STRAIN IMPROVEMENT OF *Pleurotus giganteus* (BERK.) KARUNARATHNA & K. D. HYDE VIA CONVENTIONAL MATING TECHNIQUES

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STRAIN IMPROVEMENT OF *Pleurotus giganteus* (BERK.) KARUNARATHNA & K.D. HYDE VIA CONVENTIONAL MATING TECHNIQUES

ABSTRACT

Recently, a commercial strain of *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde from China has been cultivated in Malaysia. *Pleurotus giganteus* was prone to insect infestation which led to heavy losses for farmers. The aim of this study was to generate new hybrids of *P. giganteus* that maybe resistant to pest and contamination. The commercial strain of *P. giganteus* from China (KLU-M 1227) and a wild strain from Malaysia (KLU-M 1391) were subjected to intraspecific breeding. Of the 56 combinations, 16 hybrids were obtained. All the 16 hybrids together with the parents were subjected to radial and linear growth study. Based on the median value, the hybrids were grouped into slow-growing with radial growth rate < 0.715 cm/day and linear growth rate < 0.295 cm/day, and fast-growing with radial growth rate ≥0.715 cm/day and linear growth rate ≥ 0.295 cm/day. The fast-growing strains with higher linear growth rate were tested for susceptibility to *Trichoderma saturnisporum*. The parents and three hybrids which were least susceptible to *T. saturnisporum* were cultivated. The parents and hybrids showed variation in the number of days for primordia formation and number of fruit bodies formed. Hybrid IH 32 gave the highest yield. The average biological efficiency of hybrid IH 32 for all the three generations was 479.66% higher compared to the parent KLU-M 1227 and 32.35% higher compared to the parent KLU-M 1391. The hybrid IH 32 had high nutritive values as it contained per 100g (dry weight): protein (16.3g), very high carbohydrate (6.8g), dietary fiber (2.73g), essential fatty acid, minerals, essential amino acids and vitamin B1 (7.8mg) and vitamin B3 (73.1mg).

Keywords: *Pleurotus giganteus*, hybrid, biological efficiency

PENAMBAHBAIKAN STRAIN *Pleurotus giganteus* (BERK.) KARUNARATHNA & K.D. HYDE MELALUI KAEDAH MENGAWAN KONVENSIONAL

ABSTRAK

Kebelakangan ini, satu strain komersial *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde dari China telah tanam di Malaysia. *Pleurotus giganteus* cenderung kepada serangan serangga dan ini mengakibatkan kerugian besar kepada para penanam cendawan. Tujuan kajian ini adalah untuk menghasilkan hibrid baru *P. giganteus* yang mempunyai kerentangan terhadap serangga dan kontaminasi. Strain komersial *P. giganteus* dari China (KLU-M 1227) telah dikacukkan dengan satu strain liar dari Malaysia (KLU-M 1391). Daripada 56 kombinasi kacukan, 16 hibrid telah diperolehi. Kajian kadar pertumbuhan linear telah dijalankan ke atas hibrid-hibrid baru itu bersama strain induk terbabit. Berdasarkan nilai median, kesemua hibrid baru itu dibahagikan kepada kategori kadar pertumbuhan rendah (kadar pertumbuhan < 0.295 cm/hari) dan kategori kadar pertumbuhan tinggi (kadar pertumbuhan ≥ 0.295 cm/hari). Hibrid baru dengan kadar pertumbuhan tinggi diujikan dari segi kerentangan terhadap *Trichoderma saturnisporum.* Strain induk dan tiga hibrid baru yang mempunyai kerentanan terendah terhadap *T. saturnisporum* ditanam. Strain induk dan hibrid menunjukkan variasi di antara bilangan hari bagi pertumbuhan putik cendawan dan jumlah jana buah yang dihasilkan. Hibrid IH 32 memberikan jumlah hasil jana buah tertinggi. Kadar purata kecekapan biologi bagi hibrid IH 32 untuk ketiga-tiga generasi itu adalah 479.66% lebih tinggi daripada strain induk KLU-M 1227 dan 32.35% lebih tinggi daripada strain induk KLU-M 1391. Hibrid IH 32 mempunyai nilai pemakanan yang tinggi dengan kandungan per 100g (berat kering) protein (16.3g), karbohidrat (6.8g), serat (2.73g), asid lemak penting, mineral, asid amino penting dan vitamin B1 (7.8mg) dan vitamin B3 (73.1mg).

Kata kunci: *Pleurotus giganteus*, hybrid, kecekapan biologi

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celsius
%	:	Percentage
cm	:	Centimetre
mm	:	Millimetre
g	:	Gram
w/w	:	Mass fraction
ml	:	Millilitre
PDA	:	Potato dextrose agar
ITS	:	Internal transcribed spacer
DNA	:	Deoxyribonucleic acid
rDNA	:	Ribosomal deoxyribonucleic acid
RFLP	:	Restriction fragment length polymorphism
RAPD	:	Random Amplification of Polymorphic DNA
AFLP	:	Amplified fragment length polymorphism
PCR	:	Polymerase Chain Reaction
BLAST:		Basic Local Alignment Search Tool

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CHAPTER 1: INTRODUCTION

In recent years, mushroom production has been increasing worldwide and introducing high income to the exporting countries such as Poland, China and United States (Food and Agriculture of the United Nations, 2010). According to the National Agro-Food Policy (2011-2020), Malaysia regards mushroom as one of the important food crop which possessed a high potential to contribute to food security and economic gains for the country (Ministry of Agriculture and Agro-Based Industry Malaysia, 2012). In Malaysia, *Pleurotus* species such as *Pleurotus pulmonarius* (Fr.) Quél., *Pleurotus floridanus* Singer and *Pleurotus cystidiosus* O.K. Mill. have a high demand among the consumers. As for *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde, this culinary and medicinal mushroom is still new to Malaysians.

The genus of *Pleurotus* is heterogeneous and mainly composed of edible mushrooms. It is popular for its high nutritional composition and medicinal properties (Khan & Tania, 2012; Stajić *et al*., 2005). Members of this genus are also named as oyster mushrooms due to the formation of oyster-shell like pileus which attached to the stalk (Rajarathnam *et al*., 1987).

The classification of *P. giganteus* has been revised a few times based on molecular information. It was formerly known as *Clitocybe maxima* or lowland Shiitake. Then, this species was revised as *Panus giganteus*, *Lentinus giganteus* and recently *Pleurotus giganteus* (Karunarathna *et al*., 2011) based on molecular data. In Malaysia, it has been known as "cendawan seri pagi" (morning glory mushroom).

Pleurotus giganteus consists of large fruit body and is easily recognized (Karunarathna *et al.*, 2011). It is grouped among the largest edible mushroom in the world (Klomklung *et al*., 2012). Wild *P. giganteus* is widely consumed in Sri Lanka and Thailand

(Karunarathna *et al.*, 2011; Klomklung *et al*., 2012). However, this species is still not commercialised in these two countries (Klomklung *et al*., 2012). Apart from these countries, occurrence of *P. giganteus* is reported in Vietnam, Australia (Pegler, 1983), China (Phan *et al.*, 2012; Zhishu *et al.*, 1993) and Oceania (Zhishu *et al*., 1993). *Pleurotus giganteus* is one of the popular cultivated mushrooms in China.

In Malaysia, Lee *et al.* (2006) reported that *P. giganteus* was consumed by the native residents in Perak and Selangor. Pegler (1983) also discovered this species in Sabah, Malaysia. In some indigenous villages in Negeri Sembilan and Selangor, Malaysia, wild *P. giganteus* has been found growing on soil and dead logs (Batin Long, personal communication, April 4, 2014; Batin Seturi, personal communication, July 2, 2013).

According to Mortimer *et al.* (2012), domestication of *P. giganteus* in China was accomplished in the 1980's. Recently, a variety of this species domesticated in China was grown in Malaysia. In China, this mushroom grows mainly during the summer season, which is from May to August (Dong *et al.*, 2010). However, there is another study stating that the production of *P. giganteus* is best in spring, summer and autumn seasons, i.e. from May to October (Baike baidu, 2017). According to Dong *et al.* (2010), the suitable temperature for fructification ranged from 23°C to 32°C. In Malaysia, it was reported that the best fruiting season for this mushroom species was from April to May at Fraser's Hill, where the temperature ranged from 17°C to 25°C (Thi *et al.*, 2011). Since this mushroom species is reported to grow at tropical to subtropical zone, *P. giganteus* has the potential be cultivated widely in Malaysia as a new commercial variety of edible mushroom.

Dong *et al.* (2010) stated the potential development future of *P. giganteus* as a commercial culinary mushroom. Karunarathna *et al.* (2011) also deduced that cultivation of *P. giganteus* in Thailand may introduce high income mushroom industry.

This species has a high content of magnesium, potassium, amino acids, cobalt, iron and calcium which may benefit human health (Phan *et al*., 2014). Furthermore, *P. giganteus* has been reported to have medicinal properties such as hepatoprotective (Khan & Tania, 2012; Wong *et al.*, 2012), stimulate neurite outgrowth and mitigating neurodegenerative diseases (Phan *et al*., 2012), reduce isolated skeletal muscular fatigue (Dong *et al*., 2010; Huang *et al.*, 2005), anticancer, antimicrobial, antioxidant (Khan & Tania, 2012) and inhibit growth of *Candida* species (Phan *et al*., 2013).

In China, the biological characteristics, nutritional value and cultivation methods of *P. giganteus* had been studied since year 1990 (Dong *et al*., 2010). Whereas in Malaysia, documentation and mating studies of this species are still very limited. Klomklung *et al*. (2012) reported that this mushroom species took a long time to fruit and yields were low, which may contribute to a higher market price compared to other mushroom species. Kothe (2001) elucidated the importance of mating studies and breeding programs to generate improved strains that can produce higher yields and had a high resistance to contamination. Since it is starting to gain popularity worldwide, *P. giganteus* exhibits important value for research and commercial exploitation.

In Malaysia, *P. giganteus* has not been widely commercialised so far. Recently, it was cultivated at NAS Agro Farm, Sepang (Mr. Nabil, personal communication, April 2, 2013). However, this species was reported to form long coiling stipes in substrate bag. After harvest, the stipes which remained inside the mushroom bags attracted insects and pests, leading to serious contaminations and loss in yields. This led to heavy losses to the growers. Hence, the aim of this study was to develop new intra strain hybrids of *P. giganteus* for commercial exploitation via a breeding programme to obtain better quality strains resistant to pest and diseases.

The main objectives of this study were:

a) to investigate the mating compatibility between *P. giganteus* strains from different origins.

b) to study the physiological characteristics including growth rates, yield potentials and contamination resistance of the parents and hybrids of *P. giganteus*.

c) to optimise the cultivation and analyse the biological efficiencies of the selected hybrids.

d) to compare the nutritional profiles of the parents and selected hybrids of *P. giganteus.*

CHAPTER 2: LITERATURE REVIEW

According to Guzmán (2000), difficulties in the identification of *Pleurotus* spp. taxonomy has aroused the attention of researchers worldwide. A confusion of the taxonomy of *Panus-Lentinus-Pleurotus* species has been a contradiction for a long time (Hibbett *et al.*, 2008; Hibbett & Thorn, 1994; Hibbett & Vilgalys, 1991; Hibbett & Vilgalys, 1993; Phan *et al.*, 2012; Redhead & Ginns, 1985). Pegler (1983) reported that *Lentinus giganteus* possesses many structures which are deviated from the genus of *Lentinus*. *Lentinus giganteus* is similar to some *Pleurotus* species as it has distinct lamella edge with broad, differentiated lecythiform cheilocystidia and it has soft basidiomes with short life span (Karunarathna *et al.*, 2011).

Morphological tools are commonly used to classify the mushrooms. However, Bao *et al.* (2004) clarified that factors such as climate, environmental circumstances and cultivation substratum can cause the variation of morphological characteristics in higher fungi. Karunarathna *et al.* (2011) documented the transfer of *Lentinus giganteus* to *Pleurotus giganteus* using phylogenetic ITS-1-5.8S-ITS2 rDNA sequence data. In the molecular study of Karunarathna *et al.* (2011), the Chinese and Thai *P. giganteus* strains had high similarity and might have diverged from the Sri Lankan species due to geographical barriers. Karunarathna *et al.* (2011) observed micro-morphological differences between the *P. giganteus* collections from Sri Lanka, Thailand and China and suggested to have more collections for taxonomy confirmation. Apart from the molecular techniques, biochemical tests and mating compatibility tests are also performed to determine the taxonomy of *Pleurotus* spp. (Bao *et al*., 2004).

Pleurotus giganteus (Berk.) Karunarathna & K.D. Hyde, documented by Mortimer *et al*. (2012), grows solitary most of the time, but can also be found growing in groups on

the ground (Figure 2.1). This species is saprobic, whereby it decomposes buried woody substrates (Klomklung *et al*., 2012; Mortimer *et al*., 2012). *Pleurotus giganteus* is distributed in lowland and mountain forest up to 3000 meters above sea level (Mortimer *et al*., 2012). *Pleurotus giganteus* has a strongly convex to applanate pileus which is slightly depressed in the centre. Upon maturation, the shape of this species is funnel-like and can go up to 35 cm in diameter and 28 cm in height (Mortimer *et al*., 2012). While Karunarathna *et al*. (2011) recorded the diameter measurement of pileus ranges between 6 cm to 31 cm. The occurrence of wild *P. giganteus* was reported in several regions of China, i.e. Guangdong, Fujian, Hunan, Hainan and Zhejiang (Cai, 2013).

Figure 2.1: *Pleurotus giganteus* growing in natural habitat. Bar A = 5.0 cm. (Photo by Azliza Anuar, 2016)

There are many molecular techniques being applied by researchers in fungal identity verification, for instance restriction fragment length polymorphism (RFLP), random

amplified polymorphic DNA (RAPD), small subunit ribosomal deoxyribonucleic acid (SSU rDNA) and internal transcribed spacer ITS1-5.8S rDNA-ITS2 region sequence analysis (Ro *et al*., 2007). Restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) had been carried out in genotypic identification of *Pleurotus eryngii* complex and *Pleurotus ferulae* (Urbanelli *et al*., 2007). Jasalavich *et al.* (2000) used primer ITS1-F (CCT GGT CAT TTA GAG GAA GTA A) and ITS4 for PCR amplification in one of his researches as he clarified that primer ITS1-F is specific for higher fungi.

