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Effect of Endophytic Bacteria on Growth and Suppression of *Ganoderma* Infection in Oil Palm

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ABSTRACT

Basal stem rot (BSR) caused by *Ganoderma boninense* is an important disease of oil palm in Malaysia. Control of BSR is complex, because the disease occurs in the dynamic environment at the interface of the roots with soil. Endophytes as biocontrol agents offer possible advantages as they invade and proliferate in the plants to await the pathogens and they are insulated from any adverse conditions in the soil. *Burkholderia cepacia* (B3) and *Pseudomonas aeruginosa* (P3) isolated from symptomless oil palm root tissues have shown potential to inhibit the spread of *G. boninense.* They play a role in keeping the *G. boninense* population below threshold for BSR initiation by restricting its entry and movement in the palm. When tested on 4 month-old oil palm seedlings inoculated with *G. boninense*, the bacteria singly and in a mixture suppressed the spread of the pathogen with an epidemic rate of 0.10 - 0.24 units compared to 0.52 units in the control. At 8 months after inoculation, BSR incidence was reduced by 76% in seedlings pre-inoculated with *P. aeruginosa* (P3). *B. cepacia* (B3) reduced incidence by 42% and the mixture of *P. aeruginosa* and *B. cepacia* by 54%. Inoculation of endophytic bacteria also improved vegetative growth of oil palm seedlings.

Key words: Endophytes; *Pseudomonas aeruginosa; Burkholderia cepacia; Ganoderma boninense;* Oil palm

INTRODUCTION

Oil palm, *Elaeis guineensis* is the most important plantation crop in Malaysia, producing an average palm oil yield of approximately 4 t ha^{-1} annually. Plant health is crucial in obtaining maximum production. In Malaysia, the oil palm is blessed by being largely disease free, suffering from but one major disease, Basal Stem Rot (BSR) caused by *Ganoderma* species. With no known cure at present, it is the major disease of oil palm and, therefore, of great economic importance to the Malaysian oil palm industry.

Field control of BSR by contact chemicals hve not been very successful (Soepena *et al*., 2000) even with those *in vitro* screened effective against the fungus (Khairudin, 1990; Teh, 1996). 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. Biological control need not necessarily be a cure for the disease and can be merely to arrest the disease spread by inoculation with a biocontrol agent. For example, saprophytes can be used to compete against *Ganoderma* to reduce its opportunity for colonizing oil palm.

Endophytic bacteria are organisms inhabiting plant organs that at some time in their life cycle can colonize the internal plant tissues without causing apparent harm to the host (Azevedo *et al*., 2000). 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-born 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. Two bacterial isolates *Burkholderia cepacia* (B3) and *Pseudomonas aeruginosa* (P3) were selected for evaluation in the glasshouse for their efficacy in enhancing growth and subsequent suppression of the spread of BSR in oil palm seedlings. Their selection was based on their percentage inhibition of radial growth (PIRG) values from *in vitro* screening tests and their ability to colonize and proliferate in oil palm roots (Zaiton *et al*., 2006).

MATERIALS AND METHODS

Preparation of *G. boninense* inoculum on rubber wood blocks. Fresh rubber wood blocks (12 cm x 6 cm x 6 cm, weighing approximately 450 g) were washed with water and autoclaved for 20 min at 121°C. One block each was put in heat-resistant polypropylene bags (15 cm x 33 cm x 0.05 mm thick material) and 100 mL of molten malt extract agar (MEA) added as supplementary nutrient for *G. boninense*. Since inoculation of the blocks with *G. boninense* plugs can introduce contamination, the bags were closed by drawing its open end through a 4 cm diameter polyvinyl chloride (PVC) tubing 2 cm long and the remaining hole plugged with cotton. The bags with rubber wood blocks and molten

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MEA were autoclaved at 121°C for 30 min. After sterilization and cooling, the rubber wood block in the bag was rotated to ensure that it was well covered with the agar before the latter solidified. When the agar had solidified, five 10 mm plugs taken from five day-old *G. boninense* culture were inoculated on each rubber wood block. The inoculated blocks in the bags were then incubated in a dark cabinet at 28 ± 2°C for three weeks until fully colonized by *G. boninense* mycelium.

