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Identification and factors affecting *in-vitro* growth of an indigenous mushroom, *Boletus* sp. from Bachok, Kelantan, Malaysia

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ABSTRACT

Wild mushrooms including *Boletus* are among edible mushrooms consumed by local populations. However, the species identity for many types of wild edible mushroom in Malaysia is poorly known. The present study was conducted to identify and to determine factors affecting *in-vitro* growth of an edible *Boletus* sp. (*kulat gelam*) commonly found in peat swamp forests in Bachok, Kelantan. Identification was initially done using macroscopic and microscopic characteristics of the fruiting bodies. However, due to overlapping chemical colour reactions, morphological and anatomical characteristics of the fruiting body with other *Boletus* species, species identity was confirmed using ITS region, and the mushroom was identified as *Boletus griseipurpureus*. A toxicity test indicated that *B. griseipurpureus* is an edible mushroom with low toxic levels (LC_{50}=4.33 mg/mL). From growth studies, the results suggested that potato dextrose agar (PDA), cassava dextrose agar (CDA) and yeast malt extract (YME) were the most suitable artificial media for mycelial growth of *B. griseipurpureus* at pH 6.0 and 30°C. To our knowledge, this is the first documented report on wild edible mushroom *B. griseipurpureus* in Malaysia.

Keywords: wild mushroom, *Boletus griseipurpureus, in-vitro* growth, toxicity, solid media, liquid media

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INTRODUCTION

In Malaysia, collecting wild mushrooms for food and medicine is a well-known activity among local communities (Lee & Chang, 2004). According to Lee et al. (2009), a total of 45 species of macrofungi have been reported by Bateq, Che Wong, Jakun, Semai and Temuan communities, in which 31 species were consumed as food

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and 14 species were utilised as medicine including *Auricularia* spp., *Cantharellus* spp., *Clavulina* spp., *Ganoderma* spp. *Lignosus* spp., *Russula* spp., *Schizophyllum* spp. and *Termitomyce*s spp.

Although *Boletus* is widely distributed throughout Malaysia, the consumption of *Boletus* is not common and is confined to a particular species, *B. aureomycelinus* (Lee et al., 2009) as knowledge on the edibility of unknown *Boletus* species is lacking. Moreover, cases of mushroom poisoning due to the consumption of *Boletus* species have been reported in Malaysia. The victims had confused the mushroom with a synonymous species that was inedible and poisonous (Chew et al., 2008). Thus, identification of wild *Boletus* is required to avoid poisoning from mushroom consumption.

Based on personal communication with the locals, there is an edible *Boletus* sold in local wet markets seasonally in Bachok, Kelantan, locally known as '*kulat gelam*' and recognised by its brown-grey cap and lilac-grey stipe. The fruiting bodies are commonly found in peat swamp forests where *Melaleuca cajuputi* ('*pokok gelam*') is the dominant vegetation. Due to its pleasant odour and distinctive flavour, this mushroom is a favourite dish in Malay cuisine. However, detailed information such as species identity, toxicity and growth conditions of the *Boletus* is lacking.

Therefore, the objectives of the present study were to identify the edible *Boletus* mushroom (*kulat gelam*) from peat swamp forests in Bachok, Kelantan using morphological and molecular characteristics; to determine fungal toxicity based on the brine shrimp lethality test; and to determine *in vitro* mycelial growth of the *Boletus* sp. in different media, pH and temperature.

MATERIALS AND METHODS

Sampling Area

Boletus samples were collected from an area of peat swamp forest located at 7 m above mean sea level with latitude 05° 58' 00" and longitude 102° 25' 1" in the district of Bachok, Kelantan. The forest floor was partly waterlogged and covered with a thick layer of decomposed plant litter. Soil analysis showed that the peat soil from which the *Boletus* fruiting bodies were collected was acidic (pH 3.0-4.1), having a high carbon content and low nitrogen content with sufficient levels of phosphorus, aluminium, calcium, ferrum, magnesium, potassium, sodium, manganese and zinc. Heavy metals, namely cadmium, copper, mercury, plumbum and nickel were also detected in the peat soil.

Sampling and Isolation of *Boletus*

The mushroom was seasonally fruiting from June to September every year. The first crop of fruiting bodies can be observed after a long dry season at the end of June. For this study, a sharp knife was used to lift whole fruiting bodies from the ground. The fruiting bodies were then kept in a flat basket and taken to the lab for further processing. Surface sterilisation was done by rinsing the fruiting bodies with hypochlorite solution (1% v/v)

for 10 s. After drying, a piece of tissue from the internal central stem tissue was torn off, inoculated onto a potato dextrose agar (PDA) and incubated at 27±10°C for 24 h. Sub-cultures were performed until a pure culture was obtained. The pure culture was then maintained in PDA slants and kept at 2-9°C. Seed culture was grown on a PDA containing 20 g glucose.

Morphological Identification

For preliminary identification, chemical colour reactions were performed using ammonia solution (10% v/v), potassium oxide (5% v/v), ferrous sulphate (10% v/v) and Melzer's reagent in the field as described by Smith and Smith (1973) and Moser (1983).

The macroscopic and microscopic evaluations were based on taxonomic keys and descriptions of Corner (1972), Smith and Smith (1973) and Moser (1983). The colour and grid designations were standardised based on Kornerup and Wanscher (1978). The descriptions of morphological characteristics were based on fresh mushroom samples.

