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Specialty Mushroom Production Systems: Maitake and Morels

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Foreword

Internationally there is increasing demand for both European and Asian specialty mushrooms. In particular: Maitake, a quality edible and medicinal fungi, and Morel, a fungal delicacy which is currently wild harvested. Australia is well placed to enter the market and meet demand due to its proximity to Asian markets, its multi-cultural heritage and export links with USA, Europe and Asia. Both Maitake and Morel provide opportunities for new entrants to the national mushroom growing industry.

Maitake research focussed on (i) developing a technique for the cultivation of maitake based on the Japanese bag method of cultivation; (ii) determining levels of substrate additive which enhance efficient substrate colonisation and primordia initiation; (iii) identifying maitake isolates with the best commercial potential; (iv) providing the industry with a commercially viable maitake production system.

Morel research focussed on (i) providing the industry with a wide range of Tasmanian Morel isolates; (ii) identifying Tasmanian isolates to species level; (iii) investigating triggers for sclerotia production; (iv) comparing the production of sclerotia by Tasmanian isolates with overseas reference isolates; (v) selecting Tasmanian isolates which produce the largest sclerotia for further research.

Building on the successful R&D outcomes from RIRDC projects DAT-30A (*The development of commercially viable culturing and fruiting systems for some Asian specialty mushrooms*) and UT-12A (*Preliminary investigation on "French" black morel cropping*) this project identified suitable Maitake isolates for commercialisation, determined additives to ameliorate eucalypt sawdust for substrate, and trialed a production technique. A large collection of Morel isolates was obtained and preliminary studies on their ability to produce sclerotia, a precursor to fruit body production, were completed. Extensive literature reviews about Maitake and Morels summarise both published and anecdotal information.

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Simon Hearn
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Contents

Executive Summary

The research in this report covers the period February 2000 to July 2002 for RIRDC project No. UT-30A “Specialty mushroom production systems: Maitake and Morels”.

Extensive literature reviews about *Grifola frondosa* (maitake) and *Morchella* spp. (morels) summarises both published and anecdotal information about the fungi; aspects such as their taxonomy, life cycle, sexual behaviour, nutritional and medicinal properties, environmental/nutritional requirements for fruit body production and cultivation techniques used currently or in the past. The relevance of the literature in regard to fruit body production in Australia is discussed. Most often the information available in the public domain is unreliable when subject to close scrutiny. The need for a fresh and scientifically vigorous approach to growing maitake and especially morels is only too evident.

Maitake

Commercial strains are maintained on agar that must have optimal pH and temperature to ensure vigorous and consistent hyphal growth. Temperature and pH parameters for the hyphal growth of 10 maitake isolates, including those currently approved for commercial production in Tasmania, have been determined.

Isolates showed varying responses to temperature and pH but for the majority of isolates hyphal growth was found to be optimal at 25 ºC and pH 5. The temperature selected for the spawn run of subsequent substrate trials was based on this finding. Hyphal growth was very sensitive to media pH. When selecting additives for ameliorating eucalypt sawdust their influence on substrate pH and hence hyphal growth was carefully considered.

Isolate WC808 grew better than M74 and FPC200 on agar cultures at 25 ºC and pH 5. The exponential growth of WC808, M74 and FPC200 grown in liquid medium was determined. In liquid culture M74 has slightly better growth than WC808 but both isolates could be used for the development of liquid spawn.

Maitake is grown on deciduous hardwood sawdust, a product not readily available in Tasmania, Australia. The development of a local substrate, based on eucalypt sawdust, would benefit both the mushroom and forestry industries by providing an inexpensive but essential substrate to the former and an outlet for a waste product to the latter. This ameliorated substrate needs to provide adequate nutrients for spawn run and fruit body production.

Two substrate trials were set up to determine the potential of various additives to enhance eucalypt sawdust and to investigate environmental parameters required for fruit body production using a substrate of ameliorated eucalypt sawdust. Shiitake fruited prolifically on most of the substrates and at environmental conditions tested for maitake. Maitake did not fruit as well as shittake clearly indicating that the nutritional and environmental conditions required for shiitake cultivation differ significantly to those required for maitake cultivation. Maitake must have more stringent nutritional and environmental requirements than shiitake. Differences in colonisation and fruit body formation have been described and identified for four commercial maitake isolates (FPC200, M6, M74, WC808). Isolates M6 and M74 commenced brain stage formation earlier than isolates FPC200 and WC808, independent of substrate. Supplementation of eucalypt sawdust with maize meal, rice bran or wheat bran at 10-20 % is required for maitake cultivation.

Colonisation of ameliorated eucalypt substrate takes between 31 and 42 days which is comparable to colonisation on deciduous sawdust substrates. Age and moisture content of horizontal layers within the sawdust pile have been identified as introducing unwanted variation in the potential of the substrate

for fruit body production. Due to insufficient moisture in the substrate formula supplemental watering may be required after the bags have been opened and/or increased relative humidity. In order to reduce air volume within the bag and increase CO_2 levels another method is required to seal bags, or different shaped bags/filters need to be investigated.

Current recommendations for maitake production resulting from the project are that isolate WC808 be grown on a substrate of eucalypt sawdust ameliorated with 10-20 % rice bran. Spawn run should be in the dark, at 23-25 ºC with relative humidity no greater than 70 %. Colonised bags should be left in this environment until the mycelial mat has formed and then moved to the fruiting room with no light, temperature of not more than 16 ºC, and relative humidity of 85 %. Light should be gradually increased as the fruit body develops. At the same time relative humidity should be progressively increased to 95 %. The bag should not be opened until petals are forming on the fruit body and adequate moisture for fruit body formation should be maintained.

Research priority is to conduct a semi-commercial trial at Huon Valley Mushrooms. This trial would encompass :

- **two sawdusts - *Eucalyptus* and *Nothofagus***
- **one additive combination - 10 % rice bran plus 10 % wheat bran**
- **two light combinations – wavelength and timing**
- **two types of bags.**

Laboratory trials will investigate relative humidity and CO_2.

Morels

Thirty one Tasmanian morel collections have been described. Twenty-one vegetative morel cultures are available for cultivation studies. Single spore isolates from each of five morel fruit bodies are also available for future research into morel genetics and sexual behaviour where such information could provide insight into fruit body production. A collection of Tasmanian isolates also means that IP issues in relation to isolate ownership can be avoided.

Comparisons of the potential for sclerotia development (stage in life cycle prior to fruit body production) have been made for a range of Tasmanian and overseas morel isolates. Studies on Buscot media found sclerotia production is dictated by media type and is not a function of time. This indicates that a suitable medium for rapid and abundant sclerotia production can be developed using Petri dish assays containing different media.

When inoculated on nutrient poor medium in split-plates containing both nutrient poor and nutrient rich medium a number of Tasmanian isolates formed sclerotia on both media. This differs to overseas isolates of *M. hortensis* and *M. rigida* which only formed sclerotia on the nutrient rich medium, and only when the nutrient poor medium was inoculated. This suggests that Tasmanian morels may be different species or that their production of sclerotia has developed in response to Australian soil conditions.

Two types of sclerotia developed in response to bacterial confrontation suggesting a synergy between the presence of certain bacteria and sclerotia formation. Our research indicates that the potential of bacteria, and their role in the stimulation of sclerotia/morel fruit bodies, warrants further investigation.

In conclusion, there is a significant collection of Tasmanian morel cultures available for cultivation studies. Preliminary investigations into the formation of sclerotia, by Tasmanian morels, in response to certain media and bacterial challenge have been conducted.

Research priorities identified are : characterise Australian *Morchella* isolates in relation to overseas material; **screen morel isolates on 3 standard synthetic media; screening and enzyme assays of isolates on a range of synthetic media (liquid assays and split plate trials); develop substrates having both suitable nutritional and physical properties to encourage sclerotia development; determine environmental parameters that trigger fruit body initiation and development.**

Specialty mushroom production systems: Maitake and Morels

Introduction

Maitake - Grifola frondosa

Maitake is an edible fungus valued for both its taste and claimed medicinal properties. Available information on the cultivation of maitake indicates the Japanese bag system, widely used in Japan and the USA, may be suitable for the commercial production of maitake in Australia. This cultivation system uses a substrate of sawdust, from broadleaf trees, which has been ameliorated with different percentages of rice bran, maize meal or wheat bran (Rinsanka, 1980; Mayuzumi and Mizuno, 1997; Shen and Royse, 2001). Broadleaf sawdust is not readily available in Australia but earlier studies (Wilson and Chung, 1994; Mohammed, 1998) indicated eucalypt sawdust, ameliorated with a range of additives, may be suitable for the cultivation of maitake under Australian conditions.

The objectives in regard to maitake was to:

- develop a technique for the cultivation of maitake based on the Japanese bag method of cultivation;
- determine levels of substrate additive which enhance efficient substrate colonisation and primordia initiation;
- identify maitake isolates with the best commercial potential;
- provide the industry with a commercially viable maitake production system.

Morel - *Morchella* species

Morels are keenly sought throughout the world for their excellent taste and remarkable appearance. The development of a commercial method for the cultivation of morels is the “holy grail” for both scientists and growers. Although a cultivation method has been patented in the United States of America. No one, other than the company operating the patent, has been able to commercially produce morels using the techniques described. Considerable disagreement amongst taxonomists on the identification of morel species hinders cultivation studies. It is difficult to compare the work of others due to the use of different names for the same or similar species.

Researchers must be certain they are working with isolates that have been obtained from ascocarps known to have desirable market characteristics. In Australia some work has been done on the development of sclerotia , an early stage in the cultivation of morels, but no studies have been done on morel taxonomy.

The objectives in regard to morels was to :

- provide the industry with a wide range of Tasmanian Morchella isolates;
- identify Tasmanian isolates to species level;
- investigate triggers for sclerotia production;
- compare the production of sclerotia by Tasmanian isolates with overseas reference isolates;
- select Tasmanian isolates which produce the largest sclerotia for further research.

This report is split into two sections: maitake and morel. Each section commences with a literature review, which were specific milestones within the project (Appendix 1). Progress achieved towards meeting the project objectives in 30 months is reported. The commercial term “fruit body” is used to describe a Maitake sporocarp whereas “ascocarp” is used to describe a Morel fruit body.

Literature Review: Maitake production and relevance of literature to production in Australia

Introduction

World mushroom consumption has increased both in quantity and in the variety of species (Royse, 1997b) with specialty mushrooms accounting for approximately 70 % of total world production in 1999 (Chang, 1999). Demand for both European and Asian specialty mushrooms is steadily increasing, especially in the United States of America and the South East Asian-Pacific region (Royse, 1997b; United States Department of Agriculture, 1999). In the latter region traditional producers of Asian specialty mushrooms cannot meet demand for quality edible and medicinal fungi due to increasing demand and a shift in agricultural focus away from mushroom production. In Europe numerous edible fungi cannot be produced artificially. The demand is supplied from wild harvests and there is the need to ensure that edible fungi are not over-harvested from forests. The need to move from wild harvesting to commercial production in order to meet market demand provides opportunity for new entrants to this area. Australia is well placed to enter the arena due to its proximity to Asian markets, its multi-cultural heritage and export links with USA, Europe and Asian.

Grifola frondosa is a specialty mushroom better known in Asia than globally. It is currently being promoted because it is a delicious and firm-textured fungus suitable for international cuisine whether Japanese, Chinese, or Western-style cooking (Mizuno and Zhuang, 1995) and also has nutriceutical properties (Mizuno and Zhuang, 1995; Royse, 1997b; Yamanaka, 1997). The market value of fresh *G. frondosa* at A$40/kg is at least twice the price of *Lentinula edodes* (shiitake) (Mohammed, 1998) making it an attractive product for growers to cultivate. In 2002 dried Japanese *G. frondosa* capsules sold for A$200-280 per 100 g when sold in the United States medicinal market (Maitake Products Inc., 2001; Fungi Perfecti, 2002).

Existing trade links with neighbouring Asian countries places Australia in an excellent position to supply *G. frondosa* to these markets. In Australia the domestic market for specialty mushrooms is steadily expanding. This reflects in part an increase in the number of people born in Asian countries migrating to Australia. In 1981 the total Asian born population in Australia was 0.28 million, this had grown to 0.72 million in 1991 and 1.12 million in 1999 (Australian Bureau of Statistics, 1994; Australian Bureau of Statistics, 1998; Australian Bureau of Statistics, 2001). In addition Asian cuisine is generally accepted by Australians of non-Asian background, creating consumer demand for new Asian fungi. The rich, woodsy taste and distinctive aroma of *G .frondosa* has ensured that it is adopted as a specialty culinary mushroom (Mushroom Growers' Association, 2001; Mushroom Museum, 2001).

The edible fungus *G. frondosa* is known by the common names maitake (Dancing Mushroom) (Mayuzumi and Mizuno, 1997; Stamets, 2000), Kumotake (Cloud Mushroom) (Stamets, 2000), Hen-of-the-Woods (Stamets, 2000) and the Dancing Butterfly Mushroom (Stamets, 1993a; Hobbs, 1995; Yamanaka, 1997). It is thought these references to butterflies and hens are relate to the petal like shape of the outer sections of the fruit body and that the allusion to dancing has resulted from a story, possibly apocryphal, of the dancing, engendered in a group of women who had eaten *G. frondosa* (Stamets, 2000).

Morphology

Whereas most edible fungi have a single pileus (cap with fertile layer) on a stipe (stem) *G. frondosa* has a unique shape formed by numerous overlapping pilei. The sporocarp, a large fleshy polypore of 10-30 cm in diameter, is made up of many branching stems at the end of which is a pileus of 3-7 cm diameter which overlaps with other pilei to form a mass of petaloid, or petal shaped, pilei (Bon, 1987; Jordan and Wheeler, 1995; Stamets, 2000) (Figure 1). On the underside of the pileus white pores hold spores of 4 x 6 µm in size. The pileus surface of young sporocarps is a dark grey-brown colour, which becomes a lighter grey-brown as the sporocarp matures and ages. Lighter coloured varieties exist with some becoming light yellow or whitish at maturity. The flesh of the mature sporocarp is always cream white, and has a firm and supple texture (Bon, 1987).

Distribution and natural habitat

Grifola frondosa (*sensu stricto*) is indigenous to north-eastern regions of Japan, temperate hardwood regions of China and Europe (Mizuno and Zhuang, 1995), and the north-eastern and mid-atlantic states of the USA (Stamets, 1993a). It is a white-rot saprophyte, decomposing (hemi)cellulose and lignin of dead wood and is generally found at the base of deciduous hardwoods that are dying or dead due to other causes. The most common host species include *Fagus crenata* (beech), *Quercus serrate* and *Q. crispula* (oak), *Acer* (maple), *Ulmus* (elm) and *Nyssa* (black gum) (Stamets and Chilton, 1983; Bon, 1987; Cortecuisse and Duhem, 1995; Mayuzumi and Mizuno, 1997; Stamets, 2000) but it has also been found on "Douglas fir" (Gilbertson and Ryvarden, 1987).

Taxonomic status

Grifola frondosa (Dicks: Fr.) S.F. Gray (Gray, 1821) was previously described as *Polyporus frondosus* (Dicks.: Fr.) (Fries, 1821) and earlier was known as *Boletus frondosus* (Dicks.) (Dicks, 1785). Whilst *G. frondosa* is generally accepted as being in the family Polyporaceae (Corner, 1989; Shen, 2001) it has also been placed in the family Coriolaceae (Hawksworth *et al.*, 1995), Grifolaceae (Jülich, 1981) and most recently Schizophyllaceae in the taxonomic database of the National Center for Biotechnology Information (GenBank®, Sept.7 2001). At species level Gilbertson and Ryvarden (1987), Farr *et al.* (1989) and Zhao and Zhang (1992) recognise *G. frondosa* as the only species of *Grifola.* Others consider there are a number of species in the genus such as *G. albicans* sp. nov. (Corner, 1989), *G. armeniaca* sp. nov. (Corner, 1989), *G. badia* (Gray, 1821), *G. campyla* (Cunningham, 1965), *G. cristata* (Gray, 1821), *G. eos* sp. nov. (Corner, 1989), *G. gargal* (Singer, 1969), *G. gigantea* known as "Choreimaitake" (Mizuno and Zhuang, 1995), *G. lucida* (Gray, 1821), *G. platypora* (Gray, 1821), *G. sordulenta* (Singer, 1969), *G. umbellata* known as Tonbimaitake (Donk, 1974; Corner, 1989; Mizuno and Zhuang, 1995), and *G. varia* (Gray, 1821; Shen, 2001).

In the 19th century Cooke (1892) indicated that *G. frondosa* was found in Australia but-Cunningham (1965) notes there are no Australian specimens of *G. frondosa* at Kew herbarium to substantiate this information and suggests the specimen probably referred to *G. berkeleyi*, now *Bondarzewia berkeleyi*, and not *G. frondosa*. Sporocarps found in Australia have been listed under the species *G. campyla* but they have now been moved to *Ryvardenia campyla*. *Grifola frondosa* grows on deciduous trees so it is unlikely to be found in Australia where the dominant vegetation is evergreen *Eucalyptus*.

Molecular phylogenetic analyses of rDNA, β-tubulin and a combination of datasets by Shen (2001) identified two major clades of *G. frondosa*, I - U.S. and II - Asian. These were found to be distinct groups, which have evolved independently (Shen, 2001). A third group, containing isolate WC493 from Norway, was found to share a common ancestor with Asian isolates on combined and partial β-tubulin gene sequence data but ITS rDNA sequence data indicated this isolate had a closer relationship with US isolates. Further work with isolates from a wider range of countries and sites, is needed to clarify this situation. Shen (2001) also indicated which of the isolates in the study completed a crop cycle. The commercial implication of these findings is that future breeding work can use isolates of *G. frondosa*, which are known to crop and have been genetically grouped.

Strains of *G. frondosa* are available from both public and private institutions, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ - German Collection of Microorganisms and Cell Cultures), CBS (Centraalbureau voor Schimmelcultures), FungiPerfecti and MycoSource Inc.. Strains have been indiscriminately sold and/or passed between both curator and growers. Information on origin of a particular strain may be unavailable or misleading due to poor record keeping, renaming, the passage of time and changes in personnel. This can create the situation where strain X is obtained from one source and is believed to be different from strain Y obtained for another source when in reality the strains are identical. Shen (2001) illustrates another problem area, that of mis-naming, by finding that isolate WC484, stored in the American Type Culture Collection as *G. frondosa*, was *Spongipellis detectans*.

Medicine

There is increasing interest in the medicinal benefits of edible fungi in the western world. At the same time mainstream medicine is accepting the use of mushrooms as a medicinal product (Wong 1996). History shows that *G. frondosa* has been used for its medicinal properties in China for almost 2000 years (cited in (Shen, 2001)).

In recent years the publication of research on medicinal properties of edible fungi has been steadily increasing. During the 8 year period 1983-1990 and 1991-1998 an average of 23.4 and 30.8 articles, respectively, were published each year. In the 3 year period 1999-2001 the number of articles has again significantly increased with an annual average of 44.7 articles.

Claims have been made that mushroom-derived compounds such as lentinan, from *Lentinula edodes* (Maeda *et al.*, 1971; Matsubara *et al.*, 1980; Ooi and Liu, 2000), and grifolan, from *G. frondosa*, have medicinal properties (Nanba *et al.*, 1987; Kurashige *et al.*, 1997; Ross *et al.*, 1999; Wasser *et al.*, 2000). Extracts from *G. frondosa*, entire or part of fruit body, have been stated as having anti-cancer (Nanba *et al.*, 1987; Mizuno and Zhuang, 1995; Miura *et al.*, 1996; Nanba and Kubo, 1997; Kodama *et al.*, 1999), immunopotentiating (Mizuno and Zhuang, 1995), immunomodulating (Minato *et al.*, 2001), anti-diabetic (Kubo *et al.*, 1994; Kubo and Nanba, 1996; Horio and Ohtsuru, 2001), anti-cholesterol (Kubo and Nanba, 1997; Fukushima *et al.*, 2001), hepatoprotective properties (Ooi, 1996b). It has also been stated as having improved the health of HIV patients (Nanba *et al.*, 1999). Claims have been made that combining β-glucan, extracted from the fruit body of *G. frondosa*, and indomethacin, a nonsteroidal anti-inflammatory drug, induce lethal toxicity in mice (Yoshioka *et al.*, 1998). Further work examining combination therapy, dosage and form of β-glucan is needed to clarify this interaction.

Markets

World production of cultivated mushrooms has been increasing in the last two decades (Table 1). During this period there has also been a shift in the mix of mushroom varieties being produced. The button mushroom, *Agaricus bisporus*, moved from its position of supplying 72 % of the world's mushrooms in 1981 to 31.7 % in 1997 without a decrease in production. In 1995-96 the USA supplied 7 % of world mushrooms, including all varieties, and had a value of US$ 758 million. At the same time production of specialty mushrooms in the USA accounted for 10 % of world production value, or over US$ 28.9 million. Internationally this reflected an average 15 % increase in specialty mushroom production between the years 1987-96 and a 3 % increase over the previous season (Phelps and Greenan, 1996). By 1998-99 the production value of specialty mushrooms in the USA had reached US$ 36.18 million.

Table 1 Trends in world production of cultivated mushrooms, by fresh weight (1981-1996)

Year	Total	Increase	Button mushroom*		Other**		Production of *Grifola frondosa*	
	'000 tonnes	%	'000 tonnes	%	'000 tonnes	%	'000 tonnes	% of other
1981	1257 [1]		900 [1]	72	357.2	28	- [1]	-
1986	2182 [1,] 2176 [2]	43	1227 [1] 1215 [2]	56	955 961	44	- [1]	-
1990	3763 [1]	42	1424 [1]	38	2339	62	7 [1]	0.30
1991	4264 [3]	12	1590 [3]	37	2674	63	7.6 [3,4]	0.28
1994	4909.3 [1]	13	1846 [1]	37.3	3063.3	62.3	14.2 [1]	0.46
1997	6158.4 [1]	20	1955.9 [1]	31.7	4202.5	68.3	33.1 [1]	0.79

* *Agaricus bisporus* ** All mushrooms other than *A. bisporus;* 1, (Chang, 1999); 2, (Royse, 1997b); 3, (Ooi, 1996a); 4, (Chang, 1993)

Cultivation

Under commercial cultivation saprophytic fungi are generally grown on a substrate based on straw and manure. White rot fungi, such as *G. frondosa,* grow on wood based substrates with sawdust being a suitable commercial medium. In Japan, China and Korea, fungi have been cultivated outdoors in response to the natural rhythms of the seasons for centuries, whilst in the USA outdoor cultivation of mushrooms is seen as an environment friendly method of producing fungi (Stamets and Chilton, 1983). For centuries *Agaricus bisporus* has been cultivated indoors, but many specialty fungi, which have generally been grown outdoors in Asia, have only been grown indoors relatively recently with a technique being adopted and adapted in the latter half of the 20th century.

Three methods have been used for the production of *G. frondosa* : outdoor bed culture, bottle culture and bag culture. Outdoor bed cultivation was the first method of artificial cultivation, and it is still a low cost and easy handling method. Whilst this method produces high quality fungi the disadvantage is that only one crop can be produced over the few months when environmental conditions are suitable (Mikawa, 1990). The bottle method can be readily mechanised and mass production, in automated factories, is said to have commenced in Japan in 2000 (pers. comm Kawashima 2002). This method reduces labour costs, but the small quantity of substrate, due to capacity limitations of the container, results in reduced crop size. Bag culture, which results in a pseudo-log, was introduced in the last 10 years and is now commonly used in Japan (Mayuzumi and Mizuno, 1997), USA (Stamets, 1993a; Royse, 1997a) (Ellor pers. comm. 2000) and Germany (Kirchhoff, 1996). The bag method involves more human participation than bottle culture but it is the most popular method for cultivation. This is due to the ease of substrate preparation and inoculation, and the larger sporocarps produced due to the greater substrate quantities.

Nutriceutical aspects

Edible fungi are generally thought of as being in one of two groups, European or Asian, with the latter often being attributed with having nutriceutical properties. Cultivated Asian fungi are: *Lentinula edodes* (shiitake), *G. frondosa* (maitake), *Auricularia judea* (Jew's ear fungus, Jelly fungus), *Flammulina velutipes* (Velvet Shank, Enoki) *Cordyceps sinensis* (Caterpillar fungus), *G. lucidum* (Reishi) and *Hypsizygus marmoreus* (Bunashimeji). Within this group *G. frondosa* is recognised as not only an excellent edible fungi but also as one having nutriceutical properties. Wasser and Weis (1999) state that *G. frondosa* has been commercially developed as a drug or dietary supplement purported to have the following medicinal properties: anti-viral, anti-bacterial and anti-parasitic, blood pressure regulation, cardiovascular disorders, anti-diabetic, immunomudulating. Non commercial products are stated as having therapeutic effects: antifungal, hepatoprotective and treating chronic bronchitis. Both nutritional and medicinal attributes are credited to *G. frondosa* and it is sold in various forms: fresh, dried, capsules and tea (Mizuno and Zhuang, 1995). Stamets (1993) and Royse (1997) indicate that *G. frondosa* is available in these forms internationally. Various companies such as Fungi Perfecti (USA), Gourmet Mushrooms and Mushroom Products (USA), Maitake Products Inc. (USA), GNC Co. (UK) and Immunogenic Products (Japan) use the internet to sell dried products for the gourmet food, medicinal and nutriceutical markets.

Nutrition

Fungi are known to provide beneficial nutrients to humans in the form of B-complex vitamins, riboflavin, niacin, thiamin, folic acid, pantothenic acid, and vitamins C and D. In addition they are a good source for protein and the minerals selenium, potassium and copper (Mushroom Growers' Association, 2001). *Grifola frondosa* has a protein content of approximately 27 % by dry weight, (Stamets, 1993a) is low in nucleic acid content and contains a high proportion of unsaturated fatty acids. It also contains a range of vitamins including B1, B2, C, D and niacin, and minerals/metals such as magnesium, iron, calcium and phosphorous (Table 2).

Table 2 Nutritional breakdown of 100 g of *Grifola frondosa*

Vitamins	B1	1.5 mg
	B2	1.6 mg
	Niacin	54 mg
	C	63 mg
	D	410 IU
Minerals/metals	Magnesium	67 mg
	Iron	0.5 mg
	Calcium	11.0 mg
	Phosphorus	425 mg
Protein		27 % by dry weight

Medicine

The four top medicinal fungi are *G. lucidum, C. sinensis, L. edodes* and *G. frondosa*. Since 1990 there have been 114 research papers on medicinal aspects of *G. frondosa* with the more well known medicinal fungi *G. lucidum, C. sinensis and L. edodes* having 300, 140, 154 papers respectively. In *G. frondosa* a primary polysaccharide, the MD-fraction, β1, 6-glucan having a β1, 3-side chain, has been

found and is known as grifolan. Current research has shown that the previously intangible health benefits of consuming *G. frondosa* can now be partially explained by the presence of β-glucan and grifolan**.** This is a significant step forward in our knowledge of the active components in *G. frondosa*, but further work clarifying the mechanism by which these components work, the diseases and symptoms against which they are effective and the extent to which patients benefit is required.

Grifolan has been reported as potentiating and activating helper T cells, cytotoxic T cells, delayed-type hypersensitive T cells, natural killer cells and macrophages in the treatment of cancer (Kodama *et al.*, 1999). Administered orally or by i.p. to mice *G. frondosa* has been found to inhibit breast cancer (MM 46 carcinoma), lung cancer (Lewis Lung carcinoma) and liver cancer (MH-164 carcinoma) (Kodama *et al.*, 1999). A similar response was found when 100-250 mg/day of MD-fraction containing 3-6 g *G. frondosa* powder and 5-10 mg vitamin C was administered to human cancer subjects with stage III-IV cancer. Improvement in the cancer-immune response and regression in size of tumours were reported in subjects with lung cancer (64 %), breast cancer (70 %) and liver cancer (6 %). Treatment with chemotherapy plus *G. frondosa* components has been stated as improving cancer regression rates to 70-83 % "cure" (Nanba *et al.*, 1987; Kodama *et al.*, 1999).

