cytotoxicity, apoptosis, and inhibition of tumor growth (Wang et al. 1997; Zhao et al. 2010). Lectins (agglutinins) occur in the majority of edible mushrooms, including mycorrhiza forming (*Amanita rubescens* Pers., *Lactarius deliciosus*, *L. volemus*, species from genera *Boletus* and *Laccaria*, rarely *Russula* and *Suillus*) (Seeger 1980; Seeger and Wiedman 1972; Pemberton 1994). Mitogenic lectin with an affinity to xylose and melibiose and antitumor lectin binding to a neoplastic cell-specific T-antigen disaccharide were isolated from *B. edulis* (Tardif 2000; Bovi et al. 2011). Research on the chemical composition and medicinal properties of hypogeal EEMM is limited to a few species of truffles from genera *Terfezia*, *Tirmania* and *Tuber* (Chellal and Lukasova 1995; Janakat et al. 2005; Wang and Marcone 2011; Villares et al. 2012). Desert truffles from genera *Terfezia* (*Terfezia claveryi* Chatin), *Tirmania* [*Tirmania pinoyi* (Maire) Malençon], and *Picoa* (*Picoa juniperi* Vittad.) represent a novel source of compounds with antimutagenic, anticarcinogenic, and antioxidant properties (Hannan et al. 1989; Murcia et al. 2002). An antiviral lectin cyanovirin-N (CVN) and fruiting body-specific lectin TBF-1 were described in *Tuber borchii* Vittad. (Percudani et al. 2005, Cerigini et al. 2007) (see Table 18.1).

Table 18.1 Medicinal effects of bioactive compounds reported in several EEMM

EEMM species	Bioactive compound	Medicinal effect
Boletus (=*Xerocomus*) *badius*[1–7]	Polysaccharides, polyphenolics, *N*-ethyl-γ-glutamine (L-thenine analog)	Antimitotic, antitumor, immune-modulating, antioxidant, neurotropic
Boletus edulis[2,5,8–17]	Lectin, polysaccharides, polyphenols, ergothioneine	Antitumor, immune-modulating, antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, mitogenic, neurotropic
Cantharellus cibarius[2,18–26]	Polysaccharides, cibaric acid, phenolic compounds	Antioxidant, antimicrobial, antifungal, insecticidal, nematicidal
Cantharellus tubaeformis[19,27,28]	Polysaccharides, 10-hydroxy-8-decenoic acid	Antioxidant, antimicrobial, antifungal, anti-inflammatory, insecticidal, nematicidal, hypocholesterolemic, hypoglycemic, hypotensive, antitumor
Lactarius deliciosus[11,17,21–26,29,30]	Sesquiterpenoids, lectin, phenolic compounds	Antibacterial, antifungal, cytotoxic, anti-inflammatory, insecticidal, nematocidal, antioxidant
Lactarius flavidulus[2,31,33]	Polysaccharides, flavidulols A–D	Antitumor, antibacterial, cytotoxic, anti-inflammatory, immune-suppressive
Lactarius necator[11,34]	Alkaloids necatorin and necotoron	Antibacterial, antifungal
Lactarius volemus[30,35–37]	Phenolic acids, lectin	Antioxidant
Lyophyllum decastes[38–43]	(1→3)- and (1→6)-beta-D-glucans, phenolics	Antitumor, antibacterial, hypocholesterolemic, hypoglycemic, hypotensive, anti-inflammatory, immune-modulating, radioprotective, antioxidant
**Morchella esculenta*[44]	Galactomannan (α-D-glucan)	Immune-modulating
Russula delica[45]	Lectin	Antiproliferative, antiviral
Russula paludosa[46]	Peptide	Antiviral
Rusulla virescens[47]	Polysaccharides	Antioxidant, hypoglycemic, hypocholesterolemic
Russula xerampelina[48]	Polysaccharides	Antitumor, antiparasitic
Suillus bovines[48,49]	Suillin	Immune-suppressive, antibacterial
Suillus granulatus[3,47,50–52]	Tetraprenylphenols	Antitumor
Suillus luteus[11,24,25,35–37]	Phenolics, polysaccharides	Antifungal, antioxidant, immune-modulating
Tricholoma lobayense[53]	Polysaccharides, polysaccharide-protein complex	Immune-modulating, antitumor
Tricholoma giganteum[54]	Polysaccharides	Immune-modulating, antitumor
Tricholoma matsutake[55–57]	α-D-glucan	Immune-modulating

(continued)

Table 18.1 (continued)

EEMM species	Bioactive compound	Medicinal effect
Tricholoma mongolicum[58–60]	Lectins, polysaccharide-peptide complex	Immune-modulating, antitumor, hypotensive, vasorelaxing
Tricholoma portentosum[2,11,24–26]	Polysaccharides, phenolic compounds	Antitumor, antibacterial, antifungal, fibrinolytic
Terfezia claveryi[61–67]	Peptide antibiotic	Antibacterial, antimutagenic, anticarcinogenic, antioxidant
Tirmania nivea[68]	Phenolics	Antioxidant, antiradical
Tirmania pinoyi[61,62,65,66,69]	Ethyl acetate extract	Antibacterial, antimutagenic, anticarcinogenic
Tuber aestivum[70]	Phenolic acids (hydroxycinnamic acid derivatives, *o*- and *p*-coumaric acids), flavonoids, ergosteryl ester	Antioxidant
Tuber borchii[71,72]	Lectins cyanovirin-N (CVN) and TBF-1	Antiviral
Tuber indicum [70,73]	Phenolics, flavonoids, ergosterol	Antioxidant and radical-scavenging activities
Tuber melanosporum[70,74]	Phenolic acids, ergosterol	Antioxidant, neurotropic

[1]Wegiel et al. (2001), [2]Ohtsuka et al. (1973), [3]Chauveau (1990), [4]Elmastas et al. (2007), [5]Moldavan et al. (2001), [6]Casimir et al. (1960), [7]Li et al. (2008), [8]Tang and Lu (1999), [9]Tsai et al. (2007), [10]Bovi et al. (2011), [11]Chaumont and Simeray (1982), [12]Kandefer-Szersen et al. (1980), [13]Li et al. (2009), [14]Zheng et al. (2007), [15]Vidović et al. (2010), [16]Ey et al. (2007), [17]Palacios et al. (2011), [18]Dulger et al. (2004), [19]Anke et al. (1996), [20]Cieniecka-Rosłonkiewicz et al. (2007), [21]Ribeiro et al. (2008), [22]Queirós et al. (2009), [23]Witkowska et al. (2011), [24]Ferreira et al. (2007), [25]Ferreira et al. (2009), [26]Barros et al. (2007), [27]Tsvetkova et al. (2006), [28]Pang et al. (1992), [29]Anke et al. (1989), [30]Pemberton (1994), [31]Fujimoto et al. (1993), [32]Takahashi et al. (1988), [33]Takahashi et al. (1993), [34]Bross et al. (1987), [35–36]Reis et al. (2011a, b), [37]Barreira et al. (2011), [38]Ukawa et al. (2000), [39–40]Ukawa et al. (2001a, b), [41]Ukawa et al. (2002), [42]Suzuki et al. (2001), [43]Kokean et al. (2002), [44]Duncan et al. (2002), [45]Zhao et al. (2010), [46]Wang et al. (2007), [47]Ying et al. (1987), [48]Das (2010), [49]Shirata et al. (1995), [50,51]Tringali et al. (1989a, b), [52]Geraci et al. (1992), [53]Liu et al. (1996), [54]Mizuno et al. (1996), [55]Ishihara et al. (2004), [56]Hoshi et al. (2008), [57]Byeon et al. (2009), [58–60]Wang et al. (1996a, b, 1997), [61]Murica et al. (2002), [62]Chellal and Lukasova (1995), [63–64]Janakat et al. (2004), (2005), [65]Mandeel and Al-Laith (2007), [66]Fortas and Dib-Belahouel (2007), [67]Al-Marzooky (1981), [68]Al-Laith (2010), [61,62,65,66,69]Dib-Belahouel and Fortas (2011), [70]Villares et al. (2012), [71]Percudani et al. (2005), [72]Cerigini et al. (2007), [73]Guo et al. (2011), [74]Tardif (2000), *Morchella may be a biotroph, rather than an EEMM (see Chap. 2)

18.2.1 Antitumor, Immune-Modulating, and Immune-Suppressive Activities

Antitumor and immune-modulating activities represent the most significant pharmacological and therapeutic interests associated with mushrooms. They are regarded as a potential source of antitumor polysaccharides with various branching types [(1 → 3)- and (1 → 6)-β-D-glucans] and polysaccharide-protein complexes (PSP, PSK, etc.) (Mizuno et al. 1995, 1996; Ooi and Liu 2000; Badalyan 2000). Fungal polysaccharides exert antitumor activity mainly as biological response

modifiers via activation of the immune response of the host organism (Wasser and Weis 1999; Mizuno 1999; Wasser 2002; Zhang et al. 2007; Moradali et al. 2007). Their therapeutic action includes in vivo prevention of oncogenesis and metastasis, inhibition of tumor cell proliferation, direct cytotoxicity on cancer cells, strengthening the effect of chemotherapy by decreasing drug toxicity and side effects, etc. (Poucheret et al. 2006). Polysaccharides extracted from mycelial cultures of edible ectomycorrhizal species belonging to genera *Amanita*, *Boletus*, *Cantharellus*, *Cortinarius*, *Lactarius*, *Leccinum*, *Russula*, *Tricholoma*, and *Xerocomus* revealed antitumor and immune-modulating activities against Sarcoma 180 and Ehrlich solid cancer cells (Ohtsuka et al. 1973). Polysaccharides from *Lactarius flavidulus* S. Imai inhibited the growth of both tumor cells by 100 %, whereas polysaccharides from *Boletus* (=*Xerocomus*) *badius* and *Tricholoma partentosum* by 60 % and 70 %, and 70 % and 60 %, respectively. Fungal glucans with immune-modulating, antitumor, and radioprotective effects were isolated from *Lyophyllum decastes*, *Morchella esculenta*, and *Tricholoma matsutake* (Ukawa et al. 2000, Duncan et al. 2002; Ishihara et al. 2004; Hoshi et al. 2008; Byeon et al. 2009). Immune-modulating and antitumor polysaccharides and polysaccharide-protein complexes were isolated from mycelia of other *Tricholoma* species (*Tricholoma giganteum* Massee, *Tricholoma mongolicum* S. Imai, *Tricholoma* sp.) and from cultural liquid of *Tricholoma lobayense* R. Heim (Mizuno et al. 1996; Wang et al. 1996a; Liu et al. 1996). They activated macrophages, stimulated the proliferation of T-cells, and inhibited the growth of Sarcoma 180 tumor cells in mice. Immune-stimulating activity was detected in fruiting body extracts isolated from several Boletaceae species, such as *B. edulis*, *Suillus granulatus*, *S. luteus*, *Xerocomus chrysenteron* (Bull.) Quél., *Xerocomus nigromaculatus* Hongo, *X. badius*, as well as *Amanita caesarea*, *Russula cyanoxantha* (Schaeff.) Fr., and *Russula virescens* (Schaeff.) Fr. (Ying et al. 1987; Chauveau 1990; Takahashi et al. 1992). Among them, extract from *B. edulis* has shown 100 % and 90 % growth inhibition against Sarcoma 180 and Ehrlich carcinoma cells in mice, respectively. The presence of polysaccharides and phenolic compounds in *Suillus collinitus* (Fr.) Kuntze indicates that this species is a promising source of antitumor metabolites (Ferreira et al. 2010). Tetraprenylphenol suillin and several related bioactive compounds were identified to be responsible for the cytotoxic and antitumor activities of the lipid extract obtained from *S. granulatus*. Significant antitumor effect of suillin against P-388 murine ascetic leukemia was observed (Tringali et al. 1989a; Geraci et al. 1992). The activities of 14 related compounds were revealed against KB-cells (human nasopharyngeal cancer) and NSCLC-N6 (human bronchopulmonary carcinoma) (Roussakis et al. 1991). These authors demonstrated the biological importance of prenylphenols, which possess antimicrobial, antioxidant, anti-inflammatory, antitumor, and cytotoxic activities.

Suillin, found in fruiting bodies of *Suillus bovines* (Pers.) Roussel, is regarded as a natural compound with immune-suppressive activity that preferentially perturbs B-lymphocyte function (Shirata et al. 1995). Immune-suppressive compounds flavidulols A, B, C, and D were isolated from fruiting bodies of EEMM *Lactarius flavidulus* (Fujimoto et al. 1993). It was shown that methanolic extract of *L. flavidulus*

inhibited lymphocyte proliferation in the spleen of mice (blastogenesis) caused by mutagenic factors (Takahashi et al. 1993). The immune-suppressive effect was also described in fruiting body extract of *Tricholoma populinum* (Lindequist et al. 2005).

18.2.2 Antibacterial, Antifungal, and Antiviral Activities

Fungi are well known as producers of different antimicrobial (antibacterial/antifungal/antiviral) compounds. However, the occurrence of antibiotics in Basidiomycetes mushrooms is less documented (Miles and Chang 1997; Wasser and Weis 1999). Similar to anticancer properties, antimicrobial activity can also be developed either directly against external aggressor or indirectly via immune system activation. A wide range of antimicrobial activity with a pronounced therapeutic effect was reported for over 200 mushroom species (Anke and Sterner 1991; Tringali et al. 1989b; Lee et al. 1999; Brandt and Piraino 2000; Lindequist et al. 2005). Overall, mushroom extracts from fruiting bodies and mycelia are more active against Gram-positive than Gram-negative bacteria. Antibacterial compounds against Gram-positive and Gram-negative bacteria were detected in edible desert truffles from genera *Terfezia* and *Tirmania* (Rougieux 1963; Chellal and Lukasova 1995; Janakat et al. 2005; Mandeel and Al-Laith 2007; Fortas and Dib-Belahouel 2007). Aqueous and methanolic extracts of *Terfezia claveryi* were tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Aqueous extract significantly inhibited bacterial growth, whereas methanolic extract was inactive (Janakat et al. 2004, 2005). It was shown that extract from dried fruiting bodies of desert truffle *T. claveryi* is effective for the treatment of eye diseases, such as trachoma (the causal organism is *Chlamydia trachomatis*), stomach ulcers, and open cuts (Al-Marzooky 1981; Mandeel and Al-Laith 2007). Ethyl acetate extract obtained from *Tirmania pinoyi* was tested against *Bacillus subtilis*, *Enterococcus* sp., *Escherichia coli*, P. aeruginosa, and *S. aureus* (Dib-Bellahouel and Fortas 2011). The highest activity was detected against Gram-positive bacteria *B. subtilis* and *S. aureus*. Recently, antibacterial activity was reported in *Suillus bovines* (Das 2010). Antimicrobial activity of ethyl acetate, acetone, chloroform, and ethanol extracts from *Cantharellus cibarius* against 50 tested organisms, including Gram-positive and Gram-negative bacteria, actinomycetes, yeasts, and filamentous fungi, has been described by Dulger and coauthors (2004). The extent of antimicrobial activity of flavidulol A extracted from *L. flavidulus* was reportedly different against pathogenic bacteria (*B. subtilis*, *E. coli*, *S. aureus*), yeast (*Candida albicans*), and filamentous fungi (*Trichophyton rubrum*, *T. mentagrophytes*) (Takahashi et al. 1988). Antibacterial/antifungal activities against *S. aureus*, *E. coli,* and *C. albicans* were determined in chloroform and butanol extracts from fruiting bodies of *Cantharellus tubaeformis* (Anke et al. 1996). Screening of antibacterial/antifungal activities of fruiting body extracts of *L. deliciosus* and *T. portentosum* against Gram-positive (*Bacillus cereus*, *B. subtilis, S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae*) bacteria as well as yeasts (*C. albicans*, *Cryptococcus neoformans*) showed that the

extract of *T. portentosum* was effective against Gram-positive *Bacillus* bacteria and *C. neoformans*, whereas the extract of *L. deliciosus* inhibited all Gram-positive bacteria, showing no antifungal activity (Barros et al. 2007). In vitro screening of antifungal properties of aqueous extracts from 225 Basidiomycetes and Ascomycetes mushrooms including around 44 EEMM species particularly from genera *Boletus* (*Boletus albidus* Schaeff., *B. edulis*, *Boletus luridus* Schaeff.), *Laccaria* (*Laccaria amethystine* Cooke), *Lactarius* (*L. deliciosus*, *Lactarius necator*), *Leccinum* [*Leccinum carpini* (Schulzer) Moser ex Reid, *Leccinum duriusculum* (Schulzer ex Kalchbr.) Sing.], *Russula* (*Russula fragilis* Fr., *Russula queletii* Fr.), *Suillus* (*S. luteus*), and *Tricholoma* [*T. portentosum*, *Tricholoma saponaceum* (Fr.) P. Kumm., *Tricholoma virgatum* (Fr.) P. Kumm.] revealed fungistatic effects against seven pathogenic test microfungi (*Cytospora* sp., *Fusarium oxysporum*, *Graphium ulmi*, *Rhizoctonia solani*, *Stereum purpureum*, *C. albicans*, *Aspergillus fumigatus*) (Chaumont and Simeray 1982).

Antiviral compounds obtained from mushrooms are a subject of interest in biomedical research given their potential to advance knowledge of viral replication and deliver new drugs for the treatment of viral diseases (Brandt and Piraino 2000; Piraino 2006). In vitro antiviral activity against Vaccinia virus and Tobacco mosaic virus was detected in *B. edulis* (Kandefer-Szersen et al. 1980; Li et al. 2009). The inhibitory effect on reverse transcriptase enzyme of human immunodeficiency virus (HIV) was described in *B. edulis* and *Russula paludosa* Britzelm (Zheng et al. 2007; Wang et al. 2007). A novel sugar-binding antiviral lectin cyanovirin-N (CVN) as a potent inhibitor of HIV and Ebola virus is found in *Tuber borchii* (Percudani et al. 2005).

18.2.3 Antiprotozoal, Nematicidal, and Insecticidal Activities

Until now, fungal bioactive compounds with antiprotozoal, nematicidal, and insecticidal activities have not been sufficiently investigated. Several literature sources indicate that mainly phenolic and indolic compounds, alkaloids, terpenoids, and fungal lectins possess such activities (Wang et al. 2002a; Konska 2006). However, the mechanisms underlying antiprotozoal, nematicidal, and insecticidal effects of mushroom compounds are not completely understood. One broad spectrum in vitro antiprotozoal activity of the fungal metabolite—apicidin, a cyclic tetrapeptide, has been studied (Darkin-Rattray et al. 1996). The activity appears to be due to inhibition of histone deacetylase (HDA), which induces hyperacetylation of histones in treated parasites. Other known HDA inhibitors were also investigated and found to possess antiparasitic activity, suggesting that HDA is an attractive target for the development of fungal antiprotozoal agents. Antiprotozoal activities of several edible mushrooms against *Plasmodium falciparum* and *Paramecium caudatum* were reported (Lovy et al. 2000; Badalyan 2004). Combined antiparasitic/antitumor effects were recently detected in *Russula xerampelina* (Schaeff.) Fr. (Das 2010). Insecticidal and nematicidal activities of

EEMM are rarely reported in comparison with such activities in pungent mycorrhizal species from genera *Lactarius* and *Russula* (Sterner 1995; Jonassohn 1996). Two fatty acid derivatives, cibaric acid and 10-hydroxy-8-decenoic acid, are formed in *Cantharellus cibarius* and *C. tubaeformis*, respectively. They play a role in natural protection, respond to bruising and injury of the fruiting bodies (Pang et al. 1992; Anke et al. 1996). These mushrooms are rarely infested by insects, which attack most species (Hackman and Meinander 1979), and insecticidal and antimicrobial activities of *C. cibarius* have been reported (Cieniecka-Rosłonkiewicz et al. 2007).