Pleurotus giganteus possesses a tetrapolar mating behaviour (Dong *et al*., 2010). Previous studies indicated that *Pleurotus* spp. had a typical tetrapolar mating system (Eugenio & Anderson, 1968; Kothe, 2001; Larraya *et al*., 2001; Roxon & Jong, 1977). Determination of mating-type genes is essential as these genes control the formation of a dikaryon from two monokaryotic strains (Kothe, 2001). According to Lee *et al.* (2011b), tetrapolar mating system possesses two mating-type loci. The loci contain multi-allelic genes which can result in a mating frequency exceeding 25% (Brown & Casselton, 2001). Four types of spores (AxBx, AxBy, AyBx, AyBy) are formed during meiosis. Among the mating types, AxBy and AyBx, AxBx and AyBy are compatible (Kothe, 2001). Gupta *et al.* (2011) stressed on the importance of hybridization using non-fertile homokaryotic strands in order to produce better genes. To start off hybridization, single basidiospores are the main meiotic products for the very first stage (Gupta *et al*., 2011).

Mating is the first step to initiate the sexual development of mushroom-forming fungi (Van Peer *et al*., 2011). Mating studies have been used to outline *Pleurotus* species for more than 25 years (Anderson *et al.*, 1973; Hilber, 1982). The mating process introduces the desirable characteristics from different strains into an existing germplasm (Gharehaghaji *et al.*, 2007). There are several methods used in mushroom breeding, for instance protoplast fusion, basidiospore chemical mutagenesis, basidiospores mating and

molecular genetic transformation (Lee *et al*., 2011b). Chakraborty and Sikdar (2007) raised somatic hybrids between *Volvariella volvacea* and *P. florida* through protoplast fusion. Genetic incompatibility was indicated by retarded growth of hybrid cultures, absence of clamp and basidiocarp formation, as well as secretion of white exudates and phenolic substances (Chakraborty & Sikdar, 2007). Changes in some *V. volcacea* chromosomes during spawn run might have triggered the genes to form clamps and basidiocarps in two hybrid lines (Chakraborty & Sikdar, 2007). An alternative way of mushroom breeding is the application of Buller phenomenon, a mechanism outcrossing a homokaryon and heterokaryon as described by Callac *et al.* (2006). Baral *et al*. (2017) developed *P. flabellatus* hybrids with higher nitrogen content and biological efficiency compared to the parents using conventional intraspecific mating techniques. Among these methods, conventional breeding is still widely adopted whereas the safety of genetic transformation method application is not guaranteed. Unintentional effects may be resulted from genetic engineering experiments which involve artificial method of recombinant DNA (Holdrege, 2008). Breeding, elucidated by Peberdy *et al.* (1993), is able to enhance multigenic properties such as gross nutritional value and growth rate.

The green mold, *Trichoderma* is one of the major contaminants of mushroom crops which limits commercial production and cause great loss to the mushroom growers. *Trichoderma harzianum* Rifai and *Trichoderma polysporum* (Link) Rifai produced antifungal substances and lytic enzymes which killed shiitake mushrooms (Seaby, 1998; Ulhoa & Perberdy, 1992). The occurrence of *Trichoderma* green mold had been reported attacking *Pleurotus* mushroom industries worldwide, including Hungaria (Hatvani *et al*., 2007), Korea (Kim *et al.*, 2000; Park *et al.*, 2004), Italy (Woo *et al*., 2004). According to Baars *et al*. (2013), *Trichoderma* spp. such as *T. saturnisporum* Hammill, *T. longibrachiatum* Rifai and *T. asperellum* Samuels, Lieckf. & Nirenberg are among the strong competitors of mushrooms.

According to Sonnenberg *et al.* (2005), a breeding program of *Agaricus bisporus* (J.E. Lange) Imbach was initiated in year 2002 in Netherlands, aimed to produce hybrids which can resist the two major pathogens of mushrooms, i.e. *Verticillium fungicola* (Preuss) Hassebr. and *Trichoderma aggressivum* Samuels & W. Gams. Apart from resistance to pathogens, characteristics such as pileus color, temperature tolerance, number of fruit bodies and flushing pattern can be improved by introducing new traits from wild collection into a commercial line.

Tongon and Soytong (2013) documented that the optimum condition for *P. giganteus* mycelial growth is at pH 7. While Klomklung *et al.* (2014) reported that this strain can grow in either acidic, neutral or alkaline conditions, but best at pH 5.0 to 7.0. The optimum temperature of the mycelial growth of this gigantic mushroom ranges from 15-35°C (Tongon & Soytong, 2013). Another study conducted by Klomklung *et al.* (2012) elucidated that the radial mycelia growth performed well at 25°C, 30°C and 20°C but at 35°C, the mycelium hardly grew. The mycelia can grow well on potato dextrose agar (Kumla *et al.*, 2013) and soy bean agar (Klomklung *et al*., 2014).

Soil casing is able to promote primordial production of mushroom (Noble *et al*., 2003; Kumla *et al*., 2013). In China and Thailand, *P. giganteus* is cultivated using soil casing method, which is said to be important for the fruiting stage to promote better yield (Ruan *et al*. 2013; Klomklung *et al*., 2012). Van Gerwen (2006) postulated that the mushroom size is highly impacted by the type of casing soil applied which indirectly affects the commercial value of the mushroom.

Apart from its medicinal properties, mushrooms are popular as a rich source of essential nutrients such as protein, fibre, carbohydrate and vitamin. *Pleurotus giganteus*, as reported by Qiu (2015), contains aspartate, crude protein and amino acids which are important for human body. Phan *et al.* (2014) also elucidated that this mushroom has

minerals and elements which are crucial to human health and thus it has potential to be a food source of well-balanced diet.

According to Valverde *et al.* (2015), *P. giganteus* contained higher amount of protein but also higher fatty acids content compared to *Pleurotus eryngii* and *Pleurotus ostreatus*. Since the protein level contained by mushrooms is higher than that of most of the vegetables, mushrooms are said to be a good choice for vegetarians since they also consist of essential amino acids for human health (Valverde *et al*., 2015).

CHAPTER 3: MATERIALS AND METHODS

3.1 Fungal strains

Two strains of *Pleurotus giganteus* from different origins were used in this study (Table 3.1). *Pleurotus giganteus* strain KLU-M 1227 from China was obtained from NAS Agro Farm. Whereas the wild strain KLU-M 1391 was found growing caespitose on soil within the compounds of University of Malaya, Kuala Lumpur. *Trichoderma saturnisporum* used in the susceptibility test was obtained from the Biotechnology laboratory of University of Malaya and identified by Mushroom Research Centre. All the fungal strains were maintained in Mycology and Plant Pathology Laboratory, Institute of Postgraduate Studies, University of Malaya, Kuala Lumpur, Malaysia. Macro-morphology characteristics of the commercial and wild *P. giganteus* were described and compared between each other. The colors of both the commercial and wild *P. giganteus* were described based on Kornerup and Wanscher (1963).

Table 3.1: *Pleurotus giganteus* strains collected from different origins were used in this study

Code	Place of origin	Type of strain
KLU-M 1227	China	Commercial
KLU-M 1391	Malaysia	Wild

3.2 Fungal identification

3.2.1 DNA extraction

DNA extraction was carried out using the E.Z.N.A^{TM} Forensic DNA Isolation kit (Omega Bio-tek, Inc.) with slight modification. Fresh mycelia was picked aseptically and transferred into a 2ml sterile microfuge tube. A total of 200µl of STL Buffer was added into the microfuge tube then it was vortexed to mix thoroughly. The tube was incubated in waterbath at 55°C. The tube was vortexed every 2 min and this was continued for 15 min. 25µl of OB Protease Solution was then added to the tube and vortexed to mix well. The tube was incubated in 60°C waterbath and then vortexed every 5 min for 45 min. The tube was centrifuged and then 225µl of BL buffer was added. The tube was vortexed and incubated at 60°C for 10 min followed by a short spin of centrifuge and 225µl of absolute ethanol was added into the mixture. The tube was vortexed. A HiBind ® Mini Column was inserted to a 2ml collection tube provided with the DNA kit, then 100ml of 3M NaOH (equilibrium buffer) was added into the column and allowed to sit for 4 min. The entire sample was transferred to the column. The tube was centrifuged at 8,000 x g for 1 min. The collection tube was discarded and replaced with new one. A total of 500µl of HBC Buffer (pre-diluted with absolute ethanol) was added into the column. The tube was centrifuged at 8,000 x g for 1 min. The filtrate was discarded and 750µl of DNA wash buffer (pre-diluted with absolute ethanol) was pipetted into the column. The tube was then centrifuged at 8,000 x g for 1 min. The collection tube was discarded. The column was transferred to a new 2ml collection tube. Once again, 750µl of DNA wash buffer was pipetted into the column and the tube was centrifuged at 8,000 x g for 1 min. The filtrate was discarded and the collection tube was centrifuged at maximum speed for 2 min for drying purpose. The column was transferred into a 1.5ml microfuge tube. Then, 50µl of elution buffer was added into the microfuge tube. The tube was allowed to sit for 10 min at room temperature. After that, the tube was centrifuged at 8,000 x g for 1 min and 50 µl

of elution buffer was added. The tube was allowed to sit for another 10 min at room temperature. Then the tube was centrifuged at 8,000 x g for 1 min and kept in freezer.

3.2.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed to amplify the internal transcribed spacer (ITS) region. In this study, ITS1 and ITS4 were selected as target sequence for amplification. Amplifications were carried out in a 50µl reactions consisting 1x PCR buffer (*i-Taq*^{TM} *Plus*), 0.2mM deoxynucleotide triphosphate, 0.5mM of each of the appropriate primers, 1 unit of DNA Polymerase (*i-Taq*^{TM} *Plus*), 1µl of DNA template and appropriate amount of sterile distilled water. Primer pairs of ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) from Bioneer Corporation were used. Conditions for PCR amplification of ITS sequences were: initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, elongation at 72°C for 1.5 min and final extension at 72°C for 10 min (Chew *et al.*, 2015). PCR amplification products were purified using MEGAquick-spin^{TM} PCR then separated by 1% agarose gel electrophoresis in 1xTBE with RedSafe^{TM} Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc) at 100V for 60 min. DNA bands were visualised under UV light and photographed.

3.2.3 DNA sequencing

The DNA strands were sent out to MyTACG Bioscience Enterprise, Selangor for sequencing. The sequences were assembled using ChromasPro software (Technelysium Pty Ltd) and aligned using MEGA 6 (Tamura *et al.*, 2013). Sequences were compared to the sequences in database of BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The nucleotide sequences of KLU-M 1227 and KLU-M 1391 were compared to the BLAST results of its percentage identity and E-value.

3.3 Mating Studies

3.3.1 Isolation of single spores and monokaryon cultures

Monokaryons were obtained from a small piece of lamella selected by the spore drop technique (Petersen & Bermudes, 1992). The lamella was attached under the cover of potato dextrose agar (PDA) (Difco.) plate using vaseline. The agar plate was then sealed and incubated at a slanting angle at room temperature (26±2°C) for 24 hours. The lamella was then removed. The agar plate was further incubated at room temperature until the basidiospores germinated. The plate was observed under dissecting microscope for the germinated single spores. With the help of a modified fine inoculation needle, germinated single spores were transferred onto new PDA plates. The monokaryons on PDA were kept as stock cultures in sterile distilled water and refrigerated at 4°C (Castellani, 1968).

3.3.2 Mating of monokaryons from different strains

Fast-growing monokaryotic isolates of the two parents were selected for intraspecific mating. Agar plugs of each isolate was placed on PDA plates, 10 mm apart from each other. The plates were then incubated at room temperature until the mycelia made contact. The contact zone of the isolates were cut and transferred onto a new PDA plate (Bao *et al*., 2004). A piece of mycelium was picked from 10 days old culture using a needle, mounted on a slide and then checked for clamp connection under compound microscope. Hybrids were kept as stock cultures in sterile distilled water and refrigerated at 4°C (Castellani, 1968).

3.4 Radial growth study

Seven millimetre mycelial disc was inoculated in the centre of PDA prepared in a 90mm Petri dish. The plate was incubated at room temperature 26±2°C in dark condition

(Soytong & Asue, 2012). The diameter of the colony was measured every 2 days at two perpendicular directions. The agar media inoculated with the parents served as controls. This experiment was carried out in five replicates for each parent and hybrid tested. Mid-parent heterosis was used to compare the performance of hybrids in comparison to the parents (Gixhari & Sulovari, 2010) as shown below:

$$\text{Mid Parent Heterosis} = \frac{F1 - MP}{MP} \times 100$$

Where F1 = mean of the linear growth rate of hybrid
MP = mean of the linear growth rate of the two parents

3.5 Linear growth study

Substrate consisting of 89% (w/w) sawdust, 10% (w/w) rice bran and 1% (w/w) calcium carbonate was prepared in glass race tubes (160 mm x 25 mm). The moisture content of the substrate was around 73.5±0.50%. The substrate (58.50g) was compressed to a 100 mm length in the tubes. The race tube was stoppered with non-absorbent cotton and then sterilised at 121°C for 15 mins. Mycelial disc (1cm) was taken from the growing edge of a 10 days old culture. The mycelial disc was then transferred to the substrate surface. The tubes were incubated at 26±2°C in the dark. Visible mycelial extension was recorded at two perpendicular directions every 3 days up to day 15 (Straatsma *et al*., 1989). The substrates inoculated with parental strains were used as controls. This experiment was carried out in triplicates for each parents and hybrids. The parents KLU-M 1227 and KLU-M 1391 were used as control. Mid-parent heterosis was calculated by referring to Gixhari and Sulovari (2010).

3.6 Susceptibility of the parents and hybrids of *P. giganteus* to *T. saturnisporum*

The fast-growing hybrids were chosen from linear growth study and paired interaction test with *Trichoderma saturnisporum* was conducted. Seven millimetre mycelial disc was transferred from a 10 days old culture to approximately 10 mm from the edge of a PDA plate. Seven days after the inoculation, a 7 mm mycelial disc of a 2 days old *T. saturnisporum* culture was inoculated opposite to the test strain 10 mm from the edge of the PDA plate. The parents and hybrids of *P. giganteus* inoculated individually on the PDA plates served as control. Observations were made and photographs were taken every week up to the 4th week after *Trichoderma* inoculation. Three replicates were setup for each strain. Observations made on the 4th week after *Trichoderma* inoculation were compared to the observations made on the 1st week after *T. saturnisporum* inoculation. By referring to Lee *et al*. (2008), the competitive interactions were categorised into three types: deadlock, no further growth of the parents and hybrids of *P. giganteus* and *T. saturnisporum* after mycelial contact; highly susceptible, *T. saturnisporum* overgrew the parents and hybrids of *P. giganteus*; weakly susceptible, the parents and hybrids of *P. giganteus* overgrew *T. saturnisporum* (Figure 3.1).

