ISOLATION OF ACTINOMYCETES FROM RHIZOSPHERE OF OIL PALM (*Elaeis guineensis* Jacq.) FOR ANTAGONISM AGAINST *Ganoderma boninense*

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ABSTRACT

*This study reports the isolation and screening of actinomycetes from the rhizosphere of oil palm (*Elaeis guineensis *Jacq.) for antagonism against* Ganoderma boninense*, the causal agent of basal stem rot disease of oil palm. A total of 600 isolates of spore producing and fast growing actinomycetes with different morphology were tested for their inhibitory effects on* G. boninense *mycelial growth on dual culture plates. About 13.5% of the isolates showed Percentage Inhibition of Radial Growth (PIRG) of more than 80% with 21 isolates exhibited observable abnormal growth of* G. boninense*. These 21 isolates were further tested using their culture filtrates in the form of liquid or powder (freeze-dried) for their effect towards* G. boninense *growth. Four isolates: AGA 043, AGA 048, AGA 347 and AGA 506 were highlighted for their ability to inhibit and exhibit potential metabolites against* G. boninense*. This study identified actinomycete strains with the ability to affect and inhibit the growth of* G. boninense*.*

Keyword: *Ganoderma boninense*, actinomycetes, antagonistic, biological control, *Streptomyces.*

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INTRODUCTION

Ganoderma boninense is a basidiomycete fungus that attacks old and young palms. *Ganoderma* is a cosmopolitan basidiomycete that causes white rot of hardwoods by decomposition of lignin, cellulose and related polysaccharides (Seo and Kirk, 2000). *Ganoderma* is able to grow on residual stumps and root masses, which become potential sources and parasitic colonisation of suitable hosts such as oil palm (Ariffin *et al.,* 2000). Generally, *Ganoderma* infection occurs through direct contact between healthy roots and diseased tissues remaining in the soil (Paterson, 2007).

Basal stem rot (BSR) disease is the most destructive disease of oil palm in South-east Asia where tremendous losses have been recorded. Various strategies have been recommended and subsequently applied to control the disease as well as to sustain the economic life-span of the palm. These strategies include several control measures relying on cultural practices, chemical application and biological control. The mechanical removal of diseased palm through the sanitation technique as recommended by Idris *et al.* (2005) is an effective

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method in minimising the inoculum of *Ganoderma* in plantation. This disease control technique is applicable on existing palms as well as during replanting. The technique involves mechanical removal of aerial plant parts followed by excavation of diseased roots and stumps. All these plant materials are then chipped to small fragments to reduce the inoculum potential. Through this technique, BSR incidence and risk in young replanted palms will be reduced (Idris *et al.*, 2005). The use of fungicides with appropriate application technique could possibly provide a short-term solution to BSR problem (Ariffin *et al.*, 2000). *In vitro* studies have been carried out to screen for fungicides against *G. boninense.* Numerous fungicides such as tradimefon, triadimenol, carboxin, benomyl (Jolland, 1983), hexaconazole and cyproconazole (Khairudin, 1990) were reported to strongly inhibit *G. boninense*. A more recent study by Idris (2012) showed that the application of hexaconazole (4.5 g of active ingredient) per palm with a hand knock injector had limited the spread of *Ganoderma* infection within the infected standing palms and prolonged the infected palms' life. However, treatment using fungicides was not curative as it could only delay the disease. Fungicides have also been known to cause environmental pollution. Thus, alternatives to chemical fungicides should be considered.

Biological control methods using antagonist microbes have gained much attention in research. Studies have shown that many species of beneficial microorganisms including actinomycetes, bacteria and fungi have the ability to effectively suppress plant diseases. Azizah (2003) reported that oil palm seedlings pre-inoculated with mycorrhiza were protected from *Ganoderma* infection. Biocontrol using *Trichoderma* spp. showed a high efficacy in controlling the growth and infection of *G. boninense* in glass house trials and under field conditions (Sariah *et al.*, 2005; Susanto *et al.*, 2005; Sundram *et al.*, 2008). Apart from the *Trichoderma* sp., the use of other microbes such as *Pseudomonas aeruginosa* and *Burkholderia cepacia* (Sapak *et al.*, 2008), *Gliocladium viridae* and *Bacillus* sp. (Susanto *et al.*, 2005) were also investigated. According to Tan *et al.* (2002), a *Streptomyces* genus in actinomycetes group showed a high inhibition percentage towards *G. boninense in vitro* through antibiosis and lysis of hyphae resulting in abnormal hyphal growth.