18.2.4 Antioxidant Activity

Recent studies directed to the effect of oxidative stress on the human body have become a subject of major interest. To prevent such pathological conditions, it is necessary to add a certain amount of antioxidants into the diet. Mushrooms, including mycorrhiza-forming species, are regarded as a natural source of antioxidants to prevent oxidative damage of cells and tissues (Pan and Ye 1997; Yang et al. 2002; Badalyan 2003). Antioxidant properties combined with antitumor and immune-modulating activities lead to the health-sustaining effects of mushrooms. Among mushroom-derived bioactive compounds, phenolics have been widely investigated for their antioxidant activity and various mechanisms of action proposed (Poucheret et al. 2006; Ersel and Cavas 2008). Overall, sixteen edible wild-growing mushrooms, including *L. deliciosus* and *C. cibarius* are reported as potential sources of antioxidants (Ribeiro et al. 2008; Queirós et al. 2009; Witkowska et al. 2011). Screening of their phenolic compounds (flavonoids, phenolic acids, tannins, etc.) show that phenolic composition is characterized by the presence of phenolic acids, mainly *p*-hydroxybenzoic acid (Ribeiro et al. 2008). The high total polyphenol content found in the EEMM *B. edulis*, *Boletus aurantiacus* Bull., *Boletus chrysenteron* Bull., *C. cibarius*, *L. deliciosus, Leccinum scabrum* (Bull.) Gray, *Leccinum aurantiacum* (Bull.) Gray, *Suillus grevillei*, *S. luteus*, and *T. portentosum* indicates that these species are valuable sources of antioxidants (Ferreira et al. 2007, 2009; Vidović et al. 2010). Furthermore, *B. edulis* was determined to possess the highest amount of antioxidant compound ergothioneine in fresh fruiting bodies (Ey et al. 2007). Among the different tested mushroom species, the highest antioxidant activity was expressed in a methanolic extract of dried fruiting bodies of *B. badius* (Elmastas et al. 2007). The authors reported a higher content of total polyphenolics in this species. Total phenolic and flavonoid contents in *B. edulis*, *C. cibarius,* and *L. deliciosus* and their lipid oxidation activity have recently been evaluated (Palacios et al. 2011). Tested species, particularly *C. cibarius,* possess the highest antioxidant activity. Combined antioxidant/antitumor activities have been reported in *Russula cyanoxantha* (Das 2010). The antioxidant and radical-scavenging activities related to the contents of ergosterol, phenolics, and flavonoids in several *Tuber* species (*Tuber*

melanosporum, *Tuber aestivum* Vittad., *Tuber indicum* Cook & Massee) were reported, as well (Guo et al. 2011; Villares et al. 2012). Different levels of antioxidant activity were detected in the extract of dried desert truffle *Tirmania nivea* (Desf.) Trappe. A correlation between phenolic contents and antioxidant activity was revealed (Al-Laith 2010).

18.2.5 Neurotropic and Psychotropic Effects

It is well known that mushrooms possess neurotropic and psychotropic effects (Bresinsky and Besl 1990; Guzman 2001). Edible mushrooms, including mycorrhizal species, were used in traditional medicine to treat central and peripheral nervous system disorders. Extracts of *Amanita rubescens*, *A. caesarea*, and *Lyophyllum georgii* (Clus. : Fr.) Sing. stimulate the nervous system and possess tonic and cognitive effects. Preparations obtained from *B. edulis* are widely used in homeopathy to treat epilepsy. Ascomycetes mushrooms *Morchella rotunda* (Pers.) Boud. and *T. melanosporum* possess tonic effects on the nervous system. They are widely used against asthenia, general fatigue, and other nervous disturbances (Tardif 2000). Three new neurotrophic diterpenes, tricholomalides A–C (1–3), were isolated, and their structures were elucidated from the methanolic extract of the fruiting body of *Tricholoma* sp. Tricholomalides significantly induced neurite outgrowth in rat pheochromocytoma cells (PC-12) (Tsukamoto et al. 2003). Pronounced in vitro inhibition of neuronal responses on the hippocampal slices and selective activation of definite types of receptors belonging to various types of transmitter systems in rats was shown by extracts of *A. rubescens, B. edulis*, and *X. badius* (Moldavan et al. 2001). A non-protein-forming amino acid and a derivative of glutamic acid (L-theanine analog, found in green tea *Camellia sinensis*) was separated and identified from *X. badius* (Casimir et al. 1960). A Chinese study has demonstrated a promising method for producing theanine by using submerged fermentation technology of mycelium of *X. badius* (Li et al. 2008).

18.2.6 Activity Against Metabolic Syndrome and Related Diseases

Advances in cardiovascular research and insulin resistance have led to the recognition of a concept of metabolic syndrome which is regarded as a condition associated with biochemical disturbances leading to progressive hyperglycemia, hyperlipidemia, and cardiovascular diseases (Reaven et al. 1988). The majority of well-known medicinal mushrooms produce bioactive compounds which are able to positively affect one or several of these disorders associated with metabolic syndrome (Francia et al. 1999; Ukawa et al. 2001a, b; Yang et al. 2002). Edible mushrooms

are considered cholesterol-free food. By acting on fundamental risk factors, they positively affect the very upstream of events that may lead to cardiovascular diseases, such as thromboses, as well as diabetes and obesity. Fungal polysaccharides and their protein complexes, dietary fibers extracted from fruiting bodies, mycelial biomass, and cultural broth of medicinal mushrooms were reported to possess hypoglycemic activity. Several EEMM are able to prevent cardiovascular diseases caused by high levels of sugars and blood lipids by inhibiting their accumulation in the liver and serum. Antioxidant and cholesterol- and sugar-lowering effects have been reported in *C. cibarius, R. virescens,* and *L. decastes* (Tardif 2000; Ukawa et al. 2001a, 2002; Miura et al. 2002; Das 2010). Dry powdered hot water extract from fruiting bodies of *L. decastes* significantly inhibited angiotensin-converting enzyme (ACE) activity and decreased blood pressure in spontaneously hypertensive rats (Suzuki et al. 2001; Kokean et al. 2002; Ukawa et al. 2001b). Vasorelaxant and hypotensive effects of a lectin obtained from the edible mushroom *Tricholoma mongolicum* were revealed (Wang et al. 1996b). Fibrinolytic activity in *T. portentosum* (Denisova 2005) and thrombolytic effects in *Cantharellus lutescens* Fr. and *Suillus tridentinus* (Bres.) Sing. have also been reported (Štrukelj et al. 2007).

18.3 Conclusion

This review has highlighted the medicinal importance of wild and cultivated EEMM as a natural source of antitumor, immune-modulating, antimicrobial, antioxidant, and other metabolites. The data available on chemical composition, bioactive compounds, and therapeutic potential of EEMM suggests that they can be regarded as valuable natural products to develop healthy functional food supplements and mushroom-based pharmaceuticals that can be used in the prevention and treatment of various diseases.

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Chapter 19
Insects Parasitizing Edible Ectomycorrhizal Mushrooms

Nicola Sitta and Luciano Süss

19.1 Introduction

Among the many interactions between fungi and insects, mycophagy has a great impact on the commerce and human food consumption of edible ectomycorrhizal mushrooms (EEMMs). Arthropods of different classes, and especially certain orders of insects, feed on both fresh and decaying fungal fruiting bodies, where they carry out their whole (or partial) biological cycle (Bruns 1984; Hanski 1989; Hackman and Meinander 1979; Oconnor 1984; Krivosheina 2008).

Fungal fruiting bodies differ widely in their physical and chemical features, as well as in their lifespan. Accordingly, there is great variation in the entomofauna associated with the fungal species. For instance, a wider range of insect fauna are associated with the tough, wood-like fruiting bodies of many Aphyllophorales, which can persist for several years and whose seasonal fruiting is less dependent on climatic factors, than with short-lived, fleshy mushrooms.

This study examines fungus–insect relationships, with a focus on the interactions involving the presence of parasites in the EEMMs destined for human consumption. In addition to mycophagy, we also examine predation, cannibalism, and parasitism. Predation refers to the presence in EEMMs of arthropods that are not strictly fungivorous but are predators of other arthropods. Cannibalism is a very common phenomenon among larvae and adults of Diptera within the same or similar species. Parasitoids, which occasionally can be found in the fruiting bodies of EEMMs, are a limiting factor of the pest populations.

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19.2 Insect Mycophagy

Mycophagy by soil arthropods can cause damage to young fruiting bodies, and consequently, a decrease in spore production. However, mycophagy may increase fitness through means of spore dispersal (Boddy and Jones 2008; Lilleskov and Bruns 2005), especially during the actively productive phase. A natural hierarchy of preferences or palatability appears to exist, by which certain fungal species are much more likely than others to be consumed by arthropods (Guevara and Dirzo 1999). Fungus–insect relationship is based on chemical cues (Martin 1979); fungal species with spore dispersal mechanisms involving mycophagous insects contain palatable substances, whereas the fruiting bodies of other fungal species containing toxic insecticides and repellents are less prone to arthropod attack (Bruns 1984; Hackman and Meinander 1979; Mier et al. 1996; Wang et al. 2002). Nonetheless, the presence, in a given fungus, of substances found to be toxic in the laboratory for *Drosophila melanogaster* Meigen, or for a few other insect species, does not necessarily imply that, in the natural environment, the fungus is unpalatable for mycophagous insects. For example, *Clitocybe nebularis* (Batsch) P. Kumm. and *Xerocomus chrysenteron* (Bull.) Quél. are highly sought after by fungivorous larvae of Diptera despite the presence of insecticidal lectins (Pohleven et al. 2011; Trigueros et al. 2003).

Different degrees of preferential specialization exist among mycophagous arthropods: Bruns (1984) for insect mycophagy in the Boletales and Krivosheina (2008) for the dipteran fauna of macromycetes show many analogies. Mycophagists can be divided into four categories, even though intermediate positions exist:

- Primary fungivores: They attack young fruiting bodies often causing considerable damage and frequently show preference for certain fungal hosts and, in other cases, marked specialization (e.g., they develop exclusively in a single genus); Krivosheina (2008) refers to them as obligatory macromycobionts.
- Secondary fungivores: They are also exclusively fungivorous and live on decaying hosts without damaging young fruiting bodies and are almost always polyphagous; Krivosheina (2008) calls them obligatory sapromycobionts.
- Detritivores: Like those previously mentioned, they are not to be found in young fruiting bodies but are not exclusively fungivorous as they can feed on a large variety of decaying organic matter; Krivosheina (2008) includes them among sapromycobionts.
- Predators: They are extremely widespread, and for some of them, the larvae are obligate predatory in the final instar, whereas they are fungivorous in earlier instars, for example, *Muscina assimilis* (Fallén 1823) and *Mydaea* spp. Krivosheina (2008) defines as eurybionts the arthropods that live in fungi as predators or necrophagists.

Krivosheina (2008) makes no subdivision between sapromycobionts that live solely in fungi and those capable of feeding also on other substrates, and refers to Diptera living on fruiting body surfaces (epibionts), where they feed on mycelium and spores, as facultative mycobionts.

19.3 Principal Taxa of Parasite Arthropods in EEMMs

An EEMM fruiting body, besides being a supplier of nourishment to a fungivorous species, can also become a complete miniature ecosystem, in which several species may coexist, ranging from monophagists, which are exclusive to a single fungal species, to polyphagous fungivores, predators, parasites, and other occasional presences. This chapter is focused on taxa of fungicolous arthropods that are most frequently found in major EEM species, and particular attention will be given to Diptera and Coleoptera, which represent dominating orders in insect communities associated with macromycetes.

19.3.1 Diptera

Whole families of Diptera have coevolved with fungi, exploiting fungal fruiting bodies as a source of nutrition and as a substrate for the development of larvae. Many dipteran families include selective species with specialized behaviour (e.g., way of laying eggs, development, food preferences). The phenomenon is vast; on examining only British Diptera, fungus–insect associations are currently known for about 540 species, placed in 43 different families, 23 of which, at least, include some obligate fungicoles (Chandler 2010).

The suborder Nematocera (Diptera with long antennae, e.g., mosquitoes) contains strictly fungivorous species, the so-called fungus gnats, which belong to the huge superfamily Sciaroidea. Fungus gnats (also referred to in the literature as Mycetophiloidea or Mycetophiliformia) are comprised of nine families whose phylogenetic relationships have been clarified (Amorim and Rindal 2007). According to Bechev (2000), the Sciaroidea (excluding the family Sciaridae) include more than 4,100 described species and are distributed worldwide. Alone, species in the families Bolitophilidae and Mycetophilidae account for a high percentage of primary fungivores (Krivosheina 2008). Female adults lay their eggs in various parts of fungal fruiting bodies, as tubes or gills, pileus, upper parts of the stipe (Fig. 19.2a). The larvae, whitish in color with a well-developed black head capsule (eucephalic), complete their cycle in 4 instars and reach a length of 10–12 mm. Their digging activity in the fruiting bodies accelerates the decaying process (Bruns 1984). Few species continue their development in decaying fruiting bodies; however, some are markedly polyphagous, for example, *Mycetophila fungorum* (De Geer 1776). The Sciaridae are mainly saprophagous but include fungivorous species, well known for attacking cultivated mushrooms, especially the mycelia in compost. Their larvae are quite rare in EEMMs and show a preference for decaying specimens (Locatelli et al. 2006). A rare and quite exceptional phenomenon typical of the Sciaridae is the migration of thousands of larvae in a long line. This appears to occur in response to food scarcity in the substrate.

Beyond the Sciaroidea, many other families of Nematocera, such as Limoniidae, Scatopsidae, Trichoceridae, Psychodidae, Ceratopogonidae and others, include species with fungicolous larvae. They all have eucephalic larvae, except for the Limoniidae, which are hemicephalic and have an incomplete and partly retractile head capsule.

The suborder Brachycera s.l., on the other hand, includes Diptera with short antennae and a more compact body (e.g., house flies, fruit flies). In the Anthomyiidae family, the genus *Pegomya* includes numerous primary fungivores whose development is linked to several important EEMMs (including *Boletus* section *Boletus* and *Leccinum* spp.). Their habit of laying eggs is highly specialized; in certain species, eggs are laid directly in the tubes (Hackman and Meinander 1979; Hanski 1989) (Fig. 19.2a). The large family Phoridae contains polyphagous species with predatory or parasitoid larvae; some species of the genus *Megaselia* are fungivorous and can damage artificially cultivated mushroom beds. Fungus-associated phorids attack fruiting bodies mainly in the summer months and cause serious damage (Bruns 1984; Canzanelli 1938–1939; Hackman and Meinander 1979). According to Bruns (1984), the presence of larvae of Phoridae is considerably widespread and significant in Boletales, but this factor is of lesser importance in *Boletus* section *Boletus* where statistics show that Phoridae are far less frequent than other dipteran families. The Platypezidae are very similar to the Phoridae and their larvae are frequently hosted by *Boletus* (also *Boletus edulis* Bull.), in either fresh or decaying fruiting bodies (Canzanelli 1941). The vast family Muscidae is represented in all biogeographic regions and has larvae that develop on various substrates and feed on a very wide range of organic substances, including fungi. The larval stages consist of three instars, are up to 10 mm long, and are acephalic (without an external head capsule and with their mouth part only visible when viewed under stereomicroscope). The most representative genera are *Muscina* and *Mydaea*; in the latter, fungivorous species lay their eggs mainly on the gills or in the pores, and less commonly on pileus surface (Hackman and Meinander 1979); the larvae are polyphagous and, at least in the last instar, are obligate predators of other larvae. Fungivorous species, mainly polyphagous but sometimes also specialized, are to be found in other families of Brachycera: Fanniidae, Calliphoridae, Heleomyzidae (mainly within the genus *Suillia*), Drosophilidae, and Syrphidae.

19.3.2 Coleoptera

Numerous species of beetles are habitually hosted by macromycetes both in their adult stage and as larvae. They are largely fimicolous or omnivorous detritivores, otherwise predators of fungivorous dipteran larvae, while others are strictly fungivorous and feed mostly on tough bracket fungi. Their larvae are variously shaped but can be easily distinguished from Diptera owing to the presence of six well-formed legs plainly visible in the thoracic segment.

Mycophagy among beetles is widespread in the superfamily Staphylinoidea particularly evident in the families Ptiliidae, Leiodidae, Scaphidiidae, and Staphylinidae (Newton 1984). In a study carried out by Rehfous (1955a), the Staphylinidae alone accounted for almost half of the 585 species of the fungus-associated beetles identified and for more than 2/3 of the specimens collected; the genus *Atheta* contains more than 100 species which are found on nonwoody fungi but only in an advanced stage of decomposition. Some Staphylinidae are saprophagous or predators, and others are obligate fungivores, for example, *Oxyporus* which have special morphological structures particularly suited to mycophagy (Hanley and Goodrich 1995); they also feed on young fruiting bodies of various EEM species including Boletaceae. Many species of Leiodidae feed on myxomycetes while others are specialized in hypogeous fungi (Newton 1984; Rehfous 1955a).

The Elateridae larvae are up to 2–4 cm long, reddish in color, and rigid in appearance. They are relatively common unwelcome visitors to numerous EEM species. Their development is a fairly lengthy process, and their presence in highly perishable fruiting bodies indicates a relatively tenuous link with the fungal host; this would explain their irregular, wavelike presence in the harvests. Elaterid larvae are probably predators, although it cannot be excluded that some might be fungivorous (on the basis of occasional findings in EEM specimens not attacked by dipterans). Although coprophagy is prevalent among the Geotrupidae, there are species which feed on hypogeous (truffle) fungi (Fogel and Peck 1975); moreover, adults of certain polyphagous species, especially of *Anoplotrupes stercorosus* (Scriba, 1791), can feed on different epigeous EEMMs, including *Boletus edulis* (Rehfous 1955a; Sitta and Sola 2003).

Fungivorous species of beetles are found in families Anobiidae, Ciidae, Erotylidae, Eucinetidae, Histeridae, Mycetophagidae, Nitidulidae, Tenebrionidae, and many more, but they are rarely present in EEMMs as they target mainly wood-rotting fungi, mostly Polyporaceae s.l., and in some cases puffballs (Lycoperdaceae) or slime molds (Myxomycetes).

19.3.3 Other Arthropods

Further orders of insects to be found in EEMMs are Hymenoptera and Lepidoptera. Hymenopterans are mainly parasitoids of other fungicolous insects, mainly larvae of obligate fungivorous dipterans, and belong to the superfamilies Ichneumonoidea, Cynipoidea, and Chalcidoidea (Ferrière 1955). Although their presence is sporadic, they nevertheless belong to the ecosystem formed by EEM fruiting bodies. Mycophagy in the Hymenoptera is very well known in the family Formicidae: the ants of the genus *Atta* coevolved mutualistically with saprotrophic fungi. However, other ants have a relationship with EEMMs, because they are common visitors in their fruiting bodies, where they could be facultative fungivorous or predators of dipteran larvae (Lewis and Worthen 1992). Some very small-sized species of ant have been shown to be quite common in brined and frozen Chinese porcini

(Sitta and Palumbo, unpublished data). Three taxa have been determined as belonging to genera *Pheidole* and *Cerebara*, and to the species *Pheidologeton affinis* Jerdon, 1851 (Fabrizio Rigato, *personal communication*). The genus *Pheidologeton* is peculiar for its dramatic polymorphism in the size of the worker castes (there is a super-major worker in addition to major and minor). The *Pheidologeton* species are called marauder ants: the presence of the minor workers inside the fungal fruiting bodies is certainly for predation of dipteran larvae or other fungicolous arthropods.

