AN ABSTRACT OF THE DISSERTATION OF

Charles K. Lefevre for the degree of Doctor of Philosophy in Forest Science presented on May 24, 2002.

Host Associations of *Tricholoma magnivelare*, the American Matsutake.

Abstract approved Signature redacted for privacy.

Randolph J. Molina

Beginning in the 1980s, large-scale commercial harvest of *Tricholoma magnivelare* created a need for management of forests to ensure sustainability, but little was known of the biology or ecology of this species to guide management decisions. Four of the five studies presented here explore the basic synecology of *T. magnivelare* with its hosts to determine the types of mycorrhizal associations formed and the range of potential hosts capable of supporting *T. magnivelare*. The fifth study tested an olfactory detection method, possibly simplifying large-scale surveys for *T. magnivelare* in the field. *Tricholoma magnivelare* forms ectomycorrhizae on hosts in three plant families, the Pinaceae, Fagaceae and Ericaceae, and on at least nine genera in those families. The mycorrhizae formed are consistent with types normally observed for each host, including gymnosperm and angiosperm variants of ectomycorrhizae on Pinaceae and Fagaceae, respectively, and arbutoid and monotropoid mycorrhizae on members of the Ericaceae. One host, the non-photosynthetic *Allotropa virgata*, forms a specific or nearly specific relationship with *T. magnivelare*. Ectomycorrhizae formed by *T. magnivelare* produce unusual defense responses on some hosts including accumulation of pigmented materials in the epidermis and cortex, followed by necrosis and sloughing of the cortex. In an experiment comparing responses of *Pinus contorta* seedlings to colonization by *T. magnivelare* and *A. muscaria* to test for evidence of pathogenicity, no differences were observed in biomass or root to

shoot ratio, but *T. magnivelare* stimulated lateral root branching. Synthesis environments lacking exogenous glucose failed to support *T. magnivelare* ectomycorrhizae and complete development only occurred at 10 gL^{-1} glucose. These results are consistent with other late-seral ectomycorrhizal fungi. Human olfaction used to detect *T. magnivelare* in soil produced a reasonably strong correlation with mushroom production in field plots (R^2=0.71). In the laboratory, trained and screened volunteers correctly identified 98% of samples and false-positive error rates improved as volunteers sniffed more samples. Whether improved false-positive error rates produce a net improvement in accuracy and precision requires further testing. Olfactory detection combined with knowledge of host associations creates a unique opportunity to study the ecology of *T. magnivelare* at stand or landscape scales.

Host Associations of *Tricholoma magnivelare*, the American Matsutake

by

Charles K. Lefevre

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my Dissertation to any reader upon request.

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Charles K. Lefevre, Author

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Dr. Randy Molina provided funding, facilities and critical review for all the work in this dissertation. Walter R. Müller contributed to the description of *T. magnivelare* ectomycorrhizae presented in chapter 2. Dr. Carol M. Carter provided funding and technical assistance for the study on *Allotropa virgata* presented in chapter 3. Dr. Mina McDaniel provided logistical support and advice on laboratory portions of the olfactory detection method presented in chapter 6. Dr. Daniel L. Luoma assisted with data collection and is principal investigator of the study site where the field portion of chapter 6 was conducted.

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To the ants and the corn dancer

Host Associations of Tricholoma magnivelare, the American Matsutake

Chapter 1: Introduction

Of the many wild edible mushrooms found in North America, *Tricholoma magnivelare*, the American matsutake, is among the most widespread and economically important species. It is found scattered across the length and breadth of North and Central America, and in places it makes a significant contribution to local economies. On the west coast from British Colombia through Northern California it attracts thousands of harvesters, many who migrate annually from north to south as the autumn harvest season advances. This sudden large influx of people in many otherwise remote forests combined with intensive and occasionally questionable harvest practices creates a need to manage harvests to ensure sustainability. However, because *T. magnivelare* was seldom commercially harvested on this continent before the 1980s, there was little motivation to study its unique ecology.

The Japanese common name 'matsutake', translated as pine mushroom in English, is used to refer to a number of similar mushroom species, all sharing a characteristic shape and enchanting aroma. One species, *Tricholoma matsutake*, revered for millennia in Japan, has been studied extensively since the early 20th century. Masui (1927) first showed that *T. matsutake* forms mycorrhizae, describing them as "parasitic" based on the poor condition of colonized root tissue and the death of seedlings in synthesis studies. In the 1970s and 80's Makato Ogawa described the colonies formed by all of the matsutake species, including *T. magnivelare*. He found that all produce similar mycorrhizae, which he also described as parasitic (reviewed by Ogawa, 1985) based on his observation of intracellular penetration of host cells, the blackened appearance of many roots, frequent sloughing of the root cortex and

formation of witches brooms by host root systems. Further, they all form a type of soil colony known as a 'shiro' in Japanese, which is synonymous with the terms fairy ring and ectomycorrhizal mat. These colonies consist of expanding rings of prolific mycelium and ectomycorrhizae that tend to exclude other ectomycorrhizal fungi and many other soil microorganisms (Ohara and Hamada, 1967). As these colonies expand they create a zone of bleached, dry, hydrophobic soil thought to be a site of intensive chemical weathering of the mineral substrate (Griffiths and Caldwell, 1992; Terashima and Mizoguchi, 1995). All of the matsutake species are also observed primarily on young, well drained and nutrient deficient soils (Ogawa, 1982). Thus, as close relatives, the various matsutake species appear to share some important characteristics in common, although each associates with different hosts, and lives in different climatic and edaphic environments.

In Oregon, where most of the research presented here was conducted, *T. magnivelare* is found in a variety of environments from stabilized coastal sand dunes to high elevation ridges and plateaus with widely varying soils, climates and potential hosts. Little is known about the range of acceptable conditions that *T. magnivelare* tolerates, or what factors influence its distribution and abundance. The separate studies that comprise this dissertation address questions regarding the hosts of *T. magnivelare* and methods to survey at the stand or landscape scale for *T. magnivelare* populations. Each study lays basic groundwork necessary for further ecological research on *T. magnivelare*.

Like most fungi, the vegetative state of *T. magnivelare* is cryptic and difficult to identify in the absence of reproductive structures, hindering research on anything other than its reproductive ecology. Thus it was necessary as a first step to develop means of identifying the fungal thallus. Chapter two addresses this need by providing a description of *T. magnivelare* ectomycorrhizae on *Pinus contorta*, one of its most common and widespread hosts. This description was useful in all of the subsequent studies by providing a relatively straightforward way to identify *T. magnivelare*

ectomycorrhizae with reasonable confidence. The description provided an initial screen of potential host roots during the research reported in chapters three and four, it provided baseline morphological characteristics for the ectomycorrhiza syntheses performed in chapter five, and it, along with results of chapter three, provided confirmation of the identity of the fungus in samples used to study the olfactory detection method discussed in chapter six. This description of the ectomycorrhizae will be a fundamental tool for future research on *T. magnivelare* involving the fungus itself apart from its sexual reproductive phase.

Chapter three examines the association initially observed by matsutake harvesters between *T. magnivelare* and the non-photosynthetic plant *Allotropa virgata* (Monotropoideae). Like other members of the Monotropoideae and many other achlorophyllous plants, *A. virgata* obtains fixed carbon from its mycorrhizal symbionts (Leake, 1994) rather than through photosynthesis. Soon after development of molecular methods capable of identifying fungi in ectomycorrhizal root tips, it was shown that two members of the Monotropoideae form specific relationships with individual ectomycorrhizal fungi, a level of ectomycorrhizal specificity never before observed for a plant (Cullings, et al. 1996, Kretzer et al., 2000). It seemed possible that the same level of specificity could exist between *A. virgata* and *T. magnivelare* based on the strong association observed in the field. Determining the identity of the fungal partner for such an unusual plant is interesting in itself, but the fact of its co-occurrence with *T. magnivelare* presents an excellent opportunity for study of *T. magnivelare* as well. *Allotropa virgata* can be used to precisely locate *T. magnivelare* colonies under conditions where sporocarps are not present. Further, because *A. virgata* has various types of protected status in different parts of its range, botanists have surveyed, mapped and collected habitat data for *A. virgata* populations where it exists in Oregon, Washington, Idaho and Montana. This data and the relative ease of surveying for plants are both potentially valuable for studies of *T. magnivelare* habitat and distribution. Chapter three is designed to determine whether *A. virgata* and *T. magnivelare* form mycorrhizae with each other and, if so, whether *A. virgata* is always

associated with *T. magnivelare*. The identity of the fungus was determined by morphological and molecular methods and *A. virgata* roots were sectioned to observe the anatomy of its mycorrhizae. Results of this study were invaluable for conducting the field collections in chapters four and six and knowledge of this association will undoubtedly facilitate future studies for both symbiont species.

Tricholoma magnivelare associates with a broad range of other ectomycorrhizal plant species in addition to *A. virgata*. Its sporocarps are found beneath members of the Pinaceae, Fagaceae and Ericaceae, occasionally in pure forest stands, but usually in mixed forests where the identity of its hosts are unclear. Chapter four examines host species in nine genera that form ectomycorrhizae with *T. magnivelare* in Oregon. The host and fungus were identified by morphological and molecular methods and ectomycorrhizae from members of each host genus were sectioned to observe mycorrhizal anatomy. Both partners in ectomycorrhizal symbioses exert some control over the morphology and anatomy of the association and this study presented an opportunity to observe the influence exerted by a single fungus species different hosts on the morphology and anatomy of associations with different hosts in the field. In this case the range of host species is particularly broad, encompassing host families that form four distinct types of mycorrhizae. Members of the Pinaceae and Fagaceae form variants of ectomycorrhizae and members of the Ericaceae form arbutoid and Monotropoid mycorrhizae. Because the hosts of an ectomycorrhizal fungus have a primary influence on its distribution and abundance, the knowledge gained from this study is central to understanding *T. magnivelare*'s ecology as well as its management for mushroom production.

The nature of associations that the various matsutake species form with their hosts is currently a matter of debate in the literature. Historically, matsutake species have been regarded as pathogens based on symptoms of distress in host roots. As a result, they were not considered ectomycorrhizal. However, at early stages of development the associations clearly display a mantle and Hartig net, the primary morphological

characteristics of ectomycorrhizae. The notion that matsutake species are weak pathogens is not rejected outright in the current debate, but several authors have recently cast doubt upon it or suggested that the nature of exchange changes as the ectomycorrhizae age (Wang, 1995; Koo et al., 2000; Yamada et al., 1999a; Gill et al., 2000). Chapter five is the first attempt to compare growth responses of host plants colonized by a matsutake species or a putatively mutualistic ectomycorrhizal fungus: in this case, *Amanita muscaria* (L.: Fr.) Hook. Ectomycorrhizal associations are usually considered mutually beneficial relationships, but mutual benefit cannot be assumed with the matsutake species in spite of their ectomycorrhizal anatomy.

The nature of interaction between matsutake species and their hosts is essential knowledge given their commercial status and because the group of mat-forming annulate Tricholomas that includes the matsutake species is nearly ubiquitous in Northern Hemisphere forests. As colonizers of low-productivity young and disturbed soils such as stabilized sand dunes, ridge tops, glacial till and pumice deposits they may play an important role in the early stages of soil development in many forested areas throughout the Northern Hemisphere.

Chapter six tests a survey method for *T. magnivelare* in the field. Like the previous chapters, it lays groundwork that will potentially enable a variety of studies on the ecology of *T. magnivelare* and other matsutake species. Surveying for mushrooms is inherently difficult. The abundance of sporocarps can vary two to three orders of magnitude from one year to the next depending on weather conditions, even with perennial species like *T. magnivelare*. Thus, to obtain an accurate assessment of average or potential productivity in a given location requires years of data. Further, data collection on mushrooms is complicated by their patchy distribution, the ephemeral and unpredictable nature of mushroom fruiting, and by animal and human mycophagy. Each adds variability and collectively force surveyors to establish large numbers of plots to obtain adequate sample sizes. Studies based on mushroom production also do not provide direct population measures for the fungi and cannot

separate effects due to changes in microclimate, animal mycophagy, etc. from changes in the actual fungus populations. The approach taken in chapter 6 was to measure the abundance of colonies in the soil based on the characteristic smell of matsutake mycelium. It was hypothesized that the abundance of perennial colonies would be more stable than mushroom production and that a simple assessment of colony abundance would provide a better measure of both colony abundance itself and potential mushroom production. The human sense of smell was treated as a detection instrument in this study and the first part of the chapter is devoted to measuring error associated with this instrument. The field portion of the study used olfactory detection to measure colony abundance in preexisting mushroom productivity plots to determine whether colony abundance correlates with mushroom production. Mushroom production was measured on these plots for six fall seasons prior to the soil sampling, whereas the soil sampling required nine surveyor-days.

Population surveys are essential for monitoring effects of disturbance and for effective management of mushroom harvests. They are also potentially useful for mapping productive mushroom patches to determine how various forest management activities would impact matsutake production. Mapping may be particularly important considering that the economic value of matsutake production in some areas exceeds that of surrounding timber (de Geus et al., 1992, Pilz et al., 1999).

Together, the chapters comprising this dissertation provide basic knowledge of *T. magnivelare* essential for management of commercial harvests. The information and methods presented here will also supply tools for further research necessary for a more complete understanding of its ecology.

Chapter 2: *Tricholoma magnivelare* (Peck) Redhead and *Pinus contorta* Dougl. var. *latifolia* Engelm.

Charles K. Lefevre and Walter R. Müller

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pp. CDE18.1-CDE18.4.

INTRODUCTION

The ability to identify the vegetative structures of ectomycorrhizal fungi is essential for studies of individual species, particularly considering the unpredictability and transience of sporocarp production. The following is a description of *Tricholoma magnivelare* ectomycorrhizae on *Pinus contorta* var *latifolia*.

DISTINGUISHING FEATURES:

Smooth, shiny, yellowish brown with patches of white cottony hyphae, cortex of older parts turns black and often sloughs off; thin, transparent mantle; short differentiated mycelial strands; forms mats in mineral soil; soil clings tenaciously to your roots; autofluorescent.

MORPHOLOGY (Dissection Microscope):

Ectomycorrhizal system: (Figs. 2.1-2.4)

Shape and dimensions: dichotomous to irregular systems (Figs. 2.1-2.2) 17 (7-34) mm long; tips straight or bent, 4 (0.8-7) mm by 290 (230-350) μm; mantle and cortex of older parts dies and often sloughs off, separating younger parts (Figs. 2.2, 2.4);.
Colour and texture: light yellowish-brown or light yellowish-green with areas of white; smooth or grainy, shiny or reflective; host cells visible through transparent mantle except in white areas of cottony emanating hyphae and/or adhering soil ; apices colorless, not colonized; older parts progressively blacken and lose emanating elements (Figs 2.2, 2.4).

Emanating elements:

Mycelial Strands: (Figs. 2.1, 2.2, 2.4) common, white, wefty, irregular in cross section, commonly branching, flat angle of attachment or attached by hyphal fans, short.

Hyphae: white, tortuous, cottony (Fig. 2.2), often enmeshing soil (Fig. 2.1)

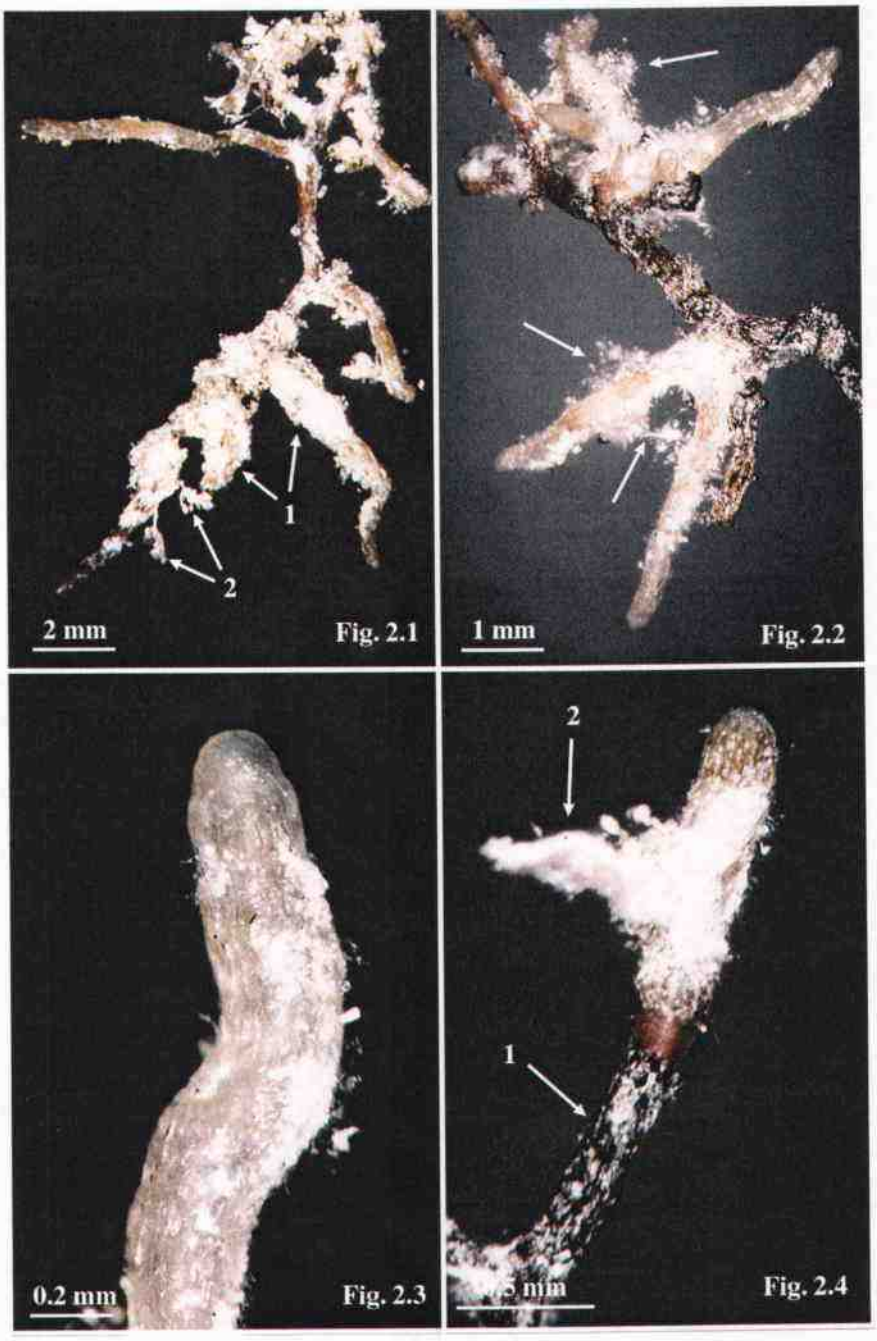

Figure 2.1 Irregular system with adhering soil (arrows 1) and mycelial strands (arrows 2). Figure 2.2 Dichotomous systems with cottony emanating hyphae (arrows). Figure 2.3 Young tip. Figure 2.4 Older tip with black cortex (arrow 1) and mycelial strand (arrow 2).

MANTLE AND EMANATING ELEMENTS IN PLAN VIEW

Mantle in plan view:

thin, one or less often two cells thick where present; Hartig net present (Fig. 2.9); specialized cells not seen; difficult to peel and observe; hyaline.
Outer Layer: felt prosenchyma with parallel hyphae forming patches of net synenchyma (Fig. 2.5), hyphae 2.4 (1.6-3.2) μm wide; hyaline, smooth, with clear to granular contents; septa common, not clamped; hyphal junctions common; junction angle 0-120°; anastomoses rare, not clamped (Fig. 2.7)
Inner Layer: felt prosenchyma with patches of net synenchyma (Fig. 2.6) or irregular interlocking synenchyma; hyphae 2.8 (2.1-3.5) μm wide; hyaline, smooth, with clear to granular contents; septa common, not clamped; hyphal junctions common, junction angle 0-120°; anastomoses not seen.

Mycelial strands in plan view:

differentiated with central, random or isolated wider hyphae (Figs. 2.10, 2.11); typical hyphae 2.1 (1.4-3.5) μm wide, with rare bulbous swellings 4.2 (3.5-4.9) μm wide near septa (Fig. 2.8), or occasionally constricted at septa (Fig. 2.11); hyphal junctions common; anastomoses rare, contact to H-shaped, without clamps; wider hyphae 5.6 (4.8-7.7) μm wide, sinuously entwined by smaller hyphae 1.9 (1.4-2.8) μm wide, branching from the wider hyphae (Fig. 2.11); all hyphae hyaline, smooth, or with crystalline ornamentation, with septa common, not clamped; with common hyphal junctions, junction angle 0-120°; individual hyphae of many strands are obscured by abundant crystals.

Emanating hyphae:

common, cells ≥ 40µm by 2.3 (2.0-3.2) µm, smooth or with crystalline ornamentation, hyaline, clear or rarely granular contents; septa common, not clamped; hyphal junctions common, junction angle 0-120°; anastomoses rare, contact to H-shaped, not clamped; swellings and constrictions at septa as seen in mycelial strands.

Cystidia: none seen

OTHER FEATURES:

Sclerotia and microsclerotia: none seen

Chlamydospores: only observed with synthesized ectomycorrhizae, globose, 11.2 (9.8-13.3) µm wide, walls 1.9 (1.4-2.1) µm thick, smooth, hyaline, terminal hyphal attachment.

Autofluorescence of whole tips: loose hyphae and rhizomorphs yellow-green at 254 nm illumination; 366 nm not tested.

Chemical reactions: KOH: no reaction of whole ectomycorrhizae, mantle or mycelial strands; Meltzers: no reaction of whole ectomycorrhizae, mantle or mycelial strands.

DNA: ITS1/NL6Bmun: AluI: 312, 297, 194, 117, 98; HinfI: 365, 340, 164, 151; RsaI: 855, 179. ITS1f/ITS4b: AluI: 291, 93; DpnII: 524, 296; HinfI: 404, 341.

Additional characters: ectomycorrhizae occur in a ring or arc, creating a mat of bleached, powdery, hydrophobic mineral soil (Griffiths and Caldwell, 1992; Ogawa, 1979), other ectomycorrhizae not typically present.

ADDITIONAL INFORMATION:

Collection and identification: collected by C.K. Lefevre, 1 Nov., 1997, Deschutes County, Oregon, near Crescent Lake Junction (430° 29' 07"N by 121° 53'56"W); described by C.K. Lefevre and W.R. Müller 3 Dec., 1997; identified by C.K. Lefevre by comparison of RFLP ectomycorrhizae and sporocarps, a distinctive odor resembling that of the sporocarps, the occurrence of ectomycorrhizae within a zone of bleached, powdery soil (Ogawa 1979), and by comparison with synthesized ectomycorrhizae; sporocarp identified using Arora (1986); DNA analysis by Charles Lefevre (ITS1f/ITS4b) and by Quentin Baldwin (ITS1/NL6Bmun); photography by Charles Lefevre (Figs. 2.1-2.3, 2.5-2.11) and Walter Müller (Fig. 2.4); accessioned in herbarium as DAVFP 25418.

