CONVENTIONAL INTERSTRAIN MATING BETWEEN *Pleurotus pulmonarius* (FR.) QUÉL AND *Pleurotus citrinopileatus* SINGER AND YIELD PERFORMANCE OF SELECTED HYBRIDS

ROSNINA BINTI ABDUL GANI

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIRMENTS FOR THE DGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA

2017

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate : Rosnina Binti Abdul Gani [redacted]

Registration/Matric No: SHC060013

Name of Degree: DOCTOR OF PHILOSOPHY

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

CONVETIONAL INTERSTRAIN MATING OF *Pleurotus pulmonarius* (Fr.) Quél AND *Pleurotus citrinopileatus* Singer AND YIELD PERFORMANCE HYBRIDS

Field of Study: **MUSHROOM BIOTECNOLOGY**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

[redacted]

Candidate's Signature

Date 10/03/2017

Dr. Noorlidah Abdullah
Profesor
Institut Sains Biologi, Fakulti Sains
Universiti Malaya
50603 Kuala Lumpur

Subscribed and solemnly declared before,

Witness's Signature [redacted]

Date 10.3.17

Name: PROFESOR DR NOORLIDAH ABDULLAH
Designation

Witness's Signature [redacted]

Date 10.3.17

Name: PROFESOR DI BARATNAM
Designation

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCES, UNIVERSITY OF MALAYA
50603 KUALA LUMPUR

ABSTRACT

Oyster mushrooms (*Pleurotus* spp.) are cultivated widely at present as it ranked second after the button mushroom. There is a growing demand for new and improved mushroom hybrids with better features including yield. The present study was conducted with the objectives to improve the characteristics and yield of *Pleurotus pulmonariius* and *P. citrinopileatus* by interstrain mating between *P. pulmonarius* and *P. citrinopileatus* monokaryon cultures. Ten single spore isolates of both parental strains were crossed in all combinations for the purpose of developing new hybrids. From a total of one hundred possible pairings, five hybrids were obtained which produced sporophores with *P. pulmonarius* dominant characteristics and six hybrids had sporophores features similar to *P. citrinopileatus*. Three dikaryotic isolates were evaluated for duration of spawn run, yield and biological efficiency. Further analysis of the mating types and compatible mating of the isolates of dikaryons from the positive mating were confirmed and differentiated. Macroscopic features of the new genotype sporophores were compared to parental strains and analyzed using DNA molecular work. The DNA sequences of five hybrids showed high genetic homology with dominant parent *P. pulmonarius* which generated a bootstrap value of 94 %. Six hybrids were found to be in the same clade as the other parent, *P. citrinopileatus* with a bootstrap value of 99 %. High bootstrap values indicated high genetic homology between hybrids and their dominant parent *P. pulmonarius* and *P. citrinopileatus*. An interspecies hybrids was shows to may have better prospects for genetic improvement in the family of *Pleurotus*. The sporophores of dominant hybrid (P19xC5) of *P. pulmonarius* showed crowded bellow lamella, the pilei similar to grey oyster with bigger in size and tougher pileus cap and less spores. These characteristics influenced the harvesting duration and shelf life of the mushroom species. While two other hybrids (P1xC9, and P3xC8) which

were similar to *P. citrinopileatus* sporophores features have less offensive aroma, improved texture and higher sporophores yield, compared to *P. citrinopileatus* have an attractive shape and yellow in colour but the fragile texture complicates packaging, and its strong aroma is unappealing to consumers. Among the selected hybrids, P19xC5 showed higher growth rate and increased sporophores yield (182.97 g) and biological efficiency (130.19%) followed by hybrids P1xC9 (70.97 %) and P3xC8 (52.14%), which were also higher than the *P. citrinopileatus* parent (35.63%). Interspecies hybrids obtained by this mating technique can lead to better strains of mushrooms with genetic improvement of *Pleurotus* species.

Keywords: Pleurotaceae, biological efficiency, sporophore, monokaryon, dikaryon

ABSTRAK

Cendawan tiram (*Pleurotus* spp.) ditanam secara meluas pada masa ini kerana ia merupakan cendawan kedua yang popular setelah cendawan butang. Permintaan yang tinggi bagi cendawan hibrid dengan ciri-ciri yang lebih baik dan penghasilan yang tinggi. Kajian ini dijalankan dengan objektif untuk meningkatkan ciri-ciri dan hasil hibrid *Pleurotus pulmonarius* (cendawan tiram kelabu) dan hibrid *P. citrinopileatus* (cendawan tiram kuning) dengan teknik kacukan antara strain (mating interstrain) antara spora tunggal cendawan tiram kelabu dan cendawan tiram kuning. Sepuluh pencilan spora tunggal dari kedua-dua strain induk disilangkan diantara satu sama lain menghasilkan hibrid baru. Hasil dari serratus pasangan yang dikacukkan, hanya 11 hibrid yang berjaya menghasilkan jana buah. 11 hibrid yang berjaya ditanam untuk memerhati perbezaan dengan ciri-ciri fizikal kedua induknya. Lebih lanjut analisis DNA dilakukan untuk melihat kesamaan dari dua strain induk. Didapati lima hibrid yang diperolehi memiliki ciri-ciri dominan dari jana buah *P. pulmonarius* dan enam hibrid memiliki ciri-ciri yang menyerupai jana buah *P. citrinopileatus*. Hasil uji DNA menunjukkan dari lima hibrid memiliki homologi genetik tinggi dengan ciri-ciri dominan dan menempati clad yang sama dengan induk *P. pulmonarius* dengan nilai bootstrap 94%, dan enam hibrid didapati pada clad yang memiliki ciri yang sama dengan *P. citrinopileatus* dengan nilai bootstrap 99%. Nilai-nilai bootstrap yang tinggi menunjukkan homologi genetik yang tinggi antara hibrid dan masing-masing kedua induk mereka *P. pulmonarius* dan *P. citrinopileatus*. Tiga dikaryotic hibrid yang menunjukkan kadar pertumbuhan miselia, penghasilan jana buah dan kadar kecekapan biologi (BE) tinggi dipilih untuk ditanam dalam sekala lebih besar masing-masing sebanyak 100 bag. Hibrid P19xC5 dominan *P. pulmonarius* menunjukkan tekstur jana buah yang dengan ukuran yang lebih besar, tekstur

topi (pileus) yang lebih kuat/liat akan mempengaruhi tempoh penuaian dan jangka hayat spesies cendawan. Manakala dua hibrid dominan *Pleurotus citrinopileatus* (P1xC9, P3xC8) yang mempunyai aroma yang kurang, tekstur yang lebih kuat dan hasil yang lebih tinggi dibanding dengan jana buah daripada induk (*P. citrinopileatus*) yang memiliki tekstur jana buah yang rapuh akan merumitkan proses pembungkusan, dan aroma yang kuat kurang disukai oleh sebagian konsumen. Antara kacukan cendawan terpilih adalah hibrid P19xC5 menunjukkan kadar pertumbuhan yang paling tinggi dan peningkatan hasil jana buah sebanyak (182,97 g) dan kecekapan biologi (130,19%) diikuti dengan hibrid P1xC9 (70,97%) dan P3xC8 (52,14%) juga lebih tinggi daripada ibu bapa *P. citrinopileatus* (35,63%). Interspecies hibrid yang diperolehi ini boleh membawa prospek yang lebih baik untuk penambahbaikan genetik dalam famili Pleurotus.

Kata kunci: Pleurotaceae, kadar kecekapan biologi, sporophore, monokaryon, dikaryon

ACKNOWLEDGEMENT

I would like to thank Profesor Dr. Noorlidah Abdullah for being a valuable mentor and a role model. She was always available for consultation on issues related to my research project and my academic achievements. A sincere appreciation is also extended to Profesor Dr. Vikineswary Sabaratnam as she shared her expertise and guided me in my research. Many thanks to her for she advice and support throughout the course of this research.

In my daily work I have been blessed with a friendly and cheerful group of fellow students and research staffs. I would like to take this opportunity to dedicate my deepest appreciation to all my friends in the Mycology Lab for their support and guidance and most importantly for contributing their unselfish time in sharing wonderful ideas and helping me carry out my research works.

I also wish to thank my friends, colleagues and the staff of department and faculty for making my time at ISB (Institute of Biological Sciences) University Malaya a memorable one. I also wish to extend my gratitude to Universitas Malikussaleh for the opportunity of allowing me to enrich my knowledge and experience during my stay in the Kuala Lumpur Malaysia.

Finally, thank you to my late father and mother and my family for their endless support, encouragements and love.

TABLE OF CONTENTS

CONTENTS **PAGE**

LIST OF FIGURES

Contents **PAGE**

LIST OF TABLES

Contents **PAGE**

LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation		Definition
ANOVA	:	Analysis of Variance
BE	:	Biological efficiency
dH_2O	:	Distilled water
Ca	:	Calcium
$CaCO_3$	:	Calcium carbonate
DMRT	:	Duncan's Multiple Range Test
DNA	:	Deoxyribonucleic acid
e.g	:	for examples
et al	:	and others
etc	:	et cetera
Fe	:	Ferum
g	:	gram
ie	:	that is (to say)
ITS	:	Internal transcribed spacers
Mg	:	Magnesium
Mn	:	Mangan
ML	:	maximum likelihood

MEA	:	Malt extract agar
mg	:	milligram
ml	:	millilitre
min	:	minute
kg	:	kilogram
MMT	:	Million metric tones
MtDH	:	Mannitol-dehydrogenase
PB	:	Parsimony bootstrap
PDA	:	Potato dextrose agar
PFAG	:	Pulsed-field agarose gel
pH	:	Potential hydrogen
RAPD	:	Random amplified polymorphic DNA
rDNA	:	Ribosomal DNA
RH	:	Relative humidity
RLFP	:	Random Amplification of Polymorphic DNA
RNA	:	Ribonucleic acid
Se	:	Selenium
TBR	:	Tree bisection reconnection
µg	:	Microgram
µm	:	Micrometre
US	:	United State
UV	:	Ultraviolet
Zn	:	Zinc
STL buffer	:	St. Louis
OB	:	oil body
BL	:	Buffer Layer
HB buffer	:	Hemaglobin

TBE	:	*Tris-Borate-Edta*
BNL	:	Block-Nested Loops
MEGA	:	five Molecular Evolutionary Genetics Analysis

CHAPTER 1

INTRODUCTION

Mushrooms play important roles in the world, being useful to humans, both as nutritious food sources and medicinal remedies (Alam *et al*., 2010). Growing mushrooms demands specific environmental conditions, and they may be cultivated simply and cheaply (Sánchez 2010). Their involvement in the bioconversion of organic wastes into forms that can enter the major nutrient cycles, there are reported greatly benefits both for humans and nature (Miles & Chang 1997). They are a potential protein source, especially in developing countries where animal proteins are scarce and expensive (Quimio, 1990; Jonathan *et al*., 2012). Also, certain species have an exotic taste, and the demand of them is steadily increasing. Mushrooms are efficient low-fat protein sources and allow for easy cultivation on a wide variety of substrates, especially on agricultural wastes and other industrial wastes (Larraya *et al*., 2000; Goltapeh & Pourjam, 2005).

Pleurotus species are commercially important as cultivated mushrooms worldwide (Kibar & Peksen, 2008). They are cultivated in many countries in Europe and Asia. One of the prominent species of this genus is *Pleurotus pulmonarius* (Fr.) Quél (1827), commonly known as grey oyster mushroom, being one of the most preferred species grown in Malaysia. The grey coloured sporophore which belongs to this species has a fleshy texture, good aroma and mild taste, high protein content, and medicinal properties such as anti-tumour, anti-inflammatory, and antimicrobial activities, (Jose *et al*. 2002; Atri *et al*., 2013). Another species from the same genus is *P. citrinopileatus* (yellow oyster mushroom) that has a bright yellow coloured pileus and fragile texture with a strong aroma. It contains useful antitumor polysaccharides (Zhang & Wang, 1994, Wang & Hu, 2005), proving antioxidant

activities (Hu, Liang *et al*., 2006), enhances immunity, and delays aging (Wang, Yan *et al*., 2001). Besides having an attractive shape and bright yellow colour, the sporophore have very fragile texture, so their packaging for transportation it is difficult. This fungus also has a strong aroma, and only a few consumers prefer the flavour. Due to these traits, the mushroom is rarely found in the market.

Pleurotus is a genus of edible mushroom which has attracted interest for improving hybrids grown with more desirable feature than the parent species. Hybridization and breeding refers to combining desired traits from different species. A breeding program will include the combination and selection of desired traits with the goal of obtaining a possible recombination of the genetic material of two different species for the production of best quality and high yield mushrooms (Miles & Chang, 1997). High quality biological sources for the spawn culture have a strong influence in mushroom sectors. The mushroom sector requires adaptive technology, particularly mushroom biotechnology, to produce and improve the quality of mushroom culturing through innovation and genetic engineering.

The production of mushrooms is dependent on the quality of spawn used for inoculating the cultures. In order to produce high-quality spawn, breeding programs to obtain cultures for strains resistant to certain diseases or able to form high-quality sporophore under standard growth conditions are necessary (Kothe, 2001). To generate new high performance of mushroom species, a range of breeding methods have been established, such as interspecific crosses reported among *P. ostreatus*, *P. florida*, *P. columbinus* and *P. sapidus* (Peberdy, 1993). There have been several reports concerning interspecific crosses involving *Pleurotus* species using protoplast fusion between interspecific hybridization between *Pleurotus cornucopiae* and *P. florida* (Yoo, 1992), *Pleurotus eous* and *P. flabellatus* by Poly-Ethylene-Glycol (PEG) – induced protoplast fusion (Parani, 2013). Dhitaphichit & Pornsuriya (2005)

reported protoplast fusion using some chemicals to remove the cell wall between *Pleurotus ostreatus* and *P. citrinopileatus*. The sporophore genotype showed recombined characteristics of both parents. Targeted manipulation at DNA level can be achieved to combine polygene from both tested parents to produce new starting material (Case *et al*., 1979; Ballance *et al*., 1983). Programmed mutations on white oyster mushroom (*Pleurotus floridae*) using gamma (^{60}Co) irradiation was carried out by Djayanegara & Harsono (2007). Sharma *et al*., (2014) carried out a strain improvement in *Pleurotus ostreatus* using UV light and ethyl methyl sulfonate as mutagens with emphasis on the reduced the number of spores produced of the sporophore.

According to McCarty (2010), it is a global challenge in cultivating *Pleurotus* spp. as the mushrooms have a short shelf-life. The desired characteristics of hybridized strains. The desired hybridized strain characteristic is can be economically beneficial for better traits, good features, higher yield, and so on. The potential of cross breeding in mating studies and molecular markers might help to identify new genotypes of distinguishing morphological characters. Some researchers have reported that some mating systems, which operate in *Pleurotus* spp., have shown successful mating. The experiments regarding the mating system conducted in this study have examined whether there is a significant operating system on the phylogenetic mating type. In detail, low potency means it can be administered to accelerate new hybrids with desired features through spore mating. Mating type genes and other compatibility factors are now used in mating-type-assisted breeding programs for producing efficient and edible mushroom species (Kothe, 2001). Moreover, Gupta (2011) reported that hybridization based on the crossing of non-fertile homokaryotic lines offer better prospects for genetic improvement. Compatible mating hybrid varieties are becoming popular as they meet consumer preference and may increase both efficiency and nutritional status.

These species, *P. pulmonarius* and *P. citrinopileatus*, have the ability to grow easily on different compost media, and allow cross breeding (hybridization). *Pleurotus citrinopileatus* has a bright yellow pileus and fragile texture and strong aromatic sporophore, while *P. pulmonarius* has a mild taste and fleshy sporophore. Although both of them have high medicinal properties and some positive characteristics such as fleshy sporophore and mild taste for *P. pulmonarius*, and bright yellow color with funnel shape for *P. citrinopileatus*. Instability of production at a relatively low level, inconsistencies of flush and appearance of sporophores, fragile texture, and strong odour of *P. citrinopileatus* affect adoption in mushroom cultivation. Thus, the development of hybrid hybrids may combine parental characteristics, which are fleshy sporophores (*P. pulmonarius*) and bright yellow colour (*P. citrinopileatus*) together with improved taste, spore less hybrid, and better features. Besides, a tougher pileus increases the yield, thus lengthening the shelf life of the hybrid. Gupta and co-researchers (2011) reported on the successful inter-strain hybridization using single spore isolation between *P. sajor-caju*, *P. florida*, *P. eous*, and *Hypsizgus ulmaris* which resulted in hybrids with shorter spawn run time periods (17 and 18 days) compared to the normal period, which takes about 25-30 days (Gupta *et al.*, 2011). Breeding techniques of mushrooms with high yield, better quality and performance in Malaysia are still limited as the introduction and procurement of imported strains from advanced countries are still ongoing. This study will fulfil the requirement for commercial production of edible mushrooms in Malaysia in view of their potential contribution to availability of a healthy food, environmental and health values.

Thus, the aim of the current study was to produce *P. pulmonarius* hybrids with thick, fleshier texture, lesser spores and increase productivity via conventional mating to improve its commercialization value. Sporophores with firmer texture minimise the risk

of damage during packaging and reduce loss. This will lead to increased productivity in the mushroom industry.

1.1 OBJECTIVES

Based on the reasons stated above, the specific objectives are as follows:

i. to produce monokaryons of *Pleurotus pulmonarius* and *P. citrinopileatus* and to determine the compatibility of hybridization
ii. to evaluate the mycelial growth rate and cultural characteristics and molecular analysis of the new hybrids compared to parental strains
iii. to obtain sporophores of new hybrids and to assess their morphology and yield compared to parental strains.
iv. to determine the productivity and morphological stability of selected hybrids through their cultivation

CHAPTER 2

LITERATURE REVIEW

2.1 World Mushroom Production

The cultivation of mushrooms has spread rapidly worldwide as a result of their remarkable natural biological features and the available versatile technology. In general, two main trends of production can be identified: private commercial enterprises operating primarily on a large and small scale for accumulation of capital, involving intensive cultivation and tend to be highly mechanized; and the rural production to fulfil regional needs, which is performed through conventional cultivation methods. Such trends will be beneficial to developing countries, in terms of its local contribution to food production, rural development, and sustainable agriculture. The most cultivated mushroom worldwide is *Agaricus bisporus* followed by *Pleurotus* spp., *Lentinula edodes, Auricularia auricula*, *Flamulina velutipes* and *Volvariella volvacea* (Aida *et al*., 2009). Button mushroom (*A. bisporus*) was widely cultivated in Europe before being exported to North America by the settlers; the Shiitake mushroom (*L. edodes*) was grown for centuries in China and other oriental countries and the oyster mushroom (*Pleurotus ostreatus*) was collected as wild specimens from forests in Florida and later actively cultivated in several countries around the world. The oriental Enoki or velvet stem mushroom (*F. velutipes*) is currently largely produced in Japan. The paddy straw mushroom (*V. volvaceae*) and ear fungus (*A. auricula*) are also gaining popularity because of their great medicinal value.

Nowadays, mushrooms have been valued as edible, medicinal provisions, pharmaceuticals and other industrialization. The worldwide mushroom production continues to increase. It is estimated that more than 10 MMT (million metric tonnes) of edible and medicinal mushrooms were produced using waste materials into a wide

diversity of products (edible or medicinal food). Cultivation of edible or medicinal mushroom presents an enormous benefit in economic growth into all over the world (Fan *et al*. 2008). The increasing of consumer's health awareness will improve the demand of edible mushroom and the production of mushrooms will continuously increase (Aida *et al*., 2009). Mushroom cultivation could also protect and regenerate the environment. The substrate used as a plant fertilizer and soil conditioner is beneficial to overall soil fertility and stability as well as vegetable yield (Levanon & Danai 1995).

According to Royse (2014), world production has increased more than 18-fold in the last 32 years, from about 350,000 metric tons in 1965 to about 6,160,800 metric tons in 1997 and consumption of mushrooms has increased at a rapid rate, especially since the mid-1990s. Not only has production and consumption increased as the world's population has increased, but per capita consumption of mushrooms has increased as well. Over a 15-year period (1997 to 2012), per capita consumption of mushrooms 0increased from about 1 kg/year to over 4 kg/year.

Asian countries produce more than 74.64% of world mushroom markets followed by Europe (19.63%) respectively in 2014 (FAO, 2015) (Fi. 2.1). In recent years, about 40% of total world mushroom are exported from China as the world's biggest producer of mushroom. However, 95% of the total China production is for domestic consumption (Zhang *et al*., 2014), In 2013, Shiitake had the best demand for mushroom consumption in China about 22.5%, followed by Grey Oyster mushroom 18.9% and Wood ear mushroom 16.8% (Li & Hu, 2014). With the largest markets, mushrooms widely cultivated only by small-scale farmers (Li and Hu, 2014). The demand for mushrooms has been phenomenal – production to meet the growing demand is a performance seldom duplicated in agriculture today.

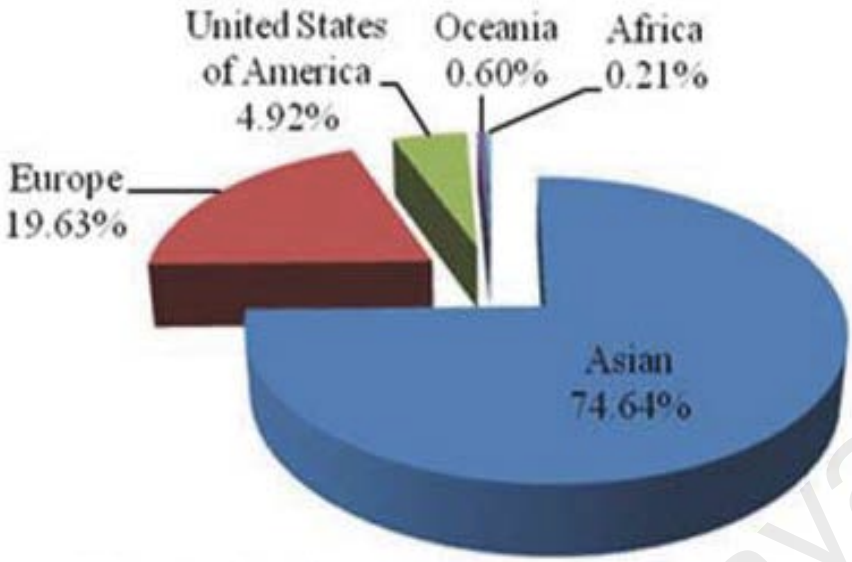


Fig. 2.1 World Mushroom Production in 2014

(Source: Food and Agriculture Organization, 2015)

Recent scenario revealed that oyster mushroom (*Pleurotus* spp.) production increased from time to time. China, South Korea, Japan, and Indonesia are the major producers. At present, *Pleurotus* spp. has become the second most important cultivated mushroom of the total world production. A considerable shift has occurred in the composite of genera that constitute the mushroom supply. China also produces more than 85 percent of all oyster mushrooms grown worldwide. Meanwhile in United States, oyster mushroom production has increased at annual rate of 14 % for last half decade (from 0.88 x 10^3 tons in 1996 to 1.65 x 10^3 tons in 2001 (USDA 2001). McCarty, (2010) & Singh (2014) reported that China's dominance has occurred even though most Chinese growers are using growing methods considered rather primitive and low technology relative to those used in the United States and other major producing countries.

2.2 Mushroom Artificial Cultivation

Cultivation of edible mushrooms is a biotechnological process for lignocellulosic organic waste recycling. It might be the only current process that combines the production of protein-rich food with the reduction of environmental pollution (Beetz & Kustudia 2004). The production of mushrooms is regarded as the second most important microbial technology, next to the yeast one, from commercial point of view (Pathak *et al*., 2009).

Generally, artificial mushroom cultivation (see Fig. 2.2) consisted of three major phases which are spawn (inoculum) production, preparation of substrate for fruiting and mushroom growing. Basically, these stages involved inoculation propagules of the specific fungus on the substrate, followed by colonisation of the substrate by the growth of the fungal mycelium and subsequently fruiting and harvesting of fruiting bodies or sporophores Wang (1999). Vegetative phase (mycelium) and reproductive phase (fruiting body formation) are the two phases of mushroom life cycle involved in this stage of mushroom cultivation. Substrates chosen for both phases should be done carefully, in order to be able to provide suitable chemical and physical requirements of requirements for good growth of mushroom. Supplementation of mushroom substrates with nitrogen sources was done by consideration the carbon and nitrogen requirements in both phases to enhance growth and also fruiting.

The history of mushroom artificial cultivation had begun since 2500 years ago when the first cultivation was done in China and the Far East. In Europe, a commercial basis of mushroom farming has begun in 1700s as the first farm in Paris was started (Stamets, 1983a). Currently, China, United States, and Netherland are the most mushrooms (mainly *Agaricus bisporus*) producing countries'. This fungus can now be easily grown on compost substrates and the waste compost can be re-used as a good source of organic fertilizer because it contains high nitrogen and other minerals.

Some mushroom can be cultivated easily and have significant worldwide markets. Over 200 species have been collected from the wild and used for various traditional medical purposes, mainly in the Far East (Sánchez, 2004). Roughly 300 mushrooms species are edible, but only 30 have been domesticated and ten grown commercially (Barny, 2009). The principal cultivated mushroom worldwide is *Agaricus bisporus* followed by *Pleurotus* spp., *Lentinula edodes*, and other mushrooms that have already an important place in the market (

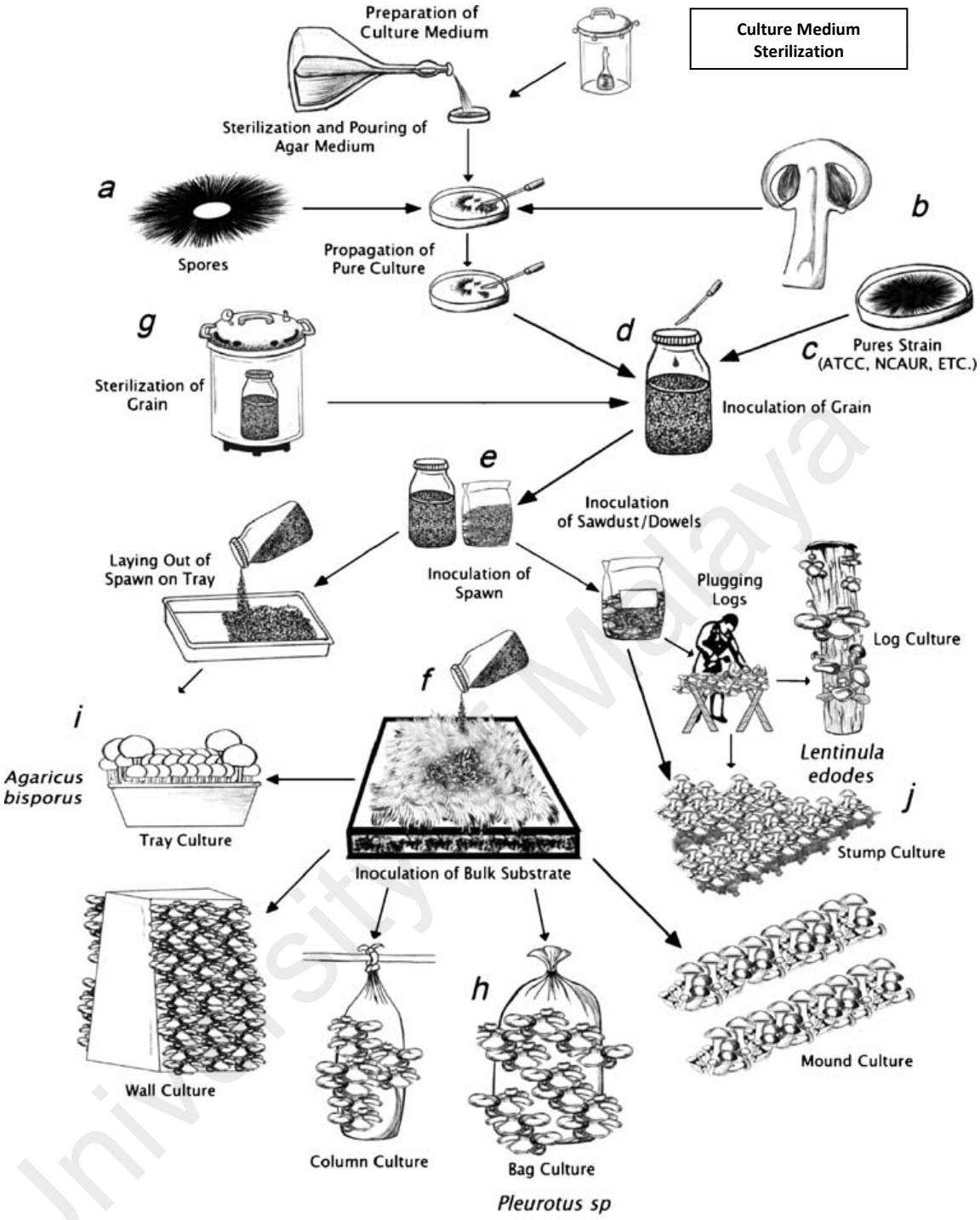


Fig 2.2 Stages of Mushroom Artificial Cultivation [Modified from Stamets (1995)]

It was reported that mushroom cultivation for global production demands a much higher level of knowledge, continuous monitoring, and timely manipulation of environmental conditions. The first stage involves obtaining pure mycelium of the specific mushroom strain. The mycelium can be obtained from spores, from a piece of the specific mushroom, or from several germplasm providers (Fig. 2.2 a-c). To obtain

inoculum, the mycelium is developed on cereal grain, e.g., wheat, rye, or millet, which is usually called the “spawn” (Fig. 2.2e). The purpose of the mycelium-coated grain is to rapidly colonize the specific bulk growing substrate (Fig. 2.2f). The success of mushroom production depends in great part on the quality of the “spawn”, which must be prepared under sterile conditions to diminish contamination of the substrate (Fig. 2.3g). Several studies have been done to improve the quality and develop new techniques for its production.

