EVALUATION ON THE CULTURAL CHARACTERISTICS AND ANTAGONISTIC ACTIVITIES OF *Cladobotryum semicirculare* AGAINST *Ganoderma boninense in vitro*

NURUL FADHILAH MARZUKI*; YIT KHENG GOH*; HUN JIAT TUNG; YOU KENG GOH* and KAH JOO GOH***

ABSTRACT

The objectives of this preliminary study were to identify the Cladobotryum*-like isolate and assess the antagonistic activity of this fungus against* G. boninense *and other fungal pathogens. Based on both morphological and molecular approaches, the unknown fungus was later described as* Cladobotryum semicirculare*. Under dual-culture assays,* Cladobotryum semicirculare *was observed to suppress radial mycelial growth of all the tested fungi with* G. lucidum *and* G. boninense *G37's growth being inhibited the most, 74.8% and 74.7%, respectively.* Clonostachys *sp. inhibited the least (12.7%). Through poison agar test, filtrates from* C. semicirculare *at 50% concentration were observed to suppress the mycelial growth of* G. boninense *G14 (35% inhibition) and* G. lucidum *(25% suppression), and with 100% filtrate concentration both* Ganoderma *species were showing more than 49% inhibition. Growth of all tested* Ganoderma *isolates and* C. semicirculare*, was faster under aerobic (2.2 to 3.8 mm per day for* Ganoderma *isolates and 4.3 mm for* Cladobotryum*) compared to anaerobic (1.6 to 2.2 mm per day for* Ganoderma *isolates and 1.4 mm per day for* Cladobotryum*) condition.* In vitro *mycoparasitism test confirmed that* C. semicirculare *reduced the re-isolation of* G. lucidum *(33.3%) and* G. boninense *(66.7%) mycelia. This study also provided the first report of* G. boninense *as the host for* C. semicirculare*.*

Keywords: antagonism, basal stem rot (BSR), fungicolous, pathogenicity, white root disease.

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INTRODUCTION

Basal stem rot (BSR) and upper stem rot (USR) diseases in oil palm (*Elaeis guineensis* Jacq.) or also known as white rot disease caused by one of the most aggressive basidiomycetous fungal pathogens - *Ganoderma boninense*, and these diseases have contributed to great economic losses annually in oil palm sector in South-east Asia (Arif *et al.*, 2011; Cooper *et al.*, 2011; Hushiarian *et al.*, 2013). BSR and USR diseases also contributed to high casualty in oil palms, yield losses for the infected palms, shortening the palm productive life-spans, and also increase in the costs for managing or treating the *Ganoderma*-infected palms (Flood *et al.*, 2002; Singh, 1991). Furthermore, isolates of *G. boninense* collected

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from different estates or locations in Malaysia were observed to show variations in aggressiveness toward the growth of oil palm seedlings and degrees of disease severity or incidences (Goh *et al.*, 2014b; Kok *et al.*, 2013). When *Ganoderma* BSR disease was first reported in 1931 by Thompson, this fungal pathogen was only found to infect the old mature oil palms planted on coastal soils (Ho and Nawawi, 1985). However, palms at the age of 1- to 2-year old have been detected with *Ganoderma* infection in the past few decades (Ho and Nawawi, 1986; Singh, 1991). Due to limited studies on the biological, physiological, and behavioural aspects of *G. boninense,* poor understanding of pathogen-host and pathogen-host-environment interactions, as well as lack of information on both aetiology and epidemiology of the disease, have made the control or management of BSR and USR very difficult (Goh *et al.*, 2014a; Hushiarian *et al.*, 2013; Rolph *et al.*, 2000). Therefore, to-date there is no known effective and satisfactory cure or treatment for BSR and USR white rot diseases (Idris, 1999; Sundram *et al.*, 2008).

For the past few years, more researches have been directed towards using a more environmental-friendly and less explored approach, namely biological control (Sundram *et al.*, 2008), to manage *G. boninense* with various bacterial and fungal candidates isolated from oil palm plantations. Common bacterial and fungal isolates investigated were *Burkholderia cepacia, Pseudomonas aeruginosa, Trichoderma harzianum,* mycorrhizae, and combinations of bacteria and mycorrhizal fungus. In addition, most of these endophytic bacteria and fungi or plant growth promoting rhizobacteria (PGPR) were reported to improve the growth of oil palms and reduce the incidence or severity of *Ganoderma* white rot diseases (Mohd Zainudin and Abdullah, 2008; Sapak *et al.*, 2008; Sundram, 2013a, b; Sundram *et al.*, 2011). Soytong (2014) reported that both *Chaetomium lucknowense* and *C. cochiliodes* were able to suppress the growth of *G. boninense* under *in vitro* conditions, and the potential use of these two fungal candidates in controlling oil palm *Ganoderma* BSR disease is still being tested by the same group as well. A list of other novel potential fungal biocontrol candidates, namely *Hendersonia, Amphinema* and *Phlebia* species, were reported to reduce the incidences and severity of *Ganoderma* BSR disease in the nursery trials (Idris *et al.*, 2012; Nurrashyeda *et al.*, 2012a, b).