Macromorphological features observed were the pileus, tube layer, stipe, context layer and any veils that were present. For pileus description, the colour, margin, shape, surface texture and size were recorded, followed by an examination of the tube attachment, pore shape and pore surface colour. For stipe description, the colour, shape, surface texture and size as well as the context colour were recorded. All observations were recorded in annotation worksheets as outlined by Lodge et al. (2004). Spore print was obtained by putting the cap with the tubes downwards on white paper, covering it with a glass bowl and leaving it overnight. The spore deposit was then observed to determine its colour.

For micromorphological observation, slides were prepared in the Histology Lab, School of Biological Sciences, Universiti Sains Malaysia, Penang. The anatomy of the hymenium, context texture, spore size and spore shape were observed. The presence or absence of the hyphal clamp was also determined.

Molecular Identification

Five *Boletus* isolates from five fruiting bodies (USMBo1, USMBo2, USMBo3, USMBo4 and USMBo5) were used for molecular identification. About 25 mg of mycelium from the seed cultures was homogenised using liquid nitrogen in a mortar. Genomic DNA was extracted using the Invisorb® Spin Plant Mini Kit (Stratec Molecular GmbH, Germany) according to the manufacturer's protocol.

For amplification of the internal transcribed spacer (ITS) region, the universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') were used (White et al., 1990). A PCR reaction was prepared in 25 µL containing, 5.0 µL PCR buffer (Promega), 4.0 µL magnesium chloride (25 mM), 0.5 µL dNTP mix (Promega), 2.5 µL each primer, 0.125 µL *Taq* polymerase (5 unit/µL, Promega),

0.5 µL genomic DNA and deionised water made up to 25 µL.

The PCR was performed using MyCycler™ thermal cycle system (Bio-Rad Laboratories Incorporation, United States) with the following cycles: An initial denaturation at 95°C for 1 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 30 s and extension at 72°C for 1 min, followed by the final extension step at 72°C for 10 min.

The PCR product was then purified using the FavorPrep™ PCR Purification Kit (Favorgen® Biotech Corporation, Taiwan) according to the manufacturer's instructions. The purified PCR product was sent to a service provider for sequencing.

For detection of the PCR product, agarose gel (1%) electrophoresis was run at 90 V and 400 mA for 90 min with the gel tank containing a 1x Tris-borate-EDTA (TBE) buffer. One kb and 100 bp DNA markers (Gene Ruler™, Fermentas) were used to estimate the size of the bands. Five µl of the PCR product and 1 µL of 6x loading dye (Fermentas) were loaded in the well of the agarose gel and stained with 0.04 µL ethidium bromide. After electrophoresis, the gel was viewed using the Molecular Imager® Gel Doc™ XR system connected to the Discovery Series™ Quantity One® 1-D analysis software version 4.6.5 (Bio-Rad Laboratories Incorporation, United States).

After sequencing, pair-wise alignment was performed using Cluster W integrated in Molecular Evolution Genetic Analysis (MEGA) version 5.1 (Tamura et al., 2011). The aligned DNA sequence was BLAST against sequences in GenBank. The Neighbour Joining (NJ) tree was applied in a phylogenetic analysis. Three *Boletus* species, namely *B. edulis* (GQ900593), *B. griseipurpureus* (JQ726594) and *B. reticulatus* (GU198973), were included for comparison with *Leccinum scabrum* (AF454585) as the out-group.

Toxicity Test

The potential of *Boletus* to be poisonous was determined by brine shrimp bioassay (Meyer et al., 1982). A total of 20 fruiting bodies from two sampling locations were rinsed with distilled water for 1 min and dried overnight in an oven at 60°C. The oven-dried mushroom was ground into fine powder and kept in a universal bottle until used.

Extraction Procedure

Five g of the mushroom fine powder was dissolved with 100 mL methanol (70% v/v) in a 250 mL conical flask. The mixture was agitated by a sonicator for 20 min. The suspension was filtered and dried in an oven at 60°C for 48 h to obtain a crude extract.

Brine Shrimp Hatching and Lethality Test

The brine shrimp eggs, *Artemia salina*, were hatched in artificial seawater prepared by dissolving 38 g sea salt in 1000 mL distilled water. After 24 h of incubation at 27±10°C with constant light, the nauplii of brine shrimp were collected.

For the lethality test, the crude extract was prepared in triplicate. An amount of 600 mg of the crude extract was dissolved in 4 mL dimethyl sulfoxide (DMSO, 4% v/v) and then, 1 mL of the extract and 2 mL of artificial seawater were pipetted into the first well of a microplate. The extract's initial concentration was 50 mg/mL. A two-fold dilution was carried out to obtain different concentrations from 50 mg/mL to 0.195 mg/mL. At the last well, a negative control was set up with 3 mL artificial seawater. Ten nauplii of brine shrimp were loaded into each concentration and the control and incubated at 27±1°C for 24 h. The number of dead nauplii was counted and recorded at the 6th and 24th hours. For positive control, the same procedure was repeated using potassium dichromate.

The percentage of brine shrimp mortality was calculated and plotted against the logarithms of concentration. The regression equation was applied to determine the lethal concentration value, LC_{50}. Using Abbott's formula, the calculation was as follows:

percentage of brine shrimp mortality (%) = $[1- (N_t / N_c)] \times 100$,

where, N_t=number of live brine shrimp in treatment, N_c=number of live brine shrimp in negative control

A linear regression analysis was carried out using IBM SPSS Statistics 20 program. The lethal concentration value, LC_{50}, was determined at 95% confidence interval using a one-way Analysis of Variance (ANOVA).