2.2.1 Spawn Preparation

Production of mushroom spawn can utilize agricultural wastes such as rice straw, sawdust mixed with sugarcane bagasse, palm oil waste and other agro-industrial waste. The sterilized agricultural wastes formulated were then inoculated with pure culture. Mushroom spawn is normally prepared in either plastic or glass containers as shown in Fig. 2.3. The spawn should be moist, white (sometimes with a brown crust) and appear rather fuzzy. Good quality spawn smells mushroom-like, not mildew or mold-like. Weed fungi and bacteria are controlled by not damaging or opening the spawn containers until the entire content is used. Spawn must be kept away from direct sunlight and temperature extremes. Storage for a month or more should be in a cool (3-4 °C) location away from direct sunlight. Spawn must not be frozen. Prior to inoculation spawn should be warmed to room temperature (25 °C) for two to five days.

Fig. 2.3 Spawn Production of Oyster Mushroom

According to Zadrazil *et al.*, (2004), spawn or mycelia running phase involved the growth of mycelium through substrate following inoculation, biodegradation of the substrates by the mycelium and at the same time the mycelia supports the formation of fruiting bodies. During this phase, it is crucial to maintain the suitable temperature and humidity for the cultivated mushroom species. Mushroom spawn is described as a medium through which the mycelium of a fruiting culture has grown and which serves as the inoculum or "seed" for the substrates in mushroom cultivation (Chang, 2001; Chang & Miles, 2004).

Good quality inoculum or spawn is characterized by vigorous mycelial growth of a pure strain on a selected medium (liquid medium, grain or sawdust) and after this vegetative propagation stage the mycelia will be used to inoculate a sterilized growing substrate. Spawn is the genetically complete mycelial tissue of fungi, which is used to propagate mushrooms, in much the same way as raspberry cuttings or seed potatoes. It can purchase spawn in a variety of forms, selection being based on intended production "substrate". Substrate is the raw materials on which the fungus grows on decayed tree, paddy straw, empty fruit bunches (EFB) of palm oil or rubber wood sawdust

2.2.2 Preparation of Growing Substrate

The basic substrate to cultivate mushroom is hardwood sawdust (a mixture of fine and course sawdust to ensure good aeration), 75-80%, supplemented with wheat bran (coarse), 20% gypsum (calcium sulfate), 1% sucrose, mixture content 60-65% and pH 5,5 – 6,5 (Chen 1999a b, Stamets 2003b).

In nature, the several mushrooms such as shiitake, oyster or other fungus propagates and spreads from spores produced by the sporophores. However, for mushroom cultivation, spore germination is too unreliable. Instead, logs are inoculated with actively growing fungus. The fungus is first adapted to wood by growing it directly on small pieces of wood.

Usually, the entire process of production takes about 15 weeks. However, production timing varies from species to species. The last few decades had noticed global increased production of mushrooms and enhanced utilization of different substrates (Stanley *et al*., 2011). However, the exhausted substrates remained unused or misused that resulted in environmental hazards. Thus, concern for environment-friendly utilization of the exhausted substrates seems important. To this regard, cultivation of *Pleurotus* species could readily be integrated within a sustainable agricultural production scheme. It should exploit and valorize agro industrial wastes of low economic value that are usually incorporated into soils, burned or disposed irrationally. Some of these exhausted are potentially hazardous to the ecosystem. Furthermore, mushroom cultivation requires relatively low economic input and energy when the raw materials and labor used are selected for adaptation to local climatic conditions and labor.

2.2.3 Sporophore formation

Success in mushroom growing and productivity depends on multiple factors. The most influential among them are the type of the substrates and composting

techniques utilized and whether the sporophores received adequate nutritional supports. Favourable nutritional and environmental conditions aids in mushrooms health withstanding against the competitors of mushroom production that can interrupt the process of mushroom cultivation.

Another important aspect is the physical factors, such as pH, temperature, aeration and light must all be set to the exact requirements for certain cultivated mushrooms. Formations of the sporophores are critically affected by the nutrient status and mycelium physiological conditions.

The optimum pH value is needed during the vegetative growth and reproductive phase of a fungus differ between species with other mushroom species. The optimum temperature range for fruiting is generally narrower than for mycelial growth. Adequate aeration is required both vegetative and reproductive phase, but reported more stringent requirements in the reproductive phase. Another physical factor that can affect mushroom growth or even failure is the accumulation of carbon dioxide occurs when good ventilation is not provided in the mushroom house. Therefore, proper aeration and ventilation in the mushroom house are very important to guarantee both the quantity and quality of the mushroom sporophores (Fig. 2.4).

Fig. 2.4 Artificial Cultivation of *P. pulmonarius* at Glami Lemi Biotechnology Research Centre (PPBGL), Jelebu

2.2.4 Biological Efficiency

Biological efficiency (BE) is used to measure the ability of fungal strains to convert the substrate material into the body of the fruit. BE is defined as the ratio of the weight of fresh harvested mushrooms by the weight of dry substrate, expressed as a percentage (Shen & Royse, 2001). The formula states that 1 kilogram of fresh mushrooms harvested from one kilogram of dry substrate has the biological efficiency of 100%. This simple formula can easily be used to determine the effectiveness of substrate used, meaning whether it is economically efficient in producing of mushrooms.

2.3 Life cycle of Basidiomycetes

The mushrooms of *Pleurotus* genus show the typical life cycle of Basidiomycetes, a major group belonging to Kingdom of Fungi (Fig. 2.5). These fungi are found throughout the world as common wood-rotting mushrooms, and are tetrapolar. Spores of mature mushroom will start to fall and disperse in the air and are carried out by the wind. Eventually, spores fall to the ground, usually along with the rain. If the environmental conditions are favourable (optimum temperature and pH, as well as moisture), the spores will germinate to form a mass of mycelium. This is the start of the vegetative phase of the mushroom. Given unrestricted amount of nutrients and favourable growing conditions, it is capable of growing infinitely. The mycelium growing from the germinating spore is the so-called primary or monokaryotic mycelium and is usually uninucleate and haploid. When two compatible monokaryotic mycelia are in close contact, they are able to establish a fertile dikaryon by hyphal fusion or plasmogamy. This dikaryon (n+n), having clamp connections and binucleate in each hyphal compartment, contains two genetically different nuclei (one from each monokaryon) throughout the mycelium.

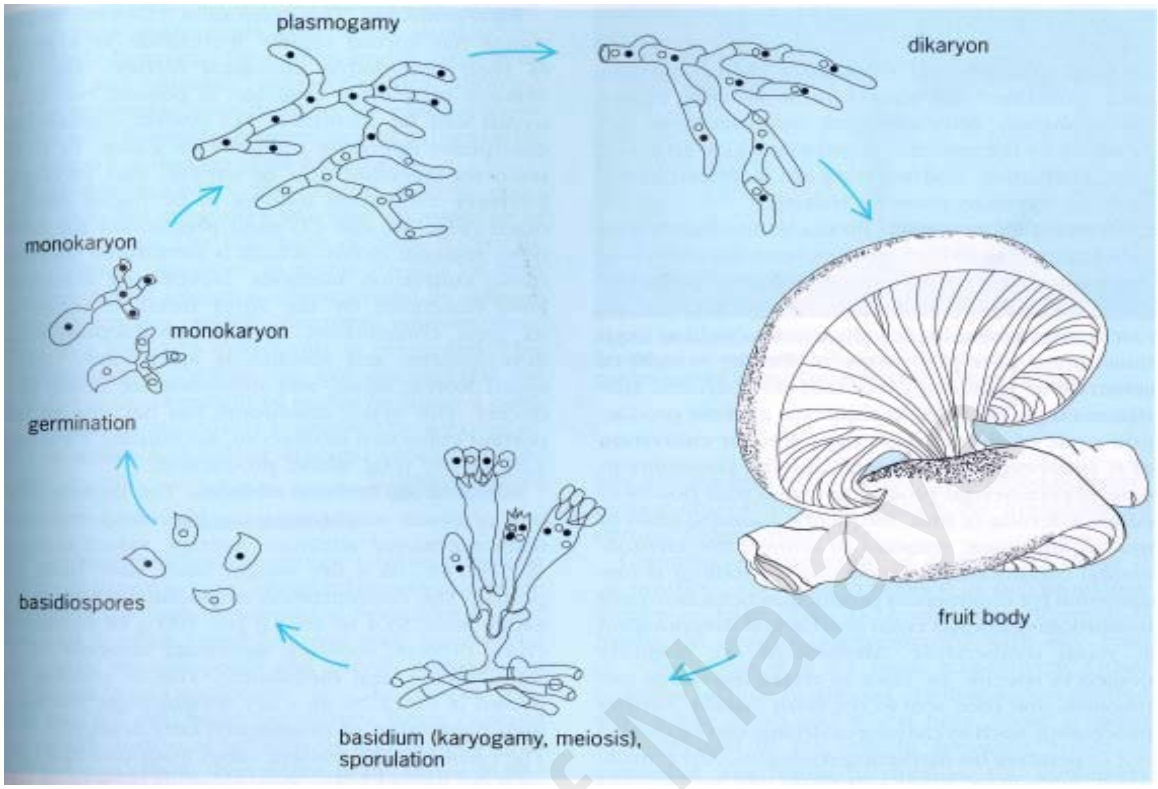


Figure 2.5. Life cycle of Basidiomycetes.

(Source: Martines-Carrera, 1998)

The dikaryotic mycelium is formed when monosporous mycelium of one mating, e.g., *A1B1*, interacts with monosporous mycelium of compatible mating type, e.g., *A2 B2* because this is a single type of nucleus in a monosporous mycelium. Fusion of hyphae of the two compatible monokaryon strains is followed by the migration of nuclei from one strain through the established hyphae of the other strain. When the migrant nucleus reaches a tip cell of the hyphae in which the nucleus invades that cell then contains then that cell will contain two compatible nuclei and will possess clamp connection (Figure 2.6). The cell is called a dikaryon. When the conditions are appropriate (temperature, light, relative humidity), the dikaryotic mycelium will differentiate into fruit bodies having specialized structures called basidia. In these club-shaped, binucleate cells, which are formed in the lamellae (hymenium) of each fruit

body, karyogamy (fusion of the paired nuclei; 2n) and meiosis (recombination and segregation) take place (Martínez-Carrera, 1998).

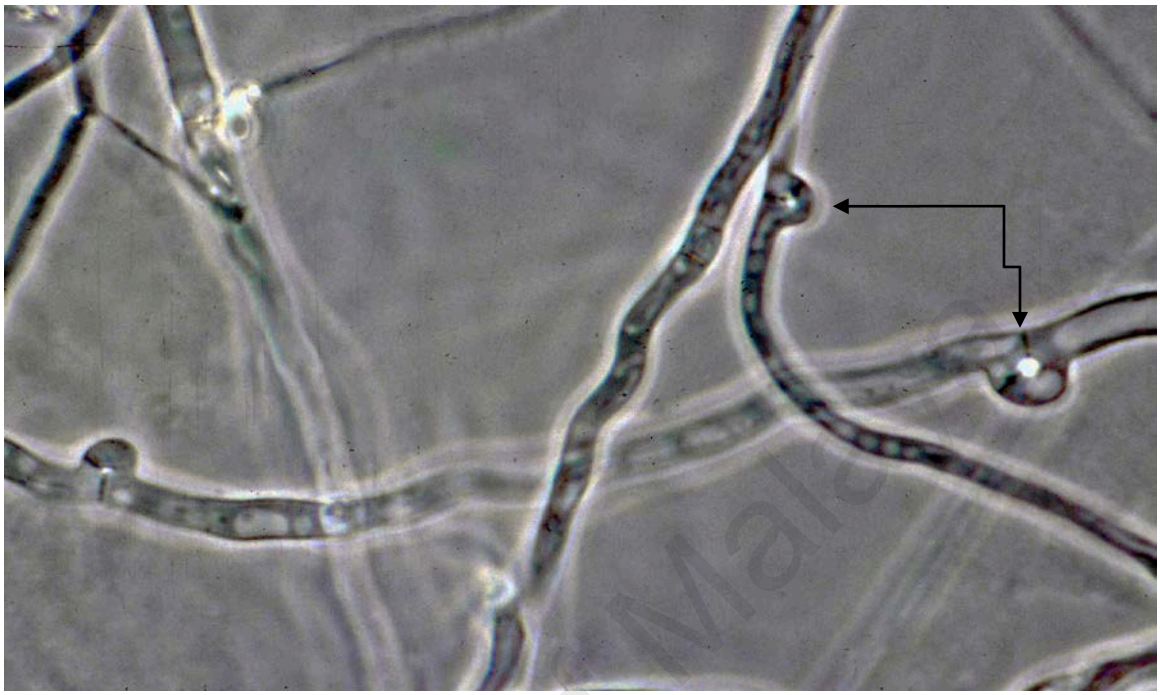


Figure 2.6. Clamp connection (arrow) formation of two different monokaryotic cultures (http://www.botany.hawaii.edu/faculty/wong/Bot201/Basidiomycota/Clamp_connection_formation.html)

The absence of a dominant trait in a gene pair will not bring out the phenotypes and will only appear when present together in pair's dominant gene. They have either a bipolar (unifactorial) or a tetrapolar (bifactorial) mating system. This results in the fact that following meiosis, the resulting haploid basidiospores and resultant monokaryons, have nuclei that are compatible with 50% (if bipolar) or 25% (if tetrapolar) of their sister basidiospores (and their resultant monokaryons) because the mating genes must differ for them to be compatible (Chang & Buswell, 1999). Moreover, there are many variations of these genes in the population, and therefore, over 90% of monokaryons are compatible with each other.

2.4 Mushroom Breeding

Mushroom breeding requires a large investment of capital and patience from both the breeder and grower. Some of the desirable properties and qualities to look for in new strain are; i) high productivity, ii) high quality and good features of sporophores, and general vigour iii) favourable culture characteristics, and pathogen disease resistance, iv) stability of strain properties during continuous sub-culture required for master spawn production.

A number of specific industry standards have been applied to grow strains available to the public for the last 2 decades. For a new strain to be successful, some modifications in culture parameters are required for optimal growth. Traditionally growers had to apply certain growing systems depending on the cultural needs such as modifying flushing regimes, watering patterns and harvesting practices to optimize strain performance. Modifying cultural practices such as frequency and timing of irrigations are required for successful future strain development.

The breeding scheme began when a promising hybrid was made through by crossing a wild monokaryon with a cultivated monokaryon. The heterokaryon had comparatively well grown and the agronomic aspect produced three successful cycles of cream-colored sporophores. Later, crosses of the collected spores with a tester monokaryon derived from commercial strain yielded segregated sporophore color with the ratio of white:cream (3:1). Subsequent crosses showed variability in sporophore color and some other traits such as pileus shape, smoothness of pileus cap and veil strength. One third of the hybrids were markedly smoother on the first cycle of production when compared to the control.

Nowadays, breeding by a combination of multispore collection, single spore isolation and the selection of chance mutation was employed; one such chance mutant could be the white sporophore colour, which predominates (Kligman, 1950).

The breeding scheme began when a promising hybrid was made through crossing a wild monokaryon with a cultivated monokaryon. The heterokaryon had generally good growth with suitable environmental conditions and produced three good "cycles" of cream-colored sporophores.

Later, crosses of the collected spores with a tester monokaryon derived from commercial strain yielded segregated sporophore color and the ratio was white: cream 3:1. Subsequent crosses showed variability in sporophore color and some other traits such as pileus shape, smoothness of pileus cap and veil strength. One third of the hybrids were markedly smoother on the first cycle of production than the control. The systematic breeding of new mushroom strain through a combination of molecular breeding techniques and specific traits has been applied to native *A. bisporus.*

Increasing the yield and quality of crops as well as resistance to pathogens are the primary goals for mushroom breeders and mushroom research. Other goals include reducing production costs and the efficient use of compost for growth. Methods of mass selection based on natural chance mutation and programmed mutation by ionizing radiations such as, X-rays and chemicals as well as cross breeding and transgenic breeding are some of the methods carried out for this purpose. However, cross and transgenic breeding are more effective and have shown greater promise and progress in the last few decades (Fan *et al*., 2006). Areas of research for mushroom breeding relate directly to commercial benefits such as problems associated with cultivation, distribution and storage, senescence-induced browning and pathogen resistance.

Another aim of breeding in mushrooms is to incorporate various improved crop growing qualities such as shorter growth cycle and prevention of spore formation. Traditional mushrooms produce billions of spores floating in the air which cause health problems such as lung allergy and fever attacks. Spores also lead to the blocking of climate installations and result in higher energy costs. A new sporeless oyster

mushroom has been developed by Plant Research International, Wageningen, Netherlands by using molecular marker technology. After crossing various oyster mushroom cultivars, this analytical technique can be used to identify progeny with the highest chance of sporelessness (Okuda *et al*., 2009). Other research areas for crop improvement in mushrooms are utilization of grain-based substrates substituted for traditional manure-based compost which will address issues related to outdoor composting and compost disposal and potentially offer sterilized substrate for biopharmaceutical manufacturing (Bechara *et al*., 2006).

To increase yield, various strategies have been carried out. Many older strains which grow profusely and produce a large number of pins but never mature are hybridized with strains with less growth. Production of hybrids with increased thickness and density of mushroom cap as well as prolific nature of the hybrid strains has increased yield potential. Two methods successfully employed for breeding in the lab are protoplast culture and spore germination (Horgen *et al*., 1991, Kerrigan *et al*., 1992).

2.4.1 Mating Compatibility in Edible Mushrooms

The sporophores of basidiomycetes achieved from plasmogamy through karyogamy and up to meiosis in reference to mating type control. In basidium, karyogamy brings together the two compatible nuclei of the dikaryon to form a diploid nucleus. This diploid nucleus undergoes meiosis resulting in the formation of four haploid nuclei. Each of these nuclei moves through a short stalk (sterigma) on the basidium into the developing basidiospores. These can be represented as the mating type loci A and B, the dikaryon can be symbolyzed as *A1B1 + A2B2*, the diploid nucleus as *A1A2 B1B2* and the meiospore as *A1B*, *A1B2 A2B1* and *A2B2*. These occur in equal frequency in a ratio 1:1:1:1, it is evident that the *A* and *B* loci are unlinked. The

spores will germinate and form homokaryotic mycelia. When mycelia form spores all possible combinations can be produced the following results occur in Table 2.1

In *Basidiomycetes* crossing is possible between monokaryons compatible in mating types (mon-mon mating) and between dikaryon and monokaryons irrespective of their mating types (di-mon mating). There are two classes of mating systems; “self-mating” (pure line) and “Crosses” which describe by hybridization within the same or between different monokaryon lines, respectively. The effectiveness of the two mating methods was evaluated by comparing the level of deviations which occurred among the groups of hybrid stock produced by “self” and “cross” mating.

Table 2.1 Compatibility of matings between different alleles *A* and *B* (Chang, 1992).

	A1B1	*A1B2*	*A2B1*	*A2B2*
A1B1	-	-	-	+
A1B2	-	-	+	-
A2B1	-	+	-	-
A2B2	+	-	-	-

\+ Represents the presence of clamp connections indicating a dikaryon (= two compatible nuclei in the same cell)

\- Represents the absence of clamp connections

For continuing of ecological balance of germ plasma availability in nature, mating is an essential step in the life cycle of sexually reproducing organisms. The function of the mating-type genes in the fungi is to impose barriers on self-mating and thereby fungi, the somatic cell fusion is sufficient for mating and no specialized cells are required. The mating type genes ensure that only nuclei from genetically different

individuals will fuse to give a diploid nucleus that will undergo meiosis prior to the formation of the sexual spores.

Self-sterile species are said to be heterothallic, whereas those that can self-mate are said to be homothallic (Blakeslee, 1904). The basidiomycete fungi are largely heterothallic, and a remarkable feature of this group is that they have evolved multiallelic mating type genes; as a result, some have many thousands of different mating types. The molecular interactions that permit mating cells to distinguish self from non-self not only are of great interest to fungal biologists but also give exciting insights into the complex interactions that control development in higher eukaryotic organisms.

Edible mushrooms contain both self-fertile and self-sterile species. Self-fertilization (homothallism or homomixis) is probably the most common mode of sexual reproduction in the fungi as a whole but in the higher fungi such as basidiomycetes, self-fertile species are in a distinct minority of only about 10%. Self-sterility (heterothallism or dimixis) is about 90%. In which 25% is bipolar and 65% is tetrapolar.

a. Heterothallism.

Sexuality in the basidiomycetes was first described by Kniep (1920) in *Schizophyllum commune* based on mating of monosporus mycelia, with the formation of clamp connections that indicate compatible reaction in these heterothallic species. Following the study of Kniep, it was discovered that heterothallism took two major forms, bifactorial and unifactorial.

i). Bifactorial, in this form two unlinked mating type factors were performed to be operative, with a heterothallic condition as both loci required for compatibility and the formation of dikaryotic condition. This was called tetrapolarity or heterothallism with bifactorial control. Cross mating between homokaryotic mycelia was required to complete the sexual cycle. Two mating systems of homothallic are commonly found

in edible mushrooms; *) bipolar mating system, in which the mating competence is determined by incompatibility factors of a single series, the A factor. Therefore, only two mating are produced in equal frequency by a single fruiting body**) Tetra polar mating system, in which the mating competence is determined by incompatibility factors of two series, A and B, which assort and segregate independently at meiosis. There are four rather than two mating types produced in equal frequency by a single fruiting body.

ii. Monofactorial. In the other type of heterothallism there is single mating type factor with a heteroallelic condition at that locus bringing about compatibility and the formation of the dikaryotic condition. This was called bipolar incompatibility (bipolarity) or referred as monofactorial control.

b. Homothallism. Fruiting structure can be produced by a single, monosporous mycelium. Potentially self-fertile fungi are not always necessarily homozygous and a variety of situation and rather imprecise regulating system can result in heterozygosity. Two types of homothalism are found among self-fertile species;

i). Primary homothalism, occurs when a homokaryotic mycelium arises from a single spore with a single post-meiotic nucleus. Homokaryotic of primary homothalism type, formed from the mycelium of a single meiosis, has the potential to develop into hetekaryotic, through the completion of the sexual cycle. *Volvariella volvaceae* has primary homothalism. In which a homokaryotic mycelium, established from single meiotic mycelium, has the potentiality to progress through hetekaryosis, to the completion of the sexual cycle.

However, the mechanisms underpinning variations among single spore isolates in successive generations are yet to be deciphered.

ii). Secondary homothalism contain two compatible nuclei and consequently a mycelium that is self-fertile. *Agaricus bisporus* is the most cultivated mushroom

incorporating this technique. In secondary homothallism of *A. bisporus*, the homokaryotic phase found in primary homothallism species is lacking. Since only those spores bearing both *A1*and *B2* alleles will give rise to fertile spores, it means that two- third of spores from two spored basidia would be self-fertile, and one-third would be self-sterile as suggested by Langton & Elliot (1980).

2.4.1.1 Study of mating inhibition

In monosporus isolates pairing, mating inhibition occurs frequently where one of the monokaryon mycelia may grow faster and outgrow the other culture. According to Esser & Blaich, 1994 unilateral or mutual interactions resulting from secretion and diffusion of inhibitory substances are the factors that causing inhibition (Fig. 2.7) Moreover, Gharehaghaji`*et al*., (2007) described that, in such circumstance, no clamp connection was ever produced in the culture.

In this case, the two types of mycelia intermingle in the contact zone and show numerous hyphal fusions via anastomosis. Another study by Fischer & Bresinsky (1992) showed that compatible reactions of hyphal fusions in *Phellinus torulosus* resulted in the formation of heterokaryotic mycelium in the contact zone. Formation of the heterokaryon was restricted to the contact zone. Then the border zone between two mycelia becomes unrecognizable through the time. The formation of clamp connections by compatible haploid isolate, indicative of dikaryon formation, is a useful *in vitro* tool for basidiomycete systematic.

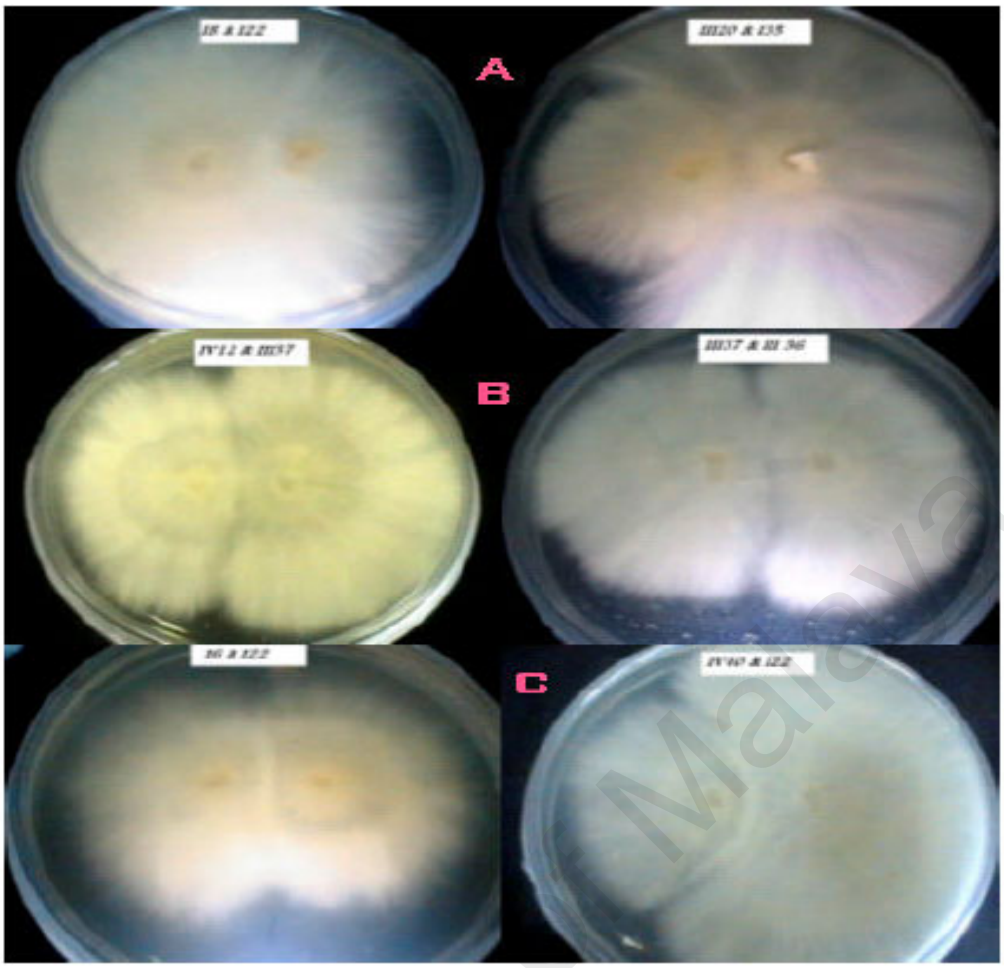


Figure 2.7 Incompatibility pairing of single spores isolation from *Pleurotus* species [(A. Surrounding mycelium by another mycelium; B: Line of demarcation, C: Barrage formation) (Larsen *et al*., 1992)].

Similar study was reported when two homokaryons carrying the same *A* factor but different *B* factors (A=B, e.g. *A1B1 + A1B2*) crossed, the result being a common-A heterokaryon with a typically aberrant morphology called flat. In A B= mating (e.g. *A1 B1 + A2 B1*), a common-B heterokaryon is formed along the region of interaction. This heterokaryon had binucleate or uninucleate apical cells and was typified by pseudoclamps, which started to develop like clamp connections but failed to fuse with the corresponding subapical cells (Raper, 1966; Parag, 1971).

In *Basidiomycetes* both of *P. pulmonarius* and *P. citrinopileatus* are tetrapolar and found throughout the world as common wood-rotting mushrooms. The events that

follow the establishment of a heterokaryotic cell by hyphal fusion between two compatible strains consist of the migration of non-resident nuclei through the mycelium of each mate. Pairing of migrant nuclei with resident nuclei is followed by conjugate nuclear division to maintain the paired nuclei within each daughter cell of the resulting dikaryon. In conjugate division a hook cell or clamp connection is formed which is diagnostic of the mycelium with paired nuclei. These events are differentially regulated by the two incompatibility factors in two distinct and complementary sequences.

Cross breeding has been carried out since 1983 in mushrooms with the production of hybrids belonging to the following genera *Lentinula* (Zhang & Molina, 1995), *Pleurotus* and *Agaricus* (Fritsche, 1983). Hybrid strains with desired traits such as fleshy texture of basidiocarps, reduced number of spore have not only given mushrooms that show resistance to pathogens and pests but also reduced the dependence and risks of cultural conditions. Hybrids obtained by pairing monosporic cultures are cultivated to evaluate the production characteristics accompanied by RAPD and RFLP analysis.

