Mycolytic Enzyme-Producing Bacteria Demonstrates Antifungal Activities Against Basal Stem Rot Disease Caused by *Ganoderma boninense*

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ABSTRACT The basal stem rot (BSR) disease of oil palm (*Elaeis guineensis* Jacq.) is caused by the white rot fungus, *Ganoderma boninense* (*G. boninense*). This study discusses the use of a biological control approach to treat BSR by using mycolytic enzymes producing bacteria as biocontrol agents against *G. boninense*. Bacteria producing mycolytic enzymes which degrade fungal cell wall were targeted. The antifungal properties of *Acinetobactor calc*oaceticus (*A. calcoaceticus*), *Chryseobacterium indologenes* (*C. indologenes*), and *Pseudomonas putida* (*P. putida*) were tested against *G. boninense*. The three strains showed the ability to inhibit the growth of *G. boninense* in dual culture test, culture filtrate test, double plate assay, and soft agar encapsulation. In dual culture test, all three test strains showed high Percentage Inhibition of Diameter Growth (PIDG) value with P. putida having the highest PIDG value of approximately 90%. As for culture filtrate test, *C. indologenes* demonstrated the highest PIDG value, approximately 85%. Double plate assay and soft agar encapsulation depicted the similar result for all three test strains which the PIDG value for both tests were 90%. The isolated strains exhibited promising results in anti-*Ganoderma* testing.

KEYWORDS: Basal stem rot, *Ganoderma boninense*, mycolytic enzymes, biological control, percentage inhibition of diameter growth (PIDG)
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INTRODUCTION

The production of palm oil has declined in recent years due to the presence of basal stem rot (BSR) disease. BSR is a type of phytopathogenic fungal disease caused by *Ganoderma boninense* (*G. boninense*). In this study, biological control was proposed to suppress the spread of the disease in the plantation as it is a natural way with limited effects towards humans and the environment as opposed to use of the chemical approach (San-Lang *et al.*, 2002). Apart from that, study also exhibits that the application of biological control has indirectly promoted plant growth and improve their yield of production (Kiewnick & Sikora, 2006).

The application of microbial biological control agents (MBCAs) is the most common approach in plant disease management. They behave in different modes of action. Some of them induce resistance of plants without the direct interaction with pathogen while the others compete for nutrients or modulating the growth conditions for pathogens (Kohl *et al.*, 2019). Antagonists interacts directly with the pathogen through antibiosis by producing secondary metabolites with inhibitory effect against phytopathogens (Raaijmakers & Mazzola, 2012).

In this study, three mycolytic enzyme producing bacteria, *Pseudomonas putida* (*P. putida*), *Chryseobacterium indologenes* (*C. indologenenes*), and *Acinetobacter calcoaceticus* (*A. calcoaceticus*) from Wee *et al.* (2021) were evaluated for their potentials as biocontrol control against *G. boninense*. The genus of *Bacillus* and *Pseudomonas* have been widely applied as antagonists in rhizosphere (Mhatre *et*

al., 2018). According to Azadeh *et al.* (2009), *Pseudomonas aeruginosa* isolated from the rhizosphere of oil palm plantation was found to have potential to become biocontrol agent against *G. boninense*.

Liu *et al.* (2007) depicted that *Acinetobacter baumannii* (*A. baumannii*) inhibited the mycelial growth of five plant phytopathogenic fungi, such as, *Botrytis cinerea*, *Cryphonectria parasitica*, *Fusarium graminearum*, *Glomerella glycines* and *Phytophthora capsici*. Isomers of iturin A, namely iturin A2, iturin A3 and iturin A6 were the antifungal metabolites produced by *A. baumannii*. These bioactive compounds can be an alternative source in plant disease management.

In addition, certain *Chryseobacterium* spp. could also show biocontrol activity against soil borne plant pathogens and promote plant growth (Sang *et al.*, 2013). *Chryseobacterium* spp. significantly reduced the severity in a 5 week old pepper plants with Phytophthora blight caused by *Phytophthora capsici* (Sang *et al.*, 2018).

Besides that, volatile organic compounds (VOCs) play a key role in antagonistic interactions between microbes in soil, as well as the inhibition of fungi by bacteria (Garbeva *et al.*, 2014). In this study, VOCs from mycolytic enzymes producing bacteria were also targeted and evaluated for their effectiveness in inhibiting *G. boninense*. VOCs can easily reach the target organism due to their low boiling point, high vapour pressure and low molecular weight by diffusing through both air- filled pores and liquid in soil. This characteristic enables VOCs to diffuse over a distance where the interactions between microbes can take place (Effmert *et al.*, 2012).

