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Basidiomata Induction and Characterization of *Ganoderma* from Oil Palm (*Elaeis guineensis*) on Three Agrowaste Substrates

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ABSTRAK

Ganoderma *ialah patogen penyebab reput pangkal kelapa sawit. Ahli-ahli genus ini menunjukkan kepelbagaian yang tinggi di tropika; tetapi adalah sukar untuk ditentukan pengertian kepelbagaian ini dari segi taksonomi kerana kestabilan ciri-ciri tertentu kulat ini masih belum diketahui. Kertaskerja ini merangkakan kaedah pengkulturan yang mengaruhkan pembentukan basidiomata* Ganoderma *dibawah keadaan terkawal, dan seterusnya membolehkan kestabilan ciri-ciri tertentu dinilaikan. Dengan menggunakan basidiomata aruhan, ciri-ciri terpilih dapat dihasilkan berulangkali tanpa perubahan diatas tiga jenis substrat, iaitu serabut kelapa sawit, serat kapas, dan habuk kayu getah. Dari segi kadar kolonisasi oleh miselia, kulat ini menunjukkan perbezaan bererti antara pertumbuhan diatas serat kelapa sawit, serat kapas dan habuk kayu getah; tetapi setelah basidioma mula terbentuk, kadar pertumbuhan dan perkembangan basidioma adalah sama, dan tidak bergantung pada jenis substrat. Selain daripada kadar pertumbuhan* Ganoderma, *kajian ini juga memberi kefahaman keatas status warna basidioma matang, potensi ciri perlekatan stip digunakan sebagai nilai taksonomi, dan keperluan kelembapan bandingan persekitaran yang tinggi untuk pengeluaran spora daripada basidiomata aruhan.*

ABSTRACT

Ganoderma *is a causal pathogen of basal stem rot of oil palm. Members of this genus are very diverse in the tropics but the significance of this diversity is difficult to relate to taxonomic levels in the genus, largely because of the lack of knowledge about the stability of particular features of the fungus. This paper outlines a culture method that induces the formation of* Ganoderma *basidiomata under controlled conditions, and thus enables the stability of characters to be evaluated. Using induced basidiomata, selected characteristics were found to be reproducible on 3 solid substrates, palm press fibres (PPF), cotton fibres (CF) and rubberwood sawdust (RSD). The rate of mycelial colonization varied significantly with different substrates but, once formed, the rates of basidioma growth and development were comparable irrespective of substrate type. Besides the growth rates of* Ganoderma, *this study also offers insight into the status of colour in mature basidioma, the potential of stipe attachment as a taxonomic character, and the requirement for high ambient RH values for spore production of induced basidiomata.*

INTRODUCTION

Species of *Ganoderma* are found worldwide as saprophytes on logs and stumps, and occasionally as parasites on trees. In Malaysia and Sumatra, species of this fungus cause serious root and basal stem rot of oil palm (*Elaeis guineensis*), for which an effective means of control is still not available. The bright and 'varnished' appearance of the fruiting body makes it easily recognizable in the field, but identification to species level is difficult.

Reports of pathogenic species on oil palm in Malaysia range from 1 species (Ho and Nawawi 1985) to at least 4 (Steyaert 1976) and as many as 8 (Turner 1981).

Conventional taxonomic approaches are based on characteristics of the fruiting body, which is also referred to as basidioma, basidiocarp, carpophore or sporophore. Current taxonomic keys are mostly concerned with temperate species, whilst those for tropical species are mainly based on dried specimens.

Members of this genus are extremely diverse in the tropics and Ryvaarden (1995) has suggested, but not yet verified, that the taxonomic characters currently used in the identification of *Ganoderma* probably vary under different growing conditions. Mycelial isolations can be made from infected palm tissues or the sporophores themselves, but there is as yet no foolproof method of verifying that these agar cultures belong to *Ganoderma*, because *Ganoderma* cultures on agar do not form fruiting bodies. In addition, all local cultures are white and remain as sterile mycelia, making hyphal characterization of limited use. This study was undertaken to establish a reliable method of inducing the formation and sporulation of *Ganoderma* fruiting bodies on solid substrates to enable the reliability of basidiomata characters to be assessed.

