SHORT COMMUNICATION

Basidiospore and Protoplast Regeneration from Raised Fruiting Bodies of Pathogenic *Ganoderma boninense*

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A b s t r a c t

Ganoderma boninense, a phytopathogenic white rot fungus had sought minimal genetic characterizations despite huge biotechnological potentials. Thus, efficient collection of fruiting body, basidiospore and protoplast of *G. boninense* is described. Matured basidiocarp raised under the glasshouse conditions yielded a total of 8.3×10^4 basidiospores/ml using the low speed centrifugation technique. Mycelium aged 3-day-old treated under an incubation period of 3 h in lysing enzyme from *Trichoderma harzianum* (10 mg/ml) suspended in osmotic stabilizer (0.6 M potassium chloride and 20 mM dipotassium phosphate buffer) yielded the highest number of viable protoplasts (8.9×10^6 single colonies) among all possible combinations tested (regeneration media, age of mycelium, osmotic stabilizer, digestive enzyme and incubation period).

K e y w o r d s: basidiocarp, basidiospore, incubation, lytic enzyme

Ganoderma boninense, a phytopathogenic fungus causes basal stem rot (BSR) disease in oil palm and thus, is deemed a prime candidate for genetic, molecular and transformation studies. Very little studies have documented the biology and molecular genetics of *G. boninense* (Shi *et al.*, 2012). The studies of fungal functional genomics are becoming more prevalent with the availability of fully sequenced genomes (Michielse *et al.*, 2005; Wang *et al.*, 2010). Currently, genome sequencing of *G. boninense* is underway (Malaysian Palm Oil Board) (http://www.genomesonline.org), hence an efficient protoplast and basidiospore production system would prepare the species to functional studies related to BSR disease. The objective of this study is to develop a highly efficient method for protoplast regeneration from basidiospores collected from raised fruiting bodies under the glasshouse conditions. A morphological description of the ideal fruiting structure which gives the best sporulating activity was determined. In order to determine the optimal condition desired for *G. boninense* protoplast isolation and regeneration, factors such as age of mycelia, type of osmoticum, lytic enzymes and enzyme incubation period were investigated.

The *G. boninense* strain T10 was provided by Applied Agricultural Resources, Sdn Bhd (AAR), Sungai Buloh. The strain was verified positive *G. boninense* as indicated by visible brown ring formation under the Ganoderma Selective Medium (GSM) amendment. The fungus was maintained on maltose extract agar (MEA; Merck) prior to usage. A rubber wood block (RWB) measuring $6 \times 6 \times 6$ cm was soaked in distilled water overnight prior to sterilization at 121°C for 30 min. The RWB was supplemented with 100 ml of molten MEA, swirled to uniform distribution. Three pieces of mycelia cubes (1×1 cm) were inoculated at random sides of the RWB, thereafter incubated in darkness at room temperature for six weeks. Sterilized soil mixture prepared at 3:2:1 topsoil/peat/sand was filled in flower pot (40×30 cm) covering 3:4 parts of the total volume. The RWB inoculum with an attached oil palm seedling (3-month-old) on its side was carefully planted into soil, at about 1–2 cm from the soil surface, positioned vertically to expose the adaxial axis