Preparation of endophytic bacteria. *B. cepacia* (B3), *P aeruginosa* (P3) and mixture (B3 + P3) suspensions were prepared using 48 h-old cultures on Nutrient Agar (NA). The inoculum suspensions were prepared and adjusted to 10^8 cfu mL^{-1}. During the preparation of mixture, equal volume of B3 and P3 suspensions were mixed.

Establishment and inoculation of the oil palm seedlings. Oil palm germinated seeds (Dura x Pisifera) supplied by Golden Hope Plantations, Banting Selangor were grown in polybags containing 1 kg soil mixture (3:2:1 v/v/v topsoil: peat: sand) in the glasshouse until they reached four to five-leaf stage. The seedlings were inoculated with the respective endophyte treatments by drenching the soil with 150 mL of the inoculum suspensions. Seedlings treated with sterile distilled water (SDW) were used as the control. A booster inoculation with the endophyte suspension was applied 5 days after the initial inoculation. Four days after the booster inoculation, the seedlings were uprooted carefully and transferred into pots containing 3 kg soil mixture. A factorial experiment was conducted with eight treatments replicated thrice with five seedlings per replicate and arranged in a CRD. Treatments 5-8 were inoculated with *G. boninense* colonized rubber wood blocks placed in contact with four randomly chosen roots (Table I). The pots were placed on benches in the glasshouse, watered daily and no supplementary organic fertilizer was applied. The experiment was repeated twice.

Effect of endophytic bacteria on BSR incidence. Disease development was monitored based on quantitative assessment measured as Disease Incidence (DI) percentage at intervals of months. DI referred to the number of seedlings visually assessed as diseased (chlorosis & necrosis of leaves, with or without production of sporophore) described by Campbell and Madden (1990) as: (number of seedlings infected/total number of seedlings assessed) x100.

A reduction in the disease incidence compared with the control would be a measure of the treatment effectiveness in suppressing the disease. This was assessed by plotting the data as disease progress curves. The Area Under the Disease Progress Curve (AUDPC) was calculated using the formula (Campbell & Madden, 1990):

$$AUDPC = \sum_{i}^{n-1} \left(\frac{Y_i + Y_{i+1}}{2} \right) \left(t_{i+1} - t_i \right)$$

Whereby n is the number of assessment times, Y is the disease incidence and t the is observation time.

The slopes of the curves were obtained by transforming the DI data using the monomolecular model (Monit) (Campbell & Madden, 1990). At the end of the experiment (8 months), the oil palm seedlings were split longitudinally to observe for root and stem decay and to visually assess the severity of the internal symptoms based on the proportion of root and bole tissues damaged by *G. boninense*. The estimation was based on the following modified scale (Breton *et al*., 2005).

0 = healthy: no internal rot, 1 = 20% rotting of tissues, 2 = 20 to 50% rotting of tissues, 3 = > 50% rotting of tissues and 4 = > 90% rotting of tissues. Disease severity (DS) for the internal symptoms was calculated by the formula of Liu *et al*. (1995) as under.

$DS_{(internal)}$ = (number of seedlings in the rating x rating number)/(total number of seedlings assessed x highest rating)x 100.The disease incidence and disease severity percentages were transformed by arcsine and analyzed using ANOVA with the means compared by the LSD ($P \leq 0.05$).

Effect of endophytic bacteria on plant vigor. The effect of the bacterial endophytes inoculation (single or in mixture) on plant vigor was also evaluated. Plant vigor was assessed based on the vegetative growth. Greater increases in plant height, stem diameter and root mass would indicate improved vigor and positive effects of the bacterial endophyte on plant health. Increases in plant height (cm) and stem diameter (mm) were monitored by measuring one cm above the soil level to the tip of the leaves. The stem diameter was taken at same height above ground. At the end of the experiment (8 months), root mass was quantified by weighing the total fresh roots of each oil palm seedling. All the growth data were analyzed using ANOVA and the means compared using LSD ($P \leq 0.05$). The vegetative growth parameters of the oil palm seedlings pre-inoculated with endophytes and challenged with and without *G. boninense* were compared using the General Linear Model.

