SHORT COMMUNICATION

Morphology and Histology Identification of Fungal Endophytes from Oil Palm Roots in *Ganoderma boninense* Endemic Area

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Endophytic fungi defined as fungi that colonize internal plant tissues without causing visible damage to their host plant. As they are internal colonisers, therefore more able to compete within the vascular systems with capacity to arrest the spread of pathogens such as *Ganoderma boninense* causal agent of Basal Stem Rot (BSR) disease in oil palm. Endophytic microbes acted against plant pathogen by antibiosis mechanism, nutrient and space competition, and induce plant pathogen resistance by producing metabolites. The objective of the present study was to identify endophytic fungi from oil palm roots in *G. boninense* endemic area Padang Halaban Estate, North Sumatera, based on morphological and histological character. At each site, five random palms were sampled. Seventy five endophytic fungi had been isolated and selected from BSR symptomless palm root. Identification of fungal endophytes were carried out by observing the reproductive structures (sexual and asexual) under a light-field microscope with camera attachment. Seventy five isolates were classified to eight genera, consisting of *Trichoderma* (20), *Fusarium* (10), *Aspergillus* (5), *Penicillium* (5), *Gliocladium* (4), *Phoma* (4), *Alternaria* (4), and *Curvularia* (3). Twenty others were unidentified due to sterile mycelia.

Key words: basal stem rot, *Elaeis guineensis*, endemic area, oil palm, root endophytic-fungi

Fungi endofit hidup dan berkoloni dalam jaringan tanaman namun tanpa menunjukkan gejala perusakan pada tanaman inang. Sebagai mikroba pengkoloni internal, akan lebih memudahkan dalam bersaing dalam system vascular dengan patogen seperti *Ganoderma boninense* penyebab busuk pangkal batang tanaman kelapa sawit. Mikroba endofitik bekerja melawan patogen tanaman melalui mekanisme antibiosis, persaingan ruang dan nutrisi untuk pertumbuhannya, dan menginduksi ketahanan tanaman melalui metabolit yang dihasilkan. Tujuan dari penelitian ini adalah mengidentifikasi fungi dari akar tanaman kelapa sawit asal kebun endemik *G.boninense* di Sumatera Utara berdasarkan karakteristik morfologi dan histologi. Lima sampel diambil secara acak dari masing-masing lokasi. Sebanyak 75 isolat diisolasi dari akar tanaman kelapa sawit sehat yang tumbuh di kebun endemik. Identifikasi morfologis dan mikroskopis fungi endofit dilakukan dengan mengamati struktur reproduksi seksual maupun aseksual menggunakan kamera terhubung dengan mikroskop. Isolat diidentifikasi ke dalam 8 genera yaitu *Trichoderma* (20), *Fusarium* (10), *Aspergillus* (5), *Penicillium* (5), *Gliocladium* (4), *Phoma* (4), *Alternaria* (4), dan *Curvularia* (3). Isolat lainnya tidak teridentifikasi secara morfologis maupun histologis, karena tidak berspora.

Kata kunci: area endemik, *Elaeis guineensis,* busuk pangkal, fungi endofit akar, tanaman kelapa sawit

Endophytes refer to a group of fungi that reside asymptomatically inside the living plant tissues as reported by Hyde and Soytong (2008). Huang *et al*. (2008) reported that recent surveys of various host plants have demonstrated that fungal endophytes are ubiquitous in plant species . Globally, there are at least one million species of endophytic fungi as reported by Ganley *et al*. (2004), which represent an important genetic resource for biotechnology. Endophytes have been recognized as potential sources of novel natural products for agricultural, industrial, and pharmaceutical uses, especially those secondary metabolites produced by fungal endophytes colonizing medicinal plants as reported by Mitchell *et al*. (2008). According to Shiomi *et al*., (2006), endophytic fungi are able to penetrate and become systemically disseminated in the host plant, actively colonize the apoplast, conducting vessels and occasionally the intracellular spaces. This colonization presents an ecological niche, similar to that occupied by plant pathogens therefore endophytic fungi are more able to inhibit plant pathogens by antibiosis mechanism, competition for nutrients and spaces for proliferation, and induce plant resistance.