Ganoderma and other basidiomycetes were found to harbour or host a wide range of fungicolous fungi. The order of *Hypocreales* (Ascomycetes) was recorded to accommodate the highest number of sporocarp- or fruiting body-inhabiting fungi, and followed by *Tremellales* (Basidiomycetes) (Gams *et al.*, 2004). *Verticillium fungicola* (Preuss) Hassebr., *Mycogone perniciosa* (Magnus) Delacr., *Cladobotryum dendroides* (Bull.) W Gams and Hooz., *Trichoderma* species, *Papulaspora byssina* Hotson, *Myceliophthora lutea* Costantin, *Sepedonium* species, and *Sporendonema purpurascens* (Bonord.) E W Mason and S Hughes infected commercial edible mushrooms and reduced their yields (Sharma *et al.*, 2007). Outbreaks of mushroom diseases due to *Cladobotryum* species were reported in Ireland and Great Britain on various commercially cultivated mushrooms (McKay *et al.*, 1999). In later years, other *Cladobotryum* species were observed in Korea and Spain, namely *C. mycophilum* was isolated from *Agaricus bisporus* and *Pleurotus eryngii,* as well as *C. varium* was reported on *Flammulina velutipes* and *Hypsizgus marmoreus* (Back *et al.*, 2012; Gea *et al.*, 2011). In another separate study, *C. semicirculare* strains were obtained from *G. tsugae* in Taiwan and from polyporicolous as well as agaricolous fungi in Cuba (Kirschner *et al.*, 2007). Furthermore, *C. semicirculare* was observed to be responsible for severe losses in *Ganoderma* production and this fungicolous parasitic pathogen can only be managed through proper sanitation or stringent hygienic approaches (pers. comm. with Dr Kirschner). *Cladobotryum semicirculare* strain was reported on polypore in Sri Lanka as well (Põldmaa, 2011). Furthermore, this hyphomycetous fungus was found to differ from other *Cladobotryum* species in terms of the presence of slightly to strongly curved to semicircular, 0–3-septate, ellipsoidal, clavate conidia (Kirschner *et al.*, 2007; Põldmaa, 2011). Morphological variability within *C. semicirculare* was observed between strains from Cuba and Taiwan. Strains from Taiwan appeared to be smaller in sizes for both conidia and conidiogenous cells, as well as looser texture and brown reverse cultures, compared to strains from Cuba (Kirschner *et al.*, 2007). Due to relatively high infraspecific morphological variability and similarity within and among *Cladobotryum* species, this has made the differentiation of species even more difficult. Therefore, both morphological and phylogenetic or genetic approaches are required to reveal the species of *Cladobotryum* (Kirschner *et al.*, 2007; McKay *et al.*, 1999; Põldmaa, 2011).

In other studies, *Ganoderma* species were found to host a few other fungal species, in particular *Sporophagomyces chrysostomus* (Berk. and Broome) K. Põldmaa and Samuels, *Hypomyces pseudo-polyporinus* Samuels and Rogerson, *Hypocrea atrogelatinosa* Dingley, *Xylogone ganodermophthora* Kang, Sigler, Y W Lee and S H Yun, and *Acremonium lindtneri* (Kirschst.) Samuels and Rogerson (Dingley *et al.*, 1981; Gams *et al.*, 2004; Kang *et al.*, 2010; Põldmaa *et al.*, 1999; Rogerson and Samuels, 1993). In addition, other fungicolous fungi reported on the substrates of *G. applanatum* and *G. carnosum* were *Albertiniella polyporicola* (Jacz.) Malloch and Cain, *Hypocrea lacteal* (Fr.) Fr., *Hypocrea rufa* (Pers.) Fr., *Rhinotrichiella globulifera, Verticillium incurvum* Helfer, and *Trichoderma polysporum* (Link) Rifai (Helfer, 1991).

In one of our previous experiments conducted to screen different oil palm planting materials for their reaction when challenged with *G. boninense* isolates, *Cladobotryum*-like fungal isolate was found to proliferate on the fruiting bodies of *G. boninense.* Therefore, the objectives of this preliminary study were to identify the *Cladobotryum*-like fungal isolate and assess the antagonistic or parasitic activity of this fungus against *G. boninense* and other fungal pathogens.

MATERIALS AND METHODS

Fungal Isolates and Growth Conditions

Mycelia and conidia of *Cladobotryum*-like AAS0114 isolate were harvested using sterilised needles from the *Ganoderma* fruiting bodies. Mycelial mass and conidia were then transferred to and maintained on Malt extract agar (MEA) (Difco, Becton Dickinson Diagnostics, Sparks, Maryland, USA) supplemented with antibiotic (100 μg litre^{-1} streptomycin sulphate) (Sigma-Aldrich, St Louis, Missouri, USA) prior to DNA extraction and *in vitro* studies. Fifteen phytopathogenic and three non-phytopathogenic (*G. lucidum, Clonostachys* sp., and *Pleurotus* sp.) fungal strains from the phyla of Ascomycota and Basidiomycota were selected (outlined in *Table 1*) and co-inoculated with *Cladobotryum*-like fungal isolate in dual-culture assays. Small subunit 18S rDNA sequences for *Ganoderma boninense* isolates G5, G8, G10, G12, and G14, and *G. australe* were obtained from GenBank (*Table 1*). Sequences for six other *Ganoderma* isolates (G30-37) and six non-*Ganoderma* isolates (AA0002-0007) were generated using primer set targeting small subunit 18S rDNA fragment outlined in Borneman and Hartin (2000) and Kok *et al.* (2013). All the small subunit 18S rDNA sequences for 12 *Ganoderma* and six non-*Ganoderma* isolates were subjected to BLAST search in NCBI to obtain their identity (*Table 1*). All the 15 phytopathogenic and three non-phytopathogenic fungal strains used were cultured and maintained on MEA prior to dual-culture assays and *in vitro* mycoparasitism test. Cultures of *G. boninense* isolates (G5, G8, G10, G12, and G14) and *G. australe* were obtained from Kok *et al.* (2013) and Goh *et al.* (2014c). In one of our previous studies, we found that a few *Ganoderma* species were

TABLE 1. IDENTITY, HOST AND ORIGIN OF ALL THE *Ganoderma* AND OTHER FUNGAL ISOLATES COLLECTED FROM VARIOUS PARTS OF MALAYSIA AND SELECTED FOR THIS STUDY

Culture/isolate		Identity of isolate	Host/origin	GenBank accession No. *
Ganoderma isolates	G14	*Ganoderma boninense*	Oil palm, Johor	JQ665238
	G8	*G. boninense*	Oil palm, Johor	JQ665233
	G5	*G. boninense*	Oil palm, Selangor	JQ665230
	G10	*G. boninense*	Oil palm, Kedah	JQ665235
	G12	*G. boninense*	Oil palm, Kedah	JQ665237
	G31	*G. boninense*	Coconut palm, Johor	KF925452
	G30	*G. australe*	Tower tree, Selangor	KF925451
	G33	*Ganoderma* sp.	Coconut palm, Perak	JN234428
	G34‡	*G. boninense* (monokaryon)	Oil palm, Selangor	NA
	G36	*G. boninense*	Oil palm, Selangor	AF255198
	G37	*G. boninense*	Oil palm, Selangor	AF255198
	G32	*G. lucidum*	Mushroom farm, Johor	FJ379281
Others	AA0002	*Schizophyllum commune*	Oil palm, Johor	X54865
	AA0003	*Clonostachys* sp.	Oil palm, Kedah	AY489695
	AA0004	*Fusarium* sp.	Oil palm, Selangor	GQ352485
	AA0005	*Pleurotus* sp.	Mushroom farm, Selangor	AY368662
	AA0006	*Phoma* sp.	Flower plant, Selangor	KF611891
	AA0007	*Cochliobolus* sp.	Flower plant, Selangor	JN941617