MATERIALS AND METHODS

Fungal Inocula

Two *Ganoderma* isolates were selected based on the different morphological appearance and growth habit of their respective sporophores in the field.

The first sample was EGSP 03, isolated from a sporophore collected from a recently infected eighteen-year-old oil palm at Sri Pelangi Estate, a coastal area in Teluk Intan, in the state of Perak. At the time of collection the EGSP 03 basidioma had a blackish-brown stipe measuring 1.7 cm in length. The main body, or pileus, measured 6.3 cm wide at the broadest part and 4.2 cm long from the distal end to the base of the pileus. The dorsal surface was a bright reddish-brown with tinges of yellow, and had conspicuous concentric growth rings. The margin was light brown. The undersurface, or pore layer, was a light cream colour. The dorsal surface of the pileus was smooth and highly laccate ('varnished') with an index of '5' on a scale of 0 to 5, where 0 indicates a complete absence and 5 the highest degree of lacca deposition. EGSP 03 was identified as *G. boninense* based on *CMI Descriptions of Pathogenic Fungi and Bacteria Paper No. 444.*

The second sample, EGUJ 02, was from Ulu Jempul, an inland area in the state of Pahang, where the sporophore was collected from an oil palm stump which had been in the oil palm plantation for nearly 3 years. The sporophore of EGUJ 02 was sessile and 8.5 cm at the widest part and 5.6 cm in length from the distal margin to the base. The sporophore shape was subungulate; the basal portion was approximately eight times thicker than the distal margin. The basal end measured 3.8 cm from top to bottom and 0.5 cm at the distal end. The dorsal surface was thick, corrugated and uneven with the presence of ridges, which also made barely discernable concentric growth rings. The pileus was uniformly dull buff brown, while the ridges on it were blackish. The pileal surface was matt, with an index of '0' on a scale of 0-5, where 0 indicates a complete absence of lacca deposition. The pore layer was a light cream colour. Based on *CMI Descriptions of Pathogenic Fungi and Bacteria Paper No. 443*, it is probably *G. cf. applanatum.*

Fungal mycelia isolated from the contextual tissues of *Ganoderma* basidioma were used as the source of inoculum. Pieces of tissues measuring 2 × 2 × 4 mm each were cut out from its contextual layer and surface-sterilized in 10% chlorox (NaOCl) for 5 min. A single piece was picked with a flamed scalpel or tweezers and placed in the

centre of a 2% malt extract agar (MEA) culture plate, which had earlier been steam-sterilized at 120°C, 25 psi for 15 min. The inoculated plates were incubated at 28°C (± 1.5°C). Pure mycelia obtained from these isolations were subcultured on MEA slopes in universal bottles as stock cultures and stored at 10°C until required.

Substrate Preparation and Inoculation

The 3 substrates used were empty fruit bunches of oil palm commonly called palm-press fibres (PPF), rubber wood sawdust (RSD) and cotton fibres (CF). PPF was obtained from oil palm plantations, CF from textile industries and RSD from furniture-processing plants. PPF were soaked overnight to saturate the fibres. Rubberwood sawdust and cotton fibres were mixed with 1:1 volume of water. All 3 substrates were then separately drained on fine wire mesh so that the water content was just at saturation point, and packed separately into 15 × 33 × 0.05 mm heat-resistant polypropylene autoclavable bags. The substrate was pressed into compact blocks of approximately 20 cm in height, 12 cm in circumference and 960-1000 g in weight. The free end of the bag was put through a PVC pipe 5 cm long and 3 cm wide to secure the bag, and a cotton ball wrapped in gauze was put through the pipe to close it. These packed bags of substrate were then steam-sterilized at 121°C, 25 psi for 45 min, and were ready for inoculation when sufficiently cooled.