Anti-viral activity has been explored by Nanba *et al.* (1999) who administered 3-6 g of *G. frondosa* powder with 5-10 mg vitamin C for one year to 35 HIV patients. It is reported that the $CD4^+$ cell count increased in 20 subjects and decreased in 8 subjects, the viral load decreased in 10 subjects and increased in 9 subjects. It is unclear whether all subjects reported their reaction to the treatment but 20 subjects reported symptom improvement. Whether 90 % of symptoms improved or 9 out of 10 people improved is unclear. An 85 % increase in the sense of "well being" was attributed to an increase in helper T cell and $CD4^+$, plus a decrease in viral load. This treatment is also stated as having a positive effect on candida-infection and on Kaposi-Sarcoma (Nanba *et al.*, 1999). Whilst these results are encouraging further work is needed to support this study and clarify findings.

In 1994 Kubo found that *G. frondosa* affected non-insulin dependent diabetes mellitus. Further investigation suggests this anti-diabetic activity is related to metabolism of absorbed glucose rather than inhibition of glucose absorption (Kubo and Nanba, 1996). Other benefits of *G. frondosa* consumption have been reported : lowered blood pressure (Adachi, 1988; Jong *et al.*, 1991; Mizuno and Zhuang, 1995; Kubo and Nanba, 1996; Kubo and Nanba, 1997) and reduced cholesterol (Kubo and Nanba, 1996; Kubo and Nanba, 1997). It has also been suggested that *G. frondosa* reduces the symptoms of chronic fatigue syndrome (Ostrom, 1992) but this has yet to be proven. The primary polysaccharide, the MD-fraction, β1, 6-glucan by which *G. frondosa* affects blood pressure has been studied by Choi *et al.* (1995) with cold water extracts of *G. frondosa* found to contain high levels of angiotensin I-converting enzyme (ACE) inhibitors.

It should be noted that a significant quantity of information about the health benefits of *G. frondosa* is untested. Studies are criticised as lacking scientific rigour, which generally reflects a different approach between scientists of different backgrounds.

The use of the term "cure" by nutriceutical proponents rather than the accepted "remission" when discussing cancer raises doubts about the validity of the science behind research that has been conducted with cancer patients. Research undertaken in Asian countries would be viewed more favourably by western medicine if the same terminology and similar methodology was used when presenting research findings. Rigorous scientific research into the potential medicinal properties of *G. frondosa*, the mechanisms by which these work and the potential benefits identified is needed. This, in turn, would enhance the acceptance level, by western medicine practitioners, of the use of fungi with medicinal properties. Criticisms, often raised, are that studies' testing the efficacy of *G. frondosa* focuses on cancer patients with a terminal condition and that no control group is established within these patients. Arguments against this are that terminal cancer sufferers are desperate for remission or cure and will try any means to control their cancer. They are therefore willing to take part in trials with medication of unknown efficacy, in this case *G. frondosa*. The setting up of a control group, given a

placebo, is considered to be playing with people's lives and therefore morally inexcusable to researchers working in this area.

Market background

Grifola frondosa is currently grown in USA, Korea, China and Japan. Detailed market and production information on "specialty mushrooms" in the USA is readily available (United States Department of Agriculture, 1999) and figures for Japan can be calculated from market information. Production and market value information from most Asian countries, the largest producers of *G. frondosa*, is not easily accessible or if available can be difficult to interpret with information provided on the basis of a country's based internal districts, and little or no explanation of terms is supplied.

A method for growing *G. frondosa* was developed in Japan between 1975 and 1980 (pers. comm. Kawashima 2002) with the first commercial cultivation commencing in Japan in 1981 (Takama *et al.*, 1981; Mayuzumi and Mizuno, 1997) with the production of 325 t. Over the next nine years world production increased to 7,000 t (1990) and more than doubled by 079 % in the following 4 years to 14,400 t (1994) (Royse, 1997b; Yamanaka, 1997). Over the same period *G. frondosa* increased its share of world production, other than *A. bisporus* by 0.79 % with Japan being the largest producer (Chang, 1996). In 2001 Japan was still the major producer and consumer of this fungus which is grown primarily in the provinces of Niigata, Nagano, Gunma and Shizuoka; (Royse, 1997b); (Matumoto, pers.comm. 2000) and sold on the local market (Daimatsu, pers.comm. 2000). It is not possible to determine the production of *G. frondosa* in most countries as hard data is unavailable on specific species of specialty fungi.

Diverse forms of market information makes it difficult to effectively compare and contrast customer profiles and to identify market opportunities for Australian growers. It is known that *G. frondosa* is a popular product with openings to supply overseas markets out of season, in particular Asia. In Australia the consumer markets of Asian population, cuisine and "health" are currently unexplored but provide significant opportunity. Whilst *G. frondosa* is not currently cultivated in Australia, market trends in other western countries, such as the USA, indicate there is an opportunity for the specialist mushroom industry to create and meet demand for fresh *G. frondosa*. In addition, demand for nutriceutical products, and the restrictions placed on imported products, indicate there is potential for local growers to establish links with manufacturers and supply this market.

Description of sporocarp development

During sporocarp development, four stages are recognised: brain, cauliflower, antler and, cluster-flower /fruiting cluster (Figure 1). As the sporocarp develops, the colour of the fungus changes from a dark grey to a lighter colour and may, at maturity, be greyish white, light brown/yellow, light grey or white, depending on the strain.

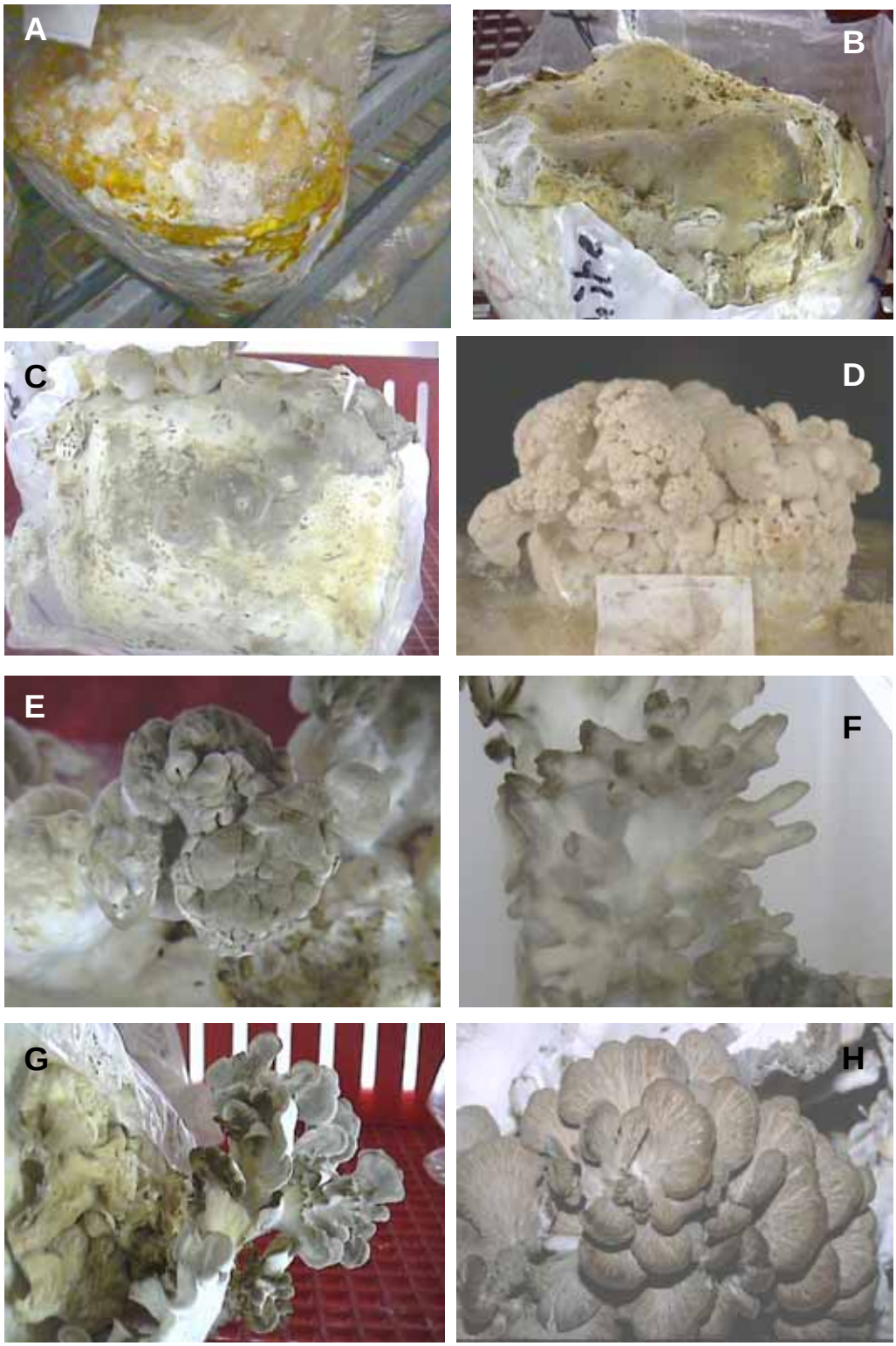
The cultivation of *G. frondosa* in bags makes it easier to control substrate moisture, relative humidity (RH) and sporocarp formation. After primordia form Stamets (1993) recommends the bag is narrowly opened at the top to encourage a single forking primordium but the stage of sporocarp formation is unclear in this and other works (Royse, 1997b; Shen and Royse, 2001). In later work Stamets (2000) describes removing the top of the bag when grey primordial mounds form, at 45-60 days after inoculation, and punching holes in the bottom of the bag to enable some gas exchange and ensure adequate drainage. No other reference to this method has been found.

Suggested temperatures at this stage of development vary considerably. Stamets (1993) and Kirchoff (1996) recommend 13-16 °C, which contrasts with Rinsanka (1980) who recommends 17-22 °C. Incubating *G. frondosa* outside the optimal temperature range affects sporocarp development with initials failing to differentiate and primordia aborting on the substrate surface, resulting in a plateau of short folds (Stamets, 2000).

A reduction in CO_2 levels and increase in light levels, once stems have grown and elongated to about 5 cm, encourages petal-shaped caps (Stamets, 1993a). At the same time it is important to ensure that RH and CO_2 levels are maintained above critical levels to encourage the opening of the petal-like structures characteristic of *G. frondosa*. At this stage of cultivation authors differ in their use of RH with ranges from 60-75 % (Chalmers, 1994), 90-95 % (Kirchhoff, 1996) and 85-90 % (Rinsanka, 1980; Stamets, 1993a; Mayuzumi and Mizuno, 1997). Stamets (1993) and Ellor (pers. comm. 2002) suggest fluctuating humidity between 80- 95 % ensures sporocarps are not deformed by excess moisture. At this stage both Stamets (1993) and Kirchhoff (1996) recommend CO_2 levels should be <800-1000 ppm with up to 8 air exchanges per day.

Stages in sporocarp development of *G. frondosa* are mycelial mat, exudate, brain, cauliflower, and petal. After full colonisation of substrate undifferentiated mycelium on the substrate surface rapidly changes to a dark grey amorphous mass which soon becomes contoured and develops into dark grey-black primordial mounds. These primordial mounds develop into ball like structures known as “brains”. A yellow or tan coloured exudate may be present both before and after this stage. The convoluted folds of the brain stage expand forming a cauliflower shape and form. This stage has highly branched lateral stems that elongate to form antlers, each of which develops a pileus. The pileus on each antler develops so that overlapping petal-shaped caps, which cluster along the elongated stems, form the mature fruiting cluster sporocarp (Stamets, 1993a; Chen, 1999b).

Figure 1 Stages in the production of *Grifola frondosa*



A, Exudate forming on surface of substrate*; B, Mycelial mat on substrate surface*; C, Grey folds forming brains structure*; D, Cauliflower formation*; E, Detail of cauliflower structure*; F, Antler formation*; G, Petals of fruit body forming ; H, Mature fruit body ready for harvest * Top of bag has been removed for photograph.

Factors influencing sporocarp production

The cultivation process for sporocarp formation of any mushroom encompasses a series of known stages: spawn run, initiation, sporocarp formation and harvest. Strain, substrate, environment and the interaction between these parameters at different times in the cultivation cycle affect all the stages. Whilst literature provides detailed information on these parameters for *A. bisporus* the conditions required for specialty mushrooms are often poorly described with conflicting information provided by authors. This reflects the comparatively small amount of research that has been undertaken with specialty fungi, in particular maitake. During the period 1990 to 2001, 538 articles have been published on edible fungi and substrate. *Agaricus bisporus*, the common button mushroom, is covered in 125 articles and the well known specialty mushroom *L. edodes Lentinula edodes* (shiitake) has 40 articles but only 5 articles refer to *G. frondosa*. This pattern is repeated with information on environmental conditions for *A. bisporus* having 131 articles, *L. edodes* 23 and *G. frondosa* 1. Strain information on *A. bisporus* can be found in 149 different articles whereas strain information on *L. edodes* and *G. frondosa* is included in the same articles that discuss environment. This highlights the difficulty in interpreting interactions both between, and within strains, substrates and environments.

Strain

The selection of a suitable strain for cultivation is not only based on the appearance and yield of the sporocarp produced but also on the ability of the fungus to colonise substrate, the environmental conditions available for cultivation and the method by which cultivation will be undertaken. Stamets (2000) evaluates strains against 28 criteria, those most important to *G. frondosa* are: recovery after inoculation, genetic stability, growth rate, quality of mycelial mat, adaptability to substrate, speed of colonisation to fruiting, photosensitivity, cold shock requirements, appearance (form, size and colour), storage capability, flavour, texture, aroma, nutritional composition and production of medicinal compounds.

Vigorous hyphal growth on agar is essential for the preparation of high quality inoculum, the first stage in the production cycle. Zenghai, Qing, Deping and Changchao (1996) found that *G. frondosa* grew well in potato-bran and potato-composite medium but poorly in potato-dextrose agar. Additional media are suggested by Stamets (2000): malt extract yeast agar, malt extract yeast peptone agar, potato dextrose yeast agar or dog food agar, with pH indicated as being between 5.5 and 6.8. This disagrees with Miyauchi, Kon, Yamauchi and Shimomura (1998) who found that *G. frondosa* grew well on YMG medium at pH 4.0 and 5.0 but poorly at pH 6.0 and higher. Unfortunately Miyauchi *et al.* (1998) does not describe YMG and it can only be assumed that this represents Yeast Malt Glucose medium but the proportions of each component are unknown.

Chen, Huang and Han (2001) indicates that strains of *G. frondosa* differ both morphologically and in their ability to produce marketable sporocarps but does not expand on suitable strains for production under different conditions. Only one study, conducted by Shen in 2001 encompasses strain and adaptability to substrate, biological efficiency (BE) and speed of colonisation-fruiting. The results indicate nine out of 23 isolates tested did not fruit, nine consistently fruiting isolates had significant differences in BE and quality. Of these, four had the highest BEs and quality but also had a longer cropping cycle. This work identified strains WC828, MO36, MO37, M040 as producing high quality sporocarps with a high biological efficiency (BE). Of these only MO40 was identified as a commercial strain.

Nutritional composition and production of medicinal compounds in the sporocarp have been studied in isolation and not on the basis of strain. Nor have there been studies, of any depth, investigating : recovery after inoculation, genetic stability, growth rate, quality of mycelial mat, photosensitivity, cold shock requirements, appearance (form, size and colour), storage capability, flavour, texture or aroma of different strains.

Substrate

Cultivated fungi are generally saprophytes, utilising substrate as primary or secondary decomposers. Primary decomposers are the first fungi onto the substrate, usually wood, and have a rapid growth rate. In this group are found woodland species *L. edodes*, *Pleurotus* spp. (Oyster), *Flammulina velutipes* (Enoki), *Hypsizygus marmoreus* (Buna-shimeji), *Ganoderma lucidum* and *G. frondosa.* Secondary decomposers, such as *Lepista nuda* and *A. bisporus* require a substrate that has been partially broken down by other microorganisms. In commercial cultivation a composted substrate is used.

Specialty fungi living off wood can be brown-rot fungi, such as *L. edodes*, degrading cellulose and hemicellulose or white-rot fungi, such as *G. frondosa*, degrading both these sources and lignin. Hyphae of *G. frondosa* grows readily on sterilised hardwood sawdust and has been grown on sawdust of various tree species such as *F. crenata*, *Q. serrate, Q. crispula*, *Alnus* spp. (alder) and *Populus* spp. (poplar) (Rinsanka, 1980; Stamets, 1993a).

Natural nutritional sources such as wheat bran, corn meal, rice bran or white millet are commonly added to *L. edodes* substrate. These have been added to sawdust at various ratios to encourage *G. frondosa* colonisation and the formation of high quality sporocarps (Appendix 2). A commercial grower would view the best additive as being one that not only produces high quality *G. frondosa* in a short period of time but one that is also cheap and readily available locally. Simple trials using maize meal, rice bran and wheat bran have been undertaken both overseas and in Australia. Sporocarps have been produced on sawdust based substrates with additives of 20 % corn meal (Kirchhoff, 1996), 10 % white millet (Chalmers, 1994), 20 % wheat bran or 20 % rice bran (Takama *et al.*, 1981; Stamets, 1993a; Lee, 1996; Royse, 1996) and a combination of 10 % wheat bran (*Panicum miliaceum*) and 20 % rye (*Secale cereale*) (Shen and Royse, 2001). Shen (2001) is the only author to provide complete botanical names for these additives. It is assumed that other authors refer to *Zea mays* (corn meal), *Oryza* spp. (rice bran), but a guess must be made as to whether *Echinochloa* or *Panicum* spp. (white millet) and *Triticum* spp. or *Panicum miliaceum* (wheat bran) are the botanical species trialed by other researchers. Research in Australian indicates that wheat bran (*Triticum sativum*) and rice bran (*Oryza sativa*) are suitable supplements (data unpublished Stott 2001). Additives can also encourage competitor fungi or have a detrimental effect on fungal growth. Rinsanka (1980) reported the presence of too much rice bran in the substrate was detrimental to sporocarps.

Gypsum ($CaSO_4$) is usually added to substrate prior to sterilisation, generally at a rate of 0.2 %. This encourages grain separation and also provides small quantities of calcium and sulphur. Only one study discusses the effect of adjusting substrate pH, finding there was no marked effect on yield of *G. frondosa* on substrates having pH 4.9-5.9 (Yoshizawa *et al.*, 1997). Further studies into the effect of substrate pH on colonisation and fruiting are required to determine the optimal range.

Moisture content of substrate is recommended as being between 60 and 65 % (Stamets, 1993a; Chalmers, 1994; Kirchhoff, 1996; Mayuzumi and Mizuno, 1997) but one author recommends a moisture content as high as 67.5 % in substrate (Chung and Joo, 1989). Rinsanka (1980) states that substrate that allows water to seep between the fingers when held is too moist and that excess moisture is undesirable in substrate. Unfortunately no percentage moisture content is attributed to this simple test. Indeed, there can be considerable variations in moisture content using this test as it depends on the strength of the individual and the amount of pressure used when squeezing substrate in the hand.

The number of hours required to sterilise substrate is a function of composition, volume, moisture content and whether cultivation is in bags or bottles. Recommended parameters for sterilisation often omit a value for pressure or temperature, making it difficult to easily determine a suitable sterilisation procedure under commercial cultivation. Sterilisation of substrate for *G. frondosa* is performed over a range of times, temperature and pressures: from 2-6 hrs at 120 °C (Kirchhoff, 1996; Mayuzumi and Mizuno, 1997); 7 hrs at 110 °C (Mayuzumi and Mizuno, 1997); 3 hrs at 117 kPa (Chalmers, 1994); and 4-5 hrs at 103 kPa (Stamets, 1993a). Again, there have been no studies to determine the optimal

sterilisation conditions for substrate, made up of selected sawdust and additives, which will be used for the cultivation of *G. frondosa*.

Raw substrate materials are extremely variable and depend largely upon locally available products. It is therefore important to find an additive that is best suited to the production of *G. frondosa* which Australian specific. To ensure a full understanding of substrate for *G. frondosa* investigation of suitable sterilisation conditions is needed, as is a more thorough study of desirable moisture content and suitable substrate pH.

Correlation has been found between the composition of sporocarps and the composition of the substrate on which they were produced. A significant correlation has been found between the protein content of sporocarps of *P. ostreatus* and the N content of the substrate but no such correlation was found with *G. frondosa* (Kawai *et al.*, 1994). In the same work sporocarps of *G. frondosa* were found to have higher concentrations of minerals than *P. ostreatus* when grown on the same substrate, respectively: K 16.51:3.2, Na 6.59:6.59, Mg 1.24:-, P 4.04:1.70, Cu 3.37:1.90, Zn 4.10:2.38 and Cd 9.26:2.75. A significant correlation was found between Cu contents of the *G. frondosa* sporocarp and the substrate and between Cd contents of the *P. ostreatus* sporocarp and the substrate (Kawai *et al.*, 1994). Whilst these findings indicate that mineral transfer from substrate by *G. frondosa* differs from *P. ostreatus* it should be noted that sporocarp data had large significant error.

The effect of substrate and its interaction with strains or its effect on taste and texture is not available in the public domain. Growers setting up in *G. frondosa* production will need to carry out stringent trials on the interaction of strain x substrate x environment in order to ensure an optimal cultivation method is developed. This work is currently being undertaken by the authors on eucalyptus sawdust based substrate.

Temperature

The reduction from optimum temperature triggers fruit body initiation in many fungi (Stamets, 2000) and slows vegetative growth. Examples of this are : *F. velutipes* (>21 °C to <10 °C), *L. edodes* (>21 °C to <16 °C), *P. ostreatus* (>24 °C to <16 °C) and >20-25 °C to <4-18 °C in *G. frondosa*.

Relative humidity

Fungi require a certain amount of moisture to metabolise, grow, and reproduce. The management of humidity is critical to *G. frondosa* sporocarp production as low humidity may result in the death of primordia (Royse, 1997a), whilst overwatering is known to encourage wet patches on sporocarps. Over supplementation of sawdust plus slightly excessive watering or humidity has also been found to encourage bacterial blotch (Stamets, 1993a).

During cultivation of *G. frondosa* recommendations for RH, an important environmental factor, vary between 70 % (Mayuzumi and Mizuno, 1997), 80 % (Kirchhoff, 1996) and 95-100 % (Stamets, 1993a). This difference in RH effects substrate moisture content, rate of substrate colonisation by hyphae and, sporocarp production and formation. The range between the lowest and highest RH is a significant value at 30 %. This may be a reflection of initial substrate moisture content, with a drier substrate requiring higher RH, or cooler temperature conditions in the growing room necessitating higher RH to achieve optimal conditions for production. In addition it is likely that different strains will require slightly different conditions for optimal production.

Light

Initiation, stem elongation and cap development of the fungal sporocarp are affected by light. The intensity and wavelength of the light is also critical as different species may have a differing response to the same light source.

Throughout spawn run light is not required (Chalmers, 1994) and can be detrimental, encouraging initiation before substrate is fully colonised and the mycelial mat formed. During primordia formation

prolonged exposure to high light levels will result in deformed sporocarps devoid of stems and/or the production of the spore producing hymenophore (Stamets, 2000). Diurnal light regimes have been used during initiation and fruiting but no trials specifically investigating the effect of the amount, and quality, of light on primordia formation of *G. frondosa* have been conducted. There are only vague descriptions in available literature of diurnal light:dark regimes, with only hours and no lux values generally provided.

Ventilation

The introduction of fresh air into the growing room supplies oxygen to the fungus, enhances the evaporation of moisture from the surface of the sporocarp and removes CO_2 . This removal of CO_2 at specific times in the cultivation cycle is essential as excess levels will be detrimental to sporocarp production. The conditions required by fungi at different stages can be expressed in terms of air changes per hour or parts per million CO_2. Isolates of *A. bisporus* have been found to produce good mycelial growth in the casing layer plus the best quality mushrooms with the highest yield when grown at 0.5 % CO_2 for seven days and 0.1 % CO_2 for 36 days during spawn run (Pahil *et al.*, 1993). The composition of substrate and its interaction with CO_2 has been found to affect formation of sporocarps (Dergham, 1993). Enriching the atmosphere with CO_2 before or during sporocarp development reduced yield of *A. bisporus* (Noble and Love, 1991). None of these areas have been investigated with *G. frondosa.*

Commercial cultivation methods

Information on cultivation is often poorly described or has some aspects of commercial-in-confidence. This results in cultivation methods that are difficult to interpret and implement in a commercial situation. The numerous temperature, humidity and light conditions described leads to further confusion for the new grower.

Outdoor bed cultivation

Commonly outdoor bed cultivation involves a substrate of deciduous broadleaf sawdust (76 %), corn bran (5-15 %), wheat bran (0-5 %) and silty loam (5 %) at pH 6-7 (Mayuzumi and Mizuno, 1997). The substrate is mixed and placed in a 2.5 kg polypropylene bag to form an artificial log. To the substrate is added 15-30 mg of mycelium liquid or grain inoculum. When outdoor temperatures reach around 15ºC, which is generally early spring, bags are placed on the ground in a plastic enclosed room. Humidity control is achieved by watering the floor and additional heating is used to control temperature as necessary. As the temperature increases outdoors the room is ventilated. The use of corn bran and soil makes the artificial log hard and compact, enabling easy bag removal when the bag is fully colonised. The colonised artificial logs are then buried next to each other in a well-drained field and covered with 3 cm of soil and leaves or black mesh sheeting (Mayuzumi and Mizuno, 1997) to prevent soil erosion by rain. This cover is also aimed at providing 85 % shade (Mayuzumi and Mizuno, 1997). Sporocarps begin to develop in early autumn when temperatures fall to 23 ºC but no upper temperature is indicated. By placing logs next to each other mycelium grows together forming a large mushroom bed which has been found to produce 1-1.5 kg of sporocarps per bed (Mayuzumi and Mizuno, 1997) but the mass of substrate is not stated. *Grifola frondosa* grown by this method are said to produce firmer and more durable sporocarps than either bag or bottle culture and are said to be similar to those found in the wild (Mayuzumi and Mizuno, 1997). Descriptions, in literature, of the cultivation method are unclear and lacking in detail with no readily available information on the environmental levels required for outdoor cultivation.

Bottle cultivation

Mayazumi and Mizuno (1997) describe bottle culture in polypropylene bottles, having a volume of 800-1000 mL, filled with 400g of substrate made up of 100 parts sawdust from deciduous broadleaf species, rice bran (15 %) and wheat bran (5 %). Whether the additive quantity is calculated by volume or weight is not stated. This is then autoclaved at 120 ºC for 6 h, allowed to cool and each bottle inoculated with 15 mL of mycelium but concentration of mycelium is not given. Inoculated bottles are

"incubated for 40 days at 23 ºC and 70 % humidity" and "maintained at a temperature of 17 ºC and 85 % humidity". It is assumed these parameters describe spawn run and fruiting conditions respectively. The room is well ventilated to ensure that high CO_2 levels do not effect cap development but no value is given to ventilation. A disadvantage of the bottle system is that the high humidity required for sporocarp formation often produces undesirable characteristics such as watery fruit bodies (Mayuzumi and Mizuno, 1997). Yield is usually 100-130 g per bottle on 400 g substrate.

Bag cultivation

Whilst bottle culture is used in some Asian countries the commercial trend in the USA is to use bag culture. Cultivation in bags started in Japan in 1980 (pers.comm. Kawashima 2002) with a patent for the bags issued in 1982 (Stamets and Chilton, 1983). The use of bag culture would also be attractive to Australian growers as this method is already used for production of *L. edodes* (Brown pers. comm. 2001). Rinsanka (1980) recommends 0.04 mm thick polycarbonate gusseted bags which are 20 x 27 cm long when folded and able to hold 1 kg substrate. Details of this method are described under sporocarp production.

Production cycle

Temperature, relative humidity, ventilation, O_2, CO_2, and light will vary during the different stages of the cultivation cycle. The environmental conditions required for the establishment of mycelium during spawn run will be different from those for initiation, pinning and fruiting. Whilst all these stages are affected by the same environmental factors the levels of each of these parameters are likely to be different at each stage.

Spawn run

Inoculation of substrate is by sawdust spawn, grain spawn or liquid spore solution on substrate surface (Mayuzumi and Mizuno, 1997). Colonisation of substrate by hyphae of *G. frondosa* passes through four recognisable stages : (1) undifferentiated white mycelia, (2) white mycelia with orange brown discolouration caused by exudate, (3) mycelial mat on substrate surface, and possibly (4) an uneven topography on the mycelial surface exuding yellow/orange-brown exudate (Chen, 1999a).

