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Research Article

Evaluation and Improvement of Protocols for *Ganoderma boninense* Protoplast Isolation and Regeneration

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ABSTRACT

Ganoderma boninense is the causal agent of basal stem rot (BSR) disease of oil palm. The BSR disease reduces oil palm yield by up to 80% of the average oil yield. Attempts to control the disease caused by this fungus in the field showed varying levels of success and cases of infection increased from year to year. Hence, the development of new efficient methods to control the spread of this fungus should be commenced promptly. To ensure a better strategy is created, more thorough research on the method deploy by this fungus to infect the host at the molecular level need to be carried out first. However, the major limitation in endeavoring into the functional analysis of virulence genes related to the pathogenicity of this fungus was hampered by the unavailability of established methods for protoplast isolation with a high regeneration rate to be used in the genetic manipulation analysis. Thus, in this paper, we report an efficient protocol for protoplast isolation and regeneration in *G. boninense* and successfully used the isolated protoplasts in PEG-mediated transformation analysis. A large quantity of protoplast was obtained using the protocol that utilizes the following parameters: 3 to 4-day-old mycelia, treated with 1% lysing enzyme and 0.02% Driselase, incubated at 30 °C in an osmotic medium containing 0.6 M mannitol at pH 5.8 for 2 h. The highest protoplast yield was in the range of 8.95×10^9 to 3.12×10^{10} cells/mL per 5 g of mycelia used. The regeneration rate ranged from 9.03% to 22.55%, depending on the regeneration media used. By using 5 µg of vector to transform into 1.0×10^7 protoplast/mL, around 3 – 10 mitotically stable putative transformants were successfully obtained and verified via PCR. This protocol will find useful applications in genetic studies to enhance insight into this poorly characterized and understood phytopathogen.

Key words: Basal stem rot, a fungal pathogen, protoplast, oil palm

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INTRODUCTION

Malaysia is the world's second-largest palm oil producer and exporter after Indonesia. With an estimated export value of RM 108.52 billion in 2021 (Parveez, 2022), the oil palm industry contributes a steady income, driving the nation's economy. However, the oil palm industry in South-East Asia (SEA) is suffering from one primary disease, known as basal stem rot (BSR), mainly caused by the phytopathogenic fungus, *Ganoderma boninense* (Isaac *et al*., 2018; Bharudin *et al*., 2022). *G. boninense* is a white-rot basidiomycete fungus, known for its capability to degrade the wood's lignin component and exercise a hemibiotrophic lifestyle (Bahari *et al*., 2018). During disease development, the fungal eventually switches from biotrophic to necrotrophic nutrition, causing host death. Young palms usually will die within 6-24 months after infection whilst the mature palm usually takes a long time to die, usually 2-3 years after the emergence of the fruiting bodies (Miller *et al*., 1999; Siddiqui *et al*., 2021). By decreasing the output of the infected palms, shortening the production period of oil palm trees, and finally burdening planters, especially smallholders, the BSR disease, which

has been brought on by this fungus for more than 80 years, poses a serious threat to the oil palm industries (Chong *et al*., 2017; Paterson, 2019).

Current strategies to combat the BSR disease, range from physical (sanitation practices), and usage of chemical fungicides up to the introduction of biological control such as *Trichoderma* sp., *Burkholderia* sp. and *Pseudomonas aeruginosa* to exert antagonistic effect toward the growth of *G. boninense* were remain stagnant. To date, there are no conclusive methods that could be used to overcome *G. boninense* infection in plantation areas (Yusoff *et al*., 2021; Bharudin *et al*., 2022). Each method needs to be reevaluated by incorporating deeper knowledge of the biological qualities of the *G. boninense* due to several factors, such as climate change and varied genetic mark-up of each isolated strain, that affect the success of each treatment (Paterson, 2019; Siddiqui *et al*., 2021).

There are three methods by which the fungus may spread. First, in plantation areas where infected and healthy palm trees come into contact with one another at the root level. Second, due to faulty sanitation management before the new planting program, through contact with secondary infection. Lastly, basidiospores can easily spread to neighboring plantation areas by winds and vectors like insects. The latter route has become one of the major causes of genetic variability of *G. boninense* (Rees *et al*., 2012; Pilotti *et al*., 2018). Due to different genetic pools, different strains of *G. boninense* exhibit different degrees of aggressiveness and capability of infecting the host (Goh *et al*., 2014). They also showed varying degrees of susceptibility to the biological control used in the fields (Goh *et al*., 2020). Therefore, to effectively treat the BSR disease brought on by this fungus, a thorough study at the fundamental level is required to understand the mechanism, which includes the ability to detect the hosts and allow stealthy entrance and colonization that results in the host's death.

Functional genomic analyses of *G. boninense* remain limited due lack of established and reliable molecular-level methods (Bharudin *et al*., 2022). Hence, in this work, we have optimized the method for protoplast isolation and subsequent regeneration. We showed that the protoplasts isolated using our optimized method were suitable for gene knock-in analyses through the protoplast PEG-mediated transformation approach. Besides molecular studies, protoplast is also a good choice for mycelial proteome analysis as it gives better protein recovery than the mycelia with intact cell walls (Bhadauria & McKee, 2010). Furthermore, strain improvement could also be generated by forming a hybrid heterokaryon protoplast containing heteroplasmic cytoplasm, overcoming the strain compatibility barriers between species (Chun *et al*., 1992; Papzan *et al*., 2021). For example, the fusion between *Trichoderma virens* and *Trichoderma harzianum* through protoplast cells resulting hybrids with improved cellulase activity (Papzan *et al*., 2021).

MATERIALS AND METHODS

Strain and cultivation

Ganoderma boninense Mg12 (monokaryotic mycelia derived from meiotic spores produced by *G. boninense* PER71 fruiting body) and *G. boninense* PER71 (dikaryotic mycelia) were kindly provided by Malaysia Palm Oil Board (MPOB). The cultures were kept on potato dextrose agar (PDA) (Merck, USA), subcultured twice per month, and stored in paraffin oil at room temperature (Sigma-Aldrich, USA) for long-term storage.

