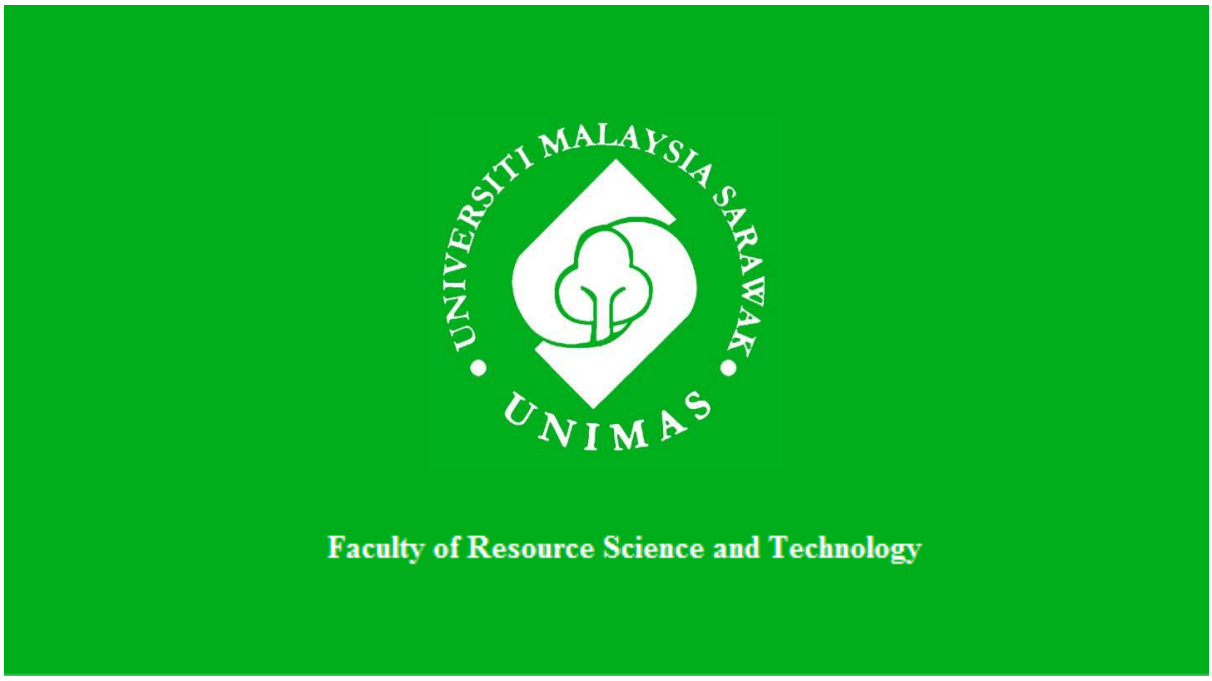

TOWARDS CONVENTIONAL AND MUTATIONAL BREEDING OF *PLEUROTUS SAJOR-CAJU*

Jessica Fung Lee Ying

Bachelor of Science with Honours
(Plant Resource Science and Management)
2015

UNIVERSITI MALAYSIA SARAWAK

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Final Year Project Report ☑

Masters ☐

PhD ☐

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TOWARDS CONVENTIONAL AND MUTATIONAL BREEDING OF *PLEUROTUS SAJOR-CAJU*

JESSICA FUNG LEE YING

A thesis submitted
In fulfilment of the requirements for the degree of Bachelor of Science with Honours in
Plant Resource Science and Management

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2015

APPROVAL SHEET

Name of Candidate: Jessica Fung Lee Ying

Title of Dissertation: Towards Conventional and Mutational Breeding in *Pleurotus sajor-caju*

"I declare that I have read this work and in my opinion this work is adequate in terms of scope and quality for the purpose of awarding Bachelor's Degree of Science with Honours (Plant Science Resource and Management Programme)."

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Supervisor

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Declaration

I hereby declare the work entitled Towards Conventional and Mutational Breeding of *Pleurotus sajor-caju* is my original work. I have not copied from any other sources except where due reference or acknowledgment is made explicitly in the text, nor has any part been written for me by another person.