2.4.2 Strain improvement by mutation

Another way to get a new strain with better features and characteristics is the using of gamma rays to induce mutation. Mutation was done by applying gamma radiation on mycelia grown on PDA Esser K (1994) by using this method the radiating ions are more evenly distributed and more easily to reach the cells of the mushroom. Furthermore, post radiation observation on the mushroom morphology was easily observed using this method.

a. Irradiation using X-ray, ultraviolet or Gamma ray –$Co_{60)}$ has been applied widely for decades to propagate agricultural crops in order to produce high quality seeds of rice, wheat etc. Recent studies have shown the same trend of using mushroom as biological

source to be experimented its efficacy and compatibility towards different types of radiation technique due to the development of diverse genotype with desired features

b. Mycelia of edible mushrooms were exposed to a Cobalt-60 source of gamma rays to mutate the genes of sporophore shape that resulted in increased mycelial growth rate and increased mushroom production. According to Esser (1994), an effective method to get positive mutation of mushroom is by applying gamma radiation in the mycelia grown on MEA.

c. Gamma radiation was done on mycelia culture occupying approximately a quarter of the agar plate (3 days after inoculation). At 15 day s after inoculation, the mycelia of white oyster mushroom were in the exponential growth period (Djayanegara & Harsono, 2007). Just like any other edible mushrooms, the study revealed that white oyster mushroom has an extensive growth pattern as long as the substrate is available. Those cells are composed of vesicles excreting many types of enzymes and polymers to support the growth at the tip of hyphae. Hyphae cells at the tip of mycelium have a high metabolism rate.

2.4.3 Hybridization of edible mushrooms by protoplast fusion

Commercial and marketing pressure has been driving forces in the development and expansion of the established areas of fungal technology. Furthermore it presented new encouragement with the realisation of a new area of technology based on isolated protoplast. Protoplast fusion has been used as a method to create mushroom hybrids, especially when the using of conventional methods can not achieve this result. Breeding of edible mushroom involving protoplast fusion is now being widely adopted in order to obtained interspecific and intergenic hybrids which produce desirable qualities of the sporophores.

According to Peberdy (1995), protoplast fusion technique practice began after the discovery of enzymes from microbial origin that can digest away the fungal cell wall liberating the hyphal protoplast as discrete units that were then allowed to fuse with another hyphal protoplast or naked cell. Enzymes digested away the fungal cell wall liberating the hyphal protoplast as discrete units of the whole provided some external osmotic support was available. Furthermore Peberdy, 1989, described the cell wall as a barrier protecting the protoplast. Without the barrier it was possible to explore a whole range of new manipulations of these naked cells. Many of which had important genetic consequences.

One of the principal advantages of protoplast fusion is hybridization among incompatible *Basidiomycetes* (Ogawa, 1975). Moreover, he described that identification of fusants obtained by protoplast fusion has generally been accomplished by nutritional complementation using auxotrophic strains. To obtain new hybrids, he carried out protoplast fusion interspecific and intergeneric fusion products that were obtained from protoplast fusion between i). *P. ostreatus* and *P. cornucopiae,* ii). *P. cornucopiae* and *Lentinus edodes*, iii) *P. ostreatus* and *L. edodes*, also between iv) *P. cornucopiae Lyophyllum decartes* (Fr.) Sing. The result showed the improvement of these *Basidiomycetes.*

Similar studies which were performed on the genus *Pleurotus* i.e. between *P. ostreatus* and *P. citrinopileatus.* Dhitaphichit & Pornsuriya (2005) reported that the only successful fusant that fruits on sawdust plastic bags, showed recombined characteristics of the parents. Its fruiting bodies were yellow in colour, which was similar to *P. citrinopileatus,* but its spore print was creamish colour, which was similar to *P. ostreatus.* Both *P. ostreatus* fruiting bodies and spore prints has creamish colour while *P. citrinopileatus* has golden yellowish fruiting bodies and pinkish grey spore prints.

Integrating several characteristics from distinguished parents in protoplast fusion method is the alternative way to introduce genetic variation. This is found more efficient to develop few strains for big scale production in mushroom industry (Peberdy, 1995). Protoplast fusion has been used as a method to create mushroom hybrids, especially when the using of conventional methods cannot achieve this result. Breeding of edible mushroom involving protoplast fusion is now being widely adopted in order to obtained interspecific and intergenic hybrids which produce desirable qualities of the sporphores. This technique has very important implications for the breeding of edible mushrooms.

2.4.4 Strain improvement by genetic manipulation

At present there are no transgenic mushroom strains commercially available but several research groups are working towards that direction with good progress. The use of recombinant DNA technique to obtain transgenic mushrooms has promoted numerous possibilities and opportunities. Importing genes from unrelated sources is now possible and it is not restricted to the searching of desirable genes only within the species. Transformation techniques used with other filamentous fungi are being adapted for the mushrooms (Van de Rhee *et al*., 1996a). Various techniques such as polyethylene glycol (PEG) (Li *et al*., 2006), electroporation and particle bombardment have been used to incorporate DNA into protoplasts, mycelium or basidiospores. An efficient homologous site-directed integration of the transformation plasmid was done by isolating the tyrosinase genes responsible for mushroom browning from *Agaricus bispous* and introducing it in antisense orientation (Van de Rhee *et al*., 1996b). However, the multinuclear nature of fertile *Agaricus* mycelia presented a problem for stable transgenic mushrooms. Another gene isolated and identified in mushrooms was the mannitol-dehydrogenase (MtDH) gene and its 3-dimensional structure has now become available (Sassoon *et al*., 2001). Isolation of this gene can allow the production of mushrooms with altered mannitol profiles and ultimately yield strains with

higher dry matter content or better pathogen resistance (Stoop & Mooibroek, 1998). The use of direct gene delivery techniques such as particle bombardment has also been carried out as an alternative method for genetic transformation in mushrooms (Li & Horgan, 1993). This process involves the bombardment of intact tissues with tungsten or gold particles coated with donor DNA and penetrating the recipient tissue. It has the advantage of being less laborious and often the problematic production and regeneration of protoplasts can be avoided. In many laboratories, attempts have been undertaken to introduce hygromycin-B resistance and other selectable markers by particle bombardment. However, this technique has not yet resulted in the selection of stable transformants or an applicable system.

2.5 Morphological characteristics and nutritional value of *Pleurotus pulmonarius* (Fries) Quél.

Pleurotus pulmonarius (Fr.) Quél. (1827) or Grey oyster is commonly also known as Indian oyster, Italian oyster or lung oyster. Sporophores of the parental *P. pulmonarius* had a somewhat depressed pileus cap, and were lung shaped to semi-circular in shape, greasy when young and fresh, fairly smooth, whitish to beige or pale tan, with the margin rolled when young, wavy and very finely lined when mature. The sporophore is grey with a fleshy texture, good aroma and mild taste. The cap of *P. pulmonarius* is normally shell-like, large. Pileus: 5-20cm, convex brown grayish with regular edges, becoming broadly convex to flat, and gills: decurrent Spore Print: White to yellowish. Stipe: Short and offset from the centre of the cap as shown in (Figure 2.8). The sphoropore's flesh is soft and juicy when young, later it becomes tough; it taste is mild and its smell pleasant. Microscopic features: Spores white to yellowish to lavender-grey when dense, more or less cylindrical, 7.5–11 × 3–4 μm. (Stamet, 2000).

According to Chang (1999), approximately 12,000 species of fungi are considered as edible mushrooms. Gregori *et al.* (2007) described that the genus *Pleurotus* (oyster mushroom) comprises some of the most popular edible mushrooms due to their favorable organoleptic and medicinal properties, vigorous growth and undemanding cultivation conditions. Moreover, *Pleurotus* species have been used by human cultures all over the world for their nutritional value, medicinal properties and other beneficial effects. This mushroom can substantiate the sufferings from malnutrition to some extent, because they produce large quantities in a short time and provide more protein per unit area than other crops (Hossain *et al*., 2007). Mushrooms are low in calories, carbohydrates, calcium and sodium. They contain high proportion of unsaturated fat but virtually no harmful lipid or cholesterol. It contains a large amount of vitamins such as Thiamine 1.4-2.2 mg (%), Riboflavin 6.7-9.0 mg (%), Niacin 60.6-73.3 mg (%), Biotin, Ascorbic acid 92-144 mg (%), Pantothenic acid 21.1-33.3 mg (%) and Folic acid 1.2-1.4 mg/100g in dry weight basis (Hossain *et al.,* 2007).

This mushroom is a good source of dietary fiber and other valuable nutrients and contents of some important minerals. Hundred grams of dried *P. ostreatus* contained Ca (35.9 mg), Fe (55.5 mg), Mg (16.4 mg), Mn (2.9 mg), Zn (26.6 mg), Se (11 µg) and As (100 µg) (Alam *et al.,* 2007). Phenolic compounds such as phenolic acids and tannins are known as major components of antioxidant in plants and mushrooms. Previous literatures reported that genus *Pleurotus* contained several types of phenolic compounds such as vanillic acid (Kim *et al.,* 2008; Puttaraju *et al.,* 2006), myricetin, naringin, homogentisic acid, 5-O-caffeoylquinic acid (Kim *et al*., 2008). In addition, Ferreira (2009) stated that most antioxidant properties that can be found in mushrooms are mainly in the form of phenolic acids and flavonoids, followed by tocopherols, ascorbic acid and carotenoids. The total phenolic content of the current mushroom extract that ranged from 38.45 to 51.94 mg TAE/g extract indicated higher

content of phenolic than Persian sour summer pomegranate (Reza *et al*., 2011). Furthermore Wang *et al*., (2007) describes *P. pulmonarius* has anti HIV activities that can inhibit HIV-1 reverse transcriptase activity by SU2 molecule having 4.5 kDa mol.

Fig. 2.8. A. Native *Pleurotus pulmonarius* (http://www.MushroomExpert.Com)

B. *Pleurotus pulmonarius* growing on sawdust substrate

2.6. Morphological characters and nutritional value of *Pleurotus citrinopileatus* Singer

Pleurotus citrinopileatus (Singer 1942), or yellow oyster mushroom, is closely allied to *P. cornucopiae* (Paulet) Roll. *P. citrinopileatus* belongs to the family *Pleurotaceae* one of edible mushrooms of bright-yellow colour. It contains high antitumor polysaccharides (Zhang *et al*., 1994 & Wang *et al*., 2005) and it has antioxidant activities (Hu *et al*., 2006). This mushroom enhances immunity and delay aging (Wang *et al*., 2001). It is rich in nutrients medicinal properties like antioxidant activities, and antitumor and other beneficial effects.

Pleurotus citrinopileatus has a brilliant yellow colour (Fig. 2.9), 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. *P. citrinopileatus* is closely allied to *Pleurotus cornucopiae* (Paulet) Roll, and is often considered a variety of it (Stamets, 2000; Zervakis & 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.

Fig. 2.9: A. *P. citrinopileatus* growing naturally on dead stump, B. Growing on sawdust substrate (http://www.fungiforum.com/uploads1245735349/galery_1_5_90870.jpg)

It contains high useful antitumor polysaccharides (Zhang *et al*., 1994 and Wang *et al.*, 2005) and it has antioxidant activities (Hu *et al*., 2006). This mushroom enhances immunity and delay aging (Wang *et al*., 2001). It is rich in nutrients, medicinal properties and other beneficial effects. This mushroom most commonly decays hardwoods and plays role in water sequestration, animal and human nutrition, soil health and ecosystem function, particularly in water-constrained environments (McGonigle 1995, Austin *et al.* 2004). The mating system of this fungus, leading to formation of a dikaryon that can produce fruiting bodies, is known to be bifactorial heterothalism (tetrapolarty) controlled by two unlinked multialletic factors, *A* and *B* (Terakawa 1960; Eguenio & Anderson 1968; Anderson *et al*. 1991).

According to Singer, *P. citrinopileatus* has monomitic hyphae, and *Pleurotus cornucopiae* has dimitic hyphae, a designation that has caused considerable confusion since Singer in 1986 used this feature as a delineating, subgeneric 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*.

P. citrinopileatus not only has good features with yellow brilliant colour but also has a good composition of nutrients that are beneficial for health if they consumed regularly. Musieba. F, *et al*, 2013 in their study of the proximate analysis contained in *P.citrinopileatus* revealed that it contains many elements of vitamins as well as protein and high fibber as shown in Table 2.2.

Table 2.2 Chemical and Minerals composition of *P. citrionopileatus*/100g dry matter (Musieba *et al.*, 2013)

Chemical composition		Minerals composition	
Nutrient	Value	Nutrient	Value
Moisture content	9.12	Potasium	2.28
Ash	7.66	Sodium	0.33
Protein	22.10	Calsium	0.02
Fat	1.32	Magnesium	0.07
Fibre	20.78	Iron	0.01
Calorific value (Kcal)	3.76	Zinc	0.00
Free fatty acids	0.48	Phosphorus	0.10
		Copper	0.00

A complex composition shows that this mushroom has good prospects for development. However, this fungus has very fragile texture that handicap in harvesting and transportation process. In addition, it has a strong odour, that some customers do not like it. There should be a way through hybridization that can improve the tuft texture and reducing a strong odour, so that the mushroom could be more preferable for many consumers.

2.7 Mushroom cultivation perspective in Malaysia

The limited production of mushroom in Malaysia is largely due to lack of understanding that mushroom industry has important roles to enhance human health by consuming mushroom as dietary food supplements, lack of reliable sources of good quality mushroom spawn for supporting the effort of breeders and local mushroom growers and support mushroom farming entrepreneurs towards promoting mushroom

growing. Increasing mushroom cultivation in Malaysia needs improving in management techniques, and providing superior spawn and diversity of mushrooms. The main type of mushrooms produced in Malaysia is grey oyster mushrooms; meanwhile another family of *Pleurotus*, brilliant yellow oyster mushrooms is not widely available in the local traditional market or supermarkets. Some mushroom farms supply mushrooms in bulk or loose with individual packaging.

From 2001-2004, Malaysian government experienced RM 556,93 million trade deficit for the importing of mushroom. It means approximately 8,100 ton of mushroom is needed and demanded by Malaysian consumers per year, and the trend will increase for the next couple years. However, local mushroom growers can produce only about 68 %. It is obvious that mushroom production needs to be increased by transferring technology and using a high-qualified mushroom spawn. Mushroom spawn is the blue print of mushroom industry in order to support and guarantee of mushroom productivity with good quality and traits.

Breeding program for mushrooms for high yielding, better quality, and performance in Malaysia has been limited to the import of strains from advanced countries for direct introduction, but the procurement and introduction of the existing commercial one. Some species are well studied and have been used to develop many commercial strains, while others are not. The creation of new strains is always required to preserve genetic diversity and meet the ever-changing consumer demands. In addition the study and improvement of mushroom spawn quality is necessary to contribute to the productivity of mushroom cultivation in order to provide high quality of mushrooms using technology and environmentally friendly materials. The materials usually use natural ingredients that utilize the recycled wastes. It offers double advantages both to environment and economy.

Production and consumption of mushroom in Malaysia is expected to increase at an accelerated rate over the years as by improved production technology through breeding of mushrooms. As economics improve in Malaysia, production of mushrooms will increase even faster than other countries like Singapore, Thailand and Indonesia. The culinary advantages offered by specialty mushroom offers a promise of advancement in the continued growth and development of the specialty mushroom industry.

2.8 Breeding strategies in *Pleurotus* spp.

Breeding has contributed much to the spread of the cultivation of *Pleurotus* species, whose world commercial production approaches annual rates of 1 MT and is the third most cultivated mushroom (Chang, 1996). *Pleurotus* species are particularly known as efficient decomposers of a large range of lignocellulosic wastes and for producing edible basidiomata of high organoleptic qualities. Similar to other species of *Pleurotus* genus, *P. pulmonarius* and *P. citrinopileatus* are also easily handled in the laboratory on culture media, spore germination is close to 100%, and mutants can be obtained by direct mutagenic treatment (*e.g.*, X rays/gamma-ray and UV radiation) of basidiospores or by hyphal fragments. These features allowed obtaining suitable combinations of selected characters through simple mating techniques. Besides the recombinant DNA technology is important in breeding oyster mushrooms, providing outstanding information and powerful tools at the molecular level. These properties significantly favoured the increase in their commercial cultivation since growth substrates are cheap and abundant resulting from agricultural, forest and related industrial activities.

The principal objective of development of strains in mushroom program is to combine the desirable characteristics from different species that gave good quality of spawn mushroom cultures. The purpose of hybridization in mushroom cultures including; morphological characters like prominent interaction in the contact zone, increased rate of mycelium growth, and better colony morphology have been used as the morphological markers for breeding purposes (Gharehaghaji *et al.* 2007; Kavousi *et al*. 2008). Strain improvement of both parents *P. pulmonarius* and *P. citrinopileatus* through mating technique to get better strain such as larger size, thicknesses and better texture with fleshy of sporophores, high yield and less of spores.

In this study, inter-species mating of two genus in family *Pleurotus* is carried out to obtain combinations characteristic of *P. pulmonarius* that has fast-growing, adaptable to the environment and fleshy basidiocarp textures that are resistant to storage, combined with characters of *P. citrinopileatus* which has a good shape and attractive colours but the texture is very fragile with strong aroma.

CHAPTER 3

MATERIALS AND METHODS

3.1 Mating compatibility between *Pleurotus pulmonarius* and *P. citrinopileatus* to obtain high quality hybrids

In this study, mating interspecies mating between different species was carried out with monokaryon cultures of *P. pulmonarius* and *P. citrinopileatus* to obtain an improved hybrid of an edible mushroom species with good quality sporophore traits.

3.1.1 Preparation of single spore isolates

Single basidiospores were obtained by cutting matured sporophores into 1cm x 1cm squares using a sterile knife. Then, the tissue (gills exposed) was attached to the underside lid of a Petri dish containing malt extract agar (MEA) as described in Appendix A) using Vaseline. The gills were hung downward and incubated at 25^{o}C in slope position for two to four hours to produce a spore drop (spore print). The basidiospores were released within 2 to 4 hours. After release of the basidiospores, the mushroom fruiting bodies were removed. These basidiospores were incubated for 1-2 days at 25°C for germination. Twenty germinated single spores of *P. pulmonarius* and *P. citrinopileatus* were picked off manually with the aid of microscope at 400x magnifications using a needle and transferred to MEA as performed by Kotasthane (2003) with modification. The mycelium was confirmed as a monokaryon by the absence of clamp connections by examination under a microscope at 400x magnification.

3.1.2 Preparation of monokaryotic cultures

Single basidiospores for each species were isolated and cultured. Twenty monokaryotic cultures of *P. pulmonarius* (designated as P1–P20), and ten selected monokaryotic cultures from *P. citrinopileatus* (hereafter called as C1–C10) were successfully prepared. Monokaryon cultures were validated by the absence of clamp connections on the hyphae. Monokaryon cultures from parental *P. pulmonarius* (P1, P3, P6, P8, P9, P13 and P19) and parental *P. citrinopileatus* (C1, C2, C5, C7, C8, C9, C10 and C13) with good cultural characteristics such as fast mycelial growth rate and high density were selected for mating studies.

3.1.3 Cross breeding of monokaryotic cultures to determine compatible mating

Monokaryotic isolates from two different species parental were transferred onto MEA and incubated at 25^{o}C for seven days. Ten cultures from each parent species were selected based on colony density and size and used in a mating system. Monokaryotic cultures of *P. pulmonarius* (Figure 3.1a) and monokaryotic cultures of *P. citrinopileatus* (Figure 3.1b) were each placed in pairs, in all combinations by placing inoculum plug mycelium (0.7cm diameter) in juxtaposition (Figure 3.1c) in the centre of a 90-mm diameter Petri dish containing MEA. The two species were located approximately 1 cm at the centre of a Petri dish (90 mm diameter) containing MEA. Then, they were left till resultant colonies overgrew the space between the inoculated and a developed contact zone, usually after five to seven days. However, pairings were not interpreted until one week after inoculation in order to establish nuclear migration and a well-formed interaction zone.

Pairings were confirmed as compatible mating if clamp connections could be observed on the hyphae, both in the contact zone and away from it, under the microscope. Mating results were interpreted as positive if clamp connections could be microscopically observed on the contact zone of a pairing. Then a small piece of about 0.5 cm of mycelium were cut off from the junction zone of encounter between the two colony cultures, then sub-cultured to a new MEA plate and incubated at 25°C for five to seven days.

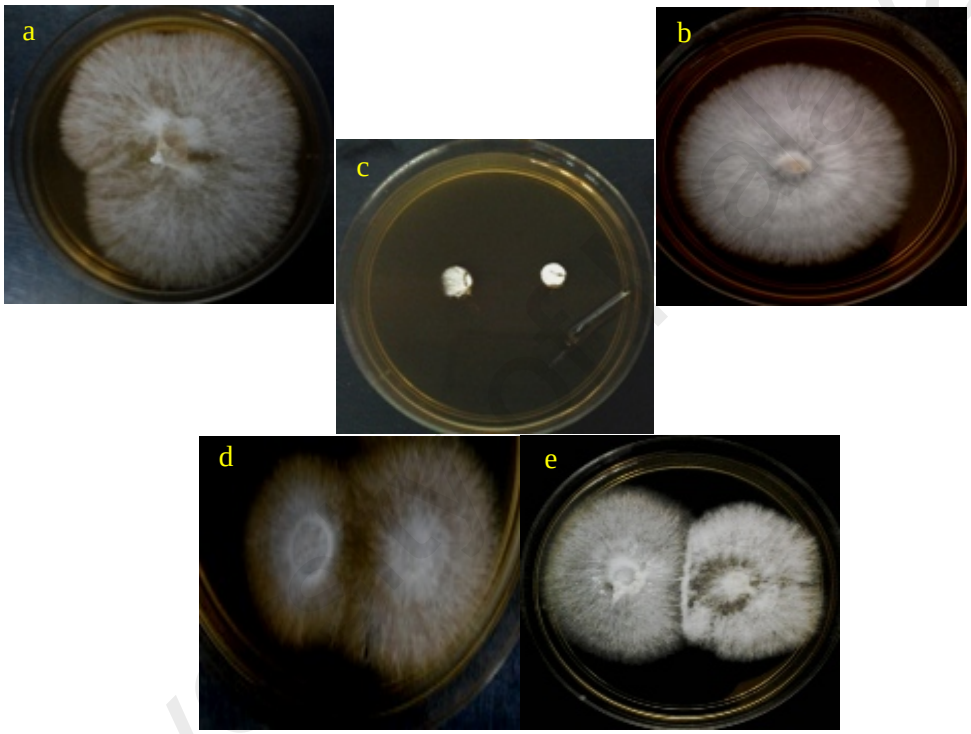


Fig. 3.1. a. Vegetative monokaryon culture of parent *P. pulmonarius*, b. Vegetative monokaryon culture of parent *P. citrinopileatus*, c. Juxta position of pairs monokaryon cultures, d. Compatible mating reaction, e. Incompatible mating reaction

3.2 Mycelium Cultural characteristics and growth rate of monokaryons and hybridized mycelium compared to the parental cultures

Mycelium cultural characteristics of compatible monokaryons from both species, dikaryon culture of hybrids and parents were evaluated. All mycelial cultures were grown on sterilized MEA in Petri dishes. A seven day-old mycelial plug of approximately 0.75 mm diameter was transferred aseptically to the centre of each Petri dish and incubated at 27^{o}C. The colonies were observed for colour, growth pattern and density. Meanwhile, mycelial growth rate was determined by measuring the average diameter of the mycelial colony every day for seven days. The average reading was plotted against time (day) to obtain the growth rate in terms of mm/day. Three repetitions were performed for each sample.

3.3 Production of sporophores, morphology and yield of parental and hybridized cultures

3.3.1 Preparation of inoculum culture

All hybridized mycelia and parental cultures *(P. pulmonarius* and *P. citrinopileatus*) were maintained on a MEA slant at 25^{o}C and sub-cultured regularly. Inoculum was prepared by inoculating a 9-mm diameter mycelium-agar plug of a young, actively growing margin of the colony on MEA plate and incubated in a growth chamber at 25°C for 7 days.

3.3.2 Preparation of growing substrate and inoculation for sporophores production

To produce sporophores of parental and hybrid cultures, cultivation on fruiting substrates polypropylene plastic bag was carried out. The fruiting substrate composed of sawdust + rice bran + calcium carbonate ($CaCO_3$) at a ratio of 100:10: 1 and 70ml of water was added to obtain a moisture content of 70%. The mixture of growing media

was then distributed into heat-resistant polypropylene bag (400g each) sealed with cotton wool and plastic cap. The substrate bags were then autoclaved at 121°C for 1 hour and allowed to cool down. After sterilization the next day, the bags were inoculated 5 plugs of mycelia inoculums. Inoculated bags were incubated for 20 days at 23^{o}C to 25^{o}C, and then transferred to the incubation chamber (preferably in the dark) for mycelium running. The temperature was then maintained at 25°C with a relative humidity at 90%. After completion of the mycelium run, the bags were opened (induction of fruiting) in a mushroom house with high humidity and ventilation.

3.3.3 Examination of sporophore morphology

The morphology of both parental and hybrids sporophores were evaluated by visual observation and measurements. After fructification, sporophores were collected. Parameters assessed includes fruiting body shape or features, stipe, colour, pileus width, thicknesses of pilei (cap) will measure, and texture of sporophore. The length and diameter of the stipe were also measured (mm) to compare with the parents.

3.4 Molecular analysis of hybridized mycelium compared to parental strains

3.4.1 Cryogenic grinding

Samples consisting of mycelium and fresh sporophores were ground into powder by applying cryogenic grinding by using mortar and pestle. Liquid nitrogen was poured onto the samples, followed by a grinding process in which the samples were ground using a circular motion with downward pressure. The pestle was held with a gloved hand and to grind, the samples were firmly press while twisting. The samples were ground into fine fragments and continued to get smaller as the grinding process continued (Izumitsu, 2012). The grinding samples were either subjected directly to DNA extraction method or stored frozen in the tube under -20°C.

3.4.2 DNA extraction

For DNA extraction purposes, Standard Forensic DNA Protocol (E.Z.N.A. Forensic DNA Extraction kit, Omega Bio-tek, Norcoss, GA, USA) was used and the isolation of DNA protocol for Dried Fungal Specimens. According to Shlyakhovenko *et al*., (2006) genomic DNA of *Pleurotus ostreatus* isolated from fresh sporpophores/fruit bodies using standardized DNA extraction protocol.

Ground samples were transferred into 1 mL microfuge tubes. 100µL of St. Louis (STL) buffer was added to the ground samples and incubated for 15 min at 55°C in a thermoblock. The mixtures were vortexed at a 2-minute interval. 25µL of oil body (OB) protease solution were added and the mixtures were homogenized by vortexing. The mixtures were incubated for 45 min at 60 °C in a thermoblock with occasional mixing. The tubes were briefly centrifuged to remove droplets from inside the lid.

Consequently, 225µL of Buffer Layer (BL) buffer was added and mixed. The mixtures were incubated at 60°C for 10 min and the droplets from the inside of the lid were removed via a centrifuge. Another 225 µL of absolute ethanol was added and mixed. The droplets from the inside of the lid were removed.

Each HiBind® DNA mini column was inserted into 2 mL collection tubes provided in the kit. The entire sample was transferred into the mini columns and centrifuged at 8000 xg for 1 min in order to bind the DNA Discard collection tube and flow-through liquid was discarded. Elution buffer was prepared at 60°C in a thermoblock. The mini columns were inserted into a second 2mL collection tube. 500µL of haemoglobin (HB) buffer was pipetted into the columns. The assembled tubes were centrifuged at 8000xg for 1 min. The flow-through was discarded and the collection tubes were reused.

The mini columns were inserted into the reused collection tubes. 750µL of wash buffer diluted with ethanol were aliquoted into the columns. The tubes were centrifuged

at 8000xg for 1 min. The collection tubes were discarded together with flow-through liquid.

By reusing the same 2 mL collection tubes, the tubes were centrifuged at maximum speed (>10,000xg) for 2 min in order to dry the mini columns. This step is required to remove residual ethanol that otherwise might interfere with downstream applications. The mini columns were inserted into 1.5mL microfuge tubes and 50µL of elution buffer preheated to 70°C was added. The tubes were allowed to settle for 3 min at room temperature. DNA was eluded from the column by centrifugation at 800xg for 1 min. Another 50µL of elution buffer was added and repeated as described in previous step. The flow-through in the 1.5mL microfuge tubes were frozen and stored in -20°C freezer.

3.4.3 Gel electrophoresis

Promega Tris/Borate/:Ethylene-Diamino-Tetra acetic Acid (EDTA) EDTA buffer solution containing a mixture of Tris-Borate-Edta TBE) stock buffer is ten times concentrated and therefore needs to be diluted. TBE buffer will be used for the gel preparation and as the electrophoresis buffer. 0.8g of agarose powder was weighed by using electronic balance and was dissolved in 80 mL of freshly prepared TBE buffer. The mixture was heated until a clear solution was obtained from the cloudy solution. 5µL of Red Safe was added into the mixture and allowed to solidified in a complete assemble of gel containers. The gel was placed in an electrophoresis tank and immersed in either fresh or recycled TBE buffer enough to completely submerge the gel.

2µL of the 100bp ladder was aliquoted into the well of the gel as reference to the size of DNA of the samples to be visualized. 2µL of loading dye was mixed with 5µL sample before inserted into the well. Once the samples were loaded into respective wells, the circuit was set so that the migration of DNA would take place from the positive terminal to the negative terminal. The voltage was set at 100V for an hour.

The samples were visualized under UV light. The appearance of smear indicates that the DNA is present in the extracted samples. Per Shlakovenko *et al*., (2006) identification of DNA in the samples was done by using pulsed-field agarose gel (PFAG) electrophoresis.