The aim of this study was to evaluate the biological control potentials of mycolytic enzymes producing bacteria isolates, *P. putida*, *C. indologenes*, and *A. calcoaceticus* against the phytopathogenic fungus, *G. boninense.*

METHODOLOGY

Dual Culture Test

Dual culture plate assay was adopted in this study (Elmahdi *et al.*, 2015). The bacteria strains were firstly streaked onto sterilised nutrient agar (NA) (Himedia, Mumbai, India) plates and then the 5-days old *G. boninense* mycelial plug was placed on the centre of the same plate. *G. boninense* used in this study identifies to *Ganoderma boninense* Pat. (ATCC 204074). The plates were incubated in the incubation room for 7 days at 35± 2ºC. The microbial inhibition was quantified on day 7 by measuring the diameter of the *G. boninense* plug of test plates in relation to the diameter of the negative control as per Himratul-Aznita *et al.* (2011). The equation of PIDG is expressed as:

$$\text{Percentage Inhibition of Diameter Growth (PIDG)} = [C\text{-}T]/C \times 100 \quad (1)$$

where C is the diameter growth of *G. boninense* in the control plate and T is the diameter growth in the test plate.

Culture Filtrate Test

For culture filtrate test, the filtrate of bacteria strains was extracted based on Elmahdi *et al.* (2015). Briefly, the 3 days culture broth (Nutrient broth at 35 ± 2 °C) (Himedia, Mumbai, India) was centrifuged at 10, 000 rpm for 10 min at 4 °C and the supernatant was filtered through a 0.22µm membrane filter (Sartorius Stedim Biotech, Goettingen, Germany). The filtrate collected were incorporated into sterilized PDA 20% (v/v) concentration; 20 ml of the amended agars were poured into each 90mm petri plate (Favorit, Malaysia) and allowed to solidify. *G. boninense* mycelial plug

was centrally placed on the solidified agar. Non-amended PDA (Himedia, Mumbai, India) was used as negative control. The diameter of the mycelial growth of *G. boninense* was measured on day 5. The antagonistic activity was expressed as PIDG in relative to the mycelia growth of *G. boninense* in the negative control.

Double Plate Assay

The antagonistic activities of mycolytic enzyme-producing bacteria against *G. boninense* was evaluated by double-plate assay (Fernando *et al.*, 2005). The test strain was streaked onto a sterilised NA plate while a 5-days old mycelial plug of *G. boninense* was placed onto the centre of another sterilised NA plate. The two plates were sealed together and incubated in the incubation room at 35± 2ºC for 7 days. The measurements were taken on day 7. The results were expressed in PIDG relative to the mycelia growth of *G. boninense* in the negative control.

Soft Agar Encapsulation

Soft agar overlay technique following Fankhauser (1994) was adopted to evaluate the *in vitro* antifungal activities of bacteria strains against plant pathogenic fungi, *G. boninense*. The test strains were inoculated to melted 0.75% NA and poured onto a thin layer of NA plate. The mycelial plug of 5-days old *G. boninense* was placed in the centre of the plate. The non- amended 0.75% of NA was used as control. Again, the results were expressed in PIDG relative to the mycelia growth of the negative control.

RESULT AND DISCUSSION

Dual Culture Test

Based on Table 1, it showed that *P. putida*, *C. indologenes*, and *A. calcoaceticus* had high PIDG rate. The PIDG for *P. putida* showed the highest PIDG amongst all isolated strains which was 88.9%, followed by *C. indologenes*, and *A. calcoaceticus* which were 88.0% and 77.0% respectively.

Pseudomonas spp. has been reported to act as biocontrol agents in previous studies. The modes of action involve the production of antibiotic (iturin, surfactin, and fengycin), the secretion of the mycolytic enzymes (chitinases, glucanases, and proteases) that have the capabilities to degrade fungi cell wall, and also volatile compounds that possess antifungal or antimicrobial properties (Arrebola *et al.*, 2010). Furthermore, *P. putida* was also found to inhibit the mycelial growth of soil borne fungal pathogen, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Ceratocystis fimbriata* (Saritha *et al.*, 2015).

Study conducted by Zhao *et al.* (2018) stated that *Enterobacter*, *Acinetobactor*, *Pseudomonas*, *Ochrobactrum* and *Bacillus* have the inhibition activities of more than 63% against the pathogenic fungus, *Phytophthora sojae*. *A. calcoacetics* demonstrated the highest inhibitory rate which was 71.14% against *P. sojae*. Such inhibiton caused the morphological abnormal changes of fungal mycelia, which include fracture, lysis, formation of a protoplast ball at the end of hyphae and split ends. Besides, acinetobactin- like siderophore produced by *A. calcoaceticus* mediated the inhibition of mycelium growth of *F. oxysporum* under iron limited conditions (Maindad *et al.*, 2014).