Lepidoptera with fungivorous behaviour do not appear to affect EEM species, as they always live off the wood-like fruiting bodies of Polyporaceae s.l. and other lignicolous mushrooms (Rawlins 1984; Rehfous 1955b).

Springtails (Collembola) are among the most frequent and are numerically the largest group of visitors to gilled fungi, especially on the hymenium surface (Yamashita and Hijii 2003) where they feed on hyphae and spores, or solely on spores (Fig. 19.2e). Springtails are six-legged arthropods belonging to the class Entognatha. They lack wings but are equipped with a "furca," that is, a forked springing organ that enables them to perform remarkable leaps. The principal fungivores are the Hypogastruridae (Castaño-Meneses et al. 2004; Greenslade et al. 2002; Nakamori and Suzuki 2005; Yamashita and Hijii 2003), often found in EEM of nearly all species. Rain stimulates their exit to seek food and to vertical migration (Sawahata et al. 2002) and explains why Collembola are more frequent and more numerous in EEMMs during rainy seasons. In *Boletus*, they penetrate mainly from the cortex of the stipe, or also directly through the hymenophore. In cases of high concentration (hundreds of specimens), they can cause serious damage because of the densely built network of short, branched, irregular tunnels, which are capable of reducing the fruiting body to a spongy pulp (Palumbo and Sitta 2007).

Finally, other arthropods that do not belong to the Esapoda can be found in EEMMs as occasional visitors or predators of fungivorous insects. In the Arachnida, the most important fungus-associated arthropods are the mites (Acari), which include a considerable number of diversified taxa. Because of their extremely small size, they can occupy ecological niches that most insects would find impossible to use. They frequently are in symbiosis not only with mushrooms but also with a third group of organisms (e.g., fungivorous dipterans in adult stage) that carry out the obligatory role of mite phoronts (Oconnor 1984). In the subphylum Crustacea and Myriapoda, larger-sized fungivorous arthropods are found. The former are terrestrial crustaceans of the order Isopoda (suborder Oniscidea) among which Canzanelli (1938–1939) reported the occasional presence of genera *Oniscus*, *Porcellio*, and *Armadillidium*. The myriapods are mostly species from the class Diplopoda, equipped with numerous pairs of small legs (two pairs per segment). As typical detritivores, they usually live in humus, under stones and wood, always in damp areas. Their presence has already been historically recognized in wild mushrooms (Farneti 1892; Canzanelli 1938–1939), and occasionally, they can be found on the stipes of frozen or brined porcini (Palumbo and Sitta 2007).

19.4 Mycophagy Predisposition in Commercially Important EEMMs

Considering the whole set of entomofauna habitually hosted by EEMMs, no prediction of parasite presence can be made through any parameters in fruiting body development. Insect contamination in batches of EEMMs destined for human consumption is bound to vary depending on the growing season, meteoclimatic conditions, biogeographic region, and, of course, mushroom species. Since a "palatability ranking" of different fungal species exists for the fungivores, this chapter focuses on single species of economically important EEMMs in relation to their propensity to be attacked by arthropods.

19.4.1 Cantharellus cibarius *s.l.*

Fresh fruiting bodies of *Cantharellus cibarius* Fr. and allied species are rarely attacked by arthropods and seldom eaten by snails. This could be explained by the presence of insecticide or repellent substances. Danell (1994) claimed that, in spite of the slow growth of the fruiting bodies, less than 1 % of *Cantharellus* are infested by dipteran larvae compared to 40–80 % of the majority of Agaricales and Boletales. The fungivores reported in literature are mainly polyphagous dipteran larvae of the family Limoniidae, followed, less frequently, by some species of *Suillia* or *Drosophila* (Krivosheina 2008). Large larvae of elaterid beetles are found quite regularly (Danell 1994).

Our experience (Sitta and Palumbo, unpublished data) is based on the parasitological analysis of 24 samples of European *C. cibarius* preserved in brine or oil. On the whole, 8.3 % (in weight) of the mushrooms had visible traces of arthropod attack; the absence of insect contamination was found in only 6 samples (25 %), whereas dipteran larvae occurred in 10 samples (42 %), coleopteran larvae in 11 samples (46 %), and mites in 10 samples (42 %). The beetle larvae were elaterid in only one case (1 cm length); in all other cases, they were small in size (2–4 mm) and proved difficult to identify as they were dead when found in preserved mushrooms and rearing was not possible.

19.4.2 Boletus edulis *and Allied Species*

Compared to other fleshy mushrooms, an exceptionally high number of specialized fungivores can be found in Boletales. These include a high prevalence of dipterans and medium to low numbers of beetle species, which are present in specimens already in a state of decomposition (Bruns 1984; Rehfous 1955a).

Species of *Boletus* sect. *Boletus* (porcini mushrooms) are attacked by Diptera belonging to several families (Canzanelli 1941), in particular fungus gnats, *Pegomyia* spp., and muscids; Phoridae and Calliphoridae larvae are much rarer. The percentage of fruiting bodies attacked varies considerably between porcini species (e.g., *B. edulis* is less subject to attack than *Boletus reticulatus* Schaeff.), as well as by environment and season of growth. Research carried out by Maroli et al. (2003) and by Locatelli et al. (1994, 2005a, b, 2006) showed that 100 % of dried porcini samples contained fungivorous dipteran larvae. Over 90 % of the larvae of fungus gnats and Anthomyiidae were found in the caps, especially in the tubes (Fig. 19.2b), which are the part of the fruiting body with the highest content of nutrients. Also the larvae of Muscidae are most common in the tubes, but they are also found in mushroom stipes more frequently than are other families (Locatelli et al. 2006).

Obligate fungivorous beetles are rare in young porcini, where elaterid larvae of various species, mainly from genera *Melanotus* and *Selatosomus* (Palumbo and Sitta 2007) and *Athous* (Rehfous 1955a), are most frequently found (Fig. 19.2c). Springtails are very often hosted by boletes, sometimes in vast numbers, though not as numerous as in gilled mushrooms. Large-sized adult arthropods (*Anoplotrupes stercorosus*, diplopods) are occasionally present.

In the course of 9 years of parasitological analysis carried out on 469 samples of dried and preserved porcini of various geographical origin, only 8 samples were found to be free of dipteran larvae (1.7 %), and nearly all of them were of South African origin (Sitta and Palumbo, unpublished data). As general rule, non-native porcini growing in artificial plantations of *Pinus* spp. in South Africa and Swaziland are not attacked by dipteran larvae or other obligate fungivores.

19.4.3 Suillus luteus *s.l.*

According to Hackman and Meinander (1979), there is a relatively clear distinction between fungivorous dipteran species, which attack *Boletus* sect. *Boletus* and *Leccinum*, and those that infest the slippery jacks (*Suillus* spp.). Nonetheless, the situation of autochthonous *Suillus luteus* (L.) Roussel closely resembles that of porcini mushrooms as far as the average presence of fungivorous dipteran larvae is concerned. In Finland, Hackman and Meinander (1979) found that 70–95 % of the fruiting bodies at an intermediate growth stage are attacked by larvae. Moreover, analogous with South African porcini, non-native *Suillus luteus* s.l. in artificial *Pinus* plantations in South America (Ecuador and Chile in particular) usually are free of Diptera larvae and other obligate fungivores. Parasitological analysis of 16 samples of brined *Suillus luteus* s.l. showed that 12 of 13 samples from South America were completely free of arthropods and in only 1 sample were fruiting bodies attacked to a small degree solely by springtails. By marked contrast, fungivorous larvae of Diptera were found in all 3 samples of European origin, with an average of 30 larvae per 100 g of mushrooms (Sitta and Palumbo unpublished). In artificial pine plantations (mainly *Pinus radiata* D. Don) in South

America, *Suillus luteus* has proliferated to an infestant level, with annual production rate far higher than in native pine stands. However, the ecosystem appears to be extremely poor in mycorrhizal fungi (only three species in certain artificial pine plantations in Ecuador). Further, *Suillus luteus* does not limit itself to the utilization of organic substances arising from the mycorrhizal symbiosis with its tree partners but adopts a saprotrophic behaviour by exploiting carbon sources which already existed in the ecosystem prior to the pine plantation event, thus contributing to a gradual carbon depletion of the soil (Chapela et al. 2001). In such an ecosystem, it seems likely that even obligate mycophagists should be absent, whereas they coevolve with *Suillus* in their native environment. The very rare cases of presence of fungus gnat larvae in South African porcini or South American slippery jacks confirm the ecological explanation for this phenomenon. For example, dipteran larvae in the tubes have been observed in one mature specimen of *Suillus luteus* (in a harvest of 12 fruiting bodies) harvested near Papallacta (Napo province, Ecuador) in a little artificial pine stand of *Pinus radiata* surrounded by parcels of autochthonous woods (Alessio Barili, personal communication).

19.4.4 Tricholoma matsutake *s.l.*

Even without specific studies on the entomofauna associated with these important EEMMs, it can be affirmed that *Tricholoma matsutake* (S. Ito & S. Imai) Singer and allied species are markedly prone to attacks by fungivorous insects. In regard to Diptera, the presence of phorids of the genus *Megaselia* (Lee and Disney 2009) and of the fungus gnat *Tetragoneura matsutakei* (Sasaki, 1935) has been reported; on the contrary, *T. matsutake* appears to be a less palatable mushroom for many collembola species (Sawahata and Narimatsu 2006). Our experience is limited to the parasitological analysis of only 2 samples of Chinese origin, both of which contained fungivorous dipteran larvae (an average number of 82 larvae per 10 g of dried mushrooms) with four families represented: Mycetophilidae, Anthomyiidae, Calliphoridae, and Phoridae (Sitta and Palumbo, unpublished data).

19.4.5 Amanita caesarea *(Scop.) Pers.*

Although we do not have data from parasitological analysis available, it is evident from direct experience that this species is highly prone to attacks by fungivorous arthropods, especially polyphagous larvae of Diptera, as reported by Hackman and Meinander (1979) for the Amanitaceae.

19.4.6 Lactarius *sect.* Dapetes, Russula *spp.*

More than 80 % of the fruiting bodies of edible *Russula* species and *Lactarius deliciosus* (L.) Gray and allied species (*Lactarius* sect. *Dapetes*) are infested by larvae of Diptera, mainly polyphagous fungus gnats (Hackman and Meinander 1979). Although, as a general rule, the acrid-tasting *Russula* are infested to a lesser degree, they are attacked by the same species present in those having a mild flavor. In many *Russula* and *Lactarius* species, beetles are absent in young specimens, while they become the predominant fauna during mushroom decomposition, with dozens of species present, and even more than 150 in *Lactarius piperatus* (L.) Pers., in the same species of fungus (Rehfous 1955a).

19.4.7 Tuber *spp.*

Mycophagy represents the major avenue of spore dispersal for hypogeous fungi. The spores of these fungi are capable of germinating even after passing through mammalian digestive systems and are dispersed primarily in the fecal pellets of small mammals that have fed on fruiting bodies (Fogel and Peck 1975; Fogel and Trappe 1978). However, whereas mammals perceive and are attracted by the characteristic odor of ripe truffles, the substances which attract obligate fungivorous arthropods must necessarily be of a different nature: for example, female adults of the beetle *Leiodes cinnamomea* (Panzer 1793) are attracted by truffles in an early growth stage (Hochberg et al. 2003) (Fig. 19.2f). The dipteran fauna associated with truffles is largely represented by different species of *Suillia* (Ciampolini and Süss 1982; Krivosheina 2008), whose female adults fly close to the surface and lay their eggs on the ground, above the truffle fruiting bodies, so that the larvae can easily reach them on hatching. Chandler (2010) reports the association with *Tuber* for Diptera of the genus *Cheilosia* (Syrphidae) as well. The fruiting bodies of truffles are very often attacked by fungivorous dipterans and beetles, which differ according to species, environment, and growth season; examination of the two species of highest commercial value definitely shows that *Tuber magnatum* Pico is more frequently attacked by larvae of Diptera (especially the fruiting bodies which grow in the summer season, going by the name of "Fiorone" in Italy), whereas *Tuber melanosporum* Vittad. is more subject to attack by beetles, particularly Leiodidae.

19.5 Behaviour of Fungivorous Arthropods in the Various Procedures of Preservation in EEMMs

During freezing, preservation in brine, or cooking, all arthropods present in fresh mushrooms die immediately with no possibility of escape from the fruiting bodies and remain inside them until the EEMMs are used as food or until the next stage in

processing. In contrast, since desiccation is a longer procedure of preservation, some arthropods (e.g., springtails) usually leave the sliced fruiting bodies during the drying process. Dipteran larvae tend to search for a more humid and suitable environment, such as internal sections of mushroom slices being desiccated, where they can have a survival time of up to several hours and can cause considerable damage to fungal tissues (Palumbo and Sitta 2007). In any event, all the arthropods present in fresh mushrooms, independent of their stage of development, will be dead at the end of preservation procedures and no longer capable of damaging the food product.

19.5.1 Drying

When mushrooms are sliced and artificially dried, the dipteran larvae that are not killed by the slicing may react to desiccation in different ways. Factors that have the greatest impact on the way the larvae behave include the method of drying employed, the species of the larvae and their size at the moment of cutting, the thickness of slices, as well as the moisture content of the fresh mushroom.

The drying method has the most significant influence on insect larvae. If drying is rapidly carried out, for example, with forced hot-air dryers, the larvae die quickly. In this case, mushroom slices will contain, on average, high numbers of dead dipteran larvae. These tend to be small in size, and fungal tissues tend to be undamaged. In porcini mushrooms, the very small larvae are mainly trapped in the long, greenish-colored tubes; from the outside of the slices, the larvae can be visible as groups of small white dots (the central part of the body) and/or black dots (the head capsules, in the case of fungus gnats). The slower the drying process (e.g., solar drying in humid weather, exceedingly thick slices, and mushrooms with elevated moisture content), the longer the larvae tend to stay alive inside, deteriorating the fungal tissues. With such a damage, dried porcini may contain numerous dried larvae, sometimes large in size, or they may be completely, or almost free from larvae, should they have managed to escape before the end of the drying process. It follows that the degree of deterioration of dried mushrooms caused by dipteran larvae can be judged solely by a macroscopic evaluation of damage to fungal tissues and not on the basis of the number of larvae present (Palumbo and Sitta 2007).

19.5.2 Freezing, Brining, and Cooking

Statistics from the parasitological analysis of 469 samples of porcini carried out by Sitta and Palumbo (unpublished data) show that mushrooms preserved in brine or frozen contain, on average, a slightly higher number of dipteran larvae than dried mushrooms; the average numbers of fungivorous Diptera larvae differ according to

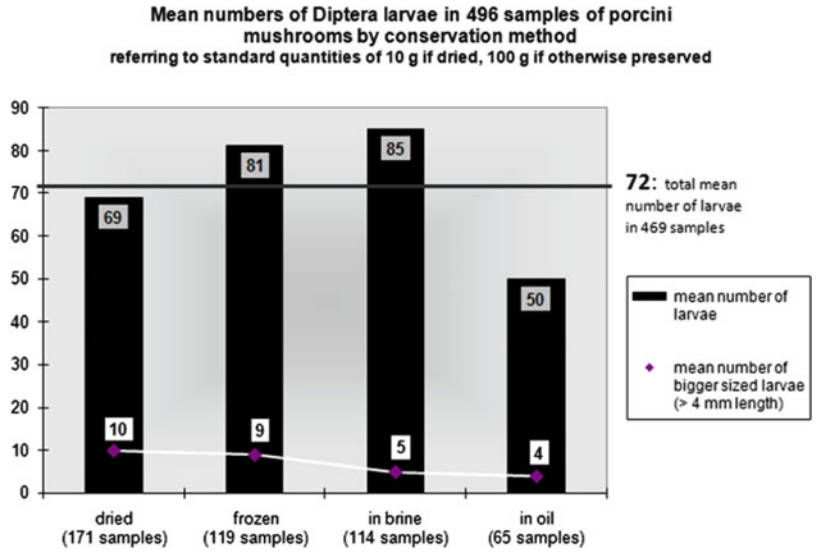

Fig. 19.1 Infestation by dipteran larvae in porcini mushrooms (*Boletus edulis* and allied species): Comparison between different conservation methods

the method of preservation (Fig. 19.1). Dried porcini contain a higher number of large-sized larvae, owing to the length of the drying process, and in mushrooms preserved in oil, which are manufactured from previously brined mushrooms, the larvae are less because some are washed away during the desalting process in running water.

Other arthropods with greater mobility than dipteran larvae are more likely to remain inside frozen and brined mushrooms, especially when the fruiting bodies are preserved whole and uncut. In frozen and brined porcini, the presence of springtails is very common (Sitta and Palumbo, unpublished data), whereas they are hardly ever found in dried EEMMs; among large-sized arthropods, a frequent problem is caused by elaterid larvae, whereas the presence of *Anoplotrupes stercorosus* or centipedes (Diplopoda) is more rare (Palumbo and Sitta 2007). The presence of snails, on the other hand, is confined to frozen mushrooms.

19.6 Post-drying Infestation: Entomofauna Living in Dried Mushrooms

Post-drying infestation in dried EEMMs is largely due to Lepidoptera, and, to a far lesser degree, to Coleoptera and Psocoptera (Palumbo and Sitta 2007). The most widespread species of Lepidoptera that can infest dried porcini is *Nemapogon granella* (Linnaeus 1758) (Fig. 19.2d); the damage, which is clearly visible and

Fig. 19.2 (**a**) Adult *Pegomyia* (Diptera Anthomyiidae) laying eggs in the tubes of Boletus edulis (photo Dimitri Gioffi). (**b**) Larvae of fungus gnats (Diptera Sciaroidea) in the tubes of Boletus edulis (photo Nicola Sitta). (**c**) A big elaterid larva (Coleoptera, Elateridae) visiting a Boletus edulis (photo Nicola Sitta). (**d**) Nemapogon granella (Lepidoptera), the most common species that can infest dried porcini (photo Nicola Sitta). (**e**) Springtails (Collembola) feeding on a gilled mushroom (photo Nicola Sitta). (**f**) Adults of Leiodes cinnamomea (Coleoptera, Leiodidae) feeding on *Tuber melanosporum* fruiting bodies (photo Serafino Fioravanti)

recognizable, is caused by the larvae alone (Süss and Locatelli 2001). Adult beetles are often long-lived and cause more damage than the larvae; many species of various families (Anobiidae, Dermestidae, Tenebrionidae, Silvanidae, Bostrychidae, etc.) can infest dried mushrooms. Dried mushrooms can also be attacked by species of psocids (e.g., genus *Liposcelis*). These insects are 1–2 mm long, are fast walkers (many species lack wings), and are able to feed on a wide range of organic substances.

19.7 Parasitological Analysis of EEMMs

Both macroscopic analysis of mushrooms and parasitological analysis involving the use of a stereomicroscope can be performed, with different aims, for the evaluation of insect contamination in EEMMs destined for human consumption (fresh, dried, or otherwise preserved).

19.7.1 Macroscopic Mushroom Analysis

Macroscopic mushroom analysis involves an inspection that is normally carried out together with the identification of the species and the sighting of possible foreign bodies present. The examination is typically performed with the naked eye, but it also may include the use of a hand lens (generally in the range of 3–10×). The analyst's proficiency requires specialized training and experience in mycological inspection and knowledge of morphology and organoleptic characters of the EEM species analyzed. For an experienced analyst, such mycological analyses are easy to perform even on relatively large quantities of fruiting bodies (100–1,000 g dry weight, 1–10 kg fresh weight). Insect contamination is assessed mainly by evaluating the condition of the fungal tissues. Results are expressed in terms of percentage weight/weight of infested fungal units. These are subdivided into at least two categories: *pitted units*, which show only holes left by the transit of arthropods, and *deteriorated units*, which also show damaged tissues. The third category of *post-drying attacked units* concerns only dried EEMMs. The final part of the test report states whether the mushroom sample is fit or unfit for human consumption.

