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Symbiotic Interaction of Endophytic Bacteria with Arbuscular Mycorrhizal Fungi and Its Antagonistic Effect on *Ganoderma boninense*

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Endophytic bacteria (*Pseudomonas aeruginosa* UPMP3 and *Burkholderia cepacia* UPMB3), isolated from within roots of oil palm (*Elaeis guineensis* Jacq.), were tested for their presymbiotic effects on two arbuscular mycorrhizal fungi, (*Glomus intraradices* UT126 and *Glomus clarum* BR152B). These endophytic bacteria were also tested for antagonistic effects on *Ganoderma boninense* PER 71, a white wood rot fungal pathogen that causes a serious disease in oil palm. Spore germination and hyphal length of each arbuscular mycorrhizal fungal (AMF) pairing with endophytic bacteria was found to be significantly higher than spores plated in the absence of bacteria. Scanning electron microscopy (SEM) showed that the endophytic bacteria were scattered, resting or embedded on the surface hyaline layer or on the degraded walls of AMF spores, possibly feeding on the outer hyaline spore wall. The antagonistic effect of the endophytic bacteria was expressed as severe morphological abnormalities in the hyphal structures of *G. boninense* PER 71. The effects of the endophytic bacteria on *G. boninense* PER 71 hyphal structures were observed clearly under SEM. Severe inter-twisting, distortion, lysis and shrivelling of the hyphal structures were observed. This study found that the effect of endophytic bacteria on *G. intraradices* UT126 and *G. clarum* BR152B resembled that of a mycorrhiza helper bacteria (MHB) association because the association significantly promoted AMF spore germination and hyphal length. However, the endophytic bacteria were extremely damaging to *G. boninense* PER 71.

***Keywords*:** arbuscular mycorrhizal fungi, endophytic bacteria, *Ganoderma boninense*, *Elaeis guineensis*

An endophyte is a bacterial, fungal or other organisms that spends the whole or part of its life cycle colonising healthy tissues of a host plant, inter- and/or intra-cellularly, typically causing no apparent symptoms of disease (Sturtz *et al.*, 2000). The relationship between the endophyte and its host plant may range from latent phytopathogenesis to mutualistic symbiosis (Strobel and Long, 1998). A more recent definition by Schulz and Boyle (2006) defines endophytes as those bacteria and fungi that can be detected at a particular moment within the tissues of apparently healthy plant hosts. They may be indigenous species occurring naturally in the soil or introduced through agricultural practices (Gordon and Okamoto, 1992). Common endophytes include a variety of bacteria, fungi, and Actinomycetes, which can be isolated from wild plants or both monocotyledonous and dicotyledonous cultivated crops (Fisher *et al*., 1992; Brooks *et al*., 1994; Liu and Tang, 1996). Arbuscular mycorrhizal fungi (AMF) are probably one of the best-researched groups of endophytic microorganisms because they enhance plant growth through improved nutrient uptake. AMF are found in the roots of 80% of vascular plants and this association is considered the classic example of mutualistic symbiosis. The symbiosis is biotrophic and mutualistic and the long-term compatible interaction is largely based on bidirectional nutrient transfer between the symbionts, along with supplementation of other factors such as drought and disease tolerance (Smith and Read, 2008).

Currently there is increasing interest in, and active research on bacterial endophytes. Another form of endophyte currently being actively researched with increasing interest is bacteria. In the early 1950s, bacteria were postulated to exist in plants without causing disease (Hollis, 1951). They have been isolated from both monocotyledons and dicotyledons and are categorised according to their potential interaction with their hosts (Whitesides and Spotts, 1991; Brooks *et al*., 1994). These categories include the common rhizosphere-colonizing bacteria such as *Bacillus* spp., *Enterobacter* spp., *Pseudomonas* spp., *Serratia* spp., and *Burkholderia* spp., which colonize the inside of plant roots (Van Loon and Bakker, 2005). These bacteria are considered endophytic, as they are reportedly capable of inducing systemic resistance in plants, suppressing disease and showing biological traits such as antibiotic activity and lysis (Barea *et al*., 1998; Viswanathan and Samiyappan, 2002; Siddiqui and Shaukat, 2003; Szczech and Shoda, 2004; Mathivanan *et al*., 2005). Recent evidence suggests that endophytic bacteria play specific and important roles in host tissues and their ability to colonize internal host tissues make them an invaluable agricultural tool to improve crop performance and protection (Siddiqui, 2005). One of these important roles is as mycorrhiza helper bacteria (MHB), in which the bacterial strains assist the mycorrhizal formation. Indeed, several bacteria that interact positively with the function of symbiosis have been reported in reviews by Garbaye (1994) and more recently by Frey-Klett *et al*. (2007).

