OPTIMISATION OF THE GROWTH CONDITIONS AND GENETIC CHARACTERISATION OF *PLEUROTUS* SPECIES

by

Chenjerayi **KASHANGURA**

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Department of Biological Sciences
Faculty of Science
University of Zimbabwe
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5 Get wisdom, get understanding: forget it not…6 Forsake her not, and she shall preserve thee…7 Wisdom is the principal thing; therefore get wisdom: and with all thy getting get understanding. (Proverbs 4: 5-7, King James Version)

DECLARATION

I hereby declare that the material contained in this thesis is my own original work and has not been submitted for a degree in any other university.

Chenjerayi **KASHANGURA**

ABSTRACT

A comprehensive genetic and optimisation of the growth conditions study of fifteen *Pleurotus* species and strains from Belgian Coordinated Collections of Microrganisms/ Mycotheque de l' Université Catholique de Louvain (Agro) Industrial Fungi and Yeasts Collection was carried out. The optimal environmental conditions for the *Pleurotus* strains were determined by establishing variability in growth response and primordium development on agar media over a range of growth parameters: osmotic potential (– 0.5 MPa to – 5.0 MPa), temperature (5 to 40 °C) and pH (2 to 12). The maximum growth rates and the optimum conditions for growth varied for the different strains. A *Pleurotus ostreatus* strain able to grow and fruit at elevated temperatures (25 °C) was identified that has potential for cultivation in the tropics. Nine strains were identified with high growth rates. Primordia were produced at low pH (4 to 6), at suboptimal growth temperatures (≤ 25 °C), and under moderate water stress (–0.5 to –3.5 MPa). An investigation into the effect of micro-solute concentration changes on the growth response of the *Pleurotus* species was carried out on agar media using micro-concentrations of polyethylene glycol (PEG) 6000, potassium chloride (KCl) and sodium chloride (NaCl). The solutes at micro-concentrations ranging from 0.35 g l^{-1} to 1.00 g l^{-1} for PEG 6000, 1.50 g l^{-1} to 3.85 g l^{-1} for KCl and 0.60 g l^{-1} to 1.54 g l^{-1} for NaCl produced a two to three fold increase in the growth rate of the *Pleurotus* strains, which has potential application in oyster mushrooms (*Pleurotus* species) spawn production and cultivation. The optimum micro concentrations of combined KCl and NaCl solutes of 2.70 g l^{-1} KCl: 1.08 g l^{-1} NaCl or 3.85 g l^{-1} KCl: 1.54 g l^{-1} NaCl at 20 °C; and 1.50 g l^{-1} KCl: 0.60 g l^{-1} NaCl or 2.70 g l^{-1} KCl: 1.08 g l^{-1} NaCl for both 25 °C and 30 °C; of PEG 6000 of 0.90 g l^{-1} (20 °C) and 0.65 g l^{-1} (25 and 30 °C) were identified. Development of alternative spawn substrate protocols was done by varying the boiling, soaking periods for the substrates (maize [*Zea mays*] grain, maize cobs, pumpkin seeds [*Cucurbita* sp.] and bean seeds [*Phaseolus vulgaris*]). Maize grain, pumpkin seeds and maize cobs with a water content of 35 to 39 %, 41 to 47 % and 64 % respectively have potential use as alternative substrates to wheat (*Triticum aestivum*) grain in *Pleurotus* species spawn production. The feasibility of creating novel media formulations for culturing *Pleurotus* strains was investigated by developing various whole wheat grain and crushed wheat grain mixtures. The novel media formulations of 1:3 (w/v) to 1:5 (whole wheat grain: distilled water) and 1:3 to 1:15 (crushed wheat grain: distilled water) were determined to be optimal for use as low-cost alternative media for culturing *Pleurotus* species for spawn production and cultivation purposes. An investigation in the use of Tyndallisation as a means of sterilising the growth media for spawn production was carried out by modifying primarily the boiling times and number of days of incubation. Tyndallisation of 30 minutes boiling daily for three days (wide-mouthed bottles) and 5 minutes boiling for four days (narrow-mouthed bottles) were optimal for production of *Pleurotus* species spawn. Alternative single substrates and substrate mixtures (1:1) were determined by growth and basidiome production on thatch grass (*Agropyron* sp.), banana fronds (*Musa sapientum*), wheat straw, sage (*Coleochloa setifera*); banana + sage, banana + grass, banana + wheat straw and sage + grass. Grass and sage were the best substrates singly and in combination with banana. Biological efficiencies greater than 140 % were obtained. Positive and negative synergistic effects on primordium development dependant on the strain and substrate mixture were noted. Genetic variability of *Pleurotus* species and strains was investigated by sequencing the internal transcribed spacer (ITS) region of ribosomal DNA. The 15 *Pleurotus* strains separated into four clusters. The internal transcribed spacer sequences analysis suggested that strain 31674 was misidentified as *P. sajor-caju* instead of *P. ostreatus*. The value of noting the history of the strain in phylogenetic analysis was proposed. This study recommends BCCM/MUCL strain 31017 (*P. sajor-caju*) for spawn production and cultivation processes due to having the characteristics of high growth rate, high biological efficiency, prolific primordial formation and moderate xerotolerance which are highly desirable for spawn production and cultivation processes.

DEDICATION

Dedicated to my dear MUM, WIFE and Son TATENDA

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'In Nature's Infinite Book of Secrecy…

A Little I Can Read.'

William Shakespeare

CONTENTS

CHAPTER 4 RESULTS

CHAPTER 5 DISCUSSION

TABLES

FIGURES

APPENDICES

CHAPTER 1

1.0 INTRODUCTION

Mushrooms and other fleshy fungi are the premier recyclers of nutrients on the planet (Stamets, 2000). Yet to date, the inherent biological power embodied within the mycelium network of mushrooms largely remains a vast, untapped resource (Stamets, 2000). Water defines the biosphere, and low water availability in arid regions of the globe limits biodiversity as well as biomass (Austin *et al*., 2004; Huxman *et al*., 2004). Saprotrophic basidiomycete macrofungi have a worldwide distribution and play critical roles in water sequestration, animal and human nutrition, soil health and ecosystem function, particularly in water-constrained environments (McGonigle, 1995; Austin *et al*., 2004).

Pleurotus is an important genus of edible basidiomycetes, especially in the tropics and sub-tropics. The biotechnological potential of *Pleurotus* species, and those of related genera, has been exploited to enhance the digestibility of animal feeds, to reduce the incidence of cancers, to synthesise fine chemicals, for bioremediation, and as part of a strategy to produce fuel ethanol from wood (Madar and Zusman, 1997; Jonsson *et al*., 1998; Rajarathnam, Shashireka and Bano, 1998; Villas-Boas *et al*., 2002; Fragoeiro and Magan, 2005, respectively). Moreover, the saprotrophic activity of basidiomycetes plays a key role in unlocking nutrients trapped in wood and plant material in the dry tropics and subtropics, helping to prevent a build-up of waste in the environment (Mswaka and Magan, 1998; Rajarathnam *et al*., 1998; Lindblad, 2000). However, nutrient cycling by these macrofungi requires an ability to grow and fruit when water availability is limited (McGonigle, 1995). Whereas the nutritional composition, ecological roles and waste-recycling potential of *Pleurotus* species have been well-studied, relatively little is known about phenotypic diversity within the

species. It is important to understand how differing water, pH and temperature conditions affect the various strains regarding primordium development and basidiome production, and whether tolerance to water stress can be selected for.

The main substrate for *Pleurotus* spawn is wheat (*Triticum aestivum*) grain (Stamets, 2000; Ogden and Prowse, 2004). In developing countries wheat is mostly a seasonal crop due to the climate and lack of equipment to cultivate wheat all year round and can occasionally be in short supply. Finding alternative substrates for *Pleurotus* spawn production will alleviate the demand for wheat grain as a substrate for *Pleurotus* spawn production.

The optimisation of the growth conditions for *Pleurotus* species and their strains will enable the future spawn producers and oyster mushroom farmers in Zimbabwe and other developing countries to use strains ideal for the conditions in the growing facility and spawn plant. To date, there is no comprehensive study on the optimum growth conditions for cultivation of oyster mushrooms in Zimbabwe. This study will identify the optimum growth conditions for *Pleurotus* species. Optimised cultivation of mushrooms will help to reduce the incidence of mushroom poisoning by providing known, well-identified, edible *Pleurotus* species as an additional source of mushrooms to the collection of wild mushroom which is prone to misidentifications and possible mushroom poisoning. The optimised cultivation of *Pleurotus* species will provide optimal standard conditions for cultivation thereby reducing the expression of morphological plasticity in *Pleurotus*.

The extreme morphological plasticity in *Pleurotus*, especially of strains able to fruit above 20 °C has been well documented (Anderson, Wang and Schwandt, 1973; Eger, Eden and Wissig, 1976; Li and Eger, 1978). This extreme morphological plasticity raises questions on the true identity of these strains. These taxonomic

ambiguities are due to initial misidentifications, absence of species typification, significant influence exercised by the environment and substratum on the morphology of most taxa, the limited existence of sound physiological characters and the lack of suitable compatibility studies (Zervakis, Sourdis and Balis, 1994; Zervakis, Moncalvo and Vilgalys, 2004). This study seeks to rectify the taxonomic dilemma faced in the identification of *Pleurotus* species and strains by DNA sequencing using universal primers designed for the **I**nternal **T**ranscribed **S**pacer (ITS) region of the ribosomal DNA (rDNA).

CHAPTER 2

2.0 LITREATURE REVIEW

The Swede Carolus Linnaeus (Carl von Linné, 1707-1778) originated the concept of binomial nomenclature and a comprehensive scheme for all nature. From Aristotle's time to the middle of the twentieth century, mostly everyone was content to divide the living world into two kingdoms, plants and animals. The opinion began to change in the 1960s, largely because of the knowledge gained by new biochemical and electron-microscope techniques (Margulis and Schwartz, 1988). In the first half of the twentieth century, 'Fungi' were generally treated as a part of the kingdom 'Plantae' in the subdivision 'Thallophyta' and placed alongside Bacteria, Lichens, and Algae (Hawksworth, 1990). The five-kingdom system of Robert H. Whittaker (1924-1980) published in 1959 (Whittaker, 1959, 1969) was rapidly adopted. This classified 'Fungi' (including myxomycetes and oomycetes) into a separate kingdom as distinct from Animalia (animals with or without backbones), Monera (bacteria and cyanobacteria), Plantae (including bryophytes and non flagellate algae), and Protista (including plasmodiophoromycetes and hyphochytridiomycetes) (Margulis and Schwartz, 1988; Hawksworth, 1990)

The fungi share with animals the ability to export to the extracellular environment hydrolytic enzymes that break down biopolymers, which can be absorbed for nutrition (Blackwell, Vilgalys and Taylor, 2005). According to the Margulis and Schwartz five kingdom classification of life on the Planet Earth published in 1988 the Kingdom Fungi is limited to eukaryotes that form spores and are amastigote (lack undulipodia) at all stages of their life cycle.

The Basidiomycota contains about thirty thousand described species, which is thirty-seven percent of the described species of Eumycota (true fungi) (Kirk *et al*.,

2001). The most conspicuous and familiar Basidiomycota are those that produce mushrooms, which are sexual reproductive structures (Weitz, 2004). A mushroom is defined as a macrofungus with a distinctive fruiting body (basidiome). The macrofungi have fruiting bodies large enough to be seen with the naked eye and to be picked up by hand (Cho, 2004).

2.1 THE GENUS *PLEUROTUS*

Fries in 1821 sorted mushrooms by their spore colour (in spore prints), so the various laterally stipitate groups were segregated in several directions (Petersen, Hughes and Psurtseva, http://fp.bio.utk.edu/Mycology/*Pleurotus*/default.htm, accessed 2007). Beginning with Patouillard in 1887 and 1900, mycologists began to revise Fries's taxonomy and to recognise increasingly larger numbers of segregate genera based on ever-more-restricted sets of characters. The catch-all genus for gilled mushrooms was *Agaricus* and one species was *Leucosporus*. The white-spored, laterally stipitate basidiomata were designated as Tribus XII. *Pleurotus* (Petersen *et al*., http://fp.bio.utk.edu/Mycology/*Pleurotus*/default.htm, accessed 2007). This name was not adopted at genus rank, but as an infrageneric level only. Within the tribe were elements of several present-day genera: *Pleurotus*, *Hypsizygus*, *Lentinus*, *Hohenbuehelia* and *Lentinellus*. Quélet in 1872 finally adopted the name *Pleurotus* at genus rank, with the core species accepted by Fries still within the genus. The type species of the genus is *Pleurotus ostreatus* (Jacq: Fr.) Kummer (1871). Pegler in 1975 and 1983 restricted *Pleurotus* to monomitic species (those lacking skeletal or binding hyphae). Corner in 1981 accepted some dimitic species in *Pleurotus.* Typification of *Pleurotus* was not clear cut until two actions were taken: (i) the generic name was conserved over the name *Pterophyllus* Lév. removing a

nomenclatural threat at the genus level and (ii) a choice of the type species was made between *P. dryinus* and *P. ostreatus* in favour of the latter (Donk, 1962).

There are many opinions on the classification of Basidiomycetes. However most mycologists and mycological litreature have adopted the scheme proposed by Ainsworth in 1973 which distinguishes basidiomycetes from all other fungi by the basidium – a microscopic club like reproductive structure from which their name is derived (Ainsworth, 1971; Ainsworth, Sparrow and Sussman, 1973). The most comprehensive modern taxonomic treatment for the Agaricales is that of Singer published in 1986. Thorn and co-researchers in 2000 suggested the introduction of the family Pleurotaceae. According to the Ainsworth classification the genus *Pleurotus* belongs to the Family Agaricaceae of the Suborder Agaricineae, of the Order Agaricales under the Subclass Holobasidiomycetidiae and the Class Basidiomycetes or Hymenomycetes which belong to the Subdivision Basidiomycotina and the Division Amastigomycota under the Kingdom Mycota or Myceteae. *Pleurotus* comes from the Greek "pleuro" which means formed laterally or in a sideways position, referring to the lateral position of the stem relative to the cap (Stamets and Chilton, 1983). Twenty-two identified *Pleurotus* species exist and the following are those widely cultivated globally (Stamets, 2000).

2.1.1. *Pleurotus citrinopileatus* Singer.

The common name is the 'Golden Oyster mushroom'. *Pleurotus citrinopileatus* has a brilliant yellow colour and it forms clusters hosting a high number of individual mushrooms (Stamets, 2000), whose stems often diverge from a single base. The stems are white and centrally attached to the caps. *Pleurotus citrinopileatus* is closely allied to *Pleurotus cornucopiae* (Paulet) Roll. and is often considered a variety of it (Stamets, 2000; Zervakis and Balis, 1996). Singer in 1986

separated *P. citrinopileatus* Singer from *P. cornucopiae* (Paulet ex Fr.) Rolland sensu Kuhn. and Rom. (= *P. macropus* Bagl.) on the basis of the arrangement of the contextual hyphae. According to Singer, *P. citrinopileatus* has monomitic hyphae, whereas *P. cornucopiae* has dimitic hyphae, a designation that has caused considerable confusion since Singer in 1986 used this feature as a delineating, sub-generic distinction. Upon more careful examination, Parmatso in 1987 found that the context was distinctly dimitic, especially evident in the flesh at the stem base. This observation concurred with Watling and Gregory's microscopic observations in 1989 of *P. cornucopiae*.

2.1.2 *Pleurotus ostreatus* (Jacq.: Fr.) Kummer .

The common names are “the oyster mushroom, oyster shelf, tree oyster straw mushroom, hiratake (Japanese for Flat mushroom)”. *Pleurotus ostreatus* has a whitish to gray to blue gray colour (colour is a light determined factor in this species) (Stamets and Chilton, 1983). Figure 1 shows *P. ostreatus* growing naturally on a tree. Some strains form clusters, while others form individual mushrooms (Stamets, 2000). *Pleurotus ostreatus* is so similar to *P. pulmonarius* that they are difficult to separate macroscopically (Stamets, 2000). *Pleurotus ostreatus* and *P. pulmonarius* differ largely in their habitat preference for conifer woods. In the Western United States, *P. pulmonarius* is found at higher altitude (1200 to 3000 m) in coniferous forests (*Abies* and *Picea*) whilst *P ostreatus* is found in the lowland, river valley. *Pleurotus floridanus* Eger and *P. columbinus* are considered by some to be varieties of *P. ostreatus* (Vilgalys *et al*., 1993).

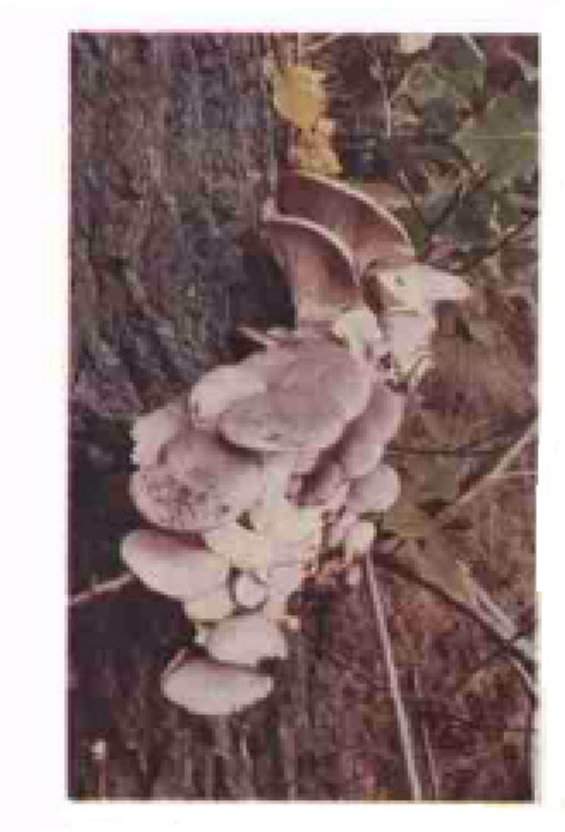


Figure 1: The oyster mushroom, *Pleurotus ostreatus* growing naturally on a tree (Photo by P. L. de Laszlo, [Ramsbottom, 1954]).

2.1.3 *Pleurotus floridanus* Singer

This species is considered to be a synonym of *P. ostreatus* because spores from each species are cross fertile, the mycelium forms clamp connections and mushrooms grown from this mating produce fertile basidiomes. However the species differs from *P. ostreatus* by its preference for warmer temperatures of 24 °C and above for fruiting (Li and Eger, 1978).

2.1.4 *Pleurotus sajor-caju* (Fries) Singer.

Pleurotus sajor-caju has a grayish white colour to beige to lilac gray to gray-brown, sometimes with pinkish or orangish tones. At high temperatures of 22 °C and above, the cap is lighter in colour whereas, the cap becomes very dark gray to grayish black at lower temperatures (Stamets, 2000). Colour varies according to the strain,

lighting and temperature conditions (Stamets, 2000). *Pleurotus pulmonarius* was first published as *Agaricus pulmonarius* Fr. in 1821. Similar to *P. ostreatus* (Jacq: Fr.) Kummer and *P. populinus* Hilber and Miller, it can be separated from them by a combination of habitat, macroscopic, and microscopic features (Eger *et al*., 1979; Petersen and Krisai-Greilhuber, 1996).

2.2 ENVIRONMENTAL FACTORS CONDITIONING FUNGAL GROWTH

2.2.1 pH

The solvent inside cells and in all extracellular fluids is water (H_2O). An important characteristic of any aqueous solution is the concentration of positively charged hydrogen ions (H^+) and negatively charged hydroxyl ions (OH^-). These ions are the dissociation products of water. They are constituents of all living systems, and they are liberated by many reactions that take place between organic molecules within cells. A fungus will grow maximally over a certain range of initial pH values of the medium and it will fail to grow at high and low extremes (Cochrane, 1958). The pH is not a unitary factor with mechanism of its action differing at different [H_3O^+]. This means one part of a pH-growth curve may reflect the effect of a low pH on enzyme systems, another high pH on metal solubilities. Actual toxicity of pH extremes is not the direct result of H^+ or OH^- concentrations, but due to the formation of increased levels of undissociated weak acids and bases (VanDemark and Batzing, 1987). These undissociated acids and bases penetrate more readily into cells and are physiologically more active and toxic than the dissociated forms (VanDemark and Batzing, 1987).

Almost any factor in the environment may change the shape of the pH-growth curve. Such factors include temperature, changes in the medium, growth factor supply, calcium and magnesium levels and nitrogen source (Cochrane, 1958).

2.2.2 Temperature

Temperature is one of the cardinal environmental factors which determine the distribution of fungi in different ecological niches (Hudson, 1986). It exerts an influence on fungi largely via its effects on enzyme-catalysed reactions (Mswaka and Magan, 1999). The overall response of a fungus to different temperatures represents the combined effect of numerous different chemical reactions, each of which exhibits its own characteristic relationships to temperature (Rayner and Boddy, 1988).

2.2.3 Water Potential

Water availability in hygroscopic materials can be measured as equilibrium relative humidity (ERH), water activity (a_w) or water potential (Ψ) (Magan and Lacey, 1988). The ERH and a_w are the same numerically except that ERH is expressed as a percentage (%) and a_w as a decimal fraction of one (Magan and Lacey, 1988). Microorganisms respond to reduced a_w by accumulating potassium, amino acids, or polyols (Christian, 1980). Fungi grow more slowly than most bacteria at high a_w, but frequently grow at much lower levels of a_w. Water potential is measured relative to a standard of pure water at atmospheric pressure and the same temperature as the system being studied. Water potential of pure water under these conditions is arbitrarily set at zero (0 megapascal [MPa]).

Water potential is linearly related to the chemical potential of water to seek equilibrium. An advantage of this system is that the various components can be partitioned or added together to create a final pressure measurement in units of pascals or bars (Badham, 1989; Mswaka and Magan, 1999). Within wood, soil and other substrates, the availability of water to the decomposer organisms is affected by two main forces, matric potential and osmotic potential (Boddy, 1983). The degree to which surfaces adsorb water molecules at atmospheric pressure is termed the matric

pressure (Bell *et al*., 1993). Matric potential is a result of forces associated with the interfaces between air and the solid matrix, and osmotic potential is a result of the presence of solutes within the water (Boddy, 1986). The sum of matric potential and osmotic potential is termed water potential (Griffin, 1977). The osmotic potential of nutrient media is commonly adjusted with sodium chloride (NaCl), potassium chloride (KCl) or glycerol (Ayres, 1977; Clarke, Jennings and Coggins, 1980; Boddy, 1983; Brownell and Schneider, 1985; Koske and Tessier, 1986; Magan, 1988; Money, Davis and Burlak, 2000). Polyethylene glycol (PEG), an inert, non ionic polymer with the formula $HOCH_2$-$(CH_2$-O-$CH_2)_X$ CH_2OH, has been widely used to maintain experimental media at predetermined matric potential values (Michel and Kaufmann, 1973; Kidd, Reid and Davidson, 1977; Brownell and Schneider, 1985; Magan, 1988).

Water is required by fungi as a solvent for metabolic processes, as a metabolite, for transport of metabolites, enzymes and organelles and via turgor pressure as a vital skelatal ingredient and the driving force behind skeletal growth (Hudson, 1986). Wood and litter-decaying basidiomycetes tend to be particularly sensitive to decreased water potential of the medium and generally grow only above – 10 MPa (Boddy, 1983; Dix and Frankland, 1987). Whereas the effect of water stress on fungal growth using KCl, NaCl and PEG species has been well-studied, relatively little is known about the effect of addition of micro-concentrations of KCl, NaCl and PEG species to conventional media on the growth of *Pleurotus* species and strains.

2.3 ENVIRONMENTAL FACTORS CONDITIONING MUSHROOM GROWTH

Light, temperature, humidity and carbon dioxide (CO_2) are the four factors that condition mushroom growth.

2.3.1. Light

The reactions of fungi to visible and ultraviolet are of three main types: inductive, inhibitive and trophic (Cochrane, 1958). *Pleurotus* species exhibit inductive and trophic responses to light (Block, Tsao and Han, 1959; Gyurko, 1972). A light intensity of 10 lux (see appendix B) is sufficient to induce the trophic responses (Zadrazil, 1978).

2.3.2 Temperature

Each species and strain has its own temperature optima for fructification, which may or may not coincide with that for vegetative growth (Quimio, Chang and Royse, 1990). In general, optimal fructification temperatures for most fungi are lower than optimum vegetative growth temperatures by a range of two to ten degrees Celsius (Quimio *et al*., 1990).

2.3.3 Humidity

Adequate water is required for the growth of mushrooms in nature and under cultivation (Badham, 1989). Maturing mushrooms have water requirements that must be met if maximum yields are to be achieved. Mushrooms which grow on substrates which do not have a casing layer applied such as *Pleurotus* species (except *Pleurotus eryngii*) draw their moisture from the substrate (Stamets and Chilton, 1983; Quimio *et al*., 1990). Uncased substrates are more susceptible to dry air and therefore require a relative humidity of 90 to 95 percent as well as periodic misting of the cropping surface.

2.3.4 Carbon Dioxide (CO_2)

Low carbon dioxide levels of the surrounding environment are required for stimulating basidiome initiation and normal basidiome development. High carbon dioxide concentrations cause elongation and branching of the stipes on the fruiting of

Pleurotus. The appearance of the basidiome often becomes bizarre because of the abnormal branching of the stipes (Quimio *et al*., 1990).

2.4 MUSHROOM CULTIVATION

Mushrooms have been used and prized as a delicacy for more than two thousand years. Over two thousand edible mushroom species have been characterised, although the number of species that have been commercialised is much smaller (Martin *et al*., 2004). The Chinese were the earliest growers of mushroom. Litreature references (Chang and Miles, 1989) indicate that *Auricularia auricula* was first cultivated in China in A.D 600. Since then many species of mushroom have been added to the ever-growing list of cultivated mushrooms. *Pleurotus* species (common name: oyster mushrooms) are no exception. *Pleurotus ostreatus* was first cultivated in 1900 whilst *Pleurotus sajor-caju* was first cultivated in 1974 (Quimio *et al.,* 1990). In the eighteenth century mushrooms were cultivated in caves where composted animal manure beds were prepared and a casing material made up of rotten leaf litter was later added to stimulate mushroom formation (Quimio *et al.,* 1990). The rapid development and growth of the mushroom industry from a primitive cave culture into one using more highly technical and controlled methods was stimulated in the 1960s. With the establishment of laboratories for research on mushroom growing, improved technologies on the use of mushroom growing houses and use of pure culture spawn resulted in the rapid and increased production of mushrooms worldwide (Flegg, Spencer and Wood, 1985).

Mushroom cultivation involves providing conditions that are suitable for the life cycle of the mushroom to occur. Each part of the life cycle requires different environmental conditions. However the basidiome (fruit body) stage is very sensitive to humidity level. Figure 2 shows the generalised life cycle of a macrofungus whilst

Figure 3 shows a summary of mushroom cultivation techniques. Stages 1 to 5 in Figure 2 and Figure 3 show the same stages in the life-cycle of the mushroom, emphasizing the point that mushroom cultivation is providing conditions suitable for the life-cycle of the mushroom to occur. Stage 1 (spore germination) is often avoided during mushroom cultivation to maintain purity of a strain (Quimio *et al.*, 1990). However the Chinese often use this method (Quimio *et al*., 1990). Stages 3, 4 and 5 occur in the mushroom growing house (Figure 3). During cultivation the mass of the initial mycelium is exponentially expanded millions of times until mushrooms can be harvested (Stamets, 2000). Stages a) to d) (Figure 3) emphasize the exponential increase in mycelium required for mushroom cultivation.

2.5 SPAWN PRODUCTION

The 'mushroom seed' is generally referred to as spawn (Oei, 1991). The spawn comprises a pure culture of the desired mycelium on a carrier material. In most cases, cereal grains (sorghum, millet, rice, wheat or rye) are used as the carrier materials (Stamets, 2000). The grain is not only a vehicle for evenly distributing the mushroom mycelium, but also a nutritional supplement. Spawn production is the limiting factor for mushroom cultivation in many countries due to the enormous capital required to setup a spawn laboratory or plant (Oei, 1991). Mushroom spawn is used to inoculate prepared substrates, which are usually agricultural wastes such as maize stover (Oei, 1991; Stamets, 2000).

2.5.1 Spawn Substrate Moisture Content.

The water content of the substrate used in spawn production is a critical parameter to the success or failure of spawn production (Stamets, 2000). Excessive water of the carrier material favours the growth of bacteria and other competitors. If the carrier material is too dry, growth of mycelium is retarded. A delicate balance

between the mass of grain and water added must be preserved to promote the production of highest quality spawn.

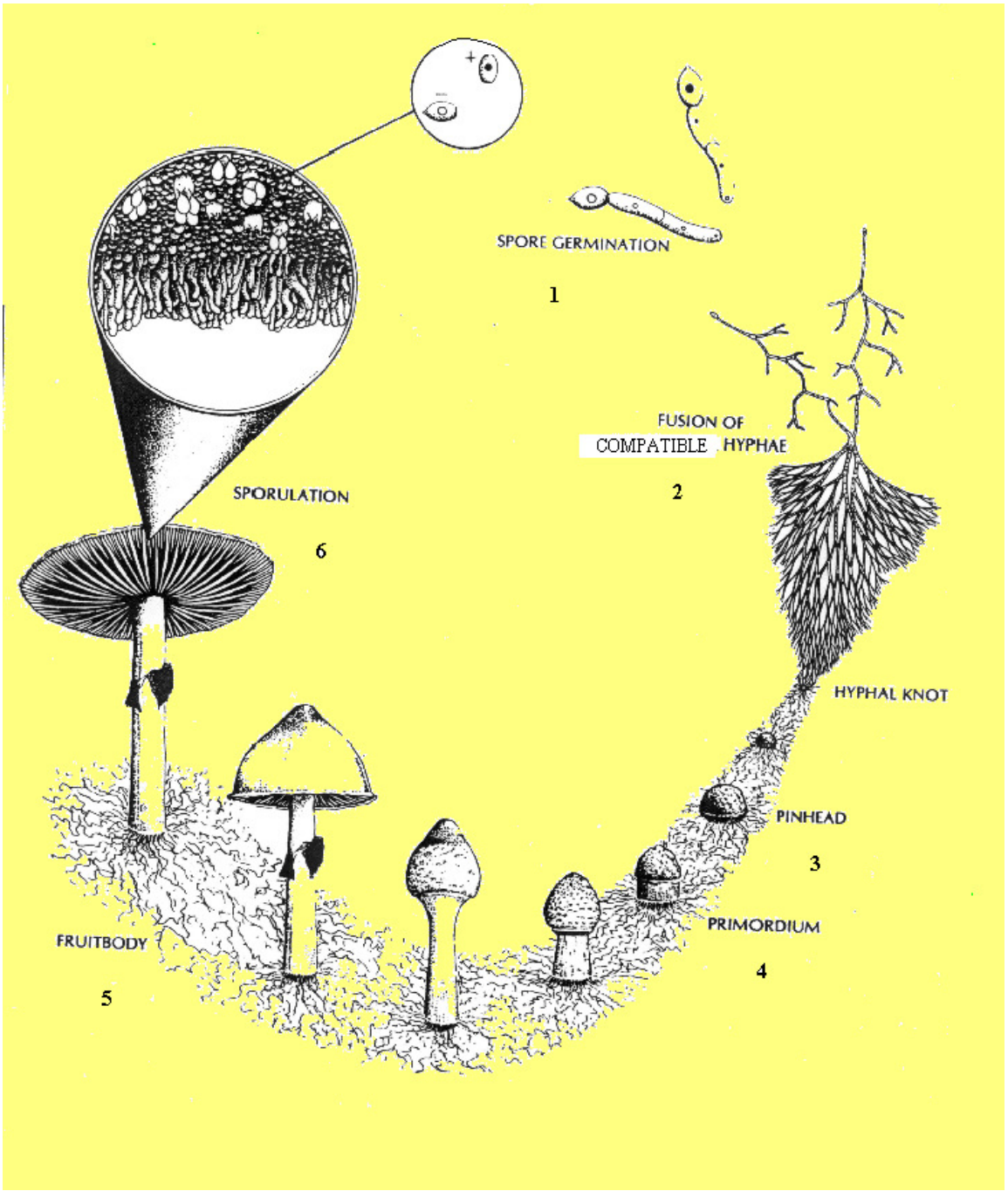


Figure 2: General life - cycle of macrofungi. Numbers 1 to 6 refer to stages in the life- cycle (Adapted from; Quimio *et al*., 1990; Stamets, 2000).

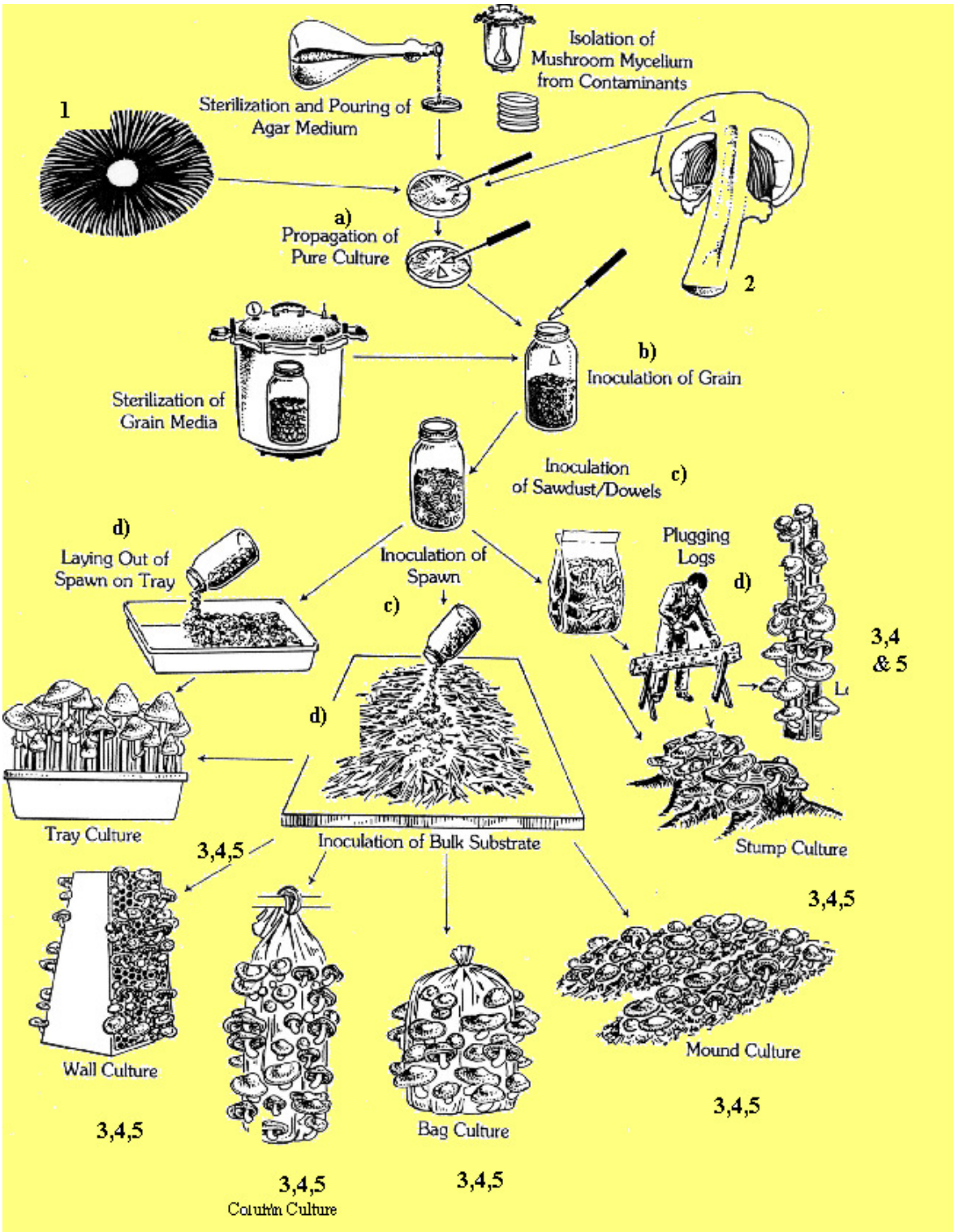


Figure 3: Summary diagram of mushroom cultivation techniques. Stages 1 to 5 correspond to those in Figure 1 whilst stages a) to d) refer to the exponential increase in mycelium required for mushroom cultivation (Adapted from Stamets, 2000).

2.5.2 Effect of Insoluble Calcium Salts on Spawn Production

Insoluble calcium salts are added in cultivation of mushrooms to increase the pH to neutrality and thereby probably reducing the bacterial contamination. The salts are also added to improve the texture and porosity of the composted or treated substrate (Hayes, 1972 cited in Chiu *et al*., 1998). Calcium carbonate, ($CaCO_3$) and gypsum, ($CaSO_4.2H_2O$) are the commonly used insoluble calcium salts. They also help in keeping the grain kernels separated. However addition of these salts to spawn is optional for most species including *Pleurotus* species (Quimio *et al*., 1990).

2.5.3 Sterilisation of Spawn Substrates

The sterilization of grain growth medium during spawn production is achieved by utilizing steam under pressure (Stamets, 2000; Ogden and Prowse, 2004). This is achieved through using autoclaves which are very expensive, with the common conditions for sterilization being one hour at 121 °C (Quimio *et al*., 1990; Oei, 1991; Stamets, 2000; Ogden and Prowse, 2004). The rate of spawn production and subsequent uptake of *Pleurotus* species cultivation is partly determined by the availability of autoclaves.

Tyndallisation is defined as intermittent sterilisation by successive boiling over a period of a few days (VanDemark and Batzing, 1987). Tyndallisation was first used by John Tyndall (1820-1983) and was employed for sterilizing many materials in the formative years of microbiology before it was eventually replaced by the use of steam under pressure termed autoclaving. Tyndallisation is a process that makes use of the fact that germinated bacterial endospores are heat-labile. The process involves boiling water at 100 °C for ten minutes, which kills most microbial pathogens and vegetative forms of bacteria but may not kill bacterial endospores (VanDemark and Batzing, 1987). By heating materials at 100 °C, vegetative microbes are killed and

endospores are heat-activated to germinate. After a period of incubation, for example twenty-four hours at ambient temperatures or 25 °C, to allow the activated endospores to germinate and begin to grow, a second exposure to 100 °C will kill these now heat-liable entities. The process is repeated once or twice to ensure all endospores have been induced to germinate and have been killed.

2.6 CULTURE MAINTENANCE

Cultivation of mushrooms requires access to agar maintained cultures in the cultivation process (Oei, 1991; Stamets, 2000). Fungal cultures are maintained by subculturing on agar (Gordon, 1952; Booth, 1971; Onions, 1971) and storing at 4 °C or under oil at room temperature or in sterile water at room temperature; by drying on anhydrous silica gel (Mehtra *et al.*, 1977), by freeze-drying (Rey, 1977) and by freezing at – 20 °C in agar slopes or at – 140 °C in liquid nitrogen or sterile 30 percent glycerol (Smith, 1983a). Media for culture maintenance of fungi is frequently out of the reach of most developing countries laboratories. Low – cost media formulations for oyster mushrooms (*Pleurotus* species) culturing, can play a key role in culture maintenance, spawn production and mushroom cultivation. The continued access to economically important *Pleurotus* species in the developing countries of the world is threatened by the high cost of maintenance of fungal cultures. Fungal strains are maintained as living cultures to replace contaminated or degraded experimental material such as deoxyribonucleic acid samples, to confirm previous results and to provide other researchers with reference strains (Smith, 1984; Weising *et al*., 1995). Fungal cultures are routinely grown on agar based media such as malt extract agar (MEA) (Blakeslee, 1915), cornmeal agar (Shear and Stevens, 1913) and yeast – peptone – dextrose agar (Van Uden and do Carmo Sousa, 1957; Weitzman and Silva – Hutner, 1967). The agar based media such as MEA and potato dextrose agar (PDA)

are media originally formulated for the isolation, cultivation and enumeration of yeasts and molds in dairy products, beverages, dried and frozen foods and meat products (Smith, 1984).