of the RWB to the open air. Soil was maintained moist under regular watering. Development of the *G. boninense* fruiting body was monitored for a period of five months throughout a hot and wet climate. The resultant fruiting body harvested at varying maturity periods were subjected to basidiospore isolation. Fruiting body was immersed into 10% Chlorox solution for 30 min followed by 70% ethanol for 10 min. A sterile razor blade was used to cut the hymenium region into small cubes of 5×5 mm each. The excised cubes (0.5 g) were dropped into 100 ml of deionized water. The mixture was subjected to shaking for 30 min at $85 \times g$ at room temperature. The mixture was filtered using a small amount of glass wool fitted into a glass tunnel. The filtrate was collected as aliquots of 2 ml. The basidiospore suspension was subjected to low speed centrifugation at $1000 \times g$ for 1 min. The concentration of the basidiospore suspension harvested from the supernatant region was determined using a haemocytometer. Direct inoculation onto PDA (39 g/l), MEA (48 g/l) and Ganoderma Complete Medium (GCM) plates using a sterile cotton swab was performed to evaluate presence/ absence of contaminants. The GCM medium was prepare following Choi *et al.* (1987) with slight modifications; glucose 30 g/l, sucrose 20 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, K_2HPO_4 1 g/l, KH_2PO_4 0.5 g/l, yeast extract agar 10 g/l, casein hydrolysate 5 g/l. Basidiospore suspensions (10^5–10^6 ml^{-1}) were subjected to serial dilution to yield 3–5 mycelial colonies in each 100 ml PD-broth. Mycelia were harvested from the liquid culture at day 3, 4 and 5 to investigate the effect of age on protoplast release. The osmotic stabilizer was prepared to constitute either sucrose or potassium chloride at 0.6 M in presence of 20 mM dipotassium hydrogen phosphate. Lytic enzyme fixed at 10 mg/ml, was diluted into the osmotic stabilizer. Commercial lytic enzymes utilized were β-(1–3) D-glucanase, lysing enzyme from *Trichoderma harzianum* and Driselase (Sigma-Adrich, USA). The mixture containing mycelia and osmotic stabilizer (1:5 weight/vol) was incubated in a water bath at 30°C for 5 h. The production of protoplast was examined at 1, 3 and 5 h throughout the incubation period. The resultant protoplasts were spread onto regeneration media consist of PDA and GCM, supplemented with 0.6 M sucrose and 20 mM phosphate buffer. The number of viable protoplasts released was observed as the number of single colonies (mycelial pellets). The number of colonies appeared on the surface of the regeneration plates at day 10 after inoculation was recorded.

At week-3 after the artificial set-up for development of *G. boninense* fruiting bodies, visible hyphal knots were observed on the surface of the potting media, suggesting the initiation of photomorphogenesis. The rates of differentiation among the pots were highly variable. This in turn suggests presence of individual niches among the pots (Kamada, 2002) affecting polarisation of hyphae into fruiting structure. Fruiting body initials were observed protruding out from the surface of RWB at week-4 onwards. The sporophore was about 1 cm, white in colour, and round in shape with no visible hymenium (Fig. 1A). Next, the single-structured sporophore budded to form two of more structures round in shape with white surface, affixed on a deep red-brownish coloured stunted stipe (Fig. 1B). At this stage, visible hymenium was observed on the underside (white surface) with poor distribution. The sporophore differentiates into primordium/basidiocarp with characteristic cap and stipe formation. The cap takes up a bracket shape with reddish to orange colour on its outer surface, white coloured on its terminal region and confluent formation of visible hymenium on its underside (Fig. 1C). As the basidiocarp continues to develop, the cap showed deep red colouration entirely with orange coloration at the sub-terminal region and white coloration at the terminal region of the bracketed shape. On the underside, even distribution of visible hymenium was observed (Fig. 1D). Finally, the bracketed shape showed a completely deep red coloration and shrunk over time. The underside was found covered with visible hymenium (Fig. 1E).

