Survival and Pathogenicity of Monokaryotic and Dikaryotic *Ganoderma boninense* following three different Preservation Methods

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ABSTRACT *Ganoderma boninense*, is the major causal agent of basal stem rot (BSR) in oil palm, causing approximately RM 1.5 billion economic losses annually. Pure dikaryotic and monokaryotic *G. boninense* cultures are frequently used for epidemiology and aetiology studies of BSR. To obtain consistent and comparable inter-experiment results, maintaining and preservation of true-to-type pure cultures of monokaryotic and dikaryotic *G. boninense* are important. Three different pure fungal culture preservation techniques, namely serial sub-culturing, on slant overlaid with mineral oil, and submersion in water, were adopted to store four *G. boninense* isolates (two monokaryons and two dikaryons) for 12 months. Viability of *G. boninense* cultures preserved on agar slant overlaid with mineral oil and submersion in water, ranged from 50 to 89%. Our results also showed that one-year-old dikaryotic *G. boninense* isolates preserved in mineral oil and water had higher pathogenicity level compared to cultures from repeated sub-culturing. Both slant with mineral oil or in water preservation methods, which are less laborious, inexpensive, and simple, can be adopted for preserving pathogenic *G. boninense*.

KEYWORDS: Basidiomycete, Mineral Oil, Pathogenicity, Sub-culturing, Viability

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INTRODUCTION

Economic losses in Malysian oil palm industry caused by *Ganoderma boninense* is estimated to be RM 1.5 billion annually (Arif *et al*., 2011). Due to its detrimental impact on the industry, various studies have been conducted to investigate and manage basal stem rot. The studies ranged from detection approach, such as using Fourier Transform Infrared Spectroscopy for early detection of the fungus in the oil palm tissues (Alexander *et al*., 2014), to disease control, i.e. using phenolic acids to treat *Ganoderma*-infected palms (Jee & Chong, 2014). It is crucial to maintain and preserve true-to-type pure cultures of *G. boninense* to obtain consistent and comparable inter-experiment, particularly the pathological characteristics of the fungal pathogen. Numerous microbial preservation methods have been studied, developed, and adopted to preserve a wide variety of fungal cultures and plant pathogens (Humber, 1997; Abd-Elsalam *et al*., 2010). The developed preservation methods ranged from relatively simple serial transfer (sub-culturing) or storing in mineral oil or water, to some of the more technologically sophisticated techniques, for instance, freeze-drying or lyophilisation. Most of the short- and long-term preservation protocols commonly adopted for preserving fungi have been summarized by Abd-Elsalam *et al*. (2010), Homolka (2014), and Humber (1997). Serial transfer (sub-culturing) and storing of cultures in mineral oil or water at ambient or above-freezing temperatures are among the relatively inexpensive and simple techniques commonly used for basidiomycetous fungi and wood-inhabiting basidiomycetes preservation (Croan *et al*., 1999; Homolka, 2014).

Serial transfer or repeated sub-culturing was, however, proposed to cause undesirable losses in fungal pathogenicity, spore proliferation, or other important characteristics (Humber, 1997; Krokene

& Solheim, 2001). Lack of selection for pathogenicity in artificial environment as a consequence of serial transfer on artificial media, as well as random genetic drift could result in genetic changes, eventually lead to the loss of pathogenicity (Sinclair & Dhingra, 1995; Krokene & Solheim, 2001). Elliott (2005) illustrated survivability and pathogenicity or virulence of *Gaeumannomyces graminis* var. *graminis* stored in conventional potato dextrose agar (PDA) slants with mineral oil and also mycelial plugs in sterile water were unaffected in terms of the morphological and pathological characteristics; however the results were dependent on storage temperature. In a separate study, de Capriles *et al*. (1993) showed that storing *Sporothrix schenckii* in water did not affect its pathogenicity. Mineral oil preservation approach was not only used in preserving plant pathogenic fungi (Borba & Rodrigues, 2000) but also basidiomycetous ectomycorrhizal cultures (Heinonen & Holopainen, 1991).

This study was conducted to assess three commonly-adopted, inexpensive and simple preservation methods, namely repeated sub-culturing on malt extract agar (MEA) medium, submersion in sterile ultra-pure water, and storing in culture slants under a layer of mineral oil for two dikaryotic and two monokaryotic mycelial cultures of *Ganoderma boninense* obtained from different fruiting bodies. Survivability of *G. boninense* cultures maintained or stored using these three methods was recorded and their pathogenicity against oil palm seedlings was evaluated in the nursery to ensure the preservation methods did not alter the infection potential of these *G. boninense* cultures (both monokaryon and dikaryon).