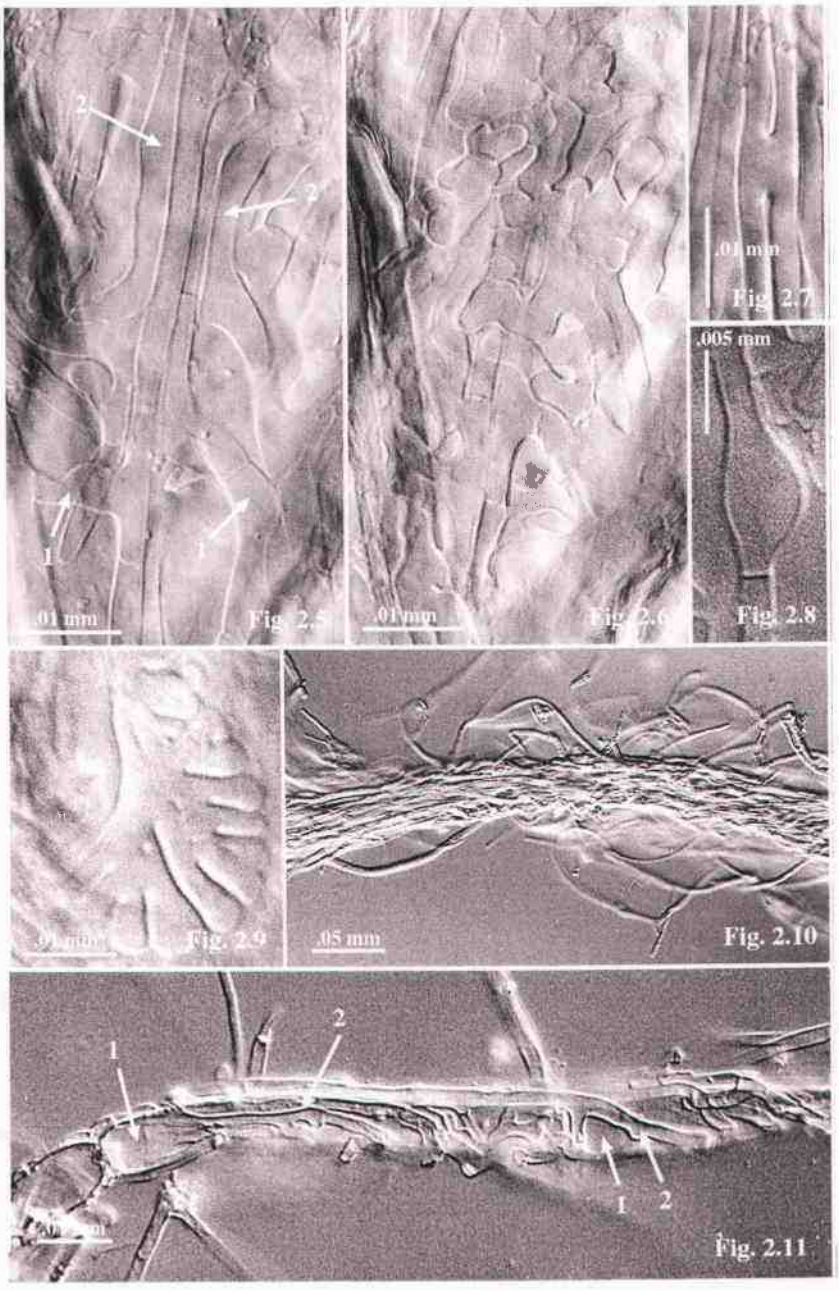

Figure 2.5. Outer mantle, felt prosenchyma (arrows 1) and net synenchyma (arrows 2). Figure 2.6. Inner mantle, a net synenchyma. Figure 2.7. Anastomosis in outer mantle. Figure 2.8. Swelling near septum in mycelial strand. Figure 2.9. Hartig net. Figure 2.10. Mycelial strand. Figure 2.11. Part of mycelial strand, large hypha (arrows 1) entwined by smaller hyphae (arrows 2).

ECOLOGY

In a 50-year-old natural pure *P. contorta* var. *latifolia* stand with no understory in the *P. ponderosa* zone of Franklin and Dyrness (1973); in mineral soil; elevation 1430 m; approximately 150 tips examined; *T. magnivelare* is distributed widely in Central and North America forming similar ectomycorrhizae with hosts in the Pinaceae, Fagaceae, and Ericaceae (Hosford et al., 1997; Redhead, 1997)

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Chapter 3: Mycorrhizal Specialization of *Allotropa virgata* (Monotropoideae) with *Tricholoma magnivelare*

Charles K. Lefevre, Carol M. Carter, and Randy Molina

ABSTRACT

Commercial harvesters of *Tricholoma magnivelare*, the American matsutake mushroom, often use the achlorophyllous plant *Allotropa virgata* (Monotropoideae, Ericaceae) as an indicator of matsutake habitat. This study is designed to determine whether the association between *T. magnivelare* and *A. virgata* is a specific association as observed with other members of the Monotropoideae. We applied morphological and molecular methods to assess the mycorrhizal associations of *T. magnivelare* and determine whether other fungi were also present. Of 65 *A. virgata* root collections representing approximately 60% of its geographic range, the mycorrhizae of 56 were morphologically similar to *T. magnivelare* ectomycorrhizae while the remaining nine were senescent and could not be morphologically characterized. Sectioned *A. virgata* mycorrhizae are monotropoid in nature. DNA from 49 of 57 collections amplified with primers ITS1f and ITS4b matched *T. magnivelare* RFLP patterns with three restriction enzymes. Ten collections from throughout the sampling area gave a single consistent unknown RFLP type. Four collections gave patterns matching *T. magnivelare* and the unknown type in separate RFLP reactions of replicate DNA extracts. Phylogenetic analysis of the unknown fungus based on a sequence of the mitochondrial large subunit rRNA gene placed it among the gilled fungi, but outside of the Amanitaceae and the genus *Tricholoma*. The nature of interaction between *A. virgata* and the unknown fungus is unclear. However, its presence throughout the sampled range suggests that a significant interaction exists between *A. virgata* and the unknown fungus. Given the lack of morphologically distinct mycorrhizae corresponding with the unknown species we consider it likely that it is not a mycorrhizal fungus and that *A. virgata* forms mycorrhizae specifically with *T. magnivelare*, although the nature and identity of the unknown species remains to be determined.

INTRODUCTION

Late in the 1980s mushroom harvesters in western Oregon observed a co-occurrence between *Allotropa virgata* Torrey & Gray *ex* Gray and *Tricholoma magnivelare* (Peck) Redhead, the American matsutake mushroom (Fig. 3.1). Discovery of the association between *A. virgata* and *T. magnivelare* allowed harvesters to precisely locate mushroom patches before the start of the mushroom season by scouting for *A. virgata* plants (Hosford et al., 1997; Moore, 1998). Despite practical application of this knowledge, the nature of interaction between these organisms was not known.

Allotropa virgata is a member of the Monotropoideae (Ericaceae), a subfamily of non-photosynthetic myco-heterotrophic plants (Leake, 1994) that form tripartite symbioses with ectomycorrhizal fungi and overstory trees (Björkman, 1960; Furman and Trappe, 1971). Its range extends from southern coastal British Columbia south to the southern Sierra Nevada of California with a disjunct population in the mountains of central Idaho and western Montana (Wallace, 1975). Its habitats vary from forested coastal sand dunes to high elevation ridges and plateaus representing a wide range of climatic and edaphic conditions and a wide variety of forest types (Copeland, 1938; Wallace, 1975; Hosford et al. 1997). In the states of Idaho and Montana it is listed as a sensitive species (Lichthardt, 1995), and in the range of the Northern Spotted owl on the west side of the Cascade Mountain range it was temporarily included on a list of old growth dependent species requiring protection by Federal land management agencies (U.S.D.A., Forest Service, U.S.D.I., Bureau of Land Management. 1994a; U.S.D.A., Forest Service, U.S.D.I., Bureau of Land Management. 1994b). Its protected status increases the urgency to study its biology and ecology, and because federal and state botanists have mapped *A. virgata* populations throughout much of its range, its legal status combined with existing habitat analyses effectively provide data on the distribution and habitat of its mycorrhizal associates (Luoma, 1987; Lichthardt and Mancuso, 1991).

The intermingling between roots of monotropoid plants with fungi and roots of overstory trees has been recognized for more than a century (e.g. Unger, 1840; Kamiensky, 1882). More recent studies have demonstrated that the Monotropoideae are physiologically linked with neighboring trees from which they receive carbon through shared mycorrhizal fungi (Bjorkman, 1960; Furman and Trappe, 1971; Vreeland, et al., 1981). The identities of the fungi involved have been the subject of great interest (Francke, 1934; Bjorkman, 1960; Boullard, 1964; Campbell, 1971; Kernan and Finocchio, 1983; Castellano and Trappe, 1985; Martin, 1985; Kasuya et al., 1995; Lichthardt, 1995; Cullings, et al., 1996; Kretzer, et al., 2000; Bidartondo and Bruns, 2001, 2002), but until recently none were known definitively.

The development of molecular techniques to identify ectomycorrhizal fungi in the absence of sporocarps has allowed scientists to identify fungal symbionts of many Monotropoid species, including *A. virgata* (Cullings et al., 1996; Kretzer et al., 2000; Bidartondo and Bruns, 2001, 2002). The apparent natural co-occurrence of *A. virgata* and *T. magnivelare* observed by mushroom harvesters presents an opportunity to test whether *A. virgata* specializes on *T. magnivelare* in the same way that specialization was observed with other members of the Monotropoideae. We used morphological and molecular methods to assess whether *T. magnivelare* forms mycorrhizae with *A. virgata* and whether *A. virgata* forms mycorrhizae with other species.

MATERIALS AND METHODS

Sampling

Sixty five soil samples containing *A. virgata* roots were collected from approximately 60% of its geographic range (Fig. 3.2). Sampling was carried out at all times of year from the fall of 1996 through the spring of 1998. Many sites were located in areas

known for *T. magnivelare* production, but we also worked with U.S. Forest Service and Bureau of Land Management botanists to locate sites known to support *A. virgata*, but not necessarily known to produce *T. magnivelare*. Where possible, samples were collected from plants with sporocarps of other ectomycorrhizal fungi growing near them to avoid biased sampling toward *T. magnivelare*.

Root samples were washed in tap water to remove loose soil and examined under a dissecting microscope for mycorrhizal characteristics. One to 30 tips from each were frozen in cetyltrimethyl ammonium bromide (CTAB) buffer for storage and subsequent DNA extraction. Roots from several locations were embedded in resin and sectioned to observe their mycorrhizal anatomy.

Morphological analysis

Morphological analysis of *A. virgata* roots was based on the description of *T. magnivelare* ectomycorrhizae on *Pinus contorta* Dougl. var. *latifolia* Engelm. in Lefevre and Müller (1998). Three primary characters were noted: characteristics of the soil surrounding the roots, appearance of whole mycorrhizae (color, texture, emanating hyphae and rhizomorphs), and mantle and rhizomorph structures. *Tricholoma magnivelare* has distinctive features at each level.

Examination of soil characteristics and mycorrhiza appearance were carried out in the process of collecting roots from the soil samples. Examination of mantle scrapes and rhizomorphs was usually completed on the same day, but not more than three days after root collection.

Molecular analysis

PCR amplification and restriction fragment length polymorphism (RFLP) protocols follow Gardes and Bruns (1993) with the exception that all DNA samples were either cleaned or extracted using GENECLEAN® II DNA purification kit (BIO101, Inc.). Reliable amplification of fungal DNA often required more than a single root tip and all amplifications that failed with an extract of a single tip were repeated with extracts of 10 tips. The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal repeat was amplified using ITS1f and the basidiomycete specific primer ITS4b. Restriction digests were performed with the enzymes HinfI, AluI and DpnII. All RFLP gels included samples from voucher sporocarps of *T. magnivelare*, *Tricholoma focale* (Fr.) Ricken and *Tricholoma caligatum* (Viv.) Ricken. These species are the closest relatives of *T. magnivelare* and occur in the same habitats within the range of *A. virgata*. Fungi amplified from *A. virgata* mycorrhizae were identified by matching RFLP band sizes with those of the voucher sporocarps. RFLP band sizes were estimated using a Alpha Imager™ 2000 (Alpha Innotech Inc.). The complete process of extraction through RFLP was performed two to three times for roots from each soil sample.

For samples that did not match the *T. magnivelare* RFLP pattern, we sequenced a portion of the mitochondrial large subunit rRNA gene for phylogenetic analysis using the methods and database presented in Bruns et al. (1998). Sequences were produced using an ABI Model 377 DNA sequencer (Perkin Elmer Corporation). Identification was based on phylogenetic analysis using the neighbor joining option in PAUP 4.0 (Swofford, 2001).

Sectioning

Pieces of fresh roots 2-5 mm long were fixed in 2.5% glutaraldehyde in 0.1M Hepes (N-2-hydroxy-ethylpiperazine-N1-2-ethane sulfonic acid) buffer at pH 6.8 for 5-8 hours followed by a rinse in the same buffer and dehydration in a graded series of ethanol solutions. Roots were embedded in soft grade LR White resin (London Resin Company Ltd.), sectioned to 1.0 to 1.5 μm with glass knives and stained with a combination of 1% toluidine blue and 1% azureII in 1% sodium borate.

RESULTS

Unlike some other members of the Monotropoideae, *A. virgata* forms a fibrous and occasionally sparse root system (Wallace, 1975) and soil samples had to be checked carefully in the field to confirm the presence of *A. virgata* roots Early in the sample collection process we obtained a number of cores taken directly below flowering stalks that contained no *A. virgata* roots and nine additional samples that contained discolored senescent roots. *Allotropa virgata* roots can be distinguished from those of overstory trees with the unaided eye by their large size, succulence, translucence, lack of suberization, and frequent presence of adventitious flower buds (Fig. 3.3) (Wallace, 1975).

We commonly observed *T. magnivelare* fruiting near *A. virgata* plants in areas known to produce both, but also on five sites located by U.S. Forest Service and Bureau of Land Management botanists where *T. magnivelare* was not known to exist. In spite of the co-occurrence and presumed symbiosis between these organisms, we rarely observed *T. magnivelare* sporocarps within 1-2 m of *A. virgata* stalks.

Sample collection could be carried out at any time of year when snow was not so deep that it covered the stalks. *Allotropa virgata* is perennial and produces stalks that

occasionally persist intact for several years, thus allowing observation of its migration in the soil. Clusters of stalks often appear several centimeters removed from the location of the previous year's stalks and as many as four separate clusters of different ages could be observed, possibly indicating the direction and rate of growth of the plant.

Morphology

The most immediate observation made with all soil samples containing *A. virgata* roots was the distinctive smell of matsutake mushrooms. This smell is a reliable indicator of *T. magnivelare* in soil cores (see Chapter 6). Many samples that lacked *A. virgata* roots also lacked the matsutake smell. Soils containing *A. virgata* roots were invariably dry, hydrophobic and "ashy" regardless of the time of year and soil moisture conditions nearby.

The appearance of the mycorrhizae was mostly consistent with the description of *T. magnivelare* ectomycorrhizae in Lefevre and Müller (1998), varying only in color of the plant tissue, branching morphology, dimensions of the roots and the lack of blackening and sloughing of cortical cells observed on *Pinus* roots. Other researchers reported *A. virgata* roots colonized by *Cenococcum geophyllum* and *Rhizopogon vinicolor* (Castellano and Trappe 1985) but we did not observe either species and, in fact, rarely observed *Cenococcum* or any other morphologically distinct ectomycorrhizae on roots of other plants in the soil cores. When other ectomycorrhizal fungi were observed on tree roots included in the soil samples it was generally in samples consisting, in part, of non-hydrophobic soils. Roots from nine collections were too old and discolored to show normal features. On some roots that appeared old or senescent we observed dark septate fungi intermingling with the white mantle hyphae.

All intact *A. virgata* roots had thin, often inconspicuous, patchy white mantles, and were found in hydrophobic soils. Fungal mantle characteristics were consistent with those observed for *T. magnivelare* on *P. contorta* (Lefevre and Müller, 1998). However, the Hartig net palmetti that were frequently visible in *P. contorta* mantle preparations were rarely observed on *A. virgata*. Rhizomorph characteristics on *A. virgata* were also consistent with *T. magnivelare* on *P. contorta*, including the occasional presence of enlarged central hyphae with smaller subtending hyphae and the frequent presence of waxy or crystalline deposits. Occasional bulbous swellings of hyphae were observed adjacent to septa on emanating hyphae, as on *P. contorta*. The majority of emanating hyphae observed lacked clamp connections, but clamped hyphae were observed on two root tips. One of the tips was part of a collection that produced an unknown RFLP pattern (discussed below). The same sample contained numerous dark septate hyphae. However, overall mantle characteristics and the majority of hyphae present on those tips were consistent with *T. magnivelare*.

Molecular analysis

Results of the molecular analyses are shown in Table 3.1. DNA was amplified from 57 of 65 root collections. Forty five of the 57 samples consistently matched *T. magnivelare*. Four additional samples matched *T. magnivelare* from at least one DNA extract, but results from at least one replicate extract from the same root collection revealed an unknown fungus. This unknown fungus appeared in a total of 10 collections in spite of typically weak amplification and it occurred throughout the sampling area. In all occurrences of the unknown type, RFLP results of collections from the same sites matched *T. magnivelare*. No other fungi were reproducibly observed. *Tricholoma focale* and *T. caligatum* produced distinct RFLPs with AluI and no RFLP patterns from *A. virgata* roots resembled them.

Phylogenetic analysis using the Bruns, et al. (1998) database placed the unknown fungus in a poorly resolved region, and allowed us only to conclude that it is a gilled fungus, but not "russuloid", not a member of the Amanitaceae, and not in the genus Tricholoma. More information is needed to narrow the identity of this fungus.

Morphology		RFLP Results				
		Consistently matched *T. magnivelare*	Matched both *T. magnivelare* and unknown species	Consistently matched unknown species	Not reproducible	Failed to Amplify
Consistent With *T. magnivelare*	56	42	2	5	1	6
Senescent	9	3	2	1	1	2
Totals	65	45	4	6	2	8

Table 3.1: Results of morphological and molecular analyses of *A. virgata* root collections.

Sectioning

Mycorrhizae of *A. virgata* are typically monotropoid, characterized by the presence of a mantle and Hartig net as in ecto- and arbutoid mycorrhizae (Fig. 3.4), but with intrusions into the epidermal cells by attenuated fungal pegs (Lutz and Sjolund, 1973; Duddridge and Read, 1982) (Figs. 3.5 and 3.6). These intrusions differ from arbutoid intracellular hyphal coils in that the hyphae do not pass through the plant cell wall, and

Figure 3.1: Three partners in symbiosis: *T. magnivelare*, *Lithocarpus densiflorus* (Hook. & Arn.), and *A. virgata* growing in unusually close proximity. Figure 3.2: Collection locations and geographic range of *A. virgata* based on Wallace (1975).

the tips of the pegs appear to "burst" releasing contents of the fungal hyphae into the epidermal cells (Fig. 3.6). Similar structures have been observed in mycorrhizae of *Monotropa hypopytis* L. (Francke, 1934; Duddridge and Read, 1982), *Monotropa uniflora* (Lutz and Sjolund, 1973), *Monotropastrum globosum* H. Andr. ex Hara (Kasuya et al. 1995) *Sarcodes sanguinea* Torrey, and *Pterospora andromedea* Nuttall (Robertson and Robertson, 1982).

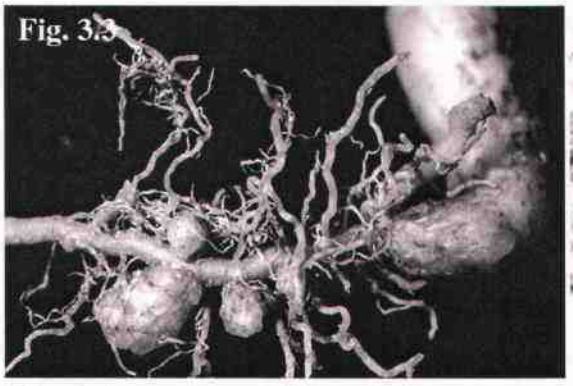

Fig. 3.5

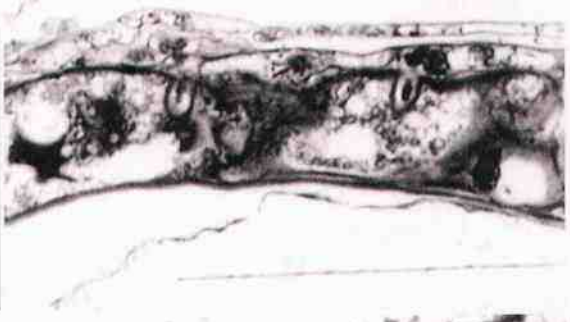

Fig. 3.4

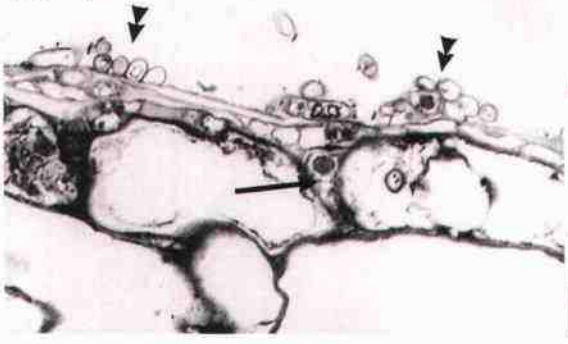

Fig. 3.6

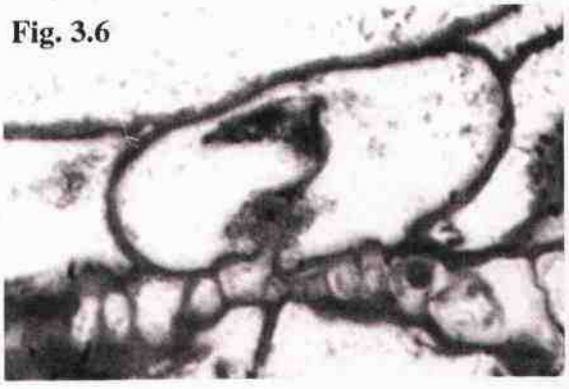

Figure 3.3: *Allotropa virgata* roots with adventitious flower buds and a developing flower stalk. Figure 3.4: *Tricholoma magnivelare* hyphae forming a rudimentary Hartig net (arrow) and mantle (double arrow) on *A. virgata*, but not penetrating epidermal cells. Figure 3.5: *Tricholoma magnivelare* mantle hyphae penetrating *A. virgata* epidermal cells on the same root as shown in Figure 3.4, but further from the tip. Figure 3.6: Fungal peg originating from the Hartig net invaginating the plant epidermal cell wall and "bursting" releasing material from the fungal cytoplasm into the plant cell.

The Hartig net in *A. virgata* is often rudimentary to nearly absent, occurring unevenly between epidermal cells and rarely penetrating the entire depth of the epidermis (Fig. 3.4). The mantle is similarly rudimentary and absent or nearly so on many roots (Figs. 3.4 and 3.5). We commonly observed root tips with no fungal hyphae evident for the first centimeter or more behind the apex, particularly in spring samples when root growth was most rapid. Even in autumn collections, fine roots commonly displayed no evidence of mycorrhiza formation for a millimeter or more behind the root apex. Progressing further down the root, however, first surface and then intercellular hyphae

become more abundant. Fungal pegs and cells containing the contents of burst pegs appear still further from the apex and increase gradually until nearly all epidermal cells show contents of burst pegs. In one November collection (partially shown in Figs. 3.4 and 3.5) this progression took place over a length of approximately 5 mm.