In order to determine the effect of fruiting body's maturity on production of basidiospores, five distinct sizes of fruiting bodies were harvested. Fruiting bodies with a basidiocarp diameter in a range from 4–6 cm (Fig. 1D) showed highest rate of basidiospore production with a yield of 83×10^3 ml^{-1} mycelial pellets. Sporophore (less than 1 cm) with no visible formation of hymenium failed to sporulate even in the presence of a distinct fruiting structure. It appeared that the best way to determine a sporulating fruiting body would be to harvest fruiting bodies with a well-developed basidiocarp (diameter > 3 cm), visible hymenium and brightly coloured (Fig. 1C-1E). Sporophore with invisible hymenium showed poor rate of sporulation in comparison to well-developed basidiocarp, which showed visible hymenium (Fig. 1F) and large pore size. Basidiocarp with a pale colouration showed poor sporulation activity with a total of 35×10^3 ml^{-1} mycelia pellets, indicates senescence period of the fungus life cycle (Fig. 1E). Amongst the regeneration media selected, PDA showed the best regeneration capability followed by GCM (Fig. 2A) whilst MEA (data not shown) showed poor ability to regenerate basidiospores. This protocol recruited a combination of both forcible spore discharge method and the density gradient method which are widely employed for basidiomycetes basidiospore isolation (An, 2005). Hymenium was selectively harvested for basidiospore isolation as this region enclosed within the hard fruiting structure, renders a highly sterile environment. The tissues were dis-
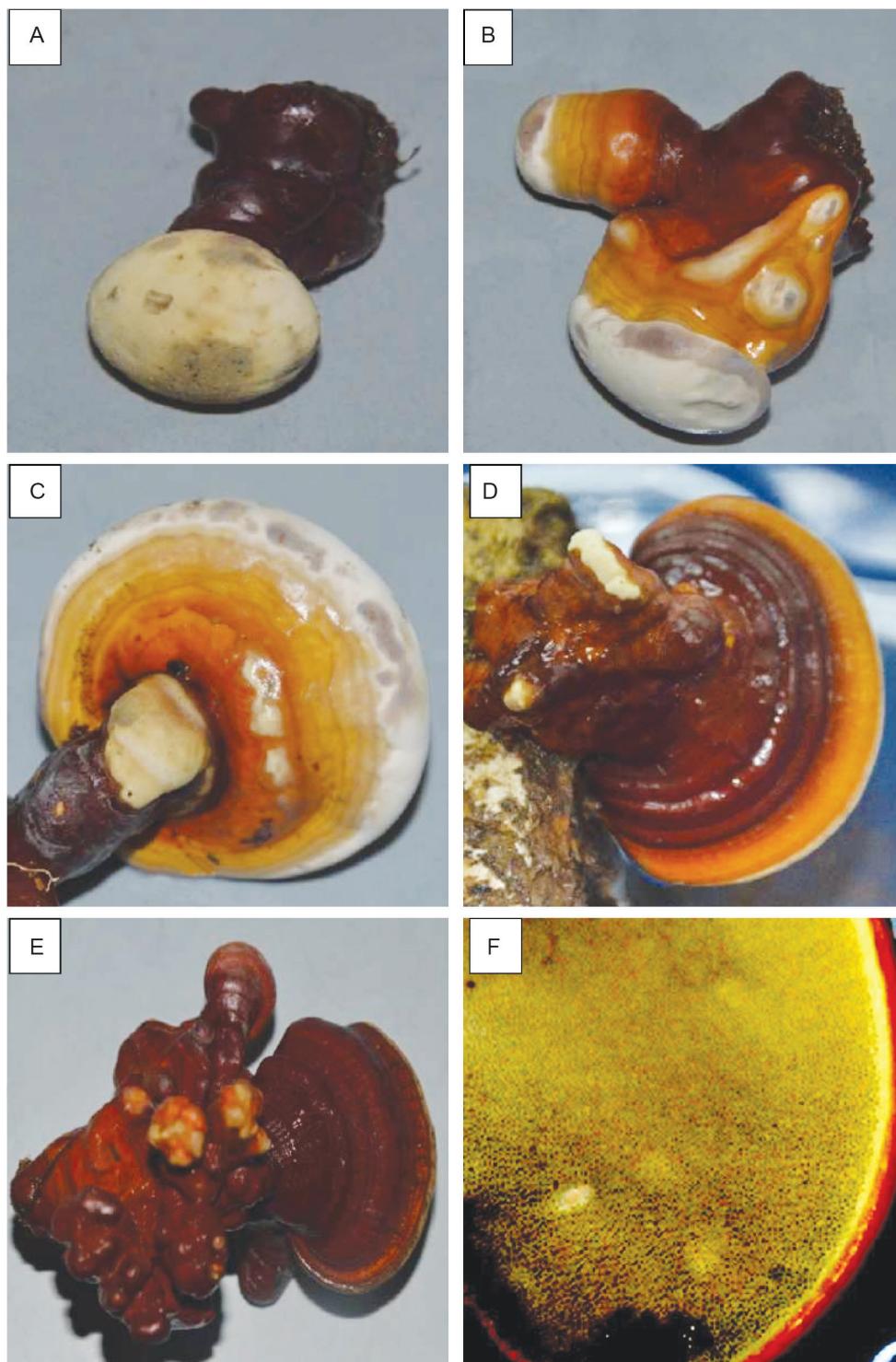
Fig. 1. Fruiting bodies of *G. boninense* harvested at different developmental stages. A) Sporophore;
B) Budding sporophores; C) Basidiocarp; D) Matured basidiocarp; E) Senescent basidiocarp;
F) Hymenium observed at underside of matured basidiocarp.