During spawn run the recommended temperature for *G. frondosa* is between 20-25 °C (Chung and Joo, 1989; Stamets, 1993a; Chalmers, 1994; Kirchhoff, 1996; Mayuzumi and Mizuno, 1997). This is a wide range of temperatures and it is likely that different strains will perform optimally at specific temperatures within this range.

The recommended CO_2 levels and air exchanges required during spawn run of *G. frondosa* would seem to vary considerably from CO_2 at <2,000 ppm (Kirchhoff, 1996) to 20,000-40,000 ppm plus 0-1 air exchanges per hour (Stamets, 1993a; Stamets, 2000). At the high levels recommended by Stamets it would be necessary to introduce more air exchanges in order to reduce CO_2 concentration in the growing room or a slowing in hyphal growth would occur.

Using bag culture, spawn run has been found to take from 28-35 days (Chalmers, 1994), 30-45 days (Lee, 1996; Royse, 1996; Royse, 1997b), 44-60 days (Stamets, 1993a) and 70 days (Mayuzumi and Mizuno, 1997). In bottle culture, Mayuzumi and Mizuno (1997) found that 40 days was sufficient for spawn run. These differences in length of spawn run reflect strain differences, substrate components and environmental conditions.

Mycelial mat

Some fungi form a tight mycelial growth, on the substrate surface, which is known as the mycelial mat. In *G. frondosa* this occurs towards the end of the spawn run appearing as whitish coloured mycelia on the surface of the substrate. As mycelia matures the mat discolours to deep yellow or orange brown, depending on the strain. At the same time the topography of the substrate surface becomes uneven and develops greyish coloured folds.

Initiation

Primordia initiation of many basidiomycetes is often triggered by one or several of the following stimuli: increased light, temperature reduction, lowered RH or lowered CO_2 levels, occurring in the growing room or sealed bag. *Grifola frondosa* is no exception as temperature reduction (cold shock) and intermittent low light is required for initiation.

Reports on temperatures required to induce primordia are conflicting. Chalmers (1994) and Rinsanka (1980) recommend a constant temperature of 20-25 °C and 22-24 °C respectively whilst cooler temperatures of 4 –18 °C (Royse, 1996; Mayuzumi and Mizuno, 1997; Royse, 1997b) or 10-15.6 °C (Stamets, 1993a) have also been indicated. Royse (1997) also suggests a cold shock at 4 °C for 1-4 days is beneficial to initiation. The temperatures suggested by Chalmers and Rinsanka would seem to be appropriate for spawn run but not a sufficient cold shock to induce initiation.

Recommended humidity levels at this time differ with Rinsanka (1980) indicating 70-80 % and Mayuzumi and Mizuno (1997) 85 %.

Descriptions of both timing and quantity of light required to initiate primordia is variable with suggestions of “intermittent” by Chalmers (1994) and “at the latter part of the culture period” by Mayuzumi and Mizuno (1997). No description of the status of the mycelial mat or exudate is included and we can only assume that light is introduced at the end of spawn run. The suggestion of light at 100-500 lux during primordia initiation and antler formation by Stamets (1993) is clearer and agrees with Wu (1997, cited (Chen, 1999b)). Miyauchi *et al.* (1998) found illumination by fluorescent light at <700 lux completely arrests mycelial growth of *G. frondosa.* This supports the light parameters already suggested as the cessation of mycelial growth is necessary before initiation can commence.

Mayuzumi and Mizuno (1997) indicate the growth chamber should be well ventilated, but the stage of the sporocarp is not mentioned. Stamets (1993) quantifies this by stating CO_2 levels should be between 2,000-5,000 ppm with 4-8 air exchanges daily at primordia and antler stages. Rinsanka (1980) and Ellor (pers. comm. 2000) indicate that *G. frondosa* should be protected from strong air movement. Therefore the balance between CO_2 levels and air exchange must be such that CO_2 parameters are met without detrimental air movement. There is conflicting information on the stage at which to ventilate the growing room. This is often indicated as being when droplets of reddish fluid exude from the fully colonised block of substrate (Kirchhoff, 1996) or much later when grey mounds of primordia, approximately 2.5-5 cm in diameter form (Chalmers, 1994). After primordia initiation Mayuzumi and Mizuno (1997) recommends the top of the bag should be cut open to ensure adequate ventilation to the fruiting block. Conversely Royse and Shen (2001) cut 20 slits in the top of for bag seven days after inoculation with two holes later cut in the top of the bag to expose the developing primordia. At the Mori Institute the top of the bag is not opened until primordia have developed.

Harvest

Sporocarps are harvested when the petals are fully mature. To prevent bacterial contamination of the sporocarp Chen (1999) recommends that misting is stopped one day before harvest. Whilst the sporocarp is large it is also extremely fragile and should be supported as the base is severed from substrate. It can then be wrapped in rice paper and stored at 1-2 ºC with a potential shelf life of 2 weeks (Stamets, 1993a; Chen, 1999b). Further information on the conditions required at harvest is unavailable.

Evaluation of cultivation methods

Biological efficiency

Yield of sporocarps can be expressed as biological efficiency (BE = (grams yield/grams initial substrate) x 100 %). The BE is influenced by substrate components, environment throughout the growing cycle and strain. BE of *G. frondosa* has been found to range from 7.5-15.5 % (Kirchhoff, 1996), 10-29 % (Stamets, 1993a), 14 % (Chalmers, 1994), 16 % (Mayuzumi and Mizuno, 1997), 16-27 % (Royse, 1997a), 25-32.5 % (Mayuzumi and Mizuno, 1997) with the highest being 35.8-39.5 % (Shen and Royse, 2001). Whilst BE is important the time taken from inoculation to harvest is equally important as gaining a higher BE but needing twice the number of days to achieve this is unsatisfactory to the commercial grower.

Extracellular enzyme analysis

Maitake is a member of the white rot fungi which are believed to be the most effective lignin-degrading microbes in nature. Investigation of extracellular enzymes found in substrate at different stages of colonisation can assess the utilisation of resources within substrate by a fungus. Activities of peroxidase, laccase and cellulase enzymes have been used to measure the utilisation of sapwood of *Chamaecyparis obtusa* (Japanese cypress) during colonisation. Differences were found in lignin and carbohydrate patterns, removed from cell walls, by the fungi *Pholiota nameko, Pleurotus ostreatus* and *G. frondosa* (Yoshizawa *et al.*, 1993).

A study of *G. frondosa* spent sawdust-bran substrate found that activities of proteinases, both acid and neutral (β-N-acetylglucosaminidase and β-1,3-glucanase) were high, whereas cellulase activity (CM-cellulase and avicelase), xylanase and amylase were low. Conversely fruiting bodies of *G. frondosa* had higher neutral proteinase than acid proteinase. The activities of chitinase and β-1,3-glucanase in the spent substrate were similar to those found in fruiting bodies whilst the hydrolytic enzyme activities in the spent substrate varied depending on the fungal strain (Terashita *et al.*, 1997).

Growing Maitake in Australia

Only broad environmental parameters are outlined in the literature for the cultivation of maitake. The interaction of substrate x additive x environment x strain is not known for maitake, but neither is this information available for other specialty mushrooms. The considerable differences between recommendations for cultivation parameters during the production cycle of maitake can be seen in the time taken for spawn run and recommended temperatures for fruiting. Available information cannot indicate one method as being optimal as the effect of substrate, cultivation method, production cycle and strain all significantly affect the time taken for production and final yield of harvest.

Overseas maitake is grown commercially on deciduous hardwood sawdust, whether this is the only suitable substrate is not known. In Australia *Eucalyptus* sawdust is a significant waste product of the timber industry and readily available for use as a commercial substrate. A study, with *L. edodes*, investigated a combination of pine, myrtle, sassafras and eucalypt substrates (Wilson and Chung, 1994) but this approach was not taken with maitake as eucalypt is the only economically viable substrate in Tasmania. An earlier study of maitake production on substrates of eucalypt woodchip/sawdust plus lipid or gypsum achieved full colonisation but did not produce fruit bodies (Mohammed, 1998). The optimal substrate additive required to make eucalypt sawdust a viable substrate alternative to deciduous hardwoods has yet to be determined. The development of an ameliorated eucalypt sawdust on which *G. frondosa* can be cultivated will have commercial implications as this will provide an affordable substrate to the fledgling Australian specialty mushroom industry.

No direct comparison of the interaction of strain x substrate x temperature on sporophore production was possible from the literature. The length of the cultivation cycle varies with information on the number of days for each part of the growing cycle expressed in different terms making comparisons

difficult if not impossible. Further work should address these issues with emphasis being placed on the response of specific strains, substrate components, environment and the resulting biological efficiency.

Anecdotal evidence, from wild collectors, suggests maitake is already growing in Australian forests, but this conflicts with opinions of plant pathologists and taxonomists. The fear of the unknown, that maitake will be pathogenic on *Eucalyptus*, has raised considerable problems with the Australian Quarantine Inspection Service currently denying import of further strains for commercial usage.

Although spawn grain is widely used in the mushroom growing industry a faster method, such as liquid spawn, also has potential. As available literature does not supply adequate information on the effects of temperature and pH on hyphal growth this study encompassed preliminary investigations looking at:

- optimal temperature and pH for hyphal growth
- growth of maitake in liquid culture.

Information on substrate and environmental parameters required for fruit body production of maitake is broad and differs between authors. Before a viable, and sustainable method, for the cultivation of maitake under Australian conditions can be developed the following areas must be investigated:

- Substrate - ameliorated eucalyptus sawdust
- Environment – relative humidity, cold shock, CO_2, ventilation and light.
- Initiation and brain formation
- Cauliflower formation
- The maturing fruit body
- Different cultivation parameters for different strains.

Preliminary studies on the development of liquid spawn of *Grifola frondosa*

Introduction

The mushroom industry currently inoculates both compost and sawdust substrates with spawn grain. Whilst this method is very effective in the largely mechanised inoculation of *Agaricus bisporus,* Australian growers of specialty mushrooms inoculate individual bags manually which is both time-consuming and expensive. The development of a liquid inoculum would enable specialty mushrooms growers to inoculate a large number of substrate bags cleanly and rapidly, reducing costs and increasing the level of production hygiene. Mayuzumi and Mizuno (1997) used a liquid spore solution to inoculate substrate, in a culture bag, by applying 30 mL to the surface of the substrate. The production of a liquid spore inoculum requires the spores to be collected from fruit bodies, a time-consuming activity which provides opportunity for contamination from the substrate, the fruit body and the environment. In contrast, a liquid hyphal inoculum has all stages under controlled and sterile laboratory conditions with larger quantities being rapidly prepared and easy to use.

Commercial strains are generally maintained on agar which should have optimal pH to ensure vigorous and consistent hyphal growth. Temperature for hyphal growth must also be optimal and this can be readily determined on agar substrate. The development of liquid spawn is a long process that requires understanding of optimal pH and temperature for hyphal growth. Determining these parameters on agar substrate can shorten this process. The aim of this initial trial was to investigate these parameters and determine when the log phase commenced.

Materials and methods

Fungal isolates

All isolates were obtained from overseas collections and imported under permits from the Australian Quarantine Inspection Service, and maintained, in rotation, on PDA and malt extract agar (MEA). Isolates FPC200, M74 and WC808 were identified in earlier work as having characteristics which made them both desirable to the marketplace and suitable for production by the industry partner (Mohammed, 1998) and were included in all trials. Isolates ATCC60304, IFO30661, NZF5198A, S49, TTI5003, WC659 and WC685 were used in temperature and pH trials but not in liquid culture.

Effect of temperature on hyphal growth

A 5 mm mycelial disc was taken from the growing edge of 10 day-old cultures on PDA and placed, with the hyphal surface in contact with the agar, at the centre of a 90 mm Petri plate containing PDA. Five replicates of each isolate were placed in the dark at 15, 20 and 25°C. The resulting fungal colony was measured along two perpendicular axes at 5, 7, 11, 14, 18 and 21 days after inoculation and the mean diameter of hyphal growth, excluding inoculum, determined.

Effect of pH on hyphal growth

Using the same method as that outlined for temperature trials each isolate was grown on PDA having a pH of 5.0, 5.5, 6.0, 6.5, 7.0 or 7.5. Three replicates of each isolate were incubated in the dark at 25°C. Hyphal growth was measured 5, 8, 12, 15 and 19 days after inoculation and the mean growth, excluding inoculum, determined.

Determination of exponential (log) phase in liquid culture

Inoculum for liquid cultures was grown on PDA, pH 5.5, in the dark at 25 °C until the agar surface was fully colonised. Precultures were obtained by inoculating mycelial fragments, which had been scraped from the agar surface of one Petri plate, into 200 mL potato dextrose broth (PDB; pH 5.5) and blending in a Waring blender six times, each for 10 seconds. Three static liquid cultures of each isolate were prepared in a 1000 mL Roux flask and incubated in the dark at 25 °C.

After 14 days incubation, isolate replicates were pooled and blended three times, each for 10 seconds, in a Waring blender. A sample of this solution was dried overnight and the mycelial mass per mL of each isolate determined. The remaining 400 mL of preculture was stored at 5 °C overnight. This was used to inoculate 250 mL erlenmeyer flasks containing 80 mL of peptone yeast glucose medium (PYG; pH 5.5), at a rate of 12.5 mg L^{-1}.

At seven-day intervals, two replicates of each isolate were removed and filtered through a fine nylon gauze. The resulting mycelial mass was placed in a pre-weighed foil boat, dried at 40 °C until constant weight was achieved and mycelial mass determined. Filtrate pH was determined with a Corning pH Meter 120.

Analysis

Data were analysed using GenStat® (Release 4.2, 5th ed. Lawes Agricultural Trust) software. ANOVA was used to analyse temperature data and REML (Repeated Measurements Analysis) to analyse pH data.

Results

Effect of temperature on hyphal growth

Due to hyphal growth reaching the perimeter of the Petri plate between days 18 and 21 no data was available for isolates S49 and WC659 at day 21. At 25 °C the majority of isolates consistently produced the greatest hyphal growth over time. Whilst incubation at 20 °C resulted in less growth than at 25 °C the difference in growth rate was not as marked between 20-25 °C as that between 15 °C and all other temperatures (Figure 2). Analysis of hyphal growth at day 18 found the effect of isolate, temperature, and their interaction, was significant (P<0.001).

Figure 2 **Hyphal growth of *Grifola frondosa* on PDA at various temperatures**
(Vertical for bars indicate ± standard deviation)

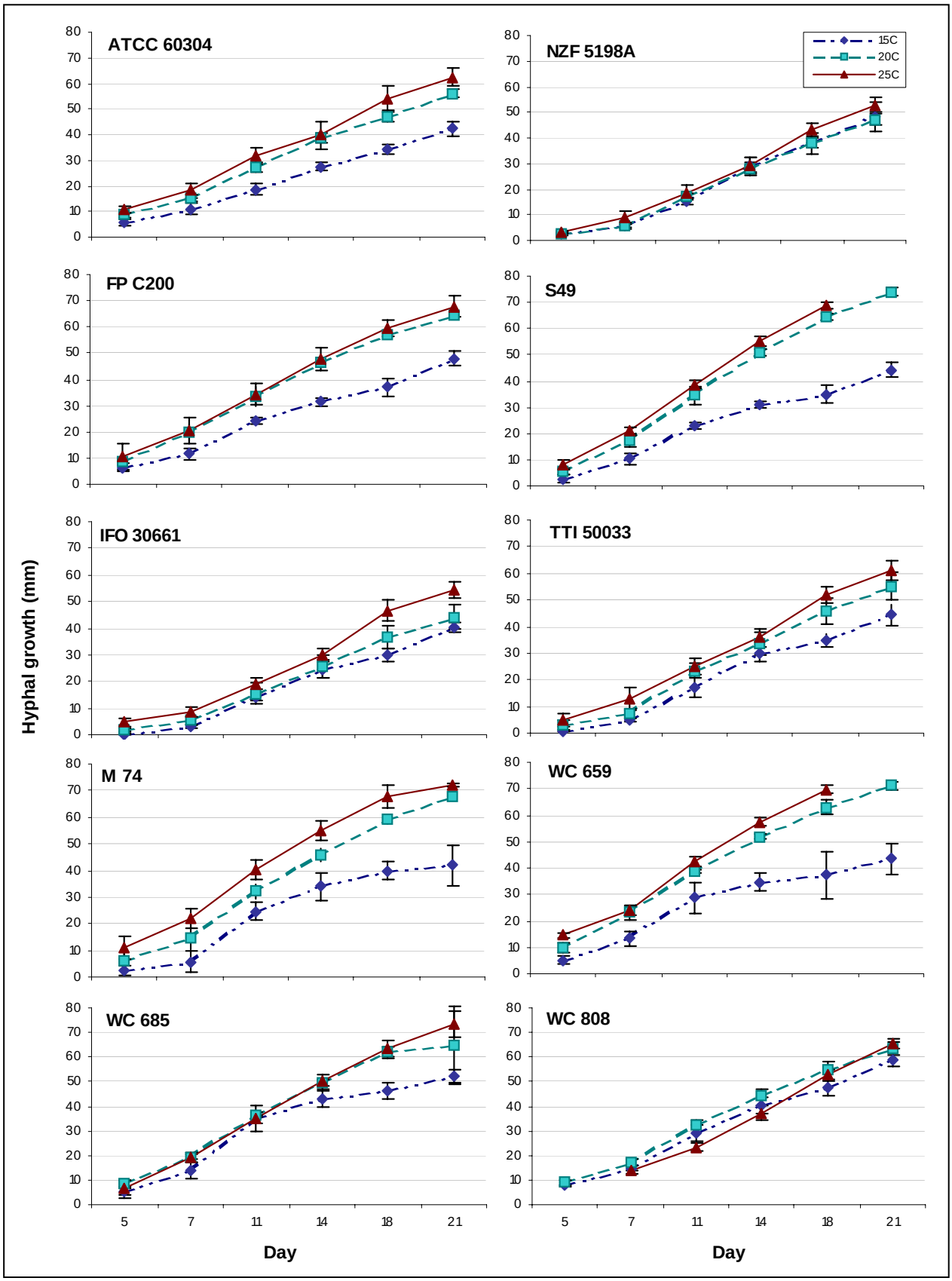
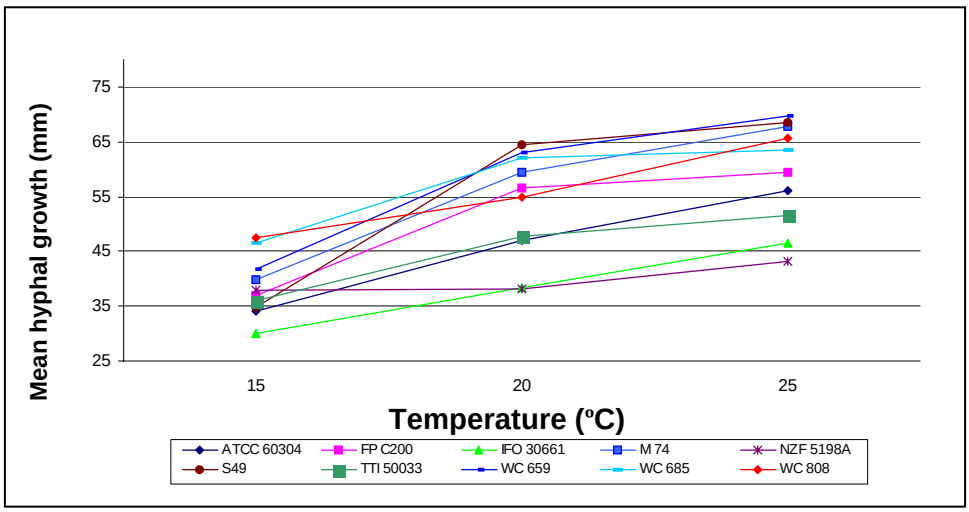


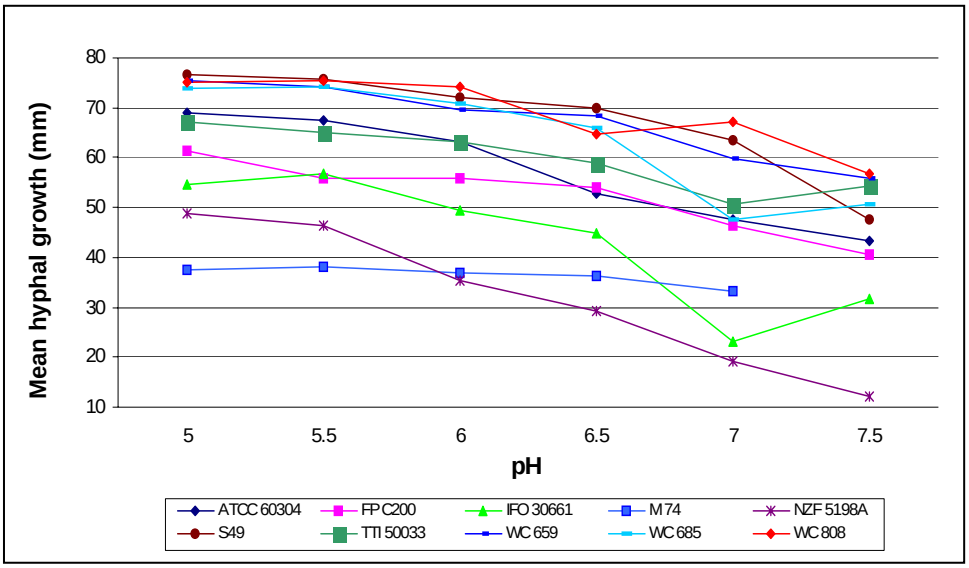
Figure 3 Hyphal growth of *Grifola frondosa* on Day 18 at various temperatures
(*P*<0.001; lsd=3.38)



Effect of pH on hyphal growth

Isolate, pH and the interaction between isolate and pH had a significant effect on hyphal growth (*P*<0.001) (Appendix 3). Whilst isolates WC808, WC685 and M74 grew optimally at pH 5.5 the least significant difference at pH 5.0 was the same. The remaining isolates grew optimally at pH 5.0. All isolates grew poorly at pH 7.5, with the exception of IFO30661 that exhibited poor growth at pH 7.0 (Figure 4).

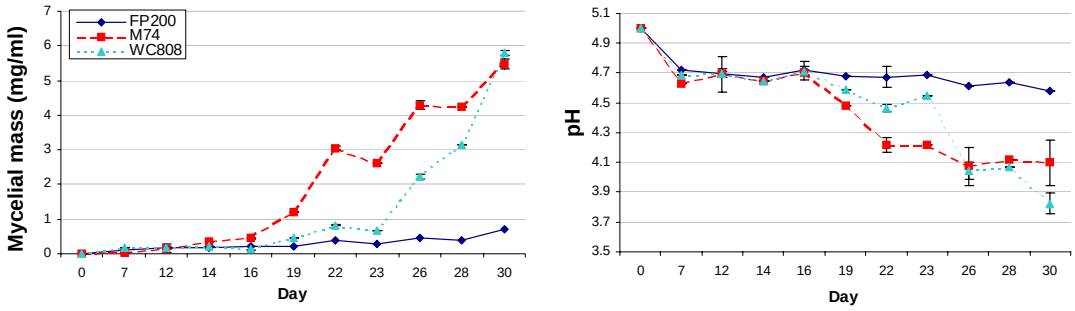
Figure 4 Hyphal growth of *Grifola frondosa* on PDA at different pH on day 19
(*P*<0.001; lsd=2.27)



Determination of exponential (log) phase in liquid culture

The exponential phase of isolate M74 commenced on day 16 with the mycelial mass of this isolate rapidly increasing until day 30. Isolate WC808 commenced the exponential phase between days 19 and 23. This trend continued until day 30, at which time, the mycelial mass of this isolate was greater than isolate M74. Isolate FPC200 grew much slower than either of these isolates with the first indication that the exponential phase had commenced on day 26 (Figure 5).

Figure 5 Mycelial mass of *Grifola frondosa* grown in PYG liquid media and pH of filtrate (Vertical bars indicate ± standard deviation)



Within the first seven days of the trial the filtrate pH for all isolates dropped from 5.0 to 4.7 and stayed within this region until day 16. When isolates M74 and WC808 commenced the exponential phase the pH of filtrate dropped. This relationship is confirmed by the correlation statistic -0.902. The commencement of the exponential phase for FPC200 at day 26 was indicated by a drop in filtrate pH on the same day.

Discussion

Vigorous hyphal growth on agar is essential for the preparation of high quality inoculum, the first stage in the production cycle. Zenghai *et al.* 1996) found that *G. frondosa* grew well in potato-bran and potato-composite medium but poorly in potato dextrose agar (PDA), whereas Shen (2001) maintained all cultures on potato dextrose yeast agar (PDYA). Other media have also been successfully used to grow *G. frondosa* : malt extract yeast agar, malt extract yeast peptone agar or dog food agar (Stamets, 2000).

No comprehensive study, on any substrate, has been undertaken to determine which temperature is optimal for hyphal growth of maitake. In this study a temperature of 25 °C was found to be optimal for all isolates with 20 °C also producing good hyphal growth and likely to achieve similar colonisation but over a longer time period. The commercial applications of this study, when applied to spawn run, suggest that temperatures of 25 °C are optimal. Where this temperature cannot be achieved the use of 20 °C is sufficient to encourage colonisation, but a temperature of 15 °C would result in poor colonisation.

Grifola frondosa was found to produce the greatest hyphal growth at pH 5-5.5 for the majority of isolates but at higher pH growth was poorer. This confirms the findings of Miyauchi *et al.* (1998) who found *G. frondosa* grew well at pH 5.Whether a pH of 4.0, as suggested by Miyauchi *et al.* (1998), would increase hyphal growth was not tested in this study. Stamets (2000) stated that *G. frondosa* grow well at pH ranges of 5.5 to 6.8 but this study shows that at pH 6.0 hyphal growth begins to markedly decline.

In liquid culture isolates M74 and WC808 have been found to commence exponential phase on day 16, whereas isolate FPC200 did not commence until day 26. The increasing mycelial mass below pH 5 indicates an initial pH of 4.5 may enhance hyphal growth and shorten the exponential phase. These findings provide the basis for further study in the development of liquid spawn.

Industry outcomes

- Temperature and pH parameters for hyphal growth of 10 maitake isolates, including those currently approved for commercial production in Tasmania, have been determined.

- Isolates showed varying responses to temperature and pH but hyphal growth was found to be optimal for most isolates at 25 ºC and pH 5. The temperature during spawn run of substrate trials was based on this finding.

- This study has found that hyphal growth is significantly affected by media pH. This was taken into account when selecting additives for studies developing a sawdust substrate

- The exponential phase of three isolates, WC808, M74 and FPC200, representative of strong, medium or poor growth on agar at 25 ºC and pH 5 has been determined. Isolates M74 and WC808 show considerable promise as suitable isolates for the development of liquid spawn.

Cultivation of Maitake on ameliorated eucalypt sawdust

Introduction

Overseas maitake is grown on deciduous hardwood sawdust, a product not readily available in Australia. The development of a local substrate, based on eucalyptus sawdust, would benefit both the mushroom and forestry industries by providing an inexpensive but essential substrate to the former and an outlet for a waste product to the latter. This ameliorated substrate would need to provide adequate nutrients for spawn run and fruit body production. The aim of substrate trials was to determine potential additives to eucalyptus sawdust and to investigate some environmental parameters.

Materials and Methods

Experimental design

Trial 1

Three *G. frondosa* isolates, M74, WC808 and FPC200 were used in this trial. A *L. edodes* isolate, HVM1, currently grown commercially on ameliorated eucalypt sawdust substrate was included for comparison. Substrates comprised of eucalypt sawdust plus 1 % $CaCO_3$ and additives of either maize meal, rice bran or wheat bran, at rates of 10 %, 20 % or 30 % w/w, plus a control without additive (Table 3). Eight replicates of each isolate x substrate were prepared and the trial arranged as randomised complete blocks of 4 isolates by 10 treatments, with each isolate representing one block.