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List of Abbreviations

EMS	Ethyl Methane Sulfonate
MMS	Methyl Methane Sulfonate
MM	Monokaryotic Mycelium
PDA	Potato dextrous agar
CD-7dpi	Colony Diameter after 7 days of post inoculation
SGCD-7	Slow Growing Colony Diameter after 7 days of post inoculation
MGCD-7	Medium Growing Colony Diameter after 7 days of post inoculation
FGCD-7	Fast Growing Colony Diameter after 7 days of post inoculation
One-way Anova	One way Analysis of Variance
SPSS	Statistical Package for the Social Sciences

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Towards Conventional and Mutational Breeding of *Pleurotus sajor-caju*

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Abstract

Currently, *Pleurotus sajor-caju* is gaining popularity due to its massive nutrient content and capability of growing on various agricultural wastes. These advantages may help in raising the economic dynamics of certain countries. *P. sajor-caju* has short shelf life. The appearance, texture, colour and taste is not consistent. There is a need to breed new strain of *P. sajor-caju* to meet the rising demands of the increasing population. Strain improvement is achievable through selection, hybridisation and mutagenesis. Unfortunately, there is limited information regarding the genetic variations of *P. sajor caju.* Therefore this study is of interest to document as much as possible the morphological variations of monokaryon cultures, to generate hybrids and generate new variations from the monokaryon culture through mutagenesis. A total of 200 monokaryotic mycelium (MM) were cultured individually on potato dextrose agar. The 200 MMs were characterized morphologically and were divided into three main groups based on macroscopic morphology which are scattered, rough and smooth. Aside from that, all the MM observed were also categorized based on their colony diameter after 7 days of post inoculation (CD-7dpi) which are slow growing colony diameter (SGCD-7), medium growing colony diameter (MGCD-7) and fast growing colony diameter (FGCD-7). In this study, 10 FGCD-7 and 10 SGCD-7 were selected for hybridization. The selected MM were hybridized within and between each other in all possible combinations without repetition. A total of 16 dikaryons were recognized. For all FGCD-7 pairings, SGCD-7 pairings, and between FGCD-7 and SGCD-7 pairings, dikaryons that is at least significantly different from one of its parents has higher CD-7dpi than the parental strains. For mutagenesis of monokaryon culture, the best concentration of Ethyl Methane Sulfonate (EMS) to mutate MM would be 1% to 2% (v/v). In this study, two candidate mutants with slower colony growth compared to control (EMS017 and EMS032) were obtained.

Keyword: *Pleurotus sajor-caju*, monokaryotic mycelium (MM), hybridization, mutagenesis, colony diameter after 7 days of post inoculation (CD-7dpi)

Towards Conventional and Mutational Breeding of *Pleurotus sajor-caju*

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Abstrak

Masa kini, *Pleurotus sajor-caju* semakin popular disebabkan oleh kandungan nutrien yang banyak serta keupayaan untuk tumbuh di pelbagai sisa pertanian. Kelebihan ini boleh membantu dalam meningkatkan dinamik ekonomi negara-negara tertentu. *P. sajor-caju* mempunyai kadar kehidupan yang singkat. Rupa, tekstur, warna dan rasa adalah tidak konsisten. Terdapat keperluan untuk membiak strain baru *P. sajor-caju* untuk memenuhi permintaan pertambahan penduduk yang semakin meningkat. Peningkatan strain boleh dicapai melalui pemilihan, penghibridan dan mutagenesis. Malangnya, maklumat mengenai variasi genetik *P. sajor-caju* adalah terhad. Oleh itu, tujuan kajian ini adalah untuk mendokumentasikan sebanyak mungkin variasi morfologi kultur monokaryon, menjana hibrid dan menghasilkan variasi baru daripada kultur monokaryon melalui mutagenesis. Sebanyak 200 miselium monokaryotic (MM) telah dikultur secara individu pada potato dextrose agar. Ciri-ciri morfologi 200MMs telah dibahagikan kepada tiga kumpulan utama berdasarkan makroskopik morfologi iaitu taburan, kasar dan halus. Selain daripada itu, semua MM juga dapat dikategorikan berdasarkan diameter koloni selepas 7 hari inokulasi (CD 7dpi) iaitu pertumbuhan koloni diameter yang lambat (SGCD-7), pertumbuhan koloni diameter yang sederhana (MGCD-7) dan pertumbuhan koloni diameter yang laju (FGCD-7). Dalam kajian ini, 10 FGCD-7 dan 10 SGCD-7 telah dipilih untuk proses penghibridan. MM yang terpilih telah dihibrid di antara satu sama lain dalam semua kemungkinan kombinasi tanpa pengulangan. Sebanyak 16 dikaryons telah dikenalpasti. Untuk semua pasangan FGCD-7, pasangan SGCD-7, dan pasangan di antara FGCD-7 dan SGCD-7, dikaryons yang mempunyai perbezaan ketara daripada salah satu induknya didapati mempunyai CD-7dpi lebih tinggi daripada strain induk. Untuk mutagenesis kultur monokaryon, kepekatan terbaik Ethyl Metana Sulfonate (EMS) untuk memutasikan MM adalah antara 1% hingga 2% (v/v). Dalam kajian ini, dua mutan yang mempunyai koloni pertumbuhan yang lebih perlahan berbanding dengan kawalan (EMS017 dan EMS032) telah diperoleh.

