Périgord black truffles

The Effect of Applied Lime and Phosphorus on the Competitiveness of *Tuber melanosporum* and other Ectomycorrhizal Fungi Found in Tasmania

by

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Abstract

Hazel (*Corylus avellana* L.) seedlings inoculated with the Périgord black truffle fungus (*Tuber melanosporum* Vitt.) are being planted in Tasmania in an attempt to culture truffles. Competition from other ectomycorrhizal fungi has a significant impact on truffle production in Europe and can be expected to have some effect on the Tasmanian industry. This thesis examines ectomycorrhizal fungi occurring in Tasmania with respect to their ability to form mycorrhizas with hazel and compete with *T. melanosporum* under various soil treatments.

Stands of hazel previously established for nut production or as ornaments were surveyed for the sporocarps of ectomycorrhizal fungi. Several species were found including species that are new either to Australia or Tasmania. The endemic species *Descomyces albus* (Klotzsch) Bougher & Castellano and *Podohydnangium* sp., previously thought to be *Eucalyptus* specific were fruiting under hazel.

T. melanosporum occurs naturally on calcareous soils in Europe. Truffières in Tasmania are heavily limed to create a calcareous soil environment. The response of selected introduced and endemic ectomycorrhizal fungi to applied lime was studied in a glasshouse experiment. Some of the endemic species, which would normally inhabit acidic soils, were unable to survive high rates of lime application and therefore should not pose a threat to the truffle industry. The introduced species were generally more tolerant to lime application.

A subsequent glasshouse experiment sought to separate the effects of pH and calcium on colonisation by *T. melanosporum*. Seedling hazels were inoculated with *T. melanosporum*. After twelve months, they were transplanted using soil amended with fourteen rates of either $CaCO_3$, $CaSO_4$, or $MgCO_3$. The seedlings

were then exposed to spores of two endemic fungal species. Applied $CaCO_3$ and $MgCO_3$ increased level of colonisation by *T. melanosporum*, whereas $CaSO_4$ had little or no effect. Soil pH appears to have a stronger influence on colonisation by *T. melanosporum* than the level of applied or exchangeable calcium. Colonisation by endemic species was low and sporadic across all treatments.

Another glasshouse experiment of similar design to that above was established to observe the effect of lime and phosphorus interaction. Applied lime significantly increased the level of colonisation of *T. melanosporum*, but phosphorus had no effect, even at very high rates of application (150 mg P / Kg soil).

A commercial truffière was surveyed for the level of colonisation by *T. melanosporum* and other ectomycorrhizal fungi.

Descriptions were compiled of the mycorrhizas of fungal species found in the glasshouse and field experiments to assist in their future identification.

The morphological identification of *Tuber* mycorrhizas was confirmed using PCR and RFLP of DNA extracted from single mycorrhizal tips.

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1 Introduction

1.1 General

Considered one of the finest edible fungi, the black truffle is prized for its unique flavour and intoxicating aroma. It is used to flavour a wide variety of dishes such as shellfish, veal, foie gras, turkey, game, beef and omelettes. Consumer demand for the Périgord black truffle greatly exceeds supply, and hence the price paid for this product is very high. In 1998, wholesale prices in France ranged from A$1250 to A$1600 per kilogram (D. Garvey, Périgord Truffles of Tasmania, pers. com.). At the same time in Hobart, Tasmania, Périgord black truffles were retailing for A$2000/kg. Such prices have created a great deal of interest in the commercial production of Périgord black truffles.

The Périgord black truffle is the fruiting body of the ectomycorrhizal fungus *Tuber melanosporum* Vitt.. *Tuber melanosporum* is an ascomycete of the family *Tuberaceae*, order *Tuberales*. It is native to southern continental Europe, occurring predominantly in the southern regions of France, and the northern regions of Italy and Spain.

The fruit bodies are found below-ground, anywhere from just below the surface to a depth of about 50 cm (Hall *et al.*, 1994). Traditionally, pigs were used to detect the truffles at the time of harvest. Nowadays, dogs are more commonly used.

Trees that are colonised by *T. melanosporum* can exhibit a brûlé. A brûlé is that area around a tree exhibiting a reduction in ground cover due to the presence of *T. melanosporum* or other fungi. There have been several proposed explanations for the presence of the brûlé including competition between the plants and the fungus for water and nutrients (Delmas, 1983), pathogenic effects of

T. melanosporum (Plattner and Hall, 1995) or the allelopathic effect of chemicals produced by *T. melanosporum* (Bonfante *et al.*, 1971; Papa and Porraro, 1978).

In addition to the Périgord black truffle, there are several other species of the *Tuber* genus that produce edible fruitbodies. The Italian white truffle, *T. magnatum*, is also in high demand and commands similar prices to the Périgord black truffle. Other *Tuber* species such as *T. uncinatum, T. himalayense, T. aestivum, T. borchii* and *T. brumale* are edible, but are of inferior quality. Some of these inferior truffles, particularly *T. himalayense* in recent times, are sold as the more valuable Périgord black truffle to unsuspecting buyers.

Other truffle species are also major competitors to the production of Périgord black truffles. Chevalier *et al.* (1982) showed that most contamination of French truffières occurred from other *Tuber* species, particularly *T. brumale* and *T. borchii*.

1.2 Background to the Study

Périgord Truffles of Tasmania Pty Ltd (PTT), have embarked on a venture to produce Périgord black truffles in Tasmania. From a marketing point of view, trufficulture is an industry well suited to Tasmania. Truffles are harvested in the European winter during December, January and February and have a shelf life of a few weeks. Truffles can be preserved in bottles or cans, but most of the flavour and aroma is lost in the process. Chefs will always use fresh produce where possible. Therefore, Tasmania has the potential to provide fresh truffles to the world market during the Southern Hemisphere winter months of June, July and August when there are few other fresh truffles available.

PTT have entered into joint venture arrangements with primary producers. As at the end of spring 1997, 45 hectares of truffières had been established on 25 sites in Tasmania. The company plans to increase the number of plantations to 200 hectares by the year 2001. With substantial capital being invested, there was a

need to commence research on how *T. melanosporum* would develop in the Tasmanian environment.

In November 1993, PTT was successful in its application to the Rural Industries Research and Development Corporation for a research grant. Part of this grant was to fund a PhD candidate to study factors affecting the establishment and proliferation of the Périgord black truffle in Tasmania. This study commenced in June 1994.

1.3 Factors Affecting the Proliferation of *Tuber melanosporum*

Factors affecting the establishment and proliferation of the Périgord black truffle involve a complex interaction between the host plant, climate, soil type and competition from other ectomycorrhizal fungi.

1.3.1 Host

Tuber melanosporum has been reported to form mycorrhizas with *Carpinus*, *Castanea*, *Cedrus*, *Cistus*, *Corylus*, *Fagus*, *Helianthemum*, *Juglans*, *Olea*, *Ostrya carpinifolia* Scop., *Pinus*, *Populus*, *Quercus*, *Salix* and *Tilia* (Hall *et al.*, 1994). PTT have chosen *Corylus avellana* (hazel) as their preferred host plant, although there have also been limited inoculations of *Quercus robur* and *Q. ilex*. The experience of truffle growers in Europe indicates that *T. melanosporum* tends to fruit earliest when associated with hazel or *Q. ilex* (D. Garvey, PTT, pers. com.). Chevalier *et al.* (1982) reported that in a truffière in Bourgogne, 73% of the hazel trees were producing *T. melanosporum* fruitbodies 3.5 years after planting, and 82% producing fruitbodies after 4.5 years.

This observation that *T. melanosporum* tends to fruit earlier when associated with hazel may be a function of tree growth rate. Shaw *et al.* (1996) found that stem

diameter was a critical factor determining fruit body production of *T. melanosporum* in symbiosis with *Quercus ilex*. This suggests that host plants may need to reach a critical biomass before being able to provide adequate carbohydrate to the fungus for fruiting. Hazels grow quickly, and the early fruiting reported under hazel may be attributed to their ability to reach this critical biomass at a younger age than other host species. Further experimentation is required to test this theory.

1.3.2 Climate

Limited studies have been made into the effect of climate on fruiting of the Périgord black truffle. These studies have concentrated on the influence of temperature and rainfall as the critical factors determining truffle production.

Temperature

Figures 1.1 and 1.2 compare the daily maximum and minimum temperatures for Périgord black truffle producing regions in France and New Zealand, and centres in Tasmania where truffières have been planted. Tasmania has a stronger oceanic influence than the Périgord black truffle growing regions of France, which tends to moderate the extremes of temperature. In summer, the maximum and minimum temperatures at the Tasmanian centres are lower than those for France. In winter, daily maximum temperatures are slightly higher in Tasmania whereas daily minimum temperatures are very similar.

Le Tacon *et al.* (1982) noted that mild oceanic climates with insufficient seasonal temperature contrast and continental climates where the winters are too cold are not suitable for truffle production. This statement appears to have been drawn from observation of the climatic distribution of the Périgord black truffle. However, truffles have been produced in Gisborne on the north island of New Zealand which has very mild winters relative to both the truffle producing regions of France and those areas being planted to truffières in Tasmania. Furthermore, Périgord black truffles were also produced from a truffière in Burgundy, France, on 4.5 year old hazels (Chevalier and Grente, 1979). Burgundy is well north of,

and consequently colder than the main truffle producing regions of Europe, and yet truffles were produced quite quickly. It would appear that the climatic requirements of *T. melanosporum* may be broader than those postulated by Le Tacon *et al.* (1982).

Montant and Kulifaj (1990) and Sourzat *et al.* (1993) have investigated the effects of climate on truffle production using poly-tunnels to control the temperature. Montant and Kulifaj (1990) found that during the early stages of truffle production (April to May in France), the temperature of the soil must not be less than 10°C, and must not exceed 35°C during the summer. Also, the soil temperature should not fall below 5°C at any time of the year. Sourzat *et al.* (1993) observed that air temperatures of 38°C for several hours did not adversely effect the mycorrhizal colonisation by *T. melanosporum*.

Montant and Kulifaj (1990) reported increased yields within the poly-tunnel relative to control treatments, presumably due to an increase in the average temperature within the greenhouse. It was also found that *T. melanosporum* fruited earlier within the poly-tunnel relative to the control treatments. This finding is corroborated by the observation that the French truffle harvest of winter 1988-89 occurred earlier than usual as temperatures were unseasonably mild (Montant and Kulifaj, 1990).

While summer temperatures in those regions of Tasmania selected for truffle production are lower than the summer temperatures of truffle growing regions in France, there is no substantial evidence to suggest that this will be a problem.

Rainfall

Rainfall or irrigation over the summer has been shown to increase truffle yields (Le Tacon *et al.*, 1982; Montant and Kulifaj, 1990; Sourzat *et al.*, 1993). Montant and Kulifaj (1990) propose that lack of soil moisture in Spring (April in Northern Hemisphere / October in Southern Hemisphere) inhibits the initiation of

primordia, whereas low soil moisture in autumn slows the growth of the fruitbody provoking imperfect maturation.

Figure 1.3. shows mean rainfall for regions of trufficulture in France, New Zealand and Tasmania. All truffières in Tasmania can be irrigated to supplement summer rainfall. Those regions with low rainfall, particularly low winter rainfall, may have an advantage in that the detrimental effects of waterlogging (Montant and Kulifaj, 1990) are less likely, and summer soil moisture levels can be carefully regulated with irrigation.

Data for Figures 1.1, 1.2 and 1.3 and climate statistics for other regions of trufficulture in France and Tasmania are given in Appendix 1.

Figure 1.1. Mean daily maximum temperatures (°C) for Périgord black truffle producing regions in France and New Zealand, and regions in Tasmania where truffières have been planted. French regions are represented by red shades, Tasmania by blue shades and New Zealand by green.

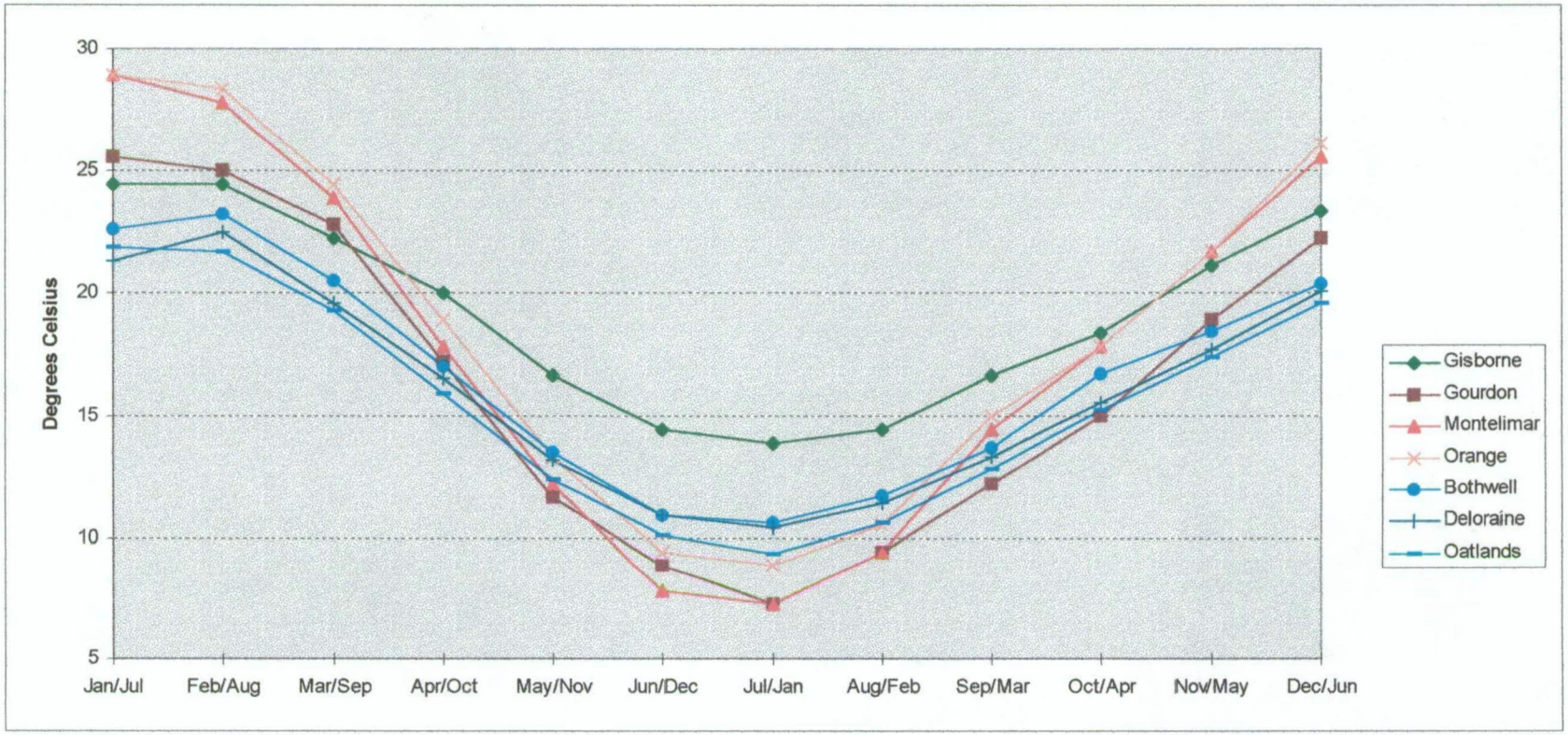


Figure 1.2. Mean daily minimum temperatures (°C) for Périgord black truffle producing regions in France and New Zealand, and regions in Tasmania where truffières have been planted. French regions are represented by red shades, Tasmania by blue shades and New Zealand by green.

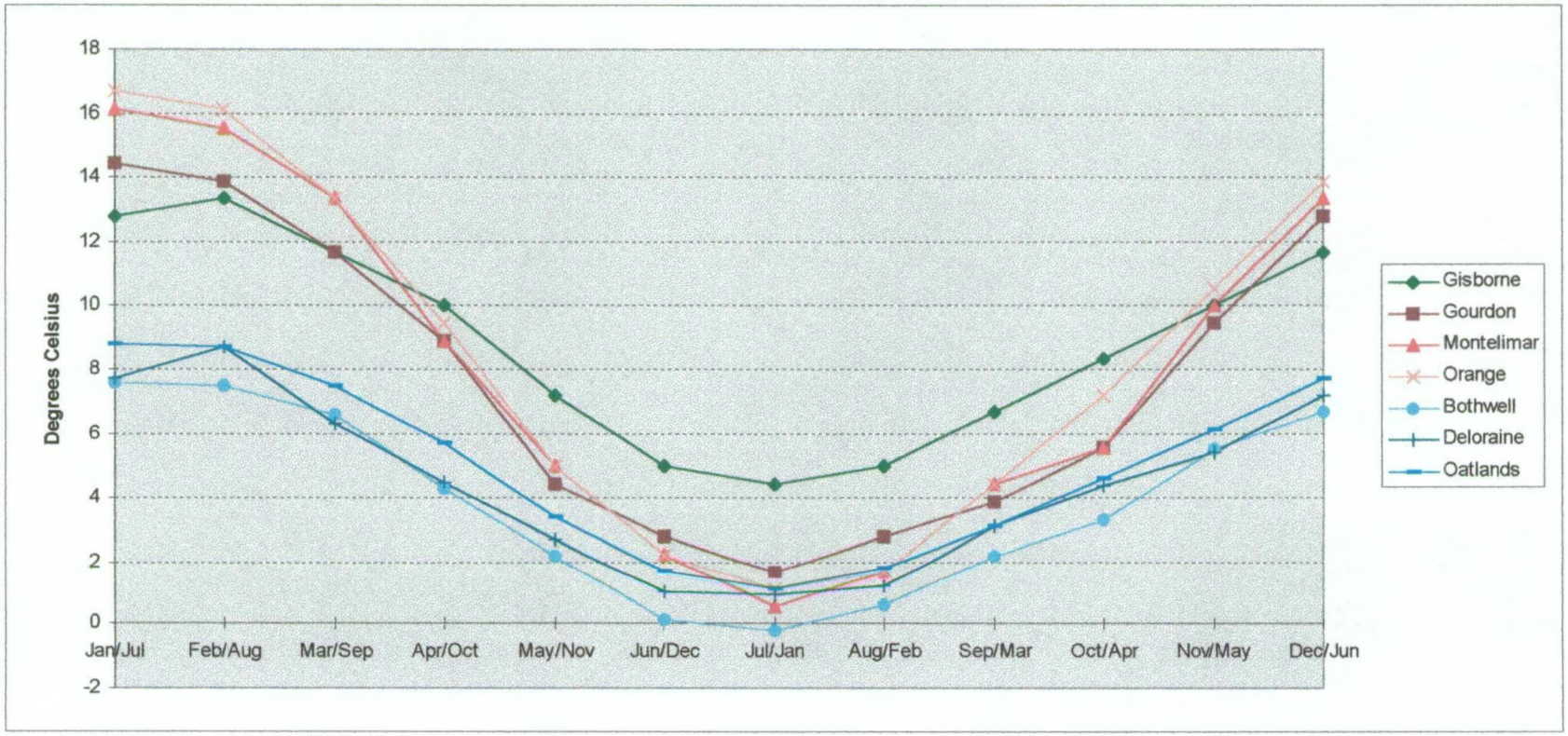
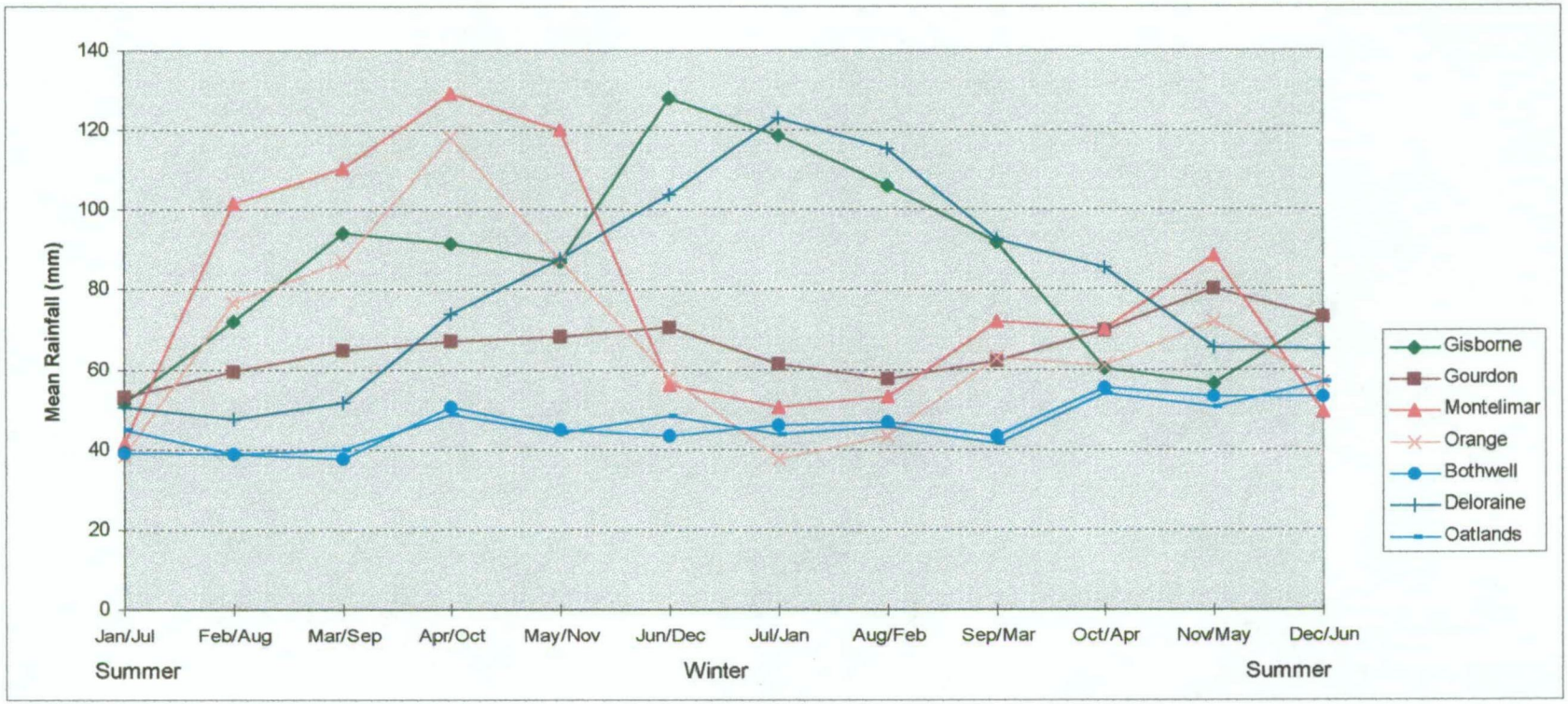


Figure 1.3. Mean rainfall (mm) for Périgord black truffle producing regions in France and New Zealand, and regions in Tasmania where truffières have been planted. French regions are represented by red shades, Tasmania by blue shades and New Zealand by green.



1.3.3 Competitive Ectomycorrhizal Fungi

The truffle growing regions of Europe and Tasmania differ greatly in the species composition of ectomycorrhizal fungi. Tasmania has a great diversity of native ectomycorrhizal fungi which have evolved in association with the indigenous flora (Molina *et al.*, 1992). Some of these fungi are found world wide and are generally considered to have a broad host range. However, the majority of native ectomycorrhizal fungi are endemic and are considered to be specific to native trees such as *Eucalyptus* (Bougher, 1995; Chilvers, 1973; Molina *et al.*, 1992). In addition to the native fungi, Tasmania has a number of introduced ectomycorrhizal fungi which are generally associated with exotic plant species.

If the majority of native ectomycorrhizal fungi are specific to *Eucalyptus* and other native hosts, *T. melanosporum* associated with hazel in Tasmania may have the advantage of an environment with few competitors. This is provided truffières are planted a sufficient distance from exotic trees that may be harbouring introduced ectomycorrhizal fungi.

In Europe there is a suite of ectomycorrhizal fungi that compete with *T. melanosporum*. These competing ectomycorrhizal fungi can reduce or prevent the production of truffles (Delmas, 1978; Sourzat *et al.*, 1993). Competitor ectomycorrhizal fungi include epigeous and hypogeous species from a range of genera including a number of species from the *Tuber* genera. The apparent disparity in fungal species composition between Tasmania and Europe has the potential to favour truffle yields in Tasmania. Consequently, further research is required to test the assumption that endemic ectomycorrhizal fungi are *Eucalyptus* specific and therefore unable to compete with *T. melanosporum* growing on hazel trees.

1.3.4 Soils

Soils are the other major area of contrast between European truffle growing regions and Tasmania. Périgord black truffles occur naturally on calcareous soils including rendzinas and brown earths (Poitou, 1988). Tasmania has a very limited area of calcareous soil. The majority of Tasmanian soils are slightly acidic or moderately acidic (Grant *et al.*, 1995). Consequently, truffières are being planted on heavily limed soils in an attempt to produce soils with calcareous characteristics. The response of *T. melanosporum* to such practice is unknown. Nor is it known whether other ectomycorrhizal fungi present in Tasmania, particularly endemic species, can adjust to such dramatic changes in soil conditions.

Phosphorus level is another soil parameter that varies considerably between the truffle soils of Europe and those soils being used for truffières in Tasmania. Due to their weathered nature, many Australian soils are inherently low in phosphorus (Grant *et al.*, 1995). The level of available phosphorus (Colwell method) in the Tasmanian truffières ranges from 10 to 52 with an average of 25.

Australian endemic ectomycorrhizal fungi appear to be adapted to low levels of soil phosphorus. For example, Bougher *et. al.* (1990) demonstrated that levels of colonisation of *Eucalypts* by *Descolea maculata* on a phosphorus deficient soil (Bray-extractable P less than 2 mg P/Kg soil) were highest at very low levels of applied P (2 to 4 mg P/Kg soil) and declined quite rapidly at higher rates of applied P. These levels of available P reported by Bougher *et al.* contrast with the P levels reported by Delmas *et. al.* (1981) for truffières in France exhibiting brûlé formation. The French soils had a range of available P (Joret Herbert method) from 6 mg/Kg to 980 mg/Kg. Delmas *et al.* also made the note that the lowest value in the range (6 mg/Kg) was much lower than average. Hence, there is some evidence that *T. melanosporum* is able to proliferate in soils with a moderate to high phosphorus content (by Australian standards) whereas Australian native ectomycorrhizal fungi prefer soils with a low phosphorus content.

In the event that native Australian fungi become established in the truffières and compete with *T. melanosporum,* there may be the potential to favour *T. melanosporum* by manipulating the soil environment, particularly in regard to lime or phosphorus application. Further research is required to determine if this is possible.

1.4 Research Aims

This thesis examines ectomycorrhizal fungi occurring in Tasmania with respect to their ability to form mycorrhizas with hazel and compete with *T. melanosporum* under various soil treatments.

The principle aims of the research project were:

1. To determine which ectomycorrhizal fungi present in Tasmania are able to colonise hazel and thereby pose a potential threat to the production of truffles in Tasmania.

2. To study the effects of applied lime on the level of colonisation by *T. melanosporum* and other ectomycorrhizal fungi found in Tasmania.

3. To study the effects of applied phosphorus and phosphorus-lime interactions on the level of colonisation by *T. melanosporum* and other ectomycorrhizal fungi.

4. To survey truffières in Tasmania to determine the level of colonisation of *T. melanosporum* and other ectomycorrhizal fungi.

2 Ectomycorrhizal Fungi Associated with Hazel (*Corylus avellana*) in Tasmania, Australia.

2.1 Introduction

The isolated nature of the Australian continent gave rise to the evolution of its peculiar indigenous flora and fauna. Often overlooked, but sharing the uniqueness and diversity of the flora and fauna with which they co-evolved, are the indigenous ectomycorrhizal fungi of Australia. For example, Bougher (1995) postulated that endemism of Australian ectomycorrhizal fungi may exceed 70%. Molina *et.al.* (1992) have suggested that *Eucalyptus* and related Myrtaceae may have the greatest diversity of genus-specific ectomycorrhizal fungi in the world.

In addition to the endemic ectomycorrhizal fungi, Australia is home to cosmopolitan fungi that are indigenous to several regions of the world including Australia, and also a number of introduced species. Tasmania is geographically isolated from mainland Australia by Bass Strait. Not all ectomycorrhizal fungi that have been found on mainland Australia are present in Tasmania and *vice versa* (May and Wood, 1997). A list of fungal genera, thought to be ectomycorrhizal, that have been reported in Tasmania is given in Table 2.1. A more detailed list giving species names can be found in Appendix 2.

The host specificity of all these ectomycorrhizal fungi has important implications for the truffle industry of Tasmania in terms of:

- whether or not they can form a mycorrhizal relationship with hazel and thereby be a potential competitor to *Tuber melanosporum* in commercial truffières of hazel trees, and
- the prevalence and distribution of the fungi which will influence their likelihood of exposing spores to commercial truffières.

2.1.1 Endemic Ectomycorrhizal Fungi

If the endemic fungi of Australia are indeed *Eucalyptus* specific, this greatly reduces the number of species that are capable of competing with *T. melanosporum*. However, the general pattern of host specificity for Australian ectomycorrhizal fungi is not well defined. There has been a broad acceptance that Australian fungi associated with *Eucalyptus* are generally not compatible with exotic forest trees (Bougher, 1995; Castellano and Bougher, 1994; Molina *et al.*, 1992). Some of this opinion has developed from work by Malajczuk *et al.* (1982) where *Hydnangium carneum* and *Descomyces albellus* (*Hymenogaster albellus*) formed mycorrhizas with several *Eucalyptus* species using a pure culture synthesis technique, but would not form mycorrhizas with *Pinus radiata*. Chilvers (1973) also noted that mycorrhizal types commonly associated with *Eucalyptus* were unable to develop on black poplars or several species of pine and fir.

With the exception of the black poplars, the studies by Malajczuk et. al. (1982) and Chilvers (1973) were both examining the ability of endemic ectomycorrhizal fungi to form relationships with exotic gymnosperms whereas the majority of ectomycorrhizal hosts in Australia are angiosperms. Further studies are required to determine whether endemic Australian ectomycorrhizal fungi will form mycorrhizal relationships with exotic angiosperm hosts such as hazel. If they do form mycorrhizal relationships with hazel, it is not known how competitive they are likely to be with *T. melanosporum*.

2.1.2 Cosmopolitan Fungi

In addition to the endemic indigenous fungi of Australia, there are ectomycorrhizal fungi found in several regions of the world including Australia. These species generally have a very broad host range. For example, *Scleroderma verrucosum* forms mycorrhizal relationships with *Eucalyptus* as well as northern hemisphere host species (Trappe, 1962). It is common throughout Tasmania and is therefore one fungus that is likely to present itself in commercial truffières.

However, it should be noted that some cosmopolitan species such as *Pisolithus tinctorius* display significant intraspecific heterogeneity of host specificity (Smith and Read, 1997). For example, carpophores of *P. tinctorius* associated with *Pinus* spp. are poor colonisers of *Eucalyptus* spp. (Cairney and Chambers, 1997). Therefore, strains of cosmopolitan fungi native to Australia may not show the same host specificity/compatibility as their European counterparts.

The prevalence of endemic and cosmopolitan ectomycorrhizal fungi in the Tasmanian landscape will mean that truffières are constantly exposed to spores of these fungi. Attempts have been made by truffle growers to reduce the exposure of the hazel trees to contaminant fungi by planting truffières on sites with long histories of pasture or crop production, and away from the rooting zone of other ectomycorrhizal hosts. Fences have been constructed to reduce the quantity of spores being carried onto the truffières by animals. However, small animals able to penetrate the fence, air-borne spores, and irrigation water still remain as potential sources of contamination.

2.1.3 Introduced Fungi

In terms of host specificity, fungi introduced to Australia can be divided into two categories: those that are able to form mycorrhizal relationships with *Eucalyptus* and those that are specific to exotic hosts. *Amanita muscaria* is one of the few examples of an introduced ectomycorrhizal fungus that is able to form a mycorrhizal relationships with *Eucalyptus* (Malajczuk *et al.*, 1982) and has also

been reported fruiting under *Nothofagus cunninghamii* in Tasmania (Fuhrer and Robinson, 1992). *Lactarius piperatus* is an example of an introduced ectomycorrhizal fungi that has not been reported to form mycorrhizas with *Eucalyptus*, but has been reported for hazel and other exotic trees (Trappe, 1962).

Cosmopolitan fungi, and introduced species that are capable of colonising *Eucalyptus*, are more likely to be the initial contaminants of the truffières as a result of their greater prevalence. Fungal species that are confined to exotic hosts are less likely to expose their spores to truffières provided that care is taken with site selection, paddock quarantine, and the preparation of inoculated seedlings.

A number of ectomycorrhizal fungi have been reported for hazel worldwide. These fungi are listed in Table 2.2. Other fungi that have been reported in truffières, but not specifically under hazel, are listed in Table 2.3. These fungi may be able to form mycorrhizas with hazel and thereby represent potential competitors. Tables 2.2 and 2.3 also indicate whether these fungi have been recorded in Tasmania.

It is also probable that some introduced species capable of competing with *T. melanosporum* have yet to be found or recorded in Tasmania. Much of the collecting of ectomycorrhizal fungi in Tasmania has been under commercially important forest trees. Less attention has been paid to the introduced flora, particularly exotic angiosperms which are likely hosts for competitors to the black truffle industry.

There is also the risk of further introductions of deleterious ectomycorrhizal fungi into Tasmania.

2.1.4 Objectives

The objectives of the study reported here were two fold:

1. To ascertain if any endemic ectomycorrhizal fungi are fruiting under hazel in Tasmania and thereby represent a potential competitor to the Périgord black truffle industry.
2. To investigate the occurrence of introduced ectomycorrhizal fungi fruiting under hazel.

Table 2.1. Genera of Ectomycorrhizal Fungi Species Reported in Tasmania and the Endemism and Habit of those Species[c]

Ascomycotina

Genus	Endemic	Introduced	Cosmopolitan	Epigeous	Hypogeous
Amylascus	*				*
Balsamia[d]		*			*
Dingleya	*				*
Elaphomyces[d]					*
Genabea	*[a]				*
Genea	*[a]				*
Hydnocystis[d]		*			*
Labyrinthomyces	*				*
Muciturbo	*				*
Paurocotylis	*				*
Ruhlandiella	*				*
Sphaerosoma	*[a]				*
Spragueola	*				*
Stephensia	*				*
Terfezia	*[a]			*	

Basidiomycotina

Genus	Endemic	Introduced	Cosmopolitan	Epigeous	Hypogeous
Alpova[d]					*
Amanita	*	*		*	
Andebbia	*				*
Arcangeliella	*[a]				*
Austrogautieria	*				*
Austroboletus[d]				*	
Boletus	*	*		*	
Boughera	*				*
Cantharellus	*[a]			*	
Castoreum	*				*
Chamonixia[d]					*
Chondrogaster	*				*
Cortinarius	*[a]			*	
Cortinomyces	*[a]				*
Cuphocybe[d]				*	
Cystangium[d]					*
Dermocybe[d]				*	
Descolea	*			*	
Descomyces	*				*
Destuntzia[d]		*			*
Diploderma	*				*
Gautieria	*[a]				*
Gummiglobus	*				*
Gymnomyces	*[a]				*
Hebeloma[d]				*	
Horakiella	*				*
Hydnangium	*				*
Hymenogaster	*[a]				*
Hysterangium	*	*			*
Hysterogaster	*				*
Laccaria[b]			*	*	

Table 2.1 continued.

Basidiomycotina

Genus	Endemic	Introduced	Cosmopolitan	Epigeous	Hypogeous
Lactarius	*	*		*	
Leucogaster[d]					*
Macowanites[d]					*
Malajczukia	*				*
Martellia[d]					*
Mesophellia	*				*
Nothocastoreum	*				*
Octaviania	*				*
Paxillus	*	*		*	
Pisolithus			*	*	
Protubera[d]					*
Quadrispora	*				*
Rhizopogon	*	*			*
Richoniella[d]					*
Rozites	*			*	
Russula	*	*		*	
Scleroderma	*		*	*	*
Sclerogaster[d]					*
Secotium	*				*
Setchelliogaster	*				*
Timgrovea	*				*
Thaxterogaster	*				*
Zelleromyces[d]					*

[a] Genus also includes introduced or cosmopolitan species that have not been reported in Tasmania.
[b] Genus also includes endemic species that have not been reported in Tasmania.
[c] The list only concerns those species that have been reported in Tasmania as per Appendix 2.
[d] Species names were not reported, hence in some cases, it is not possible to report the endemism of these fungi.
Taxonomy is consistent with the original reports. No attempt has been made to update names.

Table 2.2. Ectomycorrhizal Fungi Reported for *Corylus avellana.*

Species	Reference
Amanita rubescens (Pers. ex Fr.)	(Trappe, 1962)
Balsamia vulgaris Vitt.	(Maia *et al.*, 1996)
Boletus edulis (Bull. ex Fr.)	(Trappe, 1962)
Boletus erythropus (Fr.) Pers.	(Trappe, 1962)
Cenococcum graniforme (Sow.) Ferd. & Winge.	(Trappe, 1962)
Cortinarius anomalus (Fr. ex Fr.)	(Trappe, 1962)
Cortinarius collinitus (Pers. ex Fr.)	(Trappe, 1962)
Cortinarius multiformis (Fr. ex Secr.) Fr.	(Trappe, 1962)
Cortinarius violaceus (L. ex Fr.) Fr.	(Trappe, 1962)
Genea klotzschii Berk. & Broome	(Maia *et al.*, 1996)
Gyroporus cyanescens (Bull. ex Fr.)	(Trappe, 1962)
Hebeloma pumilum J.E.Lange	(Granetti and Angelini, 1992)
Hydnotrya tulasnei (Berk.) Berk. & Broome	(Maia *et al.*, 1996)
Hydnum repandum L. ex Fr.	(Trappe, 1962)
Hygrophorus arbustivus Fr.	(Trappe, 1962)
Hygrophorus nemoreus (Lasch.) Fr.	(Trappe, 1962)
Hygrophorus unguinosus (Fr.) Fr.	(Trappe, 1962)
Hygrophorus virgineus (Fr.) Fr.	(Trappe, 1962)
Hymenogaster citrinus Vitt.	(Bencivenga *et al.*, 1992)
Hymenogaster vulgaris	(Chevalier *et al.*, 1982)
Hypochnus cyanescens Peyr. *nom.nud.*	(Trappe, 1962)
Lactarius coryli Peyr.	(Trappe, 1962)
***Lactarius piperatus* (L. ex Fr.)**	**(Trappe, 1962)**
Lactarius pyrogalus (Bull. ex Fr.) Fr.	(Trappe, 1962)
***Lactarius subdulcis* (Bull. ex Fr.) S.F. Gray**	**(Trappe, 1962)**
Leccinum duriusculum (Schulzer in Fr.)	(Trappe, 1962)
***Paxillus involutus* (Batch ex Fr.) Fr.**	**(Malajczuk *et al.*, 1982)**
Phallus impudicus Pers.	(Trappe, 1962)
Pulvinula globifera	(Olivier and Mamoun, 1994)
Scleroderma aurantium	(Grente *et al.*, 1976)
Scleroderma laeve Loyd	(Malajczuk *et al.*, 1982)
Sphaerospella brunnea (A. & S.) Svrcek &Kubicka	(Meotto and Carraturo, 1987-88)
Strobilomyces floccopus (Vahl ex Fr.) Karst.	(Trappe, 1962)
Tuber aestivum Vitt.	(Palenzona, 1969)
T. borchii Vitt.	(Granetti, 1995)
T. brumale Vitt.	(Palenzona, 1969)
T. excavatum Vitt.	(Maia *et al.*, 1996)
T. ferrugineum Vitt.	(Fontana and Centrella, 1967)
T. griseum Pers.	(Maia *et al.*, 1996)
T. macrosporum Vitt.	(Giovanetti and Fontana, 1981)
T. magnatum Pico ex Vitt.	(Granetti, 1995)
T. melanosporum Vitt.	(Palenzona, 1969)
T. mesentericum Vitt.	(Granetti, 1995)
T. nitidum Vitt.	(Maia *et al.*, 1996)
T. rufum Pico	(Maia *et al.*, 1996)
T. uncinatum Ch.	(Granetti, 1995)
Xerocomus chrysenteron (Bull. ex St. Am.)	(Trappe, 1962)
Xerocomus subtomentosus (L. ex Fr.)	(Trappe, 1962)

Those species in **bold type** have been reported in Tasmania.
Taxonomy is consistent with the original reports. No attempt has been made to update names.

Table 2.3. Ectomycorrhizal Fungi reported in truffières where the host species was not *Corylus avellana*, or the host species was not given.

Species	Reference
Amanita echinocephala	(Ceruti and Tozzi, 1985)
Amanita ovoidea	(Ceruti and Tozzi, 1985)
Amanita solitaria	(Sourzat *et al.*, 1993)
Amanita strobiliformis	(Ceruti, 1990)
Amanita vittadini	(Ceruti and Tozzi, 1985)
Astraeus hygrometricus (Pers.) Morgan	(Baron, 1984)
Astreus stellatus	(Ceruti and Tozzi, 1985)
Boletus depilatus	(Ceruti, 1990)
***Boletus luridus* Schaeff. ex. Fr.**	(Sourzat *et al.*, 1993)
Boletus purpureus	(Ceruti and Tozzi, 1985)
Boletus regius	(Ceruti, 1990)
Boletus satanus Lenz	(Baron, 1984)
Cortinarius atrovirens	(Ceruti, 1990)
Cortinarius causticus	(Ceruti, 1990)
Cortinarius elegantior	(Ceruti, 1990)
Cortinarius flavovirens	(Ceruti, 1990)
Cortinarius largus	(Ceruti, 1990)
Cortinarius odoratus	(Ceruti, 1990)
Cortinarius olivellus	(Ceruti, 1990)
Cortinarius percomis	(Ceruti, 1990)
Cortinarius pseudosulphureus	(Ceruti, 1990)
Cortinarius rufoalbus	(Ceruti, 1990)
Cortinarius xanthophyllus	(Ceruti, 1990)
Gyroporus castaneus (Bull.) Quèlet	(Callot and Jaillard, 1996)
Hebeloma crustiliniforme	(Sourzat *et al.*, 1993)
Hebeloma edurum	(Ceruti, 1990)
Inocybe cookei	(Ceruti, 1990)
Inocybe fastigiata	(Sourzat *et al.*, 1993)
Inocybe fibrosa	(Ceruti, 1990)
Inocybe godey	(Ceruti, 1990)
Inocybe jurana (Patouillard) Saccardo	(Callot and Jaillard, 1996)
Inocybe luteipes	(Ceruti, 1990)
***Laccaria laccata* (Scop. : Fr.) Cooke**	(Ceruti, 1990)
Lactarius acerrimus	(Ceruti, 1990)
Lactarius acris	(Ceruti, 1990)
Lactarius aspideus	(Ceruti, 1990)
Lactarius cremor	(Ceruti, 1990)
Lactarius flavidus	(Ceruti, 1990)
Lactarius fulvissimus	(Ceruti, 1990)
Lactarius pterosporus	(Ceruti, 1990)
Melanogaster variegatus	(Sourzat *et al.*, 1993)
Pisolithus arrhizus (Scop) S Rauschrt	(Callot and Jaillard, 1996)
P. crassipes	(Ceruti and Tozzi, 1985)
Russula delica Fr.	(Sourzat *et al.*, 1993)
R. expallens Gillet	(Ceruti, 1990)
R. lepida Fr.	(Sourzat *et al.*, 1993)
R. luteotacta	(Ceruti, 1990)
R. maculata	(Ceruti, 1990)
R. pectinata (Bull.) Fr.	(Ceruti, 1990)

Table 2.3 continued.

Species	Reference
Russula queletii	(Ceruti, 1990)
***Scleroderma verrucosum* Vaill. ex Pers.**	(Baron, 1984)
Suillus luteus (L. ex Fr.) S.F. Gray	(Baron, 1984)
Tricholoma terreum (Schaeff. ex Fr.) Kumm.	(Baron, 1984)
Tuber maculatum Vitt.	(Ceruti, 1990)
T. himalayense	(Comandini and Pacioni, 1997)
Xerocomus rubellus Quèlet	(Callot and Jaillard, 1996)

Those species in **bold type** have been reported in Tasmania.
Taxonomy is consistent with the original reports. No attempt has been made to update names.

2.2 Materials and Methods

Over a period of three years from 1994 to 1996, fungal fruiting bodies were collected from hazel trees previously established in Tasmania for nut production or ornamental purposes. Fifteen sites were surveyed. For each site, notes were taken on the size of the plantation, distance to surrounding trees, origin and age of the hazel trees where known, soil type, soil structure, compaction and site drainage, and soil pH.

Most collections were made during late autumn, winter and spring, though some collections were made in the summer months. The fruit bodies were dried, weighed, and attempts were made to isolate a culture from each species collected.

2.3 Results and Discussion

Of the fifteen sites that were surveyed, nine recorded fruitbodies. A description of the sites where fruit bodies were harvested is given in Table 2.4. Sites that were compacted, or were poorly drained, produced few or no fruit bodies. Some of the sites which gave negative results contained only a few trees. The small number of trees may have reduced the likelihood of finding fruit bodies on these sites.

Whilst the age of the hazel plantation could be expected to influence the level of fruiting and fungal diversity, this did not appear to be the case for the fifteen sites surveyed. For example, Site 1, which was one of the most recent plantings surveyed, showed prolific fruiting of a variety of fungi. There was also no observed correlation between the abundance or diversity of fruit bodies and the proximity of other ectomycorrhizal hosts, either native or exotic.

Table 2.4. A description of sites where sporocarps of ectomycorrhizal fungi were collected under hazel.

Site Number	Name	Map Reference	Soil Type	Soil pH (1:5 water)	Associated Trees	Distance to Nearest Eucalypt (m)	Height of Nearest Eucalypt (m)	Date Planted	Origin of Trees
1	Hazelbrook Nursery, Penguin	8115: Forth DQ180471	Kraznozem	5.5	None	>200	NA	1990	Grown from suckers from Site 2
2	Hazelbrook Nursery, Penguin. Hedge near house.	8115: Forth DQ184469	Kraznozem	5.8	None	80	30	~1973	Grown from suckers imported from Victoria
3	Perth Nursery	8314: South Esk EP151968	Pansanga Sandy Loam	4.9	*Ulmus sp.*	30	9	~1980	Grown from suckers and seed from Site 4
4	Tonganah	8415 Forrester 480380	Silty Gravelly Loam	5.4	None	100	17	~ 1975	A number of varieties imported from Northern Victoria as rooted cuttings
5	University of Tasmania, Horticulture Centre	8312: Derwent EN264494	Black cracking clay on Dolerite	7.0	None	25	9	Several plantings. 1972, 1986, 1993.	As rooted cuttings and seedlings from Vic. and N.S.W. and some material from Oregon, USA.

