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Control of *Ganoderma boninense*: A Causal Agent of Basal Stem Rot Disease in Oil Palm with Endophyte Bacteria *In Vitro*

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ABSTRACT

The objective of this study was to screen the potential endophytic bacteria to be used as a biological control for *Ganoderma boninense*, the major causal pathogen of Basal Stem Rot (BSR) disease in oil palm. Twenty endophytic bacteria were isolated from symptomless oil palm roots but only seven isolates showed inhibitory effect by suppressing the mycelial growth of *G. boninense.* The isolated bacteria were screened *in vitro* by dual culture assay and culture filtrate test for their antagonistic properties towards *G. boninense.* Four bacterial endophytes (EB2, EB4, EB5 & EB6) were established to have potential as biocontrol agents based on their percentage inhibition of radial growth (PIRG) more than 50%. There was significantly difference in inhibitory effect ($p<0.05$) for dual culture test and these endophytic bacteria inhibited the fungal growth by an average of 52.78, 83.33, 67.59 and 93.52%, respectively on NA medium. Culture filtrate test likewise was showed a significant different for percentage of mycelia growth ($p<0.05$). Endophytic bacteria EB2, EB4, EB5 and EB6 suppressed the fungal growth by an average of 56.65, 91.45, 69.48 and 97.95%, respectively. Interestingly, out of four potential endophytic bacterial isolates, two, isolates namely EB4 and EB6, produced significantly ($p<0.05$) higher inhibitory effect than two more isolates of EB2 and EB5. Changes of hyphae occurred in presence of both bacteria of EB4 and EB6. Deformity and shrinkage of hyphae in EB6 was more apparent than in EB4 through mycelial growth test. Identification of species for these two bacteria was done using Biolog® System where EB6 was successfully identified as *Pseudomonas aeruginosa* but EB4 could not be unidentified. Therefore, *in vitro* activities of EB4 and *P. aeruginosa* against *G. boninense* in these studies suggested that these endophytic bacteria can be used as an effective biological control agent.

Key Words: Basal stem rot disease; Dual culture assay test; Culture filtrate test; Mycelia test; *Pseudomonas aeruginosa*

INTRODUCTION

Elaeis guineensis Jacq., which is commonly known as the oil palm is the most crucial species in the genus *Elaeis* which belongs to the family Palmae. Oil palm is truly "a golden crop of Malaysia" since it generates profitable export earnings for the country and truly nature's gifts for alleviating poverty in Malaysia (Basiron, 2007). Malaysia is currently the world's largest producer and exporter of oil palm. Areas of oil palm have increased from 54,000 hectares in 1960 to 4.05 million hectares in 2005, reflecting a compound annual growth of 10.06%. Production increased from 94,000 tones in 1960 to 15 million tones in 2005, or almost 160 times within 45 years. This represents a compound annual growth of 11.93% per year (Basiron, 2007). In Malaysia, the oil palm is blessed by being largely disease free, but suffering from one major disease, Basal Stem Rot (BSR) caused by *Ganoderma* species. BSR was first recorded in the country in 1928 (Sharples, 1928). With no known remedy at present, it is the major disease of oil palm and, therefore, of great economic importance to the Malaysian oil palm industry. BSR infects palms as young as 12-24 months after planting and is serious on palms of 4-5 years age, particularly in replanted areas (Singh, 1990).

In new oil palm planted from jungle or rubber, BSR incidence of 25% has been recorded after 25 years while in that planted from coconut, an incidence of 60% occurred after 16 years (Singh, 1991) whereas oil palm to oil palm under planting has resulted in 33% infection after 15 years. BSR infection resulted in crop loss, in severe cases turning a loss up to 45% of the yield (Singh, 1991). Oil palm has an economic life span of 25-30 years. Basal stem rot can kill more than 80% of stands by the time they are half-way through normal economic life (Abdul Razak *et al*., 2004). Field controls of BSR by contact chemicals have not been very successful even *in vitro* efficacy of fungicides have not been reported against the fungus (Soepena *et al*., 2000). This may be due to the fact that both visibly infected and subclinical palms may already have the disease established by the time treatment is applied. Control by physical methods such as clean clearing and tree surgery has had but transient effects, although there is testify that BSR can be

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dealt if all the disease inoculum is removed before planting or replanting the crop. The idea therefore is to avoid infection, instead to cure the infected palms. Thus, management of BSR in new or young oil palm areas should include: (1) using a potting mix with clean soil for growing the seedlings in polybags, (2) eradicating all disease inoculums in the field before planting or replanting and (3) applying chemical or biological control agent (Soepena *et al*., 2000).