2.7 SUBSTRATE FOR MUSHROOM PRODUCTION

The 'substrate' is the material or substance, which provides 'food' for the growing mushroom mycelium. In nature *Pleurotus* species grow on living or dead parts of plants which are generally poor in nutrients and vitamins (Chang and Hayes, 1978). *Pleurotus* species use a broad range of substrates such as all grain straws, paper, sugar cane bagasse, cottonseed hulls, wood shavings, sawdust, nutshells, and vegetable wastes (Block *et al*., 1959; Fu, Yu and Buswell, 1997). The principal substrate for oyster mushroom cultivation is wheat (*Triticum aestivum*) straw (Labuschagne *et al*., 2000). Substrate selectivity of the fungi depends on the available nutrients, pH, microbial activity, aeration, and free water available (Oei, 1991; Stamets, 2000). The environment also plays an important role: humidity of the air, ventilation, shade or sun, and temperature, together with the internal condition of the substrate, determine whether the mycelium can grow into the substrate (Oei, 1991).

Pleurotus species convert substrate mass into mushrooms with biological efficiencies often exceeding 100 percent (Stamets, 2000). Biological Efficiency is the percentage measurement of the yield of fresh mushrooms from the dry weight of the substrate as shown in Formula 1. Hundred percent biological efficiency is equivalent to saying that from a substrate with a moisture content of seventy-five percent, twenty-five percent of its mass will yield fresh mushrooms having a moisture content of ninety percent (Stamets, 2000).

$$\text{Biological Efficiency (E)} = \frac{\Delta P}{M_o}, \text{ Formula 1}$$

where ΔP is the amount in kg of edible mushrooms produced as fresh product and M_o is the initial amount (kg) of raw material introduced in the bag (as dry material) (Sanchez and Gonzalez, 1996).

Supplementation of a substrate may enhance yield of basidiomes by the *Pleurotus* species. For example, Domomdon and co-researchers in 2004 obtained enhanced yield of up to fifty-two percent of *Pleurotus pulmonarius* by supplementing wheat straw with thirty percent *Lolium perenne* grass chaff.

The source strength of a mycelial sub-unit is defined as the added rate of all processes that contribute to an increased cytoplasmic availability of a compound - uptake as well as chemical conversions (Lindahl and Olsson, 2004). The source strength of a mycelial sub-unit depends mainly on the rate with which resources may be taken by the hyphae (Lindahl and Olsson, 2004) (arrow 1, Figure 4). The uptake rate in turn depends on the quality of the colonized substrate as well as on the biomass and activity of the fungal mycelium in the substrate (arrow 2, Figure 4). The source strength is also affected by the presence of other organisms and their interactions with the mycelium (arrows 6 to 8, Figure 4). The quality of a substrate is determined by the chemical form, concentration and physical availability of the nutritionally valuable compounds in the substrates. For saprotrophic growth, a high concentration of low molecular weight compounds, such as monosaccharides or amino acids increases the quality of a substrate. Predominance of polymeric compounds, such as cellulose or chitin, which require enzymatic degradation before uptake is possible, leads to a lower substrate quality. A high concentration of lignin that physically shields the cellulose fibres also decreases the quality of a substrate. The biomass and activity of a mycelial

sub-unit influences its source strength (Lindahl and Olsson, 2004). The environmental impact of a mycelium has two components: quantitative (the amounts of mycelial biomass) and qualitative (the activity of the mycelium) (arrow 3, Figure 4). Both components are essential in determining the rate of resource uptake from a substrate.

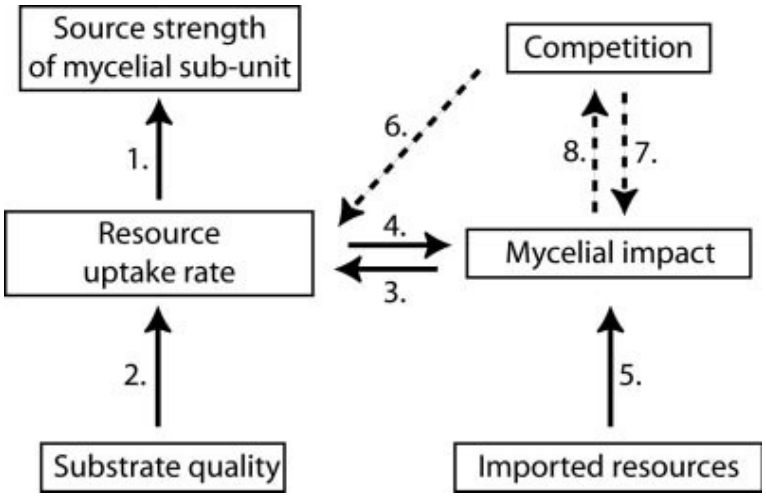


Figure 4: The major factors influencing the source strength of a mycelial sub-unit. Solid arrows indicate positive effects and dashed arrows indicate negative effects (adapted from Lindahl and Olsson, 2004).

In high quality substrates with high concentrations of low molecular weight substances directly available for uptake, the most important component of the mycelial impact is the amount of biomass (Lindahl and Olsson, 2004). The other component of the mycelial impact, the activity of the mycelium, is more important in low quality substrates that require some kind of modification before resources may be taken up. For saprotrophic growth in low quality substrates, the degradation of polymers into assimilable substances is likely to be the rate-limiting step in the exploitation of the substrate, and the mycelial impact may primarily be expressed as the activity of degrading enzymes (Lindahl and Olsson, 2004). There is a positive feedback between the uptake rate of a mycelial subunit and its impact (arrows 3 and 4,

Figure 4). The impact exerted by a mycelial subunit also depends on the amounts of resources that may be translocated from other parts of the mycelium as shown by arrow 5 in Figure 4.

Oyster mushrooms (*Pleurotus* species) cultivation is frequently done using pasteurized substrates which do not have total exclusion of other microorganisms and thus exploitation and interference competition may occur during oyster mushroom cultivation. Exploitation competition (direct competition for resources) (Lockwood, 1992) reduces the uptake rate of the mycelium and thereby the source strength (arrow 6, Figure 4). Competition for space or territory rather than directly for resources termed interference competition (arrow 7, Figure 4) frequently results in lysis of hyphae and thereby a reduction in mycelial impact of one or both of the interacting mycelia (Lindahl and Olsson, 2004). Since a successful outcome of an antagonistic interaction may result in a decreased mycelial impact of the opponent, there is a positive feedback that may eventually lead to exclusion of one of the competitors from the substrate (arrow 8, Figure 4). Pasteurisation of the substrates used in oyster mushroom (*Pleurotus* species) cultivation aids in reducing the number of competitor fungi, which may be more efficient in establishing a high mycelial impact and thus exclude the oyster mushroom from the substrate. Thus pasteurization of the substrate assists the oyster mushroom to rapidly achieve a high mycelial impact, the mycelium gaining a competitive advantage and thereby successfully exploiting the substrate resulting in the mycelial subunit turning into a net source.

The sink strength of a mycelial sub-unit can be defined as the added rate of all processes that contribute to a decreased cytoplasmic availability of a compound and represent the potential of a sub-unit to withdraw this compound from the common mobile pool. The sink strength of a mycelial sub-unit depends on the rate with which

the hyphae withdraw resources from the translocated pool and respire them, exude them or incorporate them into structural tissues. The rate of incorporation of resources into structural tissues depends on the growth rate of the mycelium, such as the build – up of quantitative mycelial impact. The build-up of qualitative mycelial impact requires exudation of enzymes and various other metabolites. The sink strength of a mycelial sub-unit is thus closely related to the build – up of mycelial impact in the substrate. In oyster mushrooms production of basidiomes contributes to the sink strength of the mycelial sub-units. Whether a mycelial sub-unit constitutes a net source or a net sink for resources depends on the relation between its source strength and its sink strength. If the uptake of resources together with mobilization from storage reserves is more rapid than the sum of exudation, respiration and incorporation of resources into structural tissues, the sub-unit is a net source and may support other parts of the mycelium with resources. If the sub-unit is a net sink, it will be dependent on translocation of resources from other parts of the mycelium to maintain its activities.

The relation between source strength and sink strength changes during the colonization and exploitation of a substrate. High initial sink strength admits the rapid build-up of a high mycelial impact within the substrate. Due to efficient exploitation of the substrate and a high competitive ability, a high mycelial impact results in high source strength (Lindahl and Olsson, 2004). There is a lag in time between the investment – high initial sink strength, and the profit – subsequent high source strength. Eventually, as the quality of the substrate decreases due to resource depletion, the source strength decreases and the resource unit is expended. By degrading structural components into mobile low molecular weight compounds and replacing the cytoplasm with vacuoles, the senescing mycelium maintains a source

strength, which, in combination with minimal sink strength, leads to net translocation out of the mycelium. By doing so, the fungus minimizes losses of valuable resources associated with mycelial death; in much the same way as deciduous trees withdraw resources from leaves before abscission (Lindahl and Olsson, 2004). Fungal growth on lignocellulosic residues and subsequent basidiome production are dependent upon the mushroom's ability to synthesize the relevant hydrolytic and oxidative enzymes which convert the individual components of the substrate (i.e. cellulose, hemicellulose and lignin) into low molecular weight compounds that can be assimilated for nutrition (Fu *et al*., 1997).

2.8 GENETIC CHARACTERISATION OF FUNGI

Fungi are becoming increasingly important for a variety of individual purposes, and many species are serious pathogens of plants, domestic animals, and humans. In several areas of research, the precise and unequivocal identification, discrimination, and characterization of fungal species, races, isolates, populations and pathotypes is of prime importance. Among the fungi, identification and classification have always presented some special challenges as highlighted below.

An essential prerequisite for mushroom breeding and cultivation procedures is the use of properly identified biological material with unambiguous relationships among its members (Zervakis *et al*., 1994). This is a difficult or even impossible task if the characterization solely relies on growth characteristics, sexual compatibility, and morphological and biochemical criteria (Weising *et al*., 1995).

A phyletic classification system is not simple for a kingdom of organisms for which the fossil record is almost non-existent, and for which present day structures may be stereotypically simple and therefore information poor (Metzenberg, 1991). Morphological data have been shown to be of limited value for fungal systematics due

to their inherent simplicity, evolutionary convergence, parallelisms and phenotypic plasticity (Bruns *et al*., 1991; Hibbett and Vilgalys, 1993; Vilgalys *et al*., 1994; Hibbett and Donoghue, 1995; Hopple and Vilgalys, 1999; Moncalvo *et al*., 2000; Wagner and Fisher, 2001; Hibbett and Thorn, 2000). Due to the unreliability of morphological characteristics in taxonomy of the genus *Pleurotus*, mating compatibility tests were introduced to aid in the taxonomy of the genus *Pleurotus* (Han *et al*., 1974; Eger *et al*., 1979; Bresinsky *et al*., 1987; Petersen and Hughes, 1993; Vilgalys *et al*., 1993; Petersen, 1995; Zervakis and Balis, 1996; Isikhuemhen *et al*., 2000; Zervakis *et al*., 2004).

Development of a natural classification system for gilled fungi and their allies (Agaricales, Basidiomycota) is increasingly relying on molecular data (Hofstetter *et al*., 2002). Classical strategies of evaluating genetic variability such as comparative anatomy, morphology, embryology, and physiology have been increasingly complemented by molecular techniques (Weising *et al*., 1995). These techniques include, for example, the study of allozymes, restriction fragment length polymorphisms (RFLPs), electrophoretic karyotypes. In the late 1980s, hybridization – based DNA fingerprinting was introduced for the analysis of fungal genomes (Scherer and Stevens, 1988; Braithwaite and Manners, 1989). Since, then, DNA profiling has become one of the favourite methods for diagnostic and epidemiological studies of human, plant and insect pathogenic fungi; for the characterization of non – pathogenic and industrially important fungi; and for the evaluation of taxonomic and phylogenetic problems. Zervakis and co-researchers in 1994 studied genetic variability and systematics of the genus *Pleurotus* based on isozyme analysis. Molecular approaches to the generic systematics of *Pleurotus* have been used since 1987 and have contributed mainly to the *Pleurotus sajor-caju* and *P. Pulmonarius*

controversy (Bresinsky *et al*., 1987; Vilgalys and Sun, 1994; Iracabal *et al*., 1995; Isikhuemhen *et al*., 2000).

2.8.1 DNA Isolation

Liquid media such as malt extract broth are used for the fast accumulation of fungal mycelia or cells for DNA isolation. In liquid culture, the division rate of fungal cells or growth of mycelia is maximal in the logarithmic phase and drops off towards the stationary phase thus cells from liquid cultures are harvested generally in the late-log or early stationary phase for maximum yield (Weising *et al*., 1995). Liquid media eliminates the problem of polysaccharides such as, agar attached to the fungal mycelia which may render DNA isolation difficult. Efficient aeration is also required for growth thus the medium should constitute no more than one fifth of the total volume of the Erlenmeyer flask used for culture (Weising *et al*., 1995).

There is no consensus on the optimal stage of growth of fungal cultures for DNA isolation. Some researchers claim that cultures should be derived from the log phase, while others prefer stationary phase cultures (Weising *et al*., 1995). The efficient disruption of the cell wall is one of the critical steps during DNA isolation. Fungal cell walls contain chitin and are often highly resistant to mechanical forces. One commonly used technique to destroy the cell walls makes use of sand or small glass beads to grind fresh mycelium in a mortar with a pestle. This is reliable and inexpensive but many hyphal segments remain intact. A more efficient method is freezing the mycelia in liquid nitrogen prior to grinding with mortar and pestle. Another technique involves cell wall-degrading enzymes to generate protoplasts prior to DNA isolation (Weising *et al*., 1995). The first comprehensively reported isolation of protoplasts from filamentous or mycelial fungi was done thirty-seven years ago (Peberdy, 1971 cited in Peberdy, 1995). Protoplast isolation avoids mechanical force

and yields highly intact genomic DNA, but it is time consuming and expensive thus it is out of the reach of many laboratories.

Deoxyribonucleic acid is recovered from cells by the gentlest possible method of cell rupture, in the presence of EDTA (ethylenediammine tetraacetate) to chelate the magnesium ions needed for DNase activity (Wilson and Walker, 1995). A large variety of methods to isolate fungal DNA have been described. However, the majority of methods belong to one of the following. The Sodium dodecyl sulphate (SDS) or triisopropylnaphthalene sulphonic acid / para-aminosalicyclic acid (TNS/PAS) – based methods which make use of strong detergents (SDS and TNS/PAS) to break the cell and organelle membranes. In addition the cetyltrimethyl ammonium bromide (CTAB) – based methods which make use of the DNA – binding detergent CTAB in the isolation buffer and the methods based on protoplast isolation (Weising *et al*., 1995). Cell disruption and most subsequent steps are performed using glassware and solutions that have been autoclaved, to destroy DNase activity (Wilson and Walker, 1995). After release of DNA from the cells, RNA can be removed by RNase (preheated to inactivate any DNase contaminants). Protein: the other major contaminant is removed by water-saturated phenol or phenol/chloroform, followed by centrifugation (Wilson and Walker, 1995). The upper aqueous phase that results contains the nucleic acid and it can further be deproteinised until the interface does not occur (Wilson and Walker, 1995). The DNA is precipitated by absolute ethanol (2 or 2.5 volumes of the aqueous phase) or isopropanol (0.5 volume) (Weising *et al*., 1995; Wilson and Walker, 1995). Optimisation strategies most importantly concern the ingredients and also the pH of the extraction buffer. The EDTA compound chelates bivalent cations and thereby inhibits metal – dependant DNases. For DNA concentration one optical density of UV absorbance at 260 nm (1 O. $D._{260}$)

corresponds to, 50 µg/ml of DNA (under standard conditions, such as 1 cm light path). The purity of DNA is obtained by the ratio of O. $D._{260}$/O. $D._{280}$, with pure DNA having a ratio ranging from 1.8 to 2.0 (Weising *et al.*, 1995). The desired degree of purity of the DNA is dependant on the intended use of the preparation. Procedures such as cloning require pure DNA, whilst a restriction enzyme analysis requires low levels of EDTA in the DNA solution. Successful polymerase chain reaction (PCR) analysis can be obtained with DNA preparations of a low degree of purity.

2.8.2 Polymerase Chain Reaction (PCR)

Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules in an afternoon. In its early days PCR was performed manually using test tubes and a source of heat. Since the PCR inception, different PCR techniques have been developed such as differential display PCR, expression PCR, hot-start PCR, long-distance PCR, multiplex PCR, nested PCR, panhandle PCR, recombination PCR and touch-down PCR (Dieffenbach and Dveksler, 2003). Primers are single-stranded pieces of DNA (oligonucleotides) with sequence complementarity to template sequences flanking the targeted region. An oligonucleotide is a short polynucleotide of typically eight to fifty nucleotides (Wetmur, 1995). To allow for exponential amplification the primers must anneal in opposite directions so that their 3′ ends face the target. Amplification is most efficient when the two primer binding sites are not further apart than about four kilobases (kb), though long template PCR systems have been devised that amplify regions of up to forty-five kilobases of DNA (Dieffenbach and Dveksler, 2003). In a typical PCR, three temperature controlled steps can be discerned which are repeated in a series of twenty-five to fifty cycles. In the denaturation step the template DNA is made single stranded by using a temperature of 94 °C. Annealing, is the second step in which the

temperature is lowered to about 25 to 65 °C (depending on primer sequence and experimental strategy) which results in primer annealing to their target sequences on the template DNA. Polymerisation (extension): is the third step where the thermostable DNA polymerase now extends the 3′ ends of the DNA primer hybrids toward the other primer binding site usually at 72 °C. Since polymerisation happens at both primers – annealing sites on both DNA strands, the target fragment is completely replicated. A cycle consists of denaturation, annealing and polymerization.

Several parameters influence the specificity of PCR, i.e. the temperature profile of the thermocycler, the annealing temperature, the activity and amount of the DNA polymerase, concentrations of primers, template DNA and magnesium ions (Mg^{2+}), and the presence of additional chemicals such as dimethylsulphoxide (DMSO). The annealing temperature is usually chosen as high as possible to prevent unspecific amplification. The efficacy of PCR is measured by its specificity (one amplification product), efficiency (more products with fewer cycles) and fidelity (highly accurate) (Cha and Thilly, 2003). The specificity, efficiency and fidelity of PCR are influenced by numerous components of PCR such as the cycling regime and buffer conditions. The specificity, efficiency and fidelity of the PCR are optimized according to the importance of each parameter for the PCR intended application (Cha and Thilly, 2003). For instance, for direct sequencing analysis, the yield and specificity of PCR is more important than the fidelity.

2.8.3 DNA Sequencing

The ultimate characterization of genetic structure is the obtaining of the DNA nucleotide sequence of an organism (Griffiths *et al.*, 2002). DNA sequences are becoming the primary currency by which we measure and study microbial biodiversity (Tautz *et al.*, 2003). Any method of DNA sequencing starts with a

population of a defined fragment of DNA labeled at one end (Griffiths *et al*., 2005). DNA sequencing provides a highly reproducible and informative analysis of data and can be adapted to different levels of discriminatory potential by choosing appropriate regions of the genome. A PCR product can be sequenced either directly or after cloning (Hoelzel and Green, 1992). Direct sequencing strategies are applied mostly to small-scale sequencing projects. In this study a direct sequencing strategy was done. Manual sequencing is comparatively difficult as well as expensive. Traditionally, radioisotopes have been (and are still) used for DNA fragment detection. Sequencing machines have become available, which make use of fluorescence – labeled primers (Smith *et al*., 1986). While the technical equipment is more expensive, DNA sequencing using fluorescence technology is cheaper and easier to perform than the traditional manual methods. Moreover, sequence data are directly transferred into a computer.

2.9 USE OF RIBOSOMAL DNA (rDNA) / rRNA GENES IN GENETIC CHARACTERISATION

The American microbiologist Carl Woese realized that differences in the genetic code could be used to differentiate between and classify microorganisms (Woese, 1967). In particular, Woese focused on the genes that code for ribosomal RNA (rRNA). Fungi contain 80S ribosomes, which consist of two subunits – the large (60S) and small (40S) subunits. Each subunit consists of rRNA as a structural molecule and a number of associated proteins, with the large subunit containing the 28S, 5.8S and 5S rRNA molecules and the small subunit containing the 18S rRNA molecule (Kennedy and Clipson, 2003). The study of the evolutionary history of organisms by tracing them to common ancestors is known as phylogenetics (Down, 2002). The general principle of making a phylogenetic tree based on similarities and differences of nucleic acid or

protein sequences is not different in kind from making a tree based on morphological differences (Metzenberg, 1991). Getting these sequences is easiest of all from rRNA genes because the rDNA is typically present in a hundred or more copies per haploid genome, and parts of the rDNA sequence are so highly conserved that they can always be detected with heterologous probes (Metzenberg, 1991). In addition, the rDNA genes are highly transcribed, and acceptably pure ribosomal RNA can generally be isolated and sequenced directly (Metzenberg, 1991). An additional advantage of the rDNA sequences is that they allow the time scale of evolution to be examined, as it were at almost any desired level of magnification – they are the calendric equivalent of a zoom lens (Sogin and Gunderson, 1987). The internal transcribed spacer – regions (ITS) – those which are present in the initial all- inclusive transcript, but are trimmed away during maturation to functional rRNAs – are subject to much more rapid evolutionary change and are appropriate to studying much more recent divergence (Metzenberg, 1991).

Although the transcribed portions of ribosomal DNA (rDNA) are the same in a given individual, the nontranscribed spacer regions between the transcribed regions can vary. The rDNA codes for the ribonucleic acid (RNA) component of the ribosome, and exists as a multigene family of similar DNA sequences (about 8 to 12 kb) arranged in tandem arrays (Figure 5) (Muir and Schlötterer; 1999; Lee, Cole and Linacre, 2000).The tandemly repeated rDNA is needed to meet the great cellular demand for their transcripts (Lodish *et al.*, 2004).

Conserved primer sequences useful for amplification and sequencing of nuclear ribosomal DNA (rDNA) from major groups of fungi (primarily Eumycota), plants and other eukaryotes have been designed (White *et al.*, 1990; Ansorge *et al.*, 1997; Vilgalys, http://www.biology.duke.edu/fungi/mycolab/primers.htm, accessed

2008). Each unit within a single array consists of the genes coding for the small and large rRNA subunits (18S and 28S). The 5.8S nuclear rDNA gene lies embedded between these genes but separated by two internal transcribed spacers; ITS1 and ITS2. The external transcribed spacer (ETS) and the intergenic spacer (IGS) separate the large and small subunit rDNAs. Several restriction sites for *Eco*R1 and *Bgl*II are conserved in the rDNA of fungi (White *et al*., 1990; Ansorge *et al*., 1997; Vilgalys, http://www.biology.duke.edu/fungi/mycolab/primers.htm, accessed 2008) (Figure 5).

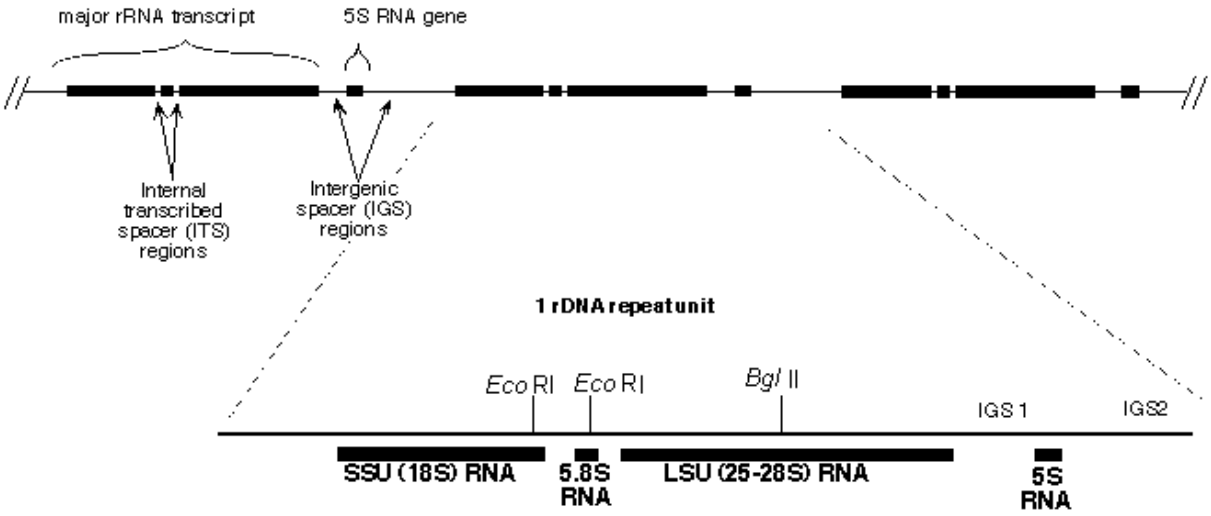


Figure 5: Schematic diagram of the nuclear regions ribosomal DNA (rDNA) region (after Vilgalys, http://www.biology.duke.edu/fungi/mycolab/primers.htm, accessed 2008). SSU is the small subunit RNA while LSU is the large subunit RNA.

Each part of the rDNA region can be employed for specific phylogenetic questions across a broad taxonomic spectrum (Hills and Dixon, 1991). The greatest amount of sequence variation in rDNA exists within the internal transcribed spacer (ITS) which has been employed for populations and congeneric phylogenies and the intergenic transcribed spacer (IGS) regions compared to the small subunit RNA (SSU). The small subunit is highly conserved and has been used to shed light on deep

evolutionary branches, for example – for relationships between Archaebacteria and Eubacteria. The large subunit RNA (LSU) regions; conserved domains within the 28S region, have been used to cover evolutionary time through the Palaeozoic and Mesozoic eras, hence variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions (Muir and Schlötterer, 1999). The smallest rDNA gene, the 5.8S, is too short to provide a robust phylogenetic signal (Muir and Schlötterer, 1999).

The ITS region is said to be the most widely sequenced DNA region in fungi (Ansorge *et al*., 1997). Consequently, PCR amplification of the ITS and IGS regions has become a popular choice for molecular systematics of fungi, plants and other eukaryotes at the species level, and even within species as for example, to identify geographic races (Vilgalys, http://www.biology.duke.edu/fungi/mycolab/primers.htm accessed 2008). Although most labs use the standard primers shown in Figure 6, several taxon-specific primers have been described that allow selective amplification of fungal sequences (Gardes and Bruns, 1993).

Application of rDNA gene analysis in the systematics of the genus *Pleurotus* has been done by several workers (Vilgalys and Sun, 1994; Neda and Nakai, 1995; Isikhuemhen *et al*., 2000). Information from studies of nucleic acids has helped refine the limits of *Pleurotus*, *Lentinus*, and *Panus* (Hibbett and Thorn, 1994). The combined study of phylogeny and biogeography provides a framework for understanding the relationship among different components of evolution at the species level, including geographic variation, genetic isolation mechanisms and morphological evolution (Vilgalys and Sun, 1994). The *Pleurotus* species and strains in this study were assessed for genetic variability by sequencing the internal transcribed spacer region of rDNA.

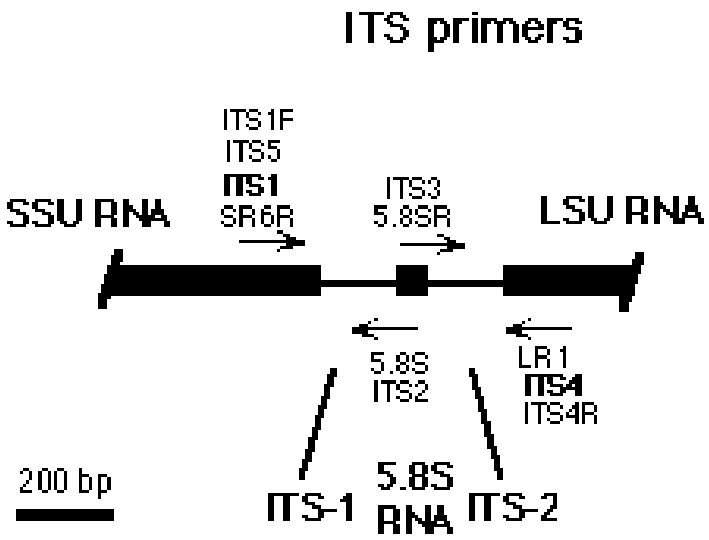


Figure 6: Diagram illustrating the routine primers used to amplify the internal transcribed spacer regions of rDNA of fungal taxa. The primers in bold were used in this study.

2.10 DNA SEQUENCE DATA ANALYSIS

Identification of a 7000 year old Neolithic polypore, *Daedaleopsis tricolour* was achieved by data analysis of the DNA recovered and sequenced (Bernicchia *et al*., 2006). Sequences of nucleic acids define the primary structure of these molecules. Sequence analysis is carried out using computer programs that implement algorithms to determine sequence properties and to compare sequences. Sequence comparison can indicate whether a region of DNA is already known (identity) or has some degree of similarity to a known sequence (Bishop, 1995). This involves the comparison of two strings of letters (either nucleotide or amino acids codes) and checking for identity requires placing the sequences side by side and counting matching letters. If one sequence is shorter than the other, the process must be repeated for every displacement of one sequence against the other (Taylor, 1995). When searching for

similarity, mismatches must be allowed, and also the possibility of relative insertions and deletions between the two sequences (referred to as indels or gaps for short).

DNA sequences are compiled in GenBank, the Data Library of the European Molecular Biology Laboratory (EMBL) and the DNA Database of Japan (DDBJ). For conventional architecture computers, program suites have been written which reduce the run time by taking short cuts. As the sequence databanks have grown, considerable effort has been put into fast alignment methods such as FASTA and BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) programs. The BLAST program is the fastest (about ten times faster than FASTA) and takes only a few minutes to search all known DNA with a 1 kb probe. Since the publication of BLAST by Altschul and co-researchers in 1990 improvements and variations to the BLAST algorithm have been incorporated such as a gapped BLAST program that runs at approximately three times the speed of the original and **P**osition – **S**pecific **I**terated BLAST (PSI-BLAST) (Altschul *et al*., 1997; Schäffer *et al*., 2001). In this study the BLAST program was used to search the DNA databases.

A central task in the study of molecular sequence data from present-day species is the reconstruction of the ancestral relationships. In the construction of phylogenetic trees, the principle of minimum evolution or maximum parsimony is often used (Saitou and Nei, 1987). The standard algorithm of the tree-making methods based on this principle is to examine all possible topologies (branching patterns) or a certain number of topologies that are likely to be close to the true tree and to choose one that shows the smallest amount of total evolutionary change as the final tree (Saitou and Nei, 1987). The most established approach to tree reconstruction is the maximum likelihood method (Hobolth and Yoshida, 2005). The neighbour-joining method (Saitou and Nei, 1987) is frequently used because of its computational speed

and has become the most widely used method for building phylogenetic trees from distances (Gascuel and Steel, 2006). In this study the neighbour-joining method was used to generate phylogenetic trees.

2.11 OBJECTIVES OF THE RESEARCH

2.11.1 The main objective of this research was to optimise the growth conditions and genetically characterise *Pleurotus* species and strains for low-cost spawn production and cultivation in the tropics and subtropical regions of the world, and for potential biotechnological applications.

The specific objectives were:

2.11.2 To determine the synergistic effects of di-substrate mixtures (1:1) on fruiting of the *Pleurotus* species and strains.

2.11.3 Assessments of low-cost media formulations on their feasibility as alternative media for *Pleurotus* species spawn production.

2.11.4 To determine the application of how differing cardinal environmental factors: water, pH and temperature conditions affect the various *Pleurotus* strains regarding primordium development and basidiome production for choice of biotechnological potential strains.

2.11.5 Assessments of the use of modified Tyndallisation protocols as an alternative to autoclaving wheat grain for *Pleurotus* species spawn production.

2.11.6 To genetically characterise the *Pleurotus* species by sequencing of the internal transcribed spacer region of ribosomal deoxyribonucleic acid.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 FUNGAL SPECIES AND STRAINS

The fifteen species and strains included in this study were obtained as freeze-dried cultures on dry ice from Belgian Coordinated Collections of Microorganisms / Mycotheque de l' Universite' Catholique de Louvain [BCCM^{TM}/MUCL] (Agro) Industrial Fungi and Yeasts Collection and are listed in Appendix C. All strains were maintained on malt extract agar (MEA; 3 % w/v malt extract and 2 % w/v agar obtained from Oxoid, UK).

3.2 OPTIMISATION OF ENVIRONMENTAL CONDITIONS

3.2.1 Osmotic Potential–Growth Relationship

Osmotic potential was varied by supplementing MEA (3 % w/v malt extract and 2 % w/v agar obtained from Oxoid, UK) with potassium chloride that was added before autoclaving at 121 ºC for 15 min over a range of concentrations (0, 1, 2, 4, 6 g l^{-1}) to obtain media with the following values: – 0.5 (control), – 1.5, – 2.5, – 3.5 and – 4.5 MPa, respectively. All media were cooled to 45 °C prior to being poured (20 ml per plate) into vented 9-cm diameter plastic Petri plates. The osmotic potentials of media were determined using a Novasina IC II water activity machine (Novasina, Pfäffikon, Switzerland) as described in the manufacturer's handbook by measuring three replicate solutions that were made up on separate occasions at 30 °C, and the variation of replicate values was within ± 0.002 water activity (Hallsworth and Nomura, 1999). The equipment was calibrated using saturated salt solutions of known water activity, supplied by the manufacturer, and values were converted to osmotic potential using a standard curve. Three replicate plates were inoculated on the circumferal edge of the media for each experimental treatment, using 4-mm diameter

plugs taken from the periphery of exponentially growing cultures of each strain grown on MEA. Plates of each medium were kept together in a sealed bag of low-density (100 µm) polyethylene obtained from Sigma Aldrich, U.S.A. to maintain a constant relative humidity and medium osmotic potential, whilst allowing gaseous exchange (Hallsworth *et al*., 2003b). Radial growth was measured daily by taking two radii readings for each replicate plate for ten days. The radii were determined by measuring colony radius minus the diameter of the inoculum disk on two perpendicular axes (Mswaka and Magan, 1999), and these data were used to establish the relationship between treatment and growth rate as described previously (Hallsworth and Magan, 1999) by analysing radius versus time plots by linear regression and the slope for each treatment recorded. Such values were called radial extension rates and these were analysed using ANOVA.

3.2.2 Temperature-Growth Relationship

The control medium (i.e. MEA with no added potassium chloride) was used to assess growth in response to environmental temperature (from 5 to 37 °C) in triplicate plastic Petri plates containing 20 ml of media each for each experimental treatment for ten days.

3.2.3 pH–Growth Relationship

For studies of pH, buffers were added to the MEA medium and the pH was adjusted using 1 M hydrochloric acid (HCl) or 5 N sodium hydroxide (NaOH) as described previously (Hallsworth and Magan, 1996). The resultant pH media was used to assess growth in response to environmental pH (from 2 to 12) in triplicate plastic Petri plates containing 20 ml media each for each experimental treatment for ten days. The pH of media was measured before inoculation using Alicit pH indicator strips (± 0.1 pH, Merck) which were more amenable for solid media use.

3.2.4 Assessment of Primordium Development and Mycelial Characteristics

Primordium development and mycelial characteristics were assessed for each treatment by eye and/ or low power microscopic examination. For strains showing no growth at certain treatments, the agar inoculum plugs were transferred to the control medium and viability, growth, primordial formation and mycelial characteristics were noted at 25 °C. Multivariate analysis (cluster analysis) of the growth data under the different stress parameters (osmotic potential, pH and temperature) was done using STATISTICA 7.1 (2006) software, StatSoft Incorporation.

3.3 OPTIMISATION OF MICRO CHANGES IN ENVIRONMENTAL CONDITIONS

3.3.1 Micro–Concentration of Polyethylene Glycol–Growth Relationship

Malt extract agar (3 % w/v malt extract and 2 % w/v agar obtained from Oxoid, UK) was supplemented with PEG 6000 (BDH Limited, UK) that was added before autoclaving at 121 °C for 15 min using the following concentrations: 0 (control), 0.10, 0.20, 0.35, 0.65, 0.75, 0.90 and 1.00 gram per litre (g l^{-1}) of the final total volume. An initial screen of growth of *Pleurotus* species and strains was carried out using PEG 1000, 6000 and 8000 (data not shown). Polyethylene glycol 6000 gave the highest growth rate, so it was selected as the representative solute. The prepared media were used to assess growth at different environmental temperatures of 20, 25 or 30 °C as in Section 3.2.1.

3.3.2 Micro–Concentrations of KCl and NaCl-Growth Relationship

MEA (Oxoid, UK) was supplemented with the solutes KCl and NaCl in the ratio 5:2 (Robinson and Stokes, 1959; Scott, 1953) before autoclaving at 121 °C for 15 min using the following respective concentrations: KCl; 0 (control), 1.50, 2.70,

3.85 and 5.00 g l^{-1} and NaCl; 0 (control), 0.60, 1.08, 1.54 and 2.00 g l^{-1} determined from a pretrial experiment (data not shown). The prepared media were used to assess growth at different environmental temperatures of 20, 25 or 30 °C as in Section 3.2.1.

3.3.3 Assessment of Primordium Development and Mycelial Characteristics

Three replicate plates were inoculated for each experimental treatment, using 4-mm diameter plugs taken from the periphery of exponentially growing cultures of each strain grown on MEA. Radial growth was measured daily as described previously (Mswaka and Magan, 1999), and these data were used to establish the relationship between treatment and growth rate as described previously (Hallsworth and Magan, 1999). Primordium development and mycelial characteristics were assessed for each treatment by eye and/ or low power microscopic examination.

3.4 OPTIMISATION OF SPAWN PRODUCTION

3.4.1 Determination of Water Content Changes of Wheat Grain during *Pleurotus* Spawn Production

3.4.1.1 The conventional method (utilising insoluble calcium salts)

Wheat grains (obtained from Grain Marketing Board [GMB], Zimbabwe) soaked overnight in tap water were washed in tap water for 5 times, then boiled in water for 5 minutes. The wheat grain was drained and allowed to cool. Wheat grain (100 g) was placed in a 250 cm^3 capacity narrow mouthed bottle. Calcium carbonate, ($CaCO_3$) and gypsum, ($CaSO_4.2H_2O$); (1 % each on weight basis) were added to the grain and thoroughly mixed into it. The mouth of each of the bottles was plugged with a 0.22 µm microporous filter plug obtained from Millipore, U.S.A. The grains were sterilised in an autoclave at 121 °C for 1 hour, prior to being cooled in a sterile room overnight which was pre-sterilised for twenty-four hours using ultra-

violet light. At each of the stages of dry, soaked overnight, boiled and autoclaved wheat grain, three samples were taken and used for determining the water content of the wheat grain: sample wheat grain of a known mass was oven dried at 150 °C for 6 hours, followed by weighing of the oven dried grain. The percentage (%) water was calculated using the Formula 2.

$$\text{percent (\%) water} = \left(\frac{\text{mass of water lost}}{\text{lost water + oven dried grain}} \right) \text{ x } 100 \quad \underline{\text{Formula 2}}$$

The respective bottled grain was agar to grain method (Stamets, 2000) inoculated with the *Pleurotus* species strain in triplicate using 5-day-old Petri plate cultures without contamination and with a margin of uncolonised media along the inside of the peripheral edge prior to incubation of inoculated bottled grain with a 2 cm gap in–between the bottles in a constant temperature room maintained at 25 ± 0.5 °C until the substrate appeared white to produce *Pleurotus* species spawn by the conventional method. Petri plate cultures (5-day-old), without contamination and with a margin of uncolonised media along the inside of the peripheral edge were used for inoculating the sterilised wheat grain. The extent of colonisation of the wheat grain by the *Pleurotus* species was noted daily by eye until the substrate was fully colonised. The day of full colonisation was recorded. Three of the sterilised grain-filled jars were left unopened and uninoculated to determine the success of the sterilisation procedure. Contamination and recovery of the mycelium on the grain kernels was visually assessed by naked eye examination after four days. Sorghum grain spawn was also prepared as above using the conventional method.

3.4.1.2 The unconventional method (omitting the boiling stage and insoluble calcium salts)

The above procedure in Section 3.4.1.1 was repeated with the modification of omitting the boiling stage and non addition of insoluble calcium salts to produce *Pleurotus* spawn by an unconventional method.

3.4.2 Assessment of the Application of Water Content Formulae in *Pleurotus* Spawn Production

Pleurotus species spawn was prepared with the amount of water added derived from water content formulae listed in appendix E. The amount of wheat grain added was kept constant. The required mass of water was added to the dry wheat grain of a known mass in a two litre capacity Erlenmeyer flask with the intention to achieve a desired water content of the wheat grain of 50 percent (Stamets, 2000). The wheat grain was sterilized, inoculated and incubated as in Section 3.4.1.1 for the conventional method. Sterilised wheat grain of each respective water content formulae was used to assess growth on Petri plates at 25 °C by plating out a single layer of grain in each Petri plate and inoculating using 10-mm diameter plugs in triplicate for each formula with the *Pleurotus* strain. The Petri plates were incubated in a non–inverted position for 14 days. Radial growth was measured daily as described previously (Mswaka and Magan, 1999), and these data were used to establish the relationship between treatment and growth rate as described previously (Hallsworth and Magan, 1999).