Zaiton (2006) studied the isolation and characterization of microbial endophytes from oil palm roots in

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Malaysia. The results showed that the fungal isolates were mainly from the genera *Fusarium* and *Trichoderma*. *Fusarium* is most ubiquitous and has been isolated from many host plants and constitutes 72.70 per cent of the isolated fungi. Detection of fungal endophytes was carried out by observation of histological oil palm root sections under light microscope (100× magnification). Fungal hyphae were stained by toluidine blue have been observed intra and inter cellularly in roots from symptomless palms, especially in the epidermal cells running parallel into the schlerenchyma cells and the cortex.

Mitrousia (2012) stated that some rhizospheric fungi are pathogens to plants such as phoma. But it is still to be confirmed if it also the case of endophytes fungi. Nur Amin *et al*. (2014) reported that endophytes *Gliocladium*, *Fusarium*, *Aspergillus*, and others have been isolated from cocoa and supposed to be beneficial for plant. Mathan *et al*. (2013) reported antimicrobial metabolites could be produced by fungi such as *Aspergillus* sp.

Basal stem rot (BSR) disease caused by *Ganoderma* species is the most destructive disease in oil palm. The use of conventional chemical fungicide has not given much green light, as the disease is known systemic. So far, cultural practices, combined to some extent with biological control, have been considered as the best approach for controlling the BSR. Recently, study on endophytic fungi as biological control agents in suppressing plant disease has gained much attention in pathological research. Some of endophytic bacteria and fungi might have potentiality to control *G. boninense* since they showed ability to inhibit *G. boninense* in vitro as reported by Wicaksono *et al*. (2011). The study of Sharaf *et al.* (2013) also reported that chitinase produced by *Trichoderma* sp. could be used as antifungal agent.

The objective of this current study was to identify as much as possible specific endophytic fungi living in BSR symptomless oil palm plant, for the first stage by morphological and histological characterizations. This study was conducted to obtain potential endophytic fungi against *G. boninense* in view of formulating indigenous biofungicide.

Root Sampling and Isolation of Fungal Endophytes. Oil palm roots were obtained from Padang Halaban Estate PT SMART, Tbk in North Sumatera, Indonesia. The age of palms was 28 years with BSR (Basal Stem Rot) symptomless at symptom areas. The roots were sampled from three sites selected for BSR infection. At each sites, five random palms were sampled with the roots diameter 0.5 cm, taken about 1.0 m away from their bases at 25-30 cm depth. Root samples from each palm were rinsed under running tap water for 20 min to remove any adhering soil from their surface, then surface sterilized using 5.25% of sodium hypochlorite. After dipping in 50, 70, and 90% of ethanol, samples were rinsed with sterilized distilled water following procedure described by Arnold *et al.* (2003). Root samples were then dried on a sterilized filter paper.

One centimeter from the ends of each section was discarded. The cuticle from the middle centimeter was removed and was cut into two sub-sections of 0.5 cm each. The sub-sections were, in turn, split longitudinally into four before transferring to the PDA media for culturing.

Purification of Fungal Endophytic Isolates. Fungal endophytes which showed emergence at cutting sample then were selected to be purified. Colonies were cut and transferred aseptically into new cultures media. Colonies which were pure then selected and transferred in slant agar and Petri dish containing PDA media. Colonies were incubated at 28±2 °C. Pure fungal isolates were then observed macroscopically and microscopically for identification.

Morphological and Histological Identification of Fungal Endophytes. Fungal endophytes were selected for further characterization and identification based on Barnett and Hunter (1998). Morphological observation was followed every 24 h during 30 d and identification of fungal isolates was done according to colony or hyphal morphology of the fungal culture, surface and reverse colony color, and colony texture. Histological identification of fungal endophytes was carried out by observing the characteristics of the spores or conidia, and reproductive structures (sexual and asexual) under a light-field microscope with camera attachment (Model Nikon Eclipse-50i, Japan).