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Ganoderma boninense is a white rot fungus that causes Basal Stem Rot (BSR) disease in oil palm (*Elaeis guineensis* Jacq.). This fungus causes severe economic losses to oil palm in Southeast Asia (Rees *et al*., 2007). The pathogen slowly rots the palm bole base and disease symptoms appear much later. This slow progression of disease makes early detection of the disease extremely difficult. Oil palm is an important perennial crop in Malaysia, being the largest producer and exporter of palm oil currently (Sumathi *et al*., 2008), requires an efficient control methodology to manage the disease effectively. The infection occurs via the roots in close proximity to an infection source, e.g., the infected stump of a felled tree (Sanderson, 2005). Historically, control of BSR involved cultural techniques such as mechanical and chemical control; however, these techniques have no demonstrable effects (Susanto *et al.*, 2005). Recent studies of biological control agents have shown some early promise (Shamala *et al*., 2008; Zaiton *et al*., 2008). Studies using AMF and endophytic bacteria to control *G. boninense* in oil palm are relatively new; recent *in vitro* and *in vivo* investigations by Zaiton *et al.* (2008) identified two endophytic bacteria – *Pseudomonas aeruginosa* UPMP3 and *Burkholderia cepacia* UPMB3 – with purported activity against the pathogen. Previous reports have also indicated extensive AMF association in the oil palm rhizosphere with *Glomus*, *Gigaspora*, and *Acaulospora* (Nadarajah, 1980; Blal and Gianizazzi-Pearson, 1990; Siti Ramlah and Tayeb, 1991), which supports this study's objective to further re-establish AMF for investigation along with endophytic bacteria.

This study aims to assess the presymbiotic effect of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 on spore germination and hyphal length of two AMF species – *Glomus intraradices* UT 126 and *Glomus clarum* BR 152B – through *in vitro* assays. It will also assess the possible roles of these bacteria as MHB by determining their effects on the germination of AMF spores. Additionally, this study attempts to investigate the antagonistic effect of these endophytic bacteria on the morphology and hyphal structures of *G. boninense* PER 71 at the microscopic level.

Materials and Methods

Endophytic bacteria

Two endophytic bacterial strains, both isolated from within oil palm roots for a previous study, were used: *Pseudomonas aeruginosa* UPMP3 and *Burkholderia cepacia* UPMB3 (Zaiton *et al*., 2008). The former is a γ-Proteobacterium and the latter is a β-Proteobacterium. The bacterial strains were isolated using the following method. Fresh root samples were cut into 3.0 cm sections. These sections were subjected to surface sterilization in 10% sodium hypochlorite for 2 min, followed by 50%, 70%, 90%, and finally 100% ethanol for 30 sec in each solution. The roots were rinsed twice in distilled water to remove the chemicals and blotted dry on sterilized filter paper. The outer layer (epidermis) of the roots was carefully removed and then the root section was cut longitudinally and transferred onto nutrient agar (NA, Oxoid, England). The emerging bacterial colonies were streaked onto fresh NA. These bacterial strains have been reported as purported antagonists of *G. boninense* (Zaiton *et al*., 2008). For the current study, these bacterial strains were prepared from stock cultures stored at 4°C and subsequently subcultured on nutrient agar when required.