3.4.3 Determination of Nutrient Changes of Wheat Grain during *Pleurotus* Spawn Production: Sterilisation of Grain Medium

The sterile grain produced by omitting the boiling stage and non addition of insoluble calcium salts (unconventional method) was used for grain nutrient analysis.

Three samples were taken after the grain was soaked overnight, after autoclaving for one hour at 121 °C and of the dry grain in 250 ml capacity Erlenmeyer flasks. Nutrients carbon (C), nitrogen (N) were analysed at the Soil Science Laboratory (Department of Biological Sciences) as described previously (Chiu *et al.*, 1998). Dried samples (10 mg) were digested and analyzed with an atomic absorption spectrometer; Na^+ and K^+ were analyzed by flame atomic emission, PO_4^{2-}, Ca^{2+} and Mg^{2+} by flame atomic absorption at the Department of Soil Science (Faculty of Agriculture) as described previously (Chiu *et al*., 1998).

3.4.4 Assessment of Alternative Substrates for *Pleurotus* Spawn Production

The normal process of spawn production (Stamets, 2000) (see Section 3.4.1.1) was modified in terms of time taken to soak and boil the substrate. The maize cobs were chopped into pieces of 15 ± 5 mm in length whilst the pumpkin seeds were sun-dried prior to soaking. Dry beans (*Phaseolus vulgaris*) were soaked for 16 hours and boiled for 5 minutes or 10 minutes as two different treatments. Maize grain (*Zea mays*) was soaked for 2, 3, 16 or 40 hours and boiled for 10, 10, 5 or 10 minutes respectively. Maize cobs (*Zea mays*) were not soaked but boiled for 5 minutes. Pumpkin seeds (*Cucurbita* sp.) were soaked for 2 or 25 hours and boiled for 5 minutes. The percentage water content of each substrate was determined after soaking, boiling and autoclaving as described above in Section 3.4.1.1. The substrates were sterilized, inoculated with the *Pleurotus* strains and incubated as described in Section 3.4.1.1

3.4.5 Assessment of Media Formulations for Culturing *Pleurotus*

3.4.5.1 Growth media

3.4.5.1.1 Formulation of whole wheat grain media

To vary the whole wheat grain media formulations MEA (3 %; obtained from Oxoid, UK), PDA (3 %; obtained from Oxoid, UK), distilled water, whole wheat grain (from Grain Marketing Board [GMB], Zimbabwe) and polyethylene glycol (PEG) 6000 (BDH Limited, UK) (concentration of 1 g per litre of distilled water) were added in different proportions to Erlenmeyer conical flasks as follows. Quantities of 1:1 (w/v) (whole wheat grain: distilled water), 1:2, 1:3, 1:4 and 1:5 were placed in 250 ml Erlenmeyer conical flasks. To sets of 1:1 to 1:5 whole wheat grain: distilled water were added MEA to result in whole wheat grain-MEA formulation, PDA (whole-wheat grain-PDA formulation), MEA and PEG 6000 (whole-wheat grain-MEA-PEG formulation) and PDA and PEG 6000 (whole-wheat grain-PDA-PEG formulation). Additional sets of media were prepared by adding PEG 6000 to MEA (3 %) and PDA (3 %) respectively. Control medium of MEA (3 %) and PDA (3 %) were also prepared. The amount of media (MEA or PDA) added was determined in relation to the amount of distilled water required for 3 % final media. The media were autoclaved at 121 °C for 20 min to obtain media with the respective whole wheat grain: distilled water ratio. The water content of the prepared media was determined as described previously in Section 3.4.1.1.

3.4.5.1.2 Formulation of crushed wheat grain media

Whole wheat grain (from GMB, Zimbabwe) was crushed using a mortar and pestle. To vary the crushed wheat grain formulations PDA (3 %; obtained from Oxoid, UK), distilled water and crushed wheat grain were added in different proportions to Erlenmeyer conical flasks as follows. Quantities of 1:2 (w/v)

(wheat grain: distilled water), 1:3, 1:5, 1:10 and 1:15 were placed in 500 ml Erlenmeyer conical flasks. To sets of 1:3 to 1:15 crushed wheat grain: distilled water were added PDA to result in crushed wheat grain-PDA formulations. The remaining sets had no PDA added to result in crushed wheat grain-distilled water formulations. The amount of PDA added was determined in relation to the amount of distilled water required for 3 % PDA media. The media were autoclaved at 121 °C for 20 min to obtain media with the respective crushed wheat grain: distilled water ratio. The water content of the prepared media was determined as described previously in Section 3.4.1.1.

3.4.5.2 Assessment of growth on wheat grain media

Three replicate Petri plates were inoculated for each experimental treatment, using 4-mm diameter plugs taken from the periphery of exponentially growing cultures of each strain growing on MEA. Plates of each medium were incubated at 25 °C in a non–inverted position for 30 days. Radial growth was measured daily as described previously (Mswaka and Magan, 1999), and these data were used to establish the relationship between treatment and growth rate. Homogeneity of variances was established by a preliminary F test (David, 1952) and Bartlett's test (Bliss, 1967). Analysis of Variance (ANOVA) was used to determine the significance of difference between means and Fisher's pairwise comparisons was used to extract which of the differences among the means were significant. Primordium development and mycelial characteristics were assessed for each treatment by eye and/ or low power microscopic examination.

3.4.6 Assessment of Feasibility of Modified Tyndallisation Protocols for Sterilising Wheat Grain

3.4.6.1 Effects of normal Tyndallisation on spawn production

Dry wheat grain (from GMB, Zimbabwe) that was washed (5 rinses in tap water) was used in all the treatments. The wide mouthed two litre capacity bottles used were washed with soap-water then rinsed three times using tap water before being dried in a drier at 80 °C for 5 minutes. Wheat grains pre-boiled for 10 minutes in tap water without draining were soaked overnight in two litre capacity bottles at ambient temperature (21 to 24 °C) monitored using a portable Hanna digital thermohygrometer model HI 8064. The following morning the grain was boiled in the same water for 10 minutes, and then bottled. A quarter of the grain was inoculated in triplicate in 500 ml capacity wide-mouthed bottles with *Pleurotus* species mycelia on 20-mm diameter agar plugs (three plugs per bottle), whilst another quarter was not inoculated. The mouth of each of the bottles was plugged with a 0.22 µm microporous filter plug obtained from Millipore, U.S.A The rest of the grain was incubated overnight at ambient temperature in the remaining water, prior to being boiled in the same water and bottled. Half of the grain was inoculated as described above whilst the other half was not inoculated. All the bottled wheat grain was incubated at 25 °C with daily monitoring for contamination.

3.4.6.2 Effects of modified Tyndallisation involving a pre-water-boiling stage on spawn production

In the second experiment wheat grain was added to tap water which was pre-boiled for 5, 20 or 30 minutes prior to a further boiling of 10 minutes, then drained into two litre bottles and incubated overnight at ambient temperatures. The following day the same grain was added to fresh tap water which was pre-boiled for 5,

20 or 30 minutes prior to boiling for 10 minutes, draining and bottling of the grain. The bottled grain was inoculated as described in Section 3.4.6.1. The bottles were immediately covered with aluminium foil and then transferred to the 25 °C incubator with daily monitoring for contamination. The remaining grain was incubated overnight, boiled for a third time, drained, bottled, inoculated and incubated at 25 °C as described in Section 3.4.6.1. Uninoculated bottled wheat grain controls were also incubated at 25 °C. Wheat grain was sterilized by autoclaving as described previously in Section 3.4.1.1. The grain was not inoculated as a control, and then incubated at 25 °C with daily monitoring for contamination.

3.4.6.3 Effects of modified Tyndallisation involving the use of disinfectant on spawn production

Dry wheat grain was washed in tap water for 5 times prior to being boiled for 5 or 10 minutes (different treatments) in tap water previously boiled for 2 minutes. The excess free water was drained and the grain was placed in covered containers previously washed in 0.0005 % (active ingredient) of a local disinfectant (Trade name, Jik) [active ingredient, Sodium hypochlorite 3.5 % m/v]. The grain was incubated overnight at ambient temperatures. The following morning the grain was boiled for 5 or 10 minutes in fresh tap water previously boiled for 2 minutes and placed in covered disinfected containers. The resultant grain was labeled bx2 (boiled twice). This process was repeated up to grain labeled bx6 (boiled six times). Water content determination as described in Section 3.4.1.1 was done for each grain treatment from bx1 to bx6. Grain (100 g) was placed in each of the narrow-mouthed disinfected bottles (500 ml capacity) for each of the grains labeled bx1 up to bx6. Half of the bottled grain was inoculated with the *Pleurotus* species mycelia on 20-mm diameter agar plugs whilst the remainder of the bottled grain was left not inoculated. The bottles were plugged using 0.22 µm microporous filter plugs obtained from

Millipore, U.S.A. All the bottles were incubated at ambient temperature with daily monitoring for contamination.

3.4.6.4 Assessment of the quality of Tyndallisation prepared spawn using banana substrate

Banana (*Musa sapientum*) fronds were chopped into small pieces less than 10 cm with a garden knife and pre-wetted overnight in a drum. The following morning, the banana substrate was boiled for one hour. *Pleurotus* species wheat grain spawn prepared by Tyndallisation of wheat grain with a pre-boiling stage (bx3, 30 min treatment, Section 3.4.6.2) and that produced by Tyndallisation with increased number of shorter boiling periods (bx4, 5 min treatment, Section 3.4.6.3) was mixed (5 percent spawning rate) into the cooled pasteurised banana substrate simultaneously with the filling of plastic bags (1000 µm thick, 310 mm diameter and 1300 mm in length) in a layered stratum. The spawning was done in triplicate bags for each *Pleurotus* strain. The mushroom spawn (wet weight) required for a 5 percent spawn rate was calculated using Formula 3 below:

mushroom spawn (wet mass) = spawn rate (decimal) x substrate (dry mass)

Formula 3

Holes measuring 3 mm in diameter were punched in the bottom and sides of the substrate bags for drainage and aeration. The substrate filled bags were hung in the mushroom-growing house using the column method (Stamets, 2000). The mushroom-growing house was constructed using glass and measured 2.35 m (height), 4.45 m (length) and 2.50 m (width) with a concrete floor. Black polythene plastic of 1 000 µm thickness was used to cover the mushroom-growing house to provide dark conditions. The substrate bags were incubated in the dark at ambient conditions with daily watering of the floor of the mushroom growing house. After three weeks larger

holes measuring 5 mm in diameter were punched to allow primordium development and basidiome production. When the substrate was colonised by the mushroom mycelium, the bags were exposed to a six hour period of normal sunlight by removal of the black polythene plastic used to cover the glass panes of the mushroom-growing house with normal air circulation during the six hours (windows were opened). Mature mushrooms were harvested by twisting off the whole cluster or single mushrooms.

3.5 OPTIMISATION OF CULTIVATION SUBSTRATE CONDITIONS

3.5.1 Substrate Mixtures – Growth Relationship

3.5.1.1 Cultivation substrate – growth relationship on a small-scale

The substrates comprised of: grass (*Agropyron* sp), banana fronds (*Musa sapientum*), wheat straw (*Triticum aestivum*, control) and sage (*Coleochloa setifera*), which were also used in combinations. Inoculum for the substrates was prepared by growing the *Pleurotus* species mycelia on malt extract agar (3 % w/v malt extract and 2 % w/v agar obtained from Oxoid, UK) at 25 °C. Whilst the mycelia were colonizing the malt extract agar media the substrates were prepared. Five grams of dry substrate was placed into 500 ml capacity wide – mouthed bottles. To prepare mixed substrates 2.5 g of each substrate were placed in the same bottle to give a ratio of 1:1 (w:w). For example, 2.5 g banana fronds plus 2.5 g grass. Water was added to immerse the substrate prior to soaking overnight. Each substrate and substrate combination were prepared in triplicate for each *Pleurotus* strain. The following morning all excess water was drained and the bottled substrates were autoclaved at 121 °C for 30 minutes. The bottled substrates were cooled overnight in a sterile inoculating room. The substrates were inoculated using five 10-mm diameter plugs taken from the periphery of exponentially growing cultures of each strain on MEA. Microporous

filter plugged-inoculated bottles were incubated in a 25 °C dark constant temperature room for 10 days, and then transferred to a standard mushroom growing house described in Section 3.4.6.4 where an Aquafog HU–SS700 humidifier and Aquafog HU–SV humidistat system were used to maintain humidity at 90 ± 1 %. A Carrier through wall air-conditioner model KCA121P was used to maintain the temperature at 25 ± 1 °C. From day 15 after inoculation the temperature was lowered to 21 ± 1 °C and artificial light was applied for a period of eight hours a day using 70 Watts light bulbs.

3.5.1.1.1 Water content and tissue analysis of substrates

Preweighed substrate samples were oven dried for two hours at 150 °C. Dry substrate oven-dried to remove the inherent water content was used in Formula 4. The percentage water of the autoclaved substrate was determined using Formula 4 below:

$$\text{Percentage (\%) water} = \frac{\text{autoclaved substrate (mass)} - \text{oven dried substrate (mass)}}{\text{autoclaved substrate (mass)}}$$

Formula 4

The macronutrients carbon (C), nitrogen (N), potassium (K) and Phosphorus (P) content were analysed using triplicate samples of each dry substrate (banana, grass, sage and wheat straw) as described in Section 3.4.3.

3.5.1.2 Cultivation substrate – growth relationship on a large-scale

Pleurotus spawn for inoculating the substrates was prepared as described in Section 3.4.1.1 using the conventional method. The substrates (grass, banana fronds, wheat straw and sage) were chopped into small pieces less than 10 cm in length with a garden knife and pre-wetted overnight in a 200 litre capacity drum. Triplicate samples of each dry substrate were used in water content determination as

described previously in Section 3.4.1.1 with dry substrate substituted for autoclaved substrate in Formula 4. The, substrates were boiled for one hour and triplicate samples of each pasteurised substrate were used for water content determination. The mass of the oven-dried wheat straw was used in Formula 4 with pasteurized substrate substituted for autoclaved substrate. Spawning was done at 5 percent rate into the cooled pasteurised substrates (two kg dry mass) in plastic bags (1000 µm thick, 310 mm diameter and 1300 mm length). Each strain was inoculated in triplicate bags. Holes measuring 3 mm in diameter were punched in the bottom and sides of the substrate bags for drainage and aeration. The bags were hung in the mushroom-growing house described in Section 3.4.6.4 using the column method. The substrate bags were incubated in the dark at 25 ± 1 °C maintained using a Carrier through wall air-conditioner model KCA121P and 90 ± 1 % relative humidity (RH) maintained using an Aquafog HU–SS700 humidifier and Aquafog HU–SV humidistat system with zero fresh air exchanges. After three weeks larger holes measuring 5 mm in diameter were punched to allow fruiting. When the substrate was completely colonised by the mushroom mycelium, the bags were exposed to a 12-hour photoperiod of 600 lux per day (Stamets, 2000) and four fresh air exchanges per hour were maintained in the mushroom-growing house using the cubic feet per minute rating of the air-conditioner (Stamets and Chilton, 1983). The air temperature was decreased to 22 ± 1 °C for basidiome initiation. Mature mushrooms were harvested by twisting off the whole cluster and cutting into individual mushrooms prior to weighing the fresh mass. The oyster mushroom (*Pleurotus* species) yield was determined over three flushes.

3.5.1.3 Additional data recorded

Formation of primordia and subsequent formation of basidiomes on the substrates were recorded. The substrate dry mass and fresh mass of the mushrooms were also recorded. These values were used to calculate the biological efficiencies of the *Pleurotus* species and strains using Formula 1 (Sánchez and Gonzaléz, 1996). The colour of the basidiome of each strain, the first harvest day and the period in-between harvests were also recorded.

3.6 GENETIC CHARACTERISATION

3.6.1 DNA Isolation, Quantification and Estimation of Purity

The *Pleurotus* species DNA was extracted using the Lee and Taylor (1990) method with certain modifications. The liquid growth medium was formulated by preparing malt extract agar (0.2 % w/v obtained from Oxoid, UK, with percentages 0.1 to 0.3 % ideal for growth of the *Pleurotus* mycelia) which resulted in the medium remaining liquid and thus did not solidify. The mycelia were grown in Nalgene® cryogenic vials of 5 ml capacity containing 2 ml of liquid medium (0.2 % malt extract agar, Oxoid) which were incubated in a horizontal position to present a larger surface area for the *Pleurotus* mycelia to grow on. The liquid medium (0.2 % malt extract agar, Oxoid) in the 5 ml cryogenic vials was drained using a sterile one ml micro-pipette, prior to washing the mycelia twice with sterile deionised water. The mycelia were then frozen in liquid nitrogen prior to being ground using a mortar to obtain a fine powder. Mycelia (80 mg) were transferred into labelled 1.5 ml micro-centrifuge tubes prior to addition of lysis buffer (400 µl) which contained 50 mM Tris-HCl (pH 7.2 at 25 °C), 50 mM EDTA, 3 % SDS and 1 % mercaptoethanol. The mixture was vortexed briefly using a VWR model G560 vortexer and incubated at 65 °C for one hour. Phenol: Chloroform (1:1) (400 µl) was added and the mixture vortexed briefly

prior to centrifugation (14 000 x g for 15 minutes) at room temperature using an Eppendorf model 5418 microcentrifuge. The upper aqueous phase (350 µl) containing the DNA was removed to a new tube and to this 3M sodium acetate (NaOAc) (10 µl, 0.03 vol) was added followed by isopropanol (175 µl, 0.5 vol, ice-cold). The solution was mixed gently by inversion and then centrifuged (14 000 x g for 15 minutes) at room temperature. The supernatant was discarded and the pellet was rinsed once with ice-cold ethanol (200 µl, 70 % v/v) and drained over a paper towel. The pellet was air dried, prior to re-suspension in sterile deionised water (50 µl) [modification to Lee and Taylor (1990) method] and then stored at 4 °C (working stock) and – 20 °C (backup stock).

For the spectrophotometric detemination of DNA concentration, stock DNA (50 µl) was diluted in TE buffer (750 µl) and absorbency readings were taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm was used to calculate the concentration of nucleic acid in the sample, using the following equation: 1-0D_{260} corresponds to approximately 50 µg/ml double stranded DNA (dsDNA) (Weising *et al.*, 1995). The purity of the DNA was estimated by the A_{260} / A_{280} ratio (pure preparations have approximately 1.8 values) (Weising *et al.*, 1995).

Electrophoresis was used to determine the degree of DNA degradation on a 0.8 % (w/v) agarose minigel using 1 x Tris Borate EDTA (TBE) electrophoresis buffer (200 ml). Electrophoresis was carried out at 5 V/cm for 2 hours. After the run was completed the gel was removed from the electrophoresis apparatus and stained for 30 minutes in a solution of ethidium bromide (0.5 µg ml^{-1}) followed by destaining for 20 minutes in distilled water. The gel was visualised under ultra-violet light using protective eye glasses.

3.6.2 PCR Optimisation and Amplification

Prevention of PCR contamination was done as previously described (Kwok and Higuchi, 1989; Dieffenbach and Dveksler, 2003). The PCR was optimized as described previously (Scheinert, Behrens and Kable, 1995) with optimization of magnesium concentration adequate for a successful PCR. The primer sequences used to amplify the internal transcribed spacer region of rDNA were as follows: ITS-1 (forward primer), 5'-TCCGTAGGTGAACCTGCGG-3' and ITS-4 (reverse primer), 5'-TCCTCCGCTTATTGATATGC-3' (White *et al*., 1990). Each PCR reaction mixture had a total volume of 25 µl and contained genomic DNA (1 µl), 1.5 mM magnesium chloride ($MgCl_2$) (1.5 µl of a 25 mM stock $MgCl_2$ solution), 0.01 % (v/v) Tween 20, 75 mM Tris-HCl (pH 8.8 at 25 °C), 20 mM ammonium sulphate [$(NH_4)_2SO_4$], 0.2 mM dNTP, 0.4 µM of the oligonucleotide primers (ITS-1 and ITS-4), 0.25 units of DNA polymerase (*Taq*) and the remainder PCR water (H_2O) (1 µl). All reagents were obtained from Roche Applied Science Division in Germany. The amplification was performed using the following conditions: initial activation of *Taq* DNA polymerase (94 °C for 4 min) and 40 cycles of 94 °C for 30 seconds, 56 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min, on a Perkin – Elmer Applied Biosystems GeneAmp PCR system 2400.

3.6.3 PCR Product Sequencing and Analysis

Amplified PCR products were checked on agarose (1.2 %) gels, and purified for sequencing using microfiltration (Ultrafree MC filters, Millipore Corp.). Approximately 50 ng of purified PCR product was sequenced using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, CA, U.S.A.). Primers used in the PCR were used in the sequencing reactions to sequence both five-prime (5′) termini. Sequencing reactions employed

fluorescent dye terminator chemistry and were run on PE Biosystems PRISM 310 Genetic Analyser according to the manufacturer's instructions.

Sequences were optimally aligned and edited using DNAMAN Sequence Analysis Software, (version 4.0, Lynnon BioSoft, Canada), CLC Workbench Sequence Analysis Software (version 3.2, CLC Bio A/S, Denmark) and ChromasPro Sequence Analysis Software (version 1.41, Technelysium Private Limited). The resultant alignments were verified and edited by eye. Homology and phylogenetic trees were produced using UPGMA (unweighted pair – group method using arithmetic averages) (Sneath and Sokal, 1973) and the neighbour-joining method (Saitou and Nei, 1987) respectively using DNAMAN Sequence Analysis Software, (version 4.0, Lynnon BioSoft, Canada). Branch robustness was evaluated with 1000 bootstrap (Felsenstein, 1985; Hillis and Bull, 1993; Song *et al*., 2001) replicate searches. The sequences were converted to FASTA format using ChromasPro Sequence Analysis Software (version 1.41, Technelysium Private Limited). Basic local alignment search tool (BLAST version 2.0) analysis of the sequences (Altschul *et al*., 1990; Altschul *et al*., 1997) was done using the following databases GenBank at the Los Alamos National Laboratory, EMBL Data Library at the European Molecular Biology Laboratory in Heidelberg, DNA Data Bank of Japan (DDBJ) and Protein Data Bank (PDB).

CHAPTER 4

4.0 RESULTS

4.1 FUNGAL SPECIES AND STRAINS MAINTENANCE

All the *Pleurotus* species and strains listed in Appendix C exhibited good growth on the culture media (MEA) used to maintain the strains.

4.2 OPTIMISATION OF ENVIRONMENTAL CONDITIONS

4.2.1 The Effects of Osmotic Potential on Growth

Strains of *Pleurotus* species were grown over a range of osmotic potentials, and their growth rates determined as an environmentally-relevant indicator of phenotypic variability (Figure 7A-C). Whereas the conditions required for optimal growth varied significantly between strains ($P < 0.05$), their growth windows (range of treatment levels that resulted in growth) were similar in relation to osmotic potential, i.e. – 0.5 to – 3.5 MPa (Figure 7A-C). All the strains showed a general decrease in the growth rate with an increase in water stress. No measurable growth occurred at – 4.5 MPa for all the strains (Figure 7A-C). Strains 28511 (*P.ostreatus*), 28683, 31674, 38047 (*P. floridanus*), 38089 and 38091 covered the mycelial agar inoculum disc at – 4.5 MPa. However no measurable extension of radii occurred. The most rapid growth (radial extension rates of $\geq$ 5 mm day^{-1}) was obtained from strains BCCM/MUCL 28511 (*P. ostreatus*), 28685, 31017, 31613, 38047 (*P. floridanus*), 38076, 38078, 38091 and 38092 (strains without scientific names are *Pleurotus sajor-caju* strains, underlined strains are shown in Figure 7A-C). Forty percent of the *Pleurotus* species strains grew optimally at the lowest water potential tested (–0.5 MPa; Figure 7A-C) and thirty-three percent of the strains tested showed a degree of xerotolerance by growing optimally at – 1.5 MPa (BCCM/MUCL 28683, 31017, 31674, 31613 and 38091 [all *Pleurotus sajor*-caju], underlined strains are shown in Figure 7A-C). For strains

exhibiting no growth at – 4.5 MPa that had the agar inoculum plug transferred to the control media, all the strains started growing.

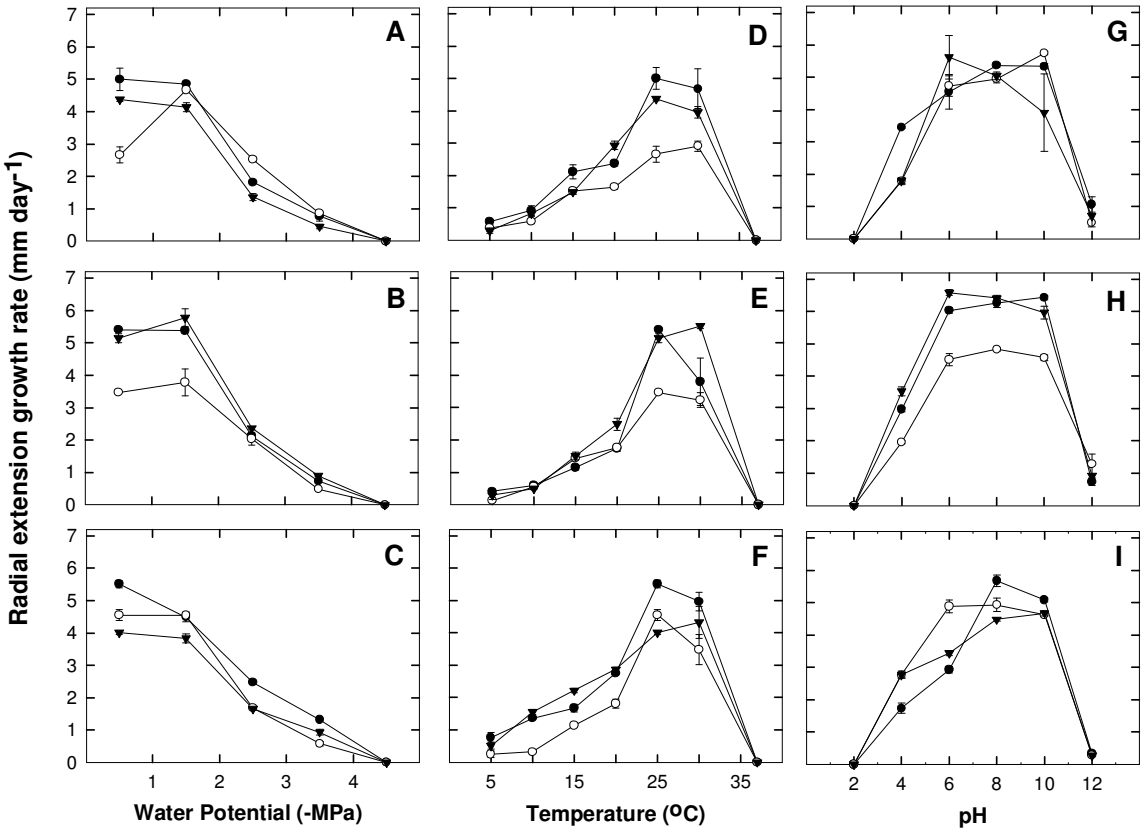


Figure 7: Growth responses of nine representative strains of *Pleurotus* spp. to different water potentials (osmotic) (A-C), temperatures (D-F) and pH (G-I). Strains BCCM 28511 (*P. ostreatus*) (●), 28683 (*P. sajor-caju*) (○) and 28684 (*P. citrinopileatus*) (▼) are shown in graphs A, D and G; strains BCCM 28685 (*P. sajor-caju*) (●), 29757 (*P. sajor-caju*) (○) and 31017 (*P. sajor-caju*) (▼) are shown in graphs B, E and H; and strains BCCM 38047 (*P. floridanus*) (●), 38071 (*P. sajor-caju*) (○) and 38089 (*P. sajor-caju*) (▼) are shown in graphs C, F and I. Bars represent the standard error of the mean (S. E. M.).

4.2.2 The Effects of Temperature on Growth

Strains of *Pleurotus* species were grown over a range of temperatures, and their growth rates determined as an environmentally-relevant indicator of phenotypic variability (Figure 7D-F). Generally growth was bell-shaped and varied significantly (Figure 7D-F). The most rapid growth (radial extension rates of ≥ 5 mm day^{-1}) was

obtained from strains BCCM/MUCL 28511 (*P. ostreatus*), 28685, 31017, 31613, 38047 (*P. floridanus*), 38076, 38078, 38091 and 38092 (strains without scientific names are *Pleurotus sajor-caju* strains, underlined strains are shown in Figure 7D-F) and the optimum temperature for growth was 25 °C for eighty percent of the strains or 30 °C for twenty percent of the strains (Figure 7D-F). The minimum growth temperature that resulted in radii extension for most strains was 10 °C. No growth occurred at 37 °C for all the strains (Figure 7D-F).

4.2.3 The Effects of pH on Growth

Strains of *Pleurotus* species were grown over a range of pH, and their growth rates determined as an environmentally-relevant indicator of phenotypic variability (Figure 7G-I). Generally strains grew only above pH 2 and below pH 12, but growth of several other strains at pH 12 occurred (Figure 7G-I). The *Pleurotus* species and strains had a wide pH range (pH 6 to pH 10) (Figure 7G-I) with the optimum pH being 6, 8, or 10 for twenty, forty-seven and thirty-three percent of the strains respectively depending on the species and strain. Radii extension rates were in the range 0 mm day^{-1} to 2.69 mm day^{-1}. The most rapid growth (radial extension rates of $\geq$ 5 mm day^{-1}) was obtained from strains BCCM/MUCL 28511 (*P. ostreatus*), 28683, 28684 (*P. citrinopileatus*), 28685, 29757, 31017, 31613, 38047 (*P. floridanus*), 38076, 38078, 38091 and 38092 (strains without scientific names are *Pleurotus sajor-caju* strains, underlined strains are shown in Figure 7G-I).

On the basis of the variation in optimal growth of the strains due to environmental parameters, optimal conditions and ranges were compiled to enable selection of strains with desirable optimal conditions for specific purposes as shown in Table 1. The results revealed that the most common optimum conditions for growth

of *Pleurotus* species are 25 °C, pH 8 and – 0.5 MPa for temperature, pH and water potential environmental parameters.

Table 1: Optimal conditions and ranges for the *Pleurotus* species and strains in the study

Strain	Species	Temp (°C)	pH	Ψ (–MPa)	Temp range (°C)	pH range	Ψ range (–MPa)
28511	*P. ostreatus*	25	8	0.5	25	8-10	0.5
28683	*P. sajor-caju*	30[a]	10	1.5[a]	–	10	–
28684	*P. citrinopileatus*	25[a]	8	0.5[a]	–	8	–
28685	*P. sajor-caju*	25	10	1.5	25	6-10	0.5-1.5
29757	*P. sajor-caju*	25[a]	8	1.5[a]	–	8	–
31017	*P. sajor-caju*	30	6	1.5	25-30	6-10	0.5-1.5
31613	*P. sajor-caju*	25*	6*	0.5*	25	6-10	0.5-1.5
31674	*P. sajor-caju*	25[a]	8[a]	0.5[a]	–	–	–
38047	*P. floridanus*	25	8	0.5	25	8-10	0.5
38071	*P. sajor-caju*	25[a]	8[a]	1.5[a]	–	–	–
Strain	Species	Temp (°C)	pH	Ψ (–MPa)	Temp range (°C)	pH range	Ψ range (–MPa)
38076	*P. sajor-caju*	25	10	0.5	25	6-10	0.5
38078	*P. sajor-caju*	25	10	1.5	25	6-10	0.5-1.5
38089	*P. sajor-caju*	30[a]	10[a]	0.5[a]	–	–	–
38091	*P. sajor-caju*	25	8	0.5	25	8-10	0.5-1.5
38092	*P. sajor-caju*	25	6	0.5	25	6 and 10	–

* Highest growth rate obtained at the respective stress parameter of all the strains in the study
a Suboptimal growth (radial extension rates of < 5 mm day^{-1}) obtained. Optimal growth defined as radial extension rates of ≥ 5 mm day^{-1}

The marked variation in growth responses to these stress parameters led to the question of how environmentally-sensitive strains are in the formation of fruiting bodies.

4.2.4 Production of Primordia and Mycelial Characteristics

The initiation of primordia as determined microscopically over a 30 day period under the conditions shown in Figure 7 showed that strains have greater variability in primordium development than in their growth responses (Table 2).

Table 2: Production of primordia (+) by different *Pleurotus* species strains over a range of growth conditions[a]

Strain[b]	Osmotic potential (MPa)				pH				Temperature (°C)			
	–0.5	–1.5	–2.5	–3.5	4	6	8	10	15	20	25	30
28511	+	−	−	−	−	−	−	−	−	−	+	−
28683	+	−	−	−	+	−	−	−	−	+	+	−
28685	−	−	+	−	+	+	+	−	+	+	+	−
29757	+	−	−	−	+	+	+	−	+	+	+	−
31017	+	+	+	−	+	+	−	−	+	+	+	−
31613	+	+	+	−	+	−	−	−	+	+	+	−
38076	−	−	−	−	−	−	−	−	−	−	−	−
38091	+	−	+	−	−	−	−	−	−	−	+	−

[a]no primordia were produced at – 3.5 MPa or temperatures below 15 ºC until after the 30 d assessment period, or at pH 2 and 12, or temperatures of 30 and 37 ºC even after 90 d (data not shown).
[b]BCCM/MUCL strain number.

The most prolific producers of primordia were strains BCCM/MUCL 28685, 29757, 31017 and 31613 [all *Pleurotus sajor-caju* strains], each of which fruited under seven to eight of the treatment conditions. For example, strain 31017 produced primordia at three different temperatures (15, 20 and 25 °C), three osmotic potentials (– 0.5, – 1.5 and – 2.5 MPa) and under two pH regimes (4 and 6) (Table 2). By contrast, strains BCCM/MUCL 28684 (*P. citrinopileatus*), 38047 (*P. floridanus*), 38076 and 38092 did not yield primordia under any conditions during the assessment period (data not shown). Generally, primordia were produced at low pH (4 to 6), at suboptimal growth temperatures (≤ 25 °C), and at moderate osmotic potentials (– 0.5 to – 3.5 MPa). On the basis of the variation in growth and fruiting behaviour of the strains, a Venn diagram (Figure 8) was compiled to enable selection of strains with desirable phenotypic characteristics for specific purposes.

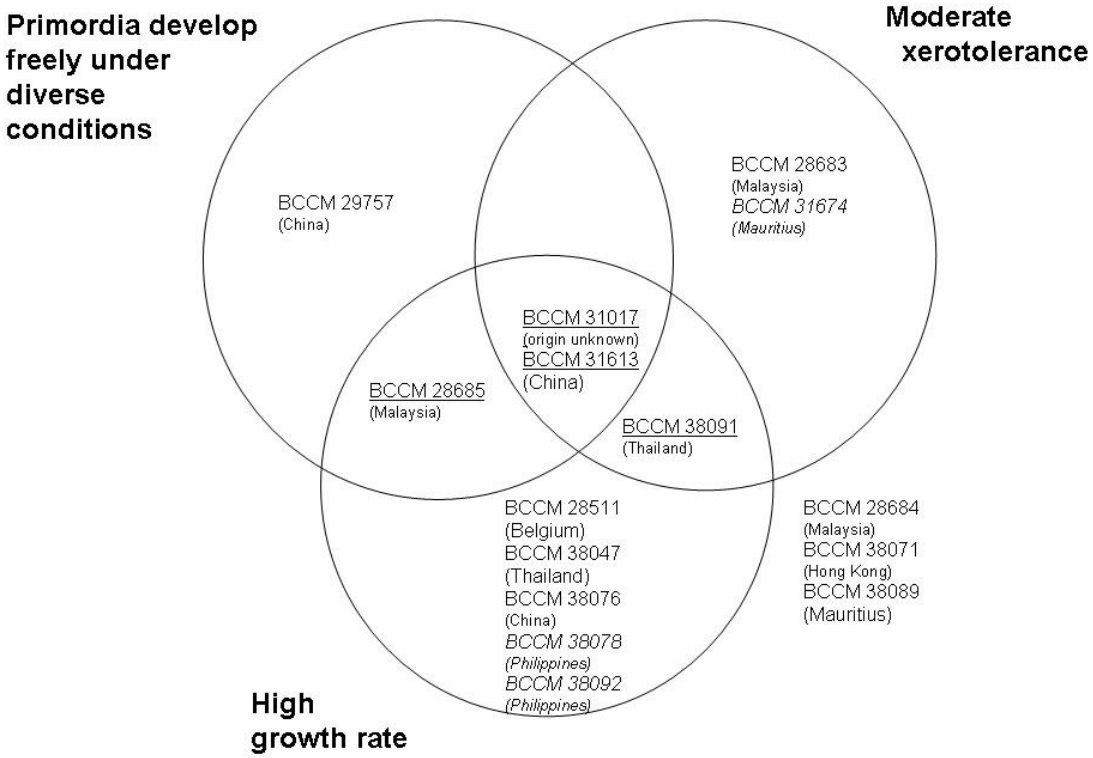


Figure 8: Venn diagram to illustrate which *Pleurotus* strains will grow and produce primordia optimally over a range of environmental conditions. Data for growth rates and primordium development were obtained from Figure 7 and Table 2, respectively, except for strains written in italics (data not shown). Strains that develop primordia readily did so under seven or more of the 12 treatments listed in Table 2; those that are moderately xerophilic grew optimally at increased water potential (-1.5 MPa; see Figure 7A-C); and those with the highest growth rate were capable of radial extension rates of ≥ 5.0 mm day^{-1} (Figure 7).

Six classes of mycelium were observed: cottony (predominant), cottony zonate, floccose, rhizormorphic, quasi-rhizormorphic and appressed (These were as described in Appendix D). When two or more mycelium types occurred, they were separated spatially with the floccose type occurring around the agar inoculum block (the old mycelium). Growth at different temperatures mainly resulted in cottony and cottony zonate mycelia. Increase in incubation temperature resulted in floccose mycelium occurring at 25 °C and 30 °C. Strains, 38071 and 38092 had appressed mycelium at 25 °C and 30 °C. Decrease in water potential (– 1.5 MPa to – 3.5 MPa)

resulted in a shift from mainly cottony zonate mycelium to cottony mycelium. Mycelium plugs transferred from – 4.5 MPa media to – 0.5 MPa media had quasi-rhizormorphic mycelium developing which was not present when mycelium discs were grown on – 0.5 MPa MEA only at 25 °C. Mycelium characteristics were mainly quasi rhizormorphic over the range of pH used in this study. Strain 28511 (*P. ostreatus*) generally produced microdroplets readily. Microdroplets were produced at – 1.5 MPa by strain 28511 (*P. Ostreatus*). Primordia and microdroplets occurred when the mycelium agar inoculum disc was transferred from – 4.5 MPa to – 0.5 MPa media.

4.2.5 Phenotypic Characteristics

Strains of *Pleurotus* species were compared on the basis of three phenotypic traits likely to influence their potential biotechnological/ecological performance, and the inherent variability in their stress responses. The traits selected were an ability to develop primordia readily over diverse environmental conditions, a high growth rate, and a degree of xerotolerance (Figure 8). Out of the 15 strains included in the study, only four (underlined) exhibited two of these three traits, and only two (BCCM/MUCL 31017 and 31613) possessed all three (Figure 8). Growth rates of *Pleurotus* species grown over a range of osmotic potentials, temperatures and pH were analysed using multivariate cluster analysis as shown in Figure 9. Strains 28511 (*P. ostreatus*) and 38047 (*P. floridanus*) separated from the other strains (Figure 9). Two clusters were produced with one cluster comprising of strains 28683, 28684 (*P. citrinopileatus*), 29757, 38071, 31674, 38089 and 38092. The other cluster comprised of most of the high growth rate strains 28685, 38078, 38076, 31613, 31017 and 38091.