Assessment of Mycelial Growth on Solid Media

Five different solid media were chosen to assess the mycelial growth of *Boletus* isolate, namely PDA, cassava dextrose agar (CDA), corn meal agar (CMA), malt extract agar (MEA) and czapek agar (CZA). All the media (1 L) were prepared manually. Preparation of PDA, CDA and CMA was adapted from Wang and Lu (2005), while that of MEA and CZA was from Sung et al. (2011). The media was sterilised at 121°C and 15 psi for 20 min; 0.05 g thiamine (vitamin B1) was added prior to use.

Before pouring into Petri plates, the media were adjusted to different pH values, pH 5.0, pH 6.0, pH 7.0 and pH 8.0. The inoculum was prepared by inoculating a mycelium disc (10 mm) from the seed culture onto each plate. The plates were then incubated at four different temperatures: 22°C, 25°C, 28°C and 30°C. Measurement of the colony diameter was carried out every 2 weeks for three months and the mean value was recorded. The experiment was conducted in a factorial design of 4x4x5 with 80 treatments, and each treatment was set up in triplicate.

Assessment of Mycelial Growth in Liquid Media

Four different liquid media, namely malt extract peptone (MEP), yeast extract peptone (YEP), yeast malt extract (YME) and potato dextrose broth (PDB) were used. All media (1 L) were prepared manually as described by Kim et al. (2002) and Xu et al. (2003) with some modifications. The liquid media

were sterilised at 121°C and 15 psi for 20 min, and 0.05 g thiamine (vitamin B1) was added prior to use.

Before pouring into 250 mL conical flasks, each medium was adjusted to different pH values, pH 5.0, pH 6.0, pH 7.0 and pH 8.0. The study was conducted in a factorial design of 4x4 with 16 treatments, and each treatment was prepared in triplicate.

A mycelium disc (1 cm) was inoculated into each conical flask containing a 100 ml culture medium. The flasks were then incubated in an incubator shaker at 27±1°C and 150 rpm. After 12 days, the mycelium was harvested using pre-weighted filter paper and dried in an oven overnight to a constant weight at 60°C. The mycelial biomass was determined and expressed as mycelial dry weight (g) per 100 mL of culture medium.

For both mycelia growth assessments, the data was analysed by applying the Univariate Analysis of Variance using IBM SPSS Statistics 20 programme. Post hoc comparison of means were performed by Duncan Multiple Range Test (DMRT) at the significant level, α=0.05.

RESULTS

Macromorphological Characteristics

Boletes fruiting bodies were found to grow singly under *Melaleuca cajuputi* trees in the peat swamp forest (Figure 1A). The pileus was 4.0 cm wide and 1.0 cm thick on average, hemispherical to broadly convex with a decurved margin. The cuticle was velvety, subtomentose, dry but slightly viscid when wet and had no scale. The pileal colour was violet white (18A2) to light violet (18A5) when young (Figure 1B) and it turned to dull lilac (15C3) with age. The white context measured 0.7 mm and was thick at the centre as well as soft and spongy. When exposed to air, the white pileus context turned to brownish orange (6C3-4). The odour was indistinctly pleasant but the taste was slightly bitter.

From the chemical tests conducted, ferrous sulphate gave a greyish yellow (4B5-6) colour on the pileus surface whereas an ammonia solution produced a mouse grey (5E3) colour on the pileus margin. No colour reactions were observed when the pileus was tested with potassium oxide and Melzer's reagent.

The hymenophore was white when young and turned pinkish white (8A2), dull red (8B3-4) or reddish brown (8E4-5) at maturity (Figures 1C and 1D) and comprised masses of adnexed or sinuate notched tubes (Figures 1E and 1F), which were soft, moist and easily detached from the context. When handled, the colour turned to bruising clay (5D5). The tubes were up to 0.5 cm deep at the centre, longitudinal and ended with tubular or angular pores that radially elongated near the stipe. On the surface, ferrous sulphate gave olive brown (4E4) and Melzer's reagent gave reddish brown (8E4) colours, respectively. No colour reactions were produced with ammonia solution and potassium oxide.

The stipe on average was 6.0 cm long, 1.0 cm wide at the apex, 1.5 cm wide in the middle and base, centric, straight