METHODOLOGY

Fungal Cultures or Isolates and Growth Conditions

Pure cultures of two monokaryotic [(122B-3(M) and 40B-5(M)] and two dikaryotic (116A-10 and 207A-4) *G. boninense* isolates were maintained on malt extract agar (MEA) (Difco) incubated at 24°C in the dark for 14 days prior to preservation.

Fungal Preservation Methods

Three different preservation methods were evaluated: 1) Repeated sub-culturing; 2) Storage on MEA slants and overlaid with a layer of mineral oil; and 3) Storage in sterile ultra-pure water, ddH_2O. For repeated sub-culturing, the four different *G. boninense* cultures were subjected to repeated sub-culturing every three months for one year (four subsequent sub-culturing). The storage method for preservation of fungi with mineral oil was prepared according to Elliot (2005), with some modifications: five mL of half-strength MEA slant in test tubes with screw caps were inoculated with 14-day-old *G. boninense* culture and the inoculated slants were incubated at 24°C in the dark for 5 to 6 days (for dikaryon) and 10 to 14 days (for monokaryon) prior to overlaying the surface with a layer of heavy mineral oil (approximately 1 cm thick). The inoculated slants with mineral oil were then stored at 10°C in the dark for one year. For the technique with storage of fungal culture in sterile ddH_2O, 4 mL autoclaved ddH_2O in sterile universal bottles with screw caps were prepared and a few mycelial plugs (approximately 5 x 5 mm) from the 14-day-old *G. boninense* cultures were transferred into the bottles. The sterile universal bottles containing ddH_2O and fungal mycelial plugs were stored in the dark at 10°C for one year. Each isolate was preserved in triplicates.

Survivability of the Fungal Cultures

Fungal mass or mycelial mass of *G. boninense* from sterile ddH_2O or slants overlaid with mineral oil were transferred to fresh MEA plates to determine the survival or viability of the fungal cultures at six separate time points i.e. 1, 2, 5, 6, 10 and 30 month-after-preservation (MAP). The experiment

was carried out in triplicates. The percent of viable *G. boninense* cultures and rate of contamination for each storage method at the selected time points were recorded.

Pathogenicity Test with Oil Palm Seedlings

Ten-week-old DxP oil palm seedlings (Dumpy x AVROS x Yangambi) were used to evaluate the pathogenicity of the revived *G. boninense* cultures (at one year after preservation). The preparation of *G. boninense*-inoculated rubber wood blocks (RWB) (at the size of 6 x 6 x 6 cm^3) , artificial inoculation of *G. boninense*-RWB inoculum on the ten-week-old oil palm seedlings in the nursery and maintenance of the seedlings (shading, planting, transplanting, watering, fertilization, and pesticide application) were carried out according to the protocols outlined in Kok et al. (2013). Presence of BSR symptoms or signs i.e. fungal fruiting body or mycelial mass and leaf yellowing or chlorosis were recorded at weekly intervals for up to 40 weeks. Disease incidence (DI) and disease severity index (DSI) were determined and calculated based on the formulae by Campbell and Madden (1990) and Sapak et al. (2008). Disease severity classes proposed by Kok et al. (2013) were adopted for DSI calculation. The experiment, with thirteen different treatments (outlined in Table 2 – three preservation methods, four *G. boninense* cultures, and one uninoculated RWB control), was carried out in randomized complete block design (RCBD) and each treatment was replicated six times (with each individual seedling as one replicate).

Statistical Analysis

Means for DSI of oil palm seedlings challenged with the four different *G. boninense* cultures and one uninoculated control at 8, 16, 25, 33, and 40 weeks after artificial inoculations were not normally distributed under Levene's test (SPSS 16.0). Therefore, separation in means of DSI among the 13 different treatments at the respective time-points were analysed with the Kruskall-Wallis test and followed by Mann-Whitney U test at $P = 0.05$ using SPSS statistical software (SPSS 16.0).