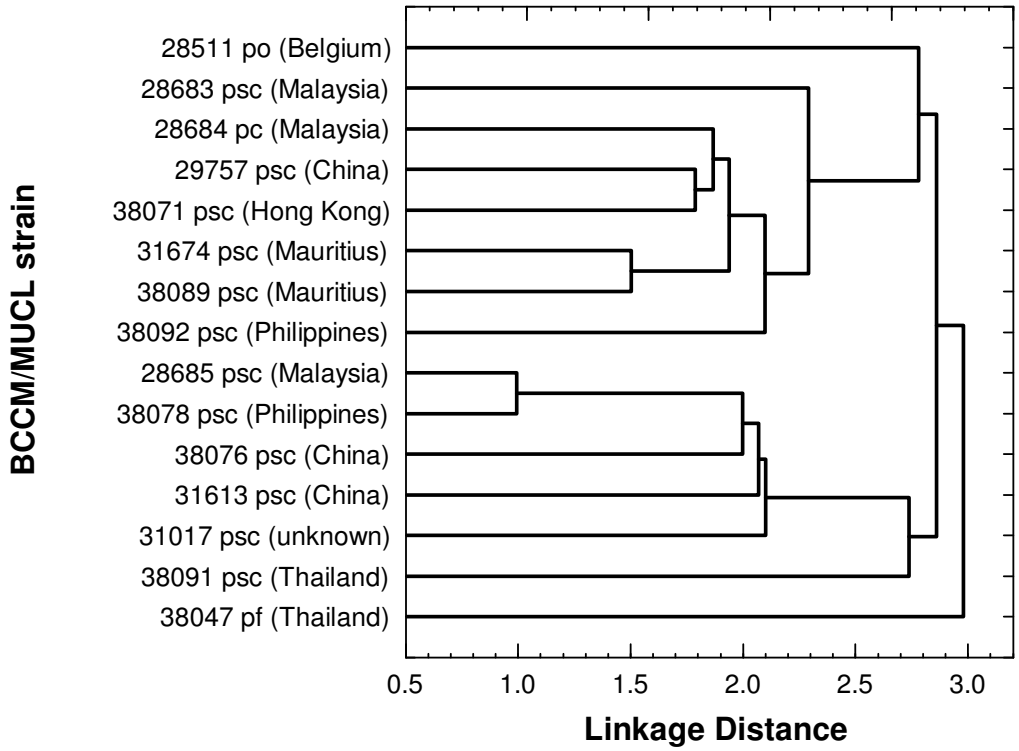


Figure 9: Cluster analysis of growth data over the stress parameters (water potential, pH and temperature) from 15 strains of *Pleurotus* species.

4.3 OPTIMISATION OF MICRO ENVIRONMENTAL CONDITIONS CHANGES

4.3.1 The Effects of Micro-Concentrations of Polyethylene Glycol on Growth

Strains were grown over a range of micro concentrations of PEG, and their growth rates determined for the *Pleurotus* strains as an investigation of micro-environmental adjustments on phenotypic variability (Figure 10). The micro-concentration of PEG required for optimal growth of the *Pleurotus* strains varied significantly between strains ($P < 0.05$). Strains exhibiting optimal growth ($\geq$ 5 mm day^{-1}) increased from 0 to 80 percent at 20 °C from 0.10 g l^{-1} to 0.90 g l^{-1} concentrations of PEG 6000. All the strains exhibited optimal growth at 0.65 g l^{-1} (25 °C and 30 °C), at 0.75 g l^{-1} (25 °C) and at 0.90 g l^{-1} (30 °C) concentrations of PEG

6000. Growth rates increased with an elevation in temperature from 20 °C to 25 °C or 30 °C from maximum growth rate at 20 °C by strain 31613 of 6.32 mm day^{-1} to 10.66 mm day^{-1} exhibited by strain 38078 at 30 °C (Figure 10A-F).

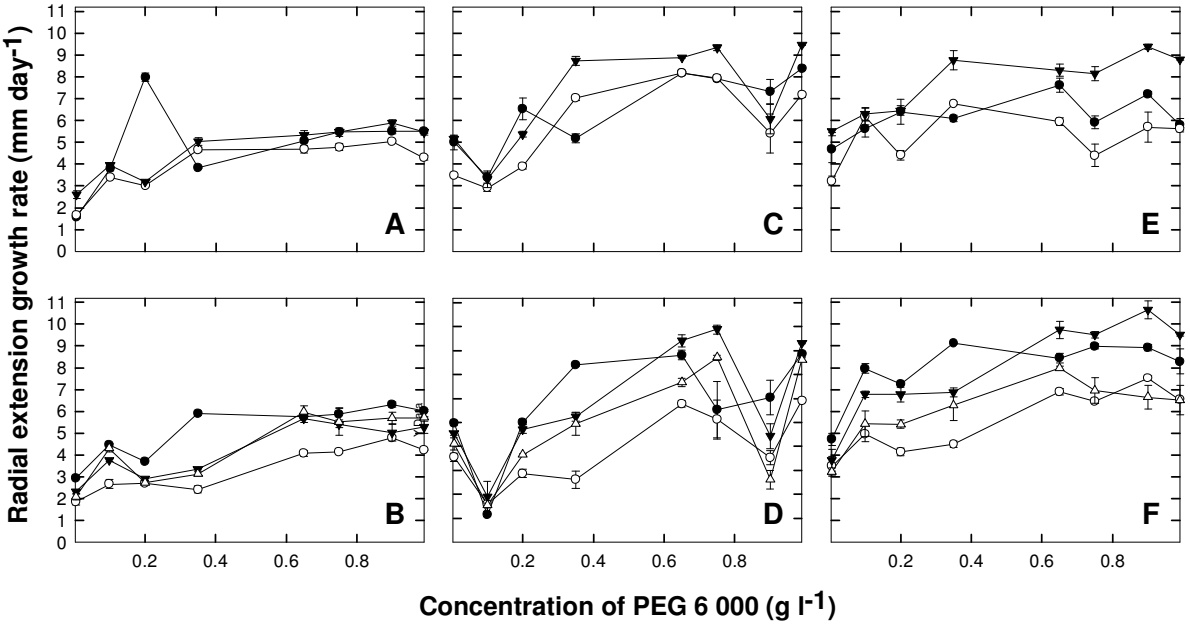


Figure 10: Growth responses of seven representative strains of *Pleurotus* species to a range of micro concentrations of PEG 6000 at different temperatures, 20 °C (A-B), 25 °C (C-D) and 30 °C (E-F). Strains BCCM 28511 (*P. ostreatus*) (●), 29757 (*P. sajor-caju*) (○) and 31017 (*P. sajor-caju*) (▼) are shown in graphs A, C and E.; strains BCCM 31613 (*P. sajor-caju*) (●), 38071 (*P. sajor-caju*) (○), 38078 (*P. sajor-caju*) (▼) and 38092 (*P. sajor-caju*) (Δ) are shown in graphs B, D and F. Bars represent S. E. M.

The most rapid growth (radial extension rates ≥ 5 mm day^{-1}) was obtained from all BCCM/MUCL strains at 20, 25 and 30 °C, apart from strains 28684, 38071 and 38076 (underlined strain is shown in Figure 10B) at 20 °C, and the optimum micro-concentrations of PEG 6000 for the majority of strains were 0.90 g l^{-1} (20 °C) and 0.65 g l^{-1} (25 and 30 °C). Rapid growth (radial extension rates of > 9 mm day^{-1}) was obtained, for example strain BCCM/MUCL 31017 had radial extension rate of 9.47 mm day^{-1} at 25 °C (Figure 10C).

4.3.2 The Effects of Micro-Concentrations of KCl and NaCl on Growth

Strains were grown over a range of micro concentrations of KCl and NaCl, and their growth rates determined for the *Pleurotus* species as an investigation of micro-environmental adjustments on phenotypic variability (Figure 11).

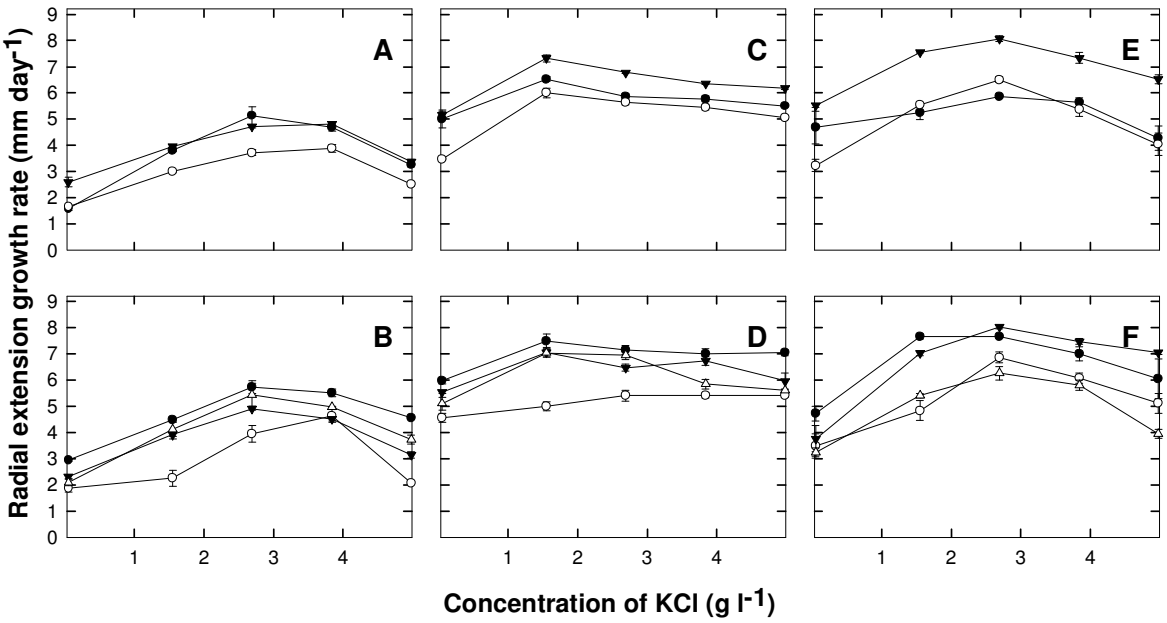


Figure 11: Growth responses of seven representative strains of *Pleurotus* species to a range of micro-concentrations of KCl and NaCl at different temperatures, 20 °C (A-B), 25 °C (C-D) and 30 °C (E-F). The concentrations of KCl were used to plot the graphs since a constant ratio of KCl: NaCl was used. Strains BCCM 28511 (*P. ostreatus*) (●), 29757 (*P. sajor-caju*) (○) and 31017 (*P. sajor-caju*) (▼) are shown in graphs A, C and E.; strains BCCM 31613 (*P. sajor-caju*) (●), 38071 (*P. sajor-caju*) (○), 38078 (*P. sajor-caju*) (▼) and 38092 (*P. sajor-caju*) (Δ) are shown in graphs B, D and F. Bars represent S. E. M.

Whereas the conditions required for optimal growth varied significantly between strains ($P<0.05$), their growth windows were similar in relation to micro-concentrations of KCl and NaCl; i.e. 0 to 5 KCl g l^{-1} (Figure 11A-F). Optimal growth was exhibited by 20 percent of the strains at 20 °C at a concentration of 2.70 KCl g l^{-1} whilst all the strains grew optimally at temperatures of 25 °C (1.50 KCl g l^{-1}) and 30 °C (2.70 KCl g l^{-1}) (Figure 11C-F). Optimal growth by greater than 90 percent of the

strains occurred at 1.50, 2.70 and 3.85 KCl g l^{-1} (Figure 11C-F). An increase in temperature resulted in growth rates increasing from 20 °C (Figure 11A-B) to higher growth rates at 25 °C and 30 °C (Figure 11C-F). The most rapid growth (radial extension rates ≥ 5 mm day^{-1}) was obtained from BCCM/MUCL 31613 and 38091 (underlined strain is shown in Figure 11A) at 20 °C and from all fifteen strains at 25 °C and 30 °C. The optimum micro concentration for all strains was 2.70 g l^{-1} KCl: 1.08 g l^{-1} NaCl or 3.85 g l^{-1} KCl: 1.54 g l^{-1} NaCl at 20 °C and 1.50 g l^{-1} KCl: 0.60 g l^{-1} NaCl or 2.70 g l^{-1} KCl: 1.08 g l^{-1} NaCl for both 25 °C and 30 °C.

4.3.3 Production of Primordia and Mycelial Characteristics

Determination of primordia as determined microscopically over a 30 day period under the conditions shown in Figures 10 and 11, showed that strains that produced primordia went on to produce basidiomes. Primordia production by the BCCM/MUCL strains occurred at 3.85 g l^{-1} KCl: 1.54 g l^{-1} NaCl (20 °C, 47 percent of the strains) and 5.00 g l^{-1} KCl: 2.00 g l^{-1} NaCl (25 °C, 20 percent of the strains and 30 °C, 47 percent of the strains) for the micro-concentrations of potassium chloride and sodium chloride; and 0.65 g l^{-1} (20 °C, 60 percent of the strains), 0.10 g l^{-1} (25 °C, 33 percent of the strains) and 0.75 g l^{-1} (30 °C, 33 percent of the strains) (data not shown). Mycelium characteristics of the *Pleurotus* strains were generally quasi-rhizormorphic or cottony mycelium as described in Appendix D.

4.4 OPTIMISATION OF SPAWN PRODUCTION

4.4.1 The Effect of Spawn Production on Wheat Grain Water Content

4.4.1.1 The conventional method (utilising insoluble calcium salts)

Pleurotus strains were grown on sterilized wheat grain produced with the conventional method and the water content of the grain was determined prior to

inoculation as an indicator for suitability for spawn production (Figure 12). The water content of the wheat grain was increased by the processes of soaking and boiling.

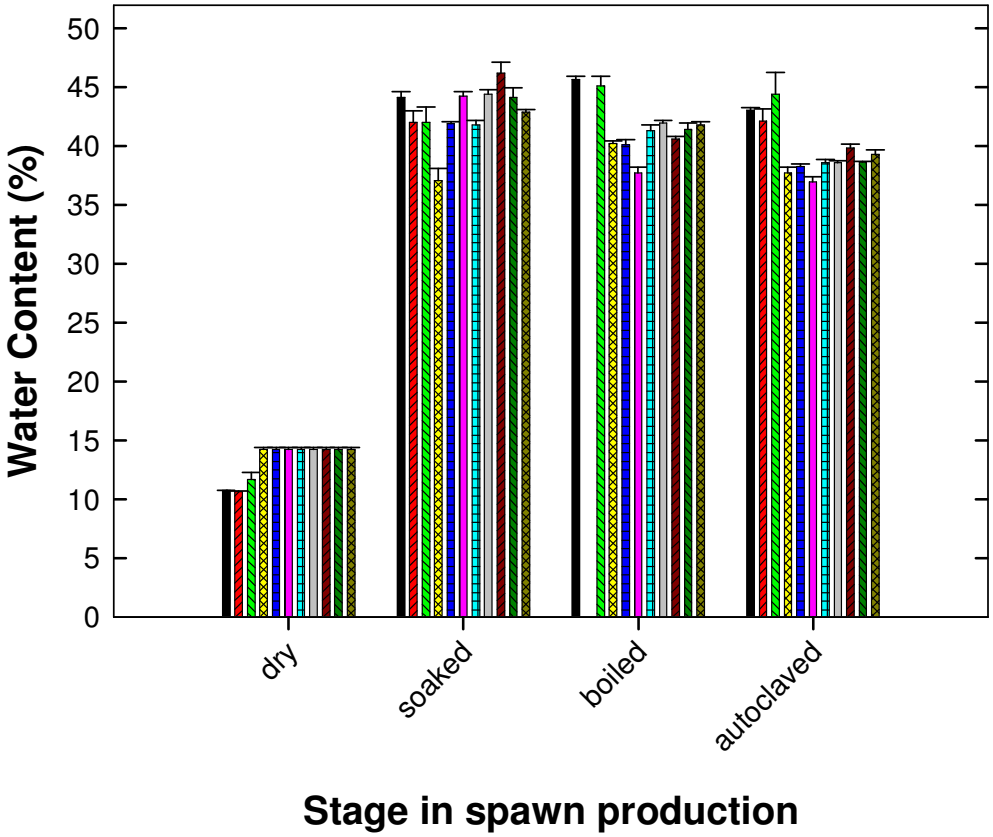


Figure 12: Water content of wheat grain produced by the conventional ( ) and unconventional ( ) methods, sorghum grain produced by the conventional method ( ) and water formulae: F1 ( ), F2 ( ), F3 ( ), F4 ( ), F5 ( ), F6 ( ), F7 ( ) and F8 ( ). Bars represent standard error of the mean (S. E. M.).

Autoclaving the grain resulted in the water content decreasing as shown in Figure 12 and the final water content deviated significantly from the desired 50 percent water content. The water content of the wheat grain varied significantly at each stage of the wheat sterilisation process (Figure 12). Sterilised sorghum grain had the highest water content of 44.4 percent.

4.4.1.2 The unconventional method (omitting the boiling stage and insoluble calcium salts)

Pleurotus strains were grown on sterilized wheat grain produced with an unconventional method and the water content of the grain was determined as an indicator for suitability for spawn production (Figure 12). The water content of the boiled and autoclaved wheat grain did not vary significantly (Figure 12). The water content of the sterilised wheat grain produced with the unconventional method did not vary significantly to the water content of sterilised wheat grain produced with the conventional method (Figure 12). The water content of the sterilised wheat grain deviated significantly from the desired 50 percent water content.

4.4.2 Application of Water Content Formulae in Spawn Production

The water content of the various sterilized wheat grain varied significantly between grain produced by the water content formulae F1 to F8 (Figure 12). All the wheat grain produced using the different water content formulae deviated from the 50 percent desired moisture content (Figure 12). The water content of the wheat grain peaked after soaking, thereafter decreasing by boiling of the grain and autoclaving the grain (Figure 12). The use of water content formulae F1 to F8 in spawn production resulted in sterilised wheat grain with significantly reduced water content in comparison with the water content of sterilised wheat grain produced by the conventional and unconventional methods (Figure 12).

Wheat grain seeds at the upper surface germinated during spawn production using formulae F1 and F2 during the soaking stage. Fewer seeds germinated during the soaking stage using formulae F3 and F4, whilst no seeds germinated during the soaking stage using formulae F5 to F8. All the available free water was absorbed by the wheat grain for formula F1, whilst the rest of the $\mathbf{V_{H2O}}$ formulae had excess water.

The excess water was least for formula F2 and the highest amount of excess water occurred for formula F8.

The most rapid growth of 7.06 mm day^{-1} radial extension rate on a single layer of wheat grain in Petri plates was obtained from strain 38091. The growth rates ranged from 4.61 to 7.06 mm day^{-1}. The *Pleurotus* strains mycelia took three weeks to fully colonise the wheat grain in the bottles. The density of the mycelia varied with the formulae F1 to F8. Sparse mycelia occurred for formulae F1 to F4 at the beginning of incubation. With further incubation the mycelia became dense for formulae F1 and F2. Dense mycelia occurred throughout the incubation period for formulae F5 to F8 with F7 and F8 having very dense mycelia. A good quality spawn characterised as sterilised grain covered with rhizomorphic mycelia or sparse cottony mycelia was obtained for the conventional, unconventional and formulae F5 to F8. The unconventional method relatively reduced the time taken by the *Pleurotus* species (*P. ostreatus*, *P. citrinopileatus*, *P. sajor-caju* and *P. floridanus*) to colonise the wheat grain in comparison with the conventional method by a range of five days (strain 38047 *P. floridanus*) to fourteen days (strain 28511 *P. ostreatus*) (data not shown).

4.4.3 Nutrient Changes of Wheat Grain during Spawn Production

Macronutrients and micronutrients levels in wheat grain produced by the unconventional method were determined as an investigation into the nutrient changes that occur during the stages of spawn production (Figure 13). The nutrient content of the wheat grain varied significantly (Figure 13). The carbon, nitrogen, potassium and sodium levels decreased from dry grain to sterilized (autoclaved) grain, whilst the calcium levels increased (Figure 13).

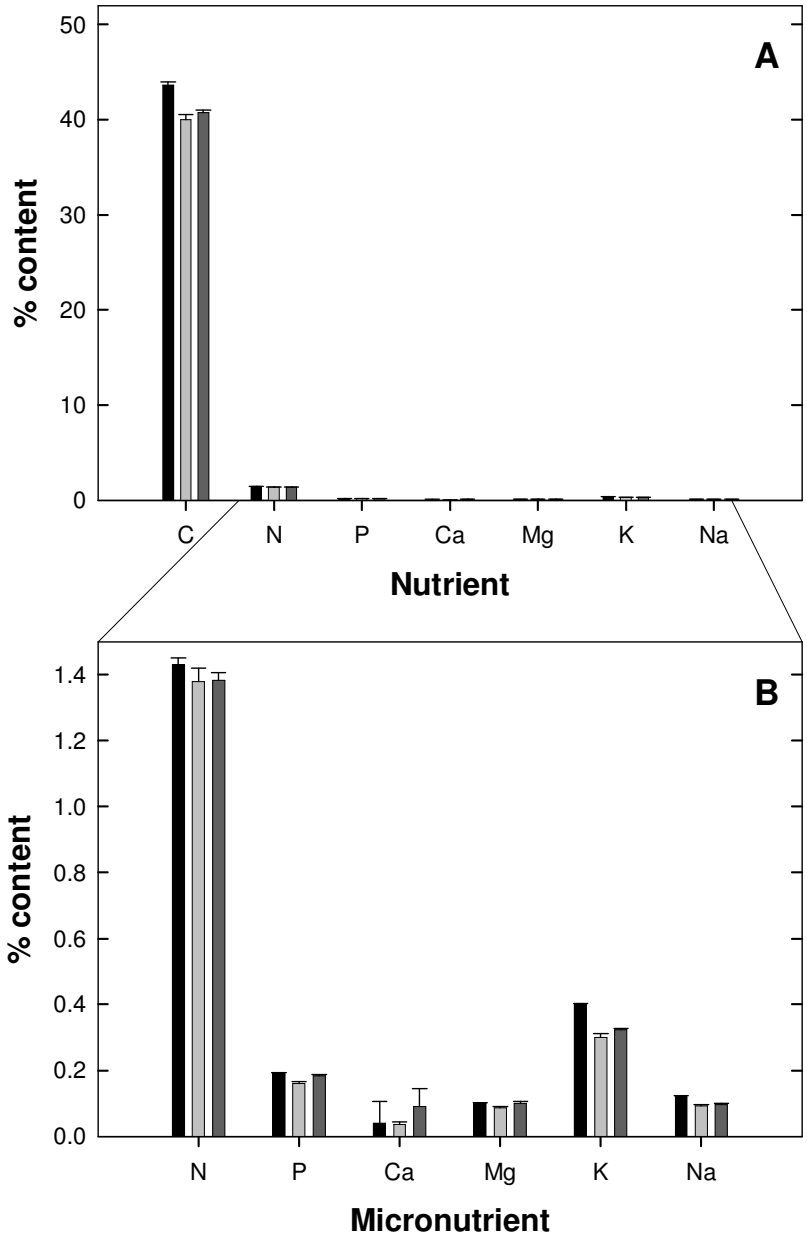


Figure 13: Changes in macronutrients carbon (C) and nitrogen (N) (graph A), and micronutrients; phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) (graph B) of wheat grain during the spawn production process [dry grain ( ), soaked grain ( ) and autoclaved grain ( )] using the unconventional method. Bars represent S. E. M.

The phosphorus and magnesium levels initially decreased for soaked grain but increased to levels that were similar to dry grain for autoclaved grain (Figure 13).

4.4.4 Water Content of Alternative Substrates and Growth Responses of Strains

Strains were grown on a range of sterilized alternative substrates. The substrates were produced using various soaking and boiling periods as shown in Figure 14 and the water content of the grain was determined as an indicator for suitability for spawn production.

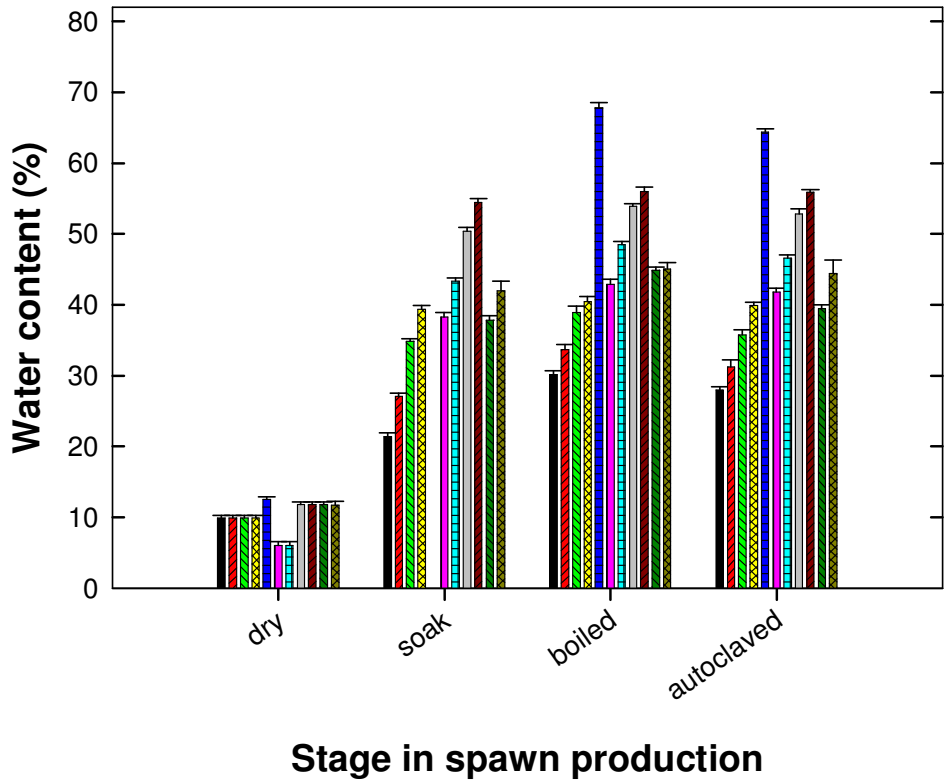


Figure 14: Water content of the different alternative substrates for spawn production produced by various soaking and boiling periods [maize grain (, 2 hours soaking {S} and 10 min boiling {B}) (, 3 hours S and 10 min B) (, 16 hours S and 5 min B) (, 40 hours S and 10 min B)], [maize cobs (, 0 min S and 5 min B)] [pumpkin seeds (, 2 hours S and 5 min B) (, 25 hours S and 5 min B)] [dry beans (, 16 hours S and 5 min B) (, 16 hours S and 10 min B)] [sorghum (, 16 hours S and 5 min B)]. Bars represent S. E. M.

Deviations occurred from the desired 50 percent moisture content of a sterilized wheat grain substrate as exemplified by sorghum grain which had a water content of 44.4

percent after autoclaving (Figure 14). Generally an increase in the period of soaking or boiling increased the water content of the respective substrate (Figure 14). Maize grain of 39.9 percent water content and pumpkin seeds of 41.8 percent and 46.5 percent water content were showing signs of colonization on the second day after inoculation. Maize cobs had the highest water content of 64.4 percent as shown in Figure 14. *Pleurotus* species failed to colonise beans successfully, the mycelia only appeared as white patches in the bottles even after six weeks of incubation. Over the same period of time other substrates such as sorghum, maize grains and maize cobs had given rise to good quality spawn characterised as sterilised substrate covered with either rhizomorphic mycelia or sparse cottony mycelia. Substrates with water content greater than 35 percent gave rise to good quality spawn apart from the beans substrate. On the third day after inoculation, beans produced a foul smell and no signs of colonization by the *Pleurotus* species were observed. Even though they were incubated at room temperatures, the bean substrate had a temperature of 43 ± 3 °C.

4.4.5 The Effects of Modified Media on Growth of *Pleurotus* Strains

4.4.5.1 Growth response on whole wheat grain PDA/MEA media formulations

Strains were grown over a range of whole wheat grain media formulations, and their growth rates determined (Figure 15). Growth rates varied significantly between strains ($P < 0.05$) as shown in Figure 15 in relation to whole wheat grain media formulation. The most rapid growth (radial extension rates of ≥ 5 mm day^{-1}) was obtained from strains BCCM/MUCL 31017, 31613, 38076 38078 and 38092. All the strains generally had a higher growth rate on the novel media formulations of 1:3, 1:4 and 1:5 than on MEA and PDA as shown in Figure 15. The

growth rate initially decreased (Figure 15) below that obtained for MEA and PDA for 80 percent of the strains.

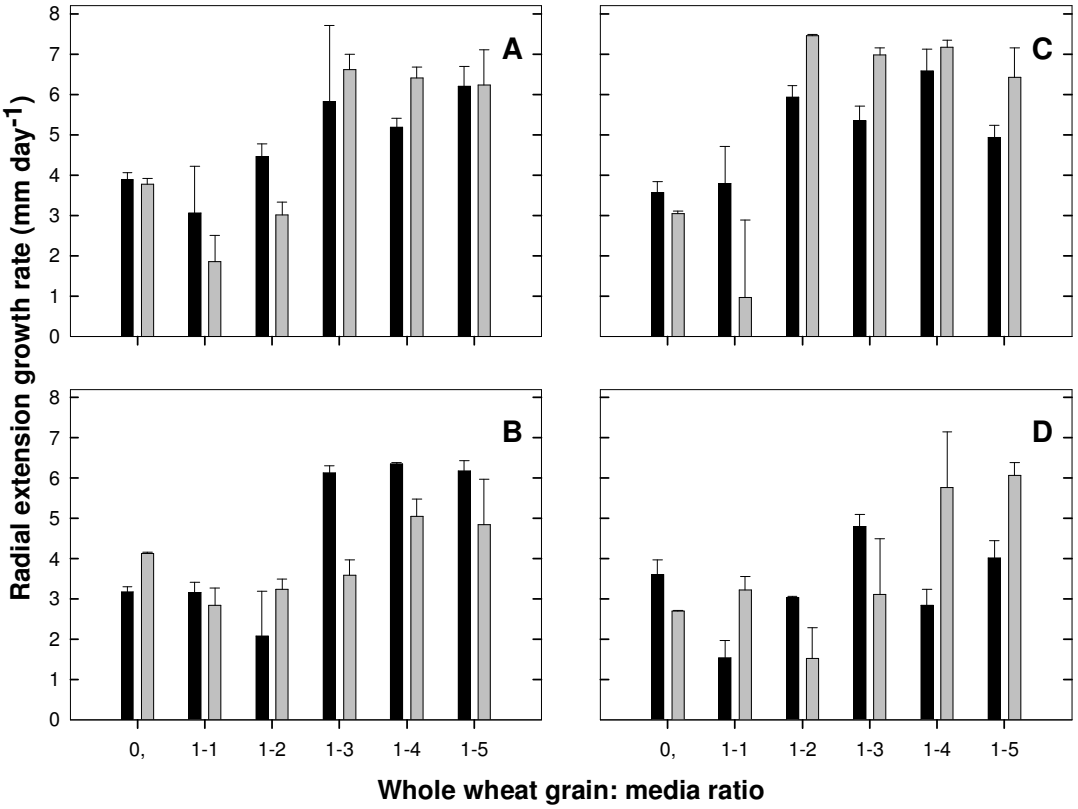


Figure 15: Growth responses of two representative strains of *Pleurotus* species to a range of different whole wheat grain: media formulations from 1:1 to 1:5. Strains BCCM 38092 (▬) and 38078 (▭) are shown for MEA (graph A), PDA (graph B), MEA + PEG (graph C) and PDA + PEG (graph D). Bars represent S. E. M.

However the growth rate increased above that for MEA and PDA for the 1:2 to 1:5 for MEA (Figure 15A) and 1:3 to 1:5 for PDA (Figure 15B). The growth rate for PEG amended MEA for formulations 1:2 to 1:5 was generally higher than that for non amended MEA (Figure 15A and C) for 53 percent of the strains. In contrast the growth rate for PEG amended PDA for formulations 1:2 to 1:5 was lower for 60 percent of the strains than that for non amended PDA (Figure 15B and D).

The wheat grain in the prepared whole wheat grain PDA/MEA formulations had a glistening appearance. The 1:1 ratio formulation of all PDA/MEA whole wheat grain formulations resulted in difficulty in dislodging the remaining grain from the conical flasks in contrast to the 1:4 ratio and the 1:1 whole wheat grain media dried by the eighth day of incubation. The water content of the media formulations were all significantly different from the water content of conventional malt extract agar (MEA, 3 %) (97.9 percent water) and potato dextrose agar (PDA, 3 %) (97.9 percent water). The water contents ranged from 14.3 percent (1:2 MEA) to 57.4 percent (1:4 PDA). There was no clear-cut trend for the water contents of the media formulations.

4.4.5.2 Growth response on crushed wheat grain media formulations

Strains were grown over a range of crushed wheat grain media formulations, and their growth rates determined as shown in Figure 16. The media without PDA did not solidify easily when compared to the media with PDA. For example, 1:15 (crushed grain: distilled water) (– PDA) did not solidify whilst 1:15 (+ PDA) was semisolid. Media from 1:2 (– PDA) to 1:5 (+ PDA) solidified. All 1:2 (– PDA) plates were contaminated by the eighth day of incubation. Growth rates of the strains varied significantly by ANOVA analysis (data not shown). Growth was highest for 1:5 (+ PDA) and lowest for 1:2 (– PDA) media (Figure 16). The water content of the crushed grain, distilled water ± PDA media increased from 75 percent (1:2) to 97 percent (1:15).

4.4.5.3 Production of primordia and mycelia characteristics

Production of primordia by the *Pleurotus* strains under the conditions shown in Figures 15 and 16 is shown in Figure 17. Numerous primordia (< 100 for

whole wheat grain) and (+ 200 for crushed wheat grain media) were produced by the *Pleurotus* strains (Figure 17 A and B).

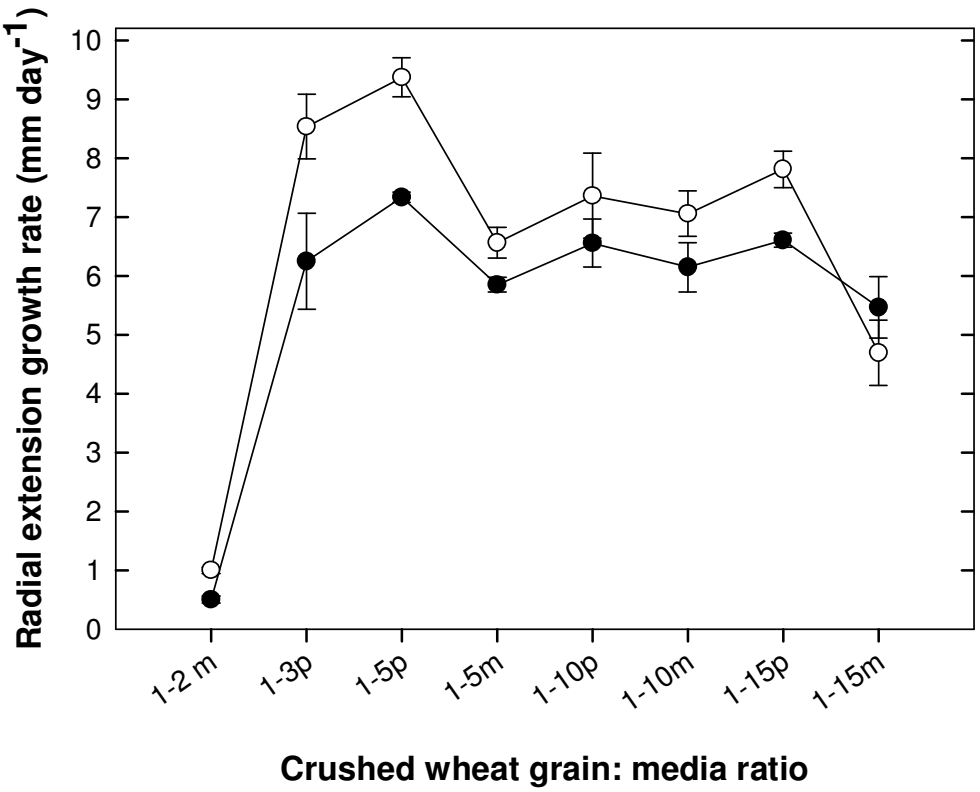


Figure 16: Growth responses of two representative strains of *Pleurotus* spp. to a range of different crushed wheat grain: distilled water ± PDA media formulations from 1:2 (- PDA) to 1:15 (- PDA). Strains BCCM 38092 (●) and 38078 (○) are shown. m in the x – axis represents without PDA and p represents with PDA added to the media formulation. Bars represent S. E. M.

The highest number of primordia and basidiomes were obtained from strains BCCM/MUCL 31017, 31613, 38076, 38078 and 38092. Three classes of mycelium were observed: cottony (predominant), cottony zonate and quasi-rhizormorphic as described in Appendix D for both the whole wheat grain media and crushed grain formulations. Mycelium covering medium was less dense than that which covered the whole wheat grains in the whole wheat grain: media formulations. The mycelium type changed from cottony to cottony zonate and finally to quasi – rhizomorphic from 1:1

to 1:5 whole wheat media. Cottony mycelia on MEA whole wheat media were denser than that on PDA.

Figure 17: Production of primordia by *Pleurotus sajor-caju* strain 38092 on the whole wheat grain PDA media 1:4 (left photo) and crushed wheat grain distilled water media 1:5 (– PDA) (right photo).

Cottony mycelia on MEA whole wheat media were denser than that on PDA. For the crushed grain media, 1:3 and 1:5 (+ PDA and – PDA) had cottony mycelium which became cottony zonate (plus quasi rhizormorphic around the agar inoculum disc) for 1:10 (+ PDA and – PDA) and 1:15 (+ PDA and – PDA). When two or more mycelium types occurred, they were separated spatially. Microdroplets were produced by the *Pleurotus* strains on the following media: whole wheat grain [MEA (1:1 to 1:3, 1:5); MEA + PEG (1:3 to 1:5); PDA (1:3 to 1:5) and PDA + PEG (1:2 to 1:5)] and crushed wheat grain [all media formulations except 1:2]. The crushed wheat grain

media formulations resulted in more luxuriant mycelial growth than for the whole wheat grain media formulations.

4.4.6 Production of *Pleurotus* Spawn on Tyndallised Wheat Grain

The water content of the wheat grain generally increased with each successive boiling, for example in the experiment using narrow-mouthed bottles and increased number of shorter boiling periods, water content increased from 39.90 percent (boiled twice) to 66.29 percent (boiled 6 times) for the 5 min boiling treatment and 41.43 percent (boiled twice) to 70.10 percent (boiled 6 times) for the 10 min boiling treatment. Burst grain occurred for the following treatments 5 min (boiled for 5 and 6 times) and 10 min (boiled for 4, 5, and 6 times) with a lot of burst grain for the boiled 6 times treatment for both the 5 min and 10 min boiling treatments.

The average number of days taken by each respective treatment to show the presence of visible contamination ranged from four days as in strain 31613 to nine days as in strain 38092. The number of days taken before contamination for all the treatments varied significantly (ANOVA, one-way $P = 0.012$ [$P < 0.05$]). The 30-minute boiling treatment resulted in the highest number of days before contamination appeared. Contamination generally occurred within the period of a week on the inoculated grain whilst it took one to two weeks on the grain not inoculated. The autoclaved grain took 30 days to show contamination. Good quality spawn characterised as sterilised wheat grain covered with either rhizomorphic mycelia or sparse cottony mycelia was obtained for the 30 min (boiled twice and thrice treatments) [assessment of sterilization of wheat grain by adding a pre-boiling stage and draining of water in Tyndallisation experiment] and the 5 mins (boiled 4 times) (Figure 18) for the assessment of sterilization of wheat grain by use of narrow-

mouthed bottles and increased number of shorter boiling periods in Tyndallisation experiment.

Figure 18: Production of *Pleurotus* (oyster mushrooms) spawn by modified Tyndallisation method using narrow-mouthed bottles and increased number of shorter boiling periods. Photo shows spawn produced by the 5 min (boiled 4 times) treatment.

4.4.7 Production of *Pleurotus* Basidiomes

Oyster mushroom (*Pleurotus*) pinheads started forming after the fourth week for the boiled thrice for 30 mins prepared spawn on banana substrate and during the fifth week for the boiled 4 times for 5 mins prepared spawn produced by Tyndallisation as a means to sterilize the wheat grain growth medium.

4.5 OPTIMISATION OF CULTIVATION SUBSTRATE CONDITIONS

4.5.1 The Effects of Substrate Mixtures on Growth

4.5.1.1 The effects of substrate on growth on a small scale

Pleurotus strains were grown on different pure substrates and substrate mixtures and their growth responses determined as an indicator of suitability of the substrate for oyster mushroom cultivation (Table 3 showing representative strains).