sected into small pieces to increase the surface required to allow the adhering basidiospores to escape into liquid solution during the shaking process. Ethanol based cleaning facilitated removal of coloured compound present on the fruiting structure. A prolonged pre-treatment of basidiospores under 10% Chlorox solution explained the resistant nature of the *G. boni-* *nense* basidiospores, which showed no adverse effect over the regeneration process. Centrifugation at relatively low speed enables large particles (contaminants) to be easily separated from basidiospores which are much smaller in size, yielding a virtually contaminant free basidiospore suspension as evident during basidiospore regeneration into mycelial colonies (Fig. 2A).
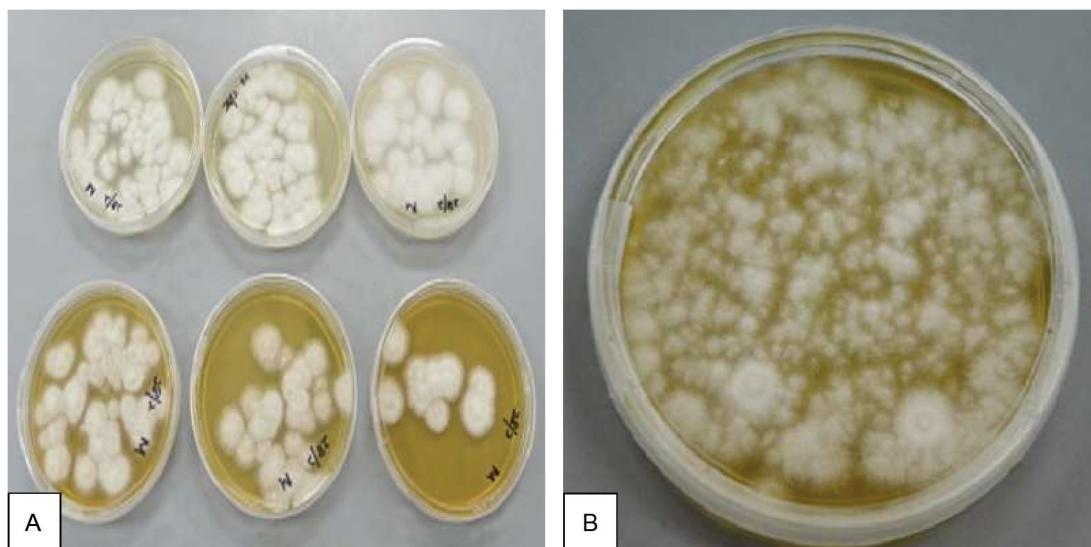
Fig. 2. A) Basidiospores (*G. boninense*) regenerated on PDA (above) and GCM (below) in absence
of antibiotics supplementation on day 14 after inoculation.
B) Single colonies regenerated on PDA suspended in phosphate buffer and 0.6 M sucrose from protoplast suspension after 3 h enzymatic
digestion using 10 mg/ml lytic enzyme from *Trichoderma harzianum* and 0.6 M KCl as osmotic stabilizer at day 10 after inoculation.