3.4.4 Determination of DNA concentration

Determination of DNA was carried out by using NanoDrop™ spectrophotometer. 10µL of sterile ddH_2O was drop on the sample reading site and cleaned with Kimberly-Clark® Kimwipes™. This step was repeated three times. 10µL of Elution Buffer was placed on the sample reading site and was set as blank.

The sample reading site was cleaned with the same steps for the sample reading. As a precaution, bubbles must not be in the samples as this would affect the reading. Per (T-009 Technical Bulletin), the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA.

3.4.5 Polymerase Chain Reaction

The reagents were assembled in PCR tubes. The following are the designated volumes for respective components in a single PCR tube as showed in Table 3.1.

Table 3.1: The volume for respective reagents

Reagent	**Volume (µL)**
dNTP	4.0
PCR buffer	5.0
Taq polymerase	0.5
ITS 1	2.5
ITS 4	2.5
DNA template	1.0
ddH_2O	34.5
Total	50.0

Once all the components were assembled, the tubes were inserted in the Eppendorf thermocycler. Table 3.2 shows the stages and respective temperature, duration, and cycle. According to Collopy *et al*., (2002) two molecular approaches (sequence analysis of internal transcribed spacers 1 and 2 (ITS-1 and ITS-2) and 5.8 S regions of the nuclear ribosomal DNA [rDNA] transcriptional unit and random amplified polymorphic DNA [RAPD]) were performed for 68 of the *Verticillium* sp.

Table 3.2 PCR stages and correspondent parameters, White *et al*., (1990)

Stage	Temperature (°C)	Time (min)	Cycle
Initial denaturation	94	5	1
Denaturation	94	1	30
Annealing	48	1	
Extension	72	1	
Final extension	72	10	1
Hold	4	-	-

The products of PCR were subjected to gel electrophoresis for the presence of DNA bands. If a single band appears brightly at the respective well, the PCR products will be subjected to purification. This is to remove the reagents that might interfere with the sequencing process.

3.4.6 DNA purification

DNA purification for this research was carried out using MEGAquick-spin™ Total Fragment DNA Purification Kit from iNtRON Biotechnology. 5 µL volume of Block-Nested Loops (BNL) buffer was added to the PCR reaction products followed by vortexing to ease mixing. For instance, if the PCR product is 30µL, 150µL of BNL

buffer is required. MEGAquick-spin™ column was inserted into a Collection Tube and the DNA mixtures were transferred to the MEGAquick-spin™ columns assembly.

DNA mixtures were centrifuged for one minute at 13,000 rpm. The flow-through was discarded and the collection tubes were reassembled. 700µL of Washing Buffer was added to columns. The columns were centrifuged at 13,000 rpm for one minute. The flow-through was discarded and the Collection Tubes were reassembled. The columns were centrifuged again at 13,000 rpm for 1 min to dry spin the membrane.

MEGAquick-spin™ columns were inserted into a clean 1.5ml micro centrifuge tubes. 50µL of Elution Buffer was applied directly to the centre of the column without having the pipette tip touches the membrane. The tubes were incubated at room temperature for 1 min. The tubes were centrifuged at 13,000 rpm for 1 min. MEGAquick-spin™ columns were discarded and the 1.5mL micro centrifuge tubes were stored at-20^{o}C.

3.4.7 Phylogenetic Analyses

Phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis MEGA) five (Tamura 2011) utilizing maximum likelihood (ML) methods, with 100 random stepwise sequence addition replicates, tree bisection reconnection (TBR), collapse of zero length branches, and. All characters were equally weighted and unordered, and gaps were treated as missing data. Parsimony bootstrap (PB) analysis was performed using 1000 bootstrap replicates, each with a limit of 1,000,000 bp rearrangements, closest taxon addition, and TBR branch swapping.

3.5 Determination of the productivity and stability of sporophore morphology of selected hybrids compared to parental strains upon large scale cultivation

3.5.1 Preparation of spawn

Wheat grains were washed, rinsed, soaked in filter water and cooked for 50 min. Excess water was drained, and then mixed with (2%) calcium carbonate ($CaCO_3$). Five hundred grams of supplemented grains were packed in polypropylene bags and autoclave sterilized (60 min at 121°C at 1.2 kg/cm^2), then allowed to cool. A seven day old mycelia plug was put on the four sides of the bag on top of the grains in polyethylene bag. Inoculated bags were transferred to an incubation cabinet and kept at room temperature for 15 to 20 days. All three selected hybrids (P19xC5, P19xC5, P3xC8) and both parental (*P. pulmonarius* and *P. citrinopileatus*) were subjected to fruiting tests.

3.5.2 Preparation of growing substrate for large scale cultivation

To evaluate morphological variation of the hybrid strains were grown on polypropylene plastic bag logs. Sawdust supplemented with rice bran was used to grow mushrooms. Sawdust was mixed with rice bran and $CaCO_3$ (100:10:1). Then 80% water was added to the mixture. Each polypropylene bag filled with 700 gram of substrate was plugged by inserting absorbing cotton into the plastic rings. The bags were autoclaved at 121°C for 1 hour and allowed to cool. Sealed bags were then sterilized at 121°C for 60 min. The bag was inoculated with spawn separately after cooling.

3.5.3 Evaluation of sporophore morphology and yield

Evaluation was carried out to determine new hybrids showing a good growth rate and mycelium density in terms of spawns run rate and biological efficiency. Cultivation was carried out in a larger scale as above and 100 bags were prepared for each hybrid. The spawn of parental strains (control) and hybrids were prepared by inoculating mycelia colonies on sterilised crushed corn grown for 2 weeks. Spawn were transferred to the top of each substrate bag using a spatula until full. This was performed in sterile conditions in a laminar air flow cabinet. Inoculated bags were transferred to an incubator and kept at room temperature at about 27°C to allow the spawn run Mycelium spawn rate to be determined by measuring mycelia extension at 4 sides of the bag at 2 day intervals for 19 to 21 days. The temperature was then maintained at 25°C and relative humidity at 90%. The average reading was plotted against time (day) to obtain the growth rate in terms of mm/day.

About 18 to 21 days after inoculation, when the mycelium completed the spawn run, the bag was open and kept at the experimental room temperature (16–17 °C) cool room equipped with a misting system. Pinheads started appearing 3 to 4 days after the cap removal. During this fruiting phase, relative humidity (RH) was maintained above 85%. This was done by spraying water in the form of fine mist using a sprinkler. The sprinkler was set to spray a fine mist for ten minutes every two hours.

After 2 to 3 days, the sporophores had matured and ready to be harvested. Three flushes were harvested with interval of 2 to 4 days. Harvesting was carried out every day for three weeks. Fresh sporophore yield produced during first, second, and third harvest was recorded. The total weight of fruiting body was taken during each harvest and weighed using a weighing scale. The number of primordia (pinhead-like appearance) was counted and recorded. Generally, *Pleurotus* spp achieved maturity within two to three days after primordia initiation. The matured fruiting body was

identified by the curve margin of the cap, as described by Amin *et al*. (2007). Mushrooms were harvested by twisting to uproot from the base.

Fully developed sporophores were counted to determine the number of effective ones; tiny and deformed sporophores were discarded at the time of counting. To obtain the average weight of yield performance, the total weight of sporophores was taken during each harvesting and weight was recorded. Mean fresh weights for the three consequent flushes of each bag were calculated for total yield per bag. The biological efficiency was determined as the yield of mushroom strain over the dry weight of each bag as described in the following section.

3.6.4 Determination of spawn run rate

Mycelial growth rate during spawn running in plastic bags were manually determined by measuring the mycelia extension from starting point using a ruler. The calculation of mycelia extension is as follows:

Mycelia extension in plastic bag (mm) = mean vertical length from 4 corners of plastic bag

3.6.5 Determination of fresh mushroom yield

Total fresh mushroom yield (from first, second and third flushes) was weighed and the average per bag was determined in grams.

3.6.6 Determination of biological efficiency (B.E)

Biological efficiency (BE) ratio is a formula used to determine the ability of a mushroom strain to convert substrate materials into fresh sporophores. At the end of the third flush, the BE of fruiting substrate was determined from each genotype by measuring the fresh weight harvest of sporophores from each bag, dividing it with the weight of bags which are uniformly 700 grams, then multiplying the value by 100%.

$$\text{Biological efficiency (\%)} = \frac{\text{Total weight of fresh sporophores produced}}{\text{Total weight of dry substrate used}} \times 100\%$$

3.7 Statistical analysis

Quantitative data such as spawn run or radial growth rate of mycelia, amount of fruit bodies, fresh weight, and dry weight of harvest were measured at least three times. All data was analysed by analysis of variance (ANOVA) using STATGRAPH, followed by Duncan's Multiple Range Test (DMRT) (P=0.05).

CHAPTER 4

RESULTS

Increasing the yield and quality of crops as well as resistance to diseases are the primary goals for mushroom breeders and mushroom research. In this study interspecies hybridization method was selected to obtain new hybrids of *P. pulmonarius* (Grey Oyster mushroom) and *P. citrinopileatus* (Yellow oyster mushroom).

4.1 Hybridization of monokaryon cultures and identification of mating types

Monokaryon cultures from parental *P. pulmonarius* (P1, P3, P6, P8, P9, P13 and P19) and parental *P. citrinopileatus* (C1, C2, C5, C7, C8, C9, C10 and C13) with good cultural characteristics such as fast mycelial growth rate and high density were selected for mating studies. Table 4.1, showed most of the crosses resulted in incompatible mating which was identified by the absence of hyphal fusion between the monosporous pairs. There were five compatible mating strains generated by A_1B_1 x A_2B_2 and six from A_1B_2 x A_2B_1 pairings.

There were five compatible mating strains generated by A_1B_1 x A_2B_2 and six from A_1B_2 x A_2B_1 pairings. Crosses designated as “NA” signifies hemi-compatibility in which an infertile, restricted, principally “monokaryotic heterokaryon” with false clamps is formed. The cultures did not produce fruiting bodies during cultivation.

Table 4.1. Mating between monokaryon isolates of *Pleurotus pulmonarius* (P) and *P. citrinopileatus* (C)

Strains			*P. citrinopileatus*							
			A_1B_1				**A_1B_2**			
			C1	C2	C5	C7	C8	C9	C10	C13
P. pulmonarius	**A_2B_2**	P9	NA	NA	NA	+	−	−	−	−
		P13	NA	NA	+	+	−	−	−	−
		P19	NA	+	+	NA	−	−	−	−
	A_2B_1	P1	−	−	−	−	NA	+	+	+
		P3	−	−	−	−	+	NA	NA	NA
		P6	−	−	−	−	NA	NA	NA	+
		P9	−	−	−	−	NA	NA	+	NA

Mating pairs were carried out in triplicate.

+, signifies full compatibility in which a non-restricted fertile dikaryon with true clamps is formed; −, signifies incompatility; NA, signifies hemicompatibility in which an infertile, restricted, principally monokaryotic heterokaryon with false clamps is formed.

Referring to Table 4.1, most of the crosses resulted in incompatible mating which was identified by the absence of hyphal fusion between the monosporous pairs. There were five compatible mating strains generated by A_1B_1 x A_2B_2 and six from A_1B_2 x A_2B_1 pairings. Crosses designated as "NA" signifies hemicompatibility in which an infertile, restricted, principally monokaryotic heterokaryon with false clamps is formed.

In the present mating studies four types of mating reaction were observed based on appearance of mycelium crossing of monokaryon culture as shown in Fig. 4.1 (a-d). Positive mating was identified by a flat and smooth mycelial mat at the junction zone (Fig. 4.1a). On the other hand the incompatible mating reactions were identified

with three categories such as overlap (Fig. 4.2b), barrage with false clamp (Fig. 4.2c) and flat (Fig. 4.2d)

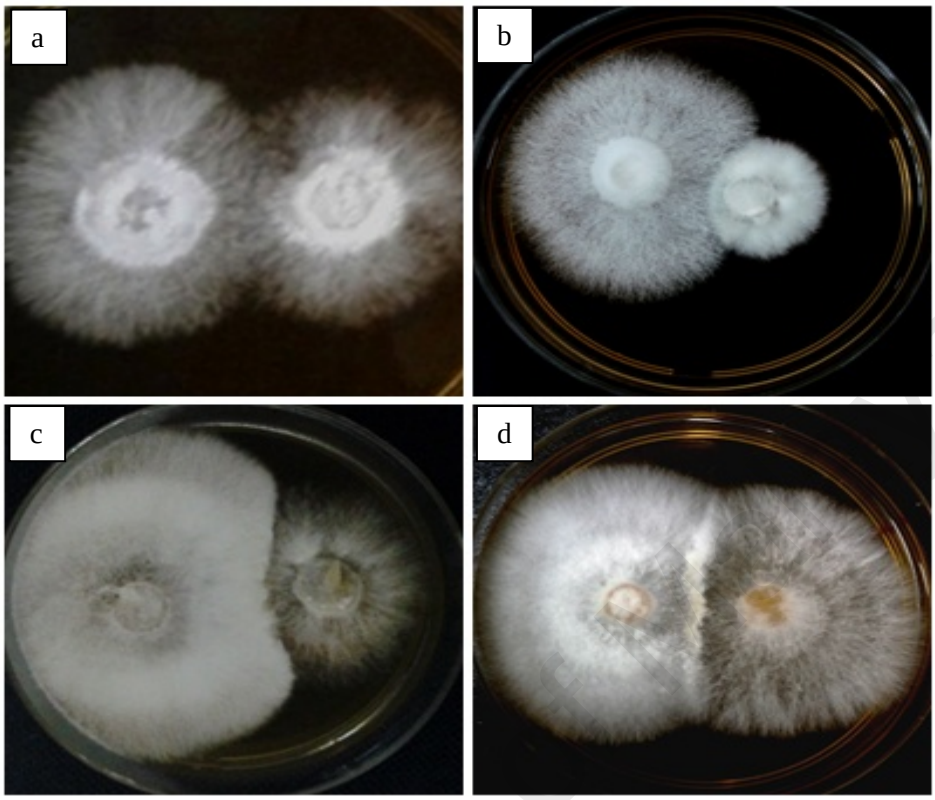


Fig. 4.2. Mycelial cultural characteristics of inter-species mating observed between some pairs of monokaryon culture isolates of *Pleurotus pulmonarius* and *P. citrinopileatus*

a. Positive mating reaction, crossing of monokaryon culture isolates (compatible) and clamp connections are detected

Incompatible mating reactions;

b. Crossing of single spore (overlap, A=B=)

c. Crossing of single spore (barrage, A≠B=, false clamps)

d. Crossing of single spore (flat, A=B≠)

Positive hybridization between two compatible homokaryotic mycelia was further validated by observing the mycelium at the contact zone between the two Hyphal nets for the presence of clamp connections (Fig 4.3b). Compatible reactions will lead to hyphal fusion resulting in the formation of heterokaryotic fertile mycelium. The

nuclear division and septum formation in this mycelium occur through clamp connections indicative of dikaryon formation.

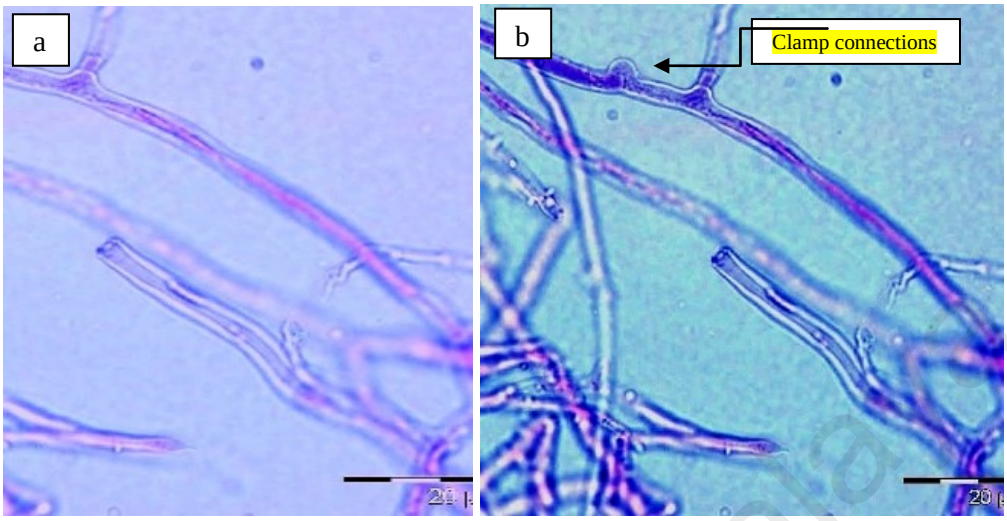


Fig. 4.3.a. Negative mating culture (homokaryon hyphae without clamp connection) and b. Positive mating culture [new dikaryotic hyphae with clamp connection (shown by arrow)]

4.2.1 Mycelial cultural characteristics of monokaryon culture isolates of *P. pulmonarius* that exhibited positive mating

Mycelial cultural characteristics of compatible monokaryon isolates of *P. pulmonarius* parental line exhibited variations as shown in Figs 4.4 (a-d). Monokaryon culture isolate P1 expressed white, loose, linear, cottony, thin, and uneven mycelia and irregular margins forming (Fig. 4.4a). Monokaryon culture isolate P3 was white, linear cottony with concentric bands of different texture and rhythmic growth (Fig. 4.4b). Monokaryon cultures isolate P6 expressed white, loose, linear, cottony, thin, uneven mycelia (Fig. 4.4c). Monokaryon culture isolate P8 was white, linear becoming cottony, moderate density of mycelia (Fig. 4.4d). Monokaryon culture isolate P9 was dull white, linear cottony, thick, uneven mycelia (Fig. 4.4e). Monokaryon culture isolate P13 exhibited as dull white, linear, cottony and thick of mycelia (Fig. 4.4f). Monokaryon culture isolate P19 was white, linear cottony, very thick and dense with concentric

bands of different texture with rhythmic growth (Fig. 4.4g). These variations illustrate genetic variations of the individual spores from *P. pulmonarius.*

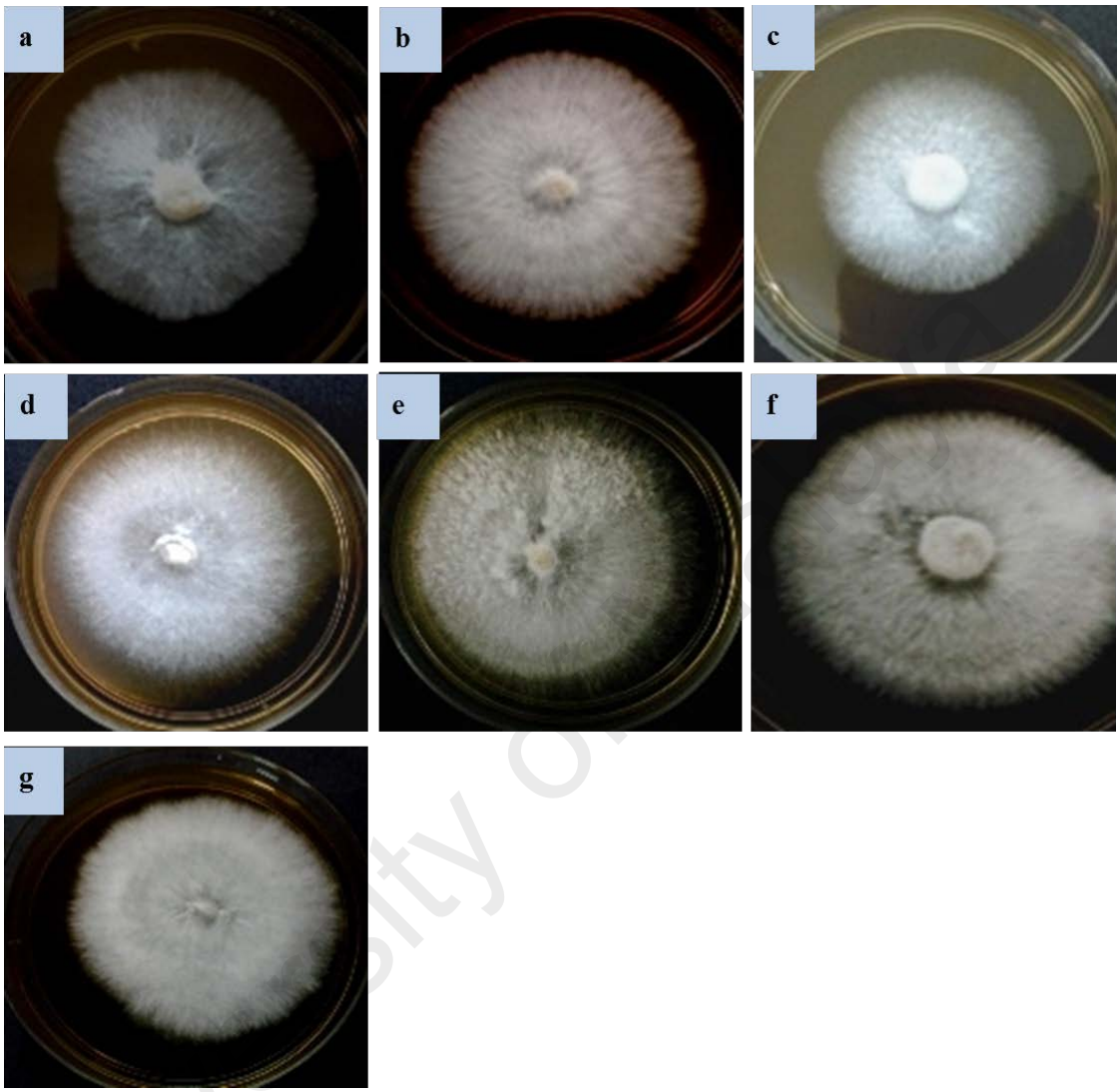
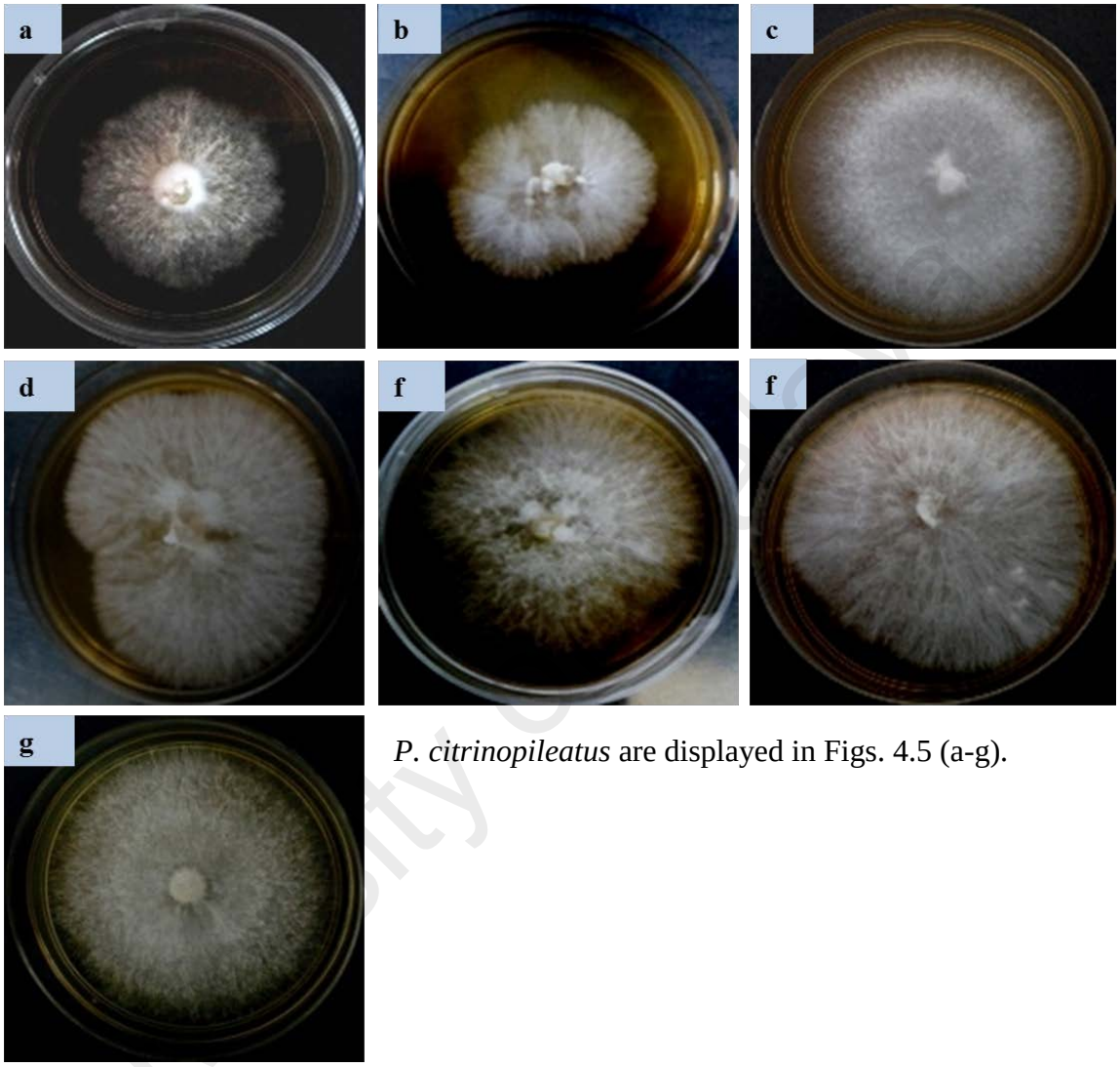


Fig. 4.4. Mycelial cultural characteristics of monokaryon isolate of parental *P. pulmonarius* (P): a. P1, b. P3, c. P6, d. P8, e. P9, f. P13, and g. P19.

4.2.2 Mycelium cultural characteristics of monokaryon isolates of *P. citrinopileatus* that exhibited positive mating

Mycelial cultural characteristics of monokaryon culture isolates of parental lines



P. citrinopileatus are displayed in Figs. 4.5 (a-g).

Fig. 4.5. Mycelial cultural characteristics of parental monokaryon isolates of *P. citrinopileatus* (C): a. C2, b. C5, c. C7, d. C8, e.C9, f. C10, and g. C13

Monokaryon culture isolate C2 expressed as whitish, thin, growth, of dense growth and run through underlying rhizomorphic strands irregular margins forming (Fig. 4.5a). Monokaryon culture isolate C5 was shown as whitish, growth become cottony whitish with yellowish tone cottony, dense growth, irregular margins forming (Fig. 4.5b). Monokaryon culture isolate C7 exhibited whitish with yellowish tone

cottony, tuft of dense growth and run through underlying rhizomorphic strands with concentric bands of uneven texture (Fig. 4.5c). Monokaryon culture isolate C8 viewed as whitish with yellowish tone cottony, tuft of dense growth, irregular margins forming (Fig. 4.5d). Monokaryon culture isolate C9 was whitish, growth and become cottony whitish with yellowish tone cottony, uneven dense growth (Fig. 4.5e). Monokaryon culture isolate C10 exhibited whitish, yellowish tone, dense growth and run through underlying rhizomorphic strands ig. 4.5f). Monokaryon culture isolate C13 expressed as whitish with yellowish tone cottony, tuft of dense growth and run through underlying rhizomorphic strands with concentric bands of different texture (Fig. 4.5.g).

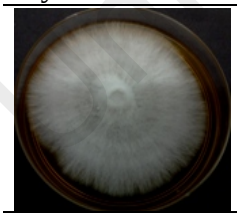
4.2.3 Mycelial cultural characteristics and growth rates of *P. pulmonarius* dominant hybrids compared to the parental *P. pulmonarius*

Mycelial culture of both *P. pulmonarius* parent hybrids dominant to *P. pulmonarius* were observed for their cultural characteristics (colour, radial growth rate and the appearance of the mycelia) as shown in Table 4.2. Dikaryon cultures of *P. pulmonarius* are white in colour, uneven, a zonate or with concentric of different texture when the growth is rhythmic. The vegetative mycelium consists of branched generative hyphae 2.5-7.0 µm in diameter. Meanwhile, the cultures of *P. citrinopileatus* are cottony, whitish in colour, often with tufts of dense growth, sometimes with yellowish tones, and occasionally run through with underlying rhizomorphic strands. The hybrid culture P19xC5 is white in colour and grows in concentric ban with irregular margins forming. The mycelium growth is rhythmic with very thick mat density. The hyphae possessed many primordial formation 3 days after inoculation. Hybrid culture of P19xC2 is white in colour with thin concentric ban. The growth is rhythmic with a medium mat density that shows tiny primordial 6 days after inoculation. The other

hybrid cultures, i.e. P9xC7, P13xC5 and P13xC7 have very thick mat density with rhythmic growth. There were no primordial formations even after completed spawn run.

Generally, the hybrid cultures grow faster and have a thicker mycelia mat compared to the parental cultures. This indicates that interspecies mating may generate good traits and quality hybrid cultures. Among these, P19XC5 showed the highest growth rate at 4.0 mm/day. Both the parent and hybrid cultures required 6-7 days to reach full growth in the Petri plates.