All the fifteen strains tested grew on grass (g), sage (s) (single substrates) and grass and sage combined with banana (b) as well as on banana combined with wheat straw (ws) (Table 3). *Pleurotus floridanus,* strain 38047 did not grow on banana and wheat straw and in the mixture of wheat straw and sage. The substrates grass, sage, and mixtures banana plus sage (b+s) and banana plus grass (b+g) had a high portion of strains fruiting as shown in Table 4. Wheat straw and banana plus wheat straw (b+ws) had few strains able to fruit on them.

Table 3: Growth of the strains after 5 days incubation at 25 °C

Strain	b	g	ws	s	b+s	b+ws	b+g	ws+s
28511	+	+	−	+	+	+	+	+
28684	+	+	+	+	+	+	+	+
31613	+	+	+	+	+	+	+	+
31674	+	+	+	+	+	+	+	+
38047	−	+	−	+	+	+	+	−
38076	+	+	+	+	+	+	+	+
38089	+	+	+	+	+	+	+	+

b = banana, g = grass, ws = wheat straw, s = sage, b+s = banana and sage mixture, b+ws = banana and wheat straw mixture, b+g = banana and grass mixture, ws+s, wheat straw and sage mixture, +: growth, −: absence of growth

The number of days taken by the *Pleurotus* strains to fruit on the different substrates ranged from three to six weeks. The results of water content and nutrients for the various substrates used in this study are shown in Figure 19. The water content of the substrates varied significantly and ranged from 74 to 84 percent (Figure 19A). Sage substrate had the highest water content of 84.3 percent, whilst wheat straw had 77.4 percent. Banana and grass had similar water contents of 74.7 and 74.4 percent respectively (Figure 19A).

The nutrient analysis of the different substrates results as shown in Table 5 and Figure 19 show that the carbon to nitrogen ratio (C:N) had varied a lot from 26:1

for grass to 112:1 for sage. Percent phosphorus was highest for grass and lowest for sage. Percent potassium was highest for wheat straw and lowest for sage, results shown in Table 5 and Figure 19).

Table 4: Basidiome production by strains on the different pure substrates and substrate mixtures

Strain	b	g	ws	s	b+s	b+ws	b+g	ws+s
28511	+	+	−	+	+	+	−	−
28684	−	+	−	+	+	−	+	+
31613	+	−	+	+	+	+	+	+
38076	+	+	+	+	+	−	+	+
38089	−	+	+	−	−	−	+	−

b = banana, g = grass, ws = wheat straw, s = sage, b+s = banana and sage mixture, b+ws = banana and wheat straw mixture, b+g = banana and grass mixture, ws+s, wheat straw and sage mixture, +: fruited, −: no fruiting

Table 5: Tissue analysis of the different substrates

Name	% Carbon	% Nitrogen	% Phosphorus	% Potassium	C:N ratio
Wheat	42.80	0.67	0.047	5.33	64:1
Grass	43.20	1.65	0.092	3.08	26:1
Sage	43.71	0.39	0.034	0.74	112:1
Banana	45.13	0.88	0.041	1.04	51:1

4.5.1.2 The effects of substrate on growth on a large scale

Strains were grown using the column culture method and among the characteristics noted was the colour of the basidiome. *Pleurotus sajor-caju* strains had basidiome colours ranging from dark gray to light gray brown. *Pleurotus ostreatus* was pale off-white whilst *Pleurotus citrinopileatus* was bright yellow in colour. Biological efficiencies greater than 75 percent on banana fronds and sage grass substrates were exhibited by 67 percent of the strains. Strains 28684 (*P. citrinopileatus*), 31017, 31674 and 38076 had biological efficiencies greater than 100

percent with strains 31017 and 31674 having biological efficiencies of 147 and 160 percent respectively.

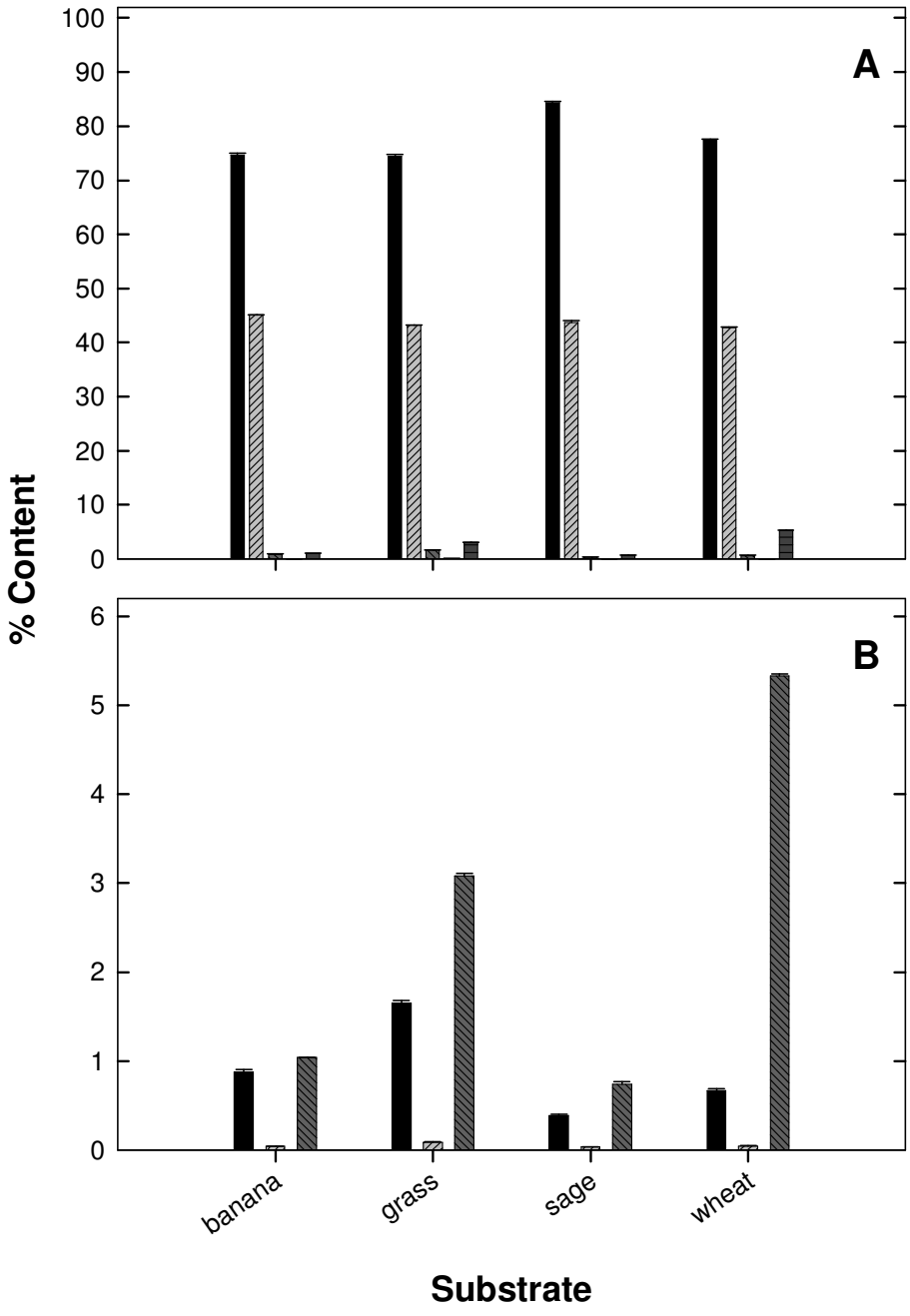


Figure 19: Water content of autoclaved substrate ( ) and tissue analysis, graph A, carbon ( ), nitrogen ( ), phosphorus ( ) and potassium ( ) of the substrates (banana, grass, sage and wheat) used in this study. Graph B shows greater detail for the nutrients nitrogen ( ), phosphorus ( ) and potassium ( ). Bars represent S. E. M.

The time between harvests took from one to three weeks. Strains 28683 and 31017 had a short time of one week between harvests. Strains 28511 (*P. ostreatus*), 31674 and 38076 took approximately three weeks between harvests whilst the strains 28684 (*P. citrinopileatus*), 29757, 31613, 38071, 38078 and 38091 generally had two weeks between harvests. The first harvest day after spawn inoculation occurred between 36 to 55 days.

4.6 GENETIC CHARACTERISATION

4.6.1 DNA Isolation

DNA from *Pleurotus* strains was successfully isolated. Single bands of intact DNA were visualised on the agarose gel (data not shown).

4.6.2 PCR Amplification

DNA isolated from *Pleurotus* strains was amplified with primers ITS-1 and ITS-4 using PCR and the products produced were visualised as a single band in agarose gels stained with ethidium bromide as shown in Figure 20. The size of the PCR fragments was on average about 800 bp in length for all strains. The PCR products migrated between the 400 and 850 bp markers as shown in Figure 20, lane 1.

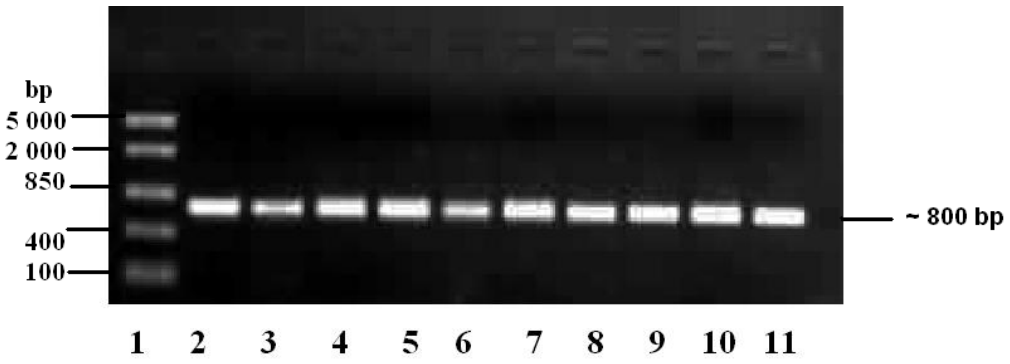


Figure 20: Amplified ITS products (using primers ITS-1 and ITS-4) of 10 *Pleurotus sajor-caju* strains, electrophoresed on a 1.2 % agarose gel. Lane (1) is Fermentas FastRuler™ DNA Ladder molecular weight marker, Lanes 2-11 are BCCM/MUCL strains: (2) 28683, (3) 28685, (4) 29757, (5) 31017, (6) 31613, (7) 31674, (8) 38071, (9) 38076, (10) 38078, (11) 38089.

4.6.3 PCR Product Sequencing Analysis

Internal transcribed spacer products of the *Pleurotus* strains obtained by PCR were sequenced using fluorescent dye terminator chemistry and BLAST analysis was done to obtain a phylogenetic tree (Appendix G). The BLAST analysis phylogenetic tree shows the *Pleurotus* species and strains were grouped with respective species apart from strain 31674 (*P. sajor-caju*) which was grouped with published *P. ostreatus*. The other *Pleurotus sajor-caju* strains were grouped with other published *P. sajor-caju* and *P. pulmonarius* strains. The full length ITS sequences obtained for all *Pleurotus* strains and sequences were submitted to the GenBank Nucleotide Database (National Center for Biotechnology Information [NCBI] U.S.A.) and their accession numbers appear in appendix G. Multiple alignment of the ITS sequences of the *Pleurotus sajor-caju* strains in 703 positions from position 23 to 726 (Appendix H) showed 63 indels (insertion [15]/deletion [48] events), 113 transition substitutions and 117 transversion substitutions. Most of the indels were obtained from strains 31674 and 38071. The transition substitutions frequency were in the following order C to T (47) > A to G (27) > G to A (24) > T to C (15) whilst the transversion substitutions frequency were in the following order C to A (24) > T to G (21) > G to T (16) > C to G (13), T to A (13) > G to C (11) > A to C (10) and A to T (9) (Appendix H). Strain 38071 had many poly (base N) indels, transition and transversion substitutions from positions 513 to 602. Multiple alignment of the ITS sequences of the *Pleurotus* species and strains in 750 positions from position 23 to 726 showed ITS sequences of strains 28684 (*P. citrinopileatus*), 38047 (*P. floridanus*), 31674 and 38071 had many indels, transitions and transversions from position 215 to 645 (Appendix H). Strain 28684 (*P. citrinopileatus*) had many sequence dissimilarities with other aligned sequences from position 32 to 173. Analysis of presence of

restriction enzyme sites on the *Pleurotus* strains ITS sequences obtained revealed that all the strains have restriction enzyme sites for the following restriction enzymes: *Acc*1, *Alu*1, *Apo*1, *Cac*81, *Cla*1, *Cvi*J1, *Dpn*1, *Fnu*4H1, *Hae*1II, *Hha*1, *Hin*f1, *Hpy*1881, *Mae*III, *Mbo*1, *Mnl*I, *Mse*1, *Mwo*1, *Nla*IV, *Rsa*1, *Scr*F1, *Sty*1, *Taq*1, *Tfi*1, *Tsp*451, *Tsp*R1 (Appendix I). The following BCCM/MUCL strains had exclusive restriction sites 28511 (*Avr*i1 and *For*1), 28684 (*Asc*1, *Kpn*1, *Msi*1, *Nhe*1, *Psi*1), 29757 (*Bsr*B1, *Hga*1), 31613 (*Tsp*GW1), 38047 (*Smi*1), 38071 (*Bsa*A1), 38076 (*Sfi*1), 38078 (*Bse*Mi1 and *Sac*II), 38089 (*Hae*II, *Nar*1, *Pfi*M1) and 38092 (*Apa*L1, *Nco*1, *San*D1). The sequences obtained were compared with sequences published in GenBank, EMBL, DDBJ and PDB using the BLAST facility. BLAST search showed significant sequence homology with other *Pleurotus* rDNA sequences (Appendix G). BLAST analysis of the ITS sequences of strains 28683, 28685, 29757, 31017, 31613, 38076, 38078, 38089, 38091 and 38092 produced identical significant alignments for the first four sequences listed (Appendix G). *Pleurotus sajor-caju* strains in this study had significant alignments with published *Pleurotus sajor-caju*, *P. pulmonarius* and *P. australis* apart from strain 31674 which had significant alignments with published *P. ostreatus* and *P. sapidus* (Appendix G). *Pleurotus ostreatus* and *P. floridanus* strains in this study had significant alignments with published *P. ostreatus* and *P. floridanus* whilst *Pleurotus citrinopileatus* in this study had significant alignments with published *P. citrinopileatus* and *P. cornucopiae* sequences.

The sequences of *Pleurotus* strains obtained by sequencing of the ITS PCR products were used to generate a phylogenetic tree produced by the neighbour-joining method (Saitou and Nei, 1987) (Figure 21). The phylogenetic tree generated (Figure 21) shows the *Pleurotus* species and strains separated into four clusters. Cluster A comprises of strains 28511 (*P. ostreatus*), 38047 (*P. floridanus*) and 31674. Cluster B

comprises of strains 38078, 38089 and 38091, whilst cluster C comprises of strains 38076 and 38092. Strains 28683 and 31017 comprise the cluster D (Figure 21).

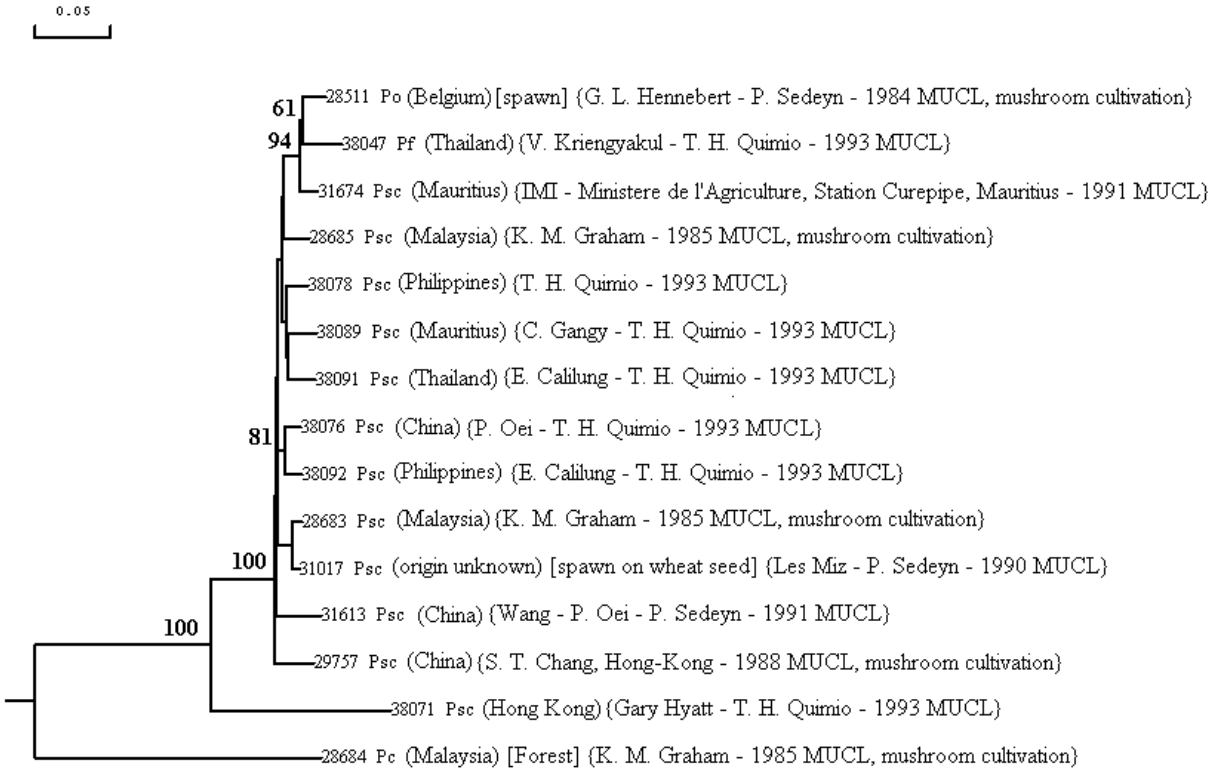


Figure 21: Phylogenetic relationships of *Pleurotus* species based on data resulting from sequencing of the ITS region of rDNA (sequence positions 26 to 776 used in analysis). Dissimilarity was calculated with the distance matrix using the neighbour joining method (Saitou and Nei, 1987) with the software DNAMAN 4.0. Abbreviations: Pc, *Pleurotus citrinopileatus*; Pf, *Pleurotus floridanus*; Po, *Pleurotus ostreatus*; Psc, *Pleurotus sajor-caju*. Bootstrap values greater than 50 % are shown above their respective branches (bootstrap values based on 1000 replicates). The history of the strain is shown in parentheses { } with hyphens representing the direct transfer of a strain between the individuals or institutions indicated. The origin of the strain, where known is shown in parentheses (country) and [substrate or habitat].

There is a good support for the separation of strain 28684 (*P. citrinopileatus*) (bootstrap value [bs] = 100 %) and strains 29757 and 31616 (bs = 100 %). There is also a good support for Cluster A (bs = 94 %). Strains 28684 (*P. citrinopileatus*), 28685, 29757, 31613 and 38071 separated from the rest of the strains (Figure 21).

The neighbour – joining phylogenetic tree obtained (Figure 21) from sequencing data of the ITS PCR products did not evidence a grouping of the strains according to their geographic origins (country).

CHAPTER 5

5.0 DISCUSSION

5.1 FUNGAL SPECIES AND STRAINS MAINTENANCE

The *Pleurotus* species and strains were successfully maintained on the chosen culture media MEA enabling this research project to proceed to completion.

5.2 OPTIMISATION OF ENVIRONMENTAL CONDITIONS

5.2.1 Effects of Osmotic Potential

Fungi are the most xerotolerant microorganisms known; some xerophilic basidomycetes can remain metabolically active up to − 7.1 MPa (Brown, 1990), although the growth limit for *Pleurotus sajor-caju* strains in the present study was − 3.5 MPa. Studies of stress tolerance in other microbial species have shown that both phenotypic plasticity and genetic recombination can be utilised to greatly increase their growth windows (Hallsworth and Magan, 1995; Ferrer *et al.*, 2003, Hallsworth *et al*., 2003a, b). Studies to optimise the growth of the oyster mushroom, *Pleurotus ostreatus*, demonstrated that it grew optimally at − 0.7 to − 1.4 MPa (water potential) (Senyah, 1987) which is not much different from the optimal ranges obtained in this study which were − 0.5 to − 1.5 MPa as shown in Section 4.2.1. Senyah's study (1987) showed no growth of the strain at − 4.0 MPa. This again is similar to the results obtained in this study where strains showed no growth at – 3.5 MPa.

5.2.2 Effects of Temperature

Most of the strains in this study exhibited mesothermic growth (minimum, optimum and maximum temperatures for growth in the region of 5, 25 and 40 °C respectively) as shown in Section 4.2.2. In common with other work, the optimum temperature for growth of wood-rotting basidiomycetes was between 20 °C and 30 °C (Humphrey, 1933). The temperature optima exhibited by the strains are ideal for

growth in tropical countries of which most comprise the developing countries of the world in areas where the temperature does not exceed 40 °C. Thus, the temperature-growth relationship obtained in this study will enable the cultivation of *Pleurotus* in the absence of environment controlling machinery such as an air-conditioner. The mycelium of the species and strains was not killed within the temperature range of 5 °C to 37 °C as seen by the growth of the species and strains when transferred to the 25 °C incubator from the 37 °C incubator. Temperatures of 40 °C and above resulted in the death of the mycelium. Thus during cultivation processes of *Pleurotus* the mycelium should not be exposed to temperatures of 38 °C and above as this might result in lack of viability of the mycelium.

5.2.3 Effects of pH

Both natural habitats and alkali-treated plant wastes used as substrates for biopulping can have a high pH, so strains with an ability to grow and fruit at under high pH conditions are potentially important as shown by strains 28511 (*P. ostreatus*), 38047 (*P. floridanus*) and 38091 (*P. sajor-caju*) which had an optimal growth pH range of 8 to 10 as shown in Section 4.2.3 (Camarero *et al*., 1998; Gramss, Ziegenhagen and Sorge, 1999). Spawn production for each strain may be optimised as the optimum initial pH has been identified for each strain (see Section 4.2.3). The pH of the grain carrier medium can be modified using the calcium insoluble salts used in spawn production. Also substrates for growth in the mushroom structures can be modified to the initial optimum pH for the respective strain. For resource – challenged mushroom farmers they can use inexpensive pH paper or litmus paper and either or both of gypsum and agricultural lime to modify the substrate pH to the initial optimum pH and thus enhance colonisation of the substrate.

5.2.4 Mycelial Microdroplets Production

The microdroplets produced by the *Pleurotus* spcies are secondary metabolic products (Stamets, 2000). Droplets that occur on mycelium have been shown to contain enzymes (Colotelo, 1973, 1978) and solutes (Colotelo, Sumner and Voegelin, 1971). Droplets produced by *Serpula lacrimans* (Wulfen: Fr) Schröter growing over a non-absorptive surface are an expression of the mass flow of water through the mycelium (Coggins, Jennings and Clarke, 1980; Brownlee and Jennings, 1981). The droplets are a reservoir for secondary metabolites, or metabolite reserves which could be readily available when needed (McPhee and Colotelo, 1977). The droplets also serve as a reservoir of water allowing hyphae, not in direct contact with liquid medium, to continue growth when the feeding mycelium might be subjected to an unfavourable water potential (Jennings, 1991). In agar cultures, aerial hyphae of *Pleurotus* produce tiny droplets of nematode toxin on minute secretory processes (Hibbett and Thorn, 1994). This toxin has been identified as trans-2-decenedioic acid (Kwok *et al*., 1992). Nematodes that come in contact with the toxin are paralysed and later invaded by the *Pleurotus* hyphae. This mode of nematode attack has been observed in *P. cornucopiae* (paulet) Rolland, *P. cystidiosus* O. K. Miller, *P. levis* (Berk. And Curt.) Sing., *P. ostreatus* (Jacq.: Fr.) Kummer, *P. populinus* (Hilber and Vilgalys (as *P. ostreatus* p.p and *P. subareolatus* Peck), and *P. pulmonarius* (Fr.: Fr.) Quél. (as *P. ostreatus* p.p.) (Thorn and Barron, 1984; Thorn and Tsuneda, 1993). The secondary metabolic products (microdroplets) do not only serve as a reservoir for water and metabolites but they also act as a nematode toxin. The *Pleurotus* species that produced microdroplets may be ideal for low-cost cultivation in arid regions.

5.2.5 Phenotypic Characteristics

The role of drought-tolerant plant species in the ecological and agricultural regeneration of arid environments has been studied for several decades (Svoray *et al*., 2004). However, the profile of fungi has received a lower priority despite their collective roles as mycorrhizal symbionts that facilitate plant growth, in improving soil health, and facilitating the development of rhizosphere microbial communities (Jeffries *et al*., 2003; Medina *et al*., 2003; Austin *et al.,* 2004). Generally microbial diversity is an underused resource (Kues and Liu, 2000; Rappe and Giovannoni, 2003): the data obtained in the present study can be utilised to select strains with the growth and fruiting characteristics required for low-cost cultivation in arid regions or for other purposes. For example, *P. sajor-caju* can be utilised to remediate soils by removing heavy metals, by harvesting and disposing of fruiting bodies that have absorbed cadmium, copper, lead, and mercury (Cihangir and Saglam, 1999). For this purpose a capacity for rapid growth and an ability to grow optimally and fruit over a range of water potentials are advantageous.

5.3 OPTIMISATION OF MICRO ENVIRONMENTAL CONDITIONS CHANGES

Generally *Pleurotus* species cultivation utilizes pasteurized substrates (Stamets, 2000; the data obtained in the present study as shown in Section 4.3 can be utilized to attain rapid growth rates of the *Pleurotus* species thereby preventing contaminating organisms to colonise the substrate at the expense of the *Pleurotus* species mycelia. The data obtained in the present study (Section 4.3) can also be utilized for spawn production to reduce the time taken for the mycelia to colonise the spawn producing substrate. Potassium chloride, sodium chloride at micro concentrations of 1.50 g l^{-1} KCl: 0.60 g l^{-1} NaCl or 2.70 g l^{-1} KCl: 1.08 g l^{-1} NaCl for both 25 °C and 30 °C and

polyethylene glycol at micro-concentrations of 0.90 g l^{-1} [20 °C] and 0.65 g l^{-1} [25 and 30 °C] where found to result in a two to three fold increase of the *Pleurotus* strains rate of growth on control media, therefore may be used as growth enhancers in *Pleurotus* species low-cost cultivation procedures. It is tempting to speculate that the level of solutes (KCl, NaCl and PEG 6000) at minute concentrations present in the media may regulate growth rate of *Pleurotus* species, by possibly taking part in switching on and off genes in as yet undiscovered way leading to the production of different isoforms that have different enzymic activities promoting more rapid hyphal tip extension and production of hyphal tips (branching).

5.4 OPTIMISATION OF SPAWN PRODUCTION

5.4.1 Effects of Spawn Production on Wheat Grain Water Content

Adequate water content of grain in spawn production would ensure the highest growth rate with the least risk of contamination and a short spawn run. The data obtained in this study (see Section 4.4.1) show that in contrary to the recommended 45 to 55 percent range (Stamets, 2000) for water content of wheat grain for spawn production, good quality spawn can be produced with water contents less than 45 percent and greater than 35 percent. The data obtained suggest that the rate of imbibition by the respective seeds affects the level of moisture content of the respective seeds, thus the water content formulae derived in this study may be modified by adding a term or terms relating to the rate of imbibition of the respective seeds. Occurrence of excess water could be due to slow imbibition (Revell and Taylor, 1998) or grain hardness (Morris and Rose, 1996). For wheat seed, grain hardness is arguably the single most important aspect of wheat utilization (Morris and Rose, 1996). The hardness of wheat grain has been assigned to the short arm of chromosome 5D (Mattern *et al.*, 1973; Law *et al.*, 1978: both cited in Morris and

Rose, 1996). The unconventional method of spawn production with the omission of boiling and omission of calcium salts may be adopted since it reduced the period for the mycelia to fully colonise the wheat grain for *Pleurotus* spawn production and is low on power (for example, electrical or gas) consumption thus ideal for a developing country spawn laboratory.

5.4.2 Spawn Production Applying Water Content Formulae

The use of the water content formulae should relate to the container for soaking taking into account the base area to the volume ratio. Containers with a lower volume to base area ratio may be used for the application of formulae F3 to F5 which result in less water being added, whilst the containers with a higher volume to base area ratio may be used for the application of formulae F6 to F8 which have more water being added to the wheat grain. In terms of growth rate and mycelia quality on wheat grain all the water content formulae are ideal with F5 to F8 giving the best results. Germination of seeds that occurred with formulae F1 to F4 is not desirable for spawn production as the germinated seed is similar to a burst grain and thus is an environment where bacteria proliferate. Germinated seed has an inferior quality due to presence of carbohydrases, proteases and other hydrolytic enzymes normally associated with germination (Morris and Rose, 1996).

5.4.3 Effects of Spawn Production on Wheat Grain Nutrients

The changes in macronutrients during the spawn production process obtained in this study show that autoclaving the soaked grain increases the level of carbon, nitrogen, phosphorous, calcium, magnesium and potassium nutrients studied. This increase in nutrient content may be attributed as the reason why the water content of the wheat grain generally decreases by autoclaving. This increase in nutrients may be

partly due to chemical reactions occurring during autoclaving that produce the compounds contributing to an increase in nutrient content of the wheat grain.

5.4.4 Spawn Production Using Alternative Substrates

The data obtained in this study as shown in Section 4.4.4 suggest that all the alternative substrates used in this study may be used in spawn production with the exception of dry beans. One of the factors that results in successful spawn production is the right water content. This is clearly illustrated by the four samples of maize grain that had different final water content; growth was poor for the two samples with water content of 28.02 percent and 31.27 percent but on the other hand colonization was very good for the other two samples with a water content of 35.67 percent and 39.92 percent. The substrates, maize grain of 39.92 percent water content and pumpkin seeds of 41.81 percent and 46.54 percent water content that showed signs of colonization on the second day may be used successfully in spawn production because they also took a relatively short time of 2 to 3 weeks for full colonization to be achieved. The anomalous behaviour of beans from all the other substrates may be due to the high nitrogen to carbon ratio due to the high protein content relative to maize, maize cobs and pumpkin seed which are rich in starch. Maize cobs had very high water content but produced good quality spawn characterised as sterilised substrate covered with either rhizomorphic mycelia or sparse cottony mycelia; on the other hand, dry beans with similar water content of 55.92 percent were poorly colonized. Though maize cobs were successfully colonised they are not easy to use in spawn production as they first have to be crushed into reasonably–sized pieces of approximately one cm in length. If a chopping device or a crushing device is used they will be the most cost effective to use since they are an agricultural waste product unlike all the other substrates. Maize cobs have similar advantages noted by Rosado,

Kemmelmeier and Da Costa in 2002 when working on cotton residue for spawn production of *Pleurotus ostreatoroseus*, as they can be stored for longer periods, have lower rates of contamination and reduced cost of production. Being a waste product, they will aid in ensuring that spawn production costs are kept low thus making available good quality, affordable spawn to small holder mushroom farmers in the developing countries of the world where these crops are grown. Pumpkin seeds were readily colonized and did not require much time for soaking (2 hours) unlike maize (40 hours) but they have a disadvantage as they are not usually available in bulk as are maize cobs and maize grains. Thus maize cobs and maize grains appear to be the only two feasible alternatives to wheat grain in spawn production of all four substrates investigated in this experiment. Maize grain has the advantage that it does not need any processing such as crushing or grinding before being used as is done for maize cobs. However maize cobs have the advantage that they are cheaper and also take a shorter time to produce spawn since they are not soaked whereas maize grains are soaked for 40 hours. Maize, pumpkin seeds and maize cobs with water content of 35 to 39 percent, 41 to 47 percent and 64 percent respectively may be used as alternative substrates to wheat in spawn production.

5.4.5 Effects of Modified Media on Growth

For *Pleurotus* species media is not only required for culture maintenance but also in the cultivation process in producing *Pleurotus* grain spawn (Oei, 1990; Stamets 2000). The *Pleurotus* mycelium is initially grown on a nutrified agar media (Ogden and Prowse, 2004). The data obtained of optimal growth of 1:3 (w/v) to 1:5 for the whole wheat grain to distilled water formulations and 1:3 to 1:15 for the crushed wheat grain to distilled water formulations in this present study can be used for low cost culture maintenance in developing countries laboratories, especially the

crushed wheat grain formulations that lack any conventional media (i.e. PDA or MEA) in their formulations. Since low quantities of PEG 6000 are required to obtain the PEG 6000 containing media formulations the media are low-cost in comparison to conventional media. The water content of the mixtures can not be used to predict the growth rates. Generally mushrooms are taken to optimally grow in media with water content of 70 percent and above (Stamets, 2000) however surprisingly growth higher than that for normal media was obtained for media with low water content such as the 6.04 mm day^{-1} growth rate obtained on 57.4 percent water content 1:4 (+ PDA) whole wheat grain media formulation for *P. sajor-caju* strain 38092 (Section 4.4.5.1, Figure 15). The increased growth rate may be attributed to the presence of nutrient rich wheat grains in the media formulations. The occurrence of contamination in the crushed wheat grain 1:2 (– PDA) formulation may be overcome by experimenting on effect of additional autoclaving time on occurrence of contamination. The mycelium types obtained in this study as shown in Section 4.4.5.3 are no different from those that occur on conventional media (MEA and PDA) (Stamets, 2000), which suggests that the media formulations produce comparable growth to conventional media. Fungal culture maintenance on agar is done three ways a) using a weak medium b) lowering the incubation temperature, or c) growing under reduced oxygen tension (Kirsop and Snell, 1984). The media formulations in this study may therefore be employed in culture maintenance of *Pleurotus* species by growth at lowered incubation temperature in Petri plates or agar slopes. Polyethylene glycol amended MEA and PDA media generally resulted in higher growth rates than non-amended media. Media formulations 1:3 to 1:5 (whole wheat grain) and 1:3 to 1:15 (crushed wheat grain) were shown to be suitable for use as low-cost alternatives to culture *Pleurotus* species. The optimal range of growth, luxuriant mycelia growth, well

developed external basidiomes and formation of numerous primordia by the *Pleurotus* strains suggest that the crushed grain: distilled water formulations are ideal for the specific culturing of *Pleurotus* species for spawn and oyster mushroom production. Production of numerous primordia by the *Pleurotus* species on the crushed grain media formulation is a very desirable trait as growth and production of basidiomes is required for bioremediation. The production of confirmed nematicidal microdroplets by the *Pleurotus* species on the novel media formulations used in this study can be used as a means of biocontrol of nematodes in the mushroom growing house (MGH) and also may allow for an extended culture maintenance period due to acting as a reservoir of water. The novel media formulations in this study presented a spatially heterogenous distribution of nutrients and growth surfaces. Growth on the novel media formulations reinforced the study by Fomina, Ritz and Gadd in 2003 which demonstrated that fungi efficiently use both 'phalanx' (dense mycelia representing an associative [constraining, exploitative] growth strategy) and 'guerrilla' (sparsely-branched mycelia representing a dissociative [expansive, explorative] growth strategy) of the mycelial system.

5.4.6 Basidiome Production Using Tyndallised Prepared Spawn

Berny and Hennebert in 1991 used Tyndallisation to sterilize media by heating three times at 100 °C for 20 minutes with two resting periods of six hours. In this study the normal Tyndallisation procedure was modified in an attempt to produce good quality spawn. This was obtained by boiling for 30 minutes daily for 3 days and for 5 minutes boiling for 4 days. The 5 minutes boiling for 4 days Tyndallisation method is low on power consumption, requiring 28 minutes of boiling and is thus ideal for developing countries spawn laboratories, whereas the 30 minutes daily boiling for 3 days Tyndallisation method requires a total of two hours of boiling. The

production of oyster mushrooms when spawn was inoculated into banana substrate showed that the wheat grain sterilized by Tyndallisation has a level of sterility comparable to the sterile wheat grain produced by the conventional autoclaving. The recommended incubation period for spawn produced by Tyndallisation is one week. This process of modified Tyndallisation is ideal for production of oyster mushroom spawn that needs to be used immediately by the end user and not for commercial oyster mushroom spawn purposes due to the small quantities produced per container. This process can be adopted in developing countries to provide nutrition required by most impoverished people, by reducing the production cost of part of spawn production and thereby possibly boosting cultivation of oyster mushrooms.

5.5 OPTIMISATION OF CULTIVATION SUBSTRATE CONDITIONS

The characteristic of producing basidiomes at high temperatures is an ideal property for cultivation of oyster mushrooms in the tropical regions of the world (Marino *et al*., 2003). The *Pleurotus ostreatus* (traditionally produces basidiomes at low-temperature) strain in this study BCCM/MUCL 28511 is recommended as a possible candidate for cultivation in the tropics and sub-tropics due to the ability to produce basidiomes at elevated temperatures, a characteristic similar to *Pleurotus ostreatus* strain Pos 98/37 studied by Marino and co-researchers in 2003. Such strains as BCCM/MUCL 28511 may play a key role in continued production of *Pleurotus ostreatus* in a world where temperatures are likely to increase due to global climate change. Basidiome colour varies with temperature and light intensity (Stamets, 2000), however it may be important for impromptu identification by the oyster mushroom cultivators especially when high temperatures are employed for basidiome production (Bugarski, Gvozdenovic and Jovicevic, 1999).

The ability of the *Pleurotus* species in this study such as *P. ostreatus* and *P. sajor-caju* (white-rot fungi) to colonise and produce basidiomes on most of the substrates is due to a high mycelial impact attributed to secretion of a range of enzymes, most notably laccases and peroxidases enabling them to grow on a range of different substrates (Toyama and Ogawa, 1974; Soden and Dobson, 2003). This study as shown in Section 4.5 reinforces that *P. sajor-caju* is much more adaptable and able to grow and produce basidiomes on a wide variety of lignocellulosic wastes in which the nitrogen content may be highly variable such as growth on sage (*Coleochloa setifera*) having a carbon to nitrogen ratio of 112:1 and grass (*Agropyron* sp.) having a carbon to nitrogen ratio of 26:1 (Fu *et al*., 1997). The use of a wide range of substrates by the fungi has been found to be due to differential regulation of laccase gene expression in *P. sajor-caju*, with four different isozymes being regulated in response to copper, manganese, nutrient nitrogen and carbon (Soden and Dobson, 2003). Therefore, although manganese – dependent peroxidase (MnP) production in *P. sajor-caju* is partially suppressed by high nutrient nitrogen levels, the effect is not nearly as marked as in another important edible mushroom, *Lentinula edodes*, where no MnP activity was recorded over a 13-week experimental period (Fu *et al*., 1997).

Mushroom mycelium does not grow in a substrate that is either too dry or too wet. Water content of sage grass shows that a substrate can have a water content outside the recommended range of 70 to 80 percent (Stamets, 2000), but still have *Pleurotus* species and strains colonizing the substrate and producing basidiomes. Sage grass takes up water readily as shown by having the highest water content of 84.3 percent and yet the dry substrate had 7.6 percent whilst grass which started with 12.4 percent water content ended up with 74.4 percent.

Studies on substrate mixtures have traditionally involved various components in excess of two, for example Marino and co-researchers in 2003 used a substrate mixture of sawdust (49.8 percent), wheat bran (20 percent), rice bran (20 percent), sugarcane bagasse (10 percent) and lime (0.2 percent), Royse in 2002 used a substrate mixture of cottonseed hulls (75 percent dry weight), 24 percent chopped wheat straw and one percent ground limestone in a research on *Pleurotus cornucopiae*. This study has diverted from the traditional various components in excess of two substrate mixtures to use substrate mixtures comprising two components. A study that involved 1:1 substrates was done by Bugarski, Gvozdenovic, and Jovicevic in 1999, and they studied the following substrates: soybean straw, sunflower husks, wheat straw 50 percent + sunflower husks 50 percent, soybean straw 50 percent + sunflower husks 50 percent and wheat straw as a control using *Pleurotus ostreatus* strain NS-77. The present study involved different substrates from the Bugarski and co-researchers study apart from the control (wheat straw), but both studies concur in that other substrates can produce better results (basidiome production) than the commonly used wheat straw substrate in the cultivation of oyster mushrooms. In the Bugarski and co-researchers 1999 study, substrate combinations with sunflower husks gave significantly higher yield suggesting synergistic effects.