Note: *The column for all the GenBank accession No. elucidates the result obtained from BLAST search in NCBI. All the sequences were generated using primer set – 817 and 1536 (Borneman and Hartin, 2000) to target for the small subunit 18S rDNA fragment. DNA extraction, PCR reaction and DNA sequencing were conducted according to the procedures outlined in Kok *et al.* (2013).
‡Culture obtained from single basidiospore of *G. boninense* G5 isolate.

able to grow under anaerobic condition as well but with slower growth rates compared to aerobic condition (pers. obs.). Therefore, linear mycelial growth rates for the *Cladobotryum*-like and all the *Ganoderma* isolates under aerobic and anaerobic (BD GasPak™ EZ anaerobic container system sachets) conditions at 24°C were studied to evaluate their capability in growing under anaerobic condition.

Morphology of *Cladobotryum*-like Fungus on Natural Substrate

Morphological studies of conidia, hyphae, mycelia, and other fungal structures for the *Cladobotryum*-like AAS0114 isolate were conducted on *Ganoderma* fruiting body and MEA under RaxVision IBS-100 microscope with a RaxVision C mount camera. Fungal materials were mounted in water with lactophenol blue dye for microscopic observations.

Molecular Analysis of *Cladobotryum*-like Isolate

Genomic DNA was extracted using FastDNA Spin Kit (MP Biomedicals, USA) as per manual instruction. Primer set ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (Gardes *et al.*, 1991; White *et al.*, 1990) targeting ITS (internal transcribed spacer) region were selected in the polymerase chain reaction (PCR). The PCR mixtures were prepared according to Kok *et al.* (2013). PCR conditions for ITS primer set was: (i) 4 min at 94ºC, (ii) 35 cycles of 20 s at 94ºC, 10 s at 56ºC and 1 min at 72ºC, and (iii) 5 min at 72ºC in Veriti™ Thermal Cycler (Applied Biosystem, USA). PCR products were run on 1% w/v agarose gel (Vivantis, USA) in 1x TBE buffer with 10x SYBR® Safe DNA gel stain (Invitrogen, USA). Purified PCR products were sent to Bioneer, Korea for sequencing. ITS sequence from the specimen (AAS0114 isolate) was submitted to GenBank.

Sequence Alignment and Phylogenetic Analyses

Sequences of ITS region from the present study (AAS0114 isolate) and 28 (*Cladobotryum* and *Hypomyces* species) sequences retrieved from GenBank were aligned using Clustal W (Thompson *et al.*, 1994), and edited in Bioedit (Hall, 1999). Alignments are available on treebase.org at the following link http://purl.org/phylo/treebase/phylows/study/TB2:S16119. Maximum likelihood (ML) analysis was performed with MEGA6 software (Tamura *et al.*, 2013). The robustness of trees was validated using bootstrap analyses with 1000 repetitions. Phylogenetic trees were prepared with sequences showing bootstrap values higher than 50%. Trees based on ITS sequences were rooted with sequence from *Hypomyces aurantius* (Pers.) Fuckel FN859425.

Dual-culture Assay

Dual-culture assays under aerobic conditions were used to evaluate percent inhibition of radial growth (PIRG) (%) for *Ganoderma* and other non-*Ganoderma* fungal isolates. Under anaerobic conditions, dual-culture assays were performed only between *C. semicirculare* AAS0114 strain and all the *Ganoderma* isolates used in this study. One cm diameter mycelial plugs excised using cork borer from both *C. semicirculare*, and 15 phytopathogenic as well as three non-phytopathogenic fungal isolates were placed approximately 4 cm apart on MEA plates. Assays were carried out in five replicates per treatment. PIRG (%) was determined according to the formula proposed by Jomduang and Sariah (1995). Radius of tested fungal colony (*Ganoderma,* phytopathogenic, and non-phytopathogenic fungi) in the direction facing towards the *C. semicirculare* AAS0114 colony was measured for PIRG analysis. Linear mycelial growth of *Ganoderma,* phytopathogenic, and non-phytopathogenic fungal strains from all the treatments were measured and recorded daily for two weeks (Goh and Vujanovic, 2010; Ouimet *et al.*, 1997).

Poison Agar Test

Filtrates from *C. semicirculare* AAS0114 isolate were prepared according to the procedures highlighted in Sundram (2013a), except potato dextrose broth (PDB) was replaced with malt extract broth (MEB). Filter-sterilised filtrates were added into Petri dishes poured with MEA to a concentration of 50% and 100%, respectively. The MEA media amended with *C. semicirculare* filtrates were inoculated with *G. boninense* mycelial plugs according to the methods outlined in Sundram (2013a) and incubated at 24°C for seven days. Only one *Ganoderma boninense* (G14) and one *G. lucidum* (G32) were selected for this experiment. The MEA plates without filtrate supplement from *C. semicirculare* acted as the control. The growth of *Ganoderma boninense* mycelia was recorded up to five days for both treated and control plates. *Ganoderma* radial growth measurements (radii in four perpendicular directions were measured from the edge of mycelial plug to the periphery of fungal colony and the average of these four readings was used) were taken to calculate percent inhibition of mycelial growth. Inhibition of *G. boninense* mycelial growth was measured and calculated accordingly to Sundram (2013a). Percent inhibition of mycelial growth (PIMG %) was calculated using the formula proposed in Skidmore and Dickinson (1976).