The so-called pitted mushroom units are more or less riddled with holes, caused by the passage of dipteran larvae in fresh fungal tissue, but without any alteration in color or tissue structure. This is not to be considered deterioration and, accordingly, pitted mushroom units, which are extremely common in nearly all species of EEMMs, are judged fit for human consumption and tolerated by some regulations (see Chap. 20).

Deteriorated mushroom units (damage caused by the parasites in the fresh mushroom, or, in the case of dried mushrooms, during the drying period) are recognizable by the darker color surrounding the holes, almost blackish in the

inner parts, with changes in texture to hollow or spongy. In dried mushrooms, the units attacked in the post-drying stage are characterized by crumbling and frequent presence of excrements, silken threads and sometimes of exuviae, pupae, larvae, and adult insects, with a different appearance if the damage is caused by larvae of Lepidoptera, beetles, or psocids. In batches of dried mushroom with presence of post-drying infestation, living arthropods can also be found.

19.7.2 Parasitological Analysis Under Stereomicroscope

This test consists of close inspection of relatively small quantities of mushrooms (10 or 15 g dry weight, 100 g fresh weight) under a stereomicroscope, with the purpose of checking the extent of insect contamination. Results are expressed in terms of the number of arthropods (or their fragments) found. In the filth-test methodology (AOAC International 2005), mushrooms are reduced to fragments with a high-speed blender, and chemical dyes are used to visualize the cuticles of the arthropods. This method of analysis originated in the USA and was meant to be applied, in all probability, to cultivated mushrooms, linked to a tolerance for a small number of larvae (FDA 1995). A simplified procedure, which consists only in the dissection of fresh, rehydrated, or thawed (and then filtered) fungal matter, allows an approximate identification of arthropods and has been standardized in Italy by the Istituto Superiore di Sanità (Maroli et al. 2003; Khoury and Bianchi 2010). In both cases, observation, determination, and count of the arthropods are made under stereomicroscope, with magnification range from 4× to 30×. Given the difficulties involved in identifying insect larvae, it is normally considered sufficient to assign the specimens found to their order or family. When it is not possible to identify the family of dipteran larvae, a subdivision into two groups is sufficient: eucephalic (fungus gnats) and acephalic larvae (various families of Brachycera, which can be defined as "Muscidae s.l.").

Samples of nearly all EEM species show a constant presence of dipteran larvae, with a completely random distribution in the fungal units, which does not correspond to the macroscopic visibility of the holes (Locatelli et al. 2005b). The highly random nature of the distribution of larvae, however, results in low repeatability of test results, and widely divergent results can be obtained in samples taken from the same batch. Furthermore, because no evaluation is made of the condition of the fungal tissues, this technique fails to give information about the state of preservation of the mushrooms and should not be used in formulating assessments of fitness for human consumption (see Chap. 20). Unfortunately, the filth test is indeed used by important regulatory authorities (FDA 1995), with a numeric tolerance for "maggots" (currently no more than 20, or no more than 5 if they over 2 mm long) and for "mites" (no more than 75), so that the fitness of EEMMs for human food consumption depends on the number of arthropods embodied in a standard weight sample.

This method is quite inappropriate for the evaluation of EEMMs destined for food consumption. For example, cannibalism and predation, which are both

widespread natural phenomena in the EEMM ecosystem, can cause the presence of an extremely large number (hundreds) of very small larvae in sound and perfectly edible fruiting bodies, whereas decomposed inedible specimens may contain far fewer. The size of the larvae, which is an important visibility factor even after food preparation, is undoubtedly a significant criterion in evaluation, but the distinction between small and large larvae, instead of the 2 mm proposed by the FDA, should be approximately 4 mm. The impact of insect contaminants smaller than 1–2 mm (e.g., mites, springtails, dipteran eggs, and larvae in their first instar) is, in any case, irrelevant.

19.8 Conclusion

Millions of people all over the world consume fresh, dried, and preserved EEMMs. Together with these EEMMs, they also ingest fungivorous larvae, springtails, mites, and other dead arthropods, and there is no evidence of harmful consequences of any sort. In preserved mushrooms, the arthropods are always dead, and in any case, they are cooked, since EEMMs, as a general rule, are cooked prior to consumption. Nonetheless, standards for the unacceptability of foods, aside from their possibility to be injurious to health, may be also based (according to its intended use) on esthetic criteria, and the sight of one or more larvae in a dish of mushrooms is likely to create a sense of disgust in consumers in Western countries (Yen 2009).

Except for *Cantharellus cibarius* and for the harvests of non-native *Boletus edulis* and *Suillus luteus* grown in exotic pine plantations, nearly all the samples of EEMMs fail to comply with the allowance-rate regulations enforced by the FDA (1995). If the systematic use of filth tests were introduced by regulatory authorities in Western countries, it would literally amount to a ban on the commerce and food consumption of EEMMs. For the evaluation of EEMM fitness for human consumption, the use of parasitological analysis under stereomicroscope should be combined with far wider, and not exclusively numeric, tolerances. Alternatively, priority should be given to mushroom macroscopic analysis, with the aim of ascertaining the state of the fungal tissues or signs of post-drying infestations, rather than the number of arthropods contained inside the fruiting bodies.

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Chapter 20
Edible Ectomycorrhizal Mushrooms: International Markets and Regulations

Nicola Sitta and Paolo Davoli

20.1 Introduction

Usage of fungal fruiting bodies for food purposes can certainly be traced "far back in the dim distance of a remote past" when our prehistoric ancestors, prompted by hunger and curiosity, discovered that some of the larger fruiting bodies of higher fungi were edible (Buller 1914). Likewise, preservation and trade of edible mushrooms must have arisen soon after, and evidence of mushroom trading dates back to the Greeks and Romans (Buller 1914).

More than a thousand of edible ectomycorrhizal mushrooms (EEMMs) are currently eaten in the world, and many other species, particularly in Africa and South America, have yet to be recorded (see Chap. 17). Despite a widespread appreciation for edible mushrooms as tasty food items, there is still some resistance to eating wild fungi, which is often based on the fear of poisonous mushrooms; such a cautious attitude toward mushrooms does limit the consumption of edible species and makes it difficult to expand local markets (Boa 2004b; see Chap. 17). A few species have well-established worldwide markets in excess of US$2 billion (Wang and Hall 2004), while many other species are important only on a local scale. Trade and private use should both be evaluated to calculate the economic importance of EEMMs for a given country (Boa 2004a). The amount of individual harvests for private use, however, is more difficult to estimate.

In many developing countries, EEMMs represent a valuable food resource and an important source of income for collectors. Collection of EEMMs also has economic importance to local people in developed countries (Finland, Italy,

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Spain, Canada, USA, etc.), at least for the most highly priced and duly sought after species, such as truffles, porcini, and matsutake (Cai et al. 2010; De Román and Boa 2006; Hosford et al. 1997; Wang and Hall 2004). Among the implications of the current economic downturn in western European countries, unemployment and increase in food and energy prices impact heavily on low-income earners. Under such circumstances, collection of EEMMs would represent a salary supplement and might well become again an important source of income, especially in marginal areas (Cai et al. 2010).

The most important EEMM species in the world, with higher prices and/or a bigger market share, are truffles (*Tuber* spp., primarily *Tuber magnatum* Pico and *Tuber melanosporum* Vitt.); matsutake (*Tricholoma matsutake* (S. Ito & S. Imai) Singer and allied species); porcini (*Boletus edulis* Bull. and allied species); chanterelles (*Cantharellus cibarius* Fr. and allied species); saffron milk caps and indigo milk caps (*Lactarius deliciosus* (L.) Gray and allied species, *Lactarius indigo* (Schw.) Fr., respectively); slippery jacks (*Suillus luteus* (L.) Roussel s.l.); Caesar's mushroom (*Amanita caesarea* (Scop.) Pers. and allied species); hedgehogs (*Hydnum repandum* L. and allied species); shoro (*Rhizopogon roseolus* (Corda) Th. Fr.); desert truffles (*Terfezia*, *Tirmania*, etc.); *Catathelasma ventricosum* (Peck) Singer; several species of *Boletus*, *Suillus*, and *Leccinum*; and *Russula virescens* (Schaeff.) Fr. and other *Russula* species (De Román and Boa 2004; Deschamps 2002; Hall et al. 2003; Härkönen et al. 2003; Ndong et al. 2011; Pérez-Moreno et al. 2008; Pilz and Molina 2002; Pilz et al. 2003; Sitta and Floriani 2008; Trappe et al. 2008; Visnovsky et al. 2010; Wang and Hall 2004; Yang et al. 2008).

20.2 Trade of Fresh Mushrooms

In addition to major EEMMs, which are traded in bulk both in national markets and on an international scale at import–export level, a great variety of other EEMM species are sold fresh in local markets, as reported in the thorough compilation by Boa (2004b) (see Chap. 1). Each geographic area has its own peculiarities; traditional usage of different fungal species for culinary purposes may vary even within the range of a few kilometers. Geographic macro-regions which share common habits also exist. For instance, in the whole of Central Africa, consumption of several species of *Cantharellus*, *Russula*, and *Lactarius* is widespread, whereas species of Boletaceae have little or no use at all and are generally feared, as blueing, which is common for most members of the family, is believed as a sign of toxicity (Ndong et al. 2011).

The price of fresh EEMMs depends on a number of different factors: how a given species is quoted on given local markets, the growth area (e.g., the mushroom harvest is more appreciated for growth areas which are traditionally considered typical of that particular species), the local and global offer (which may both vary seasonally) and related demand on the international market, and, last but not least, the merceological classification of different grades. In the case of fresh matsutake and

porcini, grading is based on similar characteristics: size, developmental stage, presence of alterations due to parasites, breakage, or other reasons. For matsutake, for instance, the highest priced categories are for the specimens which are large in size, not opened undamaged, bug-free, fresh, and with an elegant odor (Yang et al. 2008).

China represents one of the most important markets in the world for mushroom trade for overall amounts of traded mushrooms, and for the number of traded fungal species as well. The Yunnan province, in particular, which is located in southwestern China, has a major tradition of consumption and trade of EEMMs, both for the relevant domestic Chinese market and for international trade, especially for economically highly valued species such as porcini, matsutake, and truffles. A decrease in the traditional consumption of some fungal species (Arora and Shepard 2008) and an increasing trend in the export of EEMMs which are highly prized abroad, are recurring phenomena. There has also been a decline in the national production of EEMMs, which is well documented historically for countries which are traditional mushroom consumers, i.e., porcini in Italy, matsutake in Japan, and black truffle in France (Pipino 1972; Saito and Mitsumata 2008; Sitta et al. 2007a; Wang and Hall 2004; Hall et al. 2007). Declining mushroom production is also occurring more recently in some countries that have previously been major suppliers of mushrooms (Wang and Hall 2004; Yang et al. 2008). Such a decline prompts food business operators to search for new geographic areas for supplying both the domestic and export markets. Mexico and Mercosur countries (Argentina, Brazil, Paraguay, Uruguay), for instance, would have potential to exploit for export the production of porcini (*Boletus* sect. *Boletus*), *Suillus luteus* s.l. and perhaps also chanterelles, *Amanita caesarea* complex, and other EEMM species, especially by favoring the manufacture of frozen and otherwise preserved products (Deschamps 2002; Martinez-Carrera et al. 1998; Pérez-Moreno et al. 2008).

Since fresh EEMMs deteriorate quickly, import usually occurs from adjacent or neighbouring countries, by means of road or sea. Transportation times rarely exceed one day. More expensive air transportation is used only for highly priced species (truffles, matsutake) or for specific products (e.g., fresh porcini from South Africa may reach higher prices in Europe and USA as out of season delicacies). Italy's import–export for three different species of fresh EEMMs over a nine-year time span is reported in Table 20.1. The Italian domestic market requires huge quantities of fresh porcini and chanterelles. Accordingly, for these EEMMs the imported quantities mostly exceed those of exported mushrooms. Imports are from other European countries for 94–99 % of porcini and for 100 % of chanterelles. Romania and Poland are the major suppliers and export is mainly to France.

The situation for truffles is quite the opposite: the high prices which truffles command plus the great demand for black truffle in France combine to ensure that the export of truffles from Italy largely exceeds the import, and that most of the exported truffles actually originate in Italy. About 80 % of export is to other European countries, more than 40 % of which is *Tuber melanosporum* exported to France. Japan and USA are the major overseas destinations for fresh Italian truffles, in particular for the exceedingly expensive Italian white truffle (*Tuber magnatum*).

Table 20.1 Italian import–export of major EEMMs in the fresh state; values in metric tons (ISTAT-Coeweb 2012)

Year		2002	2003	2004	2005	2006	2007	2008	2009	2010
Truffles	Imp.	15	4	5	3	7	6	13	19	15
	Exp.	49	69	44	56	61	61	61	72	111
Chanterelles	Imp.	836	538	946	1,047	1,179	885	705	1,089	1,253
	Exp.	383	317	280	500	570	305	312	246	333
Porcini	Imp.	5,967	3,327	4,249	3,841	3,925	4,125	2,883	2,972	4,448
	Exp.	455	289	283	435	395	932	621	842	908

Table 20.2 Import of matsutake by Japan (in metric tons), years 1995–1999, from Yang et al. (2008)

Year	1995	1996	1997	1998	1999
From South Korea	633	170	249	355	515
From North Korea	1,141	541	615	1,086	307
From China	1,191	1,152	1,076	1,313	1,292
Total importation	3,515	2,703	3,059	3,248	2,935

Table 20.3 Import of matsutake by Japan (in metric tons), years 2007–2011, from Japan's Ministry of Finance (2012)

Year	2007	2008	2009	2010	2011
From South Korea	80	16	14	55	11
From Canada + USA	487	288	324	338	237
From China	947	854	1,177	1,561	875
Total importation	1,554	1,329	1,597	2,044	1,202

The situation is similar in Japan for the import of matsutake, even though overall quantities are less affected by climatic factors because the import occurs from all over the world by air transportation. In addition, by comparing data from Japan's ministry of Finance are in Table 20.3, and data from Yang et al. (2008), a decreasing trend in traded quantities can be observed, most likely due to a gradual decline of matsutake production even in supplying countries. The Chinese contribution increased from one-third to three-quarters of Japan's total matsutake importation (Tables 20.2 and 20.3).

In mushroom supplying countries, more strict regulations for mushroom collection may help, as in the case of China for matsutake, which it has been given a protected status and assigned exportation quotas regulated by the Endangered Species Import and Export Management Office (Yang et al. 2008). It would be most desirable if such regulations could forbid the collection and trade of immature specimens, especially for EEMMs which require digging within the outer soil layer, with the risk of damaging the litter and the underlying mycelium. Furthermore, since the reasons for the decline in production of the major EEMMs are known (Arora and Shepard 2008; Saito and Mitsumata 2008), forest management plans in most important collection areas should be structured also toward EEMM

production, in order to prevent any possible decrease in mushroom productivity. In particular, forest management plans should define methods and frequency of logging/clearing by evaluating the tree age and the ecological context that favor EEMM production and avoiding preservationist models of forest management (Arora and Shepard 2008).

Local markets for EEMMs all over the world represent a treasure trove of traditional knowledge and exploitation of biodiversity for food purposes. In most countries, however, no specific regulations for mushroom trade have been laid down, withstanding the prohibition to trade poisonous mushrooms in accordance with specific or more general laws. For instance, some European countries with long historic traditions of EEMM consumption and trade (e.g., France) do not yet possess a national regulation for wild mushroom trade and related control systems. Therefore, lists of permitted species and control protocols are defined only within a jungle of local regulations which often greatly differ from each other. Groussin and Saviuc (2009) have counted for France as many as 51 different lists of wild mushroom species that are locally marketable, for an overall number of 130 different fungal species, some of which are considered suspiciously toxic (see Sect. 20.6) or even officially forbidden, such as *Gyromitra* spp. and *Tricholoma equestre* (L.) P. Kumm. (France 1991, 2004). A specific national regulation would serve an important role in food safety, by identifying control authorities, by introducing a national positive list of marketable species, and by laying down requirements for enforcing controls and adequate training of inspectors (see Sect. 20.5).

In the few European countries having a specific regulation for wild mushroom trade and control (i.e., Italy, Finland, Spain), an official positive list of marketable species is also laid down for food safety reasons. Although official lists of marketable species are set by law in order to protect public health, in the long run they may result in a depletion of local markets, simply by ignoring—and therefore forbidding—less important mushroom species which are traditionally known and exploited only in very limited geographic areas. For example, the Spanish law combines together epigeous and hypogeous mushrooms in a positive list comprised of 43 EEMM species (Spain 2009) and considers suspiciously toxic all other species not included in the list. In Italy, by contrast, epigeous and hypogeous mushrooms are regulated by two different national laws (Italy 1985, 1995). The regulation for epigeous mushrooms lists more than 60 species (of which more than 20 are EEMMs), which can be traded fresh and allows regional additions in accordance with regional regulations (Italy 1995), bringing the overall total of marketable mushroom species to around 150. In the case of hypogeous mushrooms, only nine Italian taxa of truffles are allowed to be traded in Italy (Italy 1985), and consequently other species (especially the Asian *Tuber indicum* Cooke & Massee s. l.) are excluded from the Italian market but are legally traded in other European countries (i.e., France, Spain).

Since specific EU regulations for wild mushroom trade are also lacking, the official lists of marketable species for different countries within the EU can be conflicting. For example, *Gyromitra esculenta* (Pers.) Fr. is included in the Finnish

list of marketable species (Finland 2007) and is explicitly forbidden in France and Spain (France 1991; Spain 2009).

The Italian way of regional additions to the national positive list of marketable mushroom species might be effective to preserve the traditional usage of minor species at a local level, but is nonetheless difficult to enforce as it requires specific local regulations. In accordance with EU regulation on the hygiene of foodstuffs (EU 2004a), the best solution could be to find a specific control system for all edible species of local interest that are not included in national positive lists, in the case of "direct supply, by the producer, of small quantities of primary products to the final consumer or to local retail establishments directly supplying the final consumer." Along this line, the role of mycological inspectors should be defined by law as professional operators who have the expertise and experience for the identification of fungal species and the evaluation of their edibility (see Sect. 20.5), so that the release on the market of a greater number of fungal species, albeit restricted to small areas of traditional consumption, might not represent an increased risk for the consumers' health.

20.3 Trade of Preserved EEMMs

Preservation of EEMMs for human consumption as food can be effected by drying, freezing, brining (i.e., preservation in brine), or directly cooking fresh mushrooms.

EEMMs have different characteristics when preserved, depending on how their organoleptic properties are altered during the preservation process, and this obviously affects prices and quantities traded as preserved products. For instance, truffles and matsutake lose most of their organoleptic features after preservation. This loss of flavor and texture makes them less appreciated as preserved products and results in a conspicuous price decrease, thus favoring the trade of fresh fruiting bodies. For truffles, however, the addition of synthetic flavorings to truffle-based products is employed as compensation. Most preserved truffle-based products utilize a lower grade material (i.e., breakings and peelings) which is often used for food industry applications, even though canned and dried truffles can be found on the market. Truffle-based products such as butter, cheese, oils, and sauces are often prepared using fresh truffles. Preserved matsutake is produced mainly in China, Sichuan province (Yang et al. 2008). Dried and individually quick frozen (IQF) matsutake have a much greater market share than brined matsutake.