RESULTS AND DISCUSSION

Cultures of *G. boninense* isolates (two dikaryotic and two monokaryotic candidates) stored in water and in the MEA slant overlaid with a layer of mineral oil at 1, 2, 5, 6, 10 and 30 MAP had more than 50% viability (Table 1). This observation agrees with the results reported by Elliott (2005) where preservation methods using fungi in slant overlaid with mineral oil or in water were among the storage techniques that yielded the highest viability, in which all five strains of *G. grammininis* preserved using these methods maintained its growth rate and pathogenicity level even after ten years of preservation. Preservation in water and slant under a layer of mineral oil suppress fungal growth and reduce fungal metabolism, which are important in preventing genetic and physiological changes in the preserved cultures (Homolka, 2014). The gradual decline of viability percentage was more prominent for monokaryotic cultures compared with dikaryotic cultures. Percent of contamination for *G. boninense* cultures derived from MEA slant overlaid with mineral oil was lower than fungal mass recovered from water preservation technique (Table 1), attributed to the minimal gas exchange in the layer of mineral oil as compared to in water. Generally, contamination is quite minimal except for one of the monokaryons preserved in water.

The two monokaryotic and two dikaryotic *G. boninense* isolates were tested for their pathogenicity in oil palm seedlings. Seedling challenged with the monokaryotic isolates, namely 122B-3 and 40B-5 did not show any sign of basal stem rot, similar to observation reported by Chan *et al*. (2011). *Ganoderma boninense* in monokaryotic stage has been reported to be non-infective towards oil palm and formation of heterokaryon is required for successful fungal infection (Hasan & Flood, 2003; Rees *et al*., 2007). In this study, it was apparent that these preservation methods did not alter

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the genetic components, at least the mating genes of the monokaryons, and hence there was no sign of basal stem rot, unlike the dikaryon cultures. The two dikaryotic *G. boninense* isolates selected were infective to the oil palm (Table 2). Dikaryotic 116A-10 isolate was more aggressive compared to 207A-4 isolate. Isolate 116A-10 caused between 64 and 100% DSI at 40 week-after-inoculation compared to 30 to 56% DSI for isolate 207A-4 (Table 2). Variation in pathogenicity level among dikaryotic *G. boninense* isolates was expected as previously reported by Kok *et al*. (2013). *Ganoderma boninense* isolates used in this study were originated from different palms in the same plantation; high genetic variability can be expected in these isolates as revealed by previous studies on the *G. boninese* population using various molecular approaches (Miller *et al*., 1999; Latiffah *et al*., 2005; Rees *et al*., 2012). Differences in pathogenicity as observed in this study could then be due to this heterogeneity in the genetic backgrounds (Companile *et al*., 2004; Kok *et al*., 2013).

In this study, dikaryotic *G. boninense* cultures obtained from serial sub-culturing had lower DSI and DI compared to dikaryotic cultures derived from slants overlaid with mineral oil or in water (Table 2). Diminished pathogenicity as a result of repeated sub-culturing has been reported in *Ceratocystis polonica* (Krokene & Solheim, 2001). On the other hand, Bunny (1981), de Capriles *et al*. (1993), and Elliott (2005) reported fungi stored in water or slant with a layer of mineral oil retained their pathogenicity level (Abd-Elsalam *et al*., 2010). Our current experiment showed that *G. boninense* stored in water or slant overlaid with a layer of mineral oil were superior to serial sub-culturing, and the cultures from these two storage techniques also maintained their disease incidence and severity levels in oil palm seedlings compared to serial sub-culturing.

CONCLUSION

Two preservation methods, submersion in water and MEA slant overlaid with mineral oil yielded more than 50% recovery or viability in *G. boninense* cultures. These techniques maintained the pathogenicity of the dikaryotic cultures compared to serial sub-culturing every three months, confirmed by a decline in pathogenicity against oil palm seedlings in the nursery. Similar to other studies, there was no visual symptom of basal stem rot for seedlings challenged with monokaryotic cultures. The results highlight the importance of adopting more than one preservation method in maintaining fungal cultures, so that irreversible loss of characteristics, i.e. morphological and pathological can be avoided.

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REFERENCES

[1] Abd-Elsalam, K. A., Yassin, M. A., Moslem, M. A., Bahkali, A. H., de Wit, P. J. G. M., McKenzie, E. H. C., Stephenson, S. L., Cai, L., & Hyde, K. D. (2010) Culture collections, the new herbaria for fungal pathogens. *Fungal Diversity*, **45**, 21-32.

[2] Alexander A., Dayou, J., Chong, K. P., Sipaut, C. S., & Lee, P. C. (2014) Sensitivity analysis of the detection of *Ganoderma boninense* infection in oil palm tree using FTIR. *Transactions on Science and Technology*, **1**(1), 1-5.

[3] Arif, M. S., Roslan, A. & Idris, A. S. (2011) Economics of oil palm pests and *Ganoderma* disease and yield losses. *Proceedings of the Third MPOB-IOPRI International Seminar: Integrated Oil Palm Pests and Disease Management*. 14 November 2011, Kuala Lumpur Convention Centre, Malaysia.