AMF and spore disinfection

The two AMF species selected were *G. intraradices* UT126 and *G. clarum* BR152B. Both species were obtained from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal (INVAM) fungi, USA. They were isolated from peanut rhizosphere in Salt Lake City, Utah, and Brazil, respectively. Single spore cultures of the AMF species were received as pot contents, then transferred and maintained in pure single propagation through pot cultures using *Setaria sphacelata* as host plants under standard pot conditions (Brundrett *et al*., 1997). The spores were harvested by wet sieving and collected using a micropipette viewed under a Nikon SMZ1000 (Nikon, Japan) stereomicroscope. The spores were then surface sterilized by, first, gently stirring in sterile water twice, followed by stirring in 2% chloramine-T solution with 2 drops of Tween 20 for 10 min. The spores were rinsed again thrice in sterile water and then placed in a 0.02% streptomycin and 0.01% gentamycin solution for 10 min as described by Sylvie *et al*. (2005). Spores were stored in demineralized water at 4°C until use.

In vitro effects of endophytic bacteria on spore germination and hyphal length of AMF

The effects of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 on spore germination and hyphal length of *G. intraradices* UT126 and *G. clarum* BR152B were assessed by the Bactoagar Petri dish assay as described by Pivato *et al.* (2009). The previously surface-sterilized spores of the AMF species were used. One spore was placed at each vertex of a pentagon (i.e., five spores per Petri dish) on the surface of 1% Bactoagar (Difco, France). An aliquot (1 ml) of a bacterial suspension was mixed into the medium to obtain a final concentration of 10^7 CFU/ml. Each treatment using the AMF spores of *G. intraradices* UT126 and *G. clarum* BR152B (in the presence / absence of either one of the endophytic bacterial strains) was replicated 10 times.

After a 7-day incubation at 28°C in the dark, the percentage of AMF spore germination was determined using a Nikon SMZ1000 stereomicroscope (Nikon) fixed with a camera (Infinity2 MPX). The hyphal length of each germinated spore was measured from captured images using the software *i*Solution Lite (IMTechnology Inc.).

Scanning electron microscope (SEM) study - compatibility interaction

Spores of *G. intraradices* UT126 and *G. clarum* BR152B were surface-sterilized as described above, according to Sylvie *et al*. (2005). The endophytic bacteria were cultured separately in Luria Bertani (LB) liquid medium at 37°C with gentle shaking as described by Barea *et al*. (1998), with minor modification. After centrifugation at 3,000×g for 20 min, the supernatant from each endophytic bacterial culture was discarded and the pellet was suspended in 15 ml 0.85% sterile saline. The concentration of bacterial cells in each suspension, which was adjusted using optical density measurements, was between 10^7 and 10^8 CFU/ml. An aliquot (1 ml) of each endophytic bacterial suspension was pipetted into a micro-centrifuge tube. Five clean spores of each AMF species were submerged in the bacterial suspensions of either *P. aeruginosa* UPMP3 or *B. cepacia* UPMB3. A total of 30 AMF spores of each species were tested with each endophytic bacterium in this experiment. As a negative control, a separate batch of AMF spores was incubated in sterile saline only to rule out the possibility that the fungal surfaces were contaminated by other bacteria. The micro-centrifuge tubes were placed in an incubator shaker (INFORS HT Multitron) at 50 rpm and 37°C for 48 h. The spores were harvested and gold coated for examination under a

Hitachi S3400N scanning electron microscope with an acceleration voltage of 5 kV.

Isolation of *G. boninense* PER 71

The white wood rot basidiomycete used in this study, *G. boninense* PER 71, was isolated from an infected oil palm in Perak, Malaysia. The sporophore was carefully removed, surface-sterilized in 1% sodium hypochlorite for 15 min and rinsed thrice in sterilized distilled water. It was then blotted dry on a sterilized Whatman filter paper. Once completely dry, the sporophore was diced and placed on freshly prepared potato dextrose agar (PDA, Difco, France). The mycelium growing from the sporophore pieces was subcultured, transferred into new PDA and subsequently maintained on slants until use.