Continued over page.

Table 2.4 continued.

Site Number	Name	Map Reference	Soil Type	Soil pH (1:5 water)	Associated Trees	Distance to Nearest Eucalypt (m)	Height of Nearest Eucalypt (m)	Date Planted	Origin of Trees
6	Macquarie Plains	8212: Tyenna DN928711	Red Alluvial Sandy Loam	5.8	None	>200	NA	~1985	Cuttings from Orange & Sydney, N.S.W.
7	Police Point	8311: D'entrecasteaux EN046112	Grey podzolic	5.2	Scattered *Eucalyptus*	30	18	1986	60% of material from Site 2 as suckers. Others from Monbulk, Vic.
8	Rutherglen Bridge	8314 South Esk EQ053040	Alluvial Sandy Loam	5.6				Unknown	Unknown
9	Woodbridge Hill Road	8311: D'entrecasteaux	Clay on dolerite	5.9	*Eucalyptus*	5	8	1981	Melbourne as nuts.

Putative ectomycorrhizal fungi found fruiting under *Corylus avellana* in Tasmania are shown in Table 2.5. Some of these fungi are shown in Plate 2.1. While many of the sites had other trees growing within or adjacent to the hazels, it was clear that some of the fruit bodies collected were associated with *Corylus avellana* as they were well outside the rooting zones of any other trees. Nearby trees and the area just outside the rooting zone of the hazel were checked for the presence of sporocarps. Those fungal species found under the hazels were not found outside the rooting zone of the hazels. However, it is quite possible that some of the fungi found under the hazels were colonising surrounding trees, but were not producing fruit bodies at the times of collection.

Those areas where it could be positively concluded that the fungi could only be growing on *Corylus avellana* included the greater percentage of sites 1,2 and 6. Hence, it would appear that *Descomyces albus*, *Hebeloma crustiliniforme*, *Hymenogaster arenarius*, *Laccaria tortilla*, *Melanogaster ambiguus* and *Podohydnangium sp.* were forming mycorrhizal relationships with hazel. While it is possible that the remaining species were associated with other hosts, it is most probable that they were also associated with hazel due to the domination of hazel in the site and the proximity of the sporocarps to the hazel trees. The exceptions were *Labyrinthomyces sp.* and *Descomyces spp.* (T013H & T030H) which only occurred at sites 8 & 9 where there was a mixture of tree species including Australian native trees.

Table 2.5. Fungal collections under *Corylus avellana* in Tasmania.

Species	Collection Number	Site Number	Hemisphere of Origin
Cortinomyces sp. (large spores)	T004H	5	
Cortinomyces sp. (small spores)	T011H	3	
Descomyces albus (Klotzsch) Bougher & Castellano	T002H, T003H, T007H T009H, T021H, T022H, T027H, T028H, T029H, T031H	1,2,3,4,5,6	S
Descomyces sp. (immature, not *albus*)	T024H	3	S
Descomyces sp.	T013H	8	S
Descomyces sp.	T030H	9	S
Hebeloma crustiliniforme (Bull. : Fr.) Quél.	T005E, T009E	2,3	N
Hydnangium archeri (Berk.) Rodway	T005H	5	S
Hydnangium sp.	T001H	7	S
Hymenogaster arenarius Tul. & C.Tul.	H790, T020H	1,2,3	N
Hymenogaster australis Speg	H791	3	S
Labyrinthomyces sp.	T014H	8	S
Laccaria tortilla (Bolton) Cooke	T016E	2	N
Laccaria spp.	T010E, T013E, T014E, T015E, T016E	1	
Laccaria sp.	T018E	3	
Laccaria spp.	T001E, T010E	7	
Melanogaster ambiguus Vitt.	T006H, T008H, T012H, T023H, T025H, T026H	1,2,3	N
Podohydnangium sp.	T008E	2	S
Scleroderma verrucosum Vaill. ex Pers.	T006E, T022E	4	S/N

Plate 2.1. Some of the ectomycorrhizal fungi associated with hazel in Tasmania.

1. *Descomyces albus*

2. *Melanogaster ambiguus*

3. *Hymenogaster arenarius*

4. *Hymenogaster australis*

5. *Laccaria tortilla*

6. *Podohydnangium* sp.

2.3.1 Host Specificity of Endemic Species

Some Australian endemic mycorrhizal fungi, previously thought to be *Eucalypt* specific, appear to be forming mycorrhizal relationships with hazel. In particular, *Descomyces albus* sporocarps were found at six of the fifteen sites surveyed. At sites 1 and 2, and to a lesser extent 3, *Descomyces albus* fruited prolifically. An unidentified species of *Descomyces* (T024H) may also be mycorrhizal with hazel.

The survey findings also indicate that *Hydnangium*, which is another common Australian genus, appears to be forming mycorrhizas with hazel. *Hydnangium archeri* and an unidentified *Hydnangium* species (T001H) were both found directly beneath clusters of hazel. While it is possible that they were hosted by *Eucalyptus* in the surrounding area, the assumption that hazel was the host is supported by the subsequent collection of *Podohydnangium sp.* and *Laccaria tortilla* at site 2, and other collections of *Laccaria* at sites 1,3 and 7. *Hydnangium*, *Podohydnangium* and *Laccaria* are closely related genera (Beaton *et al.*, 1984). *Podohydnangium* is a sub-epigeal Australian genera that has characteristics intermediate to that of *Laccaria* and *Hydnangium* (Beaton *et al.*, 1984). If species of *Podohydnangium* and *Laccaria* are able to form mycorrhizas with hazel, it seems probable that *Hydnangium* would be able to do likewise.

Further to this, *Hydnangium* and other endemic genera such as *Descolea* which are commonly associated with *Eucalyptus* have been reported fruiting under *Nothofagus* (CSIRO database; Bougher, 1995). This information and the findings of this survey suggests that some genera of Australian endemic fungi such as *Hydnangium*, *Descomyces* and related genera have broader host ranges than previously reported.

Additional studies are required to determine whether those endemic ectomycorrhizal fungi apparently supported by hazels are forming mycorrhizas with a mantle and Hartig net or more superficial types of mycorrhizas. Further studies are also required to elicit the host ranges of these fungi. If they are able to

form mycorrhizas with a member of the *Corylaceae* family, then it is quite conceivable that they may also enter into relationships with other northern hemisphere hardwoods.

2.3.2 Species New to Tasmania

Hymenogaster arenarius, *Hymenogaster australis* and *Laccaria tortilla* are fungal species which have not been previously reported in Australia. *Melanogaster ambiguus* is new to Tasmania, although it has been reported on mainland Australia (May and Wood, 1997). While none of these species have been noted as being particularly aggressive to trufficulture in Europe, the discovery of these fungi indicates that there are potentially other introduced fungi, yet to be discovered, that may impact on Tasmanian truffières.

It is quite likely that *Melanogaster ambiguus, Hymenogaster arenarius* and *Laccaria tortilla* were imported from Victoria on the roots of suckers planted at Site 2 (refer to Table 2.4). The limited collections of these fungi in Tasmania can all be traced to this site. It is interesting to note that these fungi were not apparent at Site 7 where the majority of trees had been taken from Site 2 as rooted suckers. The soil at Site 7 has poor structure and was poorly drained and this may have impacted on the growth of the fungi.

2.3.3 Implications for the Truffle Industry

Of those introduced species collected in this survey, *Hebeloma crustiliniforme* is recognised as an aggressive competitor and harmful to production, particularly in older plantations (Sourzat *et al.*, 1993). *Melanogaster ambiguus* is not known as a competitor in Europe, although *Melanogaster variegatus* occurs occasionally in older truffières (Sourzat *et al.*, 1993). It should be noted however, that *M. ambiguus* was fruiting prolifically under hazel at Sites 1 and 2. If the same level of contamination were to occur in a truffière, it would represent a significant carbon drain on the trees.

With respect to competition from introduced and cosmopolitan fungi previously reported in Tasmania (Appendix 2), only three of these fungi have been reported for *Corylus avellana*. These fungi, *Lactarius piperatus*, *L. subdulcis* and *Paxillus involutus* are not cited as aggressive competitors in Europe. Furthermore, there is some doubt that *L. subdulcis* has been found in Tasmania (N. Bougher, CSIRO, pers. com.). On the other hand, *Cenococcum graniforme* is an example of a known aggressive competitor that could be reasonably expected to exist in Tasmania but appears not to have been reported as such.

Another threat to the truffle industry in Tasmania comes from those fungi that have not been specifically reported for hazel, but are expected to be capable of forming mycorrhizas with hazel (Table 2.3). Of the fungi in this category, *Boletus luridus*, *Laccaria laccata* and *Scleroderma verrucosum* have been reported in Tasmania. Sourzat *et al.* (1993) noted that those trees supporting *Boletus luridus* will not produce truffles. The competitiveness of *Scleroderma* species is subject to debate. Sourzat *et al.* (1993) suggested that *Scleroderma* species were present in most truffières, but mainly inhabited the outer edge of the brûlé and were generally not detrimental to truffle production. However, Hall *et.al.* (1994) reported that a *Scleroderma* species competed with *T. melanosporum* in New Zealand.

Amanita muscaria could possibly pose problems to the Tasmanian truffle industry as it has a broad host range and is able to form mycorrhizas with *Nothofagus* (Fuhrer and Robinson, 1992) and *Eucalyptus* (N. Malajczuk, pers. com.). While it is not mentioned as a truffle competitor in the European literature, several other *Amanita* spp. are mentioned and *A. solitaria* is noted as a very aggressive competitor (Sourzat *et al.*, 1993).

While some Australian endemic mycorrhizal fungi have the ability to form mycorrhizas with hazel, at present, there is little information regarding the compatibility of the relationship, or how competitive to *T. melanosporum* these

fungi might be in a commercial truffière. In particular, it is not known how these endemic ectomycorrhizal fungi will respond to the heavy applications of lime that are being applied to Tasmanian truffières. These issues are examined further in the subsequent chapters of this thesis.

3 The Effect of Applied Lime on *Tuber melanosporum* and other Ectomycorrhizal Fungi found in Tasmania

This section serves as a general introduction to Chapters 4, 5 and 6 which primarily concern the effects of applied lime on *T. melanosporum* and other ectomycorrhizal fungi.

Regions of truffle production in France correspond to soils formed on a calcareous substratum (Delmas, 1978). These soils include rendzinas, brown earths and lithosols (Poitou, 1988). They are typically shallow and rich in fragments of calcareous material. Most of the soils have not been strongly leached, and hence the high calcium levels give rise to high soil pH. From the results of soil surveys conducted in France, Delmas *et al.* (1981) reported that good production of Périgord black truffles occurred on soils with a pH (water) within the range 7.8 to 8.35. In addition to the high pH and high levels of calcium, truffle producing soils are free draining with a friable granular structure. The topsoil generally has a balanced texture (loams) and moderate levels of organic matter (1.5 to 8%)(Delmas *et al.*, 1981). In soils that deviate from these physical and chemical characteristics, *T. melanosporum* is likely to be replaced by other species of ectomycorrhizal fungi including other *Tuber* species (Chevalier and Poitou, 1990).

In contrast to the truffle growing regions of Europe, most Tasmanian soils are acidic (Grant *et al.*, 1995). There are only a few isolated pockets of soils formed from calcareous parent material (Davies, 1965). They include the calcareous coastal sands and terra rossa soils on the west coast of King and Flinders Islands,

loamy soils on precambrian dolerite in far north west Tasmania and podzolic soils formed on limestone in the Mole Creek region. These soils are very limited in area and are not suitable for the cultivation of truffles for a variety of reasons. These reasons differ for each soil type and include factors such as remoteness, steep topography, poor structure and poor drainage.

Consequently, truffières are being established on soils with a neutral or slightly acidic reaction and limestone is applied to increase the pH to the required level. There is little information as to how *T. melanosporum* responds to such practice. However, truffles have been produced from truffières in the USA, New Zealand and France on neutral or acidic soils that were limed to increase the pH (Hall *et al.*, 1994).

The response of native ectomycorrhizal fungi to liming is also a significant consideration. It has been shown that hazel can host some endemic fungal species such as *Descomyces albus* (refer to Section 2.3). It is reasonable to expect that these endemic fungi and naturalised strains of cosmopolitan fungi may be specifically adapted to the acidic nature of Tasmanian soils. Whether these fungi can grow at higher levels of pH needs to be determined. If these native species of fungi are not able to adapt to heavily limed soils, this further reduces the number of ectomycorrhizal fungi able to compete with *T. melanosporum*.

4 The Effect of pH on the *In Vitro* Growth of Native and Introduced Ectomycorrhizal Fungi

4.1 Introduction

It has been widely assumed that most ectomycorrhizal fungi are acidophilic (Slankis, 1974). However, there are clearly many exceptions to the rule (Hung and Trappe, 1983), including *Tuber melanosporum* (Poitou *et al.*, 1983). The response of Australian native fungi to different levels of pH is poorly understood.

This work represents a preliminary study into the pH tolerance of native and introduced ectomycorrhizal fungi found in Tasmania. Isolates of several native mycorrhizal fungi collected in Tasmania were grown *in vitro* on media adjusted to four levels of pH. The growth of these native fungi was compared to that of introduced species collected in Tasmania and elsewhere, including *Tuber* spp. from France.

It is acknowledged that *in vitro* experiments need to be interpreted with caution. For example, Hung and Trappe (1983) were able to group species of ectomycorrhizal fungi into categories depending on the number of pH units over which they exhibited good growth. However the variations in response to pH *in vitro* could not be related to the pH of the soil from which they originated. That is, those fungi that grew best *in vitro* at a high pH did not originate from high pH soils. This contrasts with work quoted by Slankis (1974) where the preferred pH for growth *in vitro* corresponded closely to the pH of soil adjacent to the sporocarps.

Erland *et al.* (1990) also emphasised the limitations of generalising from data obtained in pure culture experiments. The ectomycorrhizal fungi used in their study grew much faster when grown as symbionts than when grown in pure culture. Furthermore, pH tolerance levels of fungi grown in pure culture varied depending on the media used.

With these limitations in mind, *in vitro* experiments are still a useful tool for scanning a large number of fungi for possible trends that may influence the direction of future research.

4.2 Materials and Methods

4.2.1 Overview

Twenty seven ectomycorrhizal fungal isolates growing in culture were sub-cultured onto buffered agarose media adjusted to four levels of pH (5.6, 7.0, 8.0 and 8.5). The radial growth of the fungi was measured to give a growth rate in millimetres per day.

4.2.2 Fungal Treatments

The fungal isolates used in the experiment are listed in Table 4.1. Those fungi with the code prefixed by the letter 'T' were collected as part of the survey documented in Chapter 2. The *Tuber* spp. were provided by Mr Gérard Chevalier, INRA, France. The remainder of the isolates were obtained from the Australian Centre for International Agricultural Research collection maintained at CSIRO, Division of Forestry, Perth.

Table 4.1. List of fungal isolates.

Code	Fungal Isolate	Origin	Location	Vegetation	Soil pH*	Preferred Medium
E0412	*Amanita muscaria*	Introduced	Burnie, TAS	*Pinus radiata*	NA	Mn
H0222	*Astraeus pteridis*	Introduced	USA	NA	NA	Pach
E4432	*Boletus piperatus*	Introduced	Maydena, TAS	*Tsuga heterophylla, Pseudotsuga menziesii*	NA	Mn
H0220	*Cenococcum geophilum*	Introduced	USA	NA	NA	Mn
T011H	*Cortinomyces* sp.	Unknown	Perth, TAS	*Corylus avellana*	4.9	PDA
E4974	*Descolea recedens*	Endemic	Maydena, TAS	*Eucalyptus regnans*	NA	Pach
T003H	*Descomyces albus*	Endemic	Hobart, TAS	*C. avellana*	7.0	Pach
T007H	*Descomyces albus*	Endemic	Penguin, TAS	*C. avellana*	6.0	PDA
H2114	*Descomyces curvirostratus*	Endemic	Burnie, TAS	*E. globulus*	NA	PDA
E0784	*Hebeloma westraliense*	Endemic	Moore River, WA	*E. wandoo*	NA	Pach
E0200	*Hebeloma crustiliniforme*	Introduced	USA	NA	NA	Mn
T005H	*Hydnangium archeri*	Endemic	Hobart, TAS	*C. avellana*	7.0	Pach
T015H	*Hydnangium carneum*	Endemic	Wrens, Manjimup, WA	*E. globulus*	5.7	Pach
H1151	*Hymenogaster* sp.	Endemic	TAS	*E. globulus*	NA	Mn
E0496	*Inocybe* sp.	Introduced	Adelaide, SA	NA	NA	Mn
H0611	*Labyrinthomyces varius*	Endemic	Manjimup, WA	*E. diversicolor*	NA	PDA
E0202	*Laccaria bicolor*	Introduced	USA	NA	NA	Mn
E2092	*Laccaria* sp.	Endemic	TAS	*E. nitens*	NA	Mn
E1130	*Laccaria* sp.	Endemic	TAS	*E. nitens*	NA	Pach
H1375	*Mesophelia clelandii*	Endemic	Scamander, TAS	*E. delegatensis*	NA	Mn
E4948	*Paxillus* sp.	Endemic	Geeveston, TAS	*Nothofagus cunninghamii, E. delegatensis,*	NA	Pach
T006E	*Scleroderma verrucosum*	Cosmopolitan	Tonganah, TAS	*C. avellana*	5.4	PDA
H2000	*Scleroderma verrucosum*	Cosmopolitan	Bakers Hill, WA	*E. robusta*	NA	Mn
H1023	*Setchelliogaster sp.*	Endemic	TAS	*E. globulus*	NA	Mn
H6315	*Setchelliogaster sp.*	Endemic	Maydena, TAS	*Nothofagus cunninghamii*	NA	Mn
Mos Pey	*Tuber brumale*	Introduced	France	NA	NA	Malt
Mel 24	*Tuber melanosporum*	Introduced	France	NA	NA	Malt

- * pH of soil at the site of collection of sporocarps (if known)
- Mn = Modified Melin Norkans medium (Marx, 1969); Pach = Pachlewski medium (Pachlewski and Pachlewski, 1974); PDA = Potato Dextrose Agar; Malt = 10 g/L malt extract.
- E4948, E2092 & E1130 are assumed to be endemic because of their association with native hosts.
- WA = Western Australia; TAS = Tasmania; SA = South Australia.

4.2.3 Media

Fungal isolates were grown in 90 mm petri dishes on one of four media; modified Melin Norkans (Marx, 1969), Pachlewski (Pachlewski and Pachlewski, 1974), potato dextrose agar or malt extract. One medium would not support all the isolates, so isolates were grown on a medium on which they were known to grow strongly. The type of medium used for each isolate is given in Table 4.1. The composition of each medium is given in Appendix 3.

The biological buffers MES, HEPES, and Tricine were applied to each medium at 10 mM. The characteristics of these buffers are shown in Table 4.2.

Table 4.2. Characteristics of the biological buffers used in the media.

Buffer name	Chemical name	pK_a (25°C)	Buffering Range (25°C)
MES	2-(N-Morpholino)ethanesulfonic acid	6.1	5.5-6.7
HEPES	N-(2-Hydroxyetyl)piperazine-N-(2-ethanesulfonic acid)	7.5	6.8-8.2
Tricine	N-tris(Hydroxymethyl)methylglycine; N-(2-Hydroxy-1,1-bis[Hydroxymethyl]ethyl)glycine	8.1	7.4-8.8

Source: Sigma Chemical Company catalogue, 1996.

4.2.4 pH Treatments

Each fungal isolate was grown on buffered media at four levels of pH; 5.6, 7.0, 8.0 and 8.5. The pH of the media was adjusted using KOH as a strong alkali was needed to adjust the pH once the buffers were added. In addition to the buffered treatments, an unbuffered treatment at pH 5.6 was included to determine the effect of the buffers on fungal growth. For each treatment combination, there were four replicates.

The number of pH treatments was restricted by the limited availability of plates from which to subculture and the very slow growth rates of some species in

culture, particularly *T. melanosporum*. This made it difficult to bulk up the number of plates required for the experiment. The range 5.6 to 8.5 corresponds to the pH range dealt with in the field. The majority of soils selected for truffières in Tasmania have a pH greater than 5.6. This figure also lies between the pH usually used for the Pachlewski medium (5.4) and modified Melin Norkans (5.8). A soil pH (water) of 8.5 is approximately the maximum pH that can be reached by applying calcium carbonate.

4.2.5 Subculturing

Isolates were subcultured using a corer, ten millimetres in diameter, to cut a disk at the edge of the culture. The disk was then transferred to the centre of a treated agar plate. Five disks were taken from a single culture with one disk for each pH treatment. This process was repeated four times to obtain four replicates.

4.2.6 Measurements

Once the culture had grown approximately 20 mm from the disk, the radial growth was measured on four axis at about 90° to each other. Mean radial growth was calculated in millimetres per day.

4.2.7 Statistics

The results for each isolate were analysed using single factor ANOVA. Means were compared using the Least Significant Difference test.

4.3 Results and Discussion

Fungal growth for the buffered treatment at pH 5.6 varied quite significantly from that of the unbuffered treatment for most isolates (refer to Table 4.3). The majority of isolates showed a positive response to the inclusion of the buffers, however there were no trends between genera or between introduced and endemic species.

Table 4.3. Effect of applying biological buffers on the growth of the fungal isolates at pH 5.6.

Endemic Species			Introduced & Cosmopolitan Species		
Fungal Isolate	**Code**	**Δ Growth**	**Fungal Isolate**	**Code**	**Δ Growth**
Cortinomyces sp.	T011H	13%	*Amanita muscaria*	E412	26%*
Descolea recedens	E4974	-45%*	*Astraeus pteridis*	H222	38%
Descomyces albus	T007H	90%*	*Boletus piperatus*	E4432	18%*
Descomyces albus	T003H	5%	*Cenococcum geophilum*	H0220	44%*
Descomyces curvirostratus	H2114	-6%	*Hebeloma crustiliniforme*	E200	169%*
Hebeloma westraliense	E784	93%*	*Inocybe* sp.	E496	32%*
Hydnangium archeri	T005H	-100%*	*Laccaria bicolor*	E202	52%*
Hydnangium carneum	T015H	45%*	*Scleroderma verrucosum*	H2000	-85%*
Hymenogaster sp.	H1151	4%	*Scleroderma verrucosum*	T006E	22%
Labyrinthomyces varius	H611	-20%*	*Tuber brumale*	MosPey	42%*
Laccaria sp.	E2092	62%*	*Tuber melanosporum*	Mel 24	-79%*
Laccaria sp.	E1130	-11%			
Mesophelia clelandii	H1375	125%*			
Paxillus sp.	E4948	50%*			
Setchelliogaster sp.	H6315	77%*			
Setchelliogaster sp.	H1023	-17%*			

* denotes a significant change (P>0.05)

The following results concern the changes in growth rate between the four pH treatments for each isolate. It is not valid to compare growth rates between fungal isolates as they were grown on different media and responded quite differently to the application of the buffers. Nor is there any reason to compare the growth rate between isolates as their growth rate in culture relative to their growth rate as symbionts is likely to vary from isolate to isolate (Erland *et al.*, 1990).

Growth responses of endemic ectomycorrhizal fungi to increasing pH of the media are shown in Figures 4.1-3. Members of the Cortinariaceae family are represented in Figure 4.1. All of these isolates exhibited best growth at pH 5.6 and declined with increasing pH, some more rapidly than others. Similar responses occurred for those fungi in Figure 4.2, however the two *Laccaria* species exhibited good

growth over a broader pH range. From these two figures, it would appear that many Australian native fungi are acidophilic. On the other hand, Figure 4.3 demonstrates that some native species were largely unaffected by increasing pH. It also demonstrates that two species of a genus can react quite differently. *Setchelliogaster* sp (H6315) grew well at all levels of pH whereas *Setchelliogaster* sp. (H1023) showed no growth at pH 8 and 8.5.

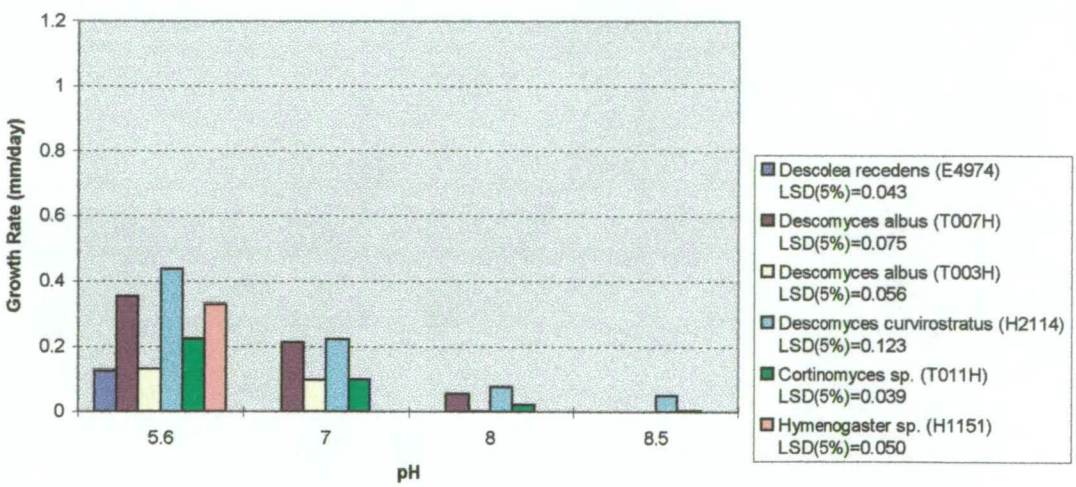
Figure 4.4 includes both introduced and cosmopolitan species exhibiting an acidophilic nature. This contrasts with Figure 4.5 where the introduced species, with the exception of the *Tuber* species, grew well at all pH treatments. *Hebeloma crustiliniforme*, *Cenococcum graniforme* and species of *Amanita*, *Astraeus*, *Boletus*, *Inocybe* and *Scleroderma* were included in the experiment as these species/genera are known competitors to *T. melanosporum* (Chevalier and Poitou, 1990; Sourzat *et al.*, 1993) and must therefore have the capacity to tolerate alkaline soils. However, comparisons between the results depicted here and observations of fungal fruiting on calcareous soils are difficult as species of the same genera, and strains within a species can respond quite differently to pH (Hung and Trappe, 1983).

For the six isolates that were collected as part of the survey reported in Chapter 2, there was information on the pH of the soil at the site of collection (refer to Table 4.1). In each case the soil was either acidic or neutral and this generally corresponded to better growth in pure culture at the pH 5.6 and 7.0 treatments (refer to Figures 4.1, 4.2 & 4.4).

The growth rate of *T. melanosporum* in culture is very slow. Growth was highest at pH 7.0 with little growth at pH 8.0 and 8.5. These results differ from those of Poitou *et al.* (1983) where *T. melanosporum* exhibited weak growth below pH 7.0 and optimal growth from pH 7.9 to 9.0. These differences may be due either to differences in the media, the alkali used to adjust the pH, or the strain of *T. melanosporum*. Poitou *et al.* (1983) used a modified Modess-Mikola medium and several different alkali to adjust pH. *Tuber brumale* grew best at pH 5.6 which is in accordance with the finding of Poitou *et al.* (1983) that *T. brumale* prefers a lower pH than *T. melanosporum*.

While there are many limitations of pure culture experiments, those native species that were collected in the field survey, such as *Descomyces albus* and *Hydnangium archeri* appear to prefer slightly acid or neutral reaction and do not grow well in alkaline media. Further studies with these species grown as symbionts are required in order to predict how these fungi will respond to the heavy lime applications occurring in Tasmanian truffières.

Figure 4.1 Growth response of endemic[1] ectomycorrhizal fungi to increasing pH.



[1] The origin of the Cortinomyces sp. is unknown.

Figure 4.2. Growth response of endemic ectomycorrhizal fungi to increasing pH

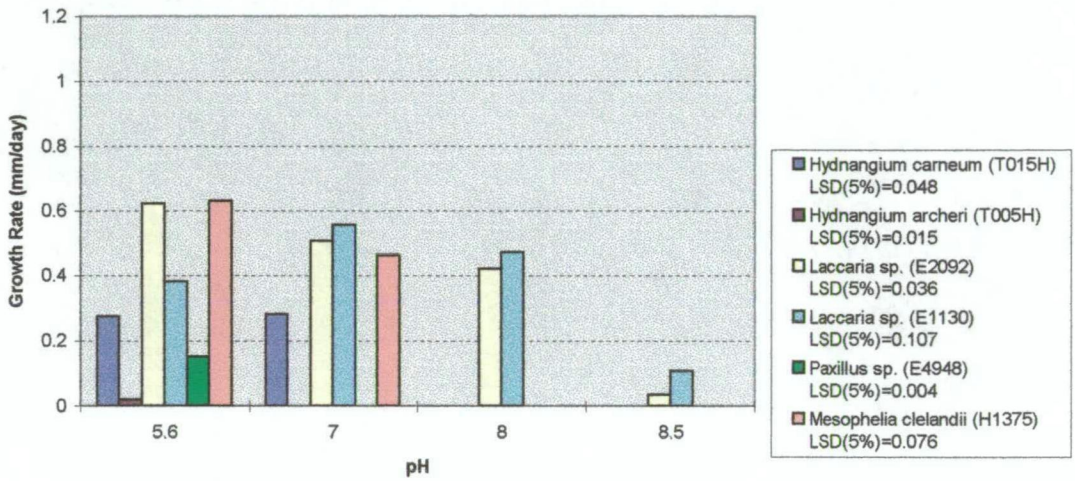


Figure 4.3. Growth response of endemic ectomycorrhizal fungi to increasing pH

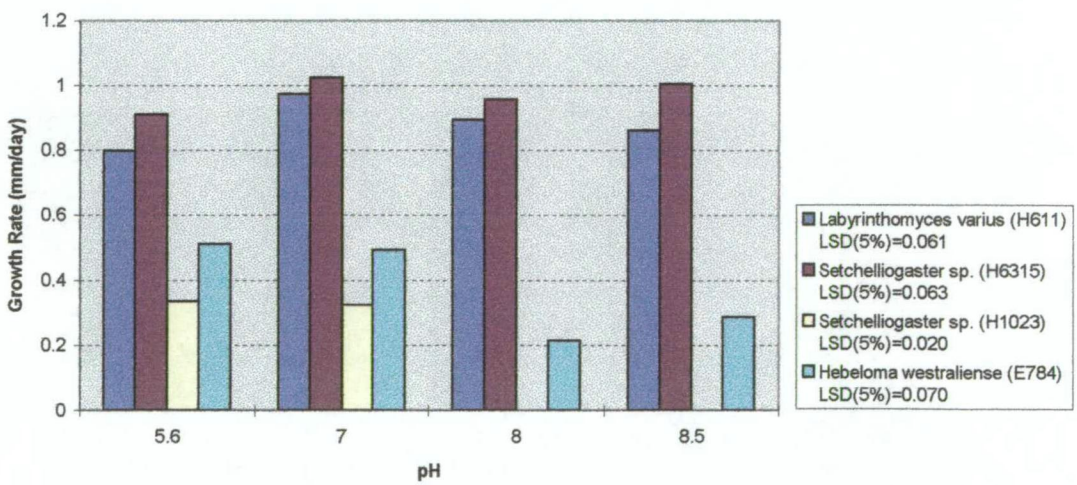


Figure 4.4. Growth response of introduced and cosmopolitan ectomycorrhizal fungi to increasing pH.

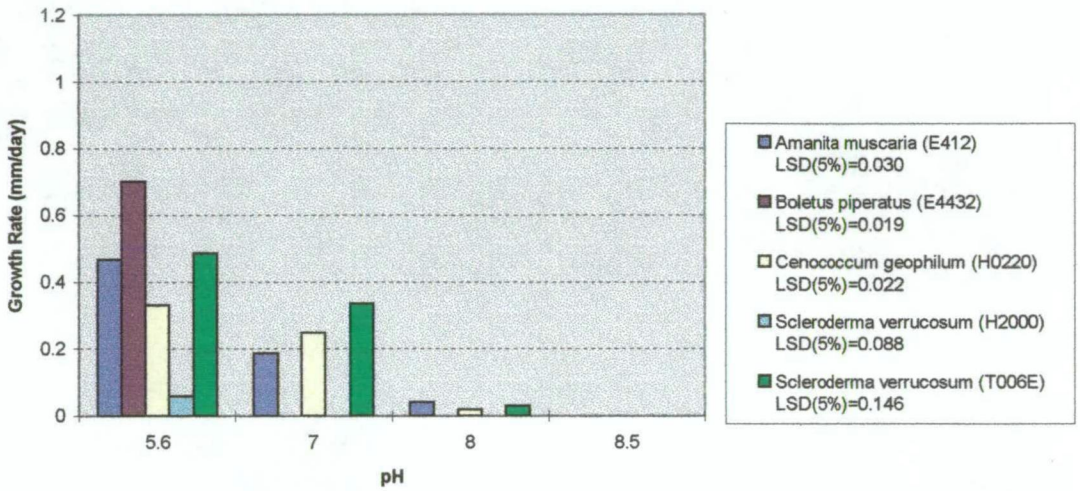
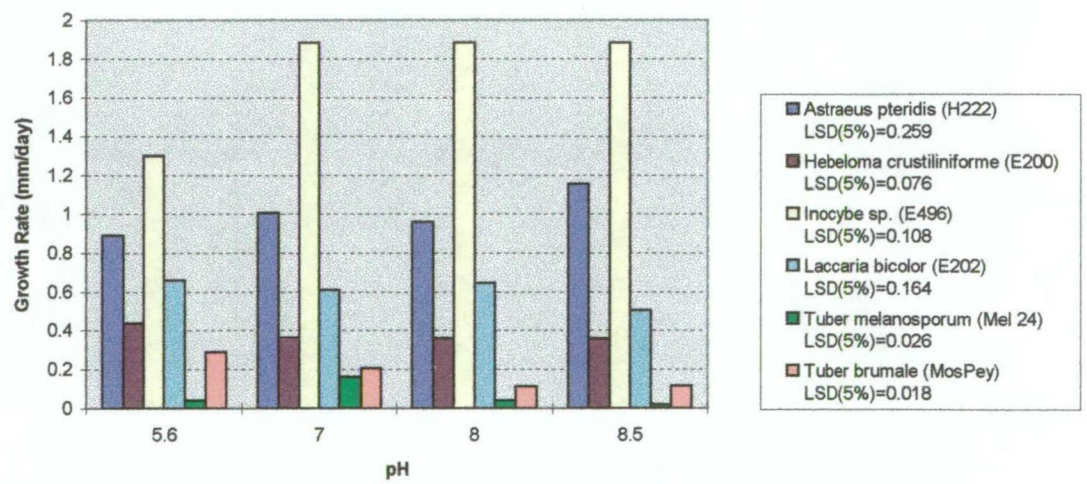


Figure 4.5. Growth response of introduced ectomycorrhizal fungi to increasing pH



5 The Effect of Applied Lime on *Tuber melanosporum* and other Ectomycorrhizal Fungi Forming Mycorrhizas with Hazel

5.1 Introduction

While it has been widely accepted that *T. melanosporum* prefers calcareous soils, there are very few published experiments looking at the effects of applied lime on *T. melanosporum*. Much of the information regarding the preferred soil ecology of *T. melanosporum* has been derived from soil surveys such as that of Delmas *et al.* (1981). From these surveys, it is very difficult to determine which soil characteristics are critical to truffle production as many soil factors are interrelated.

Delmas *et al.* (1981) surveyed soils from 144 truffières throughout the main truffle growing regions of France. One hundred and fifteen of the truffières exhibited brûlés. The remaining 29 truffières did not exhibit brûlé formation. However, there was no information given about the productivity of these sites. Some of the results of the survey are summarised in Table 5.1.

Delmas *et al.* (1981) used principal components analysis in an attempt to determine which soil parameters were responsible for the brûlé formation. The results indicated that soil types exhibiting brûlé formation were characterised by relatively high levels of fine sand and calcium carbonate, and relatively low levels of fine silt, nitrogen and organic matter. While this process was useful as a preliminary investigation, the conclusions that can be drawn regarding truffle production are limited. For example, it cannot be determined whether the putative positive

influence of fine sand and negative influence of fine silt was due to their effect on soil structure or soil chemistry. Further experimentation in controlled conditions is required to improve the knowledge base on the edaphic requirements of *T. melanosporum*.

Table 5.1. Soil parameters of French truffières as reported by Delmas *et al.* (1981).

Soil Parameter	Range for truffle soils exhibiting a brûlé	Range for all soils surveyed
pH (water)	7.8-8.35	6.7-8.45
Available P (mg/kg)	6-980	4-1380
Total P (mg/kg)	555-2531	550-3300
Total N (g/kg)	0.460-5.220	0.34-7.34
Organic Carbon (g/kg)	4.7-50	3.3-88.6
Carbon / Nitrogen Ratio	8.57-13.7	5.8-18.11
Percentage Organic Matter	0.8-8.3	0.6-12.7
Exchangeable Ca (me/100 g)	23.75-67.5	0.41-73.75
Exchangeable Mg (me/100 g)	0.425-4.422	0.23-7.65
Exchangeable K (me/100 g)	0.1-1.29	0.05-1.48
Exchangeable Na (me/100 g)	0.036-0.17	0.036-0.25

The work by Delmas *et al.* (1981) also raises the question of how to predict truffle production. Delmas *et al.* (1981) used the presence or absence of a brûlé as an indicator of truffle production. The correlation between brûlé formation and truffle production has not been studied, but the relationship does not appear to be strong. Trees without a brûlé can produce truffles and trees with a brûlé may not (Le Tacon *et al.*, 1982). Furthermore, other species of ectomycorrhizal fungi such as *Tuber aestivum* and *Scleroderma* sp. are known to produce a brûlé (Hall *et al.*, 1994).

The percentage of roots colonised by *T. melanosporum*, or the total length of mycorrhizal roots, may be a better indication of potential fruit body production than brûlé formation, although these relationships have not been examined. In most vesicular-arbuscular mycorrhizal associations, there is a close correlation between spore formation and the total length of mycorrhizal roots produced by a given host (Brundrett, 1991). However, it is possible that conditions which enable good mycorrhizal infection by *T. melanosporum* may not be suitable for fruiting. Termorshuizen and Schaffers (1989) studied correlations between environmental factors, fruiting of ectomycorrhizal fungi and mycorrhizal infection for *Pinus sylvestris*. There was a significant correlation (Spearman correlation coefficient of 0.50, P<1%) between the level of fruiting and the total number of mycorrhizas. A stronger correlation (Spearman correlation coefficient of 0.73, P<1%) existed between the level of fruiting and the number of branched mycorrhizas. However, fruitbody production was more sensitive to adverse environmental conditions (atmospheric NH_3 and SO_2 pollution) than the level of mycorrhizal colonisation.

In field experiments on established truffières, such as those conducted by Le Tacon *et al.* (1982), fruiting can be measured directly to determine the effects of various soil treatments. However, the variability of conditions in the field, combined with the extremely sporadic nature of fruiting of *T. melanosporum* may necessitate a high number of replicates and measurement of fruiting over a number of years in order to reveal treatment effects.

5.1.1. Experimental Conditions and Objectives

In Tasmania where there has been no fruiting of *T. melanosporum* to date, the level of mycorrhizal colonisation by *T. melanosporum* was chosen as the most practical indicator of potential truffle production. This experiment aims to study the effects of applied lime on the level of mycorrhizal colonisation by *T. melanosporum* and other introduced and native ectomycorrhizal fungi in controlled glasshouse conditions. The experiment was conducted as a pot experiment in a glasshouse where soil and other environmental factors can be controlled and the level of mycorrhizal colonisation can be determined more readily than in the field. This

decreases the level of variation in colonisation caused by non-treatment effects and therefore increases the potential of revealing treatment effects.

The specific objectives were:

1. To confirm that the endemic fungi collected in the survey (Chapter 2) can form mycorrhizal relationships with hazel.
2. To see if some Australian endemic species, that are normally mycorrhizal with *Eucalyptus*, are able to form mycorrhizas with hazel.
3. To determine how these endemic fungi respond to heavy lime application.
4. To examine the growth of *T. melanosporum* on an acid soil that has been heavily limed both in the absence and presence of other ectomycorrhizal fungi, either native or introduced.

5.2 Materials and Methods

5.2.1 Overview

In October 1994, 120 hazel seedlings (*Corylus avellana*) inoculated with one of eight fungal treatments were transplanted into pots in a glasshouse in Perth, Western Australia. Plants from each fungal treatment were grown at three levels of applied lime. For each lime-fungus treatment combination, there were five replicates. The pots were randomly distributed on benches within the glasshouse and the benches were rotated on a weekly basis. The experiment was non-destructively sampled three times over a period of 26 months.

5.2.2 Soil Preparation

A yellow sandy soil with a pH of 6.0 (1:5 0.01M $CaCl_2$) and Bray-extractable P of 2 mg / kg (total P 22 mg / kg) was collected from the Spearwood dune system north of Perth, Western Australia. The soil was steam sterilised twice at 80°C for

two hours and oven dried at 70°C before being sieved through a 2 mm sieve. This yellow sandy soil was selected as the growing medium for the following reasons;

- ease of handling for the processes of sterilising, drying, sieving and potting,
- known suitability for supporting native ectomycorrhizal fungi in previous pot experiments (Bougher *et al.*, 1990),
- ease with which strands of fungal hyphae and primordia can be seen growing in the soil,
- ease of removing soil from the roots for root analysis.

Fourteen centimetre plastic pots were lined with plastic bags, and each filled with 2.5 kg of dried soil. Basal nutrients were applied in solution to each pot at the following rates (mg / kg sand): K_2SO_4, 111.6; $CaSO_4.2H_2O$, 51.5; $MgSO_4.7H_2O$, 33.7; $CuSO_4.5H_2O$, 8.2; $MnSO_4.4H_2O$, 16.9; $ZnSO_4.7H_2O$, 9.2; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.46; $CoCl_2.6H_2O$, 0.34; $Na_2B_4O_7.10H_2O$, 1.10. When the soil had air-dried after the application of these solutions, 100 mg of $Ca(H_2PO_4)_2.H_2O$ was applied as a powder to each pot, together with the relevant lime application. These applications were thoroughly mixed through the soil by shaking in a plastic container. The surface of each pot was covered with reflective aluminium sheeting to limit evaporation and retard algal growth. The reflective sheeting also promotes the growth of roots and hyphae near the surface of the soil. The pots were watered to field capacity (14% w/w) with deionised water and left to equilibrate for one month prior to planting. After the equilibration period, a chemical analysis was conducted on a sample of 6 replicates from each soil treatment. Mean values from this analysis are shown in Table 5.2. The methods used for determination of soil parameters are given in Table 5.3.

Table 5.2. Mean chemical analysis of each soil treatment.

Soil Parameter	No Lime	Low Lime	High Lime
Available P (mg/kg) Bray test	3.8 a	4.6 a	5 a
Total P (mg/kg)	47.2 a	47.5 a	45.5 a
Total N (g/kg)	0.041 a	0.036 a	0.042 a
Organic Carbon (g/kg)	0.832 a	0.691 a	0.740 a
Carbon / Nitrogen Ratio	20.4 a	19.2 a	17.8 a
Percentage Organic Matter	0.14 a	0.12 a	0.13 a
Exchangeable Ca (me/100g)	0.266 c	0.515 b	0.720 a
Exchangeable Mg (me/100g)	0.146 a	0.105 b	0.085 c
Exchangeable K (me/100g)	0.026 a	0 b	0 b
Exchangeable Na (me/100g)	0.155 a	0.167 a	0.068 a

- Means that are followed by the same letter are not significantly different ($P<0.05$) by the Least Significant Difference test.
- Exchangeable bases were extracted using NH_4Cl.

Table 5.3. Methods used for determination of soil parameters.

Soil Parameter	Method	Reference
Available P (mg/kg)	Fluoride-extractable P (Bray 1-P)	Rayment & Higginson (1992)
Total P (mg/kg)	Sulphuric acid, potassium sulphate, copper sulphate	Heffernan (1985)
Total N (g/kg)	Sulphuric acid, potassium sulphate, copper sulphate	Heffernan (1985)
Organic Carbon (g/kg)	Walkley & Black	Rayment & Higginson (1992)
Percentage Organic Matter	Determined by calculation (Organic Carbon * 1.7)	Brady (1984)
Exchangeable Ca (me/100g)	1M ammonium chloride at pH 7	Rayment & Higginson (1992)
Exchangeable Mg (me/100g)	1M ammonium chloride at pH 7	Rayment & Higginson (1992)
Exchangeable K (me/100g)	1M ammonium chloride at pH 7	Rayment & Higginson (1992)
Exchangeable Na (me/100g)	1M ammonium chloride at pH 7	Rayment & Higginson (1992)

5.2.3 Fungal Treatments

Week old hazel seedlings, germinated in sterile potting mix, were inoculated with one of eight fungal treatments.

1. Control. Plants not inoculated.
2. *Tuber melanosporum* Vitt.
3. *Melanogaster ambiguus* (Vitt.) Tul.
4. *Descomyces albus* (Klotzsch) Bougher & Castellano
5. *Hydnangium carneum* Wallr.
6. *Scleroderma mcalpinei* (Rodway) Castellano
7. *T. melanosporum* and *M. ambiguus* .
8. *T. melanosporum* and *D. albus*

The inoculation procedure for *T. melanosporum* has not been documented due to the commercially sensitive nature of the information. For the remaining treatments, spore slurries of both air dried and fresh sporocarps were used to inoculate the seedlings. Ten millilitres each of both the dry spore and wet spore slurry were used per seedling. Spores slurries were obtained by blending sporocarps in distilled water. The subsequent spore slurry concentrations are shown in Table 5.4.

Table 5.4. Source and concentration of spore slurries.

Fungus	Collection Number	Collection Site	Host	Origin	Spore Slurry Concentration (spores ml^{-1})	
					Dry	Fresh
Tuber melanosporum	N/A	France	N/A	Introduced	N/A	N/A
Melanogaster ambiguus	T006H	Penguin, TAS	*Corylus avellana*	Introduced	250 000	2 775 000
Descomyces albus	T009H	Penguin, TAS	*Corylus avellana*	Endemic	595 000	418 000
Hydnangium carneum	T015H	Wrens, WA	*Eucalyptus globulus*	Endemic	1 725 000	1 240 000
Scleroderma mcalpinei	T017H	Yarnup, WA.	*Eucalyptus globulus*	Endemic	4 680 000	3 170 000

In addition to those fungi deliberately used as fungal treatments, several other ectomycorrhizal fungi became established in the experiment. Three of these contaminating fungi were particularly prominent. The mycorrhizas of these fungi were initially labelled Type 1, Type 3 and Type 9. Type 1 mycorrhizas were later associated with *Hebeloma crustiliniforme* fruitbodies and Type 9 mycorrhizas were associated with fruitbodies of *Laccaria tortilla* (refer to Section 5.3.4). Type 3 mycorrhizas did not fruit and thus remain unidentified.

