Bacillus spp: A PROMISING GRAM-POSITIVE ENDOPHYTIC BACTERIA FOR *Ganoderma* CONTROL

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This technology transfer offers the potential use of an endophytic gram-positive bacteria; *Bacillus* spp. as an active ingredient in the development of biopesticide against *Ganoderma* spp. This is because one of the biggest constraints in oil palm productivity is the challenges faced by pest and diseases (P&D) and among the P&D issues, basal stem rot (BSR) caused by *Ganoderma* spp. is of economic importance with incidences increasing over the years. The disease is being managed by a set of activities in the integrated *Ganoderma* management (IGM) that includes selected short and long-term approaches. With sustainability development goals (SDG) encouraging green approaches, biological control agents (BCA) have been actively investigated with promising results against this disease. These biological control studies include endophytic microorganisms (Zaiton *et al.*, 2006; Sundram *et al.*, 2013). Endophytic microorganisms as BCA in the field of plant protection is relatively a new field of study. Endophytic microorganisms reside internally in the plant system (Fisher *et al.*, 1992) and these microorganisms are usually from the members of prokaryotic and fungal kingdom.

Bacterial and fungal endophytes such as *Serratia* spp., *Burkholderia* spp., *Pseudomonas* spp., *Bacillus* spp., and *Fusarium* spp. have been found to induce systemic resistance in plants and show biological traits such as antibiotic activity and lysis (Adeline *et al.*, 2005; Zaiton *et al.*, 2008; Sundram *et al.*, 2011; Ramli *et al.*, 2016). This study investigates the potentially beneficial endophytic bacteria (EB) residing within the oil palm that is able to help palms thrive for survival by providing protection against P&D damages. Due to the potential hazard that can be inflicted by gram-negative bacterial cultures, this study focused on the isolation of gram-positive cultures as biocontrol agents against *G. boninense* due to their diverse biological activities (Zhao *et al.*, 2011; Khaksar *et al.*, 2016; Egamberdieva *et al.*, 2017; Li *et al.*, 2018; Deng *et al.*, 2019). Therefore, this technology describes the isolation of gram-positive EB from root tissues of the oil palm, its antagonistic activity and disease suppression on *Ganoderma boninense*, the causal fungus of BSR disease through *in vitro* assessments (applied and molecular) and nursery trials.

ISOLATION, *In Vitro* SCREENING AND EFFECTS OF GRAM-POSITIVE ENDOPHYTIC BACTERIA AGAINST *Ganoderma boninense* IN NURSERY TRIALS

The technology was first established by isolating a total of 95 strains of EB strains from oil palm root isolation. The bacterial isolates were differentiated microscopically through morphological characteristics. Dual culture assay further segregated 41 isolates according to percentage of radial inhibition (PIRG) ranging between 30 and 83%. From these 41 isolates, 13 isolates were subjected to the 16S rRNA gene-based similarity for their identification and the result is shown in *Table 1* below.

Four strains were shortlisted (*Table 1*), from which 3 were gram-positive (2 *Bacillus cereus* EB2, EB14 and *B. altitudinis* EB39) with one strain representing the gram-negative strain (*Pseudomonas aeruginosa* EB35). The strains were screened for biological control characteristics: secretion of secondary compounds (dual culture and poison food agar test), antibiotics production and iron chelating siderophores. Among the four bacterial strains, *P. aeruginosa* EB35 was able to significantly suppress *G. boninense* PER71 at the incorporation of 20%, whereas others showed hyphal growth suppression only at 40% (*Figure 1*). *Table 2* shows the results of the poison agar assay. All the bacterial strains were able to inhibit the mycelial growth of *G. boninense* PER 71 in the dual culture assay. Based

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on the plate assay of poison agar test, EB35 was noted secreting potent secondary metabolites or antibiotic production as the hyphal inhibition was noted in the 20% concentration of the agar while the remaining strains were still showing hyphal growth at 20%.

TABLE 1. IDENTIFICATION OF ENDOPHYTIC BACTERIA WITH 16S rRNA CORRESPONDING TO PIRG VALUE AGAINST *G. boninense*

No	Bacteria	Species	PIRG (%)
1	EB4	*Pseudomonas aeruginosa*	83.33
2	EB2*	*Bacillus cereus*	81.33
3	EB7	*Pseudomonas aeruginosa*	80.00
4	EB8	*Pseudomonas aeruginosa*	80.00
5	EB9	*Pseudomonas aeruginosa*	80.00
6	EB35	*Pseudomonas aeruginosa*	77.33
7	EB1	*Pseudomonas aeruginosa*	77.30
8	EB6	*Pseudomonas aeruginosa*	77.00
9	EB36	*Serratia marcescens*	74.67
10	EB39*	*Bacillus altitudinis*	76.00
11	EB46	*Pseudomonas aeruginosa*	76.00
12	EB18	*Pseudomonas putida*	76.00
13	EB14*	*Bacillus cereus*	75.33

Note: PIRG-percentage inhibition wof radial growth. *shortlisted gram-positive strains.