***In vitro* effects of endophytic bacteria on radial growth of *G. boninense* PER 71**

Two endophytic bacteria, *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3, were selected based on their high inhibition of *G. boninense* PER 71 in a dual culture assay (Zaiton *et al*., 2008). A mycelial plug from the edge of a 5-day-old PDA culture of *G. boninense* PER 71 was placed in the centre of a freshly prepared NA Petri dish. An aliquot (10 µl) of *P. aeruginosa* UPMP3 culture in LB liquid medium (adjusted to 10^8 CFU/ml) was streaked in two parallel straight lines, 6 cm apart, on the same NA plate. The same procedure was repeated for *B. cepacia* UPMB3. As a control, NA plates were streaked in a similar manner with sterile LB. All antagonistic test pairings were arranged in a completely randomized design (CRD) and incubated at 30°C in the dark with 10 replicates per pairing. The antagonistic potential of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 was assessed after 7-day incubation by measuring the radial growth of *G. boninense* PER 71 towards the endophytic bacteria (R_2). The results were transformed into percentage inhibition of radial growth (PIRG) in relation to the radial growth of *G. boninense* PER 71 in the control plate (R_1), using the formula of Jinantana and Sariah (1998):

$$\text{PIRG } (\%) = \frac{R_1 - R_2}{R_1} \times 100,$$

where R_1 and R_2 are the radii of the *G. boninense* PER 71 cultures in the control and dual culture plates, respectively.

The *in vitro* Bactoagar assay, described above for the effects of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 on AMF spore germination and hyphal length, was repeated with *G. boninense* PER 71. A mycelial plug from the edge of a 5-day-old PDA culture of *G. boninense* PER 71 was placed centrally in the Petri dish. Each *G. boninense* PER 71 and endophytic bacterial strain pairing was replicated 10 times in a CRD. After a 7-day incubation at 30°C in the dark, the radial growth of *G. boninense* PER 71 was determined.

SEM observations of mycoparasitic activity of endophytic bacteria on *G. boninense* PER 71

The hyphal interaction zones in the dual culture assays between *G. boninense* PER 71 and endophytic bacteria were carefully removed for observation by SEM (FEI Quanta 200) at Forest Research Institute of Malaysia (FRIM). The samples were fixed in 4% glutaraldehyde for 24 h at 4°C, washed in three changes of 0.1 M sodium cacodylate and post-fixed in 1% osmium tetraoxide for 2 h at 4°C. They were then washed in 0.1 M sodium cacodylate, dehydrated through an alcohol series to absolute alcohol and subjected to critical-point drying (CPD) using a Baltec 030. The samples were mounted on stubs, coated with gold and examined under a SEM with an acceleration of 5 kV.

Statistical analysis

The experiments were conducted in CRD. All data were submitted to analysis of variance (ANOVA) followed by Fisher's least significant difference test, with significant difference at $P \leq 0.05$, using SAS® Software. However, data in percentage (%) were Arcsine-Square Root transformed (Gomez and Gomez, 1984) and subjected to ANOVA with the means separated by Fisher's least significant difference test, with significant difference at $P \leq 0.05$.

Results

***In vitro* effect of endophytic bacteria on spore germination and hyphal length of AMF**

The germination rate of spores and hyphal length for both *G. intraradices* UT126 and *G. clarum* BR152B in the absence of endophytic bacterium were low (12% and 18% spore germination and average hyphal lengths of 0.76 mm and 0.87 mm, respectively). These values increased significantly in the presence of *P. aeruginosa* UPMP3 or *B. cepacia* UPMB3 (Table 1). Of the two bacterial strains, *B. cepacia* UPMB3 had a greater effect on both *G. intraradices* UT126 and *G. clarum* BR152B; this strain increased the spore germination rates to 50% and 62% and average hyphal lengths to 4.06 mm and 4.36 mm, respectively.

SEM analysis of AMF spores and endophytic bacteria

SEM revealed rod-shaped particles scattered on the surface of the spores (Fig. 1); these particles were absent from non-treated spores. The particles were either on the sloughed hyaline layer or degraded wall of the spores and appeared consistent with the shape of both *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3. *P. aeruginosa* UPMP3 was scattered on the surface of AMF spores of *G. intraradices* UT126 (Fig. 1B). Viewing and locating the rod-shaped particles on the young spores of both AMF species was difficult because the spore walls were rough and uneven, as seen in Figs. 1A and C. The endophytic bacteria were only found adhering to or embedded in the hyaline surface of spore walls that were partially eroded and their presence within the spores could not be confirmed. Spores that were incubated with only saline were consistently free of bacterial cells.