Actinomycete is a group of microorganisms with a huge potential as biocontrol agents due to their ability to produce promising secondary metabolites with biological activities such as antibiotics, anti-fungal, anti-viral, plant growth factors, enzymes and enzyme inhibitors (Berdy, 2005). Apart from that, filamentous gram positive bacteria are soil (Kim *et al.*, 1998) and rhizosphere (Sardi *et al.*, 1992) inhabitants. They are widely distributed in natural ecosystems (Srivinasan *et al.*, 1991). As they represent a large part of the rhizosphere microbial flora (Sardi *et al.*, 1992), it has also been demonstrated that they are important qualitatively or quantitatively in the rhizosphere, where they may influence plant growth and protect plant roots against invasion of root pathogenic fungi (Crawford *et al.*, 1993; Cao *et al.*, 2004). The use of actinomycetes as biological control agents of soil-borne root diseases is of interest. Various studies have shown the potential of actinomycetes as a root coloniser and fungal antagonist (Kunoh *et al.*, 2000; Cao *et al.*, 2004). It could be another potential biocontrol agent of *G. boninense.* The ability of actinomycetes from the genus *Streptomyces* and *Micromonospora* sp. isolated from mangrove area for activity against *G. boninense* was shown by Tan *et al.* (2002). In general, studies on actinomycetes isolated from oil palm plantation are very limited and not much is known. Limited information is available on the possible use of actinomycetes as a biological control agent for *Ganoderma*. Therefore, this study was undertaken to isolate, screen and identify actinomycetes from oil palm rhizosphere for activity against *G. boninense*. The aim was to screen for spore producing and fast growing actinomycetes from the rhizosphere of healthy oil palms surrounded by infected palms. The selection of potential actinomycetes in this study was based on the ability to inhibit the growth of *G. boninense.*

MATERIALS AND METHODS

Samples Collection

Soil samples were collected from the rhizosphere area of four oil palm plantations in Peninsular Malaysia. Oil palms in these plantations were more than 20 years old with severe history of *G. boninense* infection. Samples were collected based on the method described by Shahrokhi *et al.* (2005). Soil samples from 15 healthy palms were taken 1 m from oil palm bole at a depth of 20 cm below the soil surface. The top 10 cm region of the sample was excluded. The samples were air-dried at room temperature for seven to 10 days. The dried samples were crushed using mortar and pestle and sieved through a 0.2 cm wire mesh to remove root particles; the soil samples were preserved in polyethylene bags at room temperature before use. The soil pH for each sample was determined by referring to Kim *et al.* (1998). One gramme of air-dried soil was suspended in 5 ml of sterile distilled water and agitated with a vortex mixer. The pH of the samples was measured by using a pH meter with three replicates per sample.

Isolation of Actinomycetes

Isolation of actinomycetes was performed by serial dilution-spread plate technique (Crawford *et al.*, 1993). A weighed amount of 1 g air-dried soil was mixed with 9 ml of sterile distilled water. The mixture was shaken vigorously for about 5 min and allowed to settle down. A portion (1 ml) of the soil suspensions (diluted as 10^{-1}) was transferred into 9 ml of sterile distilled water and subsequently diluted to give a dilution of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Dilutions (10^{-3}, 10^{-4} and 10^{-5}) were plated onto isolation agar media consisting of yeast extract-malt extract agar (ISP2), inorganic salt-starch agar (ISP4), humic acid-vitamin-agar (HVA) (Seong *et al.*, 2001) and yeast-casamino acid-dextrose agar (YCED) (Crawford *et al.*, 1993) supplemented with 50 ppm of nystatin, 50 ppm of cycloheximide and 20 ppm of nalidixic acid to minimise fungal and bacteria growth (Sivakumar, 2008). Plates were inverted to reduce vapouration and incubated for one week in an incubator chamber set at 28°C. Colonies of actinomycetes were isolated onto fresh ISP2 plates. Repeated streaking onto fresh ISP2 plates produced pure microbial strains. All of the pure isolates were stored at 4°C until further use. Spore and mycelia suspension was maintained at -80°C in 20% (v/v) glycerol for long-term preservation.