In general, EEMMs belonging to Boletaceae are most suited for drying, as their flavor is usually enhanced through drying, thus making it an excellent and important culinary product when dried. Dried porcini mushrooms, for instance, represent an economically high-valued traditional product and are widely traded all over the world. Other preservation techniques largely utilized for porcini are brining (despite the total loss of flavor and taste) and freezing; the trade of frozen porcini, for any merceological grade, has witnessed a dramatic growth in the last 15 years, as raw material for the food industry but for retail sale as well. *Suillus* species even

Table 20.4 Italian import–export of preserved mushrooms[a]; values in metric tons (ISTAT-Coeweb 2012)

	Dried		Frozen		Brined	
Year	Import	Export	Import	Export	Import	Export
2002	2,227	387	12,936	1,431	14,294	451
2003	1,940	422	12,919	1,388	15,229	606
2004	2,061	413	16,453	1,598	14,434	349
2005	2,069	419	17,946	1,820	17,631	243
2006	1,582	580	17,165	2,317	15,964	388
2007	1,787	399	18,346	2,583	17,258	284
2008	1,958	395	15,936	2,408	14,160	337
2009	1,486	348	17,877	2,118	13,143	129
2010	1,733	381	20,646	2,300	14,455	236

[a]"Mushrooms" refer to all fungal species which are being traded, excluding the genus *Agaricus* and (only for dried mushrooms) *Auricularia* and *Tremella* species. Therefore, these data do not refer only to EEMMs but comprise also saprotrophic, mainly cultivated, species, i.e., *Pleurotus* spp., *Lentinula edodes* (Berk.) Pegler, *Pholiota nameko* (T. Itô) S. Ito & S. Imai, and *Volvariella volvacea* (Bull.) Singer. Approximately, more than 80 % dried mushrooms are represented by EEMMs, whereas for frozen and brined mushrooms the EEMMs share is reduced to 60–70 % and < 30 %, respectively

increase their value after preservation, as their slimy texture is markedly reduced; for instance, *Suillus luteus* s.l. (slippery jacks), especially from Chile and Ecuador, are usually sold dried, frozen, or brined rather than fresh. Some species of *Leccinum* and *Boletus* (in Asia mainly *Leccinum extremiorientale* (Lar. N. Vassiljeva) Singer and species belonging to *Boletus* sect. *Appendiculati*) have a good market share at local level also as dried product.

Chanterelles are well suited for preservation in brine, as they maintain an excellent texture and retain part of their flavor as well. Dried chanterelles, on the contrary, have a small market share, since rehydration is difficult and unduly lengthy. Frozen chanterelles are well traded, but freezing may sometimes modify the flavor and cause them to develop an unpleasant bitter aftertaste which, however, can be avoided by preliminary heat treatment. *Craterellus lutescens* (Fr.) Fr. and *Craterellus cornucopioides* (L.) Pers. are most suited for drying, but have a much smaller market share which is restricted to areas of traditional consumption, even though the trade of *C. lutescens* is now rapidly growing.

The firm texture of the saffron milk caps (*Lactarius deliciosus* and allied species) lends them to be dried easily, but the major trade is for otherwise preserved product (in brine, vinegar, and oil); milk caps are also traded as IQF, often after blanching. *Catathelasma ventricosum* and some species of *Russula* (i.e., *R. virescens*) are other EEMMs frequently traded in Asia as dried product.

Mushroom import–export in Italy exemplifies an important market for preserved EEMMs, as it is centered on porcini mushrooms which, as discussed above, are most suited for preservation. For Italy, figures for imported mushrooms largely exceed those of exported mushrooms (Table 20.4). It must be pointed out, however, that Italian mushroom exports consist of previously imported mushrooms that are

simply resold as such or after suitable transformation and/or packaging (Sitta et al. 2007a; Sitta and Floriani 2008).

20.3.1 Freezing and Blast Freezing

Mushroom preservation by freezing has expanded on a global scale in very recent years. Trade of frozen EEMMs is currently growing both in number of traded species as well as in quantities. Despite the high energy requirements and related costs, preservation by freezing and blast freezing maintains good to excellent organoleptic qualities of most EEM species and offers preserved mushroom products all year round with little difference from fresh mushrooms. For such reasons, mushroom preservation by means of freezing techniques represents a promising avenue of research for food technologists. Frozen mushrooms are traded in different merceological categories: sliced, in cubes, and as whole mushrooms (wholes), each with peculiar grades. Wholes frequently contain parasites and spoilage of inner fungal tissues, which are often not visible from the outside. Such defects are therefore impossible to ascertain without cutting/slicing (see Chap. 19).

Uninterruption of the cold chain represents a critical control point in all phases of production, storage, transport, and trade of frozen mushrooms and must be always kept strictly monitored by food industries within their auto-control systems based on Hazard Analysis and Critical Control Points (HACCP) principles. The presence of foreign toxic species in consignments of frozen EEMMs is rare, though not impossible, especially in batches of porcini at the youngest developmental stage, where specimens of *Amanita pantherina* (DC.) Krombh. and *Amanita muscaria* (L.) Lam. at the "egg stage" may be found occasionally. Therefore, mycological inspections must be fine-tuned to each EEMM at different developmental stages, following suitable risk assessment of the occurrence of foreign species due to manipulation errors in the course of preceding processing steps (Sitta 2007).

20.3.2 Preservation in Brine

Preparation of brined mushrooms involves brief cooking in salted water (2–3 % sodium chloride w/w), usually slightly acidified with citric acid, followed by cooling and soaking in 22 % (w/w) brine (1.5 part mushrooms and 1 part brine by weight) at 25–30 °C. After 10–15 days under such fermentation conditions, mushrooms are drained and soaked again in fresh 22 % brine. In China, the brining procedure is often different: after collection, mushrooms are pressed as such, usually without any cleaning, in (solid) salt in small barrels and transported to processing factories where they are washed in running water, graded, and soaked in strong brine (Sitta 2007).

Sodium chloride solutions used for brining are referred to as "weak brine," when salt has 10–18 % concentration (expressed as g of sodium chloride per 100 g water by weight), and "strong brine" when the sodium chloride content is higher, up to saturation (Sitta 2007). Weak brine is suited for short preservation (from weeks to a few months) and does partly preserve the mushroom flavor; by contrast, strong brine, e.g., used in China, permits a much longer preservation time (years) but completely destroys all the flavor, so that subsequent flavoring is required.

Brined mushrooms, which are not fit for human consumption as such, need to be desalted in running water, cooked, and, often, flavored. They are normally used for industrial processes, i.e., to produce pickled mushrooms in oil, sauces, or other mushroom-based products. Porcini, chanterelles, saffron milk caps, and slippery jacks are the most important EEMMs that are traded as brined.

The risk of occurrence of foreign species is higher in brined porcini at their younger stage of development, where immature species belonging to diverse taxa can be found (e.g., *Amanita*, *Cortinarius*, *Tricholoma*, *Russula*). Accordingly, brined immature porcini, commercially known as "garniture" ("garnishing") and typically employed to manufacture pickled porcini in oil, need to be subjected to closer mycological inspection. Nevertheless, the toxicity of some foreign species such as *Amanita muscaria* is removed or drastically reduced by brining and subsequent desalting and cooking, as parboiling is reported as a safe method for detoxification of *A. muscaria* (Rubel and Arora 2008). Of note, the occurrence of conspicuous amounts of stems belonging to the cultivated species *Stropharia rugosoannulata* Farl. ex Murrill has been also reported in batches of brined porcini. This would constitute fraudulent trade as stems of *S. rugosoannulata* in brined form bear quite a similar appearance to those of *Boletus* sect. *Boletus* (Sitta 2007).

The occurrence of *Clostridium perfringens* in brined mushrooms should not be considered as a real threat to consumers' health. Brined mushrooms are not edible as such. They have to be desalted and then cooked or, in the case of pickled mushrooms, acidified to pH values lower than 4.6 at which clostridia cannot develop.

20.3.3 Drying

Drying mushrooms consists of reducing their moisture content by means of moderate heating and air exchange. Freeze-drying is also possible for mushrooms and provides an excellent product with a very low moisture content, which can be quickly and almost completely rehydrated; freeze-dried mushrooms, however, are very expensive due to high production costs and therefore have a little market share.

The maximum water content for dried mushrooms is set by qualitative international standards (FAO/WHO 1981) and by some national regulations as well (Italy 1995), and must not exceed 12–14 % in weight. The drying process has important effects on color, texture, and flavor and, consequently, it affects the commercial value of dried EEMMs. A constant flow of warm dry air is required

to dry mushrooms properly, and all drying techniques can be effective if there is such an airflow and the temperature does not exceed 60 °C. Usually, EEMMs are not blanched or chemically pretreated, but only cleaned and sliced, manually or mechanically. Water or steam blanching would cause intensive color deterioration and hardness in texture (Argyropoulos et al. 2011). EEMMs can be home dried (often irregularly cut with variable thickness and dried in ovens or by solar radiation) or processed in suitable collection/processing centers, where they are cut by machine and systematically dried in large drying ovens equipped with airflow and temperature controls. Dried Chinese EEMMs are produced in villages by means of small handmade dryers, whereby slices are placed on screens or grates in the upper part of the dryer. The drying temperatures are often too high and consequently the dried mushrooms tend to become hard or rigid (and often wrinkled in a peculiar but recognizable way), with a strong browning or blackening of the flesh. Likewise, the resulting mushroom flavor is strongly affected, especially when mushrooms are dried along with other food items or goods, causing them to become smoky, slightly sour, or musty, and sometimes acquiring flavors that are reminiscent of spices, glutamate, or even tobacco (Sitta and Floriani 2008).

In dried mushrooms, water activity (A_w) values are very low and range from lower than 0.4 in laboratory dried samples (Argyropoulos et al. 2011) to 0.5–0.65 in dried porcini sold on the market (Lorini et al. 2008; Sitta et al. 2007b). Such values do not allow microbial growth and spoilage, and therefore, dried mushrooms have a long durability and can be stored and transported at temperatures below 0 °C, without the need to treat them as chilled or frozen food products. Actually, temperatures in the range of −20 to −5 °C represent the best storage conditions to ensure the organoleptic properties of dried mushrooms remain unaltered; subsequent processing and trade take place at room temperature without any deterioration. They may remain stable for some years at room temperature without any decaying phenomena, whereas organoleptic properties and especially flavor change more quickly, and sometimes an unpleasant sour smell may develop. Such a rationale is most likely to explain the maximum durability of 12 months after packaging, which has been set by Italian regulations for dried mushrooms (Italy 1995).

Nearly all fungal species can be home dried for out of season consumption, and in local markets of developing countries, all these species are sold also in dried form. In the past, this situation used to take place also in Europe, but intoxications by dried mushrooms sold on the market were rather common until the beginning of twentieth century and were difficult to avoid through market controls (Sitta et al. 2007b). In fact, species determination at inspection level is much more difficult for dried mushrooms and for many EEMMs it is unfeasible for the huge quantities that are marketed. This has ultimately resulted in a decrease in the number of species traded, in particular gilled EEMMs, and has favored mushroom species which are considered safer because they are more easily recognizable, such as *Boletus*, *Morchella*, and *Craterellus* spp. (Sitta et al. 2007b). However, the sale of many dried EEMMs still remains widespread, both in local markets in many developing countries and via the Internet. At the same time, the international trade of dried

cultivated mushrooms (i.e., *Lentinula edodes*, *Agaricus*, and *Pleurotus* spp.), either as single species or mixed (often with the addition of dried wild species), is rapidly expanding worldwide.

The greater ease of inspection of dried porcini has resulted in a clear distinction from other dried mushroom species since the early twentieth century (Sitta and Floriani 2008). Such a distinction is currently regulated by Italian law, whereby a specific positive list of marketable mushrooms in dried form has been established (Italy 1995); all the species included in this list, which contains also a dozen of EEMM species, are allowed to be traded only if packaged by food industry operators. The only exception is dried porcini, which can also be sold loose also at retail level. A separate Italian regulation specifies a commercial ranking for dried porcini only (Italy 1998), which are classified as *extra* (first quality), *speciali* (intermediate quality, with darker color and some defects), and *commerciali* (with the darkest coloration and more defects). Such a detailed regulation may appear unduly complicated and somewhat useless. However, the EEMM trade is so seasonally dependent and therefore most unsuited to business planning (in terms of available quantity and merceological quality of EEMMs) that the presence of specific regulations helps market stabilization and results ultimately in an improvement in the quality of dried products on sale to the final consumers. In France, for instance, which lacks specific regulations, the average quality of dried porcini on the market is much lower than Italy and sometimes corresponds to production waste (Sitta 2000).

Important species of dried EEMMs are traded as wholes (especially on the Asian market), sliced, diced, or powdered. Diced dried EEMMs, mainly porcini, are used for food industry applications and must have uniform appearance and organoleptic features, in addition to complying with specific microbiological and chemical parameters. They are also referred to as "kibbled mushrooms" or "fungus grits." Diced mushrooms are produced by coarsely grinding dried mushroom slices and subsequently passing them through sieves with different mesh sizes; this results also in a partial loss of product in the form of over- and undersized mushroom fragments, powder, foreign materials, and other production waste. The smaller the piece size, the higher the price of the final product. When diced or powdered products have too low prices, it is most likely that production waste has been used, but since merceological inspection is difficult, frauds are common.

Typical critical issues for batches of dried EEMMs are occurrence of foreign species, post-drying attacks by arthropods (see Chap. 19), and presence of pieces infected by moulds. Foreign species, usually edible and more frequently found in dried porcini from China, can be detected by macroscopic inspection. This task is much easier for dried porcini than for gilled EEMMs. Moulds in dried EEMMs are represented mainly by soil micromycetes or symbiotic/parasitic species of genus *Sepedonium* (see Sect. 20.7), which preexist in the fresh fruiting bodies. Storage moulds that may produce mycotoxins occur rarely in dried EEMMs; given their low A_w values, dried mushrooms are not a suitable substrate for the development of moulds (Sitta et al. 2007b). A recent study has confirmed that dried porcini generally do not contain detectable concentrations of aflatoxins and ochratoxin A,

despite the presence of *Aspergillus flavus* Link in same samples (Lorini et al. 2008). By contrast, dried *Pleurotus* mushrooms appear a better substrate for the development of moulds and for the production of aflatoxins as well (Jonathan and Esho 2010). In this respect, further research on the potential occurrence of mycotoxins in dried mushrooms in relation to different storage conditions would be recommended, especially for dried porcini of different merceological grades but also for other dried EEMMs.

The occurrence of *Bacillus cereus* in dried porcini is quite frequent and is not unusual, since in nature *B. cereus* has been demonstrated to play a role as mycorrhiza helper toward *Boletus edulis* (Wu et al. 2010). Although such an occurrence could be considered a threat to consumers' health, it should be borne in mind that dried EEMMs are to be considered fit for human consumption only after thorough cooking; moreover, to prevent any risk from microbial contamination, consumers and intermediate users (i.e., restaurants) must always follow the WHO "golden rules for safe food preparation" (WHO 1989).

20.4 Market Names and Scientific Names in EEMM Trade

In the framework of international trade, communication about mushroom products is based on commercial and/or scientific name of the species, and on a peculiar nomenclature based on qualitative grades and other merceological characters. For example, for frozen porcini or matsutake, terms such as slices, cubes, halves, or wholes are widely used and often are further subdivided according to size and grades. For any given EEM species, grade ranking is usually unequivocal as the I, II, and III grades (etc.) will always rank from higher to poorer quality.

In reference to the fungal species on the final packaging and presentation to the end consumer, usage of common/local fungal names is widespread and is usually not subjected to regulations, unless such names might sound somewhat misleading to the consumer. When labelling mushrooms, the addition of the scientific names is mandatory for some European regulations (Italy 1995; Spain 2009), either when the fungal name represents the name of the product or when it is only included within the ingredient list.

For international trade of mushrooms and mushroom products, even for exporting to countries where a specific regulation is not in force and where the use of scientific names is not strictly required, utilization of scientific names for labelling is nonetheless most welcome and appreciated (FAO/WHO 1981). Regardless of whether a current scientific name or a widespread common synonym is used, the use of Latin names will certainly help both the final consumer and the control officers designated to enforce specific regulations in matters of food safety.

For some EEMMs, the use of traditional names may be regulated by law; for example, in accordance with Italian regulation (Italy 1995), the commercial name "porcini" can be used only for designating species which belong to the genus *Boletus* section *Boletus* (Dentinger et al. 2010); likewise, the formula "*Boletus edulis* and related species" can be used to designate collectively such species.

20.5 EEMM Species Identification: The Mycological Expertise

A specific professional operator, defined by law under the name of "micologo" (i.e., mycologist) in Italy (Italy 1996), is someone suitably trained to perform species determination and evaluation of edibility for wild mushroom species. When such operators work for official control bodies (EU 2004b), they may be better referred to as "mycological inspectors." Their role is mainly to prevent mushroom poisonings. Within the services they provide for the community, free control of the edibility of mushroom species from private collectors for their own consumption is of major importance. In fact, most cases of mushroom poisoning today are caused by the consumption of privately collected mushrooms, whereas in the not too distant past they were derived from mushrooms from the market. As of 4 August 2011, in Italy, the National Registry of Mycologists set by the Ministry of Health (Italy 2003) lists 2,376 people as having received the qualification of mycologist. Also in Switzerland, those who determine mushroom species officially, both on the market and for private collectors, must be suitably trained, must have passed a specific examination, and they are recognized as mycologists by law (Swiss Confederation 1995).

According to Boa (2004b), the mycological expertise for identifying macroscopic fungi involves a microscopic examination of tissues, spores, and spore-forming structures or use of molecular tools. This kind of taxonomic expertise is bound to be performed on a single or a few fungal specimens.

Italian and Swiss mycological inspectors have different skills. They must have practical competence in species determination of huge amounts of fresh wild mushrooms for market sale and are requested to assess their fitness for human consumption; they must determine species edibility in the most diverse mushroom harvests of private collectors. Finally, they must be able to perform macroscopic control of sometimes conspicuous samples from batches of preserved EEMMs, in order to perform identity checks and physical checks as disposed by EU regulation on official food controls for the verification of compliance with feed and food law (EU 2004b).

20.6 Contrasting Evaluations of Edibility

The edibility evaluation of EEMMs is based primarily on the correct determination of genera and species, and secondly on edibility data available from the literature. While the evaluation of toxicity or edibility is quite clear cut and undisputable for *Amanita phalloides* (Vaill. ex Fr.) Link or *Cantharellus cibarius*, respectively, this is not always the case for several other EEMM species. Mushroom field guides may contain conflicting reports about edibility. For instance, some guides may recommend eating species that other guides reject as poisonous

(Boa 2004a, b). Contrasting evaluations on edibility are also found in regulations of different countries. For example, while *Gyromitra* species are regarded in Finland as culinary delicacies after careful precooking, in the USA (FDA 1984) and most of EU countries, they are considered poisonous and in some cases are strictly forbidden (France 1991; Spain 2009). Likewise, *Verpa bohemica* (Krombh.) J. Schröt. is considered edible in Europe and is widely traded in Emilia Romagna (Italy) where it has a long history of consumption, whereas in the USA, it is regarded as poisonous (FDA 1984). In this case, the contrasting evaluation is most likely due to an old misunderstanding, in the USA, with the now better documented neurological syndrome which is caused by eating excessive quantities of *Morchella* species (Berndt 2010; Pfab et al. 2008). Major epigeous EEMMs with conflicting reports on edibility are *Amanita ovoidea* (Bull.) Link and other *Amanita* belonging to sect. *Amidella* and *Lepidella*; some acrid species of genera *Russula* and *Lactarius*; some *Ramaria*, *Suillus*, and *Tricholoma* species (especially sect. *Albobrunnea*); all species of the genus *Hebeloma* and *Scleroderma*; many species of *Cortinarius*; and certainly many others. The edibility of hypogeous EEMM species is not under discussion, except for *Choiromyces meandriformis* Vittad. (= *Choiromyces venosus* (Fr.) Th. Fr.). Although this truffle is considered slightly toxic in Southern Europe, it is widely consumed and traded in Hungary, Sweden, and other North-European countries (Gógán Csorbainé et al. 2009; Wedén et al. 2009).