***In vitro* Mycoparasitism Test**

In vitro mycoparasitism tests of *C. semicirculare* AAS0114 isolate on six *Ganoderma* and four non-*Ganoderma* isolates were conducted according to

the procedures outlined in Kang *et al.* (2010), with modifications: mycelial plugs of *Ganoderma* and non-*Ganoderma* isolates were transferred and grown on MEA plates for 14 days at 24°C in the dark under aerobic conditions, and allowed to cover the whole plate. A 1-cm diameter mycelial plug from *C. semicirculare* isolate (maintained on MEA) was placed on the *Ganoderma* or non-*Ganoderma* mycelial mat for an additional 14 days at 24°C in the dark. This allowed the *C. semicirculare* to colonise or proliferate on the 2-week old *Ganoderma* or non-*Ganoderma* cultures. After two weeks of incubation, *Ganoderma* or non-*Ganoderma* mycelia plugs below the *C. semicirculare* isolate were excised and subcultured on to fresh MEA and incubated for seven days at 24°C to assess pathogenicity on the tested *Ganoderma* and other non-*Ganoderma* fungal isolates. The control plates were inoculated with *Ganoderma* or non-*Ganoderma* fungal isolates only. These plates were not inoculated with *C. semicirculare.*

Statistical Analysis

Differences in the fungal mycelial growth rates for *C. semicirculare* AAS0114 between aerobic and anaerobic conditions were analysed using *t*-test at $P = 0.05$ with Minitab 16 (Minitab Inc., State College, PA). Differences in means for the radial mycelial growth among a few selected *G. boninense* isolates under aerobic and anaerobic conditions were analysed using analysis of variance (ANOVA)-Tukey test. Whereas, differences in means for percent inhibition of mycelial growth (PIMG %) of two *Ganoderma* isolates inoculated on media amended with different concentrations of *C. semicirculare*'s filtrate were analysed using *t*-test at $P = 0.05$. *Ganoderma* radial mycelial growth rate was analysed by *Ganoderma* isolate, incubation condition (aerobic or anaerobic) and *Ganoderma* isolate × incubation condition interaction using a multi-factorial GLM ANOVA analysis. Differences in means for PIRG (%) in different *Ganoderma* or other fungal pathogens by *C. semicirculare* for different fungal isolates were analysed with ANOVA-Tukey test.

RESULTS AND DISCUSSION

Morphology and Molecular Characterisation of AAS0114 Isolate

One fungal isolate with whitish colour mycelia was found to proliferate and sporulate on *G. boninense* basidiocarps in one of the experiments (*Figures 1A* and *1B*). AAS0114 isolate was observed to form a layer of 1 to 3 mm whitish mycelial mass bearing asexual stage on hymenium (*Figures 1B* and *1D*). On the natural substrate (*Ganoderma* fruiting body), AAS0114 isolate was found to produce slightly curved to semi-circular conidia with 1-3 septates (*Figure 1E*), as well as the morphology of conidiophores, conidiogenous cells (*Figures 1F* and *1G)* and other important structures were in agreement with the description by Kirschner *et al.* (2007) and *Põldmaa (2011)*. For instance, AAS0114 isolate produced irregular or verticillate branched, septated, smooth, and hyaline conidiophores *(Figures 1F* and *1G*). Conidiogenous cells were in the whorls of two to four and with flask-like shapes (*Figure 1F*). Majority of the conidia were recorded to have two septates. In current study, conidia were found to have shorter length ranging from 14 to 22 μm and narrower width from 5.5 to 7.7 μm compared to the conidia of Cuba's strains. These measurements agree with the observations recorded in *C. semicirculare* strains isolated from Sri Lanka (Põldmaa, 2011) and Taiwan (Kirschner *et al.,* 2007). Conidia of AAS0114 isolate were observed to share similar sizes with Asia's strains but smaller compared to Cuba's strains. Fungal mycelial mass of AAS0114 was collected from *Ganoderma* basidiocarps and plated on MEA to obtain pure culture. Mycelium of AAS0114 was initially whitish in colour, turned into yellowish brown after approximately 7 to 10 days of incubation, and followed by reddish brown after two-week of incubation (*Figure 1H*), and this was in accordance to the findings reported by Kirschner *et al.* (2007) and Põldmaa (2011). Kirschner *et al.* (2007) recorded cultures of *C. semicirculare* strains from Taiwan to have looser texture and with brown reverse. Similar morphology and appearance were observed in AAS0114 culture as well after 7 to 10 days of incubation on MEA. However, there were no conidia observed on MEA. This was in agreement with the observation reported by Kirschner *et al.* (2007), who mentioned that mycelia of *C. semicirculare* strains from Taiwan were not able to sporulate on MEA. *Cladobotryum semicirculare* AAS0114 was only found to produce conidia on the *Ganoderma* fruiting bodies and on the plates co-inoculated with *Ganoderma*. ITS sequence of approximately 480 base pairs was obtained from AAS0114 isolate and subjected to BLAST search in NCBI. The sequence showed 99% similarity to *C. semicirculare* CBS705.88 isolate (accession number: FN859417 - with E-value of 0). The same sequence was also used for phylogenetic analysis. Together with 28 other ITS sequences of different *Cladobotryum* and *Hypomyces* species obtained from GenBank, a phylogenetic tree using ML with 1000 bootstrap replications was generated to show the position of AAS0114 fungal isolate (*Figure 2*). Based on the phylogenetic analysis, AAS0114 isolate was found to share the same clade as two *Cladobotryum semicirculare* isolates and the branch received more than 90% bootstrap support (*Figure 2*). AAS0114 isolate was observed to group closer to *C. semicirculare* (FN859418) (with 91% bootstrap support) from Sri Lanka compared to