Kata kunci: *Pleurotus sajor-caju*, miselium monokaryotic (MM), penghibridan, mutagenesis, diameter koloni selepas 7 hari inokulasi (CD 7dpi)

Chapter 1.0: Research Background

1.1 Introduction

Nowadays, mushrooms are becoming one the main food source which acquired more attention particularly in the Asian countries (Rosli & Solihah, 2012). The cultivation of mushroom had taken place since prehistoric times especially in the eastern countries for their nutrient content and flavor (Sadler, 2003). Mushrooms have higher protein content, fibers and minerals compared to most plants (Sadler, 2003). The content of calories, sodium, fats and cholesterol levels are low (Manzi *et al.*, 2004). Moreover, mushroom is known to contain anti-viral and anti-cancer properties (Liu *et al.*, 2005). Mushrooms are also have been used to treat and prevent high blood pressure, diabetes and constipation (Agrawal *et al.*, 2010).

The most widely cultivated mushrooms are from the genus *Pleurotus*. *Pleurotus* or oyster mushrooms with approximately 40 species was ranked second in the world as vitally cultivated mushrooms (Imran *et al.*, 2011). *P. sajor-caju*, commonly known as Dhingri oyster or grey abalone oyster mushroom, is one of the well-known cultivated *Pleurotus* species (Kumar, 2012). Due to its massive nutrient (Pala, Wani, & Mir, 2012), high dietary fibre (Schneider *et al.*, 2011), high beta-glucans (Rosli & solihah, 2012) and its capability of growing on various agricultural wastes (Pala, Wani, & Mir, 2012). *P. sajor-caju* is currently gaining popularity. In addition, *P. sajor-caju* is reported to possess medicinal values such as preventing atherosclerosis (Schneider *et al.*, 2011), lowering cholesterol level and affecting glycemic response (Rosli & solihah, 2012).

With the growing demand of *P. sajor-caju* and their huge acceptance for food products, there is a need for strains improvement (Kaul, 2001). Strains improvement can be done by conventional breeding. Conventional breeding refers to the combination of traits passable from the parent strains to the offspring by means of natural hybridization process. It is reported that

hybridisation within or between non-fertile homokaryotic strains of *Pleurotus* species gives better prospects for genetic improvement (Gupta *et al.,* 2011). In order to develop hybrids having desirable traits, the first step would be to generate and characterize single basidiospore producing monokaryotic mycelium. Not only that, the presence of variability in morphological traits and growth rate of monokaryotic mycelium is crucial for producing intra or interstrainal hybrids (Gupta *et al.,* 2011). However, the information regarding different range of polymorphisms morphology is insufficient hence affecting the hybrid production of *P. sajor-caju.* Therefore, this study is of interest to generate as much as possible monokaryon cultures with different morphologies and to generate hybrids from the monokaryon culture.

Another way of strains improvement is through mutation breeding. Mutation breeding generates genetic variation in plant species, which then leads to changes of the traits. The changes on the gene will be inherited by the younger generation (Carlile & Watkinson 1994). It is also true for edible mushrooms (Djajanegara & Harsoyo, 2008). Chemical mutagens can be used to induce mutation. Ethylmethane sulfonate (EMS) and Methylmethane sulfonate (MMS) are such chemical mutagens. EMS and MMS are alkylating agents that would cause an effect to DNA by inducing point mutation (Rhaese & Boetker, 1973). Hence, EMS and MMS can be used in generating variation of *P. sajor-caju* with desirable traits. With this, the mutants produced will be a good source of variation for future breeding purposes and genetic research. Therefore, the other purpose of this study is to generate new variant or also known as mutants from the monokaryotic hyphae through mutagenesis.

