Cultivation of edible ectomycorrhizal mushrooms

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The edible mycorrhizal mushrooms include some of the world's most expensive foods and have a global market measured in US$ billions. Despite this, few have been cultivated with any degree of success, and certainly not in volumes that are likely to reverse the catastrophic declines in production that have occurred over the past 100 years. The main obstacles to their cultivation are their need to be associated with a host plant to successfully grow and fruit, contamination with other ectomycorrhizal fungi both before and after the establishment of plantations, and a general lack of understanding of each mushroom's trophic relationships, and biotic, edaphic and climatic requirements.

Mycorrhizas are ancient symbiotic associations between specialised fungi and the fine roots of the majority of species of higher plants [1–4] (see http://www.berkeley.edu/news/media/releases/2000/09/14_funghi.html). By extending the absorptive area of the root system, mycorrhizas have their principal beneficial effect – increasing plant uptake of nutrients, in particular phosphorus [5,6]. In exchange, the host plant provides the generally obligate mycorrhizal fungus with carbohydrates and a place to live. The complexities of the mycorrhizal association have proved fertile ground for researchers, with a search of the CAB International database (http://www.cabi-publishing.org/Products/Database/Abstracts/Index.asp) finding >20 000 scientific papers in the past 15 years alone. However, mycorrhizas, and in particular ectomycorrhizas, are also of considerable interest to the chef and the gourmet because some of the basidiomycetes and ascomycetes that form them produce edible mushrooms, some of which are among the world's most expensive foods (Table 1) [7].

Falling harvests

There has been a catastrophic decline in harvests of some edible mycorrhizal mushrooms over the past century. For example, European *Tuber melanosporum* (Périgord black truffle) harvests have fallen from around 2000 tonnes at the beginning of the 20th century to rarely >150 tonnes now [8,9]. The official figures for *T. melanosporum* production in France illustrate this trend well (Fig. 1) [10,11] (M. Courvoisier, personal communication). Harvests of the Japanese delicacy matsutake [12,13] and other important species of edible mushrooms that grow in the Northern Hemisphere [14] have also fallen, necessitating the introduction of rules and regulations aimed at preventing over-harvesting [13,15,16]. Reasons advanced to account for these declines include deforestation, the loss of host plants within forests because of pests or disease, changed forest management practices such as planting more densely than occur in natural forests, the replacement of natural forests with plantations of species that are poor hosts, global warming since the last ice age, soil compaction by hordes of pickers, acid rain and the loss of expertise during two World Wars as to where and how to harvest mushrooms, particularly truffles [7–9,12–16].

Although the decline in production has triggered research into devising methods for the cultivation of mycorrhizal mushrooms, the associated scientific literature remains modest compared with other areas of mycorrhizal research. This is partly because edible mycorrhizal mushrooms take many years to fruit and other areas of mycorrhizal research provide a quicker route to a publication record. Another significant factor is that some key scientists working towards the cultivation of these potentially lucrative crops are tied by confidentiality agreements and are not permitted to publish or patent

Table 1. Approximate market information on the most important of the edible mycorrhizal mushrooms

Botanical name	Common name	Approximate in-season retail market US$	Estimated world production (t)	Approximate wholesale prices US$/kg (first grade)	Refs
Boletus edulis	Porcini	>250 million	20 000–100 000	13–198	[37]
Cantharellus cibarius	Chanterelle	1.62 billion	200 000	8–19	[75]
Tricholoma matsutake	Matsutake	500 million	2000	40–500	[12]
Tuber melanosporum	Périgord black truffle	>150 million	150	250–1200	[9]
Tuber magnatum	Italian white truffle	>150 million	50–200	1000–13 000	[38]