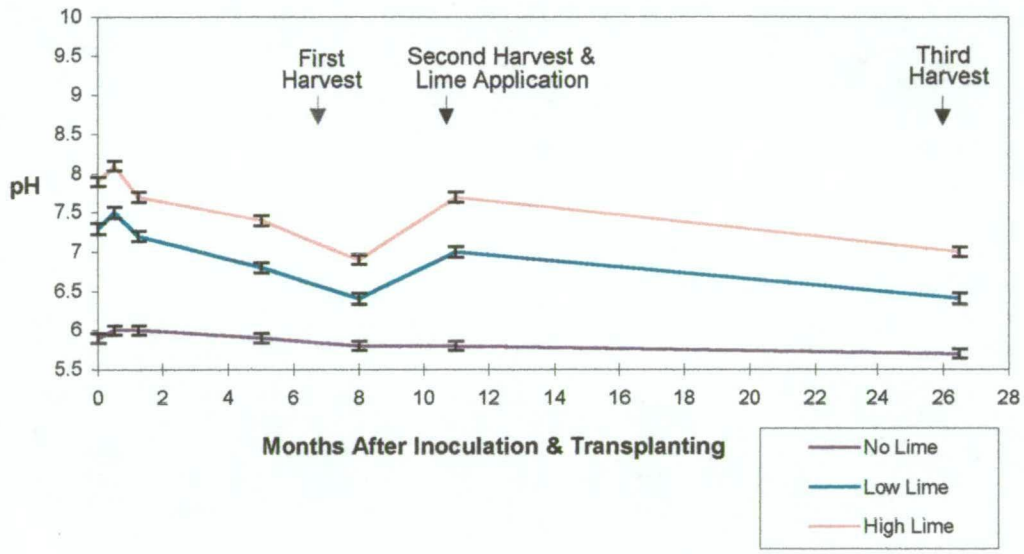
These contaminating fungi were most likely introduced as spores attached to the outside of the *M. ambiguus* and *D. albus* sporocarps collected from Penguin, Tasmania. An explanation for the presence of these fungi is given in Section 5.4.4.

5.2.4 Lime Treatments

The three lime treatments were, "no lime", "low lime" (0.25g CaCO3 / kg soil), and "high lime" (0.5g CaCO3 / kg soil). The quantity of lime required to raise the pH of the soil was determined by incubation as described in Barrow and Cox (1990). Precipitated calcium carbonate (BDH Chemicals Ltd, England) was incorporated in order to facilitate a rapid reaction with the soil. The pH (1:5 0.01M $CaCl_2$) of the lime treatments from the time of inoculation and transplanting until the third harvest are shown in Figure 5.1. The decline in pH of the soil after transplanting was most likely due to the poor buffering capacity of the sandy soil. Fluctuations in soil-soluble salts, due to environmental conditions and fertilizer inputs, cause less variation in pH values measured in 0.01M $CaCl_2$ than those measured in water (White 1969).

After the second harvest, eleven months after the commencement of the experiment, three of the five replicates from each treatment combination were randomly selected and transferred to 25 cm pots. The remaining replicates were discarded. Lime treated soil corresponding to the three treatment levels was used in transferring the plants to larger pots, hence the increase in the soil pH seen at this time.

Figure 5.1. pH ($CaCl_2$) of the lime treatments (bars show standard error).



5.2.5 Glasshouse Preparation and Maintenance

In order to limit contamination by other mycorrhizal fungi, the glasshouse and benches were steam cleaned prior to the commencement of the experiment. Plastic pots and aluminium sheeting were treated with sodium hypochlorite. During the experiment, the glasshouse floor was regularly hosed out to limit contamination from air borne dust.

5.2.6 Irrigation and Temperature Control

The plants were individually weight watered with deionised water to 70% field capacity every day (refer to Plate 5.1). Evaporative coolers restricted maximum temperatures to below 25°C. Climate statistics for Perth are shown in Appendix 4.

Plate 5.1. Watering system used to weight water the plants.

5.2.7 Harvesting and Analysis

5.2.7.1 Mycorrhizal colonisation

The level of mycorrhizal colonisation was determined using non-destructive sampling at three harvest times. The first harvest was in May 1995, seven months after inoculation, the second harvest in September 1995, eleven months after inoculation, and the final harvest in November 1996, after twenty six months. Samples were taken using a stainless steel corer, 25 mm in diameter and 100 mm in length. Three cores were taken from opposite sides of each pot at a distance of about 20 mm from the edge of the pot (refer to Plate 5.2). The corer was flame sterilised between each pot to minimise the risk of cross infection.

The percent colonisation of each mycorrhizal type was calculated on the basis of length. The lengths of each root type were determined using the grid-line intersection method (Giovannetti and Mosse, 1980; Tennant, 1975). For each sample, roots were counted as either fine roots or coarse roots. Fine roots were defined as those capable of forming mycorrhizal roots (generally less than 160 μm in diameter). For the fine root portion, the root length of each mycorrhizal type and the length of non-mycorrhizal roots was determined. These figures enabled the calculation of the following parameters:

- **Percentage Fine Roots = Length of Fine Roots / Total Root Length * 100**
- **Percentage of Mycorrhizal Roots =**
  Length of Mycorrhizal Roots / Length of Fine Roots * 100
- **Percent Colonisation of Mycorrhizal Type X =**
  Length of Type X / Length of Fine Roots * 100
- **Hence, Σ Percent Colonisation of each Mycorrhizal Type =**
  Percentage of Mycorrhizal Roots

5.2.7.2 Plant height and stem diameter

Plant height and stem diameter were measured on four occasions; 1,3,7 and 11 months after inoculation.

5.2.7.3 Sporocarp production

Fruitbody production was noted on the surface of the soil, beneath the reflective foil, and on the side of the pot by lifting the plant out of the pot whilst in the plastic bag. The pots were checked for sporocarps regularly (twice weekly) during the autumn and winter months of the first year. Two fungal species fruited prolifically. On four occasions during the first winter (8 to 10 months after inoculation), the number of primordia and fruitbodies of these two predominant fungi were recorded. Other fungal species were recorded when noticed.

5.2.7.4 Statistical analysis

- The statistical package employed for analysis was SAS for Windows version 6.11.
- The effect of lime on the level of mycorrhizal infection for each harvest period was analysed using single factor analysis of variance for each mycorrhizal type (refer to Section 5.3.1).
- Repeated measures analysis of variance was used to compare levels of mycorrhizal infection between harvests (refer to Section 5.3.1). The Greenhouse-Geisser adjustment factor was utilised whenever the sphericity test was significant ($P<0.05$).
- Two way analysis of variance was used to investigate the effects of lime and fungal treatments on variables such as percentage fine roots, plant height and stem diameter, within each harvest period (refer to Sections 5.3.2 and 5.3.3).
- Correlation analysis was used to investigate relationships between all variables within a harvest period.
- Linear regression analysis was used to compare percentage mycorrhizal roots and percentage fine roots for all harvests (refer to Section 5.3.2).
- Single factor analysis of variance was used to show the effect of fungal treatments on fruitbody production (refer to Section 5.3.4).
- The Least Significant Difference test was used to compare means.

5.3 Results

5.3.1 Mycorrhizal Colonisation

Four of the five fungal species used as inoculum formed mycorrhizal associations with the hazel. *Descomyces* mycorrhizas were formed, but it could not be conclusively shown that these mycorrhizas were derived from the inoculum (refer to Section 5.3.1.3 & 5.4.5). In addition to those used as inoculum, several other contaminant fungi formed mycorrhizas in the experiment. The main mycorrhizal types found in the experiment are described in Section 5.3.5.

The mycorrhizal fungi recorded in the experiment can be divided into three groups according to how they responded to applied lime.

1. Those that were positively affected by the application of lime. *T. melanosporum* was the only species that showed an increase in the level of colonisation when lime was applied.
2. Those that were negatively affected by the application of lime. These were *H. carneum* and *S. mcalpinei*, both of which are endemic to Australia.
3. Those that were relatively unaffected by the application of lime. This group included *M. ambiguus*, *H. crustiliniforme*, *L. tortilla* and *Descomyces* sp., all of which are introduced species with the exception of *Descomyces* sp.

More detailed results for the behaviour of each fungus are given below.

5.3.1.1 Species positively affected by lime

Tuber melanosporum colonisation

T. melanosporum was very slow to establish relative to other species in the experiment. In the treatment inoculated with *T. melanosporum* alone (refer to Figure 5.2), there was no evidence of *T. melanosporum* by the first harvest and only a slight colonisation in the 'low lime' and 'high lime' treatments by the second harvest. At the time of the third harvest, the mean level of colonisation in the 'high lime' treatment had dramatically increased to nearly 60%, the highest level of colonisation by any fungus in the experiment. This level of colonisation for the 'high lime' treatment was significantly higher ($P<0.001$) than that of the 'low lime' and 'no lime' treatments.

As is shown in Figure 5.3, a similar pattern for *T. melanosporum* colonisation occurred in the treatment inoculated with *T. melanosporum* and *M. ambiguus*. However, the responses of *T. melanosporum* to the lime treatments were not significantly different ($P>0.05$). The non significant result was due to the fact that *T. melanosporum* only established in two of the replicates of the 'high lime' treatment, thereby increasing the variation and decreasing the mean level of colonisation.

T. melanosporum did not become established in the treatment where it was co-inoculated with *D. albus*.

Figure 5.2. The level of *Tuber melanosporum* colonisation in the treatment where it was inoculated alone. Means **within** harvests that are followed by the same letter are not significantly different (P<0.05) by the Least Significant Difference test.

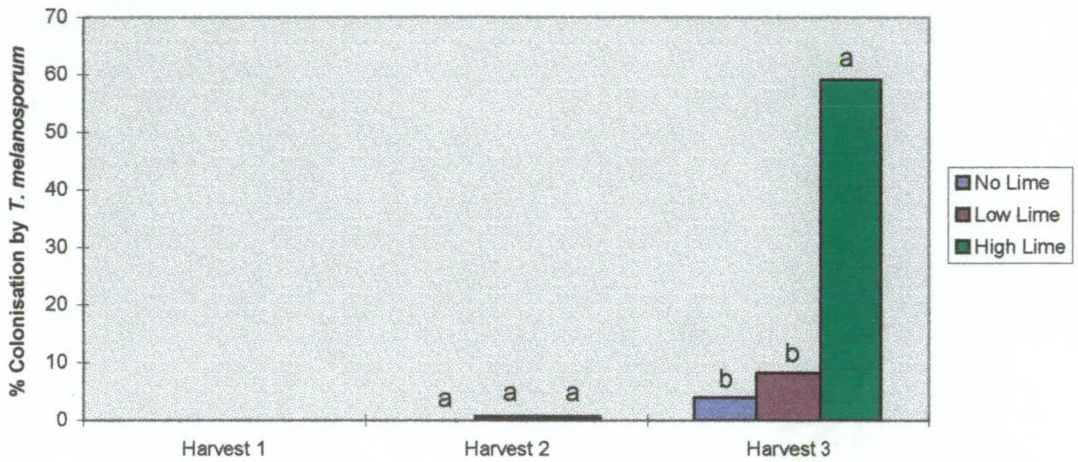
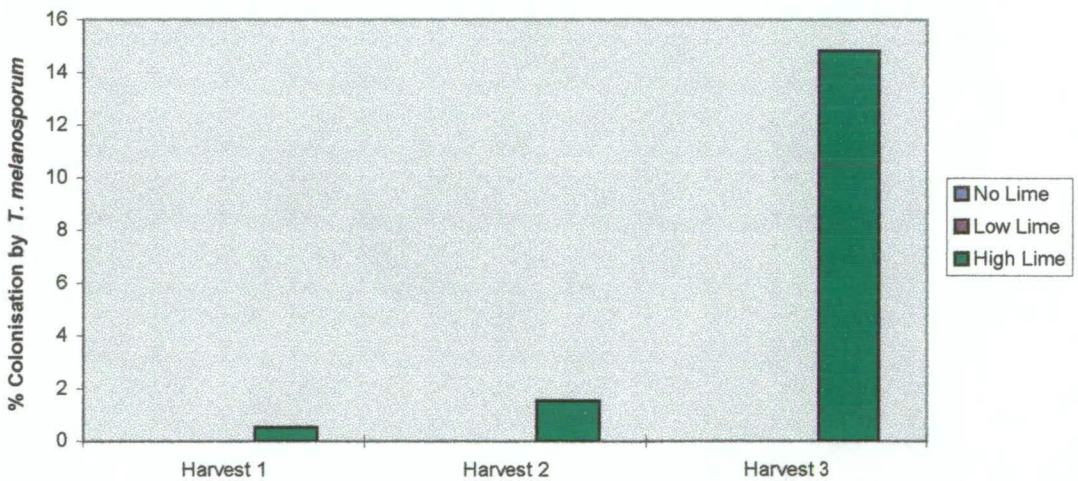


Figure 5.3. The level of *Tuber melanosporum* colonisation in the treatment where it was co-inoculated *Melanogaster ambiguus*. Means within and between harvests are not significantly different (P>0.05).

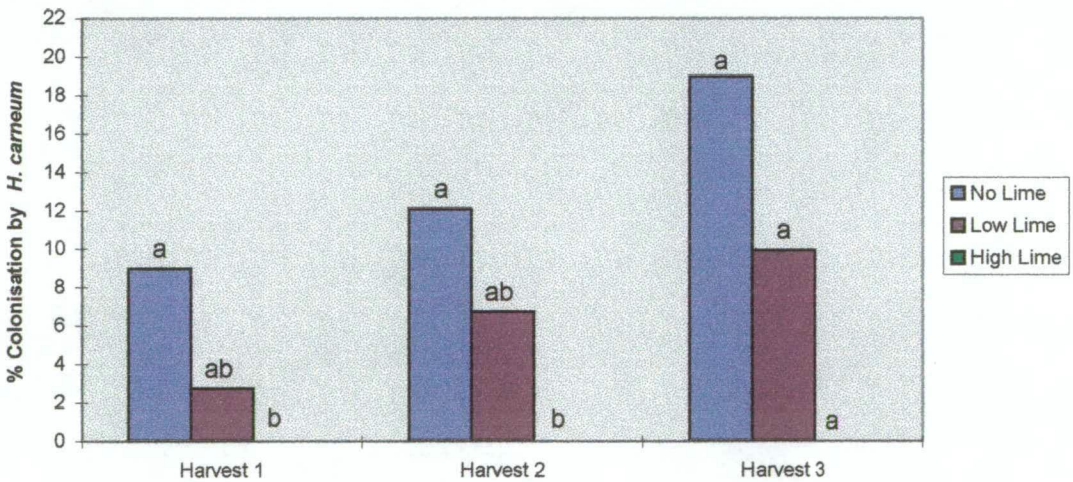


5.3.1.2 Species negatively affected by lime

***Hydnangium carneum* colonisation**

H. carneum showed moderate levels of colonisation by the first harvest, and there were no significant changes (P>0.05) in the level of colonisation in subsequent harvests. Lime had a significant effect on the level of colonisation by *H. carneum*. As shown in Figure 5.4, *H. carneum* did not establish at the 'high' level of applied lime. The 'no lime' treatment exhibited significantly higher levels of colonisation (P<0.05) than the 'high lime' treatment for the first two harvest. While this trend continued in the third harvest, the means were not significantly different (P>0.05).

Figure 5.4. The level of *Hydnangium carneum* colonisation in the treatment inoculated with *H. carneum*. Means **within** harvests that are followed by the same letter are not significantly different (P<0.05) by the Least Significant Difference test.

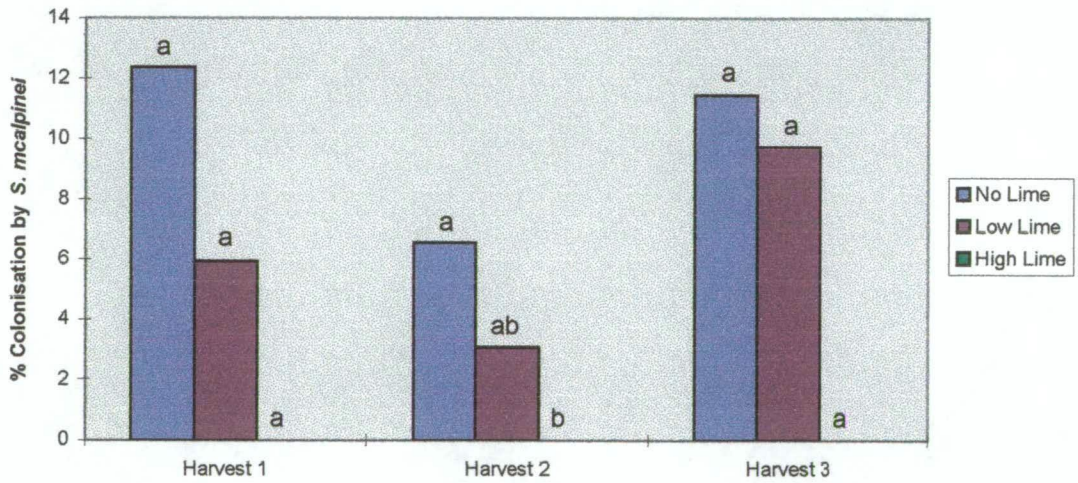


***Scleroderma mcalpinei* colonisation**

There was a general trend for the level of colonisation by *S. mcalpinei* to decrease with increasing levels of applied lime (refer to Figure 5.5). However, while *S. mcalpinei* showed no signs of colonisation at the high level of applied lime, the differences between the 'no lime' treatment and the 'high lime' treatment were only significant ($P<0.05$) in Harvest 2.

After the first harvest the mean level of colonisation did not significantly increase or decrease. That is, there were no significant differences ($P>0.05$) between harvests in the mean level of colonisation.

Figure 5.5. The level of *Scleroderma mcalpinei* colonisation in the treatment inoculated with *S. mcalpinei*. Means **within** harvests that are followed by the same letter are not significantly different ($P<0.05$) by the Least Significant Difference test.

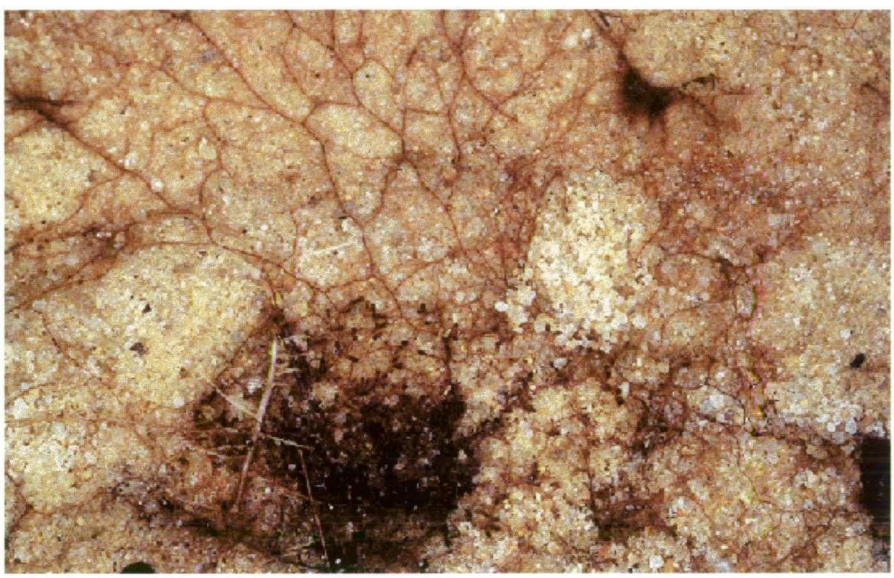


5.3.1.3 Species which were not significantly affected by lime

Melanogaster ambiguus colonisation

The level of colonisation of *M. ambiguus* was low compared to the other fungi in the experiment. While the number of mycorrhizas were low, *M. ambiguus* was noted to produce a mass of strands, visible to the eye, in the pots that it had infected. This mass of strands are shown in Plate 5.3.

Plate 5.3. Mass of strands on the soil surface produced by *Melanogaster ambiguus.*



As indicated in Figures 5.6 and 5.7, lime had no significant effect ($P>0.05$) on the level of colonisation of *M. ambiguus*. Nor was there a change in the levels of *M. ambiguus* over the harvest period. Furthermore, co-inoculation with *T. melanosporum* had no significant effect ($P>0.05$) on the level of *M. ambiguus* relative to that treatment that was inoculated with *M. ambiguus* alone.

Figure 5.6. The level of *Melanogaster ambiguus* colonisation in the treatment where it was inoculated alone. Means **within** harvests that are followed by the same letter are not significantly different (P<0.05) by the Least Significant Difference test.

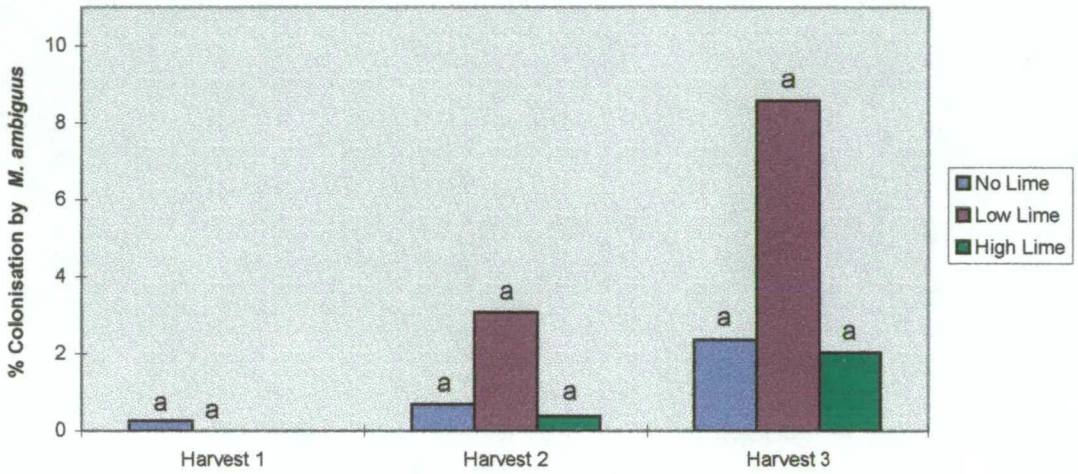
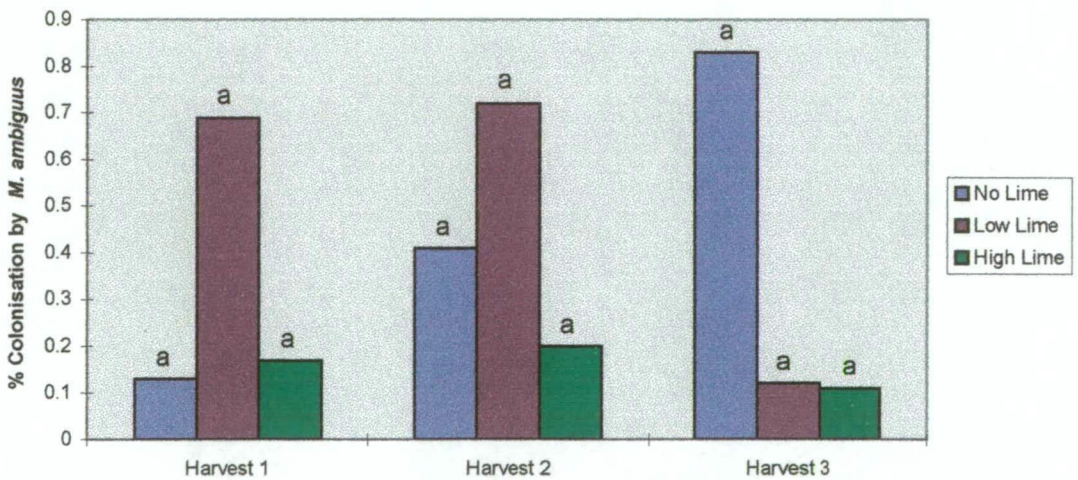


Figure 5.7. The level of *Melanogaster ambiguus* colonisation in the treatment where it was co-inoculated with *T. melanosporum*. Means **within** harvests that are followed by the same letter are not significantly different (P<0.05) by the Least Significant Difference test.



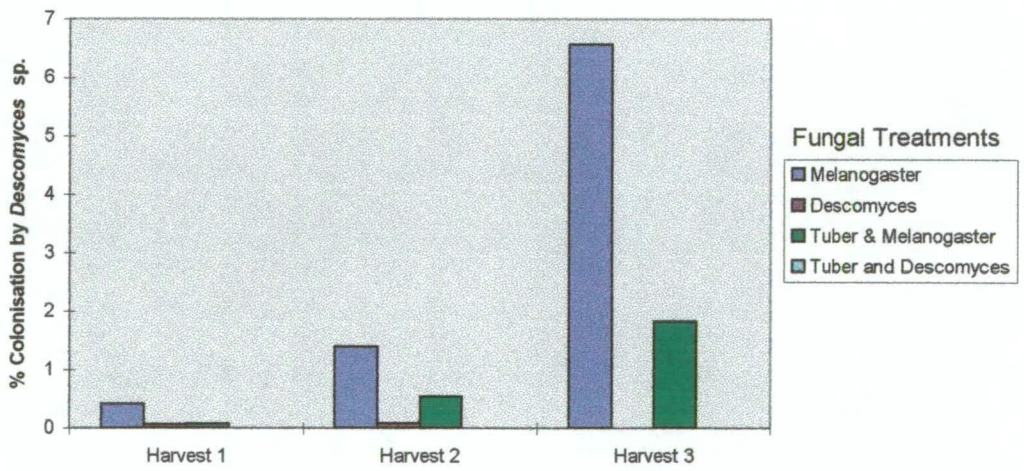
In the second harvest, *M. ambiguus* also appeared as a contaminant at a very low level in one replicate of the 'no lime', '*T. melanosporum* * *D. albus*' treatment combination.

***Descomyces* colonisation**

A *Descomyces* species appeared in those treatments inoculated with *D. albus*, but also in those inoculated with *M. ambiguus* (refer to Figure 5.8). The level of colonisation was higher in those treatments inoculated with *M. ambiguus*, but not significantly higher (P>0.05). By the second harvest, 7 of the 30 replicates inoculated with *M. ambiguus* contained the *Descomyces* mycorrhizas compared to only 1 of the 30 replicates inoculated with *D. albus*.

Lime had no significant effect (P>0.05) on the degree of colonisation of the *Descomyces* sp. for all three harvests. This can be attributed to the considerable variation within and between inoculation treatments as illustrated in Table 5.5.

Figure 5.8. The average level of *Descomyces* sp. colonisation across the three lime treatments for those treatments inoculated with *D. albus* or *Melanogaster ambiguus*. Means are not significantly different (P>0.05).



Contaminating fungi: *Hebeloma crustiliniforme*, *Laccaria tortilla* & Type 3 colonisation

Three contaminating fungi *Hebeloma crustiliniforme*, *Laccaria tortilla* & Type 3 were found at quite high levels in the experiment. In the first and second harvests, these fungi were confined almost entirely to those fungal treatments inoculated with either *M. ambiguus* or *D. albus* (refer to Figures 5.9, 5.10, and 5.11). It was only at the third harvest that *H. crustiliniforme* and *L. tortilla* mycorrhizas were found in other fungal treatments including the control treatment.

Applied lime had no significant effect ($P>0.05$) on the level of colonisation of either *H. crustiliniforme*, Type 3 or *L. tortilla*. This is illustrated for Harvest 3 in Table 5.5. Their occurrence was sporadic and did not appear to be effected by the lime treatments.

Figure 5.9. The level of *Hebeloma crustiliniforme* colonisation for each fungal treatment. Means **within** harvests that are followed by the same letter are not significantly different (P<0.05) by the Least Significant Difference test.

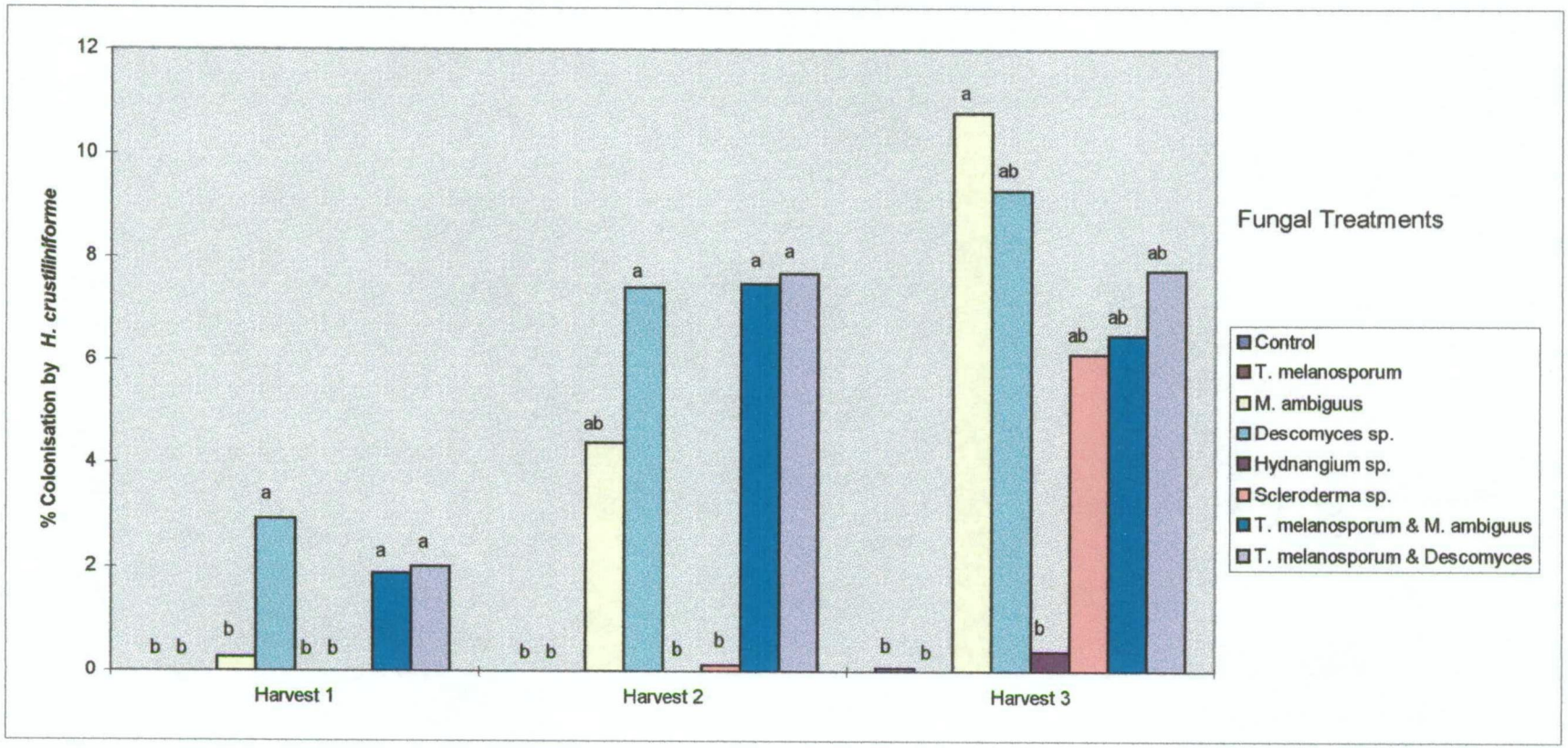


Figure 5.10. The level of Type 3 colonisation for each fungal treatment. Means **within** harvests that are followed by the same letter are not significantly different ($P<0.05$) by the Least Significant Difference test.

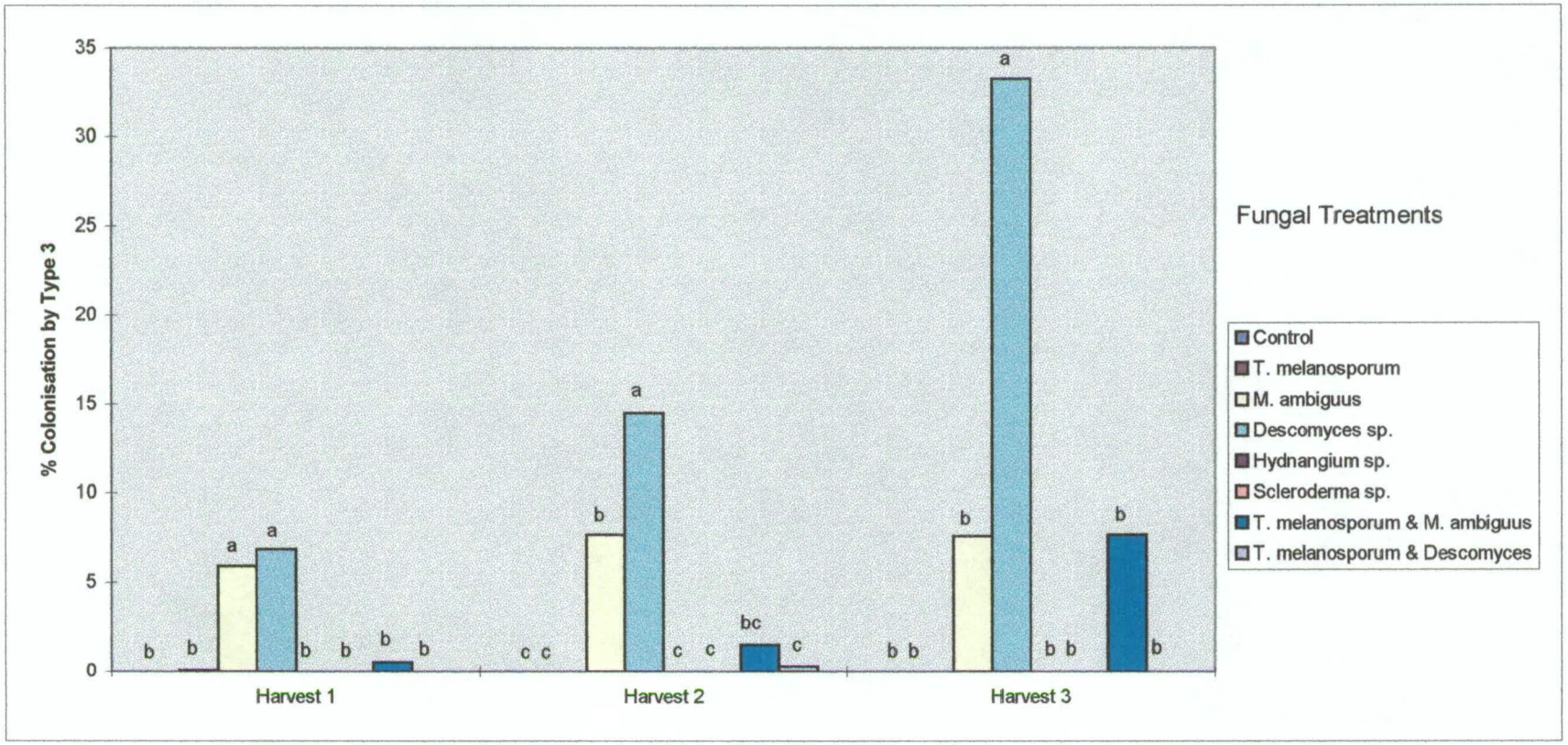


Figure 5.11. The level of *Laccaria tortilla* colonisation for each fungal treatment. Means **within** harvests that are followed by the same letter are not significantly different ($P<0.05$) by the Least Significant Difference test.

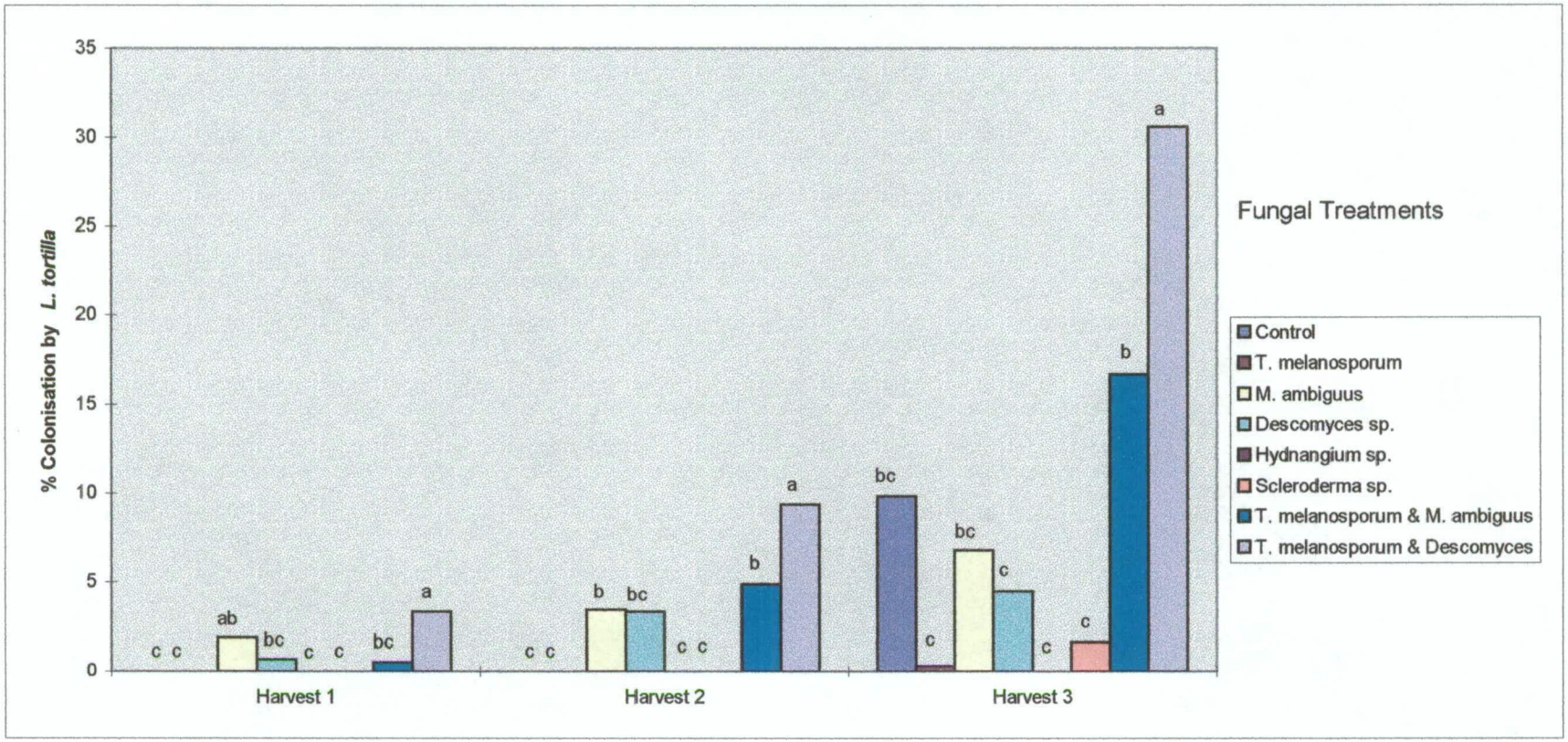


Table 5.5. The effect of lime in Harvest 3 on those mycorrhizal types found in the treatments inoculated with *Melanogaster ambiguus* or *Descomyces* sp. Values in the table are for percentage colonisation of the fungus averaged over the three replicates. The number in brackets refers to the number of replicates in which the mycorrhizal type was detected.

A. *M. ambiguus* inoculum alone

Mycorrhizas formed	No Lime	Low Lime	High Lime	LSD (5%)
T. melanosporum	0	0	0	
M. ambiguus	2.3 (2)	8.5 (2)	2.0 (1)	17.4
D. albus	11.8 (1)	0.9 (1)	7.0 (3)	25.0
H. crustiliniforme	32.3 (2)	0	0	35.0
Type 3	0	22.8 (1)	0	45.5
L. tortilla	18.3 (2)	0	2.1 (1)	19.0
Total	64.7	32.2	11.1	38.9

B. *Descomyces* inoculum alone

Mycorrhizas formed	No Lime	Low Lime	High Lime	LSD (5%)
T. melanosporum	0	0	0	
M. ambiguus	0	0	0	
D. albus	0	0	0	
H. crustiliniforme	17.3 (2)	0.35 (2)	10.1 (2)	24.6
Type 3	12.7 (1)	50.4 (3)	36.9 (3)	41.4
L. tortilla	12.4 (2)	0.27 (1)	0.21 (1)	12.5
Total	42.4	51.0	47.2	30.17

***T. melanosporum* + *M. ambiguus* inoculum**

Mycorrhizas formed	No Lime	Low Lime	High Lime	LSD (5%)
T. melanosporum	0	0	14.8 (1)	29.6
M. ambiguus	0.8 (1)	0.1 (1)	0.1 (1)	1.7
D. albus	0	5.4 (2)	0	8.0
H. crustiliniforme	0	9.5 (2)	10.0 (3)	17.6
Type 3	13.2 (1)	0	9.7 (1)	32.8
L. tortilla	25.8 (2)	22.4 (3)	1.8 (1)	34.4
Total	39.8	37.4	36.4	53.7

***T. melanosporum* + *Descomyces* inoculum**

Mycorrhizas formed	No Lime	Low Lime	High Lime	LSD (5%)
T. melanosporum	0	0	0	
M. ambiguus	0	0	0	
D. albus	0	0	0	
H. crustiliniforme	14.3 (2)	1.1 (2)	7.8 (3)	27.1
Type 3	0	0	0	
L. tortilla	26.9 (3)	26.3 (3)	38.5 (3)	41.6
Total	41.2	27.4	46.3	39.5

5.3.2 The Percentage of Fine Roots

The ratio of fine roots to coarse roots (or the percentage of fine roots), was significantly affected by the lime and fungal treatments, and there were also significant fungal-lime interactions. However, these effects could be explained by a negative correlation between the percentage colonisation and the percentage of fine roots. That is, if a treatment (lime or fungal) increased the level of colonisation, then the ratio of fine roots to coarse roots decreased.

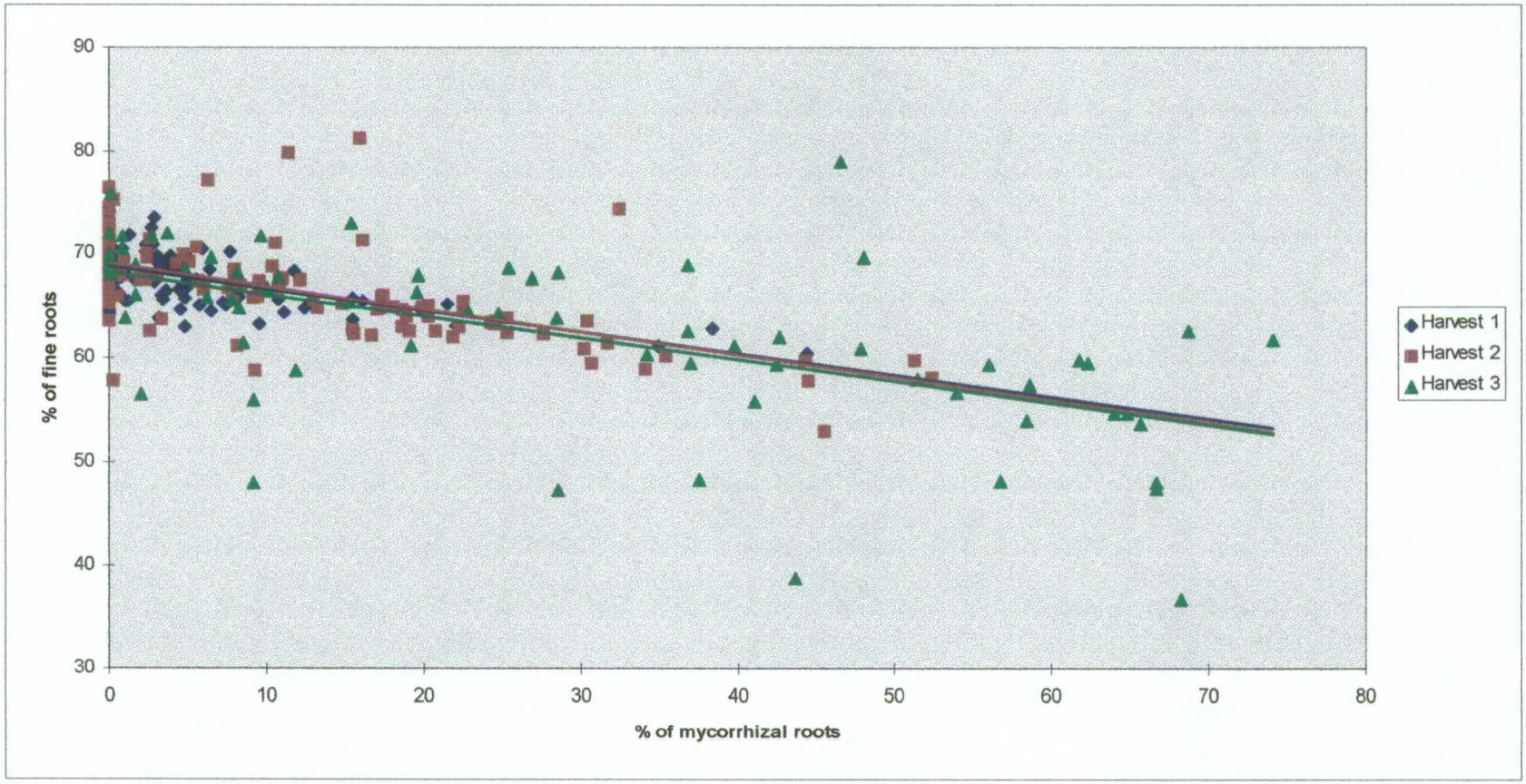
The results of the regression analysis of percentage colonisation on the percentage of fine roots are illustrated in Figure 5.12. The regression analysis was across all treatments for each of the three harvests. The parameters for the regression analysis are given in Table 5.6.

Table 5.6. Parameters for the regression analysis of percentage colonisation (x axis) on the percentage of fine roots (y axis).

Harvest	Y Intercept	Slope	Adjusted R-squared
Harvest 1	68.70	-0.209	0.32
Harvest 2	69.03	-0.219	0.37
Harvest 3	68.27	-0.210	0.36

While the adjusted R-squared values were low, the values for the Y intercept and slope were highly significant ($P<0.001$) for all three harvests.

Figure 5.12. The regression of percentage colonisation on the percentage of fine roots for Harvests 1, 2 and 3.



5.3.3 Plant Height and Stem Diameter

There were no significant treatment effects (P>0.05) on plant height or stem diameter, so data is not presented.