Table 4. 2. Evaluation of mycelial cultural characteristics, growth rate and thickness of parent and hybrid dominant to *P. pulmonarius*

Parent/Hybrids	**Mycelial Cultural Characteristics**	***Mycelial growth rate (mm/day) and Mycelia thickness**
 *Pleurotus pulmonarius*	White mycelium culture, uneven, a zonate or with concentric of different texture when the growth is rhythmic. Vegetative mycelium consists of branched generative	2.3 ± 0.21^{a} ++
 Hybrid P8xC7	White mycelium culture, very thick with rhythmic growth.	3.2 ± 0.29^{b} +++
 Hybrid P13xC5	White mycelium culture, thick, a zonate at the centre with different texture	3.3 ± 0.30^{bc} +++
 Hybrid P13x7	Mycelium culture dull white, very thick texture when the growth is rhythmic	3.6 ± 0.04^{bc} +++

	White mycelium culture, a zonate with concentric with uneven of texture when the growth is rhythmic.	3.0 ± 0.15^{b} ++
Hybrid P19xC2		
	White mycelium culture, very thick dense, a zonate with concentric of different texture. Primordial structures present.	4.0 ± 0.15^{c} +++

*Each value is expressed as mean ± standard deviation (n = 3). Different alphabet letter within the column denotes the mycelial growth rate was significantly different at $P < 0.05$.

4.2.4 Mycelial cultural characteristics and growth rates of *P. pulmonarius* dominant hybrids compared to the parental *P. citrinopileatus*

Mycelial culture of both *P. citrinopileatus* parent hybrids dominant to *P. citrinopileatus* were observed for their cultural characteristics (colour, radial growth rate and the appearance of the mycelia) as shown in Table 4.3. We observed that the vegetative monokaryon cultures of both *P. citrinopileatus* and *P. pulmonarius* had some peculiar features and were different from the parental and new dikaryotic hybrid cultures. The six hybrid cultures had different mycelial mat patterns and density, one of the characteristics responsible for adaptability to physiological parameters and high yield. For example, the hybrid strain P1xC9 had very thick, dense growth that was dull white, and zonate with concentric bands, while the P3xC8 hybrid was similar to its parental *P. citrinopileatus* with very thick, dense growth with rhizomorphic strands.

Table 4. 3. Evaluation of mycelial cultural characteristics, growth rate and thickness of parent and hybrid dominant to *P. citrinopileatus*

Parent/Hybrid	**Mycelial Cultural Characteristics**	***Mycelial growth rate (mm/day) and Mycelial thickness**
 *Pleurotus citrinopileatus*	Mycelium culture has cottony type, whitish and tufts of dense growth, with yellowish tones, and run through with underlying rhizomorphic strands.	2.3 ± 0.21^{a} ++
 Hybrid P1xC9	Mycelium culture has texture very thick dense growth, dull white, a zonate with concentric band at the centre	3.3 ± 0.30^{c} +++
 Hybrid P1xC10	Mycelium culture dull white with thick dense growth, becoming cottony.	2.3 ± 0.25^{a} ++
 Hybrid P3xC8	Mycelium culture yellowish tone, very thick dense run through underlying rhizomorphic strands	3.0 ± 0.15^{b} +++
 Hybrid P9x10	Mycelium culture uneven mycelia with a bit thin dense and irregular margin.	2.1 ± 0.29^{a} ++

 Hybrid P1xC13	Mycelium culture white, thick dense mycelia run through underlying rhizomorphic strand	3.3 ± 0.04^{c}	+++
 Hybrid P6xC13	Mycelium culture colour whitish with mycelia mat thick dense growth at the centre, and irregular margin forming	2.4 ± 0.15^{b}	++

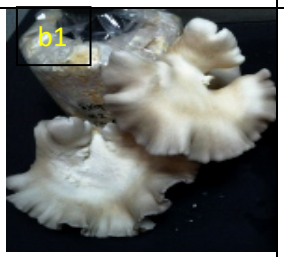
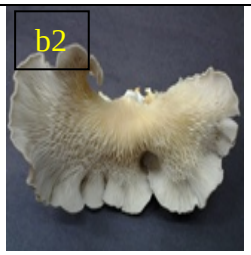
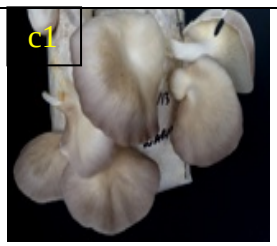
*Each value is expressed as mean ± standard deviation (n = 3). Different alphabet letter within the column denotes the mycelial growth rate was significantly different at $P < 0.05$.

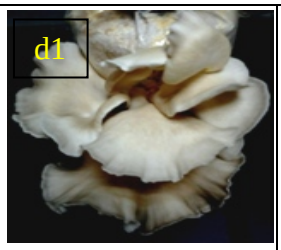
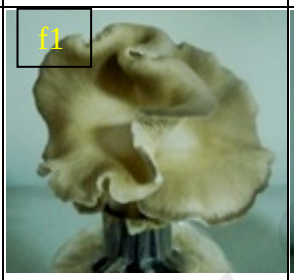
4.3 Macro-morphological description of sporophores of parental *P. pulmonarius* and hybrids dominant to *P. pulmonarius*

The morphological characteristics of sporophores for the parent and hybrid strains as well as the measurement of their stipe length and pileus width are illustrated in Table 4.4. The parent, *P. pulmonarius* had depressed pileus, lung-shaped cap with whitish to beige or pale tan. The margin enrolled when young, which later forms wavy when mature. It has very finely lined gills not split longitudinally (Stamet, 2000). Meanwhile, the other parental strain, *P. citrinopileatus* has a brilliant yellow colour which forms clusters hosting a high number of individual mushrooms, whose stems often diverge from a single base. In contrast, the five hybrid strains have considerably different morphological characteristic from their parental strains. Sporophores produced from three of the hybrid strains, i.e. P13XC5, P19XC2 and P19XC5 had blending characters between those of their parents. They had big, fleshy, grey coloured pileus which, most probably inherited from parent *P. pulmonarius* while the funnel shape characteristic

could be possessed from *P. citrinopileatus*. The margin enrolled when young and becomes wavier when matured. These hybrid strains had thicker and firmer texture with irregular and eccentrically stipe. The lamella of hybrid strains P13XC5 and P19XC2 were similar to parent *P. pulmonarius*. Interestingly, the lamella of hybrid P19XC5 was distinctive than the parental strains, *P. pulmonarius* and *P. citrinopileatus*. Instead, it resembles the lamella of *Lentinus edodes*. The pileus of hybrid strains P13XC7 and P9XC7 have lung shape pileus similar to *P. pulmonarius* but were pale grey in colour. The lamellas were seen similar to both parental strains.

Table 4.4. Comparison of Macro-morphological Description of Sporophores for Hybrid Strains Dominant to *P. pulmonarius*

Strain	**Sporophores**		***Characteristics**
	Pileus surface (dorsal)	**Lamellae (ventral)**	
P. pulmonarius			(a) 4.5 ± 0.2 (b) 7.3 ± 0.1 (c) Convex (d) Grey/fleshy
Hybrid P8XC7			(a) 4.5 ± 0.2 (b) 7.5 ± 0.2 (c) Convex (d) Grey/fleshy
Hybrid P13XC5			(a) 3.4 ± 0.2 (b) 7.8 ± 0.1 (c) Downward (d) Grey/freshier

Hybrid P13XC7			(a) 3.7 ± 0.2 (b) 8.7 ± 0.2 (c) Round (d) Grey/fleshy
Hybrid P19XC2			(a) 4.3 ± 0.1 (b) 7.0 ± 0.7 (c) Convex/wavy (d) Light grey/thin context
Hybrid P19XC5			(a) 4.2 ± 0.2 (b) 8.2 ± 0.2 (c) Convex/wavy (d) Grey/fleshier

*Characteristics of the hybrid sporophores were signified as (a) stipe length (cm); (b) pileus width (cm); (c) margin of the pileus; (d) colour/texture of the sporophores.

The (a) stipe length and (b) pileus width are expressed as mean ± standard deviation (n = 3).

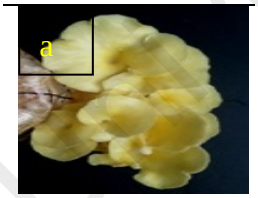
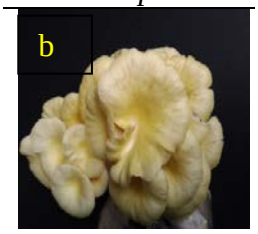
4.4 Macro-morphological description of sporophores of parental *P. citrinopileatus* and hybrids dominant to *P. citrinopileatus*

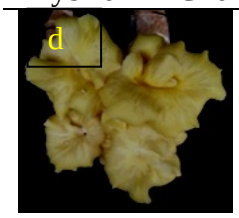
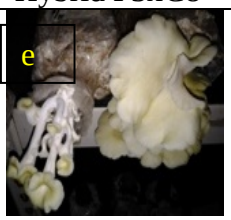
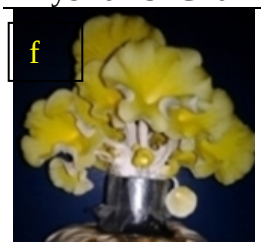
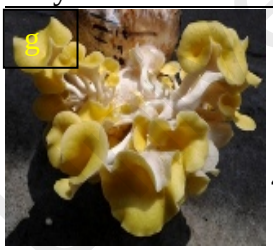
The morphological characteristics of sporophores for the parental images and hybrid strains as well as the measurement of their stipe length and pileus width are illustrated in Table 4.5. Parental strain *P. citrinopileatus* has a brilliant yellow colour

which forms clusters hosting a high number of individual mushrooms, whose stems often diverge from a single base.

All of the six hybrids sporophore had similar characteristics to *P. citrinopileatus* but showed variations in texture, colour and aroma compared to the parental stock. In general, the hybrids had a bigger pileus and a wavy pileus margin compared to the round pileus margin of their parent. The pilei of two hybrids and P6XC13 had a bright yellow colour, strong aroma and fragile texture, all undesirable characteristics. In contrast, hybrid P1XC10 and P9XC10 with a thin context and exhibited pale yellow pilei with reduced aroma and were therefore more appealing for consumption. The hybrid P1XC9 was beige in colour while hybrid P3XC8 was yellow in colour, but both were tough in texture and less pungent. In addition, their pilei were funnel shaped, larger in pileus size and grew in big clusters. Due to these characteristics, P1XC9 and P3XC8 were selected for further analysis.

Table 4.5. Comparison of Macro-morphological Characteristics and Traits of Hybrids with Parental Strains

Strain	* Stipe length/ Pileus width (Cm)		Margin of Pileus/Shape	Texture/Aroma
 *P. citrinopileatus*	3. ± 0.2[a]	4.5 ± 0.1[a]	Matured sporophores in cluster with bright yellow color	Fragile/Strong
 Hybrid P1xC9	4.10±0.1[c]	6.37±0.1[ab]	The sporophores beige in colour with undulating shape	Fleshy/Decrease

	3.43±0.1[a]	5.60±0.2[ab]	Pale yellow colour, funnel shape and smooth pileus cap	Fragile/Strong
Hybrid P1xC10				
	3.73±0.1[b]	5.93±0.1[ab]	Yellow colour with funnel shape, wavy pileus margin	Fleshy/Decrease
Hybrid P3xC8				
	4.43±0.1[d]	6.33±0.1[ab]	Pale yellow with funnel shape and smoot pileus cap	Fragile/Decrease
Hybrid P9xC10				
	3.76±0.2[ab]	4.70±0.1[ab]	Bright yellow with funnel shape Wavy of margin pileus	Fragile/Strong
Hybrid P1xC13				
	4.13±0.15[c]	5.43±0.1[ab]	Bright yellow with funnel shape Wavy of margin pileus	Fragile/Strong
Hybrid P6xC13				

*Each value is expressed as mean ± standard deviation (n = 3). Different alphabet letter within the column denotes the mycelia growth rate was significantly different at $p < 0.05$

4.5. Evaluation of sporophores yield performance of parental strains and selected hybrids

In order to demonstrate practice of the selected hybrids P19XC5 that has resembled to *P. pulmonarius*, and two hybrids P1XC9 and P3XC8 had a similar morphology to had a similar morphology to *P. citrinopileatus.* The three hybrids which have good characteristics such as higher growth rate and thick mycelium density, as well as sporophores with decreased aroma and improved texture was selected for evaluation as potential use by mushroom growers.

For fruiting in larger scale experiments, the following selected hybrids (P19XC5, P1XC9, P3XC8) and parental species were cultivated in about 100 bags were observed for their spawn run period, and yielding ability (Table 4.7). Dates of different stages of growth of the hybrid cultures and dikaryon of both parent on the substrate indicated that to attain full growth/completed spawn run 19 and 21 days after inoculation respectively. Spawn run period of hybrid culture showed 3 days faster than completed spawn run of parental cultures.

Spawn run period of hybrid culture showed 3 days faster than completed spawn run of parental cultures. Observations regarding the date of completed spawn run, and yield performance in subsequent three times of flushes were recorded as (Table 4.6).

Table 4.6 Evaluation of the radial growth rate, days required to complete the spawn run, total sphorophores yield and the biological efficiency for the parent and selected hybrid strains

Species	Mean ± Radial Growth Rate of bag (mm/day)	Completed spawn run days*	Total weight (g)	Biological Eficiency ** (%)
P. pulmonarius (P)	8.2±0.1[b**]	21±0.2[a]	103.91± 0.1[a]	74.22±0.40[b]
P. citrinopileatus (C)	7.7± 0.1[a]	21±0,3[a]	71.25± 0.1[a]	50.89±0.32[a]
Hybrid P19XC5	8.7±0.0[e***]	19±0.2[a]	182.97± 0.2[b]	130.19±0.2[a]
Hybrid P1XC9	8.5± 0.0[e**]	19±0.2[a]	141.9±0.67[c]	70.97±0.42[c]
Hybrid P3XC8	8.2±0.0[de***]	19±0.2[a]	104.3±0.28[b]	52.14±0.07[b]

S significant

*(day after inoculation)

** (weight of fresh mushroom in 4 harvest/weight of dry substrate)

In large scale cultivation, the hybrid strain P19XC5 exhibited a highest radial growth rate at 8.7 mm/day, followed by another two selected hybrid that has faster growth rate compared to the parental cultures. Hybrid P1XC9 had a mycelial growth rate of 8.5 mm/day while hybrid P3XC8 grew at 8.2 mm/day, similar to the parental *P. pulmonarius.* The growth rate of parental *P. citrinopileatus* was slower at 7.6 mm/day. Furthermore, it requires a shorter time, i.e. 19 days to colonize the substrate, that same with the two other hybrids and both their parental species respectively which was approximately 21 days the amount reported for the parental strains. Another important aspect is the increase in total yield and biological efficiency selected hybrid P19XC5 generated highest sporophores yield (182.97 g), and biological efficiency (130.19%) followed by higher rate of two another hybrid P19XC5 (141.9 g

and 70.97%), hybrid P3XC8 (104.3g and 52.14%) and parental *P. pulmonarius* (103.91g and 74.22%), *P. citrinopileatus* (71.25 g and 50.89%) species respectively. These characteristics are advantageous for the mushroom growing industry.

4.6 Comparison of spore density between parental *P. pulmonarius* and hybrid P19XC5 and phenotypic evaluation of the hybrid sporophore grown for three generations

Observation of the hybrid P19XC5 sporophores developed on do clamp connection formation results from a compatible the mycelium of *P. pulmonarius* and *P. citrinopileatus* showed differentiated macro-morphological characteristics than parental. Appearances of new hybrid (P19XC5) exhibited different features compared to parents. Special attention of characteristic had fleshier and tough of sporophores, highest of total yield and biological efficiency, hybrid P19XC5 was selected to further analysis for its spore density in comparison with the commonly cultivated grey oyster mushroom. As well as good shape this hybrid which was changed of crowded bellow lamella referred to Figure 4. The second offspring showed differentiated compared to decurrent bellow lamellae of parent *P. pulmonarius* referred to Figure 4.a2.

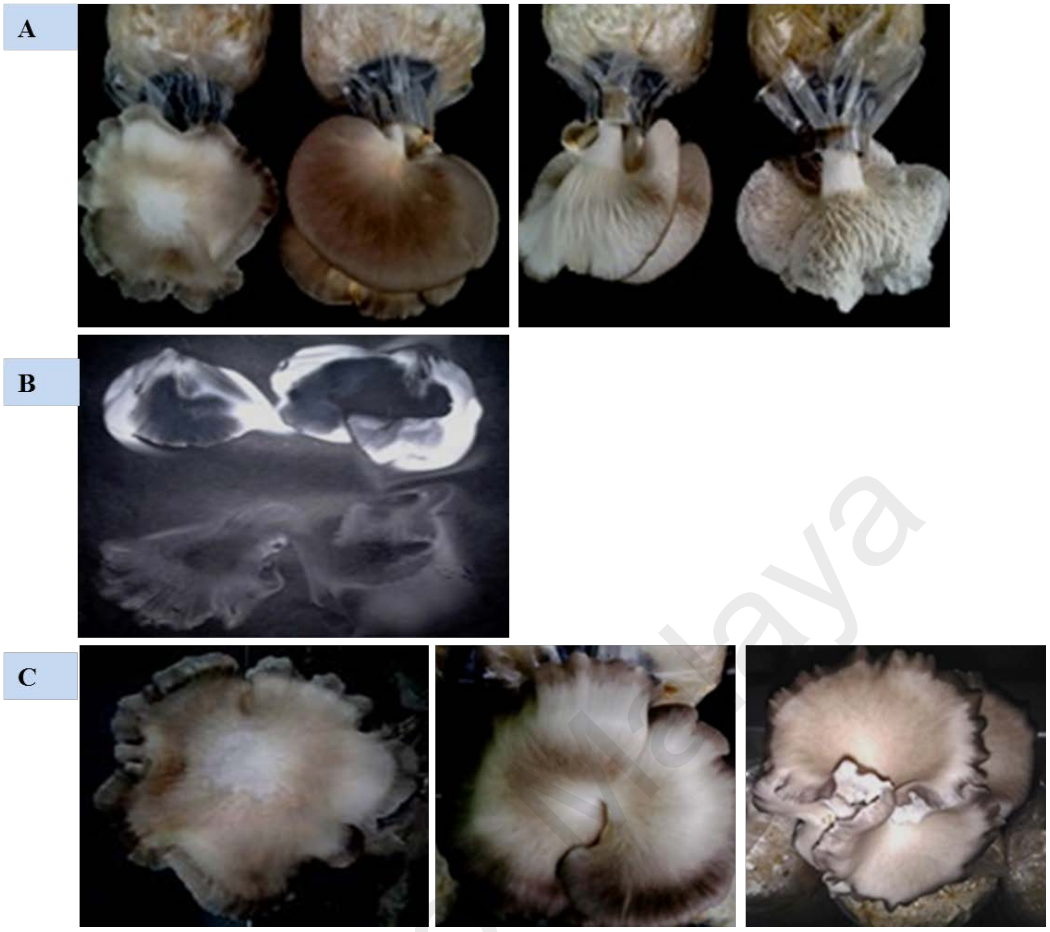


Fig. 4.6. (A) Comparison of Pileus surface (left) and Lamella ventral (right) of *P. pulmonarius* and hybrid P19XC5

(B) The spore print on art paper for *P. pulmonarius* (upper panel) and hybrid strain P19XC5 (lower panel)

(C) The sporophores for the hybrid strain P19XC5 produced in three generations (g1, g2 and g3)

4.7 Phylogenetic analysis of hybrid-dominant to *P. pulmonarius*

Further analysis, molecular identification was carried out on the two groups hybrids based on the sequencing of the internal transcribed spacer (ITS) region. PCR using ITS1 and ITS4 was performed to identify the hybrids by a molecular method compared to the parent and corresponding GenBank data of related species.

Phylogenetic tree was constructed by using two software program which are Chromas Lite version 2.1.1 and Mega 5.2. Reverse sequence was oriented into the same direction with the forward sequence by using Chromas Lite version 2.1.1. Both forward and reverse sequences were aligned by using Cluster W.

The molecular identification was performed to support visual observation of morphological characteristics of new hybrids or mushroom species. Molecular identification was carried out on the hybrids based on the sequencing internal transcribed spacer (ITS) region. Previously, Abdulmalk (2013) had used two taxon-selective primers for ITS and Random Amplification of Polymorphic DNA (RAPD-PCR) to characterize and differentiate two different species between *P. ostreatus* and *P. sapidues*. Furthermore, Nelson *et al* (2010) carried out molecular to identification on four Brazilian commercial isolates of *Pleurotus* sp. Based on sequencing of the LSU rDNA gene.

According to (Kumar *et al*. 2004) the neighbour-joining method with midpoint rooting is the type of algorithm used to create a guide tree, which is used to generate a global alignment. The guide tree serves as a rough template for clades that tend to share insertion and deletion features. Phylogenetic tree of the five *P. pulmonarius* dominant hybrid strains showing the evolutionary distance with *P. pulmonarius* and *P. citrinopileatus* was illustrated in Fig. 10.

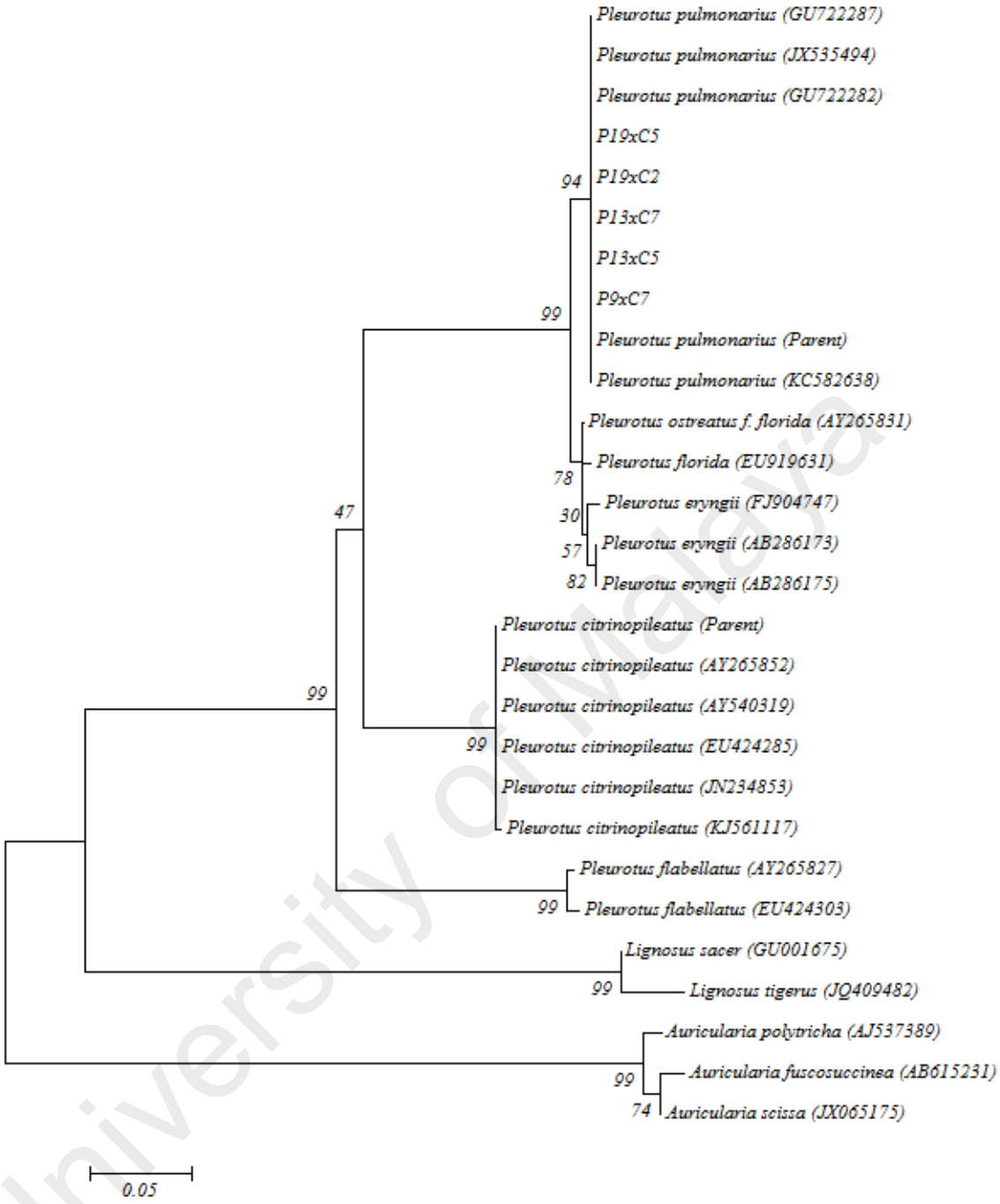


Fig.4.7. The Phylogenetic tree based on analysis of DNA gene sequences (ITS1-ITS4) of five dominant parent *P. pulmonarius* constructed by neighbour-joining tree. Bootstrap values are expressed percentages support from 1000 replicates showing the relationships among five hybrids with parental species and the homogeneous hybrids of *Pleurotus* species.

Based on molecular analysis, new hybrid P19XC5 and the other hybrid that has similar micro and macro morphological characteristics which resembled their parent *P. pulmonarius* stand in the same clade with bootstrap value at 94%. High bootstrap value indicates that high genetic homology between the new five hybrids showed dominant of parent *P. pulmonarius*.

4.8 Molecular characterisation of hybrid similar to *P. citrinopileatus*

Molecular identification was carried out on the hybrids based on the sequencing of the internal transcribed spacer (ITS) region PCR using ITS1 and ITS4 was performed to identify the hybrids using a molecular method comparing them to the parent and corresponding GenBank data of related species. There were 314 base pairs informative parsimony characters from the total 618 member of trimmed alignment. The target sequences were 100% similar with parental *P. citrinopileatus* and 14.4% difference with parental *P. pulmonarius*. As shown in the phylogenetic tree (Figure 11), hybrids P1XC9, P3XC8, P1XC10, P9XC10, P1XC13 and P6XC13 which resembled parental *P. citrinopileatus* are in the same clade with a bootstrap value of 99%. The high bootstrap value suggests high genetic homology between the hybrids and the parent *P. citrinopileatus*.

Molecular analysis of five hybrids that have characteristics and sporophore features dominant to *P. citrinopileatus* as presented in Figure 4.11.

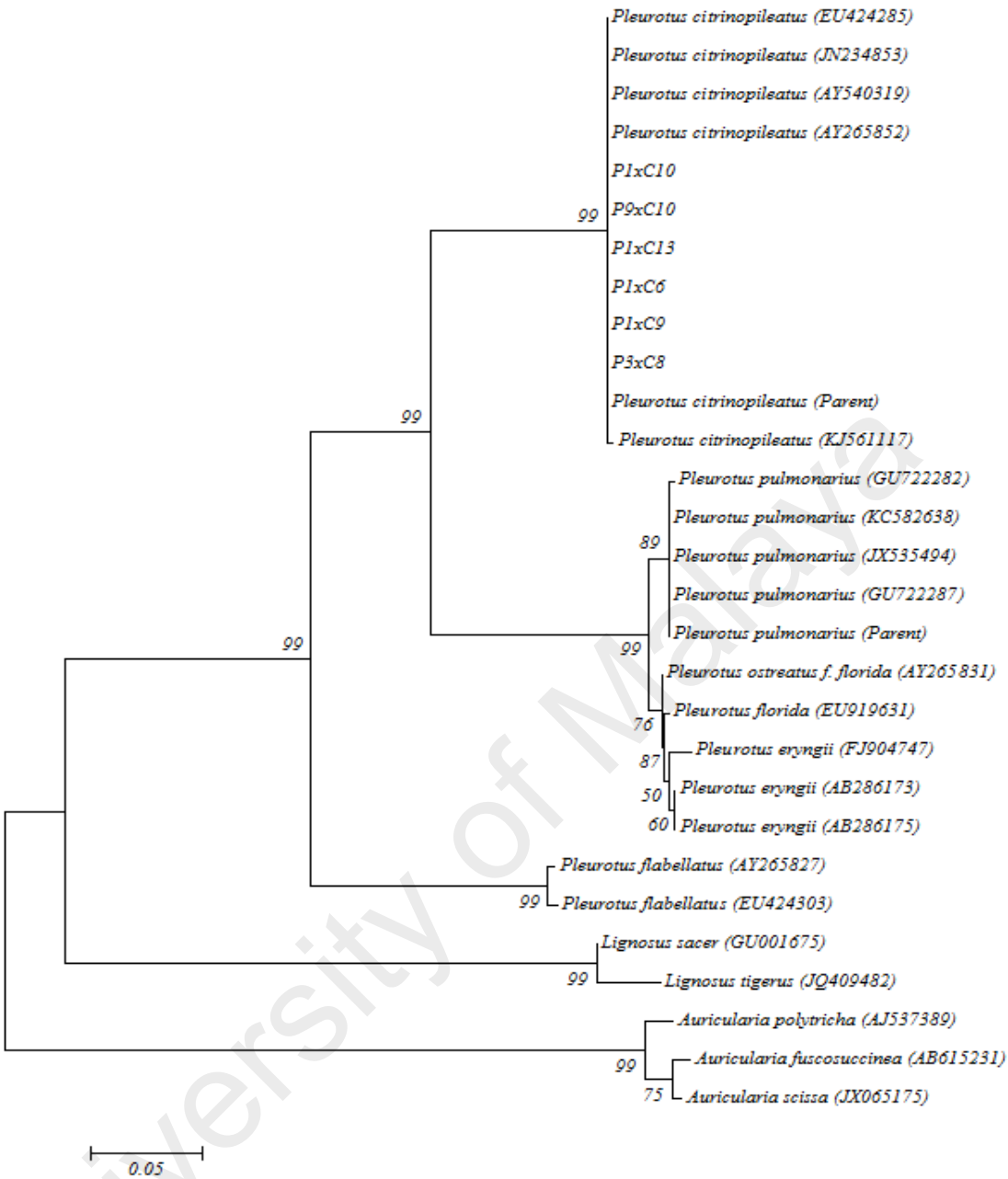


Figure 4.8. The phylogenetic tree based on analysis of DNA gene sequences (ITS1, ITS4) of hybrids similar to *P. citrinopileatus* constructed by tree. Bootstrap values are expressed as percentages from 1000 replicates showing maximum similarity with hybrids P1XC9, P3XC8 and parental strains and the homogeneous strains of *Pleurotus* species.