DISCUSSION

Taken together, the morphological evidence presented here and the molecular evidence presented here and in Bidartondo and Bruns (2002) indicates that *A. virgata* does form mycorrhizae with *T. magnivelare* and it is likely to be a highly specialized association. No mycorrhizae morphologically distinct from *T. magnivelare* were observed on *A. virgata* roots and RFLP results confirmed the presence of *T. magnivelare* in 49 of 57 samples that amplified successfully. The unknown fungus recovered from 10 collections was observed with *T. magnivelare* in four cases, and in every case other collections from the same sites contained *T. magnivelare.* If this unknown fungus is a mycorrhizal symbiont, its morphological features are indistinct from those of *T. magnivelare.*

The identity of the unknown fungus is inconclusive, but it is not a member of predominant ectomycorrhizal taxa and it falls within a clade that consists mostly of nonmycorrhizal taxa. We did observe chlamydospores and clamped hyphae unlike those of *T. magnivelare* on roots from a collection containing the unknown fungus, but these hyphae did not form typical mycorrhizal structures. Further, the characteristic smell of matsutake was present in all soil samples that contained *A. virgata* roots. The presence of the unknown fungus throughout the sampling range suggests the possibility of an unusual and perhaps specific association with *A. virgata* mycorrhizae, but the nature of the association is unclear. We also commonly observed dark septate fungi on *A. virgata* roots, though usually limited to senescent root tips and never in the absence of the typical morphological features.

Copeland (1938) presented a description of *A. virgata* roots, but did not observe fungal hyphae associated with them and concluded that they are not mycorrhizal. Ogawa (1979) also reported that *T. magnivelare* mycorrhizae lack both mantle and Hartig net on *Pinus*, *Tsuga* and *Pseudotsuga* roots. The failure of Copeland (1938) and Ogawa (1979) to notice the usual ectomycorrhizal structures is not surprising in our experience. The fungal mantle is typically thin and patchy and some roots appear to lack a fungal mantle altogether, particularly within a few millimeters of the apex. The Hartig net is similarly intermittent and often poorly developed. The unusual nature of matsutake mycorrhizae, including *T. magnivelare*, has led several authors to conclude that matsutake are more parasitic than mutualistic in their relationship with their hosts than typical ectomycorrhizae (e.g. Ogawa, 1975b, 1979; Wang, 1995). The ectomycorrhizae that *T. magnivelare* forms with a number of autotrophic plants often display evidence of host defense responses including unusual accumulation of pigmented materials and sloughing of the epidermis and cortex. (Chapter 4). Other authors have also observed intracellular penetration of host cortical cells (Ogawa, 1979; Wang, 1995, Yamada, et al., 1999). No defense responses were observed in *A. virgata* mycorrhizae.

The balance of nutrient and carbohydrate exchange between the Monotropoideae and their mycorrhizal symbionts has been discussed at length for more than a century with different authors supporting the views that the plants either benefit or parasitize the fungi. While the achlorophyllous plants take carbon from the fungi (Bjorkman,1960; Furman and Trappe, 1971; and Vreeland, et al., 1981) they also stimulate vegetative growth of the fungi (Bjorkman, 1960) facilitating colonization of greater numbers of host roots (Bidartondo et al., 2000). Gogala (1973) studied a hormonal mechanism for this growth stimulation. However, no evidence presently exists suggesting that fungal fitness is either increased or decreased.

Monotropes may utilize resources that their mycorrhizal fungi would otherwise allocate to reproduction. Matsutake harvesters observe that mushrooms are

uncommon in immediate proximity to the plants (Moore, 1998) and Amaranthus, et al. (2000) observed that while a significant spatial association exists between *T. magnivelare* and *A. virgata*, *T. magnivelare* sporocarps typically occur several meters closer to each other than to *A. virgata*. The large perennial colonies formed by *T. magnivelare* present an opportunity to test whether sexual reproduction of the fungus is suppressed by the plant.

It is arguable whether monotropes have any effect on the trees providing energy to the system since, to our knowledge, monotropes do not interact directly with the trees and may create little or no additional carbon demand. However, ectomycorrhizal fungi frequently stimulate root growth (Harley and Smith, 1983) and various authors have noted a brooming effect on host roots produced by mat-forming fungi (Ogawa, 1975a, 1979, Hintikka and Naykki, 1967). By stimulating growth of the fungi the monotropes may indirectly stimulate localized growth of the host root system, thus drawing a disproportionate fraction of the hosts resources into the monotrope's zone of influence. Bidartondo et al. (2000) noted a higher density of host tree roots in *Sarcodes* root balls than elsewhere in the soil and the density of autotrophic host roots appears similarly high among the roots of *A. virgata*. By indirectly stimulating increased root growth monotropes may effectively parasitize the autotrophic host without directly entering its operational environment. The label "epiparasitism" may correctly apply to this situation (Bjorkman, 1960; Luoma, 1987).

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Chapter 4: Host Associations of *Tricholoma magnivelare* in Nine Plant Genera.

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ABSTRACT

In order to sustain the intensive commercial harvest of *Tricholoma magnivelare* in areas throughout North and Central America, managers need to understand the ecological factors influencing its distribution and abundance. *Tricholoma magnivelare* coexists with a wide variety of potential host species in the Pinaceae, Fagaceae, and Ericaceae, including species forming ecto-, arbutoid, and monotropoid mycorrhizae, but until now, ectomycorrhizal associations have been demonstrated with only two host species: *Pinus contorta* and *Allotropa virgata*. This study is intended to assess the host range of *T. magnivelare* in western Oregon, U.S.A., and to document the different anatomies of its ectomycorrhizal associations. Host and fungal constituents of mycorrhizal root tips were identified by two RFLP methods in conjunction with observation of forest stand composition in sampling localities. After morphological examination, the mycorrhizae were sectioned to observe anatomical structures. Hosts were identified in nine plant genera and shown to form mycorrhizae typical of each genus including angiosperm and gymnosperm variants of ectomycorrhizae, arbutoid mycorrhizae, and monotropoid mycorrhizae. Morphological features controlled by the fungus including mantle structure, emanating hyphae, and rhizomorphs are consistent across host genera while factors controlled by the different hosts including dimensions of the mycorrhizae, branching morphology, and anatomical structure differed substantially among hosts. Symptoms of antagonism are evident on many hosts, including accumulation of pigmented materials in the epidermis and cortex, death of epidermal and cortical cells, and occasional sloughing of the root cortex. Despite this evidence of distress, many rootlets with dead cortices display highly nucleated stele and meristem with evidence of continued growth. Symptoms of antagonism were not observed on Ericaceous hosts in the genera *Arbutus*, *Arctostaphylos*, and *Allotropa*. Because *T. magnivelare* forms mycorrhizae with a broad range of hosts in discrete and easily located colonies where other ectomycorrhizal fungi are typically excluded, it may serve as an ideal species for the study of below-ground interplant linkages.

INTRODUCTION

Tricholoma magnivelare (Peck) Redhead, the American matsutake, is a common, and occasionally abundant ectomycorrhizal mushroom found throughout large areas of North and Central America. Intensive commercial harvest creates a need for managers to understand the major ecological factors influencing its distribution and abundance. Foremost among these factors is the range of plant species that serve as hosts. Harvesters associate *T. magnivelare* with a wide variety of host tree species and its geographic range extends beyond that of any individual host species (Redhead, 1989). In some places it grows within pure forest stands where its host can be inferred, but it is usually found in mixed forests where its host associations are unclear. Existing literature also suggests that *T. magnivelare* has a broad host range (Hosford, et al 1997; Redhead, 1997), although only two plant species have been confirmed as hosts of *T. magnivelare* ectomycorrhizae: *Pinus contorta* Dougl. and *Allotropa virgata* Torrey & Gray *ex* Gray (Ericaceae, Monotropoideae) (Ogawa, 1979; Lefevre and Müller, 1998; see Chapter 3). Among the suspected hosts are species that form different types of mycorrhizae. These include several arbutoid species and members of the Pinaceae and Fagaceae that form the typical gymnosperm and angiosperm variants of ectomycorrhizae respectively.

The objectives of this study are to determine whether *T. magnivelare* forms mycorrhizae with the potentially compatible tree and shrub species most commonly associated with it in western Oregon, and to observe the morphological and anatomical differences among its associations.

MATERIALS AND METHODS

Root samples were collected in four broad localities, all within the state of Oregon, U.S.A. These localities include the Oregon Dunes on the Pacific coast, the western

slope of the Cascades Mountains east of Eugene, the eastern slope of the Cascades Mountains northeast of Crater Lake, and the Siskiyous mountains in southwest Oregon.

Two methods were used to collect root samples. During the mushroom fruiting season, roots were collected by excavating soil samples near *T. magnivelare* sporocarps. During the rest of the year, root samples were collected by excavating soil samples near the achlorophyllous plant *Allotropa virgata* which appears to associate specifically with *T. magnivelare* (see Chapter 3). In all cases, the litter layer was removed from an area around the mushroom or *A. virgata* plant to locate the front of the expanding *T. magnivelare* colony (Ogawa, 1979). Soil samples were collected at the interface of colonized and non-colonized soil where the youngest *T. magnivelare* mycorrhizae are usually located. Roots colonized by *T. magnivelare* were collected at all times of year. All tree and shrub species known to form ecto- or arbutoid mycorrhizae within sight in the forest surrounding the sampling locations were recorded as potential hosts. Our sampling was intended to represent the range of host genera but not necessarily to identify all host species. For example, we did not sample *Pinus attenuata* Lemm., which is reputed to host *T. magnivelare* ectomycorrhizae. We included *A. virgata* and *P. ponderosa* in this study to represent the complete range of genera in Oregon and all of the anatomical forms of mycorrhizae, though members of these genera are already known to host *T. magnivelare*.

Mycorrhizae with morphological characters resembling those in the description of Lefevre and Muller (1998) were divided into subsets for molecular analysis, anatomical observation, and morphological description. In general, we sought to obtain mycorrhizae with intact surface and emanating hyphae, turgid root tissue and minimal discoloration of the root tissue. Samples for molecular analysis were placed in CTAB DNA extraction buffer and frozen for storage. Samples for anatomical observation were fixed in 2.5% glutaraldehyde in 0.1M HEPES buffer pH 6.8 for 4-7 hr then dehydrated in a graded series of ethanol solutions to 70% ethanol for storage.

Descriptions of root morphology followed the terminology of Agerer (1987). All samples were photographed and preserved for storage within three days of collection from the field.

Two restriction fragment length polymorphism (RFLP) tests were performed on the mycorrhiza samples to determine the identities of both the fungus and the host. The ITS region of fungal nuclear rDNA was amplified using the primers ITS1F and ITS4B (Gardes and Bruns, 1993) and digested using three restriction enzymes: HinfI, DpnII and AluI. RFLP results were compared with results from *T. magnivelare* sporocarps to confirm the identity of the fungus. Plant DNA was amplified using the primers KJ1 and TW14 (Cullings, 1992) and digested with the same three restriction enzymes. RFLP results were compared with samples of host species present in the stands where the samples were collected. We were unable to amplify DNA from *Lithocarpus densiflorus* (Hook. & Arn.) Rehd. and *Chrysolepis chrysophylla* using the primers KJ1 and TW14. However, we could confidently identify their mycorrhizae. Their roots could be unambiguously distinguished from neighboring gymnosperms by examining woody laterals for the presence of vessels and from both arbutoid and gymnosperm hosts from anatomical sections of mycorrhizae. Distinguishing them from each other would have been difficult, but we did not sample in areas where they co-occur. This RFLP method was also unable to distinguish the genera *Abies*, *Pinus*, and *Tsuga* from each other, nor was it useful to distinguish different species within genera. If only one species in these genera was present in a stand then RFLP results from that species could be considered unambiguous. However, many samples were discarded when host species could not be uniquely identified by this method.

Samples preserved for sectioning in 70% ethanol were eventually dehydrated to 100% ethanol, embedded in L.R. White soft grade resin (London Resin Co.), sectioned with a microtome to 1.0-1.5 μm using glass knives and stained with a solution of 1% toluidine blue and 1% azure II in 1% sodium tetraborate. Three to five root tips were

sectioned for each host species. Sectioned mycorrhizae were photographed using either standard or differential interference microscopy.

RESULTS

Tricholoma magnivelare mycorrhizae were confirmed by both morphological and molecular methods on nine plant species in nine genera (Table 4.1). These genera fall within three plant families: the Pinaceae, Fagaceae, and Ericaceae. *Tricholoma magnivelare* forms different types of mycorrhizae on these hosts, including ectomycorrhizae typical of gymnosperms (figures 4.1-4.5) and angiosperms (figures 4.6-4.7), arbutoid mycorrhizae (figures 4.8-4.10) and monotropoid mycorrhizae (figures 4.11-4.12). All types were collected in one instance within 10 cm of each other in a single soil sample, likely representing an individual *T. magnivelare* genet. In all cases, samples that were judged to be morphologically consistent with the description of *T. magnivelare* ectomycorrhizae in Lefevre and Müller (1998) also gave RFLP results consistent with *T. magnivelare* sporocarps.

Several other tree species likely serve as hosts for *T. magnivelare* in Oregon. We observed mycorrhizae morphologically consistent with *T. magnivelare* on *Arctostaphylos uva-ursi* (L.) Spreng., *Arctostaphylos patula* Greene, *Abies procera* Rehd. and *Tsuga mertensiana* (Bong.) Carr, all commonly found with *T. magnivelare*, but these mycorrhizae were neither subjected to RFLP analysis nor sectioned. The identities of the roots were clear either because *T. magnivelare* was fruiting beneath pure stands of *A. procera* or *T. mertensiana*, or in the cases of the two *Arctostaphylos* species, because they were the only woody angiosperms present in the surrounding forest.

Family	Genus	Species	Mycorrhiza type	Common Name	References
Pinaceae	*Pinus*	*contorta* vars. *contorta* and *latifolia*	Gymnosperm Ecto.	Shore pine, lodgepole pine	Ogawa, 1979; Lefevre and Müller, 1998
		ponderosa		Ponderosa pine	
	Abies	*magnifica var. shastensis*		Shasta red fir	
	Tsuga	*heterophylla*		Western hemlock	
	Pseudotsuga	*menziesii*		Douglas-fir	
Fagaceae	*Lithocarpus*	*densiflorus*	Angiosperm Ecto.	Tanoak	
	Chrysolepis	*chrysophylla*		Western chinkapin	
Ericaceae	*Arbutus*	*menziesii*	Arbutoid	Madrone	
	Arctostaphylos	*columbiana*		Hairy manzanita	
	Allotropa	*virgata*	Monotropoid	Candystick	See Chapter 3

Table 4.1: Hosts of *T. magnivelare* mycorrhizae

Host	Branching morphology	Average system length (mm)	Average diameter of main axis (mm)	Average diameter of tip base (mm)	Average diameter of tip apex (mm)	Average tip length (mm)
Pinus ponderosa	Simple to bifurcate	3.6 (1.0-6.9)	.36 (0.31-0.45)	0.34 (0.25-0.48)	0.24 (0.19-0.38)	2.8 (1.0-6.1)
Abies magnifica var. shastensis	simple, irregular or monopodial pinnate	3.7 (0.69-10)	0.33 (0.18-0.47)	0.30 (0.20-0.47)	0.26 (0.16-0.38)	2.3 (1.6-3.5)
Tsuga heterophylla	Simple, irregular, monopodial pinnate or pyramidal	8.9 (0.30-20)	0.28 (0.22-0.50)	0.38 (0.14-0.40)	0.20 (0.10-0.28)	2.2 (1.1-5.8)
Pseudotsuga menziesii	Irregular, to monopodial pinnate	14 (5.4-21)	0.5 (0.43-0.60)	0.45 (0.34-0.53)	0.42 (0.34-0.50)	2.5 (1.7-4.2)
Lithocarpus densiflorus	Simple to irregular	2.08 (0.80-4.7)	0.21 (0.14-0.30)	0.20 (0.12-0.24)	0.20 (0.14-0.24)	2.0 (0.80-4.7)
Chrysolepis chrysophylla	Simple to irregular	1.1 (0.22-3.5)	0.23 (0.18-0.28)	0.16 (0.08-0.26)	0.18 (0.060-0.32)	0.85 (0.22-2.1)
Arbutus menziesii	Simple to irregular, occasionally cruciform	3.6 (2.3-5.0)	0.21 (0.16-0.25)	0.20 (0.18-0.22)	0.20 (0.16-0.22)	2.3 (0.76-5.0)
Arctostaphylos columbiana	Simple, irregular or monopodial pyramidal	2.8 (0.82-4.5)	0.11 (0.10-0.12)	0.07 (0.04-0.10)	0.07 (0.020-0.10)	0.94 (0.20-2.0)
Allotropa virgata	Irregular, monopodial pinnate to monopodial pyramidal	>2.3cm (3.5 mm->4cm)	0.69 (0.52-0.90)	0.43 (0.28-0.80)	0.28 (0.18-0.62)	4.4 (1.3-9.2)

Table 4.2: Branching morphologies and dimensions of *T. magnivelare* mycorrhizae.

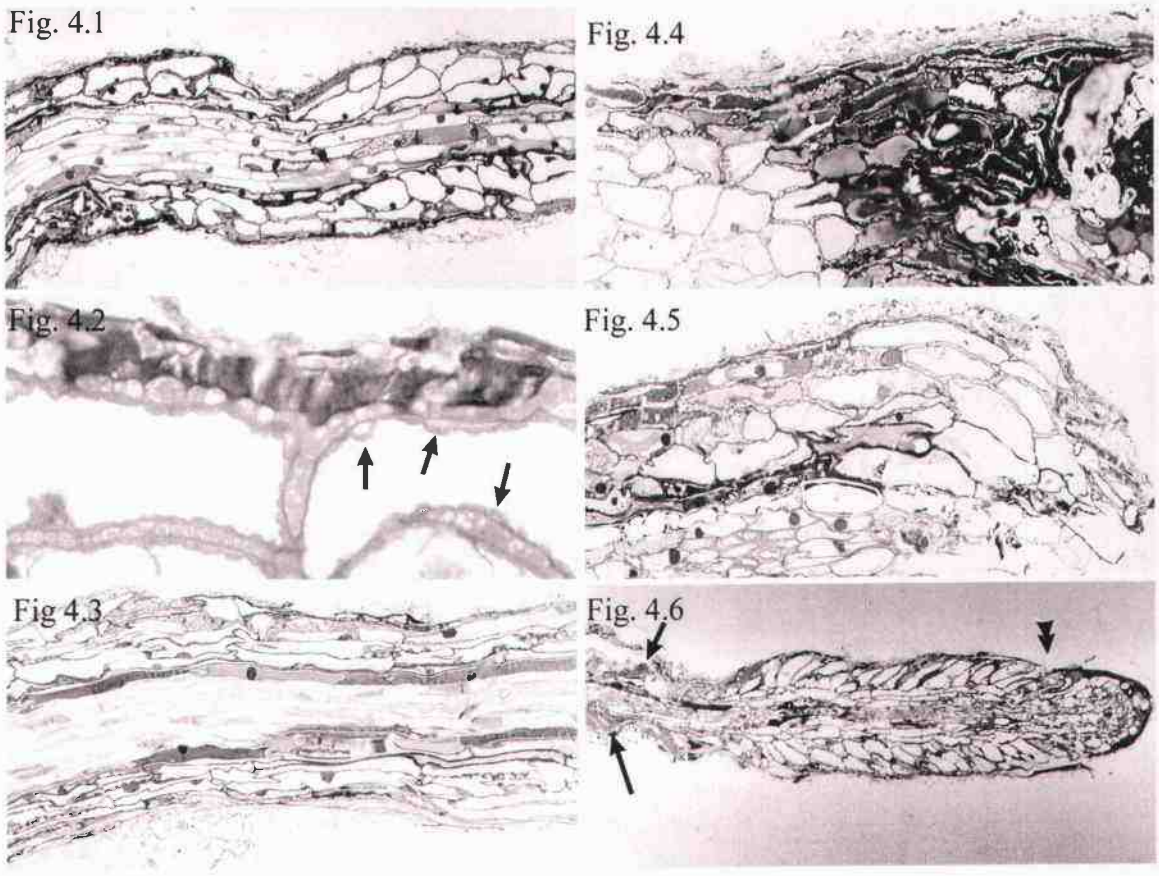

Figure 4.1 *T. magnivelare* ectomycorrhiza on *Pinus ponderosa* forming a cortical Hartig net without intracellular hyphae. Figure 4.2 *Pinus ponderosa* ectomycorrhiza showing collapsed epidermal cells filled with pigmented material and droplets of pigmented material (possibly polyphenolics) opposite Hartig net hyphae in cortical cells (arrows). Figure 4.3 *T. magnivelare* ectomycorrhiza on *Abies magnifica* var. *shastensis*. Figure 4.4 *T. magnivelare* ectomycorrhiza on *Tsuga heterophylla* forming a cortical Hartig net. Note accumulation of pigmented materials within host cortical cells. Figure 4.5 *Pseudotsuga menziesii* ectomycorrhiza showing a cortical Hartig net and accumulation of pigmented material in cortical cells. Figure 4.6 *Lithocarpus densiflorus* ectomycorrhiza showing an epidermal Hartig net and elongated epidermal cells characteristic of angiosperm ectomycorrhizae. Epidermal and cortical cells in basal region have collapsed and filled with pigmented material (arrows), whereas the apical region shows signs of continued growth (double arrow).