5.3.4 Sporocarp Production

Two mycorrhizal types initially labelled Type 1 and Type 9 fruited prolifically during the winter months. They were identified as *H. crustiliniforme* and *L. tortilla* respectively by tracing hyphal linkages to these sporocarps (refer to Plate 5.4).

The fruitbodies of *H. crustiliniforme* and *L. tortilla* were initially confined to those treatments that were inoculated with either *M. ambiguus* or *D. albus* (refer to Table 5.7) as was the case for their mycorrhizas. As a result of fruiting in the first winter, both fungi spread to other treatments .

Table 5.7. The average number of sporocarps or primordia per pot for *Laccaria tortilla* and *Hebeloma crustiliniforme* during June, July and August of 1995.

Fungal Treatment	*Laccaria tortilla* Mushrooms	*Hebeloma crustiliniforme* Mushrooms	*Hebeloma crustiliniforme* Primordia
1. Control	0 a	0 b	0 b
2. *Tuber melanosporum*	0 a	0 b	0 b
3. *Melanogaster ambiguus*	1.80 a	0.20 b	0.93 ab
4. *Descomyces albus*	2.87 a	0.20 b	3.33 a
5. *Hydnangium carneum*	0 a	0 b	0 b
6. *Scleroderma mcalpinei*	0 a	0 b	0 b
7. *T. melanosporum* & *M. ambiguus*	2.67 a	0.26 ab	0.73 b
8. *T. melanosporum* & *D. albus*	1.33 a	0.60 a	1.73 ab

Means **within** columns (sporocarp types) that are followed by the same letter are not significantly different (P<0.05) by the Least Significant Difference test.

Plate 5.4. Sporocarps produced during the experiment.

1

2

3

1. Hyphal linkages between the mycorrhizas and sporocarp of *Hebeloma crustiliniforme*.
2. *Laccaria tortilla* fruiting under hazel.
3. *Hymenogaster arenarius* fruiting under hazel.

5.3.5 Description of Mycorrhizal Types

Tuber melanosporum

Refer to Plate 5.5

Identification. Literature descriptions (Palenzona, 1969; Granetti, 1995; Meotto *et al.*, 1995; Paolocci *et al.*, 1995; Zambonelli *et al.*, 1995), DNA fingerprinting (refer to Chapter 9).

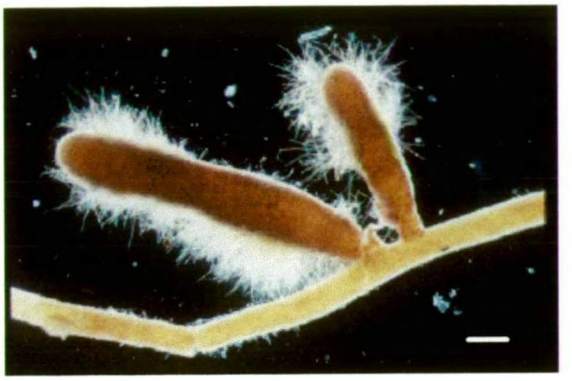
Shape. Club shaped with well rounded apices and extensive irregular branching. Tips can be up to 4.5 mm long and are normally about 0.3 mm wide.

Colour. During the dormant stage, the mycorrhiza are uniformly dark amber. When the mycorrhiza are active, the tip becomes very pale and the remainder of the mycorrhiza is a light amber colour.

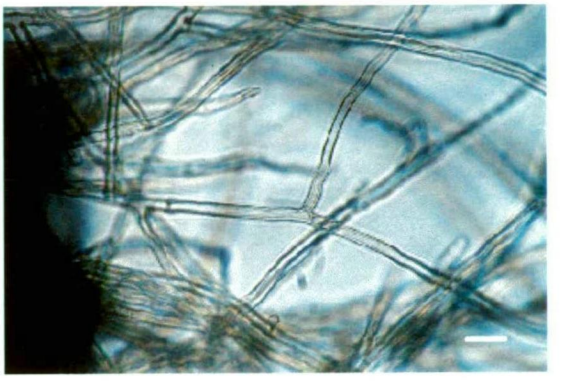
Mantle. A pseudoparenchymatous mantle where the surface cells have a puzzle-like appearance. These cells have a wavy edge with well pronounced lobes. Alternatively, the mantle can be described as irregular synenchyma with interlocking cells as per Ingleby *et.al.* 1990. The mantle is compact and smooth.

Cystidia. At certain times of the year, predominantly when the fungus is actively growing (spring, summer and autumn) these mycorrhizas can form cystidia (spinules). These cystidia are hyaline and approximately 70 to 160 µm long, with one or two transverse septa on the lower section. At the point of attachment to the mantle, the cystidia are slightly enlarged with an average 3.0 µm wide (range of 2.5 to 4.3 µm). The cell wall of the cystidia is consistently thin.

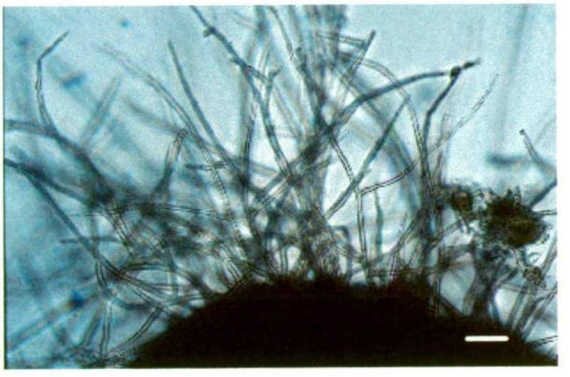
Plate 5.5. *Tuber melanosporum* mycorrhizas.



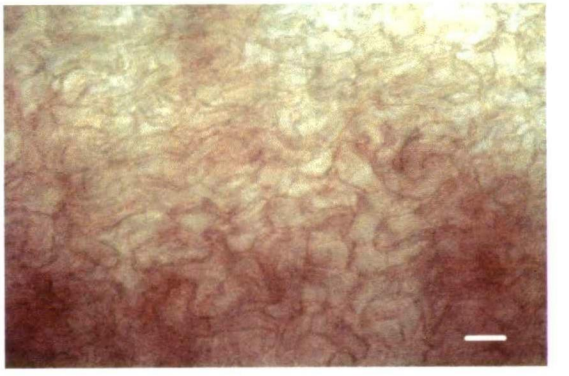
1 (bar = 200 μm)



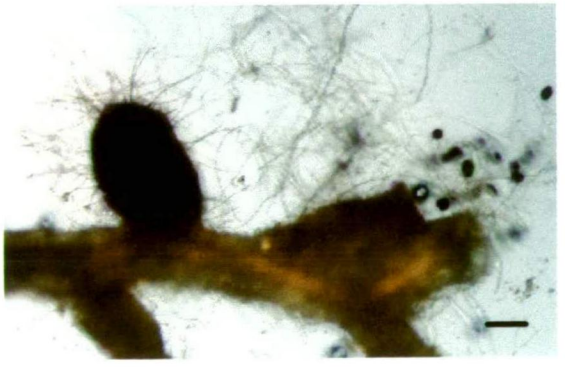
2 (bar = 10 μm)



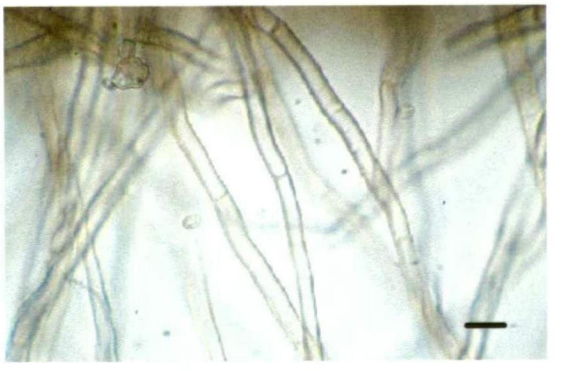
3 (bar = 25 μm)



4 (bar = 10 μm)



5 (bar = 100 μm)



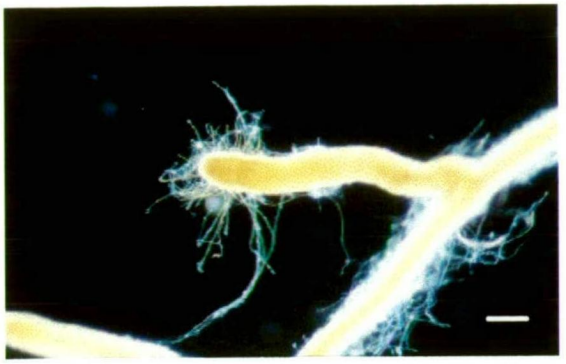
6 (bar = 10 μm)

Hyphae: During periods of active growth, the mycorrhiza produce straight rigid hyphae which have characteristic right-angled branches. This hyphae often emanates as tufts from the mycorrhiza's surface. The cell walls of the hyphae are light amber, but fade in the younger sections.

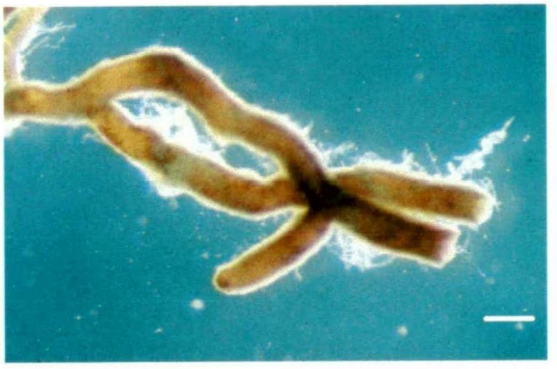
Plate 5.5. *Tuber melanosporum* mycorrhizas

1 Mycorrhiza with numerous cystidia.
2 Transverse septa of the cystidia.
3 Cystidia (spinules) showing transverse septa.
4 "Jigsaw puzzle"-like mantle.
5 Young mycorrhiza showing cystidia, emanating hyphae and germinating *T. melanosporum* spores.
6 Emanating hyphae.

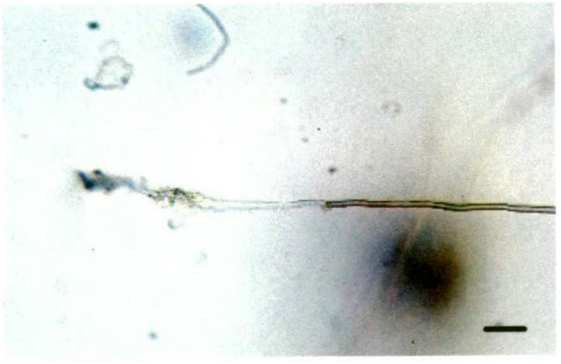
Plate 5.6. *Descomyces* sp. mycorrhizas.



1 (bar = 200 μm)

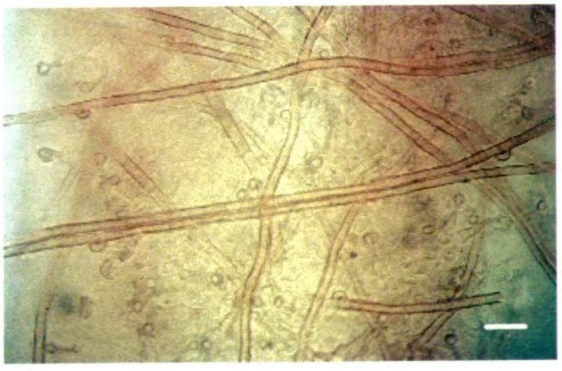


2 (bar = 300 μm)

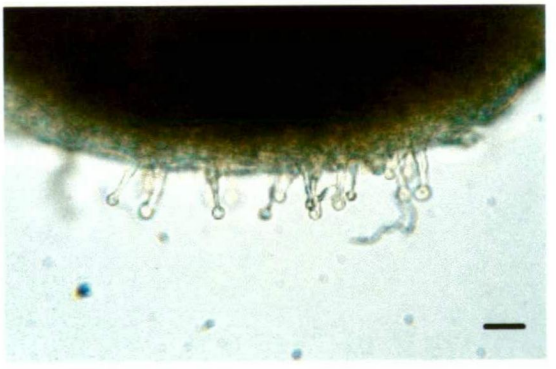


3 (bar = 20 μm)

4 (bar = 10 μm)



5 (bar = 10 μm)



6 (bar = 10 μm)

***Descomyces* sp.**

Refer to Plate 5.6

Identification. Literature descriptions of *Descomyces* species (Bougher and Malajczuk, 1985).

Shape. Mycorrhizas are fairly long, sinuate and occasionally branched.

Colour. Golden brown, even throughout the length of the mycorrhiza.

Mantle. Net prosenchyma as per Ingleby *et.al.* 1990.

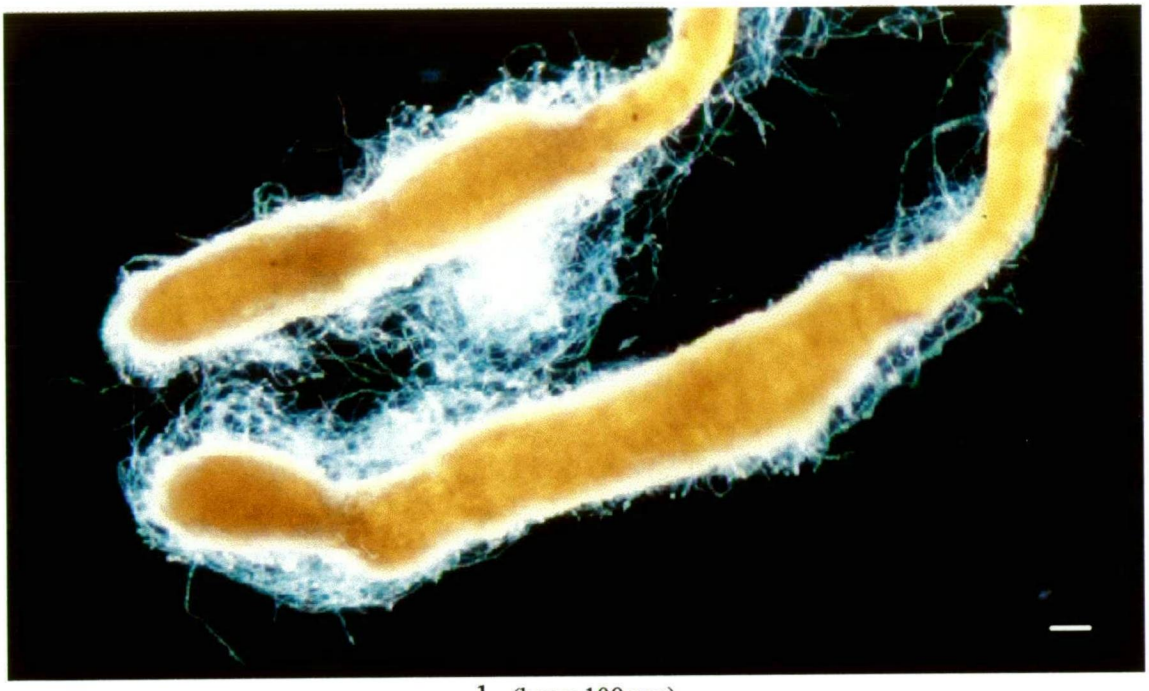
Cystidia. The cystidia are a key distinguishing feature of this mycorrhiza. They appear as a hyaline, thin-walled stalk with a swollen apex. The stalks are 6 to 12 µm long, 3.5 to 6 µm wide at the base tapering to 1 to 2 µm wide just below the swollen apex. The apices are 2 to 4 µm in diameter. The density of cystidia on the mycorrhiza can vary enormously.

Hyphae. This fungus has two distinct types of emanating hyphae. The first type is straight and rigid with clamp connections. The cell walls are thick and amber in colour. The second type of hyphae is hyaline, sinuous, with thin walls.

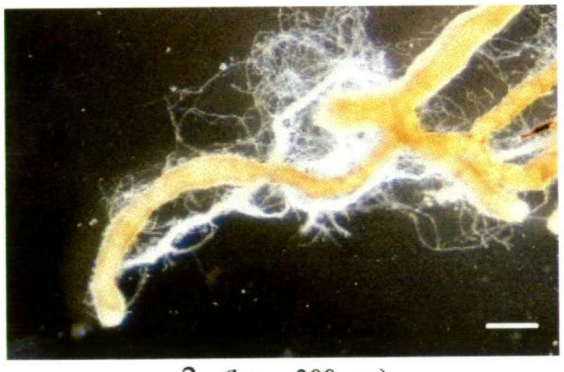
Plate 5.6. *Descomyces* sp. mycorrhizas

1. Young mycorrhiza showing the straight, rigid type hyphae emanating from the tip.
2. Mature mycorrhizas.
3. Transition at a clamp connection between the straight, rigid, amber colour hyphae and the sinuous, hyaline hyphae.
4. Mass of cystidia on the mantle surface.
5. Hyphae overlying cystidia at the surface of the mycorrhiza.
6. Cystidia emanating from the mantle.

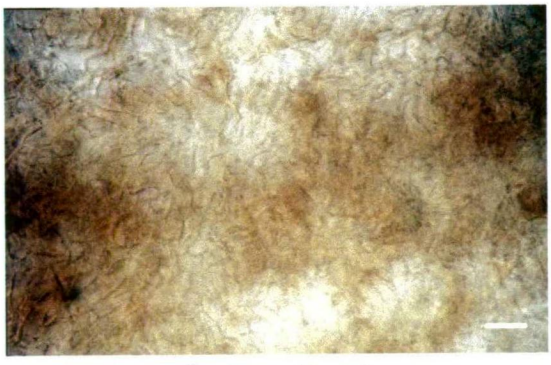
Plate 5.7. *Scleroderma mcalpinei* mycorrhizas.



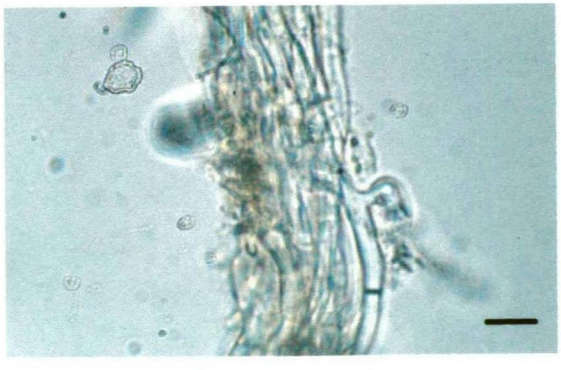
1 (bar = 100 µm)



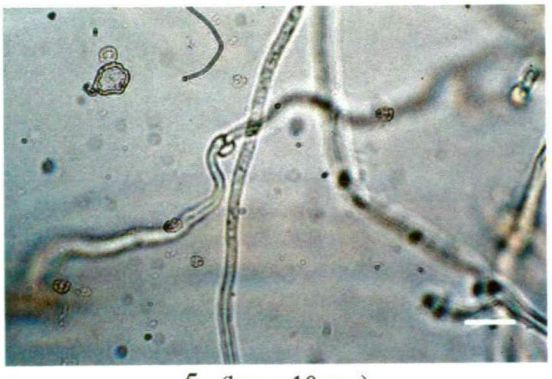
2 (bar = 300 µm)



3 (bar = 10 µm)



4 (bar = 10 µm)



5 (bar = 10 µm)

Scleroderma mcalpinei

Refer to Plate 5.7

Identification. Association with inoculant and literature descriptions of other *Scleroderma* species (Chu-Chou and Grace, 1983; Molina and Trappe, 1982).

Shape. Long, slender, tortuous and of uneven diameter.

Colour. Yellow with brown tinges.

Mantle. Net synenchyma as per Ingleby *et.al.* 1990.

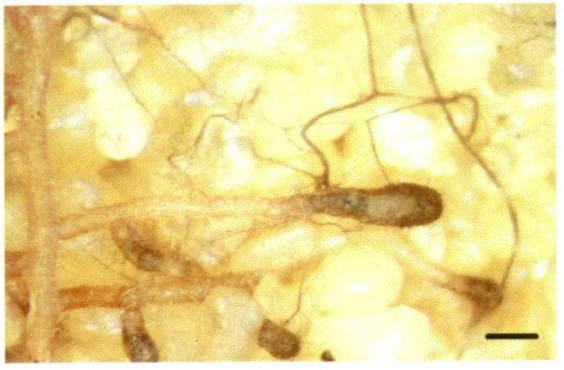
Cystidia. None seen.

Hyphae. Hyaline (2 to 3 µm in diameter) with clamp connections. Has distinct white simple strands that are 40 -50 µm in diameter.

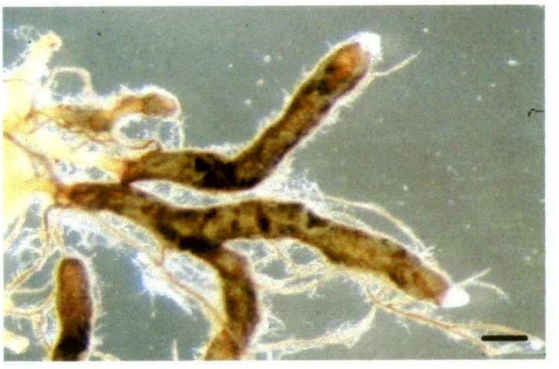
Plate 5.7. *Scleroderma mcalpinei* mycorrhizas.

1. Mycorrhizas.
2. Tortuous mycorrhizas showing hyphal strands.
3. Net synenchyma mantle.
4. Hyphal strand.
5. Extramatrical hyphae showing clamp connections.

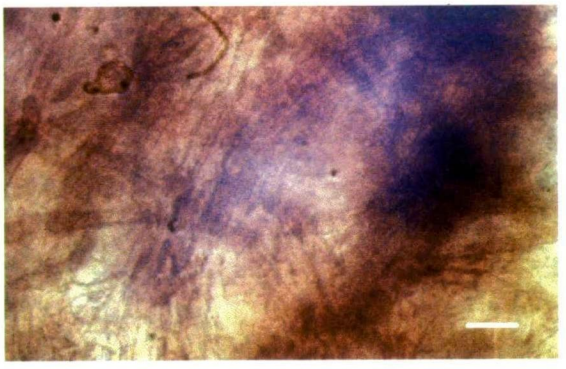
Plate 5.8. *Melanogaster ambiguus* mycorrhizas.



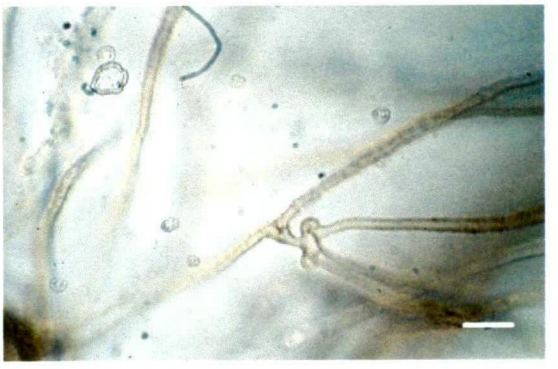
1 (bar = 300 µm)



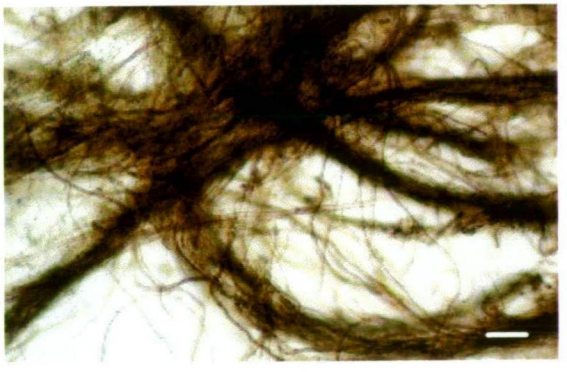
2 (bar = 300 µm)



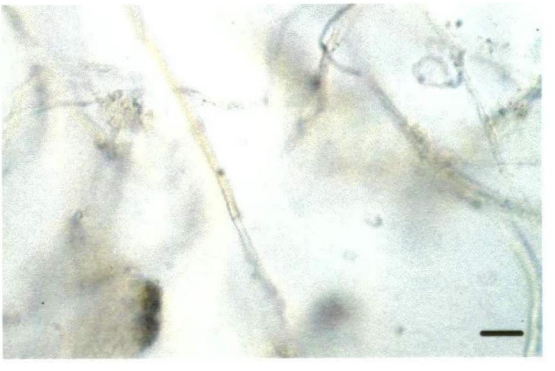
3 (bar = 10 µm)



4 (bar = 10 µm)



5 (bar = 20 µm)



6 (bar = 20 µm)

Melanogaster ambiguus

Refer to Plate 5.8

Identification. Distinct smell, association with inoculant, and literature descriptions of other *Melanogaster* species (Molina and Trappe, 1982).

Shape. Club shaped.

Colour. Dusty brown or matt brown under a stereo microscope. With the naked eye, they appear grey.

Mantle. Net synenchyma as per Ingleby *et.al.*, 1990.

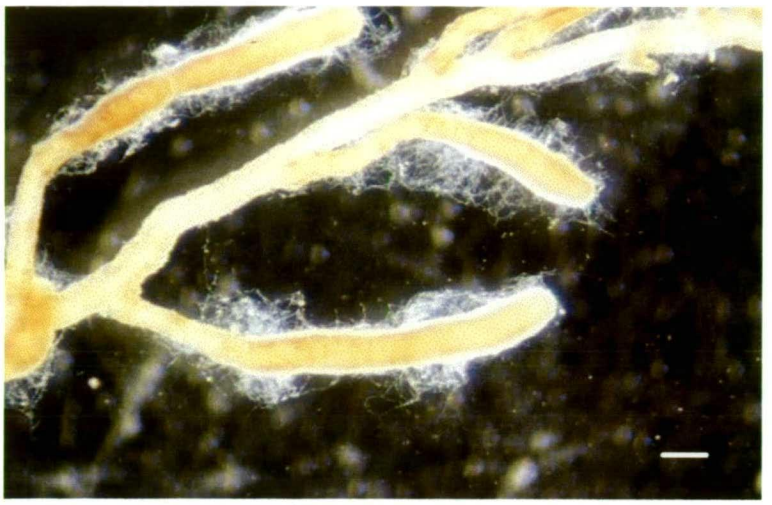
Cystidia. None seen.

Hyphae. Matt brown, up to 4µm in diameter with clamp connections. The surface of the hyphae is rough with very fine hair like structures. Crowded junctions of clamp connections have been observed (ie. two or three close together). Younger sections of hyphae are hyaline, smooth and are often associated with mucilage and bacteria. Changes from the pigmented hyphae to the hyaline hyphae are observed at hyphal septum. Large brown strands, clearly visible with the naked eye are produced in abundance. The hyphae also has the same distinct smell as the fruitbody, somewhat like chicken manure.

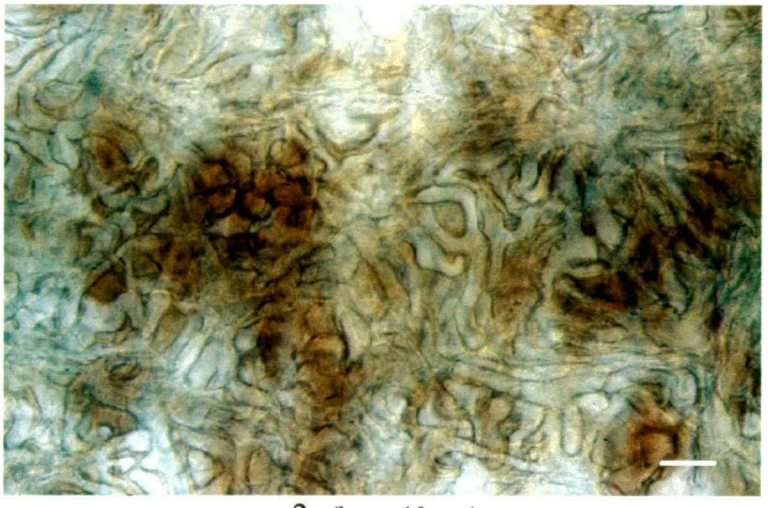
Plate 5.8. *Melanogaster ambiguus* mycorrhizas.

1. Young mycorrhizal tips growing in the yellow sand interconnected with mycelial strands.
2. Mature mycorrhizas.
3. Mantle surface.
4. Crowded junction of clamp connections.
5. Mycelial strands.
6. Septa dividing the younger hyaline hyphae from the typical brown hyphae.

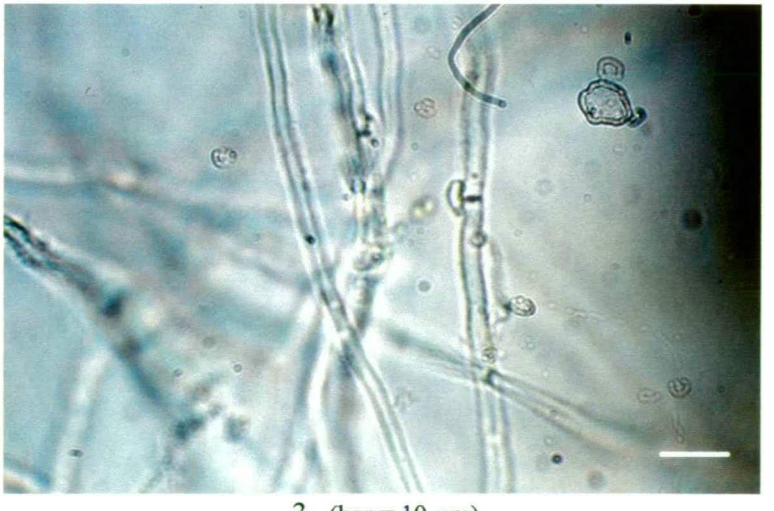
Plate 5.9. *Hydnangium carneum* mycorrhizas.



1 (bar = 200 µm)



2 (bar = 10 µm)



3 (bar = 10 µm)

Hydnangium carneum

Refer to Plate 5.9

Identification. Association with inoculant and hyphal linkages to fruitbody.

Shape. Long, straight, unbranched and fairly uniform in diameter.

Colour. Light brown.

Mantle. Irregular synenchyma not interlocking as per Ingleby *et. al.* 1990.

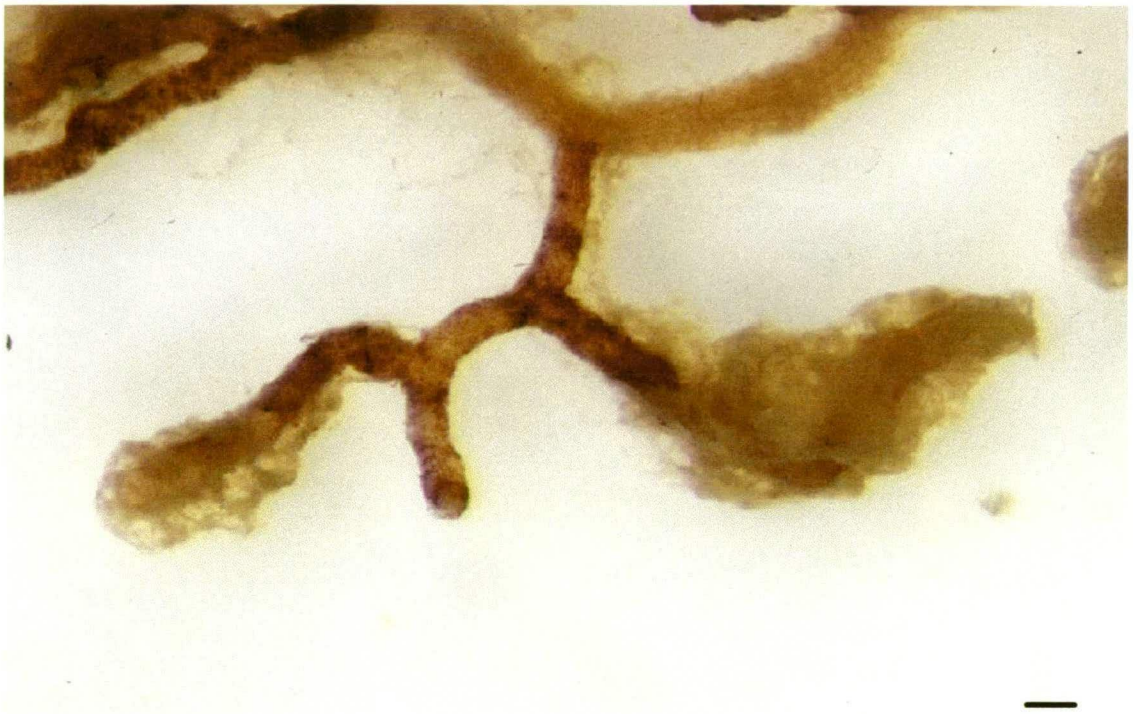
Cystidia. None seen.

Hyphae. Hyphae extends evenly from the entire surface of the mycorrhiza. This hyphae is hyaline, with clamp connections and is 2 to 3 µm in diameter.

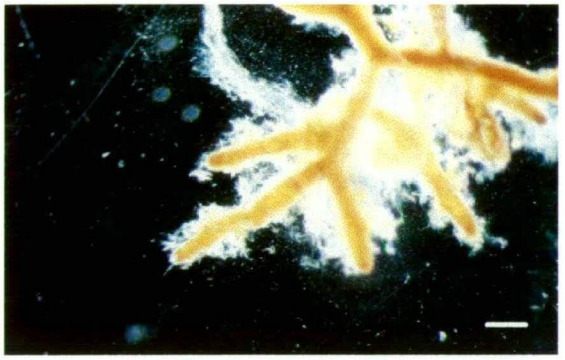
Plate 5.9. *Hydnangium carneum* mycorrhizas.

1. Buff coloured mycorrhizas.
2. Mantle surface.
3. Hyaline extramatrical hyphae showing clamp connection.

Plate 5.10. *Hebeloma crustiliniforme* mycorrhizas.

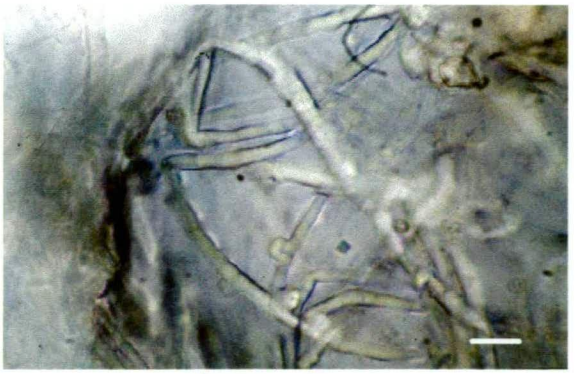


1 (bar = 150 µm)



2 (bar = 200 µm)

3 (bar = 200 µm)



4 (bar = 10 µm)

Type 1. Hebeloma crustiliniforme

Refer to Plate 5.10

Identification. Hyphal links to fruitbodies and literature descriptions (Chu-Chou and Grace, 1983).

Shape / Colour. These mycorrhiza are characterised by a mass of mucilaginous emanating hyphae. The heavy coating of mucilage on the hyphae gives the mycorrhiza its yellow colour. The mycorrhizas are typically short with little radial expansion.

Mantle. Largely undiscernible due to the mucilage.

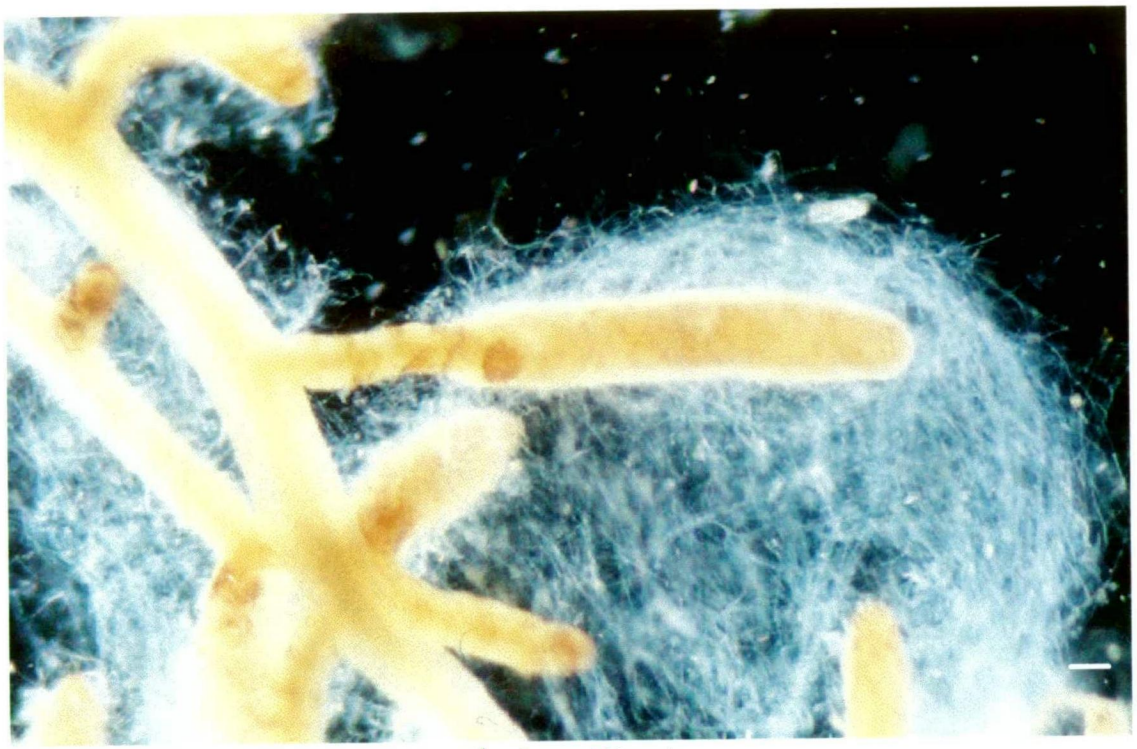
Cystidia. None.

Hyphae. The hyphae is straight, hyaline with clamp connections. The clamp connections can be very difficult to see due to the layer of mucilage. There was often a lot of bacteria and nematodes associated with this mucilaginous hyphae.

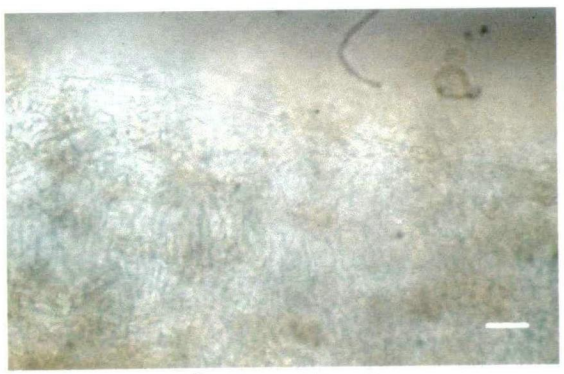
Plate 5.10. *Hebeloma crustiliniforme* mycorrhizas.

1. Mass of mucilaginous hyphae surrounding root tips with little radial expansion.
2. More developed mycorrhizas showing some radial expansion.
3. Mycorrhiza taken with differential interference contrast showing limited radial expansion.
4. Extramatrical hyphae with clamp connection surrounded by mucilage.

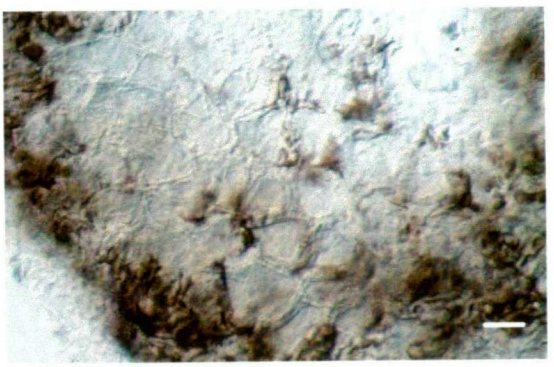
Plate 5.11. *Laccaria tortilla* mycorrhizas.



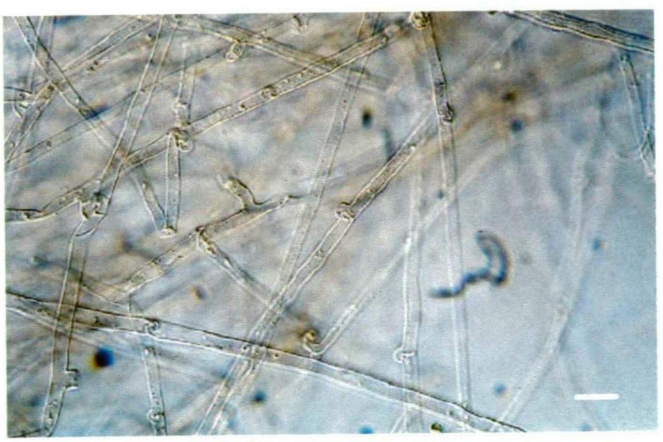
1 (bar = 100 µm)



2 (bar = 20 µm)



3 (bar = 20 µm)



4 (bar = 10 µm)

Type 9. Laccaria tortilla

Refer to Plate 5.11

Identification. Hyphal links to fruitbodies and literature descriptions (Ingleby *et. al.*, 1990).

Shape. Thin, unbranched, with a mass of emanating hyphae. Differs from *H. crustiliniforme* in that the hyphae is generally free of mucilage and there is more radial expansion.

Colour. Fawn, darkening to manilla with age.

Mantle. Loosely formed net prosenchyma becoming more compact on older mycorrhizas.

Cystidia. None seen.

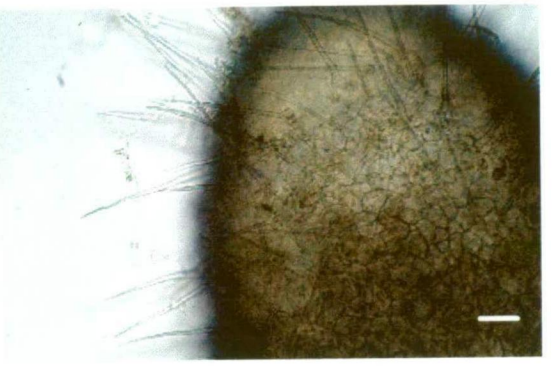
Hyphae. Hyaline, 2 to 3.5 μm in diameter with large clamp connections. At the septum the hyphae often bends towards the clamp connection to give an elbow-like appearance.

Plate 5.11. *Laccaria tortilla* mycorrhizas.

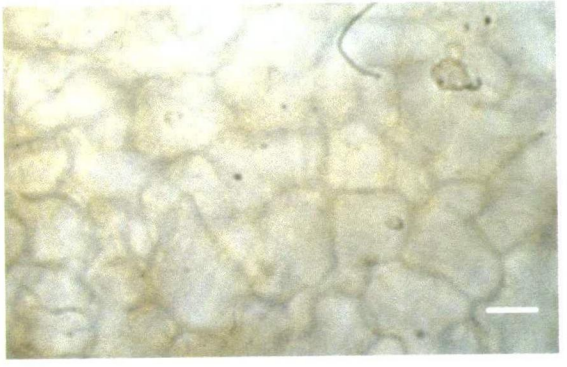
1. Fawn, unbranched mycorrhizas surrounded by extramatrical hyphae.
2. Mantle surface.
3. Surface of mycorrhiza after clearing with KOH.
4. Hyaline extramatrical hyphae with clamp connections.

Plate 5.12. Type 3 mycorrhizas.

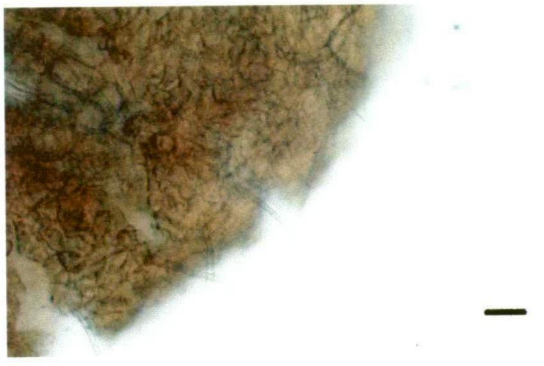
1 (bar = 300 μm)



2 (bar = 20 μm)

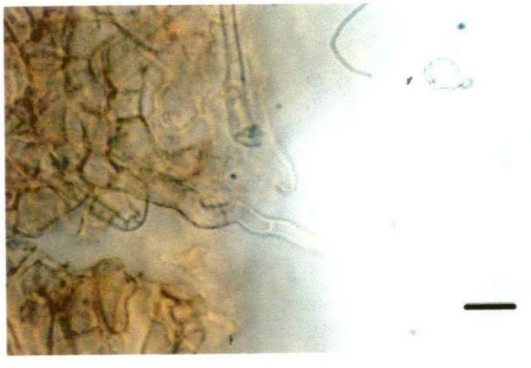


3 (bar = 10 μm)



4 (bar = 20 μm)

5 (bar = 10 μm)



6 (bar = 10 μm)

Type 3

Refer to Plate 5.12

Identification. Unknown fungus.

Shape. Unbranched, undulating and uneven in diameter. Up to 5 mm in length.

Colour. Young mycorrhizas are a manilla colour with a pale tip. Older mycorrhizas are light to medium brown colour.

Mantle. Irregular synenchyma where the cells are not interlocked.

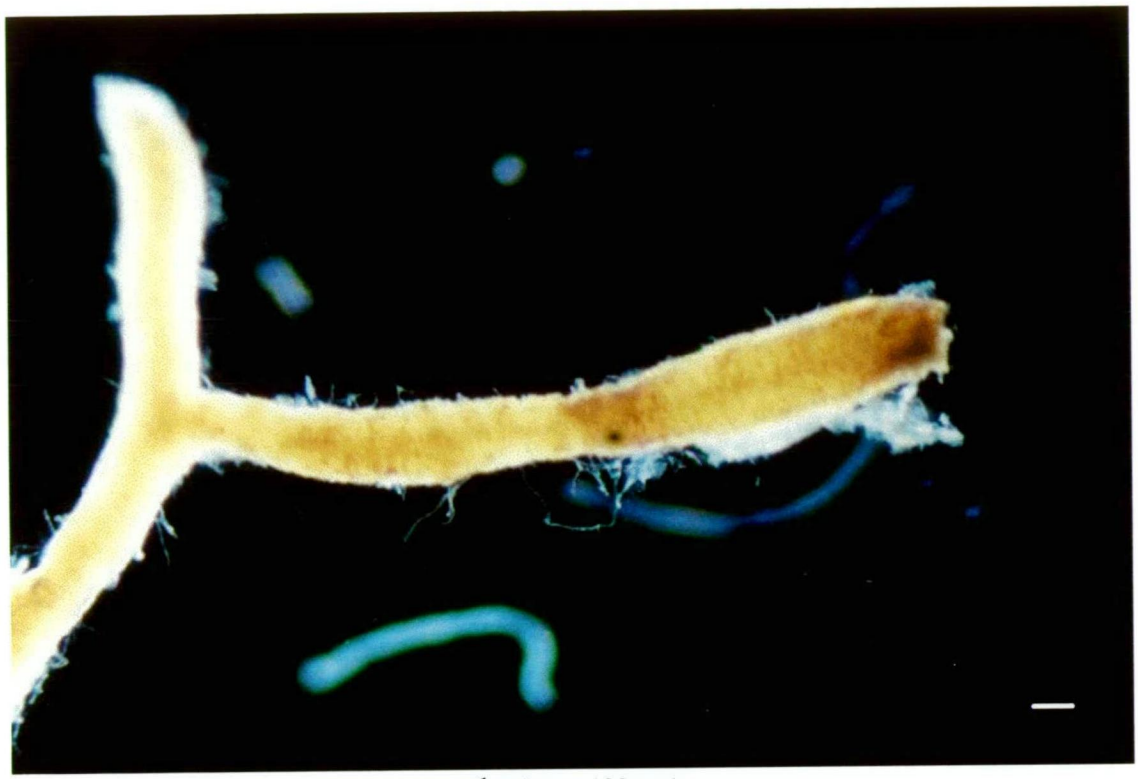
Cystidia. Awl-shaped cystidia, 70 to 100 µm in length, with one or two septum toward the base of the cystidium. The cystidia most often occur on the pale tip of young mycorrhiza.