The effects of bacterial culture filtrate on the hyphal morphology and ultrastructure of *G. boninense* PER71 were investigated by using scanning electron microscope (SEM). Morphological deformation such as wilting, shrivelling and lysis of hyphal walls of *G. boninense* were observed through scanning electron microscopy (SEM) of the poison agar cultures (*Figure 2*), indicating all the strains possess mycoparasitic characteristics and secondary metabolite/antibiotic production. Siderophores for all three gram-positive strains were almost similar to each other while the gram-negative strain EB35 gave the best siderophore production as expected. The EB strains were also tested for their phosphate solubilising ability, followed by antibiotic gene detection using PCR-based analysis. The PCR-based detection found B. *altitudinis* EB39 and *P. aeruginosa* EB35 acquiring three and two of antibiotic genes, respectively, while *B. cereus* EB2 was detected with one antibiotic gene (unpublished data).

The selected strains were then subjected to nursery studies to assess their efficacy as BCA and as vegetative enhancers in the natural environment. The study found *B. cereus* EB2 and *B. altitudinis* EB39 to be the best candidates in disease reduction with 68% and 66% (*Figure 3*) compared to the control seedlings without any treatment at 45.20% disease severity index (DSI). The same *Bacillus* spp. also gave notably higher mean for most of the vegetative growth parameters (unpublished data). The most notable and potential EB strains among the gram-positive have been demonstrated by EB2, *B. cereus* and *B. altitudinis*, EB39.

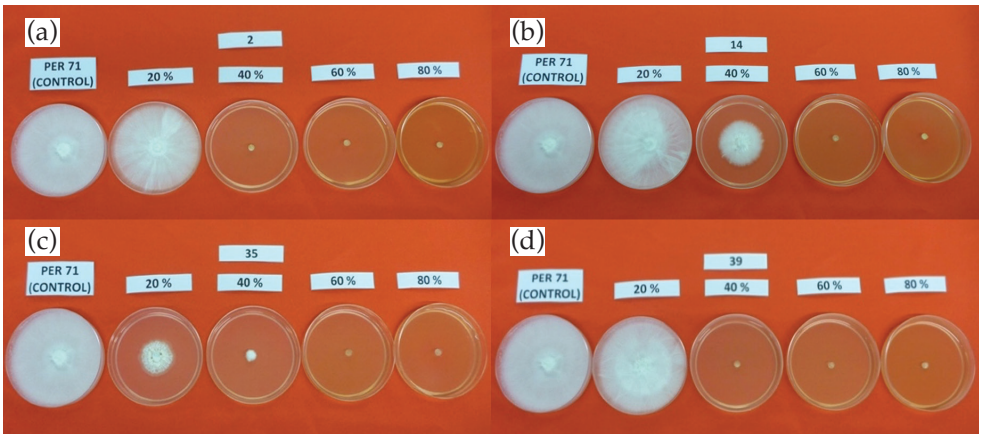
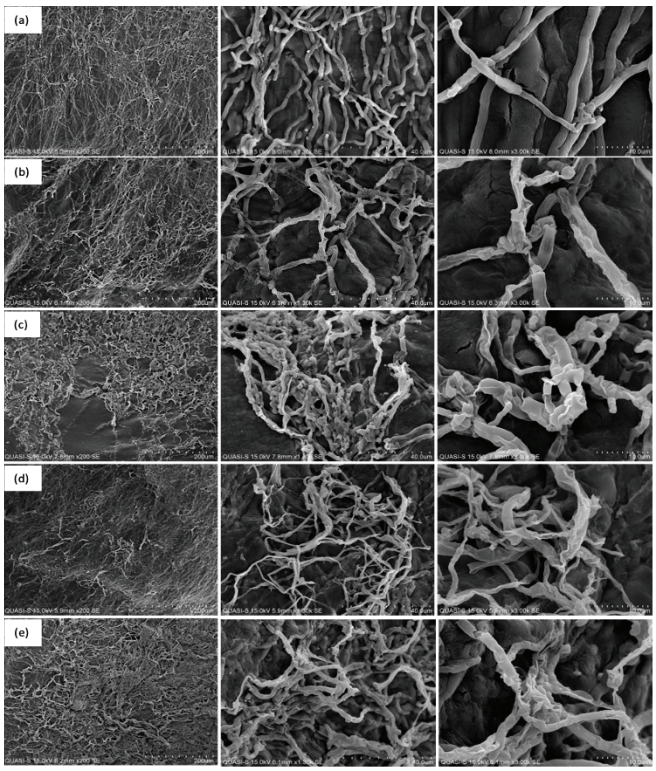