Culture medium for mycelial propagation

Ganoderma boninense was grown on complete yeast media agar (CYMA) (2% glucose, 0.2% g/L peptone, 0.1% yeast extract, 0.05% $MgSO_4$, 0.046% KH_2PO_4, 0.1% K_2HPO_4 & 1.5% bacteriological agar) supplemented with 1% of vitamins solution (0.01% pyridoxin-HCl, 0.015% thiamine-HCl, 0.075% ρ-aminobenzoic acid, 0.25% nicotinic acid, 0.25% riboflavin, 2% choline-HCl) for 7 days at 28 °C. Around 60 agar plugs of 7-day-old mycelia were inoculated into 400 mL complete yeast media broth (CYMB) (2% glucose, 0.2% g/L peptone, 0.1% yeast extract, 0.05% $MgSO_4$, 0.046% KH_2PO_4, 0.1% & K_2HPO_4) supplemented with 1% of vitamin and incubated for 4 days at 28 °C, 250 rpm to proliferate mycelia. For protoplast generation, each adopted method was tested using 3 g of day-3 mycelia.

Optimising methods for protoplast isolation

Several methods were adopted to identify the best method for protoplast liberations for *G. boninense*. The first method assessed was by Li *et al*. (2006) with modifications. The mycelia were rinsed with 0.6 M mannitol before being incubated in 15 mL of 0.6 M mannitol containing 2% lysing enzyme (Sigma-Aldrich, USA) and 0.02% Driselase (Sigma-Aldrich, USA) for 2 h at 30 °C. The protoplasts were filtered through sterile Miracloth, layered with one layer of Kimwipe, and were centrifuged at 1956 × *g*, 4 °C for 30 min. The supernatant was removed without disturbing the pellet. The pelleted protoplasts were then resuspended in 1 mL of 0.6 M of mannitol and transferred into a new sterile 1.5 mL tube and centrifuged again at 1008 × *g* for 5 min. The supernatant was removed carefully without disturbing the pellet, and this washing step was repeated twice. Lastly, the pelleted protoplasts were resuspended in 300 µL of mannitol-tris-$CaCl_2$

(MTC) solution (0.6 M mannitol, 100 mM Tris-HCl, pH 8.0, 100 mM $CaCl_2$) and counted using a hemocytometer before dilution of up to 1 × 10^8 protoplast/mL for storage or until use.

The second method was adopted by Chou & Tzean (2016) with slight modifications. The mycelia were rinsed with 0.6 M sucrose before being transferred into a 50 mL flask containing 15 mL of osmotic solution (0.6 M sucrose in 50 mM potassium phosphate buffer at pH 6, 2% lysing enzyme & 0.02% Driselase). The mixture was incubated for 2 h at 30 °C, 100 r.p.m. The lysed mycelia were filtered through sterile Miracloth, layered with one layer of Kimwipes, and were centrifuged at 1956 × *g*, 4 °C for 30 min. The pelleted protoplasts were resuspended in 1 mL of 1 M sorbitol-calcium chloride-Tris (STC) buffer (1 M sorbitol, 20 mM $CaCl_2$, 10 mM Tris buffer pH 7.5) and transferred to a new 1.5 mL tube before being centrifuged at 1008 × *g* for 5 min to remove the lysis enzymes from the sample mixtures. This washing step was repeated twice. The pelleted protoplasts were resuspended in 300 µL of STC buffer and counted using a hemocytometer before dilution of up to 1 × 10^8/mL for storage or until use.

The third method was adopted by Rodriguez and Redman (1992) with modifications. The mycelia were washed with 0.6 M $MgSO_4$ and then transferred into a 50 mL flask containing 15 mL of osmotic solution (1.2 M $MgSO_4$, 10 mM phosphate buffer pH 5.4), 2% lysing enzyme, and 0.02% Driselase®. The mixture was incubated at 30°C with shaking at 110 r.p.m. for 2 h. The protoplasts were filtered through sterile Miracloth, layered with one layer of Kimwipes, and then centrifuged at 1956 × *g*, 4 °C for 30 min. The supernatant was removed carefully to not disturb the pelleted protoplasts. To remove the remaining lysis enzymes from the sample, the pelleted protoplasts were resuspended in 1 mL of 0.6 M sorbitol, transferred into a new 1.5 mL tube, and centrifuged at 804 × *g* at 4 °C for 5 min. This step was repeated twice using 1 mL of STC solution (1.2 M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$). Lastly, the washed purified protoplasts were resuspended in 300 µL of STC buffer and counted using a hemocytometer before storage for further use.

The last method was adopted by Sun *et al*. (2001) with few modifications. The mycelia were rinsed with 0.6 M mannitol before being incubated in an osmotic solution (0.6 M mannitol & 0.1 M phosphate buffer pH 5.0) containing 2% lysing enzyme (Sigma-Aldrich, USA) and 0.02% Driselase®, 100 r.p.m. at 30 °C for 2 h. Protoplasts were isolated by filtering through sterile Miracloth, layered with one layer of Kimwipes, and then centrifuged at 1956 × *g*, 4 °C for 30 min. The supernatant was removed carefully while the pelleted protoplasts were resuspended in 1 mL of 0.6 M mannitol, transferred into a new 1.5 mL tube, and centrifuged at 1008 × *g*, 4 °C for 5 min to remove the remaining lysis enzymes. This step was repeated twice. Lastly, the pelleted protoplasts were then resuspended in 300 µL CYM broth (1% maltose, 2% glucose, 0.2% yeast extract, 0.2% tryptone, 0.05% $MgSO_4$, 0.46% KH_2PO_4, 0.6 M mannitol) and counted using hemocytometer before storage or until further use.

Table 1 showed the summary of the different parameters assessed from each attempted method. The method that offers the highest number of isolated protoplasts will be selected for further downstream optimizations.

Optimising parameters for protoplast liberation

To further increase the number of viable protoplasts, a few parameters contributing to the protoplasts' liberation such as concentration of the osmotic stabilizer, optimum pH for the osmotic solution, age of the mycelia, the use of individual and combination of lytic enzyme/s and their optimum percentage used and incubation time were further investigated. The selection of the optimum condition for each parameter was then tested for the orthogonal experiment. Each optimization step was tested using 5 g of day-3 mycelia.

Table 1. Different parameters apply to each adopted protoplast isolation technique

Methods	Osmotic stabilizer	Solution for protoplast resuspension
Li et al. (2006) with modifications	0.6 M mannitol	MTC (0.6 M mannitol, 100 mM Tris-HCl, pH 8.0, 100 mM $CaCl_2$)
Chou et al. (2015) with modifications	0.6 M sucrose in 50 mM potassium phosphate buffer pH 6	STC (1 M sorbitol, 20 mM $CaCl_2$, 10 mM Tris buffer pH 7.5)
Rodriquez and Redman (1992) with modifications	1.2 M $MgSO_4$ in 10 mM phosphate buffer pH 5.4	STC (1.2 M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$)
Sun et al. (2001) with modifications	0.6 M mannitol in 100 mM phosphate buffer pH 5.0	CYM (1% maltose, 2% glucose, 0.2% yeast extract, 0.2% tryptone, 0.05% $MgSO_4$, 0.46% KH_2PO_4, 0.6 M mannitol)

Effects of fungal age on protoplast isolation

This experiment was conducted to study enzyme digestion and fungal ages, which play a significant effect on the protoplast yield. The mycelia were grown in CYMA for 3, 4, and 5 days. After that, the mycelia were collected, washed, and weighed before mixing with the osmotic solution. Other parameters were fixed as used in the previously selected method. The protoplasts isolated were checked for quality, counted using a hemocytometer, and recorded.