In principle, when cases of gastrointestinal syndrome are reported following consumption of fungal species that are only locally eaten by a small number of people, such species should be officially regarded as "not edible." To issue such a statement, however, the number of poisoning cases and their severity must be correlated with the importance and diffusion of culinary consumption for that particular species. For example, traditional consumption in a few small areas markedly differs from widespread utilization as food by hundreds of thousands of people. The gyromitric syndrome, caused by consumption of insufficiently cooked *Gyromitra*, or the paxillic syndrome, which is due to a peculiar reaction of the immune system following consumption of *Paxillus involutus* (Batsch) Fr., are potentially deadly, yet they rarely occur and only under particular conditions and their severity is not comparable to "ordinary" gastrointestinal syndromes. The difficulty of macroscopic determination with respect to similar species of potential toxicity (i.e., genera *Cortinarius*, *Hebeloma*, and others) may also affect the issuing of an official statement of "non edibility" for a given species. On the basis of the above considerations, and taking into account any scientific update in the field of mycotoxicology, in each single country food consumption should be deterred and trade should be forbidden for the EEMMs which are regarded as unsafe. In Italy, such a task is brought into practice by mycological inspectors who operate for official control bodies, in accordance with national and European regulations (Italy 1995, 1996; EU 2002, 2004b).

20.7 Food Safety and Quality Control of Fresh and Preserved EEMMs

Beyond species identification, other food safety and quality controls must be performed on EEMMs destined for food consumption. In applying the HACCP principles, food business operators shall ensure that all stages that they are responsible for are carried out in compliance with suitable hygienic standards. Food business operators shall set up traceability systems and procedures for ingredients and foodstuffs used for food production. When a food item poses a serious risk to human health, the food business operator shall immediately withdraw that foodstuff from the market and inform users and the competent authority (EU 2002, 2004a, b). EU has established a Rapid Alert System for Food and Feed (RASFF), to provide control authorities with an effective tool for the notification of risks to human health deriving from food and feed (EU 2002) or, in any case, an efficient exchange of information (EU 2011).

In international commerce, reference to food safety and quality requirements of the recipient country is always necessary when issuing certificates that are required for the exportation of foodstuffs. Frequently, in fact, certificates that are needed for a given country might not be requested by the authorities of another country. According to the World Trade Organization (WTO), sanitary and phytosanitary measures (SPS) are defined as any measure applied to prevent or limit risks and damages from the entry, establishment, or spread of pests, diseases, and disease-carrying or disease-causing organisms and also to protect human health from risks arising from additives, contaminants, and toxins in foods and beverages (WTO 2010). SPS are often applied on the basis of bilateral agreements or protocols, and each country has the right to enforce SPS measures necessary to achieve appropriate levels of protection. However, suitable agreements (WTO 2010), international standards, guidelines, and recommendations (i.e., Codex Alimentarius, International Plant Protection Convention) have been laid down to harmonize SPS measures of different countries or customs unions and to minimize any negative effect on global trade. To apply a more stringent measure than the relevant international standard, a country must have a scientific justification for that measure and an assessment of the risk to human health to back up the measure which provides a higher level of protection. In default of a reciprocal acknowledgement of equivalence of the sanitary requirements with the supplier country, a few countries (e.g., USA, Canada, Brazil, China, and others) require their sanitary authorities to endorse the guarantee of food safety of food operators. Therefore, the authorities can perform food safety audits in the plants of the producing country, and the acknowledged plants are placed on official lists.

General guidelines on food safety and quality controls (FAO/WHO 2006) are very useful and informative, both for inspectors of official control bodies and for food business operators dealing with international commerce. More specific

guidelines also exist and can help bilateral relations (e.g., in the application of US regulations for Italian plants listed for exporting foodstuff to the USA) (Bassoli and Della Ciana 2009).

The following issues specifically concern food safety and quality control of fresh and preserved EEMMs and EEMM-based products.

20.7.1 Radioactivity

The accident at the Chernobyl nuclear power station in 1986 caused the fallout of considerable amounts of radioactive elements, mainly caesium-134 (^{134}Cs), caesium-137 (^{137}Cs), and iodine-131 (^{131}I). Whereas ^{131}I decayed quickly, ^{134}Cs and ^{137}Cs are of major concern, in particular ^{137}Cs due to its relatively long half-life (>30 years) and ease of migration through the food chain, especially in acidic soils where mobility and bioavailability are increased (Malinowska et al. 2006).

Saprotrophic fungi were contaminated initially, but in a couple of years, ectomycorrhizal species, including commercially important EEMMs, displayed higher ^{137}Cs uptake levels (Smith et al. 1993; Barnett et al. 1999), in particular *Boletus badius* (Fr.) Fr., *Craterellus lutescens*, *Craterellus tubaeformis* (Fr.) Quél., *Hydnum repandum*, and *Cortinarius caperatus* (Pers.) Fr. (Kalač 2001; Duff and Ramsey 2008); highly prized EEMMs such as *Tuber magnatum* (Lorenzelli et al. 1996), *Boletus edulis*, and *Cantharellus cibarius* were also found capable of accumulating radiocaesium, although to a minor extent. However, interspecific and intraspecific variation in radionuclide accumulation was observed, even within the same geographic region, and sporadically, samples with abnormally high values can be found in economically important species such as *Boletus edulis* (Smith et al. 1993; Barnett et al. 1999). Although values below 100 Bq/kg are usually detected in *Boletus edulis* of European origin (Sitta et al. 2007a), higher contamination levels have also been reported, e.g., from Ukraine and Sweden (Smith et al. 1993; Mietelski et al. 2002). As far as *Tricholoma matsutake* is concerned, while low ^{137}Cs uptake values were reported for specimens from Japan (Ban-Nai et al. 2004), most recently, samples from southwestern Finland have been found contaminated at levels up to 1,100 Bq/kg fresh weight that would hamper exportation to Japan (Kostiainen and Savonen 2009).

For most foodstuffs, mushrooms included, maximum permitted levels of radioactive contamination, ranging from 1,000 to 1,250 Bq/kg in terms of ^{134}Cs + ^{137}Cs, have been set by European and American regulations (EURATOM 1987; FDA 1998) and by the Codex General Standard (FAO/WHO 1995). In EU, however, the EURATOM maximum level of 1,250 Bq/kg, which concerns radioactive contamination of foodstuffs in conditions of radioactive emergency (e.g., following a nuclear accident), has been lowered to 600 Bq/kg for importation of agricultural products originating in third countries, in accordance with temporary EU regulations (see EU 2008b). A temporary limit of 500 Bq/kg has entered into force since March 2011, for the foodstuff imported from Japan, following the radioactive accident at the Fukushima nuclear power plant.

Based on an average concentration of 600–1,000 Bq/kg for ^{137}Cs and an average annual consumption of 10 kg fresh mushrooms, the effective equivalent radiation dose would correspond to 0.1–0.2 mSv/year, which can be considered negligible (Kalač 2001; Malinowska et al. 2006). Various household methods have been tested to reduce ^{137}Cs contents of mushrooms, and in most cases, they were found effective in removing most of the radioactive contamination, approximately 70–80 % (Kostiainen 2005).

Artificial radionuclides other than ^{137}Cs contribute only negligibly to the radioactivity of wild mushrooms (Mietelski et al. 2002), whereas naturally occurring potassium-40 (^{40}K) is commonly detected in mushrooms at concentrations of about 100 Bq/kg as background contamination, without specific taxonomic preferences (Baeza et al. 2004), and contributes inevitably to the overall intake of radionuclides by the human body through food consumption.

20.7.2 Heavy Metals

EEMMs have long been known to accumulate toxic heavy metals such as cadmium, lead, and mercury. Heavy metal uptake in fungal fruiting bodies displays a marked species specificity, regardless of the vicinity of potentially polluting artificial sources such as metal smelters or industrial facilities. Within a given species, metal specificity is also observed. Natural factors such as soil geology but also type and age of the forest ecosystem may also contribute to metal accumulation (Cocchi et al. 2006; Kalač et al. 2004). Tolerable intake values for such heavy metals were set by regulatory agencies and have been endorsed by specific regulations setting maximum levels of contamination in foodstuffs for food safety reasons. EEMMs are also included in some regulations (e.g., maximum level for cadmium of 1.0 mg/kg fresh weight for wild mushrooms), whereas maximum levels are set for other heavy metals (e.g., lead and mercury) only for some cultivated mushrooms (EU 2008a). International guidelines such as Codex General Standard (FAO/WHO 1995) do not set specific maximum limits for heavy metal contamination in wild mushrooms.

Cadmium is usually detected at concentrations well above 0.2 mg/kg fresh weight in commercially important EEMM species such as porcini and *Amanita caesarea* but also in *Cortinarius praestans* (Cordier) Gillet, *C. caperatus*, and *Russula vesca* Fr.; for porcini, typical values range from 0.1 to 0.5 mg/kg fresh weight; in *Cantharellus cibarius* and *Suillus luteus*, cadmium levels are usually well below 0.1 mg/kg fresh weight (Cocchi et al. 2006). Lead uptake in fruiting bodies of many wild mushrooms, including EEMMs, typically occurs at concentrations below 0.2 mg/kg fresh weight (Cocchi et al. 2006).

Among EEMMs, porcini feature a peculiar ability for mercury accumulation; values are usually in the range of 0.1–0.4 mg/kg fresh weight (Cocchi et al. 2006; Falandysz et al. 2007), even though higher levels have been reported from contaminated areas (Falandysz et al. 2007); in *C. cibarius* and *S. luteus*, by contrast,

mercury concentrations have never exceeded 0.03–0.04 mg/kg fresh weight (Cocchi et al. 2006; Chudzyński et al. 2011). However, mercury levels in foods other than fish and seafood are considered of lower concern, since mercury is not present in the most toxic form (i.e., monomethylmercury ion) which, by contrast, can make up more than 90 % of total mercury in fish and seafood. Typical mercury levels in wild mushrooms, including EEMMs, are therefore considered unlikely to pose risks to human health, given also the fact that wild mushroom species, and especially porcini, are usually quite rich in selenium (Cocchi et al. 2006), which is known to counteract mercury toxicity (Skerfving 1978).

20.7.3 Nicotine and Pesticides

In early 2009, unusually high levels of nicotine (e.g., up to 0.5–1 mg/kg) were found to contaminate dried EEMMs, in particular porcini. Such levels exceeded the default maximum residue level (MRL) of 0.01 mg/kg set by EU regulation (EU 2005). Nicotine, the major alkaloid in tobacco, occurs naturally also in species of Solanaceae such as tomato and potato, albeit in notably smaller amounts, and is still being used as insecticide for crop protection, especially in Third World countries. The most contaminated samples were dried porcini from China, but lower levels were found also in East European and South African samples of dried porcini and in other mushroom species, and almost all of the dried wild mushrooms on the market were deemed non-compliant. After a suitable risk assessment, the European Food Safety Authority (EFSA) issued alternative MRLs (EFSA 2009), albeit on a temporary basis, which were subsequently endorsed by EU regulation: the higher tolerance, 2.3 mg/kg dry weight, was set for dried porcini (EU 2010). It is most likely that the presence of nicotine in wild mushrooms results from cross contamination during processing; in particular, the use of drying ovens where tobacco leaves are also processed, sometimes simultaneously to wild mushrooms, and/or where tobacco plant waste might have been employed as fuel for heating could well explain such an occurrence in dried mushrooms, especially from China. Lower contamination levels might also result from handling by workers who are smokers, as they may pass the nicotine from their epidermis during mushroom processing. Endogenous formation of nicotine in mushrooms in the wild and/or during drying was also invoked, even in the absence of any sound biochemical evidence, but could be ruled out also from the finding that standard drying did not result in increased nicotine levels.

Most unexpectedly, synthetic pesticides have also been detected in dried EEMMs on the market. Chanterelles from East Europe were found contaminated by the insect repellant DEET at levels up to 1 mg/kg (BfR 2009), whereas pyrethroids (tetramethrin, permethrin, cypermethrin) and propoxur were found in dried porcini (Wieland et al. 2010); alphamethrin, piperonyl butoxide, chlorpyrifos, and dichlorvos were also detected in dried porcini (Sitta, unpublished data). The origin of such contamination in wild mushrooms remains unanswered at present and certainly warrants further investigations.

20.7.4 Dipteran Larvae and Other "Parasites"

The presence of dipteran larvae and other arthropods is widespread in nearly all EEMM species, and consequently, batches of EEMMs destined for human consumption are inexorably more or less contaminated by "parasites" (see Chap. 19). On a regulatory basis, however, three different phenomena, concerning parasites of fresh EEMMs, must be considered: pieces of mushroom with maggot holes (pitted units), deteriorated mushroom units, and internal or visible presence of arthropods, which are always dead in preserved EEMMs.

Pitted units are tolerated by tradition and also by several regulations; accordingly, pitted units are judged fit for human consumption, but they are considered of lower quality and used for merceological grading of fresh and preserved EEMMs. In the definition of the commercial ranking for dried porcini set by Italian law (see Sect. 20.3.3), one of the main requirements is a maximum tolerance for pitted units (from 10 % in weight in the "*extra*" grade to 25 % in the lower grade "*commerciali*"). By contrast, Italian and EU regulations do not provide for any specific tolerance for parasite insects in wild mushrooms, and both the presence of arthropods and mushroom deterioration are generally considered unfit for human consumption (EU 2002).

The definition of "maggot-damaged fungi" given by the Codex Standard (FAO/WHO 1981) coincides with pitted units, as well as for the "seriously maggot-damaged fungi," because the distinction is based only on the number of holes (4 or more) caused by maggots. Low percentages of pitted units by weight are tolerated for fresh, frozen, brined, pickled, and otherwise preserved wild mushrooms, except for dried mushrooms, for which the tolerance is raised to 20 % (FAO/WHO 1981).

The tolerance for a small number of larvae and mites, detected with the filth-test method, is set by Canada and USA regulations; the number of tolerated maggots is highly reduced if their length is >2 mm (Canada 2009; FDA 1995). The method of analysis and the tolerances are both totally inappropriate for the evaluation of EEMM fitness for human consumption; one of the reasons is the absence of any direct relationship between macroscopic parameters (percentage of pitted units and deteriorated units) and the number of parasite insects which can be found in a mushroom sample with the filth-test method (see Chap. 19). Moreover, the tolerance for 10 % in weight for "decomposition" set by US regulation (FDA 1995) is astonishing: decomposition, in fact, is equivalent to deteriorated mushroom units, as it is defined as "bacterial breakdown of the normal product tissues and the subsequent enzyme induced chemical changes, manifested by abnormal odors, taste, texture, color, etc."

The occurrence of maggots and other parasite insects in EEMMs is normal and inexorable. Such an occurrence, per se, should not be considered as unfit for human consumption, but as a peculiar feature of EEMMs. Fungal tissue deterioration, by contrast, ought not to be tolerated (Sitta et al. 2007b), unless at levels much lower than FDA specifications (FDA 1995).

20.7.5 *Presence of Species of the Genus* Hypomyces *in EEMMs*

Lobster mushroom is the common name used for a commercial wild mushroom derived from the infection by *Hypomyces lactifluorum* (Schwein.) Tul. & C. Tul. on various species of the genera *Russula* and *Lactarius*, mostly *Lactarius piperatus* (L.) Pers. and *Russula brevipes* Peck but also *Russula delica* Fr. and *Russula chloroides* (Krombh.) Bres. (Rochon et al. 2009). *Hypomyces lactifluorum* grows on the host fruiting bodies and deforms the morphology of cap, stem, and the gills, which may completely disappear; the infected fruiting bodies feature a peculiar orange to reddish orange color which accounts for the common name. The distribution of the lobster mushroom is restricted to North and Central America (Canada, USA, Mexico, Guatemala) where it is locally abundant and very appreciated. The edibility of the lobster mushroom and its fitness for human consumption is not under discussion here, even though it represents a parasitic micromycete and despite the fact that an exact identification of the host is not always feasible. In Europe, *Lactarius deliciosus* and allied species infected by *Hypomyces lateritius* (Fr.) Tul. & C. Tul. are not common enough to build up a tradition of culinary consumption and the consequent specific trade. However, mushroom pickers consider them edible and often of better quality than "normal," i.e., not *Hypomyces*-infected, saffron milk caps.

Fungicolous fungi which develop on important EEMMs include *Mycogone rosea* on *Amanita caesarea* and several *Hypomyces* species living on Boletales; these micromycetes are most common in their anamorphic forms, which are included in the genus *Sepedonium*.

Fruiting bodies of porcini, at their younger stage of development, are always accompanied by the presence of *Sepedonium* spp., whose mycelium typically occludes the pores of young specimens. Such a presence has been interpreted by taxonomists as a morphological character of *Boletus* sect. *Boletus* (Dentinger et al. 2010). Only *Sepedonium chrysospermum* (Bull.) Fr. and *Sepedonium chlorinum* (Tul. & C. Tul.) Damon are found to grow on porcini (Sitta et al. 2007b). In contrast to other *Sepedonium* species, these two species are not known to produce potentially toxic pigments such as rugulosin and other anthraquinones (Sahr et al. 1999). Fruiting bodies of porcini are not considered toxic, even when heavily infected by *Sepedonium* spp.; however, when the infection produces conspicuous deformation of and visible yellow pigmentation on fruiting bodies, porcini are not considered suitable for sale or fit for human consumption since they appear as mouldy mushrooms (Sitta et al. 2007b). Hemolytic activity has been detected and characterized in an extract of *S. chrysospermum* (Sanguineti et al. 2011); noteworthily, the isolated hemolysin was stable in high-temperature treatments, indicating that it may not be inactivated by cooking. Hence, the potential toxicity of Boletales when heavily infected by *S. chrysospermum* should be investigated in more detail (Sanguineti et al. 2011).

20.8 Conclusions

Trade of fresh and preserved EEMMs still holds ample potential for new developments, in particular for the discovery and exploitation of new geographic areas for the production of highly prized species but also for expanding international markets in the trade of other mushroom species that are currently consumed only on a local scale.

The decline in production of some prized mushroom species in countries that are traditional EEMM consumers is a well-documented issue, and this phenomenon is also widespread in other EEMM-producing countries. In the medium to long term, this might turn into an economic problem not only for people who make their living out of mushroom collection in the first instance but also for recipient countries which might have to face lower amounts of traded mushrooms available, and consequently, higher prices for lower quality material on average, given the need to import all available mushroom material including lower grades. Cultivation might represent a solution only for some EEMMs at present; forest management plans that also include production of prized EEMMs as an important non-timber forest resource are most desirable.

Controls on the edibility and fitness for human consumption for EEMMs ought to be performed by properly trained mycological inspectors, who are presently only available in some countries. The examination of batches of EEMMs to determine if they are well preserved and to evaluate the occurrence of maggot-damaged specimens should be included among these tasks and should take place on a macroscopic and organoleptic level. Laboratory analysis for the monitoring of certain contaminants (radioactivity, heavy metals, pesticide residues, etc.), of microbiological parameters and other controls in matters of food safety must always be performed to guarantee consumers' health in accordance with regulations in force. However, if regulations and standards in a given country are too strict for such a peculiar food commodity such as wild mushrooms, they may complicate the EEMM trade or render it impossible, or may result in the application of such regulations as unfeasible.