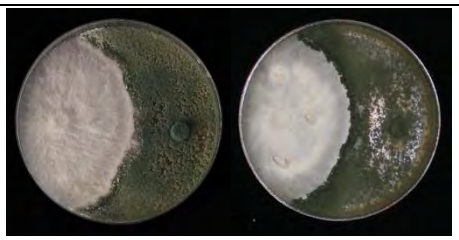
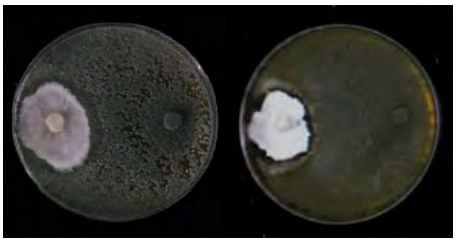
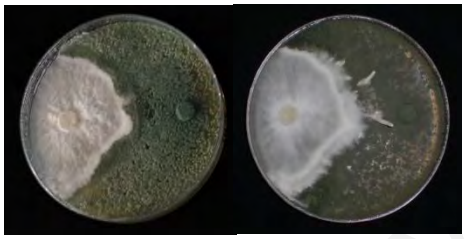
1st week 4th week	Symbol	Susceptibility
	±	**No further growth of the parent and hybrid strains of *P. giganteus* and *T. saturnisporum* after mycelial contact** **(Deadlock)**
	-	***T. saturnisporum* grew over the parent and hybrid strains of *P. giganteus*** **(High susceptibility)**
	+	**The parent and hybrid strains of *P. giganteus* grew over *T. saturnisporum*** **(Weak susceptibility)**

Figure 3.1: Different types of interaction between *P.giganteus* and *T. saturnisporum*

3.7 Cultivation of the parents and selected hybrids

Both the parents and selected hybrids of *P. giganteus* were cultivated on sawdust substrate via the conventional method practiced in mushroom farms with and without soil. Mushroom bags consisting of 73-74% of water, 89% of sawdust, 10% of rice bran and 1% of calcium carbonate were prepared in polypropylene bags. The substrate, 300g in weight, was packed tightly and a central hole was made with the help of a 50ml centrifuge tube. The bags were then stoppered with caps and sterilised at 121°C for 15 min. After sterilization, the bags were allowed to cool to room temperature for 24 hours. The bags were then inoculated with spawn prepared on wheat grains and were incubated in dark at 26±2°C. When the bag was 50% colonised by the mycelium, the wheat grains were removed from the substrate under axenic condition with a sterile spatula. In the conventional method, a week after the mycelia had completely colonised the substrate, the bags were transferred to mushroom house at Institute of Biological Sciences, University of Malaya. The relative humidity above 70% and temperature 29±6°C was maintained in the house. The caps of the mushroom bags were removed for fruiting to occur. For the soil casing method, garden soil mixed with coconut husks was bought from a nursery at Sungai Buloh, Selangor and sterilised at 121°C for 15 min. The pH value of soil was measured using a pH meter. The soil was slightly moistened with tap water and then layered on top of the substrate for about 2 cm height. The bag opening was folded down until it was 3 cm above the soil layer. Three replicates were set up for each of the parents and selected hybrids for each cultivation method. After comparing the production results of conventional cultivation method and soil casing method, soil casing method was then carried out for three generations (namely F1, F2 and F3) with ten replicates each for the parents and selected hybrids. For each generation, fruit bodies of mushroom from that generation was sub-cultured onto PDA plate and then the mycelial culture was transferred to wheat grain for spawn run and then mushroom bags for cultivation.

Data such as the time required for spawn completion, duration from stimulation to primordia emergence, duration from stimulation to harvesting, total weight of fruit bodies, pileus diameter, stipe length and stipe thickness were recorded (Klomklung *et al*., 2012). The fruit bodies were harvested before maturity (the edge of the pileus were wavy). Color descriptions of the fruit bodies were recorded by referring to Kornerup and Wanscher (1963). Total biological efficiency was calculated with the formula as below:

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

3.8 Nutritional analysis of the parents and selected hybrid

Nutritional analysis of the hybrid which had the best biological efficiency was tested. The fruit bodies of *P. giganteus* parents and the selected hybrid were freeze-dried and analysed based on AOAC methods (DXN Laboratory). Nutrient contents tested included carbohydrate, protein, cholesterol, dietary fiber, total fat, saturated and unsaturated fats, vitamin, sugar, amino acid, mineral and lipid.

3.9 Statistical analysis

Analysis of variance (ANOVA) was performed using the statistical package for the Social Sciences (SPSS) statistical software version 22 followed by Duncan's multiple range test for significant differences ($p<0.05$) between treatments.

CHAPTER 4: RESULTS & DISCUSSION

4.1 Fungal identification

The commercial strain of *P. giganteus* (KLU-M 1227) had scaly and regular pilei, moderately indented in the center. The diameter of the pilei ranged from 21-110 mm. The stipe length ranged from 32-80 mm and the stipe thickness ranged from 4-15 mm. The pilei color was yellowish brown (5F5) in the center and towards the margin yellowish white (3A2) based on Kornerup and Wanscher (1963).

The wild strain of *P. giganteus* (KLU-M 1391) was found growing caespitose on soil within the compounds of University of Malaya, Kuala Lumpur. The basidiocarps had matured and attracted insects. The pilei were regular and even, slightly depressed in the centre and the pilei surfaces were smooth with scales at the margin. The pilei margins were slightly uplifted due to over-maturation. The diameter of the pilei ranged from 35.5-85.0 mm. The stipe length ranged from 47-90 mm while the stipe thickness was 2.5-7.5 mm. The pilei color was yellowish brown (5E5) in the center and towards the margin light brown (6D6) based on the Color Handbook by Kornerup and Wanscher (1963). The lamella were pale yellowish white (2A2), subdistant spacing, decurrent with narrow breadth. The stipe ranged from 47-90 mm in length, 3-8 mm thick, color varied from greyish brown (6F3) to orange white (5A2), with solid interior and fibrous texture.

From the preliminary observation, the wild strain (KLU-M 1391) had short stipe above the ground and the pileus color was darker than that of the commercial strain (KLU-M 1227) by referring to the Color Handbook by Kornerup and Wanscher (1963). Whereas KLU-M 1227 growing in the polypropylene mushroom bag formed a long stipe coil in the space between polybag and substrate. Besides these features, no other considerable differences were noticed between the fruit bodies of these two strains. The morphological characteristics of KLU-M 1391 matched the fungal descriptions by Karunarathna *et al.*

(2011) whereby the pileus was slightly depressed in the centre with scaly surfaces, stipe length ranged from 50–90 mm, stipe thickness 7 mm and the diameter of pileus was 60-85 mm.

As environmental factors such as temperature, light intensity, carbon dioxide concentration can affect the morphological characteristics of fungi (Bao *et al*., 2004), molecular analysis was performed to verify the strain identity. According to the NCBI (National Center for Biotechnology Information) blast results shown in Table 4.1, both the parents KLU-M 1227 and KLU-M 1391 were best matched as *Pleurotus giganteus*, with expected number of chance alignments (E value) 0.0.

Table 4.1: Blast results for nucleotide sequence of KLU-M 1227 and KLU-M 1391. Only the top ten results with the best scores are shown

Parent	Name	Query Cover(%)	E value	Identity percentage (%)	Accession number	Country of origin
KLU-M 1227	*Pleurotus giganteus* MRNo556	99	0.0	100	LC068800.1	Thailand (unpublished)
	Panus giganteus P6	99	0.0	99	HM245789.1	China (unpublished)
	Panus giganteus XY3	98	0.0	99	HM245782.1	China (unpublished)
	Pleurotus giganteus MFLU08-1371	100	0.0	99	KP120919.1	Thailand (Karunarathna *et al*., 2011)
	Panus giganteus ZZ	98	0.0	99	HM245788.1	China (unpublished)
KLU-M 1391	*Pleurotus giganteus* FRI 849	97	0.0	98	KX018294	Malaysia (Karunarathna *et al*., 2016)
	Lentinus giganteus K(M)57571	97	0.0	97	JN255250.1	Malaysia (unpublished)
	Pleurotus giganteus MEL:2382605	96	0.0	98	KP012913.1	Australia (unpublished)
	Pleurotus giganteus MRNo556	96	0.0	97	LC068800.1	Thailand (unpublished)

4.2 Mating studies of *P. giganteus*

Basidiospores of the parents took 7-10 days to germinate and produce the monokaryon cultures (Figure 4.1). A total of 14 fast-growing monokaryons of KLU-M 1227 were selected to mate with 4 monokaryons isolated from the wild KLU-M 1391. To confirm the monokaryotic cultures, the mycelium was checked under compound microscope for the absence of clamp connections. In this study, 16 hybrids were obtained out of 56 combinations (Table 4.2), with a success rate of 28.6%. Clamp connections were observed as an indicator of monokaryons compatibility and dikaryon formation (Figure 4.2).

This study involved the crossing of basidiospores isolated from commercial and wild strains. This is a common breeding strategy which incorporates quantitatively inherited traits of a wild line into an existing commercial line (Sonnenberg *et al*., 2005). The mating frequency in this study was 3.6% higher than the expected mating frequency of tetrapolar basidiomycetes reported by Giraud *et al*. (2008), which was 25%. However, there were studies showing higher mating frequency in tetrapolar basidiomycetes such as *P. tuberregium* (Fr.) Singer with 55-84% mating frequency reported by Isikhuemhen *et al*. (2000), 92% of mating frequency resulted by intraspecific mating of *P. ostreatus* reported by Kumara and Edirimanna (2009), and 26.3% of mating compatibility of *Hypsizygus marmoreus* (Peck) H.E. Bigelow reported by Lee *et al.* (2011b). In the taxonomic study by Stajić *et al.* (2005), geographical isolation was said to cause genetic differences in *Pleurotus* strains. Van Peer *et al.* (2011) also added that geographical distribution caused differences on the multi-allelic nature of mating genes. However, Isikhuemhen *et al*. (2000) and Lee *et al*. (2011a) showed that tetrapolar mating using intrastock pairings from different geographical origins could achieve high mating compatibility frequency up to 70% or more. Therefore, there is possibility that the mating frequency in this study can be improved by increasing the number of basidiospores from the parental strains.

Larsen *et al*. (1992) reported that clamp connections have become important as an *in vitro* indicator for dikaryon formation. Nuclei exchange and migration resulted in clamp cell formation (Brown & Casselton, 2001). Following nuclear division and septation, clamp cell fusion occurs and results in a sexually competent dikaryon (Brown & Casselton, 2001; Gharehaghaji *et al*., 2007).

Table 4.2: Crossing between mononokaryons of *P.giganteus* (after 10 days of incubation at room temperature 26°±2°C)

KLU-M 1227 / KLU-M 1391	01	06	19	22	23	03	07	02	08	11	15	27	29	30
01	-	-	-	-	-	-	-	+	+	+	+	+	+	+
02	-	-	-	-	-	+	+	+	+	+	+	+	+	+
04	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ : formation of clamp connections; - : no clamps formed.

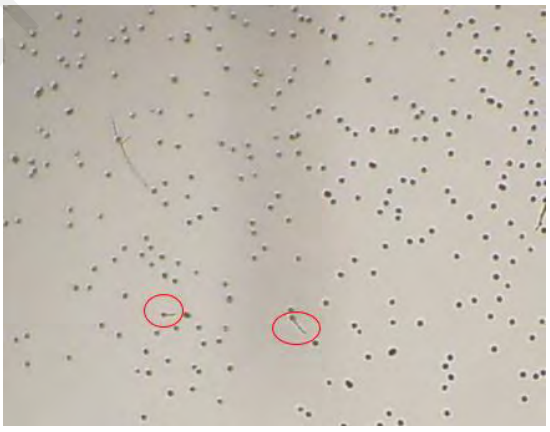


Figure 4.1: The germinated basidiospores (in red circles) on a 7-days old PDA culture

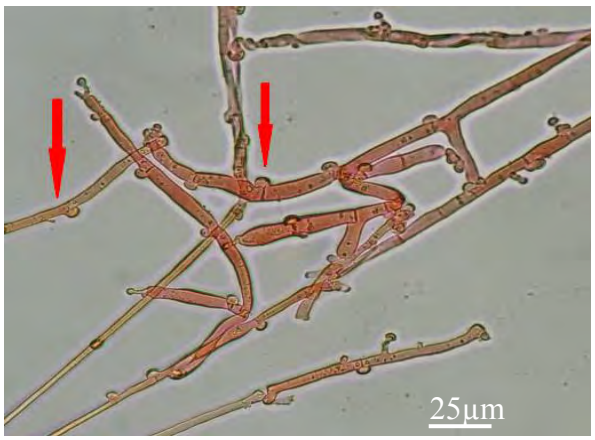


Figure 4.2: Clamp connections (shown by arrows) formed by *P. giganteus*, magnification 400x

4.3 Radial growth study of the parents and all the hybrids of *P. giganteus*

The parents KLU-M 1227 and KLU-M 1391 had low radial growth rates which were 0.40±0.05 cm/day and 0.28±0.01 cm/day respectively. Both the parents took 16 to 20 days to fully colonise the 90mm PDA plates. In contrast, 68% of the hybrids fully covered the 90 mm petri dishes on day 12 of the experiment. As shown in Figure 4.3, the hybrids IH 29 and IH 32 had the highest radial growth rates, which were 0.83±0.06 cm/day and 0.85±0.07 cm/day respectively. The hybrids were grouped into slow-growing (growth rate < 0.715 cm/day) and fast-growing (growth rate ≥ 0.715 cm/day) based on the median (M = 0.715) (Figure 4.3). All tested hybrids grew at least 42% faster than the parents on PDA based on mid-parent heterosis calculation (Table 4.3). The parents and hybrids were similar in morphological characteristics. White, fluffy, filamentous mycelia with raised elevation was observed on the surface of the parents and hybrids on PDA plates.

Nutrient medium serves as a captive environment which provides the basic supplements for fungal growth. PDA was used as the growth medium in this study because it was reported as the best media for optimum growth of *P. giganteus* (Kumla *et al*., 2013). Mycelium growth rate on media can be affected by temperature, pH of media and light

intensity (Klomklung *et al*., 2012). However, Singh and Kamal (2011) reported that radial mycelial growth showed significant negative correlation with mushroom morphological traits such as gill and stipe size. Thus, radial growth rate was not used as a parameter to select the best hybrid.