Effects of concentration of osmotic stabilizers on protoplast isolation

Different concentrations of the osmotic stabilizer were tested for the effects on the protoplast liberation at 0.4 M, 0.6 M, 0.8 M, and 1.0 M. Each reaction contained fixed parameters of the lytic enzyme, pH of the buffer, age of the mycelia and incubation time as in the previously selected method. The protoplasts isolated were checked for quality and counted using a hemocytometer.

Effects of pH on protoplast isolation

A selection of different pH buffers (pH 5, pH 5.8, pH 6.0, & pH 7.0) was prepared with 50 mM potassium phosphate buffer to determine the suitable pH for protoplast preparation. Other parameters were fixed as used in the previously selected method. The protoplasts isolated were checked for quality and counted using a hemocytometer.

Effect of incubation time on protoplast isolation

Different durations of incubation time were tested to study the influence of the incubation time on the isolation and the quality of the protoplasts. The mycelia were incubated for 1 h, 1.5 h, 2 h, and 2.5 h, respectively. Other parameters were fixed as used in the previously selected method. The number of protoplasts obtained was counted using a hemocytometer. The quality of the protoplasts was determined based on the shape and integrity of the membrane cell under low mechanical pressure.

Effects of digestive enzyme concentrations and combinations

A variety of digestive enzymes were tested at different concentrations and in different combinations. The selected enzymes were lysing enzymes from *T. harzianum* (Sigma-Aldrich, USA), Driselase® from basidiomycetes (Sigma, USA), and Celluclast® (Novozyme, USA) (Table 2). Other parameters were fixed as used in the previously selected method. The protoplasts isolated were checked for quality and counted using a hemocytometer after 2 hr of digestion.

Viability assessment of protoplast

Various osmotic stabilizers were added to the regeneration media to protect the protoplast against the osmotic pressure during regeneration, which has a major influence on the protoplast's regeneration frequency. For this purpose, various published regeneration media that have been published by Li *et al*. (2006), Priyatno (2009), Chou and Tzean (2016), Yu *et al*. (2014), Govender *et al*. (2016), Ab Wahab *et al*. (2019) and Czapek-Dox-sucrose agar (CDSC) (Zhou *et al*., 2008) with modifications, were tested for the regeneration of the protoplasts in this study. The colonies generated from protoplasts under different nutrient sources and osmotic stabilizers were compared to each other. The types of regeneration medium and different osmotic stabilizers used in this experiment were believed to affect the protoplast regeneration significantly.

The regeneration frequency was obtained by counting the colony following the plating of protoplasts in osmotically stabilized agar media. The regeneration ratio is calculated from the following equation:

$$\text{Regenaration rate (\%)} = \frac{\text{A - B}}{\text{No. of protoplast}} \times 100$$

With A: Number of colonies regenerated from the protoplasts using the selected regeneration media and B: number of colonies regenerated from protoplasts diluted with water.

Transformation analysis

Sensitivity to selectable antibiotic

To determine the suitable concentration of hygromycin to be used during the screening of the successful transformants, a minimal inhibitory concentration (MIC) assay of hygromycin was conducted on the wild-type strain (monokaryotic *G. boninense* Mg12 mycelia) in the presence of a different concentration of the hygromycin. The concentration of hygromycin tested for MIC assay was 20, 30, 40, 50, and 60 µg/mL. A single 5 mm × 5 mm PDA plug from 6-day-old cultures of *G. boninense* Mg12 was subcultured onto the amended PDA plate containing the antibiotic and the fungal growth was assessed visually after 14-day incubation at 28 °C. The concentration of hygromycin that provides 100% growth inhibition will be used for the screening of transformants.

Protoplast PEG-mediated transformation

The protoplasts obtained from the optimized method were employed in protoplast PEG-mediated transformation analysis based on methods reported by Rodriquez and Redman (1992) and Yu *et al*. (2014) with some modifications. The integrative vector used for this analysis is pUChph (Figure 1). This pUChph DNA vector comprised

Table 2. The type and composition of the cell wall lytic enzyme used in this study

	Type of individual and combination of the lytic enzymes
Composition of the lytic enzyme/s used in this analysis	a. 1.0% *Trichoderma harzianum* lysing enzyme b. 1.5% *Trichoderma harzianum* lysing enzyme c. 2.0% *Trichoderma harzianum* lysing enzyme d. 1.0% *Trichoderma harzianum* lysing enzyme + 0.02% Driselase® e. 2.0% *Trichoderma harzianum* lysing enzyme + 0.02% Driselase® f. 0.5% Celluclast + 0.02% Driselase® + 0.05% *Trichoderma harzianum* lysing enzyme g. 0.5% Celluclast + 0.02% Driselase®