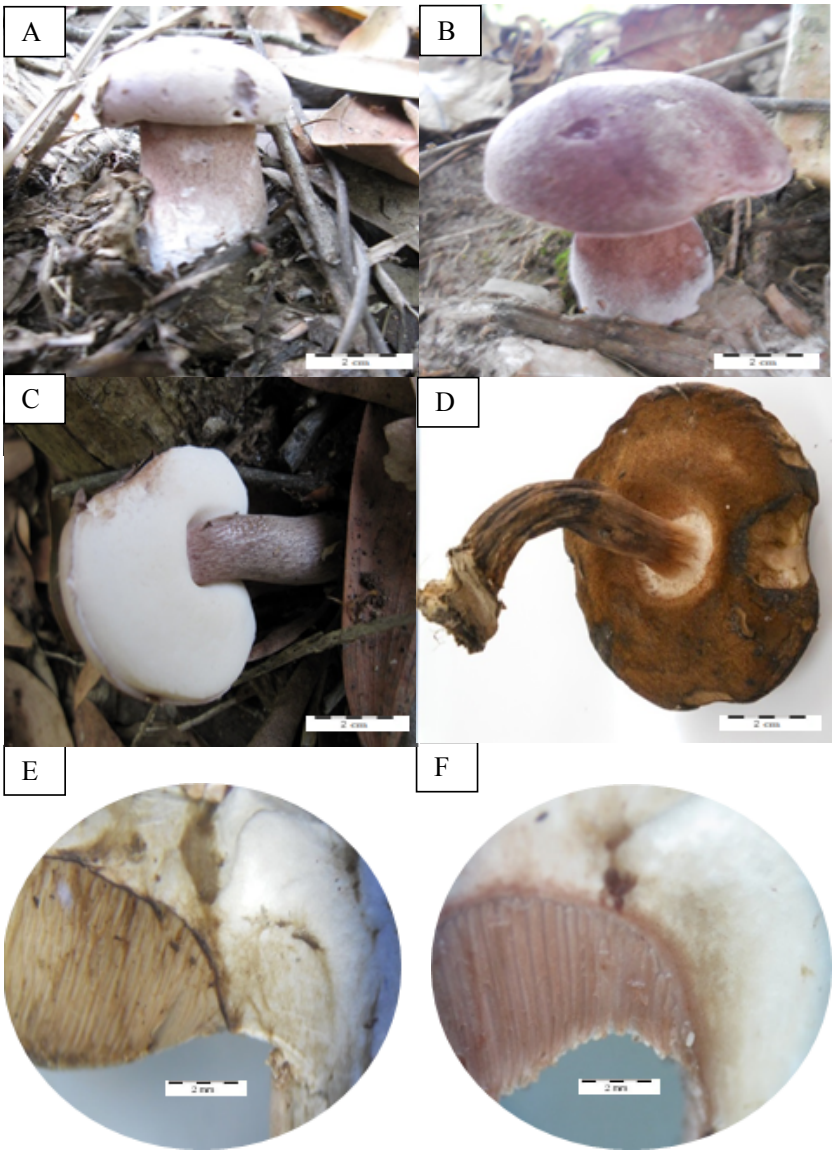

Figure 1. Macromorphological characteristics of *Boletus* sp. (A) Fruiting body of *Boletus* sp. on the forest litter. (B) Light violet young fruiting body. (C) White hymenophore when young. (D) Reddish brown (8E4-5) at maturity. (E) Adnexed tube arrangement. (F) Sinuate notched tube arrangement

or flexuous, equal or clavate with white rhizoids at the base. The annulus and volva were absent. The cuticle was smooth, glabrous, reticulated with a fine net pattern at the apex, dry and easily detached from the context. It was concolourous with the pileus when young and then turned to dull violet (15E3-4) with age, paler at the upper stipe. The white context was soft, fibrous and spongy and turned to brownish orange (6C3-4) when dried. The stipe had a pleasant odour and bitter taste as did the pileus. On the sliced context, ferrous sulphate gave olive brown (4B4-5) and Melzer's reagent gave Chinese yellow (4B7) colours, respectively. No colour reactions were observed when tested with ammonia solution and potassium oxide.

Micromorphological Characteristics

The spore print was olive brown (4E8) on white paper. The individual spore was symmetrical and ellipsoid (7-9 µm long and 2-3 µm wide) and had a smooth surface with 1 or 2 guttae (Figure 2A).

The hymenium had a row of palisade cells, some of which were basidia. The basidia were 20-40 µm long and 3-5 µm wide, fusoid-shaped with 3-4 sterigmata at the apex (Figure 2B). An abundance of pleurocystidia was found along the hymenium layer. The pleurocystidia were ventricose with a prolonged, obtuse or subcapitate apex (Figure 2C). The clavate-shaped cheilocystidia were distributed scarcely on the spore edge (Figure 2D).

The pileus surface was covered by a pile of cutis, composed of slightly erect or decumbent hyphae with cylindrical or subfusiform end cells (Figure 2E). The end cells were mostly thin-walled with vacuoles. The pileus trama was made up by interwoven hyphae without clamp connections. The tube trama showed a boletoid structure with the hyphae diverging towards the hymenium layer and the pore edge.

An abundance of caulocystidia was observed at the stipe surface. The caulocystidia were subclavate or ventricose in shape and was thin-walled with vacuoles (Figure 2F). Similar to the pileus, the stipe trama was composed of interwoven hyphae without clamps. The interwoven hyphae were more compact near the stipe surface compared to the middle.

Molecular Identification

The ITS region was successfully amplified from the five *Boletus* isolates and it showed a single band of approximately 700 bp. Based on a BLAST search, all the isolates showed 100% similarity with *Boletus griseipurpureus* (JQ726594). The nucleotide sequences of the isolates were deposited in GenBank with accession numbers from KF442405 to KF442409.