Trial 2

Four isolates of *G. frondosa*, FPC200, M6, M74 and WC808, were grown on substrates of eucalyptus sawdust plus 1 % $CaCO_3$ (limil) with additives of maize meal, rice bran or wheat bran at rates of 10 % or 20 % w/w, and a control without additive (Table 3). Eighteen replicates of each isolate x substrate were prepared and arranged as randomised complete blocks, composed of all treatments.

Table 3 Reference codes for substrate treatments

	Treatment		
Code	Additive	(%)	Eucalypt sawdust (%)
10 M	Maize meal	10	90
20 M	Maize meal	20	80
30 M	Maize meal	30	70
10 R	Rice bran	10	90
20 R	Rice bran	20	80
30 R	Rice bran	30	70
10 W	Wheat bran	10	90
20 W	Wheat bran	20	80
30 W	Wheat bran	30	70
0	Nil	-	100

Grain spawn production

In both trials the preparation of spawn grain followed a modification of the method currently used in the production of commercial *L. edodes* grain spawn at HVM (Wilson and Chung, 1995; HVM, Wilson, A., pers. comm, 2000). Petri plates, 90 mm in diameter, containing PDA, were inoculated with six replicates each isolate and incubated in the dark at 25 °C. When the surface of the agar was fully colonised grain was prepared for inoculation.

Autoclavable bags were filled with 1kg rye corn, 60 g eucalypt sawdust and 5 g $CaSO_4$ (gypsum). These components were loosely mixed within the bag before adding 850 mL tap water and leaving overnight to allow grain to take up water and for any bacterial endospores present to germinate prior to autoclaving. The final weight of each bag was 1.9 kg. After overnight soaking grain was autoclaved for 1 hr at 121 ^{o}C and 103 kPa, allowed to cool overnight and autoclaved for a further hour at 121 ^{o}C and 103 kPa. After cooling grain was inoculated under aseptic conditions using one Petri plate of isolate per bag. The open end of each bag was heat sealed and the grain incubated in the dark at 25 ^{o}C until fully colonised (Figure 6).

Figure 6 Bag of fully colonised grain spawn

Substrate preparation and inoculation

Trial 1

Substrate preparation was carried out at the premises of HVM (Glen Huon, Tasmania). A sample of all additives and eucalypt sawdust were oven dried at 105 ^{o}C for 48 hours and the dry weight of each component determined prior to mixing. Substrate of eucalypt sawdust, 1 % Ca CO_3 (limil) w/w and additives of either maize meal, rice bran or wheat bran at 10 %, 20 % or 30 % dry weight w/w plus a control of eucalypt sawdust without additive were prepared and mixed in a cement mixer. Tap water was added to achieve a final water content of ± 65 % based on dry weights. Prior to autoclaving a sample was taken from the centre of the bulk substrate and dried until constant weight to determine moisture content. One kilogram of substrate was placed into heat resistant plastic bags (100 x 100 x 490 mm) having a 65mm wide microfilter along the length of the bag, and an opening at the narrowest end. After filling bags were loosely sealed, by folding over the top, and transported to Hobart where they were autoclaved for 2 hours at 121 ^{o}C and 103 kPa.

After cooling each bag was inoculated with grain spawn, at a rate of 4 % w/w under aseptic conditions and the top of each bag sealed by heat or by rolling down the top of the bag and clamping this with a 51 mm spring loaded clip. All bags were firmly squeezed by hand to test that closure was airtight.

Trial 2

Substrate preparation, sterilising and inoculation were carried out at the premises of HVM. Substrate was mixed mechanically in a commercial hopper. As each substrate was mixed it was tested for moisture content with an infra-red moisture analyser (Mettler toledo HR73) and water added to provide a final moisture content of ±62 % prior to autoclaving. Into polypropylene bags, having a microfilter 35 x 35 mm, (Euro Bussan PLABAG, Model No. SS), was placed 2.0 kg of prepared substrate. Substrate was sterilised by autoclaving for 5 hours at 121 ^{o}C and 103 kPa.

After cooling each bag was inoculated, under aseptic conditions, with spawn grain at 4 % w/w. After inoculation bags were heat sealed and tested by firm hand pressure to ensure the seal was complete. Sixteen replicates of each isolate x substrate treatment were transported under cover, to TIAR Research Laboratories at New Town. In the growing room bags were arranged in randomised complete blocks comprised of each isolate x treatment. One set of each isolate x substrate treatment was placed in the spawn run room at HVM.

Cultivation conditions

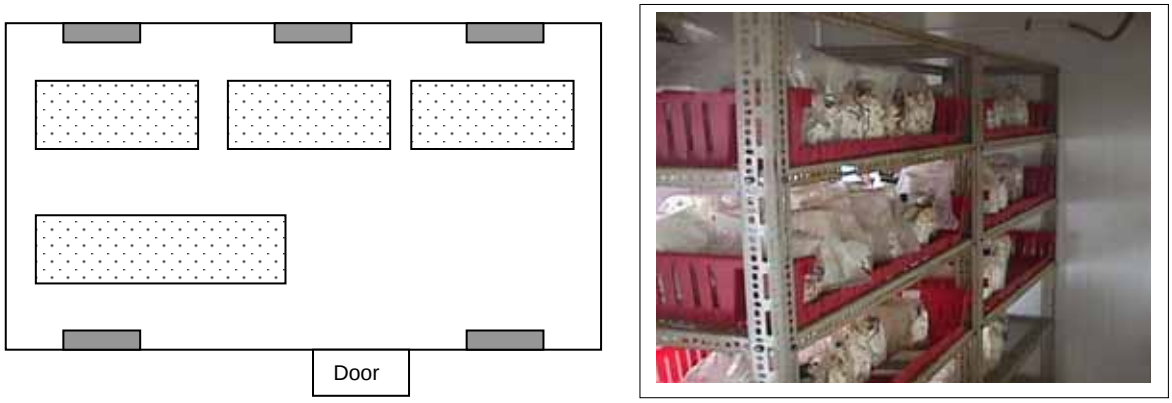
Trial 1

Inoculated bags were placed on racks in the spawn room at 25±2 ^{o}C and dark. The vertical temperature difference between upper and lower shelves did not exceed 1 ^{o}C but to ensure any differences were kept to a minimum an air space of 500 mm was allowed between the underside of the lowest bags and the floor, and the top of the highest bags and the ceiling.

Environmental parameters of temperature, relative humidity and light were altered at different stages in the cultivation cycle. From day 77 until the conclusion of the trial RH was maintained at 85 %. The lighting system was comprised of 5 cool white fluorescents (36 watt) arranged vertically around the room, at a distance of approximately 40 cm from the racks (Figure 7).

Figure 7 Trial 1 growing room – arrangement of racks and lighting

single light placement of racks in room



Trial 2

The environmental parameters of temperature, RH, ventilation of CO_2, and the diurnal cycle, were controlled by an Innotech® (Australia) system throughout the trial. Spawn run conditions were 23 ºC, 70 % RH, 5000 ppm CO_2 and dark. At cold shock temperature was reduced to 16 ºC, relative humidity

increased to 85 % and the room ventilated to achieve 3000ppm CO_2. Light was provided by eight cool white fluorescents (36 watt) arranged vertically around the room and at a distance of 30-40 cm from each rack. A diurnal light cycle of 4:20 hr light:dark was introduced to encourage initiation. This was later altered to 8:16 and humidity increased to 90 % to encourage fruit body production (Table 4). Light was increased to 72 watts each, near racks on the left of the room, on day 109 (Table 4).

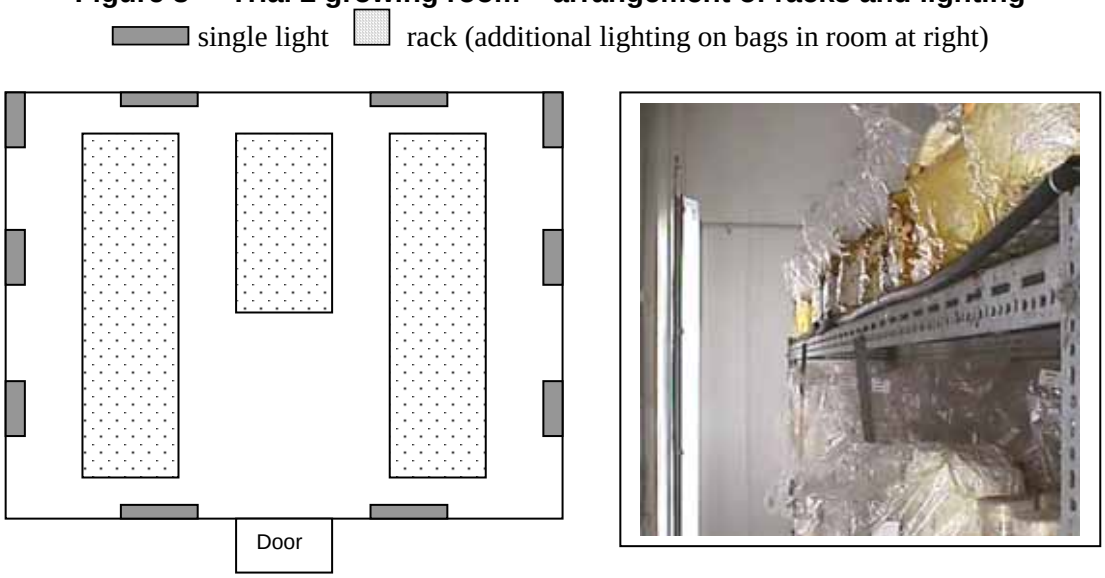
Table 4 Environmental parameters for the cultivation of Maitake in Trial 2

Stage	Day	Temperature (°C)	Relative humidity (%)	CO_2 (ppm)	Diurnal Cycle (hours)
Spawn run	0	23	Ambient	Ambient	-
	15	23	↓	5000	-
	63	23	70	5000	-
Cold shock	66	16	85	3000	-
Initiation	82	16	↓	2000	4:20
	95	16			5:19
Fruit body production	109	16	90 ↓		8:16
	113	16		1500	↓
	130	16	98	1500	

On day 68, 10 slits were placed near the filter of all bags in blocks 5 and 10, in the middle rack. At the same time isolate M6 on 10 % wheat and 20 % maize in block 5, were opened, the primordia exposed, the bags moved to and upper shelf.

Light intensity was increased on one side of the room on day 109 to identify the effect on fruit body production (Figure 8).

Figure 8 Trial 2 growing room – arrangement of racks and lighting

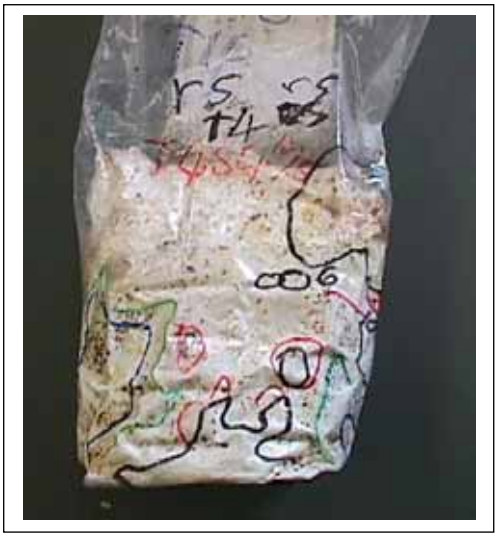


Hyphal colonisation of substrate

Trial 1

Little or no growth had occurred off the spawn grain five days after inoculation. A line was drawn at the leading edge of mycelial growth and this was used as the base line for all other growth assessments. At seven day intervals a line was drawn on the side of the bag opposite the filter to mark the mycelial front (Figure 9). This continued until substrate was fully colonised, destructively sampled, contaminated and discarded or, reached the end of the trial. Where no hyphal growth commenced from inoculum the bag was removed from the trial. At the end of the trial each bag was carefully cut away from the substrate and a rectangular section, 89 x 181 mm, representative of mycelial growth was scanned (SigmaScan®) and the surface area colonised each week determined.

Figure 9 Mycelial front marked at seven day intervals on bag



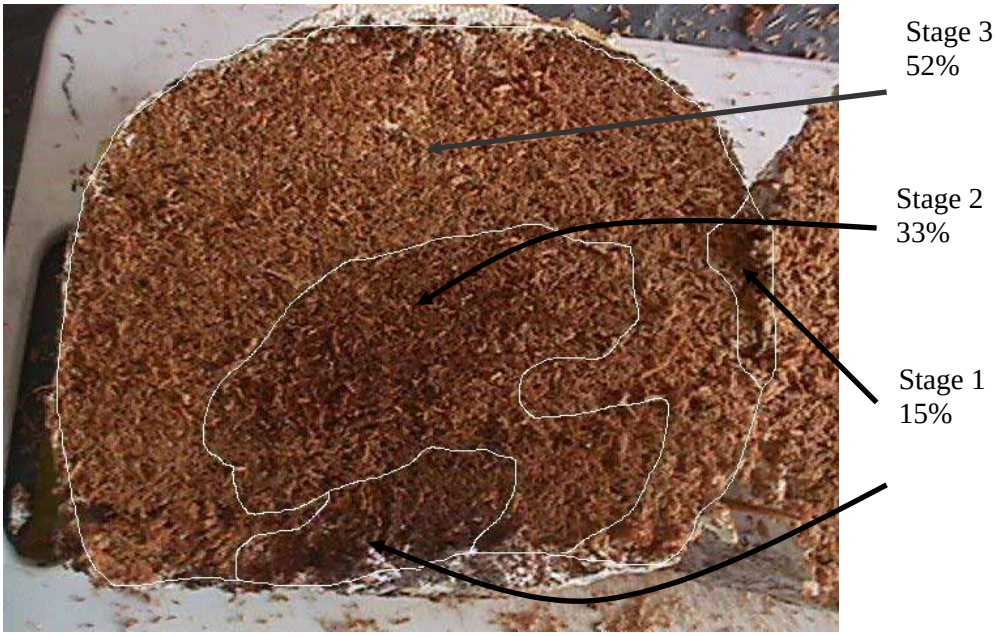
Trial 2

A complete block was removed for destructive sampling at 20, 40, 50, 60 and 100 days after inoculation. To determine hyphal colonisation through the substrate four stages were noted:

- No hyphae present, substrate dark brown in colour
- Fine density hyphae, substrate dark honey brown
- Medium density hyphae, substrate light honey brown
- Thick dense hyphae, substrate pale brown with visible white hyphae

Each log was cut vertically across the narrowest section and the different stages of colonisation at the cut surfaces traced onto clear transparency film. The two halves were then cut vertically, at right angles to the first cut, and colonisation stages at these surfaces traced. The transparency was photocopied, each stage cut out, weighed, and the different weight of each stage calculated as a percentage of the total, providing a mean value for analysis (Figure 10).

Figure 10 Cut surface of 20 % rice bran treatment colonised by M74 on day 40



Fruit body production

Trial 1 was assessed at seven day intervals with observations made of colonisation and fruit body stages.

Trial 2 was assessed at 4 and 7 day intervals. Stages recognised were: full colonisation of substrate, appearance of exudate, formation of a mycelial mat, and primordia formation. In this trial primordia formation included the stages: brain, upright, cauliflower, antler, petal and spore production.

Analysis

In Trial 1 the relative substrate densities of each treatment were calculated from the mean substrate depth of 10 bags. This was analysed by ANOVA and least significant difference (LSD) with Microsoft Excel 97®. A ratio, based on relative substrate densities, was used to convert the surface area colonised to a value that could be compared across treatments. This data was analysed with Genstat 5® (Release 4.1, 4th ed. 1998, Lawes Agricultural Trust, Rothamstead).

In Trial 2 the larger substrate volume in each bag made it unnecessary to calculate a conversion factor based on substrate depth. Substrate colonisation and stages in fruit body production were analysed by REML Variance in Components Analysis (Genstat® Release 4.2, 5th Ed., Lawes Agricultural Trust, Rothamstead).

Results

Moisture content

In Trial 1 the moisture content of each substrate was found to range between 63 % and 68 % (Table 5). The final moisture contents of treatments in Trial 2 ranged between 58-60 % (Table 6).

Table 5 Final moisture contents of substrate treatments in Trial 1

	Level of additive			
Type of additive	10 %	20 %	30 %	0 %
Control	-	-	-	65
Maize meal	63	65	64	-
Rice bran	66	65	65	-
Wheat bran	68	64	65	-

Table 6 Final moisture contents of substrate treatments in Trial 2

	Level of additive					
	10 %		20 %		0 %	
Type of additive	HVM	TIAR	HVM	TIAR	HVM	TIAR
Control	-	-	-	-	60	68
Maize meal	61	63	58	64	-	-
Rice bran	59	69	58	67	-	-
Wheat bran	61	69	58	66	-	-

Colonisation

Trial 1

All maitake isolates performed poorly on substrate without additive with the substrate not fully colonised and no fruit body production. This data was removed from all analyses.

Substrate density was found to be significantly different ($P<0.001$) with the control being the least dense and rice bran and maize meal at 30 % the most dense. To determine the rate of hyphal colonisation this value was converted, using the substrate depth ratio, to give a value that represented the proportion of the log colonised each week (Table 7).

Table 7 Substrate depth and ratio to control at day 0 (LSD 5 % = 12.59)

Treatment	Mean	Lsd	Ratio
Control	180.0	a	1
10 M	168.5	ab	1.10
20 M	158.5	bc	1.14
30 M	131.5	d	1.37
10 R	165.5	b	1.10
20 R	158.5	b	1.14
30 R	131.8	d	1.37
10 W	163.0	b	1.10
20 W	164.0	b	1.10
30 W	150.5	c	1.11

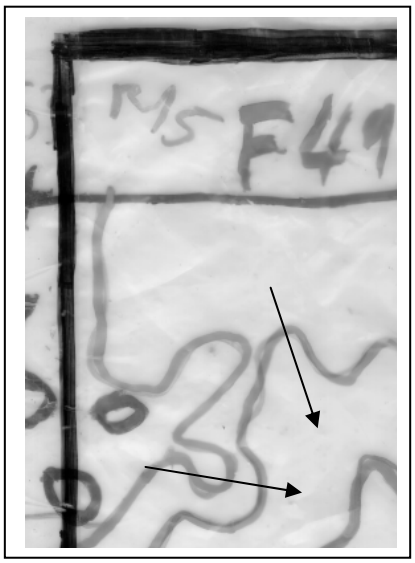
Means with same letter are not significantly different

Shiitake colonised substrates at almost double the rate of maitake and this was separated from analysis to ensure that differences between maitake isolates, across all treatments, would be clearly delineated. Shiitake rapidly colonised all substrates except 30 % wheat and rice bran. Optimal substrates for shiitake, in decreasing order, were 20 % wheat bran, 10 % rice bran and maize meal and 20 % rice bran. Under the environmental conditions used in trial 1 shiitake commenced fructification on all substrates but the control (Figure 11).

Figure 11 Shiitake fruiting on eucalypt sawdust and 10 % rice bran

Maitake had commenced colonisation of substrate in most bags by day 11. Prior to full colonisation some maitake replicates did not colonise substrate linearly but exhibited a “corner effect” (Figure 12). Where this occurred in the corner of the scanned image the actual growth rate was under represented and this data was removed from analysis until the fitted trend line had an $R^2 > 0.9$.

Figure 12 Scanned area showing "corner effect" at arrows



Significant differences in colonisation were found for all isolates (P<0.05) across all substrates with isolate WC808 colonising substrate faster than M74 whilst FP 20 its 0 colonised substrate much slower than both these isolates. All additives resulted in a significantly different colonisation rate (P<0.05) which were, in descending order, 10 % rice bran, 20 % wheat bran, 10 % maize meal, 20 % maize meal, 20 rice bran, 10 % wheat bran, control without additive, 30 % maize meal, 30 % rice bran and 30 % wheat bran. A significant interaction was found between isolate and substrate (P<0.05) (Table 8).

Table 8 Mean substrate colonisation of substrate by all isolates compared by average variance of difference

% additive	FPC200	M74	WC808	All*
10 M	gh	gh	fg	ab
10 R	h	def	de	ab
10 W	j	h	h	c
20 M	h	de	c	ab
20 R	gh	gh	cd	ab
20 W	h	cd	efg	ab
30 M	gh	de	def	ab
30 R	j	a	b	ab
30 W	k	i	j	d
Control	j	l	gh	d

* Mean of all isolates; Means with the same letter are not significantly different (P<0.05)

Trial 2

Whilst the humidifier was working at maximum capacity in Trial 2 this was not sufficient to ensure humidity >95 % was maintained and a stand-alone humidifier was placed in the room from day 113 to ensure RH of 95 %. The Innotech system was constructed to ventilate excess CO_2 but was not equipped to introduce CO_2, this reflects the situation for commercial growers. As hyphae colonises substrate CO_2 is released, increasing CO_2 levels in the growing room. This situation occurred between

days 1-14 with CO_2 levels in the growing room gradually increasing to 5000 ppm. From day 66 onwards CO_2 levels decreased as part of the natural production cycle.

Blocks sampled at day 20 were not fully colonised and collapsed on removal of the bag making tracing of colonisation stages impossible. The different areas of colonisation, in each log, were separated by hand on the basis of the stage definitions outlined. Each stage was weighed and the percentage colonisation calculated by weight. All other destructive sampling times were conducted as earlier described.

The effect of both isolate and substrate on colonisation was significant (P<0.001) but there was no interaction. Substrates containing 10 and 20 % rice bran were colonised earlier by *G. frondosa* than all other substrates (P<0.001) which showed no significant differences in colonisation rate (Table 9). The fastest coloniser was WC808 taking an average 30.76 days with FPC200 taking 34.03 days and isolates M 60 and M74 not significantly different from each other with colonisation rates of 42.38 and 41.55 days respectively began.

Table 9 Time taken for *Grifola frondosa* isolates to reach colonisation, mat, brain and upright fruit body stages

	Colonised		Mat		Brain		Upright	
Isolate	Days*		Days*		Days*		Days*	
FPC200	34.03	(b)	48.11	(b)	88.29	(c)	96.13	(b)
M74	41.55	(c)	46.38	(ab)	70.34	(a)	88.31	(a)
M6	42.38	(c)	46.49	(ab)	67.07	(a)	89.34	(a)
WC808	30.76	(a)	45.34	(a)	78.23	(b)	106.04	(c)

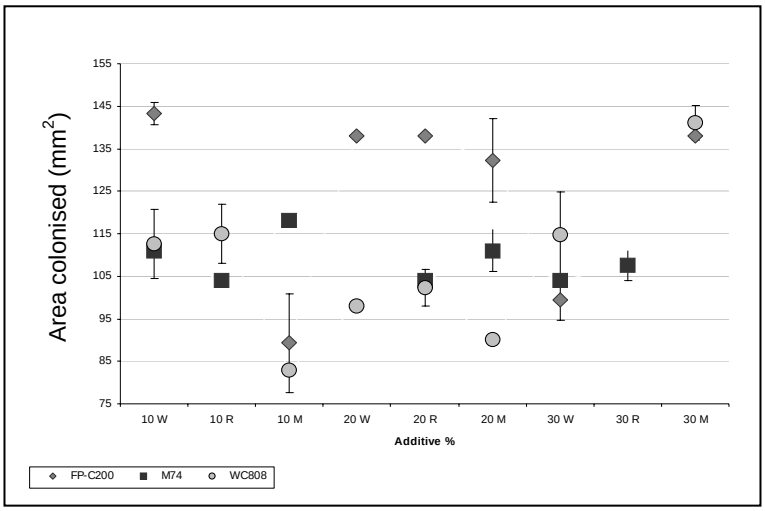
* Days after inoculation ; Means with the same letter are not significantly different (P<0.001).

Fruit body production

Trial 1

Isolate WC808, on 10 W commenced to primordia initiation first with all substrate treatments, except 30 R, which initiated primordia before day 145 (Figure 13). Isolate M74 commenced initiation on all substrates between days 105-123. The initiation of isolate FPC200 commenced between days 85 and 100 on substrate treatments 10 % wheat bran and 30 % maize meal but on treatments 10 % maize meal, 20 % maize meal and rice bran and 30 % wheat bran initiation commenced later, between 125 and 145 days.

Figure 13 Commencement of primordia initiation in Trial 1 (n<1 removed)



Initiation occurred on all treatments under the filter, and in many instances between the fabric of the filter and the external plastic making it impossible to determine further fruit body stages. Where the plastic was cut away from the filter and the flattened fruit body continued maturing the resulting fruit bodies were deformed (Figure 14). This data could not be analysed.

Figure 14 Effect of bag and filter on fruit body formation

A, fruit body forming between filter and outer plastic of bag; B, deformed fruit body growing through filter with deformed antler stage commencing

Trial 2

Initially the exudate of all isolates was lemon yellow in colour and interaction between isolate and substrate was significant (P<0.001). Whilst exudate colour of isolates WC808 and FPC200 became a deeper and stronger yellow the exudate formed by M6 and M74 progressed to deep orange and finally tan (Figure 15). At this stage interaction between these strains and substrate was not significant (Appendix 4).

Figure 15 Exudate produced Maitake on eucalypt sawdust with 10 % maize meal additive

A, Yellow exudate formed by maitake isolate FPC200; B, Orange exudate formed by maitake isolate M74.

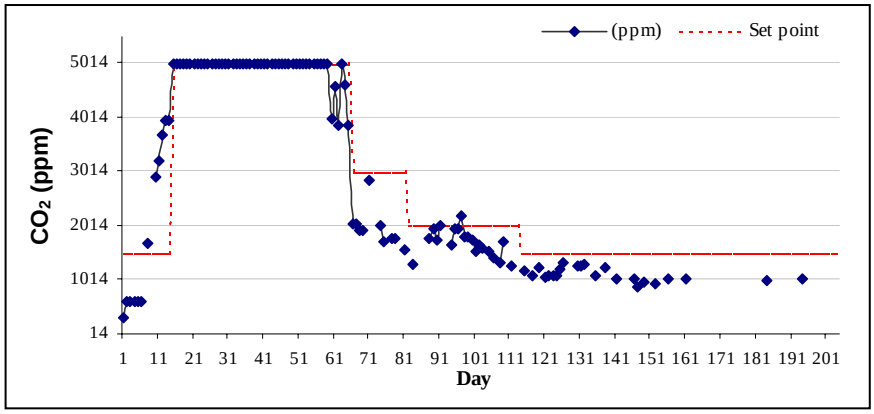
Isolates formed the mycelial mat between days 45.34 and 48.11 with significant differences (P<0.001) found between isolate WC808 (day 45.34), isolates M 60 and M74 (days 46.38 to 46.49) and isolate FPC200 (day 48.11). At this stage there was no significant interaction between isolate and supplement. All isolates but M6 produced a grey and lumpy surface on the mycelial mat but no differences were found between isolates and substrates, nor was there any interaction (data not shown).

At day 66 all bags were either fully colonised, no further hyphal growth was occurring or initiation had commenced. No interaction was found between additive level and isolate x additive at brain stage. The number of days required for brain stage formation was significantly different (P<0.001) between isolates with M6 and M74 forming brain stage at 67.07 and 70.34 days respectively, whilst WC808 formed brain stage 8 days later and a further 10 days was required for brain formation by FPC200. Some bags produced two or more brain stages with some of these were deformed (Figure 16). Bags in blocks 5 and 10, which had been slit on day 68, became very dry and primordial growth slowed considerably. The primordial growth of isolate M6 on 10 W and 20 M, which had been opened at the same time, was arrested after opening. When the temperature was reduced from 23 to 16 ºC the CO_2 levels immediately decreased (Figure 17).

Figure 16 Production of different brain stages in Trial 2

A, FPC200 at day 112 forming two brains at either end of the bag on substrate treatment 20 % maize meal; B, M6 at day 96 with deformed brain on substrate treatment 10 % wheat bran.