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Part IV
The Edible Ectomycorrhizal Mushroom Industry in the Age of "-omics"

Chapter 21
Ten Years of Genomics for Ectomycorrhizal Fungi: What Have We Achieved and Where Are We Heading?

Francis Martin and Gregory Bonito

21.1 Introduction

The science of edible ectomycorrhizal mushrooms (EEMMs) is a rapidly advancing field, as has been clearly demonstrated in the previous chapters of this book. Agronomic aspects pertaining to the practice of EEMM cultivation are adapting to new data and insights from published studies. Major achievements of the past two decades of EEMM research include both genetic tools and a phylogenetic framework to discern EM species and their populations (Hibbett and Matheny 2009; Douhan et al. 2011; Martin et al. 2011a), which have advanced our knowledge on fundamental aspects of EM biology, ecology, and evolution. As we discuss below, mycorrhizal genomics is well underway. Beginning with the genome sequencing of *Laccaria bicolor* (Maire) P. D. Orton (Martin et al. 2008) and followed by that of the black truffle *Tuber melanosporum* Vittad. (Martin et al. 2010), sequencing efforts are now underway on the genomes of more than 30 other ectomycorrhizal species (and hundreds of fungal pathogens, endophytes, and saprotrophs—see below) (Martin et al. 2011a, b; Plett and Martin 2011).

Technological advances are a major force driving science and human understanding of the natural world. Just as the invention of the telescope spawned astronomy and the microscope opened our eyes to microbiology, advances in modern technology (e.g., supercomputing, high-throughput DNA sequencing, nanotechnology) are revolutionizing global sciences. Major breakthroughs are expected in many disciplines. Yet, the emergence of genomics and related

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"-omics" sciences (i.e., transcriptomics, proteomics, metabolomics, etc.) are not ends in themselves, rather, they provide maps and tools to aid in our study and understanding of the molecular basis for EM symbiosis and ecosystem functioning. This generation of "-omics" science will address fundamental questions regarding signaling cascades and gene processes involved in the mycorrhizal phenomenon, including the formation of fungal fruiting bodies, and interacting factors (environmental, host, helper bacteria, pathogens). With such insights, new strategies for promoting mycorrhization and in developing selective host and fungal strains for EEMM production will arise.

In this volume on "Edible Ectomycorrhizal Mushrooms," the contributors provide essential background, reviews, and research on major topics concerning basic and applied facets of EEMM science. The first section of this book covers major topics pertaining to the ecology, biology, and systematics of EEMMs. In the second section, a global perspective on the propagation and cultivation of EEMM is given. Social, economic, and health aspects between EEMM and people are addressed in the third section. Here we attempt to synthesize some of the more recent advances in genomes, transcriptomics, and proteomics that are propelling EEMM science, and we assume the precarious task of speculating on the future of this field.

21.2 Where Did We Begin?

A decade ago, the Poplar Mesocosm Sequencing project, aiming to sequence the genome of three *Populus*-associated fungi, the ectomycorrhizal basidiomycete *Laccaria bicolor*, the arbuscular mycorrhizal Glomeromycete *Glomus intraradices* N. C. Schenck & G. S. Sm. (*Rhizophagus irregulare* (Błaszk., Wubet, Renker, & Buscot) C. Walker & A. Schüßler), and the poplar leaf rust *Melampsora larici-populina* Kleb., was initiated as an extension of the Joint Genome Institute (JGI) *Populus trichocarpa* Torr. & A. Gray genome project (Tuskan et al. 2006). The publication of the genome sequence of *L. bicolor* was a landmark event for the mycorrhizal community (Cullen 2008; Martin et al. 2008). It has been rapidly followed by the release of the genome of the iconic edible ectomycorrhizal (EEM) *Tuber melanosporum*, the black truffle of Périgord (Martin et al. 2010). These genome sequences have provided unprecedented knowledge about the structure and functioning of the mycorrhizal symbiont genomes and their interactions with plants. They have also led to the identification of master genes with crucial roles in symbiosis formation (Plett and Martin 2011) and mushroom development (Martin et al. 2008, 2010). The genomic era of mushroom-forming fungi is now a reality. In contemplating a vision for the future of genomics research on mycorrhizal fungi, it is appropriate to consider the remarkable path that has brought us here.

21.3 What Have We Achieved?

21.3.1 Sequencing Mushroom-Forming Fungi

The community genomics approach has generated information-rich large-scale community resource data sets and has introduced an important new dimension into fungal, and more specifically, mycorrhizal research. The first set of goals were aimed at defining reference symbiont genomes—establishing data to serve as a new and fundamental resource for genetics and molecular biology of the symbiosis and fruiting body formation. An impressive list of genomes from mushroom-forming fungi has been sequenced (Table 21.1). In addition to those of *L. bicolor* and *T. melanosporum*, the genome sequence of the oyster mushroom [*Pleurotus ostreatus* (Jacq.) P. Kumm.] (http://genome.jgi-psf.org/PleosPC15_2/PleosPC15_2.home.html) and the button mushroom (*Agaricus bisporus*) (J. E. Lange) Imbach (Morin et al. 2012) have been publicly released. These two saprotrophic basidiomycetes have been an important component of the human diet for over 200 years, and worldwide cultivation of these mushrooms forms a multibillion dollar industry. Mushroom cultivation takes place in mushroom farms and involves the large-scale biotechnological conversion of agricultural lignocellulosic wastes to high-value food with obvious extrapolations for bioenergy and biorefining.

A. bisporus is the favored model for adaptation, persistence, and growth in the humic-rich compost environment where nutrition is not readily available to primary degrading fungi (Morin et al. 2012). The comparison of its genome with those of its taxonomically related cousins *L. bicolor* and *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys, & Moncalvo highlighted genomic adaptations of these fungi to their specific ecological niche, i.e., humic-rich decaying leaf litter, herbivorous dung, and mycorrhizal root tips.

The 37-megabase genome of *C. cinerea* was sequenced and assembled into 13 chromosomes (Stajich et al. 2010). Although not edible, this species of *Coprinopsis* is a classic experimental model that provided important insights into the regulation of multicellular development in fungi, mushroom fruiting, and mating pheromones because it grows on defined media, completes its life cycle in 2 weeks, and can be manipulated at all stages in development by mutation and transformation. The wood decayer *Ganoderma lucidum* (Curtis) P. Karst. (Ling-Zhi in Chinese), one of the most famous traditional Chinese medicines, widely used as a tonic for longevity and overall health in China for thousands of years, has also been sequenced (Liu et al. 2012).

In addition to these fungi, genome sequences of 12 white-rot and brown-rot decomposers have recently been released (Floudas et al. 2012). These Agaricomycotina have been targeted for sequencing mostly for their role in lignocellulose decomposition and carbon cycle functioning, but species forming polypore- and resupinate-fruiting body have been chosen. The comparison of their gene repertoire and transcript profiles during fruiting body formation with

Table 21.1 Current sequencing status of the mycorrhizal species targeted for sequencing within the framework of the Mycorrhizal Genome Initiative (MGI)

Species	Genome	Transcriptome	Projects/ Projects/
Amanita muscaria (L.) Lam.	Complete	Complete	CSP 2011
Boletus edulis Bull.	Sequencing	Sequencing	CSP 2011
Cantharellus cibarius Fr.	DNA prep	RNA prep	CSP 2011
Cenococcum geophilum Fr.	Sequencing	Complete	FP 2010
Cortinarius glaucopus (Schaeff.) Fr.	Sequencing	Complete	CSP 2011
Gyrodon lividus (Bull.) Sacc.	DNA prep	RNA prep	CSP 2011
Hebeloma cylindrosporum Romagn.	Complete	Complete	FP 2010
Laccaria amethystina Cooke	Complete	Complete	CSP 2011
Laccaria bicolor (Maire) P. D. Orton	Published	Published	CSP 2005
Lactarius quietus (Fr.) Fr.	DNA prep	RNA prep	CSP 2011
Meliniomyces bicolor Hambl. & Sigler	Complete	Complete	CSP 2011
Meliniomyces variabilis Hambl. & Sigler	Complete	Complete	CSP 2011
Oidiodendron maius G. L. Barron	Complete	Complete	CSP 2011
Paxillus involutus P. D. Orton	Complete	Complete	CSP 2008
Paxillus rubicundulus P. D. Orton	Complete	Complete	CSP 2011
Piloderma croceum J. Erikss. & Hjortstam	Complete	Complete	CSP 2011
Pisolithus microcarpus (Cooke & Massee) G. Cunn.	Complete	Complete	CSP 2010
Pisolithus arhizus (Scop.) Rauschert (= *Pisolithus tinctorius*)	Complete	Complete	CSP 2010
Rhizopogon vinicolor A. H. Sm.	DNA prep	RNA prep	AFTOL
Rhizopogon vesiculosus A. H. Sm.	Complete	Complete	AFTOL
Rhizoscyphus ericae (D. J. Read) W. Y. Zhuang & Korf	Sequencing	Sequencing	CSP 2011
Russula sp.	DNA prep	RNA prep	CSP 2011
Scleroderma citrinum Pers.	Complete	Complete	CSP 2011
Sebacina vermifera Oberw.	Complete	Complete	CSP 2011
Suillus luteus (L.) Roussel	Complete	Complete	CSP 2011
Terfezia boudieri Chatin	Sequencing	RNA prep	CSP 2011
Thelephora terrestris Ehrh.	Sequencing	RNA prep	CSP 2011
Tomentella sublilacina (Ellis & Holw.) Wakef.	DNA prep	RNA prep	CSP 2011
Tricholoma matsutake (S. Ito & S. Imai) Singer	Complete	Complete	CSP 2011
Tulasnella calospora (Boud.) Juel	Complete	Complete	CSP 2011
Tuber melanosporum Vittad.	Published	Published	Genoscope 2005
Tuber magnatum Pico	Assembly	Complete	INRA
Tuber indicum Cooke & Massee	DNA prep	RNA prep	Genoscope 2010
Tuber aestivum Vittad.	Complete	Sequencing	Genoscope 2010

CSP JGI Community Sequencing Program, *FP* JGI Fungal Program, *AFTOL* Assembling the Fungal Tree of Life project

those of gilled mushroom- and truffle-forming EEMMs will likely provide new insights in the molecular mechanisms controlling the considerable breadth of morphological fruiting body diversity (e.g., earthstars, cannonball fungi, gilled mushrooms, resupinate fungi, and truffles).

21.3.2 *Genomic Features of* Laccaria bicolor *and* Tuber melanosporum

Comparative analysis of mycorrhizal genomes has (and will continue) to shed light on the genetic similarities between mutualistic symbionts and their saprotrophic cousins, to identify key genes in the regulation of symbiosis. Sequencing genomes of hundreds of saprotrophic and pathogenic fungi has provided an unprecedented opportunity to decipher key components that determine their various lifestyles (Arvas et al. 2007; Eastwood et al. 2011; Floudas et al. 2012). In contrast, the first ectomycorrhizal genome to be sequenced was that of *L. bicolor* (Martin et al. 2008). *L. bicolor* belongs to the Agaricales, a large order within the Basidiomycota that includes the Polyporales (e.g., *Phanerochaete chrysosporium* Burds.), Schizophyllacea (e.g., *Schizophyllum commune* Fr.), and the Psathyrellaceae [e.g., *Coprinopsis* cinerea (Schaeff.) Redhead, Vilgalys, & Moncalvo]. The genome of *L. bicolor* was found to be very large—60 Mbp with ~20,000 predicted protein-coding genes, of >85 % are expressed in free-living mycelium, ectomycorrhizal tips, or fruiting body (Martin et al. 2008). In comparison to other sequenced Basidiomycetes that cover 550 million years of evolutionary history (Floudas et al. 2012), *L. bicolor* has the largest complement of predicted clade-specific proteins to date suggesting that it was through rapid gene duplication that symbiotic genes were acquired (Martin and Selosse 2008).

However, the genome of *T. melanosporum* gave a very different picture as, despite a genome of 125 Mb (the largest fungal genome to date), it was fairly gene poor with only ~7,500 predicted protein-coding regions and only 30 pairs share >80 % amino acid identity in their coding sequence (Martin et al. 2010). Interestingly, as with *L. bicolor*, a large number of genes (~1,850) have no known homologues and thus are referred to as orphan genes. These contrasting genomes, therefore, show that the absolute size of the gene repertoires is not the prerequisite of a mycorrhizal symbiont, but rather it is likely in the high percentage of symbiosis-induced orphan genes that we may be able to define a "symbiotic toolbox"—the complement of genes used by EM fungi to broker symbiosis with plants. The sequencing of two model EM fungal species has therefore given a number of insights into the features of a symbiotic genome (Plett and Martin 2011).

21.3.3 *Nutritional Modes*

Ectomycorrhizal fungi have likely evolved from saprotrophic lineages at multiple times through convergent evolution as shown by multigene phylogenetic analyses. Switches in nutritional modes have been common throughout the evolution of Agaricomycetes. There is extensive convergence or parallelism of nutritional modes such as white rot, brown rot, or EM lifestyle. Brown rot and EM are known to have arisen independently in several clades, whereas white rot is believed

to be the ancestral condition in the Agaricomycetes. The different nutritional strategies potentially require markedly different genetic toolboxes, which imply gradual degradation of the genes not in use. It has been shown that brown rot evolves from white rot via contraction of decay-related gene families (Floudas et al. 2012). Examination of the available sequenced EM genomes, including the new *Hebeloma cylindrosporum* Romagn. and *Paxillus involutus* (Batsch) Fr. genomes, concurs with this hypothesis, in that their symbiotic lifestyle seems to be associated with a massive loss of lignocellulose-degrading genes compared to their saprophytic ancestors (Martin et al. 2008, 2010; Eastwood et al. 2011; Floudas et al. 2012; Wolfe et al. 2012), which makes EM fungi more dependent on the plant for photosynthates as a carbon source while preserving plant cell integrity. Loss of genes coding for lignocellulose-degrading enzymes is a common and striking genomic features of the two sequenced symbionts. It must be stressed, however, that such a conclusion is based upon the availability of only a handful EM genomes belonging to deeply divergent branches of the Mycota (Martin and Selosse 2008; Plett and Martin 2011). However, new research into closely related free-living and symbiotic species of *Amanita* confirms that the irreversible loss of lignocellulose-degrading enzymes marks the single origin of the ectomycorrhizal symbiosis lifestyle in this genus (Wolfe et al. 2012).

A restricted set of class II peroxidase genes and several multicopper oxidases are present in *L. bicolor, H. cylindrosporum*, and *P. involutus* genomes (Nagy et al. unpublished results) and in five different ectomycorrhizal genera, including the ecologically important *Cortinarius*, *Lactarius*, and *Russula* (Bödeker et al. 2009). These findings support the idea that EM fungi have a capacity to oxidize and degrade polyphenolic compounds, such as lignin and humic acids. In the forest humus, host photoassimilates drained by EM mycelium could be used to drive selective mobilization of humus nitrogen, mediated by ectomycorrhizal peroxidases (Bödeker et al. 2009).

Both *T. melanosporum* and *L. bicolor* share another genomic feature: their genomes have been massively invaded by transposable elements, which may play a role in gene shuffling, gene duplication, and gene neofunctionalization (Martin and Selosse 2008).

21.3.4 Single or Multiple Molecular Symbiotic Toolboxes?

One of the first, and most surprising, observations to be drawn from the comparison of these two EM genomes is that there are few similarities between genes expressed by *T. melanosporum* and *L. bicolor* to colonize the host roots and differentiate the symbiosis (Martin et al. 2010). Both species have ectomycorrhiza-specific gene expression, but in neither case are the genes expressed during symbiosis the same, except a few membrane sugar transporters and a GH5 glycosyl hydrolase (Martin et al. 2010). Thus, with the current genomic view of EM fungi that we have, a possible scenario would suggest that (1) irreversible losses of decomposition

pathways play a key role in the evolutionary stability of the ectomycorrhizal mutualisms (Wolfe et al. 2012) and (2) that each major EM fungal clade has subsequently and independently designed a symbiosis toolbox each time the ectomycorrhizal lifestyle has arisen in the tree of life—potentially through gene duplication and neofunctionalization. This hypothesis would predict that symbiosis-related gene networks ("symbiotic toolboxes") are tailor-made for each major fungal clade (e.g., Agaricales, Sebacinales, Pezizales) and may be tuned according to specific plant hosts. It remains to be seen, as more EM genomes are sequenced within the framework of the Mycorrhizal Genome Initiative (MGI), if EM symbiosis genomes are truly unique to each EM genus or if there are more similarities between EM fungi than those found to date. Several target species included in the MGI proposal are capable of forming different types of mycorrhiza, contrasting in their ability to form intracellular colonizing structures—and this plasticity depends on plant host. These additional genomes will provide additional unique opportunities to test the theory of "tailor-made symbiotic toolboxes."

Assuming that the symbiotic state arose via convergent evolution, a large number of lineage-specific gene families involved in symbiosis development are predicted. A few "master" proteins or enzymes responsible for the successful establishment of a symbiotic interaction with plants are also expected. Identification of these master genes and their role in the symbiosis will provide in-depth understanding of how these fungi colonize plant tissue and avoid plant defenses.

21.3.5 Mushroom Development

Fruiting body formation is a highly complex developmental process that has been studied in the model basidiomycetes *S. commune* and *C. cinerea* (Stajich et al. 2010; Ohm et al. 2011). After the substrate has been colonized and the application of specific environmental signals (e.g., reduced temperature), hyphae differentiate into the fruiting body where meiosis occurs. The mating type (MAT) loci are the master regulators of fruiting body development in fungi (Kues et al. 2011).

Transcript profiles expressed during the fruiting body formation have been compared in *A. bisporus*, *L. bicolor*, and *S. commune* to identify common developmental gene networks (Morin et al. 2012). Only 35 and 22 homologous genes were significantly upregulated ($p < 0.05$) in both *A. bisporus*/*L. bicolor* fruiting bodies and *A. bisporus*/*S. commune* fruiting bodies, respectively. Only thirteen genes were significantly upregulated ($p < 0.05$) in the fruiting bodies of these three species (e.g., aromatic-ring hydroxylase, glycoside hydrolase 16, FAD-linked oxidase, fatty acid desaturase), suggesting that genes induced during mushroom development are mainly clade specific.

Recently, a set of transcription factors that act downstream of the MAT loci were identified in *S. commune* (Ohm et al. 2010). These transcription factor genes have orthologs in *A. bisporus* and *L. bicolor*. Transcript profiling indicates that several of these transcription factor genes may be involved in the regulation of mushroom

formation in these three basidiomycetes. Expression profiles of the orthologs of *c2h2*, *fst3*, *fst4*, and *hom1* were similar in *S. commune*, *A. bisporus*, and *L. bicolor* (i.e., upregulation of gene expression in mushrooms compared to mycelium) suggesting that these agarics share similar master developmental switches for fruiting body formation. These genes are primary targets for a functional analysis. Studying the regulatory mechanisms underlying fructification in mushroom-forming agarics would allow the control of mushroom pin formation, considered by mushroom growers as the most important step in managing the mushroom crop.

Tuber melanosporum is the first fungus that produces hypogeous fruiting bodies (truffles) to have its genome sequenced (Martin et al. 2010). The reputation of this black truffle species as a gastronomic delicacy is long-standing (~2,000 years old), and its genome is characterized by extremely low allergenic potential, a lack of key mycotoxin biosynthetic enzymes, and the preferential overexpression of various aroma- and flavor-related enzymes in the fruiting body. Among the latter are specific subsets of sulfur assimilation and S-amino acid interconversion enzymes. These include cystathionine lyases, which are known to promote the side formation of methyl sulfide volatiles (abundant in truffles) as well as various enzymes involved in amino acid degradation through the Ehrlich pathway that give rise to known truffle volatiles and flavors (e.g., 2-methyl-1-butanal).