In the present study synergistic effects occurred with a particular strain producing basidiomes in a pure substrate and certain combinations with some substrates but failing to produce basidiomes in a combination with other substrates. For example strain 31613 did not produce basidiomes in grass but produced basidiomes in the banana plus grass mixture whilst strain 28511 produced basidiomes in both pure substrates banana and grass but failed to produce basidiomes when they were combined. This suggests substrate mixtures can induce or inhibit basidiome

production. Grass and sage seem to be the best substrates either singly or in combination with banana for both growth and fruiting. Since biological efficiency is strain and substrate specific (Stamets, 2000) the data obtained in this study can not be compared to other studies such as that of Hernandez and co-researchers in 2003 obtained biological efficiencies varying between 59.79 percent and 93 percent for *Pleurotus ostreatus* grown on a composted mixture of 70 percent grass (*Digitaria decumbens*) and 30 percent coffee pulp combined with 2 percent calcium hydroxide and biological efficiencies varying between 16.8 to 75.6 percent were obtained for strains of *Pleurotus djamor, Pleurotus ostreatus* and *Pleurotus pulmonarius* grown on barley straw by Salmones and co-researchers in 1997. However, the present study has identified strains that have a high biological efficiency on the substrates used in the present study which are readily available in Zimbabwe and most developing countries in the tropics. The data obtained in this study can be used by mushroom farmers where substrate quantities are limiting to combine various substrates for oyster mushroom cultivation.

5.6 GENETIC CHARACTERISATION

The internal transcribed spacer (ITS) region of rDNA PCR product size of approximately 800 base pairs obtained in this study is similar to that obtained by other researchers using the same primers (Martin *et al.*, 2004). There has been much discussion about the acceptability of bootstrap proportions as a measure of phylogenetic support (Sanderson, 1989; Bremer, 1994), Although Felsenstein (1985) advocated using traditional P values ($P \geq 0.95$ and $P \geq 0.90$) to indicate clade robustness based on boot-strapping, Hillis and Bull (1993) later suggested that much lower levels (to 0.70) may be acceptable and recently levels of > 0.50 have been used (Hofstetter *et al.*, 2002) as an indicator of branch robustness. Sequencing of the ITS

regions of ribosomal DNA of the *Pleurotus* species enabled differentiation between species and strains. The data obtained in this study has augmented the worldwide *Pleurotus* DNA database which aids in the identification of unidentified *Pleurotus* species by comparing the PCR amplified ITS sequences to those in the *Pleurotus* DNA database. This makes identification of the unknown species possible as for example, species identification of Chenopodiaceae (Zhou *et al*., 2007). This study has shown that the most frequent mutation within the ITS region of *Pleurotus sajor-caju* strains rDNA is a transition substitution from cytosine to thymine, and this may be due to presence of rare base analogues (Griffiths *et al*., 2002). The mutations obtained in this study may be due to DNA replication errors by DNA polymerase due to loop formation and errors in the addition of bases, especially in the non-coding regions of DNA such as the ITS region (Weising *et al*., 1995; Lodish *et al*., 2004). Strain specific primers may be designed using the variable regions (Vainio and Hantula, 2000) obtained in this study. The exclusive restriction enzymes identified may be used in a PCR-RFLP of rDNA strategy for identifying the respective strains. Other investigators using a combination of PCR and RFLP of rDNA region have determined the differentiation of strains or isolates (Henrion *et al*., 1994; Glen *et al*., 2001; Hughes *et al*., 2001). The clustering of the *Pleurotus sajor-caju* strains in this study with *Pleurotus pulmonarius* and other *Pleurotus sajor-caju* strains by BLAST analysis of DNA databases supports previous work that assigns *P. sajor-caju* strains as the cultivated strains of *P. pulmonarius* (Zervakis *et al*., 1994; Chiu *et al*., 1998). *Pleurotus pulmonarius* has been reported to have positive monokaryon mating crosses with *Pleurotus sajor-caju* (50-100 % compatibility) and *Pleurotus sapidus* (63-100 % compatibility). Thus, some of the *Pleurotus sajor-caju* strains are actually *Pleurotus pulmonarius* with other investigators saying *Pleurotus sajor-caju* strains are the

cultivated strains of *Pleurotus pulmonarius* whilst others have the notion that only some of the strains of *Pleurotus sajor-caju* are *Pleurotus pulmonarius* (Zervakis and Balis, 1996).

Bridge and co-researchers in 2003 published an article on the growing problem of taxonomic misidentification in the public DNA databases. In their study they attributed the causes to misidentified original cultures, differences in taxonomy between specialists, contamination by other fungi during culture and PCR-based errors. This study has produced DNA sequences that were classified in the *Pleurotus* genus as evident in Appendix G (BLAST search tree of all sequences in Genbank, EMBL and DDBJ) eliminating the possible misidentification of the original cultures up to the genus level. However, at the species level strain 31674 is controversial. Strain 31674 produced significant alignments with *P. ostreatus* and *P. sapidus.* Looking at the history of each strain (see Figure 21) all the other strains in this study were assigned to a certain individual before accession in the Belgian Coordinated Collections of Microorganisms/ Mycotheque de l'Université Catholique de Louvain (Agro) Industrial Fungi and Yeasts Collection. The controversy in taxonomic identification of strain 31674 may be due to an initial misidentification of the strain at the respective institute or erroneous switching of cultures from one species to another with the culture submitted to BCCM/MUCL being either a *P. ostreatus* or *P. sapidus* strain. It may be necessary in future due to the problem of misidentification (Bridge *et al.*, 2003) for submissions in DNA databanks to include the history of the strain if the source organism is from a culture collection.

The clustering of the strains in this study demonstrates that though rDNA phylogenies can be used for understanding patterns of evolution and speciation as in the Vilgalys and Sun (1994) study, their use in this context within a species that is

globally cultivated has to be taken with caution. In this study geographical boundaries between the *Pleurotus* strains are not strict, as rare events of long distance dispersal may have occurred with strains originating from as far apart as Mauritius and Thailand being grouped into the same cluster. This study demonstrates that determining phylogeny of a cultivated edible species in the context of biogeography may be problematic as the original source culture deposited in the culture collections may have been developed from an imported basidiome bought at a market place. Chiu and co-researchers in 1998 studied 19 cultivated strains of shiitake (*Lentinula edodes*) used in China by PCR and discovered genetic homogeneity of the cultivated strains, contrary to the claims by the Chinese agricultural institutes that the cultivated strains were different strains. The investigators proposed that institutes in China may readily exchange cultures and the receiving institute assigns a different strain number since intellectual property rights are not a major concern in China (Chiu *et al*., 1998). This study has shown a high level of genetic variability of strains from any single country of origin as shown in Section 4.6.3, thus the possibility of exchange of cultures is minimised.

Adaptation to natural conditions is considered to be the main factor causing the divergence of natural *Pleurotus* populations and eventually allopatric speciation (Shnyreya and Shtaer, 2006). The data obtained in this study suggests that phylogenetic analysis of globally cultivated edible mushroom species, such as *Pleurotus* may be done by utilising strains that have a history of being collected from the natural habitat with a history of transfer from individual to individual before accession in a culture collection, and avoiding strains such as those developed from spawn seed or from a specimen bought at a market place. In a first broad systematic treatment study of the euagarics as they have recently emerged in phylogenetic

systematics (Hibbett and Thorn, 2000), Moncalvo and co-researchers in 2000 produced one hundred and seventeen clades of the euagarics. In the 2000 Moncalvo study *Pleurotus citrinopileatus*, *P. floridanus* and *P. sajor-caju* were not included. Thus this study adds to the body of knowledge on the systematics of *Pleurotus*.

The present study has demonstrated the high genetic variability within the species *P. sajor-caju*, and hence considerable breeding potential for the development of qualitatively improved cultivars by exploitation of the genetic variability between genotypes. Sequencing of the internal transcribed spacer regions of rDNA of *Pleurotus* species and strains has been able to resolve the clusters of strains and to separate *P. citrinopileatus*, *P. ostreatus* and *P. floridanus* from *P. sajor-caju* strains. However the taxonomic position of strain 31674 is still uncertain.

5.7 GENERAL DISCUSSION

An additional economic burden becomes inevitable in countries continually struggling against disease and malnutrition (Hossain *et al*., 2003). Although mushrooms are increasingly being recognised as important food products for their significant role in human health, nutrition and disease, their consumption in many developing countries is extremely limited (Hossain *et al*., 2003). The most important medicinal mushrooms include the Reishi (*Ganoderma lucidum*), Shiitake (*Lentinus edodes*), Maitake (*Grifola frondosa*), Jews ear (*Auricularia auricular*) and oyster mushrooms (*Pleurotus* species) (Sadler, 2003). Properties of *Pleurotus ostreatus* and *P. sajor-caju* have significantly increased their commercial value in the last years (Marino *et al*., 2003). Owing to their considerable enzymatic versatility *P. ostreatus* and *P. sajor-caju* have also become the focus of increasing attention for their possible utility in biobleaching and bioremediation applications (Yara *et al*., 2006). *Pleurotus ostreatus* satisfactorily colonised non-sterilised soils (sand, peat [forest] and basalt and marl

mixed till [field]) as an evaluation of its potential use in soil bioremediation applications (McErlean, Marchant and Banat, 2006). A water-soluble extract from *P. ostreatus* produced cytotoxicity on human androgen ribonuclease-independent prostrate cancer PC-3 cells (Gu and Sivam, 2006) and anti-HIV properties of *P. ostreatus* have been demonstrated (Baker, 2002). *P. ostreatus* has also been shown to have antiproliferative activity toward leukaemia L1210 cells (Xia, Chu and Ng, 2005).

Pleurotus ostreatus is extensively produced in localities with average temperature of 15 °C (Marino *et al*., 2003). There is, however, a variety of *P. ostreatus* from Thailand, which grows in temperatures above 25 °C and is considered to be *P. ostreatus* var. Florida. This study has identified a *Pleurotus ostreatus* strain from Belgium that is able to grow at elevated temperatures of 30 °C. This study has produced data that may be applied to increase the global production of the economic and medicinally important oyster mushrooms especially in the developing countries of the world and arid environments. This can be exemplified by the testing and successful cultivation of strain BCCM/MUCL 28511 (*P. ostreatus*) in Buhera district in Manicaland province of Zimbabwe by mushroom farmers, a region that is in agro-ecological regions III, IV, and V characterised by mid-season dry spells and high temperatures (region III), periodic seasonal droughts and severe dry spells during the rainy season (region IV) and receives erratic rainfall (region V) with summer temperatures and winter temperatures for Buhera district being 27.5 to 32.5 °C (summer) and 22.5 to 27 °C (winter) (Marongwe *et al*., 1998). The data obtained in this study may also be useful in the case when global climatic temperatures increase due to global warming by providing a nutritional food source at elevated temperatures in the form of oyster (*Pleurotus*) mushrooms. Knowing the temperature parameters enables the mushroom cultivator to speed or slow development. Lower temperatures

can be used to postpone or lengthen the harvesting period and allow for maximum quality control (Stamets and Chilton, 1983). High temperatures serve to shorten the cropping period by promoting rapid, intense flushes (Stamets and Chilton, 1983).

Biodegradation is essential for cycling carbon, nitrogen, phosphorous, oxygen, and several other elements in the biosphere (Graves, 1995). Bioremediation is the application of biodegradative processes to the treatment of undesirable wastes for the purpose of restoring, reclaiming, or remediating soil, sediment, air, and water that has been negatively impacted by the presence of wastes (Graves, 1995). Among microorganisms that degrade the lignocellulosic compounds of wood, only white-rot filamentous fungi such as *Phanerochaete chrysosporium, Pleurotus ostreatus, P. sajor-caju*, *Phlebia radiate* and *Trametes versicolor* (Gianfreda, Xu and Bollag, 1999; Tekere *et al*., 2001a, b), mineralise lignin to carbon – dioxide and water. The enzymatic lignolytic system of white – rot fungi which is essential for such processes as biodegradation and bioremediation includes lignin peroxidase (LiP), manganese – dependant lignin peroxidase (MnP) and laccase (Lomascolo *et al*., 2001). Laccases have numerous biotechnological applications including pulp biobleaching and waste water treatment in the paper industry (Luisa, Gonclaves and Steiner, 1996), synthesis of polymers by reticulation of phenolic compounds (Figueroa – Espinoza and Rouau, 1998) and soil bioremediation (Rama *et al*., 1998). The practical application of laccase in biotechnology requires large quantities of enzyme (Lomascolo *et al*., 2001); hence the data for optimal growth of the *Pleurotus* species obtained in this study may be employed to enhance the biotechnological applications of *Pleurotus ostreatus* and *Pleurotus sajor*-caju. The growth data may also be utilised by researchers and oyster mushroom cultivators. High mycelial growth rate is related to insusceptibility to the green molds belonging to the genus *Trichoderma* (Sharma and Vijay, 1996) is

positively correlated with basidiome yield (Larraya *et al*., 2002) and thus is an important characteristic for selection in oyster mushroom breeding programs. This study has identified high growth rate strains that can be used to reduce *Trichoderma* colonisation of substrates during oyster mushroom cultivation. The study presents for the first time a comprehensive application and modification of Tyndallisation in *Pleurotus* species cultivation that may be employed for the increased production of oyster mushrooms, especially in the developing countries of the world to provide alternative relish and the much-needed nutrition particularly for HIV/AIDS patients.

Analysis of phenotypic diversity is a powerful tool for both the taxonomic classification and ecophysiological characterisation of heterogenous taxa (Rainieri *et al*., 1999). In exploring potential ecological significance and developing the industrial and biotechnological potential of species, identification and classification of the fungal species is an important step (Martin *et al*., 2004). Matching molecular phylogeny data on *Pleurotus* to the growth responses data shows general agreement, but there are some conflicts. This study has generated data that demonstrates that assessment of genetic and phenotypic diversity is necessary to confidently distinguish genotypes of *Pleurotus sajor-caju* when seeking traits of interest and to identify strains with high yield potential. It is widely considered that genetic diversity within a species can be correlated with fitness of that species (Avery, 2005). Diversity enhances the chances of a species persisting during change, such as may arise from environmental perturbations (Avery, 2005). With global climatic change occurring, *Pleurotus* species seem fit to survive the likely increase in global temperatures. High genetic and phenotypic variability also ensure less production risk, especially, in cases of biotic or abiotic stress (Avery, 2005). The high level of phenotypic diversity obtained was reflected in the high level of genetic variability obtained in this study

and thus ensures less production risk for the *Pleurotus* species in this study. The study has shown the need to carefully select strains that have a history of transference from individual to individual and also that have been collected from the natural habitat when analysing phylogeny of fungal species that are globally cultivated.

Sustainable development is the maintenance of economic, social and industrial growth while preserving the integrity of the biosphere (Venturella, Zervakis and Raimondo, 2002). In addition to the income generated by wild edible mushrooms sales in local markets, cultivation and consumption of edible *Pleurotus* species growing on lignocellulosic wastes is an additional sustainable source of income for communities in developing countries of the world. The optimised cultivation of edible *Pleurotus* species will aid in the achievement of sustainable development in the developing parts of the world and will reduce incidents of mushroom poisoning by providing an additional source of mushrooms to the seasonal wild mushroom markets. As most of the *Pleurotus* strains in cultivation today require low temperatures (below 18 °C) for at least a few hours daily (Zervakis, Dimou and Balis, 1992), the property of the *Pleurotus* species and strains in this study to produce basidiomes of high organoleptic quality under elevated temperatures (25 °C) is especially useful to cultivation in the tropics as the need to acquire air-conditioners or costly cooling equipment to lower temperatures to below 18 °C in the mushroom growing structures is reduced, together with the risk of serious losses because of unexpectedly warm weather.

6.0 CONCLUSIONS

This study has generated a wealth of information on the phenotypic and genetic diversity, optimal growth conditions, novel media formulations and substrate mixtures for *Pleurotus* species which can be applied to further harness the biotechnological potential of *Pleurotus* species. The optimal conditions identified in this study may enhance worldwide production of oyster mushrooms, especially in the developing countries. Polyethylene glycol 6000, potassium chloride and sodium chloride at minute concentrations are recommended as growth enhancers in *Pleurotus* spawn and cultivation procedures. This study has identified alternative substrates (maize grain and maize cobs) and developed modified protocols for oyster mushroom spawn production which may assist in the increased production of oyster mushrooms. Low-cost novel media formulations have also been developed that may be used as low-cost alternatives to culture *Pleurotus* species. Modified Tyndallisation protocols for sterilising wheat grain for spawn production have been developed and may aid in the increased uptake of oyster mushroom cultivation, particularly in the developing regions of the world. Substrate combinations that result in high yields have been identified in this study and strains with a high biological efficiency have also been identified. The use of molecular techniques brought out the need to choose strains for phylogenetic analysis by noting their history and their substrate or habitat of origin in addition to the common criteria of biogeography. BCCM/MUCL strain 31017 is overall recommended for spawn and cultivation purposes due to the high growth rate, high biological efficiency, moderate xerotolerance and production of primordia under diverse environmental conditions. Optimisation of the growth conditions for *Pleurotus* species has been achieved and the species and strains have been

successfully genetically characterised by DNA sequencing of the internal transcribed spacer region of ribosomal DNA.

- Strains 28511 (*P. ostreatus*), 28685, 31017, 31613, 38047 (*P. floridanus*), 38076, 38078, 38091 and 38092 have been identified as high growth rate strains.
- A high temperature *P. ostreatus* strain has been identified which has potential application as a candidate strain for cultivation in the tropics.
- The optimum micro concentrations of combined KCl and NaCl solutes of 2.70 g l^{-1} KCl: 1.08 g l^{-1} NaCl or 3.85 g l^{-1} KCl: 1.54 g l^{-1} NaCl at 20 °C; and 1.50 g l^{-1} KCl: 0.60 g l^{-1} NaCl or 2.70 g l^{-1} KCl: 1.08 g l^{-1} NaCl for both 25 °C and 30 °C; of PEG 6000 of 0.90 g l^{-1} (20 °C) and 0.65 g l^{-1} (25 and 30 °C) have been determined and these can be employed to enhance the growth of *Pleurotus* strains for biotechnological applications.
- This study has shown that wheat grain with water content below the minimum recommended 45 percent results in good quality spawn.
- The formulae for water content of wheat grain developed in this study may be used to standardise the amount of water added during production of oyster mushroom spawn.
- Optimal protocols for production of maize grain, pumpkin seeds and maize cobs oyster mushroom spawn were developed which can assist in the rapid uptake of oyster mushroom cultivation in the developing countries of the world.
- Optimal (1:3 to 1:5 [whole wheat grain PDA/MEA] and 1:3 to 1:15 [crushed wheat grain: distilled water ± PDA]) novel media formulations were

developed which have potential applications in short-term culture maintenance, spawn production, bioremediation and ecological regeneration.

- This study for the first time comprehensively applied Tyndallisation to the production of spawn and developed optimal modified Tyndallisation protocols using 30 minutes boiling for three days (wide-mouthed bottles) and 5 minutes boiling for four days (narrow-mouthed bottles). These Tyndallisation protocols have potential in augmenting oyster mushroom spawn production for *Pleurotus* species cultivation in the developing countries of the world.
- This study has identified grass and sage singly and in combination with banana fronds as the best alternative substrates to wheat straw for cultivation of *Pleurotus* species.
- Synergistic effects of substrates on basidiome production by *Pleurotus* species have been demonstrated by this study.
- This study has generated internal transcribed spacer DNA sequence data of *Pleurotus* strains all of which had not yet been sequenced.
- The ITS sequence data demonstrated the need to include such criteria as specimens collected from a natural habitat and proper history of the strain for phylogenetic studies.
- Strain BCCM/MUCL 31674 ('*P. sajor-caju*') was shown to be closely related to *P. ostreatus* and thus its taxonomic status requires further work.

7.0 FUTURE RESEARCH

This study has pioneered the study of the effects of micro-concentrations of solutes on the growth of *Pleurotus* species. Future studies are necessary to determine the active enzyme molecules and mRNA profiling of the *Pleurotus* species grown under different micro-concentrations of polyethylene glycol 6000, potassium chloride and sodium chloride as a means of gaining an insight into why the growth rate of *Pleurotus* species increases in such a manner obtained in this study. In this aspect also nutritionally heterogenous environments such as the agar droplet tessellations method used by Jacobs and co-researchers in 2002 can also be employed for further determining the effects of PEG on growth of *Pleurotus* species and strains in a nutritionally heterogeneous environment. Future work may also look at carbon-dioxide specificity of primordia of the strains and also measure relative humidity changes at the substrate-mycelial interface when the air relative humidity is low. Research on how feasible Tyndallisation is in production of spawn in a situation that requires transportation of the spawn to mushroom farmers should be researched. A physiological study of the *Pleurotus* species grown on various substrate mixtures may aid in understanding the synergistic effects of substrate combinations. A genetic analysis of more strains and different DNA regions may elucidate in more detail the taxonomic placing of strains such as BCCM/MUCL strain 31674.

8.0 REFERENCES

Ainsworth, G. C. (1971) *Ainsworth and Bisby's Dictionary of Fungi, including the Lichens*. International Publications Service, New York

Ainsworth, G. C. (1973) Introduction and Keys to higher taxa. In *The Fungi: An Advanced Treatise* **IVB, 1-7** (G. C. Ainsworth, F. K. Sparrow and A. S. Sussman, eds), Academic Press, New York

Ainsworth, G. C., Sparrow, F. K. and Sussman, A. S. (1965 – 1973) *The Fungi*, 4 vols. Academic Press, New York.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Anderson, N. A., Wang, S. S. and Schwandt, J. W. (1973) The *Pleurotus ostreatus-sapidus* species complex. *Mycologia* **65**: 28-35.

Ansorge, W., Voss, H. and Zimmermann, J., eds (1997) *DNA Sequencing Strategies*: *automated and advanced approaches*. John Wiley and Sons, New York.

Austin, A.T., Yahdjian, L., Stark, J.M, Belnap, J., Porporato, A., Norton, U., Ravetta, D.A., and Schaeffer, S.M. (2004) Water pulses and biogeochemical cycles in arid and semiarid ecosystems. *Oecologia* **141:** 221-235.

Avery, S. V. (2005) Phenotypic diversity and fungal fitness. *Mycologist* **19**: 74-80.

Ayres, P. G. (1977) Effect of water potential of pea leaves on spore production by *Erysiphe pisi* (Powdery Mildew). *Transactions of the British Mycological Society* **68**: 97–100.

Badham, E. R. (1989) Influence of water potential on growth of shiitake mycelium. *Mycologia* **81**: 464–468.

Baker, L. (2002) Anti-HIV properties of oyster mushrooms (how mushrooms will save the world) Salon Media Group, Inc, http://www.salon.com.

Bell, M., Murphy, P., Parker, P. and Ridge, I. (1993) Plants and water. In *Plant Physiology* (I. Ridge, ed), Hodder and Stoughton, The Open University, U.K.

Bernicchia, A., Fugazzola, M. A., Gemelli, V., Mantovani, B., Lucchetti, A., Cesari, M. and Speroni, E. (2006) DNA recovered and sequenced from an almost 7000 y-old Neolithic polypore, *Daedaleopsis tricolor*. *Mycological Research* **110**: 14-17.

Berny, J. F. and Hennebert, G. L. (1991) Viability and stability of yeast cells and filamentous fungus spores during freeze-drying: effects of protectants and cooling rates. *Mycologia* **83**: 805-815.

Bishop, M. (1995) Sequence analysis. In *Molecular Biology and Biotechnology*: *A comprehensive desk reference* (R. A. Meyers, ed), Wiley-VCH, New York.

Blackwell, M., Vilgalys, R. and Taylor, J. W. (2005) Fungi, Eumycota: mushrooms, sac fungi, yeast, molds, rusts, smuts, etc. Version 14 February 2005 (under construction). http://tolweb.org/Fungi/2377/2005.02.14 in The Tree of Life Web Project, http://tolweb.org/

Blakeslee, A. F. (1915) Lindner's roll tube method of separation cultures, *Phytopathology* **5**: 68-69.

Bliss, C. I. (1967) *Statistics in Biology.* Vol I. McGraw-Hill, New York.

Block, S. S., Tsao, G. and Han, L. H. (1959) Experiments in the cultivation of *Pleurotus ostreatus*. *Mushroom Science* **4**: 309-325.

Boddy, L. (1983) Effect of temperature and water potential on growth rate of wood – rotting basidiomycetes. *Transactions of the British Mycological Society* **80**: 141–149.

Boddy, L. (1986) Water and decomposition processes in terrestrial ecosystems. In *Water, Fungi and Plants* (P.G. Ayres and L.Boddy, eds), Cambridge University Press, Cambridge.

Booth, C. (1971) In *Methods in Microbiology* (C. Booth, ed) Vol 4, Academic Press, New York.

Braithwaite, K. S. and Manners, J. M. (1989) Human hypervariable minisatellite probes detect DNA polymorphisms in the fungus *Colletotrichum gloeosporioides*. *Current Genetics* **16**: 473-475.

Bremer, K. (1994) Branch support and tree stability, *Cladistics* **10**: 295-304.

Bresinsky, A., Fischer, M., Meixner, B. and Paulus, W. (1987) Speciation in *Pleurotus*. *Mycologia* **79**: 234-245.

Bridge, P. D., Spooner, B. M., Roberts, P. J., Panchal, G. (2003) On the unreliability of published DNA sequences. *New Phytologist* **160**: 43-48.

Brown, A.D. (1990) *Microbial Water Stress Physiology- Principles and Perspectives.* Wiley, Chichester, pp. 20.

Brownell, K. H. and Schneider, R. W. (1985) Roles of matric and osmotic components of water potential and their interaction with temperature in the growth of *Fusarium oxysporum* in synthetic media and soil. *Phytopathology* **75**: 53-57.

Brownlee, C. and Jennings, D. H. (1981) Further observations on tear or drop formations by mycelium of *Serpula lacrimans*. *Transactions of the British Mycological Society* **77**: 33-40.

Bruns, T. D., White, T. J. and Taylor, J. W. (1991) Fungal molecular systematics. *Annual Review of Ecology and Systematics* **22**: 524-564.

Bugarski, D., Gvozdenovic, D. J. and Jovicevic, D. (1999) Influence of substrates on fructification of oyster mushroom strain NS-77 (*Pleurotus ostreatus*). In *Balkan Symposium on Vegetables and Potatoes. International Society for Horticultural Science (ISHS) Acta Horticulturae* **579**.

Camarero S., Barrasa J.M., Pelayo M., and Martinez A.T. (1998) Evaluation of *Pleurotus* species for wheat-straw biopulping. *Journal of Pulp and Paper Science* **24**: 197-203.

Cha, R. S. and Thilly, W. G. (2003) Specificity, efficiency and fidelity of PCR. In *PCR Primer*: *A laboratory manual* (C. W. Dieffenbach and G. S. Dveksler, eds), Cold Spring Harbor Laboratory Press, New York.

Chang, S. T., and Hayes, W. A. (1978) *The Biology and Cultivation of Edible Mushrooms*. Academic Press, New York.

Chang, S. T. and Miles, P. G. (1989) *Edible mushrooms and their cultivation*. CRC Press, Inc., Boca Raton, FL.

Chiu, S. W., Chan, Y. H., Law, S. C., Cheung, K. T. and Moore, D. (1998) Cadmium and manganese in contrast to calcium reduce yield and nutritional values of the edible mushroom *Pleurotus pulmonarius*. *Mycological Research* **102**: 449-457.

Cho, S. B. (2004) What is Mushroom, In *Oyster Mushroom Cultivation*. MushWorld, Seoul, Korea.

Christian, J. H. B. (1980) *Microbial Ecology of Foods: Factors affecting Life and Death of microorganisms*. Agarikon Press, Washington.

Cihangir, N., and Saglam, N. (1999) Removal of cadmium by *Pleurotus sajor-caju* basidiomycetes. *Acta Biotechnologica* **19:** 171-177.

Clarke, R. W., Jennings, D. H. And Coggins, C. R. (1980) Growth of *Serpula lacrimans* in relation to water potential of substrate. *Transactions of the British Mycological society* **75**: 271–280.

Cochrane, V. W. (1958) *Physiology of Fungi*. John Wiley and Sons, New York.

Coggins, C. R., Jennings, D. H. and Clarke, R. W. (1980) Tear or drop formation by mycelium of *Serpula lacrimans*. *Transactions of the British Mycological Society* **75**: 63-67.

Colotelo, N. (1973) Physiological and biochemical properties of the exudate associated with developing sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary. *Canadian Journal of Microbiology* **19**: 73-79.

Colotelo, N. (1978) Fungal exudates. *Canadian Journal of Botany* **24**: 1173-1181.

Colotelo, N., Sumner, J. L. and Voegelin, W. S. (1971) Chemical studies on the exudate and developing sclerotia of *Sclerotinia sclerotionum* (Lib.) de Bary. *Canadian Journal of Microbiology* **17**:1189-1194.

Corner, E. J. H. (1981) The agaric genera *Lentinus, Panus* and *Pleurotus*. Beih. *Nova Hedwigia* **69**: 169pp.

David, H. A. (1952) Upper 5 and 1 % points of the maximum F-ratio. *Biometrika* **39**: 422-424.

Dieffenbach, C. W. and Dveksler, G. S. (2003) *PCR Primer*: *A laboratory manual*, Cold Spring Harbor Laboratory Press, New York.

Dix, N. J. and Frankland, J. C. (1987) Tolerance of litter-decomposing agarics to water stress in relation to habitat. *Transactions of the British Mycological Society* **88**: 127-129.

Domomdon, D. L., He, W., De Kimpe, N., Hofte, M. and Poppe, J. (2004) Beta-adenosine, a bioactive compound in grass chaff stimulating mushroom production. *Phytochemistry* **65**: 181-187.

Donk, M. A. (1962) The generic names proposed for Agaricacea. *Beihafte Nova Hedwigia* **5**: 1-320.

Down, G. (2002) Fungal family trees-finding relationships from molecular data. *Mycologist* **16**: 51-58.

Eger, G., Eden, G. and Wissig, E. (1976) *Pleurotus ostreatus* – breeding potential of a new cultivated mushroom. *Theoretical Applied Genetics* **47**: 156-163.

Eger, G., Li, S. F and Leal-Lara, H. (1979) Contribution to the discussion on the species concept in the *Pleurotus ostreatus* complex. *Mycological Research* **71**: 577–588.

Felsenstein, J. (1985) Confidence limits of phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-791.

Ferrer M., Chernikova T.N., Yakimov M.M., Golyshin P.N., and Timmis K.N. (2003) Chaperonins govern growth of *Escherichia coli* at low temperatures. *Nature Biotechnology* **2:** 1266-1267.

Figueroa-Espinosa, M. C. and Rouau, X. (1998) Oxidative cross-linking of pentosans by a fungal laccase and horseradish peroxidase: mechanism of linkage between feruloylated arabinoxylans. *Cereal Chemistry* **75**: 259-265.

Flegg, P. B., Spencer, D. M. and Wood, D. A. (1985) *The Biology and Technology of the Cultivated Mushroom*. John Wiley and Sons, Inc., New Jersey.

Fomina, M., Ritz, K. and Gadd, G. M. (2003) Nutritional influence on the ability of fungal mycelia to penetrate toxic metal-containing domains. *Research* **107**: 861-871.

Fragoeiro, S. and Magan, N. (2005) Enzymatic activity, osmotic stress and degradation of pesticide mixtures in soil extract liquid broth inoculated with *Phanerochaete chrysosporium* and *Trametes versicolor*. *Environmental Microbiology* **7:** 348-355.

Fries, E. M. (1821) *Systema Mycologicum*. **1**: 520pp. Lund.

Fu, S. Y., Yu, H., Buswell, J. A. (1997) Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by *Pleurotus sajor-caju*. *FEMS Microbiology Letters* **147**: 133-137.

Gardes, M. and T. D. Bruns. (1993). ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113-118.

Gascuel, O. and Steel, M. (2006) Neighbour-joining revealed (review). *Molecular Biology and Evolution* **23**: 1997-2000.

Gianfreda, L., Xu, F. and Bollag, J. M. (1999) Laccases: a useful group of oxidoreductive enzymes. *Bioremediation Journal* **3**: 1-25.

Glen, M., Tommerp, I. C., Bougher, N. L. and O'Brien, P. A. (2001) Specificity, sensitivity and discrimination of primers for PCR – RFLP of larger basidiomycetes and their applicability to identification of ectomycorrhizal fungi in *Eucalyptus* forests and plantations. *Mycological Research* **105**: 138-149.

Gordon, W. L. (1952) *Canadian Journal of Botany* **30**: 209-251.

Gramss G., Ziegenhagen D., and Sorge S. (1999) Degradation of soil humic extract by wood- and soil-associated fungi, bacteria, and commercial enzymes. *Microbial Ecology* **37:** 140-151.

Graves, D. (1995) Biodegradation of organic wastes. In *Molecular Biology and Biotechnology*: *A comprehensive desk reference* (R. A. Meyers, ed), Wiley-VCH, New York.

Griffin, D. M. (1977) Water potential and Wood - decay fungi. *Annual Reviews Phytopathology* **15**: 319-329.

Griffiths, A. J. F., Gelbart, W. M., Lewontin, R. C., Miller, J. H. (2002) *Modern Genetic Analysis*. W. H. Freeman, New York.

Griffiths, A. J. F., Wessler, S. R., Lewontin, R. C., Gelbart, W. M., Suzuki, D. T., Miller, J. H. (2005) *An Introduction to Genetic Analysis*. W. H. Freeman, New York.

Gu, Y. H. and Sivam, G. (2006) Cytotoxic effect of oyster mushroom *Pleurotus ostreatus* on human androgen-independent prostrate cancer PC-3 cells. *Journal of Medical Food* **9**: 196-204.

Gyurko, P. (1972) Die rolle der belichtung bei dem Anbau des Austern seitlings (*Pleurotus ostreatus*). *Mushroom Science* **8**: 461-469.

Hallsworth, J.E., and Magan, N. (1995) Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiol.-SGM* **141:** 1109- 1115.

Hallsworth, J. E., and Magan, N. (1996). Culture age, temperature and pH affect the polyol and trehalose contents of fungal propagules. *Applied and Environmental Microbiology* **62**: 2435-2442.

Hallsworth, J. E., and Magan, N. (1999) Water and temperature relations of growth of three entomogenous fungi *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology* **74**: 261-266.

Hallsworth, J.E., and Nomura, Y. (1999) A simple method to determine the water activity of ethanol-containing samples. *Biotechnology and Bioengineering* **62:** 242-245.

Hallsworth, J.E., Heim, S., and Timmis, K.N. (2003a). Chaotropic solutes cause water stress in *Psuedomonas putida*. *Environmental Microbiology* **5:** 1270-1280.

Hallsworth, J.E., Prior, B.A., Iwahara, M., Nomura, Y. and Timmis, K.N. (2003b). Compatible solutes protect chaotrope (ethanol)-induced, nonosmotic water stress. *Applied and Environmental Microbiology* **69:** 7032-7034.

Han, Y. H., Chen, K. M. and Cheng, S. (1974) Characteristics and cultivation of a new *Pleurotus* in Taiwan. *Mushroom Science* **9**: 167-174.

Hawksworth, D. L. (1990) The fungal dimension of biodiversity: magnitude, significance and conservation. *Mycological Research* **95**: 641-655.

Henrion, B., Chevalier, G. and Martin, F. (1994) Typing trffle species by PCR amplification of the ribosomal DNA spacers. *Mycological Research* **98**: 37-43.

Hernandez, D., Sanchez, J. E. and Yamasaki, K. (2003) A simple procedure for preparing substrate for *Pleurotus ostreatus* cultivation. *Bioresource Technology* **90**: 145-150.

Hibbett, D. S. and Donoghue, M. G. (1995) Progress toward a phylogenetic classification of the Polyporaceae theough parsimony analyses of mitochondrial ribosomal DNA sequences. *Canadian Journal of Botany* **73** (supplement 1): S853-S861.

Hibbett, D. S. and Thorn, R. G. (1994) Nematode trapping in *Pleurotus tuberregium*. *Mycologia* **86**: 696-699.

Hibbett, D. S. and Thorn, R. G. (2000) Basidiomycota: Homobasidiomycetes. In *The Mycota Vol. VII. Part B. Systematics and Evolution* (D. J. McLaughlin, E. G. McLaughlin and P. A. Lemke, eds), Springer-Verlag, Berlin.

Hibbett, D. S. and Vilgalys, R. (1993) Phylogenetic relationships of *Lentinus* (Basidiomycota) inferred from molecular and morphological characters. *Systematic Botany* **18**: 409-433.

Hillis, D. M. and Bull, J. J. (1993) An empirical method of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**: 182-192.

Hills, D. M. and Dixon, M. T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly Review of Biology* **66**: 411 – 446.

Hobolth, A. and Yoshida, R. (2005) Maximum likelihood estimation of phylogenetic tree and substitution rates via generalized neighbour-joining and the EM algorithm. *Algebraic Biology*: 41-50.

Hoelzel, A. R. and Green, A. (1992) Analysis of population – level variation by sequencing PCR – amplified DNA. In *Molecular Genetic Analysis of Populations*: *A Practical Approach* (A. R. Hoelzel, ed), IRL Press, Oxford.

Hofstetter, V., Clémençon, H., Vilgalys, R. and Moncalvo, J. (2002) Phylogenetic analyses of the *Lyophylleae* (Agaricales, Basidiomycota) based on nuclear and mitochondrial rDNA sequences. *Mycological Research* **106**: 1043-1059.

Hopple, J. S. and Vilgalys, R. (1999) Phylogenetic relationships in the mushroom genus *Coprinus* and dark-spored allies based on sequence data from the nuclear gene coding for the large ribosomal subunit RNA: divergent domains, outgroups and monophyly. *Molecular Phylogenetics and Evolution* **13**: 1-19.

Hossain, S., Hashimoto, M., Choudhury, E. K., Alam, N., Hussain, S., Hasan, M., Choudhury, S. K. and Mahmud, I. (2003) Dietary mushroom (*Pleurotus ostreatus*) ameliorates atherogenic lipid in hypercholesterolaemic rats. *Clinical and Experimental Pharmacology and Physiology* **30**: 470-475.

Hudson, H.J. (1986) *Fungal Biology*. Edward Arnold, London.

Hughes, K. W., Petersen, R. H., Johnson, J. E., Moncalvo, J., Vilgalys, R., Redhead, S. A., Thomas, T. and McGhee, L. L. (2001) Infragenic phylogeny of *Collybia s. str.* Based on sequences of ribosomal ITS and LSU regions. *Mycological Research* **105**: 164-172.

Humphrey, C. J. (1933) Temperature relations of wood – destroying fungi. *Journal of Agricultural Research* **47**: 997 – 1008.

Huxman, T.E., Smith, M.D., Fay, P.A., Knapp, A.K., Shaw, M.R., Loik, M.E., Smith, S.D., Tissue, D.T., Zak, J.C., Weltzin, J.F., Pockman, W.T., Sala, O.E., Haddad, B.M., Harte J., Koch, G.W., Schwinning, S., Small, E.E., and Williams, D.G. (2004). Convergence across biomes to a common rain-use efficiency. *Nature* **429:** 651-654.

Iracabal, B., Zervakis, G. and Labarère, J. (1995) Molecular systematics of the genus *Pleurotus*: analysis of restriction length polymorphisms in ribosomal DNA. *Microbiology* **141**: 1479-1490.

Isikhuemhen, O. S., Moncalvo, J., Nerud, F. and Vilgalys, R. (2000) Mating compatibility and phylogeography in *Pleurotus tuberregium*. *Mycological Research* **104**: 732-737.

Jacobs, H., Boswell, G. P., Ritz, K., Davidson, F. A. and Gadd, G. M. (2002) Solubilisation of calcium phosphate as a consequence of carbon translocation by *Rhizoctonia solani*. *FEMS Microbiology Ecology* **40**: 64-71.

Jeffries P., Gianinazzi S., Perotto S., Turnau K., and Barea J.M. (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biology and Fertility of Soils* **37:** 1-16.

Jennings, D. H. (1991) The role of droplets in helping to maintain a constant growth rate of aerial hyphae. *Mycological Research* **95**: 883-884.

Jonsson L.J., Palmqvist E., Nilvebrant N.O., and Hahn-Hagerdal B. (1998) Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. *Applied Microbiology and Biotechnology* **49:** 691-697.

Kennedy, N. and Clipson, N. (2003) Fingerprinting the fungal community. *Mycologist* **17**: 158-164.

Kidd, F. A., Reid, C. P. P. and Davidson, R. W. (1977) Culture growth inhibition of *Ceratocystis montia* by induced water stress. *Mycologia* **69**: 615-618.