Colonization and establishment of endophytic bacteria in the host tissues. The colonization and establishment efficiency of *B. cepacia* (B3) and *P. aeruginosa* (P3) were determined in the root, bole and stem tissues, as well as in the rhizosphere (root surface) of the oil palm seedlings. The populations of B3 and P3 were estimated quantitatively by direct plating (colony forming unit (cfu) count). To estimate the populations in the rhizosphere, 1 g (fresh weight) of roots with attached soil were macerated using a mortar and pestle and suspended in 9 mL sterile distilled water by shaking vigorously with a vortex mixer. A series of suspension dilution was performed and 0.1 mL aliquot from each was plated on nutrient agar with four replicates and incubated at 28 ± 2°C in the dark for 48 h for growth of the bacterial colonies. B3 and P3 were distinguished by the color of their colonies on the nutrient agar plates; B3 forming yellow, non-fluorescent and P3 brown colonies. The results were expressed in cfu g^{-1} and analyzed using ANOVA and the means compared by LSD at $P \leq 0.05$.

RESULTS

Effect of endophytic bacteria on BSR incidence. Generally, disease developed much more slowly in the seedlings pre-inoculated with endophytes than in the seedlings from the negative control (Fig. 1). A lower DI would indicate some disease suppression by the endophytes. For the seedling pre-inoculated with *P. aeruginosa* (T6), DI at 5 months was 0%, demonstrating a total absence of external symptoms. However, yellowing of leaves and sporophore production gradually appeared after six months when the DI was 13.3%. At the end of the experiment (8 months after challenged inoculation with *Ganoderma*), DI was only 26.7%, suggesting good disease suppression by *P. aeruginosa*. The seedlings treated with mixed *P. aeruginosa* and *B. cepacia* (T7) also showed a reduced DI, but the disease suppression was less than by *P. aeruginosa* alone (T6). At seven and eight months, T7 had DI of 33.3% and 46.7%, respectively. *B. cepacia* alone (T5) was the least effective in suppressing *Ganoderma* with DI of 46.7% and 60.0% at seven and eight months. As expected, the seedlings without endophytes recorded the highest DI of 80.0% and 86.7% at seven and eight months, respectively.

The disease development was also evaluated by area under disease progress curve (AUDPC). The ability of the bacterial endophytes to reduce BSR damage was expressed as the percentage disease reduction (% DR) derived from values of AUDPC. The AUDPC values indicate the amount of disease developed in each treatment where the lowest AUDPC values correspond to the effectiveness of the bio-control in reducing disease (Table II). The seedlings pre-inoculated with *P. aeruginosa* (T6) gave lowest AUDPC of 46.67, followed by the mixed inoculum with AUDPC of 90.00 and *B. cepacia* with 113.33. Thus, *P. aeruginosa* was the most effective in suppressing BSR, followed by *B. cepacia* and mixture of *P. aeruginosa* with *B. cepacia* based on the disease reduction (DR). The seedlings pre-inoculated with *P. aeruginosa* alone (T6), showed 76.27% DR with the lowest epidemic rate at 0.10 units. The lowest DR was recorded in T5 at 42.2% with the highest epidemic rate of 0.24 units. This suggested that *B. cepacia* was not very effective in disease control. The mixture of *P. aeruginosa* and *B. cepacia* was also not very effective in controlling BSR in the oil palm seedlings.

At the end of the experiment destructive samples of seedlings were collected to assess the extent of root rot and decay of their bole tissues. Severe root rot was seen in all seedlings suffering from foliar desiccation. Longitudinal sections of the affected seedlings revealed extensive rotting of the bole extending into the stem. However, the healthy seedlings showed no root rot or stem decay. This analysis of disease severity based on the extent of root rot and bole decay showed that the seedlings pre-inoculated with *P. aeruginosa* (T6) demonstrated significantly lower disease severity (16.33% & 8.33%, respectively) and are shown in Fig. 2. This suggested that *P. aeruginosa* had good potential as a biocontrol agent against *G. boninense*. This was supported by the ability of *P. aeruginosa* to colonize the seedlings and proliferate throughout the plants to better inhibit the entry of *G. boninense* to the phloem and xylem vessels. *B. cepacia* was less effective in disease control than *P. aeruginosa* alone or in mixture. As expected, the seedlings in the negative control suffered the highest root rot and developed the most necrotic lesions at 68.5% and 51.6%, respectively.