Growth of Fungal Endophytes. Only a few of pure cultures of fungi started to grow out of the surface of sterilized root sections after 24 h of incubation on PDA at 28±2 °C, with 1.1 to 2.6 cm of diameter. Almost all of them started growing after 48 h with diameter range from 0.4 to 5.3 cm. Some were fast growing and others were even very slow growing fungi, which started to grow after 168 h (2.0 and 9.0 cm of diameter respectively). Seventy-five endophytic fungi isolates were obtained from palm roots. According to morphology and histology characters, the isolates of endophytic fungi belonged to genera *Trichoderma* (20), *Fusarium* (10), *Aspergillus* (5), *Penicillium* (5),

Gliocladium (4), *Phoma* (4), *Alternaria* (4), and *Curvularia* (3). Twenty others could not be identified due to sterile mycelia.

Trichoderma sp. *Trichoderma* sp. were typically fast growing observed on PDA at 28 °C. Colonies were transparent to white at beginning on agar media, which was developed 24 h after incubation, with diameter of 1.5-2.0 cm, and becoming compact in time. The surface of colony color was white and scattered greenish patches become visible as the conidia were formed starting from 72 h and concentric rings were slowly formed in time. On the reverse side, the color was pale to yellowish. A yellow pigment seemed clearly to be secreted into the agar (Fig 1A).

Taxonomy of *Trichoderma* were based largely on histological character such as conidial form, size, color and ornamentation, branching pattern with short side branches, short inflated phialides and the formation of sterile, and fertile hyphal elongation from conidiophores. Microscopic of *Trichoderma* showed the presence of septate hyaline hyphae of around 200 μm. Phialides were hyaline, branched, and flask-shaped, inflated at the base, represented in cluster, and attached to the conidiophores at right angles. Conidiophores were also hyaline and branched. Conidia were unicellular, ellipsoidal form, green in color, smooth walled, with an average diameter of 5 μm, and were grouped in sticky heads at the tips of the phialides (Fig 1B).

The genus *Trichoderma* (Ascomycetes, Hyprocreales) contains species that are of vast economic importance owing to their production of antibiotics, industrial enzymes, and ability to act as biological control agents (BCA) against plant pathogens since 1920s. Some *Trichoderma* strains have been developed and widely applied in agricultural practices as bio-control agents against plants' fungal diseases. The various mechanisms include antibiosis, parasitism, inducing host-plant resistance, and competition. Strains producing enzymes such as chitinase and glucanase have been selected as criteria for BCA.

Fusarium sp. Morphological observation of *Fusarium* sp. showed that these cultures were fast growing starting to grow after 24 h, and produced woolly and cottony spreading colonies. From the front, the color of the colony were observed creamy white. From the reverse side, it was almost colorless. Some species also produce distinctly different conidia in the aerial mycelium (referred to as microconidia). Aerial mycelium represented the growth of hyphae above the agar surface, often forming a convex shape, with a cottony or somewhat ropey texture (Fig 2A). Species of *Fusarium* typically produce both macro- and microconidia from slender phialides. Macroconidia were hyaline, two- to three-celled, comma shaped, mostly with an elongated apical cell and pedicellate basal cell. Microconidia were formed from 1- to 2-celled, hyaline and ovoid, straight or curved. Chlamydospores were present in this *Fusarium* culture.