Astute observations of the low incidence of disease due to pathogenic *Ganoderma* species in some natural stands, suggest that the disease is most likely kept under control by some biological means. Due to these observations, recent control measures to overcome the *Ganoderma* problem are now focused on the use of biological control agents. For example, saprophytes can be used to compete against *Ganoderma* to reduce its opportunity for colonizing oil palm roots. Several promising antagonists such as *Trichoderma* (Shukla & Uniyal, 1989; Wijesekera *et al*., 1996; Sariah, 2003) and *Penicillium* (Dharmaputra *et al*., 1989) have shown *in vitro* antagonistic activity against *G. boninense*. Some of endophytic microorganisms such as *Serratia* spp., *Burkholderia* spp., *Pseudomonas* spp., *Bacillus* spp. and *Fusarium* spp. have been found to induce systemic resistance in plants and shown biological traits like antibiotic activity and lysis (Kloepper *et al*., 1992: Dorworth & Callan, 1996). They have been isolated from a wide range of hosts including wild and cultivated crops, such as woody plants (Bills, 1996), banana (Pan *et al*., 1997) and grass (Clay, 1998). Endophytic microorganisms are therefore a relatively new field of study in biological disease control. Endophytic bacteria live in the plant tissues without doing substantive harm or gaining benefit other than residency (Kobayashi & Palumbo, 2000; Zaiton *et al*., 2008).

Introducing endophytic bacteria to the roots to control plant disease is to manipulate the indigenous bacterial communities of the roots in a manner, which leads to enhanced suppression of soil-borne pathogens. The use of endophytic bacteria should thus be preferred to other biological control agents as they are internal colonizers, with better ability to compete within the vascular systems, limiting *Ganoderma* for both nutrients and space during its proliferation. This study was therefore, undertaken with following objectives; to isolate endophytic bacteria from symptomless oil palm roots and to screen the potential endophytic bacteria against *G. boninense in vitro* and identify the most potential bacteria using Biolog® System.

MATERIALS AND METHODS

Root sampling: Oil palm roots were obtained from University Putra Malaysia, Bintulu Sarawak Campus. Healthy palms were randomly selected and sampled with the roots taken about 0.2 m from their bases at 20 cm depth. Each palm was sampled at three points around its bases and both primary and secondary roots were collected. All the roots from each palm were bulked and stored in refrigerator at 4ºC before isolation of microbial organisms.

***Ganoderma boninense* culture:** *G. boninense* pure culture plates were taken from Malaysian Palm Oil Board (MPOB) at Bangi, Selangor. The pure culture was initially isolated from infested oil palm plantation situated in Miri and Bintulu Division, Sarawak. The plates of *G. boninense* culture were then sub-cultured to mass multiply a pure culture of the pathogen for this study (Fig. 1).

Isolation of endophyte bacteria: Endophyte bacteria were isolated from the oil palm roots and cultured on nutrient agar (NA) medium. Root samples from each palm 10 cm long were taken and rinsed under tap water for 5 min to remove any adhering soil from their surface. Then 0.5 cm from both ends was discarded and the remaining root divided into three sections of three cm in length. The sections were surface sterilized by dipping in 10% Clorox for two minutes, then in 50, 70, 90 and 100% ethanol for 30 s each (Schena *et al*., 2003). They were then rinsed three times with sterilized distilled water to remove all the chemicals and allowed to dry on a sterilized filter paper. One cm from the ends of each section was discarded. The cuticle from the middle section was removed and it was cut into two sections of 0.5 cm each. Then, the root sections placed in sterilized distilled water of 250 mL conical flasks and allowed to agitate for one day at 150 rpm. Afterwards, one mL of the original sample was taken out and added to 9 mL of sterilized distilled water to give 10^{-1} dilution of original sample. Similarly, serial dilution of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were prepared. Later on, 10 µL of 10^{-6} dilution was placed in each Petri dishes and hockey stick was used to flatten out the suspension on NA media for bacteria. Then, Petri dishes were incubated at 30ºC. Endophyte bacterial colonies were then further isolated by sub culturing single bacterial colony on NA media plates. Pure cultures were maintained in refrigerator at 4ºC for further study.