[4] Borba, C. M. & Rodrigues, K. F. (2000) Viability and sporulating capability of Coelomycetes preserved under a range of different storage regimes. *Revista Iberoamericana de Micología*, **17**, 142-145.

[5] Bunny, F. J. (1981) Variation in the pathogenicity of *Gaeumannomyces graminis* after storage. *Australasian Plant Pathology*, **10**, 22.

[6] Campanile, G., Giove, S.L. & Luisi, N. (2004) Genetic and morphologic variability of *Phellinus torulosus* isolates in some oak woods of Southern Italy. *Journal of Plant Pathology*, **86**, 105-115.

[7] Campbell, C. L. & Madden, L. V. (1990) *Introduction to Plant Disease Epidemiology*. USA: John Wiley and Sons.

[8] Chan, J. J., Latiffah, Z., Liew, K. W. & Idris, A. S. (2011) Pathogenicity of monokaryotic and dikaryotic mycelia of *Ganoderma boninense* on oil palm seedlings and germinated seeds in Malaysia. *Australasian Plant Pathology*, **40**, 222-227.

[9] Croan, S. C., Burdsall, Jr., H. H. & Rentmeester, R.M. (1999) Preservation of tropical wood-inhabiting basidiomycetes. *Mycologia*, **91**, 908-916.

[10] de Capriles, C. H., Essayag, S. M., Lander, A. & Camacho, R. (1993) Experimental pathogenicity of *Sporothrix schenckii* preserved in water (Castellani). *Mycopathologia*, **122**, 129-133.

[11] Elliott, M. L. (2005) Survival, growth and pathogenicity of *Gaeumannomyces graminis* var. *graminis* with different methods of long-term storage. *Mycologia*, **97**, 901-907.

[12] Heinonen, T. H. & Holopainen, T. (1991) Maintenance of ectomycorrhizal fungi. *In*: Norris, J. R., Read, D. J., and Varma, A. K. (eds.). *Methods in microbiology* (vol. 23). London: Academic, 413-422.

[13] Hasan, Y. & Flood, J. (2003) Colonization of rubber wood and oil palm blocks by monokaryons and dikaryons of *Ganoderma boninense*- implications to infection in the field. *The Planter*, **79**, 31-38.

[14] Homolka, L. (2014) Preservation of live cultures of basidiomycetes – Recent methods. *Fungal Biology*, **118**, 107-125.

[15] Humber, R. A. (1997) Fungi: Preservation of cultures. *In*: Lacey, L. A. (ed.) *Manual of techniques in insect pathology*. California, USA: Academic Press, Inc., 269-280.

[16] Jee, W. R. & Chong, K. P. (2014). Effect of phenolic acids to *Ganoderma* viability in oil palm tissues and soil. *Transactions on Science and Technology*, **1**(1), 43-49.

[17] Kok, S. M., Goh, Y. K., Tung, H. J., Goh, K. J., Wong, W. C. & Goh, Y. K. (2013) *In vitro* growth of *Ganoderma boninense* isolates on novel palm extract medium and virulence on oil palm (*Elaeis guineensis*) seedlings. *Malaysian Journal of Microbiology*, **9**, 33-42.

[18] Krokene, P. & Solheim, H. (2001) Loss of pathogenicity in the blue-stain fungus *Ceratocystis polonica*. *Plant Pathology*, **50**, 497-502.

[19] Latiffah, Z., Harikrishna, K., Tan, S.G., Faridah, A. & Ho, Y.W. (2005) Random amplified polymorphic DNA (RAPD) and random amplified microsatellite (RAMS) of *Ganoderma* from infected oil palm and coconut stumps in Malaysia. *Asia Pacific Journal of Molecular Biology and Biotechnology*, **13**, 23-34.

[20] Miller, R.N.G., Holderness, M., Bridge, P.D., Chung, G.F. & Zakaria M.H. (1999) Genetic diversity of *Ganoderma* in oil palm plantings. *Plant Pathology*, **48**, 595-603.

[21] Rees, R.W., Flood, J., Hasan, Y. & Copper, R.M. (2007) Effects of inoculum potential, shading and soil temperature on root infection of oil palm seedlings by the basal stem rot pathogen *Ganoderma boninense*. *Plant Pathology*, **56**, 862-870.

[22] Rees, R.W., Flood, J., Hasan, Y., Wills, M. A. & Copper, R.M. (2012) *Ganoderma boninense* basidiospores in oil palm plantations: evaluation of their possible role in stem rots of *Elaeis guineensis*. *Plant Pathology*, **61**, 567-578.