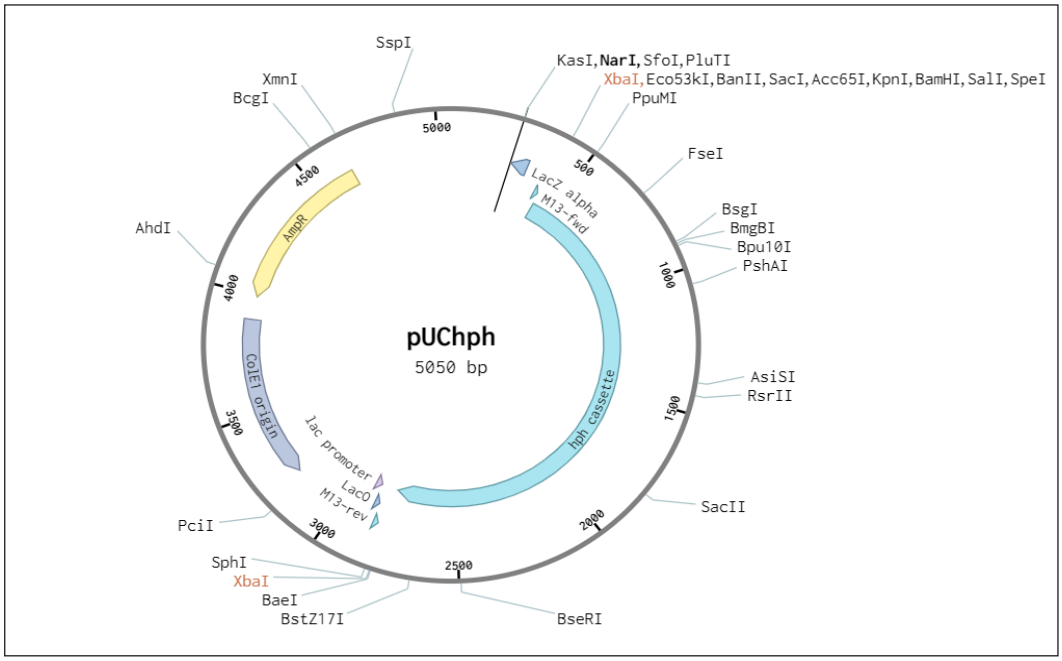


Fig.1. The pUChph vector. Image illustrates the components of the vector; ColE origin for autonomous replication of the vector inside the cell, AmpR conferring resistance towards ampicillin, and hph cassette.

pUC19 as the backbone vector, and a hygromycin phosphotransferase (*hph*) cassette derived from the pN1389 vector. The *hph* gene was used as the selectable marker and the expression of this gene was under the control of the *Aspergillus niger* glucoamylase *(glaA)* promoter and *Aspergillus nidulans trpC* terminator. Transformants harboring this vector will exhibit hygromycin resistance traits on the selection plate.

For each transformation procedure, approximately 100 µL of 1 × 10^7 protoplast/mL were employed, and the transformation analysis was carried out in three biological replicates. For the Rodriquez and Redman (1992) procedure, sample tubes containing protoplasts were filled with 25 µL of STC solution (1.2 M sorbitol, 10 mM Tris-HCl pH 7.0, 10 mM CaCl2) and incubated at room temperature for 30 min. This solution contained 5 µg of the pUChph vector. The mixture was then added 400 µL of PEG solution (1.0 M sorbitol, 20% PEG 6000, 50 mM Tris-HCl buffer; pH 7.5, 50 mM $CaCl_2$), incubated for 30 min, and then centrifuged at 736 × *g* for 8 min at 4 °C. The pellet of the protoplast was slowly resuspended in 1 mL of yeast extract-malt extract-sucrose broth (YMSB) (0.4% yeast extract, 0.4% peptone, 0.8% malt extract, 1.2% glucose, and 0.6M sucrose) after the supernatant had been properly removed. Second centrifugation of the protoplast suspension at 736 × *g* for 8 min at 4 °C was performed. This process was carried out twice. Finally, 10 mL of YMSB regeneration broth (0.4% yeast extract, 0.4% peptone, 0.8% malt extract, 1.2% glucose, 0.6 M sucrose, and 2% agar) was added before the pellet of the protoplast was resuspended in it. After being properly put into the Petri dish, the mixture was incubated for two days at 28 °C. After two days of incubation, melted PDA containing 50 g/mL of hygromycin was applied to the transformation plate, where it was incubated for an additional seven to ten days at 28 °C.

For the transformation method adopted by Yu *et al*. (2014) with modification, 20 µL of PTC buffer (60% PEG 4000, 10 mM Tris-HCl buffer; pH 7.5,

50 mM $CaCl_2$) containing 5 µg of pUChph vector were added into the sample tube containing 100 µL of 1 × 10^7 protoplast/mL and left to incubate for 20 min at room temperature. Then, 10 mL of CYM regeneration broth and 10 mL of heated CYM regeneration agar were combined, then put onto the Petri plate after 10 min at room temperature. Before being covered with PDA supplemented with hygromycin and left to continue incubating for 10 days, the plate was incubated for 2 days at 28 °C.

The putative transformants exhibiting hygromycin resistance traits and growing on the selection media containing the hygromycin will be assessed.

Validation of the putative transformants

For three generations, all of the potential transformants were subcultured onto PDA that contained 50 µg/mL of hygromycin. The putative transformants will next be subcultured onto non-selective PDA media for three generations before being subcultured onto PDA containing 50 µg/mL once again to screen for putative transformants that are mitotically stable. The putative transformants exhibiting hygromycin resistance trait (HygR) will be validated for the presence of the hygromycin gene in the genome using the following primer pair: FhygB - 5'- GAACTCACCGCGACGTCTG-3' and RhygB - 5'- CACTAGGCAACCATGGTTACTATT-3', and the amplified amplicon will be sent for sequencing analysis.

RESULTS

Numerous techniques for producing protoplasts from fungus mycelia have been documented. However, due to the variations in cell wall compositions between each species or strain, not all documented procedures are appropriate for all fungi. As a result, this work assessed a few techniques for producing protoplasts from the phytopathogenic fungus *G. boninense* Mg12 (monokaryotic mycelia). When 3 g of mycelia was utilized as the starting material, the results showed that the method adopted from Sun *et al*. (2001) with minor adjustments had the most protoplasts isolated, 3.24 × 10^7 cell/mL as compared to other assessed methods (Table 3). As a result, optimization of variables impacting protoplast liberation has been done. The age of the mycelium and the type of culture medium that affects the fungal cell wall compositions are only two examples of the many variables that might affect protoplast development. The type and concentration of osmotic stabilizers, the pH of the osmotic medium, temperature, the type and composition of the digestive enzymes, and preparation methods are some other external aspects to take into account.

Fungal ages

The age of the mycelia, which directly impacts the makeup and thickness of the fungal cell wall, is one of the most important elements in influencing the development of protoplasts (Eyini *et al*., 2006). The mycelia of different ages provided varying protoplast counts from this experiment under the same digesting conditions. It was found that the optimal protoplast yield was obtained using 3 and 4-day-old cultured mycelia with the total protoplast 9.3 × 10^7 and 9.8 × 10^7 protoplast/mL, respectively. Utilizing the 5-day-old cultured mycelia resulted in a considerable decrease in the number of separated protoplasts (Figure 2A). According to a study by Wei *et al*. (2010), as the number of isolated protoplasts will substantially drop as exponential growth progresses, the optimal mycelia to be used for protoplast generation should be taken during the fungus' log phase. It might be a result of the younger mycelium's looser cell walls, which allowed protoplasts to escape more easily when exposed to the lytic enzyme (Ren *et al*., 2018). The ideal age of the mycelia for protoplast formation needs a preliminary analysis because different fungal species or strains may have varied growth rates. For example, 4-day-old cultured mycelia for *Ganoderma multipileum* gave the highest isolated protoplasts compared to 30 h of cultured mycelia for *Antrodia cinnamomea* (Chou & Tzean, 2016; Wu & Chou, 2019).