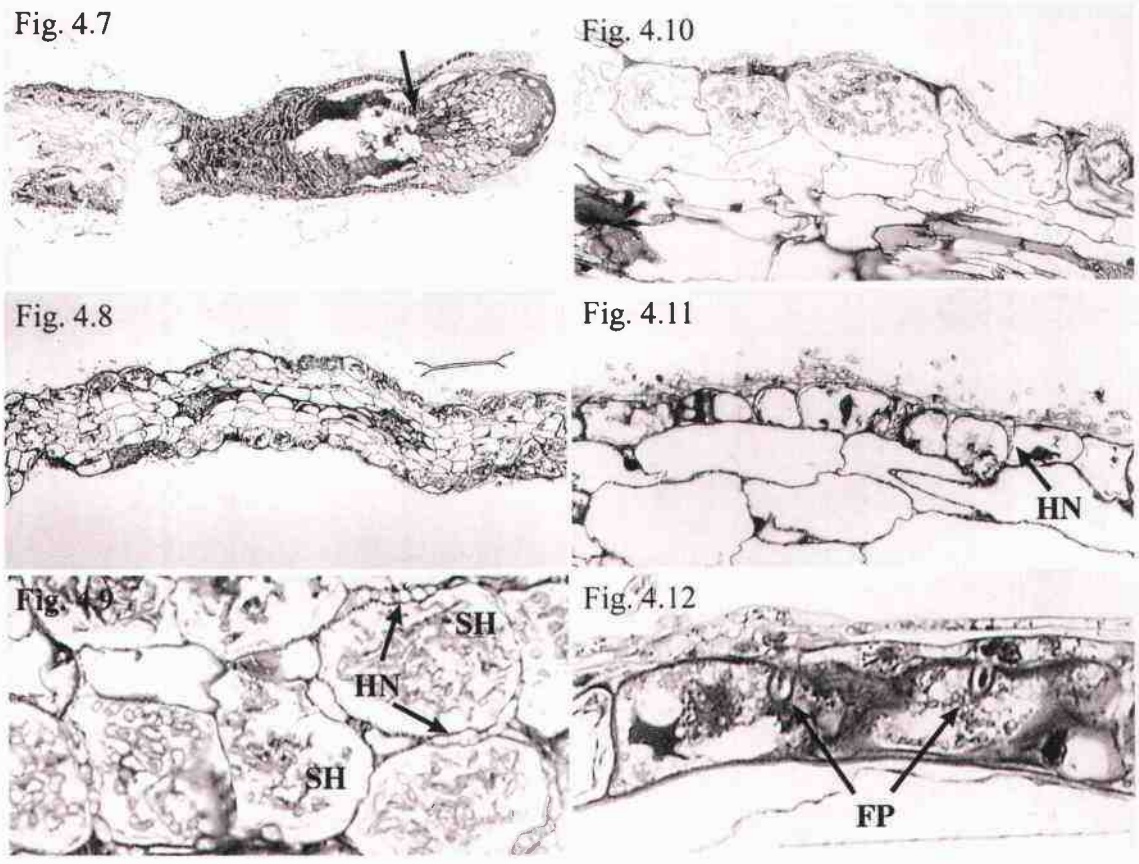

Figure 4.7 *Chrysolepis chrysophylla* ectomycorrhiza showing an epidermal Hartig net and elongated epidermal cells. The apical meristem is highly nucleated and shows signs of continued growth (arrow) despite heavy accumulation of pigmented materials in nearby epidermal cells (arrows). Gaps in the section resulted from poor infiltration into highly pigmented regions of root tissue. Figure 4.8 *Arbutus menziesii* mycorrhiza showing typical arbutoid anatomy with mantle, Hartig net (occasionally present) and intracellular penetration of epidermal cells. Figure 4.9 Intracellular colonization of *Arbutus menziesii* with discontinuous Hartig net (HN). Intracellular hyphae in some host cells appear to have senesced (SH) without accompanying signs of host defense responses. Figure 4.10 Arbutoid anatomy on *Arctostaphylos columbiana* showing mantle and intracellular colonization of host cells, but Hartig net is poorly developed (not present in this figure). Figure 4.11 Apical region of *Allotropa virgata* mycorrhiza showing mantle and poorly developed Hartig net (HN), but lacking fugal pegs penetrating host cells. Figure 4.12 Basal region of the same *Allotropa virgata* mycorrhiza showing fungal pegs (FP) penetrating host cells that appear to contain material discharged from the hyphae.

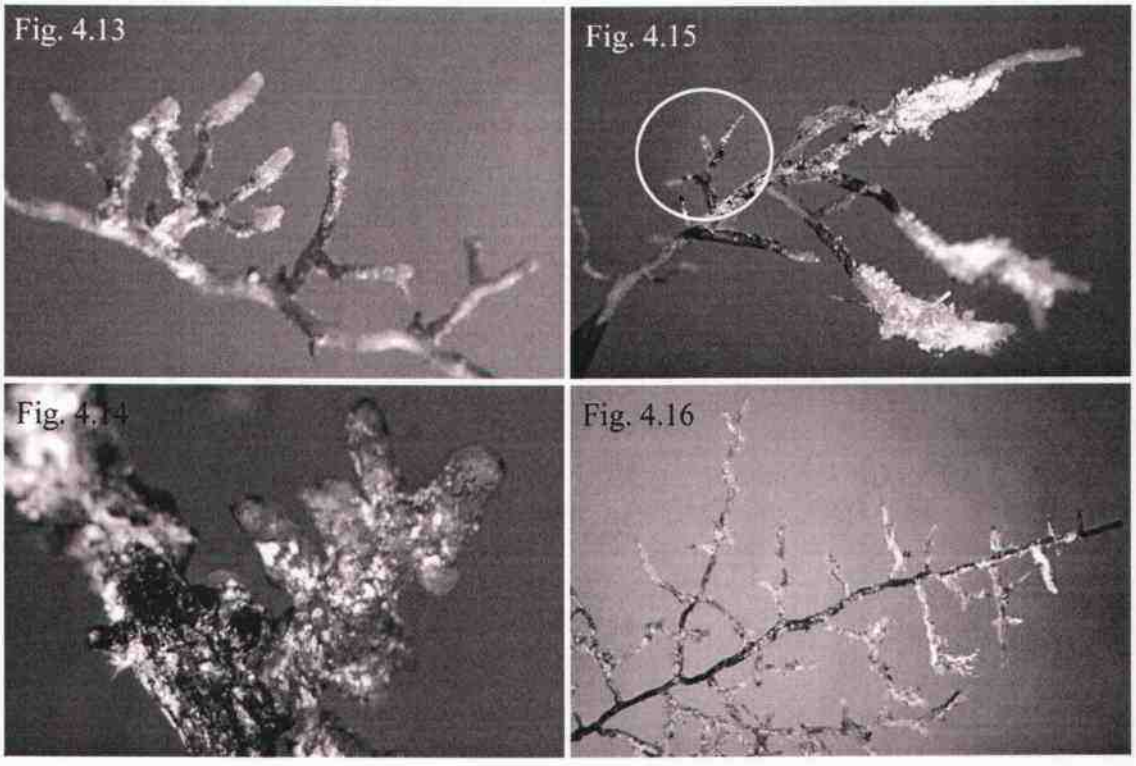

Figure 4.13 *Pinus ponderosa* showing characteristic bifurcate branching morphology. Figure 4.14 *Pseudotsuga menziesii* showing monopodial pinnate branching morphology characteristic of the genus. Figure 4.15 *Arbutus menziesii* showing irregularly branching mycorrhizae, but with a single cruciform mycorrhiza in the background (circled). Figure 4.16 Monopodial pinnate system on *Tsuga heterophylla*.

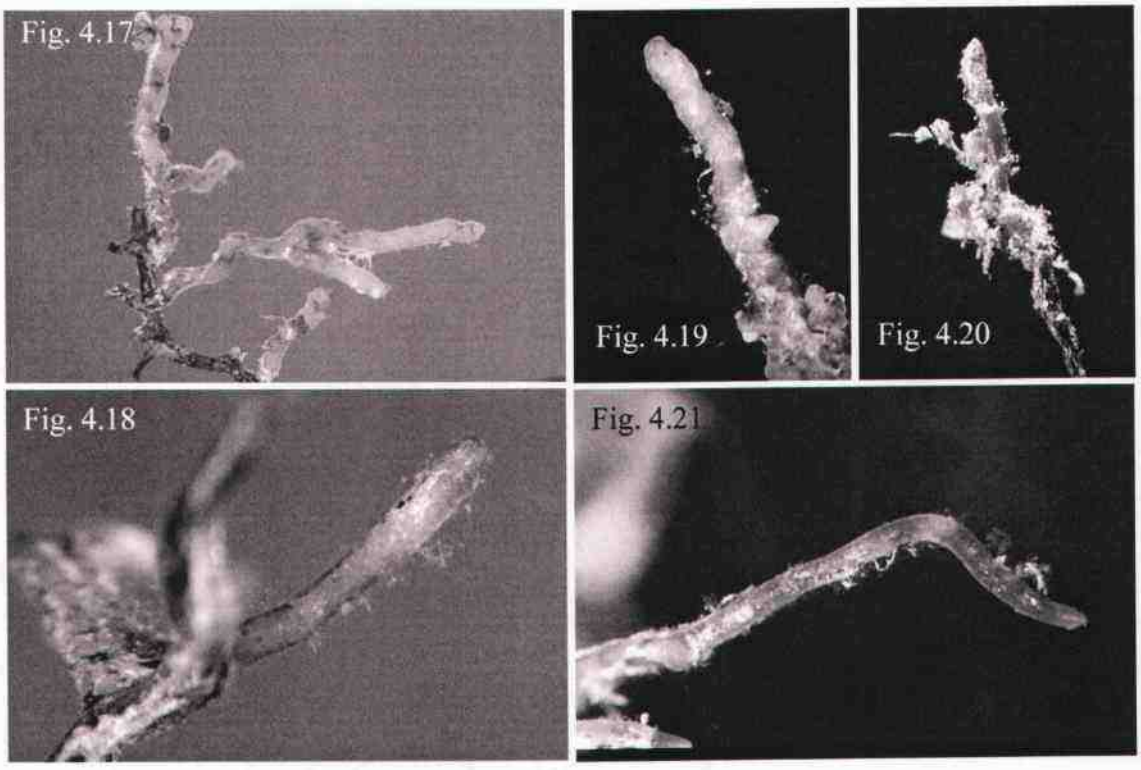

Figure 4.17 Irregular ramification of mycorrhizae on *Chrysolepis chrysophylla*. Figure 4.18 Simple *Lithocarpus densiflorus* mycorrhiza. Figure 4.19 *Arctostaphylos columbiana* mycorrhiza. Figure 4.20 *Abies magnifica* var. *shastensis* mycorrhiza. Figure 4.21 *Allotropa virgata* mycorrhiza showing characteristic translucence.

We were unable to obtain healthy mycorrhizae of some host species despite repeated collection attempts throughout the growing season (figures 4.4, 4.5 and 4.7). The majorities of fine roots in many soil cores were blackened and lacked surface or emanating hyphae, but nevertheless contained intact *T. magnivelare* DNA. Blackened roots often displayed a beaded appearance with translucent and turgid root apices, suggesting that root growth continued despite death of the epidermis and cortex (e.g. figure 4.7). Sections of these roots show that the stele and apical meristem contained intact nuclei. Accumulations of pigmented materials, possibly tannins or polyphenolics, were observed in cortical and/or epidermal cells of most host species, with the exception of the arbutoid and monotropoid hosts. In the samples we examined, these Ericaceous hosts lacked the characteristic blackened appearance and collapse of epidermal and cortical cells common on other hosts.

External fungal structures including the mantle, emanating hyphae and rhizomorphs did not vary among hosts apart from effects of age and senescence. The size and branching morphology of mycorrhizal roots did vary among hosts, although branching morphologies were perhaps less distinct than usual for these species (Table 4.2). In general *T. magnivelare* mycorrhizae are either simple or irregularly branched with occasional systems forming the unique branching morphologies characteristic of individual hosts. For example, most *Pseudotsuga menziesii* (Mirb.) Franco. ectomycorrhizal systems branched irregularly, but occasionally formed the characteristic monopodial pinnate systems common to that species (figure 4.14) (Molina and Trappe, 1982b). Similarly, *Arbutus menziesii* Pursh. formed simple or irregular mycorrhizae with an occasional system showing its characteristic cruciform branching morphology (figure 4.15) (Molina and Trappe, 1982a). *Pinus ponderosa* Dougl. ex Loud. was unusual in forming fewer irregular systems and more bifurcating systems typical of *Pinus* species (figure 4.13), whereas *Pinus contorta* Dougl. formed relatively few bifurcating systems (Lefevre and Müller, 1998). Mycorrhizae on all hosts were typically bent, but ranged from straight to tortuous.

The morphology of *T. magnivelare* mycorrhizae shows elements of control by the fungus and by the hosts. Structures formed by the fungus including the mantle, emanating hyphae and rhizomorphs were consistent across host genera and are described in Lefevre and Müller (1998). The hosts appear to have characteristic root dimensions, anatomical structure, and branching morphologies, although the fungus also appears to have some influence over branching morphology. In general, the mycorrhizal anatomies formed by the different plants are typical of those species. On arbutoid hosts *T. magnivelare* forms an epidermal Hartig net with intracellular hyphal coils (figures 4.8-4.10). On gymnosperms *T. magnivelare* forms a cortical Hartig net without intracellular penetration (figures 4.1-4.5). On members of the Fagaceae *T. magnivelare* forms an epidermal Hartig net, also without intracellular penetration (figures 4.6-4.7). And on *Allotropa virgata*, *T. magnivelare* forms a typical monotropoid mycorrhiza with epidermal Hartig net and penetration of epidermal cells

by fungal pegs that 'burst' releasing material into host epidermal cells (figures 4.11-4.12)(Lutz and Sjolund, 1973; Duddridge and Read, 1982; Robertson and Robertson, 1982). The Hartig net on members of the Ericaceae was rudimentary and infrequently observed between epidermal cells (figures 4.9-4.12). Where present, it seldom penetrated the entire depth of the epidermis.

DISCUSSION

Tricholoma magnivelare forms anatomically diverse mycorrhizae with a wide range of hosts. Where it was possible to distinguish plants by RFLP analysis *T. magnivelare* was found on all potential hosts present in sampled stands. *Tricholoma magnivelare* sporocarps, and mycorrhizae morphologically consistent with *T. magnivelare* were also observed beneath pure stands of both *Tsuga mertensiana* and *Abies procera,* and mycorrhizae morphologically consistent with *T. magnivelare* were observed on *Arctostaphylos uva-ursi* and *A. patula.* Some potential hosts could not be uniquely identified because the RFLP method used was unable to distinguish host species within genera. Potential hosts that were not observed in the absence of other species in the same genera include *Abies amabilis* (Dougl.) Forbes, *Abies grandis* (Dougl.) Lindl., *Pinus monticola* Dougl. ex D. Don and *Pinus lambertiana* Dougl. Elsewhere in North America, *T. magnivelare* is found in association with a variety of other species that likely serve as hosts including members of the genera *Picea* and *Quercus.*

In some cases we were only able to locate *T. magnivelare* ectomycorrhizae that appeared dead or distressed in spite of repeated collection attempts at different times of year. In our experience, *T. magnivelare* mycorrhizae are extremely abundant near the advancing edge of colonies, but most *T. magnivelare* mycorrhizae present at a given time appear damaged, suggesting that the root epidermis and cortex deteriorate rapidly, but the root remains alive persisting in a distressed condition. Despite death

of epidermal and cortical cells, the stele and meristem of distressed fine roots were often highly nucleated and displayed evidence of continued growth (Figure 4.7).

Interestingly, *T. magnivelare* does not appear to elicit defense responses from the Ericaceous hosts. In the *Arbutus*, *Arctostaphylos*, and *Allotropa* samples examined for this study we did not see accumulations of pigmented materials or death of host cells associated with *T. magnivelare* hyphae (figures 4.8-4.12). This apparent lack of antagonism on Ericaceous hosts is curious given the putative pathogenicity of *T. magnivelare* and other matsutake species (Ogawa, 1979, 1985). The hyphae forming intracellular coils on arbutoid hosts were themselves senescent in some roots without obvious distress on the part of the host (figure 4.9). This lack of antagonism is perhaps predictable for the specialized parasitism of *A. virgata* on *T. magnivelare*. However, *Arbutus* and *Arctostaphylos* are receptive to an broad range of mycorrhizal fungi (Molina and Trappe, 1982a) and would not be expected to show specialized adaptation to *T. magnivelare*. Further anatomical and ultrastructural research on the ontogeny of mycorrhizal development would be valuable to clarify the nature of interactions between *T. magnivelare* and its various hosts.

Despite the compatibility of *T. magnivelare* with a broad range of hosts, its abundance appears to be greatest beneath certain hosts in mixed stands. For example, in the mixed conifer stands dominating the pumice soils north east of Crater Lake, *T. magnivelare* appears to reach its greatest abundance beneath *A. magnifica* var. *shastensis*. It forms ectomycorrhizae with, and fruits beneath, most other ectomycorrhizal species in the area, but commercial harvesters focus their attention on individual *A. magnifica* var. *shastensis* trees and stands with higher concentrations of *A. magnifica* var. *shastensis*, regardless of other habitat factors like slope position and aspect. Similar associations are observed by harvesters with *L. densiflorus* in southwest Oregon and with *C. chrysophylla* and *A. menziesii* on the west slope of the Cascades Mountains and in the Oregon Coast Range. It is not clear whether these associations reflect host preferences or coincidental habitat requirements. A case may

be made for host preference with *A. magnifica* var. *shastensis* because the area northeast of Crater Lake is uniformly blanketed with young pumice soils that reduce soil heterogeneity and *T. magnivelare* appears to be clustered with individual *A. magnifica* var. *shastensis* trees in habitats dominated by other host species. Further, although host specificity in ectomycorrhizal fungi often segregates at the host genus level (Molina and Trappe, 1982b), *T. magnivelare* seems to discriminate among hosts within genera. For example, it fruits abundantly beneath at least two *Pinus* species in Oregon, but its fruiting is not spatially correlated with *Pinus monticola* (Amaranthus et al., 2000). Thus, by discriminating within and between compatible taxa *T. magnivelare* appears to exhibit a more restrictive 'ecological specificity' (Harley and Smith, 1983) despite otherwise broad host compatibility.

The occurrence of *T. magnivelare* mycorrhizae on a broad range of hosts within individual *T. magnivelare* colonies presents an opportunity to study belowground links between plants. In the case of *Allotropa virgata, T. magnivelare* is clearly providing a net flow of carbon to the achlorophyllous plant (Bjorkman, 1960; Leake, 1994). Similar net flow of carbon may benefit other hosts like *Arctostaphylos* spp. that tend to live in the forest understory (Simard, et al., 1997). *Tricholoma magnivelare* excludes other mycorrhizal fungi from the roots of its hosts within its colonies, and colony boundaries tend to be visible on the surface of the mineral soil. These features simplify study of *T. magnivelare* mycorrhizae and may provide an ideal system to study interplant linkages in the field by a single known fungus.

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Chapter 5: Growth Response of *Pinus contorta* to *Tricholoma magnivelare* and *Amanita muscaria* ectomycorrhizae Synthesized In-Vitro.

Charles K. Lefevre and Randy Molina

ABSTRACT

All matsutake and closely related species within the genus *Tricholoma* form ectomycorrhizae with thin, discontinuous mantles, intermittent Hartig net and evidence of antagonism in host epidermal and cortical cells. These species are often described as weak pathogens, but this conclusion is debated in the literature, and, until now, no experimental evidence existed to evaluate their effect on host trees. Methods to synthesize matsutake ectomycorrhizae are necessary to conduct controlled experiments, but researchers have experienced a history of failure both synthesizing and maintaining matsutake ectomycorrhizae under controlled conditions. This research examines the ability of *Tricholoma magnivelare*, the American matsutake, to form ectomycorrhizae with *Pinus contorta* var. *latifolia* using three synthesis systems: 1) open pots using spore inoculum, 2) a non-aseptic petri plate system using excised natural ectomycorrhizae and colonized agar plugs as inoculum, and 3) an aseptic system with three concentrations of added glucose. In the aseptic system *Amanita muscaria* was included as a control allowing comparison of host growth responses to the two fungi. *Tricholoma magnivelare* failed to colonize host roots in synthesis systems lacking added glucose, but with 10 g L^{-1} glucose roots colonized by *T. magnivelare* appeared similar to natural *T. magnivelare* ectomycorrhizae. These results are consistent with the behavior of late successional ectomycorrhizal fungi in synthesis studies. As in natural *T. magnivelare* ectomycorrhizae, host epidermal and cortical cells contained large amounts of pigmented materials and epidermal cells were frequently dead, but no statistically significant differences were observed in root, shoot, or total biomass, root to shoot ratio, or stem caliper among seedlings colonized by *T. magnivelare* and *A. muscaria*. Branching of lateral roots was significantly more frequent on seedlings inoculated with *T. magnivelare*, which is consistent with observations of witches brooms on root systems naturally colonized by matsutake species and other ectomycorrhizal mat-forming fungi. The evidence of antagonism between host and fungus observed with matsutake species is similar to ectomycorrhizae formed by a variety of ectomycorrhizal mat-forming fungi known for

their intensive chemical weathering of recalcitrant soil substrates. The biology of matsutake species may be more clearly understood within the broader context of ectomycorrhizal mat-forming fungi.

INTRODUCTION

Mushroom species collectively known as matsutake and other annulate members of the genus *Tricholoma* form unusual ectomycorrhizae. Despite numerous publications discussing and describing these ectomycorrhizae (see reviews by Hosford et al. 1997; Wang et al., 1997; and Redhead, 1997) the nature of interaction between the fungi and their hosts remains unclear and it is questionable whether the association is mutually beneficial.

Like other matsutake species, *Tricholoma magnivelare* (Peck) Redhead has been described as forming "parasitic mycorrhizae" (Ogawa, 1979). Masui (1927) originally described *Tricholoma matsutake* (S. Ito et. Imai.) Sing. mycorrhizae as parasitic based on observations of intracellular hyphal penetration and damping-off of seedlings in synthesis experiments. Subsequently, various authors have described one or more of the following unusual characteristics of matsutake ectomycorrhizae: brooming of root systems, absent or poorly developed mantle and Hartig net, accumulation of tannins or polyphenolics in host epidermal and cortical cells, intracellular penetration of epidermal and cortical cells, host cell wall thickening adjacent to hyphae, eventual blackening, collapse and sloughing of the root cortex, and occasional death of naturally colonized and inoculated seedlings (Gill et al. 1999, 2000; Lefevre and Müller, 1998; Koo et al., 2000; Ogawa, 1975, 1977, 1979, 1985; Ohara and Ogawa, 1982; Terashima, 1993; Wang, 1995; Yamada et al., 1999a;b). This evidence has been interpreted in different ways. Several authors describe matsutake ectomycorrhizae as parasitic or pathogenic (Ogawa, 1975, 1979, 1985; Masui, 1927; Terashima, 1993; Shimazono et al., 1979; and Smith and Read, 1997). Wang (1995)

proposes that these fungi may act as mutualists, pathogens, or saprotrophs at different developmental stages. Koo, et al. (2000) suggest that the nature of interaction changes from mutualistic to parasitic from the leading to trailing edges of expanding colonies. Others find it premature to label *T. matsutake* a parasite based on normal ectomycorrhizal anatomy at early stages of development and anatomical and morphological similarities with other ectomycorrhizal fungi (Yamada et al., 1999a,b; Gill et al., 1999, 2000). This ambiguity in the literature results from features consistent with both pathogen infection and ectomycorrhizal colonization. Understanding the ecophysiology of matsutake species and their relatives is essential because of their status among the world's most valuable commercially harvested wild mushrooms as well as their widespread presence and localized abundance in forests throughout the northern hemisphere. However, until now, no experimental evidence existed to evaluate their putative pathogenicity.

Experimental examination of matsutake ecophysiology is hindered by the difficulty of synthesizing and artificially maintaining their ectomycorrhizae. Because matsutake have significant commercial value numerous laboratory syntheses have been attempted throughout the past century, but clear evidence of success has only been published relatively recently (see Reviews by Wang et al., 1997, and Yamada et al., 1999b). A complicating factor in laboratory synthesis systems that have succeeded to date with matsutake species is inclusion of exogenous carbohydrates in the growth medium. Duddridge (1986) found that glucose caused aggressive fungal colonization of root tissue by ectomycorrhizal fungi leading to anatomical abnormalities, exaggerated host defense responses and successful syntheses between otherwise incompatible partners. She cautions that exogenous carbohydrates may cause anomalies in host specificity studies and such synthesis systems should be avoided. However, late-seral ectomycorrhizal fungi such as *Amanita muscaria* (L. ex Fr.) Hook. and *Lactarius pubescens* (Boud.) Pat. Hooker appear to require more energy for growth and survival than seedlings can provide and systems lacking sufficient exogenous carbohydrate fail to support formation of their ectomycorrhizae (Gibson

and Deacon, 1990; Hutchison and Piche, 1994; Fox, 1983, 1986). Further, ectomycorrhizae of late-seral species fail to proliferate on seedling root systems when the supply of exogenous carbohydrate is withdrawn (Eto, 1990; Fleming, 1985; Last et al., 1985; Wang, 1995). Like *A. muscaria*, matsutake species appear beneath mature trees and likely require exogenous carbohydrates to colonize seedlings. The ability to synthesize and maintain matsutake ectomycorrhizae creates the potential for controlled experiments exploring the nature of exchange between matsutake species and their hosts, but exogenous carbohydrates may exaggerate the antagonisms such experiments would be designed to measure. Thus, the ability to synthesize matsutake ectomycorrhizae without exogenous carbohydrates would be desirable for plant pathological and host specificity studies, though such syntheses may not be easily achieved.