Classification of Isolates Based on Colour Grouping

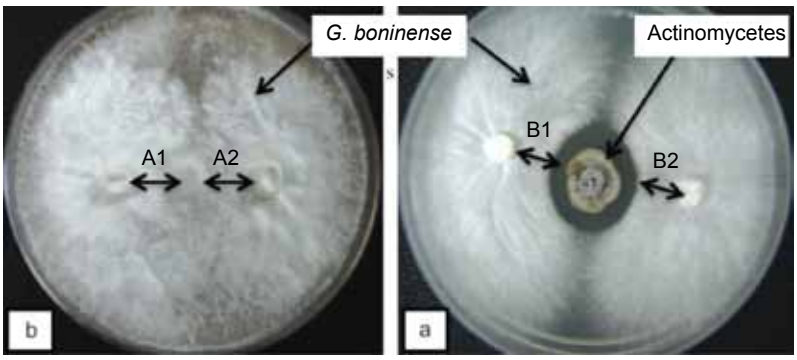
Actinomycetes suspected colonies were isolated onto ISP2 agar and incubated for seven days to obtain pure culture. Isolates were preliminarily classified through naked eyes and with the aid of stereo microscope. Colouration of aerial mycelium on the surface, substrate mycelium (underside of plate) and diffusible pigment were observed. Colour determination was done by referring to Ndonde and Semu (2000) by using colour names as compiled by Pridham (1965).

Screening of Actinomycetes for Antagonistic Activity against *G. boninense*

The isolates were screened for antagonistic activity against *G. boninense* isolate PER 71 following the method described by Getha and Vikineswary (2002). Plates were inoculated with actinomycetes five days prior to *G. boninense* inoculation to allow for the actinomycetes to establish. A mycelia disc of actinomycetes was placed at the centre of PDA plate surface by using 5 mm sterile straws and incubated at 28°C. When these isolates were visibly growing, 5 mm diameter of *G. boninense* mycelia plugs were transferred equidistantly 2 cm away on each side of the actinomycetes plug. A PDA plate inoculated with *G. boninense* isolate PER 71 was included as a negative control. Two actinomycetes known to produce anti-fungal metabolites, *Streptomyces noursei* (ATCC 11455) and *Streptomyces griseus* (ATCC 15395), were used as positive control. After two and four days of incubation at 28±2°C, the plates were examined for inhibition of growth. The antagonistic activity of the actinomycetes against *G. boninense* was determined by observing the distance of inhibition between the actinomycetes and the *G. boninense* colony margin. The width of inhibition zone between the actinomycetes and *G. boninense* was measured by using a digital caliper and applied to the Percent Inhibition of Radial Growth (PIRG) formula as follows;

$$\text{PIRG}\ (\%) = \frac{(\text{A} - \text{B})}{(\text{A})} \times 100\%$$

In which, *A* is the average width of *Ganoderma* without a biological control agent (A_1 and A_2); *B* is the average width of *Ganoderma* with a biological control agent (B_1 and B_2) (*Figure 1*).



Note: A1 and A2 is the length of *G. boninense* mycelia without biocontrol; B1 and B2 is the length of *G. boninense* mycelia with biocontrol.

Figure 1. Measurement of radial growth of G. boninense *in dual culture plate. a) Control plate and b) dual culture plate.*

Screening of the Culture Filtrate for Activity against *G. boninense*

Preparation of culture filtrates. Isolates with PIRG value higher than 90% in the preliminary antagonistic screening were selected and inoculated into 20 ml ISP2 medium (0.4% glucose, 0.4% yeast extract, and 1% malt extract, pH 7.2) in 150 ml Erlenmeyer flask for seed inoculum preparation. After incubation at 28±2°C for two days in a rotary shaker at 150 rpm, 10 ml of seed inoculum was transferred into 100 ml (in 500 ml Erlenmeyer flask) of molasses-starch broth containing 1% cane molasses, 1.5% soluble starch, 1% cottonseed flour, 1% glucose, 1 ml of trace element solution. The trace element solution contained 0.1 g ferrous sulphate heptahydrate ($FeSO_4.7H_2O$), manganese chloride tetrahydrate ($MnCl_2.\ 4H_2O$), zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) in 100 ml of distilled deionized water. The broth was prepared in distilled water and adjusted to pH 7. The flasks were incubated with continuous shaking at 150 rpm in 28±2°C for seven days. The culture was then centrifuged at 10 000 rpm for 15 min and the supernatant was filtered through a 0.2 μm membrane filter (Minisart, Sortorius) in a sterile condition and kept at 4°C until further use (Getha and Vikineswary, 2002; Prapagdee *et al.,* 2008).