Hyphae. Thick, frequently septate hyphae usually 4 to 5 µm in diameter, with a maximum diameter of 8 µm. Occasionally the hyphae were covered in mucilage. No clamp connections.

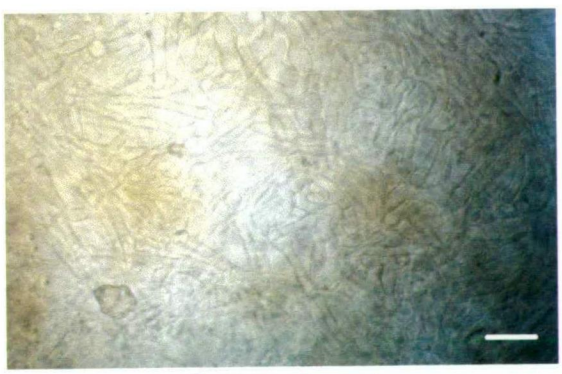
Plate 5.12. Type 3 mycorrhizas.

1. Mycorrhiza with cystidia evident on the pale tip.
2. Mycorrhizal tip showing cystidia and mantle pattern.
3. Mantle surface.
4. Straight unbranched cystidia.
5. Thick emanating hyphae showing septum.
6. Base of cystidia.

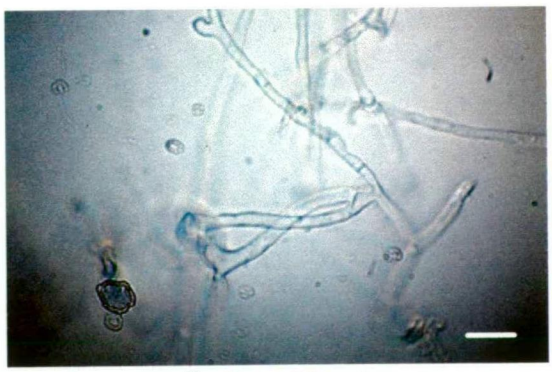
Plate 5.13. *Hymenogaster arenarius* mycorrhizas.



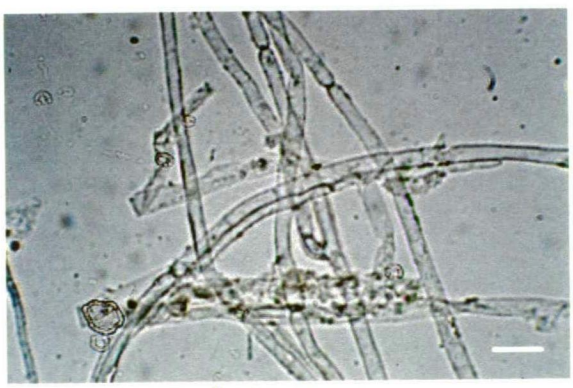
1 (bar = 100 µm)



2 (bar = 10 µm)



3 (bar = 10 µm)



4 bar = 10 µm

Hymenogaster arenarius

Refer to Plate 5.13

Identification. Hyphal links to fruitbodies.

Shape. Unbranched and uneven in diameter. Up to 4 mm in length.

Colour. Manilla coloured with a reddish brown mesh when seen under a stereo microscope.

Mantle. Net synenchyma. Short truncate branching and elbow structures seen in the emanating hyphae are also present in the mantle.

Cystidia. None seen.

Hyphae. Hyphae is about 2-4 μm in diameter, smooth, uneven, hyaline, thin walled, with short truncate branching and elbow type bending. Hyphae are constricted at the septa.

Plate 5.13. *Hymenogaster arenarius* mycorrhizas.

1. Manilla coloured mycorrhiza with reddish brown mesh appearance.
2. Mantle surface.
3. Extramatrical hyphae showing short truncate branch at the top of the photo.
4. Extramatrical hyphae showing constriction at the septa.

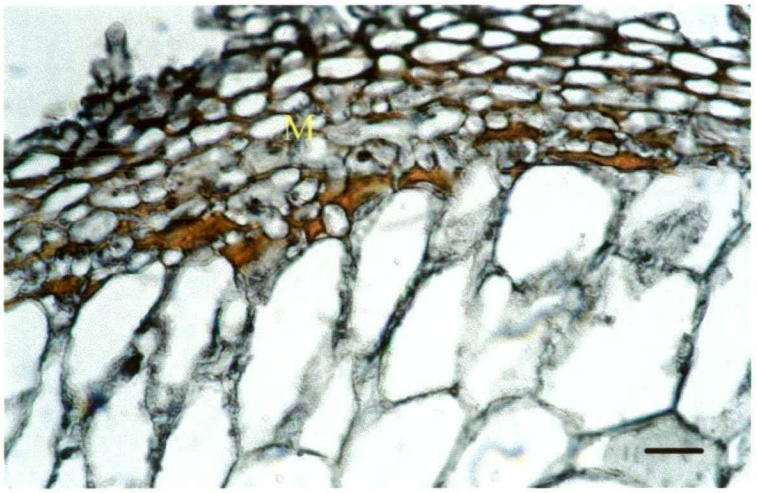
Sections of mycorrhizas

Plate 5.14 shows mantle formation for those species described in the experiment. Hartig net formation was evident for, *H. crustiliniforme, H. carneum, L. tortilla, S. mcalpinei* and Type 3. *Tuber melanosporum* also had a Hartig net, but it is not shown clearly in the section.

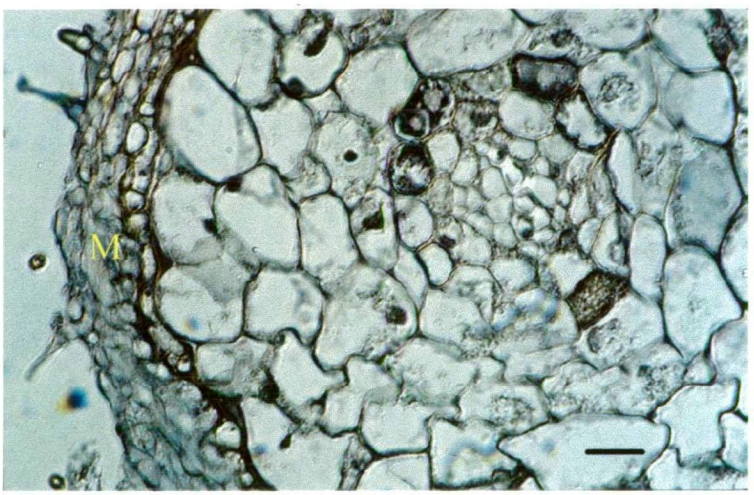
Spore types

Plate 5.15 shows the spores of fungi in the experiment.

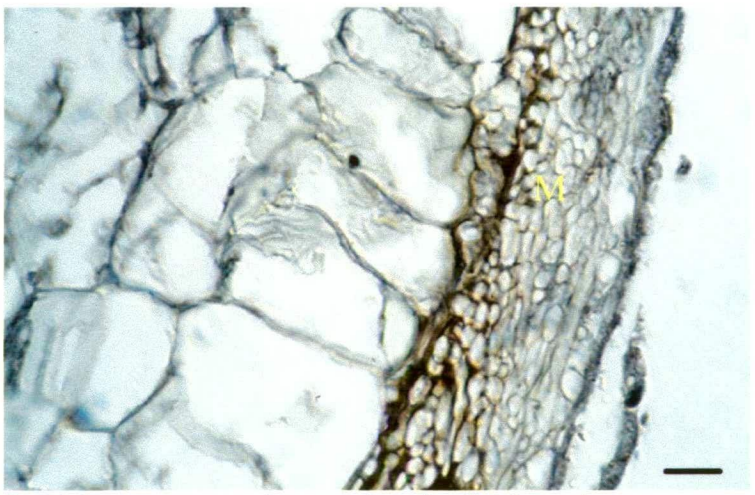
Plate 5.14. Sections of mycorrhizas stained with Chlorazol Black E showing mantle and Hartig net (scale bar = 10 μm). M = mantle, H = Hartig net.



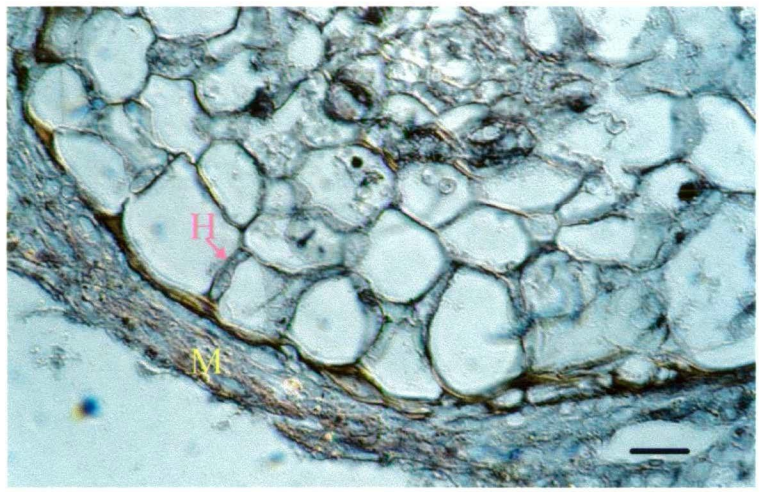
1. *Tuber melanosporum*, cross section.



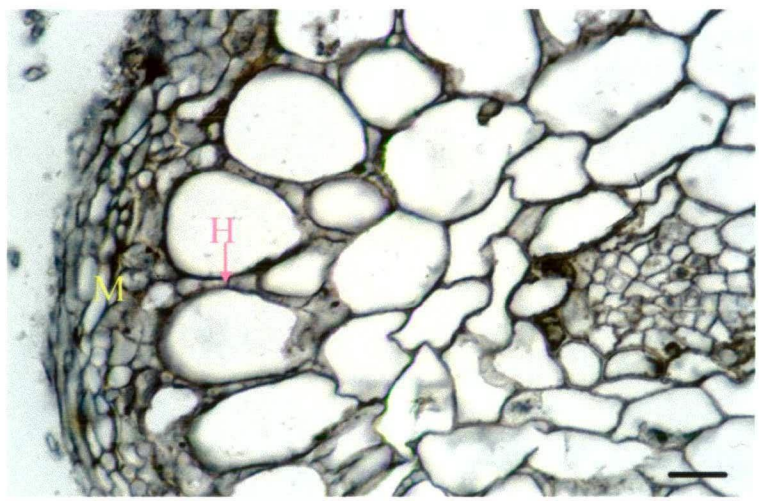
2. *Descomyces* sp., cross section.



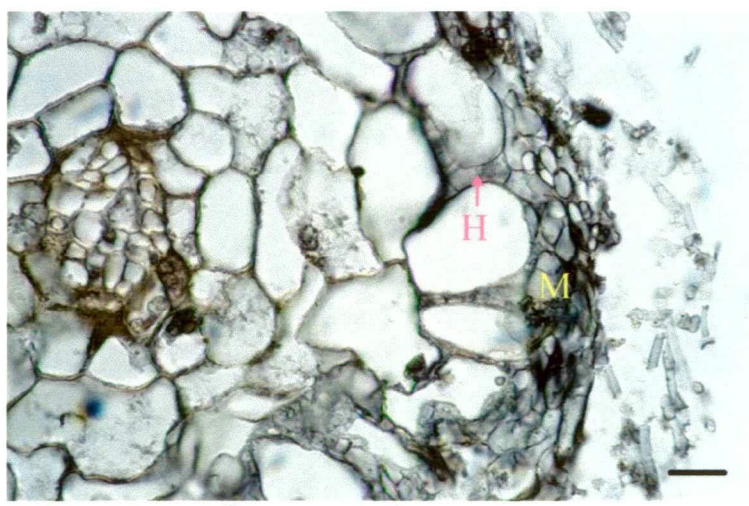
3. *Melanogaster ambiguus*, longitudinal section.



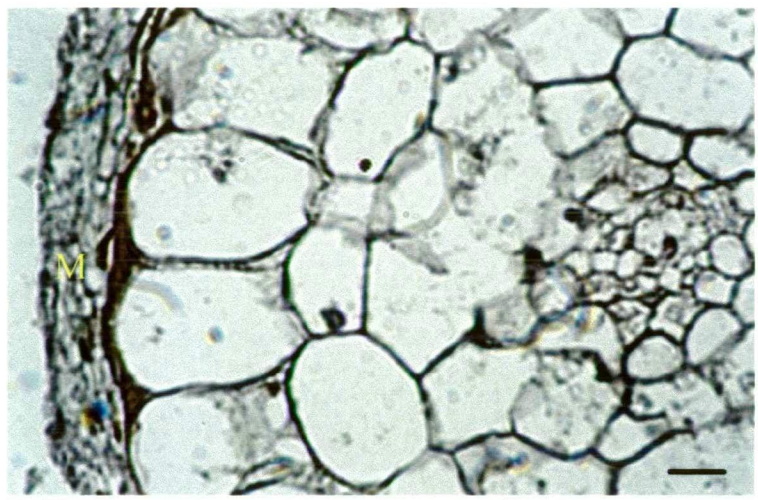
4. *Hebeloma crustiliniforme*, cross section.



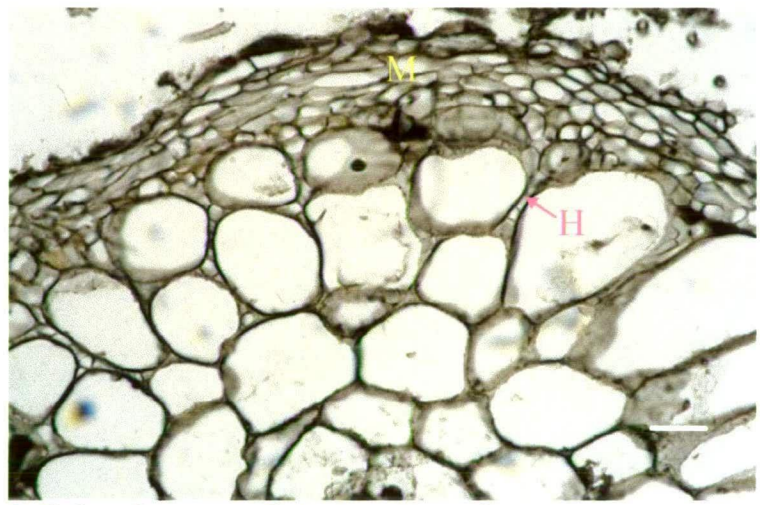
5. *Hydnangium carneum*, cross section.



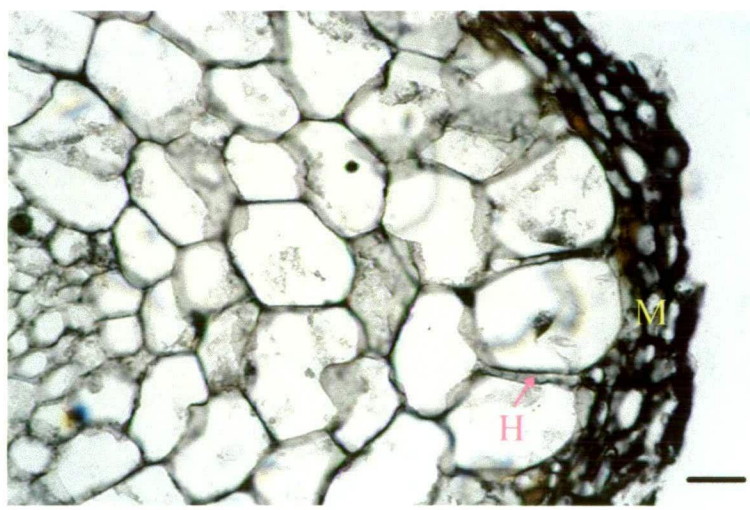
6. *Laccaria tortilla*, cross section.



7. *Hymenogaster arenarius*, cross section.

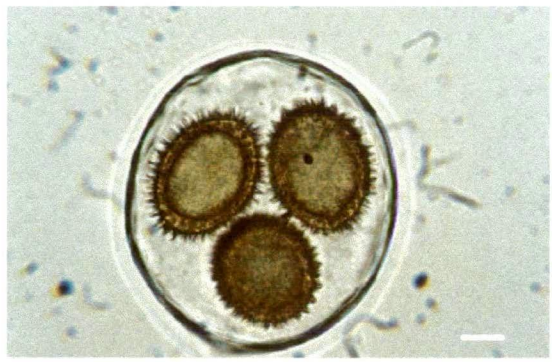


8. *Scleroderma mcalpinei*, cross section.

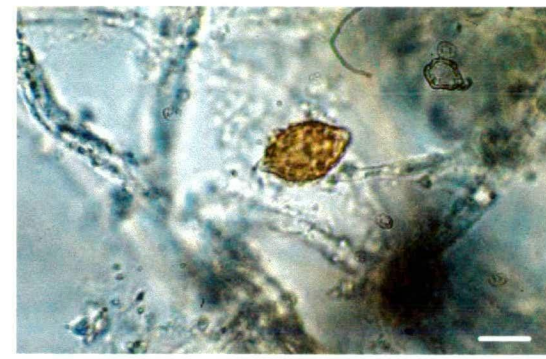


9. Type 3, cross section.

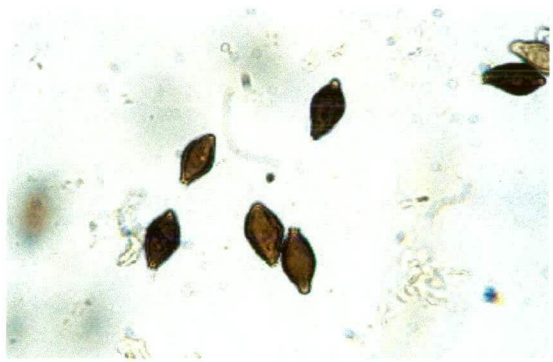
Plate 5.15. Spores of fungi in the experiment (scale bar = 10 μm).



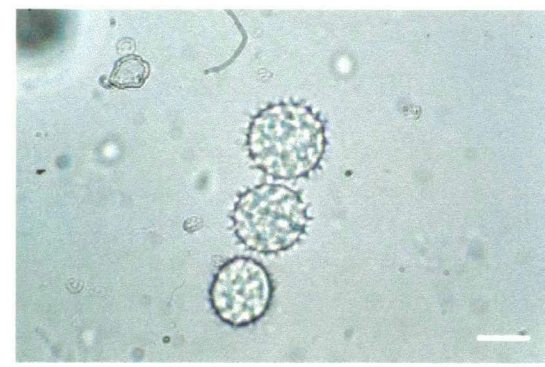
1. *Tuber melanosporum* spores in ascus.



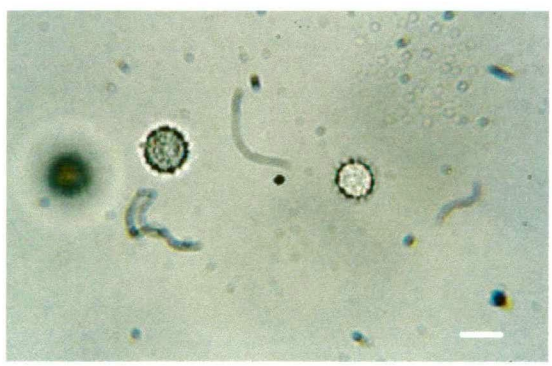
2. *Descomyces albus* spores & mycelium



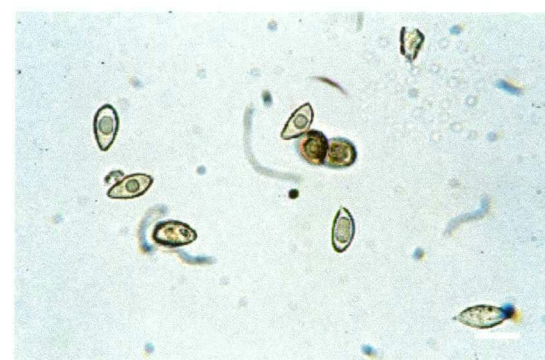
3. *Melanogaster ambiguus* spores



4. *Hydnangium carneum* spores



5. *Laccaria tortilla* spores



6. *Hebeloma crustiliniforme* spores

5.4 Discussion

5.4.1 The Response of *Tuber melanosporum* to Applied Lime

The results of the experiment show that *T. melanosporum* will colonise hazel growing on an infertile Australian sand with a pH of 6, albeit very slowly and at low levels of colonisation. When lime is applied, colonisation by *T. melanosporum* increases. Strong colonisation by *T. melanosporum* (~ 60% of fine roots) was obtained at the highest level of lime application. This demonstrates that *T. melanosporum* prefers calcareous soils.

Most of the research in Europe suggests that for good fruitbody production the texture of the soil needs to be a balanced mix of sand, silt and clay, and that the soil should have a granulose structure (Delmas *et al.*, 1981). By comparison, the soil used in this experiment was a structureless sand. Other differences are demonstrated in Table 5.8 which compares the levels of soil parameters of the high lime treatment with a range for which Delmas *et al.* (1981) observed brûlé formation on a number of truffières throughout France.

Tuber melanosporum was able to colonise hazel in a soil that would generally be considered to be unsuitable for truffle production. Possible explanations for this are:

1. Soil conditions that are capable of supporting strong mycorrhizal development may not be suitable for fruit body production. That is, high levels of colonisation can occur in soils that would not normally be expected to furnish good truffle yields.
2. A soil may not be optimal for *T. melanosporum*, but lack of competition from other fungi can allow colonisation and potential for fruit formation.

The first point could be tested by studying the correlation between fruitbody production and the level of mycorrhizal infection by *T. melanosporum* in mature truffières. If there were soil types that supported good mycorrhizal infection but

produced few or no fruit bodies over a number of years, then it would suggest that the basic soil conditions required for fruiting are more stringent than those required for good vegetative growth.

The second point could be tested by growing hazels inoculated with *T. melanosporum* for several years in the 'High Lime' treatment soil type to see whether truffles are produced. That is, grow them in pots large enough to support a mature tree, prevent contamination from other mycorrhizal fungi, provide climatically favourable conditions, then monitor for possible fruitbody production.

Table 5.8. Soil parameters for the 'High Lime' treatment and for soils of French truffières exhibiting brûlés as reported by Delmas *et al.* (1981).

Soil Parameter	French truffle soils producing a brûlé	High Lime treatment ~ 60% colonisation
pH (water)	7.8-8.35	7.8[1]
Available P (mg/kg)	6-980	5
Total P (mg/kg)	555-2531	45.5
Total N (g/kg)	0.460-5.220	0.042
Organic Carbon (g/kg)	4.7-50	0.74
Carbon / Nitrogen Ratio	8.57-13.7	17.8
Percentage Organic Matter	0.8-8.3	0.13
Exchangeable Ca (me/100 g)	23.75-67.5	0.72
Exchangeable Mg (me/100 g)	0.425-4.422	0.085
Exchangeable K (me/100 g)	0.1-1.29	0
Exchangeable Na (me/100 g)	0.036-0.17	0.068

[1] At the time of the third harvest.

For the majority of soil parameters, the levels differ greatly between the High Lime treatment and the range reported by Delmas *et al.* (1981). This indicates that the levels of these parameters are either not critical to the vegetative growth of *T. melanosporum*, or, that the range required for vegetative growth is much wider

than the range given by Delmas *et al.* (1981). Parameters of interest include exchangeable calcium, total and available P, total nitrogen, percent organic matter. and the carbon/nitrogen ratio as these parameters have been considered to have an influence on truffle production (Chevalier and Poitou, 1990; Delmas *et al.*, 1981; Delmas and Poitou, 1978; Le Tacon *et al.*, 1982; Mamoun and Olivier, 1991). The effect of available phosphorus, exchangeable calcium and exchangeable magnesium are discussed further in Chapters 6 and 7.

On the other hand, those parameters in Table 5.8 showing corresponding levels may be important for the vegetative growth of *T. melanosporum*. The pH and exchangeable Na were the only parameters where this was the case. It is unlikely that exchangeable sodium would play a significant role in a soil dominated by calcium. However, the pH of the 'high lime' treatment is one parameter that is likely to have a significant role. The role of pH is discussed further in Chapter 6.

It was also evident that *T. melanosporum* suffered a dramatic decline in its frequency of colonisation when other fungi were present at the time of inoculation such as in the co-inoculated treatments. These competitor fungi established quickly and appear to prevent primary infection by *T. melanosporum*. This emphasises the requirement to eliminate inoculum of competitor ectomycorrhizal fungi when commercially inoculating seedlings with *T. melanosporum*, and during the time the plants are in the nursery. When the seedlings are exposed to other ectomycorrhizal fungi at the time of planting in the field, *T. melanosporum* should have colonised much of the root system and be more resilient to competition.

5.4.2 Host Specificity of Australian Native Ectomycorrhizal Fungi

Examination of the associations formed by *Descomyces* sp., *H. carneum* and *S. mcalpinei* prove that these Australian native fungi, normally associated with Eucalypts, are forming mycorrhizal relationships with *Corylus avellana*. It is reasonable to expect that other members of the *Hydnangium*, *Descomyces and*

Scleroderma (native species) genera may also be capable of forming mycorrhizal relationships with hazel as well as species from other native genera.

5.4.3 The Response of Australian Native Fungi to Applied Lime

Hydnangium carneum and *S. mcalpinei* were not able to adapt to the high level of applied lime. Therefore, some of the competition to *T. melanosporum* from native ectomycorrhizal fungi can be reduced by the application of high levels of lime. On the other hand, the *Descomyces* sp. appeared to be unaffected by the lime treatments. However, the very sporadic establishment of *Descomyces* in the experiment may have prevented lime effects being recognised. The possible causes for the sporadic development of the *Descomyces* mycorrhizas are discussed below in Section 5.4.5.

Further research with more fungal species, soil types, and levels of applied lime would offer more information as to the likely competition to *T. melanosporum* by endemic ectomycorrhizal fungi.

It should also be noted that in a field situation where there may be a number of potential ectomycorrhizal competitors present, reducing the number of competitor species may not reduce the competition experienced by *T. melanosporum*. It is probably the competitiveness of the fungi present, not the number of competitive species that restrict the production of *T. melanosporum*. For example, in New Zealand where there are very few ectomycorrhizal fungi, some of the newly established truffières are being affected by competition from just one or two species including a *Scleroderma* sp. and another *Tuber sp.* (I. Hall, pers. com.). However, the fewer number of species in Tasmania that are able to establish and grow strongly at high levels of applied lime, the less likely the trufferies are to become infected with contaminant fungi.

5.4.4 The Response of Introduced Fungi to Applied Lime

The introduced species which occurred in the experiment included *M. ambiguus*, *H. crustiliniforme* and *L. tortilla*. These species did not appear to be affected by the lime treatments. *M. ambiguus* and *H. crustiliniforme* are common world-wide. Their broad distribution indicates an ability to grow under a wide range of soil conditions. As mentioned in Chapter 2, *H. crustiliniforme* is a known competitor to the Périgord black truffle in Europe. While *Melanogaster vulgaris* has been reported as being found infrequently in old trufferies, *M. ambiguus* has not been reported as a serious competitor. However, the prolific fruiting of *M. ambiguus* that occurred at the Penguin site and its ability to grow in heavily limed soils means it cannot be discounted as being a possible competitor to *T. melanosporum* in Tasmania.

Hebeloma crustiliniforme and *L. tortilla* were both contaminants. The most likely explanation for the presence of these contaminant fungi in the experiment is that their spores were attached to the peridium of the *M. ambiguus* and/or *D. albus* sporocarps collected for inoculum from Penguin, Tasmania. Evidence to support this presumption include:

1. In the first and second harvests, *H. crustiliniforme*, *L. tortilla* and Type 3 were confined almost entirely to those fungal treatments inoculated with either *M. ambiguus* or *D. albus*. It was only at the third harvest that *H. crustiliniforme* and *L. tortilla* mycorrhizas were found in other fungal treatments including the control treatment. Their spread would have been facilitated by the fruiting of these two fungi more than a year prior to the third harvest. The Type 3 mycorrhizas, which did not produce fruiting bodies, remained confined to those treatments inoculated with either *M. ambiguus* or *D. albus*.

2. The sporocarps of *M. ambiguus* or *D. albus* were collected at the same site at Penguin, Tasmania, whereas the *H. carneum* and *S. mcalpinei* sporocarps were collected from two different sites in Western Australia, and the

T. melanosporum inoculum came from France.

3. In addition to the three main contaminants of the experiment, mycorrhizas and fruit bodies of *H. arenarius* were found in a pot inoculated with *M. ambiguus*. *H. arenarius* and *L. tortilla* are rare in Australia and all collections of these fungi in Tasmania can be traced back to the Penguin site.

4. *D. albus* contaminated pots inoculated with *M. ambiguus* and vice versa. Yet neither of these fungi were found in any of the other treatments.

While the *D. albus* and *M. ambiguus* sporocarps were not surface sterilised, they were cleaned thoroughly so one would expect the number of spores of the contaminants to be insignificant compared to the spore mass of the sporocarp. This presumption was supported by examinations of the spore slurries. Three separate and extensive examinations of the *D. albus* and *M. ambiguus* spore slurries failed to reveal any contaminating spores, suggesting that their concentration in the spore slurry must have been very low.

5.4.5 Origin of the *Descomyces* Mycorrhizas

Their was no definite link between inoculation with *D. albus* spores and the development of *Descomyces* mycorrhizas. *Descomyces* mycorrhizas were more common in those treatments inoculated with *M. ambiguus* (7 out of 30 pots) than those inoculated with *D. albus* (1 of the 30 pots). Possible explanations for the results include:

- The *D. albus* inoculum was not viable and the *Descomyces* mycorrhizas formed were from contaminant inoculum carried on the *M. ambiguus* and *D. albus* sporocarps. The spores from the *D. albus* sporocarps may not have been viable due to a dormancy factor.
- The *Descomyces* mycorrhizas were more commonly found in the *M. ambiguus* treatments because there was more contaminant inoculum (spores or hyphae) on the *M. ambiguus* sporocarps.

- The higher level of colonisation of *Descomyces* mycorrhizas in the *M. ambiguus* treatments may be a function of variability. Whilst the mycorrhizas were more commonly found in the *M. ambiguus* treatments than the *D. albus* treatments, the difference was not significant (P>0.05). This high variability may have been the result of competition from other species, mainly *H. crustiliniforme*, *L. tortilla* and Type 3, affecting primary infection by *Descomyces*.
- Both the *D. albus* inoculum and contaminant inoculum formed mycorrhizas of *D. albus*. That is, the contaminant inoculum was *D. albus*.

Whether the mycorrhizas were formed solely from contaminant inoculum or a combination of *D. albus* inoculum and contaminant inoculum, it is highly likely that the inoculum came from the Penguin site. The only *Descomyces* species found at this site was *D. albus* and it was found in abundance. Therefore it is quite likely that the *Descomyces* mycorrhizas observed in the experiment were that of *D. albus*.

5.4.6 Variation in Colonisation

A high level of variation in colonisation was evident in the experiment, particularly in those treatments dominated by the contaminant fungi, *H. crustiliniforme*, *L. tortilla* and Type 3 (refer to Table 5.5). In each replicate, one or more of these contaminant species established quickly creating competition for available root tips. This appeared to result in a decline in the proportion of replicates that were colonised by the inoculated species, particularly *T. melanosporum*.

Failure of primary infection was also evident in those treatments not affected by contaminants. For example, two of the five replicates of the high lime treatment inoculated with *T. melanosporum* alone showed no sign of colonisation. However, the remaining three replicates produced quite high levels of colonisation. Similarly, a portion of the 'no lime' and 'low lime' replicates in the *S. mcalpinei* treatment showed no sign of colonisation which may have precluded statistically significant lime treatment effects for this fungus.

5.4.7 Root Structure

The negative correlation observed between mycorrhizal colonisation and the percentage of fine roots can be attributed to the fact that root tips which are sheathed by a mycorrhizal fungus are usually shorter and grow more slowly than an unsheathed tip (Harley and Smith, 1983).

However, there may be another factor contributing to the negative correlation observed. The percentage of fine roots (or the ratio of fine roots to coarse roots) is a reflection of specific root length (metres of root / gram of root). Specific root length varies considerably between plant species and is correlated to mycorrhizal dependency (Brundrett 1991). Those plants with high mycorrhizal dependency tend to have low specific root length. Conversely, those plants with low mycorrhizal dependency have a high specific root length.

Variation within species in the capacity to host *T. melanosporum* has also been observed by several authors (Boutekrabt *et al.*, 1990; Guinberteau *et al.*, 1990; Mamoun and Olivier, 1996). It is possible that the correlation observed between mycorrhizal colonisation and the percentage of fine roots may also be attributable to variation between individual hazel plants in their mycorrhizal dependency / receptivity. That is, some plants were poorly colonised and had extensive fine root systems because they were inherently less dependent on mycorrhizal fungi and therefore less receptive.

While there is generally less variation in root structure within species than there is between species (Brundrett, 1991), the possibility that some individuals may be better hosts than others should be explored further. This idea is discussed at greater length in the general discussion.

5.4.8 Sporocarp Production

Of the nine fungal species found in the experiment, four produced sporocarps; *Hebeloma crustiliniforme*, *Laccaria tortilla*, *Hydnangium carneum* and *Hymenogaster arenarius*. *Hebeloma crustiliniforme* and *L. tortilla* fruited frequently in the experiment. For both these species there were significant correlations between colonisation and fruiting. These findings reflect the work of Termorshuizen and Schaffers (1989) who found significant correlations between the level of fruiting and the total number of mycorrhizas on *Pinus sylvestris*.

The frequency of fruiting of *H. arenarius* and *H. carneum* was too low to observe correlations between fruiting and mycorrhizal colonisation.

5.4.9 Mycorrhizal Descriptions

Descriptions of mycorrhizal types have been included as a reference tool to aid the future identification of ectomycorrhizal fungi in Tasmanian truffières. As new mycorrhizal types are discovered in the truffières, they should be described and added to the collection.

6 The Effect of pH and Calcium on Competition between *Tuber melanosporum* and Other Ectomycorrhizal Fungi.

6.1 Introduction

The two most prominent effects on soil chemistry of applying calcium carbonate are an increase in soil pH and an increase in the levels of soil calcium as described by the equation below.

$$CaCO_3 + H^+ + OH^- \leftrightarrow Ca^{++} + HCO_3^- + OH^-$$

There have been no controlled glasshouse or field experiments that have sought to separate the effects of pH and calcium on the growth of *T. melanosporum* as a symbiont. In an *in vitro* experiment, Poitou *et al.* (1983) used different bases containing either calcium, magnesium, potassium or sodium to adjust media on which *T. melanosporum* was grown. Growth was best for calcium with little or no growth in the magnesium and potassium treatments. While this may suggest that calcium plays an important role in the growth of *T. melanosporum*, extrapolation from *in vitro* results to the field is very difficult (refer to Section 4.1).

Most other publications regarding the roles of pH and calcium are based on observations of the preferred soil characteristics of *T. melanosporum*:

- Delmas and Poitou (1973) and Delmas (1978) state that truffle producing soils are always calcareous or at least very rich in exchangeable calcium. There was no supporting data.

- Delmas and Poitou (1974) note that while many truffle producing soils have a calcareous substratum, a dolomitic substratum is equally as favourable. That is, soils with high levels of both calcium and magnesium do not represent an obstacle to truffle production.
- As previously discussed in Chapter 5, Delmas *et al.* (1981) conducted soil analysis on a number of truffle producing soils. They recorded the levels of several soil parameters for soil types exhibiting a brûlé. The parameters included pH, total calcium carbonate, 'active' calcium carbonate and exchangeable calcium as well as other parameters listed in Table 5.1. Due to the complexities of chemical interactions in soils, few conclusions could be drawn as to the relative importance of each parameter.
- More recent papers reviewing the edaphic requirements of *T. melanosporum* appear to be largely based on the work of Delmas *et al.* (1981) and Poitou *et al.* (1983). These include Poitou (1988) and Poitou (1990).

While most truffle producing soils have an alkaline pH and are calcareous or at least dolomitic, the relative importance of pH and calcium to *T. melanosporum* growing as a symbiont has yet to be established.

The objectives of this experiment were two fold:

1. To determine the relative importance of pH and calcium on the colonisation of hazel by *T. melanosporum,* and
2. To study the effects of pH and calcium on competition between *T. melanosporum* and native ectomycorrhizal fungi.

6.2 Materials and Methods

6.2.1 Overview

In a glasshouse experiment, seedling hazels (*Corylus avellana*) were inoculated with *T. melanosporum* and planted into 125 mm pots. After a year, these plants were transplanted from the 125 mm pots into 250 mm pots with soil treated with either $CaCO_3$, $CaSO_4$, K_2CO_3 or $MgCO_3$. Each soil treatment was applied at fourteen levels, giving a total number of 56 soil treatment combinations. Two trees were treated with each soil treatment combination giving a total of 112 trees. A month after transplanting, one of the two trees from each of the 56 soil treatment combinations was inoculated with spores of native ectomycorrhizal fungi. One year after transplanting the trees were analysed for the level of colonisation by *T. melanosporum* and native ectomycorrhizal fungi.

6.2.2 Soil Type and Preparation

The soil used to transplant the trees into the larger 250 mm pots was taken from a site in the Huon Valley, Tasmania, which was to be established as a commercial truffière. This site was devoid of native trees and had been used for grazing for more than 10 years. Prior to this, the site was an apple orchard, hence the inoculum load of ectomycorrhizal fungi was expected to be low (apples have endomycorrhizas). The A horizon used for the experiment was a grey sandy loam. A chemical analysis of the soil is shown in Table 6.1.

The soil was sterilised in large plastic lined bins with methyl bromide. This treatment was expected to kill any source of inoculum of ectomycorrhizal fungi. The soil was then left in the bins for two months before being sieved through a 2 mm sieve.

Table 6.1. Chemical analysis of the soil.

Soil Property	Level
pH (H_2O 1:5)	6.3
pH ($CaCl_2$ 1:5)	5.4
Electrical Conductivity (dS/m)	0.02
Available P (mg/kg) Colwell test	8
Available K (mg/kg)[1]	105
Total P (mg/kg)	127
Total N (g/kg)	0.93
Organic Carbon (g/kg)	23
Carbon / Nitrogen Ratio	24.7
Exchangeable Ca (me/100 g)	5.11
Exchangeable Mg (me/100 g)	0.14
Exchangeable Na (me/100 g)	0.04
Exchangeable K (me/100 g)	0.21

Exchangeable bases were extracted using NH_4Cl.

[1] Using the same bicarbonate extraction as for available P.

6.2.3 Soil Treatments

The rate of $CaCO_3$, $MgCO_3$ and K_2CO_3 application was determined by developing titration curves for the soil (Barrow and Cox, 1990). The titration curve method developed by Barrow and Cox is used to determine the liming requirement of a soil. The liming material was mixed with the soil at a range of rates. The soil was moistened to the sticky point then heated at 60°C for three days. After the incubation period the pH was measured and plotted against the liming rate. This process was carried out for $CaCO_3$, $MgCO_3$ and K_2CO_3. These titration curves were used to determine how much liming material to apply in order to obtain the desired pH.

The rates of application of each soil amendment are shown in Table 6.2. Also listed are the corresponding rates of applied calcium and carbonate. $CaCO_3$, $MgCO_3$ and

K_2CO_3 were applied at rates to give a range of pH levels gradually increasing from the initial pH of 5.4 (refer to Figure 6.1). For the $CaCO_3$ treatment, the curve flattens out at approximately 3.33 g $CaCO_3$ / kg soil (level 9) where the soil has become saturated with $CaCO_3$ and dissociation ceases. Subsequent increases in the rate of applied $CaCO_3$ (levels 10-14) were included to determine the effect of an increasing reserve of $CaCO_3$. For treatment levels 1 to 8, $MgCO_3$ and K_2CO_3 were applied at a rate such that the quantity of applied carbonate was equivalent to that of the $CaCO_3$ treatment. Consequently the pH for these three treatments for levels 1 to 8 were very similar. From level 9 onwards, the rates of $MgCO_3$ and K_2CO_3 were modified to give a modest incremental increase in pH.

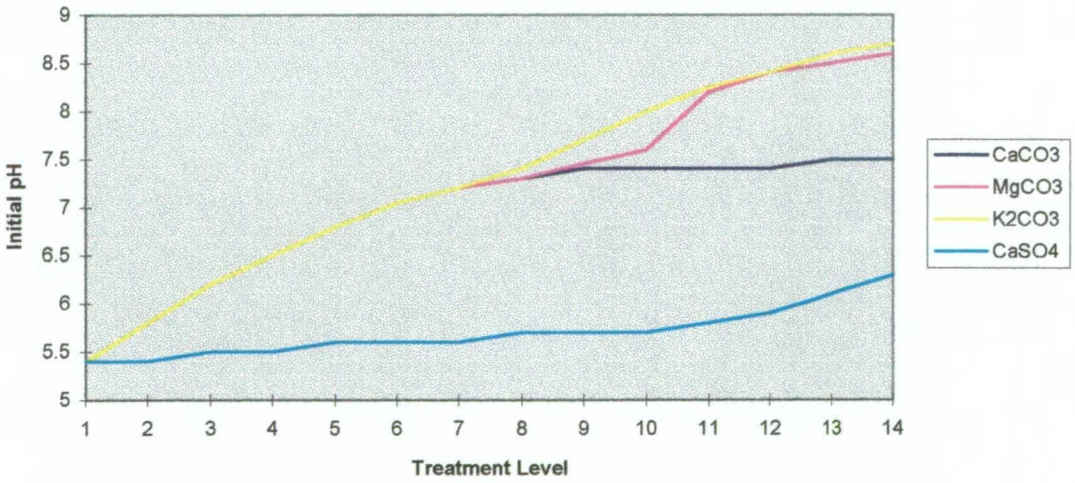
$CaSO_4$ was applied at a rate that allowed the level of applied calcium to correspond to the level of calcium in the $CaCO_3$ treatment.

Each soil amendment was thoroughly mixed through the soil prior to transplanting the trees.

Table 6.2. Rate of application of the four soil treatments.

	Calcium Carbonate			Magnesium Carbonate		Potassium Carbonate		Calcium Sulphate	
	$CaCO_3$ g/kg soil	CO_3 g/kg soil	Ca g/kg soil	$MgCO_3$ g/kg soil	CO_3 g/kg soil	K_2CO_3 g/kg soil	CO3 g/kg soil	$CaSO_4$ g/kg soil	Ca g/kg soil
1	0	0	0	0	0	0	0	0	0
2	0.33	0.2	0.13	0.28	0.2	0.46	0.2	0.57	0.13
3	0.67	0.4	0.27	0.56	0.4	0.92	0.4	1.15	0.27
4	1	0.6	0.4	0.84	0.6	1.38	0.6	1.72	0.4
5	1.33	0.8	0.53	1.12	0.8	1.84	0.8	2.29	0.53
6	1.67	1	0.67	1.4	1	2.3	1	2.86	0.67
7	2	1.2	0.8	1.69	1.2	2.76	1.2	3.44	0.8
8	2.5	1.5	1	2.11	1.5	3.45	1.5	4.3	1
9	3.33	2	1.33	2.53	1.8	4.14	1.8	5.73	1.33
10	4.17	2.5	1.67	2.95	2.1	4.83	2.1	7.16	1.67
11	6.25	3.75	2.5	3.51	2.5	5.75	2.5	10.74	2.5
12	8.33	5	3.33	4.22	3	6.9	3	14.32	3.33
13	12.5	7.5	5	7.03	5	9.2	4	21.48	5
14	25	15	10	10.54	7.5	11.5	5	42.96	10

Figure 6.1. Initial pH ($CaCl_2$ 1:5) of soil treatment combinations.



6.2.4 Transplanting

The plants were removed from the 125 mm pots and most of the potting mix gently worked free of the root system. The plants were then repotted in the 250 mm pots with 4 kg of treated soil per pot. The surface of each pot was covered with reflective aluminium sheeting to limit evaporation and retard algal growth. The plants were then placed in the glasshouse.

6.2.5 Fungal Treatments

Each tree was inoculated with *T. melanosporum* three weeks after germination. These seedlings were placed in a glasshouse and watered with overhead sprinklers. A year after inoculation the level of colonisation by *T. melanosporum* was determined by analysing a core sample of the root system for each tree. The levels of infection were low but relatively homogeneous (mean % colonisation = 2.65, standard deviation = 1.51). After being transplanted into the larger pots as year old trees, one of the two trees from each soil treatment combination was inoculated with *Descomyces albellus* (T038H) and *Hydnangium carneum* (T037H).

The two stage inoculation process was utilised to reflect the situation occurring within the Tasmanian truffle industry. PTT minimises the risk of early contamination from native ectomycorrhizal fungi by growing inoculated seedlings in sterilised medium within poly-tunnels. Most exposure to inoculum of native ectomycorrhizal fungi occurs when the trees are planted in the field, which can be up to a year after inoculation. Hence, the split inoculation process was seen as a way to more accurately assess the effects of competition from endemic ectomycorrhizal fungi. This method differs from the inoculation procedure employed in Chapter 5 where the trees were inoculated with *T. melanosporum* and competitor fungi simultaneously.

Inoculation with the native fungi was carried out using a fresh spore slurry. The fruitbodies used for the slurry were collected under a *Eucalyptus nitens* plantation at Gould's block, Dover, Tasmania (8311:D'entrecasteaux EN002071). Sporocarps were surface sterilised with a 5% solution of sodium hypochlorite for 3

minutes before rinsing thoroughly with distilled water. After the treatment with the sodium hypochlorite, the efficacy of the sporocarps was checked by successfully isolating the fungus in pure culture from several regions of the gleba. Sporocarps were then blended with distilled water to give a spore slurry concentration for *D. albellus* and *H. carneum* of 3,430,000 and 3,950,000 spores / ml respectively. Ten millilitres of each slurry were combined to inoculate each pot. Those plants inoculated with *T. melanosporum* and native fungi were separated from those inoculated solely with *T. melanosporum* to limit the risk of cross-contamination.