Concentration of osmotic stabilizer

The osmotic stabilizer used in this experiment is mannitol, tested at a concentration of 0.4 M, 0.6 M, 0.8 M, and 1.0 M to determine the most suitable concentration to avoid shrinking or rupturing the protoplast cells. Mannitol has a modest infusion rate, is biologically inert, and was not metabolized by the protoplast (Pongchawee *et al*., 2007; Mastuti & Rosyidah, 2018). The protoplasts'

Table 3. Comparison of different methods on the release of protoplast in *G. boninense*

Method (with modifications)	No. of the protoplast (× 10^6 cell/mL)
Li *et al*. (2006)	4.2
Chou and Tzean (2016)	0.087
Rodriquez and Redman (1992)	21
Sun *et al*. (2001)	324

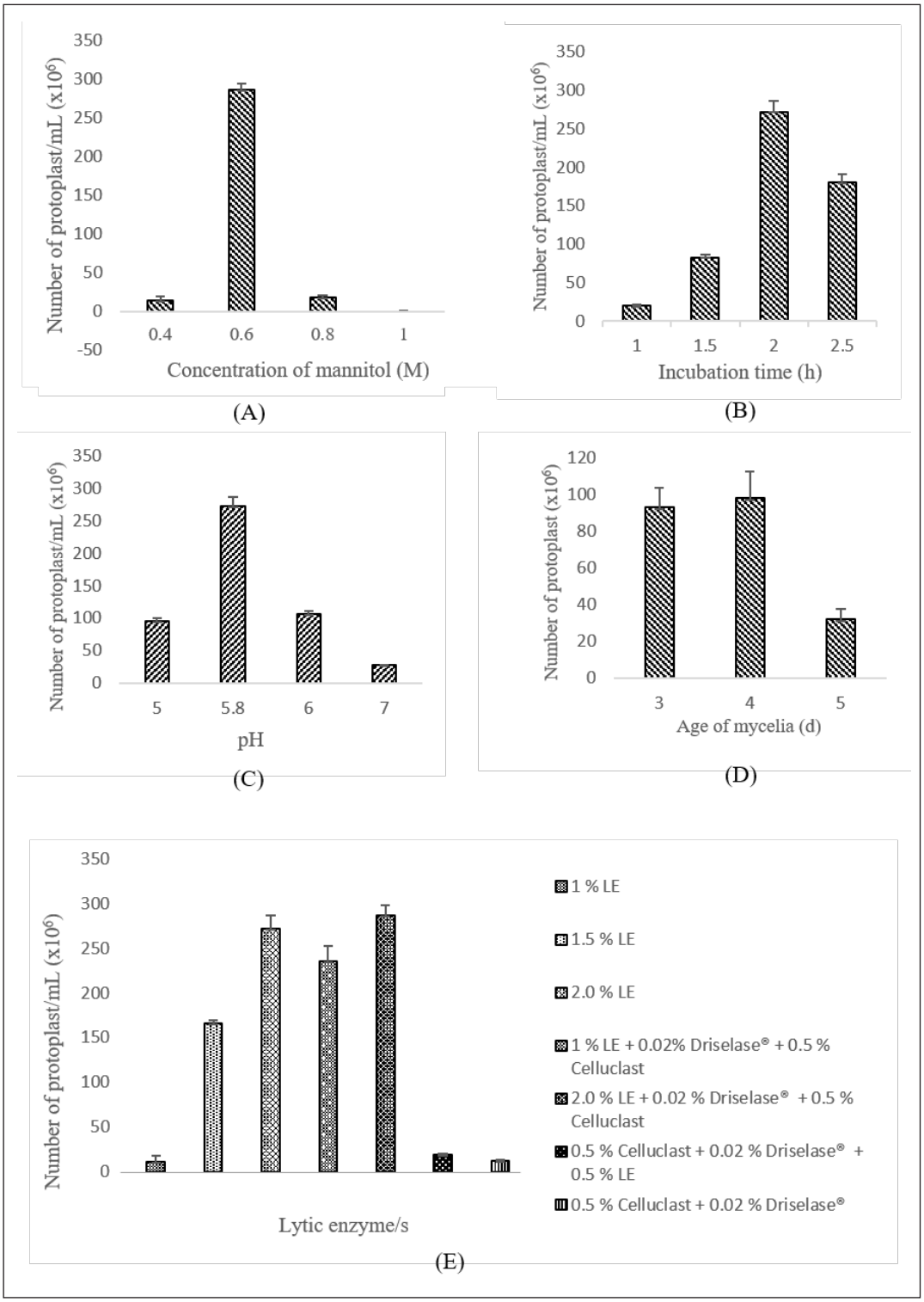


Fig. 2. Optimization of the parameters for protoplast generation. Effect on the protoplast generation when different (A) age of the mycelia, (B) concentration of osmotic stabilizer, (C) pH of the osmoticum, (D) incubation time, and (E) composition of lytic enzyme/s were used in this experiment.

spherical shape was extremely well preserved, and the results showed that osmotic pressure of 0.6 M was the optimal concentration for isolating them (Figure 3). The protoplasts' form serves as a clue to understanding whether the situation being evaluated is appropriate. The removal of the cell wall causes the naked cell to lose its form and become more vulnerable to environmental factors, which can cause plasmolysis and the disintegration of protoplasts. Appropriate osmotic stabilizers guard against plasmolysis in the naked cells. High osmoticum concentration resulted in shrinking protoplasts with decreased protoplast volume, and low osmoticum concentration resulted in ruptured protoplasts with enlarged protoplast volume. However, the balanced pressures between the protoplast's internal hydrostatic pressure and its external osmotic pressure caused the stable spherical protoplast to form (Mastuti & Rosyidah, 2018). Compared to other concentrations, the number of protoplasts isolated at 0.6 M mannitol was also the highest (Figure 2B). Other experiments revealed that different species of fungi displayed different optimum concentrations of osmotic stabilizer, with 0.4 M sucrose for *Ustilago esculenta*, 0.6 M KCl for *Coprineus cinerea*, and 0.6 M sucrose for *Ganoderma lucidum* (Choi *et al*., 1987; Yu *et al*., 2015). According to Cheng and Bélanger (2000), there was no clear reason why various strains or species needed various quantities and kinds of osmoticum to produce protoplasts at their best.