Table 1. Effects of the endophytic bacteria *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 on spore germination and hyphal length of *G. intraradices* UT126 and *G. clarum* BR152B

Treatment	Germination (%)[a]	Hyphal length (mm)[a]
UT126	12±4.42 a	0.76±0.33 a
BR152B	18±4.67 a	0.87±0.37 a
UT126+UPMP3	34±6.00 b	3.52±0.78 b
UT126+UPMB3	50±3.33 c	4.06±0.57 b
BR152B+UPMP3	48±6.80 bc	3.97±0.55 b
BR152B+UPMB3	62±3.06 c	4.36±0.67 b

[a] Values are Mean±Standard error of ten replications. Means in the same column with the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference test.

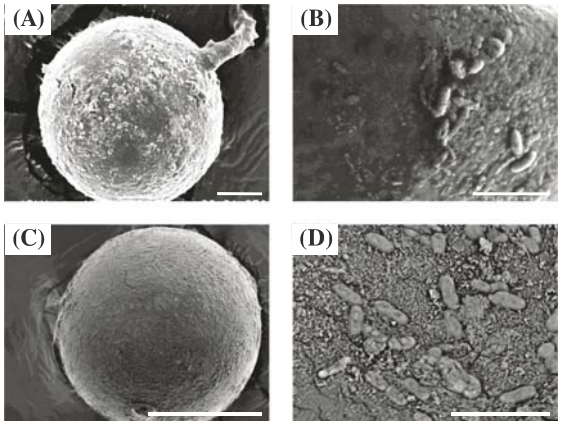

Fig. 1. Scanning electron microscope (SEM) images of spore surfaces of *G. intraradices* UT126 and *G. clarum* BR152B. (A) Young spores of *G. intraradices* UT126 with sloughed and eroded hyaline layer of spore wall. Scale bar=20 μm. (B) Higher magnification image of rod-shaped *P. aeruginosa* UPMP3 bacteria attached on eroded spore wall of *G. intraradices* UT126. Scale bar=5 μm. (C) Young spore of *G. clarum* BR152B with sloughed and eroded outer hyaline layer of spore wall. Scale bar=100 μm. (D) Rod-shaped *B. cepacia* UPMB3 bacteria found embedded on surface hyaline layer of *G. clarum* BR152B spore. Scale bar=5 μm.

Table 2. Effects of the endophytic bacteria *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 on radial growth of *G. boninense* PER 71

Treatment	Dual culture assay (%PIRG)[a]	Bactoagar assay (mm)[a]
PER 71 (control plate)	0 a	36.07±1.26 b
PER 71+UPMP3	86.12±0.82 c	0.66±0.05 a
PER 71+UPMB3	74.35±1.24 b	0.73±0.07 a

[a] Values are Mean±Standard error of ten replications. Means in the same column with the same letter are not significantly different (P≤0.05) according to Fisher's least significant difference test. PIRG, percentage inhibition of radial growth.

In vitro effect of endophytic bacteria on radial growth of *G. boninense* PER 71

Both *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 inhibited the growth of *G. boninense* PER 71 in the dual culture assay. There was a significant difference in the PIRG of *G. boninense* PER 71 for *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3, with values of 86.12% and 74.35%, respectively, compared with the growth of *G. boninense* PER 71 in the control plates without endophytic bacteria.

The Bactoagar assay (Pivato *et al*., 2009) using the endophytic bacterial strains revealed almost complete inhibition of *G. boninense* PER 71 radial growth after a 7-day incubation (Table 2). Less than 1 mm radial growth of *G. boninense* PER