6.2.6 Glasshouse Preparation, Irrigation and Experimental Maintenance

To reduce the risk of contamination by other ectomycorrhizal fungi, the glasshouse walls and floors, benches and pots were treated with sodium hypochlorite prior to the commencement of the experiment.

Plants were irrigated using an overhead sprinkler system. Plants were maintained at as close as possible to 70% field capacity. Evaporative coolers restricted maximum temperatures to below 25°C. Climate statistics for Hobart are shown in Appendix 5.

Every four weeks, nutrients were applied in solution to each pot at the following rates (mg / kg soil): NH_4NO_3, 121.5; $CuSO_4.5H_2O$, 0.012; $MoNa_2O_4$, 0.0038; $ZnSO_4$, 0.032; $MnCl_2$, 0.27; H_3BO_3, 0.43; FeNaEDTA, 0.049.

6.2.7 Harvesting and Analysis

6.2.7.1 Mycorrhizal colonisation

One year after transplanting the trees into the 250 mm pots, mycorrhizal colonisation was determined by examining the combined contents of two core samples (25 mm diameter by 100 mm depth) taken 10 mm from the edge of the pot. Roots were removed from the soil cores by placing the cores over a 1 mm sieve and gently washing the soil through the sieve leaving the roots behind. The

various mycorrhizal types were identified by examination under light microscopes. The identity of *T. melanosporum* was later confirmed using PCR and RFLP as described in Chapter 9. The lengths of each root type and calculation of root parameters were determined as per Section 5.2.7.1.

6.2.7.2 Plant height, stem diameter and fruitbody production

Plant height and stem diameter were measured at the time of transplanting into the larger pots and at harvest. The pots were checked regularly (twice weekly) for fruitbody production.

6.2.7.3 Soil analysis

At harvest, each soil treatment combination was analysed to determine pH ($CaCl_2$ & H_2O) and exchangeable bases.

6.2.7.4 Statistical analysis

Several regression models including straight line, polynomial and asymptotic models were used to test for a relationship between the level of colonisation of *T. melanosporum* and independent variables such as applied $CaCO_3$, $CaSO_4$ and $MgCO_3$.

The response of *T. melanosporum* to pH was described using linear regression.

The statistical package used for the analysis was SAS for Windows version 6.11.

Statistical analysis was not applied to the following dependent variables; percent colonisation by *Descomyces albellus* and percent colonisation by *Hydnangium carneum*, as mycorrhizas of these fungi occurred very infrequently.

6.3 Results

6.3.1 Percent Colonisation of Hazel by *Tuber melanosporum*

6.3.1.1 Effect of applied $CaCO_3$, $MgCO_3$ and K_2CO_3

Application of either $CaCO_3$ and $MgCO_3$ increased the level of colonisation of *T. melanosporum* as shown in Figure 6.2. The results for K_2CO_3 are not presented; soon after the commencement of the experiment, it was noted that many of the plants in the K_2CO_3 treatment were affected by potassium toxicity.

An asymptotic regression model provided the best fit for the response of *T. melanosporum* to independent variables such as applied $CaCO_3$ and $MgCO_3$ (refer to Figure 6.2)

The model used was:

$$y = \alpha - \beta(\gamma)^x$$

where

y = percent colonisation by *T. melanosporum*

α = the asymptote corresponding to $X \rightarrow \infty$

β = the range of the response between X=0 and X=∞

γ = the rate which Y changes from its initial value at X=0 to its final value at α

x = dependent variable

The response of *T. melanosporum* to $CaCO_3$ and $MgCO_3$ can be described by Equations 1 and 2 respectively.

Equation 1 $y = 60.3 - 47.6(0.510)^x$ Root mean square residual = 17.4

Equation 2 $y = 53.2 - 39.8(0.796)^x$ Root mean square residual = 13.1

Where y = percent colonisation by *T. melanosporum*

x = level of soil treatment (mg/Kg soil)

Tuber melanosporum showed a greater response to $CaCO_3$ than an equivalent quantity of $MgCO_3$ and the maximum predicted response for $CaCO_3$ was higher than that for $MgCO_3$.

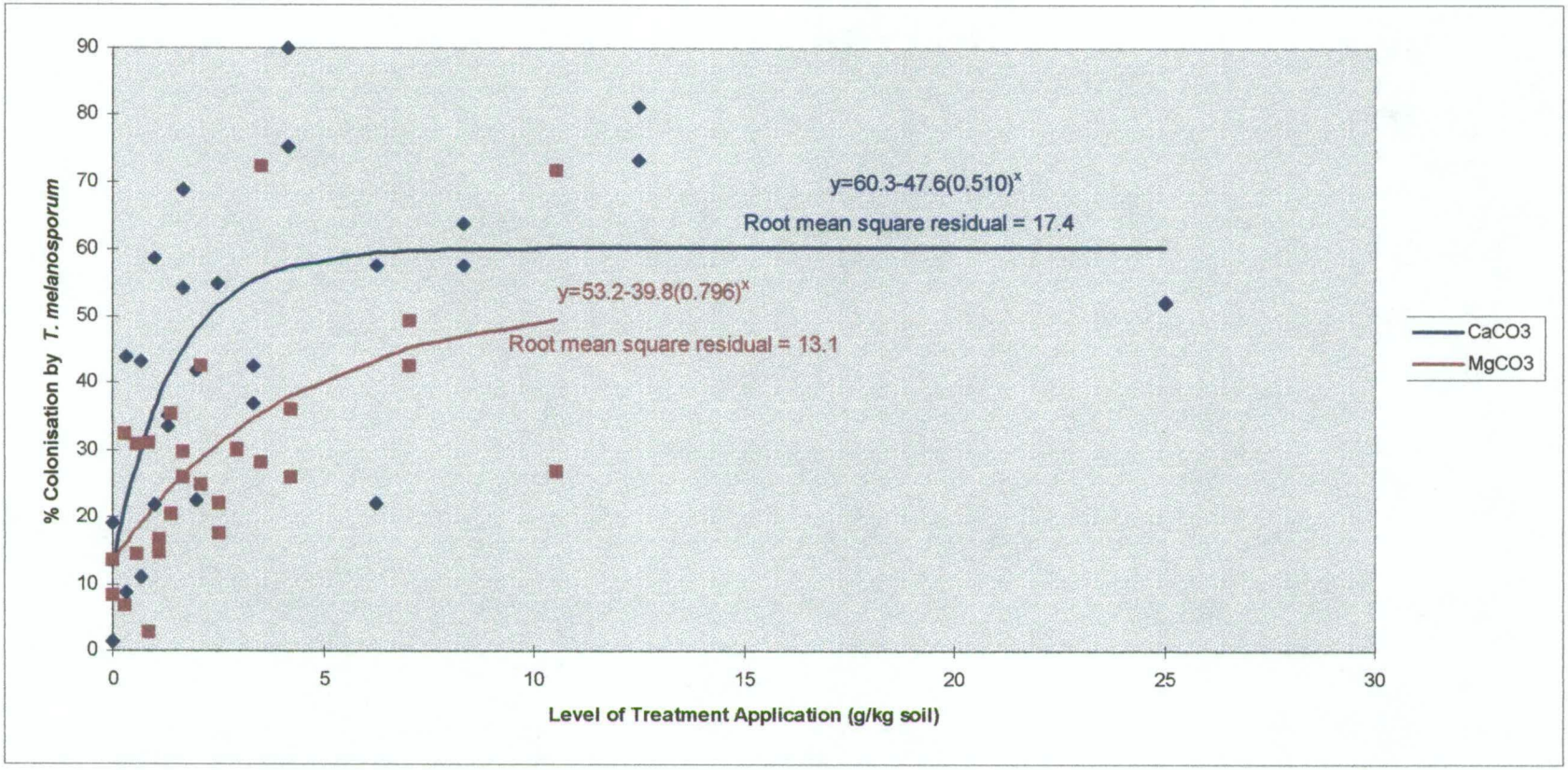
An alternative way to consider the results is to look at the response of *T. melanosporum* to $CaCO_3$ and $MgCO_3$ at equivalent levels of applied CO_3 (refer to Figure 6.3). The regression equations are essentially the same with the exception of the gamma parameter. Gamma changes in magnitude with the change in scale of the x axis but also the relative difference between gamma for $CaCO_3$ and gamma for $MgCO_3$ changes because $CaCO_3$ and $MgCO_3$ contain a different percentage by weight of carbonate.

It was observed that at high levels of $MgCO_3$ application (>2.11g/kg soil), root growth was retarded. Both coarse roots and fine roots were affected.

6.3.1.2 Effect of applied calcium

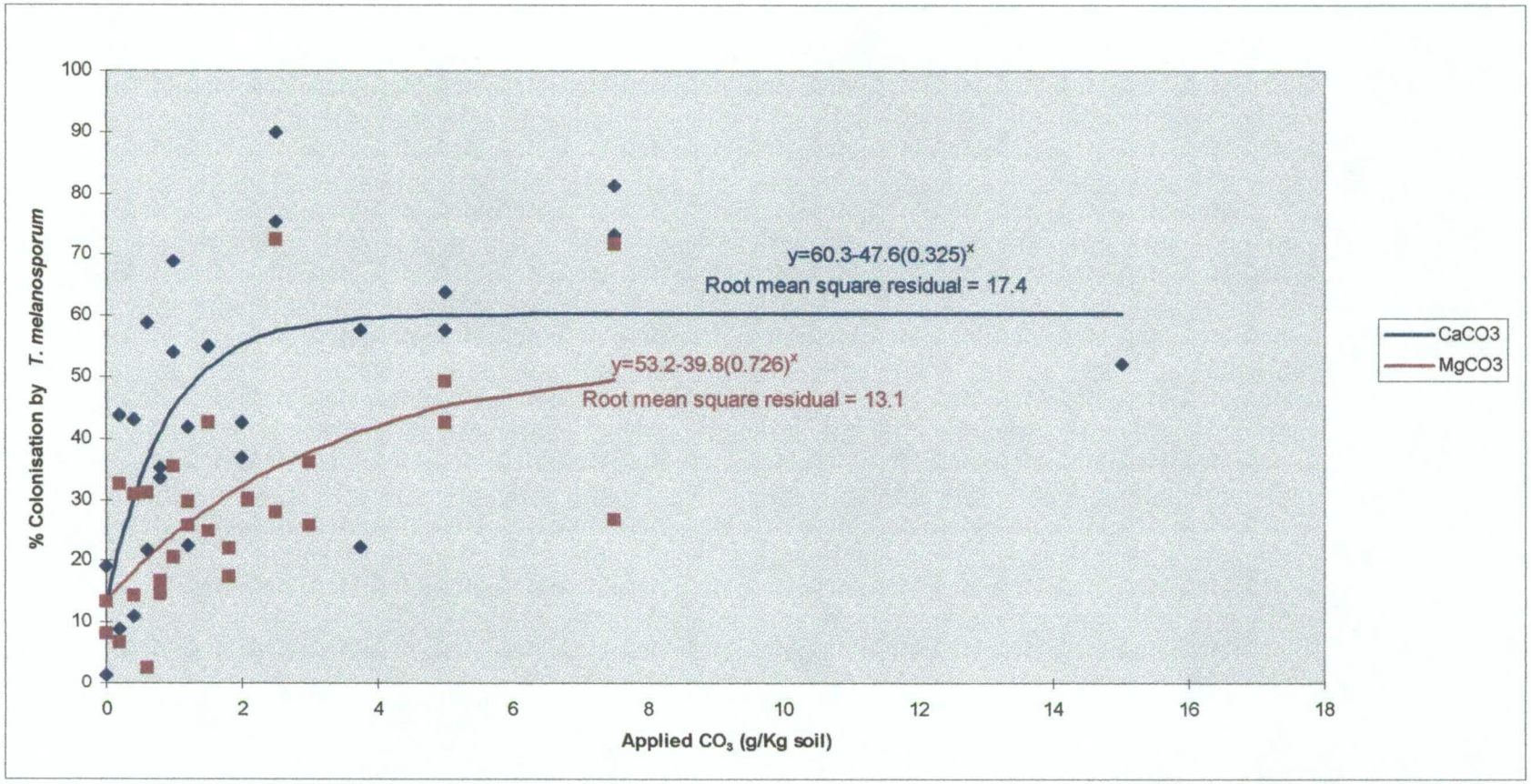
Figure 6.4. compares the response of *T. melanosporum* to $CaCO_3$ and $CaSO_4$ at equivalent levels of calcium application. The response of *T. melanosporum* to $CaSO_4$ can be depicted by the equation, $y = 19.8-17.6(0.0038)^x$. This asymptotic model appeared to give a slightly better fit than a straight line ($y = -0.67x+18.8$, $P>0.05$).

Figure 6.2. Effect of applied $CaCO_3$, and $MgCO_3$ on colonisation of hazel by *Tuber melanosporum*.



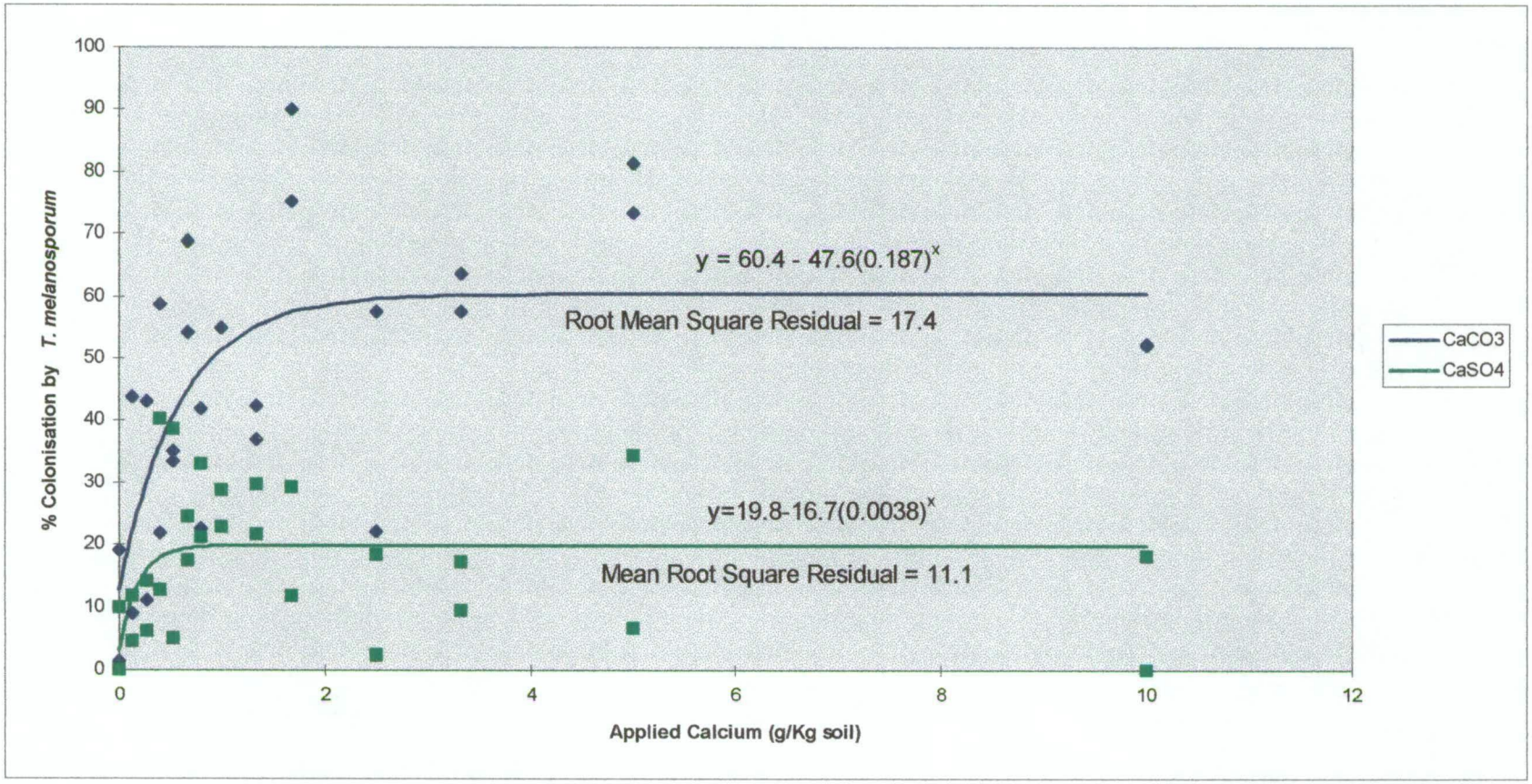
Note. All replicates formed *T. melanosporum* mycorrhizas. Where there is only one point per treatment level, the two replicates had the same value.

Figure 6.3. The response of *Tuber melanosporum* to $CaCO_3$ and $MgCO_3$ at equivalent levels of CO_3.



Note. All replicates formed *T. melanosporum* mycorrhizas. Where there is only one point per treatment level, the two replicates had the same value.

Figure 6.4. The response of *Tuber melanosporum* to $CaCO_3$ and $CaSO_4$ at equivalent levels of calcium application.



Note. All replicates formed *T. melanosporum* mycorrhizas. Where there is only one point per treatment level, the two replicates had the same value.

6.3.1.3 Effect of exchangeable bases

Exchangeable bases were initially analysed separately for each soil treatment group ($CaCO_3$, $MgCO_3$ and $CaSO_4$). In the $CaCO_3$ treatment, exchangeable Ca was significantly correlated to percent colonisation by *T. melanosporum* ($P<0.05$, $r^2=0.198$). Similarly, in the $MgCO_3$ treatment, exchangeable Mg was significantly correlated to percent colonisation by *T. melanosporum* ($P<0.01$, $r^2=0.324$). In the $CaSO_4$ treatment, there was no correlation, neither significant nor observable, between percent colonisation by *T. melanosporum* and exchangeable Ca.

In the $CaCO_3$ and $MgCO_3$ treatments, the level of the exchangeable Ca and exchangeable Mg respectively, were closely correlated to pH. Hence, it is difficult to gauge whether the response by *T. melanosporum* was a function of the level of the exchangeable base or of pH. The fact that exchangeable Ca was not correlated to percent colonisation by *T. melanosporum* in the $CaSO_4$ treatment (where there was also no correlation between exchangeable Ca and pH) suggests that pH was the factor responsible.

Exchangeable bases were also analysed across all the soil treatments combined. Neither the individual exchangeable bases (Ca, Mg, K or Na) nor total exchangeable bases were significantly correlated ($P>0.05$) to the level of colonisation by *T. melanosporum*.

6.3.1.4 Effect of pH

Figure 6.5 shows the pH ($CaCl_2$) for each soil treatment at the time of harvest. $CaCO_3$ and $MgCO_3$ significantly increased the pH ($P<0.001$) whereas $CaSO_4$ had no significant effect on pH ($P>0.05$). For the soil type used in this experiment, the maximum pH ($CaCl_2$) obtainable by liming with $CaCO_3$ was 7.3. To convert pH ($CaCl_2$) to pH (H_20) for the soil used in this experiment, refer to Appendix 6.

When analysed across all soil treatments, pH explained 38% of the observed variation in the percent colonisation of hazels by *T. melanosporum* as described by the equation below.

$$y = 16.6x - 75 \qquad r^2=0.38$$

Where y = percent colonisation by *T. melanosporum*

x = pH ($CaCl_2$)

This regression was highly significant ($P<0.001$) and is depicted in Figure 6.6 as a black line.

When the effect of pH on *T. melanosporum* was compared for each soil treatment, it was noted that $CaCO_3$ gave a greater response than $MgCO_3$ at an equivalent pH level as shown in Figure 6.6.

Figure 6.5. The pH ($CaCl_2$) of each soil treatment at the time of harvest.

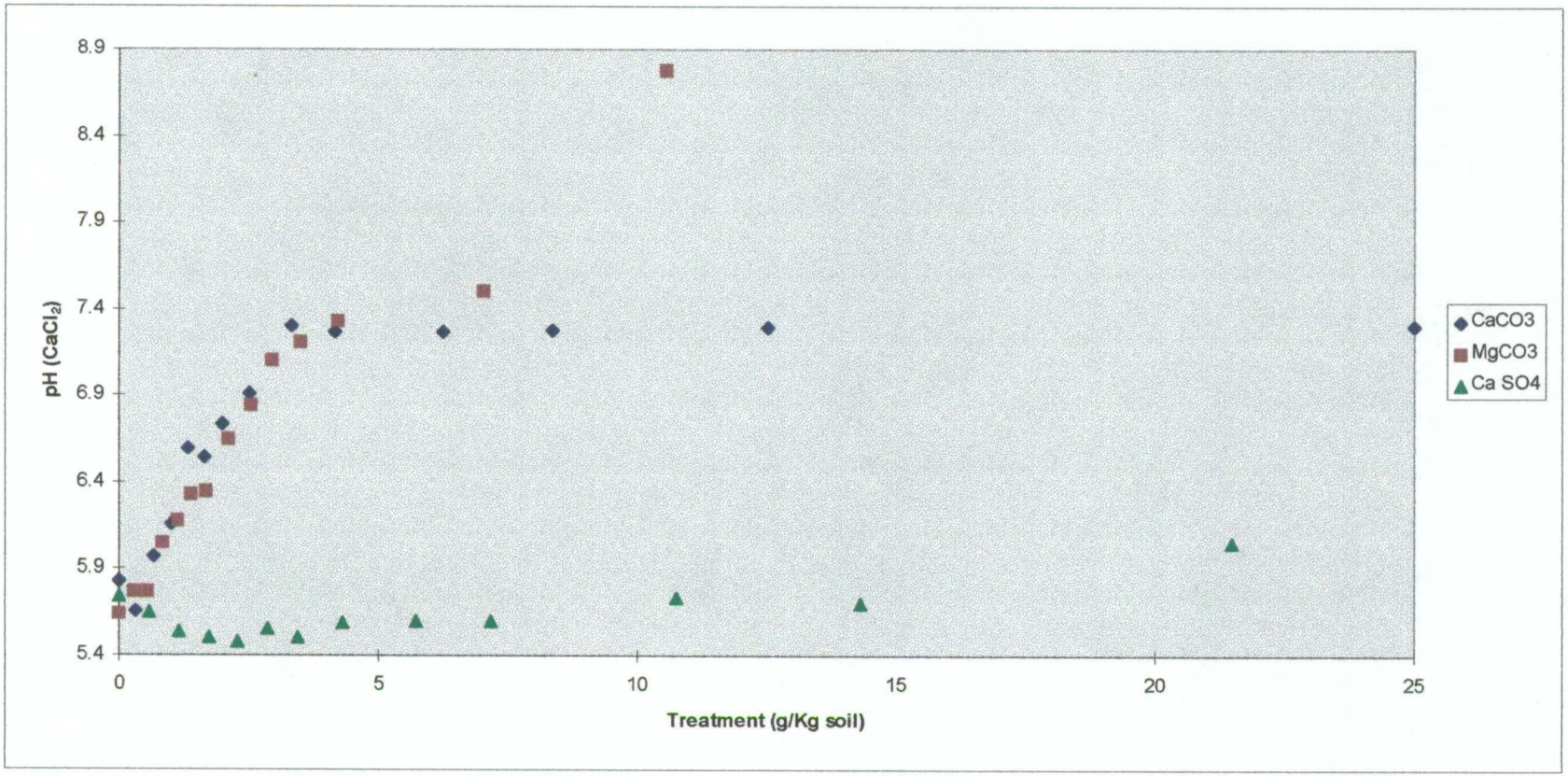
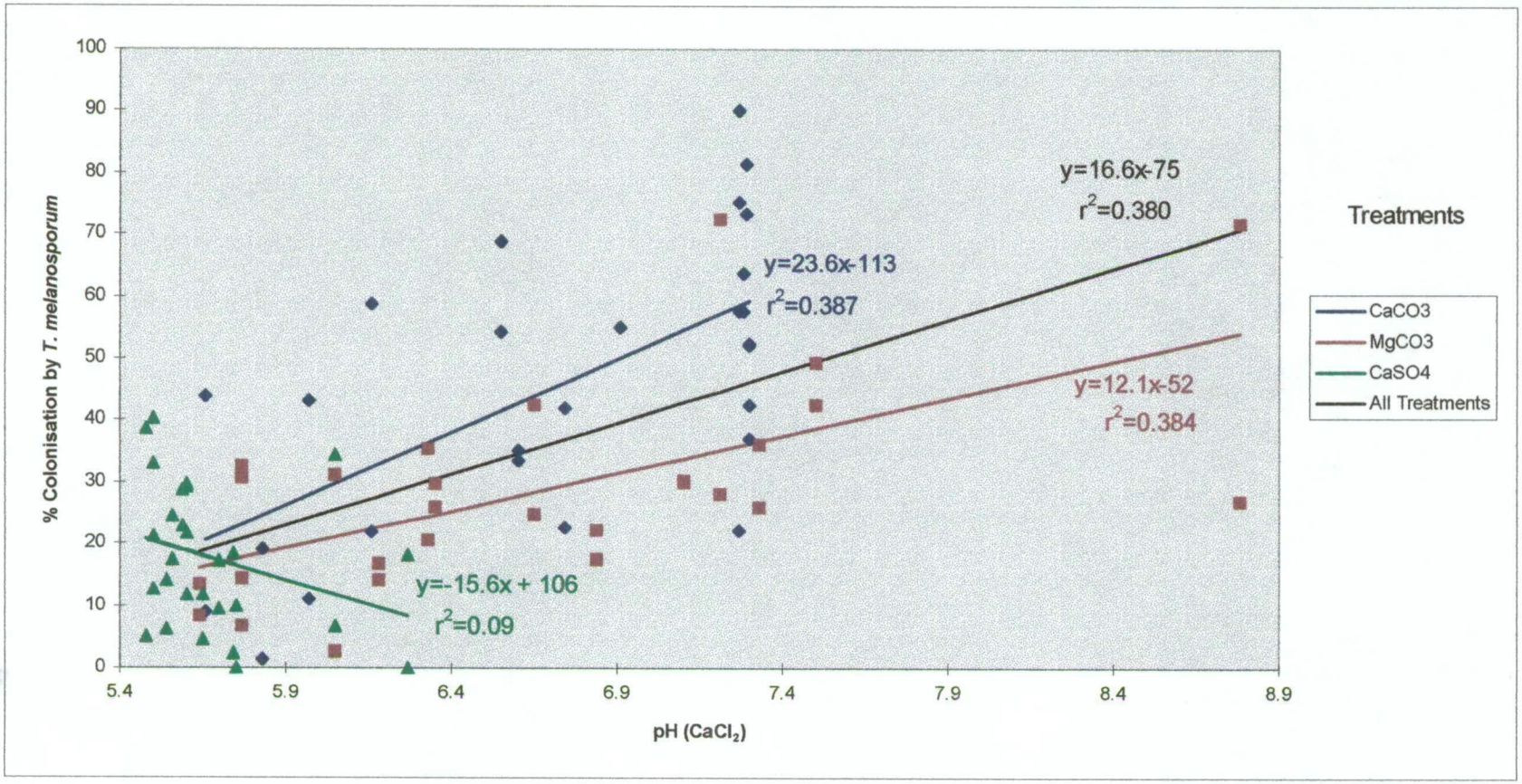


Figure 6.6. The effect of pH on colonisation of hazel by *Tuber melanosporum* for each soil treatment.



6.3.1.5 Other variables

The level of colonisation by *T. melanosporum* at the time of harvest was not significantly correlated to plant height, stem diameter, or to the initial level of colonisation by *T. melanosporum* prior to the addition of the soil treatments.

6.3.2 Percent Colonisation of Hazel by *Descomyces albellus* and *Hydnangium carneum*

Both *D. albellus* and *H. carneum* occurred infrequently and at low levels of colonisation with the exception of one tree which where *D. albellus* colonised 41.5% of the fine roots. The occurrence of *D. albellus* and *H. carneum* is outlined in Tables 6.3 and 6.4 respectively.

Table 6.3. Pots in which *Descomyces albellus* was found.

Pot Number	Soil treatment applied to the pot (mg/Kg soil)			Percent Colonisation by *D. albellus*	pH ($CaCl_2$)
	$CaCO_3$	$MgCO_3$	$CaSO_4$		
114	0	0	0	41.5	5.83
182	0	1.69	0	0.84	6.35
198	0	0	0	0.98	5.75
200	0	0	0.57	0.75	5.65
204	0	0	1.72	2.4	5.5
208	0	0	2.86	1.69	5.5
218	0	0	10.74	1.82	5.56
220	0	0	14.32	0.37	5.74

Table 6.4. Pots in which *Hydnangium carneum* was found.

Pot Number	Soil treatment applied to the pot (mg/Kg soil)			Percent Colonisation by *H. carneum*	pH ($CaCl_2$)
	$CaCO_3$	$MgCO_3$	$CaSO_4$		
178	0	1.12	0	3.33	6.18
182	0	1.69	0	0.85	6.35
202	0	0	1.15	1.55	5.54
210	0	0	3.44	0.847	5.5
214	0	0	5.73	1.69	5.6

While the levels of colonisation by *D. albellus* and *H. carneum* were not significantly correlated to any other variable, it was noted that these fungi did not occur in pots treated with high rates of either $CaCO_3$ or $MgCO_3$ (ie. pots with a high pH).

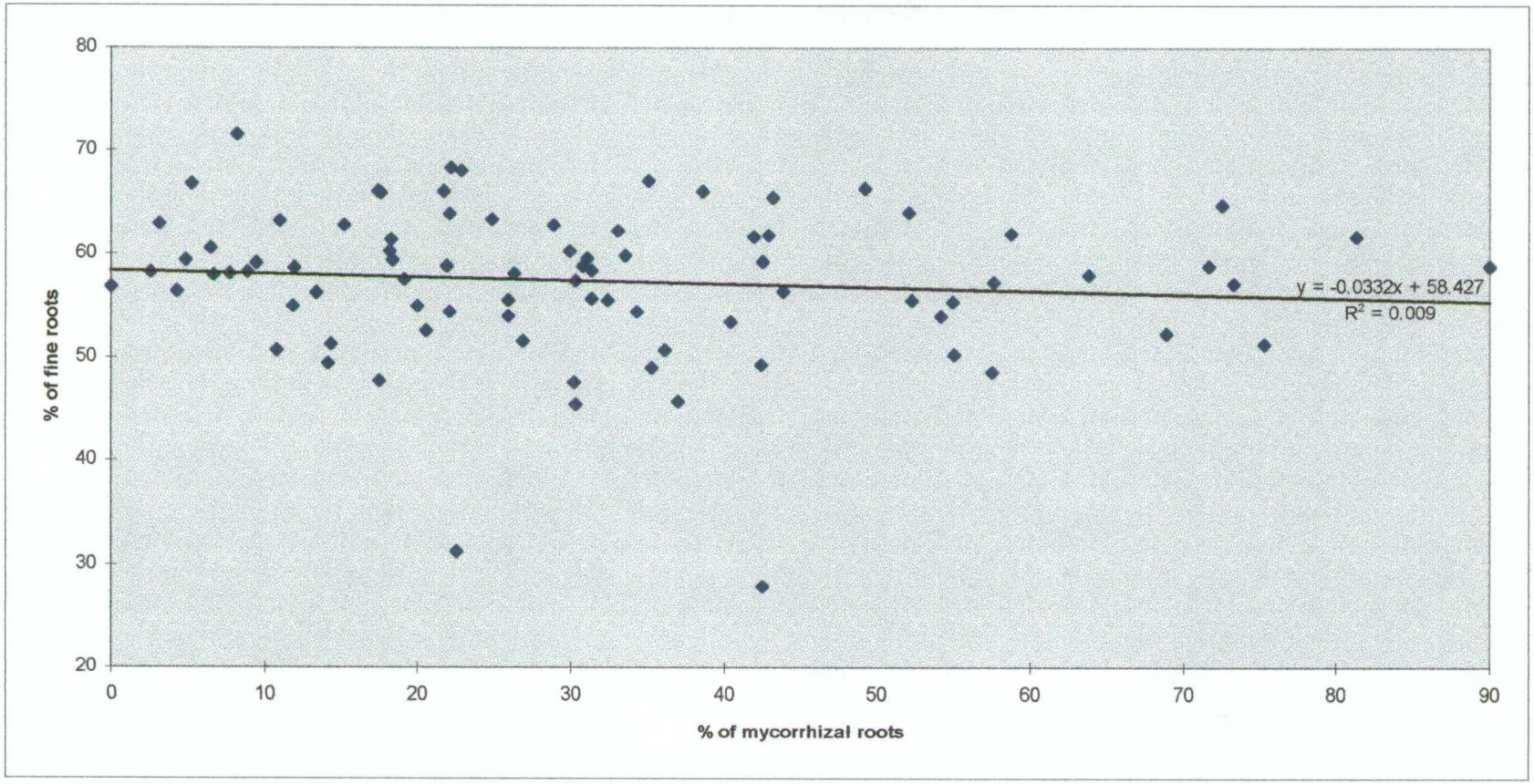
6.3.3 The Percentage of Fine Roots

There was no significant ($P>0.05$) correlation between the total level of mycorrhizal colonisation and the percentage of fine roots (ratio of fine roots to coarse roots) as is illustrated in Figure 6.7.

6.3.4 Sporocarp Production

In a pot exhibiting 41.5% mycorrhizal colonisation by *D. albellus*, a sporocarp of this fungus and several primordia were found in April, 1997.

Figure 6.7. The regression of percentage of mycorrhizal roots on the percentage of fine roots.



6.4 Discussion

6.4.1 The Effect of pH and Calcium on Colonisation of Hazel by *Tuber melanosporum*

In Chapter 5, it was observed that applying lime to an acid soil increases root colonisation by *T. melanosporum*. In this experiment, applying liming materials increased root colonisation up to a point where further additions of liming material did not affect the level of colonisation. That is, the response was asymptotic in nature. It is likely that the asymptotic curves gave the best fit for $CaCO_3$ and $MgCO_3$ because of the influence of $CaCO_3$ and $MgCO_3$ on soil factors such as pH and exchangeable bases. Applying $CaCO_3$ and $MgCO_3$ continues to increase the pH and level of exchangeable bases up to the point when the soil becomes saturated with either $CaCO_3$ or $MgCO_3$ and dissociation of these liming substances discontinues.

There were several findings that suggest the response of *T. melanosporum* to applied lime was primarily a function of pH. Applied calcium or exchangeable calcium may have a secondary role. The factors pointing to this conclusion include:

- The liming materials $CaCO_3$ and $MgCO_3$ (which increase pH) increased root colonisation by *T. melanosporum*.
- There was a highly significant regression ($P<0.001$) between root colonisation by *T. melanosporum* and soil pH.
- $CaSO_4$, which does not significantly effect pH, had little if any effect on root colonisation by *T. melanosporum*.
- There was no clear relationship between either applied calcium or exchangeable calcium, and root colonisation by *T. melanosporum*.

While applied $MgCO_3$ increased root colonisation by *T. melanosporum*, the response appeared to be weaker than an equivalent application (by weight) of $CaCO_3$. This is despite the fact that per unit weight, $MgCO_3$ increases pH slightly more than does $CaCO_3$. There are two possible explanations for this:

- The weaker response to $MgCO_3$ may have been due to an imbalance of Ca and Mg in the soil affecting plant/fungus nutrition. For example, high levels of Mg relative to Ca can induce a calcium deficiency (Leeper and Uren, 1993). Furthermore, when the calcium/total cation ratio is low, root growth is inhibited (Marschner, 1995).
- Alternatively, the stronger response by *T. melanosporum* to $CaCO_3$ may have been a result of an additional positive influence of high calcium levels over and above the primary response to pH.

Tuber melanosporum also appears to have shown an asymptotic type response to $CaSO_4$. There are several possible explanations for this response.

1. *Tuber melanosporum* may have been calcium deficient over the first three treatments levels. Applying $CaSO_4$ alleviated the deficiency and thereby resulted in a positive response in colonisation over the initial range of 0 to 1 grams of calcium.
2. The low levels of colonisation by *T. melanosporum* for the first three treatments levels may have been coincidental. If these points were the result of experimental variation, the straight line model ($y = -0.67x+18.8$) would have been a more appropriate model than the asymptotic model depicted in Figure 6.4. That is, applied $CaSO_4$ has not influenced the colonisation of *T. melanosporum* and the average level of colonisation for the $CaSO_4$ treatment (ca. 18%) represents a base level of colonisation for the soil.
3. *Tuber melanosporum* shows a positive response to increasing levels of calcium as depicted by the response within the range 0 to 1 grams of applied calcium. At higher rates, an inhibitory effect of sulphate negates the positive effect of calcium. This explanation is unlikely as it does not account for the two highest $CaSO_4$ treatments where one replicate in each showed a relatively high level of colonisation.

6.4.2 Practical Implications

Since the response of *T. melanosporum* to $CaCO_3$ appears to be primarily a function of pH, management guidelines for lime application can be simplified.

Before planting trees, truffle growers should incorporate sufficient lime so as to reach the maximum pH obtainable with $CaCO_3$. In the experiment, this pH level corresponded to maximum root colonisation by *T. melanosporum*. Growers need not be overly concerned with parameters such as exchangeable calcium or total calcium, but simply refer to soil pH. Truffières should be monitored over time. As pH begins to decline, more lime can be applied.

6.4.3 The Effect of $MgCO_3$ on Root Growth

As previously mentioned in section 6.4.1, when the calcium/total cation ratio is low, root growth of plants can be inhibited (Marschner, 1995). This may explain the poor root growth observed for those plants treated with high rates of $MgCO_3$ (>2.11 g/kg).

It is unlikely that the reduction in root growth was related to pH. As shown in Figure 6.5, at the time of harvest, the pH of the treatment levels for $MgCO_3$ were very similar to those for $CaCO_3$, with the exception of the highest rate of $MgCO_3$ (treatment level 14, 10.54 g/kg) with a pH of 8.8. Reductions in root growth for the $MgCO_3$ treatment were visually observed from treatment level 8 (2.11 g/kg). However, there were no corresponding effects in the $CaCO_3$ treatment even though the pH levels were similar (except treatment level 14).

6.4.4 The Effect of pH and Calcium on Colonisation of Hazel by Two Species of Australian Endemic Ectomycorrhizal Fungi

Descomyces albellus can be added to the list of endemic ectomycorrhizal fungi capable of colonising hazel and fruiting under hazel.

Due to the sporadic occurrence of both *D. albellus* and *H. carneum*, it was not possible to determine if they were significantly affected by the soil treatments. While neither fungus occurred at a pH ($CaCl_2$) higher than 6.35, further experimentation is required to determine if they are able to colonise hazel at higher levels of pH. This experiment is being maintained to determine if there are any

changes in the occurrence and level of colonisation by *D. albellus* and *H. carneum* over time.

6.4.5 The Percentage of Fine Roots

In the experiment described in Chapter 5 there was a significant negative regression between percentage of mycorrhizal roots and the percentage of fine roots. This contrasts with the results of this experiment where there was no relationship between percentage of mycorrhizal roots and the percentage of fine roots.

One possible explanation is that fungal species may influence the relationship between colonisation and the percentage of fine roots. The trees in this experiment were colonised predominantly by *T. melanosporum*, whereas in Chapter 5, *T. melanosporum* only comprised a small proportion of the overall mycorrhizal colonisation. Mamoun and Olivier (1996) have observed that the relationship between percent colonisation and root volume for *T. melanosporum* was different to that of other mycorrhizal fungi.

6.4.6 Variability in the Colonisation of Hazel by *Tuber melanosporum*

The results demonstrated substantial variation in the colonisation of the hazel trees by *T. melanosporum* that could not be explained by the soil treatments or any other measured variable. Soil pH only accounted for 38% of the observed variation. A possible explanation for the remaining variation is proposed in the general discussion.

7 The Effects of the Interaction Between Lime and Phosphorus on Competition Between *Tuber melanosporum* and other Ectomycorrhizal Fungi.

7.1 Introduction

Soil pH has a significant influence on the availability of soil phosphorus. As the soil pH increases above 7.2, the form of P in the soil solution changes from $H_2PO_4^-$ to HPO_4^{2-}. Plant uptake of HPO_4^{2-} is much slower than $H_2PO_4^-$ (Tisdale *et al.*, 1993). Furthermore, in alkaline soils, orthophosphate ions can react with calcium to produce sparingly soluble calcium phosphate compounds which decreases the quantity of plant available P (Brady, 1984).

Improving the uptake of soil nutrients, particularly P from sparingly soluble sources, is one of the major functions of mycorrhizal fungi (Smith and Read, 1997). Much of this benefit is achieved due to the ability of mycorrhized plants to explore a greater volume of soil than that of non-mycorrhized plants (Bolan, 1991). In addition to this increase in spatial availability of P, the hyphae of mycorrhizal fungi utilise several other uptake mechanisms to improve P acquisition (Marschner and Dell, 1994). An example of interest is the ability of some ectomycorrhizal fungi to produce significant quantities of oxalic acid (Lapeyrie *et al.*, 1987), which in calcareous soils, may mobilise P from insoluble calcium phosphates. At the same time, oxalic acid may prevent calcium intoxication of the plant host by the formation of calcium oxalate at the fungus-soil interface (Lapeyrie

et al., 1987). Another example is that mycorrhizal fungi may be able to influence the rhizosphere pH through differences in absorption of cations and anions and thereby increase the availability of P (Bolan, 1991).

While mycorrhizal fungi can improve the uptake of P from P deficient soils, high levels of soil P can result in the depression of ectomycorrhizal fungal colonisation. This depression may be due to the effects of high concentrations of plant P reducing the supply of soluble carbohydrates to the root system (Harley and Smith, 1983). The degree of depression varies from species to species (Marschner and Dell, 1994). Australian native ectomycorrhizal fungi appear to exhibit maximum levels of mycorrhizal colonisation at relatively low levels of soil phosphorus. For example, in a study by Bougher *et al.* (1990) two isolates of *Descolea maculata* and one isolate of *Laccaria laccata* showed maximum levels of mycorrhizal infection with the addition of less than 4 mg P / kg soil to a phosphorus deficient soil (available P < 2 mg/kg, total P ≡ 22 mg/kg). Similar trends for three ectomycorrhizal fungi of *Eucalyptus diversicolor* were noted in unpublished data from Grove, Dell and Malajczuk (Brundrett *et al.*, 1996). The apparent inability of Australian native ectomycorrhizal fungi to cope with modest levels of applied P is probably a reflection of the low phosphorus status of many Australian soils.

Some authors have noted that high levels of soil P may depress *T. melanosporum*.

- Delmas and Poitou (1974) state that an excess of P or N is detrimental to the formation and maintenance of *T. melanosporum* mycorrhizas. The evidence for this statement is not given in the paper but reference is made to another paper by the same authors that was 'in print'. However the journal for the paper in print was not given, and the paper cannot be found.
- Delmas (1978) and Delmas *et al.* (1981) state that an excess of phosphorus leads to a reduction in colonisation by *T. melanosporum* but do not provide supporting data or references.

While phosphorus may depress the growth of *T. melanosporum*, there is evidence to suggest that *T. melanosporum* may be more tolerant to higher levels of P than Australian native species. For example:

- Grente *et al.* (1976) found that at low levels of total P (40 mg/kg), applied P initially increased the level of colonisation by *T. melanosporum*. Maximum colonisation was attained at a total P level of 530 mg /kg. However, at 775 mg/kg and above, colonisation by *T. melanosporum* was reduced to very low levels. By comparison, the maximum colonisation by Australian native species in the experiment of Bougher *et al.* (1990) occurred at a total P level of approximately 26 mg/kg (2 to 4 mg/kg applied P plus the initial total P of the soil of about 22 mg/kg). While total P is probably not the best measure of P for predicting mycorrhizal colonisation, there appears to be a marked difference in the level of P required for maximum colonisation of *T. melanosporum* relative to the native species.
- The range of available P reported by Delmas *et al.* (1981) for 'good truffle production' is much higher that the available P levels of most Tasmanian soils. For instance, the available P levels of those soils being used for truffières in Tasmania range from 10 to 52, whereas the range reported by Delmas *et al.* was 6 to 980 mg / kg.
- Le Tacon *et al.* (1982) applied P and N treatments to trees within a truffière. Phosphorus was applied once at a rate of 200 kg P / Ha and the level of fruiting was recorded in the following two winters. Very low levels of ascocarp production made statistical analysis of the experiment difficult, however, the authors made the provisional conclusion that the applied P had no perceptible effect either positive or negative on the level of fruiting. Unfortunately, they did not investigate the effects of the applied P on the level of mycorrhizal colonisation.
- Sourzat *et al.* (1990) applied P to hazels within a truffière at rates up to 140 kg / Ha. The treatments were repeated for three consecutive years. The phosphorus treatment did not significantly increase or decrease the level of mycorrhizal colonisation by *T. melanosporum*. However, there were only four

trees per treatment and only one root core sample taken per tree which would dramatically reduce the likelihood of detecting significant changes.

- Chu-Chou and Grace (1987) noted that a *Tuber* sp. forming mycorrhizas with *Pinus radiata* was significantly more prevalent on agroforestry sites than conventional forest sites. They postulated that this may be due to the preference or tolerance of the *Tuber* sp. to the higher levels of soil fertility of the agroforestry sites. These sites were especially high in P.

If there are negative effects of excess P on the growth of *T. melanosporum*, it appears that this may happen at higher soil P levels than it does for Australian native species. The difference in response to P may be another way to manipulate the soil environment to favour *T. melanosporum* over native competitors. Therefore, the objectives of this experiment is to study the effects of applied phosphorus and the interaction between lime and phosphorus on colonisation of hazel by *T. melanosporum* and Australian native ectomycorrhizal fungi.

7.2 Materials and Methods

7.2.1 Overview

Seedling hazels (*Corylus avellana*) were inoculated with *T. melanosporum* and planted into 125 mm pots. After a year, these plants were transplanted from the 125 mm pots into 250 mm pots with soil treated with different rates of lime and phosphorus. Lime was applied at four levels. At each level of lime application, phosphorus was applied at fourteen levels hence a total of 56 soil treatment combinations. Two trees were treated with each soil treatment combination giving a total of 112 trees. A month after transplanting, one tree from each of the 56 soil treatment combinations was inoculated with spores of native ectomycorrhizal fungi. One year after transplanting the trees were analysed for the level of colonisation by *T. melanosporum* and native ectomycorrhizal fungi.

7.2.2 Soil Type and Preparation

Refer to section 6.2.2.

7.2.3 Soil Treatments

The four rates of lime application and corresponding initial pH ($CaCl_2$) of the soil are shown in Table 7.1.

Table 7.1. Level of lime application and corresponding pH of the soil used to transplant the year old plants.