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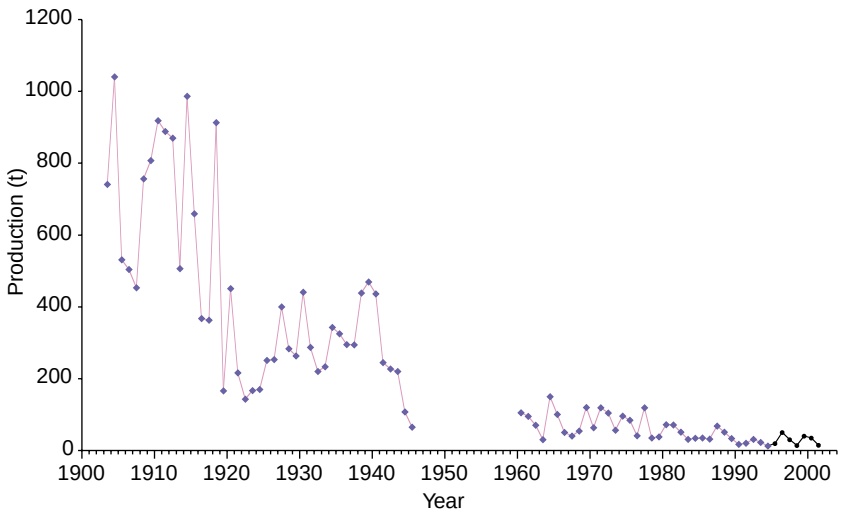

Fig. 1. *Tuber melanosporum* and *Tuber brumale* truffle production in France 1903–2001 (from Refs [10,11] and M. Courvoisier, personal communication).

their research findings for fear that vital intellectual property will be lost.

Cultivation of *Tuber* spp

Joseph Talon, who lived in France during the first part of the 18th Century, devised the first method for cultivating an edible mycorrhizal mushroom [7]. He discovered that if self-sown seedlings growing under Périgord black truffle-producing oaks were used to establish truffle plantations (truffières), 5 to 20 years later these plantations would also produce truffles. During Talon's lifetime this 'dirty technique' became the mainstay of the truffle industry and continued to be used until the 1970s. Similar techniques were also used to cultivate *Tuber uncinatum* (Burgundy truffle) [17] and the Japanese delicacy honshimeji (*Lyophyllum shimeji*) [18]. However, Talon's technique produces plants contaminated by other soil flora and fauna, including other potentially competing ectomycorrhizal fungi, such as *Scleroderma*, and possibly pathogens, nematodes and insect pests. Consequently, many of the resulting trees never produced truffles and the technique was considered unreliable (see G. Chevalier, http://www.mykopat.slu.se/mycorrhiza/edible/proceed/chevalier.html) [7]. However, beginning in the late 1960s and early 1970s more reliable methods were devised to artificially infect plants with *Tuber* spp. in greenhouses either with spores [19–22] or segments of infected root [22] (and I.R. Hall, unpublished data based on [21]).

Although dozens of nurseries around the world now produce seedlings inoculated with *T. melanosporum* and *T. uncinatum* [7–9,17,23], many details that ensure success, such as the amount, quality and treatment of inocula as well as watering, fertilisation, temperature, light levels, potting medium formulation and pH, remain trade secrets [24,25]. Another crucial factor is the control of contaminating saprobic and ectomycorrhizal fungi, such as *Thelephora* spp., *Sphaerosporella brunnea* and *Pulvinula constellatio*, that compete with the truffle mycelium for space on the host's roots in the greenhouse and later in the field. Consequently, poor seedling quality remains a serious problem in *Tuber*-inoculated plants (see http://www.mykopat.slu.se/mycorrhiza/edible/proceed/bencivenga.html) [26]. As Hall and Wang point out, there is an important difference between inoculating a plant (i.e. applying the inoculum to the roots) and producing a plant well-infected solely with the correct ectomycorrhizal fungus [24]. Considerable effort has been devoted to devising quality control standards (see http://www.mykopat.slu.se/mycorrhiza/edible/proceed/chevalier.html) [27,28], but their implementation has been limited. Despite these problems, more than half of all *T. melanosporum* truffles are now harvested from artificial plantations.