Bacterial gram staining: One droplet of distilled water was dropped onto a slide and smeared with bacterium. The bacterial smear was air dried and heated. The slide was then stained with crystal violet for one to two minutes. The slide was flooded with iodine for one to two minutes after stained with distilled water to remove the acetone alcohol. The slide was then washed thoroughly with distilled water to remove the acetone alcohol. Safranin was flooded onto the slide for two minutes followed by distilled water to remove safranin colour. The excess water was blotted and dried over Bunsen flame. The slide was then examined under compound microscope to identify the cell morphology and group of the bacteria.

***In vitro* screening of endophyte bacteria against *G. boninense*:** The bacterial isolates were screened for their antagonistic activity against *G. boninense in vitro* by the dual culture and culture filtrate tests, based on the percentage inhibition of radial growth (PIRG) (Jinantana & Sariah, 1997).

Dual culture assay test: To evaluate the antagonistic activity of the endophytic bacteria isolates, a five mm diameter agar disc was taken from the five day-old PDA culture of *G. boninense* and plugged centrally in nutrient agar plate and then colonies of endophytic bacteria was streaked three cm away from *G. boninense* plug. The PIRG was obtained after seven days incubation and the most promising bacteria with more 50% PIRG kept for further study.

All the test antagonistic pairings were incubated at 28±2ºC. The ability of the endophyte bacteria to inhibit the growth of *G. boninense* was assessed after seven days incubation by measuring the radius of the *G. boninense* colony in the direction towards the antagonist colony (R2). The data were later transformed into percentage inhibition of radial growth (PIRG) in relation to the radial growth of *G. boninense* in the control plate (R1) using the formula:

$$\text{PIRG} = \frac{R1 - R2}{R1} \text{ x } 100\%$$

Culture filtrate test: The endophytic bacteria that gave good results in the dual culture test were inoculated in 250 mL nutrient broth and maintained at 28±2ºC in the dark for seven days. The culture was then centrifuged at 10000 rpm for five min, supernatant collected and pellet discarded. The supernatant was filtered through a 0.25 µm membrane filter in sterile conditions. The filtrate was incorporated into sterilized double strength PDA in ratio 2:1, 20 mL of the amended agar was poured into each Petri plate and allowed to solidify. A *G. boninense* mycelial plug was centrally inoculated in each of the plate. Non-amended PDA was used as the control. The diameter of the mycelial growth of *G. boninense* was measured over seven days. The antagonistic activity was expressed as PIRG in relation to the mycelia growth of *G. boninense* in the control plate.

Preparation of bacterial suspension: Twenty five mL of NB was prepared for each 150 mL conical flask. NB in the conical flasks was then autoclaved and cooled. One loop of each bacterium screened from the pure culture and was transferred into each conical flask and shaken for 24 h at 160 rpm.

Mycelia growth test: A mycelial plug was taken from the edge of six day old culture of *G. boninense* and dipped into selected bacterial suspension for 30 min and air dried in the laminar airflow chamber. Treated mycelia plugs were placed on Petri dishes containing PDA medium. Fungal plugs dipped in sterile distilled water served as a control. The PDA plates were then incubated at 28±2°C for seven days. Hyphal strands at the end of the fungal colony were removed and examined under a compound microscope for abnormalities, if any.

Identification of endophyte bacteria: Potential endophytic bacterial isolates were identified by Biolog® identification system which followed the Biolog procedures. The procedures for identification utilized 96 wells of microplate containing 95 different dried carbon sources plus control. Bacterial isolates were initially determined for gram reaction, and oxidase test for categorizing into enteric or non-enteric bacteria. Enteric bacteria are gram negative and negative oxidase. Non-enteric bacteria are gram negative and oxidase positive. Bacteria were then cultured onto biolog universal growth (BUG) medium for 24 h at 26±2°C to promote growth and retention of its metabolic activity. Bacterial suspension was prepared in the inoculants solution (0.1 g Gellan Gum, 4 g NaCl, 0.3 g Pluronic F-68 & 1 L distilled water) with concentration for non-enteric bacteria at 52%, enteric bacteria at 63% and positive gram bacteria at 25-28% transmission using biolog's spectrophotometer. Bacterial suspension was inoculated into GN or GP micro plates depending on gram reaction cluster, 145 µL per well using the 8 channel repeating pipette. Microplate was covered with its lid and incubated at 28 to 30°C for 24 h to allow the utilization of carbon sources. Reading result was directly done after inserting the incubated microplate into the Biologs reader apparatus for identifying the bacteria up to species level.