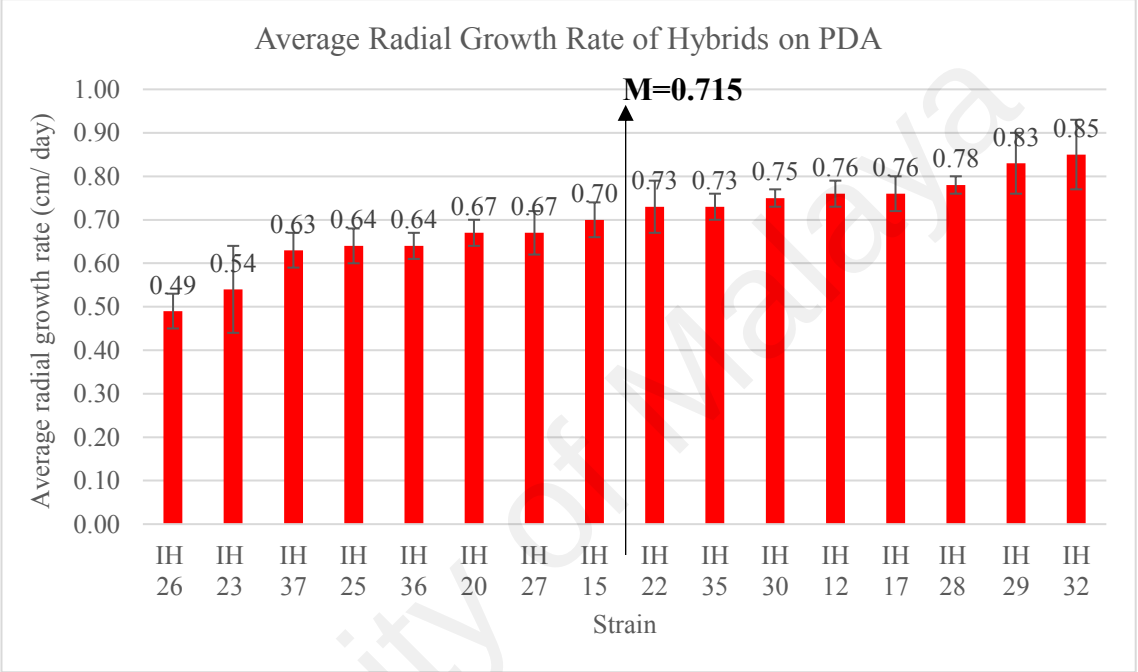


Figure 4.3: Average radial growth rate of hybrids of *P. giganteus* on PDA at 26±2°C. Average radial growth rate of the parent KLU-M 1227 was 0.40 cm/day and the parent KLU-M 1391 was 0.28 cm/day. Value means of 3 replicates (p<0.05)

Table 4.3: Mid parent heterosis of the hybrids based on the radial growth rate

Strain	Mid Parent heterosis
IH 26	42.03
IH 23	56.52
IH 37	82.61
IH 25	85.51
IH 36	85.51
IH 20	94.20
IH 27	94.20
IH 15	102.90
IH 22	111.59
IH 35	111.59
IH 30	117.39
IH 12	120.29
IH 17	120.29
IH 28	126.09
IH 29	140.58
IH 32	146.38

4.4 Linear growth study of the parents and selected hybrids of *P. giganteus*

Both parents and selected hybrids took 24 to 49 days to fully colonise the substrate in the race tubes. Among the hybrids tested, 37.5% of them formed primordia in the race tube even before the mycelium had fully colonised the substrate (Figure 4.4). As shown in Figure 4.5, the fastest-growing hybrids based on the linear growth rate were IH 15 and IH 32. Both of these hybrids had a linear growth rate at 0.33±0.00 cm/day. Whereas the parents had low linear growth rates, of 0.18±0.00 cm/day (KLU-M 1227) and 0.19±0.00 cm/day (KLU-M 1391), which were 83% and 73% lower than both IH 15 and IH 32 respectively. The hybrids were grouped as slow-growing (growth rate < 0.295 cm/day) and fast-growing (growth rate ≥ 0.295 cm/day) based on the median (M = 0.295) (Figure 4.5). All the tested hybrids grew at least 13% faster than the parents on the substrate based on mid-parent heterosis calculation (Table 4.4).

Linear growth study was carried out to compare the mycelial growth rate between hybrids in a substrate composition practiced in the mushroom farms in Malaysia.

According to Kumara and Edirimanna (2009), the genetic differences inherited within different strains may contribute to variation in mycelium colonization rate of a medium. In this study, the hybrids showed a significant (p<0.05) difference in linear growth rates (Figure 4.5) but no variation was observed in the mycelium density on the substrate (Figure 4.6). This observation was similar to that reported by Mensah and Obodai (2014) that not all the fast-growing strains had dense mycelium growth. Furthermore, Mensah and Obodai (2014) reported that mycelia growth rate and density depend on the media used.

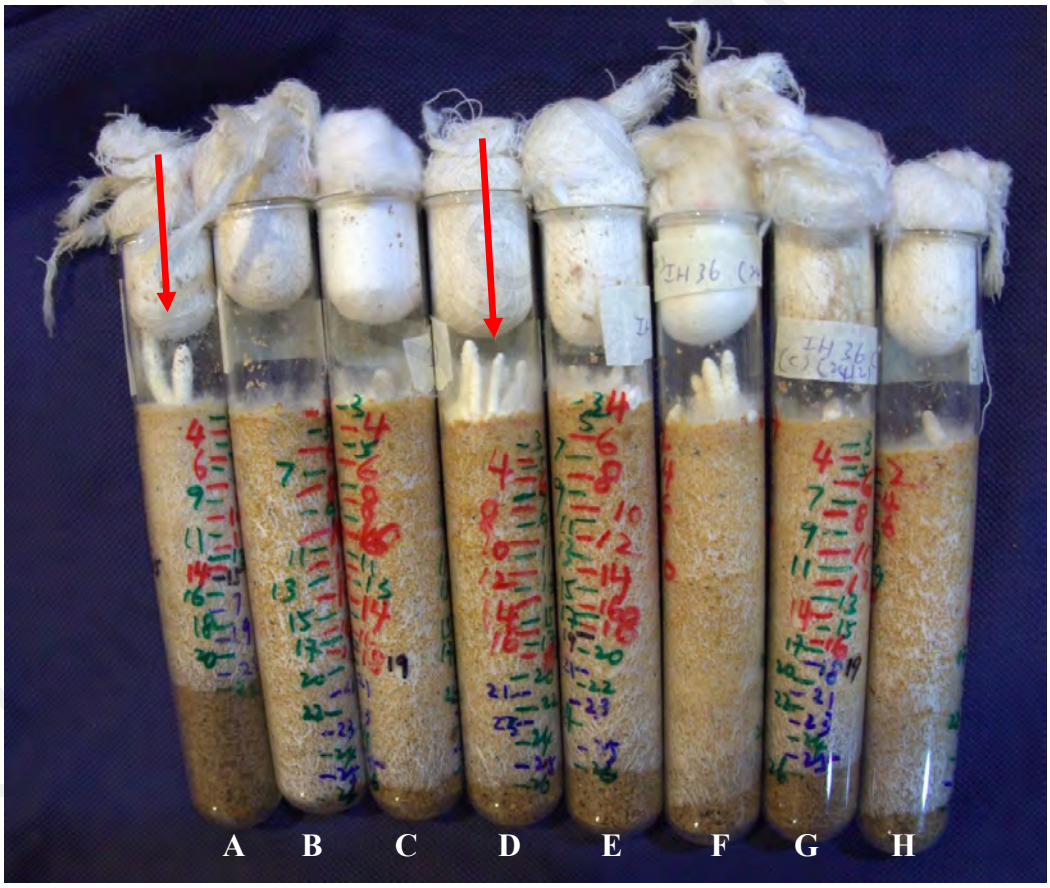


Figure 4.4: Primordia formed by hybrids: (A) IH 35 (replicate 1); (B) IH 32 (replicate 1); (C) IH 22 (replicate 1); (D) IH 22 (replicate 2); (E) IH 15 (replicate 1); (F) IH 36 (replicate 1); (G) IH 36 (replicate 2); (H) IH 30 (replicate 1); on day 26 during mycelium colonization of sawdust substrate, incubated at room temperature 26±2°C

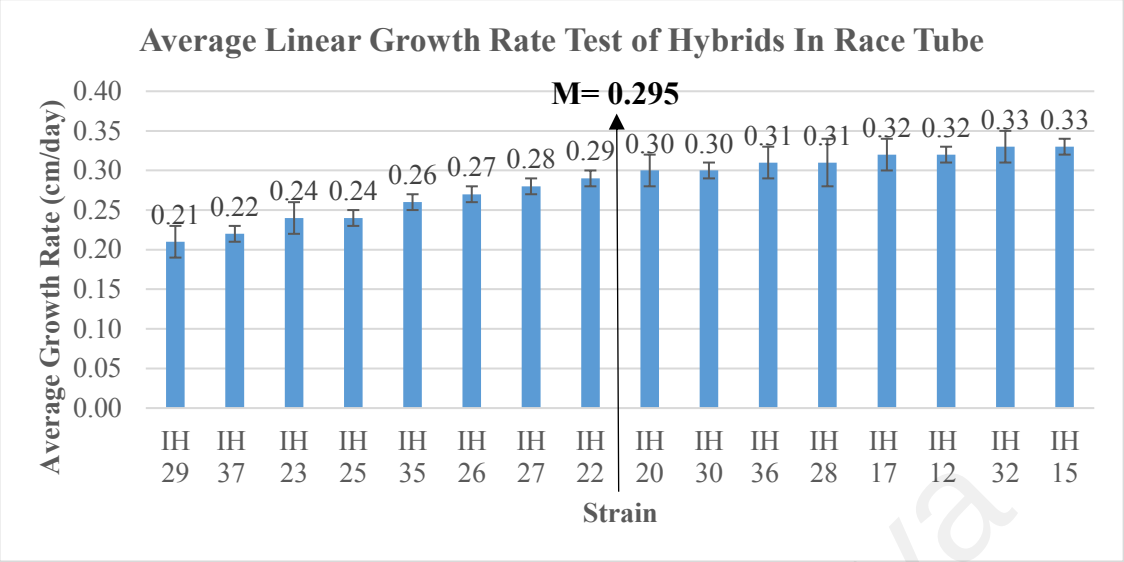


Figure 4.5: Average linear growth rate of hybrids of *P. giganteus* [at 26±2°C in dark condition; substrate: 89% (w/w) sawdust, 10% (w/w) rice bran, 1% (w/w) calcium carbonate]. Average linear growth rate of the parent KLU-M 1227 was 0.18 cm/day and the parent KLU-M 1391 was 0.19 cm/day. Value mean of 3 replicates (p<0.05)

Table 4.4: Mid parent heterosis of the hybrids based on the linear growth rate

Strain	Mid-parent heterosis
IH 29	13.51
IH 37	18.92
IH 23	29.73
IH 25	29.73
IH 35	40.54
IH 26	45.95
IH 27	51.35
IH 22	56.76
IH 20	62.16
IH 30	62.16
IH 36	67.57
IH 28	67.57
IH 17	72.97
IH 12	72.97
IH 32	78.38
IH 15	78.38

Figure 4.6: Linear growth study of the parents and hybrids of *P. giganteus*: (A) KLU-M 1227; (B) KLU- M 1391; (C) IH 12; (D) IH 17; (E) IH 30; (F) IH 29; (G) IH 35; (H) IH 28; (I) IH 36; (J)IH 37; on day 24 during mycelium colonization of sawdust substrate, incubated at room temperature 26±2°C)

In this study, the average radial growth rate on PDA and linear growth rate on substrate exhibited by the hybrids of *P. giganteus* did not correlate (Table 4.5). According to Singh and Kamal (2011), the radial growth rate on media cannot serve as a selection criterion for commercial mushroom cultivation as it has no significant association with the yield of mushroom. However, in contrast, the downward linear growth showed a significant relationship with the yield of mushroom (Singh & Kamal, 2011). Thus in this study, hybrids were selected based on the linear growth rate on substrate which resembles the composition of substrate used for *Pleurotus* mushrooms in most of the mushroom farms in Malaysia.

Table 4.5: The radial growth rate and linear growth rate of the parents and hybrids of *P. giganteus*

Strain	Radial growth rate (cm/day)	Linear Growth Rate (cm/day)
KLU-M 1227	0.40±0.05^{b}	0.18±0.01^{a}
KLU-M 1391	0.29±0.02^{a}	0.19±0.01^{a}
IH12	0.76±0.03f^{g}	0.32±0.01^{hi}
IH15	0.70±0.04^{def}	0.33±0.01i
IH17	0.76±0.04^{fg}	0.31±0.02^{ghi}
IH20	0.67±0.03^{de}	0.29±0.02^{fg}
IH22	0.73±0.06^{efg}	0.29±0.01^{fg}
IH23	0.54±0.10^{c}	0.24±0.02^{c}
IH25	0.64±0.04^{d}	0.24±0.01^{c}
IH26	0.45±0.04^{c}	0.27±0.01^{de}
IH27	0.68±0.05^{de}	0.28±0.01^{ef}
IH28	0.79±0.02^{gh}	0.31±0.03^{ghi}
IH29	0.83±0.07^{h}	0.21±0.02^{b}
IH30	0.75±0.02^{fg}	0.30±0.01^{fgh}
IH32	0.85±0.08^{h}	0.33±0.02^{i}
IH35	0.73±0.03^{efg}	0.26±0.01^{cd}
IH36	0.65±0.03^{d}	0.31±0.02^{gh}
IH37	0.63±0.04^{d}	0.22±0.01^{b}

Values are mean ± standard deviation of three replicates. Values in a row followed by the same letter indicate no significant difference of growth rate at $p<0.05$ level by Duncan's multiple range test.

4.5 Susceptibility of the parents and selected hybrids of *P. giganteus* to *T. saturnisporum*

Trichoderma saturnisporum is a fast-growing fungus species and it could fully colonise a 90 mm PDA plate in two days (Figure 4.7a). By comparison, control plates containing either the parent or the hybrids of *P. giganteus* fully covered the PDA plates in 5 weeks after inoculation. As such, the experiment was designed as described herein to avoid overgrowth of *T. saturnisporum* over the *P. giganteus* cultures. The *P. giganteus* cultures were cultivated on PDA plates for seven days, followed by inoculation of *Trichoderma* sp. (Lee *et al*., 2008). A total of eight hybrids (IH 12, IH 15, IH 17, IH 20, IH 28, IH 30, IH 32, IH 36) were tested for their susceptibility towards *Trichoderma* sp. In all pairings, *T. saturnisporum* grew over the *P. giganteus* colonies on the second day after inoculation of *T. saturnisporum*. This observation was similar to the results reported by Lee *et al*. (2008). A week after inoculation of *T. saturnisporum*, 62.5% of the hybrids started to grow over the contaminant. Both of the parents and hybrids were not fully overgrown by *Trichoderma* colony during the observation period.