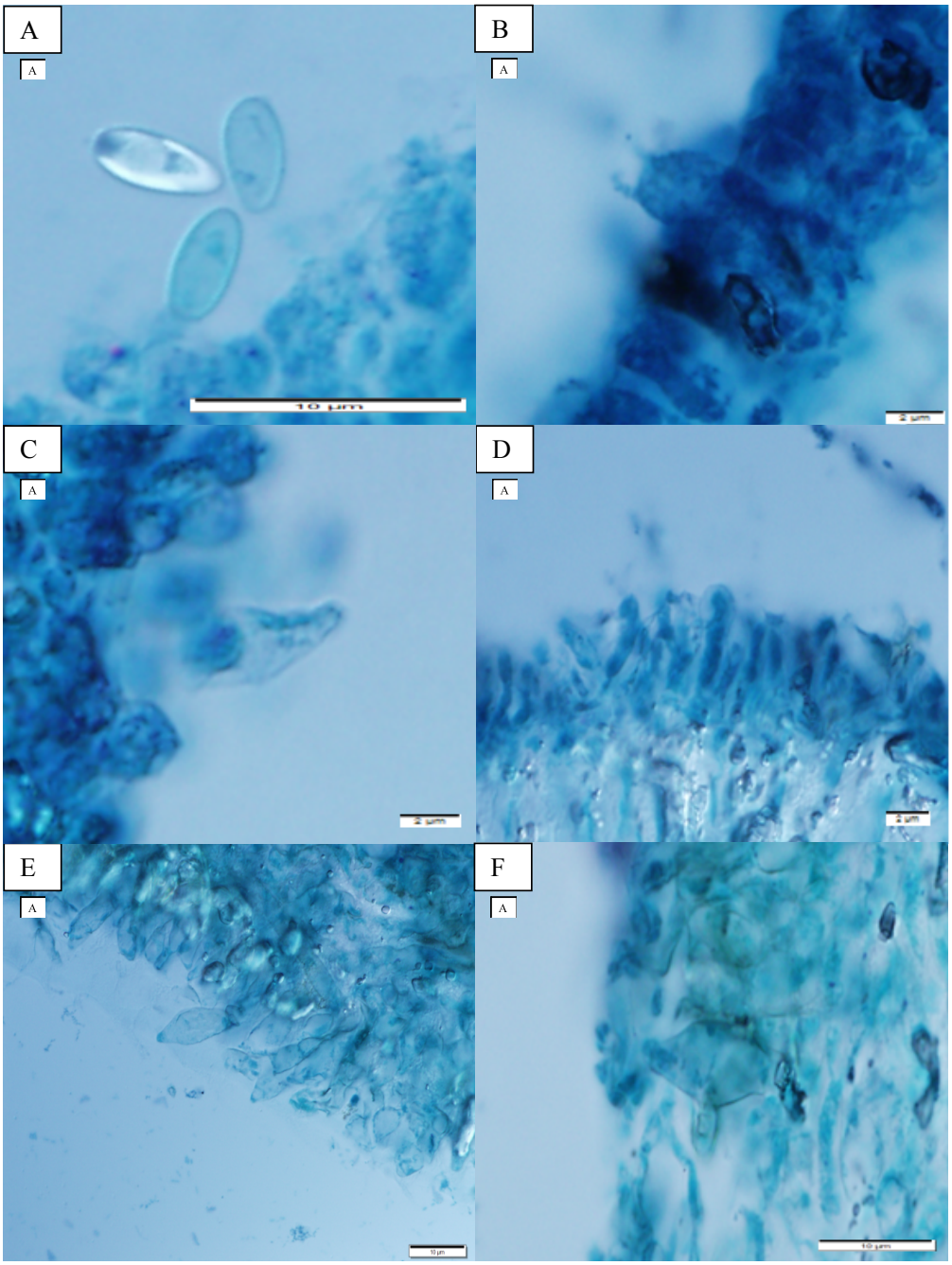

Figure 2. Micromorphological characteristics of *Boletus* sp. (A) Ellipsoid spore. (B) Fusoid basidium. (C) Subcapitate apex pleurocystidia. (D) Clavate cheilocystidia. (E) Subfusiform end pileocystidia. (F) Ventricose caulocystidia

The NJ tree generated is shown in Figure 3, and can be divided into two main clades, I and II. Main clade I consisted of the five *Boletus* isolates, grouped with *B. griseipurpureus* (JQ726594). *Boletus edulis* (GQ900593) and *B. reticulatus* (GU198973) were grouped in main clade II.

Figure 3. Neighbour-joining tree showing the relationship of *B. griseipurpureus* isolates (USMBo1, USMBo2, USMBo3, USMBo4 and USMBo5) and other *Boletus* spp. *Leccimum scabrum* is the out-group

Toxicity Test

In the lethality test, the maximum mortality of brine shrimps took place at a concentration of 100 mg/mL while minimum mortality was at 0.195 mg/mL. After 6 hours of incubation, the extract showed lethal concentration values, LC_{50} of 4.33 mg/mL (Figure 4). The toxicity increased twofold after 24 hours of incubation and the extract had LC_{50} at 2.38 mg/mL (Figure 5). Potassium dichromate acted as a positive control and showed the highest toxicity (LC_{50}=0.74 mg/mL) after 6

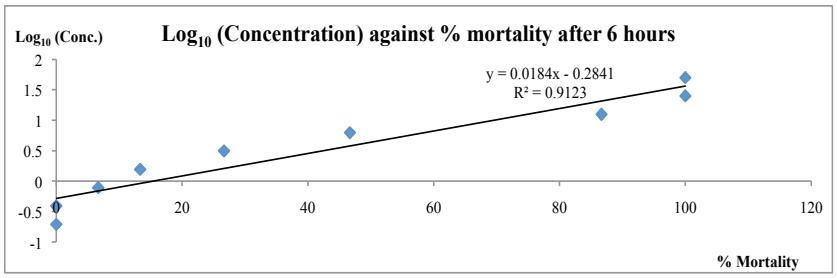

Figure 4. Toxicity of mushroom extract after 6 hours of incubation using brine shrimp bioassay. Each data point represents the mean value of three replicates per concentration level

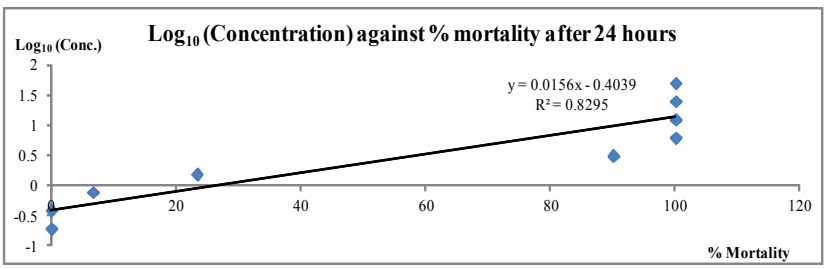

Figure 5. Toxicity of mushroom extract after 24 hours of incubation using brine shrimp bioassay. Each data point represents the mean value of three replicates per concentration level

hours of incubation. All the brine shrimps were dead after 24 hours of exposure to the solution.