The evaluation of the culture filtrate for activity against *G. boninense* was done by using liquid and powder form of the culture filtrate. The powder form was prepared by freeze drying 50 ml of the filtrate using a freeze dryer until a powder was formed. A 1 mg sample of the powdered filtrate was dissolved in 1 ml of sterile distilled water prior to assay. To evaluate the bioactivity of the culture filtrate, 40 μl of liquid or powdered filtrate in water was loaded onto a 6 mm sterile paper disc (Whatman, Whatman Ltd). The loaded paper disc was air-dried under laminar flow hood for 20 min before placing on the assay plates. Sterile disc without filtrate was used as negative control. The culture filtrates of *S. nourseii* (the standard strain of nystatin producer) together with the standard polyene anti-fungal, nystatin was used as positive control. The polyene anti-fungal, nystatin was obtained from Sigma. While, standard strain of *Streptomyces nourseii* (polyene producer) was purchased from ATCC (United States).

Bioassay of Culture Filtrates against *Ganoderma boninense*

a. ***Modified disc assay.*** *G. boninense* was cultured at both halves of the potato dextrose agar (PDA) plate and incubated in an incubator (Memmert, Germany) at 28±2°C for three days. Then, the disc loaded with culture filtrate sample (as described above) was placed 1.5 cm away from *G. boninense* plug. The bioactivity of the culture filtrate against *G. boninense* was obtained by referring to the method for PIRG calculation at two and four days after incubation at 28±2°C.

b. ***Modified plate assay.*** *Ganoderma* supplemented agar was used in the modified plate assay. The agar was prepared by referring to Lee *et al.* (2011) with modification. Fine mycelia of *G. boninense* were obtained through static culture by inoculating five plugs of mycelial discs into 50 ml potato dextrose broth (PDB) and incubated at 28±2°C. Newly generated mycelia surrounding the original plug were washed with sterile distilled water and crushed by using sterile Potter-elvehjem tissue grinder (Cole Parmer, USA). The crushed mycelia were filtered through a sterile cotton cloth sieve (25-30 μm). Two ml of filtered *G. boninense* mycelia was inoculated into 18 ml of sterile PDA agar at 50°C and mixed well by a gentle shake. The mixture was poured into sterile plates and left to solidify. All of the plates were incubated at 28±2°C for three days before placing the disc loaded with culture filtrate samples. Plates were re-incubated at 28°C. The inhibition ability was evaluated by measuring the diameter of inhibition zones (DIZ mm) (Baniasadi *et al.,* 2009) as; +++ = >20 mm , ++ = 11-19 mm, + = 2-10 mm, ± = ≤ 1 mm, - = 0 mm (Kunoh *et al.,* 2000; Taechowisan *et al.,* 2003).

RESULTS AND DISCUSSION

Actinomycete Isolates from the Rhizosphere of *Elaeis guineensis*

Various types of bacteria, actinomycetes and fungal colonies were visible on isolation plates three to seven days after incubation at 28°C. Differentiation into bacteria, actinomycetes and fungi was done based on their morphological characteristics on agar surface. Bacteria were observed as slime colonies compared to actinomycetes and fungi which were furry to woolly in appearance. Fungi were differentiated from actinomycetes based on the colony size. Through observation, fungi mycelia and colony thickness were relatively larger compared to actinomycetes. In this study, 15 rhizosphere soils of healthy palms standing in BSR disease foci from coastal, inland, laterite and peat areas were used to isolate actinomycetes. The frequency of actinomycetes recovered from these mature palms was higher in peat (47.1%) and laterite (31.6%) compared to inland (15.7%) and coastal (6.6%) (*Table 1*). The high occurrence of actinomycetes in this study may be related to different chemical composition of the different soils (Tian *et al.,* 2004) and may well account for the presence of different actinomycetes groups in different soils studied (Alimuddin *et al.,* 2011). Actinomycetes have been demonstrated to grow extensively in soils rich in organic matter and moisture content (Jayasinghe and

TABLE 1. NUMBER OF ACTINOMYCETES ISOLATED FROM DIFFERENT SOIL TYPES AND AREA

Area of collection	Colony forming unit g^{-1} (CFU) of soil	Percentage of occurrence
Peat	1.54×10^5	47.06
Laterite	1.0×10^5	30.61
Inland	2.17×10^4	15.73
Coastal	5.15×10^4	6.61

Parkinson, 2007). Peat soil is rich in organic matter and moisture content, compared to other soil types used in this study; this explains the high occurrence of actinomycetes recovered from peat soil.

Among the four media used, ISP2 and YCED were the most preferred media in the isolation of actinomycetes. Both media contain low levels of organic nutrients compared to other media used for the isolation of actinomycetes in this study. The data proved the fact that actinomycetes were capable of growing on simple media without requiring any special growth factors (Crawford *et al.,* 1993). The other isolation media used in this study supported the growth of actinomycetes, but with fewer colonies and had the plates overgrown with bacteria compared to ISP2 or YCED. The use of these media supplemented with anti-fungal and anti-bacterial agents retarded the growth of bacteria and fungi and sufficiently allowed actinomycetes to grow and sporulate.