According to Summerel *et al.* (2003), *Fusarium* were characterized by the production of slimy, hyaline, canoe-shaped conidia (known as macroconidia) that in most species are produced in fruiting-structures called sporodochia. Some *Fusarium* species produce morphologically distinct conidia in the aerial mycelium, which are usually smaller and have fewer septa than the macroconidia produced in sporodochia. Therefore, they are called microconidia (Fig 2B) showing that macroconidia was a type C, straight. Microconidia were represented in comma shaped or ellipsoidal. Chlamydospores were produced singly or in pairs. Monophialide is phialide that produces conidia from one opening only. *F. oxysporum*-non pathogenic have been developed as bio-control agents against plant pathogen by antibiosis and mycoparasitism mechanism (Rubini *et al.* 2005).

Aspergillus sp. *Aspergillus* sp. characteristics which are essential in genera identification are the growth rate, colony color and thermo-tolerance. Texture of colonies varies from downy to powdery. The colonies were fast growing, with average diameter of 1.5 cm at 24 h and 6.0-9.0 cm at 168 h on PDA at 28 °C. Surface colony color was black with powdery aspect, and the reverse was mostly pale yellow (Fig 3A). The only thermo-tolerant *Aspergillus* which can grow at temperature range of 20 °C to 50 °C is *A. fumigatus*.

Histological microscopic of *Aspergillus* showed that the hyphae of these genera were septate and hyaline. Conidiophores arised from the basal foot cell found at the supporting hyphae and terminate in a vesicle at the tip. Vesicle is typically for the genus *Aspergillus*. Phialides were flask-shaped attached to the vesicle via a supporting cell, metula. Conidia were attached over the phialides forming radial chains (Fig 3B). Conidia were round with 1 μm of diameter in average.

Penicillium sp. *Penicillium* sp. is comparable to *Aspergillus*. The genus *Penicillium* falls into the order Eurotiales. In this order, organisms produce asci within cleistothecia. *Penicillium* is often reffered to as Deuteromycetes, or Fungi imperfecti. The name *Penicillium* comes from the word "brush"; this refers to

the appearance of spores in *Penicillium*. Bancerz *et. al.* (2005) found that this species was one of the best lipase producers among other fungi they studied in the arctic tundra. *Penicillium* has high enzymatic activity and has the ability to produce alpha-amylase. Most fungi contain secondary metabolites. These were used to produce antibiotics such as penicillin. Certain components of fungal genetic structure that create these secondary metabolites are common across not just species, but across orders (Carlile *et al.* 2001).

Penicillium is known as filamentous fungi. The colonies of *Penicillium* were rapid growing, flat, filamentous, and velvety in texture. The colonies were initially white and became pinkish in time. The plate reverse was yellowish and pale (Fig 4A). They had branched conidiospores. Conidia were observed round and unicellular. *Penicillium* culture seemed to have small hyphae. Glucans are reported common in the cell walls of *Penicillium* species.

Penicillium hyphae were septate hyaline (1.5 to 5 μm in diameter), simple or branched conidiophores, metulae, phialides, and conidia were observed. Metulae were secondary branches that were formed on conidiophores. The metulae carried the flask-shaped phialides. The organization of the phialides at the tips of the conidiophores was very typical. They form brush-like clusters, which referred to as "penicilli". The conidia (2.5-5 μm in diameter) were rounded, unicellular, and visualized as unbranching chains at the tips of the phialides (Fig 4B).

Gliocladium **sp.** *Gliocladium* sp. is most close related to *Penicillium* and *Paecilomyces*. Colonies on potato dextrose agar were white initially, but typically become green, granular, poorly demarcated, and grew across the entire plate, resembling a "green lawn" (Fig 5A). Phialides were branched and tapered at tips. Spherical conidia were gathered at tip of phialides in a tight,ball-shaped cluster and were often somewhat larger than *Trichoderma* (Fig 5B). Conidiophores had penicillate branches and conidial masses collecting in a mucilaginous droplet. Spores were smooth-walled. Small spines, which were microscopically observable, distribute sparsely on the main stem of conidiophores.