	Applied $CaCO_3$ g/kg soil	Initial pH ($CaCl_2$)
1	0	5.4
2	1.25	6.7
3	2.5	7.3
4	12.5	7.5

The level of lime application was determined by developing a titration curve for the soil (Barrow and Cox, 1990). The aim was for the third level of application to be at the point where the soil is virtually saturated with calcium and additional applications of lime result in little or no increase in pH. The second level is an intermediate level between level 3 and the natural pH of the soil. The fourth level represents a massive excess of undissociated lime in the soil.

For each lime treatment, phosphorus was applied as calcium phosphate monobasic at the following rates: 0, 2, 4, 8, 12, 16, 20, 28, 36, 48, 60, 80, 110 and 150 mg P / kg soil.

Each soil amendment was thoroughly mixed through the soil prior to transplanting the trees.

7.2.4 Transplanting

Refer to section 6.2.4.

7.2.5 Fungal Treatments

Refer to section 6.2.5.

7.2.6 Glasshouse Preparation, Irrigation and Experimental Maintenance

Refer to section 6.2.6. Potassium phosphate was excluded from the maintenance nutrient solution.

7.2.7 Harvesting and Analysis

7.2.7.1 Mycorrhizal colonisation, plant height, stem diameter and sporocarp production

Refer to Section 6.2.7.

7.2.7.2 Soil analysis

At harvest, each lime treatment was analysed to determine pH ($CaCl_2$ & H_2O) and exchangeable bases. Each soil treatment combination was analysed for available phosphorus using the Colwell method (Colwell, 1963).

7.2.7.3 Statistical Analysis

Regression models including straight line, polynomial and asymptotic models were used to test for a relationship between the level of colonisation of *T. melanosporum* and applied $CaCO_3$.

Linear regression analysis was used to study the relationship between the dependent variable, percent colonisation by *T. melanosporum*, and other variables including applied P, available P, fungal treatment, plant height and stem diameter. The statistical package used for the analysis was SAS for Windows version 6.11.

Statistical analysis was not applied to the following dependent variables; percent colonisation by *Descomyces albellus* and percent colonisation by *Hydnangium carneum,* as mycorrhizas of these fungi occurred very infrequently.

7.3 Results

7.3.1 The Effect of Lime and Phosphorus on Colonisation by *Tuber melanosporum*

Tuber melanosporum colonisation was extremely variable. The response of *T. melanosporum* to the four levels of applied lime is shown in Figure 7.1.

An asymptotic regression model best described the response of *T. melanosporum* to applied $CaCO_3$.

The model used was

$$y = \alpha - \beta(\gamma)^x$$

where

y = percent colonisation by *T. melanosporum*

α = the asymptote corresponding to $X \rightarrow \infty$

β = the range of the response between X=0 and X=∞

γ = the rate which Y changes from its initial value at X=0 to its final value at α

x = dependent variable

The relationship between applied lime and percent colonisation by *T. melanosporum* can be described by the regression equation

$$y = 53.3 - 37.8(0.691)^x$$

The root mean square residuals for the regression was 16.7.

Neither applied P nor available P had any significant effect (P>0.05) on the percent colonisation of hazel by *T. melanosporum*. Nor was there a lime/phosphorus interaction. The nil effect of applied phosphorus on the percent colonisation by *T. melanosporum* is depicted in Figure 7.2 where the response of *T. melanosporum* to increasing rates of applied P is given for the four levels of applied lime. The slope of the regression line for each level of applied lime is not significantly different to zero (P>0.05).

The remaining variables including initial level of *T. melanosporum* colonisation, plant height and stem diameter also had no significant effect (P>0.05) on the level of colonisation by *T. melanosporum*.

Figure 7.1. Regression analysis for the effect of lime on the level of colonisation of *Tuber melanosporum*.

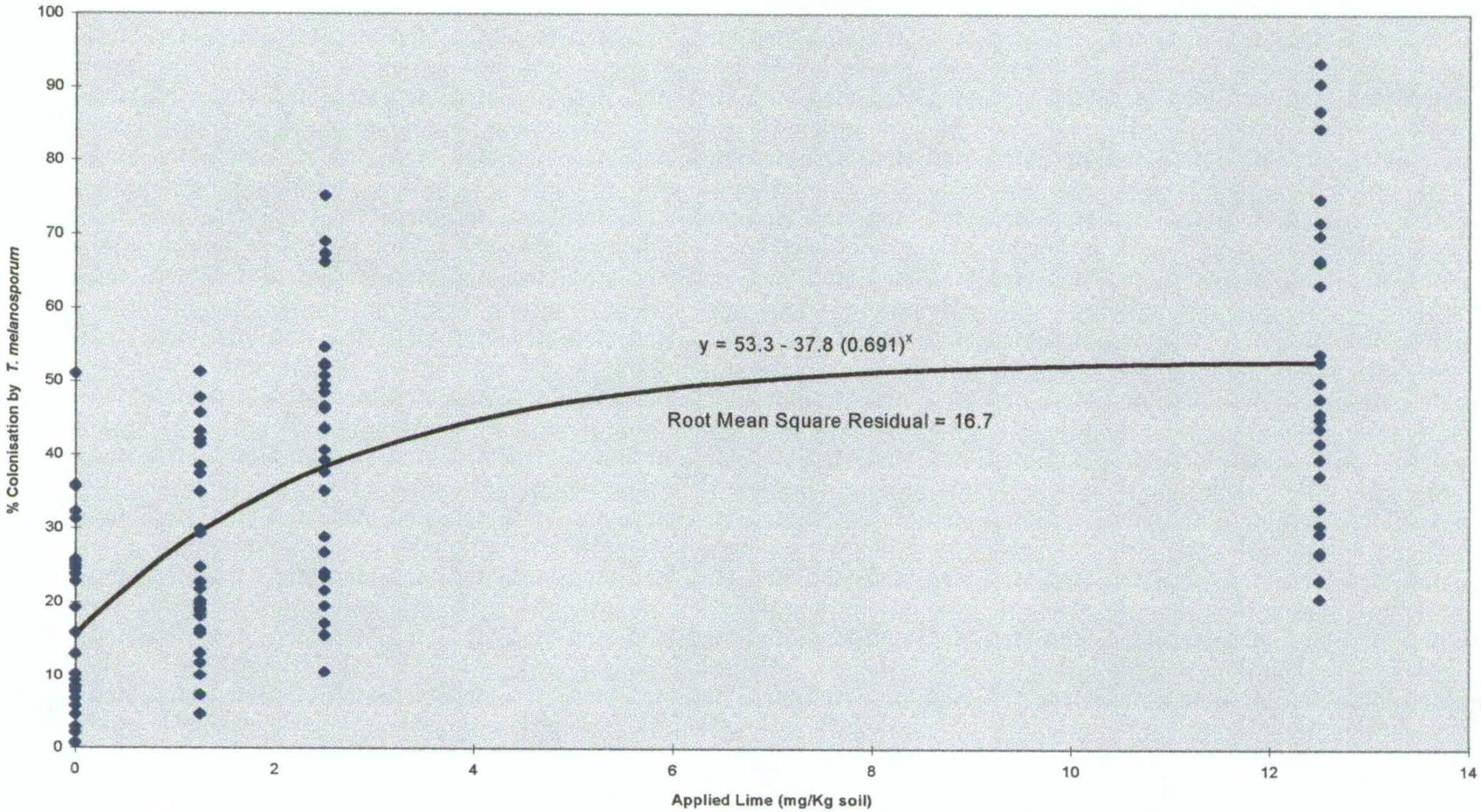
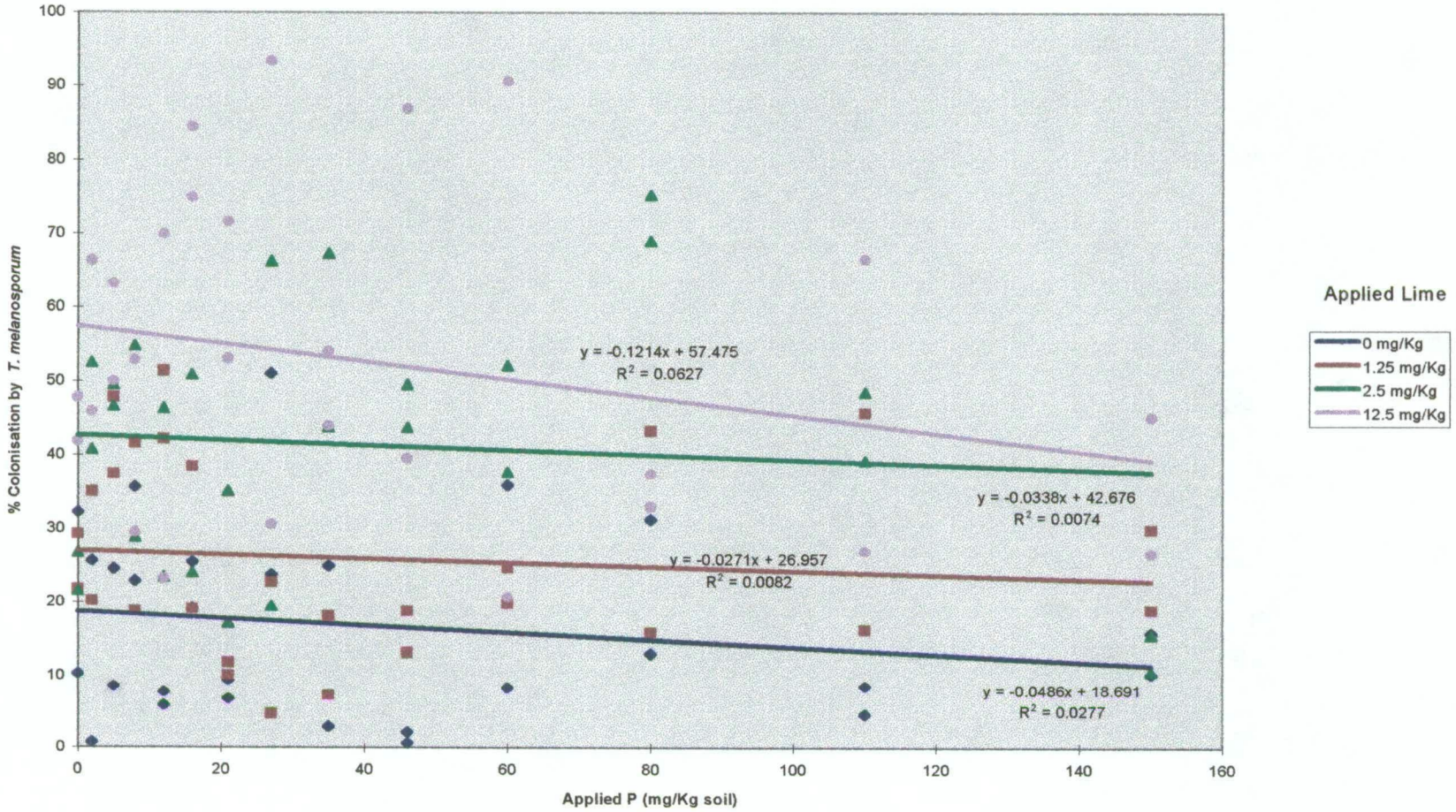


Figure 7.2. The effect of applied P on the level of colonisation of *Tuber melanosporum* at four levels of applied lime.



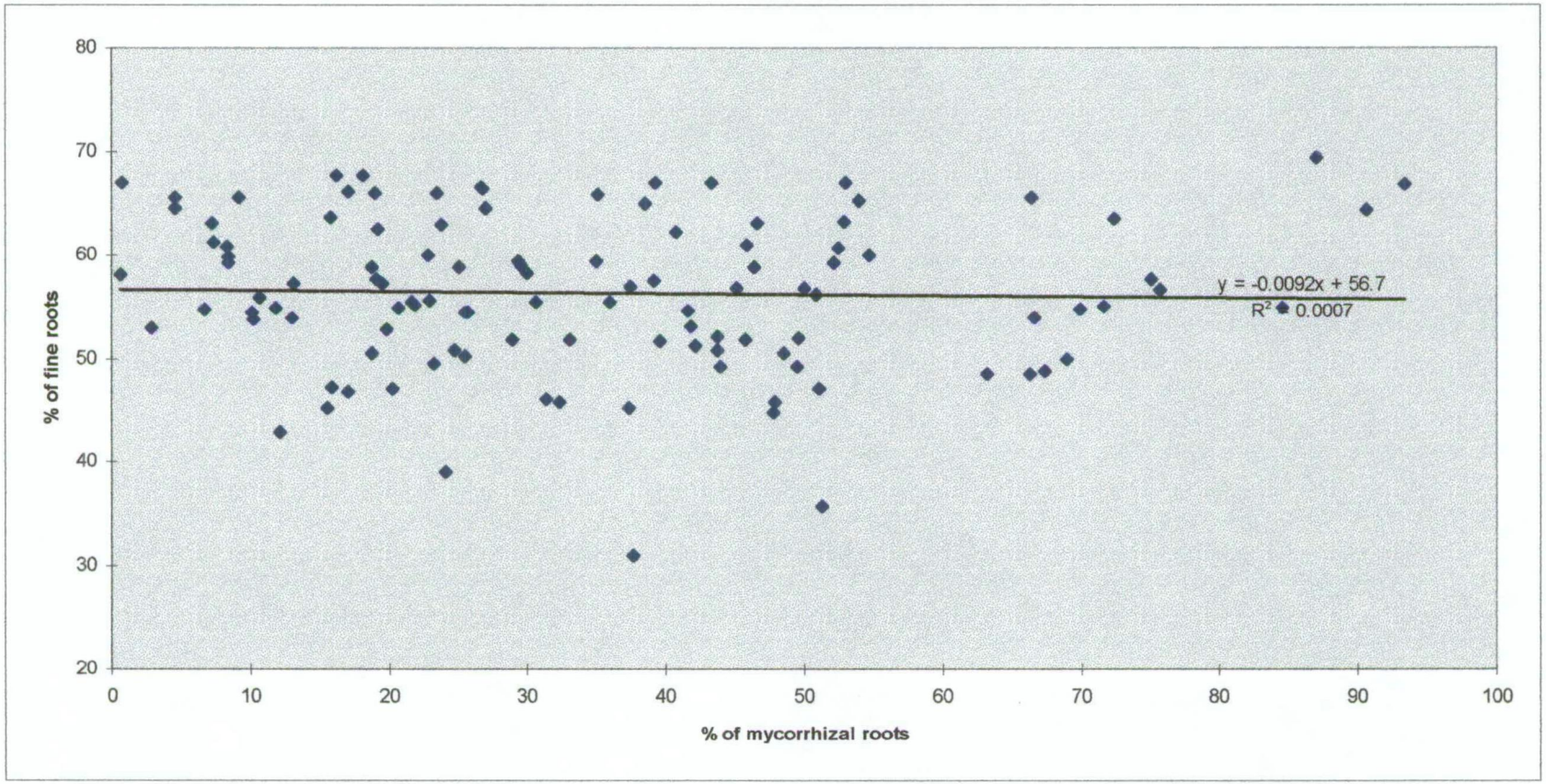
7.3.2 The Effect of Lime and Phosphorus on Colonisation by *Descomyces albellus* and *Hydnangium carneum*

D. albellus was present in only one pot which had been treated with 5 mg/Kg of P and no lime. The level of colonisation was extremely low (1%). Similarly, *H. carneum* appeared in only one pot which had been treated with 8 mg/Kg of applied P and no lime. Once again, the level of mycorrhization was very low (3.5%).

7.3.3 The Percentage of Fine Roots

There was no significant ($P>0.05$) correlation between the total level of mycorrhizal colonisation and the percentage of fine roots (ratio of fine roots to coarse roots) as is illustrated in Figure 7.3.

Figure 7.3. The regression of percentage of mycorrhizal roots on the percentage of fine roots.



7.4 Discussion

7.4.1 The Response of *T. melanosporum* to Applied Lime

The response of *T. melanosporum* colonisation to applied lime was very similar to that observed in Chapter 6. The levels of variation in *T. melanosporum* colonisation explained by applied lime also corresponded closely to that observed in Chapter 6 (root mean square residual equals 16.7 verses 17.4).

7.4.2 The Response of *T. melanosporum* to Applied Phosphorus

There were no significant effects of applied or available P on *T. melanosporum* colonisation at any of the four levels of applied lime. It is possible that the high levels of variation may have masked a possible treatment effect. However, it would appear that *T. melanosporum* is unresponsive to applied P up to quite high levels of application (150 mg/kg soil).

7.4.3 The Percentage of Fine Roots

There was no relationship between percentage of mycorrhizal roots and the percentage of fine roots in this experiment. This is a similar result to that observed in Chapter 6, but contrast with Chapter 5 where there was a positive correlation. A possible explanation for these differences has been given in Section 6.4.5.

7.4.4 The Response of Australian Native Fungi to Applied Phosphorus

The poor establishment of the native species *D. albellus* and *H. carneum* in this experiment precludes any conclusions as to their response to P. However, comparisons can be made between the results for *T. melanosporum* from this experiment and previous work on native species. As discussed in the introduction, Bougher *et al.* (1990) and Brundrett *et al.* (1996) reported maximum levels of mycorrhizal colonisation of Australian native ectomycorrhizal fungi at levels of

applied P of less than 4 to 6 mg/kg soil. As P application rates increased, the level of colonisation of the native fungi declined rapidly. If these results are representative for majority of Australian native ectomycorrhizal fungi, then there is a considerable difference between the response of *T. melanosporum* and native fungi to applied P.

If native ectomycorrhizal fungi are found to compete with *T. melanosporum* in a Tasmanian truffière, it would be worthwhile to conduct further field based experiments to determine if applying P could decrease competition from the native fungi while not reducing *T. melanosporum* colonisation or truffle yields.

The poor establishment of *D. albellus* and *H. carneum* in Chapters 6 and 7 may have been a function of the staggered inoculation. While the level of colonisation of *T. melanosporum* was quite low at the time of inoculation of the native species, it is possible that the presence of *T. melanosporum* may have been sufficient to inhibit primary infection by *D. albellus* and *H. carneum*. To verify this would require a similar experiment including another fungal treatment where *D. albellus* and *H. carneum* were inoculated in the absence of *T. melanosporum*.

8 Survey of Ectomycorrhizal Fungi in a Tasmanian Truffière.

8.1 Introduction

Of the Australian endemic ectomycorrhizal fungi included in the glasshouse experiments of Chapters 5,6 & 7, most were unable to colonise hazel at high levels of lime application with the exception of *D. albus* which showed sporadic colonisation. These findings support the hypothesis that most Australian endemic ectomycorrhizal fungi are adapted to acidic soils and are unlikely to be strong competitors to *T. melanosporum* colonising hazel on calcareous soils.

To further test this theory, a survey was conducted of the first truffière established by Périgord Truffles of Tasmania. As it is the oldest truffière, it has had the longest time with which to become contaminated by other ectomycorrhizal fungi. The truffière was planted at Bream Creek (Map Reference 8412:PROSSER EN659591) in October, 1993, and at the time of the survey was about 2.5 years old. While the truffière has only been recently established, it is expected that the hazel trees have been exposed to spores of endemic ectomycorrhizal fungi by the following mechanisms:

- The truffière is surrounded by a mesh fence, but small animals could still disperse spores on the truffière.
- The truffière is irrigated from a nearby dam, the water from which would be expected to contain spores from a range of endemic ectomycorrhizal fungal species.
- A small cluster of *Eucalyptus*, close to the southern edge of the truffière and other nearby *Eucalyptus* would host epigeal ectomycorrhizal fungi whose spores could be readily blown onto the truffière.

The objectives of the survey were:

- To determine the number of species contaminating the truffière and the extent of contamination.
- To identify the major contaminants where possible.
- To determine the level of colonisation by *T. melanosporum*.

8.2 Materials and Methods

The site is an ex-dairy pasture with an easterly aspect. The soil, a podzolic on sandstone, had a pH (1:5 H_2O) of 6.3. Before planting the truffière, 30 tonnes of limestone were applied to increase the pH to 7.85. A soil test for the site taken after the initial lime application is shown in Table 8.1.

Table 8.1. Chemical analysis of the soil at the Bream Creek truffière.

Soil Property	Level
pH (1:5 H_2O)	7.85
Conductivity (dS/m)	0.073
Available P (mg/kg) Colwell test	19
Exchangeable K (me/100g)	0.48
Exchangeable Mg (me/100g)	2.22
Exchangeable Ca (me/100g)	7.25
Total Ca (mg/kg)	3688
Organic Carbon (g/kg)	34
Organic Matter (g/kg)	58.6
Total Nitrogen (g/kg)	3
Carbon/Nitrogen	11.33

Exchangeable bases were extracted using NH_4Cl.

The survey was conducted in May 1996. Sixty trees, randomly selected from within the truffière, were surveyed. A single root sample was taken using a stainless steel corer 10 cm in diameter and 20 cm long. The core was taken 30 cm

from the base of the tree. Roots were removed from the soil cores by placing the cores over a 1 mm sieve and gently washing the soil through the sieve, leaving the roots behind. The various mycorrhizal types were identified by examination under stereo and compound light microscopes. The identities of *T. melanosporum* and *T. brumale* were later confirmed using PCR and RFLP as described in Chapter 9. The lengths of each root type and calculation of root parameters were determined as per Section 5.2.7.1.

The height and basal diameter of each tree were recorded.

Correlation analysis was used to investigate possible relationships between tree height and basal diameter and the level of colonisation of each mycorrhizal type.

8.3 Results

8.3.1 Level of Colonisation by *Tuber melanosporum* and Contaminants

The level of mycorrhizal colonisation by *T. melanosporum* and the major contaminant, *T. brumale* are shown in Table 8.2. *T. melanosporum* had colonised all the trees sampled in the survey, and the level of mycorrhizal colonisation was high, averaging 70% colonisation of the fine root system. *T. brumale* was present in the sample of 15% of the trees. These trees were evenly distributed over the area surveyed. For those trees contaminated with *T. brumale*, the mean level of colonisation by *T. brumale* was 13%.

Six mycorrhizal tips of an unidentified basidiomycete were found in one core sample. No other contaminants were found.

There was no correlation between the level of colonisation by *T. melanosporum* or *T. brumale* and the tree height or basal diameter ($P>0.05$).

Table 8.2. The level of mycorrhizal colonisation by *Tuber melanosporum* and *Tuber brumale.*

Parameter	*T. melanosporum*	*T. brumale*
Percentage of trees colonised	100	15
Maximum level of colonisation (%)	100	39
Minimum level of colonisation (%)	5	0
Mean level of colonisation across all trees sampled (%)	70 (S.D.=22)	3 (S.D.=9)
Mean level of colonisation for those trees contaminated by *T. brumale* (%)	65 (S.D.=23)	13 (S.D.=14)

S.D. = Standard Deviation

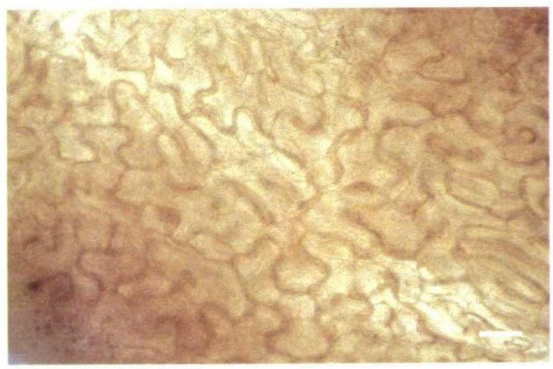
Hyphae. The emanating hyphae is simple or branched, tortuous, thin walled, faint pale yellow or hyaline, and about 4.5 μm in diameter.

Plate 8.1. *Tuber brumale* mycorrhizas.

1. Mycorrhizas covered with cystidia. One mycorrhiza is unusual, exhibiting a tuft of hyphae emanating from the tip.
2. Mantle surface with interlocking lobes.
3. Surface of mycorrhiza showing the density of the cystidia.
4. Robust cystidia with well rounded tip and wide base.
5. Cystidia showing pale yellow walls.

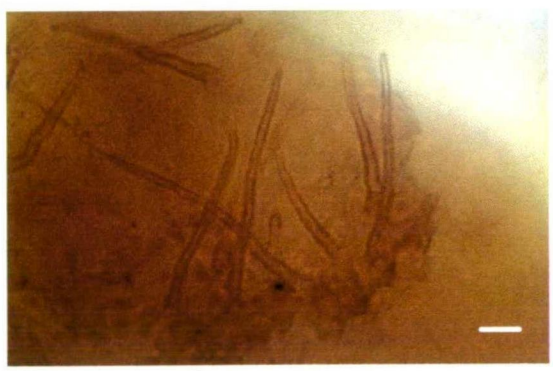
Plate 8.1. *Tuber brumale* mycorrhizas.

1 (bar = 100 µm)

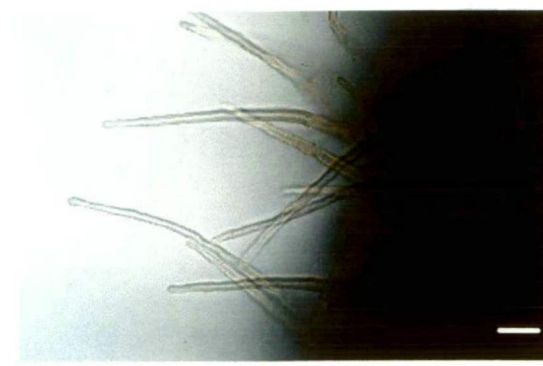


2 (bar = 10 µm)

3 (bar = 50 µm)



4 (bar = 10 µm)



5 (bar = 10 µm)

8.4 Discussion

The strong level of mycorrhizal colonisation by *T. melanosporum* in the field confirms the findings of the glasshouse experiments that *T. melanosporum* will readily colonise hazel on soils of non-calcareous origin provided adequate lime is applied. At this early stage of development of the industry, such consistently high levels of colonisation by *T. melanosporum* is a very positive outcome.

Both the number of contaminant species, and the levels of contamination were very low. By comparison, Chevalier *et al.* (1982) noted that young truffières in France (2 to 7 years) were often contaminated by several species, and the levels of contamination were moderate to high. Values for the level of contamination were not given.

That *T. brumale* was the only significant contaminant lends weight to the argument that competition to *T. melanosporum* in Tasmanian truffières is likely to come from introduced or cosmopolitan ectomycorrhizal fungi rather than endemic fungi. This emphasises the need to prevent contamination from introduced species by measures such as attention to inoculation procedures (surface sterilising sporocarps) and subsequent rearing of inoculated trees in the nursery, and careful paddock selection.

In a survey of French truffières conducted by Chevalier *et al.* (1982), most contamination occurred from other *Tuber* species, particularly *T. brumale* which was widespread and abundant under hazel. Chevalier *et al.* (1982) also notes a personal communication from L. Riousset that hazel has a propensity to host *T. brumale*. While the fruit body of *T. brumale* is edible, its value is considerably less than that of *T. melanosporum*.

Several papers have reported studies of the ecological preferences of various *Tuber* species and competition between *T. melanosporum* and other *Tuber* species (Bencivenga and Granetti, 1988; Chevalier and Frochot, 1990; Granetti and

Angelini, 1992; Mamoun and Olivier, 1993a; Mamoun and Olivier, 1993b). These papers indicate that there are opportunities to manipulate environmental conditions such as the soil moisture content to favour the development of *T. melanosporum* over other *Tuber* species (Mamoun and Olivier, 1993a; Mamoun and Olivier, 1993b).

Further surveys at the Bream Creek site are required to determine whether the levels of contamination of *T. brumale* and other contaminants increase or decrease, and at what rate. To obtain a greater appreciation of the nature of competition within Tasmanian truffières, a number of truffières need to be surveyed over an extended period of time.

9 Identification of *Tuber* Mycorrhizas Using DNA Fingerprinting

9.1 Introduction

The mycorrhizas of different *Tuber* species can be difficult to identify morphologically. This is particularly the case when characteristic features such as cystidia are absent. Polymerase chain reaction (PCR) combined with restriction fragment length polymorphism (RFLP) has been successfully developed to identify *Tuber* species from their mycorrhizas (Amicucci *et al.*, 1996; Henrion *et al.*, 1994; Lanfranco *et al.*, 1995; Mello *et al.*, 1996).

The main objective of this work was to confirm the morphological identification of *Tuber* mycorrhizas in all glasshouse and field experiments using PCR and RFLP of DNA extracted from single mycorrhizal tips. It is important that identification can be made from a single tip. In the work by Mello *et al.* (1996), 15 to 20 tips were used for the extraction. It is quite possible that one or more of these tips may have been a different species to the remainder and was not identified. This is particularly the case with *Tuber* as the PCR products (when using the primer pairs ITS1 and ITS4) are approximately the same length for most *Tuber* species, with the exception of *Tuber brumale*. RFLP of the PCR product is required for identification. By this stage the bands of the contaminant fungi could be so faint as to be unrecognisable.

Another objective was to simplify the DNA extraction process. The extraction process used by Henrion *et al.* (1994), Lanfranco *et al.* (1995), Mello *et al.* (1996) and Amicucci *et al.* (1996) was based on the method developed by Henrion *et al.* (1992). Any simplification of this method will increase the likelihood of its adoption to identify fungal species in commercial truffières and nurseries.

9.2 Materials and Methods

9.2.1 General

Mycorrhizas morphologically identified as *T. melanosporum* were collected from hazels in the glasshouse experiments described in Chapters 5,6 and 7 and from hazels in the truffière at Bream Creek, Tasmania (Chapter 8). DNA extracted from these mycorrhizas was compared using PCR and RFLP to DNA from a pure culture isolate of *T. melanosporum* (Mel 24) supplied by Gérard Chevalier (Unité de Mycologie, Institut National de la Recherche Agronomique, Clermont-Ferrand, France). Similarly, DNA was extracted from mycorrhizas found at the Bream Creek site morphologically identified as *T. brumale*. This DNA was compared with DNA extracted from a pure culture isolate of *T. brumale* (Mos Pey) also supplied by Gérard Chevalier.

9.2.2 DNA Extraction

The methods for DNA extraction were modifications of procedures used by Shane Herbert at Murdoch University, WA. The procedures developed by Shane Herbert have not been published.

DNA extraction from mycorrhizal roots

Extraction was carried out both on fresh tips and tips stored in 100% ethanol. A single mycorrhizal tip was placed in a 1.5 ml microcentrifuge tube with 30 µl of SDS extraction buffer (0.5% Sodium Dodecyl Sulfate, 100 mM tris HCl pH 7.8, 50 mM EDTA, 500 mM NaCl and 0.2% β-mercaptoethanol). The sample was frozen in liquid nitrogen then thawed in a water bath at 65°C. This freezing thawing process was repeated three times before the tip was ground with a plastic pestle in the same microcentrifuge tube. Cellular debris was precipitated with the addition of 15 µl of 3M sodium acetate (pH 5.5) and left stand on ice for 10 minutes. After centrifuging for 10 mins at 13000 rpm the supernatant was pipetted into a clean tube. The DNA was precipitated with 0.7 volumes of ice-cold isopropanol and left on the bench for 10 minutes before centrifuging and pouring

off the supernatant. The sample was then placed in a vacuum drier (Heto Lab Equipment DNA Mini) to remove the remaining moisture around the pellet. The pellet was resuspended in 10 µl of sterile distilled water and let stand for 20 minutes before storing on ice or at -20°C. The quantity of DNA extracted could not be reliably quantified using a fluorometer. The final suspension was diluted 10 times and 100 times and 1 µl of each dilution used in the PCR reaction.

DNA was extracted from non-mycorrhizal hazel roots using the same method as for mycorrhizal roots.

DNA extraction from pure cultures

Tuber melanosporum and *T. brumale* were grown on malt extract media as described in Appendix 3. Approximately 400 mg of sterile fungal tissue and agar were ground in a mortar with liquid nitrogen to form a fine powder. Further grinding with 3 ml of SDS extraction buffer produced a slurry which was transferred to microcentrifuge tubes and incubated at 60°C for 10 minutes. Cellular debris was precipitated with the addition of 1.5 ml of 3M sodium acetate (pH 5.5) and placed at -20°C for 10 minutes. After centrifuging for 10 mins at 13000 rpm the supernatant was pipetted into a clean tube. Samples were then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and the upper aqueous layer transferred to new tubes. The DNA was precipitated with 0.7 volumes of ice-cold isopropanol and left on the bench for 10 minutes before centrifuging and pouring off the supernatant. The pellet was washed in ice cold 70% ethanol. The remaining ethanol was aspirated before resuspending the pellet in 20 µl of sterile distilled water and storing at -20°C. The quantity of DNA was determined using a fluorometer (Hoefer Scientific Instruments TKO 100).

9.2.3 PCR amplification of the ITS region

The primer pairs ITS1 and ITS4 (Bresatec Pty Ltd) used to amplify the internal transcribed spacer (5' to 3' sequence: TCCGTAGGTGAACCTGCGG and TCCTCCGCTTATTGATATGC, respectively) have been described by White *et al.* (1990). Amplification reactions were carried out in a final volume of 25 µl

consisting of 10 mM Tris-HCl (pH 9.0 at room temp.), 50 mM KCl, 2 mM $MgCl_2$, 10 pmol of each primer, 1 unit of Pharmacia Biotech *Taq* DNA Polymerase, 120 μM each of dATP, dCTP, dTTP and dGTP, 5% glycerol, 2.5 μg Bovine Serum Albumin (BSA), and either 0.1 ng to 1 ng of DNA template extracted from the pure cultures or 1 μl of each DNA dilution (10X & 100X) for mycorrhizal and non-mycorrhizal roots. Control tubes with no DNA were included for each series of reactions. Charged tubes were then place in a Corbett Research FTS-960 Thermal Sequencer. Thermal cycling parameters were as described by Henrion *et al.* (1994), that is, initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 2 minutes, annealing at 50°C for 25 s, and extension at 72°C for 10 minutes with a final extension at 72°C for 10 minutes.

9.2.4 RFLP of Amplified DNA

RFLP reactions were carried out in a final volume of 20 μl consisting of 10 μl of PCR product, 2 μl of buffer and 5 units of Hinf I (New England Biolabs). The restriction digest reactions were run for 1.5 hours at 37°C.

PCR and RFLP products were size-fractionated on 1.5% agarose gels stained with ethidium bromide.

9.3 Results and Discussion

ITS amplified fragments from the pure culture of *T. melanosporum* were about 620 bp (Lane 1 for Fig. 9.1, 9.3 and 9.5). As most *Tuber* species, with the exception of *T. brumale*, occur in the range 600 to 680 bp (Henrion *et al.*, 1994), it is necessary to use RFLP of the ITS products to identify species (Amicucci *et al.*, 1996; Henrion *et al.*, 1994; Mello *et al.*, 1996). When digested with Hinf I, the PCR products of *T. melanosporum* mycelium are cut in half to give overlapping bands at about 300 bp (Lane 1 for Fig. 9.2, 9.4, and 9.6).

T. melanosporum / hazel mycorrhizas showed bands corresponding to *T. melanosporum* mycelium for both the PCR reactions (Lane 2 for Fig. 9.1, 9.3 and 9.5) and RFLP (Lane 2 for Fig. 9.2, 9.4, and 9.6). These bands representing the fungal fraction of the mycorrhiza were much more prominent than those of the plant fraction (hazel). The PCR product for hazel was approximately 720 bp (Root tip - Lane 3 for Fig. 9.1, 9.3 Lane 5 for Fig. 9.5; Mycorrhizas - Lane 2 for Fig. 9.1, 9.3 and Lanes 2 & 4 for Figure 9.5). After digesting with Hinf I, two bands were visible at approximately 270 bp and 180 bp (very faint on the photos).

The amplified ITS for *T. brumale* mycelium was approximately 970 bp (Lane 3, Fig. 9.5). When digested with Hinf I, two of the resulting fragments were visible at about 300 bp and 430 bp (Lane 3, Fig. 9.6). *T. brumale* / hazel mycorrhizas (Lane 4, Figs. 9.5 & 9.6) showed bands corresponding to both organisms. Once again, the bands for hazel were faint relative to those for the fungus.

The results reflect previous studies by Henrion *et al.* (1994) and Mello *et al.* (1996). They confirm the morphological identification of *T. melanosporum* in the glasshouse experiments (Chapters 5,6 & 7) and *T. melanosporum* and *T. brumale* at the Bream Creek truffière (Chapter 8). The procedure used for DNA extraction and amplification of *Tuber* / hazel mycorrhizas represents a significant simplification and time saving over the method used by Henrion *et al.* (1994).

Even with the time savings made using this DNA extraction technique, DNA fingerprinting of mycorrhizas is still a very laborious and expensive means of quantitatively identifying mycorrhizas. Its main use in population studies would be to assist in the initial identification of a mycorrhizal type, and to test the accuracy of subsequent morphological identifications.

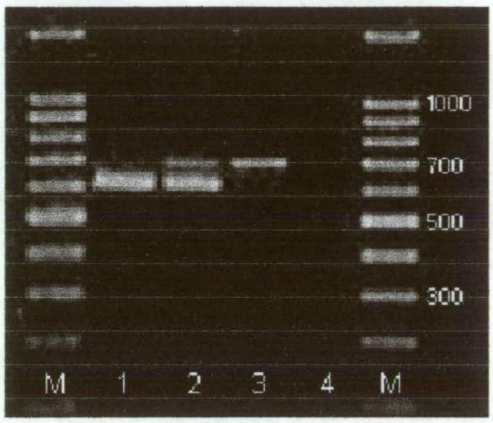


Figure 9.1. Amplification products with primers ITS1 and ITS4 of DNA extracted from the following: Lane 1, pure culture of *Tuber melanosporum*; Lane 2; mycorrhiza of *T. melanosporum*/hazel from the Perth glasshouse experiment described in Section 5.3.5; Lane 3, non-mycorrhizal hazel root tip; Lane 4, control; Lane M, marker (100 bp).

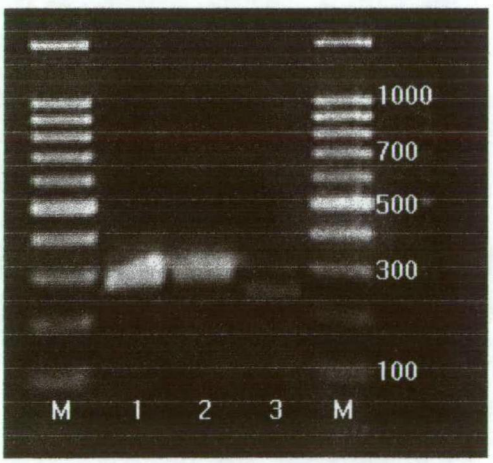


Figure 9.2. Digestion of ITS amplification products with Hinf I from the following: Lane 1, pure culture of *Tuber melanosporum*; Lane 2; mycorrhiza of *T. melanosporum*/hazel from the Perth glasshouse experiment described in Section 5.3.5; Lane 3, non-mycorrhizal hazel root tip; Lane M, marker (100 bp).

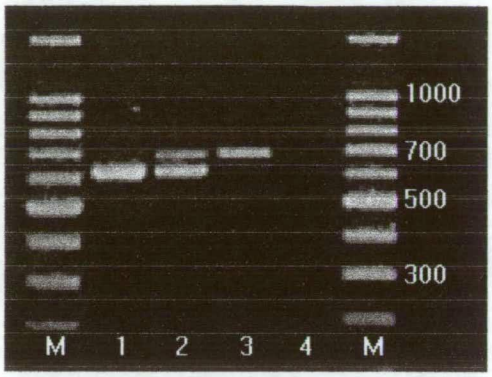


Figure 9.3. Amplification products with primers ITS1 and ITS4 of DNA extracted from the following: Lane 1, pure culture of *Tuber melanosporum*; Lane 2; mycorrhiza of *T. melanosporum*/hazel from the Hobart glasshouse experiments (Chapters 6 & 7); Lane 3, non-mycorrhizal hazel root tip; Lane 4, control; Lane M, marker (100 bp).

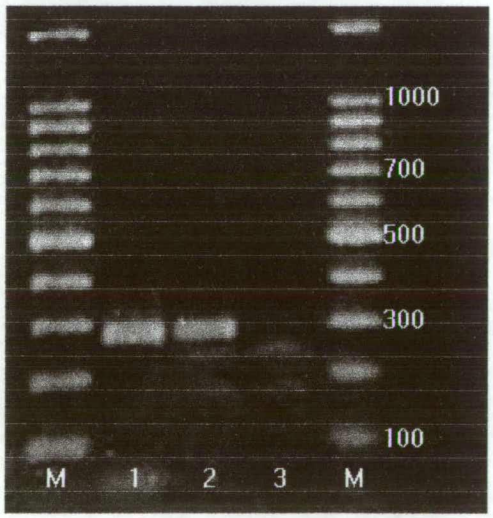


Figure 9.4. Digestion of ITS amplification products with Hinf I from the following: Lane 1, pure culture of *Tuber melanosporum*; Lane 2; mycorrhiza of *T. melanosporum*/hazel from the Hobart glasshouse experiments (Chapters 6 & 7); Lane 3, non-mycorrhizal hazel root tip; Lane M, marker (100 bp).

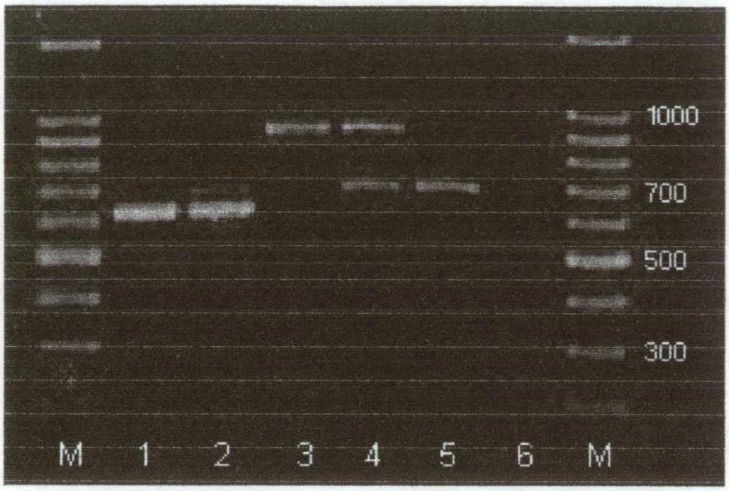


Figure 9.5. Amplification products with primers ITS1 and ITS4 of DNA extracted from the following: Lane 1, pure culture of *Tuber melanosporum*; Lane 2; mycorrhiza of *T. melanosporum*/hazel from Bream Creek; Lane 3, pure culture of *T. brumale*; Lane 4, mycorrhiza of *T. brumale*/hazel from Bream Creek; Lane 5, non-mycorrhizal hazel root tip; Lane 6, control; Lane M, marker (100 bp).

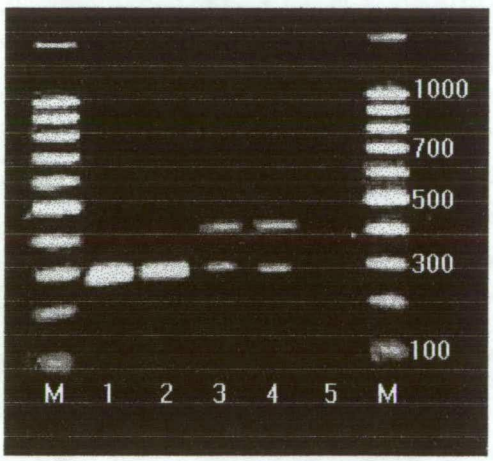


Figure 9.6. Digestion of ITS amplification products with Hinf I from the following: Lane 1, pure culture of *Tuber melanosporum*; Lane 2; mycorrhiza of *T. melanosporum*/hazel from Bream Creek; Lane 3, pure culture of *T. brumale*; Lane 4, mycorrhiza of *T. brumale*/hazel from Bream Ck; Lane 5, non-mycorrhizal hazel root tip; Lane M, marker (100 bp).

General Discussion and Future Research Perspectives

Establishment of a truffle industry in Tasmania involves transplanting *Tuber melanosporum*, and a suitable host, into a foreign environment on the other side of the world. Such a venture entails both opportunity and uncertainty.

There exists the opportunity to provide Périgord black truffles to the world at a time when there are no other fresh black truffles on the market with the exception of those being cultivated in New Zealand. Prices are expected to be higher than during the European harvest due to the limited supply. Also, *T. melanosporum*, like so many other species of plant and animal introduced to Australia, may find itself in an environment with less competition than in its region of origin. Without its natural competitors *T. melanosporum* may be more productive in Tasmania. Hence, there is the potential for higher yields. The combination of yield and price has the potential to produce a very lucrative industry.

The other side of the ledger is the uncertainty and subsequent risk of introducing *T. melanosporum* into a foreign environment. There may be unforeseen factors that limit or negate the viability of the Tasmanian truffle industry. The areas of greatest concern are the lack of knowledge about the edaphic requirements of *T. melanosporum* and possible competition from Australian native ectomycorrhizal fungi.

The objective of this thesis was to reduce some of the uncertainty about truffle production in Tasmania by providing more information concerning the host specificity of Australian endemic ectomycorrhizal fungi and the edaphic requirements of both *T. melanosporum* and potential competitor fungi.

The initial aim of the thesis was to test the assumption that Australian endemic ectomycorrhizal fungi are *Eucalyptus* specific and therefore unable to compete with *T. melanosporum* whilst colonising hazel trees. A survey of established hazels in Tasmania indicated that several endemic ectomycorrhizal fungi species were colonising hazel. This was confirmed in subsequent glasshouse experiments. Further research is required to determine the boundaries of host specificity of Australian native ectomycorrhizal fungi, though this research is not a priority for the Tasmanian truffle industry. The reasons for this are explained below.

The next stage of the project was to investigate the potential competitiveness of endemic ectomycorrhizal fungi to *T. melanosporum*. It was considered that native fungi, which are accustomed to the acidic nature of most Tasmanian soils, may not be able to adapt to the large quantities of lime being applied to Tasmanian truffières. In controlled glasshouse experiments, native ectomycorrhizal fungi colonising hazel were generally adversely affected by the application of large quantities of lime to soils with an acid pH. This finding was supported by the fact that native fungi were not detected in a survey of Tasmania's first truffière. The exception to the rule was a *Descomyces* species which sporadically colonised hazel growing in soils treated with high rates of lime. However, in these isolated cases, the level of root colonisation was very low.

While the results indicate that some endemic ectomycorrhizal fungi cannot adapt to the combined change in host and soil conditions, the Tasmanian truffle industry should continue to monitor truffières across the state for contamination. Other species of endemic ectomycorrhizal fungi not tested in this thesis may be able to colonise hazel under calcareous soil conditions. Surveying the truffières is the most practical way to respond to this threat. In addition, surveying will increase understanding of how well *T. melanosporum* has colonised the truffière and whether there has been contamination from introduced or cosmopolitan species of mycorrhizal fungi. Further glasshouse experiments to determine which endemic fungi will form mycorrhizal associations with hazel, and how they respond to applied lime are not warranted. They are expensive to conduct, and can only test a limited number of species.