Figure 17 Carbon dioxide levels inside the growing room during Trial 2

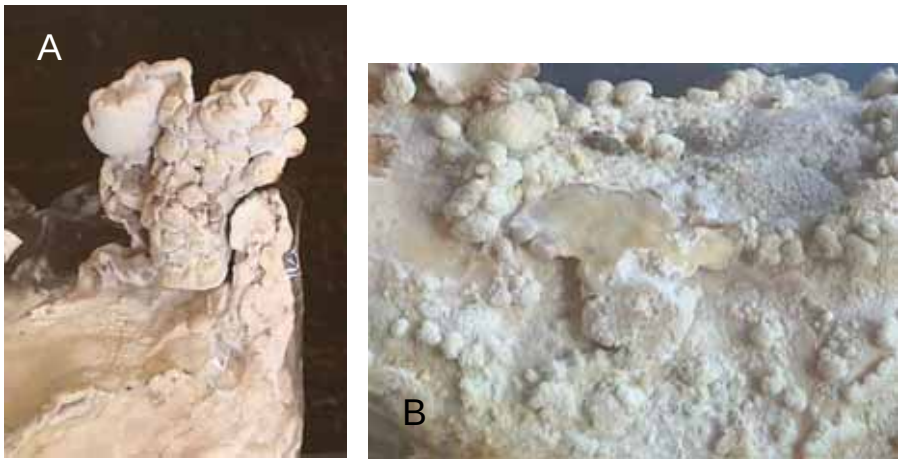


An additional interim stage, upright primordia, was included in observations and describes the commencement of the change between brain and cauliflower stage. This was characterised by the appearance of the brain extending vertically. The interaction between isolate and substrate was significant (P<0.001).

The interaction between isolate and substrate additive was significant at the upright stage (P<0.001) with this stage occurring between 81.6 and 101.9 days. Treatment WC808 on 10 % wheat taking the longest to form upright fruit bodies at 161.7 days.

Increased light on one side of the room was not beneficial encouraging some fruit body expansion but also encouraged the formation of pores on both the upper and lower surfaces (Figure 18).

Figure 18 Light induced initiation of pore structures on fruit bodies and mycelial mat



A, Pores forming on upper and lower surface of maitake fruit body structure and mycelial mat; B, Pores on surface of mycelial mat and initiation structure

Table 10 Relationship between substrate additive and stages in the cultivation cycle

Additive level	Colonised Days*		Mat Days*		Brain Days*	
10 M	39.43	(b)	49.85	(c)	82.81	(c)
10 R	34.94	(a)	44.56	(a)	72.95	(ab)
10 W	38.28	(b)	47.08	(b)	80.58	(bc)
20 M	37.80	(b)	47.09	(b)	75.37	(abc)
20 R	34.57	(a)	44.62	(a)	68.90	(a)
20 W	38.07	(b)	46.28	(ab)	75.29	(abc)

*Days after inoculation ; Means with same letter are not significantly different (P<0.001)

Table 11 Mean number of days for *Grifola frondosa* isolates to reach colonisation, mat, brain and upright fruit body stages

Isolate	Colonised Day		Mat day		Brain Day	
FPC200	34.03	(b)	48.11	(b)	88.29	(c)
M74	41.55	(c)	46.38	(ab)	70.34	(a)
M6	42.38	(c)	46.49	(ab)	67.07	(a)
WC808	30.76	(a)	45.34	(a)	78.23	(b)

Means with same letter are not significantly different (P<0.001)

Discussion

Substrate moisture

Whilst moisture contents can be specified in literature, in the commercial situation it is, in general, only possible to obtain a moisture content within the desired range. This is caused by variations in the moisture content of raw materials. In a commercial situation sawdust is stored outside without cover. Fresh sawdust delivered to the farm is usually placed on top of this existing sawdust pile, creating horizontal layers of various ages. The moisture content within each layer can vary considerably depending on the age of the sawdust, whether the older sawdust has been rained on, and how often, the state of sawdust decomposition.

The final moisture content of substrates in both trials differed from the desired 62 % by +4 % in Trial 1 and –4 % in Trial 2. Whilst this is likely to have affected production no direct relationship was found between moisture content and any of the production stages in either trial. The difference in moisture content did not appear to adversely effect colonisation, indicating maitake can tolerate moisture contents ranging from 59-69 %. This falls within, what has been recommended by others (Chung and Joo, 1989; Stamets, 1993a; Chalmers, 1994; Kirchhoff, 1996; Mayuzumi and Mizuno, 1997). Until the optimal moisture content for maitake fruit body production is known growers need to be that additional moisture may be required. This may take the form of increasing RH after the bags have been opened and/or supplemental watering.

Spawn run and colonisation

The effect of substrate density was taken into account in trial 1 as the depth of substrate was greater than the surface area. In trial 2 this was reversed with the larger surface area making it unnecessary to compensate for any effect of substrate density on colonisation data.

Colonisation of substrate in trial 1 was slower than in trial 2 which is believed to have been caused by the dramatic reduction in substrate moisture, resulting from the large filter on the bags, and insufficient relative humidity in the room. Trial 1 found the addition of 30 % rice bran to eucalyptus sawdust was optimal for colonisation by isolates WC808 and M74. In a commercial situation the difference between the effect of 30 % and 20 % maize meal, rice bran or wheat bran does not merit of the expense of using 30 % additive. In the second trial rice bran at 10 and 20 % levels produced the best colonisation across all isolates but the number of days between the fastest and slowest colonised substrates only by 4.86 days overall. This pattern was similar for mycelial mat formation.

Shiitake colonised eucalypt sawdust without additive whereas maitake grown on this substrate resulted in poor growth of all isolates in both trials confirming that eucalypt sawdust alone is insufficient to support hyphal growth of maitake. The implications of these findings to industry are that eucalyptus sawdust must be ameliorated with either maize meal, rice bran or wheat bran at 10-20 %.

The colonisation of ameliorated eucalypt substrates occurred between 30.76 and 42.38 days in trial 2 which falls within the lower ranges found by others : 30-45 days on a range of sawdust substrates (Lee, 1996; Royse, 1996; Royse, 1997b). This is much faster than the 44-60 days stated by Stamets (1993). This result falls well within the range indicated by others for the cultivation of maitake on substrates naturally suited to this fungus and confirms ameliorated eucalyptus sawdust is suitable for the commercial cultivation of maitake. This is of significant benefit to Australia where eucalyptus sawdust is the only readily available material for specialty mushroom cultivation.

Exudate and mycelial mat

The appearance of exudate, and its colour, is often referred to in literature (Chen, 1999a; Stamets, 2000). This study shows that the colour of exudate markedly changes during cultivation and between isolates. As the appearance of exudate marks the beginning of the “resting phase” (Stamets, 2000) the use of colour to identify the commencement of this phase is misleading due to differences which will

occur over time and between isolates. Supplementation with rice bran at 10-20 % encourages rapid formation of the mycelial mat but the difference between these and other additives was similar across all treatments except 10 % maize meal which took 2.5 more days than all other treatments to form the mycelial mat.

Initiation and brain formation

The formation of the brain is the first stage in the production of a sporocarp and is induced by environmental changes. A temperature reduction to 16 ºC, increased humidity at 85 % and reduction in CO_2 contributed to brain formation. In this study some isolates formed the brain stage before or at the time of these changes. For this to occur within an average of 24 hours, as indicated for some isolates, is extremely rapid and indicates that another trigger had occurred prior to day 66 that contributed to the commencement of brain formation. Future analysis of extracellular enzymes, produced between days 20 and 100, may clarify this trigger which perhaps results from biochemical changes in substrate. If this is found to occur then a substrate formula which encourages colonisation, initiation and fruit body development could be more readily determined.

Strain differences are to be expected, and this is often beneficial in a commercial environment, allowing growers to select strains that are suitable to available substrate material, their cultivation practices and the demand of the marketplace. The differences between strains was the most marked, in terms of the time required to commence production of a marketable commodity, at brain stage. The formation of the brain stage of isolates M74 and M6 commenced earlier than isolates FPC200 and WC808, independent of substrate. With the development of the upright stage isolate x substrate treatments were exhibiting marked differences. In trial 2 on 10 % rice bran and 10 % wheat bran both isolates M74 and M6 commenced production of the upright stage between days 81 and 83. During the next 20 days all treatment combinations, except isolate WC808 on 10 % wheat bran, had begun to produce upright primordia. Whilst isolate WC808 appears to have required an average of 161.37 days to produce upright primordia this disagrees with the formation of the cauliflower stage which was found to occur at 152.8 days. The difference between these two values reflects fewer bags being available for assessment as the trial progressed which affected mean values.

Cauliflower formation

In trial 2 the interaction of isolate x additive was found to be significant in the formation of both cauliflower and antler stage ($P<0.001$). It should be noted the number of bags had been reduced by this time due to contamination. In addition, not all isolate x supplemented substrates produced these stages, or the number of bags that did were of such low numbers as not to be considered as having sufficient data for a valid analysis.

The timing for the introduction of light is not clearly explained in literature, but the slow introduction of light has been said to encourage petal formation (Stamets, 2000). Light was introduced and CO_2 reduced to 3000 ppm on day 82. The change in the diurnal cycle to 5:19 on day 95 appeared to encourage cauliflower formation in some treatments. Whilst the introduction of light could have induced cauliflower formation it would seem that at this stage, as at the formation of brain stage, other environmental or biochemical triggers such as CO_2 , RH or moisture content are involved.

The maturing fruit body

The successful fruiting of shiitake in trial 1 indicates that substrates and conditions required for fruit body production by shiitake significantly differs from that of maitake. These conditions are not believed to have been the cause of maitake producing performed fruit bodies, but the result of the large filter at the side of the bag. The bag was removed from shiitake colonised substrate when the "popcorn" stage was reached and normal fruit bodies formed, indicating a suitable environment for production. Maitake also commenced fruiting but this occurred at the filter and as the bag was only partially opened around the emerging fruit body this resulted in the formation of deformed fruit bodies. In trial 2 the production of maitake fruit bodies was also affected by the bags which were sealed across the upper edge creating a large airspace above the substrate. It is thought that this,

coupled with the filter placed at the centre of the bag, resulted in reduced CO_2 levels inside the bag, particularly under the filter which would have affected fruit body formation.

Considerable contamination problems occurred during trial 1 which affected the results of the trial. This is believed to have been caused by poor farm hygiene and this was exacerbated by the large filter on the bags. Improved farm hygiene and the use of different bags in trial 2 minimised contamination problems experienced in trial 1.

At mycelial mat and brain stage there was no significant interaction between strain and substrate but significant differences were found in all the following production stages. This would seem to reflect differences in strain rather than substrate.

Whilst temperature, relative humidity, ventilation and see $C0_2$ levels have been shown to affect maitake fruit body formation. Another important parameter, light, has been shown to rapidly progress the sporocarp into sporulation. This parameter requires further investigation to clarify acceptable light levels during fruit body maturation.

Isolates

Isolate WC808 was found to be the best coloniser across all substrate treatments in both trials 1 and 2. In trial 2 FPC200 was found to be a better coloniser than both M74 and M6. This result differs from trial 1 where M74 appeared to be the better coloniser. This can be explained by the rapid colonisation of M74 on 30 % rice bran in trial 1, which affected the analysis. Isolate M6 was not used in trial 1. Of the four isolates studied in trial 2 WC808 performed well at production of mycelial mat, brain and upright primordia stages. While data at cauliflower and antler formation was considered insufficient for analysis observation of these stages indicated this isolate performed well. This was also observed in the fruit bodies at HVM.

The different environmental regimes recorded in earlier studies in Tasmania could well reflect strain variability. Whilst there is very general information on environmental parameters used during the cultivation cycle there is little hard evidence on the quantity of sporophores produced as a result of these regimes.

Industry outcomes

- The conditions required for shiitake cultivation differ significantly to that required for maitake cultivation.
- Age and moisture content of horizontal layers within the sawdust pile have been identified as introducing another undefinable variable into the substrate formula.
- Due to insufficient moisture in the substrate formula supplemental watering may be required after the bags have been opened and/or increased RH.
- Differences in colonisation and fruit body formation have been identified in four commercial maitake isolates (FPC200, M6, M74, WC808).
- Isolates M6 and M74 commenced brain stage formation earlier than isolates FPC200 and WC808, independent of substrate.
- Supplementation of eucalypt sawdust with maize meal, rice bran or wheat bran at 10-20 % is required for maitake cultivation.
- Colonisation of ameliorated eucalypt substrates takes between 31 and 42 days which is comparable to colonisation on the deciduous sawdust substrates.

- In order to reduce air volume within the bag and increase CO_2 levels another method is required to seal bags, or different shaped bags/filters investigated. The different results between trial 1 and 2 indicates that a different type of bag may optimise fruiting.

- Material put aside for assay of extracellular enzymes produced during the cultivation cycle will provide further information on maitake's ability to utilise e*ucalyptus* sawdust. The effect additives are having on colonisation, initiation and fruit body formation will also be analysed.

Literature Review: Research strategies for the cultivation of edible *Morchella* (Morel)

Introduction

The fruit bodies of the ascomycetous fungi *Morchella* spp. (morels) are highly prized for their edibility and appearance, which is similar to "a sponge on a stick". They are amongst the most sought after edible fungi in world markets, with a premium demanded by suppliers, and paid by consumers. Due to their complex life cycle and the lack of knowledge surrounding ascocarp formation, these delicacies are not artificially cultivated and can only be supplied to the market by wild harvesting. In the wild, morel fruit bodies are generally found growing in forests for a few short weeks in early spring. The morels harvested during this short period are the only fruit bodies to reach the market.

Information on the life cycle, cultivation and classification of morels to species level is available but is often extremely variable in content, with areas of conflicting information or the omission of valuable data. This is a common situation amongst most edible ectomycorrhizal fungi that cannot be artificially cultivated. In part this is due to the short, and often sporadic, fruiting period which makes it difficult to observe and compare morel size and colour. Resulting taxonomic confusion negatively impacts scientific research. Morels are attributed to different species, solely on the basis of morphological characters, when they may actually be the same biological species. This review critically discusses the available literature on the cultivation of morels and related areas, such as life cycle and ecology. It provides a synopsis of current knowledge, providing a basis for research into morel identification and cultivation.

Edibility

There are indications that morels have been used as a food product since the 15th century (Kaul, 1997). Today they are still popular, reflected by the numerous articles written on their culinary properties (McIlvaine and Macadam, 1973; Carluccio, 1995) and their high price in the marketplace. A number of morel species have been identified as excellent edible fungi (Appendix 5). These are well known to wild collectors in various countries but by different common names.
When preparing morels for cooking it is essential to wash the ascocarps carefully as the many pits, formed by numerous asci, readily hold dirt and insect debris. Morels must be cooked slowly to release the volatiles that give these mushrooms their excellent flavour. Where this is not possible the mushrooms can be blanched for one or two minutes prior to use. Cooking also removes the risk of stomach upsets that have been reported after the consumption of raw morels. Fortunately the reported symptoms of nausea, vomiting and abdominal pain disappear within a day (Benjamin, 1995).

Existing and potential markets

In 1992 the wild harvest of morels in the northwest region of the USA yielded 90,000 kg of fresh morels. This harvest was destined for the USA and European markets in fresh or dried form (Rowe, 1997). In Australia, the wholesale market pays from A$ 60-120/kg for morels, dependant upon quality (pers. comm. Peter Smedley 2002). Due to their scarcity and value the locations from where these morels have been harvested is often a closely kept secret. Currently the importation of dried morels, from suppliers all over the world, and fresh morel fruit bodies, from nearby countries such as New Zealand, is allowed by the Australian Quarantine and Inspection Service. The combination of fresh Australian morels and fresh or dried imports is still not enough to meet market demand. In Australia, fresh product is in high demand and the Australian mushroom industry is keen to develop cultivation techniques in order to reduce the dependence upon seasonal wild fungi of variable quality and to establish a new product.

Morel research to develop an artificial cultivation technique has not been well funded and pressure for "quick-fix" results has been considerable. Essential and rigorous scientific investigation has been lacking. Attempts at cultivation techniques by both scientists and industry have been often founded on the poorly reported and superficial work of others, another form of "re-inventing the wheel". In addition, the high market value of morels and the involvement of commercial partners, has resulted in scientists being reluctant, or unable, to publish their best results.

Identification and Genetic Variation

Black (*M. angusticeps* (Peck) Boudier, *M. costata* (Vent.) Boudier, *M. conica* (Pers.) Boudier) and yellow (*M. esculenta* Pers.: St. Amans, and *M. deliciosa* Fr.) morels are the most popular edible species and the species most commonly investigated. Classical taxonomy relies on accepted morphological characters that have been ascribed to species. Whilst this is effective for many basidiomycetes, morels exhibit considerable morphological diversity and there is considerable disagreement on the identification of morel species. Identification keys for morels vary greatly. Yoon, Gessner and Romano (1990) clearly outlines the difficulties that are caused by the use of ephemeral ascocarp characters to determine taxonomic species such as colour and size, which rapidly change as a result of age and environment. Grey, tan and large tan forms of *M. esculenta* have been found to be one species (Foster *et al.*, 1993). Jacquetant (1984) proposes 28 species and 14 varieties but recent studies support the view that there are relatively few morel species but that considerable phenotypic variation occurs (Volk and Leonard, 1989b; Yoon *et al.*, 1990; Bunyard *et al.*, 1994; Wipf *et al.*, 1996; O'Donnell *et al.*, 1997; Wipf, 1997).

There is considerable debate over 4 species: *M. costata, M. elata* Fr.Fr., *M. esculenta* and *M. crassipes* (Vent.:Fr.) Pers.. Cortecuisse and Duhem (1995) state that it is common to confuse *M. costata* with *M. elata* Fr.Fr. (=*M. conica* (Pers.) Boudier). However the differences they point out do not seem great to the non-mycologist; *M. elata* has an ascocarp with more uneven ridges than *M. costata* and generally is found in coniferous forest and orchards (Jacquetant, 1984; Cortecuisse and Duhem, 1995) whereas *M. costata* is found in orchards and rubbish heaps (Cortecuisse and Duhem, 1995). Some mycologists believe that *M. esculenta* and *M. crassipes* are a single species (Volk, 2000). Researchers tend to collect morel fruit bodies local to the region in which they work. This ensures that they have viable material over which no other group has any claim. These researchers are not mycologists and the potential for misidentification with such a variable genus is evident.

The problem of misidentification also applies to vegetative isolates obtained from fruit bodies. Any appellation will be linked to and bear any morphological misidentification originating from the ascocarp. Misidentified cultures may be lodged in culture collections and made available to others. Researchers who obtain different cultures from a collection may believe they are making comparative studies of distinct and different species, whereas they are working with only one species. Although all parties have acted ethically, unwittingly erroneous results may reach the public domain.

In summary the lack of clarity in morel taxonomy can lead to significant amounts of time and money being expended on only one species when a research project was intended to investigate a wider range of species. Another major problem is the difficulty faced when trying to compare work between authors as the comparison may or may not be valid depending upon the veracity of the taxonomic identification.

The tools of molecular biology offer another more discriminative method by which to confirm species identity. Studies of *Morchella* species have been undertaken using horizontal starch gel enzyme electrophoresis (Royse and May, 1990; Yoon *et al.*, 1990), sequencing of rDNA ITS (Wipf *et al.*, 1996; O'Donnell *et al.*, 1997), PCR-RFLPs of rDNA ITS and IGS (Wipf *et al.*, 1999) and micro-satellite primed PCR (Bunyard *et al.*, 1995).

Three “grey forms”, which had previously been referred to as *M. deliciosa* were found (using enzyme electrophoresis) to be *M. esculenta* (Yoon *et al.*, 1990). Allelic variation found during this study of isolates from different locations may in part explain the variation in colour and form expressed by this morel species. A study of rDNA ITS RFLPs suggested that the commonly recognised black and yellow morel groups are two distinct taxonomic groups; subdivision of each group into 3 species (*M. angusticeps, M. elata, M. conica* and *M. esculenta, M. crassipes, M. deliciosa*) is not valid (Bunyard *et al.*, 1994; Wipf *et al.*, 1996).

Genetic variation between geographically isolated populations of the same species may be as marked as that between two distinct species (Bunyard *et al.*, 1994). Goldway, Amir, Goldberg, Hadar and Levanon (2000) report that DNA-PCR fingerprints of *M. conica* isolates from the Dan Nature Reserve, Israel, have been found to be different to any morel isolates from a neighbouring region including *M. conica* isolates.

Habitat and distribution

Morels are known to produce fruit bodies under two distinct ecological environments: undisturbed and disturbed. Stable undisturbed ecosystems produce a limited number of fruit bodies each spring with production continuing over many years. Disturbed habitats produce numerous fruiting bodies in the spring following the disturbance but production rapidly declines over following years.

It is suggested that morels form an association with tree roots in stable ecosystems and it is this association that ensures long-term annual fruiting (Buscot *et al.*, 2000). This association may be ectomycorrhizal as was shown by Mayr (1982) with *M. conica* and *Pinus sylvestris.* More research in this area is needed to confirm such a relationship.

There are many examples of morels acting as pioneers on recently disturbed sites with fruit bodies appearing in the spring following the disturbance. This type of habitat is believed to have high nutrient availability and low levels of competition as a result of disturbance by mechanical or chemical means (Fourre 1985, cited in Buscot (1987)), fire damage (Stamets, 1993b; Volk, 2000) or after volcanic eruption (Carpenter *et al.*, 1987). *Morchella esculenta*, referred to as the natural morel, has been said to fruit in forests two or three years after disturbance caused by tree felling, as long as sufficient rain has occurred (Rowe, 1997).

In the18th-century German peasant women are said to have mimicked nature by deliberately starting forest fires to encourage morel production. In the 20th century morels were found in England on World War II bomb sites and after fires in Yellowstone National Park, USA. Rowe (1997) refers to two species (*M. esculenta* var. *atromentosa* and *M. conica* var. *nigripes*) as fire morels, stating that these species regularly fruit after major forest fires west of the Rocky Mountains, USA. An example is given of 10,000 kg of fire morels being harvested after a forest fire covering 1500 hectares in northeast Washington State, USA - an average of 6.7 kg per hectare (Rowe, 1997). Another illustration is the occurrence of morels in Malheur National Forest, Oregon, in 1991, after a forest fire the previous year (Volk, 2000). A two-year study investigated the persistence of morel basidiospores and sclerotia in both burnt and unburnt forest areas (Miller *et al.*, 1994). More sclerotia were found in the burnt than unburnt forest. Both basidiospores and sclerotia persisted in the soil for over two years. A large number of ascocarps occurred initially after disturbance in burnt forest but this rapidly declined in following years.

Association with plant tissue

Buscot and Roux (1987) found that fruit bodies of *M. rotunda* were connected by mycelial strands to subterranean compact mycelial masses surrounding the roots, which they termed “mycelial muffs”. These muffs were always attached to the roots of living trees such *Fraxinus excelsior, Ligustrum vulgare, Ulmus campestris, Quercus robur, Corylus avellana* or *Cornus sanguinea*, or herbaceous plants: *Equisetum hiemale, Illium ursinum* and occasionally *Taraxacum* spp. and *Eupatorium*

cannabinum (Buscot, 1987). Muffs form during summer and autumn and are purported to become dormant over winter, producing fruit bodies in spring. The starting point for the morel vegetative cycle is proposed as beginning in the spring when new muff associations are formed with roots (Buscot, 1989; Buscot and Bernillon, 1991; Buscot, 1993).

Hyphae from the muffs formed by *M. rotunda* penetrate the roots intercellularly, invading cells of the periderm, cortical parenchyma and young secondary phloem but not the xylem (Buscot, 1987). This strongly indicates *M. rotunda* can form a symbiont relationship with plant roots but the role this symbiosis plays in the morel life cycle is not known.

Sclerotia of *M. elata* have been reported to be associated with ectomycorrhizal root tips of *Picea abies* (L.) Karst (Norway spruce), although the nature of this association is unclear (Buscot, 1994). In the laboratory isolates of *M. elata* have been said to form ectomycorrhizal structures (mantle and Hartig net) with *Larix ocidentalis* (larch), *Pinus contorta* (lodgepole pine), *Pinus ponderosa* (ponderosa pine) and *Psuedotsuga meniesii* (Douglas-fir) but not with *Arbutus menzisii* (madrone) (Dahlstrom *et al.*, 2000). Whilst many fungi form ectomycorrhizae on the same host plant, and the same root tip has been found to host several ectomycorrhizal infections (Trappe, 1962), this relationship does not mean that an ectomycorrhizal relationship is essential for either the morel life cycle or ascocarp production.

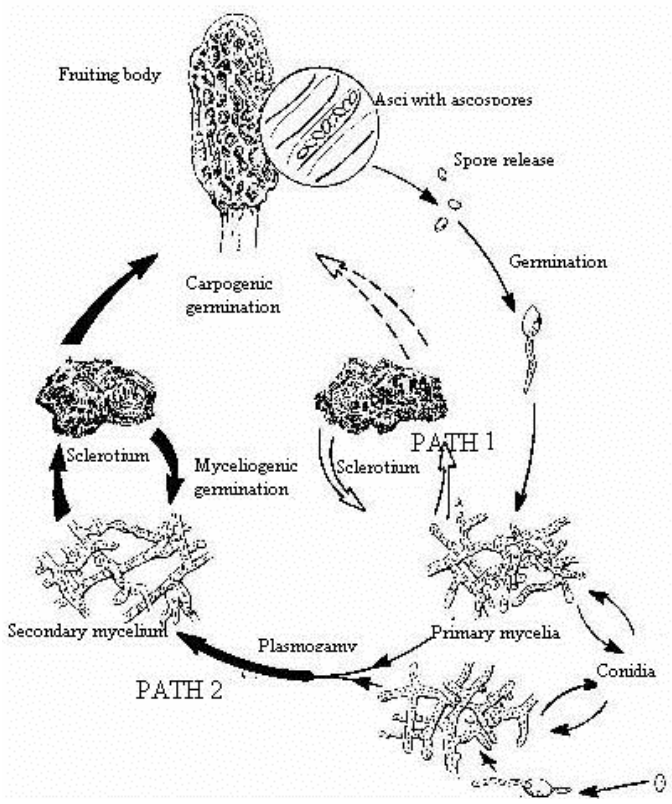
Life cycle and sexual behaviour

Morels are ascomycetes, fungi that generally have both asexual and sexual reproductive phases. Conidia result from asexual reproduction and ascospores result from sexual reproduction (Alexopoulos *et al.*, 1996). Each morel ascocarp is made up of numerous asci - each ascus containing eight spores.

The ascospores are ejected simultaneously from the ascus and disseminated by wind. Ascospores of *M. esculenta* germinate readily, within 24 hours at 20 ºC on 2 % MEA, 0.3 % yeast-extract filter-paper agar but poorly on 2 % bacto-agar (Hervey *et al.*, 1978). Vegetative hyphae rapidly colonised complete yeast malt agar (CYM) agar at 22-25 ºC, growing at a rate of 0.4-0.5 mm h^{-1} (Hervey *et al.*, 1978).

Volk and Leonard produced their representation of the *Morchella* life cycle, based on cytological observations, in 1989 (Figure 19).

Figure 19 *Morchella* life cycle proposed by Volk and Leonard (1989)



Whilst a conidial stage of *Morchella* species has been reported (Costantin, 1936) and sometimes obtained in culture (Molliard, 1904; Callieux, 1968; Ower, 1982; Buscot, 1987) others disagree that conidia are formed.

The hyphae of *M. esculenta* are relatively large in diameter (5-10 µm), multinucleate, septate, and have a tendency to anastomose (Hervey *et al.*, 1978; Volk, 1990). Morel mycelium is generally brownish in colour and has a characteristic growth pattern. Further information on morel hyphae, such as the presence, absence, or quantity of chitin and cellulase in morels has not been investigated.

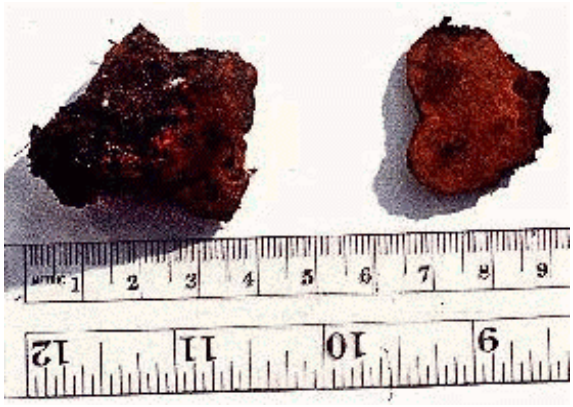
Two mycelial types have been distinguished in *M. esculenta* by Hervey *et al.* (1978), namely, "downy", or "fluffy", characterised by a fluffy rich growth of aerial hyphae, and, "granular" or "flat" lacking aerial hyphae. Hervey's study found some ascocarps only produced either "downy" or "granular" mycelium from SSIs whereas other ascocarps produced SSIs with both types of mycelia in an approximately equal ratio.