Effect of pH

The lytic enzymes' activity was adversely affected by various osmotic stabilizers to variable degrees. Therefore, various pHs of the osmotic medium were investigated to improve the activity of the lytic enzymes. The pH of the osmotic medium would affect how the lytic enzyme's active site was shaped (Bowman *et al*., 2020). The number of protoplasts generated can be used to determine the pH value of the osmotic media that will boost the lytic enzyme activity. The ideal pH for protoplast production was 5.8, as shown in Figure 2C. The pH value indirectly affects the formation of protoplasts due to an effect on enzyme activity, hence higher or lower pH values will have a negative impact on protoplast yield (Ren *et al*., 2018).

Incubation time

Another important element for the release of protoplasts is the duration of the lytic enzyme incubation. The best time for lytic enzyme treatment depends on the culture's age and various phases, such as immature germlings, old mycelium, or conidia, and varies between fungal species and strains. Figure 2D showed that the protoplast yields rose as the digestion time increased, reaching a maximum of 2.72×10^8 protoplast/mL at 2 h. However, fewer protoplasts were produced when incubation durations were prolonged. The lack of regeneration primers and increased sensitivity to mechanical pressure caused by excessive protoplast digestion may be the reason for this (Ren *et al*., 2018). Prolonged incubation also may cause the early-formed protoplasts to degenerate (Kirn *et al*., 2000). Shin *et al*. (2019) reported that the generation of *Collectotrichun scovillei* protoplasts revealed a similar observation. After 3 h of incubation in the mixture of 2% lysing enzyme and 0.8% Driselase, the isolated protoplasts of *C. scovillei* had assumed a sphere-like shape. The diameter of the protoplasts increased from 8.82 ± 0.06 µm to 11.09 ± 0.07 µm following a prolonged incubation of up to 5 h, nevertheless. Additionally, the lysed protoplasts were seen after a 5-hr incubation (Shin *et al*., 2019).

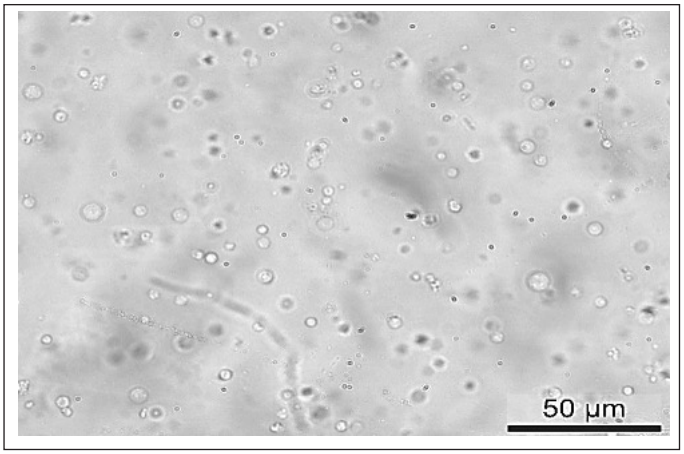


Fig. 3. Microscopic observation of the protoplast generated when 0.6 M of mannitol was used as osmotic stabilizer.

Cell wall lysis by lytic enzyme/s

The cell wall provides both stiffness and defense against outside influences. To acquire intact protoplasts, they must be extracted without harming the cell membrane. It has been observed that more protoplasts can be released by a mixture of enzymes than by a single enzyme. The number of protoplasts liberated was shown in Figure 2E dependent on the kind and concentration of the lytic enzyme(s) utilized. Maximum release of protoplasts was achieved when the mycelia were treated with 2.0% lysing enzyme, 0.02% Driselase, and 0.5% Celluclast.

Optimized protocols for protoplast generation and liberation

Based on the parameters that were optimized for protoplast generation and liberation, it was discovered that using mycelia that were 3- or 4-day old, 0.6 M mannitol with a pH of 5.8 as the osmotic solution, 2 h at 30 °C, and using 2.0% of lysing enzyme, 0.02% of Driselase, and 0.5% of Celluclast with a total yield of protoplasts isolated 5.2 to 8.2 × 10^{10} cells/mL. However, the protoplasts isolated were poorly stable and ruptured easily under low mechanical pressure. The concentration of the lytic enzyme/s may directly affect the release of protoplasts. Although the number of protoplasts increased as the concentration of the lytic enzyme increased, high concentrations of the enzyme were occasionally harmful, leading to the lysis of protoplasts shortly after they appeared, possibly because the protoplasts' structure was weakened, and indicating the toxic levels of the lytic enzymes (Lalithakumari, 2019). Therefore, to acquire more stable and viable protoplasts, the combination and composition of the lytic enzymes used were 1.0 % lysing enzyme, 0.02 % of Driselase, and 0.5% of Celluclast with a total yield of protoplasts obtained 8.95 × 10^{9} up to 3.12 × 10^{10} cell/mL.

Regeneration media

Regenerating protoplasts on six different nutritional media with various osmoticum (0.6 M of sucrose, mannitol, or sorbitol) led to variable regeneration rates. The best regeneration media for *G. boninense* was MYGMA (Li *et al*., 2006) with modification (1% maltose, 0.4 % glucose, 0.4 % yeast extract, 1% malt extract, 0.6 M mannitol) with a regeneration rate of 22.55 ± 3.92% (Table 4). It was determined that the modified MYGMA medium which contains 2% malt extract content was essential since it boosted the regeneration rate by up to 200% when compared to MYGMA without the previously indicated carbon source.