The objectives of this research were to test three different synthesis systems with *T. magnivelare*. The first was an open pot inoculation of seedlings in a growth chamber with *T. magnivelare* spores. The second tested the ability of *T. magnivelare* to colonize seedling roots from excised natural ectomycorrhizae and colonized agar plugs placed in contact with non-colonized seedling fine roots using a modification of the method described in Unestam and Stenström (1989). The third was an aseptic synthesis method (Molina, 1979) using three levels of added glucose. As a secondary objective to the aseptic synthesis, *A. muscaria* was included in parallel with *T. magnivelare* to compare host growth responses to the two fungi.

MATERIALS AND METHODS

Open pot spore inoculations

Nineteen *Pinus contorta* Dougl. var. *latifolia* Engelm., 17 *Pinus ponderosa* Dougl. ex Loud. and 9 *Abies magnifica* Murr. var. *shastensis* Lemm. seedlings were germinated and grown in steam pasteurized soil collected from the natural habitat of these species in an area known for production of *T. magnivelare*. These tree species are hosts to *T. magnivelare* ectomycorrhizae (Lefevre and Molina, unpublished data). Seeds were stratified for one month at 4° C before planting. The seedlings were grown in 66 cm^3 pots (Ray Leach Cone-tainers) at 20° C under 150 μmol m^{-2} s^{-1} PAR with a 16 hr light, 8 hr dark photoperiod in a growth chamber for ten weeks prior to inoculation and seven months following inoculation. Seedlings received 15 ml of deionized water twice weekly: an amount sufficient to supply water throughout the pot, but without drainage and consequent nutrient leaching. No fertilizers were used. To collect spore inoculum mature *T. magnivelare* sporocarps were stored resting on their caps overnight at room temperature allowing mature spores to accumulate between the gills. Gill tissue was then scraped off, homogenized in sterile deionized water and filtered through a 15 μm nylon mesh. Spores were collected by this tissue slurry method to provide for possible "helper bacteria" that might be present within sporocarps (Duponnois and Garbaye, 1991). The final spore concentration was 3.3 * 10^6 spores per cm^3. Inoculations were performed by injecting 10 ml of spore suspension into the soil with a syringe and a 10 cm long needle. The pots were injected progressively as the needle was inserted to distribute spores evenly between the soil surface and a 10 cm depth. The spores were four days old at the time of inoculation. Five seedlings of each *Pinus* species and two *A. magnifica* var. *shastensis* seedlings received 10 ml injections of sterile deionized water to serve as controls. Upon harvesting, soil was gently washed from the roots and all fine roots were examined to assess the presence or absence of ectomycorrhizae.

Petri plate synthesis

Pinus contorta var. *latifolia* seedlings were grown in 14 cm plastic petri dishes containing a 1:1 mix of pumice and sphagnum peat moss with their roots separated from potting mix by a 50 μm nylon mesh (Unestam and Stenström, 1989). Seedling rootlets were inoculated individually by placing field collected *T. magnivelare* ectomycorrhizae or MMN (Marx, 1969) agar plugs colonized by *T. magnivelare* in contact with them. The colonized agar plugs were rinsed in sterile distilled water for one hour to leach excess nutrients before using them as inoculum. A total of 40 seedlings in 20 petri dishes were grown for 10 weeks prior to inoculation. Seedlings were grown in a 20° C clean room at 150 μmol * m^{-2} s^{-1} PAR with a 16 hr light, 8 hr dark photoperiod. *Tricholoma magnivelare* ectomycorrhizae were collected beneath a pure stand of *Pinus contorta* var. *latifolia* in the Cascade Mountains of Central Oregon. Soil was freed from roots with running tap water and healthy, non-discolored (Lefevre and Müller, 1998), well-colonized ectomycorrhizae were selected individually under a dissecting microscope. Ectomycorrhizae were placed in contact with seedling roots within one day of collection from the field. A total of 10 ectomycorrhizae of two unidentified species were used as controls to inoculate seedling roots in the same petri dishes. In all, excised ectomycorrhizae were placed next to 15-20 seedling fine roots per petri dish and colonized agar plugs were placed next to 5-10 fine roots per petri dish. Petri dishes were wrapped in foil to exclude light from the root zone and seedling shoots were loosely covered with clear plastic bags to conserve moisture. Colonization progress was checked twice weekly for four weeks without removing the Petri dish covers.

Aseptic synthesis

Aseptic syntheses were based on the method of Molina (1979). Syntheses were conducted in 30 x 300 mm test tubes containing 110 cm^3 of 1:11 sphagnum peat moss

: medium grade vermiculite. Tubes were capped with inverted 50 ml glass beakers. The liquid nutrient media consisted of 70 ml MMN formulated with three levels of glucose: 0, 2.5 and 10 g L^{-1}. The tubes were autoclaved for 25 min., cooled to room temperature and aseptically planted with *P. contorta* var. *latifolia* germinants.

Pinus contorta var. *latifolia*. was chosen because it is among the most widespread hosts for *T. magnivelare* in North America. Seeds were agitated in 30% H_2O_2 for 45 minutes, stratified for three weeks at 4° C and germinated at room temperature in petri dishes containing MMN agar medium. Seeds that developed fungal or bacterial contamination were discarded. Uncontaminated germinants with 10-15 mm long radicles were transferred to the synthesis tubes. The tubes were immersed to 10 cm in slowly running water to maintain temperatures below 20° C around the roots. Seedlings were grown for ten weeks prior to inoculation at 300 µmol * $m^{-2}s^{-1}$ PAR under a combination of fluorescent cool white and red-end grow lights and 25 W incandescent lights with a 16 hr light: 8 hr dark photoperiod (Figure 5.1).

Two fungal cultures were used for this study. The *T. magnivelare* culture (Boswell-1) was obtained from a sporocarp collected in the Siskiyous mountains in Oregon, USA within one year of its use in this study. The *A. muscaria* culture (L-1029) was obtained from the USDA Forest Service PNW station ectomycorrhizal fungus culture collection in Corvallis, Oregon. Both species were maintained on MMN agar. To produce inoculum, the fungi were transferred to fresh MMN agar plates. Both species grow slowly in culture and colonies were approximately 1 cm in diameter after one month at room temperature. At that time new growth on each plate was excised, cut into tiny cubes (~2 mm^3) and transferred into 1000 ml flasks containing 400 ml liquid MMN medium. After three months at room temperature without agitation the mycelium from each flask was filtered onto sterile filter paper. The mycelia were concentrated by resuspension from the filter papers into beakers containing 200 ml sterile MMN (0 g L^{-1} glucose). The inocula were homogenized for 30 s at medium speed in sterile Waring blenders then distributed in 10 ml aliquots from the blenders to

the synthesis tubes. The blenders were used in brief (~0.5 s) low-speed spins between aliquots to keep the mycelium in suspension. Non-inoculated controls received 10 ml aliquots of 0 gL^{-1} glucose MMN. Seedlings were grown for seven months prior to harvest.

The experimental design is shown in Table 5.1. Six replicate synthesis tubes were established for each treatment combination. Seedlings were assigned randomly to tubes, inoculation treatments were randomly assigned to tubes within glucose levels, and tubes receiving a particular inoculation treatment were inoculated in random order. Six non-inoculated controls were grown with 10 g L^{-1} glucose, but were not included in
statistical analyses because seedling growth is strongly affected by fungal metabolism of glucose in a CO_2 limited environment (Jumpponen and Trappe, 1998).

Species	Glucose Level (g L^{-1})		
	10	2.5	0
T. magnivelare	6	6	6
A. muscaria	6	6	6

Table 5.1: Experimental design showing the number of synthesis trials for each treatment combination. The standard glucose concentration in MMN is 10 g L^{-1}. One tube inoculated with *T. magnivelare* at 10 g L^{-1} glucose became contaminated and was excluded from statistical analyses.

Harvest and data collection

Seedling root systems were gently washed in running water to remove most of the substrate. Final cleaning was done by hand under a dissecting microscope with forceps and a fine brush. Data collected included total number of root tips, number of colonized tips, shoot caliper, and numbers of primary, secondary and higher order lateral roots. Colonized tips were defined as fine roots with fungal hyphae on the root

surface. We did not determine the proportion of roots with a mantle that lacked a Hartig net. Harvest order was randomized to avoid serial bias in the data collection process. After collecting fresh data, 5-15 colonized root tips from each tree were fixed for 5-8 hours in 2.5% formaldehyde and 2.5% glutaraldehyde in O.1 M HEPES buffer (pH 6.8). Fixed roots were dehydrated in a graded series of ethanol solutions and embedded in Spurr's resin. Roots from 2-3 trees representing each glucose level were sectioned with glass knives to 1.0 to 1.5 μm thickness and stained with a solution of 1% toluidine blue and 1% azure II in 1% sodium tetraborate. The shoots and remaining roots were dried separately for 48 hr. at 60° C and weighed on an analytical balance. The weight of root tips preserved for sectioning was considered negligible. Host nutritional status was not measured. Dry weights, root to shoot ratios, root branching, and shoot calipers were compared between fungus species by ANOVA including glucose level and its interaction with fungus species as covariates.

RESULTS

Open pot spore inoculations

At the time of harvest, the seedlings had reached heights of 3-6 cm and, in spite of constant growth chamber conditions, all seedlings had set bud. All root tips were examined under a dissecting microscope with oblique illumination to highlight hyphae, but no fungi were observed in association with any seedling roots.

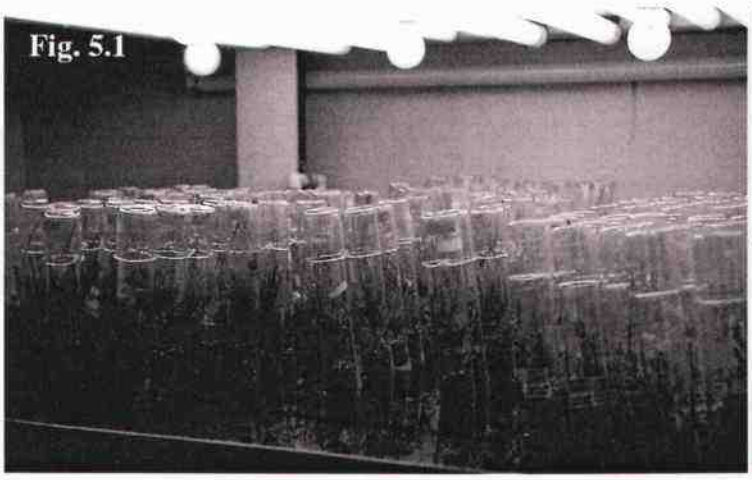
Petri plate synthesis

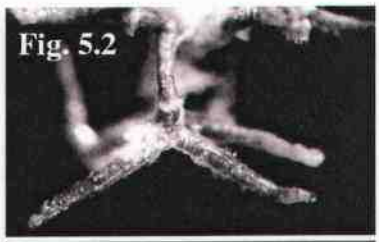
Field-collected *T. magnivelare* ectomycorrhizae bristled with white hyphae within three days of inoculation, but no structures resembling a fungal mantle formed on

adjacent seedling roots within the four week observation period. Little or no growth of *T. magnivelare* was observed on the agar plugs and no adjacent seedling fine roots were colonized by *T. magnivelare*. Most rootlets continued to grow after inoculation and all but one rootlet inoculated with *T. magnivelare* ectomycorrhizae or agar plugs developed root hairs. One of the two unknown ectomycorrhiza types colonized seedling roots forming a well developed mantle on three of five inoculated rootlets within the first week. After the first week all field ectomycorrhizae appeared blackened and decomposed, agar plugs grew sporulating contaminants, and no further colonization from field-collected control species occurred.

Aseptic synthesis

Both *T. magnivelare* and *A. muscaria* formed compoundly bifurcating ectomycorrhizae at 10 g L^{-1} glucose (Figures 5.2 and 5.3). Colonization was less frequent at 2.5 g L^{-1} glucose and infrequent at 0 g L^{-1} glucose (Figures 5.4 and 5.5), although a larger fraction of root tips was colonized by *A. muscaria* at 2.5 g L^{-1} glucose (Figure 5.5). Seedling biomass also increases with higher glucose levels (Figures 5.6 and 5.7), although this is likely a result of fungal metabolism in the CO_2 limited environment of the synthesis tubes (Jumpponen and Trappe, 1998; Fortin et al., 1983). In another study using the same synthesis system seedling growth was not affected by glucose concentration in noninoculated controls (Jumpponen and Trappe, 1998). However, seedling growth is stimulated by nonmycorrhizal contaminants in control tubes containing glucose (data not shown; Fortin, et al., 1983). Two tubes in this study became contaminated: one noninoculated control and one inoculated with *T. magnivelare* at 10 g L^{-1} glucose. Neither seedling was included in statistical analyses.





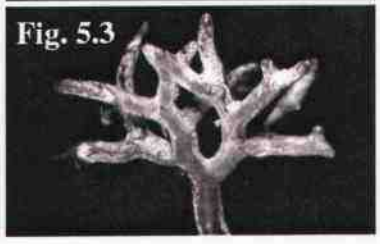


Figure 5.1: Aseptic synthesis system. Figure 5.2: *Tricholoma magnivelare* ectomycorrhizae synthesized with *P. contorta*. Figure 5.3: *Amanita muscaria* ectomycorrhizae synthesized with *P. contorta*.

After transforming response variables as necessary to meet ANOVA assumptions and accounting for glucose levels and their interaction with fungus species, there were no statistically significant differences in shoot caliper ($F(\text{caliper})_{1,29}=1.7$, $p=0.21$), root dry weights ($F(\text{log root dry weight})_{1,29}=0.071$, $p=0.79$), shoot dry weights ($F(\text{shoot dry weight})_{1,29}=0.005$, $p=0.95$), total dry weights ($F(\text{total dry weight})_{1,29}=0.081$, $p=0.78$) or root to shoot ratios ($F(\text{inverse of root to shoot ratio})_{1,29}=0.11$, $p=0.74$) between trees inoculated with *A. muscaria* and *T. magnivelare*. (Figures 5.6, 5.7, 5.8, 5.9, 5.10). However, lateral roots branched more frequently on seedlings inoculated with *T. magnivelare* ($F(\text{log number of lateral root branches})_{1,29}=13.4$, $p=0.001$) (Figure 5.11). Control seedlings grown at 10 g L^{-1} glucose were smaller than inoculated seedlings grown at the same glucose concentration and comparable to inoculated seedlings grown at 0 g L^{-1} glucose, except in the one tube that became contaminated. The seedling in the contaminated tube was substantially larger than other control seedlings.

Figure 5.4

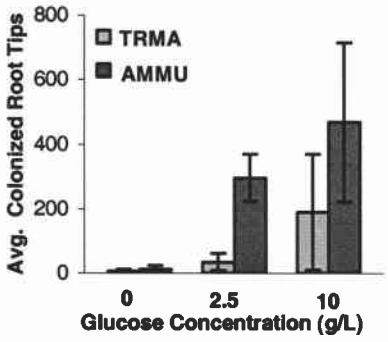


Figure 5.6

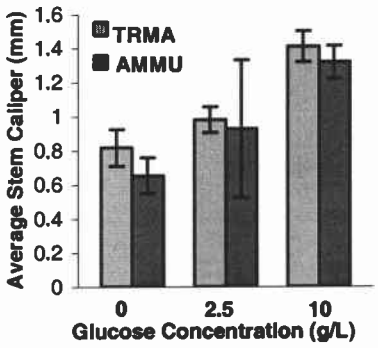


Figure 5.5

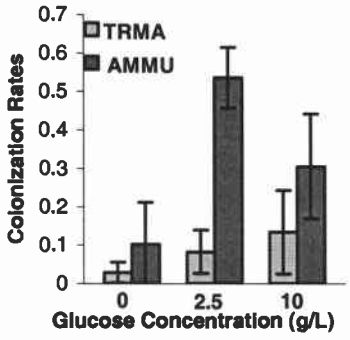


Figure 5.7

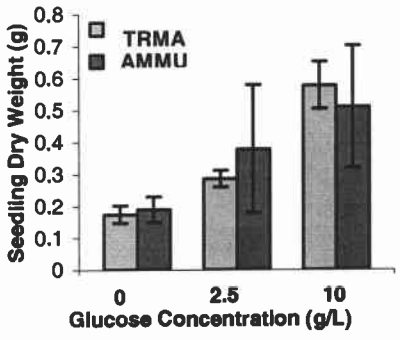


Figure 5.4: Average numbers of *P. contorta* root tips colonized by *T. magnivelare* and *A. muscaria* in the aseptic synthesis system. Figure 5.5: Colonization rates of *P. contorta* root tips by *T. magnivelare* and *A. muscaria* in the aseptic synthesis system. Figure 5.6: Stem caliper at the root crown of *P. contorta* seedlings inoculated with *T. magnivelare* and *A. muscaria* in the aseptic synthesis system. Figure 5.7: Dry weight of *P. contorta* seedlings inoculated with *T. magnivelare* and *A. muscaria* in the aseptic synthesis system.

Figure 5.8

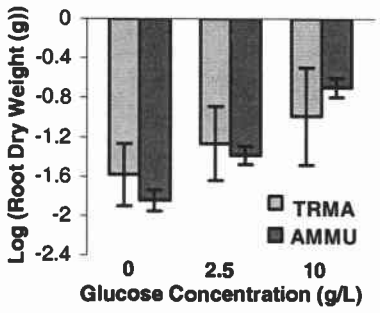


Figure 5.10

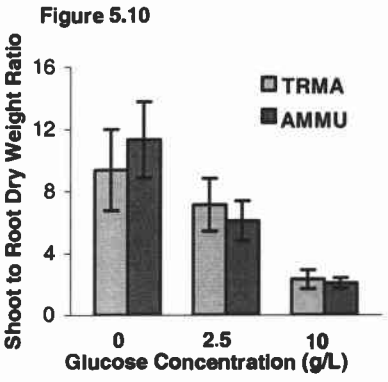


Figure 5.9

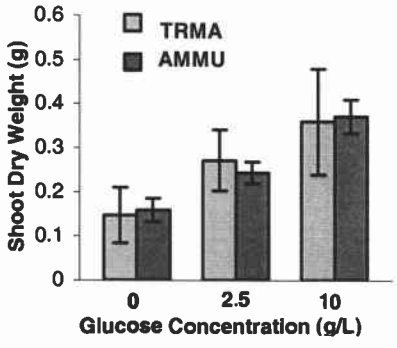


Figure 5.11

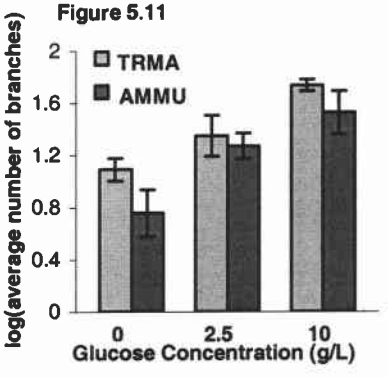


Figure 5.8: Root dry weight of *P. contorta* seedlings inoculated with *T. magnivelare* and *A. muscaria* in the aseptic synthesis system. Figure 5.9: Shoot dry weight of *P. contorta* seedlings inoculated with *T. magnivelare* and *A. muscaria* in the aseptic synthesis system. Figure 5.10: Root to shoot dry weight ratios of *P. contorta* seedlings inoculated with *T. magnivelare* and *A. muscaria* in the aseptic synthesis system. Figure 5.11: Number of lateral root branches on *P. contorta* seedlings inoculated with *T. magnivelare* and *A. muscaria* in the aseptic synthesis system.

The mantle of *T. magnivelare* ectomycorrhizae synthesized at 10 g L^{-1} glucose was sparse and discontinuous as in field collections (Lefevre and Müller, 1998). At 2.5 g L^{-1} glucose the abundance of surface hyphae was reduced and at 0 g L^{-1} glucose few hyphae were associated with the roots (Figures 5.12, 5.13, 5.14). Similarly, *T. magnivelare* developed a Hartig net at 10 g L^{-1} glucose, but Hartig net was not

observed at 2.5 or 0 g L^{-1} glucose (Figures 5.12, 5.13, 5.14). Overall, *T. magnivelare* ectomycorrhizae synthesized at 10 g L^{-1} glucose appeared similar to natural collections, but reducing the glucose concentration prevented formation of normal ectomycorrhizal structures. *Amanita muscaria* formed a relatively thick continuous mantle as observed in other studies (Molina and Trappe, 1982; Ingleby et al. 1990), but its thickness also diminished with lower glucose concentrations (Figures 5.15, 5.16, 5.17). Unlike *T. magnivelare*, *A. muscaria* formed Hartig net at all glucose concentrations. The formation of Hartig net by *A. muscaria* in the absence of glucose contrasts with results of other studies (Gibson and Deacon, 1990; Hutchison and Piche, 1994; Fox, 1983, 1986). Both fungi penetrated host cells in single instances. *Tricholoma magnivelare* penetrated cells in a root tip at 2.5 g L^{-1} glucose (Figure 5.18) and *A. muscaria* penetrated epidermal cells in a single root at 10 g L^{-1} glucose (Figure 5.19).

As in field collections of *T. magnivelare*, most epidermal cells had collapsed and contained large amounts of tannins or other pigmented materials. This cell damage and accumulation of pigmented material decreased at lower glucose concentrations, but occurred even without glucose and with little contact between host and fungus (Figure 5.14). Pigmented materials did not accumulate in roots of noninoculated controls (Figure 5.20). Roots colonized by *A. muscaria* at 10 and 2.5 g L^{-1} glucose lacked unusual amounts of pigmented material, but at 0 g L^{-1} glucose cell damage and accumulation of pigmented material did occur (Figure 5.17).

Poor infiltration of resin into *T. magnivelare* ectomycorrhizae synthesized at 10 g L^{-1} glucose caused interior portions of most roots to disintegrate immediately upon sectioning. In many cases one or two cortical cell layers and intercellular hyphae remained intact, but host cells were often filled with pigmented material and disintegration of sections seemed more severe in roots containing more of this material. Resin infiltration was adequate at lower glucose concentrations and with *A. muscaria* ectomycorrhizae. Tissue integrity was improved by substituting L.R. White soft grade resin (London Resin Company Ltd.) for Spurr's resin even in heavily

pigmented portions of natural *T. magnivelare* ectomycorrhizae (Lefevre and Molina, unpublished data).

Synthesized *T. magnivelare* ectomycorrhizae display several unusual features including chlamydospores (Figure 5.21), hyphal coils (Figure 5.22), and distorted hyphae (Figure 5.22). These features are observed in *T. magnivelare* MMN agar cultures, but have not been observed in collections of natural *T. magnivelare* ectomycorrhizae (Lefevre and Müller, 1998; Lefevre and Molina, unpublished data). An aroma similar to that of matsutake mushrooms is also detectable from colonized root systems. Rhizomorphs were common on synthesized *T. magnivelare* ectomycorrhizae (Figure 5.23), but unlike field collections, none produced differentiated central hyphae (Lefevre and Müller, 1998). A single *T. magnivelare* primordium showing initial stages of tissue differentiation developed in the rhizosphere of a seedling at 10 g L^{-1} glucose (Figures 5.24 and 5.25).