Fungal sclerotia are hard subterranean structures that are believed to act as a resting stage, resistant to unfavourable environmental or physiological conditions. They remain dormant until favourable conditions occur at which time they form new mycelium, or an ascocarp, and continue the life cycle (Alexopoulos *et al.*, 1996; Nelson, 1996). Morel sclerotia are believed to enhance survival over winter (Volk, 1990) but the conditions that trigger ascocarp formation arising from sclerotia are not clearly understood. The presence of a sclerotial stage in morels may be a precursor for ascocarp formation but could also simply be a nutrient storage organ waiting for favourable conditions for ascocarp production. Some fungal sclerotia are highly differentiated with complex rind and medulla tissue. Volk and Leonard in 1990 however suggested that morel sclerotia are undifferentiated and refer to them as pseudo-sclerotia. They describe these pseudo-sclerotia as ranging in size from 1-10 mm in diameter and composed of large cells with thick walls.

Volk and Leonard (1989) state that a true sclerotial stage is never formed under conditions of artificial cultivation. This statement is incorrect. Both the sporocarp and sclerotium of *Coprinus cinereus* (Kues, 2000) and *Pleurotus tuber-regium* have been observed in the laboratory or under conditions of artificial cultivation respectively (Fasidi and Ekuere, 1993; Ude *et al.*, 2001).

Morel sclerotia grown in artificial culture have been reported to be far larger than those described by Volk and Leonard in 1990 (Figure 20). These authors themselves, in 1992, describe *M. esculenta* and *M. crassipes* sclerotia as having the size and feel of a walnut (Volk and Leonard, 1992). The sclerotia of *M. angusticeps* are, according to Stamets (1993) much smaller.

Figure 20 Morel sclerotia grown under artificial conditions



Ascomycetes may be homothallic or heterothallic. Individuals of homothallic species are self-compatible, capable of forming fruit bodies, and have no mating types. Heterothallic species require the mating of two compatible individuals, of different mating types, for ascocarp production to occur. In both the ascomycete genera *Neurospora* and *Sordaria* a single species can show either homothallic or heterothallic behaviour depending on unknown events in the life cycle (Pöggeler, 1999). Pseudo-homothallism also occurs, arising from the compartmentalisation of two nuclei, of opposite mating types, in a single spore (Nelson, 1996). No evidence of mating types has been reported in morels, although Gessner, Romano and Shultz (1987) discussed this possibility in their population biology study. In summary, it is not known whether or which morel species are homothallic or heterothallic or whether a single species can present both types of sexual behaviour.

Under natural conditions, morel ascospores germinate readily within a few days of release, with each spore forming a mycelium of a single nuclear type. According to Volk and Leonard (1989), hyphae of different SSIs anastomose frequently and hyphal compartments have been found to contain 40-50 nuclei with sub-terminal “cells” having an average of 10-15 nucleii per cell and newly-partitioned tip “cells” having 1-2 nuclei. Under *in vitro* conditions compatible SSIs of *M. conica* have been found to form a “stable but partial” heterokaryon in which only a limited number of cells are heterokaryotic (Arkan, 1992). These heterokaryotic cells have several pairs of nuclei per cell (Volk and Leonard, 1992). This heterokaryotic pairing of nuclei has also been observed in sclerotia and in the sterile cells of the fruit bodies. It is not known if the heterokaryotic condition is required for morel ascocarp formation although heterokaryotic cells present in vegetative hyphae, sclerotia and fruit bodies indicate that this is the case. Buscot *et al.* (2000) discuss the possibility that sclerotia produce fruit bodies in a stable ecosystem are heterokaryotic; those sclerotia present in an ecosystem that only gives rise to fruit bodies after disturbance are homokaryotic and act primarily as storage organs.

Both Hervey *et al.* (1978) and Volk and Leonard (1990) found the contact zone where self-crosses of *M. esculenta* SSIs met to be indistinguishable from the morphology of the original SSI. However all non-self crosses of SSIs from the same ascocarp produced a barrage like concentration of aerial hyphae, often dark in colour, at the contact zone. These barrages were later termed "mycelial meld" (Volk and Leonard, 1990). This barrage formation indicates genetic variation amongst the SSIs from the same ascocarp. Hervey *et al.* (1978) concluded that for this to occur either the ascus fusion nuclei are heterozygous for certain genetic factors or the asci of a single ascocarp do not all arise from the same pair of nuclei.

Determining the life cycle and sexual behaviour of *Morchella* will contribute to our understanding of sclerotia formation and ascocarp production. Information to date is scant and inconclusive.

Factors influencing sclerotia formation

Early encrusting and late isolated sclerotia

Buscot (1993) working with both SSIs and a polyspore isolate from a single ascocarp of *M. esculenta* confirmed the findings of Mayr (1982) and identified two forms of sclerotia forming on single or multi-spore cultures, on agar medium A (g l^{-1}: agar, 10; malt extract, 5; glucose, 10) and agar medium B (g l^{-1}: agar, 10; yeast extract, 4; starch, 15; glucose, 10: KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5). These sclerotia, early encrusting sclerotia (EES) and late isolated sclerotia (LIS), were differentiated on the basis of morphogenetic and other characteristics. When hyphal growth was interrupted abundant EES were simultaneously produced at the site of interruption ie. the colony margin. The sclerotia were 0.2-0.5 mm diameter and rapidly aggregated into circular crusts that became pigmented. EES (as do young fruit bodies) form at low temperatures and have similar types and quantities of mycosporins (Buscot and Bernillon, 1991). LIS form natural subterranean mycelial masses connected to ascomata (Buscot and Bernillon, 1991). Under artificial conditions, LIS formed in relation to the age of the culture, anywhere on the mycelium, were few in number, remained separate and grew to a diameter of 5-8 mm with only the centre becoming pigmented (Buscot, 1993). It is suggested that LIS are equivalent to subterranean mycelial masses (mycelial muffs?) found in nature, acting as storage and frost-resistant organs which do not form an ascocarp (Buscot, 1993).

Analysis of mycosporins and related compounds has been suggested as a good method to characterise natural mycelial structures and to correlate between field and cultural forms exhibiting different morphological characteristics (Buscot and Bernillon, 1991). Similar mycosporin content in EES and ascocarps has been found. However the suggestion that this indicates EES are an early stage in fruiting development (Buscot, 1993) is not strongly borne out by available data.

Polyspore (vegetative) cultures on Buscot medium B formed radial lines of aerial mycelium that delimited into two to six irregular sectors. On these sectors: no sclerotia formed, either EES or LIS formed, or both EES and LIS formed. Where both EES and LIS formed this was not synchronous on the different sectors and EES were not confined to the colony margins (Buscot, 1993). In contrast monospore cultures on medium B did not form sectors. Sclerotia formation took longer, with EES forming in three weeks but less abundantly and not at the mycelial margin. LIS formed later and continued to form for several months (Buscot, 1993). Buscot grouped single ascospore cultures on the basis of: presence of EES, presence of LIS only, or absence of sclerotia. Confrontation studies between ascospore cultures from different ascocarps formed a line of aerial mycelium in all non-self pairings both between isolates from the same ascocarp and "between" isolates from different ascocarps. This finding was similar to that found between polyspore cultures (Hervey *et al.*, 1978; Volk and Leonard, 1989a).

Cultivation

Fruit body formation

Claims that morels have been cultivated have been made for more than a century (Table 12).

Table 12 Morel cultivation in the last 150 years

Year	Substrate	Author
1883	*Helianthus tuberosus*, Jerusalem Artichoke	Roze
1904	Apple compost	Molliard
1953	Burned leaves	Ramsbottom
1959	Cymbidium orchids	Baker and Mathin
1986	Soil and sand	Ower
1990	*Begonia tuberosus*, Tuberous Begonia.	Volk and Leonard

Singer and Harris (1987) state *M. hortensis, M. esculenta* and *M. costata* have been cultivated and that "cultivation of the morel is most similar to the cultivation of *Volvariella volvacea*, the only difference being a great dependency on the presence of trees, a slight difference in nutritional requirements....and very much lower temperature optima". This is a very vague description but it supports Molliard (1904) who is said to have grown wild *M. hortensis* under cherry trees in open ground. Molliard also grew *M. hortensis* in a pot filled with earth and some compost from apples. The resulting fruit bodies in the pot were said to have been small and few in number. Further anecdotal observations suggest that morels grow on apple residue covered with paper waste or dry leaves (Singer and Harris, 1987; Kaul, 1997).

Nutrition

As with other fungi, morels have been found to have specific nutrition requirements. This specificity indicates that nutritional sources should be determined for each species of morel.

There is scant information on the preferred pH for hyphal growth or production of sclerotia. In Australia morels have been found growing in soils of pH 4.29, 4.64 and 5.61 (Faris *et al.*, 1996). This is lower than the pH at morel sites in Denmark (Petersen, 1985), Sweden (Petersen, 1985) and India (Kaul, 1975). Singer and Harris (1987) also mention pH (Brock 1951, cited in (Singer and Harris, 1987)) stating that *M. esculenta* has a preferred pH of 6.93. How this was determined is not described.

Most fungi form sporocarps when under nutritional stress. Morels form sclerotia when nutritional stress, such as moving from a nutrient rich to a nutrient poor media, is encountered by hyphae. This stress can be replicated by using split-plates that are divided in two by a plastic barrier. Each half of the plate contains a different media, for example, nutrient rich in one half and nutrient poor in the other half. This method is commonly used to determine sclerotia formation in response to different media.

A decrease in mycelial biomass has been found to be accompanied by an increase in sclerotial biomass indicating strong translocation in the direction of the developing sclerotia (Amir *et al.*, 1993; Amir *et al.*, 1994). In a study of *M. esculenta* it is suggested this process occurs via turgor driven mass flow that can be affected by metabolic activities in both the sclerotia and hyphae (Amir *et al.*, 1995). A study in split-plates, with both rich and poor medium, found the nutrient status and availability in a poor medium affected the water potential between hyphae and sclerotia. The addition of hexose in the poor medium created a high turgor potential resulting in a large quantity of sclerotia (Amir *et al.*, 1992; Amir *et al.*, 1995).

Asparagine, urea, peptone, glutamic acid, ammonium salts, sodium nitrite, sodium nitrate and potassium nitrate provide good sources of nitrogen for the hyphal growth of *M. conica, M. deliciosa,*

M. hybrida, M. esculenta, M angusticeps and *M. crassipes* in liquid culture (Brock 1951, cited in (Singer and Harris 1987)). Starch was the best carbon source for *M. esculenta*, and was closely followed by maltose, d(-) fructose, d(-) turanose, d(+) glucose and sucrose (Brock 1951, cited in (Singer and Harris 1987)). Buscot *et al.* (2000) found L-fructose to be a poor source of carbon and confirmed D-fructose was a good carbon source for hyphal growth.

The addition of potassium nitrate and starch to boiled wheat grain, 1:4:400 respectively, was found to produce high yields of *M. esculenta* sclerotia within 35 to 40 days when grown at a temperature of 25 ^{o}C without light (Sharma *et al.*, 1997). Other grains suggested as supplying the nutrition morels require to produce sclerotia are: ryegrass, rape, hemp and rice (Ower *et al.*, 1986; Mushroompeople, 1996).

In summary information available on the nutritional requirements for the production of hyphae, sclerotia and ascocarp production is little more than anecdotal.

Patented method for fruit body formation

In 1982 Ower published a method for cultivating morels (Ower, 1982). This is the only method to have been commercialised. In conjunction with Neogen, a company affiliated with Michigan State University, Ower developed Patent no. 4,594,809 (Ower *et al.*, 1986). Unfortunately Ower died just before the patent was granted in 1986. Two Neogen scientists, Gary Mills and Jim Malachowski continued the study and a second patent, no. 4,757,640, was granted in 1989 (Ower *et al.*, 1989).

In 1988 Company M was formed by partners Neogen, Domino=s Pizza, Skandigen Incorporated of Sweden, Kuhn Champignon of Switzerland and Salk Institute Biotechnology Associates of La Jolla, California. A pilot plant was built in Mason, Michigan and has been said to produce approximately 50 morels in a soil substrate over an area of 1 square foot (Volk and Leonard, 1992). In 1992 the company Morel Mountain, Michigan, was said to produce 500 lbs of morels each week using the patented method. The patent was later sold to Terry Farms and production moved to Auburn, Alabama, where 5,000 lbs of morels are said to be produced each week (2002). Whilst this would seem to indicate that morel production is well established with this one company in the USA, there have been comments that morels grown under this process lack the taste and size of wild morels. Many people have tried, without success, to repeat the cultivation process described by Ower (Ower, 1982; Ower *et al.*, 1986; Ower *et al.*, 1989). The patent has been copied and sclerotia produced but ascocarp production has been unsuccessful.

Substrates enhancing sclerotia production

In studies by Singh *et al.* (1999) *M. esculenta*, isolate ME-1, was inoculated on a variety of substrates: sawdust, wheat straw, paddy straw, sand, farmyard manure, garden soil or sand plus garden soil which had been pre-soaked or impregnated with a 2 % dextrose solution. To these substrates was added a nutrient rich mixture of boiled wheat berries, CaCO3 (0.5 %), $CaSO_4$ (2 %), asparagine (250mg/kg), yeast extract (0.01 %) and peptone (0.1 %). Sand plus the nutrient rich mixture produced the greatest number and weight of sclerotia. Further analysis of data indicated that whilst the addition of sand significantly increased the number and weight of sclerotia the mean weight of sclerotia was actually slightly less (3.46g) than that resulting from the nutrient rich mixture without sand (3.5g). Growing two different isolates of the same species together on nutrient mixture plus sand produced more and heavier sclerotia, than substrate inoculated with a single isolate (Singh *et al.*, 1999).

Stimulation of sclerotia production

The patented method for growing morels indicates that a container, typically a 500ml volume jar, is filled to between 40-80 % of its volume with wheat or other vegetative material which has been supplemented with organic and inorganic nitrogens, minerals, vitamins and carbohydrates. This is covered with a perforated liner of plastic film or metal foil and the remaining 20-60 % of the container filled with moist soil. The jar is then covered and autoclaved but little detail is given. After autoclaving the soil is inoculated with ascospores, vegetative hyphae or small pieces of sclerotia, and

the container sealed and incubated at 18-22 ºC in the dark. The mycelium is said to grow through the substrate and on reaching the nutrient rich layer nutrients are translocated into the older mycelia, where they are stored as lipids in sclerotia. When sclerotia reach maturity the nutrient rich substrate is removed and water percolated between the sclerotia. Small ascocarps are said to appear at 10-12 days with mature morels forming in a further 12-15 days if conditions are favourable (Volk, 1990) but these conditions are not detailed. A similar technique using trays has also been reported (Mushroompeople, 1996). Images of fruit bodies produced using this technique show very small ascocarps of poor colour and shape.

Volk and Leonard (1989) investigated the suitability of components for the nutrient rich layer. Boiled rye grain, with peptone, yeast extract, trace elements and "Casamino acids", benefited sclerotial formation, as did the addition of asparagine or aspartic acid. The addition of a range of sugars and starch had no significant effect.

It is clear that sclerotia production requires both a nutrient poor and nutrient rich medium. The conditions required to ensure transition from sclerotia to ascocarp are not understood but a physiological shock, such as water, and/or temperature, seems to be the key element.

Temperature

The appearance of morels in spring, after the cold temperatures of winter, indicate that changes in temperature and soil moisture are possible triggers for ascocarp production. At the same time as temperature increases water availability also increases and this is likely to assist nutrient translocation. The change from cold to warm weather plus increased water availability may be the trigger required for ascocarp production.

In the Dan Nature Reserve, Israel, *M. conica* were produced during an eight-month period when temperatures and day length varied considerably (Goldway *et al.*, 2000). The dryness of the ground or the transition from saturated soil to relative arid conditions was suggested as being a major factor in the induction of morel ascocarps. This might explain the fruiting of morels in spring, when higher temperatures begin to dry the ground, or in early spring when night temperatures fall below freezing thus reducing water availability.

If sclerotia are an over wintering structure, then the suggestion that sclerotia be placed at 3.3-4.4 ºC for a period of two weeks to induce fruiting (Mushroompeople, 1996) may well be beneficial. Sclerotial studies found that EES form at temperatures ranging from 10-25 ºC and LIS form at temperatures >20 ºC. After a cold shock of both EES and LIS at +/-1 ºC for periods ranging from two to seven days only LIS were able to regenerate mycelium at 25 ºC with the resulting mycelium producing a higher number of EES than the original culture (Buscot, 1993). To initiate ascocarp formation it has been suggested that sclerotia are washed in water, placed in soil, and incubated at a temperature of 18 ºC (Buscot *et al.*, 2000). The washing of sclerotia in water may have the same effect as percolating water through substrate, as mentioned in patents. The unanswered question is whether the water is triggering ascocarp formation by imbibition, temperature change or removal of an inhibitor to ascocarp formation.

Conclusion

The taxonomy of *Morchella* is in disarray. Cooperation is urgently required between interested parties, both amateur and professional, with skills in taxonomy, genetics and molecular biology.

Numerous factors have been suggested as affecting sclerotia and their formation - the age of hyphae, the translocation of nutrients from mycelia to sclerotia, temperature and water. The optimal sclerotia size for ascocarp production is as yet unknown. After initiation young primordia have been found to be prone to abort unless environmental conditions are altered to enhance ascocarp development (Volk, 2000). These conditions remain largely undefined and it is obvious that a specific interaction of CO_2, temperature, humidity and nutrition is required for the formation of morel ascocarps.

Most of the research investigating artificial cultivation of morel's has focussed on detailing its life cycle, sclerotia production and the replication of existing patents. These patents and a large bank of anecdotal evidence provide little support for developing a cultivation technique for morels. There are two distinct steps to growing morels. The first should focus on the environmental and nutritional requirements for producing reliable and abundant sclerotia. The second step should investigate triggers and conditions required for ascocarp initiation and maturation.

Establishment of the Tasmanian Morel collection

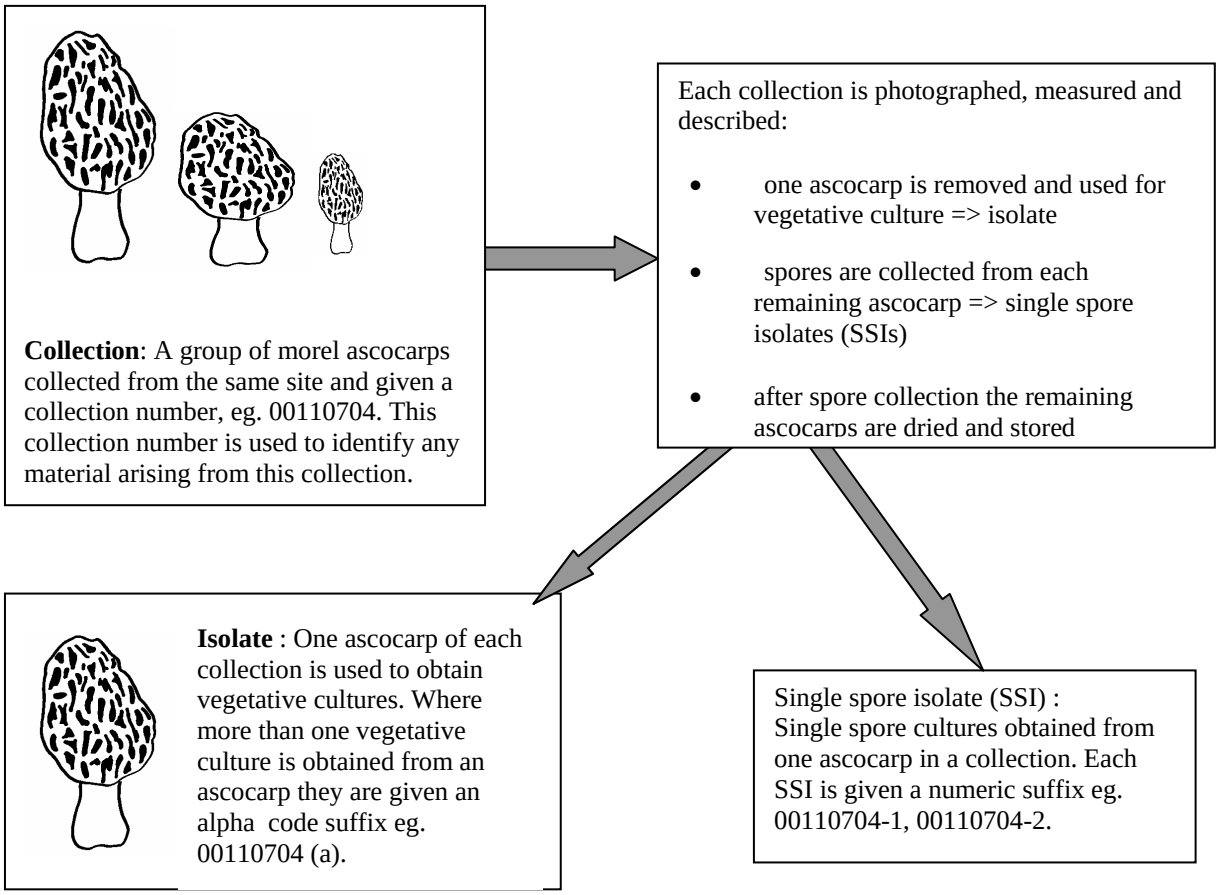
Introduction

Existing vegetative isolates were no longer viable, or the description of the fruit bodies from which they had been isolated was incomplete. In order to commence a study of development stages in the morel life cycle, and to contribute knowledge of cultivation techniques, a new collection of morels was needed.

Materials and methods

Thirty one collections of wild morels were made in Tasmania. Vegetative isolates were obtained from 21 collections and single spore isolates were obtained from 5 of these collections (Figure 21).

Figure 21 Description of collection, isolate and single spore isolate



Field sampling of wild Morels

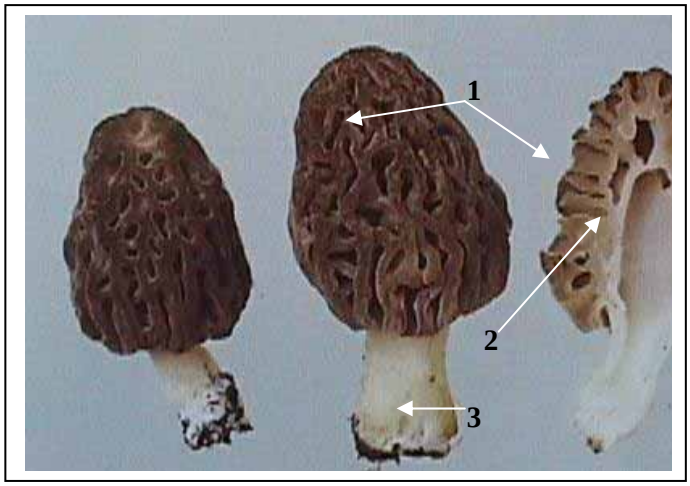
To ensure a variety of genetic material was available, morels were collected throughout Tasmania, Australia. Where possible, three ascocarps at different developmental stages, were collected from each site and given a collection number (Figure 21). Each ascocarp was removed with the first 15 mm of

substrate attached to the base of the stipe and loosely wrapped in paper prior to insertion of each ascocarp into a separate paper bag. In the laboratory each collection was photographed, measured and described and a sporulating ascocarp selected for spore collection. To ensure that the ascocarp did not dry out too quickly and to enhance spore collection, the sporulating structure was wrapped in greaseproof paper, aluminium foil and placed in a separate paper bag until spore collection was complete, or the ascocarp had dried.

Vegetative cultures

Axenic vegetative cultures were obtained from collections of Tasmanian morels (Figure 21). Thin sections of fleshy tissue, approximately 1 x 1 mm, were aseptically removed using a scalpel. These sections were taken from three regions of the ascocarp (Figure 22) and placed on one plate each of 0.5 malt extract agar (0.5 MEA; g l^{-1}: agar, 15; malt extract, 10) and 0.5 potato dextrose agar (0.5 PDA; g l^{-1}: agar, 7.5; potato dextrose agar, 20) in 90 mm disposable Petri plates and incubated in the dark at 25 °C until growth was established.

Figure 22 Sites of vegetative culture tissue samples



1, between the ribs on the upper section of the ascocarp; 2, at the core of the ascocarp; 3, towards the fleshy section at the base of the stipe

When a pure hyphal colony covered approximately 75 % of the surface area of a Petri plate, 5 x 5 mm sections were removed from the growing edge and placed, hyphal surface down, onto 0.5 MEA slopes, 5 replicates each, and incubated at 25 °C in the dark. These slopes became mother cultures and were designated *transfer 1* (*T1*). The *T* designation indicates subculture of an isolate whilst the numeral indicates the number of subcultures from the original wild specimen. Morels are known to rapidly lose their viability. In order to minimise this problem cultures were stored at 8 ºC and transferred at 6 monthly intervals to fresh slopes alternating between 0.5 PDA and 0.5 MEA.

Single spore isolates

All single spore isolates were grown from freshly germinated ascospores taken from one ascocarp of collections: 00110101, 00110304, 00110401, 00110404 and 00110801. Where possible, 50 single spore isolates (SSIs) were obtained from a single ascocarp (Figure 21). Spores were placed in sterile distilled water, and a dilution series prepared. At each step the spore suspension was vortexed for 20 sec, 1 mL removed and aseptically transferred to 0.5 MEA in a 90 mm Petri plate, and spread over the agar surface. Plates were incubated in the dark at 25 ºC with spore germination checked at 24 hours after inoculation and then at 12 hourly intervals. As soon as germination was evident SSIs were removed from plates which had less than six germinating spores. These SSI were designated *T1*, transferred to 0.5 PDA in a 90 mm Petri plate, and incubated in the dark at 25 ºC for three weeks. A

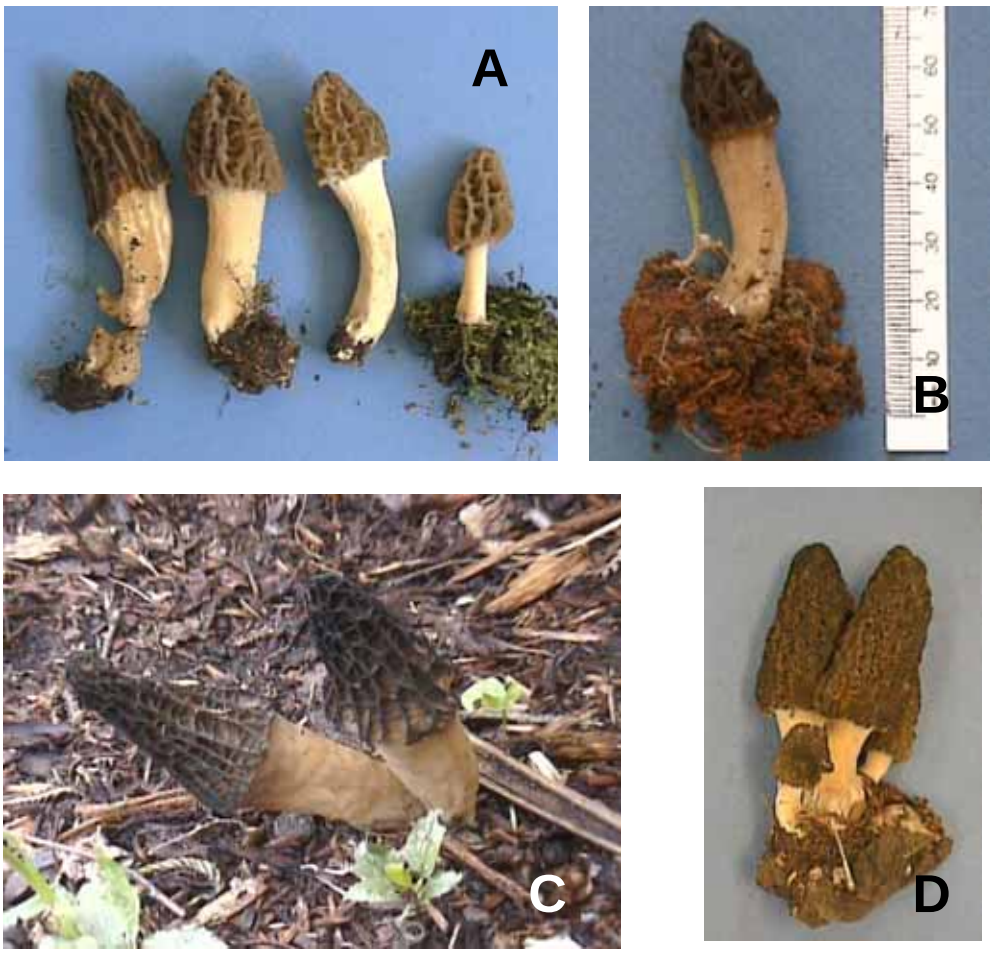
five mm disc was taken from the growing edge of each SSI (*T2*) and placed, with the hyphal surface in contact with the agar, at the centre of a 60 mm Petri plate containing 0.5 MEA. Three replicates of each isolate were placed in the dark at 25°C. The resulting fungal colony was observed at days 10, 30, 50 and 60 and SSIs of each collection grouped according to colony morphology and pattern of sclerotia production. Where groups, from different collections, exhibited similar colony morphology and sclerotia production, they were given the same group nomenclature.