Table I. Treatments of bacterial endophytes applied to oil Palm Seedlings

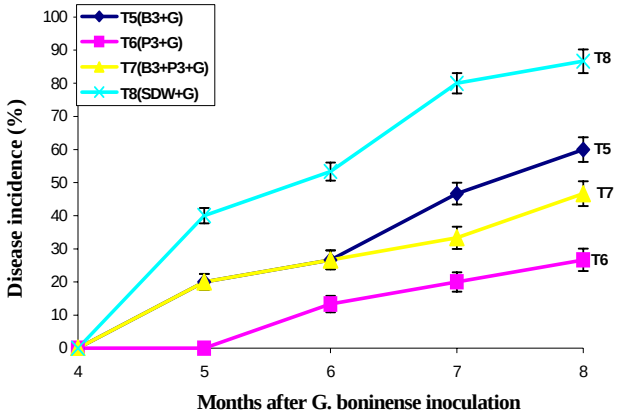
Treatment		Description
Without *G. boninense*	T1	B3 (*B. cepacia*)
	T2	P3 (*P. aeruginosa*)
	T3	B3 + P3 (*B. cepacia* + *P. aeruginosa*)
	T4	Positive control (SDW)
With *G. boninense*	T5	B3 (*B. cepacia*)
	T6	P3 (*P. aeruginosa*)
	T7	B3 + P3 (*B. cepacia* + *P. aeruginosa*)
	T8	Negative control (SDW)

Table II. The effect of endophytes on BSR development in oil palm seedlings after 8 months

Treatment	AUDPC[1] (units[2])	ER[2] (unit month^{-1})	DR[3] (%)
T5 (B3 + *G. boninense*)	113.33[1]	0.24[1]	42.20
T6 (P3 + *G. boninense*)	46.67	0.10	76.27
T7 (B3 + P3 + *G. boninense*)	90.00	0.13	54.24
T8 (SDW + *G. boninense*)	196.67	0.52	-

[1] Area Under Disease Progress Curve (AUDPC); [2] Epidemic Rate (ER); [3] %Disease Reduction(DR).

Fig 1. Basal stem rot development in oil palm seedlings pre-inoculated with bacterial endophytes and after challenged with *G. boninense*. Bars represent the standard deviation.



Effect of endophytic bacteria on plant vigor. *P. aeruginosa* (P3), *B. cepacia* (B3) and mixture of P3 and B3 significantly increased the plant height and stem diameter over those of the control seedlings. However, the advantages shown appeared to decrease gradually over time, possibly due to the impoverished soil as no fertilizer was applied throughout the experiment. The seedlings treated

with *P. aeruginosa* (T2) grew faster than the seedlings in T1 (*B. cepacia*) and T3 (*P. aeruginosa* + *B. cepacia*) (Fig. 3). The production of root hairs and increase in root mass were similarly enhanced. T3 also improved the vegetative growth of the seedlings. The seedlings treated with *B. cepacia* alone recorded the lowest vegetative growth. Generally, the control seedlings challenged with *Ganoderma* suffered stunted growth and recorded the lowest increase in height, stem diameter and root mass (Figs. 4A & 4B). Seedlings pre-inoculated with endophytes (T5, T6 & T7) suffered only slight retardation in their vegetative growth and showed no significant difference with the control seedlings in T1, T2 and T3 (pre-inoculated with endophytes but not challenged with G. *boninense*). This suggested that the endophytes had contributed to the plants in sustaining their growth over the damage caused by *G. boninense*.

Colonization and establishment of endophytic bacteria in the host tissues. The colonization and establishment of B3 (*B. cepacia*) and P3 (*P. aeruginosa*) in the tissues and rhizosphere of the oil palm seedlings were quantified by direct plating. The numbers of colony forming units per g tissue (cfu g^{-1}) were estimated from the colonies recovered on NA. B3 formed yellow non-fluorescent pigment and P3 brown colonies. The number of endophyte colonies recovered differed with the tissue used for assessment with the highest populations for B3 and P3 recovered from the roots, followed by the bole, rhizosphere and stem. The higher populations in the roots could have been attributed to the roots being the site of entry by the endophytes with the necessary nutrients and space for their proliferation and establishment. The populations of B3 and P3 were generally higher in the seedlings without *G. boninense*, possibly, because there was less competition for nutrients and space. The population of P3 was higher than that of B3 with 10^5 $cfug^{-1}$ tissue versus 10^4 cfu g^{-1} tissue, respectively. However, with the mixed inoculum, the population of P3 was lower than from P3 applied alone at 10^4 cfu g^{-1}, while the B3 population was 10^3 cfu g^{-1}. This could be attributed to competition by the endophyte for nutrients and space, mutually limiting their growth. Histological study showed B3 to be faster in colonizing the plant tissues than P3. At 4 days after inoculation, B3 could already be detected in the xylem and pith. However, the populations of the bacterial endophytes appeared to decrease gradually over time. From the initial inoculum load of 10^8 cfu mL^{-1} applied in the soil drench, only 10^{-4} to10^{-5} cfu g^{-1} were recovered from the host tissues at the end of experiment after challenging with *G. boninense*. The cause of decreased population is not known, although it could be due to dilution effect encountered using dilution technique to estimate population count.