Chryseobacterium spp. had been found to effectively control *Phytophthora capsici* in a dual culture test (Yang *et al.*, 2012). *Chryseobacterium* spp. also significantly reduced the severity of Phytophthora blight caused by *P. capsici* in 5-weeks old pepper plants compared to the inoculated plants with $MgSO_4$ solution.

Culture Filtrate Test

The evaluation of secondary metabolites produced by the test strains was performed by culture filtrate test. The results indicated that *C. indologenes*, *P. putida,* and *A. calcoaceticus* possess anti-*Ganoderma* properties by secreting extracellular metabolites which inhibited the growth of *G. boninense*. According to Table 1, *C. indologenes* exhibited the highest PIDG value, 85.0 ± 4.0% followed by *P. putida*, and *A. calcoaceticus* with PIDG value, 56.0 ± 4.0% and 35.0 ± 4.0% respectively. From Table 1, there was a big difference of PIDG value of *P. putida* and *A. calcoaceticus* in culture filtrate test compared to dual culture assay. It may be caused by the discontinuous secretion of secondary metabolites from the bacteria. In addition, different growth rates of bacteria could be one of the reasons of low PIDG were obtained in comparison to the dual culture method as the formation of secondary metabolites is regulated by enzyme induction, feedback control, nutrients, enzyme inactivation, and growth rate (Davati & Najafi, 2013).

Double Plate Assay

Double plate assay was done to access the effectiveness of bacterial volatile compounds by test strains against *G. boninense*. Based on Table 1, the results suggested that all three bacteria did produce volatile compounds in inhibiting the mycelial growth of *G. boninense*. *P. putida* showed the highest percentage inhibition of *G. boninense* mycelial growth by 92.0 ± 0.4% while *A. calcoaceticus,* and *C. indologenes* showed 89.0 ± 3.0% and 90.2 ± 0.3% respectively. It was previously noted that *Pseudomonas* spp. does produce antifungal volatile compounds, such as cyclohexanol, dimethyl trisulfide, nonanal, benzothiazole, n-decanal and 2-ethyl-1-hexanol that inhibit sclerotium formation in *Sclerotinia sclerotiorum* (Fernando *et al.*, 2005). Antifungal volatile compounds produced by *C. indologenes* was found to have strong inhibition on plant pathogenic fungi, such as *Fusarium oxysporum*, *Fusarium culmorum*, *Verticillium albo- atrum*, *Mucor hiemalis*, *Chaetomium* sp., *Pythium ultimum* and *Rhizoctonia solani* (Garbeva *et al.*, 2014). In addition, volatile organic compounds (VOCs) produced by *Acinetobacter* spp. inhibited the mycelial growth of *P. capsici* (Syed-Ab-Rahman *et al.*, 2019).

Soft Agar Encapsulation

Based on Table 1, the 3 tested strains exhibited promising results. *A. calcoaceticus*, *C. indologenes,* and *P. putida* had the same PIDG value of 91.0 ± 0.2%, 90.9 ± 0.5% and 90.9 ± 0.5% respectively. This indicated that the microbes were able to secrete enough mycolytic enzymes when the conditions are suitable for growth.

Table 1. Percentage Inhibition of Diameter Growth (PIDG) values of three tested strains against *G. boninense* in four different experiments with their standard deviations.

Assay	PIDG, % Test strain		
	A. calcoaceticus	***C. indologenes***	***P. putida***
Dual culture test	77.0 ± 4.0	88.0 ± 4.0	88.9 ± 0.8
Culture filtrate test	35.0 ± 4.0	85.0 ± 4.0	56.0 ± 4.0
Double plate assay	89.0 ± 3.0	90.2 ± 0.3	92.0 ± 0.4
Soft agar encapsulation	91.0 ± 0.2	90.9 ± 0.5	90.9 ± 0.5

CONCLUSION

Due to limitations of the control methods to combat the BSR disease, an eco-friendly approach was explored to control this phytopathogen. The three bacterial strains, *P. putida*, *C. indologenes*, and *A. calcoaceticus* isolated from Sarawak's soil samples that are able to produce mycolytic enzymes demonstrated their anti-fungal properties against phytopathogenic fungus, *G. boninense*. The findings obtained in this study describes the promising results of the use of the mycolytic enzyme producing bacteria as biological control agents towards *G. boninense*. Further study can be explored to identify the volatile compounds and secondary metabolites produced by these bacteria strains. In addition, pot trial is also suggested for further evaluation on the effectiveness of mycolytic enzyme producing bacteria in oil palm plantation settings.

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