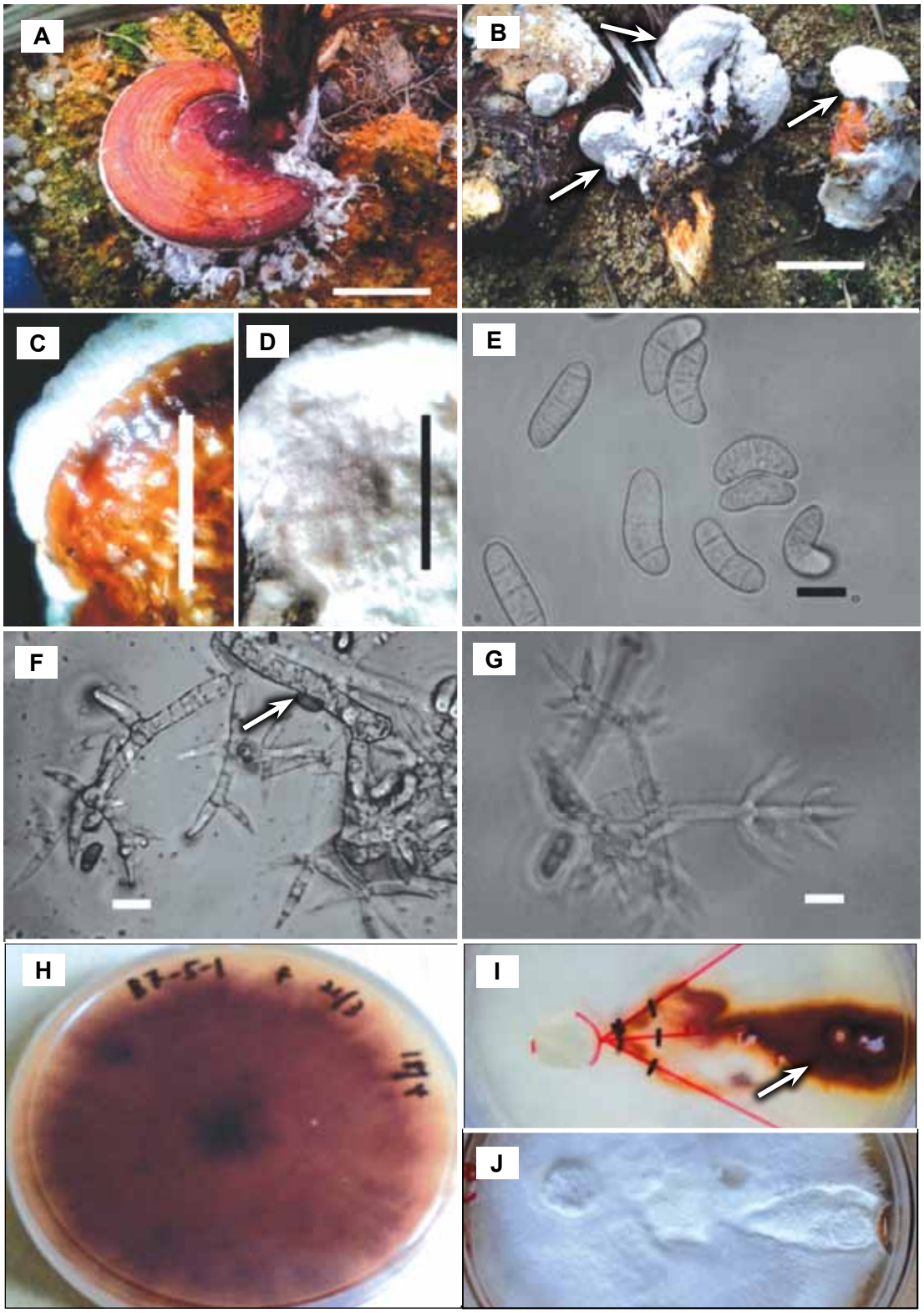


Figure 1. Cladobotryum semicirculare *AAS0114. (A-B) Whitish mycelia with conidia on fruiting bodies of* Ganoderma boninense *(hymenium) (arrows); (C-D) on hymenium of* G. boninense*; (E) microscopic observation of* C. semicirculare *conidia and both conidiophores and conidiogenous cells (F-G) (basidiospores of* G. boninense *- arrows); (H) colony characteristic;* G. boninense *(G14 isolate) melanised organs or strucutures (arrows) induced by* C. semicirculare*: (I) bottom view and (J) top view. Scale bars: 3 cm for A-B; 1 cm for C-D; and 10 µm for E-G.*

C. semicirculare (FN859417) from Cuba on polypore and agaric (*Figure 2).* Based on both morphological examination and phylogenetic analysis, we classify the unknown AAS0114 fungal isolate as *C. semicirculare* AAS0114. Furthermore, this is the first report on the isolation of *C. semicirculare* from fruiting bodies of *G. boninense.*

In vitro Growth under Aerobic and Anaerobic Conditions

Radial mycelial growth rates for *C. semicirculare* AAS0114 and a few selected *G. boninense* isolates under aerobic and anaerobic were assessed. Under aerobic condition, *C. semicirculare* AAS0114 was found to grow significantly faster compared to anaerobic condition (*Figure 3A*). The analysis of variance for assessing fungal radial mycelial growth rates showed a significant effect on *Ganoderma* growth for each of the main factors tested (incubation conditions – aerobic and anaerobic; and different *Ganoderma* isolates), with the incubation condition demonstrating the greatest effect ($F = 90.3$; $P < 0.001$). There was also a significant *Ganoderma* isolate × incubation condition interaction ($P < 0.001$). Consequently, means of radial mycelial growth rate for different *Ganoderma* isolates were then analysed separately based on the incubation conditions (*Figure 3B*). *Ganoderma* radial mycelial growth rate was faster under aerobic compared to anaerobic and this result was in accordance to a previous observation reported by Goh *et al.* (2014c) on *G. australe.* Survival and growth of *Ganoderma* and other fungal pathogens were reported to be reduced under anaerobic conditions in a few previous studies. For instance, flood-fallow or flooding method has been highlighted before as one of the control measures and used to weaken or kill the *Ganoderma* inoculum in the soils (Singh, 1991) and increase the carbon dioxide level or induce anaerobic conditions in the soils to reduce the survival of *Fusarium oxysporum* (Newcombe, 1960). In addition, control measures, such as submerging the wood substrates in the water have been proposed to reduce the post-harvest colonisation or infection by wood-decaying fungi – *Ganoderma* species (Chang, 2003). The above studies described the use of water to induce high carbon dioxide and establish anaerobic conditions for reducing survival of soil-borne fungal pathogens.