Cultivation of other species

Plants infected following inoculation with sporal suspensions [24,29] or pure cultures prepared either from fruiting bodies [30] or mycorrhizal root tips [22] have led to the formation of edible mycorrhizal mushroom fruiting bodies in the field, including *Lactarius deliciosus* [31,32], *Lyophyllum shimeji* ('Kyoto scientists grow hon-shimeji mushrooms', http://www.kippo.or.jp/KansaiWindowhtml/News/1996-e/19961119_NEWS.HTML), *Rhizopogon rubescens* [31], *Suillus granulatus* [32], *Terfezia* (A. Gutierrez, unpublished) [33] and various species of *Tuber*, including *T. borchii* (bianchetto) [34], *T. melanosporum* [7,8] and *T. uncinatum* [17]. Danell and Camacho also produced *Cantharellus cibarius* in pots in the greenhouse [35]. But despite these successes, fewer than a dozen of the many hundreds of edible mycorrhizal mushrooms have ever been cultivated with any degree of success [36], and this includes important commercial species such as *Boletus edulis* [37], *Tricholoma matsutake* [13] and the Italian white truffle (*Tuber magnatum*) [26,38]. Consequently, supplies of most commercially important edible mycorrhizal mushrooms are still restricted to those that can be harvested from the wild during autumn or winter. For these we are left to speculate on the reasons why we have not

been able to establish viable infections on a suitable host plant, why what appear to be adequate infections subsequently fail or are displaced by other ectomycorrhizal fungi, or why fruiting bodies simply fail to form.

Symbionts, saprobes and pathogens

One possible reason why we have been unsuccessful in cultivating some 'mycorrhizal' species is because we simply assume that they are mycorrhizal when their relationship with their hosts is more complicated. Mycologists classify fungi as symbionts (e.g. lichens and mycorrhizas), saprobes (those that live in dead animal or plant remains), and pathogens (those that gain their nutrition from living plants or animals). However, on closer inspection some fungi do not fit neatly into these man-made categories and instead a continuous gradation of behaviour can be seen [39,40], with many fungi occupying zones within the triangle defined by the terms 'symbiont', 'saprobe' and 'pathogen' (Fig. 2) (Y. Wang, unpublished) [12]. For example, many pathogens such as *Armillaria mellea*, a close relative of *T. matsutake*, can live on in the host tissues long after the host has died. By contrast, the 'mycorrhizas' of non-photosynthetic plants hardly seem to fit the classical functional definition of mycorrhiza, as the flow of nutrients seems to be in the direction of the host [6,41,42].

Many mycorrhizal fungi exhibit marked saprobic abilities [43], and the 'mycorrhizal' fungus *Tricholoma matsutake*, although initially appearing to be mycorrhizal on young roots, soon becomes pathogenic, sometimes completely destroying the root cortex, before finally exhibiting some saprobic ability by continuing to live in the host tissues perhaps even after its host tree has been milled for timber [12,13]. Even 'typical' mycorrhizal fungi such as *Tuber melanosporum* and *T. uncinatum* will kill all the vegetation around the host tree, producing the 'burnt' area known as the brûlé [7,44], whereas *Lactarius deliciosus* can invade host cortical cells (Y. Wang, unpublished). Some mycorrhizal fungi can also maintain infections and fruit in soils containing high concentrations of available phosphorus [17,45,46], which would markedly depress the growth of other mycorrhizal fungi [4].

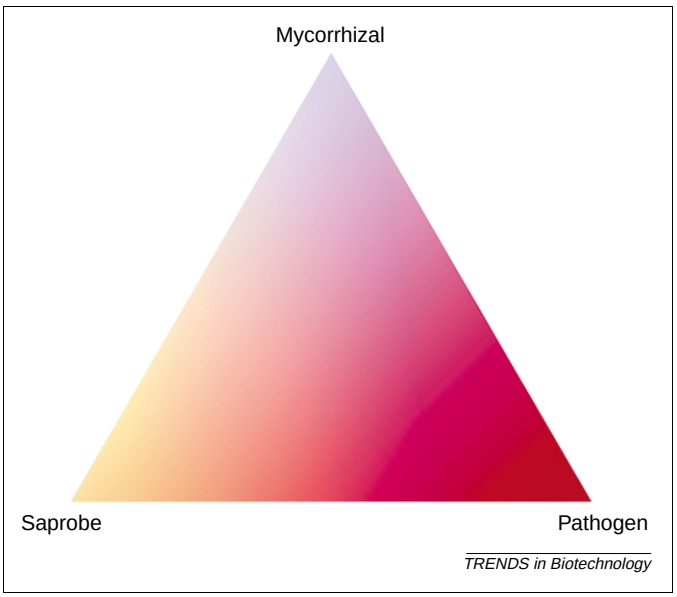

Fig. 2. Although many fungi fit neatly into the terms 'saprobe', 'pathogen' or 'mycorrhizal', others, including some 'mycorrhizal' fungi, do not occupy precise zones within the triangle defined by these terms. In some cases these zones might shift depending on the phase in the life cycle of a fungus.