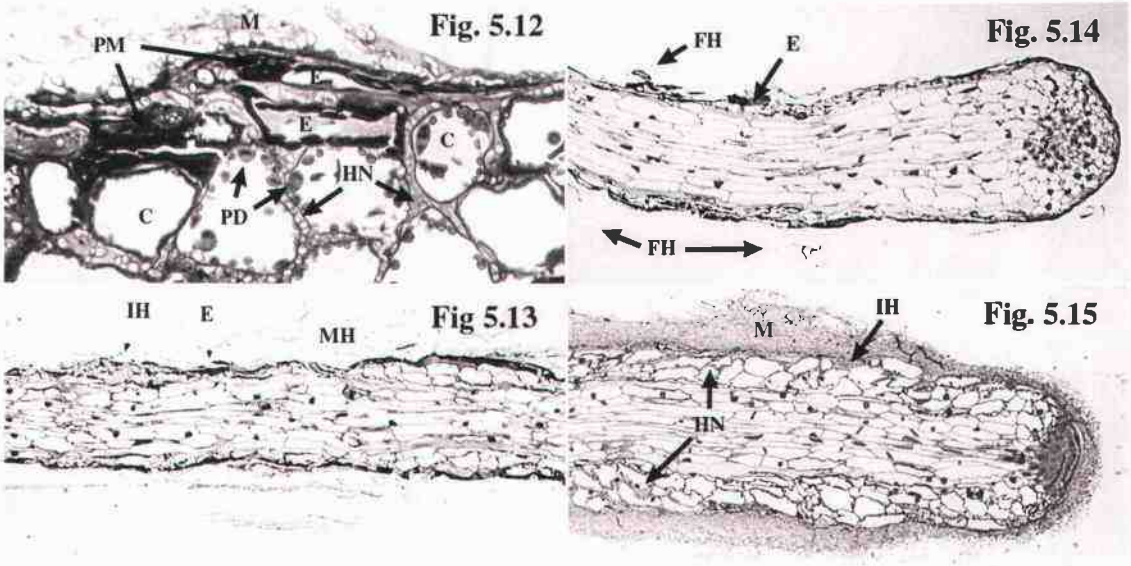
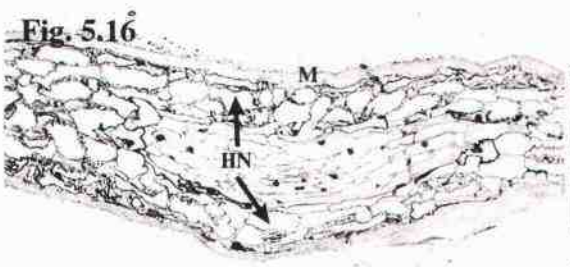
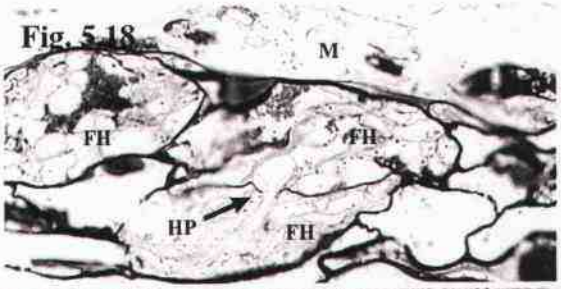
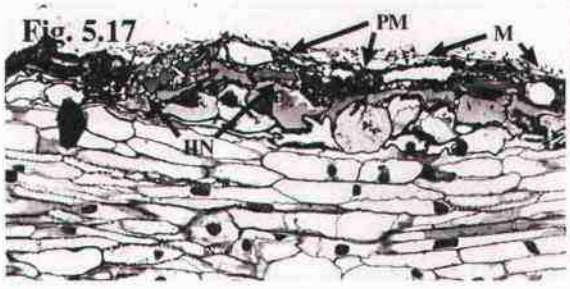
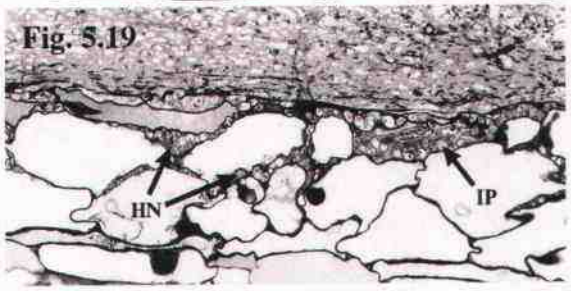


Figure 5.12: Glancing section of a *T. magnivelare* ectomycorrhiza synthesized at 10 g L^{-1} glucose. Pigmented materials (PM) are present in epidermal (E) and cortical (C) cells and polyphenolic droplets (PD) are present within cortical cells opposite the Hartig net (HN). Some epidermal cells have collapsed. Figure 5.13: Longitudinal section of a *P. contorta* fine root colonized by *T. magnivelare* at 2.5 g L^{-1} glucose. This root, like others colonized by *T. magnivelare* at 2.5 g L^{-1} glucose, lacks a Hartig net and mantle hyphae (MH) are sparse. Several host cells contain intracellular hyphae (IH) and many epidermal cells (E) contain pigmented materials. Figure 5.14: Longitudinal section of a *P. contorta* fine root with loosely associated *T. magnivelare* hyphae (FH) grown at 0 g L^{-1} glucose. Many epidermal cells (E) contain pigmented materials and some have collapsed. Figure 5.15: Longitudinal section of an *A. muscaria* ectomycorrhiza synthesized at 10 g L^{-1} glucose showing a relatively thick fungal mantle (M) and well-developed Hartig net (HN). A few host cells near the apex contain intracellular hyphae (IH) visible in serial sections (not shown).









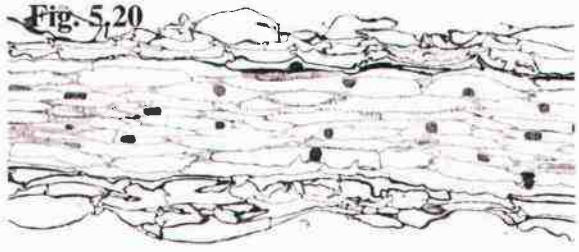


Figure 5.16: Longitudinal section of an *A. muscaria* ectomycorrhiza synthesized at 2.5 g L^{-1} glucose. The fungal mantle (M) and Hartig net (HN) are both well-developed, although the mantle is less so than at 10 g L^{-1} glucose. Figure 5.17: Longitudinal section of an *A. muscaria* ectomycorrhiza synthesized at 0 g L^{-1} glucose. Note the thin fungal mantle (M), presence of Hartig net (HN), accumulation of pigmented materials (PM) and occasional collapse of epidermal cells. Figure 5.18: *Tricholoma magnivelare* ectomycorrhiza grown at 2.5 g L^{-1} glucose showing a fungal mantle (M), but no Hartig net. Note penetration by a hypha (HP) through the cell wall separating two host cells, both filled with fungal hyphae (FH) (shown at lower magnification in figure 13). Figure 5.19: Intracellular penetration (IP) of host cells by *A. muscaria* at 10 g L^{-1} glucose (Shown in figure 14 at lower magnification) distinguished from Hartig net (HN) by observation of serial sections (not shown). Figure 5.20: Longitudinal section of a non-inoculated control fine root showing intact epidermis and cortex without accumulation of pigmented materials.

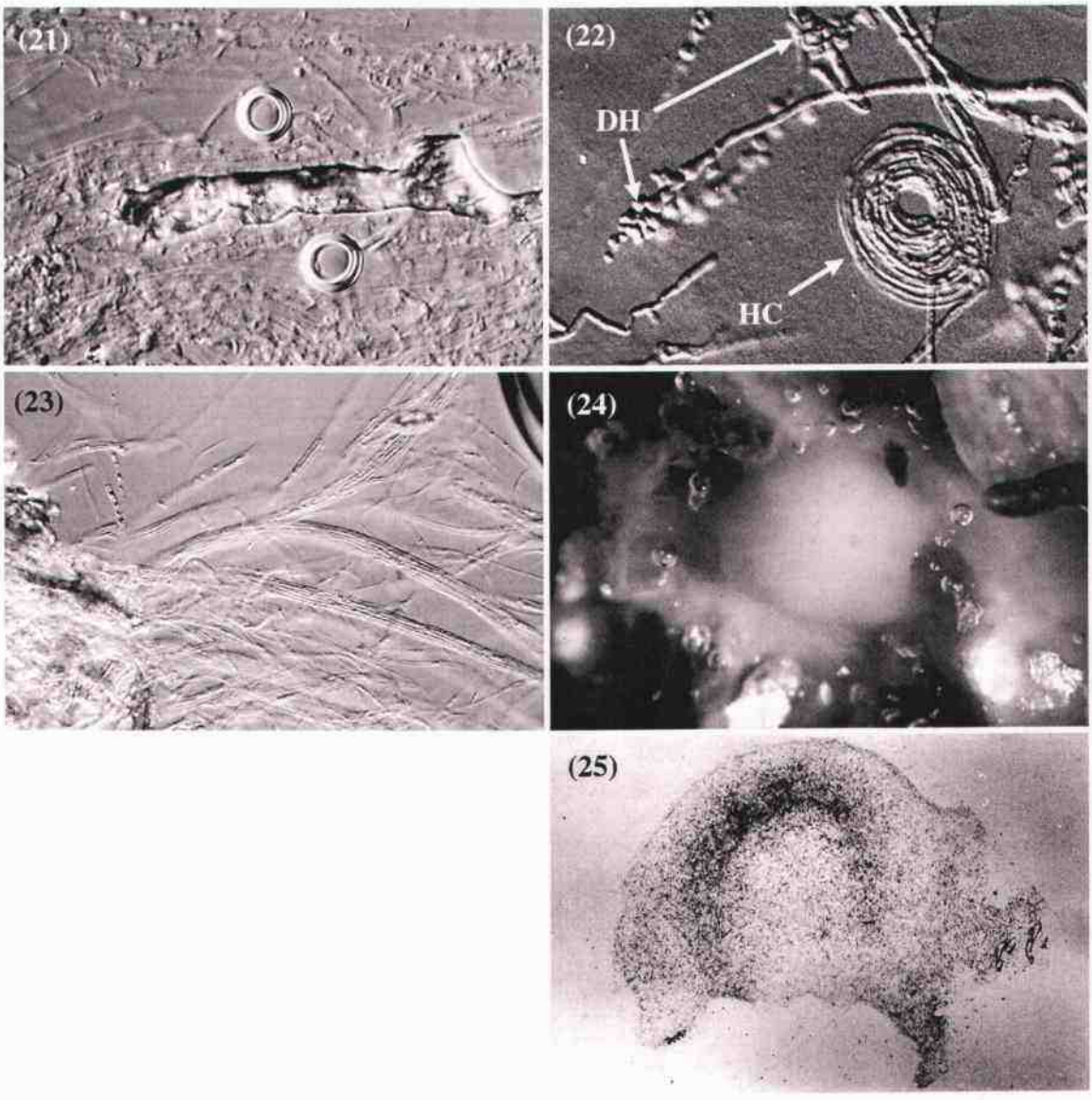


Figure 5.21: *Tricholoma magnivelare* chlamydospores associated with rhizomorph hyphae. Figure 5.22: *Tricholoma magnivelare* hyphal coil (HC) and distorted hyphae (DH) associated with ectomycorrhizae synthesized at 10 g L^{-1} glucose. Figure 5.23: *Tricholoma magnivelare* rhizomorphs associated with ectomycorrhizae synthesized at 10 g L^{-1} glucose. Figure 5.24: *Tricholoma magnivelare* primordium produced in the rhizosphere of a single seedling grown at 10 g L^{-1} glucose. Figure 5.25: Transverse section of the primordium shown in figure 5.22 showing initial stages of tissue differentiation.

DISCUSSION

If *T. magnivelare* is indeed a pathogen it might be expected to affect the growth of its hosts differently than a presumably mutualistic species like *A. muscaria*. In this case, however, no statistically significant differences were observed among trees colonized by *T. magnivelare* and *A. muscaria* in the various biomass measures, biomass allocation between roots and shoots, or stem caliper, with or without an exogenous supply of glucose. Improved nutritional status, particularly in the form of added glucose, enhances virulence of pathogenic fungi (Entry, et al., 1991; Sinclair, et al., 1987; Weinhold, et al., 1969), presumably by facilitating penetration of host defenses (Entry, et al., 1991). Duddridge (1986) recommends against use of glucose in synthesis media because it stimulates ectomycorrhizal fungi to interact more aggressively with host plants. The environment in a synthesis tube is highly artificial and does not necessarily reflect the behavior of these species in the field, but despite conditions in this study known to enhance pathogenic behavior, *T. magnivelare* did not reduce growth of its hosts relative to *A. muscaria*.

The only statistically significant difference in plant growth was increased frequency of lateral root branching on seedlings inoculated with *T. magnivelare*. These results are consistent with the apparent brooming of root systems caused by *T. magnivelare* and related species in the field (Ogawa, 1975, 1979, Ohara and Ogawa, 1982; Terashima, 1993). Other authors have concluded that brooming is evidence of a general stimulation of root growth (Ogawa, 1975, 1979, 1985; Ohara and Ogawa, 1982; Terashima, 1993), but we did not observe differences in root biomass or root to shoot ratio corresponding with differences in lateral root branching.

The two fungi did cause dramatic anatomical differences within fine roots. Apart from expected differences in mantle thickness and depth and regularity of Hartig net penetration, *T. magnivelare* appears to damage root cells and elicit heavy production of tannins or other pigmented materials within the epidermis and cortex (Figure 5.12).

Similar cell damage and host defense responses are typical of natural *T. magnivelare* ectomycorrhizae (Lefevre and Molina, unpublished) as well as ectomycorrhizae of other annulate Tricholomas (Wang, 1995; Gill et al, 2000; Yamada, 1999a). Intracellular colonization of host cells was observed for both fungi in this study, but only in single instances for each species (Figures 5.18 and 5.19). Wang (1995) observed intracellular penetration of hyphae into epidermal and cortical cells of synthesized matsutake ectomycorrhizae, but rarely in natural ectomycorrhizae of the same species. Because intracellular colonization is seen with a wide variety of ectomycorrhizal fungi under natural conditions, particularly in senescent roots (Smith and Read, 1997), and some ectomycorrhizal fungi penetrate host cell walls when supplied with exogenous glucose (Duddridge, 1986), intracellular penetration by *T. magnivelare* in this study likely says little about the normal relationship between host and fungus given the highly artificial conditions. Except for arbutoid and monotropoid types, no similar penetration of host cells was observed in roots of nine host genera colonized naturally by *T. magnivelare* (Lefevre and Molina, unpublished). Ultrastructural analysis is needed to determine whether symptoms of antagonism in matsutake ectomycorrhizae are exacerbated by inclusion of glucose in aseptic synthesis systems. Other components of nutrient media can also influence ectomycorrhizal anatomy as noted by Yamada (1999b).

Unusual features of *T. magnivelare* hyphae including chlamydospores, hyphal coils, and somewhat convoluted and highly branched hyphae could serve as useful characters to distinguish this fungus in culture. Similar features have been observed with other matsutake species. Chlamydospores have been observed in culture and in field collections of other matsutake species (Wang, 1995; Yamada, et al., 1999b; Gill et al., 2000; Terashima, 1993). Hyphal coils were observed for *T. matsutake* by Wang (1995) who speculates that they may serve as nematode traps. The formation of primordia in culture has also been observed for *T. matsutake* (Wang, 1995; Kawai and Ogawa, 1976, 1981; Ogawa and Hamada, 1975), though none have developed into mature sporocarps.

Despite reports of seedling mortality after inoculation with *T. matsutake* (Wang, 1995; Masui, 1927), matsutake species have a reputation for resisting laboratory synthesis attempts (e.g. Kawai and Ogawa, 1981) and display little propensity to colonize seedlings. This could be interpreted as resistance to infection on the part of seedlings or as an inability of seedlings to support matsutake ectomycorrhizae. The mid- to late-seral status of matsutake species is well established (reviewed by Redhead, 1997) and may explain the behavior of these species in synthesis studies. In instances where *T. matsutake* ectomycorrhizae were successfully synthesized either under aseptic conditions or by planting seedlings on the margins of existing colonies, the associations almost always failed to persist after the seedlings were transplanted (Eto, 1990; Kareki and Kawakami, 1985; Kawai and Ogawa, 1981). In the present study, as well as Wang (1995) and Yamada et al. (1999b), attempts to synthesize matsutake ectomycorrhizae only succeeded with glucose in the synthesis medium. Both the history of failure to establish matsutake colonies artificially and the results of the different synthesis methods tested in this study and by Wang (1995) and Yamada (1999b) are entirely consistent with results using other late successional ectomycorrhizal fungi (Fox, 1983, 1986; Gibson and Deacon, 1990; Hutchison and Piche, 1994; Last, et al., 1985; Deacon et al., 1983; Fleming, 1985). Apart from occasional death of seedlings, which could easily occur for other reasons, matsutake species are not known to cause mortality of their hosts, and host trees typically outlive the fungus. The observation that matsutake species appear during a discrete successional stage and are then supplanted by other ectomycorrhizal fungi is more consistent with the behavior of ectomycorrhizae than pathogens.

If *T. magnivelare* is a pathogen, it appears to fit within the category of host-dominated diseases described by Kommedahl and Windels (1979), forming tissue-specific long lasting associations that may cause little harm to the host. On a continuum of pathogenicity these fungi approach a "near-symbiotic" relationship and may only reduce fitness of the host when conditions favor the fungus (Kommedahl and Windels, 1979). However, in spite of the symptoms of antagonism evident in matsutake

ectomycorrhizae, the nature of the relationship should not be viewed outside the context of the demanding edaphic environment where matsutake species occur. In the young, nutrient deficient soils where matsutake species thrive, energetic costs to obtain resources are likely to be high. Signs of physiological stress resulting from greater demands on the host might be expected in association with fungi well adapted to nutrient uptake from such soils. The term 'shiro' used to describe the fairy rings formed by matsutake species is synonymous with the term 'ectomycorrhizal mat' used to describe colonies of fungi in numerous genera including *Gautieria*, *Hydnellum*, *Ramaria*, *Catathalasma* and others (Hintikka and Näykki, 1967; Griffiths and Caldwell, 1992; Ogawa, 1985). Mat-forming fungi are known for their ability to create locally extreme chemical environments by a variety of means where Fe, Ca, P, Mg and other nutrients are liberated from recalcitrant soil substrates (Hintikka and Näykki, 1967; Terashima and Mizoguchi, 1995; Cromack et al., 1979; Griffiths et al., 1994), and some mat-forming fungi, in addition to matsutake species, appear to stimulate host defense responses within ectomycorrhizal rootlets (Malajczuk, et al., 1987; Ogawa, 1985; Agerer, 1996; also discussed by Yamada et al, 1999a). If the characteristics of matsutake ectomycorrhizae that resemble symptoms of disease are common to fungi forming ectomycorrhizal mats the key to understanding matsutake ecophysiology may depend on an understanding of the chemical foraging strategy employed by this diverse array of fungi.

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Chapter 6: Sniffing for Matsutake at Landscape Scales

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ABSTRACT

American matsutake mushrooms provide an important source of income for mushroom harvesters and certain rural communities from Canada to Mexico. In places its commercial value exceeds that of the timber above it, but consideration of matsutake production in forest management decisions is limited by inadequate data. Mushroom surveys are problematic on large scales for a variety of reasons including unpredictable seasonal production, ephemeral fruiting, dramatic variation in annual yields, and unauthorized harvests of edible species. The abundance of perennial mycelial colonies in the soil is largely unaffected by these factors, but laboratory methods to detect soil fungi are also impractical at large scales. This research employed the human sense of smell as a detection instrument for matsutake mycelium in soil, enabling rapid and reasonably accurate data collection from large numbers of samples. In three laboratory studies, trained and untrained volunteers easily distinguished soil samples that contained or lacked matsutake mycelium. After an exercise intended to cause olfactory fatigue, trained volunteers correctly identified 98% of samples. False-positive errors were more frequent with samples that contained mycelium of other fungi. Panelists were more likely to correctly identify matsutake than they were to erroneously detect it in samples containing other fungi. In an 8.3 km^2 field survey mushroom production was correlated with detection rates in soil cores (R^2=0.71), and the sampling process was rapid enough to apply at landscape scales.

INTRODUCTION

Measuring the distribution and abundance of wild mushrooms is essential for both conservation and management of commercial harvests. Research is underway around the world to quantify production of various species. Yet, in spite of occasionally high economic value and growing awareness of their ecological importance, systematic

surveys for wild mushrooms are difficult and seldom attempted at landscape scales. This study focuses on *Tricholoma magnivelare* (Peck) Redhead, a wild edible species found in many forested areas throughout North and Central America (Redhead, 1989; Redhead, 1997; Hosford et al., 1997). It is known by a variety of common names including the American matsutake, pine mushroom, tanoak mushroom, or white matsutake. In the past twenty years, income generated by *T. magnivelare* harvests has contributed substantially to the economies of a number of rural communities from Canada to Mexico. In some areas its commercial value approaches or exceeds that of surrounding timber (De Geus et al., 1989; Pilz et al., 1999). In the interest of capitalizing on the economic potential of forest lands selected for commercial activity while simultaneously maintaining values associated with intact forest ecosystems, *T. magnivelare* production should be accommodated in forest management decisions. To do so, managers would need to locate and quantify *T. magnivelare* production, possibly at the stand or smaller scale of resolution. However, the inherently unpredictable nature of mushroom fruiting presents serious challenges to surveyors.

Mushroom production is typically measured by establishing random plots where each mushroom is counted, measured or weighed. For most species, patchy fruiting increases the number and size of plots required to adequately assess productivity and complicates sampling designs (Thompson, 1992; Vogt et al., 1992; Amaranthus and Pilz, 1996; Pilz and Molina, in press). Because yields can vary two to three orders of magnitude from one year to the next (Amaranthus et al., 2000), studies with numerous large plots designed to capture patchy production in low-yield years can become overwhelming in high-yield years (Pilz et al., 1998; Pilz and Molina, in press). Also, because most mushrooms are ephemeral and the timing and duration of the fruiting season is somewhat unpredictable, thorough surveys require multiple and frequent visits each year for a period exceeding the typical fruiting season. Given large numbers of plots, labor-intensive data collection, and multi-year commitments with multiple visits per year, surveys for individual species are often considered impractical at large scales. Additionally, such surveys are frequently compromised by

unauthorized harvest of commercially valuable species (Pilz et al., 1996; Pilz et al., 1999) and researchers have gone to great lengths to protect study areas including placing study plots in remote areas and seeking assistance from law enforcement authorities (Pilz and Molina, in press).

An alternative to sporocarp collection is to measure the abundance of mycelium in soil. Studies utilizing direct detection in soil could sidestep weather conditions, microsite characteristics, mycophagy and other factors that confound sporocarp surveys. Because many ectomycorrhizal fungi form relatively stable perennial colonies their abundance in the soil may provide a better estimate of typical or potential sporocarp production than can be obtained from several years of actual sporocarp production. Direct detection of mycelium might also address questions regarding the status of fungal colonies: their size, distribution, abundance, and changes over time without inference based on sporocarp production.