Kirk, P. M., Cannon, P. F., David, J. C. and Stalpers, J. (2001) *Ainsworth and Bisby's Dictionary of the Fungi*. CAB International, Wallingford, UK.

Kirsop, B. E. and Snell, J. J. (1984) *Maintenance of Microrganisms: A manual of laboratory methods*, Academic Press, London.

Koske, R. E. and Tessier, B. (1986) Growth of some wood and litter-decay Basidiomycetes at reduced water potential. *Transactions of the British Mycological Society* **86**: 156-158.

Kües, U. and Liu, Y. (2000) Fruiting body production in basidiomycetes. *Applied Microbiology and Biotechnology* **54**: 141-152.

Kwok, S. and Higuchi, R. (1989) Avoiding false positives with PCR. *Nature* **339**: 237-238.

Kwok, O. C. H., Plattner, R., Weisleder, D. and Wicklow, D. T. (1992) A nematicidal toxin from *Pleurotus ostreatus* NRRL 3526. *Journal of Chemical Ecology* **18**: 127-136.

Labuschagne, P. M., Eicker, A., Aveling, T. A. S., de Meillon, S. and Smith, M. F. (2000) Influence of wheat cultivars on straw quality and *Pleurotus ostreatus* cultivation. *Bioresource Technology* **71**: 71-75.

Larraya, L. M., Idareta, E., Arana, D., Ritter, E., Pisabarro, A. G. and Ramirez, L. (2002) Quantitative trait loci controlling vegetative growth rate in the edible basidiomycete *Pleurotus ostreatus*. *Applied and Environmental Microbiology* **68**: 1109-1114.

Lee, J. C., Cole, M. and Linacre, A. (2000). Identification of members of the genera *Paneolus* and *Psilocybe* by a DNA test: A preliminary test for hallucinogenic fungi. *Forensic Science International* **112**: 123-133.

Lee, S. B. and Taylor, J. W. (1990) Isolation of DNA from fungal mycelia and single spores. In *PCR Protocols: A guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, eds), Academic Press, San Diego.

Li, S. and Eger, G. (1978) Characteristics of some *Pleurotus* strains from Florida, their practical and taxonomic importance. *Mushroom Science* **10**: 155-169.

Lindahl, B. D. and Olsson, S. (2004) Fungal translocation – creating and responding to environmental heterogeneity. *Mycologist* **18**: 79-88.

Lindblad, I. (2000) Host specificity of some wood-inhabiting fungi in a tropical forest. *Mycologia* **92:** 399-405.

Lockwood, J. L. (1992) Exploitation competition. In *The Fungal Community: Its organization and role in the ecosystem* (G. C. Carroll and D. T. Wicklow, eds), Marcel Dekker, New York.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., Zipursky, L., Darnell, J. (2004) *Molecular Cell Biology*. W. H. Freeman, New York.

Lomascolo, A., Cayol, J., Roche, M., Guo, L., Robert, J., Record, E., Lesage-Meessen, L., Ollivier, B., Sigoillot, J. and Asther, M. (2001) Molecular clustering of *Pycnoporus* strains from various geographic origins and isolation of monokaryotic strains for laccase hyperproduction. *Mycological Research* **106**: 1193-1203.

Luisa, M., Gonclaves, C. and Steiner, W. (1996) Use of laccase for bleaching of pulps and treatment of effluents. *Enzyme Pulp and Paper Process* **665**: 197-206.

Madar Z. and Zusman I. (1997) The role of dietary factors in prevention of chemically-induced cancers. *International Journal of Oncology* **11:** 1141-1148.

Magan, N. (1988) Effects of water potential and temperature on spore germination and germ-tube growth in vitro and on straw leaf sheaths. *Transactions of the British Mycological Society* **90**: 97-107.

Magan, N. and Lacey, J. (1988) Ecological determinants of mould growth in stored grain. *International Journal of Food Microbiology* **7**: 245-256.

Margulis, L. and Schwartz, K. V. (1988) *Five Kingdoms*: *An illustrated guide to the phyla of life on Earth*. W. H. Freeman, New York.

Marino, R. H., da Eira, A. F., Kuramae, E. E. and Queiroz, E. C. (2003) Morphomolecular characterization of *Pleurotus ostreatus* (Jacq. Fr.) Kummer strains in relation to luminosity and temperature of fructification. *Scientia Agricola* **60**: 531-535.

Marongwe, N., Made, J.M., Dengu, E., Mukahanana, M., Vhudzijena, V. and Moyo, M, (1998) Land Resources. In *The State of Zimbabwe's Environment 1998* (M. Chenje, L. Sola and D. Paleczny, eds), Government of the Republic of Zimbabwe, Ministry of Mines, Environment and Tourism, Harare.

Martin, P., Muruke, M., Hosea, K., Kivaisi, A., Zerwas, N. and Bauerle, C. (2004) A rapid PCR-RFLP method for monitoring genetic variation among commercial mushroom species. *Biochemistry and Molecular Biology Education* **32**: 390-394.

McErlean, C., Marchant, R. and Banat, I. M. (2006) An evaluation of soil colonisation potential of selected fungi and their production of ligninolytic enzymes for use in soil bioremediation applications. *Antonie Van Leeuwenhoek* **90**: 147-158.

McGonigle, T.P. (1995) The significance of grazing on fungi in nutrient cycling. *Canadian Journal of Botany- Revue Canadienne Botanique* **73:** S1370-S1376 Suppl. 1 E-H.

McPhee, W. J. and Colotelo, N. (1977) Fungal exudates I. Characteristics of hyphal exudates in *Fusarium culmorum*. *Canadian Journal of Botany* **55**:358-365.

Medina A., Probanza A., Manero F.J.G., and Azcon R. (2003) Interactions of arbuscular-mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin). *Applied Soil Ecology* **22:** 15-28.

Mehtra, S. K., Dayal, H. M. and Agarwal, P. N. (1977) *Indian Journal of Experimental Microbiology* **15**: 81-82.

Metzenberg, R. L. (1991) Benefactors' lecture: the impact of molecular biology on mycology. *Mycological Research* **95**: 9-13.

Michel, B. E. and Kaufmann, M. R. (1973) The osmotic potential of polyethylene glycol 6000. *Plant Physiology* **51**: 914-916.

Moncalvo, J. M., Lutzoni, F., Rehner, S. A., Johnson, J. and Vilgalys, R. (2000) Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Systematic Biology* **49**: 278-305.

Money, N. P., Davis, D. J., and Burlak, C. (2000) Osmotic pressure of fungal compatible osmolytes. *Mycological Research* **104**: 800-804.

Morris, C. F. and Rose, S. P. (1996) Wheat. In *Cereal Grain Quality* (R. J. Henry and P. S. Kettlewell, eds). Chapman Hall, London.

Mswaka, A.Y, and Magan, N. (1998) Wood degradation, and cellulose and ligninase production, by *Trametes* and other wood-inhabiting basidiomycetes from indigenous forests of Zimbabwe. *Mycological Research* **102:** 1399-1404.

Mswaka ,A. Y. and Magan, N. (1999) Temperature and water potential relations of tropical *Trametes* and other wood-decay fungi from the Indigenous forests of Zimbabwe. *Mycological Research* **103**: 1309-1317.

Muir, G. and Schlötterer, C. (1999). Limitations to the phylogenetic use of ITS sequences in closely related species and populations- a case study in *Quercus petraea* (Matt.) Liebl. In *Which DNA Marker for which purpose? Final Compendium of the Research Project Development, optimisation and validation of Molecular tools for assessment of biodiversity in forest trees in the European Union DGX11 Biotechnology FW IV Research Programme Molecular Tools for Biodiversity.* (E. M. Gillet, ed.), URL http://webdoc.sub.gwdg.de/ebook/y/1999/whichmarker/index.htm

Neda, H. and Nakai, T. (1995) Phylogenetic analysis of *Pleurotus* based on data from partial sequences of 18SrDNA and ITS – 1 regions. *Proceedings of the 14th International Congress on the Science and Cultivation of Edible Fungi* (T. J. Elliott, ed) *17-22 September, Oxford, United Kingdom*: **Volume 1**: 161-168.

Oei, P. (1991) *Manual on Mushroom Cultivation:Techniques, species and Opportunities for Commercial Application in Developing Countries*. TOOL (Transfer of Technology for Development) Publications, Amsterdam.

Ogden, A and Prowse, K. (2004) How to make oyster mushroom grain spawn in a simple way. In *Oyster Mushroom Cultivation: Mushroom growers handbook 1* (MushWorld), Heineart Inc, Korea.

Onions, A. H. S. (1971). In *Methods in Microbiology* (C. Booth, ed) Vol 4, Academic Press, New York.

Parmatso, E. (1987) The species problem in fungi. In *Problems of Species and Genus in Fungi* (E. Parmatso, ed) (English Summary) *Scripta Mycologia* **15**: 25-28.

Patouillard, N. (1887) *Les Hyménomycètes d'Europe: Anotomie générale et classification des champignons supérieurs*. Librairie Paul Klincksieck, Paris.

Patouillard, N. (1900) *Essai taxonomique sur les familles et les genres d'Hyménomycètes.* Lons-le-Saunier, Paris.

Peberdy, J. F. (1995) Fungal protoplasts. In *The Mycota, A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research Volume II* (U. Kück, ed), Springer-Verlag, Berlin.

Pegler, D. N. (1975) *Pleurotus* (Agaricales) in India, Nepal and Pakistan. *Kew Bulletin* **31**: 501-510.

Pegler, D. N. (1983) The genus *Lentinus*. *Kew Bulletin (Additional Series)* **10**: 281pp.

Petersen, R. H. (1995) Contributions of mating studies to mushroom systematics. *Canadian Journal of Botany* **73**: 831-842.

Petersen, R. H. and Hughes, K. W. (1993) Intercontinental interbreeding collections of *Pleurotus pulmonarius*, with notes on other species. *Sydowia* **45**: 139-152.

Petersen, R. H. and Krisai – Greilhuber, I. (1996) An epitype specimen for *Pleurotus ostreatus*. *Mycological Research* **100**: 229-235.

Petersen, R. H., Hughes, K. W. and Psurtseva, N. (internet) *Biological Species in Pleurotus* http://fp.bio.utk.edu/Mycology/Pleurotus/default.htm.

Quélet, L. (1872-1876) Les Champignons du jura et des Vosges. *Mem. Soc. Emul. Montbeliard* (**Part 1, 1872).**

Quimio, T. H., Chang, S. T. and Royse, D. J. (1990) Technical Guidelines for Mushroom Growing in the Tropics*: FAO Plant production and Protection Paper* **106**, Rome.

Rainieri, S., Zambonelli, C., Hallsworth, J. E., Pulvirenti, A. and Giudici, P. (1999) *Saccharomyces uvarum*, a distinct group within *Saccharomyces sensu stricto, FEMS Microbiology Letters* **177**: 177-185.

Rajarathnam, S., Shashireka, M.N., and Bano, Z. (1998) Biopotentialities of the basidiomycetes. *Advances in Applied Microbiology* **37:** 233-361.

Rama, R., Mougin, C., Boyer, F. D., Kollmann, A., Malosse, C. and Sigoillot, J. C. (1998) Biotransformation of benzo[a]pyrene in bench scale reactor using laccase of *Pycnoporus cinnabarinus*. *Biotechnology Letters* **20**: 1101-1104.

Ramsbottom, J. (1954) *Mushrooms and Toadstools : A study of the activities of fungi*. Collins, London.

Rappe, M.S., and Giovannoni, S.J. (2003) The uncultured microbial majority. *Annual Reviews of Microbiology* **57:** 369-394.

Rayner, A. D. M. and Boddy, L. (1988) *Fungal Decomposition of Wood*. John Wiley and Sons, New York.

Revell, C. K. and Taylor, G. B. (1998) Imbibition and germination of yellow serradella seeds. *Proceedings of the 9th Australian Agronomy Conference Agronomy – Growing a Greener Future,* Wagga Wagga: 195-198.

Rey, L. R. (1977). In *Development in Biological Standardisation. International symposium on freeze – drying of biological products* (V. J. Cabusso and R. H. Regamey, eds) **Vol 36**, S. Krager, Basel.

Robinson, R. A. and Stokes, R. H. (1959) *Electrolyte Solutions*. Academic Press, New York.

Rosado, F. R., Kemmelmeier, C. and Da Costa, S. M. (2002) Alternative method of inoculum and spawn for the cultivation of the edible Brazilian mushroom *Pleurotus ostreatoroseus* Sing. *Journal of Basic Microbiology* **42**: 37-44.

Royse, D. J. (2002) Influence of spawn rate and commercial delayed release nutrient levels on *Pleurotus cornucopiae* (oyster mushroom) yield, size and time to production. *Applied Microbial Biotechnology* **58**: 527-531.

Sadler, M. (2003) Nutritional properties of edible fungi. *British Nutrition Foundation Nutrition Bulletin* **28**: 305-308.

Saitou, N. and Nei, M. (1987) The neighbour-joining method: a new method for reconstruction of phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425.

Salmones, D., Gaitin-Hernandez, R., Perez, R. and Guzman, G. (1997) Studies on genus *Pleurotus* VIII. Interaction between mycelial growth and yield. *Rev Iberoam Micol* **14**: 173-176.

Sánchez, C. and Gonzaléz, G. V. (1996) Detection of highly productive strains of *Pleurotus ostreatus* by their tolerance to 2-deoxy-D-glucose in starch-based media. *Mycological Research* **100**: 455-461.

Sanderson, M. J. (1989) Confidence limits on phylogenies: the bootstrap revisited. *Cladistics* **5**: 113-129.

Schäffer, A. A., Aravind, L., Madden, T. L., Shavirin, S., Spouge, J. L., Wolf, Y. I., Koonin, E. V. and Altschul, S. F. (2001) Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucleic Acids Research* **29**: 2994-3005.

Scheinert, P., Behrens, B. and Kable, D. (1995) Optimising DNA amplification protocols. Biospektrum Special Publication.

Scherer, S. and Stevens, D. A. (1988) A *Candida albicans* dispersed, repeated gene family and its epidemiological applications. *Proceedings of the National Academy of Science U. S. A.* **85**: 1452-1456.

Scott, W. J. (1953) Water relations of *Staphylococcus aureus* at 30°C. *Australian Journal of Biological Sciences* **6**: 549-564.

Senyah, J. K. (1987) *Cultivation of the oyster mushroom Pleurotus ostreatus on cocoa shell waste.* Ph. D. Thesis. University of Reading, Berks. U. K.

Sharma, S. R. and Vijay, B. (1996) Yield loss in *Pleurotus ostreatus* caused by *Trichoderma viride*. *Mushroom Research* **5**: 19-22.

Shear, C. L. and Stevens, N.E. (1913) Cultural characters of the chestnut blight fungus and its relatives, *Circular U. S. Department of Agriculture, Bur, Plant Ind,* **131**: 3-18.

Shnyreva, A. V. and Shtaer, O. V. (2006) Differentiation of closely related oyster fungi *Pleurotus pulmonarius* and *P. ostreatus* by mating and molecular markers. *Genetika* **42**: 667-674.

Singer, R. (1986) *The Agaricales in Modern Taxonomy*. Koeltz Scientific Books, Koenigstein, FDR.

Smith, D. (1983) *Transactions of the British Mycological Society* **80**: 360–363.

Smith, D. (1984) Maintenance of fungi In *Maintenance of Microorganisms*, Academic Press, London.

Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. B. H. and Hodd, L. E. (1986) Fluorescence detection in automated DNA sequence analysis. *Nature* (*London*) **321**: 674-679.

Sneath, P. H. A. and Sokal, R. R. (1973) *Numerical Taxonomy (The principles and practice of numerical classification)*, W.H. Freeman and company, San Francisco.

Soden, D. M. and Dobson, A. D. W. (2003) The use of amplified flanking region-PCR in the isolation of laccase promoter sequences from the edible fungus *Pleurotus sajor-caju*. *Journal of Applied Microbiology* **95**: 553-562.

Sogin, M. L. and Gunderson, J. H. (1987) Structural diversity of eukaryotic small subunit ribosomal RNAs, Evolutionary implications. *Annals of the New York Academy of Sciences* **503**: 125-139.

Song, J., Weon, H., Yoon, S., Park, D., Go, S. and Suh, J. (2001) Phylogenetic diversity of thermophilic actinomycetes and *Thermoactinimyces* spp. Isolated from mushroom composts in Korea based on 16S rRNA gene sequence analysis. *FEMS Microbiology letters* **202**: 97-102.

Stamets, P. (2000) *Growing Gourmet and Medicinal Mushrooms*. Ten Speed Press, Berkelely.

Stamets, P. and Chilton, J. S. (1983) *The Mushroom Cultivator*. Agarikon Press, Olympia, Washington.

StatSoft, Inc. (2006). STATISTICA (data analysis software system), version 7.1. www.statsoft.com.

Svoray, T., Gancharaski, S.B.Y, Henkin, Z., and Gutman, M. (2004) Assessment of herbaceous plant habitats in water-constrained environments: predicting indirect effects with fuzzy logic. *Ecological Modelling* **180**: 537-556.

Tautz, D., Arctander, P., Minelli, A., Thomas, R. H. and Vogler, A. P. (2003) A plea for DNA taxonomy. *Trends in Ecology and Evolution* **18**: 70-74.

Taylor, W. R. (1995) Sequence alignment of proteins and nucleic acids. In *Molecular Biology and Biotechnology*: *A comprehensive desk reference* (R. A. Meyers, ed), Wiley-VCH, New York.

Tekere, M., Zvauya, R. and Read, J. S. (2001a) Ligninolytic enzyme production from selected sub-tropical white rot fungi under different culture conditions. *Journal of Basic Microbiology* **41**: 97-111.

Tekere, M., Mswaka, A. Y., Zvauya, R. and Read, J. S. (2001b) Growth, dye degradation and ligninolytic activity studies on Zimbabwean white rot fungi. *Enzyme and Microbial Technology* **28**: 420-426.

Thorn, R. G. and Barron, G. L. (1984) Carnivorous mushrooms. *Science* **224**: 76-78.

Thorn, R. G. and Tsuneda, A. (1993) Interactions between *Pleurotus* species, nematodes and bacteria on agar and in wood. *Transactions of the Mycological Society of Japan* **34**: 449-464.

Thorn, R. G., Moncalvo, J. M., Reddy, C. A. and Vilgalys, R. (2000) Phylogenetic analyses and the distribution of nematophagy support a monophyletic Pleurotaceae within the polyphyletic pleurotoid-lentinoid fungi. *Mycologia* **92**: 241-252.

Toyama, N. and Ogawa, K. (1974) Comparative studies on cellulolytic and oxidizing enzymes of edible and inedible wood rotters. *Mushroom Science* **9**: 745-760.

Vainio, E. J. and Hantula, J. (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycological Research* **104**: 927-936.

VanDemark, P. J. and Batzing, B. L. (1987) *The Microbes: An Introduction to their Nature and Importance*. Benjamin Cummings, Menlo Park.

Van Uden, N. and do Carmo Sousa, L. (1957) Yeasts from the bovine caecum. *Journal of General Microbiology* **16**: 385-395.

Venturella, G., Zervakis, G. and Raimondo, F. M. (2002) Mycology in sustainable development: The case of *Pleurotus nebrodensis* from Sicily (Southern Italy). Journal (Anon.).

Vilgalys, R. (internet) *Conserved primer sequences for PCR amplification and sequencing from nuclear ribosomal RNA*. http://www.biology.duke.edu/fungi/mycolab/primers.htm and agaricphylogeny start. Html

Vilgalys, R. and Sun, B. L. (1994) Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. *Proceedings of the National Academy of Sciences, USA* **91**: 4599-4603.

Vilgalys, R., Hopple, J. S. and Hibbett, D. S. (1994) Phylogenetic implications of generic concepts in fungal taxonomy: the impact of molecular systematic studies. *Mycologia Helvetica* **6**: 73-91.

Vilgalys, R., Smith, A., Sun, B. L. and Miller, O. K. (1993) Intersterility groups in the *Pleurotus ostreatus* complex from the continental United States and adjacent Canada. *Canadian Journal of Botany* **71**: 113-128.

Villas-Boas S.G., Esposito E., Mitchell D.A. (2002) Microbial conversion of lignocellulosic residues for production of animal feeds. *Animal Feed Science and Technology* **98:** 1-12.

Wagner, T. and Fisher, M. (2001) Natual groups and a revised system for the European poroid Hymenochaetales (Basidiomycota) supported by nLSU rDNA sequence data. *Mycological Research* **105**: 773-782.

Watling, R. and Gregory, N. M. (1989) Crepidotaceae: Pleurotaceae and other pleurotoid agarics. British Fungus Flora. *Agarics and Boleti* **6**. Royal Botanic Garden, Edinburgh.

Weising, K., Nybom, H., Wolff, K. and Meyer, W. (1995) *DNA fingerprinting in Plants and Fungi*, CRC Press, Inc.

Weitz, H. J. (2004) Naturally bioluminescent fungi. *Mycologist* **18**: 4-5.

Weitzman, L. and Silva – Hutner, M. (1967) Non – keratinous agar media as substitutes for the ascigerous state in certain members of the Gymnoascaceae pathogenic for the man and animals. *Sabouraudia* **5**: 335–340.

Wetmur, J. G. (1995) Nucleic acid hybrids, formation and structure of. In *Molecular Biology and Biotechnology*: *A comprehensive desk reference* (R. A. Meyers, ed), Wiley-VCH, New York.

White, T. J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR*

Protocols, *A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, eds), Academic Press, New York.

Whittaker, R. H. (1959) On the broad classification of organisms. *Quarterly Review of Biology* **34**: 210-226.

Whittaker, R. H. (1969) New concepts of kingdoms of organisms. *Science* **163**: 100-102.

Wilson, K. and Walker, J. (1995) *Practical Biochemistry* (*Principles and techniques*). Cambridge University Press, Cambridge.

Woese, C. R. (1967) *The Genetic Code: the molecular basis for genetic expression*. Harper and Rowe, New York.

Xia, L., Chu, K. T. and Ng, T. B. (2005) A low – molecular mass ribonuclease from the brown oyster mushroom. *Journal of Peptide Research* **66**: 1-8.

Yara, R.. Maccheroni, W. Jr, Horii, J. and Azevedo, J. L. (2006) A bacterium belonging to the *Burkholderia cepacia* complex associated with *Pleurotus ostreatus*. *Journal of Microbiology* **44**: 262-268.

Zadrazil, F. (1978). Cultivation of *Pleurotus*. In *The Biology and Cultivation of Edible Mushrooms* (S. T. Chang and W. A. Hayes, eds), Academic Press, New York.

Zervakis, G. and Balis, C. (1996) A pluralistic approach in the study of *Pleurotus* species with emphasis on compatibility and physiology of the European morphotaxa. *Mycological Research* **100**: 717-731.

Zervakis, G., Dimou, D. and Balis, C. (1992) First record of the natural occurrence in Europe of the basidiomycete *Pleurotus cystidiosus* on a new host. *Mycological Research* **96**: 874-876.

Zervakis, G., Soudis, J. And Balis, C. (1994) Genetic variability and systematics of eleven *Pleurotus* soecies based on isozyme analysis. *Mycological Research* **98**: 329–341.

Zervakis, G. I., Moncalvo, J. and Vilgalys, R. (2004) Molecular phylogeny, biogeography and speciation of the mushroom species *Pleurotus cystidiosus* and allied taxa. *Microbiology* **150**: 715-726.

Zhou, L. J., Pei, K., Zhou, B. and Ma, K. (2007) A molecular approach to species identification of Chenopodiaceae pollen grains in surface soil. *American Journal of Botany* **94**: 477-481.

9.0 APPENDICES

APPENDIX A: List of publications

APPENDIX B: Conversion factors and units

APPENDIX C: *Pleurotus* species and strains used in the study

APPENDIX D: Description of terms used in describing mycelia texture

APPENDIX E: Water content formulae

APPENDIX F: Output of genetic characterisation of the *Pleurotus* species using ITS rDNA

APPENDIX G: ITS rDNA sequences analysis data of the *Pleurotus* species and strains

APPENDIX H: Multiple alignment of ITS rDNA sequences of the *Pleurotus* species and strains

APPENDIX I: Restriction enzyme analysis of the ITS rDNA sequences of the *Pleurotus* species and strains

APPENDIX A: List of Publications

This thesis is based on the following list of publications

1. **Low-cost media formulations for culture maintenance and prolific fruiting of oyster mushrooms (*Pleurotus* spp.)**. **Kashangura, C.** (2007) *Proceedings of the Research Council of Zimbabwe 8th Symposium on Science and Technology Knowledge based development for Zimbabwe, 31 January-1 February, University of Zimbabwe, Harare, Zimbabwe* **8**: 1-13.

2. **Phenotypic diversity amongst strains of *Pleurotus sajor-caju*: implications for cultivation in arid environments**. **Kashangura, C**., Hallsworth, J. E. and Mswaka, A. Y. (2006) *Mycological Research*. **110**: 312-317.

3. ***Manual for Mushroom Cultivation (Especially for growers with limited financial and material resources)***. **Kashangura, C**., Kunjeku, E. C., Chirara, T., Mabveni, A.R.S., Mswaka, A. and Dalu, V. (2005) Biotechnology Trust of Zimbabwe. Harare, Zimbabwe. 64 pages.

4. **Oyster mushroom spawn production: Tyndallisation as an alternative medium for sterilization of growth medium. Kashangura, C**. (2004) *Proceedings of the Research Council of Zimbabwe 7th Symposium on Science and Technology-Impact of Innovative Science and Technology on National Wealth Creation, 1-3 September, Harare International Conference center, Sheraton hotel, Harare, Zimbabwe*. **7**: 242 – 244.

5. **Polyethylene glycol 6000: a possible growth enhancer for *Pleurotus sajor-caju* cultivation. Kashangura, C**., Mswaka, A. Y., Tekere, M. and Sithole-Niang, I. (*Manuscript*).

6. **Genetic variability of *Pleurotus sajor-caju* based on sequencing of the ITS region of rDNA. Kashangura, C**., R. Mswaka, Tekere, M. and Sithole-Niang, I. (*Manuscript*).

APPENDIX B: Conversion Factors and Units

1 bar = 10^6 dyne cm^{-2} = 10^5Pa

1 MPa = mega pascal (Pa) = 10^6 Pa = 1.0 µN μm^{-2} = 10 bars = 9.9 atmospheres

1 atm = 1.01325 bar = 1.0332 x 10^4 kg m^{-2} = 760 torr = 101 325 Pa

1 lumen = 7.9577 x 10^{-2} candle power

lux = 1 lumens m^{-2} (illumination unit = 1x = $1m/m^2$)

1 molal solution = 1 mol per 1000g of solvent

1 molar solution = 1 mol of solute in 1 litre of solution

1M = 1 mol dm^{-3}

Sources: Eckert, Randall and Augustine, (1988); Mahan and Myers, (1987); Moore, (1961) and, Shriver, Atkins and Langford, (1992).

APPENDIX C: *Pleurotus* Species and Strains used in the Study

***Pleurotus* species and strains used in the study.**				
Strain	Species	Origin	Collector	Accession year in BCCM/MUCL
28511	*P. ostreatus*	Belgium	G. L. Hennebert	1984
28683	*P. sajor-caju*	Malaysia	K.M. Graham	1985
28684	*P. citrinopileatus*	Malaysia	K.M. Graham	1985
28685	*P. sajor-caju*	Malaysia	K.M. Graham	1985
29757	*P. sajor-caju*	China	S.T. Chang	1988
31017	*P. sajor-caju*	----	Les Miz	1990
31613	*P. sajor-caju*	China	Wang	1991
31674	*P. sajor-caju*	Mauritius	----	1991
38047	*P. floridanus*	Thailand	V. Kriengyaku	1993
38071	*P. sajor-caju*	Hong Kong	G. Hyatt	1993
38076	*P. sajor-caju*	China	P. Oie	1993
38078	*P. sajor-caju*	Philippines	T.H. Quimio	1993
38089	*P. sajor-caju*	Mauritius	C. Gangy	1993
38091	*P. sajor-caju*	Thailand	E. Calilung	1993
38092	*P. sajor-caju*	Thailand	E. Calilung	1993

APPENDIX D: Description of Terms Used in Describing Mycelia Texture

Cottony: Erect, rather long (3-5 mm) mycelium spreading in all directions. Appears like tufts of cotton.

Floccose: Consisting of cottony or woolly mycelium aggregated to form small erect tufts.

Linear: Is arranged as diverging, longitudinal strands. The mycelium emanates from the centre of the Petri-plate as a homogeneously forming mat.

Rhizomorphic: Similar to linear mycelium and composed of braided, twisted strands, often of varying diameters.

Quasi-Rhizomorphic: Similar to rhizomorphic mycelium in appearance, but lacks braided, twisted strands

Zonate: Cottony mycelium often shows concentric circles of dense and light growth, or zones.

Matted or Appressed: Carpet like appearance which is highly dense.

Subfelty: With mycelium appressed to agar, forming a thin barely visible mat.

APPENDIX E: Water Content Formulae

The first term, the fraction, represents a proportion of the water needed to reach the desired 50 % moisture content of the grain (Stamets, 2000) with the numerator a subtraction of the inherent water content in the grain from the desired 50 % moisture content. This proportion of water required is then converted to the total amount of water required for a total sterilised grain with 50 % moisture content. The formulae were developed by the author.

$$V_{water} = \left(\frac{DM\% - \%\ \text{water in DG}}{DM\%} \right) \quad X \quad (\text{mass of TGR x } [DM\%, \text{ as a decimal }])$$

DM%: desired moisture percentage

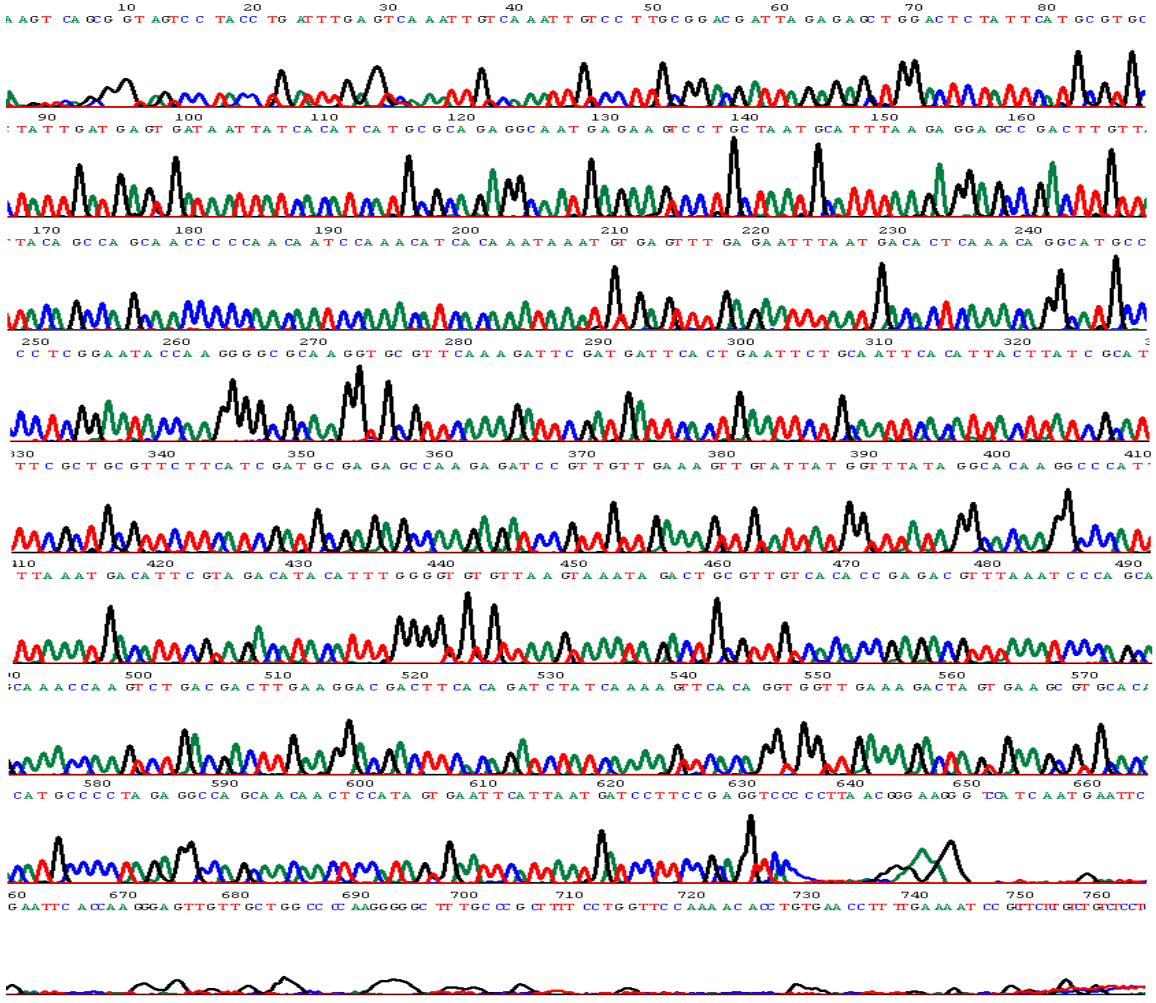
DG: dry grain

TGR: total sterilised grain required for spawn production.

Formulae for water content of wheat grain used in preparing *Pleurotus* spawn		
Formula name	V_{H2O} X scalar	Calculation of dry grain[a]
F1	V_{H2O} X 1.0	Mass of TGR – (V_{H2O} X 1)
F2	V_{H2O} X 1.5	Mass of TGR – (V_{H2O} X $^2/_3$)
F3	V_{H2O} X 2.0	Mass of TGR – (V_{H2O} X $^1/_2$)
F4	V_{H2O} X 2.5	Mass of TGR – (V_{H2O} X $^2/_5$)
F5	V_{H2O} X 3.0	Mass of TGR – (V_{H2O} X $^1/_3$)
F6	V_{H2O} X 3.5	Mass of TGR – (V_{H2O} X $^2/_7$)
F7	V_{H2O} X 4.0	Mass of TGR – (V_{H2O} X $^1/_4$)
F8	V_{H2O} X 4.5	Mass of TGR – (V_{H2O} X $^2/_9$)

APPENDIX F: Output of Genetic Characterisation of the *Pleurotus* Species Using ITS rDNA

Example of output from the automated PE Biosystems PRISM 310 Genetic Analyser of DNA sequencing of the internal transcribed spacer region of ribosomal DNA of BCCM/MUCL strain 28683 (*Pleurotus sajor-caju*) using ITS-4 primer is shown below.



APPENDIX G: ITS rDNA Sequences Analysis Data of the *Pleurotus* Species and Strains

GenBank accession numbers and sequence base composition of the *Pleurotus* ribosomal DNA, internal transcribed spacer region sequences obtained in this study

Strain	Species	GenBank accession number	Base composition (%)[a]			
			A	C	G	T
28511	*P. ostreatus*	EF458642	27.92	23.72	23.85	24.51
28683	*P. sajor-caju*	EF458644	28.47	23.19	22.05	26.29
28684	*P. citrinopileatus*	EF458640	26.28	27.65	26.51	19.56
28685	*P. sajor-caju*	EF458645	28.02	23.09	22.57	26.32
29757	*P. sajor-caju*	EF458646	25.78	29.27	19.93	25.02
31017	*P. sajor-caju*	EF458647	27.78	23.36	22.83	26.03
31613	*P. sajor-caju*	EF458648	28.72	23.05	22.80	25.43
31674	*P. sajor-caju*	EF458649	29.01	22.54	23.66	24.79
38047	*P. floridanus*	EF458641	27.86	24.05	23.03	25.06
38071	*P. sajor-caju*	EF458650	29.53	22.68	22.22	25.57
38076	*P. sajor-caju*	EF458651	26.35	24.55	22.37	26.73
38078	*P. sajor-caju*	EF458652	28.02	22.56	23.56	25.86
38089	*P. sajor-caju*	EF458653	23.03	24.58	26.03	26.36
38091	*P. sajor-caju*	EF458643	28.59	22.35	23.66	25.40
38092	*P. sajor-caju*	EF458654	29.92	22.00	23.43	25.28

BLAST search results of the GenBank, EMBL data library, DNA Data Bank of Japan and Protein Data Bank		
Strain	Scientific Name	Sequences producing significant alignments[a]
28511	*Pleurotus ostreatus*	gi|69145181|gb|DQ077884.1| *Pleurotus ostreatus*
		gi|34582623|gb|AY368665.1| *Pleurotus ostreatus*
		gi|30039383|gb|AY265839.1| *Pleurotus ostreatus*
		gi|34582621|gb|AY368663.1| *Pleurotus floridanus*
31674	*Pleurotus sajor-caju*	gi|47087812|gb|AY540332.1| *Pleurotus ostreatus*
		gi|47087805|gb|AY540325.1| *Pleurotus ostreatus*
		gi|47087807|gb|AY540327.1| *Pleurotus sapidus*
		gi|47087806|gb|AY540326.1| *Pleurotus sapidus*
28683, 28685, 29757, 31017, 31613, 38076, 38078, 38089, 38091 and 38092	*Pleurotus sajor-caju*	gi|47087809|gb|AY540329.1| *Pleurotus sajor-caju*
		gi|47087808|gb|AY540328.1| *Pleurotus sajor-caju*
		gi|38426286|gb|AY450349.1| *Pleurotus pulmonarius*
		gi|37222347|gb|AY315765.1| *Pleurotus australis*
28684	*Pleurotus citrinopileatus*	gi|30039396|gb|AY265852.1| *Pleurotus citrinopileatus*
		gi|30039230|gb|AY265816.1| *Pleurotus cornucopiae*
		gi|47087799|gb|AY540319.1| *Pleurotus citrinopileatus*
		gi|47087798|gb|AY540318.1| *Pleurotus citrinopileatus*
38047	*Pleurotus floridanus*	gi|47087802|gb|AY540322.1| *Pleurotus floridanus*
		gi|47087803|gb|AY540323.1| *Pleurotus floridanus*
		gi|49357478|gb|AY636055.1| *Pleurotus ostreatus*
		gi|30039375|gb|AY265831.1| *Pleurotus ostreatus f. florida*
38071	*Pleurotus sajor-caju*	gi|47087809|gb|AY540329.1| *Pleurotus sajor-caju*
		gi|47087808|gb|AY540328.1| *Pleurotus sajor-caju*
		gi|56699215|gb|AY696300.1| *Pleurotus pulmonarius*
		gi|37222347|gb|AY315765.1| *Pleurotus australis*

BLAST analysis phylogenetic tree of 100 sequences generated by minimum evolution method.

APPENDIX H: Multiple Alignment of ITS rDNA Sequences of the *Pleurotus* Species and Strains

Multiple alignment of the edited sequences of all the BCCM/MUCL *Pleurotus* strains used in this study is shown below. Bases different from other bases at the respective position in the sequence are depicted by small caps.