Phoma **sp.** *Phoma* sp. is a cosmopolitan, dematiaceous filamentous fungus that inhabits the soil and plant material. Colonies growth rate was rapid, starting from 48 h, texture powdery to velvety, spreading and frequently submerged in the PDA medium. Surface colony color was initially white becoming olive gray, sometimes with a tint of pink while reverse color was purple to yellow diffusible pigment.

Histological microscopic showed that hyphae of this genera were septate and hyaline to brown (Fig 6B). Conidia were hyaline, oval shaped, unicellular and each conidium usually had two oil droplets inside. Chlamydospores were brown and appear in long chains.

Alternaria **sp.** *Alternaria* sp. grew rapidly and the colony size reached a diameter of 3 to 9 cm after incubation at 28 °C for 7 d on potato dextrose agar. The colony was flat, downy to woolly and was covered by grayish, short, aerial hyphae in time. The surface was grayish white at the beginning, which later darkened and became greenish black or olive brown with a light border (Fig 7A). The reverse side was typically brown to black due to pigment production. *Alternaria* spp. had septate, brown hyphae. Conidiophores were also septate and brown in color, occasionally producing a zigzag appearance. They beared simple or branched large conidia (7-10 × 23-34 μm) which have both transverse and longitudinal septations. These conidia were observed singly or in acropetal chains and may produce germ tubes (Fig 7B). They were ovoid to obclavate, darkly pigmented, muriform, and smooth or roughened. The end of the conidium which were the nearest the conidiophore was round while it tapered towards the apex. This gave the typical beak or club-like appearance of the conidia.

Curvularia **sp.** *Curvularia* sp. produced rapidly growing, woolly colonies on potato dextrose agar at 28 °C. From the front, the color of the colony was white to pinkish gray initially and turned to olive brown as the colony matured. From the reverse, it was dark brown to black (Fig 8A).

Curvularia hyphae were septates brown, of which brown conidiophores, and conidia were visualized. Conidiophores were simple or branched and bent at the points where the conidia originated. This bending pattern was called sympodial geniculate growth. The conidia (8-14 × 21-35 μm), which were also called the poroconidia, were straight or pyriform, brown, multiseptate, and had dark basal protuberant hila. The septa were transverse and divided each conidium into multiple cells. The central cell was typically darker and enlarged compared to the end cells in the conidium. The central septum may also appear darker than the others. The swelling of the central cell usually gave the conidium a curved appearance (Fig 8B).

The number of the septa in the conidia, the shape of the conidia (straight or curved), the color of the conidia (dark vs pale brown), existence of dark median septum, and the prominence of geniculate growth pattern were
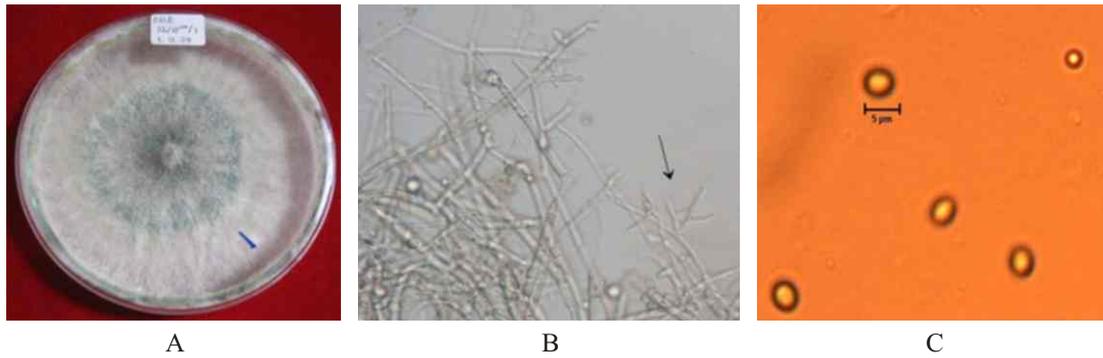
| A | B | C |