Dual-culture Assays and Poison Agar Test

A total of 12 *Ganoderma* and six other fungal isolates were selected to challenge with *C. semicirculare*

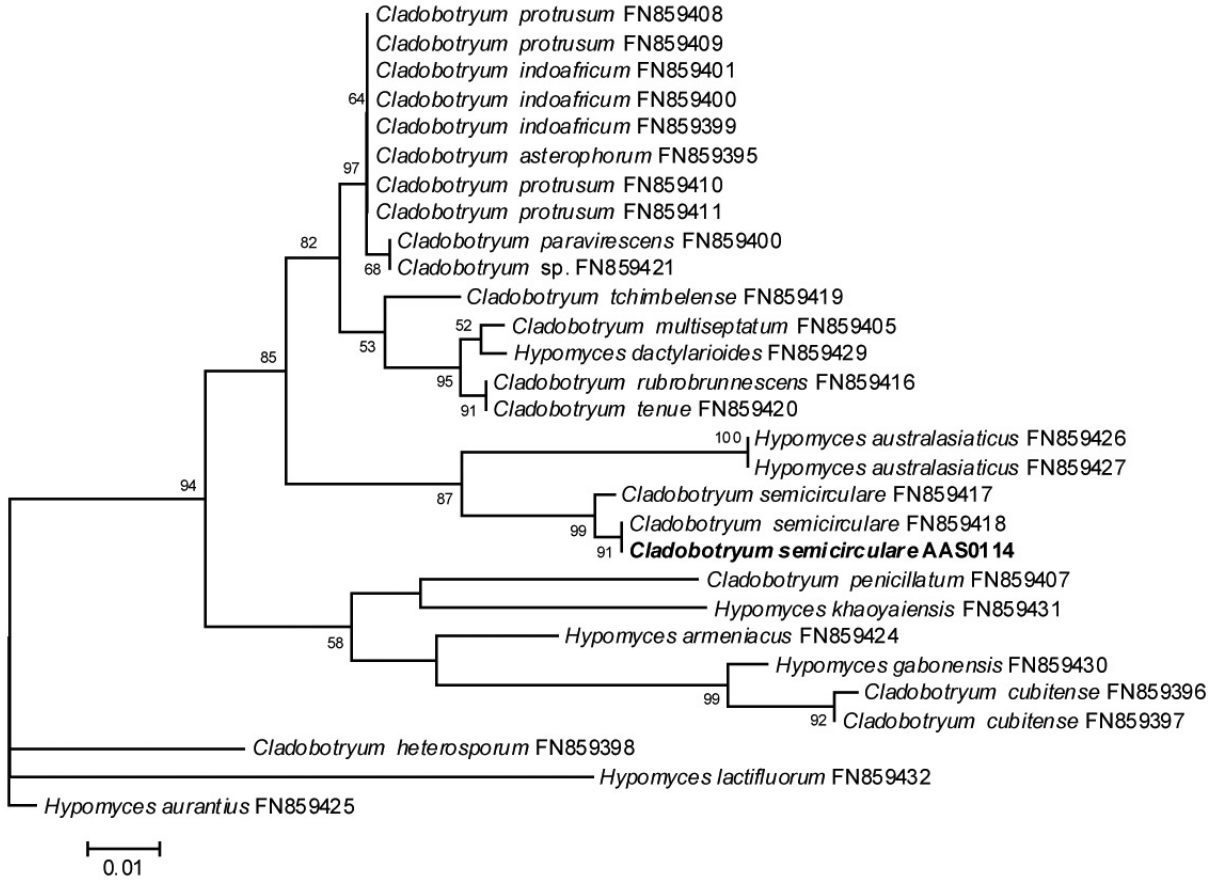
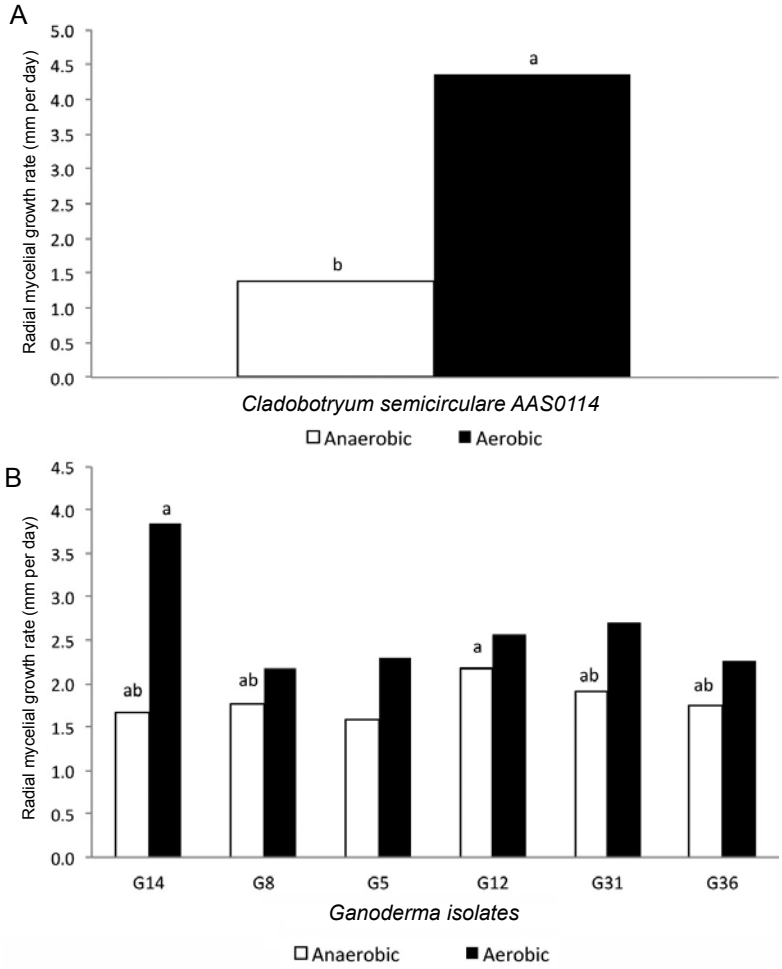


Figure 2. Phylogenetic tree based on internal transcribed spacer (ITS) sequences showing the position of Cladobotryum semicirculare *AAS0114 isolate (in bold) using Maximum Likelihood (ML) analysis. Bootstrap values of 50% or higher obtained from 1000 bootstrap replications are outlined in the tree.*



Note: In *Figure 3A*, means between aerobic and anaerobic followed by the same letter are not significantly different at P = 0.05 after t-test. Whereas, in *Figure 3B*, both aerobic and anaerobic conditions were analysed separately, and means within each incubation conditions for different *Ganoderma* isolates followed by the same letter are not significantly different at P = 0.05 after ANOVA-Tukey test. There were five replicates per treatment.