Assessment of Mycelial Growths on Solid Media

Medium, pH and temperature have significant effect on the mycelial growth. There was a significant interaction between pH and incubation temperature. The mycelia grew well in acidic conditions (pH 5.0 and pH 6.0) and reached maximum growth at 30°C.

At this optimal condition (pH 6.0 and 30°C), the mycelial growth was significantly different depending on the medium (Figure 6). CZA promoted the highest mycelial diameter growth with shortest incubation time (four weeks), followed by CDA and CMA, which produced the maximum growth in the sixth week. Maximum mycelial growth on MEA and PDA was achieved after eight weeks. Full-plate growth was not observed on CDA, CMA, MEA and PDA but was present for CZA.

Assessment of Mycelial Growth in Liquid Media

The results showed that the type of liquid medium has a significant influence on the production of mycelia. There was no significant interaction between medium and initial pH. The mycelia can grow well over a wide range of pH values (pH 5.0-pH 8.0) without significant difference in biomass production.

YME and YEP tend to be the most suitable liquid media to support *Boletus* growth in acidic conditions (pH 5.0 and pH 6.0) after two weeks of incubation (Figure

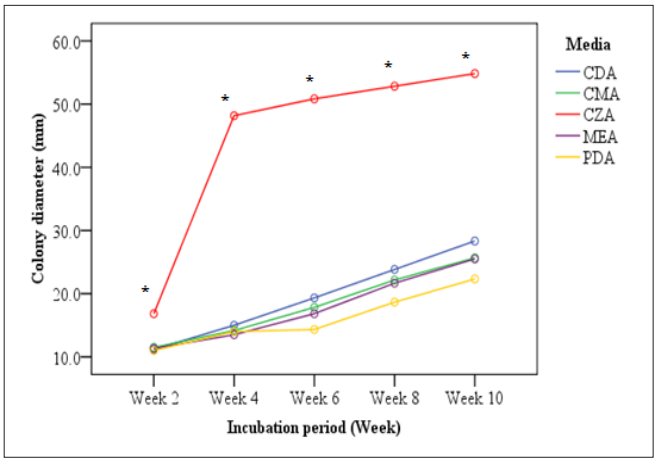

Figure 6. Mycelial growth on different solid media at pH 6.0 and 30°C, 10 weeks of incubation
* indicate significant difference (n=3, $p<0.05$) within the same incubation period

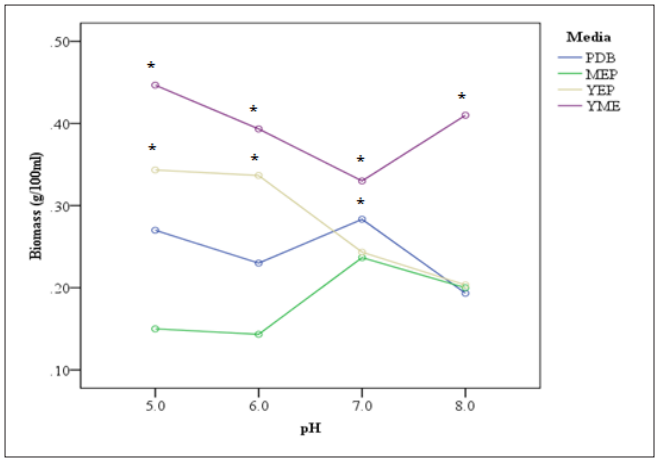

Figure 7. Effect of liquid media on mycelial biomass after 12 days of incubation
* indicate significant difference (n=3, p<0.05) within the same pH

7). YME produced the highest biomass (0.45 g/100 mL), followed by YEP with an average biomass of 0.34 g/100 mL at pH 5.0. The mycelial biomass was the lowest (about 0.20 g/100 mL) in MEP, PDB and YEP at pH 8.0.

DISCUSSION

Colour reactions on the fruiting bodies acted as a preliminary identification in the field. Among the four chemical reagents, potassium hydroxide produced the most consistent result since all the mushroom samples gave no colour changes. Similar to the present observation, the majority of *Boletus* species described by Bessette et al. (2007) reacted to ferrous sulphate and gave olive brown on the stipe contexts. The stipe context also responded to Melzer's reagent, but the yellow colour produced did not match the green-blue colour of *B. rhodopurpureus*, *B. torosus*, *B. xanthocyaneus* and *B. xanthopurpureus* (Hills, 1997). On the pileal surface, the mushroom samples showed a greyish yellow with ferrous sulphate and the result was similar to those in the studies conducted by Bessette et al. (2007) and Chantorn et al. (2007), in which the colour changes ranged from olive green to olive grey.

According to Chantorn et al. (2007), ammonia solution produced orange red on the pileal surfaces of *B. subvelutipes*, *B. laetissimus* and *B. obscureumbrinus*. It differed from the mouse grey colour of the *Boletus* pileal surface in the present study. For the temperate *Boletus* species, the colour reactions can be green or blue on *B. badius*, olive or yellowish on *B. carminiporus* and green or orange-red on *B. innixus* (Bessette et al., 2000; Bessette et al., 2007). These observations suggested that the *Boletus* species responded inconsistently in an ammonia solution to give distinctive colour changes. Melzer's reagent gave a reddish brown on the hymenophore; this

dextrinoid reaction can be attributed to the high lipid content of the spores (Watling, 1971). Similar results were also reported on *B. rhodopurpureus* and *B. edulis* (Smith & Thiers, 1971; Hills, 1997). However, there were no comparable reports on *Boletus* species of olive brown hymenophore by ferrous sulphate.