Deadlock occurred in 25% (n = 2) of pairings of the hybrids and *T. saturnisporum* while 37.5% (n = 3) of the hybrids were overgrown by *T. saturnisporum* (highly susceptible) on the 4th week of incubation (Table 4.6). Among the eight hybrids tested, hybrid IH 17 was the least susceptible to *T. saturnisporum* on PDA. The hybrid grew over *T. saturnisporum* and covered three quarters of the medium on the 4th week (Figure 4.7b). Further, hybrids IH 15 and IH 32 partially grew over *T. saturnisporum* on the 4th week (Figures 4.7 c & d). In contrast, both of the parents KLU-M 1227 and KLU-M 1391 were highly susceptible to *T. saturnisporum* (Figures 4.7 e & f). *T. saturnisporum* gradually grew over the parents throughout the observation period (1st to 4th week) but did not fully colonise the parents.

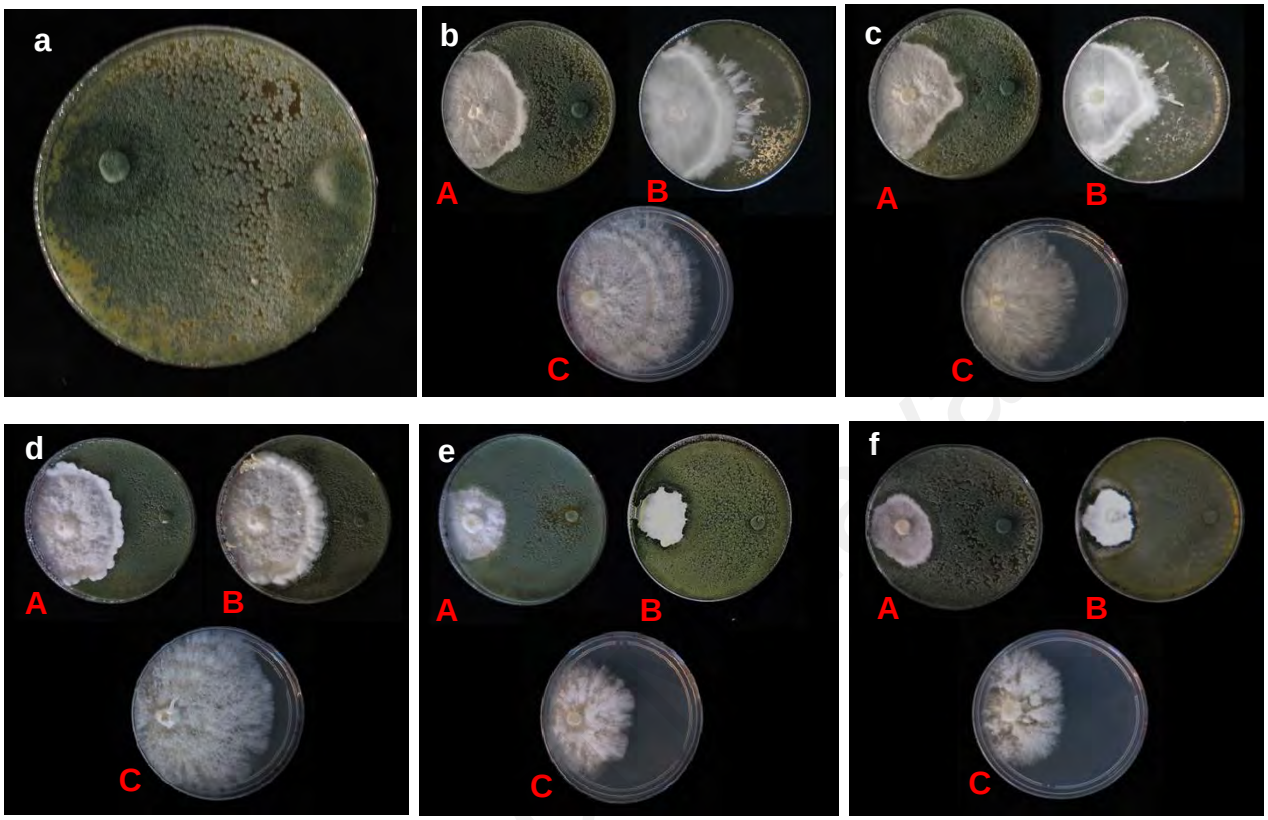


Figure 4.7: Susceptibility of *P. giganteus* to *T. saturnisporum* (a) *T. saturnisporum* fully covered the PDA plate on 2nd day after inoculation; (b) IH 17; (c) IH 15; (d) IH 32; (e) KLU-M 1227. (f) KLU-M 1391. (A) 1st week; (B) 4th week and (C) Control one week after *T. saturnisporum* inoculation

Table 4.6: Susceptibility of the hybrids of *P.giganteus* to *T. saturnisporum*

Interaction / Strain	Deadlock	High susceptibility	Weak susceptibility
	IH 28	IH 12	IH 15
	IH 36	IH 20	IH 17
		IH 30	IH 32

In this study, the susceptibility of the *P. giganteus* strains did not correlate with the linear growth rate. Some hybrids grew slower than the others but were able to grow with *T. saturnisporum*, in accordance with the study by Albert *et al*. (2011). Hybrid IH 12, though it had a higher linear growth rate than other hybrids, it was highly susceptible to *T. saturnisporum*. In contrast to hybrid IH 12, IH 36 and IH 28 had lower linear growth rate than IH 12 but were able to grow with *T. saturnisporum* (deadlock was observed). An obvious antithetic line was formed in the deadlock interaction zone. Dark green pigments with abundant conidiation of *T. saturnisporum* were observed at the interaction zone. Some studies suggested that this could be related to the production of laccase and melanin compounds as a response to the attack by antagonists (Badalyan *et al*., 2004; Flores *et al*., 2009; Komoń-Zelazowska *et al*., 2007). *Trichoderma* will form lytic enzymes during antagonism with other fungus. This triggers the laccase production or laccases isoform induction of a basidiomycete, as a defensive response against the mycelial invasion (Badalyan *et al*., 2004; Flores *et al*., 2009).

Trichoderma saturnisporum colonised the media faster than *P. giganteus*. *Trichoderma* is one of the most aggressive contaminants which competes with mushroom strains for nutrients and space (Beyer *et al*., 2000). This green mold produces dense white mycelium which turns into green in color with dense sporulation during composting (Rezaei *et al*., 2001). The attack by green mold often occurs half way during mushroom composting in cultivation. In this study, even though all the parent and hybrid strains were overgrown by *T. saturnisporum* on the first week after the introduction of *Trichoderma* isolate, some hybrids were able to grow over the *T. saturnisporum* at the later stage (4 weeks after inoculation of *Trichoderma* isolate). So during the mushroom bag composting in mushroom farm, the improved strains may be able to compete with the contaminant, and may eventually overgrow it. Mushroom bags with serious contamination, however, are not encouraged to be retained in the mushroom house. Strain IH 17, IH 15 and IH 32

were selected to proceed with cultivation as the strains had high linear growth rate and low susceptibility towards *T. saturnisporum.*

4.6 Cultivation of the parents and selected hybrids

Cultivation of the parents and selected hybrids was initially carried out using the conventional method. For conventional cultivation substrate composing of 89% of sawdust, 10% of rice bran and 1% of calcium carbonate in polypropylene bags were prepared. This is a common practice in *Pleurotus* mushroom cultivation among mushroom growers in Malaysia. However in foreign countries such as Thailand and China, soil-casing method was widely applied for the cultivation of *P. giganteus* (Klomklung *et al*., 2012; Ruan *et al*., 2013). Thus, soil-casing method was then studied to compare the yield as well as the physiological characteristics of the mushroom. All the cultivated parents and hybrids were cased with soil one week after complete mycelium colonization of substrate. The comparisons between fruit body production using two different cultivation methods are discussed as below.

4.6.1 Conventional cultivation method

Spawn running: Time required for the completion of mycelium colonization in substrate of the parents and hybrids of *P. giganteus* was 2 to 4 weeks (Table 4.7).

Primordia formation: The parent KLU-M 1391 formed primordia 2 weeks after spawn run, which was about three times faster than the hybrid IH 15 (Table 4.7).

Fruit body production: Long coiling stipes were formed by all the cultivated parents and hybrids in the mushroom bags using conventional cultivation method (Figure 4.8a i-iii). After harvesting, the remaining stipes inside the mushroom bags attracted insects and this eventually led to bag contamination. The parents formed 1 to 2 fruit bodies whereas the hybrids formed averagely 2 to 3 fruit bodies.

Biological efficiency: As presented in Table 4.7, all the hybrids showed at least two times higher biological efficiency compared to the parents.

Number of flush: The crop of *P. giganteus* was harvested in 1 to 2 flushes.

4.6.2 Soil-casing cultivation method

Spawn running: Similar to the results of conventional cultivation method, spawn run of the parents and hybrids of *P. giganteus* was 2 to 4 weeks after inoculation.

Primordia formation: The parent KLU-M 1227 was the fastest to show primordia compared to the other parent and hybrids. It took 7 days to form primordia after soil-casing, which was about three times faster than the hybrid IH 17.

Fruit body production: The soil-casing cultivation method had prevented the formation of the long coiling stipe problem formed by the fruit body of *P. giganteus*. All cultivated parents and hybrids formed short stipes using soil-casing method (Figure 4.8b). The parents formed on average 3 to 6 fruit bodies whereas the hybrids formed around 4 to 11 fruit bodies.

Biological efficiency: The parent KLU-M 1227 showed the lowest biological efficiency, which was only 10%. However, the parent KLU-M 1391 had the second highest biological efficiency compared to all other cultivated parent and hybrids. The biological efficiency of the hybrids ranged from 38-42% (Table 4.7).

Number of flush: All the cultivated parents and hybrids formed 2 flushes on average.

For both cultivation methods, there was no distinct variation among the parents and hybrids in terms of morphological characteristics such as the color of fruit body and pileus margin (Figure 4.9). For all cultivated strains, the pilei were regular and even, slightly depressed and the pilei surfaces were smooth with scales at the margin. The pilei margins

were slightly uplifted when the mushrooms were over-matured. The pilei color was ranged from yellowish brown (5E5) to dark brown (6F7) in the center, towards the margin yellowish white (4A2) to light brown (6D6). The lamella were pale yellowish white (2A2), subdistant spacing, decurrent with narrow breadth. The stipe ranged from 47-90mm in length, 3-8mm thick, color varied from greyish brown (6F3) to orange white (5A2), with solid interior and fibrous texture. Color descriptions were based on Kornerup and Wanscher (1963). The morphological characteristics of all the cultivated parents and hybrids matched the descriptions by Karunarathna *et al*. (2011).

Obvious differences were observed in the physiological characteristics of fruit bodies produced using conventional cultivation method and soil-casing method. Overall, the parents and hybrids of *P. giganteus* with soil-casing took shorter time for primordia formation and formed higher number of fruit body, higher biological efficiency as well as higher number of flush compared to the fruit body production using conventional cultivation method.

A major problem encountered throughout the period of cultivation in this study, whether the conventional or soil-casing method was applied, was that the parent KLU-M 1227 only formed primordia after an average of 18 days which remained as primordia and failed to mature as a pileus. The primordia were subsequently contaminated by green mold a month later (Figure 4.9b). However, the parent KLU-M 1227 was able to differentiate into a relatively small but deformed pileus (Figure 4.9 c & d) when the room temperature ranged from 23-32°C and humidity ranged from 27-99%. Whereas when the room temperature was within the range of 22-35°C and humidity ranged from 18-99%, none of the primordia of the parent KLU-M 1227 formed pileus. Among the parents and selected hybrids, the parent KLU-M 1227 was the only one with retarded development of mushroom pilei. It was reported that factors such as inadequate light and ventilation, chemical vapours and overheated substrate during spawn run might lead to malformed

fruit bodies (Royse, 2003). The abnormal maturation of the parent KLU-M 1227 in this study could be related to the high sensitivity of the strain and also due to high temperatures in the mushroom house with no environment control facilities. This happens quite often in tropical countries. Apart from that, this strain being a commercial strain may have degenerated, caused by genetic alteration and/or changes in environmental conditions.

During the incubation period, though the mushroom house had two humidifiers, due to the distinct changes in the external environmental conditions, the temperature of the mushroom house varied from 23-38°C while the humidity ranged from 14-99%. The dramatic climate change resulted in a long period of "mushroom hibernation" as the primordia were not formed in the conventional method without casing layer. In contrast, substrate cased with soil layer showed shorter period for primordia emergence even under such inconsistent circumstances.

In this study, the soil was sterilised before it was used as casing layer. Cho *et al.* (2008) reported that in comparison to sterilised casing layer, non-sterilised casing layer contains more microorganisms which may help to promote the formation of fruit bodies. However, in Malaysia, the tropical climate with high humidity favours the growth of contaminants such as *Trichoderma* sp. Thus, non-sterilised casing layer may be a suitable habitat for unwanted microorganisms. This should be taken into considerations for the study of cultivation method using soil casing layer. The pH value of the soil used in this study was pH 5. Mycelium started to colonise the soil layer 2 days after the substrate was cased, in accordance with the study by Klomklung *et al*. (2014) and Kumla *et al*. (2013), who reported that the best mycelial colonization and density of *P. giganteus* was observed at pH 5-7.

Soil layer functioned as a medium for humidity retention and helped to maintain the humidity which promotes the growth of the fungal strain (Van Gerwen, 2006). Van Gerwen (2006) reported that the casing soil serves as a room for gaseous and water exchange from the compost through evaporation. The casing layer does not function as nutrient sources for the mushroom but it provides spaces for the transition of mushroom from vegetative to reproductive stage (Gülser & Pekşen, 2003).

The cultivation method using soil casing showed that the soil layer promoted the growth of primordium in the substrate in a shorter period, in agreement with previous reports that indicated that soil casing helped in improving the growth of *Pleurotus* mushrooms (Cho *et al.*, 2008; Kumla *et al.*, 2013) and promoting primordial production of mushroom (Kumla *et al*., 2013). In China and Thailand, *P. giganteus* is cultivated using soil-casing method, which is said to be important for the fruiting stage to promote better yield (Klomklung *et al.*, 2012; Ruan *et al*., 2013). Since soil-casing cultivation performed better results in all aspects such as biological efficiency and total number of flushes, this method was carried out for all the parents and hybrids with ten replicates each and for three generations.