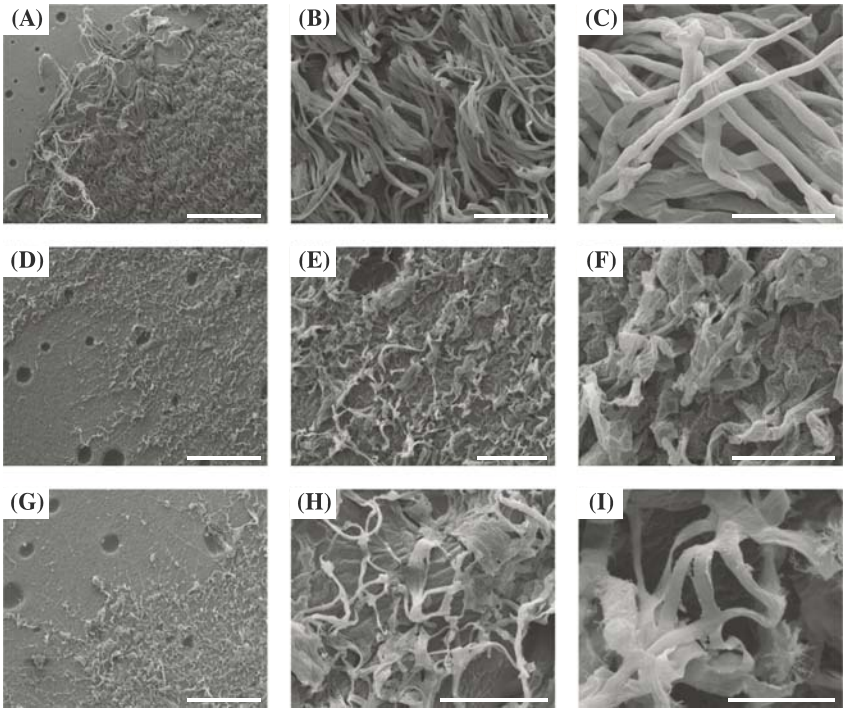

Fig. 2. Antagonistic effects of the endophytic bacteria *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 on *G. boninense* PER 71. (A), (B), and (C): Lower to higher magnification images, respectively, of the healthy and dense hyphal network of *G. boninense* PER 71 from a control plate cultured in the absence of endophytic bacteria. Scale bars=400, 50, and 20 μm, respectively. (D), (E), and (F): Lower to higher magnification images, respectively, of the highly deformed, distorted, and clumpy hyphal network of *G. boninense* PER 71 in the presence of *P. aeruginosa* UPMP3. Scale bars=200, 50, and 20 μm, respectively. (G), (H), and (I): Lower to higher magnification images, respectively, of the highly shrivelled and flattened hyphal network of *G. boninense* PER 71 in the presence of *B. cepacia* UPMB3. Scale bars: 200, 40, and 10 μm, respectively.

71 was observed under the light microscope (the measurement was carried out using the *i*Solution Lite, IMTechnology Inc.). On the contrary, the results of this assay on AMF by the endophytic bacterial strains promoted the germination and hyphal length of both AMF species (*G. intraradices* UT126 and *G. clarum* BR152B).

SEM analysis of mycoparasitic activity of endophytic bacteria on *G. boninense* PER 71

SEM images of *G. boninense* PER 71 prepared from the interaction zone of the dual culture plates showed severe morphological abnormalities in hyphal structure compared with the control (*G. boninense* PER 71 with no endophytic bacteria). Fig. 2C (control plate) shows a healthy, dense and branched hyphal network of *G. boninense* PER 71 that was free from abnormality. The network is thick and each hyphal strand appears healthy, with no deformity. The effects of *P. aeruginosa* UPMP3 (Figs. 2D, E, and F) and *B. cepacia* UPMB3 (Figs. 2G, H, and I) on the hyphal morphology of *G. boninense* PER 71 were observed. Treatment with these strains caused the hyphal structure to become highly deformed, flattened, disaggregated, and shrivelled to a looser mass (Figs. 2F and I). The hyphal network was uneven and clumpy, especially in Fig. 2F, probably due to lysis activity mediated by *P. aeruginosa* UPMP3. *B. cepacia* UPMB3 caused slightly different effects, with more shrivelling and flattening of the hyphae. The damage wrought on the hyphal network eventually inhibited the growth of *G. boninense* PER 71. This effect was also observed in a Bactoagar assay (Pivato *et al*., 2009) where radial growth of *G. boninense* PER 71 was highly inhibited when samples were treated with endophytic bacteria.