The inoculum starters were prepared by subculturing the isolate from slope cultures on 2% MEA culture plates, incubated at 28°C. A 7-day-old culture colony was selected and aseptically macerated with a flamed scalpel. The PVC tube was pulled off, the bag opened, and the macerated mycelia transferred on to the upper surface of the compacted substrate. The tube was put back in place and the culture bag subsequently closed with the cotton plug, ready for incubation.

Growth and Development on Solid Substrates

Both EGSP 03 and EGUJ 02 isolates were used in this experiment. Data collection was confined to EGSP 03 only, but induced basidiomata from both cultures were used for the morphological study. Mycelial inoculum was prepared as outlined above. One culture was used as inoculum starter for one bag. Ten replicates were made per isolate, per substrate type, and all were incubated at 28°C (±1.5°C). The bags were placed upright to allow maximum colonization of the surface area and inspected daily for mycelial extensions from the top to the lower portion of the bag. When fully colonized, a 2 × 4 cm slit was made in the side of the plastic bag to expose the mycelia, which was then transferred to the mushroom nursery to stimulate basidioma development.

The mushroom nursery consisted of an enclosure surrounded by fine-mesh plastic netting to keep out insects and pests. The ceiling was fitted with a fine-volume water sprinkler, which produced a mist-like spray. The sprinkler automatically turned on for 15 min at 6-hour intervals to maintain the nursery at 90-95% relative humidity (RH). A thermohygrograph was placed in the nursery to check that the ambient temperature was 27-28°C and the RH value was 90-95% throughout the day and night. Once spores were produced, the whole bag was placed in a 20 × 30 × 15 cm perspex box, in the bottom of which was placed a white paper lining to gauge spore production. The top was taped with a muslin cloth to confine spores within the box.

Data collection of EGSP 03 development included rates of fungal growth on 3 substrate types at 3 distinct developmental stages; namely, (i) mycelial establishment

on the substrate, (ii) time taken for basidioma formation and (iii) duration of spore production until senescence sets in.

Morphological Characteristics

Three morphological characteristics were examined in induced basidiomata of EGSP 03 and EGUJ 02. They were: colour of basidioma from stipe to pileus margin, degree of lacca deposition, and attachment of the stipe. Comparisons of these characteristics were made among replicates on PPF, between replicates on different substrates, and between a representative of induced EGSP 03 and EGUJ 02 basidioma and the original fruiting bodies obtained from the field. Another series of observations was made to compare these characteristics in mature basidioma (i.e. aged 3-7 weeks) with young basidioma (aged under 3 weeks, which had not yet produced spores).

RESULTS AND DISCUSSION

Growth on Solid Substrates

EGSP 03 completely colonized all 3 solid substrates at a rate of 13.8^a mm/day on PPF, 12.5^b mm/day on CF and 9.2^c mm/day on RSD (figures with different letters denote a significant difference at $p = 0.05$, using Duncan's multiple range test (DMRT) in the analysis of variance). The colonies were smooth, white and dense on all 3 substrates but on exposure turned brown and crustose and primordial buds arose. The total incubation time needed before the basidioma buds (or primordium) started to appear was fastest on PPF (with a mean of 21.5^a days), followed by CF (at 27.3^b days) and slowest on RSD (35.5^c days).

The buds appeared as a raised, dome-shaped, velvety white structure measuring 5-10 mm at the widest point. The time taken for the primordium to elongate into a slender stipe and to reach a constant length from its initial formation was 3.5^a days on PPF, 3.7^a days on CF and 3.7^a days on RSD. The stipe lengths reached a constant length of 2.5-3.1 cm, and each bore a white tip, the primordium, now reduced in size. From this point onwards the tip expanded to form a flat, bracket-like pileus. The time taken for the basidioma to reach a constant size was 19.2^a days on PPF, 19.3^a days on CF and 19.3^a days on RSD.

Spore Production

Basidioma maturity was marked by spore production, which occurred once the fruiting body reached a constant size. Spores were strongly ejected from the pore layer and fell on the box lining, as well as all over the basidioma surface. The 'spore deposits' were light brown and powder-like on the paper lining; they were also found on the upper surface of the basidioma, giving it a velvety brown appearance.