Molecular identification was carried out on the hybrids based on the sequencing of internal transcribed spacer (ITS) region. The analysis is important to perform molecular identification for confirmed characterization, and to support visual observation of morphology characteristics new hybrids or mushroom species. It was described that morphological characteristics of mushroom are inconsistent and unstable criteria because they are strongly influenced by the environmental conditions (Bresinsky, 1977).

CHAPTER 5

DISCUSSION

5.1. Strain Improvement of *Pleurotu*s spp through Interspecies Mating Technique

In this study, hybrids dominant to *P. pulmonarius* and *P. citrinopileatus* were successfully obtained through mating technique to get better strains with high yielding characteristics such as larger in size, thick and fleshy sporophores, and less spores. These findings contributed to the generation of good traits and quality hybrids cultures.

For evaluating the mating behaviour of the test-crosses, each monokaryon culture was designated as one of four types: *A1B1*, *A2B2*, *A1B2*, and *A2B1*. Usually, the single basidiospores of *Pleurotus* are sterile in nature and able to form dikaryotic mushroom only after mating with the other compatible isolate (Brown & Casselton, 2001). Mating compatibility will be occur if two single cells of different mating types have fused to combine the different proteins in one cytoplasm. As most heterokaryotic mushrooms, mating type in *Pleurotus* is controlled by two genetic loci named as A and B. Mating type genes and other compatibility factors are now used in mating-type-assisted breeding programs for producing economical and edible mushroom species Kothe (2001).

The mating system of this fungus, leading to formation of a dikaryon that can produce fruiting bodies, is known to be bifactorial heterothallism (tetrapolarty) controlled by two unlinked multialletic factors, *A* and *B* (Terakawa 1960; Eguenio & Anderson 1968; Anderson *et al*. 1991). According to Esser and Blaich, (1994), that mating of monokaryon isolates from both different species *P. pulmonarius* and *P. citrinopileatus* also had a tetra polar pattern similar to the other basidiocarps.

We found 11% successful mating between different species of *P. pulmonarius* and *P. citrinopileatus*. Our finding is a little lower than those of

Bahukhandi and Sharma (2002) who had successful mating rate in the range of 15.38–20.59% in different combinations of hybridisation among *P. sajor-caju*, *P. sapides* and *P. cornocupiae*. However, our current finding is relatively higher than that of our preliminary study of interspecies mating between *P. sajor-caju* and *P. flabellatus* where we achieved a success of 7% for the presence of clamp connection (Rosnina *et al*. 2007). The present findings indicate that to some extent, species of the same genus tend to have the same mating type.

Fischer and Bresinsky (1992) showed that compatible reaction of two types of mycelium intermingles in the contact zone and form numerous hyphal fusions via anastomosis. In the present study, a compatible reaction of hyphal fusion between *P. pulmonarius* and *P. citrinopileatus* might have resulted in the formation of heterokaryotic mycelium in the contact zone. More importantly, formation of the heterokaryon was restricted to the contact zone. As time passed, the border zone between two mycelia became unrecognizable and this feature is supported by some previous findings (Esser & Blaich, 1994). Moreover, Esser & Blaich (1994) showed that nuclear exchange is not inhibited in all barrages known so far, but in most cases two types of mycelia form abnormal and even lethal fusions. The hyphal tips may branch profusely. A clear line of contacts appear with increasing age of the cultures. Barrage formation is mainly found in intra specific mating.

5.2 Analysis of mycelial growth of hybrid cultures

In general mycelia of hybrids produced grew faster with thicker mat than those of parent cultures. This is one of the most important adaptive characteristic that determines suitability towards neo-physiological condition and high yield production (Gharehaghaji *et al*, 2007). Our findings are compatible with those of Gharehaghaji *et al* (2007) who used morphological characters such as prominent

interaction in the contact zone, increased rate of mycelium growth, better colony morphology as the morphological markers for breeding purposes.

In the present study, the hybrid P19xC5 showed significantly higher growth rate of 8.7 mm/day, followed by hybrid P3xC8 than both parents (*P. pulmonarius* and *P. citrinopileatus*) at 8.2 mm/day and 7.7 mm/day, respectively. Interestingly, the hybrid P1xC9 showed the similar radial growth rate of its parent *P. pulmonarius*. In case of *P. pulmonarius*, the highest mycelia growth rate (8.1mm/day) was observed on the substrate compost of 45 % sawdust (Liang *et al*. 2011). Radial growth of dikaryons has been reported to depend on the growth of the monokaryons, the dikaryon composing partners (Wang and Anderson, 1972). Our finding is comparable with those of Musieba *et al*. (2012) who reported that *P. citrinopileatus* took up to 13 days to colonize 1 kg rice straw substrate and 21 days to colonize 1 kg rice sawdust substrate. In a different study, Alemu *et al*. (2014) had noticed that the primordia appeared 20 days after scratching depending on the type of substrate and the sporophores matured 48 hours after the appearance of primordia. In the current study, hybrid sporophores were ready for harvest after approximately 2–3 days.

The growth of the heterokaryotic mycelia differed from each other. The colony radius of hybrids and parental cultures was measured and compared among each other based on every seven days' performance. Time required for completed spawn run of our hybrids' was 19-20 days. This time period was a little slower compared to those of Gupta *et al*. (2011) who reported the minimal period for completed spawn run to be 17-18 days in dikaryons of *P. sajor-caju.* Importantly, our findings were faster compared to the normal time range (25-30 days) required for spawn running (Gupta *et al* 2011). Furthermore, the hybrid sporophores were ready for harvesting after approximately 2–3 days. Both of our findings are compatible with those of Alemu (2014) who reported that primordia appeared 20 days after scratching depending

on the type of substrate and the sporophores matured, generally 48 hours after the formation of primordia.

5.3 Comparison of macromorphological features of hybrids and parental sporophores

Macro morphological characteristics hybrid sporophores varied in their morphological characteristics and features. The variation between the hybrids dominant to *P. pulmonarius* and differ in their macro-morphological features as presented in both monokaryon cultures from different parental lines produced sporophores which is possible only when mycelia of two single basidiospores of opposite mating type fuse and form the new dikaryon (Brown & Casselton, 2001).

Pleurotus citrinopileatus, parent possesses an attractive shape and bright yellow colour. The sporophore is extremely bitter and tangy with an unpleasant flavour when lightly cooked and is hence disdained by many people. In addition, sporophores are brittle increasing loss during harvesting and packaging. However, when they are crisply cooked, a strongly appealing cashew-like flavour eventually develops Stamets (2000). The main compounds responsible for the odour of yellow oyster mushroom are 3-octanol, 3-octanone and 1-octen-3-ol. (Zawirska-Wojtasiak, *et al* 2009).

They were grey in colour with semi-circular with, bigger in size and even fleshier of sporophores than the parent *P. pulmonarius.* Another similarity in feature was the funnel shape at the centre of pileus cap as possessed by the parent *P. citrinopilratus*. At the young stage, the margin was very fine line shaped and the edge of the margin appeared dark grey. As they matured, the margins became more wavy in shape, thicker and firmer in texture and the stipe became irregular and eccentric. Also, the two lamellae were similar to those of the parent *P. pulmonarius*

The margin enrolled when young and very finely lined, dark grey colour of edge margin and more wavy when matured, thicker and firmer texture, irregular, and eccentrically stipe. Special attention of characteristic of hybrids bellow lamella of hybrid P19XC5 referred Fig 4.f2. The crowded bellow lamella which were changed similar to lamella of *Lentinus edodes* showed differentiated compared to decurrent lamellae of both parent *P. pulmonarius* see Fig. 4.a2 and *P. citrinopileatus* Fig. 4.5.

The hybrid P8XC7 and hybrid P13XC7 were lung shape similar to the parent *P. pulmonarius* with a little variation in color such as pale grey at the center of pileus cap (Fig. 4.b2) and their lamellae were similar to those of the parent (Fig. 4.d2). On the contrary, the lamella of the hybrid P19XC5 was different than those of both of the parental strains, *P. pulmonarius* and *P. citrinopileatus*. Instead, it resembled the lamella of *Lentinus edodes*. The pileus of the hybrid strains P8XC7 and P13XC7 were lung shape similar to those of the *P. pulmonarius* but pale grey in colour (Fig 4.b1). Their lamellae were similar to both of the parental strains (Fig.4.a2). Generally, the hybrids contain fleshy and thicker sporophore compared to both of the parental strains. This feature might add sporophore yield and contribute to their longer and durable shelf life.

5.4.2 Cultivation and sporophore yield performance of selected hybrid P19XC5

Knowledge of the morphological and genetic differences along with the mating type of the monospores provides the basis for selection of the isolates for developing high yielding hybrids. In this study, development and evaluation of the selected hybrid P19XC5 were performed for three generations to confirm the consistency of the sporophore features.

For fruiting in larger scale experiments, the following selected hybrid P19XC5 and parental species were cultivated in about 100 bags and they were observed for their spawn run period, and yielding ability (Table 4.6). Dates of different stages of growth of

the hybrid cultures and dikaryon of both parents on the substrate indicated that to attain full growth/completed spawn run 19 and 21 days after inoculation respectively. Spawn run period of hybrid culture showed 3 days faster than completed spawn run of parental cultures.

Akinmusire, (2011) reported that faster spawn run of *P. pulmonarius* was observed on rice straw substrate compared to sawdust substrate. It took an average of 22 days for the spawn run in rice straw substrate, compared to 35 days for the sawdust substrate. Furthermore, Musieba (2012) reported *P. citrinopileatus* took 13 days to colonize 1 kg rice straw substrate compared to 21 days required to colonize 1kg sawdust substrate. It has taken same 21 days of completed spawn run for both parental cultures.

Generally, spawn run completion is achieved between 20 and 30 days and primordial formation occurred on average between 3 and 4 days after opening the cap. Sporophores of *Pleurotus* species were usually formed between 3 to 4 days after primordial formation. Alemu (2014) reported primordial formation appeared 20 days after scratching depending on types of substrate, and the sporophores matured generally 48 hours after primordial appearance. The development of hybrids sporophores took roughly 2-3 days before they could be harvested. Observations regarding the date of completed spawn run, and yield in subsequent three times of flushes were recorded as (Table 4.6).

The third generation underwent evaluation at larger scale in about 100 mushroom bags. Compared to the parental strains, significantly increased average total yield and biological efficiency (BE) were observed in case of the hybrids (Table 4.6). Among others, the hybrid P19xC5 possessed the highest total yield of 182.97 g and BE of 130.19 %. Its other important features included fleshier sporophores and better trait expression as demonstrated by the less spores compared to the enormous spores of parental species (Figure 9B).

Appearances of new hybrid (P19XC5) exhibited different features compared to parents. As presented in Fig. 9. All the three generations of hybrid had consistence which exists that crowded bellow lamella, bigger and thicker of pileus cap referred Fig. 9A (right) and dark grey cycle and wavy edge of the pileus cap see in Fig. 9A (left). The taste is mild with smell pleasant and less spores of the sporophores. The characteristics are prospective for hybrid improvement to produce the quality of mushroom culture. This correlates with the biological productivity of hybrid with high eficiency, this is a positive trait desired in excellent yield and quality spawn. The selected that have a thick pileus cap and others positive characteristics to developed for a new hybrid.

All members of the third generation belonging to hybrid P19XC5 showed consistency as all of them possessed the same crowded lamellae, bigger and thicker pileus cap, and dark grey and wavy edge of the pileus cap (Figure 49C g1–g3). They had pleasant smell and less spores in the sporophores (Figure 9C). The characteristics of the hybrid P19xC5 are prospective for both hybrid improvement and for development of mushroom cultivation.

5.4.3 Evaluation of morphological characteristics and traits of selected hybrids similar to *P. citrinopileatus*

Two out of six hybrids showed morphological characteristics similar to those of the parental *P. citrinopileatus* i.e hybrid P1XC9 (Figure 4.5b) had pale yellow colour and the hybrid P3XC8 (Figure 4.5d) changed texture from fragile to tough with decreased aroma. Two other hybrids were pale yellow in colour (hybrid P1XC10, Figure 4.5c and hybrid 9X10,Fig. 4.5e), funnel shaped with fragile texture and strong aroma like those of their parent *P. citrinopilratus*. Two hybrids (P1XC13 and hybrid P6XC13, Fig. 4.5f)

were bright yellow in colour and possessed the similar characteristics and traits (Fig.4.5g).

Sporophores of the selected hybrids (P1XC9 and P3C8) had similar morphology to those of the parental *P. citrinopileatus.* Their morphological characteristics included bigger size, greater thickness of pileus cap, decreased aroma and better texture than the parent *P. citrinopileatus*. Hybrid P1xC9 had beige yellow colour and yellow colour of hybrid P3xC8 was branched in large clusters, irregular, funnel shaped with a bright yellow colour, with the stipe off-centre, and a larger pileus margin that was wavy when mature. Both hybrids had less pungent aroma compared to the parental *P. citrinopileatus.*

5.3.4 Sporophore yield performance of selected hybrid P1xC9 and P3xC8

Of six hybrids, two hybrid cultures (P1XC9 and P3XC8) exhibited thicker mycelium, high colony density, and faster growth rate compared to the parental cultures. Hybrid P1Xc9 had the highest mycelial growth rate of 8.5 mm/day, followed by hybrid P3XC8 which had same mycelia growth rate (8.2 mm/day) of parent *P. pulmonarius*. Performance of our hybrid was better than that one of Liang et al. (2011), who reported the 8.1 mm/day mycelial growth rate of *P. pulmonarius* on the substrate containing 45 % sawdust.

On large scale cultivation, both hybrids completed the spawn run at 19 days compared to the 21 days of the parental strains. Primordia of the hybrids appeared 2 days after completed spawn run. Another important finding was the increased total yield and biological efficiency of both of the selected hybrids (Table 4.6). Hybrid P1XC9 had highest total production and biological efficiency while hybrid P3XC8 had higher rates than parental *P. pulmonarius* and *P. citrinopileatus* (Table 4.6).

The sporophores of the selected hybrids were analysed for important morphological traits that could potentially add value for commercialisation. Special

attention was given to the texture and decreased aroma of hybrids. The two *P. citrinopileatus* dominant hybrids, P1XC9 and P3XC8 had less pungent odour. To confirm the strong aroma, ten volunteers were asked to smell the sporophores. Out of 30 responses from the public, 25 of them confirmed that the hybrids exhibited less aroma. Due to better traits such as less aroma and texture of the sporophores, these two hybrids (P1xC9 and P3xC8) were selected for further analyses and larger cultivation.

Kong (2004) described the aroma of *P. citrinopileatus* sporophores as similar to that of wheat flour. The main odorants of the mushrooms are eight carbon (C8) compounds, mainly 1-octen-3-ol (Drawert *et al.,* 1983). Gas chromatography olfactometry has been used to detect and measure odour dilution and aroma extract dilution used to detect and measure odour dilution and aroma extract dilution analysis had shown that sulphur and nitrogen-containing components, C8 ketones and C8 aldehydes are responsible for the aroma of *P. citrinopileatus* (Miyazawa *et al*., 2011). Similar studies on the aroma of several *Pleurotus* species revealed that compound 1-octen-3-ol was found in relatively high concentrations (1.14 - 2.83 mg/100 g) of fresh weight (Zawirska-Wojtasiak *et al.*, 2009).

5.4. Evaluation of molecular characterisation of parental and hybrid strains

Molecular identification was carried out on the hybrids based on the sequencing of internal transcribed spacer (ITS) regions. The analysis is important to confirm characterization, molecular identification and to support visual and morphological characteristics of the parental and new hybrids of mushroom species. The reason is that morphological characteristics are inconsistent and unstable criteria because they are strongly influenced by the environmental conditions according to Bresinsky *et al*. (1977) and Petersen & Hughes, (1999).

As shown in the phylogenetic tree (Fig. 4.10) the two groups of hybrid sporophores resembled each parental stains *P. pulmonarius* and *P. citrinopileatus*. The similarities of these hybrids were further tested using molecular markers. It is important to perform molecular identification for characterization of mushroom species other than observation on morphology alone. The six hybrids (P1xC10, P3xC8, P9xC10, P1xC9, P1xC13 & P6xC13) exhibiting characteristic similar to *P. citrinopileatus* (controls) are grouped together in the same clade with bootstrap value of 99% (Fig. 411).

Abdulmalk (2013) used two taxon-selective primers for the ITS region and random amplification of polymorphic DNA (RAPD-PCR) to characterise and differentiate between *P. ostreatus* and *P. sapidus*. DNA molecular markers that were strictly specific to identify a species did not differentiate the hybrid from the parental strain. Gupta *et al*. (2010) found that combination of both morphological and genetic markers provided a clear picture of the diversity of the isolates. Future investigations should be extended to other genera with the aim of establishing new varieties of edible basidiomycetes.

The selected hybrid gave better trait in terms of fewer spores and higher yield, increased spawn productivity, total weight of fresh fruit body, biological efficiency and production rate compared to their parental hybrids. Comparison of macro-morphological characteristics and data production showed differences between the new hybrids and the parental hybrids. Molecular analysis identification showed high bootstrap value of 100% which indicates high genetic homology between the hybrids and *P. citrinopileatus*. Our seminal findings entailing improved sporophore features, higher yield and characteristics pertaining to longer shelf life are potent indicators of these mushrooms booming commercialization. Importantly, DNA molecular markers that are strictly specific to identify a species could not differentiate the hybrid and the

parental hybrid. However, further study should be extended to other genera with the aim of establishing new variety on *Pleurotus* species.

CHAPTER 6

CONCLUSIONS

This results form a basis of further research to see whether the two fungal species cross the impact on the quality of grey mushroom (*Pleurotus pulmonarius*) and *P.citrinopileatus*, i.e. nutritional content, the content of secondary metabolites (Pleuran, fiber, etc.) or other characters (taste, shelf life, and etc.) and medicinal importance. Basically, it can be concluded that recombination of two fungal species belonging to genus *Pleurotus* does not cause mutations in the grey oyster mushroom (*P. pulmonarius*) as indicated by the sequencing data. But the production data presented in tables show the increased productivity of new strains compared with those of the parental strains of both species.

The selected hybrid culture of bag showed faster growth rate and thicker mycelial mat compared to those of parental. Radial growth of dikaryons depends on the growth of their constituting monokaryons. The yield performance improvement was carried out by cross bred of *P. pulmonarius* with *P. citrinopilieatus*. The morphological and molecular characterization of the hybrids established their true variation from their parents. The results obtained from this study have shown the improved performance and traits of selected hybrids over both of the parental species.

In this study, selected hybrid dikaryon P19xC5 sporophore had bigger and fleshier pileus than the parental strains. Molecular analysis showed high genetic homology between the hybrids and *P. pulmonarius*. The hybrid P19xC5 had good features, fewer spores content and higher yield in terms of total weight of fresh fruiting body and biological efficiency. The characteristics of fleshy sporophores and less spores will increase shelf life of the mushroom itself and reduce respiratory threat to the mushroom growers. This would pave new vista for enhanced commercialization of

mushroom and mushroom-based products. These attributes, among others, also lie at the ground of mushroom strain development. It's far reaching commercial potentiality. It's far reaching potentially for commercialization.

Special attention was given to the texture and decreased aroma of hybrids. The selected *P. citrinopileatus* dominant hybrids P1XC9 and P3XC8 had less pungent odour. Other than that, hybrids P1XC9 and P3XC8 showed better features and high yield in terms of total weight of fresh fruiting body and biological efficiency than the parent strains. They produced less offensive aroma and contained characteristics similar to those of *P. citrinopileatus.* Due to pungent odour, *P. citrinopilieatus* was not favourite to the local consumers in Malaysia. Odour test involving ten respondents confirmed declined pungent odour in the produced hybrid sporophores *P. citrinopileatus.* The hybrids had desirable traits as well as higher productivity and biological efficiency and thus could have optimistic commercialisation potential. Molecular analyses showed high genetic homology between the hybrids and the parental *P. citrinopileatus.* Improved characteristic traits including firmer texture bears their suitability for storage and distance transport. These traits are expected to increase palatability and to determine people to consume yellow oyster mushrooms in the future.

REFERENCES:

Abdulmalk, H. W. (2013). Rapid Differentiation of *Pleurotus ostreatus* from *Pleurotus Sapidus* Using PCR Technique. *A-Science*, 153.

Aida, F.M.N.A., Shuhaimi, M., Yazid, M., & Maaruf, A. G. (2009). Mushroom as a potential source of prebiotics: a review. *Trends in Food Science & Technology, 20*(11), 567-575.

Akinmusire, O. O., Omomowo, I. O., & Oguntoye, S. I. K. (2011). Cultivation performance of *Pleurotus pulmonarius* in Maiduguri, North Eastern Nigeria, using wood chippings and rice straw waste. *Advances in Environmental Biology*, 2091-2095.

Alam N., Yoon, K. N., Lee, K. R., Shin, P. G., Cheong, J. C., Yoo, Y. B., . . . Lee, T. S. (2010). Antioxidant activities and tyrosinase inhibitory effects of different extracts from *Pleurotus ostreatus* fruiting bodies. *Mycobiology, 38*(4), 295-301.

Alemu, F. (2014). Cultivation of *Pleurotus ostreatus* on *Grevillea robusta leaves* at Dilla University, Ethiopia. *Journal of Yeast and Fungal Research, 5*(6), 74-83.

Amin, S.M.R., Rahman, M. M., Hossain., M. M., Haque, M. M., & Sarker, N. C. (2007). Effect of different substrates on the growth and yield of five selected oyster mushrooms. *Bangladesh Journal Mushroom, 1*(2), 21-25.

Anderson, N. A., Furnier, G. R., Wang, A. S., & Schwandt, J. W. (1991). The number and distribution of incompatibility factors in natural populations of *Pleurotus ostreatus* and *Pleurotus sapidus*. *Canadian Journal of Botany, 69*(10), 2187-2191.

Ardekani, S., Reza, M., Hajimahmoodi, M., Oveisi, M. R., Sadeghi, N., Jannat, B., . . . Moridi, T. (2011). Comparative antioxidant activity and total flavonoid content of Persian pomegranate (*Punica granatum* L.) cultivars. *Iranian Journal of Pharmaceutical Research*, 519-524.

Atri, N. S., Sharma, S. K., Joshi, R., Gulati, A., & Gulati, A. (2013). Nutritional and neutraceutical composition of five wild culinary-medicinal species of genus *Pleurotus* (Higher *Basidiomycetes*) from Northwest India. *International Journal of Medicinal Mushrooms, 15*(1) 49-56.

Austin, A.T., Yahdjian, L., Stark, J. M., Belnap, J., Porporato, A., Norton. U., Ravetta, D. A., Schaeffer, S. .M. (2004). Water pulses and biogeochemical cycles in arid and semiarid ecosystems. *Oecologia, 141*(2), 221-235.

Bahl, N.I.T.A. (1983). *Medicinal Value of Edible Fungi*. Proceedings of the International Conference on Science and Cultivation Technology of Edible Fungi. *Indian Mushroom Science*, (2), 203-209.

Ballance, D. J., Buxton, F. P., & Turner, G. (1983). Transformation of *Aspergillus nidulans* by the orotidine-5′-phosphate decarboxylase gene of Neurospora crassa. *Biochemical and Biophysical Research Communications, 112*(1), 284-289. Date retrived 12 June 20015

Bahukhandi, D., & Sharma, R., K. (2002). Interspecific hybridization in *Pleurotus* species. *Indian Phytopathology, 55*(1), 61-66.

Barney, D., L. 2009. Growing mushrooms commercially: risk and opportunities ETSE. In http://www.naturalresource.msstage.edu/resources/mushroom.html

Bechara, M. A., Heinemann, P., Walker, P. N., Romaine, C. P., & Heuser, C. W. (2004). *Novel methods of cultivating Agaricus bisporus.* Paper presented at the 2004 ASAE Annual Meeting.

Blakeslee, A., F. (1904). *Sexual reproduction in the Mucorineae.* Paper presented at the Proceedings of the *American Academy of Arts and Sciences, 40*(4), 205-319.

Bresinsky, A., Hilber, O., & Molitoris, H. P. (1977). The genus *Pleurotus* as an aid for understanding the concept of species in Basidiomycetes.

Brown, A. J., & Casselton, L. A. (2001). Mating in mushrooms: increasing the chances but prolonging the affair. *Trends in Genetics, 17*(7), 393-400.

Cai, Y. J., Chapman, S. J., Buswell, J. A., & Chang, S. T. (1999). Production and distribution of endoglucanase, cellobiohydrolase, and β-glucosidase components of the cellulolytic system of *Volvariella volvacea*, the edible straw mushroom. *Applied and Environmental Microbiology, 65*(2), 553-559.

Case, M. E., Schweizer, M. K., Sidney R, & Giles, N. H. (1979). Efficient transformation of Neurospora crassa by utilizing hybrid plasmid DNA. *Proceedings of the National Academy of Sciences, 76*(10), 5259-5263.

CFR Ferreira, Isabel, A. V., Josiana, Vasconcelos, M. H., & Martins, A. (2010). Compounds from wild mushrooms with antitumor potential. *Anti-Cancer Agents in Medicinal Chemistry* (*Formerly Current Medicinal Chemistry-Anti-Cancer Agents*), *10*(5), 424-436.

Chang, A.C. (1992). *Genetics and breeding of edible mushrooms*: *Chinese University of Hong Kong* (CRC) Press.

Chang, S. T. (1996). Mushroom research and development-equality and mutual benefit. *Mushroom Biology and Mushroom Products,* 1-10.

Chang, S.T. (1999). Global impact of edible and medicinal mushrooms on human welfare in the 21st century: nongreen revolution. *International Journal of Medicinal Mushrooms, 1*(1).

Chang, S. T. (2001). *Mushrooms and Mushroom Cultivation*. *eLS*.

Chang, S. T., Buswell, J. A., & Chiu, S. W. (1993). *Mushroom Biology and Mushroom Products* p392. Paper presented at the *International Conference on Mushroom Biology and Mushroom Products1993,* Chinese University of Hong Kong.

Chang, ST., & Buswell, J. A., (1999). *Ganoderma lucidum* (Curt.: Fr.) P. Karst. (*Aphyllophoromycetideae*) A Mushrooming Medicinal Mushroom. *International Journal of Medicinal Mushrooms,1*(2). 139-146

Chen, Alice W. (1999a). Cultivation of the medicinal mushroom *Ganoderma lucidum* (Curt.: Fr.) P. Karst. (*Reishi*) in North America. *International Journal of medicinal mushrooms, 1*(3).

Chen, Alice W. (1999b). A Practical Guide for Synthetic-Log Cultivation of Medicinal Mushroom *Grifola frondosa* (Dick.: Fr.) SF Gray (Maitake). *International Journal of Medicinal Mushrooms, 1*(2).

Chen, J. N., Wang, Y. T., & Wu, J. S. (2009). A glycoprotein extracted from golden oyster mushroom *Pleurotus citrinopileatus* exhibiting growth inhibitory effect against U937 leukemia cells. *Journal Agricultural Food Chemistry, 57*(15), 6706-6711.

Cohen, R., Persky, L., & Hadar, Y. (2002). Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*. *Applied Microbiology and Biotechnology, 58*(5), 582-594.

Collopy, P. D., Largeteau-Mamoun, M. L., Romaine, C. P., & Royse, D. J. (2001). Molecular Phylogenetic Analyses of Verticillium fungicola and Related Species Causing Dry Bubble Disease of the Cultivated Button Mushroom, *Agaricus bisporus*. *Phytopathology, 91*(9), 905-912.

Dhitaphichit, P., & Pornsuriya, C. (2005a). Protoplast Fusion between *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. and *P. citrinopileatus* Singer. *International Journal of Medicinal Mushrooms, 7*(3).

Dhitaphichit, P., & Pornsuriya, C. (2005b). Protoplast fusion between *Pleurotus ostreatus* and *P. djamor*. *Songklanakarin J. Sci. Technology, 27*(5), 975-982.

Djajanegara, I., & Harsoyo, A. (2007). Mutation study on white oyster mushroom (*Pleurotus floridae*) using gamma (^{60}CO) irradiation. *Journal. Chem. Natural Resource. Enginee, 4*(1), 12-21.

Drawert, F., Berger, R. G., & Neuhauser, K. (1983). Biosynthesis of Flavour Compounds by Microorganisms 4. Characterization of the major Principles of the Odour of *Pleurotus eusmus*. *European Journal of Applied Microbiology and Biotechnology, 18*(2), 124-127.

Esser, K. (1971). Application & importance of fungal genetics for industrial research. *Radiation & radioisotopes for industrial microorganisms. IAEA, Viena*, 83-91.

Esser, K., & Blaich, R. (1994). Heterogenetics incompatibility in fungi. Wessels/Meinhardt (Eds). The Mycota I. *Growth, Differentation and Sexuality*.

Esser, K., & Meinhardt, F. (1984). Barrage formation in fungi. *In Cellular Interactions* (pp. 350-361): Springer.