The analysis of genes implicated in the mating process, including pheromone response, meiosis, and fruiting body development, showed that most sex-related components identified in other ascomycetes are also present in *T. melanosporum* (Martin et al. 2010). Sexual reproduction in ascomycete filamentous fungi is partly controlled by two different MAT genes that establish sexual compatibility: one *MAT* gene codes for a protein with an alpha-box domain, whereas the other encodes a high-mobility group (HMG) DNA-binding protein. The sequenced Mel28 strain contains the *HMG* locus, and the opposite linked *MATa* locus was identified in another natural isolate (Rubini et al. 2011a), confirming that *T. melanosporum* is heterothallic and thus an obligate outcrossing species (see Chap. 4). This result has major implications for truffle cultivation, which will be improved by the use of host plants harboring truffle strains of opposite mating types (Rubini et al. 2011b).

21.3.6 Large-Scale Genome Sequencing and Resequencing Endeavors

The above-mentioned genome sequencing projects have been accompanied by the development, organization, and management of consortia to carry out these large-scale scientific endeavors; the establishment of dedicated databases and tools to navigate these genomes, such as the JGI Portal MycoCosm (http://genome.jgi.doe.gov/programs/fungi/index.jsf); and the development of many novel approaches and tools for data analysis. These factors have jointly established a maturing model for analyzing genomes and for making resulting data broadly available. They have also

had a profound impact on the entire biological community by promoting collaboration and establishing goals for quantifying, standardizing, and sharing all types of biological data. We are now ready for sequencing the Fungal Tree of Life within the framework of the 1000 Fungal Genome Project (F1000) (http://1000.fungalgenomes.org/home/, see below).

Interwoven advances in comparative genomics, high-throughput transcriptomics, and bioinformatics are providing mycologists with a markedly improved repertoire of research tools that will allow the functioning of EEMMs to be analyzed and comprehended at an unprecedented level of molecular detail. Our ability to explore genome function is increasing in specificity as each subsequent EEMM genome is sequenced. Microarray technologies and Illumina RNA-Seq have allowed studying the expression of tens of thousands of genes in a few days.

Sequencing technologies have also made a strong contribution to understanding genetic variation in EEMMs. The first two draft EEMM genome sequences were completed in 2008 and 2012 (Martin et al. 2008, 2010), and the first *T. melanosporum* and *L. bicolor* genomes were resequenced in 2011 at a fraction of the cost using next-generation sequencing (NGS) technology (Murat and Martin, unpublished results). Six new complete *T. melanosporum* genomes—originating from Spain, Italy, and France—have been resequenced (Payen et al. 2012). In comparing the genome of the seven *T. melanosporum* isolates (the reference genome and the six resequenced isolates), more than 500,000 single nucleotide polymorphisms (SNPs) were detected. The density of SNPs was ~4,000 SNPs per Mbp. The accumulation of SNPs in genes coding for the enzymes of the sulfur metabolic pathways leading to VOCs suggests that the observed genetic variation may underpin differences in truffle aromatic properties.

Sixteen genomes of *L. bicolor* strains of different geographic origins (Australia, North America, Europe) have also been resequenced and that number is expected to rise exponentially in the next several years as costs fall. These studies have identified millions of SNPs and indicate that individual genomes may differ by kilobases of sequence because of structural variation, including insertions, deletions, and inversions (Martin and Grigoriev, unpublished results). SNP discovery arising from the genome sequencing is particularly relevant to EEMMs. Large panels of SNP markers will enable linkage maps, more precise mapping of quantitative trait loci (QTL), and eventually the implementation of genomic selection in EEMM breeding programs.

Most amazing to us is the emerging role of transposable elements in evolutionary change. This became apparent through the comparative resequencing of the *L. bicolor* and *T. melanosporum* genomes, which revealed the dynamic nature of these genomes as well as the role of transposable elements in this process. The abundant transposable elements accumulated in mycorrhizal fungal genomes and other biotroph genomes [e.g., *Blumeria graminis* (DC.) Speer (Spanu et al. 2010), *M. larici-populina*, and *Puccinia graminis* Pers. (Duplessis et al. 2011)] are predicted to have an important role in the evolution of gene regulatory networks.

21.4 Where Are We Heading?

Genomic analyses of mycorrhizal symbionts have so far been limited to two model fungal species, *L. bicolor* and *T. melanosporum*. We are currently experiencing a massive shift toward the inclusion of environmental and evolutionary perspectives in genome sequencing initiatives. The successful *L. bicolor* and *T. melanosporum* projects inspired new larger-scale genome projects to sequence a diversity of symbiotic fungi and forest soil fungal metagenomes (Martin et al. 2011a). These projects will produce catalogs of reference genomes of fungi that can be found in forests as well as genomic markers of fungi that are found in forest soils.

21.4.1 Mycorrhizal Genomics Initiative

Of interest to those working on EM fungi (and EEMMs), a massive, multi-partner, international JGI and INRA effort attempting to understand the evolution and functioning of plant-fungus symbioses through genome sequencing has been launched in 2011. This project, referred as to the Mycorrhizal Genomics Initiative (MGI), targets a set of 30 fungal species (Table 21.1) that are able to form various types of mycorrhizal symbioses (e.g., EM, ericoid). Major aims of the MGI are to identify the genetic mechanisms that underpin the establishment of mycorrhizal symbioses, to determine whether certain genes are selectively associated with particular symbiotic patterns, and to decipher the evolution of ecologically and economically important symbioses in terrestrial ecosystems. As several of the species selected for this project produce edible mushrooms (*Boletus edulis* Bull., *Cantharellus cibarius* Fr., *Suillus luteus* (L.) Roussel, *Terfezia boudieri* Chatin, *Tricholoma matsutake* (S. Ito & S. Imai) Singer), this initiative will undoubtedly impact the research on edible fruiting body development.

The mycorrhizal fungal species sequenced within the framework of the MGI have been selected for (1) their phylogenetic novelty, (2) their ability to establish different types of mycorrhizal symbiosis (ericoid, ectendomycorrhizas, EM), and (3) their taxonomic relationships with already sequenced EM genomes to explore the intraclade variability in symbiosis gene repertoire. The sequenced species are dominant members in their ecological settings (e.g., *Cenococcum geophilum* Fr., *Meliniomyces* spp.). Several of them are currently used in the commercial forestry industry to inoculate conifer (e.g., *L. bicolor*) or hardwood seedlings (*Pisolithus* spp.) for lumber, bioenergy, and landscape trees. As of this writing, genomes have been sequenced for most Tier 1 and several Tier 2 MGI species [*Amanita muscaria* (L.) Lam., *C. geophilum, H. cylindrosporum, Laccaria amethystina* Cooke, *Meliniomyces bicolor* Hambl. & Sigler, *Oidiodendron maius* G. L. Barron, *Paxillus rubicundulus* P. D. Orton, *Piloderma croceum* J. Erikss. & Hjortstam, *Pisolithus microcarpus* (Cooke & Massee) G. Cunn., *Pisolithus arhizus* (Scop.) Rauschert (= *Pisolithus tinctorius*), *Scleroderma citrinum* Pers., *Sebacina vermifera* Oberw.,

S. luteus, T. matsutake, Tulasnella calospora (Boud.) Juel—see Table 21.1]. This genome sequence data will be released by the end of 2012 (see also the MGI Web portal: http://mycor.nancy.inra.fr/IMGC/MycoGenomes/index.html).

The MGI genomes are sequenced using the Illumina platform (shotgun and paired end fragment sequencing). All general aspects of library construction and sequencing can be found at the JGI Web site (http://www.jgi.doe.gov/). Each fastq file is filtered for artifact/process contamination and subsequently assembled with Velvet (Zerbino and Birney 2008). The resulting assembly is used to create a long mate-pair library with 3,000-bp insert, which is then assembled together with the fragment library using ALLPATHS-LG (Gnerre et al. 2011). This approach results in $>100\times$ coverage assemblies but with hundreds of scaffolds.

The MGI is promoting a series of achievements that would lead to substantial advances in mycorrhizal (and EEEMs) research and its applications to the sustainable mushroom production and related industry.

21.4.2 *Comparative Genomics of Truffle Species and the Evolution Ectomycorrhizal Symbiotic Genomes*

Within the project TUBEREVOL—"*Comparative Genomics of Truffle Species and the Evolution of Ectomycorrhizal Symbiotic Genomes*"—the genomes of four truffle species (*T. melanosporum, Tuber indicum* Cooke & Massee, *Tuber aestivum* Vittad., and *Tuber magnatum* Pico) are being sequenced to provide an opportunity to study genome evolution in the Pezizomycotina, a basal clade of this Kingdom Fungi. Analyses will focus on the genomic bases of their differing fruiting body, mycorrhiza physiologies, and host specificity while investigating their common biology. These studies are expected to yield new insights into eukaryotic genome evolution, the evolution of symbiosis, the evolution of sex and reproductive structure, the role of conserved sequence elements in gene regulation, and the role of transposons in shaping these genomes.

The comparison of the genomes and transcriptomes of *Tuber's* EM plant symbionts and between saprotrophic, pathogenic, and symbiotic fungi will provide insights into symbiosis mechanisms and differences in evolutionary processes developed by Ascomycota. This project will provide unprecedented insights into the molecular bases of symbiosis, sex and fruiting, and adaptation. A deeper understanding of the genome of one of the worldwide recognized icons of the European gastronomy and culture will have an exceptional social and cultural impact. An understanding of the genome polymorphism and evolution within the *Tuber* phylum will also contribute to the development of forensics to control serious frauds caused by less valuable or illicit truffle species. The genome sequences of white and black truffles produced through TUBEREVOL will enhance (1) the *T. melanosporum* genome annotation, (2) our understanding of the mechanisms responsible for the organization and composition of the

Pezizomycotina and *Tuber* genomes, (3) our understanding of the evolutionary processes controlling divergence of the *T. melanosporum* genome from that of other *Tuber* species (e.g., role of transposons), and (4) investigations on the genetic and developmental basis of species differences. These genomes will also lay the groundwork for whole genome approaches to the study of molecular and phenotypic population variation within the *Tuber* model system. As of this writing, genomes have been sequenced for *T. magnatum* and *T. aestivum*. As observed in *T. melanosporum*, the genomes of these other *Tuber* species are characterized by a proliferation of transposable elements that account for >60 % of their genome, which leads to very large genome sizes, i.e., >125 Mbp.

A crucial step in making genome resources useful to the scientific community is through generating gene annotations. We will perform gene finding for MGI genomes using the JGI (Grigoriev et al. 2012) or Genoscope annotation pipelines and will combine the output within the MycoCosm and MycorWeb databases. One major focus will be the large-scale structure of fungal genomes, focusing on the degree of syntenic conservation at different scales within these genomes. We will also carry out a number of traditional analyses of genome content using the MGI genomes, focusing on repeated sequences, clade-specific expanding gene families, secretome, membrane transporters, transcriptional factors, signaling pathways, and secondary metabolism. These analyses include the evolution of repeat families and patterns of TE proliferation. We will compare the repeat family content within mycorrhizal genomes and with other soil saprotrophs. Additionally, we will conduct analyses of gene family evolution within major clades of EM to identify specific genes and other functional elements, including the identification of ultraconserved regions, with a special focus on those sequences that could have been gained or lost both within a specific lineage and in comparison to the other relevant lineages that are now available for investigation.

Several fungal species selected in the present sequencing projects (e.g., *C. geophilum, S. vermifera, Meliniomyces* spp.) are among the most abundant taxa found in large-scale surveys of soil ribosomal DNA (Buée et al. 2009). The sequenced MGI genomes will therefore be used as in silico anchors for sorting through thousands of metagenomic repertoires and categorizing them by sequence alignments. This will likely lead to a better understanding of the spatiotemporal dynamics of functional diversity in EM communities.

21.4.3 1000 Fungal Genomes (F1000)

Even more ambitious, the overarching goal of the F1000 project is to inform all areas of fungal biology by providing broad, genomic coverage of Kingdom Fungi (Mycota). The sampling design is based on a phylogenetic framework developed by the Assembling the Fungal Tree of Life (AFTOL) project (Hibbett and Matheny 2009) and is focused on covering all major subordinal groups (clades) of Fungi. Currently, there are approximately 140 orders of Fungi and over 550 families.

The F1000 goal over the next 5 years is to facilitate the sampling of fungal genomes so that at least two representatives are sequenced from every family or family-level clade of Fungi. As in similar gigantic efforts, the F1000 project members recognized that data coordination is critical to move forward productively and to ensure that resulting data are available to the community in a reasonable time frame. We indeed acknowledge the critical importance of early, unfettered access to genomic data in achieving maximum community benefit.

21.4.4 Elucidating the Structure and Function of Genomes

The available genome sequences of EM fungi will represent foundational information for understanding the symbiosis and mushroom development. Embedded within this as yet poorly understood genome structure of EEMMs are the genetic instructions for the entire repertoire of cellular components, knowledge of which is needed to unravel the complexities of fruiting body formation. Elucidating the structure of genomes and identifying the function of the encoded elements will allow connections to be made between genomics and complex developmental processes, such as sex and fruit body formation. For this, new conceptual and technological approaches will be needed to develop a comprehensive catalog of all of the components encoded in a few of selected "model" species genome, e.g., *T. melanosporum*, and to determine how the genome-encoded components (protein-coding genes, regulatory *cis*- and *trans*- factors) function in an integrated manner to perform cellular and organismal functions.

Comparison of genome sequences from evolutionarily diverse species has emerged as a powerful tool for identifying functionally important genomic elements in fungi (Floudas et al. 2012). Further comparisons of sequences derived from multiple species, especially those occupying distinct evolutionary positions (e.g., *T. melanosporum* and *T. magnatum*), should lead to significant refinements in our understanding of the functional importance of conserved sequences or clade-specific gene families. Thus, the generation of additional genome sequences from several well-chosen species within the framework of the JGI 1000 Fungal Genomes project is crucial to the functional characterization of EEMM genomes.

Effective identification and analysis of functional genomic elements that control mushroom development will require increasingly powerful computational capabilities, including new approaches for tackling ever-growing and increasingly complex data sets and a suitably robust computational infrastructure for housing, accessing, and analyzing those data sets. Defining the complex, intermingled metabolic and developmental pathways that, taken together, give rise to the workings of fruiting body production and determining their properties and interactions are crucial to understanding this complex biological system. There will be a greatly increased need for the collection, storage, and display of the data in robust and user-friendly databases, such as the JGI MycoCosm and INRA MycorWeb warehouses (Martin et al. 2011a, Grigoriev et al. 2012).

Complementing the computational detection of functional elements will be the generation of additional experimental transcriptomic data by high-throughput RNA-Seq methods. This will allow the identification of genetic networks and protein pathways, the "hubs" connecting these networks, and the master switches turning them on or off. It will be important to develop and refine genetic engineering techniques that modulate gene expression, such as gene knockout and knockdown methods to establish the temporal and cellular expression pattern of individual proteins and to determine the functions of those proteins (Grimaldi et al. 2005; Kemppainen et al. 2005). This is a key first step toward assigning all genes and their products to functional pathways.

21.4.5 Understanding of Genetic Variation in Model Mushroom Organisms

This approach would facilitate studies to establish relationships between genotype and biological function. The study of particular variants and how they affect the functioning of specific proteins and protein pathways will yield important new insights about physiological mechanisms leading to environmental adaptations. The study of interspecies sequence comparisons is important for identifying functional elements in the genome. Determining the sequence differences between species, such as *T. melanosporum*, *T. aestivum*, and *T. magnatum*, will provide insight into the distinct anatomical, physiological (e.g., synthesis of volatile organic compounds), and developmental features of different organisms and will help to define the genetic basis for speciation.

Such strategies should enable the EEMM research community to (1) achieve the identification of genes and pathways with a role in fruiting body formation and determine how they interact with environmental factors for improved yields and (2) develop, evaluate, and apply genome-based diagnostic methods for the spatiotemporal tracing of introduced EEMM inoculum in soil fungal communities (molecular taxonomy); the identification of key biological markers, such as sex compatibility genes that are affecting mushroom production; the prediction of response to environmental factors in natural settings (truffle grounds) and/or mushroom farms; and the prediction of susceptibility to disease.

21.4.6 Developing Links with the Bio-Industry

The past decade has witnessed the impact of genomes on mycorrhizal research. We also have more confidence than we had 10 years ago in the premise that genomes will bring new applications and be useful to the bio-industry. In the coming years, we believe genomics of EEMMs will still primarily concentrate on deciphering and

understanding the molecular mechanisms driving mycorrhiza and mushroom development, but using genomes in molecular ecology and environmental genomics will become especially important. Only a few applications are currently being developed by virtue of EEMM genomics; it is therefore essential that work focuses on innovation in experimental technologies. The patenting of a robust molecular diagnostic kit for the identification of truffle sexual compatibility *MAT* genes that can be used in truffle grounds (Martin et al. 2011b) is a good example of the ability of genomics to rapidly translate in advanced tools for truffle growers and industry. Without commercialization, most diagnostic and biotechnology advances will not reach the truffle grounds, mushroom farm settings, and bio-industry, where they can benefit producers. Thus, we need to develop policy options for data access and for patenting, licensing, and other intellectual property issues to facilitate the dissemination of genomics data.

21.5 Outlook

The genomes of two model EM fungi (*L. bicolor* and *T. melanosporum*) (Martin et al. 2008, 2010) have given us new insights into the genes that are involved in scavenging for nutrients, avoidance of plant defenses and control of plant immunity, remodeling of the root architecture, sex mechanisms, formation of the fruiting body, and much more (Martin and Selosse 2008; Plett and Martin 2011). These genomes have also shown how, through reduction in the sizes of different gene families (e.g., cellulases), EM fungi have become more reliant on their plant hosts. Transcriptomic profiling has highlighted a number of different genes that may be involved in the establishment and maintenance of the symbiosis and fruiting body formation. Induced expression of genes coding for membrane transporters and SSPs during the symbiotic interaction and the lack of expression of hydrolytic enzymes acting on PCW polysaccharides are hallmarks of the ectomycorrhizal *L. bicolor and T. melanosporum* transcriptome. These findings extend conserved evolutionary patterns of gene expression observed in obligate biotrophic pathogens (Spanu et al. 2010; Kemen et al. 2011) and the obligate AMF symbiont *G. intraradices* (Tisserant et al. 2011).

The availability of >30 new mycorrhizal genomes and terabases of expressed sequenced tag (EST) collections of phylogenetically diverse mycorrhizal symbionts will represent the core foundation for deciphering genome functionality in fungal symbionts of major tree species of ecological and economical relevance in the coming decade. Comprehensive repertoires of symbiosis-related genes will provide a basis for future research in environmental genomics and for accessing symbiosis-related functional features throughout the Fungal Tree of Life.

The comparative genomics and transcriptomics of mycorrhizal genomes and transcriptomes would be a great advantage because of the in-depth phylogenetic positions, their ecological interactions, and their economic and social importance.

Studying the symbiotic genomes and transcriptomes will provide us with new glimpses including but not limited to:

- A better understanding of the tree mesocosm (i.e., interactions of the host plant with its cortege of endophytic, symbiotic, and pathogenic microbes)
- A basis for studying the protein-coded cross talk between symbiotic partners involving mycorrhizal effectors
- A molecular definition of mechanisms leading to mushroom initiation and development
- The metabolic pathways controlling the nutrient transport and assimilation in the symbiosis and fruiting body
- Bioinformatic exploration of important symbiotic gene networks and master transcriptional factors—the mycorrhizal genetic landscape(s)
- Comparative transcriptomics with the other economically important saprobic and pathogenic fungi

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