Figure 3. Radial mycelial growth rates for Cladobotryum semicirculare *AAS0114 (A) and a few selected* Ganoderma boninense *isolates (B) under aerobic and anaerobic conditions.*

AAS0114 on MEA and incubated under aerobic condition up to nine days to evaluate the antagonistic activity. Conidia production were checked and recorded up to two weeks. Almost all the tested *Ganoderma* isolates had more than 50% of PIRG value, with *G. lucidum* being the highest (74.75%) and followed by *G. boninense* G37 isolate (74.72%) (no significant difference from *G. lucidum*) (*Table 2*). Among the six other non-*Ganoderma* fungal isolates tested, *Pleurotus* sp. had the highest percent of mycelial inhibition (66.94%), followed by *Cochiobolus* sp. with *Clonostachys* sp. being the lowest (12.72%). Among all the fungal isolates tested under dual-culture assays, *Clonostachys* sp. showed the lowest percent inhibition of radial growth by *C. semicirculare* (12.72%) (*Table 2*). This could be due to metabolites produced by *Clonostachys* sp. In some of our previous studies, *Clonostachys* sp. was observed to produce yellowish diffusible metabolites either cultivated alone on MEA or co-inoculated with other fungal isolates, and *Clonostachys* sp. also illustrated strong antagonistic activity toward other microbes as well (data not shown). Both antagonistic activity and metabolite production could be partially responsible for low percent inhibition of radial growth in *Clonostachys* sp. by *C. semicirculare*.

A total of six *Ganoderma* isolates, five *G. boninense* from oil palm and one *Ganoderma* species from coconut were selected to determine the degree of inhibition for radial mycelial growth by *C. semicirculare* under anaerobic condition. When challenged with *C. semicirculare*, *G. boninense* G14 isolate was suppressed the least (0.14%) compared to other *Ganoderma* isolates, ranging from 35% to 68% (*Table 2*). This could be due to G14 being one of the most pathogenic and fast growing isolates (Kok *et al.*, 2013). Whereas, G8 and G12 isolates were ranked as moderate to least pathogenic toward the oil palm seedlings in a previous study. Therefore, both G8 and G12 had relatively high percent of radial growth

TABLE 2. PERCENT OF RADIAL GROWTH INHIBITION (%) BY *Cladobotryum semicirculare* AAS0114 UNDER BOTH AEROBIC AND ANAEROBIC CONDITIONS, PATHOGENICTY TEST, AND OTHER OBSERVATIONS ON DUAL-CULTURE ASSAYS

Isolate		Aerobic					Anaerobic
		PIRG (%)‡	Conidia†	PLS*	*In vitro* mycoparasitism		PIRG (%)‡
					No.	%c	
G14	*G. boninense*	63.77±2.25 abc	P	P	4a/6b	66.7	0.14±0.13 c
G8	*G. boninense*	54.51±1.69 bcd	P	P	4/6	66.7	68.15±6.02 a
G30	*G. australe*	52.27±5.19 bcd	P	P	6/6	100	-
G31	*G. boninense*	52.19±5.49 bcd	P	P	6/6	100	-
G32	*G. lucidum*	74.75±1.99 a	P	P	2/6	33.3	-
G5	*G. boninense*	49.92±1.89 cd	P	-	-	-	37.63±0.56 b
G10	*G. boninense*	58.00±1.86 abcd	P	-	-	-	-
G12	*G. boninense*	50.36±1.44 bcd	P	-	-	-	47.66±7.59 a
G33	*Ganoderma* sp.	52.12±2.74 bcd	P	-	-	-	35.96±2.53 b
G34	*G. boninense* (monokaryon)	46.58±4.06 d	P	-	-	-	-
G36	*G. boninense*	49.10±2.61 cd	P	-	-	-	35.02±4.04 b
G37	*G. boninense*	74.72±2.61 a	P	-	-	-	-
AA0002	*Schizophyllum commune*	49.44±3.57 cd	-	-	6/6	100	-
AA0005	*Pleurotus* sp.	66.94±3.60 ab	-	-	6/6	100	-
AA0007	*Cochiobolus* sp.	63.15±1.69 abcd	-	-	6/6	100	-
AA0003	*Clonostachys* sp.	12.72±2.25 e	-	-	-	-	-
AA0004	*Fusarium* sp.	55.42±1.91 bcd	-	-	-	-	-
AA0006	*Phoma* sp.	60.22±0.71 abcd	-	-	-	-	-

Note: ‡PIRG (percent of inhibition in radial growth) (%) (± standard error) for all the tested fungi under both aerobic and anaerobic conditions were analysed separately, and means of PIRG (%) within each incubation conditions for different fungal isolates followed by the same letter are not significantly different at $P = 0.05$ after ANOVA-Tukey test. There were five replicates per treatment.
†With (P) or without (A) the presence/production of conidia produced by *Cladobotryum semicirculare* AAS0114 in dual-culture assay on the tested fungi.
*PLS indicates pseudosclerotia-like structure formed by *Ganoderma* isolates when challenged with *C. semicirculare* in pathogenicity test.
[a]Number of plates with mycelia of *Ganoderma* or tested fungi that were viable/re-isolated/recovered.
[b]Number of plates where the mycelia of *Ganoderma* or tested fungi were inoculated with *C. semicirculare* mycelial plugs.
[c]Percent of viability/re-isolation/recovery of the mycelial plugs excised from the cultures of *Ganoderma* and other tested fungal isolates.

suppression by *C. semicirculare* (*Table 2*). Percent of radial mycelial growth inhibition on *Ganoderma* isolate under anaerobic by *C. semicirculare* appeared to be lower compared to aerobic condition, except *G. boninense* isolate G8. This might contribute to lesser efficiency in suppressing *Ganoderma* growth under anaerobic condition. The suppression of *G. boninense* G8 isolate was higher under anaerobic (68.15%) compared to aerobic (54.51%) condition (*Table 2*). G8 isolate might be more susceptible to *C. semicirculare*. However, the mechanism and biology behind this phenomenon are uncertain.