The overlapping morphological characteristics shown among related *Boletus* species have led to the difficulties in identification and classification. For instance, *Boletus* in the present study showed a purplish pileus, which resembled *B. kluzakii*, *B. xanthopurpureus* and *B. regius* (Thiers, 1975; Hills, 1997; Šutara & Špinar, 2006). However, these three species showed diverse colours of the stipe and hymenophore. Several species such as *B. appendiculatus*, *B. badius*, *B. inedulis* and *B. luridiformis* have contexts that stained blue when exposed to air (Bessette et al., 2000; Bessette et al., 2007; Læssoe, 2010) whereas *Boletus* in this study showed brownish orange. Besides that, most *Boletus* species did not show a pinkish pore surface even though olive brown spores had been reported by Corner (1972).

Because of these overlapping morphological characteristics, the ITS region was used to assist in species identification. Based on the ITS region, the *Boletus* isolates were identified as *B. griseipurpureus*. From the phylogenetic tree, all the *B. griseipurpureus* isolates (USMBo1, USMBo2, USMBo3, USMBo4 and USMBo5) were grouped in the same clade and separated from other *Boletus* species with a high bootstrap value (100%). In Peninsular Malaysia, Corner (1972) was the first to record the occurrence of *B. griseipurpureus* (Mycobank: 309689)

Based on Corner (1972) descriptions of *B. griseipurpureus*, the pileus is convex to plane-shaped, measuring 3.5-10 cm wide, is fleshy and its colour ranges from purple-mauve to purple-grey. The stipe is subclavate, measuring 6-15 cm long and 5-9 mm wide and it is around 7-15 mm at the base. It is finely reticulated, purple-grey but white at the apex of the stipe. However, the white to pale yellow-green pore surface of *B. griseipurpureus* contrasted with pinkish white to reddish brown colour as observed in this study. Corner (1972) further described the basidia of *B. griseipurpureus* to have 3-4 sterigmata, with each bearing a smooth and boletoid spore. The pleurocystidia are ventricose with a prolonged, obtuse or subcapitate apex, often thin-walled. Although several characteristics such as ventricose pileocystidia and clavate cheilocystidia conformed well to the descriptions by Corner (1972), pale red spores in potassium oxide and red-brown pleurocystidia in alcohol formalin were not observed in the present study.

The morphological characteristics of *B. griseipurpureus* in the present study were very similar to the *B. griseipurpureus* reported by Seehanan and Petcharat (2008) in Thailand. The morphological similarities included convex to plane pileus, light purple subtomentose cuticle, white context, pinkish or pale brown tubes, purple cylindrical stipe, clavate basidia and pale brown smooth spores.

According to Meyer et al. (1982), a crude extract that shows LC_{50} lower than 1.0 mg/mL may possess cytotoxic effects. In this study, the mushroom extract exhibited LC_{50} of 4.33 mg/mL. The toxic levels increased twofold after 24 hours of incubation with LC_{50} of 2.38 mg/mL. LC_{50} higher than 1.0 mg/mL implied that the *B. griseipurpureus* collected from the sampling areas were non-toxic. Similar results have been reported for *Agaricus* sp. and *Termitomyces letestui* (Nyigo et al., 2005). In contrast, several edible mushrooms such as *Cantharellus* spp., *Hypsizygus tessulatus* and *Pleurotus ostreatu*s showed LC_{50} below 1.0 mg/mL (Faridur et al., 2010; Kidukuli et al., 2010; Monira et al., 2012). These comparative data suggested that edible mushrooms normally are low in toxicity.

The edibility of *B. griseipurpureus* can be affected by its growing habitat. Studies on mushroom toxicity showed a correlation between fungal metal content and sources of metal pollution, in which the metal content of edible mushrooms sometimes exceed poisonous levels (Carvalho et al., 2005; Stihi et al., 2011). This can be attributed to the accumulation of heavy metals in fruiting bodies (Li et al., 2011). In the present study, the peat soils were slightly contaminated with copper, mercury and plumbum, which can pose risk of mushroom poisoning. In addition to heavy metals, dispersal of toxic pesticides on the growing site can also cause hazardous effects on mushrooms (Pacioni & Lincoff, 1982). The residue of pesticides may persist on the mushroom surfaces for a long time and consequently, pose a health risk for humans if consumed.

Wild *Boletus* produced a high level of secondary metabolites including ascorbic acids, phenolic acid, terpenes, tocophenols and steroids (Tsai et al., 2007; Grangeia et al., 2011; Heleno et al., 2011). These antioxidant substances may also be present in *B. griseipurpureus*, resulting in the bitter taste of the mushroom. However, some edible *Boletus* such as *B. aereus*, *B. borrowsii*, *B. edulis* and *B. reticulatus* are well known for their sweet taste and pleasant smell (Dentinger et al., 2010). This aromatic property may be due to the volatile substances of arabitol, myo-inositol, mannitol and trehalose (Tsai et al., 2008). Any form of processing of fresh mushrooms is expected to alter the composition of these volatile substances, which can be chemically reactive (Manning, 1987). Therefore, new compounds may be formed and this may be the cause of the sweet taste of the mushrooms after cooking.