1.2 Problem statement

With the growing popularity of *P. sajor-caju*, there is a need for strain improvement. Strain improvement is achievable through selection (Gharehaghaji et al., 2007). In order to have more choices of strains to select from, the information on genetic variations (eg. morphological variations) of *P. sajor-caju* is needed. Unfortunately, there is relatively little knowledge about the genetic variations of this species (Kashangura et al., 2006).

Hybridisation is another approach for strain improvement. This strategy will create new variations by manipulating genetic combination of *P. sajor-caju* with interesting traits. To achieve this, the information on morphological variations and growth rate of monokaryotic strain is crucial for producing intra or interstrainal hybrids (Gupta *et al.*, 2011). Unfortunately, systematic documentation on the genetic variations of monokaryotic strain for *P. sajor-caju* is lacking.

Finding the genetic variations of monokaryotic strain is possible through conventional screening or alternatively by generating the genetic variations through mutagenesis. There is yet a protocol for mutating the mycelium of *P. sajor-caju* using Ethyl Methane Sulfonate. The generated variations can be used to select for new superior strain or used to improve the available wild type strains.

1.3 Research Objectives

A) To document as much as possible the morphological variations of monokaryotic cultures.

B) To generate hybrids from the monokaryon culture.

C) To generate new variants (mutants) from the monokaryotic hyphae.

Chapter 2.0: Literature Review

2.1 What is edible mushroom?

Edibility can be defined as the nonexistence of poison and are fit to be eaten which would not bring any disastrous effects on human being (Arora, 1986). Mushroom is defined as "macrofungus with a distinctive fruiting body which can either be epigenous or hypogenous and large enough to be seen with the naked eye and to be picked by hand" (Chang & Miles, 1991). Hence, edible mushrooms are a type of fungi which are fleshy and having edible fruiting bodies.

Edible mushrooms are found in their natural habitat or cultivated. It is known that more than 2000 species of mushrooms are edible but only a number of them are being chosen to be cultivated commercially (Meng & Li, 1997). In general, the edible mushrooms are cultivated for their nutritional value as well as medicinal value. Edible mushrooms that are fresh have low fat content (Chye, Wong & Lee, 2008), indicating low calorific value i.e. healthy. Edible mushrooms also have certain amount of fibre and β-glucan. The presence of β-glucan contributes to antioxidant properties of edible mushrooms (Synytsya *et al.*, 2008).

2.2 The genus *Pleurotus*

Pleurotus or commonly known as oyster mushroom and is place under the Family Pleurotaceae. The common characteristics of *Pleurotus* mushrooms are the presence of gills and having kidney-shaped caps that either attached to wood in direct contact or connected via rudimentary stem. Oyster mushrooms usually have a loose or dense clusters way of growing forming umbrella-like assemblage (Kuo, 2005).

The most cultivated species after the genus *Agaricus* and *Lentinus* is from the genus *Pleurotus.* The increase in production of *Pleurotus* species causes the worldwide production of both

Agaricus and *Lentinus* mushrooms to decline (Chang, 1993). This is because the cost involved in manufacturing the products of *Pleurotus* as well as managing the stock inventory is lower and the nutrient content of *Pleurotus* is not destroyed during the production process (Patil, 2013). Therefore, *Pleurotus* are becoming the choice of cultivated edible mushroom throughout the world (Upadhyay & Singh, 2010). This is also due to the facts that *Pleurotus* species has higher content of vitamin C, minerals and protein (Manzi, *et al*., 1999; Caglarirmak, 2007). It is also observed that oyster mushrooms are rich in potassium to sodium ratio (Mandhare, 2000) making *Pleurotus* a suitable food intake for those having high blood pressure and cardiovascular complication (Rai *et al.,* 1998). In addition, *Pleurotus* is able to lower down the level of cholesterol (Scheider *et al.,* 2011), prevents high glucose level in blood and irritation in adipose tissue (Kanagasabapathy *et al.,* 2012).

2.3 *Pleurotus sajor-caju*

One of the most popular *Pleurotus* species is *Pleurotus sajor-caju*. This mushroom is commonly cultivated around the world for food or food component complimenting certain dishes (Rosli & Solihah, 2012). *P. sajor-caju* is known to contain all the crucial protein needed by an adult human. *P. sajor-caju* is a provider of riboflavin, niacin and patothenic acid (Rosli & Solihah, 2012).