There was no conidia production by *Cladobotryum semicirculare* AAS0114 when inoculated for a month on MEA. However, conidia were observed on mycelial mat of a few *Ganoderma* species in dual-culture assay under aerobic condition after two to three weeks after inoculation (*Table 2*). No conidia were spotted on dual-culture assays with other non-*Ganoderma* isolates (*Table 2*). In previous studies, *C. semicirculare* was only found to grow and sporulate on a few members of Agaricales and Polyporales, as well as *G. tsugae* (Kirschner *et al.*, 2006; Põldmaa, 2011). In addition, a few other mycoparasites or fungicolous fungi were reported to sporulate on their respective hosts, namely *Melanospora, Sphaerodes* and *Persiciospora* on various *Fusarium* species (Goh and Vujanovic, 2010; Harveson and Kimbrough, 2001), and this phenomenon was proposed to be one of the criteria in determining host ranges. Furthermore, growth factors or substances and nutrients from a range of specific hosts were crucial for mycoparasitic or fungicolous fungi to growth or sporulate (Jeffries and Young, 1994).

In the poison food experiment, media amended with filtrates from *C. semicirculare* were inoculated with two *Ganoderma* isolates, and results were summarised in *Table 3*. Filtrates from *C. semicirculare*

TABLE 3. PERCENTAGE INHIBITION OF MYCELIAL GROWTH (PIMG%) OF *Ganoderma* ISOLATES IN POISON AGAR TEST WITH MEDIUM AMENDED WITH FILTRATES FROM *Cladobotryum semicirculare* AAS0114

Isolates		PIMG (%)‡	
		50%	100%
G14	*Ganoderma boninense*	35.04±3.41 [a]	55.02±2.42 [a]
G32	*G. lucidum*	24.98±3.16 [a]	49.08±5.24 [a]

Note: ‡PIMG (percentage inhibition of mycelial growth) (%) for the selected *Ganoderma* isolates inoculated on MEA supplemented with *C. semicirculare* filtrates at the concentrations of 50% and 100% were analysed separately, and means of PIMG (%) within each column for different *Ganoderma* isolates followed by the same letter are not significantly different at $P = 0.05$ after t-test. There were five replicates per treatment.

were observed to suppress the growth of two *Ganoderma* species (*Table 3*). Percent inhibition of mycelial growth (PIMG%) in *G. boninense* G14 isolate was higher when inoculated in MEA with both 50% or 100% of *C. semicirculare* filtrates compared to *G. lucidum* (*Table 3*). Fungal radial mycelial growth rates were significantly lower in the media amended with *C. semicirculare* filtrates compared to the control (MEA) for all the three isolates tested ($P<0.05$, t-test) (data not shown). Tezuka *et al.* (1994) reported that metabolites extracted from *Cladobotryum varium* were able to suppress growth of *G. lucidum.* Furthermore, various anti-fungal metabolites were reported in a few *Cladobotryum* species that showed antagonistic effects to other fungi (Holdenrieder, 1984; Kellock and Dix, 1984; Wagner *et al.*, 1995).

In vitro Mycoparasitism Test

Cladobotryum semicirculare was observed to reduce viability or re-isolation of a few *Ganoderma* colonies, namely *G. boninense* G14 and G8 isolates, and *G. lucidum* G32 (*Table 2*). *In vitro* mycoparasitism of *G. lucidum* by *Xylogone ganodermophthora* was initiated by Kang *et al.* (2010) and they found that *X. ganodermophthora* was able to suppress the regeneration of *Ganoderma.* In addition, melanised structures produced by *Ganoderma* (*Figure 2J*) were noted in the *in vitro* mycoparasitism test two-week after being inoculated with *C. semicirculare* (*Table 2*). Formation of melanised structures might serve as survival or resistant organs for *Ganoderma* to counter the antibiosis effects and damage by *C. semicirculare.* Melanised structures and melanised pseudo-sclerotia were proposed in previous study to carry many different functions, namely protecting the *Ganoderma* or other fungal pathogens from plant defence mechanisms, dehydration and other microbial agents or antagonists (Henson *et al.*, 1999; Rees *et al.*, 2009). In addition, chlamydospore or resting/resistance structure formation in other fungi were also reported to be induced by various antagonistic fungi or bacteria (Camyon and Gerhardson, 1997; Goh *et al.*, 2009).

CONCLUSION

The fungal isolate that was found to thrive on the fruiting bodies of *Ganoderma boninense* was identified as *Cladobotryum semicirculare* in this study based on morphological descriptions and phylogenetic analysis. To the best of our knowledge, this is the first report of *C. semicirculare* recorded on *G. boninense.* Furthermore, *C. semicirculare* was found to produce conidia on a few *Ganoderma* species, namely *G. boninense, G. lucidum* and *G. australe. Cladobotryum semicirculare* was able to inhibit the growth of most of the *Ganoderma* isolates (with PIRG ranging from 46% to 75%). Filtrates from *C. semicirculare* were also found to suppress the growth of *Ganoderma* isolates in poison agar experiment, 25% to 35% and 49% to 55% mycelial growth inhibition with 50% and 100% concentration of the filtrates, respectively. PIRG for most of the tested *Ganoderma* isolates was higher under aerobic compared to anaerobic condition, except for *G. boninense* G8 isolate. In addition, *C. semicirculare* reduced the regeneration or recovery of *Ganoderma* mycelia in *in vitro* mycoparasitism test. These features elucidate the biological control potential of this fungus. However, more studies are needed to evaluate the capability of *C. semicirculare* in reducing *G. boninense* infection in oil palm in the fields.

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