A total of 1050 isolates were recovered from the rhizosphere of oil palm and then classified into streptomycete-like strains (abundant aerial mycelium with powdery spores) and non-streptomycete-like strains (small and compact, or slimy colonies, orange, red-brown or brown to black) (Getha and Vikineswary, 2002). Most of the actinomycetes isolated were streptomycete-like, where they showed typical morphology of *Streptomyces* and possessed an earthy odour. The predominance of *Streptomycetes* in any actinomycete population is well-known (Crawford *et al.,* 1993; Getha and Vikineswary, 2002; Khamna *et al*., 2009) as they are present in all rhizosphere soils suggesting their wide distribution in association with plants in the natural environment (Khamna *et al.,* 2009). In addition, there were no special pre-treatment techniques used in the isolation method to enhance the isolation of non-streptomycete in this study. The streptomycete-like isolates were categorised into seven colour series according to the colour of their mature sporulated aerial mycelium. Some of the main colours observed were grey, white, brown, green, blue and red. The colour of aerial spore mass and substrate mycelium varied which reflected the diversity or variability of *Streptomyces* isolates. In this study, the grey streptomycete-like isolates showed highest in occurrence (48.7%) compared to other colours (*Table 2*). Similar results were also obtained by other researchers (Alimuddin *et al*., 2011; Ndonde and Semu, 2000).

Antagonistic Activity of Actinomycetes against *G. boninense*

A total of 600 isolates of actinomycetes consisting of streptomycete-like and non-streptomycete-like isolates with different morphology were selected and screened for bioactivity against *G. boninense.* The levels of inhibition were calculated by using the equation as previously mentioned and isolates with PIRG value of more than 80% were considered as potential isolates against *G. boninense*. Growth of *G. boninense* on assay plates was compared to growth on control plates. Based on observation, the *in vitro* interactions between the tested actinomycetes and the pathogen resulted in either the production of a clear distance between them, contact inhibition or no inhibition at all (*Figure 2*). Eighty-one isolates (13.5%) showed a positive inhibition with PIRG value of more than 80%. A total of 148 isolates (24.7%) showed inhibition with PIRG value between 50% to 80%, and 371 isolates with no activity against *G. boninense*. Amongst 81 isolates with the promising inhibition activity, 21 isolates showed highly antagonistic effect towards *G. boninense.* The presence of these 21 isolates caused a complete or highly inhibited growth of the fungal colony (*Figure*

TABLE 2. NUMBER OF ACTINOMYCETES ISOLATED FROM RHIZOSPHERE OF OIL PALM (*Elaeis guineensis* Jacq.) ACCORDING TO AERIAL COLOUR

Group	Colour of aerial mycelia							Total
	Grey	White	Brown	Green	Blue	Red	Other colours (violet, pink, orange, black)	
Streptomyces	511	257	104	28	3	13	55	971
Non-streptomyces	0	1	7	0	0	2	69	79
Total								1 050

Figure 2. In vitro *assay plates of actinomycetes isolates against* G. boninense*; a) clear distance observed between tested actinomycetes and* G. boninense*, b) contact inhibition between tested actinomycetes and* G. boninense *and c) no inhibition between tested actinomycetes and* G. boninense.