Complex biotic interactions

Bacteria, particularly *Pseudomonas*, can be intimately associated with ectomycorrhizas and appear to aid the infection process. As a consequence, they have been referred to as 'mycorrhiza helper bacteria' [47–49]. Bacteria, again typically *Pseudomonas*, are also routinely found in the fruiting bodies of *Cantharellus cibarius*, although they do not seem to be mycorrhiza helper bacteria in this situation [50]. Bacteria, in particular *Pseudomonas* spp., have also been isolated from *Tuber* fruiting bodies [51,52], and are involved in the suppression of competing ectomycorrhizal fungi [25,53]. Most interesting is the antimycotic activity of *Pseudomonas* spp. against fungal contaminants from ascocarps of *Tuber borchii* [53]. In this work some of the bacteria tested were observed to release metabolites that affected the growth of *T. borchii* mycelia and morphogenesis in culture [54], suggesting that they might have a role in ascocarp development and in the formation of mycorrhizas. *T. borchii mycelia* also contain a *Cytophaga-Flexibacter-Bacteroides* phylogroup bacterium, although its role has yet to be determined [55].

Several ectomycorrhizal fungi are also regularly found associated with saprobic [56] or other ectomycorrhizal fungi, for example *Suillus bovinus* and *Rhizopogon* with *Gomphidius roseus*; *Suillus* spp. with *Chroogomphus* spp. [57–59]; and *Boletus parasiticus* with *Scleroderma citrinum* [60]. Similarly, *Boletus edulis* is regularly found in the same locations and at the same time of year as *Amanita muscaria* or *Amanita excelsa* in Austria, England, Italy, New Zealand, Sweden and USA. Following closer investigation, Wang and Stringer (A. Stringer, unpublished) found that *A. muscaria* or *A. excelsa* and *B. edulis* hyphae and rhizomorphs were often closely

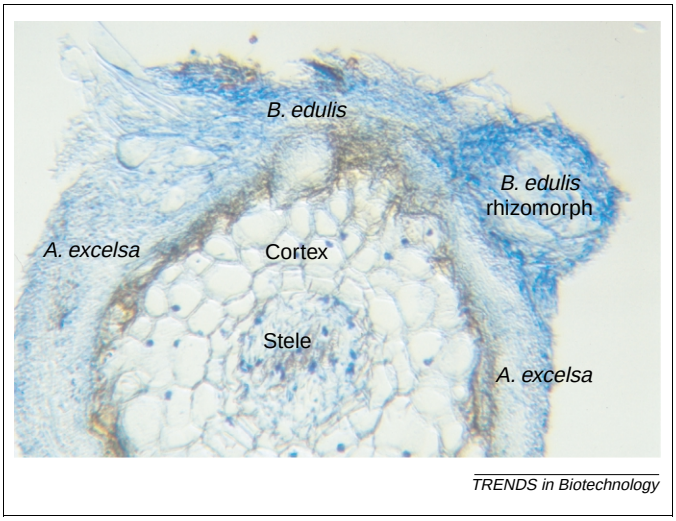

Fig. 3. The sometimes intimate association between species of mycorrhizal fungi is illustrated by this transverse section of a *Pinus radiata* mycorrhiza in New Zealand formed by *Amanita excelsa* and *Boletus edulis*.

interwoven and formed composite mycorrhizas (Fig. 3) similar to those described by Agerer for *Suillus bovinus* and *Rhizopogon* with *Gomphidius roseus* [58]. Some useful tools that could be used to investigate this further would be *in situ* DNA hybridisation [61,62] or immunological techniques [63].