Besides its nutritional properties, *P. sajor-caju* has some medicinal properties through which the active component can help in lowering down hypertension (Alam *et al*., 2008). *P. sajor-caju* also is rich in fibers and has low fat content making them suitable to prevent the narrowing of arteries (Scheider *et al.,* 2011).

In terms of cultivation, *P. sajor-caju* is popular to be cultivated due to its capability to grow at a wider range of temperatures and various agro wastes (Baysal *et al.,* 2003) viz. "wheat straw, paddy straw, stalks and leaves of sorghum, pearl, millet and maize" (Asghar, Tariq, & Rehman,

2007) and rice hulls combined with leftovers of cotton (Chang *et al.*, 1981). *P. sajor-caju* also secretes some enzymes such as laccases and peroxidases that enable this mushroom to grow on agricultural wastes that act as a substrate (Toyama & Ogawa, 1974).

2.4 Life cycle of *Pleurotus sajor-caju*

To manipulate a species to achieve certain breeding aim needs a complete understanding of the life cycle of a species. The life cycle of *P. sajor-caju* involves three cardinal events (Figure 1) which are plasmogamy, karyogamy and meiosis (Miles, 1993). Starting from the haploid spore, when the spore lands on a suitable habitat, the spore will germinates to produce hyphae. When the hyphae encounter a mate, plasmogamy takes place enabling the protoplast of two compatible monokaryotic hyphae to fuse with each other leading to the formation of dikaryotic mycelium with two different nuclei in the same cell (Figure 1). Clamp connection observed via microscope is used as an evidence of the heterokaryotic condition (Miles, 1993). During the simultaneous division of the compatible nuclei, formation of clamp connections happened in the developing hyphal tip that eventually becomes a hook. The function of the hook cell is to provide a temporary place for one of the daughter nuclei in order to retain the dikaryotic condition in the apical cell (Miles, 1993). Hence, the occurrence of clamp connection indicates the heterokaryotic condition with two nuclear types (Petersen & Bermudes, 1992). The fusion of the two different nuclei is known as karyogamy. The diploid nucleus formed then undergoes meiosis to produce four haploid spores (Martnez, 1998; Ramirez *et al.*, 2000; Larraya *et al.*, 2003).