Results

Field sampling of wild Morels

Thirty one Tasmanian morel collections were made in habitats ranging from native forests to domestic gardens. On the basis of morphological characters collections were placed into one of the following species: *M. esculenta* var. *angusticeps* (syn. *crassipes*), *M. elata, M. esculenta, M. deliciosa/elata* or *M. deliciosa* (Figure 23). The same problems experienced by other authors (Jacquetant, ; Yoon *et al.*, 1990), that of considerable variability in shape, colour and size of fruit bodies meant that it was only possible to give 16 collections a temporary classification. The remainder shared characters of different species making it impossible to give them even a temporary classification. These were grouped under *Morchella* spp. (Table 13).

Figure 23 Morel species collected in Tasmania



A, *Morchella deliciosa*; B, *Morchella deliciosa/Morchella elata*; C, *Morchella esculenta* var. *angusticeps* (syn. *crassipes*); D, *Morchella elata*.

Table 13 Species, origin and habitat of Tasmanian Morel collections

Morphological species	Collection[1]	No. [2]	Origin	Habitat
Morchella deliciosa	00110703	4	Hobart (Site 4)	Native bush
	00110704	3	Hobart (Site 4)	Native bush
*Morchella deliciosa/elata**	00110404	2	Southern Tasmania	Unknown
	00110702	2	Molesworth	Native grass
	00110802	2	Hobart (Site 6)	Native grass
	00110803	2	Hobart (Site 6)	Native grass
Morchella elata	00110302	4	Old Beach	Domestic garden
	00110402	3	Blackmans Bay	Domestic garden
	00110405	2	Mount Stuart	Bark mulch
	00110601	4	West Hobart	Bark mulch
Morchella esculenta var.	00110303	2	Old Beach	Domestic garden
*angusticeps***	00110304	2	Campbelltown	Domestic garden
	00110401	2	Taroona	Bark mulch
	00110602	2	West Hobart	Domestic garden
Morchella sp.	00082801	3	Sandy Bay	Domestic garden
	00101802	2	Hobart (site 1)	Native bush
	00101803	3	Hobart (site 1)	Native bush
	00101804	2	Hobart (site 2)	Native bush
	00101805	2	Hobart (site 3)	Native bush
	00101806	3	Hobart (site 3)	Native bush
	00101807	3	Hobart (site 3)	Native bush
	00103101	2	Southern Tasmania	Unknown
	00110102	1	Southern Tasmania	Unknown
	00110103	1	Southern Tasmania	Unknown
	00110403	2	Southern Tasmania	Unknown
	00110101	3	South Hobart	Bark mulch
	00110301	2	Old Beach	Domestic garden
	00110305	2	Campbelltown	Domestic garden
	00110705	2	Hobart (Site 5)	Native grass
	00110801	3	Hobart (site 6)	Native bush
	00111201	2	Lenah Valley	Native bush

* These isolates share morphological characters similar to both *M. deliciosa* and *M. elata*, ** Some authors would classify this species as *M. esculenta* var. *crassipes*; (1) Collection, a group of morel ascocarps collected from the same site; (2) No., number of dried ascocarps in a collection.

Vegetative cultures

Vegetative isolates were obtained from 21 collections, with some specimens yielding more than one isolate. Where several different isolates were obtained from the same fruit body, the isolates were given the suffix a, b, c, etc. Where a collection had multiple isolates confrontations were made between isolates in the collection, duplicate isolates eliminated, and the most vigorous isolate selected for later studies.

Single spore isolates

As cultures of SSIs often initiated sclerotia between day 30 and 50, assessment of SSI colony morphology was based on appearance at day 60 (Table 14). SSIs from the same fruit body were grouped on the basis of morphological characters such as: hyphal appearance; colour; texture; pattern

of sclerotia production; sclerotia colour; and exudate. Due to the variability in both colony morphology and sclerotia production, it was not possible to compare most groups between collections.

Table 14 Single spore isolates of *Morchella*, grouped within each collection, by colony morphology at day 60 on 0.5 MEA

Group	Collection				
	00110304 n =45	00110401 n=60	00110404 n=54	00110801 n=58	00110101 n=36
1	2.2	18.3	18.5	12.1	13.9
2	4.4	13.3	0.0	8.6	19.4
3	0.0	6.7	14.8	5.2	13.9
4	0.0	8.3	0.0	0.0	8.3
5	4.4	26.7	0.0	12.1	19.4
6	51.1	0.0	48.1	22.4	8.3
7	20.0	26.7	11.1	13.8	8.3
8	17.8	0.0	0.0	8.6	8.3
9	0.0	0.0	7.4	17.2	0.0

Group, SSIs within each collection grouped by colony morphology; n=, number of single spore isolates obtained from a single ascocarp in a collection; Values represent the percentage of each type of colony morphology found within each collection.

Discussion

The variability between species descriptions, and the difficulties in ascribing appropriate or adequate species identification, makes it extremely difficult to compare the results of this study with the work of others. In fact this is unlikely to be beneficial as previous research is of relatively little benefit. In essence, the focus of research in Tasmania is to establish a Tasmanian morel collection and to investigate cultivation techniques with these isolates, not to complete a taxonomic study.

Single spore isolates from one ascocarp in five different collections have been obtained with SSIs placed into groups, within each ascocarp, on the basis of hyphal morphology and sclerotia production. It was not possible to compare these groups across collections as colony morphology, in particular colour and density of hyphae varied considerably.

Industry outcomes

- Thirty one morel ascocarp collections have been made within Tasmania providing a considerable amount of material for research ensuring that IP issues relating to isolate ownership can be avoided.

- Twenty one vegetative morel isolates have been established and are available for cultivation studies.

- Single spore isolates of five morel fruit body collections are available for future research

Sclerotia production by Tasmanian *Morchella* species

Introduction

Previous studies with Australian morel isolates have found the development of sclerotia is a significant process in the life cycle of morels. In this study, isolates from both Tasmania, Australia, and overseas were tested to determine techniques which enhanced sclerotia production, the type of sclerotia produced, and differences between isolates.

Australian morel studies investigated mycelial growth as a function of temperature and the formation of sclerotia *in vitro* (Faris *et al.*, 1996). Morels were collected from three sites, the Grampians and Mount Pilot in Victoria, and Cowra in New South Wales. The pH of soils at these morel fruiting sites was 4.29, 5.61 and 4.64 respectively. Temperatures of 20-25 ºC were found to be optimal for hyphal growth, whereas 5, 10, 15 and 30 ºC produced poorer growth. The application of a heat-shock of 50 ºC for 3-7 days did not benefit hyphal growth nor sclerotia production. In a split-plate study with nutrient poor carnation leaf agar (CLA), and nutrient rich malt extract agar (MEA) sclerotia only formed on the medium on which the inoculum was placed. The formation of sclerotia was enhanced when mycelium grew from the nutrient poor to the nutrient rich medium.

Materials and methods

Isolates

Tasmanian morel isolates were obtained from collections made in the year 2000 (Table 13). Morel isolates, sourced from overseas and used in this study were imported under an AQIS permit (Table 15).

Table 15 Species, identifying code and source of overseas Morel isolates

Species	Code	Source
Morchella angusticeps	M 18	Fungi Perfecti, USA
Morchella angusticeps	Ma 92-23	CBS 288-63
Morchella angusticeps	WC186	Penn.State Uni., USA; West Kettle River BC Canada
Morchella angusticeps	WC198	Penn.State Uni., USA; Juliet Creek #1 BC Canada,
Morchella angusticeps	WC331	Penn.State Uni., USA, State College PA
Morchella angusticeps	WM145	Northwest Mycological Consultants, OR, US
Morchella conica	Mcn 92-1	INRA, France
Morchella costata	Mc 88-4	INRA, Pont de la Maye, Gironde
Morchella crassipes	Mcr 92-24	CBS 289-63
Morchella deliciosa	Md 92-25	CBS 276-88
Morchella elata	Me 92-22	CBS 170-73
Morchella esculenta	M 11	Fungi Perfecti, USA
Morchella esculenta	Mes 92-6	INRA, Suisse
Morchella hortensis	Mh 88-7	INRA, Provence
Morchella intermedia	Mi 93-6	United States of America
Morchella rigida	Mri 93-7	INRA, Ste Anastasie, Cantal
Morchella rotunda	Mr 87-7	INRA
Morchella umbrina	Mu 91-8	Ardes sur Couze, Puy de Dôme, under hazel or oak
Morchella vulgaris	Mv 87-8	INRA; black

CBS, Centraalbureau voor Schimmelcultures; INRA, Institut National de la Recherche Agronomique

Buscot media

Vegetative isolates of Tasmanian morel collections: 00101806, 00101807, 0110101, 00110301(b), 00110302(a), 00110303(a), 00110304(a), 00110401(a), 00110401 (c), 00110402 (a), 00110403(a), 00110403(b), 00110404(a), 00110405(a), 00110702(a), 00110703(a), 00110703 (c), 00110703 (d), 00110704(b), 00110704(d) (Table 13), were studied to determine whether they formed sclerotia, an integral stage in the morel life cycle, on Buscot A and Buscot B media (Buscot, 1993). Five mm diameter discs were taken from the growing edges of vegetative cultures and placed, with the hyphal surface in contact with the agar, at the centre of 90 mm Petri plate containing either Buscot medium A (g l^{-1}: agar, 10; malt extract, 5; glucose, 10) or Buscot medium B (g l^{-1}: agar, 10; yeast extract, 4; starch, 15; glucose, 10; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5). Three replicates of each isolate were placed in the dark at 25°C. The resulting fungal colony was observed at weekly intervals, with the day of first sclerotium formation and the region where sclerotia first formed: inoculum, perimeter or all over agar surface noted, until no further sclerotia developed.

Influence of substrate nutrients

Vegetative isolates of Tasmanian isolates 0082801, 00101806, 00110302(a), 00110403(a), 00110702(a), 00110703 (Table 13) and overseas isolates Mh 88.7, Mr 87.7b (Table 15) were studied to determine the formation of sclerotia in split-plates containing both nutrient rich and nutrient poor media. The two different media were nutrient rich malt extract agar (MEA; g l^{-1}: agar, 15; malt extract, 20), and nutrient poor distilled water agar (DWA; g l^{-1}: agar, 15). Both the poor or the rich medium was inoculated with each isolate tested. Five mm discs, taken from the growing edges of vegetative isolates were placed, with the hyphal surface in contact with the agar. Three replicates for each isolate on each medium type were placed in the dark at 25°C. Formation, location and type of sclerotia were assessed at weekly intervals.

Response to bacteria

Isolates

During, sub-culturing, some morel isolates were found to be contaminated with bacteria, presumably associated with original fruit body habitat or fruit body specimen. Some of these bacteria appeared to be enhancing sclerotia production. Eight different bacteria, which had been isolated from vegetative and SSI morel isolates, were used in interaction studies (Table 16).

Three morel vegetative isolates were selected for study on the basis of their sclerotia formation in culture: 00110601(b) was a fast coloniser and formed sclerotia in large numbers; 00110703(b) formed sclerotia in moderate numbers, and; 00111201(a) was a poor producer of sclerotia.

Table 16 Origin of bacterial cultures

Bacteria	Collection No.	Source
B1	00101807-AA	Vegetative isolate of *Morchella* No. 00101807(a)
B2	00101807-AB	Vegetative isolate of *Morchella* No. 00101807(a)
B3	00110101-18-A	Single spore isolate *Morchella* No. 00110101-18
B4	00110101-19-AA	Single spore isolate *Morchella* No. 00110101-19
B5	00110101-19-AB	Single spore isolate *Morchella* No. 00110101-19
B6	00110801-53-A	Single spore isolate *Morchella* No. 00110801-53
B7	00110703(d)-A	Vegetative isolate of *Morchella* No. 00110703(d)
B8	00110703(d)- B	Vegetative isolate of *Morchella* No. 00110703(d)

Characterisation of bacteria

Three replicates of each of the following treatments were prepared, incubated in the dark at 25 ºC and growth assessed at day 7 after inoculation. Each of the 8 bacteria was streaked onto 0.5 MEA (g l^{-1}: agar, 15; malt extract, 10), MYA, PDA and nutrient agar (NA) in 90 mm Petri plates, and into 10mL of potato dextrose broth (PDB) or nutrient broth (NB). The ability of bacteria to produce cytochrome oxidase, catalase, and their oxidative/fermentative utilisation of glucose in Hugh and Liefson medium was determined. Bacteria were differentiated by colony shape and colour on NA streak plates using a combination of colony morphology and microscopy to determine the presence of extracellular polymeric substances. The gram reaction and cell morphology were determined by Gram stain whilst motility was determined using phase contrast optics on an Olympus compound microscope.

Morel-bacteria confrontation

The eight different bacteria and three *Morchella* isolates, 00110601(b), 00110703(b) and 00111201(a), were used for interaction studies, which were undertaken on 90 mm Petri plates. A line was drawn on the base of each plate to mark the plates into two halves. The centre of one half was inoculated with a 6mm disc, taken from the growing edge of morel vegetative culture, and placed, with the hyphal surface in contact with agar, on NA and MEA and incubated in the dark at 25 ºC. After incubation for 48 hours seven-day-old bacterial broth cultures, which had been gently vortexed for 10 seconds, were spread, on one half of the plate opposite *Morchella*, using a sterile cotton bud. Three replicates of each treatment were incubated in the dark at 25 ºC for a further 5 days. Plates were visually assessed for growth of aerial hyphae and distribution of sclerotia, hyphal colonisation, sclerotia formation, sclerotia distribution (bacteria or inoculum side of the plate or on inoculum disc).

Morphological variations of sclerotia, between different treatments, were examined using a Zeiss dissection microscope to observe sclerotia, hyphae and aerial hyphae. Squash preparations of sclerotia and hyphae from Petri plates (0.5 MEA) were stained and mounted in lactophenol cotton blue beneath a coverslip and examined under x 100 magnification.

Analysis

Data from the Buscot media trial was analysed using a Chi Squared technique (Genstat® (Release 4.2, 5th Ed., Lawes Agricultural Trust, Rothamstead)). The number of days for all isolates and treatments to form sclerotia at the inoculum was analysed by ANOVA (Genstat®). Comparisons between the treatment days until sclerotia formation was tested by least significant difference (LSD <0.05).

Results

Buscot media

The effect of medium on the region of the Petri plate where sclerotia formed was significant (P<0.001) (Table 17). Buscot medium A was more likely to result in sclerotia formation at the agar perimeter whereas on Buscot medium B, sclerotia were more likely to form at the inoculum. Different strains were found to form sclerotia at different locations (P<0.001) but no interaction between strain and medium was found.

Table 17 Time and location of sclerotia formation on Buscot Media

Isolate	Medium A		Medium B	
	Region	Day	Region	Day
00101806	I	52	I	16
00101807	A	56	I	56
00110101	P	7	P	52
00110301(b)	P	8	-	-
00110302(a)	P	7	A	11
00110303(a)	P	7	I	52
00110304(a)	I	8	I	8
00110401©	P	8	I	8
00110401(a)	P	12	I	11
00110402(a)	P	7	I*	11*
00110402(a)	-	-	P**	13**
00110403(a)	P	7	P	7
00110403(b)	P	8	P	10
00110404(a)	P	7	P	4
00110405(a)	P**	24**	I**	8**
00110405(a)	I*	8*	A*	24*
00110702(a)	P	7	I	10
00110703(a)	P	20	-	-
00110703(d)	-	-	I	8
00110703(c)	-	-	I	8
00110704(b)	A	47	I	-
00110704(d)	I	24	I	24

I, sclerotia formed at inoculum; A, sclerotia formed all over agar, P, sclerotia formed at agar perimeter. Unless otherwise shown values represent the mean of three replicates; * one replicate; ** mean of two replicates; -, data not available.

After day 200 no further sclerotia production was observed. Sclerotia had developed all over the agar surface in medium A. Sclerotia development on medium B was less consistent and only a few isolates had developed sclerotia over the entire agar surface. On medium B, some isolates did not form sclerotia, sclerotia formed at either the inoculum or at the perimeter of the dish and in some cases, at both the inoculum and perimeter.

Influence of substrate nutrients

The production of sclerotia on DWA or MEA differed between ascocarp collections with some isolates producing sclerotia only on either DWA or MEA and others on both or neither (Figure 24) (Table 18).

Figure 24 ***Morchella*** **forming sclerotia on split-plates containing DWA and**

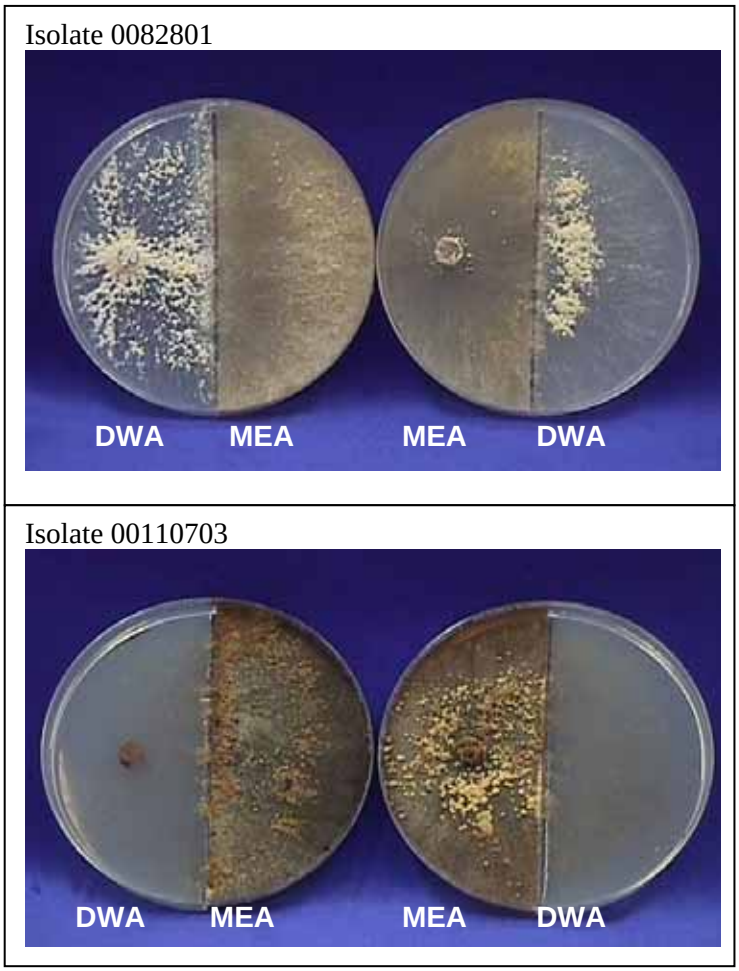


Table 18 Sclerotia formation by Morels in response to high and low nutrient concentrations

Isolate	Medium Inoculated	Sclerotia DWA	Sclerotia MEA
00082801	DWA	+	+
	MEA	+	+
00101806	DWA	-	-
	MEA	-	-
00110302(a)	DWA	+	-
	MEA	+	-
00110403(a)	DWA	+	-
	MEA	+	-
00110702(a)	DWA	+	-
	MEA	+	-
00110703	DWA	-	+
	MEA	+	-
Mh 88.7	DWA	-	+
	MEA	-	-
Mr 87.7b	DWA	-	+
	MEA	-	-

Response to bacteria

Characterisation of bacteria

The colony morphologies of bacteria on NA were distinctly different (Table 19) with tests and observations distinguishing different bacterial isolates (Table 20). Bacteria 8 was the only isolate capable of utilising glucose.

Table 19 Descriptive characterisation of bacterial colonies on NA.

Bacteria	B1	B2	B3	B4	B5	B6	B7	B8
Colour	pink orange	yellow	cream	apricot	yellow	peach	white	v. faint yellow
Opacity	opaque	translucent	opaque	opaque	translucent	opaque	Translucent	opaque
Form	circular	circular	circular	circular	irregular	circular	circular	circular
Elevation	flat	flat	convex	umbonate	flat	flat	Umbonate	convex
Margin	entire	entire	entire	entire	filamentous	lobate	entire	erose
Other	slow growing	slow growing			slow growing		Digests agar	

Table 20 Characterisation of bacterial isolates

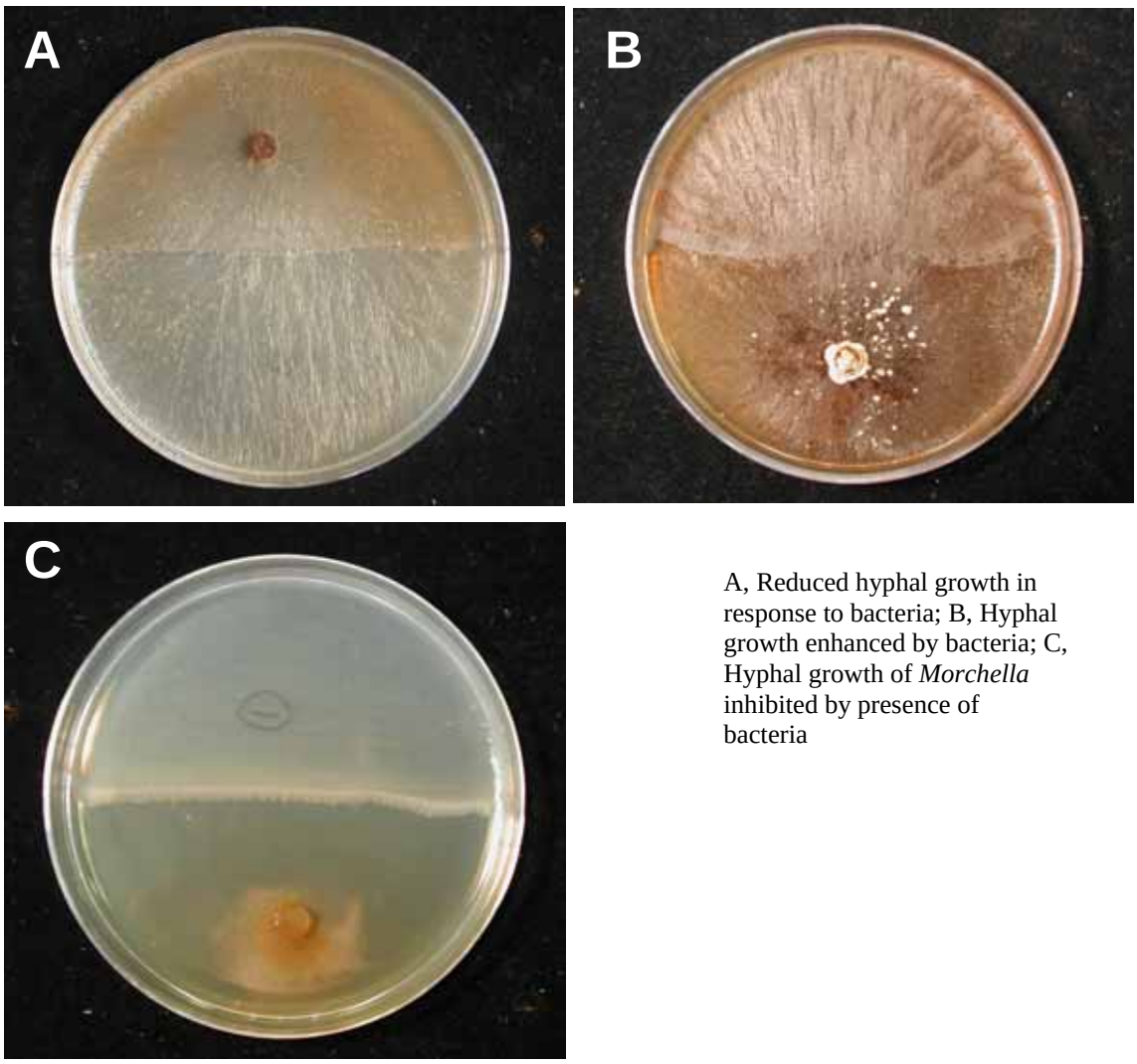
Test	B1	B2	B3	B4	B5	B6	B7	B8
Gram	-	-	+	+	+	+	-	-
EPS	N	N	Y	Y	Y	Y	N	NR
Cell shape	rod	rod	rod	rod	rod	rod	rod	rod
Cell form	rounded	elongated narrow	pleomorphic	NR	NR	short	NR	NR
Motility	N	N	NR	Y	Y	NR	NR	Y
Catalase	+	+	+	NR	NR	+	+	+
Oxidase	-	-	NR	NR	NR	-	NR	NR
Glucose Utilisation	no	no	no	no	no	no	no	oxidative
PDA	NR	NR	NR	+	+	NR	NR	NR
MYA	+	+	+	+	+	+	+	+
MEA	reduced growth	reduced growth	+	+	+	+	+	+
NA	+	+	+	+	+	+	+	+
NB	pellicle	+	+	+	pellicle	+	+	+
PDB	poor	poor	Good	good		good	good	NR

NR, no result; -, negative result; +, positive result; EPS, extracellular polymeric substances; pellicle, formed pellicle on broth.

Morel-bacteria confrontation

All observations were made on 0.5 MEA as growth of *Morchella* on NA was extremely poor. Three effects of bacteria on hyphal growth of *Morchella* were noted: increasing hyphal density, reduction of hyphal growth or inhibition of hyphal growth by antagonism. All bacterial isolates against 00110601(b) produced isolated sparse aerial hyphae, with the exception of B8 which developed dense aerial hyphae over the whole of the plate giving it a fluffy appearance. Aerial hyphae of other isolates were dense and in concentrated, localised regions. Where *Morchella* 00110703(b) came in contact with bacterial isolate B3 dense hyphal growth was encouraged (Figure 25) but no other interactions were found. On 0.5 MEA hyphal growth of all *Morchella* isolates was reduced by bacterial isolate B8. Bacterial isolate B7 reduced hyphal growth of 00110703(b) and 00111201(a) whilst B6 only reduced hyphal growth of 00111201(a).

Figure 25 Hyphal growth of *Morchella* in response to bacteria



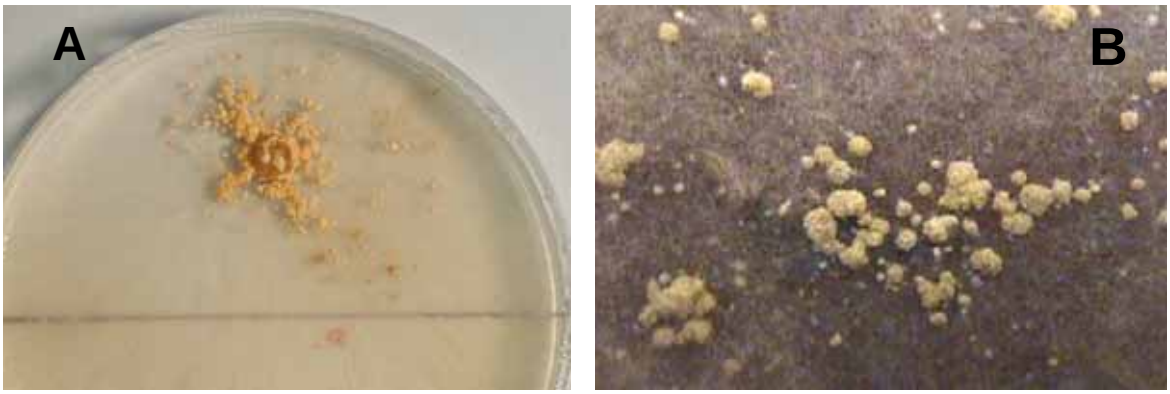
A, Reduced hyphal growth in response to bacteria; B, Hyphal growth enhanced by bacteria; C, Hyphal growth of *Morchella* inhibited by presence of bacteria

Sclerotia formation

Two types of sclerotia were produced, dependent on the interaction of *Morchella* with bacteria. These were designated sclerotium type 1 (ST1) and sclerotium type 2 (ST2). Sclerotia type 1 formed first, generally at or before ten days, often aggregated at or near the inoculum and had few sclerotia forming on the side of the plate opposite to inoculum. These sclerotia matured slower than ST2 and exhibited a colour change from light to dark, usually from white to brown or orange at maturity (Figure 26). ST1 were confined to the surface of the medium and were "fluffy" in appearance. ST2 initiated after ST1, were dispersed across the surface of the agar and matured faster than existing ST1, becoming a darker uniform colour at maturity (Figure 27).