Several methods exist to detect specific ectomycorrhizal fungi in soil. These include morphological examination of ectomycorrhizae (Agerer, 1987, 1991; Goodman et al., 1996; Ingleby et al., 1990), immunological probes (Kough et al., 1983; Zambonelli et al.. 1993; Neuner-Plattner et al., 1999), DNA fingerprinting (Gardes and Bruns, 1993; Gardes and Bruns, 1996) and specific DNA probes (Bruns and Gardes, 1993; Rubini et al., 1998; Lee, et al., 1999; Murata, et al. 1999; Dunstan et al., 2000; Kikuchi et al., 2000). These methods potentially eliminate constraints from weather conditions, seasonality, mycophagy, and harvest pressure. They are also essential to confirm the identity of fungi in the absence of reproductive structures. However, because most individual species likely occupy a small proportion of the forest floor even in productive areas, surveys will typically require thousands and perhaps tens of thousands of samples to achieve adequate precision over large areas. Each of the above methods involves some degree of laboratory analysis and typically requires considerable time and expense to process large numbers of samples. Thus, none will simplify mushroom surveys at large scales.

We have taken a different approach. The primary appeal of all matsutake mushroom species is their enticing aroma (Zeller & Togashi, 1934; Smith, 1979; Yajima et al., 1981; Aurora, 1986; Kytovuori, 1989; Hosford et al., 1997; Wang et al., 1997). Components of this aroma are produced by *T. magnivelare* mycelium in soil (Dr. William F. Wood, Humboldt State University, CA, unpublished data) and can be detected in soil cores using the human sense of smell. By using olfaction as a detection instrument one surveyor can collect data from several hundred samples per day. In our experience, morphological or molecular analysis of a similar number of samples would require a month or more with the additional costs of equipment and reagents. While detection of volatile organic compounds could be conducted more precisely using gas chromatography, processing large numbers of samples in the laboratory would be similarly time consuming. Portable instruments could be designed to detect specific volatile compounds produced by matsutake mycelium and they might ultimately provide more precise results than human olfaction. In any case, the ability to quickly process large numbers of samples in the field may, for the first time, provide a cost-effective method to survey for *T. magnivelare* and possibly other matsutake species at landscape scales.

This study addresses two main objectives. The first is to measure rates of detection error in the laboratory with known samples. The second is to determine whether the frequency of detection in soil cores correlates with *T. magnivelare* production in field plots of known productivity.

The known samples used for the first objective are necessary to demonstrate whether humans actually can smell *T. magnivelare* in soil cores and distinguish it from the smells of other fungi. The laboratory studies included in the second objective also measured olfactory fatigue and the performance of panelists screened for olfactory acuity relative to unscreened panelists. The second objective will determine whether detection of mycelium can be used to accurately predict productivity in the field. It will also help to assess the precision of estimates derived from this survey method.

Results of this research will determine whether sniffing for *T. magnivelare* can be an effective survey method and provide data on the sampling intensity necessary to adequately estimate production.

This study is a first step in developing an olfactory survey procedure for *T. magnivelare*. As with other methods applied to resource inventories at large scales, such as the Bitterlich method of variable sized plots used to survey timber (Bitterlich, 1947), this method may overcome inherently high variability by providing practical means to obtain large sample sizes.

METHODS

Laboratory tests

The laboratory portion of this study consisted of three tests. In each test, volunteer panelists were asked to identify soil samples that either contained or lacked *T. magnivelare* mycelium. The "positive" samples (containing *T. magnivelare* mycelium) were collected directly beneath either *T. magnivelare* sporocarps or beneath stalks of the achlorophyllus plant *Allotropa virgata* Torrey & Gray *ex* Gray. This plant forms mycorrhizae specifically with *T. magnivelare* (see Chapter 3). When *A. virgata* was used to locate the fungus, the soil samples were checked to confirm the presence of its roots. Within *T. magnivelare* colonies other ectomycorrhizal fungi are rarely observed (Lefevre and Müller, 1998).

Sporocarps and *A. virgata* stalks provided convenient markers to locate positive samples. It was more difficult with "negative" samples to confirm the absence of *T. magnivelare*. Fortunately, *T. magnivelare* forms ectomycorrhizal mats characterized by dense mycelial colonies with visible boundaries in the soil (Ogawa, 1979; Griffiths

and Caldwell, 1992). Soil within a colony has a whitish cast compared with soil outside of the colony. The abrupt edge between colonized and non colonized soil allowed us to obtain most negative samples by collecting 10-20 cm outside colony boundaries. Some negative samples were collected beneath sporocarps of other mat-forming fungi including *Ramaria* spp. and *Tricholoma focale* (Fr.) Ricken. Both are common in our study area and *T. focale* is the closest relative to *T. magnivelare* found in Central Oregon.

Perhaps the closest relative of *T. magnivelare* in the Western Hemisphere is *Tricholoma caligatum* (Viv) Ricken. Unlike *T. focale*, one variety of *T. caligatum* produces a matsutake-like aroma that might easily be confused with that of *T. magnivelare* (Smith, 1979). Although not found in central Oregon, it is found in other parts of Oregon and throughout much of North America. Because it is not found locally and it is generally uncommon where it does occur in the Western United States we were unable to include it in this study.

In all of the laboratory tests, panelists commented on the qualities and intensities of smells they detected in the soil samples. These comments were not rigorously solicited, but they were nevertheless useful for understanding differences in results among the different tests. The lead author administered the laboratory tests and references to panelist comments are his informal tally.

Triangle tests

The first laboratory study was a series of triangle tests designed to measure the abilities of untrained panelists to distinguish the smells of positive and negative samples. A triangle test consists of three samples, two that are identical and one that is different. The identical samples were taken from one soil sample. In all, six different pairs of positive and negative soil samples comprised six triangle tests. In

three tests the "different" sample was positive and in three it was negative. Positive and negative samples were presented in all possible orders. Each panelist was randomly assigned to one of the six triangle tests with instructions to identify the sample that smelled different than the other two. Samples were presented in foil covered wine glasses. Because colonized soil is typically lighter colored than noncolonized soil, cotton batting was placed over the samples to eliminate visual cues. A total of 96 panelists participated in the study – sixteen assigned to each of the six triangle tests. The soil samples were collected in mid-June and used in the triangle tests within three days. Positive samples were collected beneath *A. virgata* stalks. The data were analyzed with a Chi-square test of the null hypothesis that untrained panelists are unable to smell differences between positive and negative samples. In tests where no detectable differences exist there is a one-third probability of choosing any particular sample.

Sample series tests

We performed two tests in which panelists were asked to sniff a series of samples scoring them as a "hit" if they smelled *T. magnivelare* or a "miss" if they did not smell matsutake. We will refer to the first as the "September" test and the second as the "October" test. These tests were designed to assess error rates by panelists exposed to a large number of samples where olfactory fatigue might influence results. The samples were presented in 50 ml beakers capped with foil. Each beaker contained approximately 20 ml of loose soil. The panelists were briefly trained to distinguish smells of positive and negative reference samples. Additionally, they were told that in the field the proportion of positive samples is typically small, but they were not told how many positive samples they would encounter in the beakers. They were asked to work through the samples smelling each and identifying the positive samples. All of the samples were assigned random three digit numbers to identify them in a way that

would not introduce bias. Panelists were allowed to refer back to the reference samples (just as field surveyors might carry reference samples).

Two types of detection error are possible corresponding with Type I and Type II error: detecting *T. magnivelare* in samples where it is not present (false-positive) and failing to detect *T. magnivelare* in samples where it is present (false-negative). Where the numbers of positive and negative samples are unknown, Bayes' theorem (Mood, 1974) can be used to estimate the likelihood that positive and negative responses are correct given some estimate of the false-positive and false-negative error rates.

$$P(\mathrm{M}\mid +)=\frac{P(+\mid \mathrm{M})P(\mathrm{M})}{P(+\mid \mathrm{M})P(\mathrm{M})+P(+\mid \mathrm{A})P(\mathrm{A})}$$

In the above formulation of Bayes' theorem, + indicates a hit, M indicates that *T. magnivelare* is present and A indicates that *T. magnivelare* is absent. The critical question in field surveys for *T. magnivelare* is to determine the proportion of observed hits that are actually false-positive errors, and by re-writing the above formula, the proportion of misses are actually false-negative errors. Data from the September and October tests were used to estimate these error proportions.

September test

The September test consisted of 55 soil samples: five positive and 50 negative. The samples were collected early in September near the beginning of the mushroom season. All of the positive samples were collected beneath *T. magnivelare* sporocarps. Twenty-nine panelists evaluated all 55 samples

October test

Panelists in the October test were presented with two sets of samples. The first set was intended as training or practice for panelists to familiarize themselves with the process, to allow their olfactory system to adapt to the smells, and to cause olfactory fatigue. The second set was intended to allow measurement of error probabilities after the panelists had adapted to the smells and the process.

Both sets of October samples were presented to two groups of volunteer panelists. The first group consisted of 25 unscreened volunteers. The second group consisted of 12 panelists screened for high olfactory acuity from the 29 volunteers who participated in the September test. The screening criterion was simply to include only panelists who correctly identified all of the samples in September. Both groups were made up of University students, faculty and staff.

Six positive and 24 negative samples, collected in late October (near the end of the mushroom season), were subsequently divided into identical portions: two for each positive sample and four for each negative sample. The beakers containing the samples were then separated into two sets, each set containing one portion of each positive sample and two replicate portions of each negative sample. To avoid bias from identical numbers of samples, one positive and three negative samples were randomly removed from the first set. Thus, the first set consisted of 50 samples, five positive. The second consisted of 54 samples, six positive. The sets consisted of nearly identical samples to minimize variability resulting from different soil cores.

Seven of the 24 original negative soil cores included mycelium of other ectomycorrhizal mat-forming fungi. Four were collected beneath *T. focale* sporocarps and three were collected beneath *Ramaria* spp. sporocarps. Like the other negative soil cores, these cores were split into four portions and divided between the sets giving each set a total of eight *T. focale* samples and six *Ramaria* spp. samples.

Both groups of panelists were briefly trained to identify the smell of positive and negative reference samples. For the screened panelists this exercise was simply to refresh their memories of the smells. All panelists worked through the first set of samples before beginning on the second set. Within each set, panelists were allowed to start with any sample, but they were asked to record the order that they proceeded.

False-positive errors were tallied over both sample sets and plotted as a function of the number sniffed for the 16 panelists who indicated the sequence that they worked through the samples. Groups of 13 samples were combined to eliminate values of zero.

Chi-square tests were performed using data from the combined data sets and from both *T. focale* and *Ramaria* spp. samples to test 1) whether false positive error rates from ordinary negative samples differed from false positive error rates from the other mat-forming fungi, and 2) whether false positive error rates from the other mat-forming fungi differed from positive response rates from positive samples.

Field test

Field sampling was conducted in Central Oregon, USA on the Chemult Ranger District of the Winema National Forest. The study site consists of three adjacent 2.78 km^2 sections placed in an area known for high *T. magnivelare* production. Each section contains three randomly placed large (five ha) plots, which each contain six randomly placed 50 m x 2 m permanent strip plots (nine total large plots and 54 total strip plots). Mushroom surveys occurred twice weekly over the duration of four fall seasons (approximately two months each) between 1993 and 1996. On each strip plot, the cap diameter of every sporocarp was measured to estimate fresh weight (log (fresh weight(g)) = -2.62 (SE: 0.43) + 2.29 (SE: 0.24) * log (cap diameter (mm)), $R^2 = 0.86$; Pilz et al., 1999). Mushrooms were left in place to minimize disturbance, and were

marked to avoid repeat measurements. By measuring every mushroom when it was first located the survey assessed "biological productivity" (Pilz et al., 1999). This differs from commercial productivity because pickers probably miss or ignore a portion of the crop (Pilz et al., 1999) and it is not an estimate of maximum biomass because mushrooms were typically measured before reaching full size.

In late October, 1998, soil samples were taken using long handled garden bulb planters at one meter intervals along both sides of each strip plot. The samples were taken 10 cm outside the strip plots (and promptly replaced) to avoid disruption of the ongoing productivity study. Immediate detection of the *T. magnivelare* aroma was recorded as a hit; otherwise the sample was scored as a miss. A single surveyor conducted most of the sampling. Hit frequency data were summed across the six strip plots within each large plot. The relationship between hit frequencies and average annual productivity was determined by regression. Log transformation was necessary to address skewed variability in the untransformed data. Because the three sections of the study area are at slightly different elevations early models included section as a covariate, but neither section or its interaction with average percent hits were significant at the $\alpha = 0.05$ level in F-tests and were removed stepwise.

RESULTS

Laboratory tests

Triangle tests

Eighty five of the 96 panelists (89%) who participated in the triangle tests correctly identified the odd sample, where only 32 correct responses (33%) would be expected

if there were no detectable differences ($p<0.001$, $\chi^2=263$, df=1). Many panelists commented that the difference was obvious and that the positive samples smelled "fruity", "sweet" or "fungal", whereas negative samples had little or no noticeable smell. A small number of panelists had difficulty distinguishing the smells and a few found the smell unpleasant.

September test

Nineteen of the 29 panelists who participated in the September test correctly identified all of the samples. Data from two panelists were excluded from the analysis. One panelist misunderstood directions and the other was unable to detect positive samples. The ability to distinguish positive and negative samples would be a basic criterion for selection of field personnel and this panelist would not have passed an initial screening. Only one other panelist made a false-negative error. The explanation given by that panelist for missing that sample was that its smell was much stronger than the other positive samples. Apart from the two excluded panelists, eight panelists made between one and four errors, averaging 1.9 errors each. Seven panelists made false-positive errors. Error rates after excluding the two panelists are shown in Table 6.1. Overall, panelists correctly identified the samples in 99.% of cases. The average probability of making a false-positive error on an individual negative sample was 0.01. The average probability of making a false-negative error was 0.0075. Using Bayes' theorem, the probability that samples recorded as hits actually contained *T. magnivelare* was $P(\mathrm{M} \mid +) = 0.91$. Similarly, re-writing the equation for negative samples, the probability that a sample scored as a miss actually lacked *T. magnivelare* was $P(\mathrm{A} \mid -) = 0.999$.

Response	Positive samples	Negative samples
Hit	0.99	0.010
Miss	0.0075	0.99
Overall Correct Responses = 99%		

Table 6.1: Response rates for the September samples series.

Many panelist commented that the difference in smells was obvious or that most of the positive samples had a strong smell whereas most negative samples had little or no smell. Some also commented that smells of different positive samples had different qualities and intensities. A few panelists expressed a lack of confidence in their ability to smell the positive samples. These panelists nevertheless correctly identified all of the positive samples, but appeared more likely to falsely detect *T. magnivelare* in samples that lacked it.

October test

Rates of correct responses in the October test are shown in Table 6.2. False-positive and false-negative error rates are shown graphically in Figure 6.1. Overall response rates are shown in Table 6.3.

	Set 1	Set 2	Overall
Inexperienced Group	91%	94%	93%
Experienced Group	98%	98%	98%

Table 6.2: Rates of correct sample identification for the October test

Figure 6.1

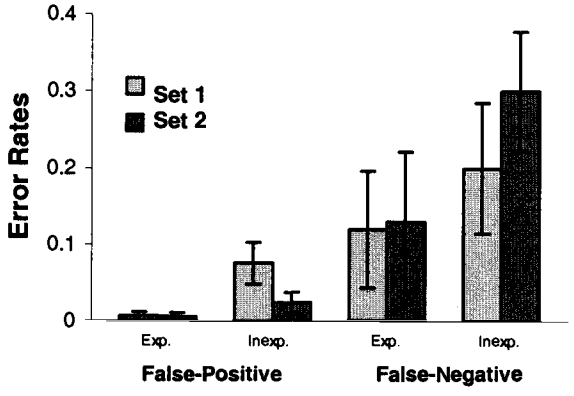

Figure 6.1: Error rates in the October test with 95% confidence intervals.

Group	Response	Set 1 Samples		Set 2 Samples	
		Positive	Negative	Positive	Negative
Experienced	Hit	0.88	0.0056	0.88	0.0052
	Miss	0.12	0.99	0.12	0.99
Inexperienced	Hit	0.80	0.076	0.70	0.024
	Miss	0.20	0.92	0.30	0.98
Combined	Hit	0.83	0.053	0.76	0.019
	Miss	0.17	0.95	0.24	0.98

Table 6.3: Response rates for the October test

Results from Bayes' theorem are shown in Table 6.4. Probabilities that samples scored as hits actually contained *T. magnivelare* mycelium were higher for the experienced group in both sample sets. Probabilities that samples scored as misses actually lacked *T. magnivelare* mycelium were slightly higher for the experienced group.

Group	Hits		Misses	
	Set 1	Set 2	Set 1	Set 2
Experienced	0.95	0.95	0.99	0.99
Inexperienced	0.53	0.78	0.98	0.96

Table 6.4 Probability that responses were correct on the October test.

On the whole, scores were lower in October than in the September. Only 16% of panelists (6 of 37) scored perfectly on the first set in October while in September 70% of panelists (19 of 27) scored perfectly. Five of the six perfect scores in October came from screened panelists. Most comments on the October test suggested that the positive samples were "faint" or "weak" or otherwise difficult to detect while fewer indicated that they were "strong" or "obvious".

The other mat-forming fungi were more likely to be confused with *T. magnivelare* than other negative samples, but they were nevertheless easily distinguished from samples actually containing *T. magnivelare* mycelium. Panelists were more likely to falsely detect *T. magnivelare* in samples containing the other mat-forming fungi than they were to falsely detect it in ordinary negative samples. The allocation of false-positive errors depended on the identity of the samples (χ^2=32.1, df=1, p<0.001). In spite of the higher positive response rates with *T. focale* and *Ramaria* spp. samples, panelists easily distinguished *T. magnivelare* samples from samples containing the other mat-forming fungi. The allocation of hits was clearly dependent on whether the sample was one of the other mat-forming fungi or whether it actually contained *T. magnivelare* (χ^2=776, df=1, p<0.001).

As panelists sniffed more samples they were less likely to erroneously detect matsutake (Figure 6.2). Because positive samples were represented in low numbers

we were unable to obtain sufficient data to construct a similar curve for false-negative rates.

Figure 6.2

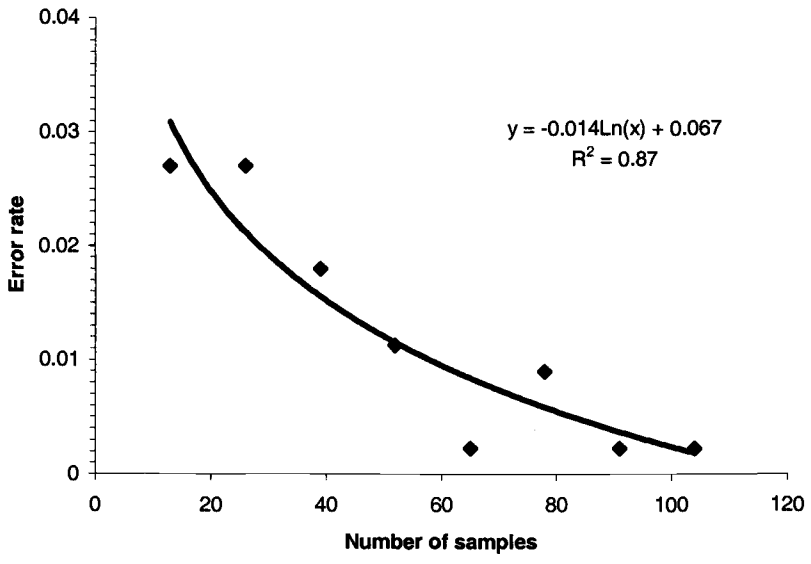

Figure 6.2: Change in false-positive responses as a function of the number of samples sniffed measured over intervals of 13 samples.

Field study:

The field survey required approximately 9 person-days including time spent locating and measuring plots, sampling, and recording data.

Sixteen of the 54 strip plots failed to produce mushrooms between 1993 and 1996. Of these, seven produced zero hits and nine produced an average of 2.0% hits. Four strip plots that did produce mushrooms failed to produce hits. The maximum hit frequency for any strip plot was 7.8% and the maximum hit frequency for any large plot averaged over the six strip plots was 4.3%. The hit frequency for the study site as a whole was 2.1%.

Average annual *T. magnivelare* productivity was strongly related to average hit frequency in the plots ($F_{1,7} = 13$, p-value = 0.0046, R^2=0.71, log (average annual productivity of plots (Kg/ha)) = 0.23 (SE=0.28)+ 0.49 (SE=0.12)* % hits), suggesting that the area of the soil occupied by *T. magnivelare* colonies explains a substantial part of the variability in production among plots (Figure 6.3).

Figure 6.3

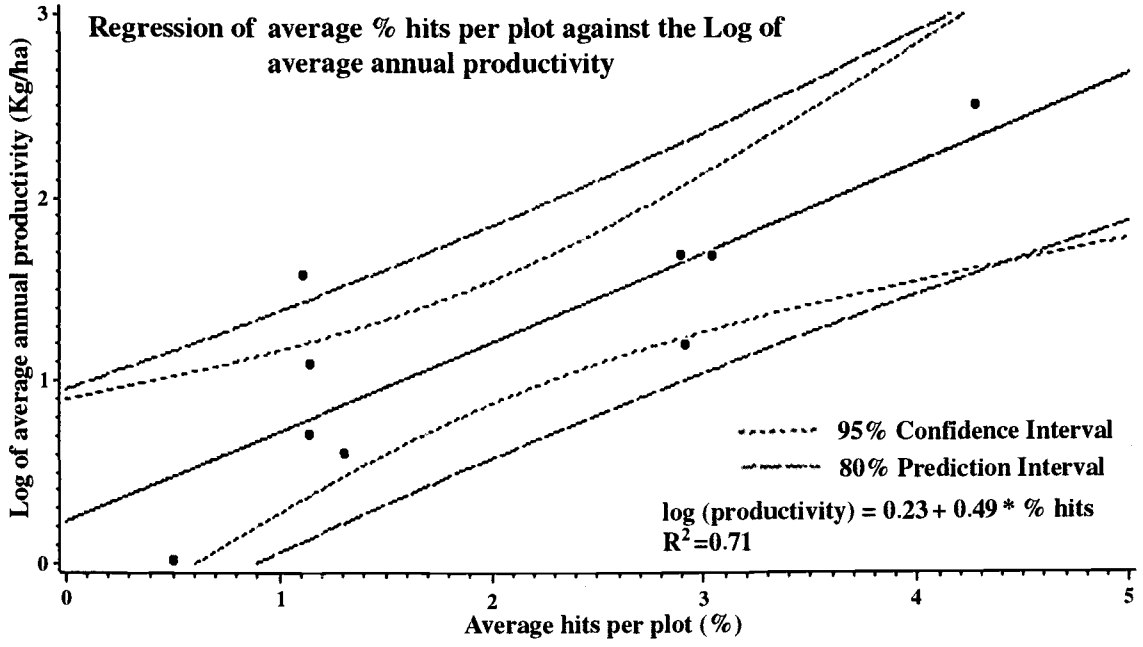

Figure 6.3: Regression of log average annual productivity as a function of hit frequency for each of the large plots including the 95% confidence interval and the 80% prediction interval.