Protoplast PEG-mediated transformation analysis

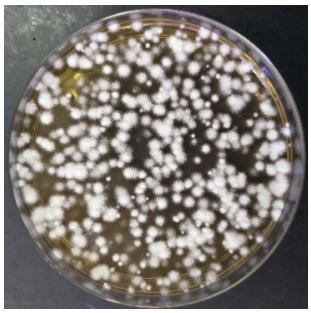
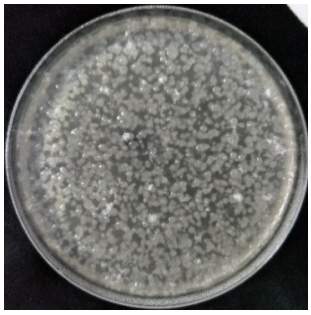
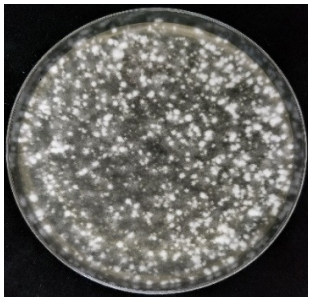
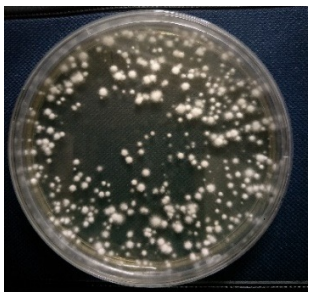
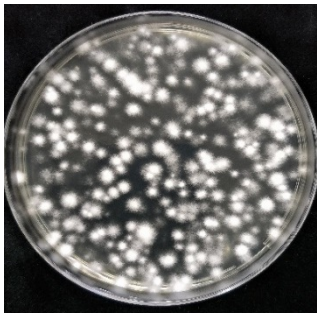
Two PEG-mediated techniques have been modified to examine the isolated protoplast's capacity for transformation with foreign DNA. The integrative circular vector used in this transformation analysis was pUChph of which carries the *hph* cassette as a reporter gene and is useful during the selection of the successful transformants. However, to make the selection of successful transformants possible, the determination of MIC of hygromycin needs to be done before proceeding with the transformation analysis. According to the MIC assay, hygromycin needed to be present at a minimum concentration of 50 g/mL to stop the growth of *G. boninense* Mg12 (Figure 4). Because of this, 50 g/mL of hygromycin was added to each screening plate to help identify the potential transformants that were carrying pUChph.

The first protoplast PEG-mediated transformation method was adopted by Rodriquez and Redman (1992) with modifications. More than 300 putative transformants exhibited the hygromycin resistance trait (HygR) on the transformation plate. About 100 of the potential transformants were chosen at random and subcultured onto fresh PDA plates with hygromycin at a concentration of 50 g/mL. Only 10 of the putative transformants, however, exhibit sustained hygromycin gene expression and continue to exhibit the HygR phenotype after three generations. The second PEG-mediated protoplast transformation technique was adapted from Yu *et al*. (2014). Also, more than 300 potential transformants were obtained. Nevertheless, only 3 out of 100 randomly chosen and subcultured putative transformants on the PDA plates with 50 g/mL of hygromycin maintained the HygR phenotype. Table 5 summarises the number of potential transformants that showed HygR characteristics up until three generations. Through PCR and sequencing, the hygromycin gene's existence in the genomes of all the potential transformants was successfully verified. The amplicon for hygromycin was predicted to be about 1000 bp in size (Figure 5).

DISCUSSION

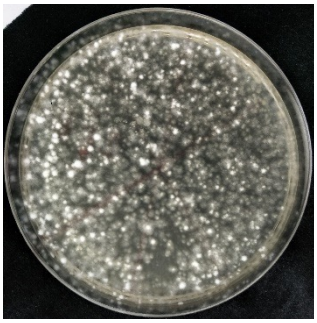
Protoplasts are cells without a cell wall and they make excellent candidates for manipulation in molecular genetics analyses. In general, the protoplast-based technique is widely used in gene manipulation studies in fungi. For instance, the genes knocked-out analysis in *Botrytis cineria*, a necrotrophic plant pathogen, was frequently performed via protoplasts PEG-mediated

Table 4. Rates of protoplast regeneration on various regeneration media

Type of regeneration media	Regeneration rate (%)	Morphology of the colonies on regeneration media
Yeast extract-maltose-sucrose agar (YMSA) (0.6 M sucrose) (Chou & Tzean, 2016)	18.96 ± 1.22	
Potato dextrose sorbitol agar (½ PDA) (Priyatno, 2009) (½ PDA containing 0.6 M sorbitol)	14.95 ± 2.11	
Potato dextrose sucrose agar (PDASP) (Govender *et al*., 2016) (PDA containing 0.6 M sucrose)	12.90 ± 2.93	
Czapek-Dox-sucrose agar (CDSC) (Zhou *et al*., 2008) with modifications (3.34% Czapek Liquid modified, 1% casamino acid, 0.6 M sucrose and 2% agar)	9.03 ± 0.57	
Complete yeast extract mannitol agar (CYMNA) (Yu *et al*., 2014) (1% maltose, 2% glucose, 0.2% yeast extract, 0.2% tryptone, 0.05% $MgSO_4$, 0.46% KH_2PO_4, 0.6 M mannitol and 2% agar)	19.98 ± 3.42	

continued...

...Table 4 continued

Maltose-yeast-glucose-mannitol agar (MYGMA) (0.6 M mannitol) (Li *et al*., (2006) with modification) (1% maltose, 0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.6 M mannitol, and 2% agar)	22.55 ± 3.92	
Minimal media-KCl (MM-KCL) (Ab Wahab *et al*., 2019) (5.0% 20X salt solution, 0.5% 2.25 M $MgSO_4$, 0.1% Hunter's trace element, 1% glucose, 0.6 M KCl, and 2% agar)	no regeneration of protoplasts was observed	

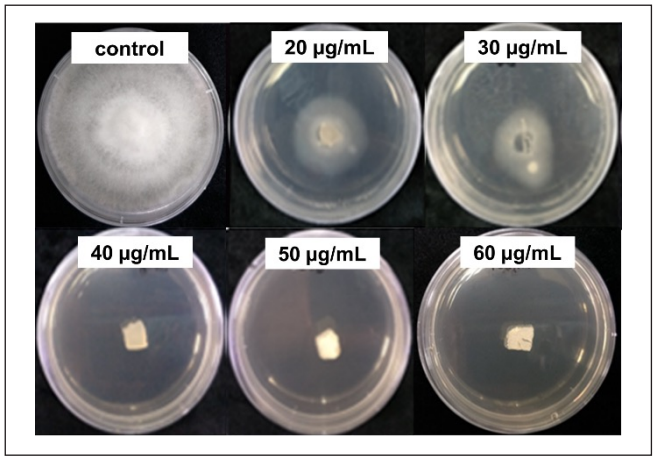


Fig. 4. Hygromycin MIC assay for *G. boninense* Mg12. Negative control is unsupplemented PDA. The mycelia growth rate was affected by the presence of hygromycin in the agar. At 50 µg/mL of hygromycin, the growth of the *G. boninense* was totally inhibited.