Fig 1 Culture of *Trichoderma* growing on potato dextrose agar. The white areas did not contain spores, while the green areas were covered with dense masses of spores (conidia) (A). *Trichoderma* was repeatedly branched conidiophores, irregularly verticillate, bearing clusters of divergent, irregularly bent, flask-shaped phialides (B). Conidia/spores (C).
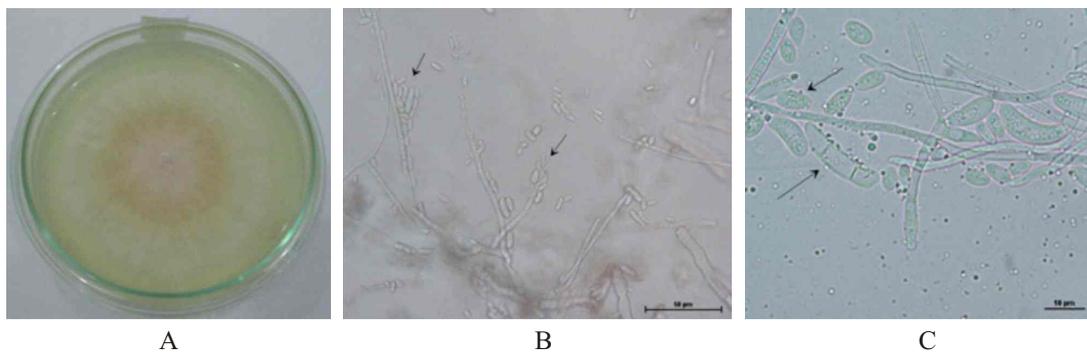

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Fig 2 Culture of *Fusarium* growing on potato dextrose agar (A). Microconidia, macroconidia (2 - 4 septates), septate hyphae, and chlamydospores were present (arrows showed in B).
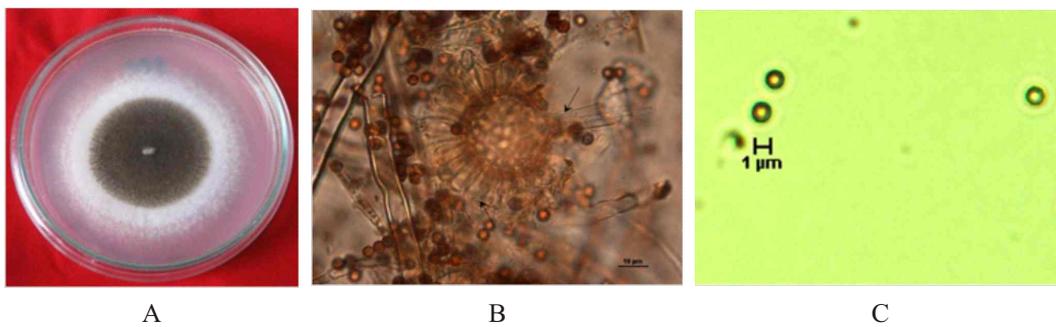

| A | B | C |

Fig 3 Culture of *Aspergillus* growing on potato dextrose agar. The spores come in black color (A); Microscopic histology of *Aspergillus*. Phialides attached to the vesicle via a supporting cells called metula (arrow showed in B). Conidia/spores (C).
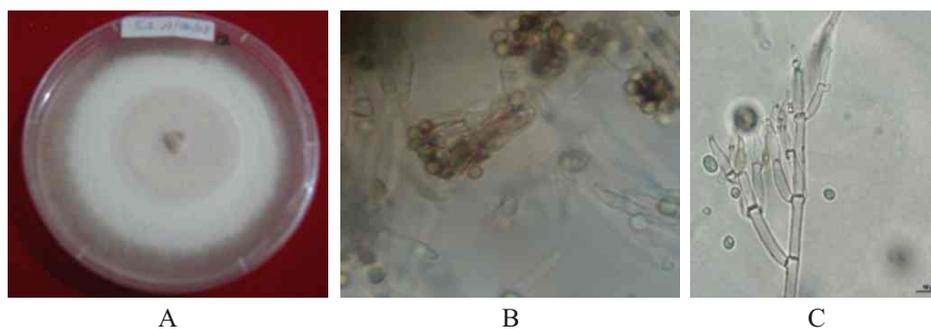

| A | B | C |

Fig 4 Culture of *Penicillium* growing on potato dextrose agar. Colonies texture are very powdery (A); Conidia, phialides and metula are present. Phialides are attached to the vesicle via a supporting cells called metula (B).