The number of protoplasts regenerated under all possible combinations tested in this study ranged from $2–89 \times 10^5$ single colonies ml^{-1} (Table I). To assign the best possible combination for efficient protoplast regeneration, treatments with mycelial colonies ranging at $10^6–10^5$ were designated as high, $10^4–10^2$ being moderate and below 10^2 as poor yield. Each treatment was evaluated using mycelium at different ages subjected to varying lytic enzymes, osmotic stabilizers and growth media under selected incubation periods. Mycelia were harvested at day 3, 4 and 5 to identify optimal age for protoplast production. Day 1 and 2 were not utilized since no visible mycelium mass were observed. Mycelia aged 3-day-old produced the highest viable protoplast yield (89×10^5 single colonies ml^{-1}) on PDA when treated with lytic enzyme from *T. harzianum* at an incubation period of 3 h (Fig. 2B) followed by mycelia aged 4-day-old (14×10^3 single colonies ml^{-1}) under a similar treatment conditions. Moderate protoplast yield was observed on mycelia aged 5-day-old (35×10^2 single colonies ml^{-1}) with peaked protoplast yield at an incubation period of 5 h. The protoplast production observed during 3 h incubation period was relatively moderate to high for 3 and 4-day-old mycelia ($3 \times 10^2–78 \times 10^5$ single colonies ml^{-1}). In contrast, the 5-day-old mycelia ($5 \times 10^2–84 \times 10^3$ single colonies ml^{-1}) recruited a prolonged incubation period, 5 h for an optimal digestion of a more intricate cell wall structure. The structure of fungal cell wall remodels throughout its growth. Newly synthesized cell walls are generally more thin and smooth in comparison to older tissues which comprise of primary and secondary layers which are heavily deposited with amorphous matrix materials (Annamalai and Lalithakumari, 1991; Lalithakumari, 1996).

Incubation period was observed as limiting factor throughout the three intervals. Mycelial age was found to affect the protoplast productions. Generally, a similar trend was observed on the incubation period tested among all treatment combinations. Poor to moderate protoplast production was observed in the first hour ($2–76 \times 10^2$ single colonies ml^{-1}), however, protoplast yield increased rapidly and peaked at 3 h ($8–89 \times 10^5$ single colonies ml^{-1}). At 5 h, protoplast yield declined ($2 \times 10^2–84 \times 10^3$ single colonies ml^{-1}) probably indicates the proteolytic activity of the pre-existing protoplast which affects the production of newly-formed protoplast (Robinson and Deacon, 2001). Enzyme incubation period of 3 h was found effective for maximal protoplast production from *G. boninense* mycelia of 3-day-old (89×10^5 single colonies ml^{-1}) and 4-day-old (14×10^3 single colonies ml^{-1}). In basidiomycetes, the fungal cell wall constitutes α-1-3-glucan, xylose and hemicelluloses (Adams, 2004). Three different commercial lytic enzymes from different origin exhibited variable enzymatic enzyme-cell wall digestibility of *G. boninense*. The lytic enzyme from *T. harzianum* contains β-glucanase, chitinase, cellulase and protease activities. This preparation has been predominantly employed for protoplasing filamentous fungi (Liu *et al.*, 2010; Rivera *et al.*, 2014), basidiomycetes (Shi *et al.*, 2012) and yeast (Kelly and Nurse, 2011). Driselase originates from basidiomycetes sp. containing laminerase, xylanase and cellulase activities. It has also shown efficient digestibility on other fungal species (Souza *et al.*, 2014). The β-(1–3) D-glucanase

Table I
Effect of incubation time and lytic enzymes (10 mg/ml) on protoplast yield/ml from *G. boninense* mycelia treated
in different osmotic stabilizer and regeneration media