Table 5. Number of putative transformants obtained from each transformation method adopted

Method of protoplast PEG-mediated transformation	Total of putative transformants	Total of putative transformant exhibiting HygR on PDA containing 50 µg/mL of hygromycin for the first generation	Total of putative transformant exhibiting HygR on PDA containing 50 µg/mL of hygromycin for the second generation	Total of putative transformant exhibiting HygR on PDA containing 50 µg/mL of hygromycin for the third generation
Rodriquez and Redman (1992)	>300	95	55	10
Yu *et al*. (2014)	>300	52	23	3

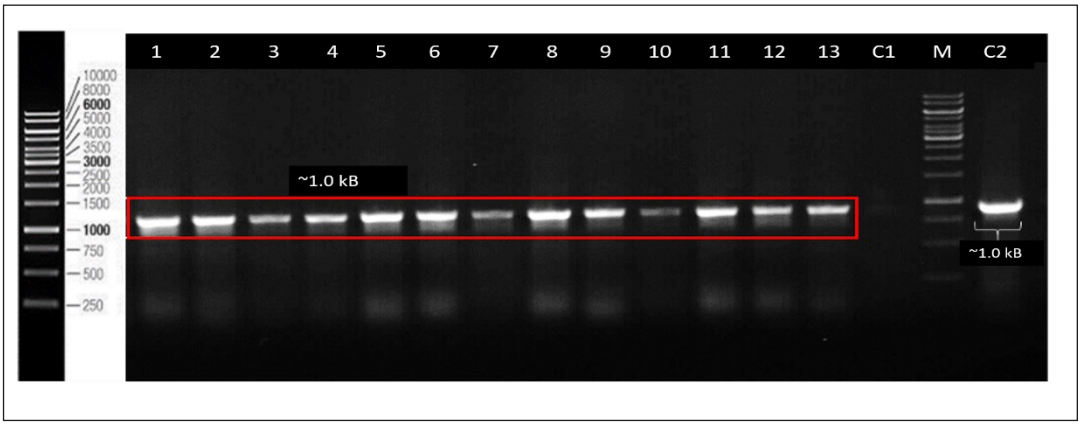


Fig. 5. Validation of the putative transformations via PCR. All the putative transformants (lane 1 – 13) showed the presence of hph gene depicted by the DNA band with the size of ~1.0 kb. Negative control (lane C1), which was the untransformed *G. boninense* showed no band for hph gene. The positive control used for this analysis is pUChph vector carrying the hph gene. M: GeneRuler 1 kb marker (Thermo Scientific, USA)

transformation (Kars *et al*., 2005; Rodenburg *et al*., 2018), as was the case with *Verticillium dahlia*, one of the most destructive plant pathogens that affects more than 400 plant species (Rehman *et al*., 2016), and *Magnaporthe oryzae*, the rice pathogenic fungus (Shimizu *et al*., 2019). The virulence factors were typically the genes that were chosen for the knocked-out analysis. The function of these genes in the pathogenesis of fungi will be clarified by their deletion. For this reason, it is vital to develop an efficient and reproducible protocol for protoplast generation. In the case of *G. boninense,* optimized techniques for protoplast generation will envisage future functional genomic studies. However, to our knowledge, only two reported methods for *G. boninense's* protoplast generation have been published with the number of protoplasts obtained not more than 10^7 protoplasts/mL (Govender *et al*., 2016; Lim *et al*., 2021). A higher number of protoplasts obtained will increase the transformation process and success rate, which is an advantage for a high throughput analysis.

Ganoderma boninense is a white-rot basidiomycete fungus and will only form basidiospores within basidiocarps. Growing the fungus is very time-consuming; hence, using laboratory-grown mycelia is the best alternative for generating protoplasts. The advantage of using grown mycelia is that only the same strain will be used for analysis compared to basidiospores, which carry different genetic materials and perhaps affect the composition of the cell wall and eventually affect the results obtained.

The protoplast concentrations acquired under different methods were different, ranging from 10^4 to 10^{10} protoplast/mL. The best method is from Sun *et al*. (2001) with modifications. The protoplasts obtained by Chou and Tzean (2016) were less than 10^5 protoplast/mL. The microscopic observation under 100× magnification revealed the protoplast condition was inferior, with big vacuoles and highly fragile. This condition is perhaps due to the hypotonic solution used during protoplast generation. In contrast, the protoplast generated using Sun *et al*. (2001) with modifications, other than generating protoplast up to 10^8, likewise had a highly robust membrane and small to medium-sized vacuoles.

The protoplast release efficiency depends on the chemical composition and rigidity of the cell wall, at different stages of growth. Although the elements of the cell wall were similar, the chemical makeup varies depending on the developmental stage. Hence, a suitable lytic enzyme that can enhance the release of the protoplasts is needed. In most cases, a mixture of lytic enzymes performed better than a single lytic enzyme. Other than that, a suitable type and concentration of osmoticum should be used to protect the released protoplasts from being shrunk or ruptured. To ensure the number of protoplasts obtained was optimum, suitable age of mycelia, preferably during log phase growth, and adequate incubation time veraciously needed to determine. In this study, the number of protoplasts obtained when the experiment was done under all optimized parameters was very high, up to 8.2 × 10^{10} protoplasts/mL, however, the protoplasts condition was unstable and easily broken. Thus, the best-optimized working protocol is treating the 3 to 4-day-old mycelia with 1.0% lysing enzyme, 0.02% Driselase, and 0.5% Celluclast or 2% lysing enzyme as individual enzyme for 2 h in 0.6 M mannitol, pH 5.8 with 110 r.p.m. at 30 °C. Even though the number of protoplasts produced was a little lower than when the lytics enzyme was used at its optimal concentration, it was still better than

the non-optimized condition and sufficient for the downstream study. The ability of the protoplast to be viable—able to develop, divide, and revert to the parent culture — is the most crucial aspect of producing protoplasts.

Transformation analysis has been done utilizing protoplast PEG-mediated transformation methods to gauge the isolated protoplasts' capacity to absorb foreign DNA. PEG is used as a cell fusion promoter during transformation analysis because it creates a molecular bridge between cells and DNA, which helps with adhesion, changes membrane permeability, and facilitates the entry of exogenous DNA into cells (Ruiz-Dez, 2002; Li *et al*., 2017). Due to its low cost, simplicity, and effectiveness without the need for expensive equipment, the protoplast PEG-mediated procedure is one of the most popular techniques employed in genetic transformation for fungi (Li *et al*., 2017). Based on the transformation result obtained, the isolated protoplasts were successfully transformed and produced stable transformants. However, more optimization on the transformation parameters such as PEG concentration, temperature, and incubation time needs to be done in the future to increase the transformation efficiency.

CONCLUSION

In conclusion, we manage to optimize an effective method for the isolation of the protoplasts from *G. boninense* Mg12 monokaryotic mycelium with a high regeneration rate. Future molecular studies of this phytopathogenic fungus should be able to be accelerated by adopting the protoplast isolation and regeneration produced by this improved approach,

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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