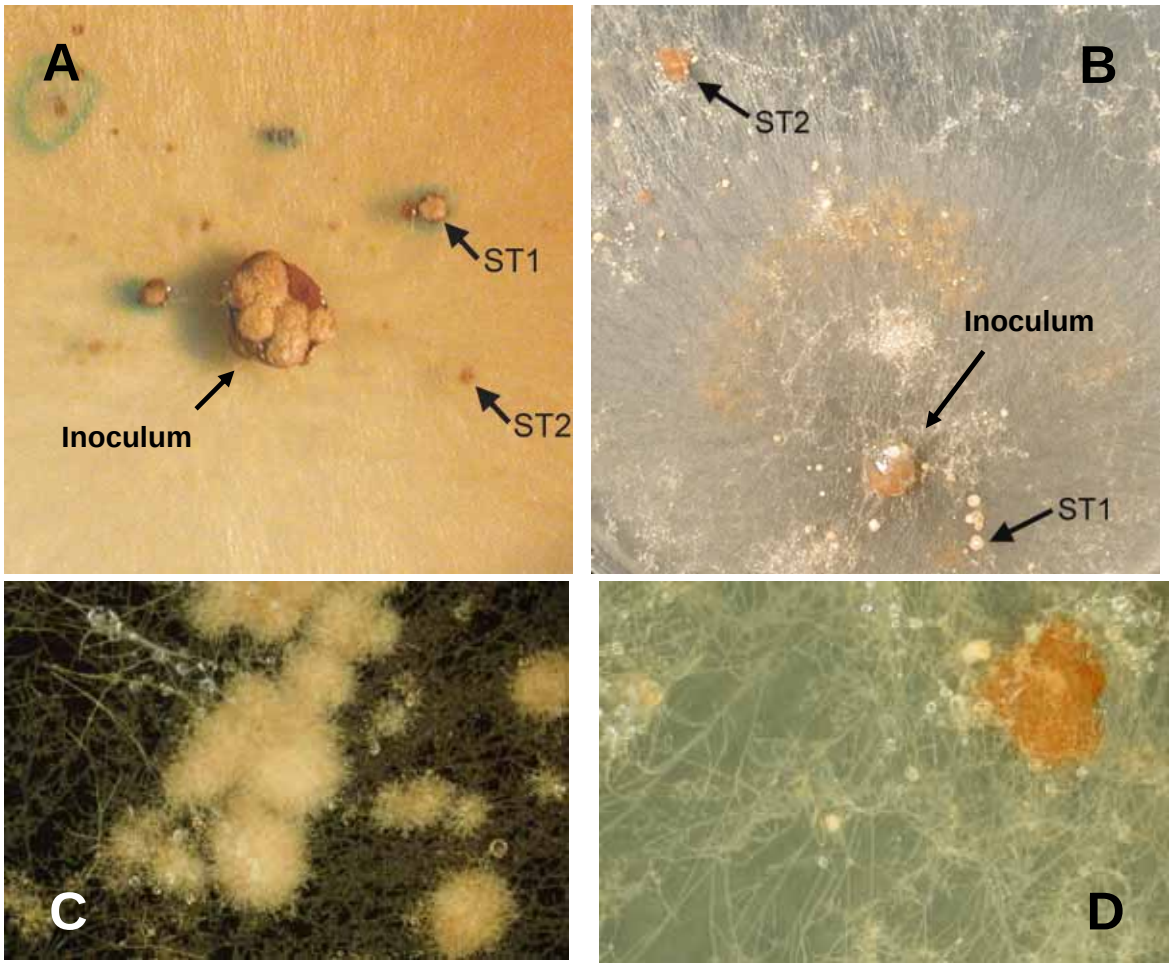
No differences were found in the microstructure of ST1 and ST2. Microscopic examination of the main body of sclerotia revealed cells to be swollen and reduced in length when compared to hyphae. Those hyphae at the perimeter of the sclerotial structure were swollen and densely packed together. The “fluffy” appearance of sclerotia appeared to be caused by longer hyphae extending from the sclerotium surface (Figure 28).

Figure 26 Sclerotia types 1 and 2



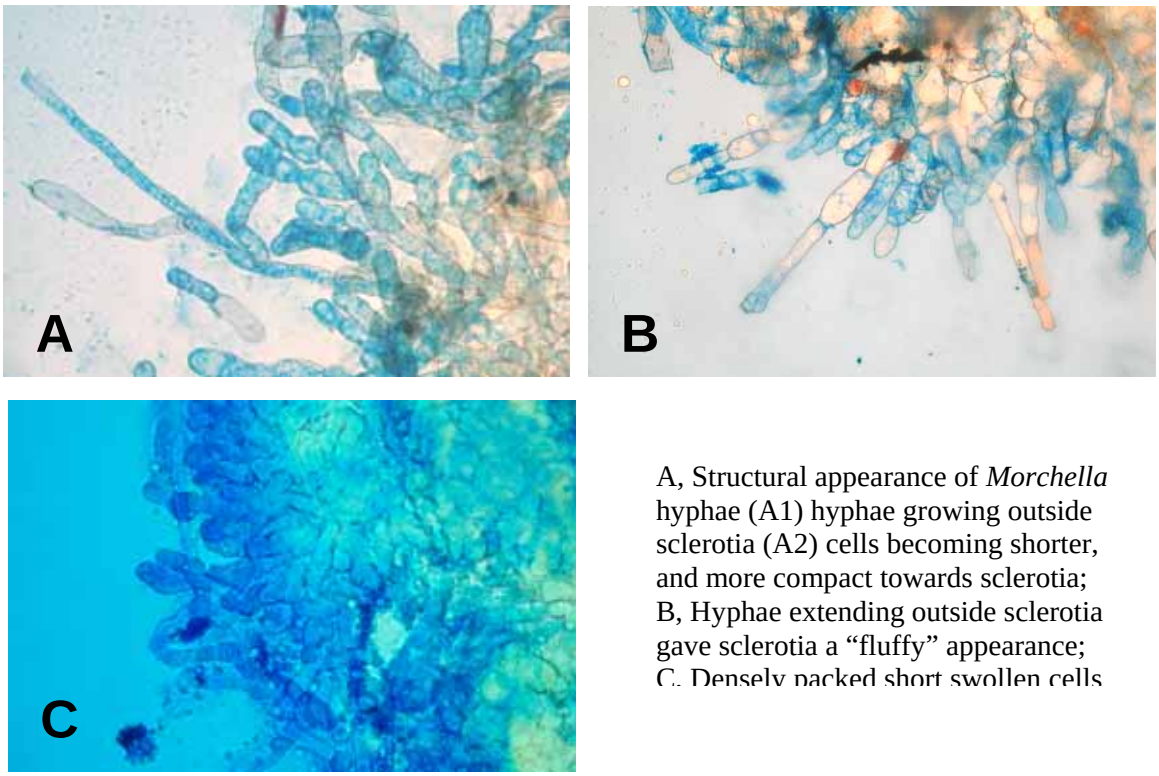
A, ST1 forming near inoculum; B, Young ST2

Figure 27 Position of sclerotia types 1 and 2 in relation to agar surface



A and B, ST1 forming on agar surface and ST2 forming in agar; C, Sclerotial initials forming on aerial hyphae; D, Sclerotia forming on hyphae at agar surface

Figure 28 Microstructure of sclerotia



A, Structural appearance of *Morchella* hyphae (A1) hyphae growing outside sclerotia (A2) cells becoming shorter, and more compact towards sclerotia; B, Hyphae extending outside sclerotia gave sclerotia a "fluffy" appearance; C, Densely packed short swollen cells

Dependant on the *Morchella* isolate ST2 formed on the surface of the medium, 00110601(b), below the surface of the medium, 00110703(b), or both above and on the surface of medium, 00111201(a). Initially, the sub-surface sclerotia appeared as concentrated masses of long thin hyphae with no swollen cells. Whilst ST1 were present on at least one replicate of all three *Morchella* x bacteria treatments, ST2 occurred on most bacteria confrontations with 00111201(a) (Table 21). Bacterial isolate B8 caused a reduction in growth of all *Morchella* isolates with a significant reduction in both size and number of ST1 with 00110703 and 00111201(a).

Table 21 Occurrence of ST1 and ST2 on *Morchella*- bacteria confrontation

Morchella	B1	B2	B3	B4	B5	B6	B7	B8	Control
00110601(b)	1	1	1	1	1	1	1	1,2	1
00110703(b)	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1	1,2
00111201(a)	1	1,2	1,2	1,2	1,2	1	1	1	2

1 = ST1; 2 = ST2.

Bacterial isolate B8 affected growth of all *Morchella* isolates, and was the only bacteria able to utilise the glucose in the Hugh and Liefson experiments. Treatments with reduced hyphal growth had smaller sclerotia: 00110703(b) on B7, 00111201(a) on B6, B7 and B8, which did not increase in size over time.

Discussion

Buscot media

For the first time the formation of sclerotia on Buscot media has been statistically analysed: medium A favours the formation of sclerotia at the perimeter of agar whilst medium B favours the formation of sclerotial at the inoculum. Considerable variation in the timing, and location, of sclerotia formation was found between isolates on the different media. The use of Buscot=s terms “late” and “early” to describe sclerotia formation is misleading as sclerotia were found to form, in response to media and not as a function of time. Little similarity was found with the early encrusting or later isolated sclerotia described by Buscot and Bernillon (1991) and the sectoring they describe with polyspore cultures, did not occur with vegetative isolates used in this study. Studies by other authors (Philippoussis and Balis, 1995; Faris *et al.*, 1996) have also found that *Morchella*, from different ascocarps, did not form EES or LIS.

Influence of substrate nutrients

In this study overseas isolates *M. hortensis* and *M. rigida* only produced sclerotia on the nutrient rich medium when nutrient poor medium was inoculated. Philippoussis and Balis (1995) found the same result for a Greek isolate of *M. esculenta*. Three Tasmanian isolates produced sclerotia on both nutrient poor and nutrient rich media but only when the inoculum was placed on the nutrient poor medium. This finding is similar to a previous study of Australian *Morchella* isolates in which sclerotia production was enhanced when isolates grew from CLA, a poor nutrient substrate, to MEA, a rich substrate (Faris *et al.*, 1996). One Tasmanian isolate produced sclerotia on both nutrient rich and nutrient poor media irrespective of which medium was inoculated whilst another isolate did not produce any sclerotia. It has been suggested that a nutrient poor medium is not required for sclerotia production (Singh and Vema, 2000) but this study agrees with Buscot (1993) and Faris *et. al.* (1996) that a nutrient poor substrate is necessary for the production of sclerotia.

The variety in sclerotial responses to different nutrient media, again illustrates that little is known about why and how sclerotia are formed. Philippoussis and Balis (1995) hypothesised that their results, which differed from virtually all previous work, were due to the different isolates and cultural conditions in Greece. Similar variation was found with Tasmanian isolates.

Morels and bacteria

The fungi *A. bisporus* and *Glomus versiforme* have had their relationship to associated bacteria scrutinised. In the absence of the soil bacteria *Pseudomonas putida* basidiocarp initiation of the edible mushroom *A. bisporus* has been found to be delayed by one to two weeks with less initials forming (Rainey *et al.*, 1990). Where bacteria were allowed to grow with germinating spores of *G. versiforme* more spores were found to germinate (Mayo *et al.*, 1986). *Morchella* have been found in the root zone of a plant with *Bacillus* spp. (Buscot, 1992) and within the fungal cells of a Hartig net with an undetermined endobacteria (Buscot, 1994). No other information is available on the relationship between bacteria and *Morchella*.

A selection of eight bacteria, found growing in association with Tasmanian *Morchella*, have been characterised. Two types of sclerotia were formed by *Morchella* in association with bacteria, ST1 and ST2. ST1 occurred on the surface of the agar and tended to aggregate near the inoculum which is different to Buscot’s description of early encrusting sclerotia that form at the margin. The later forming ST2 were dispersed across the agar surface with sclerotia forming in, on or above agar

dependent on the morel isolate grown. This pattern of sclerotia formation is similar to Buscot's late isolated sclerotia but whether late isolated sclerotia form in, on or above medium is not known.

Industry outcomes

- Comparisons of sclerotia production have been made between a range of Tasmanian and overseas morel isolates.

- A number of Tasmanian isolates produced sclerotia on both nutrient poor and nutrient rich medium only when inoculated on nutrient poor medium. This response differs to overseas isolates indicating the mechanism of sclerotial formation differs and/or Tasmanian morel collections are different species.

- Studies on Buscot media found the formation of sclerotia at the inoculum or perimeter is a response to media and not time. This indicates that a suitable medium may be developed for rapid sclerotia production, shortening the time required for one phase in the morel life cycle.

- Two types of sclerotia formed in response to bacterial confrontation suggesting there is much more to be learned about the interaction of morel and other organisms found growing in association with it in the wild.

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Appendix

Appendix 1 Project Milestones

General Tasks	Milestone	Reporting Date	Performance Indicator
1. Existing maitake and morel isolates subcultured from collection	Overview of maitake and morel isolates available for research by 1 April 2000	30th November 2000	List of maitake and morel isolates available and description of vitality
2. Evaluation of mushroom growing facilities at HVM, TIAR (Newtown) and University of Tasmania	Detailed description of all possible experimental facilities, including any (within budget) modifications necessary by 1st June 2000	30th November 2000	List and description of facilities, clearly defining access and possible modifications plus costing
3. Modifications to existing mushroom growing equipment for CO_2 and RH control	Availability of equipment with stringent control of CO_2 and RH by1st September 2000	30th November 2000	Progression to further and successful experimental research
4. Up to date review of relevant literature	Acquisition of all relevant background information by 1st July 2000	30th November 2000	Report for industry partner Review article submitted to scientific journal
5. Contacting national and international mushroom growers • Visit to USA morel and maitake growers (TBA) • ISMS conference in Holland, May 2000 • Visit to Japanese maitake growers (early 2000)	Increased access to current and relevant (unpublished) information by 1st October 2000	30th November 2000	List and contact details (including web sites) of contacts made and possible contacts appended to industry report Separate travel reports for USA, Holland and Japan indicating travel benefits
6. Major review of progress with maitake and morel fruiting	Major review completed by 15th September 2002	15th September 2002	Peer reviewed report

Maitake Tasks	Milestone	Reporting Date	Performance Indicator
7. Liquid spawn trials for maitake inoculation	Initiate trials 1st June 2000	30th November 2001	Description of details of experimental design of research and exact time line
8. Complete liquid spawn trials for maitake inoculation	Complete by 1st June 2001	30th November 2001	Industry report, assessment and cost analysis of technique.
9. Testing of Japanese maitake bag method	Trial initiated 1st September 2000	30th November 2001	Trials established - report including detail of experimental design
10. Testing effectiveness of additives to substrate re maitake production	Trials initiated 1st September 2000	30th November 2002	Trials established - report including background and exact time line to detailed experimental design
11. Testing of influence of environmental factors on maitake production	Trials initiated 15 March 2001	30th November 2001	Trials established - report including background and exact time line to detailed experimental design
12. Completion testing of Japanese bag method.	Testing of Japanese maitake bag method completed by 1st October 2001	30th November 2001	Yield and other data collected, collated and statistically analysed. Critical analysis of technique submitted to industry partner
13. Complete testing effectiveness of additives to substrate re maitake production	Complete by 1st March 2002	30th November 2002	Yield and other data collected, collated and statistically analysed
14. Complete testing of influence of environmental factors on maitake production	Complete by 1st March 2002	30th November 2002	Yield and other data collected, collated and statistically analysed
15. Completion of maitake project	Commercially viable technique for matiake production developed	30th November 2002	Final reporting on maitake production in Tasmania

Morel Tasks	Milestone	Reporting Date	Performance Indicator
16. Locating morel fruiting sites and selection of five sites to be monitored over a five year period	Field work start Feb. 2000	30th November 2000	Map of location of sites, detailed description of sites to be monitored
17. Additional isolates obtained from local morel fruitbodies	Isolations start in tandem with field work Feb. 2000	30th November 2001	List of morel isolates in culture associated with morphological description of fruit body
18. Taste testing of morel fruitbodies	Taste tests with chefs organised (depending on availability of fruitbodies) by 15th August 2000	30th November 2001	Isolates obtained from fruitbodies with known gourmet qualities
19. Monitoring of morel sites over 5 years	Data loggers installed at each site by 30 Sep.2000 (if fruitbodies present)	30th November 2000	List of observations to be recorded and monitoring times for 5 year period
20. Start DNA identification of morel isolates	DNA analysis of morel isolates initiated 15th February 2001	30th November 2001	DNA analysis started by growing isolates and DNA extraction
21. Complete DNA identification of morel isolates	Analyses completed 15th October 2001	30th November 2001	Scientific publication on the identity of species of morel present in Tasmania
22. Trials to increase morel sclerotia size	Experiments initiated 1st April 2001	30th November 2002	Description of details of experimental design of research
23. Completion of morel increasing sclerotia experiments	Completed by 1st April 2002	30th November 2002	Data collected and statistically analysed

Morel Tasks	Milestone	Reporting Date	Performance Indicator
24. Testing of influence of environmental factors on morel production (initiation and development)	Trials initiated 1st December 2002	30th November 2004	Trials established - report including background and exact time line to detailed experimental design
25. Completion of morel site monitoring	Completed by 1st November 2004	30th November 2004	Data collected and analysed. Report on possible environmental influences on fruiting base on observations at 5 sites
26. Completion of evaluation of different treatments to sclerotia to obtain fruiting	Completed by 1st October 2004	30th November 2004	Data collected and analysed
27. Testing of influence of environmental factors on morel production (initiation and development)	Completed by 1st November 2004	30th November 2004	Data collected and analysed
28. Completion of morel research	Fruiting techniques developed for morel species in culture	31st January2005	Final report

Appendix 2 Base substrate and additives investigated for the cultivation of *Grifola frondosa*

Base substrate	Additive	Ratio (v/v)	Author
Beechwood sawdust	Corn meal Wheat bran Wheat bran : corn meal	8:2 9:1 85:10:5	Kirchoff (1996)
Wood chips : oak or alder sawdust	White millet : $CaCO_3$	64:25:10:1	Chalmers (1994)
Hardwood sawdust - fine and coarse 3:1	Wheat bran (coarse) : sucrose : lime or gypsum	75:23:1:1	Chen (2001)
Broadleaf sawdust (coarse) Broadleaf sawdust (fine) + rice hull	Rice bran or wheat bran	10:1 or 10:1-2 7:3:1 or 7:3:1-2	Rinsanka (1980)
Broadleaf sawdust	Rice bran : wheat bran	20:3:1	Royse (1996)
Oak + poplar sawdust Larch sawdust Sawdust : woodchips (0.5-4")	Corn waste Rice bran Rice bran (20% of biomass) : $CaSO_4$	15:5:2 unstated 20:10:8:1-1.4	Stamets (1993)
Deciduous broadleaf sawdust	Rice bran : wheat bran	20:3:1	Mayuzumi and Mizuno (1997)
Sawdust	Wheat bran or rice bran	5:1	Takama *et al.* (1981), Lee (1996)
Mixed oak sawdust - Mostly *Quercus rubra*	Wheat bran : millet : rye Wheat bran : rye	7:1:1:1 7:1:2	Shen (2000)

Appendix 3 Hyphal growth response of *Grifola frondosa* at day 19 on PDA at different pH Means with the same letter are not significantly different ($P<0.001$)

Isolate	Ph	Mean (mm)	
ATCC60304	5	69.1	(efg)
	5.5	67.5	(fgh)
	6	63.3	(kl)
	6.5	52.8	(rs)
	7	47.5	(uuv)
	7.5	43.2	(xy)
FPC200	5	61.5	(lm)
	5.5	56	(pq)
	6	56	(pq)
	6.5	54	(qr)
	7	46.3	(vw)
	7.5	40.6	(yz)
IFO30661	5.5	56.8	(op)
	5	54.5	(pqr)
	6	49.5	(tuu)
	6.5	44.7	(wx)
	7	23	(E)
	7.5	31.6	(CD)
M74	5	37.5	(A)
	5.5	38.1	(zA)
	6	36.8	(A)
	6.5	36.3	(A)
	7	33.2	(BC)
	7.5	-	-
NZF5198A	5	48.8	(tuu)
	5.5	46.3	(vw)
	6	35.3	(AB)
	6.5	29.3	(D)
	7	19.2	(F)
	7.5	12.2	(G)

Isolate	Ph	Mean (mm)	
S49	5	76.5	(a)
	5.5	75.8	(ab)
	6	72.2	(cd)
	6.5	69.8	(def)
	7	63.5	(jkl)
	7.5	47.7	(uuv)
TTI50033	5	67.2	(ghi)
	5.5	65	(ijk)
	6	63.2	(kl)
	6.5	59	(no)
	7	50.7	(st)
	7.5	54.2	(qr)
WC659	5	75.5	(ab)
	5.5	74.3	(abc)
	6	69.7	(ef)
	6.5	68.3	(fg)
	7	59.7	(mn)
	7.5	55.7	(pq)
WC685	5.5	74.3	(abc)
	5	74	(bc)
	6	70.8	(de)
	6.5	65.8	(hij)
	7	47.7	(uuv)
	7.5	50.8	(st)
WC808	5	75.0	(ab)
	5.5	75.3	(ab)
	6	74.2	(abc)
	6.5	64.7	(jk)
	7	67.2	(ghi)
	7.5	56.8	(op)

Appendix 4 Interaction between *Grifola frondosa* and substrate additives at mycelial mat, brain, upright, cauliflower and antler stages in the cultivation cycle

		Mycelial mat			**Brain**			**Upright** (*P<0.001*)			**Cauliflower** (*P<0.001*)			**Antler** (*P<0.001*)		
Isolate	**Level**	mean[(1)]	no. [(2)]	avd [(3)]	mean[(1)]	no. [(2)]	avd [(3)]	mean[(1)]	no. [(2)]	Avd [(3)]	mean[(1)]	no. [(2)]	Avd [(3)]	Mean[(1)]	no. [(2)]	avd [(3)]
FPC200	1M	51.72	6	(fg)	94.65	6	(hi)	101.84	6	(bc)	100	2	(abcd)	187.3	1	(c)
FPC200	10R	47.39	13	(cdef)	91.25	13	(hi)	101.58	11	(bc)	101	6	(abcd)	136.4	7	(b)
FPC200	10W	48.52	11	(defg)	100.55	12	(i)	97.94	7	(abc)	104.4	5	(abcd)	112.4	3	(ab)
FPC200	2M	49.9	13	(efg)	77.44	12	(cdefgh)	85.46	13	(ab)	99.4	13	(abcd)	105.5	11	(a)
FPC200	20R	43.61	14	(abc)	79.87	13	(defgh)	96.88	9	(abc)	111.1	8	(abcdefg)	105.2	2	(ab)
FPC200	20W	47.5	11	(cdef)	86.01	12	(efghi)	93.06	9	(abc)	106.2	7	(abcdefg)	117.3	6	(ab)
M74	1M	47.09	15	(cdef)	72.84	11	(bcdef)	92.72	8	(abc)	115.9	5	(cdefghi)	*	*	*
M74	10R	45.76	15	(cd)	64.48	13	(abc)	82.55	12	(a)	106.6	7	(abcdefg)	81.3	1	(a)
M74	10W		14	(cdef)	68.2	13	(abcd)	83.18	10	(a)	101.4	4	(abcd)	99.1	3	(a)
M74	2M	45.38	16	(cde)	75.1	11	(bcdefg)	92.94	5	(abc)	104.6	3	(abcde)	99.2	3	(a)
M74	20R	47.09	15	(cdef)	77.01	12	(cdefgh)	89.19	5	(abc)	106	3	(abcdef)	*	*	*
M74	20W	45.76	15	(cd)	64.41	13	(abc)	89.3	9	(abc)	98.3	5	(bd)	104	2	(ab)
M6	1M	47.3	13	(cdef)	75.1	13	(bcdefg)	99.22	10	(bc)	131.7	3	(ij)	*	*	*
M6	10R	45.03	14	(cde)	61.5	14	(ab)	82.93	10	(a)	127.4	7	(fi)	*	*	*
M6	10W	46.67	15	(cde)	63.48	13	(abc)	81.62	10	(a)	116.2	4	(bcdefgh)	*	*	*
M6	2M	47.51	12	(cdef)	69.84	12	(abcd)	94.28	10	(abc)	90.2	3	(a)	*	*	*
M6	20R	45.76	15	(cd)	61.64	13	(ab)	89.1	11	(abc)	109.2	2	(abcdefhi)	*	*	*
M6	20W	46.67	15	(cde)	70.87	12	(abcde)	88.87	11	(abc)	129.5	4	(fgi)	*	*	*
WC808	1M	53.27	4	(g)	88.66	10	(fghi)	95.43	7	(abc)	118.8	4	(defghi)	122.9	3	(ab)
WC808	10R	40.07	15	(a)	74.56	12	(bcdefg)	98.25	10	(bc)	124.3	9	(efgi)	121.2	6	(ab)
WC808	10W	45.96	13	(cd)	90.08	11	(ghi)	161.37	9	(d)	152.8	5	(j)	173.7	7	(c)
WC808	2M	45.56	9	(cde)	79.11	9	(cdefgh)	96.16	9	(abc)	106.6	4	(abcdefg)	101 .8	7	(a)
WC808	20R	42	15	(ab)	57.11	13	(a)	94.52	9	(abc)	98.4	8	(bcd)	107.6	7	(a)
WC808	20W	45.18	13	(cde)	79.89	12	(defgh)	90.49	5	(abc)	127.7	3	(efghi)	109.5	3	(ab)

(1) Mean number of days; (2) Number of observations for data; (3) Average variance of difference * Insufficient data

Appendix 5 Attributed species, on the basis of morphological characters, of commonly studied morels

Species	*M. angusticeps*	*M. costata*	*M. conica*	*M. esculenta*	*M. deliciosa*
Authority	Peck (w)	(Ventenat) Persoon (1822) W	Peerson: Fries	Linnaeus ex St.-Amans (W)	Fries (Syst. Mycol. 2: 8. 1822)
Synonym				*M. rotunda* (Pers.) Boudier	
Common name	Black Morel, Conic Morel, Early Morel, Ko Togari, Amigasa Take, Morel, Peck=s Morel	Morel	Comic Morel, Black Morel, Spitzmorchel, Spugnola bruna	Common Morel, Amigasa take, Amigasa dake, Brain Mushroom, Gray Morel, Morel, Sponge Mushroom, White Morel, Yellow Morel, Yangdujun, Speismorchel, Spugnolla gialla	Delicious Morel
Size		5-8 x 4-6 cm	6-12 cm	10-20 cm	
Sporocarp		Conical, long; pale fawn brown, sometimes olive with browner edges to ribs; numerous elongated honeycomb-like pits separated by darker parallel ribs	Conical or cylindrical; radiantly arranged longitudinal ribbing; less parallel ribs than *M. costata.* Sometimes exceeds height of *M. crassipes*	Round to oval in shape; ochraceous yellow regularly rounded pits, darker yellow brown at bottom of pits	
Stipe		5 x 2 cm, short, white	5-7 x 1-2 cm; almost cylindrical; ochraceous grey, conspicuously furfuraceous; hollow	5-15 x 1-2 cm; pale; hollow; enlarged at base	
Habitat		Orchards, rubbish heaps	Beneath poplars and pines, in forests	Sandy, open ground; near tulip-poplar, ash, dead elm, old apple orchards	