Mycelial age	Lytic enzyme/ Source	Media	Osmotic stabilizer	Protoplast regenerated as single colonies /ml		
				1 h	3 h	5 h
3-day culture	Driselase	PDA	Sucrose	50	56×10^5	23×10^2
			KCl	80	78×10^5	40×10^2
		GCM	Sucrose	40	34×10^5	11×10^2
			KCl	20	46×10^5	7×10^2
	β-(1-3) D-glucanase	PDA	Sucrose	21×10^2	22×10^4	3×10^2
			KCl	34×10^2	34×10^4	4×10^2
		GCM	Sucrose	18×10^2	17×10^4	2×10^2
			KCl	26×10^2	17×10^4	5×10^2
	T. harzianum	PDA	Sucrose	3×10^2	72×10^5	11×10^2
			KCl	13×10^2	89×10^5	28×10^2
		GCM	Sucrose	10×10^2	65×10^5	14×10^2
			KCl	15×10^2	69×10^5	21×10^2
4-day culture	Driselase	PDA	Sucrose	81×10	17×10^2	17×10^2
			KCl	74×10	34×10^2	21×10^2
		GCM	Sucrose	68×10	18×10^2	34×10^2
			KCl	76×10	26×10^2	18×10^2
	β-(1-3) D-glucanase	PDA	Sucrose	23×10	3×10^2	26×10^2
			KCl	17×10	13×10^2	3×10^2
		GCM	Sucrose	9×10	10×10^2	13×10^2
			KCl	15×10	15×10^2	10×10^2
	T. harzianum	PDA	Sucrose	58×10^2	11×10^3	15×10^2
			KCl	76×10^2	14×10^3	16×10^2
		GCM	Sucrose	67×10^2	12×10^3	17×10^2
			KCl	65×10^2	12×10^3	15×10^2
5-day culture	Driselase	PDA	Sucrose	23	93	8×10^2
			KCl	17	88	6×10^2
		GCM	Sucrose	18	45	7×10^2
			KCl	12	82	5×10^2
	β-(1-3) D-glucanase	PDA	Sucrose	5	9	13×10^2
			KCl	6	2	19×10^2
		GCM	Sucrose	2	8	27×10^2
			KCl	3	8	35×10^2
	T. harzianum	PDA	Sucrose	45	23×10^2	76×10^3
			KCl	46	35×10^2	84×10^3
		GCM	Sucrose	38	11×10^2	57×10^3
			KCl	42	29×10^2	63×10^3

was utilized to study the effect of single enzyme treatment over the *G. boninense* cell wall digestion. The lytic enzyme from *T. harzianum* showed the best efficiency (89×10^5 single colonies ml^{-1}) for *G. boninense* protoplast production indicated β-1, 3 glucanase, protease and chitinase are synergistically effective in degradation of *G. boninense* cell wall. The finding complements with the fungi polysaccharides cell wall compositions which constitute mainly cellulose and chitin (Yan *et al*., 2004; Wang *et al*., 2010; Souza *et al*., 2014). This is followed by Driselase (78×10^5 single colonies ml^{-1}), which showed better enzyme activity in comparison to β-(1–3) D-glucanase (34×10^4 single colonies ml^{-1}). Driselase acted selectively on both xylan hemicellulose and glucose polymers which constitute *G. boninense* cell wall (Mendoza, 1992). The results demonstrated stable integration of several compositions in fungi cell wall, thus, the usage of only one lytic enzyme is not

favourable for protoplast release of *G. boninense*. Protoplasts release was fairly high on both potassium chloride and sucrose supplemented osmotic stabilizers under most treatments. Potassium chloride treated protoplasts regenerated ($3–89 \times 10^5$ single colonies ml^{-1}) at a higher rate than sucrose treated protoplasts ($2–72 \times 10^5$ single colonies ml^{-1}) under all treatment combinations (Table I). The findings, however, contradicted with protoplast production from *G. lucidum* which demonstrated sucrose as optimal osmotic stabilizer (Choi *et al.*, 1987). Osmotic stabilizer composed of inorganic sugar KCl was consistently more efficient than the organic sugar, sucrose. PDA was the most suitable regeneration medium for protoplast as number of single colonies regenerated in PDA ($5–89 \times 10^5$ single colonies ml^{-1}) was consistently higher than GCM ($2–69 \times 10^5$ single colonies ml^{-1}) in all treatment combinations (Table I). Medium used for regeneration of protoplast was consistent with basidiospore regeneration which evident PDA as the highly efficient medium for reversion into filamentous morphology.

In conclusion, usage of inorganic sugar for lysing procedure and organic sugar for selection facilitates isolation and regeneration of *G. boninense* protoplast respectively. Next, raised fruiting body for collection of actively growing mycelia (exponential phase) is a fundamental key attribute to protoplast regeneration procedure. Our study is the first to demonstrate isolation of contaminant free *G. boninense* basidiospore and its subsequent regeneration into mycelium, which was directly used for protoplast regeneration. The method described here detailing on the protoplast and basidiospore preparation would render *G. boninense* strain receptive to advanced technologies which ultimately creates avenue towards high-throughput researches.

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