Statistical analysis: The experiments in dual culture and culture filtrate test were conducted in Completely Randomized Design (CRD) with four replicates. Recorded data were analyzed with SAS® Software. The significant data was determined using Duncan's Multiple Range Test at 5% probability level. The percentage data were transformed into Arcsine transformation before subjected to ANOVA.

RESULTS

Isolation of endophytic bacteria: Twenty endophytic bacteria groups were successfully isolated from the symptomless oil palm roots. The separation of bacterial endophytic was carried out on the basis of morphological characteristics such as colony colour, elevation, the margin of colony and colony surface. The pure culture of each isolates was maintained for screening test against *G. boninense.* Gram staining was carried out for each endophytic bacterial to determine whether it is in Gram-positive or negative group. All the bacteria were gram negative (Table I). From the statistical analysis EB4 and EB6 had the maximum inhibitory effect against *G. boninense* and both bacteria were Gram-negative and rod-shaped.

In vitro* Screening of Microbial Bacteria against *G. boninense

Dual culture assay test: The dual culture test is an established method used to distinguish isolates with antagonistic potential from large populations. All the microbial endophytes tested gave different degrees of inhibition toward the mycelial growth of *G. boninense.* The bacterial isolates had a significantly higher antagonism than the fungal isolates with the exception of *Trichoderma*. However, as *Trichoderma* are aerobic fungi with their endophytic nature yet to be established, they were not further evaluated. Furthermore, in these studies the focus

Table I: The graming and shape of isolated bacteria from symptomless oil palm roots

Bacterial isolate	Gram	Shape
EB1	Negative	Rod
EB2	Negative	Rod
EB3	Negative	Rod
EB4	Negative	Rod
EB5	Negative	Rod
EB6	Negative	Curved rod
EB7	Negative	Rod
EB8	Negative	Rod
EB9	Negative	Rod
EB10	Negative	Rod
EB11	Negative	Rod
EB12	Negative	Rod
EB13	Negative	Rod
EB14	Negative	Rod
EB15	Negative	Rod
EB16	Negative	Rod
EB17	Negative	Rod
EB18	Negative	Rod
EB19	Negative	Rod
EB20	Negative	Rod

Table II: Antagonistic potential of endophytic bacteria in dual culture test against *G. boninense in vitro*

Isolates	Inhibitory effect (PIRG)*
EB1	48.15^{de}
EB2	52.78^{d}
EB3	35.19^{ef}
EB4	83.33^{b}
EB5	67.59^{c}
EB6	93.52^{a}
EB7	21.30^{f}

Table III: Antagonistic potential of endophytic bacteria in culture filtrate test against *G. boninense in vitro*

Isolates	Inhibitory effect (PIRG)*
EB2	56.65^{d}
EB4	91.45^{b}
EB5	69.48^{c}
EB6	96.95^{a}

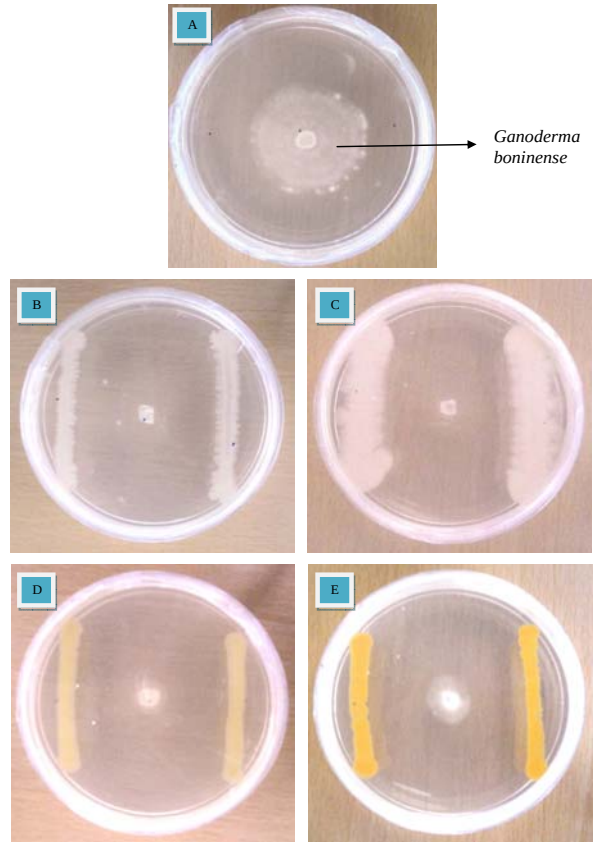
Means in the same column with different alphabet(s) are significantly different ($p < 0.05$) according to DMRT