[23] Sapak, Z., Meon, S. & Ahmad, Z. M. A. (2008) Effect of endophytic bacteria on growth and suppression of *Ganoderma* infected in oil palm. *International Journal of Agriculture & Biology*, **10**, 127-132.

[24] Sinclair, J.B. & Dhingra, O.D. (1995). *Basic Plant Pathology Methods* (2nd edition). United States: Lewis Publishers.

Table 1. Survivability or viability of *Ganoderma boninense* cultures or fungal mass retrieved from two different preservation methods at six separate time points and their respective percent of contamination during recovery.

Dikaryotic cultures

Method of preservation		Storage or preservation duration (months)					
		1	2	5	6	10	30
MEA slant with mineral oil	Viability (%)	88.89	100.00	100.00	100.00	100.00	88.89
	Contamination (%)	0.00	0.00	0.00	0.00	0.00	0.00
Water	Viability (%)	55.56	88.89	88.89	100.00	100.00	66.67
	Contamination (%)	0.00	0.00	0.00	0.00	0.00	0.00

Monokaryotic cultures

Method of preservation		Storage or preservation duration (months)					
		1	2	5	6	10	30
MEA slant with mineral oil	Viability (%)	100.00	100.00	100.00	87.50	87.50	50.00
	Contamination (%)	0.00	0.00	0.00	0.00	12.50	0.00
Water	Viability (%)	55.56	100.00	88.89	77.78	100.00	66.67
	Contamination (%)	0.00	0.00	0.00	42.85	33.33	0.00

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Table 2. Disease severity index (DSI) and disease incidence (DI) of oil palm seedlings at five different time points for thirteen separate treatments.

	Treatment		**Disease scorings**									
			DSI (%)‡φ					**DI (%)‡‡**				
	Method	**Isolate**	**8 WAI†**	**16 WAI**	**25 WAI**	**33 WAI**	**40 WAI**	**8 WAI**	**16 WAI**	**25 WAI**	**33 WAI**	**40 WAI**
1	Serial sub-culturing	116A-10	0 a	16.7 (13.6) a	44.4 (20.5) ab	50 (22.4) bc	63.9 (20.4) bc	0	33.3	50	50	66.7
2		207A-4	0 a	0 a	20 (12.2) abc	30 (20) cd	30 (20) cd	0	0	40	40	40
3		122B-3	0 a	0 a	0 c	0 d	0 d	0	0	0	0	0
4		40B-5	0 a	0 a	0 c	0 d	0 d	0	0	0	0	0
5	Slants with mineral oil	116A-10	0 a	8.3 (5.7) a	50 (20.2) a	91.7 (8.3) a	100 a	0	33.3	66.7	100	100
6		207A-4	0 a	11.1 (7.0) a	50 (22.4) a	50 (22.4) bc	50 (22.4) bc	0	33.3	50	50	50
7		122B-3	0 a	0 a	0 c	0 d	0 d	0	0	0	0	0
8		40B-5	0 a	0 a	0 c	0 d	0 d	0	0	0	0	0
9	Fungus in water	116A-10	0 a	11.1 (8.2) a	58.3 (20.1) a	77.8 (16.5) ab	77.8 (16.5) ab	0	33.3	66.7	83.3	83.3
10		207A-4	5.6 (5.6) a	16.7 (13.6) a	38.9 (19.6) ab	38.9 (19.6) c	55.6 (20) bc	16.7	33.3	66.7	80	83.3
11		122B-3	0 a	0 a	0 c	0 d	0 d	0	0	0	0	0
12		40B-5	0 a	0 a	0 c	0 d	0 d	0	0	0	0	0
13	Control*		0 a	0 a	0 bc	0 d	0 d	0	0	0	0	0

*Control represents treatment with the non-inoculated rubber wood block (RWB)

‡Disease severity index (DSI) was calculated based on the formula proposed by Sapak et al. (2008).

‡‡Disease incidence (DI) = (Number of diseased seedlings / number of seedlings per treatment set) x 100%.

†WAI refers to week-after-inoculation.

φDSI for respective treatments at 8, 16, 25, 33, and 40 WAI (week-after-inoculation) presented in mean of 6 replications and numbers in the bracket were standard error. DSI at the respective time points were analysed separately. Means within each column of weeks after inoculation followed by the same letter are not significantly different at $P = 0.05$ after Kruskal-Wallis test followed by Mann-Whitney test.