Fig 5 Culture of *Gliocladium* growing on potato dextrose agar (A); microscopic structure of phialides with branches and
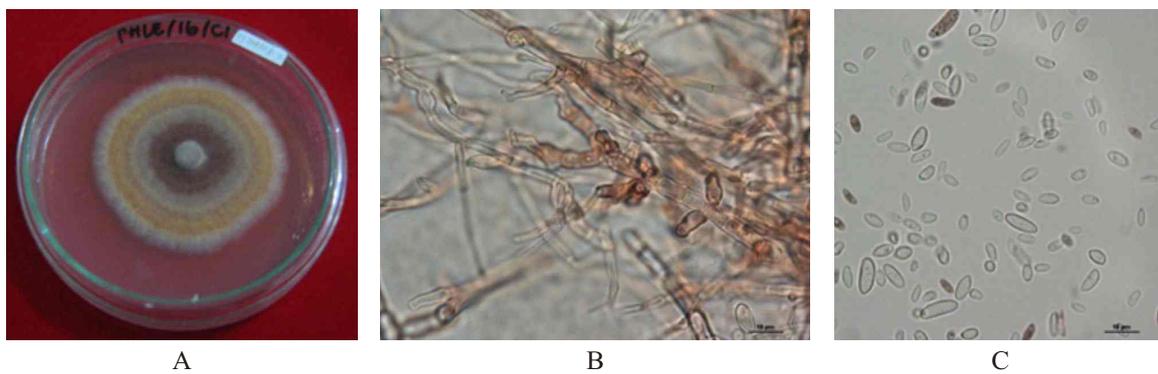tapered at tips (arrows showed in B).

Fig 6 Culture of *Phoma* growing on potato dextrose agar. Septate hyaline hyphae and hyaline conidia were present (B).

Fig 7 Culture of *Alternaria* growing on potato dextrose agar (A); Conidia darkly pigmented with germ tubes (B).
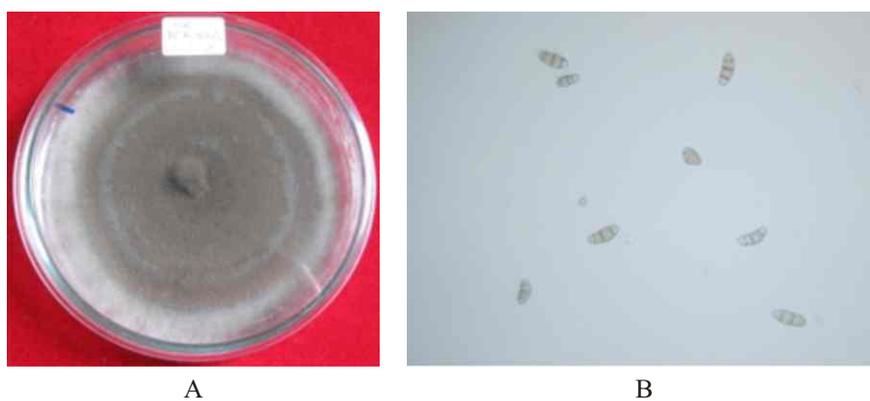

Fig 8 Culture of *Curvularia* growing on potato dextrose agar (A); Conidia are brown and multiseptate (B).

the major microscopic features that help in differentiation of *Curvularia* spp. among each other. For instance, the conidia of *C.lunata* have 3 septa and 4 cells, while those of *C. geniculata* mostly have 4 septa and 5 cells.

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