Table 4.7: Fruit body production of *P. giganteus* using conventional (C) and soil-casing (SC) cultivation methods

Strain	Number of days for spawn completion		Number of days to show primordia		No. of fruit bodies		Biological efficiency		Total no. of flushes	
	C	SC	C	SC	C	SC	C	SC	C	SC
KLU-M 1227	26.3± 1.6^{c}	28.0± 0.0^{c}	28.3± 8.3^{abc}	7.3± 1.3^{a}	1.6± 0.3^{a}	3.0± 1.0^{ab}	11.8± 1.8^{ab}	10.1± 1.2^{ab}	1.0± 0.0^{ab}	2.3± 0.3^{c}
KLU-M 1391	24.0± 1.0^{bc}	23.0± 0.0^{bc}	15.6± 15.6^{abc}	12.0± 2.0^{abc}	0.6± 0.6^{a}	6.0± 0.5^{b}	5.9± 5.9^{a}	40.4± 1.8^{c}	0.3± 0.3^{a}	2.0± 0.0^{c}
IH 15	16.0± 1.5^{a}	16.0± 1.5^{a}	47.3± 6.6^{c}	19.0± 1.7^{abc}	1.6± 0.6^{a}	3.6± 1.6^{ab}	28.1± 2.8^{c}	41.5± 1.9^{c}	1.0± 0.0^{ab}	2.0± 0.0^{c}
IH 17	18.3± 0.3^{ab}	16.3± 0.3^{a}	46.0± 23.1^{bc}	20.3± 4.9^{abc}	2.1± 1.4^{ab}	4.0± 1.0^{ab}	24.3± 12.8^{bc}	37.9± 2.9^{c}	1.0± 0.5^{ab}	2.0± 0.0^{c}
IH 32	16.0± 5.0^{a}	14.0± 0.0^{a}	26.6± 15.2^{abc}	10.0± 0.0^{ab}	2.8± 0.7^{ab}	11.0± 2.4^{c}	36.2± 6.5^{c}	39.8± 0.6^{c}	1.6± 0.3^{bc}	2.0± 0.0^{c}

Values are mean ± standard error of three replicates. Values in a row followed by different letters indicate significant differences by Duncan's multiple range test (p<0.05)

Figure 4.8: Fruit bodies of the cultivated *P. giganteus*. (a) Conventional cultivation method: a(i) long stipe formed at the bottom of mushroom bag; a(ii) long coiling stipe formed in the mushroom bag; a(iii) long stipe formed by fruit bodies; (b) absence of long stipe using in soil-casing cultivation method

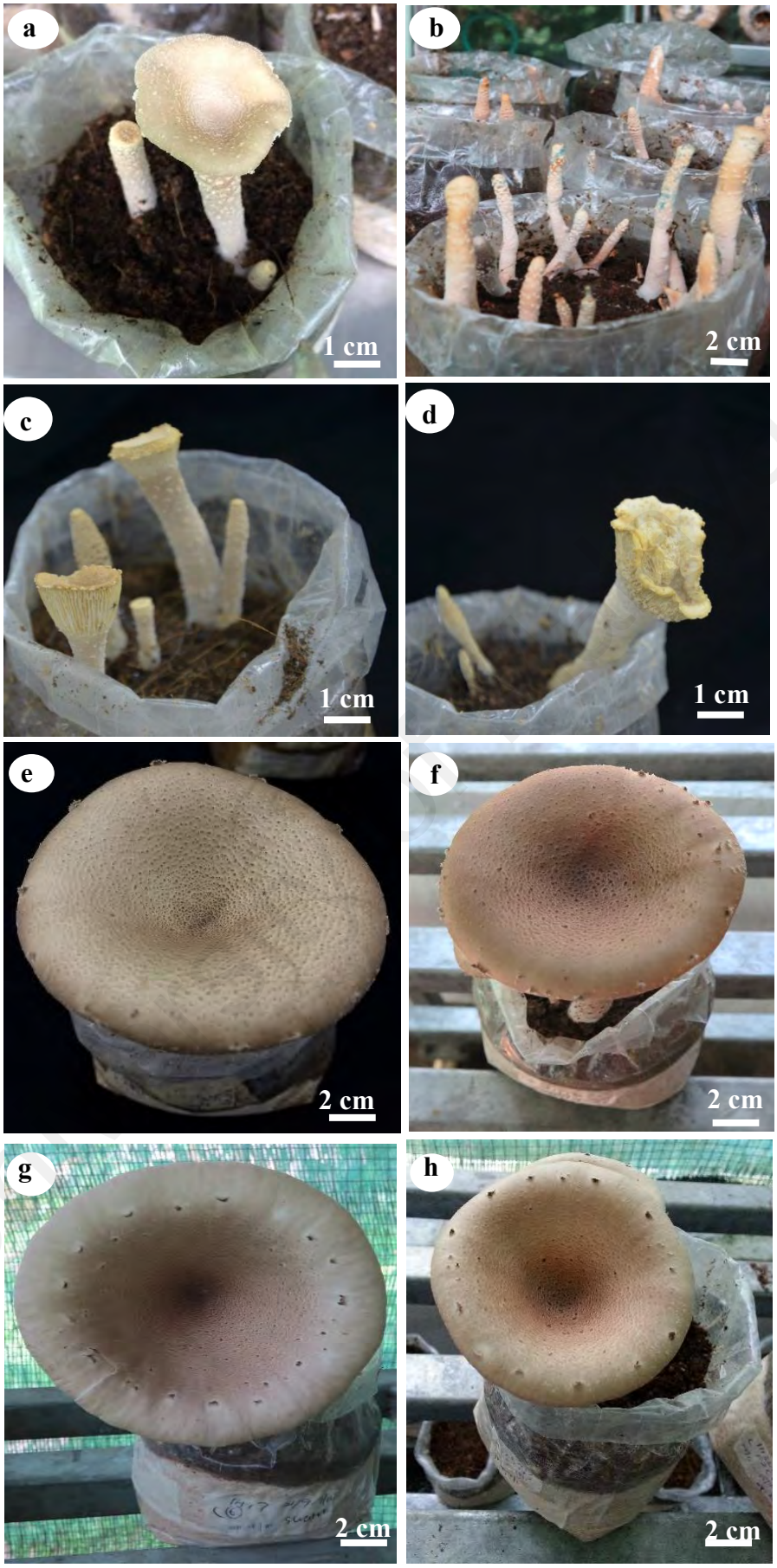


Figure 4.9: Fruit bodies of the cultivated *P. giganteus* parents and hybrids in soil-casing cultivation; (a - d) The parent KLU-M 1227 had undeveloped primordia and deformed caps; (e) KLU-M 1391; (f) IH 15; (g) IH 17; (h) IH 32

4.6.3 Cultivation of the parents and hybrids of *P. giganteus* using soil-casing cultivation method

First generation: As shown in Table 4.8, both the parent strains took 24 to 27 days for spawn run completion. Whereas the hybrids took 2 weeks to fully colonize the substrate in mushroom bags. The parent KLU-M 1227 was the fastest to show primordia formation among all the parents and hybrids. The primordia formation of KLU-M 1227 was three times faster than that of IH 17. The parent KLU-M 1227 formed the lowest number of fruit bodies (1.1±0.5) among all the parents and hybrids. Whereas IH 32 formed the highest number of fruit bodies, which was ten times higher than that of KLU-M 1227. The parents and hybrids had an average of 1 to 2 flushes. Due to the retarded growth, KLU-M 1227 formed the smallest pileus (1.0±0.4cm) among all the cultivated strains and hybrids of *P. giganteus*. The pileus size of IH 15 (7.2±0.4) and IH 17 (7.2±0.3) was seven times larger than that of KLU-M 1227. The stipe thickness of all the parents and hybrids ranged from 0.5 to 0.9cm, whereas the stipe length ranged from 2.8cm to 6.4cm. During the fruiting period of the F1 generation (temperature was 23-35.4°C and humidity was 39-99%), all hybrids fruited except that 60% of the parent KLU-M 1227 and 10% of the parent KLU-M 1391 did not fruit. The biological efficiency of the parent KLU-M 1227 was the lowest (3.73%) among all parents and hybrids tested (Figure 4.10). Whereas the hybrid IH 15 had the highest biological efficiency, which was eleven times higher than that of the commercial strain KLU-M 1227.

Second generation: All the parents and hybrids showed no considerable difference in the number of days for spawn run completion which were from 17 to 19 days (Table 4.8). The hybrid IH 32 formed primordia one week after soil-casing, which was about three times faster than both the parents and also the hybrid IH 15, but eight times faster than the hybrid IH 17. The parent KLU-M 1227 and the hybrid IH 15 formed an average of two fruit bodies during F2 generation. Hybrid IH 32 formed the highest number of fruit

bodies (5.4±0.8) among all the parents and hybrids. The parents and hybrids had an average of two flushes. IH 17 formed the largest pileus (10.3±2.4cm) among all the cultivated parents and hybrids, which was around three times of the pileus size of the parent KLU-M 1227. Despite of the small pileus size, KLU-M 1227 had the thickest stipe (2.3±0.8cm), which was two times thicker than that of other parent and hybrids. On the other hand, KLU-M 1227 also formed the longest stipe (7.7±1.0cm) among all the cultivated parents and hybrids. During the F2 generation (temperature was 20-38°C and humidity was 14-99%), all parents and hybrids fruited except 20% of the hybrid IH 15. Biological efficiency of IH 32 was the highest among all the hybrids (56.79%), which was four times higher than that of KLU-M 1227 (Figure 4.10).

Third generation: The parent KLU-M 1391 took the shortest time for spawn run completion among all the cultivated strains and hybrids of *P. giganteus* (Table 4.8). Whereas the parent KLU-M 1227 was the slowest to complete spawn run in 25 days in the mushroom bags. All the hybrids took around three weeks for spawn run completion. IH 32 was the fastest among all the hybrids tested to show primordia formation after soil-casing. It took about 20 days for primordia formation, which was 55% faster than that of IH 15 and IH 17. Both the parents tested formed only two fruit bodies in this generation. Whereas the number of fruit bodies formed by the hybrids ranged from two to five. The parents and hybrids gave an average of two to three flushes. IH 15 formed the largest pileus (8.4±0.6cm) among all the cultivated parents and hybrids, which was about four times larger than that of KLU-M 1227. All the parents and hybrids formed similar stipe thickness in this generation, ranging from 0.7 to 0.9cm. KLU-M 1227 and IH 15 formed the longest stipes among all the cultivated *P. giganteus*, which were 7.2±0.3cm and 7.7±0.5cm respectively. During the F3 generation (temperature was 21.5-35.7°C and humidity was 14-99%), all the parents and hybrids fruited. IH 32 showed the highest

biological efficiency (47.12%) among all the hybrids, and it was about six times higher than that of the parent KLU-M 1227 (Figure 4.10).

Table 4.8: Comparison of the fruit body production of the parents and hybrids in three generations

Strain	Generation	No. of days for spawn run completion	No. of days for primordia formation*	No. of fruit bodies	Total no. of flush	Pileus size (cm)	Stipe thickness (cm)	Stipe length (cm)
KLU-M 1227	1	23.7± 1.2^{b}	7.9± 1.3^{a}	1.1± 0.5^{a}	1.4± 0.2^{a}	1.0± 0.4^{a}	0.5± 0.2^{a}	2.8± 1.2^{a}
	2	19.1± 0.8^{a}	19.8± 5.9^{a}	2.0± 0.2^{a}	1.7± 0.1^{a}	2.8± 0.1^{b}	2.3± 0.8^{b}	7.7± 1.0^{b}
	3	25.2± 1.7^{b}	13.3± 3.7^{a}	2.0± 0.2^{a}	1.9± 0.1^{a}	1.9± 0.3^{ab}	0.9± 0.1^{ab}	7.2± 0.3^{b}
KLU-M 1391	1	26.8± 0.9^{b}	15.0± 4.1^{a}	4.0± 0.7^{b}	1.5± 0.2^{a}	5.4± 0.8^{a}	0.8± 0.1^{a}	4.5± 0.5^{a}
	2	17.9± 0.3^{a}	21.1± 2.7^{ab}	3.8± 0.5^{b}	2.1± 0.2^{ab}	7.6± 0.4^{b}	0.8± 0.0^{a}	5.9± 0.2^{b}
	3	18.5± 1.3^{a}	32.7± 6.1^{b}	1.8± 0.1^{a}	2.7± 0.2^{b}	7.2± 0.3^{b}	0.9± 0.0^{a}	6.4± 0.1^{b}
IH 15	1	16.5± 0.4^{a}	17.2± 1.3^{a}	5.5± 0.8^{b}	1.9± 0.1^{a}	7.2± 0.4^{a}	0.8± 0.0^{a}	6.4± 0.3^{a}
	2	19.7± 0.5^{b}	20.4± 3.6^{a}	2.0± 0.4^{a}	2.0± 0.3^{a}	6.6± 1.2^{a}	0.8± 0.1^{a}	5.8± 0.9^{a}
	3	20.3± 0.9^{b}	45.6± 3.4^{b}	1.9± 0.2^{a}	2.0± 0.1^{a}	8.4± 0.6^{a}	0.9± 0.0^{a}	7.7± 0.5^{a}
IH 17	1	16.7± 0.3^{a}	25.8± 5.4^{a}	4.4± 0.7^{a}	1.8± 0.1^{a}	7.2± 0.3^{a}	0.9± 0.0^{b}	6.0± 0.2^{a}
	2	17.4±. 1.1^{a}	59.3± 4.5^{c}	3.6± 0.4^{a}	1.7± 0.1^{a}	10.3± 2.4^{a}	1.0± 0.0^{b}	5.4± 0.1^{a}
	3	21.6± 1.3^{b}	45.6± 3.7^{b}	2.8± 0.2^{a}	1.8± 0.2^{a}	6.6± 0.3^{a}	0.8± 0.0^{a}	5.9± 0.3^{a}
IH 32	1	14.9± 0.5^{a}	10.0± 0.4^{a}	10.3± 1.2^{b}	2.0± 0.0^{a}	5.3± 0.3^{a}	0.7± 0.0^{a}	5.1± 0.1^{a}
	2	17.2± 1.1^{a}	6.9± 1.1^{a}	5.4± 0.8^{a}	2.4± 0.1^{a}	7.1± 0.5^{b}	0.9± 0.0^{b}	6.1± 0.2^{b}
	3	23.9± 1.8^{b}	20.5± 4.4^{b}	4.7± 1.0^{a}	2.4± 0.2^{a}	6.9± 0.2^{b}	0.7± 0.0^{a}	6.3± 0.3^{b}

*After spawn run. Values are mean ± standard error of ten replicates. Values in a row followed by different letters indicate significant differences by Duncan's multiple range test (p<0.05).