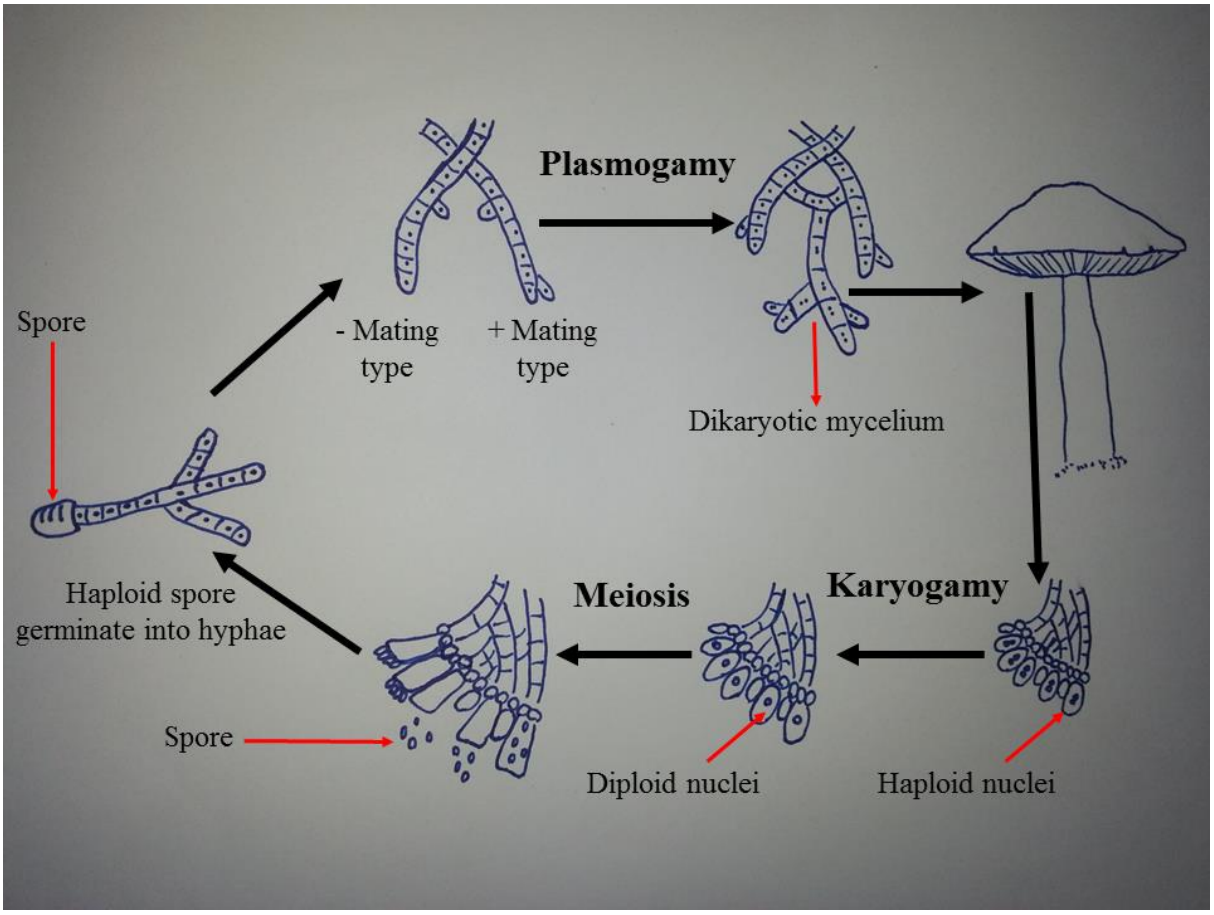

Figure 1: Life cycle of *P. sajor-caju*

2.5 Methods used to induce mutation on mushrooms.

Researchers mainly induced mutation on mushrooms for strain improvement that include higher productivity, more nutritional value, desirable colour and lower spore count. In the past, the attempt to mutagenically improve mushroom were done on *Coprinus cinerus* (Takemaru and Kamada 1972; Kanda *et al*.1989), *Pleurotus ostreatus* and *Pleurotus pulmonarius* (Imbernon and Labarere, 1989), and *Agrocybe cylindracea* (Murakami 1993; Kiuchi 1998).

The two methods used to induce mutation on mushrooms can be divided into physical and chemical method of mutagenesis. Physical method mainly involved the use of ionizing radiation such as ultraviolet radiation, X-rays and gamma rays. Example of mutated mushroom that has been generated in the past using ionizing radiation was *Agaricus bisporus* (Elliot, 1982).

On the other hand, chemical method involves the use of chemicals mutagens such as Ethyl Methanesulfonate (EMS) and Methyl Methanesulfonate (MMS). EMS is widely used to induce mutation in plants (Talebi, Talebi, & Shahrokhifar, 2012) by adding ethyl group to guanine bases through the process of ethylation consequently causes the alkylated G to pair wrongly with T consequently leading to G/C-A/T transitions (Bhat *et al.,* 2007). MMS modify DNA through the addition of methyl group at nucleophilic sites on DNA bases particularly guanine and adenine (Wyatt & Pittman, 2006), this process is known as methylation process and it is believed that this action can stop the function of replication folks which helps to unwind the double stranded DNA (Lundin *et al.*, 2005).

Chapter 3.0: Materials and Methods

3.1 Monokaryotic strain screening and hybridization

3.1.1 Source of spores and monokaryotic strain culture

The strains of *P. sajor-caju* were obtained from a mushroom cultivation site at Kampung Tanah Putih, Siburan Sarawak. Spore print of *P. sajor-caju* that were printed on clean papers were scrapped off and put into an Eppendorf tube containing one ml of sterile distilled water forming a suspension of spore. Each spore print was treated separately and the concentration of spore suspension was adjusted to 50 spores/200µL to be pipetted into PDA media. Spores that germinated were picked and cultured individually on PDA to obtained 200 monokaryotic mycelium denoted as MM001 – MM200 (Figure 2).

3.1.2 Characterization and morphological observation of monokaryotic strain

The 200 MM were observed and characterized into 3 main groups which were smooth, rough and scattered group with the appearance of the mycelium being the basis of classification (Figure 2). Under each main group, range of morphological variations, colour and colony diameter after 7 days of post inoculation (CD-7dpi) of the MM were observed.

3.1.3 Replication of MM

All different range of morphologies observed in each group were selected to reconfirm the observed traits based on an experiment with 3 replicates (Figure 2). The data on CD-7dpi obtained were analyzed using One-way Anova using SPSS software.