DISCUSSION

The lower DI%, DS% and ER and higher values in DR% and vegetative growth from the seedlings pre-inoculated with endophytes (T6, T7 & T5) over the control

Fig.2. Basal stem rot developments in oil palm seedlings pre-inoculated with bacterial endophytes eight months and after challenged with *G. boninense*. Means with same letter are not significantly different within treatments ($LSD_{0.05}$); T5: B3 (*B. cepacia*) +*G. boninense*; T6: P3 (*P. aeruginosa*) + *G. boninense*; T7: B3+P3+ *G. boninense*; T8: Control (SDW) + G. boninense

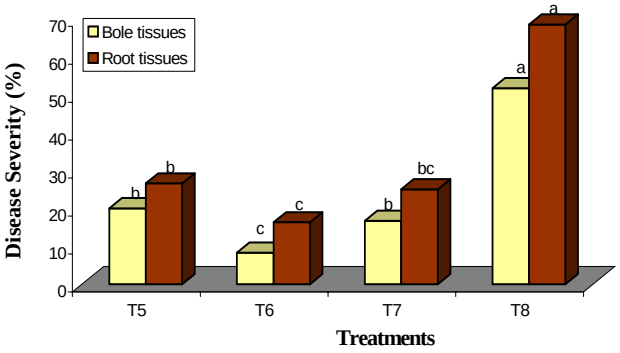
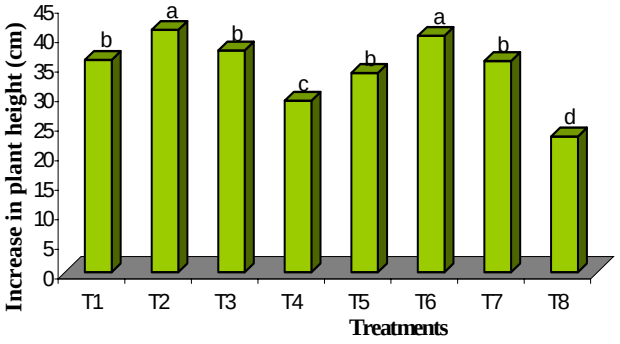
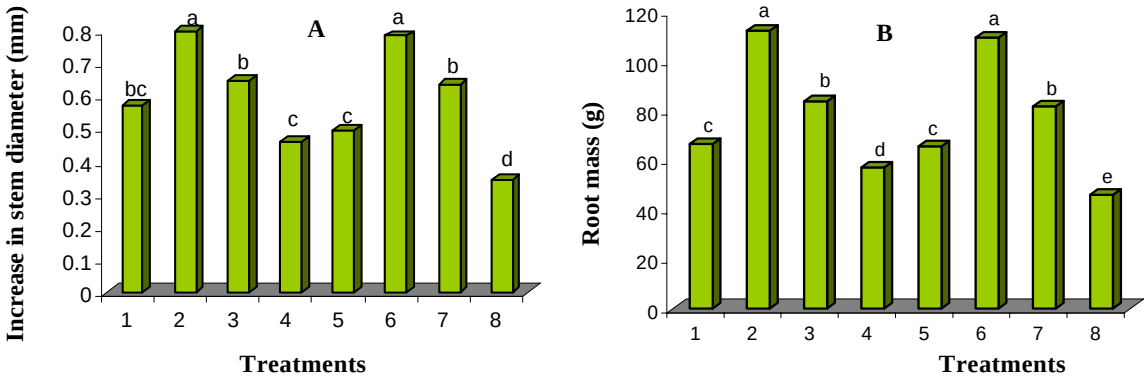