DISCUSSION

Our laboratory and field results suggest that olfaction is an effective detection method for *T. magnivelare* mycelium in soils. The aroma of *T. magnivelare* mycelium was

easily detected by untrained panelists who correctly identified samples nearly 90% of the time. Panelists who were minimally trained or screened for high olfactory acuity correctly identified samples more than 90% of the time (Tables 6.1 and 6.2). In the field, olfactory detection explained 71 % of the variability in mushroom production among plots (Figure 6.3), despite several known sources of error in the soil sampling and mushroom collection data. That *T. magnivelare* can be distinguished from other fungus smells is demonstrated by the field results where other fungi were frequently encountered, and by successful discrimination between *T. magnivelare* and other mat-forming fungi in the laboratory.

An initial concern in the planning stages of this study was the possibility that olfactory fatigue and adaptation could limit the capacity of surveyors to accurately evaluate large numbers of samples. The relatively strong correlation between olfactory detection of *T. magnivelare* colonies and mushroom production in the field survey after sniffing approximately 600 samples per day allayed these concerns. In addition, we observed a decline in false-positive error rates as the number of samples sniffed increased (Figure 6.2). Two factors likely contributed to this decline in error. First, our panelists undoubtedly became more discerning as they worked through the samples, making fewer errors as they progressed. Second, fatigue and adaptation likely impaired the sensitivity of panelists to other smells in the samples that could be confused with *T. magnivelare*. Thus, by simultaneously reducing both sensitivity to *T. magnivelare* and sensitivity to other smells, the olfactory impairment resulting from fatigue and adaptation may have both detrimental and beneficial effects. Reduced sensitivity to *T. magnivelare* decreases signal while reduced false-positive error decreases noise. The relative weights of these effects require further study to determine whether precision and accuracy increase or decrease as a result. The decrease in false-positive error illustrated in Figure 6.2 appears to approach stabilization near the end of the test, suggesting that the factors leading to the decline are eventually overcome. Stabilization of sensitivity to *T. magnivelare* can also be hypothesized by the strong correlation with mushroom production observed in the

field study, although we did not determine how much exposure to the smells is necessary to overcome the initial conditions.

Decreased false-positive rates as well as superior performance by panelists screened for olfactory acuity produce marked improvements in the likelihood that *T. magnivelare* is actually present in samples in which it is detected, particularly when the probability of encountering *T. magnivelare* is low (Figure 6.4).

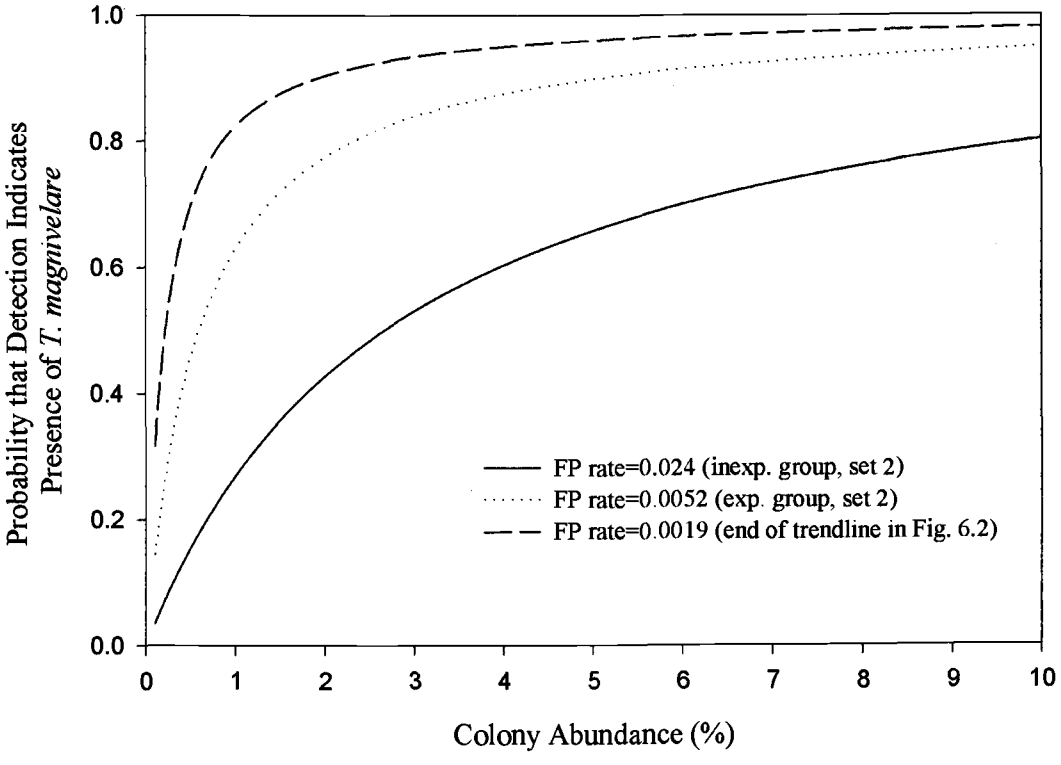

Figure 6.4: Probability that detection indicates presence of *T. magnivelare* as a function of hypothetical colony abundances at false-positive error rates observed for the inexperienced and experienced groups in set 2 of the October test and at the end of the trend line in Figure 6.2.

The decrease in false-positive error is particularly significant considering the low frequency that *T. magnivelare* is actually encountered in the field. When *T.*

magnivelare colonies are rare, even small numbers of false-positive errors can make up a large proportion of overall detections. Thus, even a small decrease in false-positive error rates can significantly improve the likelihood that samples where T. magnivelare is detected actually contain *T. magnivelare*. For example, Figure 6.4 shows that at 2% colony abundance (similar to our field results), the 0.0058 decrease in the probability of false positive errors between sets 1 and 2 created a 19% increase in the likelihood that samples actually contained *T. magnivelare* when they were scored as hits. By the end of the trend line the error rate had fallen by 0.0087 from the average of set 1 with a corresponding 30% increase in the likelihood (0.63 to 0.90) that samples scored as hits actually contained *T. magnivelare* mycelium. Our laboratory tests did not involve enough samples to determine whether, or at what level the false-positive error rate stabilizes, but it appears to continue decreasing beyond the point that we measured, suggesting that false-positive errors may become a minor source of variability even at relatively low *T. magnivelare* colony abundance.

Screened panelists were consistently more accurate than unscreened panelists, suggesting that olfactory abilities vary and that rigorous training and screening of surveyors, even beyond the simple training and screening procedures employed in this study might further reduce detection error. These laboratory results suggest that screening may have improved error rates in the field where no such screening procedure was used to select the lead surveyor.

Other sources likely contribute as much or more to overall variability in the field. Because our field survey was ancillary to an ongoing productivity study we were unable to collect soil cores within the actual strip plots where mushroom productivity was measured, and resorted to sampling approximately 10 cm outside their boundaries. Our survey was based on the assumption that hit frequencies outside the strip plots would be spatially correlated with colony and sporocarp abundances within the strip plots, although this was undoubtedly a source of error. Further, our productivity data were between two and six years old at the time of the soil sampling.

The forest on the site is approximately three hundred years old and is not changing rapidly; and *T. magnivelare* colonies may live for several decades (Ogawa, 1975, 1979). Nevertheless, more concurrent productivity data might better correlate with hit frequencies. Our study also suffered from mycophagy and unauthorized harvest of mushrooms, as in the study by Pilz et al., (1999) that analyzed data from the same site. Weather conditions during the years sporocarp productivity data were collected may be another source of variability. The years 1993 through 1996 were moderate to poor years for *T. magnivelare* production. Variability due to microsite characteristics might be reduced during the infrequent high-yield years in which a larger proportion of the colonies on a site may experience the conditions they require to produce sporocarps. Finally, toward the end of our field survey the temperature fell as low as -13° C and a crust of ice had cemented the top few centimeters of soil. The smell of *T. magnivelare* was detected in soil cores under these conditions, but surveyor sensitivity could easily have been affected.

Taken together, the factors listed above could contribute substantial variability to our results. The regression in Figure 6.3 includes those error sources as well as random error from both the sporocarp survey and the soil survey. Surprisingly, only 29 % of the variability in sporocarp production remained unexplained in the regression against detection frequency.

Speed and simplicity are the primary advantage of this method. The survey described here required nine person-days to process approximately 5500 samples. The study plots covered a total of 45 ha, from which inference is extended to an area of 8.3 km^2. A crew of two working with the same sampling design and at the same rate over the course of a three month field season would cover 6.5 km^2, allowing inference to an area of 120 km^2. Surveys conducted on a systematic grid might better capture the typical patchiness of *T. magnivelare* distribution than the plot design in this study. Also, sampling intensity could easily be increased to improve precision over smaller areas, stratification could be employed to account for habitat variation, geostatistical

approaches could be tested to predict productivity of non-sampled areas and adaptive designs could be used to better account for patchy distribution (Thompson, 1992).

An additional concern not explored in this study is the possibility of seasonal variation in the intensity and quality of smells associated with *T. magnivelare* colonies. Our only evidence for such variation is the informal tally of comments made by panelists during the three laboratory studies that took place in June, September and October. In both June and September panelists frequently reported "strong" or "obvious" differences, whereas more "weak" and "faint" comments occurred in October. Comments on the nature of the *T. magnivelare* aroma in soils also differed with more describing a "stale" quality in October in contrast to descriptions like "sweet", "fruity" and "fungal" in June and September. Declining intensity in the aroma of *T. magnivelare* mycelium corresponding with seasonal changes could explain the higher rates of failure to detect *T. magnivelare* by inexperienced panelists in late October than in September (Tables 6.1 and 6.2).

If the aroma intensity undergoes an autumnal decline, our field survey conducted in late October may reflect more error than it might have earlier in the year. Reduced detectability would have the effect of reducing signal against the noise of false-positive responses, resulting in a weaker correlation between colony detection and mushroom production. Had we performed the survey between June and September we may have reduced the prediction interval in Figure 6.3, allowing greater precision in our ability to predict production based on detection frequencies.

This survey method is potentially useful to quantify colony abundance anywhere that matsutake species occur. However, relationships between mushroom production and colony abundance likely vary with climatic and edaphic conditions and mushroom surveys may be necessary to calibrate the soil surveys in different environments.

A useful application of this method would be systematically mapping productive areas. Mapping is possible using sporocarp occurrences, but it is complicated by the same factors that confound productivity surveys: seasonality, unpredictable timing and abundance of fruiting, the fact that not all patches fruit equally well in a given year, and competition from commercial harvesters and animals. Soil sampling allows direct quantification of colony abundance free from these confounding factors.

Such mapping of patches would make it possible to determine precisely where logging, road building and other disturbances would impact *T. magnivelare* production. Mushrooms are generally concentrated in patches rather than evenly dispersed across the landscape. Although Pilz et al. (1999) found that the value of *T. magnivelare* on our study site as a whole was somewhat less than that of the timber, 56% of the production occurred on 17% of the transects. They did not address economic implications of patchy distribution, but their results suggest that the economic value of *T. magnivelare* production was substantially greater than that of the trees within productive patches. Because production is patchy, impacts are determined more by where disturbances occur than by the size of the disturbed area. The ability to map patches may allow substantial protection of *T. magnivelare* production with minimal effect on other forest uses.

Detection of the fungus in soil samples also may lend itself to monitoring the status of populations over time. Because measurement of typical sporocarp production requires years of data, population changes may require decades to observe. Population monitoring may be particularly useful for habitat manipulation studies, allowing distinctions between effects resulting from changes in microclimate on existing colonies and changes in the actual number of colonies. The ability to monitor populations would have improved the habitat manipulation study described by Weigand (1998), which did not include baseline data because of the time and expense required to collect it.

Developing the procedure described here into a survey method will require a screening procedure similar to the sample series tests performed in this study to select survey personnel. It will also require assessment of the method's reproducibility in the field using different surveyors and possibly the calibration or calculation of error coefficients for individual surveyors to avoid systematic error in the explanatory variable. Hand-held gas detectors currently used to hunt for culinary truffles (Talou et al., 1988) may provide an alternative to reduce the potential for error in the explanatory variable.

The results of this study suggest that human olfaction is a useful tool to survey for *T. magnivelare*. The correlation between detection frequencies and mushroom production is reasonably strong and several potentially important sources of variability in this study can be eliminated in future surveys. Larger sample sizes and other sampling designs can also easily be employed to reduce variability. Because sniffing for *T. magnivelare* is dramatically faster and cheaper than mushroom collection or soil assays using molecular probes, it may for the first time allow surveys for *T. magnivelare* at landscape scales.

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Chapter 7: Conclusion

Tricholoma magnivelare clearly forms ectomycorrhizae with its hosts. On several hosts the mantle and Hartig net are poorly developed, but they are always present. *Tricholoma magnivelare* forms arbutoid and monotropoid mycorrhizae on members of the Ericaceae, often intermingled with ectomycorrhizal roots that are likely colonized by the same fungal individual. These observations support the suggestion that arbutoid and monotropoid mycorrhizae are sub types of ectomycorrhizae (Molina and Trappe, 1982a).

However, despite its normal ectomycorrhizal anatomy, *T. magnivelare* may not fit a functional definition of ectomycorrhizae that includes the concept of mutual benefit (i.e. Trappe, 1996). Roots colonized by *T. magnivelare* frequently contain accumulations of pigmented materials in the epidermis and cortex, dead epidermal and cortical cells, and on many roots the epidermis and cortex blacken and slough off. Colonized root systems also become bushy (described by Ogawa (1979) as witches brooms), and roots colonized in pure culture branched more frequently than the roots of seedlings inoculated with *A. muscaria*. These observations are consistent with some symptoms of pathogen infection, but no other evidence supports the idea that *T. magnivelare* and other matsutake species are pathogens. In the pure culture synthesis experiment detailed in chapter five, no differences were observed in several growth parameters including total dry weight and root to shoot ratio of seedlings colonized by *T. magnivelare* and *A. muscaria* and neither fungus species caused seedling mortality. Pathogens do not necessarily reduce the size of their host or cause mortality and these results therefore do not rule out the possibility that *T. magnivelare* harms its hosts. However, the distress symptoms associated with matsutake species are observed in a variety of other mat-forming fungi and may be a product of the chemical environment produced by the fungi (i.e. Cromack et al. 1979) or of the energy-intensive process of obtaining nutrients from the infertile soils where these fungi predominate. Mat

forming fungi are known for their ability to weather mineral nutrients from recalcitrant soil substrates (Griffiths and Caldwell, 1992) and this process may place high energy demands on the host. Thus, the symptoms of antagonism in associated roots raise the possibility that matsutake species are weak pathogens, but this evidence is not sufficient in itself to indicate that these fungi are taking more than they are giving.

Allotropa virgata, on the other hand, is clearly deriving all or most of its resources from *T. magnivelare* and it seems unlikely that the fungus receives benefit in return. Based on the results of chapter 3, *A. virgata* appears to specialize on *T. magnivelare*, although another basidiomycete was present in a significant number of collections taken throughout the sampling range and it is not clear what form of relationship exists between this unknown species and *A. virgata*. In all cases where the unknown fungus was present, *T. magnivelare* was also present either in roots from the same soil core or on collections taken nearby at the same sampling sites. The unknown fungus also did not form mycorrhizal structures distinct from those of *T. magnivelare*, at least in the samples examined for this study. The unknown species is a member of the gilled fungi, and not a member of the Amanitaceae or the genus Tricholoma. The possibility that this fungus is another mycorrhizal symbiont cannot be ruled out, but it could also be a pathogen or saprotroph. Overall, throughout the studies comprising this dissertation, roots were sampled from approximately 200 *A. virgata* plants, many used to locate *T. magnivelare* colonies when mushrooms were not fruiting. In every case when *A. virgata* roots were present in a soil sample, the soil possessed all of the characteristics of a *T. magnivelare* colony including whitish, dry, hydrophobic soil, the smell of matsutake mushrooms and the presence of ectomycorrhizae morphologically consistent with *T. magnivelare*. The presence of *T. magnivelare* was also confirmed by molecular methods on the roots of overstory trees in many samples located using *A. virgata* for chapter 4. It seems unlikely, therefore, that this unknown fungus serves the same role as *T. magnivelare* in the life cycle of *A. virgata*. However, the fact of its co-occurrence with *A. virgata* throughout at least 60% of its geographic range suggests that some form of specialization may exist between these two species.

In addition to *Allotropa*, *T. magnivelare* ectomycorrhizae were found on hosts in eight other genera distributed among the Pinaceae, Fagaceae and Ericaceae. These families and genera represent the range of hosts observed in association with *T. magnivelare* in Oregon. Other potential host genera that may exist in similar habitats with *T. magnivelare* in Oregon are *Picea* and *Larix*, although no sites were located containing both *T. magnivelare* and either of these genera. Outside of Oregon *T. magnivelare* is found in forests containing *Picea* spp., *Quercus* spp. and other ectomycorrhizal angiosperms.

The anatomies of *T. magnivelare* mycorrhizae correspond with the normal types formed by each host group. Members of the Pinaceae and Fagaceae form the gymnosperm and angiosperm variants of ectomycorrhizae, respectively, and members of the Ericaceae form arbutoid and monotropoid mycorrhizae. Mycorrhiza branching morphologies are also influenced by the host, although not completely controlled by the host. The characteristic branching morphologies of some host species, such as the monopodial pinnate systems common on *Pseudotsuga menziesii*, were observed on those hosts, but *T. magnivelare* shows its influence in the preponderance of irregularly ramifying systems. One exception to the generally irregular systems was *P. ponderosa*, which consistently formed bifurcate ectomycorrhizae.

Elements of the ectomycorrhizae controlled entirely by *T. magnivelare* include the mantle and emanating hyphae. These structures were consistent across all hosts. Features of the fungus that were only observed in culture include formation of hyphal coils and chlamydospores. The coils shown in chapter five resemble nematode traps as pointed out by Wang (1995) who saw similar structures formed by *T. matsutake* in culture. The formation of hyphal coils may be stimulated by depletion of nutrients in the growth medium, which could explain their apparent rarity in the field. However, the possibility that matsutake species form nematode traps seems reasonable considering the oligotrophic soils where they are usually found. Formation of thick-

walled chlamydospores by *T. magnivelare* may provide a means to endure extended adversity such as the loss of host trees through fire or other disturbances that preclude sexual reproduction. This possibility has management implications since it suggests that basidiospores are not the only means of reproduction available to *T. magnivelare* and that reestablishment of *T. magnivelare* on a site recovering from disturbance may not require an immediate source of airborne sexual spores. Asexual reproduction may also reduce the short-term danger of over harvesting if it is a significant source of recruitment of new colonies. However, asexual reproduction would not reduce other harmful effects of over harvesting such as the loss of *T. magnivelare* as a food source for wildlife.

Effective management for *T. magnivelare* depends on developing means to measure its abundance at a stand or landscape scale. Chapter six discussed the difficulty of surveying for mushrooms resulting from the unpredictability of weather conditions, the patchy distribution of colonies, and competition from wildlife and mushroom harvesters. Because of the time and expense required for mushroom surveys it is not currently practical for managers to monitor mushroom production or to assess changes in *T. magnivelare* populations, nor is it practical to balance matsutake production against other forest uses that may occasionally generate less economic benefit and cause greater disruption of forest ecosystems. The survey method proposed and tested in chapter six offers a rapid and relatively inexpensive alternative to mushroom harvests for locating and quantifying matsutake populations. Use of the human sense of smell as a detection instrument raises questions regarding the sensitivity, stability and accuracy of detection which require further testing. However, the preliminary results in chapter six suggest that the method is reasonably accurate. Further, field results suggest that it is sufficiently sensitive and stable to obtain useful correlations with mushroom production despite confronting the olfactory system of surveyors with up to 600 samples per day. The effort required to conduct the field survey was a fraction of that required for a single season of mushroom surveying and it explained 71% of the variability in mushroom production observed between plots over four

seasons. Use of this method to predict mushroom productivity in other habitats will require mushroom surveys to establish the relationship between colony abundance and mushroom production in those habitats, but other applications can be applied without preliminary studies. These possible applications include mapping of productive patches as a factor in forest management planning, assessing matsutake populations in areas considered for forest management activities, measuring effects of disturbance on matsutake populations over time, measuring correlations between colony abundance and various habitat variables, and ground-truthing habitat models or predictions based on remote sensing data.

The commercial value of matsutake mushrooms has drawn attention to wild harvests and most of the research effort underway on *T. magnivelare* concerns its ecology and harvest. However, the possibility of cultivating matsutake has also attracted great interest around the world. As ectomycorrhizal species, the primary focus of cultivation research is on the controlled synthesis of matsutake ectomycorrhizae on seedlings as a precursor to establishment of plantations in the field. This approach has succeeded with various culinary truffles, but it may be more difficult with matsutake species. Matsutake species reach their greatest abundance in mature forests and are rarely, if ever, found in young stands. Like some other mushrooms associated with mature trees, matsutake species appear to require nutritional stimulation in an aseptic environment to colonize seedlings and they may fail to persist in association with seedlings when they are removed from the aseptic environment. Results of chapter five and other published reports concerning syntheses with matsutake species are consistent with the behavior of other late-seral ectomycorrhizal fungi and this fact may explain the continuing failure of scientists to devise methods for cultivating matsutake species.

A number of interesting questions arose over the course of this research. Among the most important for forest managers, as well as for a basic understanding of *T.*

magnivelare's ecology is the possibility that *T. magnivelare* has host preferences that exert influence over where *T. magnivelare* occurs in the forest. For example, it appears likely that *T. magnivelare* clusters around *Abies magnifica* var. *shastensis* within forests comprised largely of other suitable hosts. Mushroom harvesters already seek-out stands with high concentrations of *A. magnifica* var *shastensis* and demonstration of a host preference would be useful for habitat modeling and possibly remote sensing applications. Few other examples of host preference among ectomycorrhizal fungi exist in the literature.

The olfactory detection method explored in chapter six also merits further development. Results of the field survey suggest that error rates stabilize and at levels that allow accurate measurement of colony abundance at large scales, but those levels will have to be measured to allow assessment of confidence associated with detection frequencies. It will also be important to determine whether the smell of matsutake in soil undergoes seasonal changes and, if so, to find the optimal time of year for olfactory surveys. This method could be used now without further research for certain types of studies, particularly mapping of populations, but quantitative surveys would benefit from more thorough measurement of error rates. Because of the time and expense required for mushroom surveys, development of this olfactory method creates a unique opportunity to study the distribution, abundance and ecology of a mushroom at stand and landscape scales.

The study of *Allotropa virgata* should also be followed-up to determine the identity of the unknown fungus found on its roots in 12% of collections from throughout the sampling range. Whether or not this fungus forms mycorrhizae with *A. virgata*, it likely has some specific interaction with it that could be helpful for understanding *A. virgata's* synecology.

Allotropa virgata also demonstrates the ability of *T. magnivelare* to provide physiological links connecting understory plants to the roots of overstory trees.

Without the ability to produce sugars through photosynthesis, *A. virgata* depends on *T. magnivelare* to provide photosynthates obtained from overstory trees. Results from Simard, et al. (1997) suggest that achlorophyllous species are not alone in benefiting from this below ground source of carbon. *Tricholoma magnivelare* associates with at least two *Arctostaphylos* species commonly found in the forest understory that may be excellent candidates for receiving fixed carbon from overstory trees via ectomycorrhizal links. Because *T. magnivelare* forms large, easily located colonies that tend to lack other ectomycorrhizal fungi, it may provide an opportunity to study the role of a known ectomycorrhizal fungus in carbon-sharing between plants in the field.

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