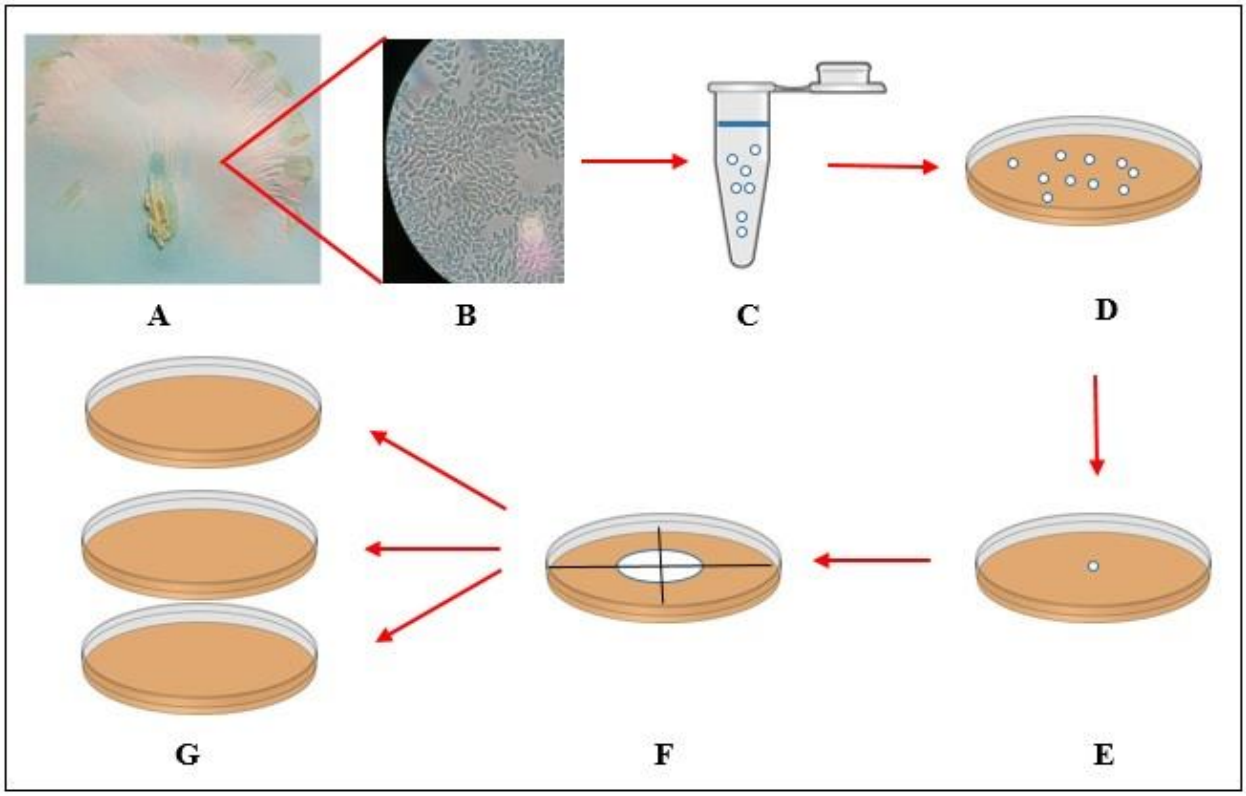

Figure 2: Source of spores and monokaryotic strain culture, characterization and morphological observation, and replication of monokaryotic mycelium. **A**: Spore Print of *P. sajor-caju* **B**: Spores observed under compound microscope **C**: Eppendorf tube containing 1ml sterile distilled water forming spore suspension **D**: 50 spores/200µL on PDA media **E**: 200 Spores cultured individually on PDA media (Monokaryotic mycelium obtained denoted as MM001-MM200) **F**: 200 MM characterized into 3 main groups which were scattered, rough and smooth. Under each main group, range of morphological polymorphism, colour and CD-7dpi of the MM were observed. **G**: 3 Replicates to reconfirm the observed traits

3.1.4 Selection and inoculation of desirable MM

Ten fast growing colony diameter (FGCD-7) and ten slow growing colony diameter of MM after 7 days of post inoculation (SGCD-7) selected randomly from 200MM were subjected to two point inoculation. The two point inoculations of the selected MM were performed with every possible combination without repetition. Pairs of the MM were placed 3 cm apart from each other in a Petri dish containing PDA (Figure 3). The plate was placed in room temperature until the two mycelia formed a contact zone.