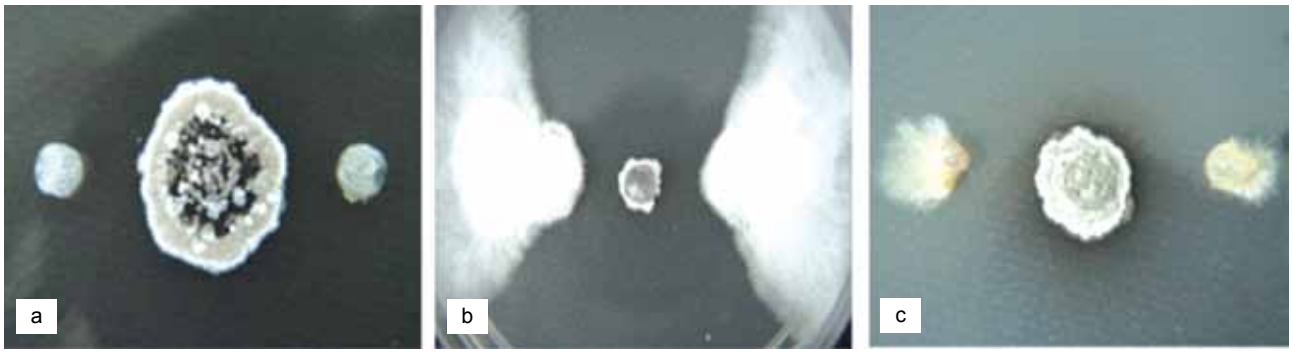


Figure 3. Three growth pattern of G. boninense *observed on* in vitro *plates from 21 potential isolates with highly antagonistic effect towards* G. boninense*. a) No formation of new mycelia from the* G. boninense *plug on both sides; b) new mycelia generated from the added* G. boninense *plug with high inhibition on area facing actinomycetes and, c) very little formation and sparse growth of new* G. boninense *mycelia generated from the plug.*

3). Plates containing only *G. boninense* plug from the same source without any biological control agent showed a normal growth with the appearance of white cottony mycelia growth. Although 61.8% of the actinomycetes isolates did not show any antagonistic activity against *G. boninense* in this study, they may produce other useful compounds.

The pre-inoculation of actinomycetes in antagonism assay was done to allow growth of the isolates prior to inoculation of *G. boninense*. According to Adebola and Amadi (2010), early establishment of the biological control agent will be able to provide sufficient time for it to grow and colonise surrounding substrate which then provides a competitive ability towards the pathogen. Results obtained demonstrated the competitive ability of actinomycete isolated from the rhizosphere of oil palm against *G. boninense*. Viability test of the affected *G. boninense* hyphae in inhibition zones with some of these potential isolates showed that the hyphae could no longer recover when transferred onto fresh medium. This reflects a non-culturability or local death of hyphae caused by anti-fungal substances produced by the biological control agent (Yuan and Crawford, 1995). It indicates that the tested isolates are producing extracellular diffusible bioactive substances affecting the growth of *G. boninense*. Generally, antagonistic effect by actinomycetes might be due to the production of bioactive compound such as antibiotic, enzyme and anti-fungal substances (Crawford *et al.,* 1993; Prapagdee *et al.,* 2008). Potent inhibitory ability can be observed from the inhibitory percentage of *G. boninense* on assay plate.

Bioactivity of the Culture Filtrates for Activity against *G. boninense*

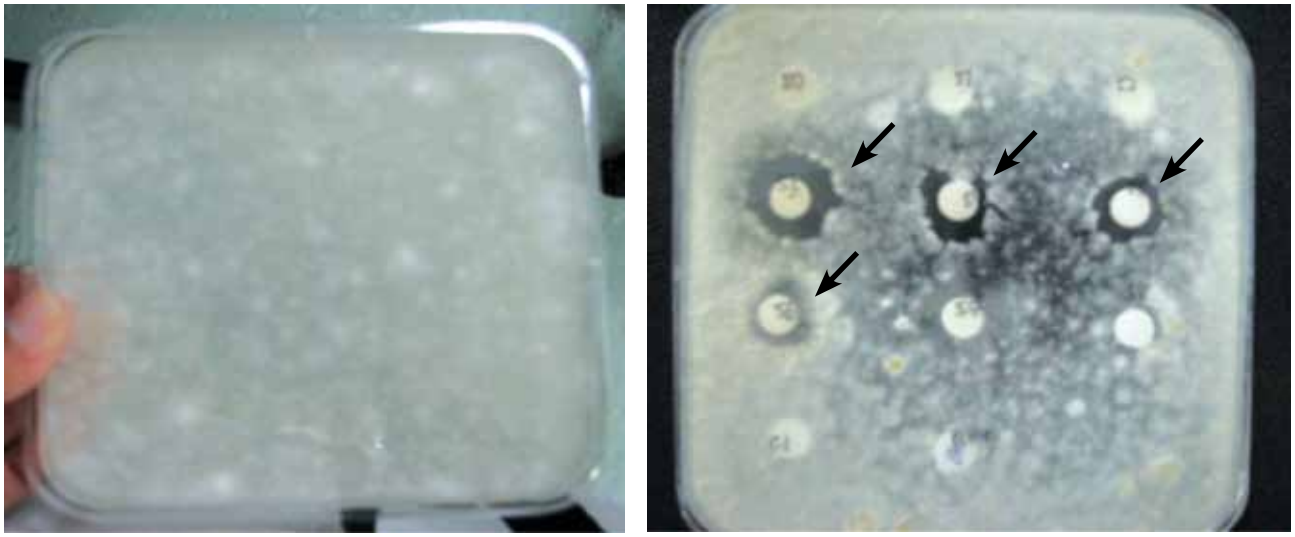
A liquid culture was obtained from submerged cultures of 21 isolates with the highest inhibition against *G. boninense* in dual culture assay. Culture filtrates of the fermentation product may contain anti-microbial compounds and enzyme which may correlate with the biocontrol activity.