Among the five solid media tested, CDA promoted the highest colony diameter (about 30 mm), suggesting high soluble starch-induced mass production of mycelium. CMA and MEA contain moderate amounts of starch and thus, allow good mycelial growth. Dung et al. (2012) reported that fungal strains that actively degrade starch always grow well on starch-containing media. Although full-plate growth was observed on CZA, poor utilisation of sucrose by *B. griseipurpureu*s resulted in a sparse appearance of the mycelium.

A similar result was reported by Hughes and Mitchell (1995) and Hatakeyama and Ohmasa (2004).

CMA and MEA contained yeast extract and peptone, respectively. Since the mycelia took a relatively longer time (8 weeks of incubation) to achieve maximum growth, media enriched with high nitrogenous nutrients are not recommended. Mycelial biomass of *Boletus* spp. is also reduced in high nitrogen content (Hatakeyama & Ohmasa, 2004). Contrasting results were shown by *Lentinus subnudus*, which grew well in CMA and MEA without the presence of yeast extract and peptone (Gbolagade et al., 2006). According to Koike et al. (2001), C:N ratio in the range of 15 to 20 provides a balance between carbon and nitrogen sources, which encourages mycelial growth and maintains stable metabolite production. When nitrogen sources became the limiting factor, *B. griseipurpureus* grew better in CDA and PDA in response to the high consumption of carbohydrates (Paustian & Schnürer, 1987).

Temperature and pH are important factors determining fungal growth on synthetic media. In the present study, the mycelia reached optimum growth at 30°C, which suggested that high temperature is required for the enzyme activities of *B. griseipurpureus*. The results contrasted with the optimal temperature (20°C) for the vegetative growth of *B. reticulatus* (Yamanaka et al., 2000). The mycelial growth of the *B. griseipurpureus* was enhanced at pH 6.0. Several single-factor designed experiments showed that temperature ranging from 25°C to 30°C and pH value from pH 3 to pH 6 are the most suitable cultural conditions for mycelial growth of some cultivated mushrooms such as *Volvariella volvacea*, *Agaricus brasiliensis* and *Pleurotus* sp. (Akinyele & Adetuyi, 2006; Calauto et al., 2008; Zharare et al., 2010).

Regardless of the type of liquid media tested, *B. griseipurpureus* grew well over a wide range of pH from pH 5 to pH8. Although most edible mushrooms are found to tolerate a broad pH range in a submerged culture, the optimal pH can be varied depending on the mushroom species. Kim et al. (2005) obtained the highest mycelial biomass of *Agrocybe cylindracea* at pH 4. Xu and Yun (2003) reported that *Auricularia polytricha* achieved the maximum growth at pH 5. The optimal pH of *Agaricus bisporus* and *B. edulis* was pH 6 (Kurbanoglu et al., 2004; Baptista & Nogueira, 2009). These observations suggested that different species prefer different pH values from acidic to neutral.

Effects of different carbon sources on the biomass production in a submerged culture have been studied (Papaspyridi et al., 2010; Smiderle et al., 2012) and it has been suggested that glucose is an excellent monosaccharide for mycelial growth. In the present study, all the liquid media were provided with the same amount of glucose, and thus the variation of mycelial biomass might be attributed to the nitrogen sources. Among the four liquid media, YME significantly produced the highest yield of mycelium, followed by YEP. A similar result

was reported by Lin and Chen (2007), who showed that liquid media supplemented with peptone and yeast extract enhanced mycelial growth of *Antrodia cinnamomea*. Several studies have also found that media containing yeast extract gave the highest mycelial yield when glucose was used as the main carbohydrate source (Feng et al., 2010; Lee et al., 2013).

MEP produced lower mycelial biomass indicating that *B. griseipurpureus* grew slowly in the absence of yeast extract. Pokhrel and Ohga (2007) reported that 1% yeast extract in liquid media stimulated maximum mycelial growth of *Lyophyllum decastes*. This stimulatory effect of yeast extract may be due to its high protein, amino acid and vitamin content. The mycelial biomass was the lowest in basal medium PDB, suggesting the deficiency of nitrogenous compounds in the medium as complex organic nitrogen generally increases the biomass production for the submerged culture of higher fungi (Papaspyridi et al., 2010). Corn steep powder, soybean meal, malt extract, tryptone and casein were found to be good nitrogen sources in submerged culturing of wild edible mushrooms such as *Agrocybe cylindracea*, *Lentinus subnudus* and *Cordyceps militaris* (Kim et al., 2005; Gbolagade et al., 2006; Shih et al., 2007) and thus, may also be suitable for the mycelial growth of *B. griseipurpureus*. However, there are many other factors that can influence mycelial growth in a submerged culture including inoculum volume, agitation rate, aeration rate, incubation duration, salinity and light intensity (Cho et al., 2006; Miao et al., 2006; Chen et al., 2008; Vargas-Isla & Ishikawa, 2008). The combined effect of these factors on the growth of *B. griseipurpureus* needs further investigation.

CONCLUSION

The mushroom locally known as '*kulat gelam*' from a peat swamp area in Bachok, Kelantan was identified as *B. griseipurpureus*. A scientific name of wild mushroom is important as it can provide information on the edibility as well as the nutritional and medicinal properties of the mushroom. A toxicity test suggested that this species is an edible mushroom with a low toxic level (LC_{50}=4.33mg/mL). At optimal cultural conditions (30°C and pH 6.0), CDA promoted the highest colony diameter on plates while YME was the most suitable medium to obtain a good biomass production for submerged culturing of *B. griseipurpureus*. The results obtained from the present study provide the basic information of substrate formulation to cultivate wild edible *B. griseipurpureus*.

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