Figure 1. Percentage inhibition of radial growth (PIRG) of Ganoderma boninense *PER71 in endophytic bacteria poisoned PDA media. (a)* Bacillus cereus *EB2; (b)* B. cereus *EB14; (c)* Pseudomonas aeruginosa *EB35; (d)* B. altitudinis *EB39.*

TABLE 2. PERCENTAGE INHIBITION OF RADIAL GROWTH (PIRG) ON *Ganoderma boninense* PER71 IN ENDOPHYTIC BACTERIA POISON AGAR MEDIA

Bacteria	Concentration of bacteria (%) in PDA			
	20	40	60	80
EB2	0.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
EB14	0.00 ± 0.00	58.65 ± 1.89	100.00 ± 0.00	100.00 ± 0.00
EB35	42.00 ± 1.60	96.23 ± 0.58	100.00 ± 0.00	100.00 ± 0.00
EB39	0.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Note: The values represent mean ± standard error. EB2 & EB14: *Bacillus cereus*; EB35; *Pseudomonas aeruginosa* and EB39: *B. altitudinis*.



Note: Same strain in a row, all images were from lower to higher magnification (left to right), scale bar = 200 μm, 40 μm, 10 μm, respectively.

Figure 2. Effect of the culture filtrate of endophytic bacteria on the morphology and ultrastructure of Ganoderma boninense *observing severe morphological deformation; wilting, shrivelling and lysis of hyphal walls: (a) negative control (arrow showing clamp connection of hyphae); (b)* Bacillus cereus *EB2; (c)* B. cereus *EB14; (d)* Pseudomonas aeruginosa *EB35; (e)* B. altitudinis *EB39.*

CONCLUSION

B. cereus EB2 and *B. altitudinis* EB39, both gram-positive bacterial spp. were identified as promising BCAs for further exploration in the management of *Ganoderma* BSR in oil palm. These gram-positive *Bacillus* spp. strains are offered as technology transfer to be fortified into a biopesticide for the control of *Ganoderma*.

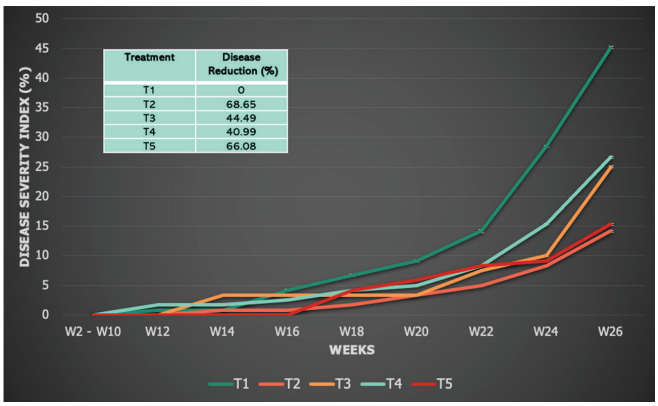


Figure 3. The disease reduction (%) of the oil palm seedlings treated with endophytic bacteria based on disease severity index (DSI) for T1: control; T2: Bacillus cereus *EB2; T3:* B. cereus *EB14; T4:* Pseudomonas aeruginosa *EB35 and T5:* B. altitudinis *EB39 whereby the values represent the mean of 20 replicates.*

BENEFITS OF THE TECHNOLOGY

The use of gram-positive bacteria as a BCA in agriculture shows advantageous potential because gram-positive bacteria is generally safer than gram-negative bacteria while the spore forming characteristic of *Bacillus* species lends a longevity advantage to its use as an agricultural agent especially for the development of formulation and mass production of biopesticide product of oil palm BSR disease.

PROSPECTUS CLIENTS AND ECONOMIC ANALYSIS

This technology will be valuable for interested clients in developing a biocontrol product against oil palm BSR disease. The breakdown of economic analysis is as shown in *Table 3* below.

TABLE 3. ECONOMIC ANALYSIS OF ENDOPHYTIC BACTERIA MASS PRODUCTION

Economic Analysis	Value
Net present value (NPV)	RM5.5 million
Internal rate of return (IRR)	20.39%
Payback period	3.7 years
Benefit cost ratio (B:C)	1.06

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