*Percentage inhibition of radial growth of *G.boninense* after seven days of incubation

was only on endophytic bacteria rather than endophytic fungi. Isolated bacteria with inhibitory characteristics were selected and screened by means of dual culture assay test. Out of 20 endophytic bacteria, only seven showed inhibitory effect by suppressing the mycelia growth of *G. boninense* on NA media subsequently after seven days of incubation at 28±2°C. From the seven potential endophytic bacteria, only four isolates gave PIRG > 50%, while the other three bacterial isolates EB1, EB3 and EB7 gave PIRG < 50%. Interestingly, these four isolates namely EB2, EB4, EB5 and EB6 (*P. aeruginosa*) produced significantly ($p<0.05$) higher inhibitory effect 52.78, 83.33, 67.78 and 93.33%, respectively than the other isolates (Table II), with respect to the control after seven days of incubation (Fig. 2).

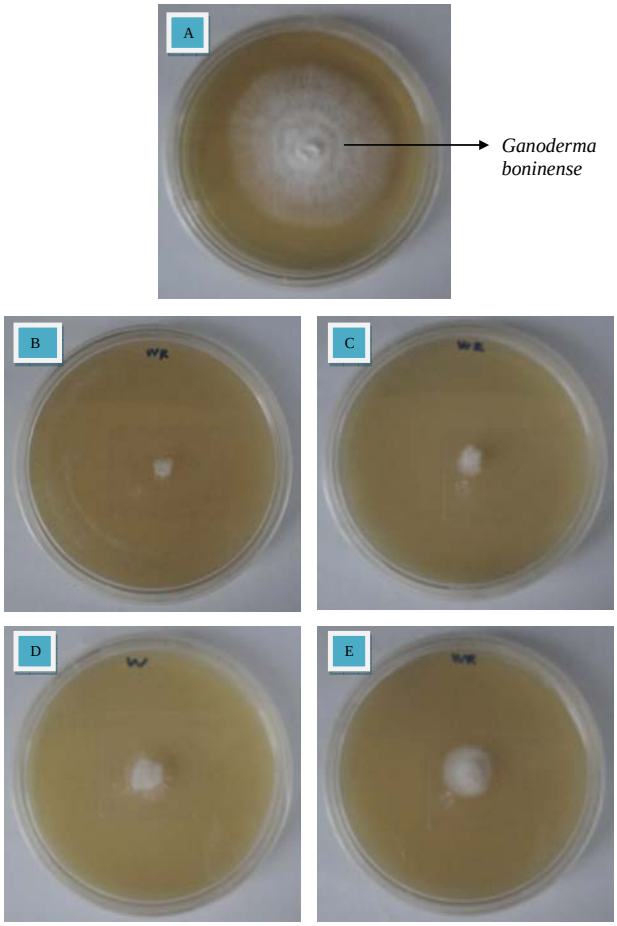
Fig. 1: A pure culture of *G. boninense* taken from MPOB, Bangi, Selangor, Malaysia

Fig. 2: Effect of (B) EB6 (*Pseudomonas aeruginosa*), (C) EB4, (D) EB5, (E) EB2 on the radial growth of *G. boninese* in the dual culture test at seven days incubation. (A) *G. boninense* in control plate



Therefore, four isolates from this category were selected for further screening test based on culture filtrate test.