Spore discharge was light in the first 2 days, becoming very heavy from the third day onwards. Spores were produced between midnight and 3 a.m. Sporulation was profuse for an average of 7 weeks, irrespective of substrate type. By the 8th week, spore production started to decrease and had stopped completely by the 10th week.

Morphological Characteristics

Induced EGSP 03 basidiomata were stipitate for all replicates on all 3 substrates. A mean reading for 5 induced EGSP 03 mature basidiomata showed that stipe length was 1.7 (±0.02) cm. The pileus measured 5.3 (±0.13) cm at the widest part and was 3.4 (±0.08) cm long from the distal margin to the base of the pileus. The margin was slightly rounded ('flabelliform'), measuring 8 (±0.12) cm in thickness increasing to 1.75 (±0.07) cm at the pileus base.

Induced EGSP 03 had a blackish-brown stipe. The pileus was smooth, flat

and dimidiate. The pileus was reddish-orange with a very wide white margin. This white margin disappeared completely in mature basidioma. The dorsal surface of mature basidioma was a bright reddish-brown with a band of yellow on the outermost part. It had conspicuous concentric growth rings and a light red-brown margin. The pore layer was light cream. The young basidioma (pre-spore production) was more rounded in shape. The dorsal surface in both young and mature basidiomata was highly laccate with an index of 5. The colour of mature basidiomata was similar for all replicates on PPF, was reproducible between replicates on all 3 substrates and matched those collected from the field. The basidioma gradually darkened in colour once spore production had stopped. By the 12th week, the sporophore had lost its original colour and turned uniformly blackish-brown; the dorsal surface became dull, and the laccate index decreased from 5 to 1. The dead basidioma was dull, dry and brittle.

Induced EGUJ 02 basidiomata were sessile and applanate (i.e. of uniform thickness from base to pileus) on all 3 substrates. The young basidioma (pre-spore production) was a buff light brown colour from base to pileus with a distinct white margin all round. In mature basidioma, the pileus was the same buff, light-brown colour, but the white margin had disappeared. The dorsal surface was matt, scoring '0' on the laccate index in both young and mature basidiomata. EGUJ 02 was of approximately uniform thickness from base to margin in both young and mature basidiomata and measured 8.6 (±0.05) mm in thickness in the latter. The basidiomata colour of induced EG UJ 02 did not vary significantly within replicates and between replicates on different substrates, nor in young and mature basidiomata, and matched those collected from the field. However, there was a complete mismatch in shape for EGUJ 02. All induced EGUJ 02 were applanate irrespective of substrate type, but the original specimen collected from the field was subungulate with the basal portion approximately 8 times the thickness at the margin. Except for the reduction in size for EGSP 03, no such morphological differences were observed.

CONCLUSION

All 3 substrates supported the growth and development of *Ganoderma* basidiomata. The rates of mycelial colonization varied significantly from one substrate to the other but the rate of basidiomata growth and development was relatively uniform and independent of substrate type.

Morphological examinations showed that size and shape of *Ganoderma* basidiomata are not good taxonomic characters. Basidioma colour holds potential but has to be treated with caution as the character is not stable in young, pre-sporulating *Ganoderma boninense*, whilst it is homogeneously blackish in old, post-sporulating specimens.

However, in mature and actively-sporulating *Ganoderma boninense* basidioma (i.e aged 3-7 weeks), colour is a constant and reproducible characteristic, regardless of substrate type. Attachment of stipe holds potential as a taxonomic character. Steyaert (1976) observed that attachment of stipe may be dependent on point of insertion, whilst Ryvaarden (1994) observed that it seemed to be a consistent character in the *Ganoderma lucidum* group in Europe.

Spore production in induced basidiocarps occurs only once in its life span. Under normal circumstances, it shows a diurnal pattern of spore release, which continued for approximately 7 weeks. An RH value exceeding 90% at an ambient temperature of 28°C was essential to

stimulate spore production.

In conclusion, development of a culture technique to induce basidioma formation on solid substrates allows investigation into the growth habits of *Ganoderma* and provides a means of verifying whether particular characters are stable and are of taxonomic value.

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