Maximum Length: 750 bases, bases 26 to 776 of the full ITS region sequences A = adenine, C = cytosine, G = guanine and T = thymine
Po = *Pleurotus ostreatus*, Psc = *Pleurotus sajor-caju*, Pc = *Pleurotus citrinopileatus*, Pf = *Pleurotus floridanus*

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STRAIN     SEQUENCE                                                base

28511_Po   CAT.ACCTGATTTGAGTC..AAATTGTCAAATTGTC..CT                 35
28683_Psc  -c-.--------------..----------------..--                 35
28684_Pc   -c-.--------------t.----g------aa-ctgt-c                 38
28685_Psc  ---.------------ctct----------------..--                 37
29757_Psc  -c-.--------------..----------------..--                 35
31017_Psc  -c-.--------------..----------------..--                 35
31613_Psc  tga.ca------------tgt---------------t.--                 38
31674_Psc  -c-.--------------..----------------..--                 35
38047_Pf   g-c.--a---------ctct----------------..--                 37
38071_Psc  ---g------------ctcat---------------..--                 38
38076_Psc  -c-.--------------..----------------..--                 35
38078_Psc  -c-.--------------..----------------..--                 35
38089_Psc  -c-.--------------..----------------..--                 35
38091_Psc  -c-.--------------..----------------..--                 35
38092_Psc  -c-.--------------..----------------..--                 35

28511_Po   TGCGGACGATTGGAGAGCTGGA...CTCTAT......TCA                 66
28683_Psc  -----------a----------...------......---                 66
28684_Pc   gaaa---a----a--------gact-c-a--aagatcctt                 78
28685_Psc  -----------a----------...------......---                 68
29757_Psc  -----------a----------...------......---                 66
31017_Psc  -----------a----------...------......---                 66
31613_Psc  -----------a----------...------a....tc--                 71
31674_Psc  -----------a----------...------......---                 66
38047_Pf   -----------a----------...------......---                 68
38071_Psc  -----------a----------...------......c--                 69
38076_Psc  -----------a----------...------......---                 66
38078_Psc  -----------a----------...------......---                 66
38089_Psc  -----------a----------...------......---                 66
38091_Psc  -----------a----------...------......---                 66
38092_Psc  -----------a----------...------......---                 66

28511_Po   TGCGTGCTATTGATGAGTGATA.ATTATCACATCATGCG.                104
28683_Psc  ----------------------.----------------.                104
28684_Pc   g----agggcc-ggcgtaa---t---t----cc-c--gta                118
28685_Psc  ----------------------.----------------.                106
29757_Psc  ----------------------.----------------.                104
31017_Psc  ----------------------.----------------.                104
31613_Psc  ----------------------.----------------.                109
31674_Psc  ----------------------.----------------.                104
38047_Pf   ----------------------.----------------.                106
38071_Psc  ----------------------.----------------.                107
38076_Psc  ----------------------.----------------.                104
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38078_Psc  ---------------------.----------------.    104
38089_Psc  ---------------------.----------------.    104
38091_Psc  ---------------------.----------------.    104
38092_Psc  ---------------------.----------------.    104

28511_Po   .CAGAGGCAATGAGAAGT.CCTGC.TAATG.CATTTAAGA    140
28683_Psc  .-----------------.-----.-----.---------    140
28684_Pc   c--a------cacc--a-t--c--a-----g-t-----a-    158
28685_Psc  .-----------------.-----.-----.---------    142
29757_Psc  .-----------------.-----.-----.---------    140
31017_Psc  .-----------------.-----.-----.---------    140
31613_Psc  .-----------------.-----.-----.---------    145
31674_Psc  .-----------------.-----.-----.---------    140
38047_Pf   .-----------------.-----.-----.---------    142
38071_Psc  .-----------------.-----.-----.---------    143
38076_Psc  .-----------------.-----.-----.---------    140
38078_Psc  .-----------------.-----.-----.---------    140
38089_Psc  .-----------------.-----.-----.---------    140
38091_Psc  .-----------------.-----.-----.---------    140
38092_Psc  .-----------------.-----.-----.---------    140

28511_Po   GGAGCCGACCT...GTCAAGGCCAGCAGCCCCCAACAATC    177
28683_Psc  ---------t-...--t-ca-------a------------    177
28684_Pc   --------a--cgg-gac-a---g---c-------a----    198
28685_Psc  ---------t-...--t-ca-------a------------    179
29757_Psc  ---------t-...--t-ca-------a------------    177
31017_Psc  ---------t-...--t-ca-------a------------    177
31613_Psc  ---------t-...--t-ca-------a------------    182
31674_Psc  ----------...--------------------------    177
38047_Pf   ----------...--------------------------    179
38071_Psc  ---------t-...--t-ca-------a------------    180
38076_Psc  ---------t-...--t-ca-------a------------    177
38078_Psc  ---------t-...--t-ca-------a------------    177
38089_Psc  ---------t-...--t-c--------a------------    177
38091_Psc  ---------t-...--g-ca-------a------------    177
38092_Psc  ---------t-...--t-ca-------a------------    177

28511_Po   CAAACATCACAATTGGAAAAAAACCAAAGTGAGTTT...G    214
28683_Psc  ------------a-a..........--t--------...-    204
28684_Pc   ------ct------tccggc---g-----g---gg-tga-    238
28685_Psc  ------------a-a..........--t--------...-    206
29757_Psc  ------------a-a..........--t--------...-    204
31017_Psc  ------------a-a..........--t--------...-    204
31613_Psc  ------------a-a..........--t--------...-    209
31674_Psc  ---------------------.-------------...-    213
38047_Pf   -------------------g---------------...-    216
38071_Psc  ------------a-a..........--t--------...-    207
38076_Psc  ------------a-a..........--t--------...-    204
38078_Psc  ------------a-a..........--t--------...-    204
38089_Psc  ------------a-a..........--t--------...-    204
38091_Psc  ------------a-a..........--t--------...-    204
38092_Psc  ------------a-a..........--t--------...-    204

28511_Po   AGAATTTAATG.ACACTCAAACAGGCATGCCCCTCGGAAT    253
28683_Psc  -----------.----------------------------    243
28684_Pc   ---------g-.--c-------g---t-----t---a---    277
28685_Psc  -----------.----------------------------    245
29757_Psc  -----------.----------------------------    243
31017_Psc  -----------.----------------------------    243
31613_Psc  -----------.----------------------------    248
31674_Psc  -----------.----------------------------    252
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38047_Pf   ----------.--------------------------    255
38071_Psc  ----------.--------------------------    246
38076_Psc  ----------.--------------------------    243
38078_Psc  ----------.--------------------------    243
38089_Psc  ----------g--------g-------------------    244
38091_Psc  ----------.--------------------------    243
38092_Psc  ----------.--------------------------    243

28511_Po   ACCAAGGGGCGCAAGGTGCGTTCAAAGATTC..GATGATT    291
28683_Psc  ------------------------------..-------    281
28684_Pc   ---------g---g--g-----a---aga--caaga----    317
28685_Psc  ------------------------------..-------    283
29757_Psc  ------------------------------..-------    281
31017_Psc  ------------------------------..-------    281
31613_Psc  ------------------------------..-------    286
31674_Psc  ------------------------------..-------    290
38047_Pf   ------------------------------..-------    293
38071_Psc  ------------------------------..-------    284
38076_Psc  ------------------------------..-------    281
38078_Psc  ------------------------------..-------    281
38089_Psc  ------------------------------..a------    282
38091_Psc  ------------------------------..-------    281
38092_Psc  ------------------------------..-------    281

28511_Po   CACTGAATTCTGCAATTCACATTAC.TTATCGCATTT.CG    329
28683_Psc  ------------------------.-----------.--    319
28684_Pc   -c------c-g---t-----t----c-----------c--    357
28685_Psc  ------------------------.-----------.--    321
29757_Psc  ------------------------.-----------.--    319
31017_Psc  ------------------------.-----------.--    319
31613_Psc  ------------------------.-----------.--    324
31674_Psc  ------------------------.-----------.--    328
38047_Pf   ------------------------.-----------.--    331
38071_Psc  ------------------------.-----------.--    322
38076_Psc  ------------------------.-----------.--    319
38078_Psc  ------------------------.-----------t--    320
38089_Psc  ------------------------.-----------.--    320
38091_Psc  ------------------------.-----------t--    320
38092_Psc  ------------------------.-----------.--    319

28511_Po   CTG.CGTTCTTCATCGATGC...GAGAGCCAAG..AGATC    363
28683_Psc  ---.----------------...----------..-----    353
28684_Pc   ---g---c--c--------gcga---------aga-----    397
28685_Psc  ---.----------------...----------..-----    355
29757_Psc  ---.----------------...----------..-----    353
31017_Psc  ---.----------------...----------..-----    353
31613_Psc  ---.----------------...----------..-----    358
31674_Psc  ---.----------------...----------..-----    362
38047_Pf   ---.----------------...----------..-----    365
38071_Psc  ---.----------------...----------..-----    356
38076_Psc  ---.----------------...----------..-----    353
38078_Psc  ---.----------------...----------..-----    354
38089_Psc  ---.----------------...----------..-----    354
38091_Psc  ---.----------------...----------..-----    354
38092_Psc  ---.----------------...----------..-----    353

28511_Po   CGTTGTTGA..AAGTTGTATTATGGTTTATAGGCACAAGG    401
28683_Psc  ---------..----------------------------    391
28684_Pc   -cg--g---ga----g----a-g-----ta-tagggc---    437
28685_Psc  ---------..----------------------------    393
29757_Psc  ---------..----------------------------    391
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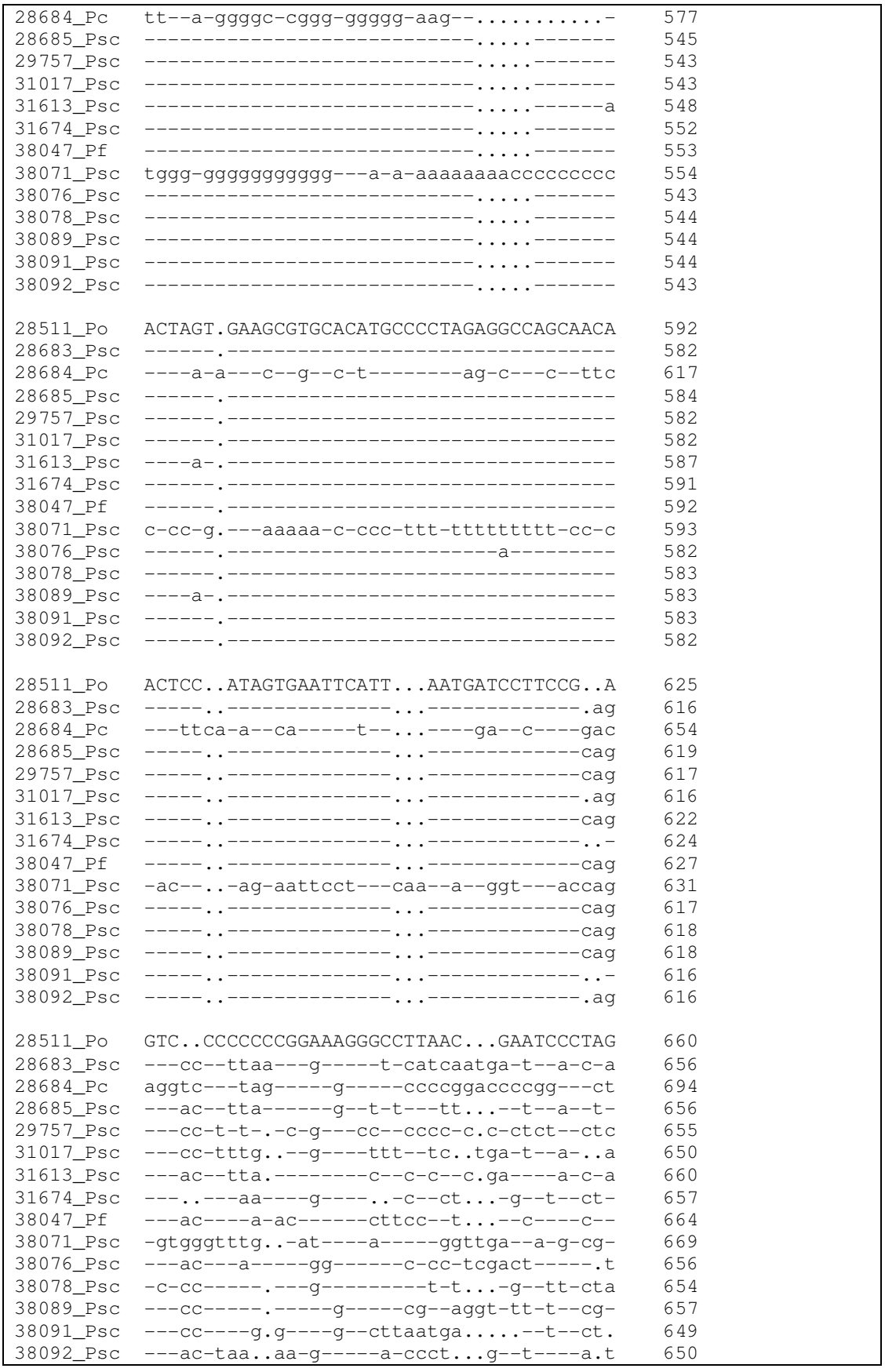
31017_Psc  ---------..--------------------------    391
31613_Psc  ---------..--------------------------    396
31674_Psc  ---------..--------------------------    400
38047_Pf   ---------..-----------------a----------    403
38071_Psc  ---------..--------------------------    394
38076_Psc  ---------..--------------------------    391
38078_Psc  ---------..--------------------------    392
38089_Psc  ---------..--------------------------    392
38091_Psc  ---------..--------------------------    392
38092_Psc  ---------..--------------------------    391

28511_Po   CCCATTAAATGACATTCGTAGACATACATTTGGGGTGTGT    441
28683_Psc  ----------------------------------------    431
28684_Pc   -...-gggccc-t--aaa-gagac-t-cg-aaaca-ac--    474
28685_Psc  ----------------------------------------    433
29757_Psc  ----------------------------------------    431
31017_Psc  ----------------------------------------    431
31613_Psc  ----------------------------------------    436
31674_Psc  ----------------------------------------    440
38047_Pf   ----------------------------------------    443
38071_Psc  ----------------------------------------    434
38076_Psc  ----------------------------------------    431
38078_Psc  ----------------------------------------    432
38089_Psc  ----------------------------------------    432
38091_Psc  ----------------------------------------    432
38092_Psc  ----------------------------------------    431

28511_Po   TAAGTAAATAGACTGTGTTGTCACACCGAGACGTTTAAAT    481
28683_Psc  ---------------c----------------------    471
28684_Pc   -gg-gggggta-gg--ta-aag--c--cc--a---ac-g-    514
28685_Psc  ---------------c----------------------    473
29757_Psc  ---------------c----------------------    471
31017_Psc  ---------------c----------------------    471
31613_Psc  ---------------c----------------------    476
31674_Psc  ---------------c----------------------    480
38047_Pf   .--------------c--a--------------------    482
38071_Psc  ---------------c----------------------    474
38076_Psc  ---------------c----------------------    471
38078_Psc  ---------------c----------------------    472
38089_Psc  ---------------c----------------------    472
38091_Psc  ---------------c----------------------    472
38092_Psc  ---------------c----------------------    471

28511_Po   CCCAGCAAACCAAGTCTGACGACTTG...AAAGACGACTT    518
28683_Psc  -------------------------...--g--------    508
28684_Pc   gt--c-gg...g--g-gac--t---t...ccg-gaa-tc-    548
28685_Psc  -------------------------...--g--------    510
29757_Psc  -------------------------...--g--------    508
31017_Psc  -------------------------...--g--------    508
31613_Psc  -------------------------...--g--------    513
31674_Psc  -------------------------...-----------    517
38047_Pf   --------.-----------------...-g---------    518
38071_Psc  ------------------------tttggga-aa-ggg    514
38076_Psc  -------------------------...--g--------    508
38078_Psc  -------------------------...--g--------    509
38089_Psc  ----a--------------------...--g--------    509
38091_Psc  -------------------------...--g--------    509
38092_Psc  -------------------------...--g--------    508

28511_Po   CACAGATCTATCAAAAGTTCACAGGTGG.....TTGAAAG    553
28683_Psc  -------------------------.....-------    543
```


28684_Pc tt--a-ggggc-cggg-ggggg-aag--..........- 577
28685_Psc ----------.....------- 545
29757_Psc ----------.....------- 543
31017_Psc ----------.....------- 543
31613_Psc ----------.....------a 548
31674_Psc ----------.....------- 552
38047_Pf ----------.....------- 553
38071_Psc tggg-gggggggggg---a-a-aaaaaaaaccccccccc 554
38076_Psc ----------.....------- 543
38078_Psc ----------.....------- 544
38089_Psc ----------.....------- 544
38091_Psc ----------.....------- 544
38092_Psc ----------.....------- 543
28511_Po ACTAGT.GAAGCGTGCACATGCCCCTAGAGGCCAGCAACA 592
28683_Psc ------.---------- 582
28684_Pc ----a-a---c--g--c-t--------ag-c---c--ttc 617
28685_Psc ------.---------- 584
29757_Psc ------.---------- 582
31017_Psc ------.---------- 582
31613_Psc ----a-.---------- 587
31674_Psc ------.---------- 591
38047_Pf ------.---------- 592
38071_Psc c-cc-g.---aaaaa-c-ccc-ttt-ttttttttt-cc-c 593
38076_Psc ------.----------a--------- 582
38078_Psc ------.---------- 583
38089_Psc ----a-.---------- 583
38091_Psc ------.---------- 583
38092_Psc ------.---------- 582
28511_Po ACTCC..ATAGTGAATTCATT...AATGATCCTTCCG..A 625
28683_Psc -----..----------...----------.ag 616
28684_Pc ---ttca-a--ca-----t--...----ga--c----gac 654
28685_Psc -----..----------...----------cag 619
29757_Psc -----..----------...----------cag 617
31017_Psc -----..----------...----------.ag 616
31613_Psc -----..----------...----------cag 622
31674_Psc -----..----------...----------..- 624
38047_Pf -----..----------...----------cag 627
38071_Psc -ac--..-ag-aattcct---caa--a--ggt---accag 631
38076_Psc -----..----------...----------cag 617
38078_Psc -----..----------...----------cag 618
38089_Psc -----..----------...----------cag 618
38091_Psc -----..----------...----------..- 616
38092_Psc -----..----------...----------.ag 616
28511_Po GTC..CCCCCCCGGAAAGGGCCTTAAC...GAATCCCTAG 660
28683_Psc ---cc--ttaa---g-----t-catcaatga-t--a-c-a 656
28684_Pc aggtc---tag-----g-----ccccggaccccgg---ct 694
28685_Psc ---ac--tta------g--t-t---tt...--t--a--t- 656
29757_Psc ---cc-t-t-.-c-g---cc--cccc-c.c-ctct--ctc 655
31017_Psc ---cc-tttg..--g----ttt--tc..tga-t--a-..a 650
31613_Psc ---ac--tta.--------c--c-c--c.ga----a-c-a 660
31674_Psc ---..---aa----g----..-c--ct...-g--t--ct- 657
38047_Pf ---ac----a-ac------cttcc--t...--c----c-- 664
38071_Psc -gtgggtttg..-at----a-----ggttga--a-g-cg- 669
38076_Psc ---ac---a-----gg------c-cc-tcgact-----.t 656
38078_Psc -c-cc-----.---g---------t-t...-g--tt-cta 654
38089_Psc ---cc-----.-----g-----cg--aggt-tt-t--cg- 657
38091_Psc ---cc----g.g----g--cttaatga.....--t--ct. 649
38092_Psc ---ac-taa..aa-g-----a-ccct...g--t----a.t 650

```
28511_Po   GGGG..TGTGGT..GGCCT.TAGGGGATGT              685
28683_Psc  ---agt---t-ctg-c--c.a-----gct-              685
28684_Pc   ct--ctcc---cgc-c---t-c---tc-tc              724
28685_Psc  ---...---t--..---tc.c-----t---              680
29757_Psc  tc-cgt-cct-ctt-c-tc.-c-ctcc-t-              684
31017_Psc  t--agt---t-cgg-c--c.a..---gctg              677
31613_Psc  ccacttg--t-ctg-c--c.cg---ct--c              689
31674_Psc  ---...--gt--..--g-c.c-----g-tg              681
38047_Pf   ----..---c-gcg----c.c-----c-t-              691
38071_Psc  --ttct-tg--ggg-c--c.cccaaccat-              698
38076_Psc  ----gt-----cgg-c--c.ag---cc-tg              685
38078_Psc  t---gg------gg-c-tc.ag----t---              683
38089_Psc  ----gt--g--ggg-c--c.ag---ct--g              686
38091_Psc  ..--gagtg--gcg---tc.cg---ct---              676
38092_Psc  --a-..-----ggg-c--..ag---ctgtg              676
```

Multiple alignment of the edited sequences of all the BCCM/MUCL *Pleurotus sajor-caju* strains used in this study is shown below. Bases different from other bases at the respective position in the sequence are depicted by small caps

Maximum Length: 704 bases, bases 23 to 726 of the full ITS sequences, A = adenine, C = cytosine, G = guanine and T = thymine

Po = *Pleurotus ostreatus*, Psc = *Pleurotus sajor-caju*, Pc = *Pleurotus citrinopileatus*, Pf = *Pleurotus floridanus*

STRAIN SEQUENCE base

```
28683_Psc  CCTAC.CTGATTTGAGTCA..AATTGTCAAATTGTC.CTT     36
28685_Psc  -a---.----------ctcta--------------.---     38
29757_Psc  -----.-------------..---------------.---     36
31017_Psc  -----.-------------..---------------.---     36
31613_Psc  -tg--a------------tgt--------------t---     40
31674_Psc  -----.-------------..---------------.---     36
38071_Psc  -a-gac----------ctcat--------------.---     39
38076_Psc  -----.-------------..---------------.---     36
38078_Psc  -----.-------------..---------------.---     36
38089_Psc  -----.-------------..---------------.---     36
38091_Psc  -----.-------------..---------------.---     36
38092_Psc  -----.-------------..---------------.---     36

28683_Psc  GCGGACGATTAGAGAGCTGGACTCTAT..TCATGCGTGCT     74
28685_Psc  --------------------------..----------     76
29757_Psc  --------------------------..----------     74
31017_Psc  --------------------------..----------     74
31613_Psc  -------------------------atc----------     80
31674_Psc  --------------------------..----------     74
38071_Psc  --------------------------..c---------     77
38076_Psc  --------------------------..----------     74
38078_Psc  --------------------------..----------     74
38089_Psc  --------------------------..----------     74
38091_Psc  --------------------------..----------     74
38092_Psc  --------------------------..----------     74

28683_Psc  ATTGATGAGTGATAATTATCACATCATGCGCAGAGGCAAT    114
28685_Psc  ------------------------------------    116
```

```
29757_Psc  ------------------------------------    114
31017_Psc  ------------------------------------    114
31613_Psc  ------------------------------------    120
31674_Psc  ------------------------------------    114
38071_Psc  ------------------------------------    117
38076_Psc  ------------------------------------    114
38078_Psc  ------------------------------------    114
38089_Psc  ------------------------------------    114
38091_Psc  ------------------------------------    114
38092_Psc  ------------------------------------    114

28683_Psc  GAGAAGTCCTGCTAATGCATTTAAGAGGAGCCGACTTGTT    154
28685_Psc  ------------------------------------    156
29757_Psc  ------------------------------------    154
31017_Psc  ------------------------------------    154
31613_Psc  ------------------------------------    160
31674_Psc  ----------------------------------c---c    154
38071_Psc  ------------------------------------    157
38076_Psc  ------------------------------------    154
38078_Psc  ------------------------------------    154
38089_Psc  ------------------------------------    154
38091_Psc  ----------------------------------------g    154
38092_Psc  ------------------------------------    154

28683_Psc  ACAGCCAGCAACCCCCAACAATCCAAACATCACAAATA..    192
28685_Psc  ------------------------------------..    194
29757_Psc  ------------------------------------..    192
31017_Psc  ------------------------------------..    192
31613_Psc  ------------------------------------..    198
31674_Psc  -ag-------g-----------------------t-gga    194
38071_Psc  ------------------------------------..    195
38076_Psc  ------------------------------------..    192
38078_Psc  ------------------------------------..    192
38089_Psc  --g---------------------------------..    192
38091_Psc  ------------------------------------..    192
38092_Psc  ------------------------------------..    192

28683_Psc  .......AATGTGAGTTTGAGAATTTAATG.ACACTCAAA    224
28685_Psc  .......----------------------.---------    226
29757_Psc  .......----------------------.---------    224
31017_Psc  .......----------------------.---------    224
31613_Psc  .......----------------------.---------    230
31674_Psc  aaaaacc--a-------------------.---------    233
38071_Psc  .......----------------------.---------    227
38076_Psc  .......----------------------.---------    224
38078_Psc  .......----------------------.---------    224
38089_Psc  .......----------------------g--------g    225
38091_Psc  .......----------------------.---------    224
38092_Psc  .......----------------------.---------    224

28683_Psc  CAGGCATGCCCCTCGGAATACCAAGGGGCGCAAGGTGCGT    264
28685_Psc  ------------------------------------    266
29757_Psc  ------------------------------------    264
31017_Psc  ------------------------------------    264
31613_Psc  ------------------------------------    270
31674_Psc  ------------------------------------    273
38071_Psc  ------------------------------------    267
38076_Psc  ------------------------------------    264
38078_Psc  ------------------------------------    264
38089_Psc  ------------------------------------    265
38091_Psc  ------------------------------------    264
```

```
38092_Psc  ------------------------------------    264

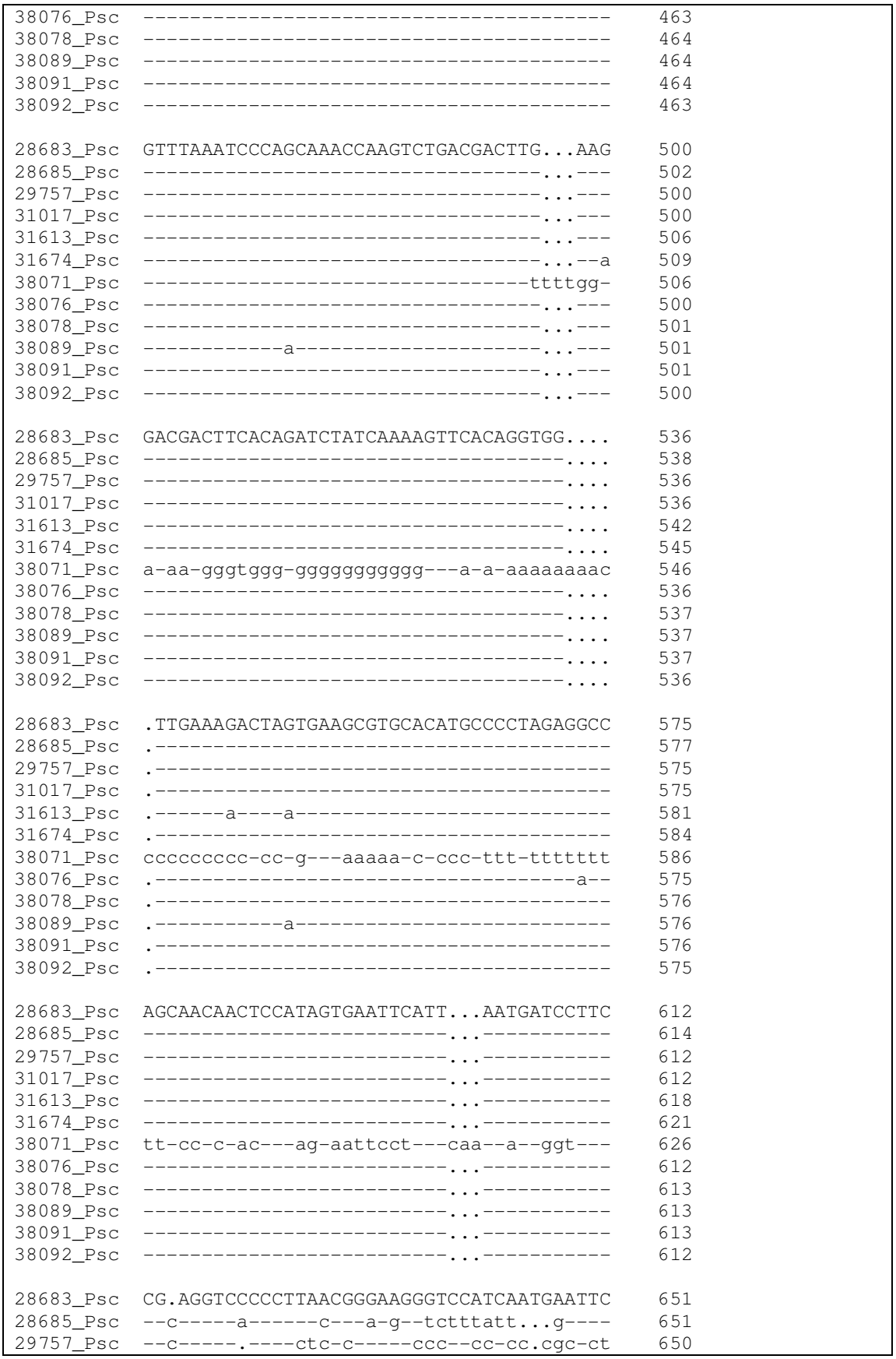
28683_Psc  TCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT    304
28685_Psc  ------------------------------------    306
29757_Psc  ------------------------------------    304
31017_Psc  ------------------------------------    304
31613_Psc  ------------------------------------    310
31674_Psc  ------------------------------------    313
38071_Psc  ------------------------------------    307
38076_Psc  ------------------------------------    304
38078_Psc  ------------------------------------    304
38089_Psc  ----------a-------------------------    305
38091_Psc  ------------------------------------    304
38092_Psc  ------------------------------------    304

28683_Psc  ACTTATCGCATTT.CGCTGCGTTCTTCATCGATGCGAGAG    343
28685_Psc  -------------.-------------------------    345
29757_Psc  -------------.-------------------------    343
31017_Psc  -------------.-------------------------    343
31613_Psc  -------------.-------------------------    349
31674_Psc  -------------.-------------------------    352
38071_Psc  -------------.-------------------------    346
38076_Psc  -------------.-------------------------    343
38078_Psc  ------------t-------------------------    344
38089_Psc  -------------.-------------------------    344
38091_Psc  ------------t-------------------------    344
38092_Psc  -------------.-------------------------    343

28683_Psc  CCAAGAGATCCGTTGTTGAAAGTTGTATTATGGTTTATAG    383
28685_Psc  ------------------------------------    385
29757_Psc  ------------------------------------    383
31017_Psc  ------------------------------------    383
31613_Psc  ------------------------------------    389
31674_Psc  ------------------------------------    392
38071_Psc  ------------------------------------    386
38076_Psc  ------------------------------------    383
38078_Psc  ------------------------------------    384
38089_Psc  ------------------------------------    384
38091_Psc  ------------------------------------    384
38092_Psc  ------------------------------------    383

28683_Psc  GCACAAGGCCCATTAAATGACATTCGTAGACATACATTTG    423
28685_Psc  ------------------------------------    425
29757_Psc  ------------------------------------    423
31017_Psc  ------------------------------------    423
31613_Psc  ------------------------------------    429
31674_Psc  ------------------------------------    432
38071_Psc  ------------------------------------    426
38076_Psc  ------------------------------------    423
38078_Psc  ------------------------------------    424
38089_Psc  ------------------------------------    424
38091_Psc  ------------------------------------    424
38092_Psc  ------------------------------------    423

28683_Psc  GGGTGTGTTAAGTAAATAGACTGCGTTGTCACACCGAGAC    463
28685_Psc  ------------------------------------    465
29757_Psc  ------------------------------------    463
31017_Psc  ------------------------------------    463
31613_Psc  ------------------------------------    469
31674_Psc  ------------------------------------    472
38071_Psc  ------------------------------------    466
```


38076_Psc ---------- 463
38078_Psc ---------- 464
38089_Psc ---------- 464
38091_Psc ---------- 464
38092_Psc ---------- 463
28683_Psc GTTTAAATCCCAGCAAACCAAGTCTGACGACTTG...AAG 500
28685_Psc ----------...--- 502
29757_Psc ----------...--- 500
31017_Psc ----------...--- 500
31613_Psc ----------...--- 506
31674_Psc ----------...--a 509
38071_Psc ----------ttttgg- 506
38076_Psc ----------...--- 500
38078_Psc ----------...--- 501
38089_Psc ----------a----------...--- 501
38091_Psc ----------...--- 501
38092_Psc ----------...--- 500
28683_Psc GACGACTTCACAGATCTATCAAAAGTTCACAGGTGG.... 536
28685_Psc ----------.... 538
29757_Psc ----------.... 536
31017_Psc ----------.... 536
31613_Psc ----------.... 542
31674_Psc ----------.... 545
38071_Psc a-aa-gggtggg-gggggggggg---a-a-aaaaaaaac 546
38076_Psc ----------.... 536
38078_Psc ----------.... 537
38089_Psc ----------.... 537
38091_Psc ----------.... 537
38092_Psc ----------.... 536
28683_Psc .TTGAAAGACTAGTGAAGCGTGCACATGCCCCTAGAGGCC 575
28685_Psc .---------- 577
29757_Psc .---------- 575
31017_Psc .---------- 575
31613_Psc .------a----a---------- 581
31674_Psc .---------- 584
38071_Psc ccccccccc-cc-g---aaaaa-c-ccc-ttt-tttttttt 586
38076_Psc .----------a-- 575
38078_Psc .---------- 576
38089_Psc .----------a---------- 576
38091_Psc .---------- 576
38092_Psc .---------- 575
28683_Psc AGCAACAACTCCATAGTGAATTCATT...AATGATCCTTC 612
28685_Psc ----------...---------- 614
29757_Psc ----------...---------- 612
31017_Psc ----------...---------- 612
31613_Psc ----------...---------- 618
31674_Psc ----------...---------- 621
38071_Psc tt-cc-c-ac---ag-aattcct---caa--a--ggt--- 626
38076_Psc ----------...---------- 612
38078_Psc ----------...---------- 613
38089_Psc ----------...---------- 613
38091_Psc ----------...---------- 613
38092_Psc ----------...---------- 612
28683_Psc CG.AGGTCCCCCTTAACGGGAAGGGTCCATCAATGAATTC 651
28685_Psc --c-----a------c---a-g--tctttatt...g---- 651
29757_Psc --c-----.----ctc-c-----ccc--cc-cc.cgc-ct 650

```
31017_Psc  --.--------t--g..-------t-ttt--..-------    647
31613_Psc  --c-----.a--c-t----a----cc--tcacc.---a--    656
31674_Psc  --..a---..--ca-c--------cct...act.-g----    653
38071_Psc  acc---gtgggt--g..-at----ac-ttaggt----aa-    664
38076_Psc  --c-----a---cacc----g----c--tc-ct.cg-c-t    651
38078_Psc  --c---c-.---cccc---------c-tt-at...gga-t    649
38089_Psc  --c-----.---cccc---a-g---c--gaa-ggtgt--t    652
38091_Psc  --ag.....t--cccc----g-a--g-ttaat...g-a-t    645
38092_Psc  --.-----.a---a--a--a-g--ac--c-g....g----    646

28683_Psc  ACCAAGGGAGTTGTTGCTGGCCCC                   675
28685_Psc  --ttg---...-----tg-ct--a                   672
29757_Psc  c--tctc-c---cc----t---t-                   674
31017_Psc  --..-t----------g------                    669
31613_Psc  -----ccact-g-----------                    680
31674_Psc  c-tgg--....--g--tg-----a                   673
38071_Psc  g--gg--ttc--tgg-gg------                   688
38076_Psc  c--tt---g-----g--g------                   675
38078_Psc  t--t-t--g-g---g-tg----t-                   673
38089_Psc  c--gg---g----gg-gg------                   676
38091_Psc  c--t..--gag--gg--g---t--                   667
38092_Psc  c-atg-a-...---g-gg-----a                   667
```

APPENDIX I: Restriction Enzyme Analysis of the ITS rDNA Sequences of the *Pleurotus* Species and Strains

Example of a restriction enzyme map of the ITS rDNA sequence of BCCM/MUCL strain 28683 (*Pleurotus sajor-caju*) is shown below.

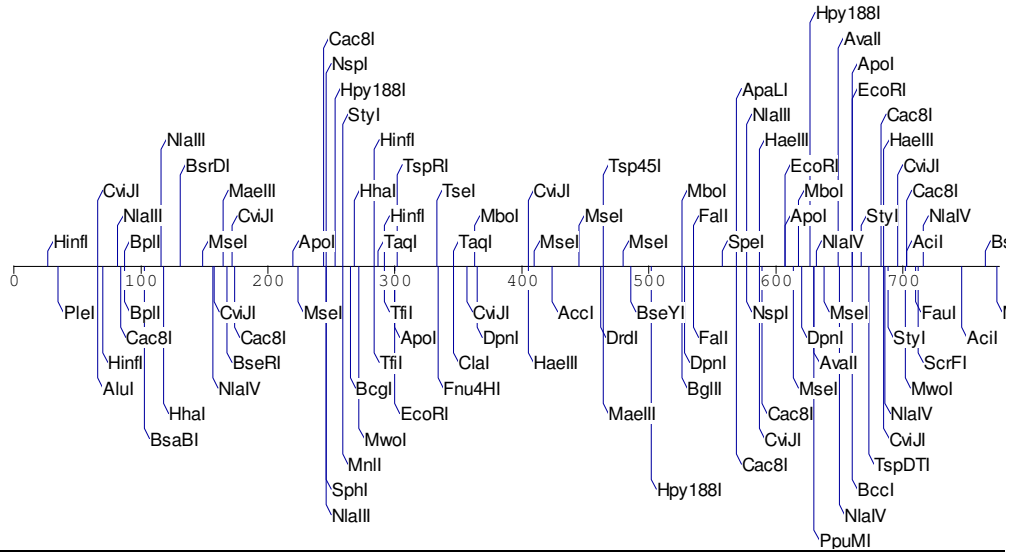


Table to show restriction enzymes that have sites in the ITS sequences of rDNA of *Pleurotus* species and strains in this study	
Restriction Enzyme	BCCM/MUCL strain
*Acc*1, *Alu*1, *Apo*1, *Cac*81, *Cla*1, *Cvi*J1, *Dpn*1, *Fnu*4H1, *Hae*1II, *Hha*1, *Hin*f1, *Hpy*1881, *Mae*III, *Mbo*1, *Mnl*I, *Mse*1, *Mwo*1, *Nla*IV, *Rsa*1, *Scr*F1, *Sty*1, *Taq*1, *Tfi*1, *Tsp*451, *Tsp*R1	All strains in this study (AS)
Strains listed are exceptions to the respective restriction enzyme (do not have restriction site for respective restriction enzyme	
Restriction Enzyme	BCCM/MUCL strain
*Aci*1	38091, 28684
*Apa*L1, *Bse*R1, *Bsr*D1, *Drd*1	38089, 28684
*Bcg*1, *Bgi*II, *Bsa*B1, *Bse*Y1, *Eco*R1, *Nla*III, *Nsp*1, Sph1, *Tse*1	28684
*Ava*II	28685, 31613, 31674, 38047, 38071, 38076, 38078, 38091

Restriction Enzyme	BCCM/MUCL strain
*Ava*I	28511, 28683, 28685, 31017, 31674, 38047, 38071, 38092
*Bbv*1	28683, 28684, 28685, 31017, 31613, 38071, 38076, 38078, 38089, 38091, 38092
*Bcc*1	28511, 28684, 31017, 31613, 31674, 38047, 38071, 38076, 38078, 38091, 38092
*Bpi*1	38089
*Fau*1	28684, 28685, 31674, 38047, 38071, 38076, 38078, 38091, 38092
*Hpa*II	28683, 38047, 38071, 38092,
*Ple*1	28685, 38047, 38071, 38089
*Spe*1	28684, 31613, 38071, 38089
*Tau*1	28683, 29757, 31613, 38047, 38071, 38076, 38078, 38091
*Fal*1	28684, 28511, 31674, 38047

Strains listed have exclusive restriction site(s) for the restriction enzymes listed

Restriction Enzyme	BCCM/MUCL strain
*Apa*1	28684, 31017, 31674, 38047, 38076, 38092
*Alw*N1	31017, 38092
*Avr*i1, *For*1	28511
*Bsm*A1	28683, 28685, 31017, 31613
*Bse*Mi1, *Sac*II	38078
*Dde*1	28684, 28511,38078, 38089
*Bsr*B1, *Hga*1	29757
*Asc*1, *Kpn*1, *Msi*1, *Nhe*1, *Psi*1	28684
*Hae*II, *Nar*1, *Pfi*M1	38089
*Apa*L1, *Nco*1, *San*D1	38092
*Nae*1	28684, 38089
*Bci*V1	38078, 38091
*Sfi*1	38076
*Bsa*A1	38071
*Xmn*1	28684, 38047
*Smi*1	38047
*Stu*1	31674, 38089
*Tsp*GW1	31613
*Sma*1	28684, 31613, 38076, 38078, 38089, 38091

Restriction Enzyme	BCCM/MUCL strain
*Hph*1	28684, 31613, 38071, 38089
*Dra*1II	28684, 31613
*Bst*X1	31613, 31674
*Mbo*II	28684, 31017, 38089
*Bsr*1	31017, 31674
*Bgi*1	28684, 31017, 31613, 38047, 38076, 38089
*Rsa*1	28684, 29757, 38089
*Pfo*1	28684, 29757
*Taq*II	28685, 29757
*Sac*1	28685, 38047
*Hpy*991	28685, 29757, 38047
*Hin*41	28684, 28685, 31613, 38047
*Eco*N1	28685, 29757, 31613, 38047, 38071, 38076, 38078
*Bst*EII	28685, 31613, 38047, 38076, 38092
*Tsp*DT1	28511, 31613, 38089, 38091
*Ppu*M1	28683, 28684, 29757, 31017, 38092
*Xcm*1	28511, 28684, 31613
*Mfe*1	28511, 28684, 31674, 38047