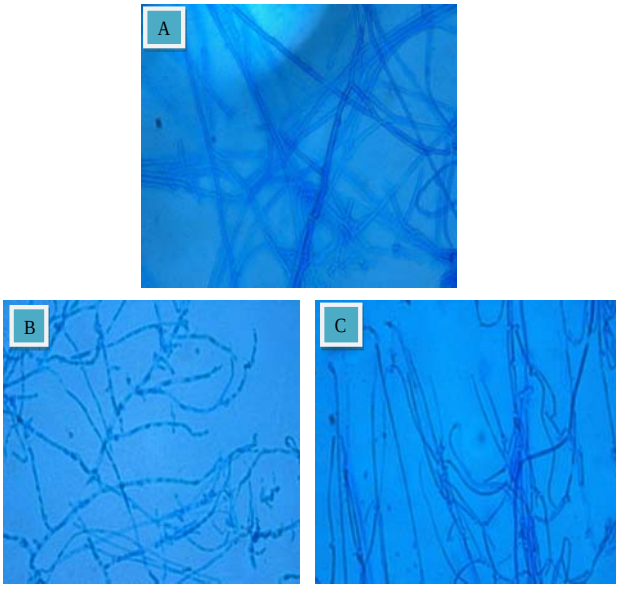
Fig. 3: Effect of (B) EB6 (*Pseudomonas aeruginosa*), (C) EB4, (D) EB5, (E) EB2 on the radial growth of *G. boninese* in the culture filtrate test at seven days incubation. (A) *G. boninense* in control plate



Culture filtrate test: Four isolates from dual culture test were selected for culture filtrate study based on PIRG value greater than 50%. These bacteria i.e., EB2, EB4, EB5 and EB6 (*Pseudomonas aeruginosa*) were significantly different at 5% probability level. Isolates EB6 (*P. aeruginosa*) showed maximum percentage inhibition (96.95%) of radial growth value against *G. boninense* at seven days of incubation with respect to control followed by EB4, EB5 and EB2 with 91.45, 69.48, 56.65%, respectively (Table III). Hence, both EB4 and EB6 were suggested as effective biological control agent *for G. boninense in vitro* since both showed effective result in dual culture and culture filtrate test (Fig. 3). Generally isolates with high PIRG values from the dual culture also exhibited greater activity in the culture filtrate test and thus have higher potential as bio control agents against *G. boninense.*

Mycelia growth test: The effect of the suppression of potential endophytic bacteria against *G. boninense* was further investigated using compound microscope. The changes of hyphal tips of *G. boninense* treated with EB4 and EB6 (*P. aeruginosa*) were observed under 400 magnifications. Malformation of hyphae occurred in the presence of EB6 (*P. aeruginosa*) (B) compared with hyphae in the control plate. Hyphae of *G. boninense* treated with EB4 were shrunk as observed in (Fig. 4C). Moreover, the treated fungus also showed the reduction in the coverage of spore produced.

Fig. 4: Observation of hyphae abnormalities of *G. boninense* at 400 magnifications as treated with EB4 and EB6. (A) Normal hyphae in control treatment. (B) Malformation of hyphae by EB6 (*P. aeruginosa*). (C) Shrinkage of hyphae by EB4



Bacterial identification using biolog® system: From Biolog system endophytic bacteria EB6 was successfully identified to species level; it was *Pseudomonas aeruginosa* but EB4 species could not be identified or unidentified due to limitation of the method used.

DISCUSSION

The abundance of red stained Gram-negative endophytic bacteria in the oil palm roots was supported by the frequency of their isolation. In other plants (cereals, vegetables and woody plants), the bacteria isolated from the xylem tissues are mainly (about 78 to 84% of the population) Gram-negative (Gardner *et al*., 1982; Bell *et al*., 1995). In this case, 100% (20 out of 20) of isolates were Gram-negative. Some of the bacterial endophytes from the genera *Pseudomonas*, *Burkholderia* and *Serratia* might have the potential to control *G. boninense*, as they were mostly found in healthy roots from symptomless palms. They also occur mostly in the vascular systems adjacent to the phloem and xylem vessels and are uniform throughout the cortex, which suggests that they may play a role in inhibiting penetration by *Ganoderma* and their movement to

the vascular systems. The role of endophytes in protecting plants against pathogens has been mentioned by several authors. For instance, Clay (1998) speculated that the interaction between a host plant and its endophytic microorganisms is one of the mechanisms of disease resistance. Some studies have shown that functioning communities of endophytes in plants contribute to their resistance to pathogens e.g., microbial endophytes in potato controlling bacterial soft rot (Sturz *et al*., 1999). Furthermore, Reiter *et al*. (2002), studying the response of endophytic bacterial communities in potato, suggested that disease may be inhibited by a diverse endophyte community maintaining the pathogen population below the threshold for expression of pathogenicity.