Eugenio, C. P., & Anderson, N. A. (1968). The genetics and cultivation of *Pleurotus ostreatus*. *Mycologia*, 627-634.

Fan, L. Pan., H., Soccol, A. T., Pandey, A., & Soccol, C. R. (2006). Advances in mushroom research in the last decade. *Food Technology and Biotechnology, 44*(3), 303-311.

Fan, L., Soccol, C. R., & Pandey, A. (2008). Mushroom production *Current Developments in Solid-state Fermentation* (pp. 253-274): Springer.

Fischer, M., & Bresinsky, A. (1992). *Phellinus torulosus*: Sexuality and Evidence of Intersterility Groups. *Mycologia, 84*(6), 823-833.

Food, & Staff, Agriculture Organization. (2015). *OECD-FAO Agricultural Outlook, 2015-2024*: OECD.

Fritsche, G. (1983). Breeding *Agaricus bisporus* at the Mushroom Experimental Station, Horst. *Mushroom Journal* (122), 49-53.

Gharehaghaji, A.N., Goltapeh, E. M., Masiha, S., & Gordan, H. R. (2007). Hybrid production of oyster mushroom *Pleurotus ostreatus* (Jacq: Fries) Kummer. *Pakistan Journal of Bbiological Sciences: PJBS, 10*(14), 2334-2340.

Gregori, A., Šavagelj, M., Pahor, B., Berovič, M., & Pohleven, F. (2008). The use of spent brewery grains for *Pleurotus ostreatus* cultivation and enzyme production. *New Biotechnology, 25*(2), 157-161.

Gregori, A., Šavagelj, M., & Pohleven, J. (2007). Cultivation techniques and medicinal properties of *Pleurotus* spp. *Food Technology & Biotechnology, 45*(3).

Goltapeh, E. M.,& Pourjam, E. (2005). Principles of Mushroom cultivation. *Tarbiat Modares University Press* 625

Gupta, B. Reddy, B. N., & Kotasthane, A. S. (2011). Molecular characterization and mating type analysis of oyster mushroom (*Pleurotus* spp.) using single basidiospores for strain improvement. *World Journal of Microbiology & Biotechnology, 27*(1), 1-9.

Horgen, P.A., Jin, T, & Anderson, J.B. (1991). *The use of protoplast production, protoplast regeneration and restriction fragment length polymorphisms in developing a systematic and highly reproducible breeding strategy for Agaricus bisporus.* Paper presented at the Proceedings of the First International Seminar on Mushroom Science: Genetics and Breeding of Agaricus, Horst (Netherlands), 14-17 May 1991.

Hossain, M. S., Alam, N., Amin, S.M.R., Basunia, M. A., & Rahman, A. (2007). Essential fatty acid contents of *Pleurotus ostreatus*, *Ganoderma lucidum* and *Agaricus bisporus*. *Bangladesh Journal Mushroom, 1*, 1-7.

Hu, S. H., Liang, Z. C., Chia, Y. C., Lien, J. L., Chen, K. S., Lee, M. Y., & Wang, J. C. (2006). Antihyperlipidemic and antioxidant effects of extracts from *Pleurotus citrinopileatus*. *Journal of Agricultural and Food Chemistry, 54*(6), 2103-2110.

Izumitsu, K., Hatoh. K., Sumita, T., Kitade, Y., Morita, A., Tanaka, C., . . . Neda, H. (2012). Rapid and simple preparation of mushroom DNA directly from colonies and fruiting bodies for PCR. *Mycoscience, 53*(5), 396-401.

Jonathan, S.G., Popoola, K.O.K., Olawuyi, O. J., Ajiboye, M., & Oyelakan, A. O. (2012). Insect and fungal pests of some mushrooms collected from university of Ibadan, Nigeria campus. *Nature and Science, 10*(9), 142-147.

Jose, N. Ajith, T. A., & Janardhanan, K. K. (2002). Antioxidant, anti-inflammatory, and antitumor activities of culinary-medicinal mushroom *Pleurotus pulmonanus* (Fr.) Quel.(*Agaricomycetideae*). *International Journal of Medicinal Mushrooms, 4*(4).

Kavousi, H. R., Farsi, M., & Shahriari, F. (2008). Comparison of random amplified polymorphic DNA markers and morphological characters in identification of homokaryon isolates of white button mushroom (*Agaricus bisporus*). *Pakistan Journal of Biological Sciences, 11*(14), 1771.

Kerrigan, R. W., Royer, J. C., Baller, L. M., Kohli, Y., Horgen, P. A., & Anderson, J. B. (1993). Meiotic behavior and linkage relationships in the secondarily homothallic fungus *Agaricus bisporus*. *Genetics, 133*(2), 225-236.

Kibar, B., & Peksen, A. (2008). Modelling the effects of temperature and light intensity on the development and yield of different *Pleurotus* species. *Agric Trop Subtrop, 41*(2), 68-73.

Kim, M. Y., Seguin, P., Ahn, J.K., Kim, J. J., Chun, S. C., Kim, E. H., . . . Park, Y. J. (2008). Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. *Journal of Agricultural and Food Chemistry, 56*(16), 7265-7270.

Kligman, A.M., (1950). Handbook of mushroom culture at Agris Records US201300639125 http://agris.fao.org/aos/records/US201300639125

Kniep, Hans. (1920). Untersuchungen über den Antherenbrand (*Ustilago violacea* Pers.). Ein Beitrag zum Sexualitätsproblem: JSTOR.

Kong, W. S. (2004). Descriptions of commercially important *Pleurotus* species. Oyster mushroom cultivation. Part II. Oyster mushrooms. *Seoul: Heineart Incorporation*, pp 54-61.

Kothe, E. (2001). Mating-type genes for basidiomycete strain improvement in mushroom farming. *Applied Microbiology and Biotechnology, 56*(5-6), 602-612.

Kotasthane, A. S., & Singh, U. S. (2000). Simple technique for single ascospore isolation and a modified two point inoculation technique of mating in test tubes. *Journal of Mycology and Plant Pathology, 30*(3), 366-369

Kumar, Deepak, Patro, Sunita, Ranjan, Rajiv, Sahoo, Dipak K, Maiti, Indu B, & Dey, Nrisingha. (2011). Development of useful recombinant promoter and its expression analysis in different plant cells using confocal laser scanning microscopy. *PLoS One, 6*(9), e24627.

Kumara, K. W., & Edirimanna, I.C.S. (2009). Improvement of Strains of Two Oyster Mushroom Cultivars Using Duel Culture Technique. *World Applied Sciences Journal 7*(5), 654-660.

Liang, Z. C., Wu, K. J., Wang, J. C., Lin, C. H., & Wu, C.Y. (2011). Cultivation of the Culinary-Medicinal Lung Oyster Mushroom, *Pleurotus pulmonarius* (Fr.) Quel. (*Agaricomycetideae*) on Grass Plants in Taiwan. *International Journal of Medicinal Mushrooms, 13*(2), 193-199.

Martínez-Carrera, D., Aguilar, A., Martínez, W., Morales, P., Sobal, M., Bonilla, M., & Larqué-Saavedra, A. (1998). A sustainable model for rural production of edible mushrooms in Mexico. *Micologia Neotropical Aplicada, 11*, 77-96.

Miles, P. G., & Chang, S. T. (1997). *Mushroom biology: concise basics and current developments*: World Scientific, p329.

Miles, P. G., & Chang, S. T. (2004). *Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact* (477p): CRC press.

Miyazawa, M., Dejima, Y., Takahashi, T., Matsuda, N., & Ishikawa, R. (2011). Characteristic Odor Components of Essential Oil from Dried Fruiting Bodies of Golden Oyster Mushroom (*Pleurotus citrinopileatus*). *Journal of Essential Oil Research, 23*(3), 58-63.

Musieba, Fredrick, Okoth, Sheila, Mibey, Richard K, Wanjiku, Stella, & Moraa, Knight. (2012). Suitability of Locally Available Substrates for Cultivation of the Kenyan Indigenous Golden Oyster Mushroom (*Pleurotus citrinopileatus* Singer). *Agricultural Journal, 7*(4), 240-244.

Musieba, F, O., Sheila, M., Richard K., Wanjiku, S., & Moraa, K. (2013). Proximate composition, amino acids and vitamins profile of *Pleurotus citrinopileatus* Singer: An indigenous mushroom in Kenya. *Am Journal Food Technology, 8*, 200-206.

Oei, P., & Maas, M. (2003). *Mushroom cultivation: appropriate technology for mushroom growers*: Backhuys.

Ogawa, K. (1993). *Interspecific and intergeneric hybridization of edible mushrooms by protoplast fusion.* Paper presented at the Genetics and breeding of edible mushrooms.

Okuda, Y., Murakami, S., & Matsumoto, T. (2009). Development of STS markers suitable for marker-assisted selection of sporeless trait in oyster mushroom, *Pleurotus pulmonarius*. *Breeding Science, 59*(3), 315-319.

Parag, Y. (1965). Common-B heterokaryosis and fruiting in *Schizophyllum commune*. *Mycologia, 57*(4), 543-561.

Parag, Y., & Koltin, Y. (1971). The structure of the incompatibility factors of *Schizophyllum commune*: constitution of the three classes of B factors. *Molecular and General Genetics MGG, 112*(1), 43-48.

Parani, K., & Eyini, M. (2013). Interspecific hybridization between *Pleurotus eous* and *Pleurotus flabellatus* by PEG-induced protoplast fusion. *International Journal of Research in Chemistry and Environment, 3*(1), 144-146.

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

Patel, Y., Naraian, R., & Singh, V. K. (2012). Medicinal properties of *Pleurotus* species (oyster mushroom): a review. *World Journal of Fungal and Plant Biology, 3*(1), 1-12.

Pathak, R., Joshi, N., & Dwivedi, R. R. (2009). Eco-friendly production of *Agaricus bisporus* (lange) imbach (white button mushroom). *National Science, 6*, 57-60.

Peberdy, J. F. (1979). Fungal protoplasts: isolation, reversion, and fusion. *Annual Reviews in Microbiology, 33*(1), 21-39.

Peberdy, J. F., Hanifah, A. H., & Jia, J. H. (1993). New perspectives on the genetics of *Pleurotus* (pp. 55-62): *The Chinese University Press*, Hong Kong, China.

Peberdy, JF. (1995). Fungal protoplasts *Genetics and Biotechnology* (pp. 49-60): Springer.

Peberdy, J. F. (1989). Presidential address: fungi without coats—protoplasts as tools for mycological research. *Mycological Research, 93*(1), 1-20.

Perry, M. D., White, P. L., & Barnes, R. A. (2014). Comparison of four automated nucleic acid extraction platforms for the recovery of DNA from *Aspergillus fumigatus*. *Journal of Medical Microbiology, 63*(9), 1160-1166.

Petersen, R. H., & Hughes, K.W. (1999). Species and Speciation in Mushrooms Development of a species concept poses difficulties. *Bioscience, 49*(6), 440-452.

Puttaraju, N. G., Venkateshaiah, S. U., Dharmesh, S. M., Urs, S.M.N., & Somasundaram, R. (2006). Antioxidant activity of indigenous edible mushrooms. *Journal of agricultural and food chemistry, 54*(26), 9764-9772.

Quélet L (1872) *Pleurotus pulmonarius* (Fr.) Quél. Mém Soc Émul Montbéliard Sér 2 5:11

Quimio, T. H., Chang, S. T., & Royse, D. J. (1990). *Technical guidelines for mushroom growing in the tropics*. 155p

Raper, J. R. (1966). Genetics of sexuality in higher fungi. *Genetics of sexuality in higher fungi*. 532p.

Royse, D. J. (2014). *A global perspective on the high five: Agaricus, Pleurotus, Lentinula, Auricularia & Flammulina*. Paper presented at the Proceedings of the

8th International Conference on Mushroom Biology and Mushroom Products (ICMBMP8).

Rühl, M., Fischer, C., & Kües, U. (2008). Ligninolytic enzyme activities alternate with mushroom production during industrial cultivation of P*leurotus ostreatus* on wheat straw-based substrate. *Current Trends Biotechnol Pharmceutical*, *2*(4), 478-492.

Sánchez, C. (2004). Modern aspects of mushroom culture technology. *Applied Microbiology and Biotechnology*, *64*(6), 756-762.

Sanchez, C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnology Advaces*, *27*(2), 185-194.

Sanchez, C. (2010). Cultivation of *Pleurotus ostreatus* and other edible mushrooms. *Applied Microbiology and Biotechnology*, *85*(5), 1321-1337.

Sassoon, J., Hoerer, S., Stoop, J., Mooibroek. H., & Baumann, U. (2001). Crystallization and preliminary crystallographic analysis of mannitol dehydrogenase (MtDH) from the common mushroom *Agaricus bisporus*. *Acta Crystallographica Section D: Biological Crystallography*, *57*(5), 711-713.

Singh, M. (2014). *Proceedings of 8th International Conference on Mushroom Biology and Mushroom Products (ICMBMP8), New Delhi, India, 19-22 November 2014. Volume I & II.* Paper presented at the Proceedings of 8th International Conference on Mushroom Biology and Mushroom Products (ICMBMP8), New Delhi, India, 19-22 November 2014. Volume I & II.

Singer, R (1942) *Pleurotus citrinopileatus* *Annales Mycologicia* 40:149

Sharma, R., & Sharma, B. M. (2014). Strain improvement in *Pleurotus Ostreatus* using UV light and ethyl methyl sulfonate as mutagens. *African Journal of Microbiology Research*, *8*(5), 432-436.

Shen, Q., & Royse, D. (2001). Effects of nutrient supplements on biological efficiency, quality and crop cycle time of maitake (*Grifola frondosa*). *Applied Microbiology and Biotechnology*, *57*(1-2), 74-78.

Shlyakhovenko, V., Kosak, V., & Olishevsky, S. (2006). Application of DNA from mushroom *Pleurotus ostreatus* for cancer biotherapy: a pilot study. *Experimental Oncology*, *28*(2), 132-135.

S.T., Chang, & Miles, P.G. (1997). *Mushroom biology concise basics and current development*: Singapore (SG): *World Scientific* Publishing.

Stamets, P. (2000). Growth Parameters for Gourmet and Medicinal Mushroom Species, Growing gourmet and medicinal mushrooms: Ten Speed Press, Berkeley, California, USA. page

Stamets, P. & Chilton, JS. (1983a). The mushroom cultivator. *First Washington*.

Stamets, P., & Chilton, J. S. (1983b). The mushroom cultivator: a pratical guide to growing mushrooms at home.

Stamets, P. E. (2005). Notes on nutritional properties of culinary-medicinal mushrooms. *International Journal of Medicinal Mushrooms, 7*(1&2).

Stanley, H. O., Umolo, E. A., & Stanley, C. N. (2011). Cultivation of oyster mushroom (*Pleurotus pulmonarius*) on amended corncob substrate. *Agriculture and Biology Journal of North America, 2*(10), 1336-1339.

Stoop, J.M.H, & Mooibroek, H. (1999). Advances in genetic analysis and biotechnology of the cultivated button mushroom, *Agaricus bisporus*. *Applied Microbiology and Biotechnology, 52*(4), 474-483.

Tamura, K., Peterson, D., Peterson, N., Stecher, Glen, N. M., & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*(10), 2731-2739.

Terakawa, H. (1960) The incompatibility factors in *Pleurotus ostreatus*. *Scientific Papers of the College of General Education, University of Tokyo, 10*(1)

Timothy P. McCarty (2010) Mushroom Industry & Trade Summary., Office of *Industries United States International Trade Commission* Washington, DC 20436

USDA, National Agricultural Statistics Service (2001) Arkansas Cropland Data Layer. *Geospatial Data Presentation Form:* raster digital data *Publication Information: Publication Place:* USDA, NASS Marketing and Information Services Office, Washington, D.C.

Van de Rhee, Miranda, D., Graca, Paula, M. A., Huizing, H. J., & Mooibroek, H. (1996a). Transformation of the cultivated mushroom, *Agaricus bisporus*, to hygromycin B resistance. *Molecular and General Genetics MGG, 250*(3), 252-258.

Van de Rhee, Miranda D., Mendes, Odette, W., Marc, W.T., Huizing, H. J., & Mooibroek, Hans. (1996b). Highly efficient homologous integration via tandem exo-β-1, 3-glucanase genes in the common mushroom, *Agaricus bisporus*. *Current genetics, 30*(2), 166-173.

Wang, H-H. (1999). Development and/or reclamation of bioresources with solid state fermentation. *Proceedings of the National Science Council, Republic of China. Part B, Life sciences, 23*(2), 45-61.

Wang, J. C., Hu, S.H., Liang, Z.C., & Lee, M. Y. (2005). Antigenotoxicity of extracts from *Pleurotus citrinopileatu*s. *Journal of the Science of Food and Agriculture, 85*(5), 770-778.

Wang, J., Wang, H. X., & Ng, T. B. (2007). A peptide with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom *Russula paludosa*. *Peptides, 28*(3), 560-565.

Wang, Q., Yan, L. L, Wang, Y. L., & Zhang, Y. J. (2001). Submerged culture of *Pleurotus citrinopileatus* and its determination. Paper presented at the Proceedings of the 5th Cross-Strait Conference on *Mycology*, 8-12.

Wang, S. S., & Anderson, N. A. (1972). A genetic analysis of sporocarp production in *Pleurotus sapidus*. *Mycologia*, 521-528.

White, T. J., Bruns, T., Lee, S.J.W.T., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications, 18*, 315-322.

Yoo, Y. B. (1992). Interspecific hybridization between *Pleurotus cornucopiae* and *Pleurotus florida* following protoplast fusion. *The Korean Journal of Mycology, 20*(2), 118-125.

Yoo, Y. B., Lee, K. H., & Kim, B. G. (2002). Characterization of somatic hybrids with compatible and incompatible species by protoplast fusion in genera *Pleurotus* (Fr.) P. Karst. and *Ganoderma P*. Karst. by RAPD-PCR analysis. *International Journal of Medicinal Mushrooms, 4*(2).

Zhang, Y., & Molina, F. I. (1995). Strain typing of Lentinula edodes by random amplified polymorphic DNA assay. *FEMS Microbiology letters, 131*(1), 17-20.

Zawirska-W., Renata, S, M., Mildner-Szkudlarz, S., & Wąsowicz, E. (2009). Studies on the aroma of different species and strains of *Pleurotus* measured by GC/MS, sensory analysis and electronic nose. *Acta Scientiarum Polonorum, Technologia Alimentaria 8*(1), 47-61.

Zervakis, G., & 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*(6), 717-731.

Zhang, J., Wang, G., Li, H., Zhuang, C., Mizuno, T., Ito, H., . . . Li, J.. (1994). Antitumor polysaccharides from a Chinese mushroom,"*yuhuangmo*" the fruiting body of *Pleurotus citrinopileatus*. *Bioscience, Biotechnology, and Biochemistry, 58*(7), 1195-1201.

LIST OF PUBLICATIONS

World J Microbiol Biotechnol (2016) 32:18
DOI 10.1007/s11274-015-1959-2

ORIGINAL PAPER

Morphological and molecular characterization of yellow oyster mushroom, *Pleurotus citrinopileatus*, hybrids obtained by interspecies mating

A. G. Rosnina[1,2] · Yee Shin Tan[1] · Noorlidah Abdullah[1] · S. Vikineswary[1]

Received: 24 March 2015 / Accepted: 29 October 2015 / Published online: 8 January 2016

Abstract *Pleurotus citrinopileatus* (yellow oyster mushroom) has an attractive shape and yellow colour but the fragile texture complicates packaging, and its strong aroma is unappealing to consumers. This study aimed to improve the characteristics and yield of *P. citrinopileatus* by interspecies mating between monokaryotic cultures of *P. citrinopileatus* and *P. pulmonarius*. Ten monokaryon cultures of the parental lines were crossed in all combinations to obtain hybrids. Eleven compatible mating pairs were obtained and cultivated to observe their sporophore morphology and yield. The selected hybrid, i.e. P1xC9, was beige in colour while hybrid P3xC8 was yellow in colour. Their sporophores had less offensive aroma, improved texture and higher yield. The DNA sequences of these hybrids were found to be in the same clade as the *P. citrinopileatus* parent with a bootstrap value of 99 %. High bootstrap values indicate high genetic homology between hybrids and the *P. citrinopileatus* parent. The biological efficiencies of these hybrids P1xC9 (70.97 %) and P3xC8 (52.14 %) were also higher than the *P. citrinopileatus* parent (35.63 %). Interspecies hybrids obtained by this mating technique can lead to better strains of mushrooms for genetic improvement of the *Pleurotus* species.

Keywords Pleurotaceae · Biological efficiency · Sporophore · Monokaryon · Dikaryon

✉ Noorlidah Abdullah
noorlidah@um.edu.my

[1] Mushroom Research Centre, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

[2] Department of Agroecotechnology of Agriculture Faculty, University of Malikussaleh, Lhokseumawe, Aceh, Indonesia

Introduction

Pleurotus citrinopileatus (Singer 1942), or yellow oyster mushroom, is a popular edible mushroom with an attractive shape and bright yellow colour. This mushroom is extremely bitter and tangy with an unpleasant flavour when lightly cooked and is hence disdained by many people. In addition, the sporophores are brittle, which increases loss during harvesting and packaging. However, when they are crisply cooked, a strongly appealing cashew-like flavour eventually develops (Stamets 2000). The main compounds responsible for the odour of the yellow oyster mushroom are 3-octanol, 3-octanone and 1–46 octen-3-ol (Zawirska-Wojtasiak et al. 2009). *Pleurotus pulmonarius* (Fr.) Quél. (1827) had depressed pileus, lung-shaped cap with whitish to beige or pale tan. The margin enrolled when young, which later forms wavy when mature. It has very finely lined gills not split longitudinally (Stamets 2000). The sporophore is grey with a fleshy texture, has a good aroma and mild taste. Sporophores of the parental *P. pulmonarius* show a pileus cap which is somewhat depressed, lung-shaped to semi-circular in shape, greasy when young and fresh, fairly smooth, whitish to beige or pale tan in colour, with a margin rolled when young, but wavy and very finely lined when mature (Stamets 2000). *P. citrinopileatus* has a bright yellow pileus and fragile texture and strong aromatic sporophore while *P. pulmonarius* has a mild taste and fleshy sporophore.

Based on these special characteristics, mating studies were carried out on the two species in order to obtain better traits, that is high-yield, tough texture and decreased aromatic odour. The mating of *Pleurotus* spp. which leads to the formation of a dikaryon that can produce sporophores, is determined by bifactorial (tetrapolar) heterothallism controlled by two unlinked multi-allelic factors, A and B.

Mating-type genes and other compatibility factors are now used in mating-type-assisted breeding programs for producing economical and edible mushroom species (Kothe 2001).

Gupta et al. (2011) reported that hybridisation based on crossing non-fertile homokaryotic lines offers developed inter-strainal hybrids for the genetic improvement of different species. In their study, inter-strain hybridisation using single spore isolation between *P. sajor-caju*, *P. florida*, *P. eous* and *Hypsizygus ulmaris* resulted in shortened spawn run periods of 17 and 18 days after inoculation compared to the normal period of a spawn run which is about 25–30 days. In addition, dikaryons of hybrids showed a better trait in terms of their high yield, which could be the basis for strain improvement. The main objective of this study was to produce *P. citrinopileatus* hybrids with a firmer sporophore texture to facilitate harvesting and reduce the risk of damage during packaging and with a less pungent smell to improve acceptability for consumption. We compared the morphological characteristics and molecular characterisation of the hybrids with those of the parental strains.

Materials and methods

Hybridisation of monokaryon cultures and mating compatibility

Single spore cultures (monokaryon) were obtained by attaching a small piece of pileus with Vaseline to the inner surface of a Petri dish lid with gills the facing the solidified malt extract agar (MEA) medium. The plates were incubated at 25 °C in a sloping position for three to 4 h to obtain the spores. Single basidiospore isolation was done according to Gupta et al. (2011). The spores took 2–3 days to germinate. Germinating spores were picked off manually using a fine needle with the aid of a microscope under 400× magnifications and then transferred onto malt extract agar, MEA (Oxoid, Cat. No. CM 0059) and incubated for 7 days at 25 °C. The mycelium was confirmed as a monokaryon by the absence of clamp connections.

Ten monokaryon cultures with good cultural characteristics such as fast mycelial growth rate and density were selected for mating studies from the parental *P. pulmonarius* (P1–P10) and *P. citrinopileatus* (C1–C10). A dual culture technique was used to cross the monokaryotic cultures (Fig. 1a, b) by placing actively growing mycelial agar plugs (7 mm diameter) 1 cm apart on MEA media in petri dishes (90 cm diameter; Fig. 1c). A compatible mating reaction was identified by cutting a small piece (about 0.5 cm^2) from the junction of the contact zone and sub-culturing it on a new MEA plate incubated at 25 °C for 7 days. Figure 1d indicates the compatible mating reaction and Fig. 1e the incompatible mating reaction observed on plate cultures. The compatible mating reaction, indicated by clamp connections, is shown in Fig. 1f, while the incompatible mating reaction, which lacked clamp connections on the mycelium, is shown in Fig. 1g. These reactions were observed under a microscope at 400× magnification. The cultural characteristics and growth rate of the dikaryotic hybrid cultures were determined by measuring the average diameter of the mycelial colony every day for 7 days. The average reading was plotted against time (day) to obtain the growth rate in mm/day. Three replicate plates were prepared for each dikaryon.

Fruiting substrate preparation and cultivation of hybrids

The fruiting substrate was sawdust supplemented with 10 % rice bran (as a nitrogen source) and 2 % (dry weight) calcium carbonate (CaCO3). Water (80 % of dry weight of the substrate) was added and thoroughly mixed. The substrate (600 g) was then put into polypropylene bags and sealed. The bags were sterilised at 121 °C with 15 psi pressure for 60 min. After cooling, the substrates were inoculated with 3–5 plugs of 7-day-old mycelium discs (10 mm diameter) from the hybrids and parental strains. Three bags were prepared for each hybrid. Inoculated bags were kept at room temperature (27–28 °C) to allow mycelium colonisation. After the substrate had been fully colonised (19–21 days), the bags were opened to induce fruiting and placed in the mushroom house with ample ventilation and humidity at 25 °C. Mushrooms were harvested every day for 3 weeks. Parent and hybrid sporophores were examined for size, colour, shape, pileus texture, margin and aroma of the pileus.

Molecular characterisation and phylogenetic analyses of hybrids and parental strains

Molecular technique

The entire region of the internal transcribed spacers (ITS-1 and ITS-2), including the 5.8Ss rRNA gene, was analysed in this study. Genomic DNA was extracted from dried herbarium material using an E.Z.N.A. Forensic DNA kit (Omega 118 Bio-tek Inc., Doraville, GA, USA), and amplified using primer pairs ITS-1 and ITS-4 (White et al. 1990). The amplification reaction mixture (50 µl) typically contained 4.0 µl of dNTP, 1.25 µl of $MgCl_2$, 1.25 µl of ITS-1, 3.5 µl of ITS-4, 1.9 µl of BSA, 0.5 µl of taq polymerase, 5.0 µl buffer, 1.0 µl of DNA template and 34.5 µl distilled water. The amplification protocol consisted of 5 min for 1 cycle at 94 °C, followed by 30 cycles of 5 min denaturation temperature at 94 °C, annealing 60 s at 48 °C and elongation at 72 C for 1 min, followed by a final extension of 1 min and 30 cycles at 72 °C. Sequencing

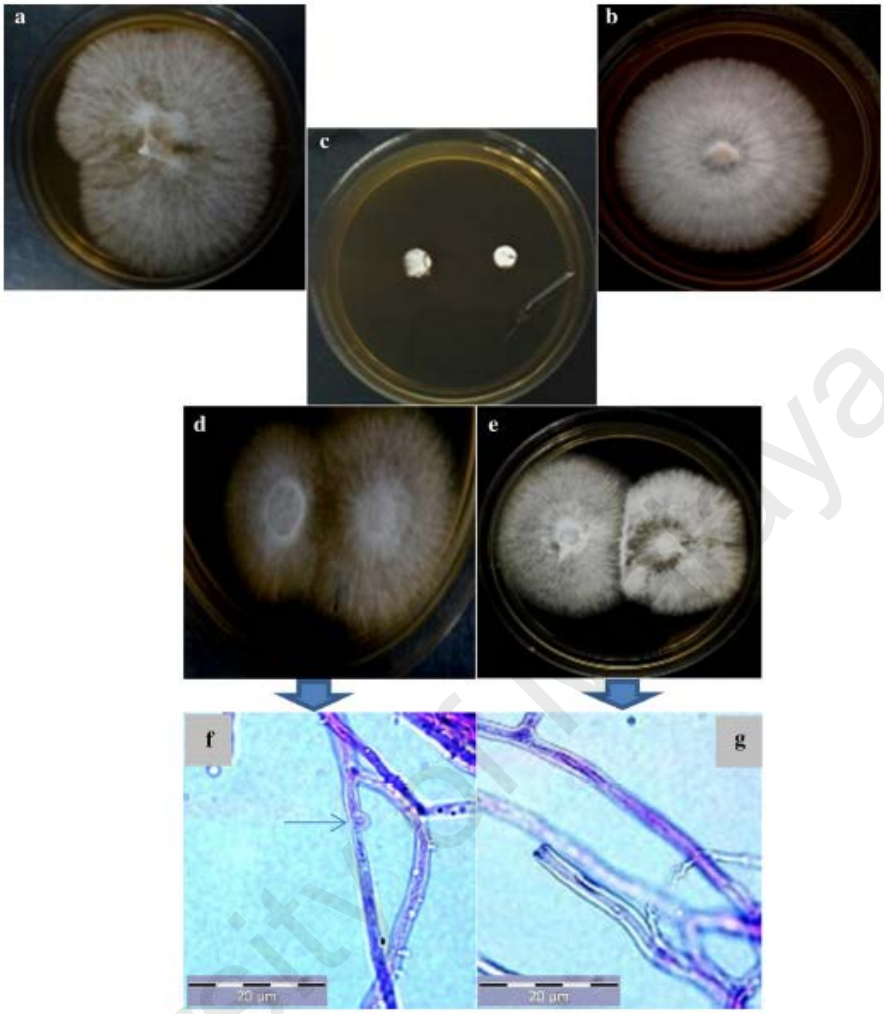


Fig. 1 Compatibility mating by dual culture technique. Monokaryotic of parental strains culture **a** *P. citrinopileatus*, **b** *P. pulmonarius*, **c** juxta position inoculation of monokaryotic cultures, **d** compatible mating reaction, **e** incompatible mating reaction, **f** dikaryon hyphae with clamp connection (shown by *arrow*), **g** homokaryon hyphae or unclamp hypha

reactions utilized the same primers as PCR and were purified using an isopropanol precipitation with 125 µl of 65 % isopropanol added to the sequencing reactions, vortexed and centrifuged at 15,000 rpm for 35 min. The supernatant was removed, then added with 125 µl of 75 % isopropanol, vortexed and centrifuged at 15,000 rpm for an additional 10 min. The supernatant was again removed and the pellet was air-dried for 30 min. Clean sequencing reactions were suspended in 15 µl Hi-Di formamide (Applied Biosystems) and visualized on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

Apart from DNA sequence of six hybrid and parental strains were included as a reference. All sequences were aligned using Mega 5, following standard multiple alignment, as well as visual adjustment. Sequence length was

made uniform, and regions showing ambiguous alignments were excluded. Sequences were processed using Chromas lite considering empty regions derived from alignments as additional character states. The method was performed based on the genetic distance between sequences according to Tamura et al. (2011). Neighbour-joining trees were identified using the heuristic search option, and the tree bisection and reconnection (TBR) algorithm$_{25}$. Neighbour-joining (NJ) analysis was performed using 1000 bootstrap replicates, each with a limit of 1,000,000 rearrangements, closest taxon addition and TBR branch swapping.