Clearly the relationships between some edible ectomycorrhizal mushrooms and other ectomycorrhizal fungi and bacteria are anything but casual. The trophic relationships between some are likely to be more complex than the simple triangular model (Y. Wang, unpublished) Wang envisaged. If these complex tripartite relationships are essential then it will be necessary to ensure that all the components are present to ensure stable infections of some edible ectomycorrhizal mushrooms on their host plant. This might, for example, account for the collapse of *Boletus edulis* ectomycorrhizas when well-infected plants are transferred into open pots in the greenhouse (Y. Wang, unpublished). One way such potential problems could be overcome would be to use 'dirty' techniques similar to those developed by Talon for *Tuber melanosporum*, as they offer the opportunity of establishing the full necessary biota required for an edible mycorrhizal fungus to infect and fruit perhaps long before we understand the intricate biotic interactions involved.

Correct ecological conditions – the challenge

In a newly planted *T. melanosporum* truffière, where there is little or no canopy cover, the fungus survives rapid diurnal swings in temperature and fluctuating soil moisture [46]. It is also capable of fruiting under similar conditions or, like *T. uncinatum* [17] and *L. deliciosus* [31], where there is almost a complete canopy [46]. By contrast, *Tricholoma matsutake* does not appear in *Pinus densiflora* stands until the trees are 20-years-old and there is 75% canopy cover. This fungus then disappears once its pioneer host plant begins to succumb to competition from trees later in the succession [12]. These and other edible mycorrhizal mushrooms have a distinct set of edaphic and climatic conditions that they will either tolerate or require to both survive in the soil and ultimately fruit. However, apart from *T. melanosporum* and *T. uncinatum* [7,8,17], our understanding of the ecological conditions favoured by edible ectomycorrhizal mushroom is restricted to a handful of fungi and a paucity of publications (A. Gutierrez, unpublished) [12,17,33,37,38,50]. Even so, the published information is rarely complete and for *T. melanosporum* gaps in knowledge are often filled by growers' and scientists' opinion. Clearly there is a need for considerable study in this area to determine whether an edible mycorrhizal mushroom is found on young or old trees – so-called early or late-stage mushrooms [64,65]; define the optimum edaphic and climatic conditions tolerated by the fungus; monitor the effect of changing environmental conditions on mycorrhizal community structures [66]; their relationship with other below-ground organisms [43]; and identify the factors required to trigger fruiting [46]. Biochemical and molecular studies might well prove crucial to our understanding of these issues.

Molecular tools

Whether the starting point in the cultivation of an edible mycorrhizal mushroom is a pure culture, spores or segments of infected root, the use of molecular tools is almost essential to ensure that the inoculum used is of the correct species and avoid the problems that have been encountered in attempts to cultivate *Tuber magnatum* [26]. Molecular tools provide more accurate methods for identification than the few characters afforded by traditional morphological features. These tools can also be used to detect contaminant species [67] as well as the persistence on a plant of an inoculant fungus, so it is likely that their use will soon become widespread.

The molecular strategies developed in recent years for edible mycorrhizal mushrooms are primarily PCR-based, using specific oligonucleotides deduced from internal transcribed spacer regions [68,69] and sequence characterised amplified region (SCAR) markers obtained from the random amplified polymorphic DNA (RAPD) technique [70–72]. The RAPD technique provides DNA fingerprints from which it is possible to select and sequence fragments to use as SCAR markers.

Specific primers are already available for *Tuber magnatum*, *T. melanosporum*, *T. borchii* and other *Tuber* species belonging to the group of the so-called 'bianchetti' (whitish truffles) [70]. These techniques, in particular multiplex PCR [73], allow the identification of inoculant and contaminant mycorrhizal fungi on a single root tip both in the nursery and in plantations. The use of molecular strategies will be crucial in following the survival of inoculant fungi, the initiation of infection [74], the degree of competition from contaminant mycorrhizas, presence of hyperparasites, browsing by insects, and initiation of fruiting [74], perhaps in long-term experiments in which, for example, soil composition, pH, fertility, moisture and temperature are varied.

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