Fig. 3. Effect of endophytic bacteria on height of oil palm seedlings eight months after challenged or not challenged with *G. boninense*. Means with the same letters between treatments are not significantly different ($LSD_{0.05}$).T1: B3 (*B. cepacia*); T2: P3 (*P. aeruginosa*); T3: B3+P3; T4: Control (SDW); T5: B3 + *G. boninense*; T6: P3 + *G. boninense*; T7: B3+P3+ *G. boninense*; T8: Control (SDW) + *G. boninense*



(T8) suggested that the former seedlings had acquired some form of tolerance to the physical damage caused by *G. boninense* from the bacterial endophytes. Disease suppression could be mainly due to induction of the host defense mechanisms, such as formation of structural barriers, like lignified cell walls, to keep out the pathogen and production of antifungal metabolites to slow down the infection progress and improved growth and plant vigor (Hammerschmidt & Kuc, 1995). In this study, *P. aeruginosa* showed the potential for suppressing BSR incidence in the oil palm seedlings. The reduction of DI% and DS% in the oil palm seedlings pre-inoculated with *P. aeruginosa* after challenged inoculation with *Ganoderma* suggested that the bacteria could play a significant role in inhibiting the penetration of *Ganoderma* into vascular

Fig. 4. Effect of endophytic bacteria on (A) stem diameter and (B) root mass of oil palm seedlings eight months after challenged or not challenged with *G. boninense*. Means with the same letters between treatments are not significantly different (LSD $_{0.05}$). T1: B3 (*B. cepacia*); T2: P3 (*P. aeruginosa*); T3: B3+P3; T4: Control (SDW); T5: B3 + *G. boninense*; T6: P3 + *G. boninense*; T7: B3+P3+ *G. boninense*; T8: Control (SDW) + *G. boninense*



systems. This was supported by observation in histological study that showed bacteria endophytes being more concentrated in vascular systems of the roots taken from symptomless palms (Zaiton *et al*., 2006). *P. aeruginosa* and *B. cepacia* have reported as bio-control agents for controlling pathogen in oil palm, *Schizopyllum commune* Fr (Dikin *et al*., 2003).

Interaction between plants and beneficial bacteria can also have profound effects on the crop health and therefore yield (Kloepper *et al*., 1989; Sturz *et al*., 2000). The mechanisms involved in supporting plant growth and health include increasing soil nutrient availability, improving soil structure, inducing the plant defense mechanisms, producing antibiotics, out competing pathogens and providing growth-stimulating substances or enzymes (Van Loon & Bakker, 2004). Some endophytic bacteria have been classed as plant growth-promoting bacteria (PGPB). The positive effects of PGPB on plant growth are always correlated with remarkable increase in the root morphology such as lateral root length, root hair number and also shoot length and yield. It is generally assumed that these developmental responses are triggered by phytohormones such as auxins, cytokinins and gibberellins produced by the bacteria (Persello-Cartieaux *et al*., 2003). *P. aeruginosa* has been shown to improve plant growth and is classified as a Plant Growth Promoter Bacterium (PGPB). It is known to synthesize growth-stimulating plant hormones. Plant hormones produced by *Pseudomonas* include auxins and cytokinins, as well as volatile signals such as ethylene 2, 3-butanediol and acetonin (Lambrecht *et al*., 2000; Persello-Cartieaux *et al*., 2003; Ryu *et al*., 2003). Plant hormone syntheses have been shown to stimulate root growth. In this study, *P. aeruginosa* significantly increased the seedling plant growth and root mass. *B. cepacia* can also control *Ganoderma*, although less effectively than *P. aeruginosa*. A combination of biocontrol agents can offer advantages over a single agent alone in suppressing pathogens (Lemanceau *et al*., 1993; Pierson & Weller, 1994; Crump, 1998). However, the combination of *P. aeruginosa* with *B. cepacia* was less effective than *P. aeruginosa* alone. This may be due to some incompatibility between them as bio-control agents are typically selected based on their individual antagonistic behavior towards pathogens rather than for their combined potency (Leeman *et al*., 1996; Meyer & Roberts, 2002). Therefore, the compatible bacteria should first be determined before they are used in combination.

Although promising results were obtained from using endophytic bacteria, especially *P. aeruginosa* to control *G. boninense*, field studies must be done to confirm their efficacy *in vivo*. There is also need to determine the required population density of the endophytes to apply and the best method of application. The effects of agrochemicals on the endophytic bacteria for good management of BSR by the bacteria also need to be investigated.

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