Discussion

The results from this study suggest that *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 have the potential to be used in combination with AMF as biological control agents. This conclusion is based on the following results: (i) these endophytic bacteria do not exhibit detrimental effects on the germination and hyphal length of AMF species *G. intraradices* UT126 and *G. clarum* BR152B; (ii) they behave as predicted for MHB association; and (iii) they have extremely adverse effects on the white wood rot pathogen *G. boninense* PER 71. The first part of the study assessed the effects of the endophytic bacteria on AMF growth *in vitro* and the second part of the study assessed the effects of the endophytic bacteria on *G. boninense* PER 71. The information from this study is important for the subsequent investigation of the potential use of these endophytic bacteria and AMF in combination as biological control agents against *G. boninense*. The study consisted of *in vitro* assessments of the presymbiotic compatibility of AMF spores and endophytic bacteria through Bactoagar assays and SEM imaging of AMF spores after 48 h of incubation with endophytic bacterial suspensions. The antagonistic effect of the endophytic bacteria was assessed via dual cultures, Bactoagar assays and SEM imaging of the *G. boninense* PER 71 mycelial network in the dual culture assay.

A presymbiotic effect was observed by *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 where they promoted AMF spore germination and hyphal length significantly ($P \leq 0.05$). The effects of the bacterial strains on the AMF spores were consistent with those of MHB, which are defined as "bacteria associated with mycorrhizal roots and mycorrhizal fungi that selectively promote the establishment of mycorrhizal symbiosis" (Garbaye, 1994). Neither *P. aeruginosa* UPMP3 nor *B. cepacia* UPMB3 has been previously reported as MHB. This MHB-like role was clear based on the significantly lower ($P \leq 0.05$) spore germination and hyphal length recorded *in vitro* in the absence of the endophytic bacteria. Incubation with *B. cepacia* UPMB3 resulted in the highest germination and hyphal length for both AMF species. Previous reports have indicated that indigenous bacterial groups preferentially associated with AMF symbiosis belong to the order Burkholderiales (Bianciotto and Bonfante, 2002; Offre *et al*., 2007). Bacterial promotion of *in vitro* and *in vivo* mycorrhization was also found to be specific and induced by a limited number of bacteria in work carried out by Pivato *et al.* (2009), where the compatibility of eight bacterial strains was tested against two AMF species, *G. mosseae* and *Gigaspora rosea*. When AMF spores in the current study were submerged in bacterial suspensions for 48 h, SEM analysis revealed no evidence of deformation of the AMF spores post-incubation. Rod-shaped bacteria were found scattered on the spore walls, especially on the older spores with a thin hyaline layer. Previously, other authors (Macdonald and Chandler, 1981; Roesti *et al*., 2005) suggested that the bacteria associated with the spore wall of AMF were possibly feeding on the surface hyaline layer. The saprophytic activity of these bacteria on the hyaline layer is suggested based on the fact that the hyaline layer is composed mainly of chitin, a straight-chain polymer of N-acetylglucosamine (Sbrana *et al*., 1995). We were unable to confirm the presence of bacterial cells within AMF spores.

Bacterial groups associated with AMF have been isolated by many researchers, who found that the association is beneficial for mycorrhization and eventually leads to health and growth improvements for host plants (Gamalero *et al*., 2002; Selim *et al*., 2005; Hildebrandt *et al*., 2006; Offre *et al*., 2007; Pivato *et al*., 2009). One of the early works by Mosse (1962) found that some MHB's culture filtrates were able to stimulate spore germination of *G. mosseae*. A more recent work by Xavier and Germida (2003) reported that direct contact between spores and bacteria was necessary for the induction of spore germination in *G. clarum*, possibly indicating a ligand-receptor interaction between the two microbes. As reviewed by Garbaye (1994) and Frey-Klett *et al.* (2007), mycorrhizal promotion by MHB is at least partly ascribed to the stimulation of presymbiotic fungal growth, which will eventually lead to an increase in root-fungus contacts and colonization.