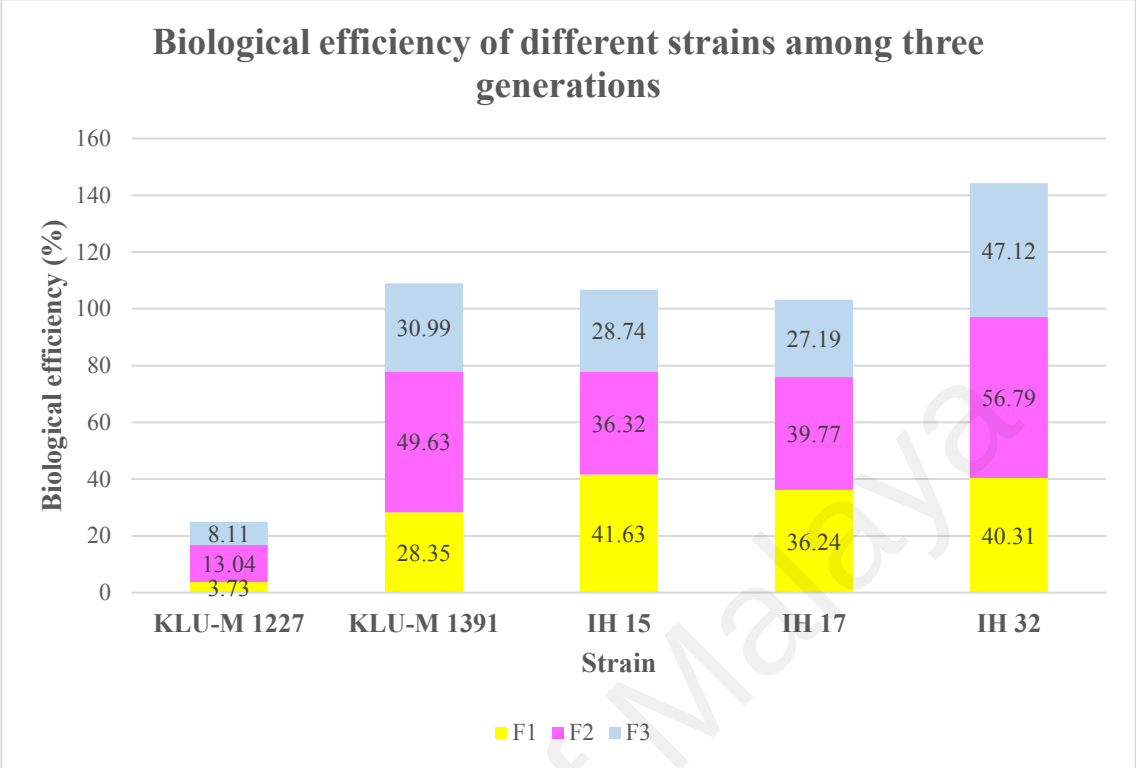


Figure 4.10: Biological efficiency (%) of the parents and selected hybrids of three generations (F1-F3)

Generally, all the cultivated parents and hybrids took a longer period for spawn run completion compared to the first and second generations except for the parent KLU-M 1391. However, all the cultivated parents and hybrids produced the highest number of flushes in the F3 generation (Table 4.8). Both the parents and hybrids took a longer period for primordia formation during the second generation except for IH 32. The average measurements for fruit body production and measurements of all the cultivated *P. giganteus* are presented in Table 4.9 and 4.10.

All the hybrids took an average of 18 days for spawn run completion, which was 12% and 18% faster than KLU-M 1391 and KLU-M 1227 respectively (Table 4.9). Variability in time to form primordia was observed among the strains. Among the hybrids, IH 32 was the fastest to fruit, which was significantly (p<0.05) different from the other hybrids

tested. Primordia of hybrid IH 32 grew after an average of 12.4±4.1 days after the bag was opened. Whereas IH 15 and IH 17 took a longer period for primordia formation compared to both of the parents KLU-M 1227 and KLU-M 1391. The parent KLU-M 1227 and KLU-M 1391 took only 13.7±3.4 days and 22.9±5.1 days respectively for primordia formation after spawn run was completed and the bags were opened. However, the hybrid IH 15 and IH 17 took 27.7±8.9 days and 43.5±9.7 days respectively to form primordia after the bag were opened. This study was similar to the previous study by Kumla *et al.* (2013) who reported the primordia formation of *P. giganteus* was after two weeks of spawn run/incubation. However, in this study, the cultivated *P. giganteus* parents and hybrids only took 3-5 days for the primordia to develop into matured fruit body. This result is different from the study by Kumla *et al*. (2013) who reported that the maturation of fruit bodies of wild *P. giganteus* took 10-15 days.

Hybrid IH 32 produced the highest number of fruit bodies compared to parents and hybrids in all three generations investigated. The average number of fruit bodies formed by IH 32 (6.8±1.7) was about four times that of the parent KLU-M 1227 (1.7±0.3) and two times that of the parent KLU-M 1391 (3.2±0.7). All the parents and hybrids produced an average of 1 to 2 flushes in the three generations.

The pileus size of the cultivated mushrooms negatively correlated with the number of fruit bodies formed whereby the mushroom bags with higher number of fruit bodies formed smaller pileus. Even though hybrid IH 32 had the highest number of fruit body formed during the cultivation, but its pileus size (6.5±0.5cm) was relatively small when compared to the other hybrids. The average pileus size of the cultivated strains was 6-8cm, except for parent KLU-M 1227 which showed retarded growth with an average of 1.9±0.5 cm for pileus size (Table 4.10). However, fruit bodies of KLU-M 1227 had the highest stipe thickness (1.2±0.5cm) among all the cultivated parents and hybrids despite its retarded pileus development.

The hybrid IH 32 had the highest biological efficiency among all the cultivated parents and hybrids throughout all three generations with a mean value of 48.07%. When comparing the fruit body production within the three generations, the mushroom yield and biological efficiency during F2 generation were generally higher (Figure 4.10). The highest biological efficiency of Hybrid IH 32 was 56.79±11.98 (%) during F2 generation. The biological efficiency of IH 32 during the F2 generation was about 335% higher than the parent KLU-M 1227 and 14% higher than the parent KLU-M 1391. On the other hand, although the other hybrids generally showed lower yield than the parent KLU-M 1391, all the hybrids had remarkably higher biological efficiency (at least four-fold higher) than the parent KLU-M 1227. This study is in contradiction to Cai (2013) and Dong *et al.* (2010) which stated that the optimum temperature for fruit body formation of *P. giganteus* is 23-32°C. Even though the temperature of the mushroom house reached the maximum of 38°C during the F2 generation, the mushroom yield was the highest during this period.

Further, KLU-M 1227 showed the least biological efficiency due to its retarded growth. This parent had a BE of 3.73±1.58 (%)-during the F1 generation. The mean value of the biological efficiency of the hybrids in this study ranged from 34.4-48.07%. However, the hybrid with the best yield (IH 32) performed 479.66% and 32.35% higher in biological efficiency respectively when compared to the parent KLU-M 1227 and KLU-M 1391.

Biological efficiency, defined as the percentage conversion of the medium into a more usable form, which is the fruit bodies on a dry weight basis (Bisaria *et al*., 1987; Kumara & Edirimanna, 2009). Biological efficiency is an indication of the yield potential of a strain from a medium. In this study, hybrid IH 32 showed the highest biological efficiency among the cultivated strains. So, this hybrid had the highest capability to utilize the saw dust based medium compared to the other cultivated strains tested in this study. The mean value of the biological efficiency of the hybrids in this study ranged from 34.4-48.07%, which was considered low if compared to the previous studies of *Pleurotus ostreatus*

hybrid with biological efficiency 34.10-54.10% (Kumara & Edirimanna, 2009), and also the study by Valencia del Toro and Leal-Lara (2002) who obtained a 40-73% of biological efficiency of *Pleurotus* hybrids, but considered high if compared to the study by Avin *et al*. (2016) which obtained hybrid of *P. pulmonarius* with biological efficiency 15-26%.

Cultivation is the most important stage to evaluate if the breeding study had produced improved hybrids that gave better yield and morphological characteristics such as larger pilei and better taste (Rebeca *et al*., 2011). Formation of fruit body confirmed that there was a cross of the monokaryons isolated from both parents. In this study, an improved hybrid IH 32 had been produced as it showed higher biological efficiency (40.31-56.79%) , number of fruit body (4.7-10.3) and total number of flush (2 flushes) ,as well as shorter period for spawn run completion (15-24 days) and primordia formation (7-21 days) in comparison to both of the parents.

Table 4.9: Fruit body production of the parents and selected hybrids (of 30 replicates for each strain) in three generations

Strain	No. of days for spawn completion	No. of days for primordia formation	No. of fruit body	Total no. of flush
KLU-M 1227	22.6±1.8^{a}	13.7±3.4^{a}	1.7±0.3^{a}	1.6±0.1^{a}
KLU-M 1391	21.0±2.8^{a}	22.9±5.1^{ab}	3.2±0.7^{a}	2.1±0.3^{a}
IH 15	18.8±1.1^{a}	27.7±8.9^{ab}	3.1±1.1^{a}	1.9±0.0^{a}
IH 17	18.5±1.5^{a}	43.5±9.7^{b}	3.6±0.4^{ab}	1.7±0.0^{a}
IH 32	18.6±2.6^{a}	12.4±4.1^{a}	6.8±1.7^{b}	2.2±0.1^{a}

Values are mean ± standard error of ten replicates. Values in a row followed by different letters indicate significant differences by Duncan's multiple range test (p<0.05)

Table 4.10: Fruit body measurements of the parents and selected hybrids (of 30 replicates for each strain) in three generations of cultivation

Strain	Pileus size (cm)	Stipe thickness (cm)	Stipe length (cm)
KLU-M 1227	1.9±0.5[a]	1.2±0.5[a]	5.9±1.5[a]
KLU-M 1391	6.7±0.6[b]	0.8±0.0[a]	5.6±0.5[a]
IH 15	7.4±0.5[b]	0.8±0.0[a]	6.6±0.5[a]
IH 17	8.0±1.1[b]	0.9±0.0[a]	5.8±0.1[a]
IH 32	6.5±0.5[b]	0.8±0.0[a]	5.8±0.3[a]

Values are mean ± standard error of ten replicates. Values in a row followed by different letters indicate significant differences by Duncan's multiple range test ($p<0.05$)

4.7 Nutritional analysis of the parents and selected hybrid

4.7.1 Nutrient content

The approximate nutrients content of dried *P. giganteus* (g/100g) is shown in Table 4.11. The hybrid IH 32 contained 68g/100g of carbohydrate, which was 1.17% and 9.55% higher than that of both the parents KLU-M 1227 and KLU-M 1391 respectively. The parent KLU-M 1227 presented the highest content of calories and dietary fiber but the lowest amount of total fats when compared to KLU-M 1391 and IH 32. KLU-M 1227 had 364 Kcal of calories (in 100g dried matter) but it contained a very low amount of total fat in comparison to KLU-M 1391 and IH 32, which was 3.7g/100g. The total fat of KLU-M 1391 was 58% higher than the parent KLU-M 1227 and 28% higher than the hybrid IH 32 (Table 4.11). The total fat content of *P. giganteus* in this study was relatively low as compared to the carbohydrate and protein content (Wani *et al*., 2010). According to Wani *et al*. (2010), fats in all mushroom fruit body was mainly comprised of unsaturated fatty acids.

The dietary fiber of KLU-M 1227 was 10.64% higher than KLU-M 1391 and 18.14% higher than IH 32. Both the parents and also hybrid IH 32 of *P. giganteus* in this study

contained ample amount of dietary fiber in accordance with the recommended daily intake adapted from the National Health and Medical Research Council (2017), which ranged from 25-30g daily intake for both male and female. On the other hand, mushroom fiber was also reported to have cholesterol-lowering effects (Fukushima *et al*., 2001).

The parents and selected hybrid of *P. giganteus* in this study exerted higher content of carbohydrate in comparison to other *Pleurotus* mushrooms reported by Alam *et al*. (2008). *β*-glucans, the naturally occurring carbohydrate polymer in mushrooms are known to have anti-cancer, anti-oxidant and neuroprotective properties (Valverde *et al*., 2015). Polysaccharides extract of *P. pulmonarius* was reported to have direct impact on colon cancer cell viability and progression (Lavi *et al*., 2010). In this study, the hybrid IH 32 might perform better than the parents in terms of the medicinal value since it had higher content of *β*-glucans.

The wild strain of *P. giganteus* (KLU-M 1391) contained the highest amount of protein (17g/100g) and total fat (8.9g/100g) compared to the parent KLU-M 1227 and hybrid IH 32. The protein content of both the parents and the hybrid IH 32 was three times lower than that of RDI by National Health and Medical Research Council (2017). The protein content of *P. giganteus* in this study was ranged from 15.4-17.0g in 100g dried matter, which was relatively lower than that of other oyster mushrooms reported by Akyuz and Kirbag (2010), Alam *et al*. (2008) and Chang *et al*. (1981) in which the protein content was ranged from 20-42g in 100g dried matter. However, the protein content of *P. giganteus* strains in this study was slightly higher than that of *P. tuber-regium* (13-17g/100g) as reported by Fasidi and Ekuere (1993). The protein composition of mushrooms can be varied according to chemical composition of substrates (Chang *et al*., 1981; Nunes *et al*., 2012), pileus size, harvest time and mushroom species (Bernaś *et al*., 2006).