In the event that endemic ectomycorrhizal fungi do become significant competitors, application of P to the truffières should be investigated as a possible method of control. The results of Chapter 7 suggest that mycorrhizal colonisation by *T. melanosporum* is tolerant to moderately high rates of phosphorus application (150 mg/kg soil). Previous studies on endemic species demonstrated a rapid decline in the percentage of roots colonised at very low levels of applied P (greater than 4 to 6 mg/kg soil) (Bougher *et al.* 1990, Brundrett *et al.* 1996). There would appear to be the opportunity to use P application to decrease the level of colonisation of endemic species while not adversely affecting colonisation by *T. melanosporum*.

There are two important aspects which must be considered before P application could be used to reduce competition from endemic ectomycorrhizal fungi. Firstly, while moderate levels of applied P do not appear to decrease colonisation of *T. melanosporum*, its effect on sporocarp production is not fully understood. Secondly, applying P to truffières with low levels of soil P may result in increased growth rates of the trees and subsequent increases in growth rate of the root system. Chevalier and Poitou (1989) noted that if the root system of the host plant grows too quickly, *T. melanosporum* cannot keep pace with the rate of root growth. This leaves the outer edge of the root system uncolonised by *T. melanosporum* and open to colonisation by contaminants.

The phenomenon of the root system growing faster than *T. melanosporum* has not been observed in Tasmania. Even in the summer when truffières are being irrigated and the root system is actively growing, *T. melanosporum* rapidly colonises the fine roots as they form at the edge of the root system. The fact that *T. melanosporum* appears to be able to colonise actively growing root systems and not be displaced by competitors may be due to a lack of competitors in the Tasmanian environment.

The growth rate of the tree and its effect on root colonisation and fruiting of *T. melanosporum* is an area that warrants further research. Shaw *et al.* (1996)

found that stem diameter was a critical factor determining fruit body production of *T. melanosporum* in symbiosis with *Quercus ilex*. This suggests that host plants may need to reach a critical biomass before being able to provide adequate carbohydrate to the fungus for fruiting. The observation that *T. melanosporum* tends to fruit earlier when associated with hazel may be a function of tree growth rate. Hazels grow quickly, and the early fruiting reported under hazel may be attributed to their ability to reach this critical biomass at a younger age than other host species.

Research is required to determine whether increased growth rate of trees through plant nutrition can be used to induce early sporocarp production. Such experiments would entail monitoring such factors as; colonisation of the root system by *T. melanosporum*, competition from other ectomycorrhizal fungi, the effect of plant fertilizers on soil parameters that are considered to be important to truffle production such as the C/N ratio, and fruiting of *T. melanosporum*.

Competition from introduced and cosmopolitan species of ectomycorrhizal fungi is likely to present a greater risk to the Tasmanian truffle industry than competition from endemic species. *Tuber brumale* has become established in at least one truffière and is the only competitor identified in Tasmanian truffières to date. Other species have the potential to be a problem. In these studies, *Hebeloma crustiliniforme, Laccaria tortilla* and *Melanogaster ambiguus* were able to colonise hazel in soil treated with high rates of lime, and largely prevented colonisation by *T. melanosporum*. Other exotic fungi reported in Tasmania such as *Boletus luridus* and *Scleroderma verrucosum* also have the potential to contaminate the truffières. Furthermore, it is likely that there are other known antagonistic ectomycorrhizal fungi that exist in Tasmania but have not been reported.

Should a truffière become infected with a problem ectomycorrhizal fungus such as has occurred at Bream Creek, efforts should be made to isolate the contaminant within that truffière. People moving from one truffière to the next are at risk of spreading the contaminant. Footwear, vehicle tyres and equipment should be

cleaned and disinfected before moving to the next truffière. Owners of a contaminated truffière wishing to expand their area of trufficulture should be advised to consider a new site rather than expanding the existing site.

Having said this, it may be unreasonable to expect that fungal diversity within the truffières can be restricted to one species. In natural ecosystems it is normal for a wide diversity of ectomycorrhizal fungi to colonise the roots of a single host tree (Mason *et al.* 1987). However, Chevalier *et al.* (1982) have found that some trees in plantations were abundantly colonised by *T. melanosporum* to the exclusion of all other mycorrhizal fungi. In their study of fungal diversity in French truffières, Chevalier *et al.* (1982) found the number of mycorrhizal fungi (including *T. melanosporum*) varied from 1 to 8 per tree and the degree of contamination increased with the age of the trees.

Furthermore, the assumption that the presence of other ectomycorrhizal fungi in a truffière is detrimental may not always be correct. It is possible that some ectomycorrhizal fungi can co-exist with *T. melanosporum* without influencing production or even be of benefit to *T. melanosporum*. For example, Sourzat *et al.* (1993) reported that a *Scleroderma* sp. was prevalent at almost all their sites of experimentation, even those sites with good truffle production. The *Scleroderma* sp. was generally observed at the edge of the brûlé where the soil was reportedly richer in organic matter. Sourzat *et al.* proposed that *Scleroderma* may have a useful role in altering the soil to a state that is more favourable for the growth of *T. melanosporum*.

In another experiment, Mamoun and Olivier (1993) found that competition between *T. melanosporum* and *T. brumale* could be influenced by the rate of irrigation. Under moderate irrigation, *T. melanosporum* colonisation declined in the presence of *T. brumale*. However, the presence of *T. brumale* at high rates of irrigation appeared to increase the competitiveness of *T. melanosporum*.

Another area for consideration is how to measure the effects of competition between species of ectomycorrhizal fungi. It has been shown in field studies

(Gardes and Bruns, 1996; Taylor and Alexander, 1989; Vogt *et al.*, 1992) that in the short term, there appears to be little correspondence between fruit body production and the population structure of mycorrhizal fungi measured in terms of mycorrhizal colonisation. For example, Taylor and Alexander (1989) found that those species which produced the most sporocarps, each formed less than 5% of the mycorrhizal types examined. However, within species, positive correlations have been reported between the frequency of fruiting and the level of colonisation (Termorshuizen and Schaffers, 1989). Positive correlations between the frequency of fruiting and the level of colonisation were also observed for *H. crustuliniforme and L. tortilla* in Chapter 5.

Gardes and Bruns (1996) postulated that those fungi that are able to fruit prolifically from a comparatively short length of mycorrhizal root may be particularly efficient in the transfer of carbon from the roots to the fruit bodies. Similarly, it was observed in Chapter 5 that *Melanogaster ambiguus* was able to produce a mass of mycelium from very few mycorrhizas. It seems unlikely that the carbon required to maintain such an extensive mycelial network could have been supplemented by saprotrophic sources as the soil used in the experiment was extremely low in organic matter (0.14%).

For the Périgord black truffle industry, the competitiveness of other ectomycorrhizal fungi can be considered as their ability to reduce the yield of *T. melanosporum* fruitbodies. There are a number of ways that this may occur and many of them are interrelated. They would include:

- competing with *T. melanosporum* for carbohydrate from the host,
- competing for positions on the root system on which to form a mycorrhizal relationship with the host,
- competing for soil nutrients and soil water, and
- allelopathic effects.

Fungal diversity, spatial variation, succession, and competition between ectomycorrhizal fungal species within truffières are complex fields of study that have so far received little attention. Further research in these areas will no doubt bring about advances in the management of truffières.

While native ectomycorrhizal fungi were generally adversely affected by applied lime, *Tuber melanosporum* showed strong colonisation of hazel in soils of non-calcareous origin provided adequate lime was applied. *Tuber melanosporum* was able to colonise hazel growing in acidic soil, but the levels of colonisation were very low. The benefit of applied lime appears to be largely a function of increased pH, although applied calcium may also have an additional positive effect or a positive interaction with pH. Maximum root colonisation appears to coincide with the maximum soil pH obtainable by applying lime. This point occurs when the soil is saturated with calcium, and dissociation of $CaCO_3$ discontinues. Applying additional lime increases the reserve of undissociated lime within the soil. This reserve is useful in that it is likely to buffer the soil against pH decline bought about by leaching of calcium from the soil profile.

One of the most interesting aspects of the results was the magnitude of variation observed in the colonisation of hazel by *T. melanosporum* under controlled glasshouse conditions. The variation in colonisation by *T. melanosporum*, which could not be accounted for by the treatments, was most easily recognised in Chapters 6 and 7. In these experiments, all the replicates were colonised by *T. melanosporum*, but the soil treatment effects only explained about one third of the variation in colonisation.

The observed variation is important for two reasons.

1. It reveals the high level of root colonisation which can be achieved by *T. melanosporum*. If it is possible to explain why one plant might have 80% of its fine roots colonised while another plant under similar conditions only supports 20% colonisation, then it may be possible to manipulate the system such that all plants support 80% colonisation or higher.

2. In any experimentation, such large variation will reduce the likelihood of obtaining significant treatment effects.

One possible explanation for the variation observed in Chapters 6 and 7 may be variation in soil moisture from plant to plant. The plants from these experiments were watered using overhead sprinklers. The distribution pattern of the sprinklers was tested, but it is quite possible that there were differences between pots in soil moisture content due to shading and variation in water use rates of the plants as a consequence of their different sizes and growth rates.

It has been shown that soil moisture content does affect root colonisation by *T. melanosporum* (Mamoun and Olivier, 1990; Mamoun and Olivier, 1993a). Mamoun and Olivier (1993) demonstrated that in the absence of other mycorrhizal fungi, *T. melanosporum* colonisation was 24% lower at a high rate of irrigation (31% soil moisture) than at a medium rate of irrigation (21% soil moisture), but the difference was not significant ($P>0.05$). Therefore, while variations in soil moisture content may have explained some of the variation in *T. melanosporum* colonisation observed in Chapters 6 and 7, it seems unlikely to be the major contributor.

It is more likely that the variation in colonisation by *T. melanosporum* can be attributed to the heterogeneity of both the plant and fungal material. Variation in the capacity of individual plants to host *T. melanosporum* has been observed by several authors (Boutekrabt *et al.*, 1990; Guinberteau *et al.*, 1990; Mamoun and Olivier, 1996). Mamoun and Olivier (1996) showed that variation in *T. melanosporum* colonisation could be reduced by using cloned hazel plants. Furthermore, the clone that they used in their experiment showed consistently higher colonisation than seedling hazels. Mamoun and Olivier (1996) also suggested that individual plants may vary in their capacity to host different species of ectomycorrhizal fungi thus offering the potential to select clones that show a propensity to host *T. melanosporum* rather than competitor fungi. Strong genetic control of ectomycorrhizal traits has also been observed for different genotypes of

Pinus elliottii var. *elliottii* inoculated with *Pisolithus tinctorius* (Rosado *et al.*, 1994).

The influence of plant genotype on colonisation by *T. melanosporum* provides great potential for selecting individual trees to clone for commercial truffle production. It is also possible that there would be a host/fungus/environment interaction such that eventually, different clones could be used for different environments within Tasmania.

Selecting and cloning superior hosts is an area that should be pursued by the Tasmanian truffle industry. Care should be taken not to excessively reduce the genetic diversity of host plants within a truffière as one or several of the clones may be susceptible to disease or some other deleterious factor. Selection would be initially based on the ability of the tree to support mycorrhizal colonisation by *T. melanosporum*, but once truffles are produced in Tasmania, trees would most likely be selected on the basis of truffle production.

Finally, the correlation between percent root colonisation by *T. melanosporum* and truffle production requires further investigation. It has been assumed that there is a positive correlation, but it has never been tested for *T. melanosporum*. A knowledge of the relationships between root colonisation and truffle production is required to verify the value of experiments which look at treatment effects on root colonisation.

Knowledge of the relationship between truffle production and root colonisation may also prove to be a valuable tool for predicting truffle production. It may be possible to combine root colonisation with other parameters such as, the number of branched mycorrhizas, brûlé formation and stem diameter to develop an equation to predict the potential truffle yield of a tree.

Summary of Outcomes

- Some species of Australian endemic ectomycorrhizal fungi, previously thought to be *Eucalyptus* specific, are forming mycorrhizal relationships with hazel.
- Of the endemic fungi that were forming mycorrhizas with hazel, most were unable to colonise hazel in soils that were heavily limed. This reduces the likelihood of these fungi having a detrimental effect on truffle production in Tasmania.
- *Tuber melanosporum* was able to colonise hazel in an infertile, moderately acidic, structureless Australian soil albeit at very low levels. When lime was applied to the soil the level of *T. melanosporum* colonisation increased significantly. Maximum colonisation corresponds to the maximum pH of the soil obtainable by liming with $CaCO_3$.
- The effect of applied lime on *T. melanosporum* appears to be primarily a function of pH. Calcium may have a secondary role.
- The level of colonisation of hazel by *T. melanosporum* was not significantly affected by high rates of phosphorus application.
- *Tuber melanosporum* exhibited very strong colonisation of hazel in a Tasmanian truffière. The diversity and abundance of other ectomycorrhizal fungi in the truffière was very low.
- Guidelines for the management of Tasmanian truffières have been developed based on the above outcomes.

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Appendices

Appendix 1 Climate statistics for Périgord black truffle producing regions in France and New Zealand, and regions in Tasmania where truffières have been planted

Mean daily maximum temperatures (°C) for Périgord black truffle producing regions in France and New Zealand, and regions in Tasmania where truffières have been planted.

	Gisborne, N.Z.	Avignon, France	Gourdon, France	Montelimar, France	Montpellier, France	Orange, France	Valence, France	Bothwell, Tas.	Deloraine, Tas.	Grove, Tas.	Oatlands, Tas.	Westbury, Tas.
Jan/Jul	24	31	26	29	28	29	29	22.6	21.3	22.2	21.9	23.4
Feb/Aug	24	31	25	28	28	28	28	23.2	22.5	22.3	21.7	23.4
Mar/Sep	22	26	23	24	24	24	24	20.5	19.6	20.3	19.3	21
Apr/Oct	20	20	17	18	20	19	18	17	16.5	17.5	15.9	17.1
May/Nov	17	13	12	12	15	13	12	13.5	13.2	14.4	12.4	13.8
Jun/Dec	14	10	9	8	11	9	8	10.9	10.9	11.9	10.1	10
Jul/Jan	14	9	7	7	11	9	7	10.6	10.4	11.6	9.4	9.7
Aug/Feb	14	12	9	9	12	11	9	11.7	11.4	12.8	10.6	11.9
Sep/Mar	17	15	12	14	15	15	14	13.7	13.3	14.6	12.8	14.5
Oct/Apr	18	19	15	18	18	18	18	16.7	15.5	16.9	15.2	16.9
Nov/May	21	23	19	22	21	22	22	18.4	17.7	18.3	17.4	19.4
Dec/Jun	23	28	22	26	26	26	26	20.4	20.1	20.1	19.6	21.8
Ann	19	20	17	18	19	19	18	17.5	16.1	16.8	15.4	16.9

Mean daily minimum temperatures (°C) for Périgord black truffle producing regions in France and New Zealand, and regions in Tasmania where truffières have been planted.

	Gisborne, N.Z.	Avignon, France	Gourdon, France	Montelimar, France	Montpellier, France	Orange, France	Valence, France	Bothwell, Tas.	Deloraine, Tas.	Grove, Tas.	Oatlands, Tas.	Westbury, Tas.
Jan/Jul	13	16	14	16	16	17	16	7.6	7.7	9.3	8.8	8.2
Feb/Aug	13	16	14	16	16	16	16	7.5	8.7	9.3	8.7	8.5
Mar/Sep	12	14	12	13	14	13	13	6.6	6.3	7.9	7.5	7.2
Apr/Oct	10	9	9	9	9	9	9	4.3	4.5	6.3	5.7	4.7
May/Nov	7	5	4	5	6	5	5	2.2	2.7	4.2	3.4	2.4
Jun/Dec	5	3	3	2	2	2	2	0.1	1	2.3	1.7	1
Jul/Jan	4	1	2	1	1	1	1	-0.2	0.9	1.9	1.1	0.6
Aug/Feb	5	2	3	2	2	2	2	0.6	1.2	2.7	1.8	1.7
Sep/Mar	7	5	4	4	5	4	4	2.2	3.1	3.9	3.1	3
Oct/Apr	8	7	6	6	8	7	6	3.3	4.4	5.6	4.6	4.4
Nov/May	10	11	9	10	11	11	10	5.5	5.4	7	6.1	5.8
Dec/Jun	12	14	13	13	14	14	13	6.7	7.2	8.6	7.7	7.5
Ann	9	9	8	8	9	8	8	4.4	4.4	5.7	5	4.5

Mean rainfall (mm) for Périgord black truffle producing regions in France and New Zealand, and regions in Tasmania where truffières have been planted.

	Gisborne, N.Z.	Avignon, France	Gourdon, France	Montelimar, France	Montpellier, France	Orange, France	Valence, France	Bothwell, Tas.	Deloraine, Tas.	Grove, Tas.	Oatlands, Tas.	Westbury, Tas.
Jan/Jul	51.6	32.5	53	41.25	19.75	38.5	43.25	39.3	50.3	48.1	44.8	44.8
Feb/Aug	72	44.25	59.6	101.5	39.5	76.75	62	38.7	47.3	44.8	38.7	47.9
Mar/Sep	94.2	64	64.9	110.25	98.5	86.75	127	37.8	51.4	47.2	40	47.6
Apr/Oct	91.3	81.75	67	129	93.5	118	96.5	50.4	73.9	66.9	48.4	65.6
May/Nov	86.8	73.75	68	120	69	87.5	98.5	44.6	87.8	63.6	44	78.4
Jun/Dec	127.8	52.25	70.4	56	88.5	58	61	43.4	104	62.5	48.3	86
Jul/Jan	118.5	22.75	61.5	50.25	55.25	37.5	51.25	46	123	77.1	43.7	103.9
Aug/Feb	106.2	31.5	57.4	53.25	29.5	43.25	41.25	46.6	115.3	76.7	45.5	95
Sep/Mar	91.7	47.25	62.2	71.75	71	63	62	43.2	92.5	73.3	41.4	79.6
Oct/Apr	60.2	53.25	69.7	70	55.25	61	58	55.2	85.6	69.4	54	76.2
Nov/May	56.3	62	80	88.5	46.25	71.75	71.75	53	65.4	68.9	50.5	56.7
Dec/Jun	73.5	41.25	73	49.25	32.5	57	59	53	65.2	66.3	56.7	57.3
Ann	1033.1	607.5	787.2	940	697.5	800	832.5	551.1	961.7	764.7	555.8	839.1

Appendix 2. Fungi known to occur in Tasmania and presumed to be ectomycorrhizal.

Taxonomy is consistent with the original reports. No attempt has been made to update names.

Ascomycotina

Species	Reference	Presumptive Mycorrhizal Host
Amylascus sp.	(Castellano and Bougher, 1994)	
Amylascus tasmanica: see *Terfezia tasmanica*		
Balsamia sp.	(Castellano and Bougher, 1994)	
Dingleya tectiascus Trappe, Castellano & Malajczuk	(Trappe *et al.*, 1992)	
Dingleya phymatodea (Zhang & Minter) Trappe, Castellano & Malajczuk	(Trappe *et al.*, 1992)	*Leptospermum* sp.
≡ *Labyrinthomyces phymatodeus* Zhang & Minter		
Dingleya tectiascus Trappe, Castellano & Malajczuk	(Trappe *et al.*, 1992)	
Elaphomyces spp.	(Castellano and Bougher, 1994)	
Genabea tasmanica Massee & Rodway	(Castellano and Trappe, 1992a)	
Genea pazschkei Bresadola	(Castellano and Trappe, 1992a)	
Hydnocystis convoluta McAlpine	(McAlpine and Rodway, 1896)	
≡ *Hydnotrya convoluta* (McAlpine) McLennan		
≡ *Peziza jactata* Bursdall & Korf		
Hydnocystis echinospora: see *Sphaerosoma tasmanica*		

Hydnotrya convoluta: see *Hydnocystis convoluta*		
Labyrinthomyces sp.	(Castellano and Bougher, 1994)	
Labyrinthomyces phymatodeus: see *Dingleya phymatodea*		
Labyrinthomyces varius: see *Stephensia varia*		
Muciturbo reticulatus Talbot	(Warcup, 1991)	*Eucalyptus obliqua*
Paurocotylis niveus Rodway	(Rodway, 1920)	
Peziza jactata: see *Hydnocystis convoluta*		
Ruhlandiella berolinensis Hennings	(Warcup, 1991)	*Eucalyptus obliqua*
Sphaerosoma mucida: see *Spragueola mucida.*		
Sphaerosoma tasmanica Rodway	(Rodway, 1920)	
≡*Hydnocystis echinospora* Rodway		
Spragueola mucida Rodway	(Rodway, 1920)	
≡ *Tremellodiscus mucidus* (Rodway) Lloyd		
≡ *Sphaerosoma mucida* (Rodway) Hansford		
Stephensia varia Rodway	(Rodway, 1898)	
≡ *Labyrinthomyces varius* (Rodway) Trappe		
Terfezia tasmanica Rodway	(Rodway, 1926)	
≡ *Amylascus tasmanica* (Rodway) Trappe		
Tremellodiscus mucidus: see *Spragueola mucida.*		

Basidiomycotina

Species	Reference	Presumptive Mycorrhizal Host
Agaricus recedens: see *Descolea recedens*		
Alpova spp.	(Castellano and Bougher, 1994)	
Alpova clelandii: see *Rhizopogon clelandii.*		
Amanita ananiceps (Berk.) Sacc.	(May and Wood, 1997)	
Amanita grisea Massee & Rodway	(May and Wood, 1997)	
Amanita grossa Sacc.	(May and Wood, 1997)	
Amanita muscaria (L. : Fr.) Lam.	(Shepherd and Totterdell, 1988)	
Amanita umbrinella E.J. Gilbert & Cleland	CSIRO database	
Andebbia pachythrix (Cooke & Massee in Cooke) Trappe, Castellano & Amaranthus	(Trappe *et al.*, 1996a)	*Eucalyptus*
≡ *Diploderma pachythrix* Cooke & Massee		
≡ *Mesophellia pachythrix* (Cooke & Massee) Lloyd		
Arcangeliella alveolata: see *Octaviania alveolata.*		
Arcangeliella ellipsoidea Zeller & Dodge	(Castellano and Trappe, 1990)	
Arcangeliella glabrella Zeller & Dodge	(Castellano and Trappe, 1990)	
≡ *Hydnangium glabrellum* (Zeller & Dodge) Cunningham		
≡ *Zelleromyces glabrellus* (Zeller & Dodge) Singer & Smith		
Arcangeliella nana: see *Hymenogaster nanus.*		

Arcangeliella seminuda: see *Gymnomyces seminudus.*		
Arcangeliella tasmanica: see *Hydnangium tasmanicum.*		
Arcengeliella violacea: see *Cortinomyces violaceus*		
Austrogautieria clelandii Stewart & Trappe	(Stewart and Trappe, 1985)	
≡ *Gautieria clelandii* Cunningham		
≡ *Gautieria tasmanica* Cunningham non Rodway		
Austrogautieria costata Stewart & Trappe	(Johnson, 1994)	*Eucalyptus tenuiramus, Acacia dealbata, Exocarpus cupressiformis*
≡ *Gautieria costata* Cunningham, nom. nud.		
Austrogautieria rodwayi: see *Hymenogaster rodwayi.*		
Austroboletus sp.	CSIRO database	
Boletus luridus Schaeff. : Fr.	(Rodway, 1898)	
Boletus piperatus	CSIRO database	Douglas Fir
Boletus megalosporus Berk.	(Rodway, 1898)	
Boletus tasmanicus Hongo and A.K.Mills	(Hongo and Mills, 1988)	
Boughera spp.	(Castellano and Bougher, 1994)	
Cantharellus cibarius Fr. : Fr.	(Rodway, 1898)	
Cantharellus cinereus (Pers. : Fr.) Fr.	(Rodway, 1898)	
Cantharellus pusio Berk.	(Rodway, 1898)	
Cantharellus strigipes Berk.	(Rodway, 1898)	
Castoreum cretaceum: see *Diploderma cretaceum*		

Castoreum radicatum Cooke & Massee	(Cooke, 1886)	
≡ *Diploderma radicatum* (Cooke & Massee) Lloyd		
Castoreum tasmanicum Cunningham	(Castellano and Trappe, 1990)	
Chamonixia spp.	(Castellano and Bougher, 1994)	
Chondrogaster spp.	(Castellano and Bougher, 1994)	
Cortinarius archeri Berk.	(May and Wood, 1997)	
Cortinarius globuliformis Bougher	CSIRO database	*Eucalyptus globulus*
Cortinarius magellanicus Speg.	(May and Wood, 1997)	
Cortinarius ochraceus: see *Cortinarius sinapicolor*		
Cortinarius parochraceus var. *australiensis*: see *Cortinarius sinapicolor*		
Cortinarius rotundisporus Cleland & Cheel	CSIRO database	*Nothofagus cunninghamii*
Cortinarius sinapicolor Cleland	CSIRO database	*Eucalyptus*
≡ *Cortinarius ochraceus* Cleland		
≡ *Cortinarius parochraceus* M.M.Moser var. *australiensis* M.M.Moser		
Cortinomyces luteus (Massee) Bougher & Castellano	(Bougher and Castellano, 1993)	*Eucalyptus*
≡ *Protoglossum luteum* Massee		
≡ *Hymenogaster luteus* (Massee) Cunningham		
= *Hysterangium atratum* Rodway		
= *Hymenogaster atratus* (Rodway) Zeller & Dodge		
Cortinomyces violaceus (Massee & Rodway) Bougher & Castellano	(Bougher and Castellano, 1993)	
≡ *Hymenogaster violaceus* Massee & Rodway		

≡ *Arcengeliella violacea* (Massee & Rodway) Dodge

≡ *Dendrogaster violaceus* (Massee & Rodway) Cunningham

≡ *Arcangeliella violacea* (Massee & Rodway) Zeller & Dodge

≡ *Gymnoglossum violaceum* (Massee & Rodway) Cunningham

Cortinomyces viscidus (Massee & Rodway) Bougher & Castellano (Bougher and Castellano, 1993)

≡ *Hysterangium viscidum* Massee & Rodway

≡ *Hymenogaster viscidus* (Massee & Rodway) Dodge & Zeller

Cuphocybe sp. CSIRO database

Cystangium spp. (Castellano and Bougher, 1994)

Cystangium rodwayi: see *Secotium rodwayi.*

Cystangium sessile: see *Secotium sessile.*

Dendrogaster fulvus: see *Hymenogaster fulvus.*

Dendrogaster violaceus: see *Cortinomyces violaceus*

Dermocybe sp. CSIRO database

Descolea recedens (Cooke & Massee) Singer CSIRO database

≡ *Agaricus recedens* Cooke & Massee

≡ *Pholiota recedens* (Cooke & Massee) Sacc.

≡ *Pholiotina recedens* (Cooke & Massee) Singer

≡ *Pholiotina filaris* (Fr.) Singer var. *recedens* (Cooke & Massee) Singer

Descolea phlebophora E. Horak CSIRO database

Descomyces albellus (Massee & Rodway) Bougher & Castellano (Bougher and Castellano, 1993) Myrtaceae

≡ *Hymenogaster albellus* Massee & Rodway

≡ *Hymenogaster zeylanicus* Petch

≡ *Hymenogaster maideni* Rodway

= *Hymenogaster maurus* Maire

= *Hymenogaster weiblianus* Maire

Descomyces albus (Klotzsch) Bougher & Castellano (Bougher and Castellano, 1993) Myrtaceae

≡ *Hymenangium album* Klotzsch

≡ *Hymenogaster albus* (Klotzch) Berk. & Br.

≡ *Hymenogaster klotzschii* Tul.

≡ *Splanchnomyces albus* Corda *emend.* Zobel

Destuntzia sp. (Castellano and Bougher, 1994)

Diploderma castoreum Lloyd (Castellano and Trappe, 1990)

Diploderma cretaceum Lloyd (Castellano and Trappe, 1990)

≡ *Castoreum cretaceum* (Lloyd) Cunningham

≡ *Nothocastoreum cretaceum* (Lloyd) Beaton

Diploderma dehiscens Lloyd (Castellano and Trappe, 1990)

Diploderma glaucum: see *Mesophellia glauca*

Diploderma pachythrix: see *Andebbia pachythrix.*

Diploderma parvispora: see *Mesophellia glauca.*

Diploderma radicatum: see *Castoreum radicatum.*

Elasmomyces rodwayi: see *Secotium rodwayi.*

Elasmomyces sessile: see *Secotium sessile.*		
Gautieria albida: see *Hymenogaster albidus.*		
Gautieria clelandii: see *Austrogautieria clelandii.*		
Gautieria costata: see *Austrogautieria costata.*		
Gautieria microspora Rodway	(Rodway, 1929)	
Gautieria monospora Beaton, Pegler & Young	(Johnson, 1994)	*Eucalyptus tenuiramus, Acacia dealbata, Exocarpus cupressiformis*
Gautieria rodwayi: see *Hymenogaster rodwayi.*		
Gautieria tasmanica Rodway	(Rodway, 1929)	
Gautieria tasmanica Cunningham: see *Austrogautieria clelandii.*		
Gigasperma clelandii: see *Horakiella clelandii*		
Gummiglobus agglutinosporus (Beaton in Beaton & Weste) Trappe, Castellano & Amaranthus	(Trappe *et al.*, 1996a)	
≡ *Mesophellia agglutinospora* Beaton		
Gymnoglossum fulvum: see *Hymenogaster fulvus.*		
Gymnoglossum violaceum: see *Cortinomyces violaceus*		
Gymnomyces flavus Rodway	(Rodway, 1918)	
≡ *Octaviania flava* (Rodway) Cunningham		
≡ *Octavianina flava* (Rodway) Singer & Smith		
≡ *Stephanospora flava* (Rodway) Beaton, Pegler & Young		
Gymnomyces flavus* f. *tetraspora Rodway	(Rodway, 1924a)	
Gymnomyces megasporus Rodway	(Rodway, 1926)	

≡ *Octaviania megaspora* (Rodway) Cunningham		
Gymnomyces pallidus Massee & Rodway	(Castellano and Trappe, 1990)	
≡ *Octaviania pallida* (Massee & Rodway) Cunningham		
Gymnomyces seminudus Massee & Rodway	(Castellano and Trappe, 1990)	
≡ *Arcangeliella seminuda* (Massee & Rodway) Zeller & Dodge		
≡ *Octaviania seminuda* (Massee & Rodway) Cunningham		
Gymnomyces solidus Rodway	(Rodway, 1921)	
Hebeloma sp.	CSIRO database	
Horakiella clelandii (Rodway) Castellano & Trappe	(Castellano and Trappe, 1992b)	
≡ *Hydnangium clelandii* Rodway		
≡ *Leucophlebs clelandi* (Rodway) Zeller & Dodge		
≡ *Octaviania clelandii* (Rodway) Cunningham		
≡*Gigasperma clelandii* (Rodway) Horak		
Hydnangium alveolatum: see *Octaviania alveolata.*		
Hydnangium archeri: see *Octaviania archeri.*		
Hydnangium carneum Wallroth	(Johnson, 1994)	*Eucalyptus tenuiramus, Acacia dealbata, Exocarpus cupressiformis*
≡ *Octaviania carnea* (Wallroth) Corda		
Hydnangium clelandii: see *Horakiella clelandii.*		
Hydnangium densum Rodway	(Rodway, 1920)	
≡ *Octaviania densa* (Rodway) Cunningham		

Hydnangium glabrellum: see *Arcangeliella glabrella.*

Hydnangium glabrum Rodway (Rodway, 1921)

≡ *Octaviania glabra* (Rodway) Cunningham

Hydnangium hinsbyi Rodway (Rodway, 1924b)

≡ *Octaviania hinsbyi* (Rodway) Cunningham

Hydnangium microsporium Rodway (Rodway, 1920)

Hydnangium tasmanicum Kalchbrenner (Massee, 1890)

≡ *Arcangeliella tasmanica* (Kalchbrenner) Zeller & Dodge

≡ *Maccagnia tasmanica* (Kalchbrenner) Zeller & Dodge

≡ *Octaviania tasmanica* (Kalchbrenner) Lloyd

≡ *Octavianina tasmanica* (Kalchbrenner) Pegler & Young

Hymenangium album: see *Descomyces albus.*

Hymenogaster albellus: see *Descomyces albellus.*

Hymenogaster albidus Massee & Rodway (Castellano and Trappe, 1990)

≡ *Gautieria albida* (Massee & Rodway) Zeller & Dodge

≡ *Gautieria albida* (Massee & Rodway) Cunningham

Hymenogaster albus: see *Descomyces albus.*

Hymenogaster atratus: see *Cortinomyces luteus.*

Hymenogaster aureus Rodway (Rodway, 1924b)

Hymenogaster barnardi Rodway (Rodway, 1921)

Hymenogaster fulvus Rodway (Rodway, 1918)

≡ *Dendrogaster fulvus* (Rodway) Cunningham

≡ *Gymnoglossum fulvum* (Rodway) Cunningham

Hymenogaster fusisporus: see *Hysterangium fusisporum.*

Hymenogaster klotzschii: see *Descomyces albus.*

Hymenogaster levisporus Massee & Rodway (Rodway, 1912)

≡ *Octaviania levispora* (Massee & Rodway) Rodway

≡ *Thaxterogaster levisporus* (Massee & Rodway) Beaton

Hymenogaster luteus: see *Cortinomyces luteus.*

Hymenogaster macrosporus: see *Timgrovea macrospra.*

Hymenogaster maideni: see *Descomyces albellus.*

Hymenogaster nanus Massee & Rodway (Castellano and Trappe, 1990)

≡ *Arcangeliella nana* (Massee & Rodway) Zeller & Dodge

Hymenogaster reticulatus: see *Timgrovea reticulata.*

Hymenogaster rodwayi Massee (Castellano and Trappe, 1990)

≡ *Gautieria rodwayi* (Massee) Zeller & Dodge in Dodge & Zeller

≡ *Gautieria rodwayi* (Massee) Zeller & Doge in Cunningham

≡ *Austrogautieria rodwayi* (Massee) Stewart & Trappe

Hymenogaster tasmanicus Cunningham (Cunningham, 1934)

≡ *Hysterogaster tasmanicus* (Cunningham) Beaton, Pegler & Young

Hymenogaster violaceus: see *Cortinomyces violaceus*

Hymenogaster viscidus: see *Cortinomyces viscidus.*

Hymenogaster zeylanicus: see *Descomyces albellus.*		
Hysterangium affine Massee & Rodway	(Castellano and Trappe, 1990)	
Hysterangium affine* var. *irregulare Massee	(Castellano and Trappe, 1990)	
Hysterangium affine* var. *tenuispora Rodway	(Rodway, 1912)	
Hysterangium aggregatum Cribb	(Johnson, 1994)	*Eucalyptus tenuiramus, Acacia dealbata, Exocarpus cupressiformis*
Hysterangium atratum: see *Cortinomyces luteus.*		
Hysterangium burburianum Rodway	(Rodway, 1918)	
Hysterangium clathroides Vitt.	(Rodway, 1898)	
Hysterangium fusisporum Massee & Rodway	(Castellano and Trappe, 1990)	
≡ *Hymenogaster fusisporus* (Massee & Rodway) Cunningham		
≡ *Hymenogaster fusisporus* (Massee & Rodway) Zeller & Dodge		
≡ *Hysterogaster fusisporum* (Massee & Rodway) Zeller & Dodge		
≡ *Hysterogaster fusisporum* (Massee & Rodway) Beaton, Pegler & Young		
Hysterangium inflatum Rodway	(Rodway, 1918)	
Hysterangium membranaceum Vitt.	(Rodway, 1898)	
Hysterangium neglectum Massee & Rodway	(Castellano and Trappe, 1990)	
Hysterangium obtusum Rodway	(Rodway, 1920)	
Hysterangium pumilum Rodway	(Rodway, 1918)	
Hysterangium viscidum: see *Cortinomyces viscidus.*		
Hysterogaster spp.	(Castellano and Bougher, 1994)	

Hysterogaster fusisporum: see *Hysterangium fusisporum.*		
Hysterogaster tasmanicus: see *Hymenogaster tasmanicus.*		
Inoderma arenaria: see *Mesophelia arenaria*		
Laccaria laccata (Scop. ex Fr.) O. Berk. et Br.	(Shepherd and Totterdell, 1988)	
Lactarius deliciosus (L.Fr.) Gray	CSIRO database	*Pinus radiata*
Lactarius piperatus *(L.:Fr.) Pers.*	(Rodway, 1898)	
Lactarius sepiaceus McNabb	(Hongo and Mills, 1988)	
Lactarius stenophyllus Berk.	(Rodway, 1898)	
Lactarius subdulcis (Pers.:Fr.) Gray	(Rodway, 1898)	
Leucogaster sp.	(Castellano and Bougher, 1994)	
Leucophlebs clelandi: see *Horakiella clelandii.*		
Maccagnia tasmanica: see *Hydnangium tasmanicum.*		
Macowanites spp.	(Castellano and Bougher, 1994)	
Malajczukia spp.	(Castellano and Bougher, 1994)	
Martellia alveolata: see *Octaviania alveolata.*		
Martellia spp.	(Castellano and Bougher, 1994)	
Mesophellia agglutinospora: see *Gummiglobus agglutinosporus.*		
Mesophellia arenaria Berkeley ≡ *Inoderma arenaria* (Berkeley) Berkeley	(Trappe *et al.*, 1996b)	*Eucalyptus regnans*
Mesophellia castanea Lloyd	(Trappe *et al.*, 1996b)	*Eucalyptus*
Mesophellia clelandii Trappe, Castellano & Malajczuk	(Trappe *et al.*, 1996b)	*Eucalyptus*
Mesophellia glauca (Cooke & Massee) Reid	(Trappe *et al.*, 1996b)	*Eucalyptus*

≡ *Diploderma glaucum* Cooke & Massee

≡ *Potoromyces loculatus* Mueller

≡ *Diploderma parvispora* Lloyd

Mesophellia oleifera Trappe, Castellano & Malajczuk (Trappe *et al.*, 1996b) *Eucalyptus*

Mesophellia pachythrix: see *Andebbia pachythrix*

Nothocastoreum spp. (Castellano and Bougher, 1994)

Nothocastoreum cretaceum: see *Diploderma cretaceum.*

Octaviania alveolata Cooke & Massee (Rodway, 1920)

≡ *Octavianina alveolata* (Cooke & Massee) O.Kuntze

≡ *Hydnangium alveolatum* (Cooke & Massee) Rodway

≡ *Arcangeliella alveolata* (Cooke & Massee) Zeller & Dodge

≡ *Martellia alveolata* (Cooke & Massee) Smith

Octaviania archeri Berkeley (Castellano and Trappe, 1990)

≡ *Octavianina archeri* (Berkeley) O. Kuntze

≡ *Hydnangium archeri* (Berkeley) Rodway

≡ *Hydnangium archeri* (Berkeley) Zeller & Dodge

Octaviania carnea: see *Hydnangium carneum.*

Octaviania clelandii: see *Horakiella clelandii.*

Octaviania densa: see *Hydnangium densum.*

Octaviania flava: see *Gymnomyces flavus.*

Octaviania glabra: see *Hydnangium glabrum.*

Octaviania hinsbyi: see *Hydnangium hinsbyi.*		
Octaviania levispora: see *Hymenogaster levisporus.*		
Octaviania megaspora: see *Gymnomyces megasporus.*		
Octaviania pallida: see *Gymnomyces pallidus.*		
Octaviania seminuda: see *Gymnomyces seminudus.*		
Octaviania tasmanica: see *Hydnangium tasmanicum*		
Octavianina flava: see *Gymnomyces flavus*		
Octavianina alveolata: see *Octaviania alveolata.*		
Octavianina archeri: see *Octaviania archeri.*		
Octavianina tasmanica: see *Hydnangium tasmanicum.*		
Paxillus involutus (Batsch : Fr.)Fr.	A.K. Mills, pers. com.	
Paxillus muelleri Berk.	CSIRO database	*Eucalyptus globulus*
Pholiota recedens: see *Descolea recedens*		
Pholiotina recedens: see *Descolea recedens*		
Pholiotina filaris var. *recedens*: see *Descolea recedens*		
Pisolithus tinctorius (Mich. ex Pers.) Coker et Couch	(Shepherd and Totterdell, 1988)	
Potoromyces loculatus: see *Mesophellia glauca.*		
Protoglossum luteum: see *Cortinomyces luteus.*		
Protubera spp.	(Castellano and Bougher, 1994)	
Quadrispora musispora Bougher & Castellano	(Bougher and Castellano, 1993)	*Nothofagus cunninghamii, N. gunnii*
Rhizopogon clelandii Cunningham	(Johnson, 1994)	*Eucalyptus tenuiramus, Acacia dealbata, Exocarpus cupressiformis*

≡ *Alpova clelandii* (Cunningham) Beaton, Pegler & Young

Rhizopogon colossus	CSIRO database	*Pseudotsuga*
Rhizopogon hawheni	CSIRO database	*Pseudotsuga*
Rhizopogon rodwayi McAlpine	(McAlpine, 1895)	
Rhizopogon rubescens (Tul.) Tul. & C.Tul.	(Rodway, 1912)	
Richoniella sp.	(Castellano and Bougher, 1994)	
Rozites armeniacovelata Bougher, Fuhrer & Horak	(Bougher *et al.*, 1994)	*Nothofagus*
Rozites foetens Bougher, Fuhrer & Horak	(Bougher *et al.*, 1994)	*Nothofagus*
Rozites fusipes Horak & Taylor	(Bougher *et al.*, 1994)	*Nothofagus, Eucalyptus*
Rozites metallica Bougher, Fuhrer & Horak	(Bougher *et al.*, 1994)	*Nothofagus*
Rozites occulta Bougher, Fuhrer & Horak	(Bougher *et al.*, 1994)	*Nothofagus*
Rozites roseolilacina Bougher, Fuhrer & Horak	(Bougher *et al.*, 1994)	
Russula alutacea (Pers.:Fr.) Fr.	(Rodway, 1898)	
Russula coccinea Massee	(Rodway, 1898)	
Russula compacta Frost & Peck	(Hongo and Mills, 1988)	
Russula emetica (Schaeff.:Fr.) Gray	(Rodway, 1898)	
Russula purpurea Gillet	(Rodway, 1898)	
Russula semicrema Fr.	(Rodway, 1900)	
Scleroderma parodoxum Beaton	CSIRO database	*Eucalyptus*
Scleroderma verrucosum Vaill. ex Pers.	CSIRO database	*Eucalyptus*
Sclerogaster sp.	(Castellano and Bougher, 1994)	
Secotium ochraceum Rodway	(Rodway, 1920)	

Secotium rodwayi Massee	(Castellano and Trappe, 1990)	
≡ *Elasmomyces rodwayi* (Massee) Zeller		
≡ *Cystangium rodwayi* (Massee) Smith		
Secotium sessile Massee & Rodway	(Rodway, 1912)	
≡ *Elasmomyces sessile* (Massee & Rodway) Rodway		
≡ *Cystangium sessile* (Massee & Rodway) Singer & Smith		
Setchelliogaster sp.	(Castellano and Bougher, 1994)	
Splanchnomyces albus: see *Descomyces albus.*		
Stephanospora flava: see *Gymnomyces flavus.*		
Timgrovea macrospra (Cunningham) Bougher & Castellano	(Bougher and Castellano, 1993)	
≡ *Hymenogaster macrosporus* Cunningham non Knapp & Soehner		
Timgrovea reticulata (Cunningham) Bougher & Castellano	(Bougher and Castellano, 1993)	*Eucalyptus*
≡ *Hymenogaster reticulatus* Cunningham		
= *Hymenogaster reticulatus* Zeller & Dodge		
= *Gymnoglossum reticulatum* Cribb		
Thaxterogaster spp.	(Castellano and Bougher, 1994)	
Thaxterogaster levisporus: see *Hymenogaster levisporus.*		
Zelleromyces spp.	(Castellano and Bougher, 1994)	
Zelleromyces glabrellus: see *Arcangeliella glabrella.*		

Appendix 3. The composition of the media used in Chapter 4.

Ingredient	MMN	Pach
Maltose (g/L)		5
Glucose (g/L)	10	20
Malt extract (g/L)	3	
Ammonium tartrate (mg/L)		500
KH_2PO_4 (mg/L)	500	1000
$(NH_4)_2HPO_4$ (mg/L)	250	
$MgSO_47H_2O$ (mg/L)	150	500
$CaCl_22H_2O$ (mg/L)	50	50
NaCl (mg/L)	25	
Fe EDTA (mg/L)	20	20
H3BO3 (mg/L)		2.8
MnCl22H2O (mg/L)		3
ZnSO47H2O (mg/L)		2.3
CuCl22H2O (mg/L)		0.63
Na2Mo42H2O (mg/L)		0.27
Thiamine HCl (μg/L)	0.1	0.1
Agar (g/L)	10	10
pH	5.8	5.4

MMN = modified Melin Norkans media

Pach = Pachlewski media

The potato dextrose agar media used was a commercial preparation by Merck, Germany.

The malt extract media contained 10 g/L of food grade malt extract manufactured by Mauri Foods Division, Australia. Agar was incorporated at 10 g/L.

Appendix 4. Climate statistics for Perth, Western Australia.

PERTH

Latitude: 31.99 S Longitude: 115.82 E Elevation: 6.1 m State: WA

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Ann
Mean Daily Max Temp (deg C)	29.5	30.0	27.6	23.8	20.7	18.5	17.5	18.2	19.5	21.6	24.4	26.9	22.8
Mean Daily Min Temp (deg C)	17.6	18.1	16.5	13.3	10.8	9.7	8.5	8.4	9.7	11.2	13.6	15.8	12.5
Mean Rainfall (mm)	7.8	12.1	17.4	50.3	110.8	186.8	170.3	114.3	70.3	49.2	19.4	12.6	821.2
Mean 9am Relative Humidity (%)	56	58	66	69	76	79	80	76	71	64	58	61	69

Appendix 5. Climate statistics for Hobart, Tasmania.

HOBART

Latitude: 42.89 S Longitude: 147.33 E Elevation: 50.5 m

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Ann
Mean Daily Max Temp (deg C)	21.5	21.6	20.1	17.2	14.3	11.8	11.6	12.9	15.0	16.9	18.5	20.2	16.8
Mean Daily Min Temp (deg C)	11.8	11.9	10.8	8.9	6.9	5.1	4.5	5.2	6.3	7.7	9.2	10.7	8.2
Mean Rainfall (mm)	48.3	39.8	45.7	52.9	47.9	54.8	53.8	52.8	51.7	62.8	54.8	58.2	623.7
Mean 9am Relative Humidity (%)	59	63	66	70	76	79	78	73	66	62	60	59	68

Appendix 6. Graph to convert pH ($CaCl_2$) to pH (H_2O) for the soil used in Chapters 6 and 7.