Modified disc assay. From the 21 isolates tested, only six samples showed inhibition in assay using both liquid and dried filtrates with PIRG between 50% to 80%. None of the samples showed inhibition with more than 80% PIRG. Most of the plates were overgrown by *G. boninense*. As expected, the bioassay of the filtrate without the cells against *G.*

boninense would give a low PIRG value compared to the whole cells. This may be related to either: (i) lack of active substances produced in the filtrate towards *G. boninense,* or (ii) the available concentration of active compounds in the filtrate was too low to cause inhibition towards *G. boninense*. To prove this assumption, a polyene anti-fungal, nystatin and a standard strain known as the nystatin producer were used as control. Use of nystatin powder at three different concentrations showed inhibition towards *Ganoderma* with different PIRG values. Based on the observation, nystatin at concentration of 10 mg ml^{-1} gave an inhibition with more than 80% PIRG value. At a concentration of 1 mg ml^{-1} and 0.1 mg ml^{-1}, the PIRG values observed were 67.7% and 31.7%, respectively. The powdered form of culture filtrate obtained from *S. nourseii* showed inhibition towards *G. boninense* at 63.3% with no inhibition seen on liquid culture filtrate. By referring to the data obtained, the required amount of standard polyene anti-fungal agent to cause more than 80% inhibition towards *G. boninense* is 10 mg ml^{-1}. Nystatin at 0.1 mg ml^{-1} and 1 mg ml^{-1} showed inhibition with PIRG lower than 80%. Culture filtrate of *S. nourseii*, when applied as liquid was not able to inhibit *G. boninense*. However, when challenged in a concentrated form (powdered), the filtrate showed inhibition with 63.3% PIRG value. The result obtained was similar to the inhibition of *G. boninense* by 1 mg ml^{-1} of nystatin. It indicates that the active compound was present, however it was affected by the concentration, as it may need 1 mg ml^{-1} and above to successfully inhibit *G. boninense in vitro.* Most of the culture filtrates assayed against *G. boninense* showed negative inhibition. Only four isolates showed inhibition towards the tested pathogen with PIRG between 50% to 80%. They were AGA043, AGA048, AGA347 and AGA506. Both, the liquid and powdered form of filtrate from these four isolates showed better activity against *G. boninense* compared to nystatin at 0.1 mg ml^{-1}.

Modified plate assay. The diameter of the inhibition zone (DIZ, mm) was measured based on the halo zone observed on PDA supplemented with *G. boninense* (*Figure 4*). In this study, five liquid culture filtrates from five individual isolates showed inhibition with 12.5 to 23.5 mm range of the halo zone. At the same time, 10 powdered culture filtrates from 10 individual isolates showed inhibition with various sizes of the halo zone. Out of these numbers, only four filtrates showed inhibition towards *G. boninense* in both liquid and powder forms. Nystatin powder at concentration of 10 mg ml^{-1} and 30 mg ml^{-1} showed inhibition with diameter of inhibition zone of 25 mm and 25.5 mm, respectively. At concentration of 1 mg ml^{-1} and 0.1 mg ml^{-1} of nystatin, the sizes were 3.25 mm and 0.2 mm, respectively. The powdered form of culture filtrate obtained from *S. nourseii* showed inhibition towards *G. boninense* with 15.63 mm ± 3.3 mm diameter of inhibition zone; no inhibition zone was seen on liquid culture filtrates. The result was expected since nystatin contained a concentrated amount of anti-fungal agent. These results indicated that, each filtrate may contain less than 10 mg ml^{-1} of active compounds against *G. boninense*.

Based on the antagonist dual culture evaluation, most of the antagonistic activities of different isolates of actinomycete against *G. boninense* depend mainly on their metabolites. According to Moussa *et al.* (2011), the mechanism of action for most of actinomycetes is mainly focused on the production of bioactive



Note: *Arrow indicates the halo zone measured and recorded in this study.