In this study, 20 endophytic bacteria were isolated from symptomless oil palm roots and isolation of bacterial endophytic were carried out based on morphological characteristics such as colony colour, colony surface, elevation, margin of colony and colony surface. These isolated bacteria were known as EB1, EB2, EB3, EB4, EB5, EB6, EB7, EB8, EB9, EB10, EB11, EB12, EB13, EB14, EB15, EB16, EB17, EB18, EB19, EB20. Out of 20 endophytic bacteria isolated, only seven of them (EB1, EB2, EB3, EB4, EB5, EB6 & EB7) showed inhibitory effect against the mycelia of *G. boninense*. From these seven potential endophytic bacteria, only four isolates (EB2, EB4, EB5 & EB6) gave PIRG greater than 50% on dual culture assay test. These four isolates produced higher inhibitory effect than the other isolates. According to the culture filtrate test, out of these four potential endophytic bacteria only two bacteria (EB4 & EB6) produced very high PIRG value. Moreover, the effect of the inhibition of potential endophytic bacteria against *G. boninense* was investigated using compound microscope.

The changes of hyphal tips of *G. boninense* happened once treated with *Pseudomonas aeruginosa* and EB4 were observed under 400 magnifications. A great malformation of hyphae occurred in the presence of *P. aeruginosa* as compared with hyphae in the control plate. Hyphae of *G. boninense* treated with EB4 were shrunk compared with control. These bacteria were screened to determine the best candidate to control Basal Stem Rot disease in oil palm. Result of this study showed that there are potential endophytic bacteria to control BSR disease caused by *G. boninense*.

The most efficacious biocontrol agents identified in this study were EB4 and EB6. These endophytic bacteria were identified using the Biolog® system and one of the isolates (EB6) has been successfully identified to the species level to be *Pseudomonas aeruginosa* and one more bacterium (EB4) could not be identified. This might be a new species and no data concerning this species (EB4) were in the list of Biolog data base. The results of the *in vitro* screening supported this speculation as the bacterium with the maximum PIRG value in the dual culture tests with 93.52 and 83.33% meanwhile for culture filtrate tests with 96.95 and 91.45% were *Pseudomonas aeruginosa* and EB4, respectively. *Pseudomonas* is known as growth-promoting bacteria and inducer of systemic resistance against fungal, bacterial and viral diseases (Liu *et al*., 1995; Chen *et al*., 2000) by producing metabolites with antimicrobial activity against pathogens (Dikin *et al.*, 2005). They are also known to produce siderophores that restrict pathogen growth by limiting iron availability in the soil. This obligate aerobic is also an opportunistic pathogen causing harm to human and animals (Ndip *et al*., 2005). Oil palm roots support large and more diverse taxa of endophytes including those with antagonistic properties against *G. boninense*. Since *Ganoderma* infection is often initiated at the roots, an endophyte isolated at the 'target' site would have lesser problem in adapting and colonizing the host plant, leading to a more efficient disease control.

It has been demonstrated that the isolated endophytic bacteria are antagonistic to the pathogen *in vitro* (Dikin *et al.*, 2005) but their ability to colonise the plant is important to test. To improve this study, *in vivo* or field study should be performed to confirm their efficacy. This is supported by Jubina and Girija (1998), on already established nursery plants of black pepper where one of the Bacilli isolates showing poor inhibition of the fungal pathogen in the dual culture, exhibited the maximum disease suppression in the *in vivo* biological control assay.

In conclusion, BSR caused by *G. boninense* is the most destructive disease of oil palm in Malaysia. The use of conventional contact fungicides has not given much control, as the disease is systemic. Thus, hygienic cultural practices with biological control are being explored to control the disease. The control of soil-borne pathogens is particularly complex, because the diseases occur in the dynamic environment at the interface of the roots with the soil. Although endophytic bacteria, especially *P. aeruginosa* showed biological control potential against BSR, further studies must be conducted to verify their effectiveness in the field. The population density of the endophytes to be applied must also be determined, as well as the best method of application. The effects of agrochemicals on the bacteria have also to be evaluated so as not to compromise their effectiveness against BSR disease.

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