Table 4.11: Nutrients in freeze-dried mushrooms (g/100g) and Reference Daily Intake (RDI) for male and female aged 19 to 50 years old

Parameter	KLU-M 1227*	KLU-M 1391	IH 32	RDI	
				Male	Female
Carbohydrate	67.20	61.50	68.00	NA	NA
Calories/Energy (kcal)	364.00	335.00	340.00	2440-2460**	2000-2180**
Cholesterol	ND	ND	ND	NA	NA
Dietary Fiber	33.35	29.80	27.30	30***	25***
Protein	15.40	17.00	16.30	62**	55**
Total Fat	3.70	8.90	6.40	NA	NA
Saturated fat	0.97	2.30	1.60	NA	NA
Monounsaturated fat	1.97	5.20	3.60	NA	NA
Polyunsaturated fat	0.78	1.40	1.20	NA	NA
Trans fat	ND	ND	ND	NA	NA

*Phan *et al*. (2014).
**National Coordinating Committee on Food and Nutrition (2005).
***National Health and Medical Research Council (2017).
RDI: Reference Daily Intakes
ND: not detected.
NA: not available

4.7.2 Mineral content

The mineral content of the parents and selected hybrid of *P. giganteus* is shown in Table 4.12. The highest content of potassium was in IH 32 (2216.1 mg/100g) whereas the lowest amount presented in KLU-M 1227 (1346 mg/100g). The potassium level of the hybrid IH 32 is high and close to the RDI for female aged 19-50 years old recommended by the National Health and Medical Research Council 2017 (2800-3800mg/day). Phosphorus level was similar for both the parents (527.0 – 578.9 mg/100g) but relatively high in hybrid IH 32 (780.9 mg/100g). Magnesium content of the mushrooms studied was ranged from 67.0 mg/100g to 96.1 mg/100g. Calcium level was the highest in KLU-M 1391 (145.9 mg/100g), which was 8-fold higher than that of IH 32 and 25-fold higher

than that of KLU-M 1227. Sodium level was similar for KLU-M 1391 and IH 32 and was about 72% higher than that of KLU-M 1227. However, KLU-M 1227 showed a higher amount of manganese, which was 85% higher than that of KLU-M 1391 and 82% higher than that of IH 32. KLU-M 1391 contained the highest amount of iron, which was two fold higher than that of IH 32 and 3.5-fold higher than that of KLU-M 1227 (Table 4.12). The mushrooms studied had a similar amount of zinc, ranged from 2.6 to 2.7 mg/100g. Copper level was low in the domesticated strain KLU-M 1227 (0.06 mg/100g) but higher in the wild strain KLU-M 1391 (0.5 mg/100g) and hybrid IH 32 (0.7 mg/100g).

Edible mushrooms are rich in minerals which are good for human health. Bernaś *et al*. (2006) stated that mushroom pileus usually contain more minerals than stipe. According to Chang *et al*. (1981), mineral content of mushroom vary according to types of substrate used. The sodium level of mushrooms studied was ranged from 5.8 to 21.7 mg/100g, which was considerably low compared to the sodium level of recommended RDI by the National Health and Medical Research Council 2017 and also sodium level contained in *P. ostreatus* and *P. sajor-caju* reported by Akyuz and Kirbag (2010). The low sodium level of mushrooms was said to be beneficial to human health as mushrooms with high concentration of sodium might have an impact on the K/Na pumps of cell membranes and have harmful effects on patients with high blood pressure (Vetter, 2003). Akyuz and Kirbag (2010) also added that mushroom can serve as an anti-hypertensive diet for its low level of sodium but high level of potassium. Since both the parents and the hybrid in this study had low level of sodium and high level of potassium compared to the RDI reported by the National Health and Medical Research Council (2017), these strains can serve as a good choice of food for hypertension patients. Magnesium content of *P. giganteus* in this study was similar to that reported earlier (Akyuz & Kirbag, 2010). However, calcium and iron content of mushrooms in this study were relatively low when compared to the previous study by Akyuz and Kirbag (2010) and Alam *et al*. (2008).

Table 4.12: Minerals of freeze-dried mushrooms (mg/100g) and Reference Daily Intake (RDI) for male and female aged 19 to 50 years old

Parameter	KLU-M 1227*	KLU-M 1391	IH 32	RDI** Male	Female
Potassium	1346.00	2070.40	2216.10	3800.0	2800.0
Phosphorus	527.00	578.90	780.90	1000.0	1000.0
Magnesium	67.00	84.40	96.10	400.0-420.0	310.0-320.0
Calcium	5.80	145.90	18.10	1000.0	1000.0
Sodium	5.80	21.30	21.70	460.0-920.0	460.0-920.0
Manganese	4.10	0.60	0.70	5.5	5.0
Iron	1.90	6.70	3.90	8.0	18.0
Zinc	2.70	2.60	2.60	14.0	8
Copper	0.06	0.50	0.70	1.7	1.2
Selenium	ND	ND	ND	70000.0	60000.0

*Phan *et al*. (2014).
**National Health and Medical Research Council (2017).
RDI: Reference Daily Intakes
ND: not detected.

4.7.3 Lipid content

Lipid content of the tested strains is shown in Table 4.13. Generally, the commercial strain KLU-M 1227 had the lowest percentage of all the fatty acids among the three tested strains. The palmitic acid content of KLU-M 1391 and IH 32 was similar, and was about 97% more than that of KLU-M 1227. Palmitoleic acid level of the mushrooms tested was about the same level, ranged from 0.01 to 0.30%. Whereas the stearic acid level of KLU-M 1391 and IH 32 was 98% higher than that of KLU-M 1227. The domesticated strain KLU-M 1227 had only 0.1% of oleic acid, which was 99.8% lower than that of the wild strain KLU-M 1391 and the hybrid IH 32. Linoleic acid content was ranged from 0.50-19.50% in the parents and hybrid. Out of the three tested strains, only the wild strain KLU-M 1391 contained a small amount of α-Linolenic acid (0.2%). Arachidic acid content was 0.6% in KLU-M 1391 and 0.4% in IH 32 but was not detectable in KLU-M 1227 (Table 4.13).

Lipids serve as the source of metabolic energy for digestion process in human body (Ribeiro *et al*., 2009). The unsaturated fatty acid, oleic acid dominates the lipid content of the parent KLU-M 1391 and hybrid IH 32. Oleic acid is categorised under omega-9 family and is reported to reduce cholesterol (Tomás *et al*., 2001).

Linoleic acid and α-Linolenic acid are two essential fatty acids for humans but their uptake can only be from food sources. The content of linoleic acid in this study is relatively low in comparison to other oyster mushrooms like *P. ostreatus* and *P. eryngii* (Reis *et al*., 2012). Both the reported *P. ostreatus* and *P. eryngii* contained 69% of linoleic acid, which is greatly different from that of the tested strains in this study. However, both the oyster mushrooms analysed by Reis *et al*. (2012) had a similar percentage of α-Linolenic acid with the wild strain KLU-M 1391. Even though linoleic acid was said to reduce cardiovascular diseases (Zhao *et al*., 2004), but the replacement of saturated fats with linoleic acid was said to increase death risk of cardiovascular disease and coronary heart disease (Ramsden *et al*., 2013). The predominant saturated acid in the tested strains was palmitic acid, which was similar to the study by Khan and Tania (2012) and Phan *et al*. (2014), in which palmitic acid was the major fatty acid constituent of glycolipids and phospholipids in *P. florida* (Khan & Tania, 2012) and *P. giganteus* (Phan *et al*., 2014).

Table 4.13: Fatty acids composition of freeze-dried mushrooms (%) and Reference Daily Intake (RDI) for male and female aged 19 to 50 years old

Parameter	KLU-M 1227*	KLU-M 1391	IH 32	RDI**	
				Male	Female
Palmitic acid (C16:0)	0.38	17.60	17.50	NA	NA
Palmitoleic acid (C16:1)	0.01	0.20	0.30	NA	NA
Stearic acid (C18:0)	0.11	7.90	7.10	NA	NA
Oleic acid (C18:1C)	0.10	58.20	55.20	NA	NA
Linoleic acid (C18:2C)	0.50	15.30	19.50	13.0g	8.0g
α-Linolenic acid (C18:3N3)	ND	0.2	ND	NA	NA
Arachidic acid (C20:0)	ND	0.60	0.40	1.3g	0.8g

*Phan *et al*. (2014).
**National Health and Medical Research Council (2017).
RDI: Reference Daily Intakes
ND: not detected.
NA: not available.

4.7.4 Vitamin content

Vitamin content of the cultivated mushrooms is presented in Table 4.14. Vitamin C was only detectable in a little amount in the domesticated strain KLU-M 1227 and not detected in the wild parent KLU-M 1391 and hybrid IH 32. Vitamin B3 (niacin) was the primary vitamin found in the tested mushrooms, ranging from 0.0090 to 0.0731g/100g. The amount of vitamin B1 (thiamine) contained by KLU-M 1391 and IH 32 was 94% higher than that of the domesticated strain KLU- M 1227. Whereas three of the tested strains presented similar amount of vitamin B2 (riboflavin) which ranged from 0.009 to 0.0014g/100g.

Pleurotus mushrooms were said not to contain high level of all vitamins (Khan & Tania, 2012). This study is in agreement with Khan and Tania (2012) as vitamin B and C only existed in a very minimal amount in dried samples (Table 4.14). Vitamin B3 (niacin) showed highest amount in the mushroom, similar to the study by Phan *et al*. (2014). However, the vitamin B3 level of the wild parent KLU-M 1391 and hybrid IH 32 was 87% higher than that of the commercial strain (KLU-M 1227) and wild strain of *P. giganteus* as reported by Phan *et al*. (2014) and 57% higher than that of *P. ostreatus* as reported by Bautista *et al*. (1998). In this study, KLUM-1391 and IH 32 had 77-80% higher content of vitamin B3 than the RDI reported by the National Coordinating Committee on Food and Nutrition (2005). Bautista *et al*. (1998) stated that oyster mushrooms could be taken as a source of complex B vitamins. Vitamins are unstable and the amount can be affected by heating process and contact with chemicals (Furlani & Godoy, 2008). Vitamin D is absent in the mushrooms analysed. This is in line with the result reported by Mattila *et al.* (2001) that vitamin D was almost absent in the studied mushrooms. Breene (1990) also stated that vitamin D is uncommon especially in cultivated species.

Table 4.14: Vitamins of freeze-dried mushrooms (mg/100g) and Reference Daily Intake (RDI) for male and female aged 19 to 50 years old

Parameter	KLU-M 1227*	KLU-M 1391	IH 32	RDI**	
				Male	Female
Vitamin C	0.3	ND	ND	70	70
Vitamin B1	0.4	7.6	7.8	NA	NA
Vitamin B2	0.9	1.4	1.2	NA	NA
Vitamin B3	9.0	72.4	73.1	16	14

*Phan *et al*. (2014).
**National Coordinating Committee on Food and Nutrition (2005).
RDI: Reference Daily Intakes
ND: not detected.
NA: not available.

4.7.5 Amino acid content

According to Table 4.15, the major constituent of essential amino acids were lysine (1.45-5.20g/100g), followed by leucine (1.50-2.30g/100g). Apart from lysine and leucine, the wild strain KLU-M 1391 showed the highest content of valine (3.80g/100g) and isoleucine (1.30g/100g) compared to the domesticated parent KLU- M 1227 and hybrid IH 32. Glutamine level of KLU-M 1391 was relatively high, which was 46% higher than that of IH 32. The presence of glycine in KLU-M 1227 was 79% higher than that of KLU-M 1391 and IH 32. Among the non-essential amino acids present in the tested mushrooms, aspartic acid and glutamic acid were of high concentrations especially in KLU-M 1391 and IH 32. Aspartic acid concentration of the three tested mushrooms ranged from 0.69 to 3.50g/100g whereas glutamic acid was 1.57 to 4.30g/100g (Table 4.15).

The parent KLU-M 1227 contained 8 essential amino acids and in agreement with Ruan *et al*. (2013), but the wild parent KLU-M 1391 and hybrid IH 32 only contained 6 types of essential amino acids. The conditionally essential amino acid, glutamines existed in considerably high amount (3.7-6.9g/100g) except for KLU-M 1227 in comparison with other amino acids found in the mushrooms. In accordance to Dong *et al*. (2010) who reported that essential amino acids comprised of 45% of the total amount of amino acids

in freeze-dried mushrooms, the essential amino acids of the tested mushroom strains were 42-46% of the total amino acids. The high content of essential amino acids of *P. giganteus* shows that this mushroom can be a component in a healthy diet for humans to gain the external source of amino acids since they cannot be produced in human body.

Table 4.15: Amino acids composition of freeze-dried mushrooms (g/100g) and Reference Daily Intake (RDI)

Parameter	Amino Acid	KLU-M 1227*	KLU-M 1391	IH 32
Essential	Histidine	1.54	ND	ND
	Isoleucine	0.77	1.30	1.30
	Leucine	2.04	2.30	1.50
	Lysine	1.45	5.20	3.70
	Methionine	0.31	ND	ND
	Phenylalanine	1.64	0.80	0.80
	Threonine	0.53	1.20	2.10
	Tryptophan	ND	ND	ND
	Valine	0.80	3.80	1.50
Conditionally essential	Arginine	1.14	1.20	1.10
	Glutamine	ND	6.90	3.70
	Glycine	2.90	0.60	0.60
	Serine	1.11	ND	ND
	Tyrosine	0.58	ND	ND
Non-essential	Alanine	1.11	ND	ND
	Asparagine	1.52	1.50	1.70
	Aspartic acid	0.69	3.50	3.50
	Glutamic acid	1.57	3.80	4.30

*Referred to Phan *et al*. (2014).
ND: not detected.

CHAPTER 5: CONCLUSIONS

This study showed that *Pleurotus giganteus* strains from two geographic regions, i.e. China and Malaysia were compatible and mated successfully with a frequency of 28.6%. The linear growth rate of *P. giganteus* did not correlate to the susceptibility to *T. saturnisporum.* Three hybrids with high linear growth rates and least susceptibility to *T. saturnisporum* were selected for cultivation and to compare their biological efficiency. In this study, soil casing cultivation method successfully solved the long coiling stipe formation problem of *P. giganteus*. Due to the high variation of environmental conditions in Malaysia, there was an inconsistency in the fruit body production by the cultivated strains during the three generations of cultivation. Incubation of the mushroom bags in a controlled room would probably increase the mushroom yield. Cultivation of *P. giganteus* was carried out in small scale in this study. Thus, optimization of the cultivation method of this species should be carried out in large scale and in farm conditions prior to commercialisation of this mushroom in Malaysia. To my knowledge, this is the first mating study of *P. giganteus* in Malaysia. Perhaps more mating studies with wild germplasms can be carried out in order to produce improved mushroom strains with better performance in many aspects such as the yield and growth rate, as well as to increase the income of the mushroom growers in Malaysia. Hybrid IH 32 showed high content of essential amino acids, potassium, niacin (vitamin B3), protein and dietary fibre. The high nutritional content and favourable morphological and physiological characteristics obtained in this study showed that this hybrid maybe a potential mushroom for commercialisation. Further large scale trials are essential to enable the transfer of this technology to mushroom growers.

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