The antagonistic activity of the endophytic bacterial strains on *G. boninense* PER 71 was clearly demonstrated via the dual culture assay. This antagonistic effect was confirmed through the use of the same Bactoagar assay performed on the AMF (Pivato *et al*., 2009). SEM analysis of the interaction zone in the dual culture assay between the endophytic bacteria and *G. boninense* PER 71 detected severe morphological abnormalities, with shrivelling and distortion of the fungal hyphae; we hypothesise that these abnormalities were mediated by antibiosis due to the lytic activity of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3. Many reports have suggested that the antagonistic effect, whether *in vitro* or *in vivo*, involves

mechanisms such as competition for iron through siderophore production, which limits the iron availability to the pathogen, production of antibiotics and secretion of lytic enzymes (Siddiqui, 2005; Van Loon and Bakker, 2005). In addition to these mechanisms, there is also the release of volatile non-specific inhibitors, such as hydrogen cyanide (HCN), enzymes and phytohormones, which hamper the activity of pathogenic microorganisms (Schippers *et al*., 1991; Gupta *et al*., 2001). Members of the *Pseudomanas* genus are known to restrict pathogens by producing metabolites with antimicrobial activity (Chen *et al*., 2000) and *B. cepacia* has been reported to produce several potential compounds for biocontrol, such as pyrrolnitrin and pyoluteolin (Lieven *et al*., 1989).

Although they positively stimulate AMF spores, an adverse effect was clearly observed when the endophytic bacterial strains were plated together with *G. boninense.* The possible antifungal compounds produced by *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 that caused deleterious effects on *G. boninense* were ineffective against the AMF spores; instead, these bacteria promoted AMF spore germination and increased hyphal length. A similar observation was reported by Budi *et al.* (1999), who found that *Paenibacillus* sp., which has antagonistic activity against the pathogenic fungus *Phytophora parasitica*, was inactive against *G. mosseae* and even stimulated its development within roots. This hypothesis was critically examined by Selim *et al.* (2005), who determined that the antagonistic factor of *Paenibacillus* sp. was caused by three peptides separated by HPLC. The three peptides caused the antagonistic activity against other bacteria and pathogenic soil-borne fungi but were inactive against the mycorrhizal fungus *G. mosseae*. Barea *et al.* (1998) also reported that a *Pseudomonas* strain producing the antifungal metabolite 2, 4-diacetylphloroglucinol (DAPG) stimulated mycorrhizal formation of *G. mosseae.* Frey-Klett *et al.* (2007) suggested that MHB could have evolved with respect to mechanisms of interaction with their microbial surroundings, having neutral or positive effects on their host mycorrhizal association but negative effects on root pathogens that might threaten their habitat. Specialised activities such as the production of vitamins, amino acids, and hormones may be operating in microbe-microbe interactions and may account for the stimulatory effects (Barea *et al*., 1998).

The *in vitro* promotion of AMF spore germination and hyphal length by endophytic bacteria occurred despite the fact that the AMF species used in this study was not indigenous. The selection of AMF was made from *Glomus* sp. (*G. intraradices* UT126 and *G. clarum* BR152B) because previous bioprospecting of naturally existing AMF species in the oil palm rhizosphere indicated that AMF are extensively present in the oil palm rhizopshere, with the highest number of species recorded from the genera Glomus, followed by Gigaspora and Acaulospora (Nadarajah, 1980; Blal and Gianizazzi-Pearson, 1990; Siti Ramlah and Tayeb, 1991). The *in vitro* assessment of AMF established a presymbiotic phase with the endophytic bacteria, which was necessary before carrying out the *in vivo* assessment in the future. Although the endophytic bacterial strains mimic an MHB association, the complexity of the AMF-plant symbiosis warrants an *in vivo* investigation to confirm the level of mycorrhization and growth-promoting effects in the presence and absence of these endophytic bacterial strains in the oil palm rhizosphere. Further studies are also required to identify the compounds produced by the endophytic bacteria. Nevertheless, the use of these endophytic bacterial strains as biocontrol agents to increase plant protection in combination with AMF opens up the possibility of using dual bacteria-fungal inoculation as a promising sustainable approach for controlling basal stem rot of oil palm.

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