Figure 4. Anti-fungal effect of potential actinomycetes culture filtrate against G. boninense*, a) control plate of* G. boninense *supplemented agar at Day 7 after incubation; b)* G. boninense *supplemented agar with paper discs of tested culture filtrates at Day 7 after incubation.*

compounds, such as antibiotics, cell wall degrading enzymes and competition for nutrients. However, in this study, the culture filtrates of potential isolates showed different activity towards *G. boninense* compared to the antagonism test. From 21 culture filtrates tested against *G. boninense* through modified disc assay and *Ganoderma* plate assay, most of the isolates did not show activity in liquid medium. It was observed that 15 of the isolates that exhibited strong inhibitory activity against *G. boninense* in dual culture failed to cause inhibition in the culture filtrate assay. Out of the 21 isolates which showed strong antagonism, only four isolates (19.95%) exhibited significant activity in culture filtrate (*Table 3*). Similar results were observed by other researchers (Ndonde and Semu, 2000; Thakur *et al*., 2007) where some of the active isolates did not show any the activity in the liquid medium. Variation of results observed in antagonism and filtrate assays might be affected by the medium composition used. Different medium composition used in submerged culture may cause the production of different bioactive compound. Research by Yuan and Crawford (1995) showed that different types of carbon sources in the growth medium gave important impact on production of different bioactive compound by Streptomycetes. Furthermore, in this study, no solvent was used to extract the active compounds produced in the submerged culture. Alimuddin *et al.* (2000) demonstrated that the use of solvent in extraction possessed a higher anti-fungal potential as the extracellular anti-fungal compound was related to the type of solvent used. It is also possible that the concentration and potency of active compounds in the supernatant were very small and not enough to cause inhibition towards *G. boninense*. For example, about 23.8% to 28.7% of the filtrates tested in this study showed activity against *G. boninense* when

TABLE 3. BIOACTIVITY OF 21 CULTURE FILTRATES OF ACTINOMYCETES ISOLATED FROM RHIZOSPHERE OF OIL PALM (*Elaeis guineensis* Jacq.) TOWARDS *G. boninense in vitro*

Isolates number	Modified disc assay (PIRG value,%)		Microbial plate assay (Halo zone size, mm)	
	Liquid culture filtrates	Powdered culture filtrates	Liquid culture filtrates	Powdered culture filtrates
AGA01	OG	OG	NI	11 ± 0
AGA039	56.52	47.48	NI	11 ± 1.4
AGA043*	52.94	51.30	16 ± 1.41	35 ± 1.4
AGA048*	56.64	65.12	18.5 ± 4.95	15.5 ± 0.7
AGA66	OG	OG	23.5 ± 0.71	NI
AGA67	OG	OG	NI	6 ± 8.5
AGA98	OG	**51.16**	NI	NI
AGA124	OG	OG	NI	22.15 ± 1.6
AGA156	OG	OG	NI	NI
AGA199	OG	OG	NI	NI
AGA328	OG	OG	NI	NI
AGA346	OG	OG	NI	NI
AGA347*	56.82	62.50	12.5 ± 0.71	34 ± 1.4
AGA353	OG	OG	NI	NI
AGA392	OG	60.00	NI	22.25 ± 1.1
AGA425	OG	48.72	NI	18.75 ± 0.4
AGA506*	58.47	67.44	20.5 ± 2.12	16.75 ± 2.2
AGA523	OG	OG	NI	NI
AGA547	OG	OG	NI	NI
AGA430	51.79	OG	NI	NI
EX-095	OG	OG	NI	NI
S. nourseii	OG	63.33	NI	15.65 ± 3.3

Note: PIRG - Percent Inhibition of Radial Growth. OG- no inhibition and overgrown by *G. boninense*; NI - no Inhibition, 0 mm of halo zone observed.
*Isolates with number AGA043, AGA048, AGA347 and AGA506 were selected for identification.

40 μl of the supernatant was used. However, when used at a higher concentration (1 mg ml^{-1} w/w of freeze-dried filtrates), about 42.9% to 52.8% of the filtrates showed inhibition towards *G. boninense*.

CONCLUSION

The potential of actinomycetes in controlling the growth of *G. boninense* was demonstrated in this study. The dual culture assay was carried out to determine the ability of the isolates to inhibit the growth of *G. boninense*. The screening by using culture filtrate was designed to narrow down the number of potential isolates for further investigation. Bioactivity of each isolate was screened by using the whole-cell and released compounds against *G. boninense in vitro.* In this study, the ability of actinomycetes to affect the growth of *G. boninense* was proven. Different growth pattern of *G. boninense* was observed on assay plates. Growth pattern from none to very little formation of new mycelia generated from the added plug was observed on the antagonistic assay plate. Four isolates were found to give inhibitory activity towards *G. boninense in vitro*. Potential *Streptomyces* isolates will be identified using the molecular and classical approaches. These potential isolates will be further developed into a biological formulation, and evaluated for activity against *G. boninense in vivo*. More detailed investigation on characterisation of active compounds, siderophore and indole acetic acid (IAA) production are required.

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