Cultivation and biological efficiency of selected *P. citrinopileatus* hybrids

Pleurotus citrinopileatus dominant hybrids (P1xC9 and P3xC8), which had better traits such as higher growth rate and thick mycelium density, decreased aroma and improved texture, were selected to determine spawn run rate and biological efficiency. Cultivation was carried out as above and 100 bags were prepared for each hybrid. The mycelium spawn rate was determined by measuring mycelial extension on four sides of the bag at 2-day intervals for 19–21 days. The average reading was plotted against time (day) to obtain the growth rate in mm/day. Pinheads started appearing 3–4 days after the cap was removed. The environment was watered two to three times every day during the fruiting phase. After 4–5 days, the sporophores had matured and were ready to be harvested. Three flushes were harvested at intervals of 2–4 days. The biological efficiency was worked out as yield of mushroom strain against the dry weight of each bag as follows:

$$\text{Biological efficiency} = \frac{\text{Total weight of fresh fruit bodies (g)}}{\text{Dry weight of fruiting substrate (g)}} \times 100$$

Statistical analysis

One-way analysis of variance and Duncan's multiple range test were used to test for 159 significant differences among the means of radial growth rate ($p = 0.05$). The data were in triplicate. All calculations were performed using the STATGRAPH plus 3.0 statistical software.

Results

Compatibility of mating between *Pleurotus citrinopileatus* and *P. pulmonarius*

Interspecies mating between vegetative monokaryon cultures of *P. citrinopileatus* and *P. pulmonarius* was successful in 11 out of 100 combinations (Table 1). Success was confirmed by the presence of clamp connections on the hyphae of the hybrids, indicating sexual compatibility (alleles A and B are different).

Table 1 Combinations of two monokaryotic isolates used in interspecies breeding of *P. pulmonarius* (P) and *P. citrinopileatus* (C)

Pc (C)	Pp (P)						
	A1B1				A2B2		
	C8	C9	C10	C13	C2	C5	C7
A1B1							
P19	−	−	−	−	+	+	−
P13	−	−	−	−	−	+	+
P8	−	−	−	−	NA	NA	+
A2B2							
P1	NA	+	+	+	−	−	−
P3	+	−	NA	NA	−	−	−
P6	NA	−	NA	+	−	−	−
P9	NA	NA	+	NA	−	−	−

+ Compatible mating producing *P. citrinopileatus* dominant hybrids
+ Compatible mating producing *P. pulmonarius* dominant hybrids
− Incompatible mating
NA not applicable

Cultural characteristics and sporophore morphology of parental *P. citrinopileatus* compared to the hybrids

Characterisation of mycelial colony

Successful mating between *P. citrinopileatus* and *P. pulmonarius* resulted in 11 hybrids of which six had *P. citrinopileatus*—dominant morphology. We observed that the vegetative monokaryon cultures of both *P. citrinopileatus* and *P. pulmonarius* had some peculiar features and were different from the parental and new dikaryotic hybrid cultures. The six hybrid cultures had different mycelial mat patterns and density, one of the characteristics responsible for adaptability to physiological parameters and high yield (Fig. 2). For example, the hybrid strain P1xC9 (Fig. 2d) had very thick, dense growth that was dull white, and zonate with concentric bands, while the P3xC8 hybrid was similar to its parental *P. citrinopileatus* with very thick, dense growth with rhizomorphic strands (Fig. 2c).

Macromorphological characters

Buchalo et al. (2009) described sporophores of *P. citrinopileatus* as branched, growing in large clusters with a small pileus size, irregular and cylindrical in shape and stipe off-centre (Fig. 3a). Sporophores of the parental *P.*

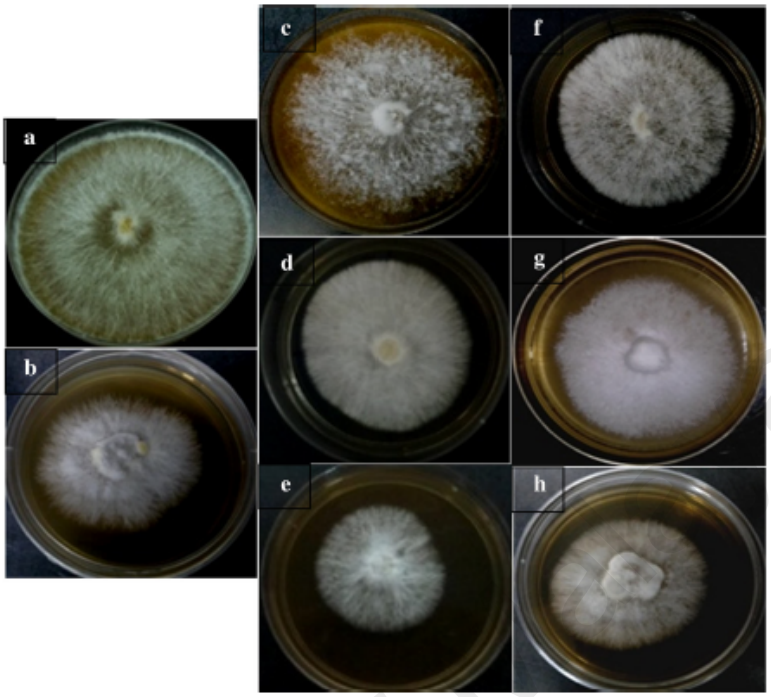


Fig. 2 Comparison of mycelial colonies cultures of parental strains and hybrids; **a** *P. citrinopileatus*, **b** *P. pulmonarius*, **c** hybrid P3xC8, **d** hybrid P1xC10, **e** hybrid P9xC10, **f** hybrid P1xC9, **g** hybrid P1xC13, **h** hybrid P6xC13

pulmonarius had a somewhat depressed pileus cap, and were lung shaped to semi-circular in shape, greasy when young and fresh, fairly smooth, whitish to beige or pale tan, with the margin rolled when young, wavy and very finely lined when mature (Fig. 3b). All of the six hybrids sporophore that had similar to *P. citrinopileatus* characteristics showed variations in texture, colour and aroma compared to the parental stock. In general, the hybrids had a bigger pileus and a wavy pileus margin compared to the round pileus margin of their parent. The pilei of two hybrids P1xC13 (Fig. 3g), and P6xC13 (Fig. 3h) had a bright yellow colour, strong aroma and fragile texture, all undesirable characteristics. In contrast, hybrid P1xC10 and P9xC10 (Fig. 3d, e), with a thin context and a pliable texture exhibited pale yellow pilei with reduced aroma and were therefore more appealing for consumption. The hybrid P1xC9 (Fig. 3c) was beige in colour while hybrid P3xC8 (Fig. 3f) was yellow in colour, but both were tough in texture and less pungent. In addition, their pilei were funnel shaped, larger in pileus size and grew in big clusters. Due to these characteristics, P1xC9 and P3XC8 were selected for further analysis.

Molecular characterisation of parental and hybrid strains

Molecular identification was carried out on the hybrids based on the sequencing of the internal transcribed spacer (ITS) region PCR using ITS1 and ITS4 was performed to identify the hybrids using a molecular method comparing them to the parent and corresponding GenBank data of related species. There were 314 base pairs informative parsimony characters from the total 618 member of trimmed alignment. The target sequences were 100 % similar with parental *P. citrinopileatus* and 14.4 % difference with parental *P. pulmonarius*. As shown in the phylogenetic tree (Fig. 4), hybrids P1xC9, P3xC8, P1xC10, P9xC10, P1xC13 and P6xC13 which resembled parental *P. citrinopileatus* are in the same clade with a bootstrap value of 99 %. The high bootstrap value suggests high genetic homology between the hybrids and the parent *P. citrinopileatus*.

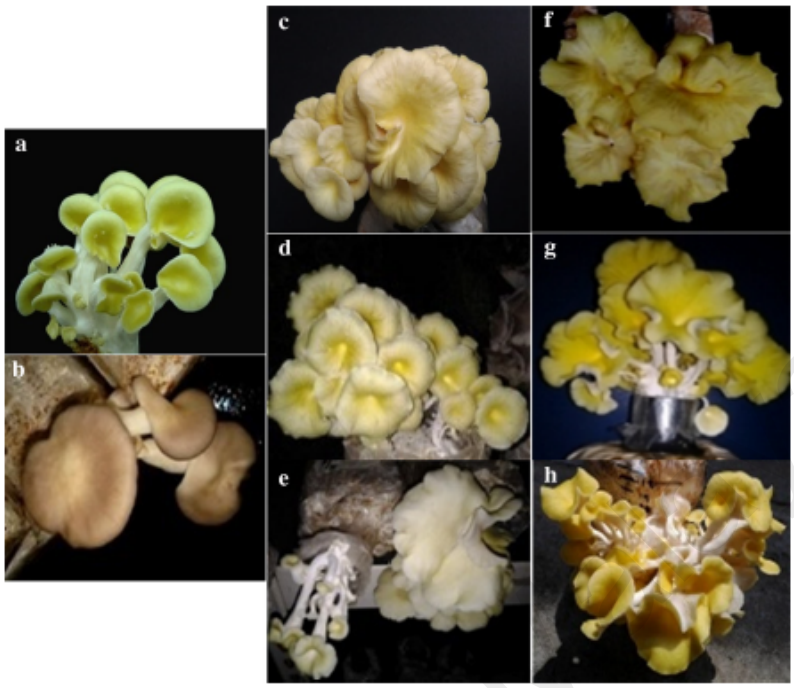


Fig. 3 Morphological characteristics of both parental strain and hybrids sphorophores; **a** *P. citrinopileatus*, **b** *P. pulmonarius* and **c** hybrid P1xC9, **d** hybrid P1xC10, **e** hybrid P9xC10, **f** hybrid P3xC8, **g** hybrid P1xC13, **h** hybrid P6xC13

Cultivation and biological efficiency of parental and selected *P. citrinopileatus* hybrids

Of six hybrids, two (P1xC9 and P3xC8) exhibited a thicker mycelium, high colony density, and faster growth rate compared to the parental cultures. Hybrid P3xC8 had a mycelial growth rate of 8.5 mm/day while hybrid P1xC9 grew at 8.2 mm/day, similar to the parental *P. pulmonarius* (Table 2). The growth rate of parental *P. citrinopileatus* was slower at 7.6 mm/day. The sporophores of hybrids P1xC9 and P3xC8 had a similar morphology to *P. citrinopileatus*. More details about the variations in hybrid sporophores and the sporophores of both parental species are presented in Table 2. In large scale cultivation, both hybrids had a faster to completion spawn run at 19 days and the primordia appeared 2 days after the completed spawn run, while both parental strains completed spawn runs at 21 days (Table 3). Another important aspect is the increase in total yield and biological efficiency of both selected hybrids. Hybrid P1xC9 had the highest total production and biological efficiency and hybrid P3xC8 had higher rates than the parental *P. pulmonarius* and *P. citrinopileatus* (Table 3). Table 3 shows that hybrid P3xC8 has the highest mycelia growth rate at 8.5 mm/day, meanwhile hybrid P1xC9 had the same mycelia growth rate as parent *P. pulmonarius* at 8.2 mm/day compared to the lowest mycelia growth rate of the other parent *P. citrinopileatus* at 7.6 mm/day.

Discussion

Table 1 shows successful mating at 11 %, which is lower than the successful mating rates of 15.38–20.59 % to that recorded by Bahukhandi and Sharma (2002) for different combinations of hybridisation between *P. sajor-caju* and *P. sapides* and *P. cornucopiae*. A preliminary study of interspecies mating between *P. sajor-caju* and *P. flabellatus* exhibited a lower success rate of 7 % for the presence of clamp connection Rosnina et al. (2007).

Our findings indicate that, to some extent, species of the same genus tend to have the same mating type. All of the six new dikaryon cultures from compatible mating had thicker mycelial mat patterns and faster growth compared to the parental cultures. The dikaryons of hybrids were able to form new sporophores. Brown and Casselton (2001)

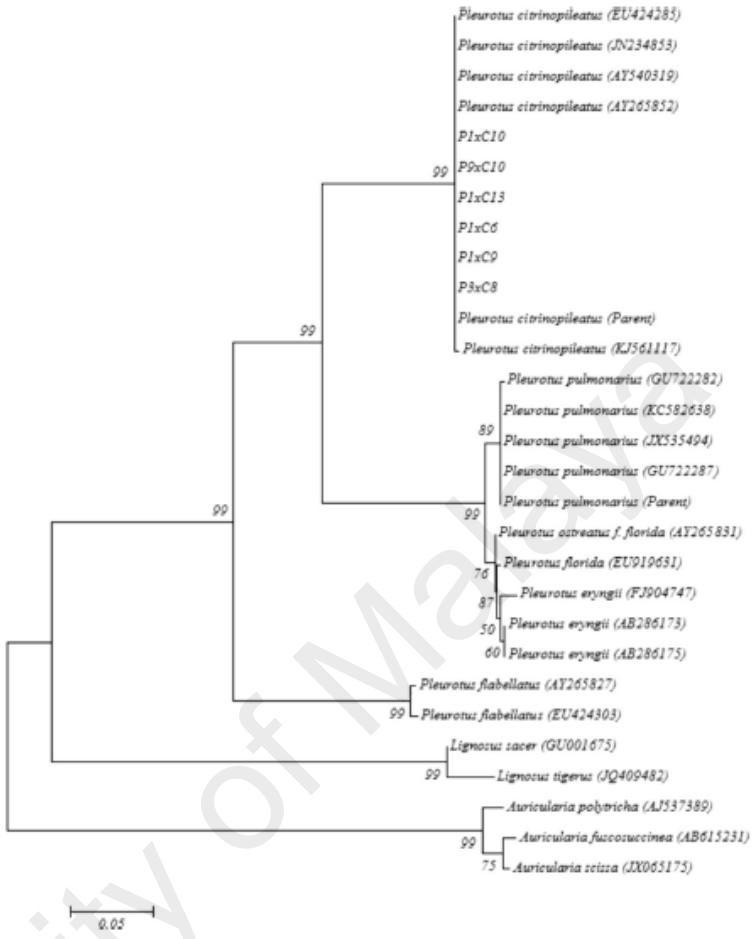


Fig. 4 The phylogenetic tree based on analysis of DNA gene sequences (ITS1–ITS4) of hybrids P1xC9, P3xC8 and parent *P. pulmonarius* and *P. citrinopileatus* constructed by neighbour-joining tree. Bootstrap values are expressed percentages support from 1000 replicates showing maximum similarity with hybrids P1xC9, P3xC8 and parental strains and the homogeneous strains of *Pleurotus* species

Table 2 Comparison of mycelial growth rate in bag, completed spawn run, production and biological efficiency of parental strains and selected hybrids

Strain	Mycelial growth rate in bag (mm/day)	Completed spawn run (mm/day)	Total harvest (g)	Biological efficiency (%)
P. pulmonarius (P)	8.2 ± 0.04^{b}	21 ± 0.20^{a}	103.9 ± 0.46^{b}	51.96 ± 0.40^{b}
P. citrinopileatus (C)	7.7 ± 0.06^{a}	21 ± 0.06^{a}	71.2 ± 1.15^{a}	35.63 ± 0.32^{a}
Hybrid P1xC9	8.5 ± 0.04^{c}	19 ± 0.25^{b}	141.9 ± 0.67^{c}	70.97 ± 0.42^{c}
Hybrid P3xC8	8.2 ± 0.02^{b}	19 ± 0.02^{b}	104.3 ± 0.28^{b}	52.14 ± 0.07^{b}

Each value is expressed as mean ± SD of three replicate analyses. The homogenous group represented in alphabet; different letter denotes significantly different at the level of 0.05 ($p < 0.05$)

Table 3 Comparison of macromorphological characteristics and traits of hybrids with parental strains

Parent and hybrid strains	Stipe length (cm)*	Pileus width (cm)*	Margin of pileus/shape	Sporophores texture	Typical type of stipe	Aroma/ Odor
Parent 1 (P)	4.60 ± 0.10^{d}	7.37 ± 0.05^{ab}	Grey colour with undulating shape like an oyster	Fleshy	Rarely forms cluster	Pleasant (Mild taste)
Parent 2 (C)	3.77 ± 0.15^{ab}	4.60 ± 0.08^{a}	Golden Yellow and funnel shaped	Fragile	Eccentrically attached to the pileus with large clusters	Strong odour
Hybrid P1xC9	4.10 ± 0.10^{c}	6.37 ± 0.12^{ab}	Beige with undulating shape	Fleshy	Eccentrically attached to the pileus with large cluster	Decreased
Hybrid P3xC8	3.73 ± 0.15^{b}	5.93 ± 0.12^{ab}	Yellow colour and funnel shaped with wavy pileus margin	Fleshy	Eccentrically attached to the pileus with large cluster	Decreased

* Each value is expressed as mean ± standard deviation (n = 3). Different alphabet letter within the column denotes the mycelia growth rate was significantly different at $p < 0.05$

found that the single basidiospore of *Pleurotus* was sterile but it was able to form a dikaryotic mushroom when mating occurred with another compatible single spore isolate. The fruiting trials demonstrated that hybrids resemble their parent *P. citrinopileatus* sporophores. The hybrid sporophores varied in their features. The similarities of these hybrids were further tested using DNA molecular work. It is important to perform molecular identification for characterization of mushroom species rather than depend on observation of the morphology alone. The reason is that morphological characteristics are inconsistent and unstable criteria because they are strongly influenced by environmental conditions according to Bresinsky et al. (1987). Zhang et al. (2012) reported the genetic diversity of *P. citrinopileatus* using ISSR and SRAP marker and concluded that morphological analysis had advantage in species identification and gauging growth performances while molecular markers were more reliable for assessing genetic diversity and give more accurate information on effective genetic distances.

As mentioned in Fig. 4, six hybrids (P1xC10, P3xC8, P9xC10, P1xC9, P1xC13 and P6xC13) that exhibited characteristics similar to *P. citrinopileatus* (controls) were grouped together in the same clade with a bootstrap value at 99 %. During the breeding process, a bias segregation might favour to one of the mating-type allele. This phenomenon was observed in *Pleurotus djamor* as reported by James et al. (2004).

Future investigations should be extended to other genera with the aim of establishing new varieties of edible basidiomycetes. The hybrid P3xC8 had the highest mycelia growth rate, followed by hybrid P1xC9, which had the same mycelia growth rate as parent *P. pulmonarius,* while the lowest rate was for *P. citrinopileatus,* as presented in Table 3. In a similar study, Liang et al. (2011) reported that the mycelia growth rate of *P. pulmonarius* on a substrate compost of 45 % sawdust was 8.1 mm/day. Musieba et al. (2012) reported that *P. citrinopileatus* took up to 13 and 21 days to colonize 1 kg rice straw substrate and 1 kg rice sawdust substrate, respectively. This is comparable with the results obtained in the current study.

Furthermore, Alemu (2014) reported that primordia appeared 20 days after scratching depending on the type of substrate, and that the sporophores matured generally 48 h after the appearance of the primordia. In this study, hybrid sporophores were ready for harvest after approximately 2–3 days. Strain improvement, using a mating technique for enhancing yield, which was better in texture and decreased the aroma of the sporophores of the hybrids, was achieved through interspecific mating using monokaryotic culture isolates. Both of the selected hybrids were analysed for important morphological traits that could potentially add value for commercialisation. Special attention was given to the texture and decreased aroma of the hybrids.

The selected *P. citrinopileatus* dominant hybrids, P1xC9, and P3xC8, had a less pungent odour. To confirm the reduced aroma, 30 volunteers were asked to smell the sporophores. Out of the 30 responses, 25 of them confirmed that the hybrids exhibited fewer aromas. Kong (2004) described the aroma of *P. citrinopileatus* sporophores as being similar to that of wheat flour. The main odorants of the mushroom are eight carbon (C8) compounds, mainly 1-octen-3-ol (Drawert et al. 1983). Gas chromatography olfactometry and aroma extract dilution analysis has shown that sulphur and nitrogen-containing components, C8 ketone and C8 aldehyde are responsible for the aroma of *P. citrinopileatus* (Miyazawa et al. 2011). Similar studies on the aroma of several *Pleurotus* species revealed that the compound 1-octen-3-ol was found in relatively high concentrations from 1.14 to 280 mg/100 g of fresh weight (Zawirska-Wojtasiak et al. 2009). The two hybrids obtained in this study had desirable traits as well as higher productivity and biological efficiency and less offensive aroma, thus good commercialisation potential.

Conclusion

A high quality mushroom can be obtained through interspecies breeding that produces desirable traits and characteristics. The hybrid strains produced in our study had a less offensive aroma and gave a higher yield, in terms of the total weight of fresh fruiting body and biological efficiency, than the parent strains. Molecular analysis showed high genetic homology between the hybrids and *P. citrinopileatus*. The hybrids P1xC9 and P3xC8 have good characteristics and a high yield that has commercial potential.

Acknowledgments The authors would like to thank University of Malaya for Grant IPPP/UPDiT/Geran (PPP) S239/2008 C and RP005A-13AFR.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

Alemu F (2014) Cultivation of *Pleurotus ostreatus* on *Grevillea robusta* leaves at Dilla University, Ethiopia. J Yeast Fungal Res 5(6):74–83. doi:10.5897/JYFR2014.0139

Bahukhandi D, Sharma RK (2002) Interspecific hybridization in *Pleurotus* species. Indian Phytopathol 55(1):61–66

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

Brown AJ, Casselton LA (2001) Mating in mushrooms: increasing the chances but prolonging the affair. Trends Genet 17(7):393–400. doi:10.1016/s0168-9525(01)02343-5

Buchalo A, Volz PA, Nevo E (2009) Microstructures of vegetative mycelium of Macromycetes in pure cultures. Altepress, Kiev

Drawert F, Berger RG, Neuhauser K (1983) Biosynthesis of flavor compounds by microorganisms 4. Characterization of the major principles of the Odor of *Pleurotus eusmus*. Eur J Appl Microbiol Biotechnol 18(2):124–127. doi:10.1007/bf00500836

Gupta B, Reddy BPN, Kotasthane AS (2011) Molecular characterization and mating type analysis of oyster mushroom (*Pleurotus* spp.) using single basidiospores for strain improvement. World J Microbiol Biotechnol 27(1):1–9. doi:10.1007/s11274-010-0419-2

James TY, Liou S-R, Vilgalys R (2004) The genetic structure and diversity of the A and B mating-type genes from the tropical oyster mushroom, *Pleurotus djamor*. Fungal Genet Biol 41:813–825

Kong WS (2004) Descriptions of commercially important *Pleurotus* species. In: Mushworld (ed) Mushroom grower's handbook - Oyster mushroom cultivation. Heineart Incorporation, Seoul, pp 54–61

Kothe E (2001) Mating-type genes for basidiomycete strain improvement in mushroom farming. Appl Microbiol Biotechnol 56(5–6):602–612

Liang Z-C, Wu K-J, Wang J-C, Lin C-H, Wu C-Y (2011) Cultivation of the culinary-medicinal lung oyster mushroom, *Pleurotus pulmonarius* (Fr.) Quel. (Agaricomycetideae) on grass plants in Taiwan. Int J Med Mushrooms 13(2):193–199

Miyazawa M, Dejima Y, Takahashi T, Matsuda N, Ishikawa R (2011) Characteristic odor components of essential oil from dried fruiting bodies of golden oyster mushroom (*Pleurotus citrinopileatus*). J Essent Oil Res 23(3):58–63

Musieba F, Okoth S, Mibey RK, Wanjiku S, Moraa K (2012) Suitability of locally available substrates for cultivation of the Kenyan indigenous Golden oyster mushroom (*Pleurotus citrinopileatus* Singer). Agric J 7(4):240–244

Rosnina AG, Noorlidah A, Vikineswary S (2007) Strain improvement of edible mushrooms by mating. In: Proceedings of the Asian mycological congress 2007 & Xth international marine and freshwater mycology symposium. December 2007. Penang, Malaysia, p 266

Stamets P (2000) Growth parameters for gourmet and medicinal mushroom species, growing gourmet and medicinal mushrooms. Ten Speed Press, Berkeley

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739

White TJ, Bruns T, Lee SJWT, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc Guide Methods Appl 18:315–322

Zawirska-Wojtasiak R, Siwulski M, Mildner-Szkudlarz S, Wąsowicz E (2009) Studies on the aroma of different species and strains of Pleurotus measured by GC/MS, sensory analysis and electronic nose. Acta Sci Pol Technol Aliment 8(1):47–61

Zhang Q, Xu B, Liu L, Yuan Q, Dong H, Cheng X, Lin D (2012) Analysis of genetic diversity among Chinese *Pleurotus citrinopileatus* Singer cultivars using two molecular marker systems (ISSRs and SRAPs) and morphological traits. World J Microbiol Biotechnol 28:2237–2284

Morphological and Molecular Characterization of *Pleurotus pulmonarius* (Fr.) Quél Hybrids with Improved Sporophore Features and Higher Biological Efficacy

Rosnina Abdulgani[1,2], Ching Ching Lau[1], Noorlidah Abdullah[1*] and Vikineswary S.[1]

[1]*Mushroom Research Centre, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur. Malaysia.*

[2]*Department of Agroecotechnology of Agriculture Faculty, University of Malikussaleh Lhokseumawe, Aceh, Indonesia*

[*]**For correspondence:** noorlidah@um.edu.my

Abstract

To improve the sporophore characteristics and production yield of *Pleurotus pulmonarius* by interspecies hybridization between monokaryotic cultures of *P. pulmonarius* and *P. citrinopileatus*, a total of a hundred possible pairings were done. Five hybrids were obtained producing sporophores with morphological characteristics dominant to those of *P. pulmonarius*. DNA analysis confirmed the high genetic affiliation of the hybrids with *P. pulmonarius* generating a bootstrap value of 99%. Among the hybrids, P19xC5 strain exhibited good traits such as higher mycelial growth rate, thicker mycelium density as well as generating sporophores with fleshier texture and bigger pileus. Further analysis by spawning showed that it had faster growth rate at 8.7 mm/day as opposed to 8.2 and 7.7 mm/day for *P. pulmonarius* and *P. citrinopileatus*, respectively. It generated a higher sporophores yield (182.97 g) and biological efficiency (130.19%) which was approximately twice that of the parental strains. Its sporophore was found to contain lower spore density, a criteria favourable for the health and work conditions of farm workers. The good features imposed by the hybrid P19xC5 may indicate its better adoption in the mushroom growing industry and greater commercialization value.

Keywords : Pleurotaceae; mushroom breeding; sporophore yield; biological efficiency

The paper has been submitted to IJAB.

APPENDICES

Appendix A. Fungal growth media

Media was sterilized by autoclaved at 121 ^{o}C for 15 minutes

Malt extract agar (MEA) (per litre)

MEA (Difco)	39 g
Distilled water	1000 ml

Appendix B- Buffer and reagents

Potassium hydroxide (KOH)

Potassium hydroxide	3 g
Distilled water	97 ml

10x TBE Buffer (Tris-Borate-EDTA)

Tris base 890 mM	108 g
Boric acid 890 mM	55 g
Na_2 EDTA 20 mM	7.44 g

Add ddH2O to 1 liter. Adjust pH to 8.3