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Proficiency of biocontrol agents as plant growth promoters and hydrolytic enzyme producers in *Ganoderma boninense* infected oil palm seedlings*

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ABSTRACT

Basal stem rot (BSR) is a major disease encountered by Malaysian oil palm caused by *Ganoderma* species. *Pseudomonas aeruginosa* has been shown to improve plant growth and is classified as a Plant Growth Promoter Bacterium (PGPB) while *Trichoderma* species has been reported as the most common biocontrol agents (BCAs) of oil palm rhizosphere. Therefore, based on preliminary trials *P. aeruginosa* (UPM P3) and *Trichoderma asperellum* (UPM29) were selected as BCAs to control *Ganoderma* infection in oil palm. Both BCAs were screened for their antagonistic properties against *G. boninense* (UPM13), plant growth promoting traits and enzymatic activities. The result of dual culture test demonstrated that *P. aeruginosa* and *T. asperellum* were able to inhibit *G. boninense* growth with the percentage of inhibition radial growth (PIRG) values of 71.42% and 76.85%, respectively. Besides that, both showed positive results for phosphate solubilizing activity and indole acetic acid (IAA) production. However for siderophore production test, only *T. asperellum* exhibited positive siderophore production. These BCAs were also tested for their ability inproducing hydrolytic enzymes such as chitinase, cellulose, and 1, 3, β-glucanase.

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a monocotyledon from the family Arecaceae (formerly Palmae) within the subfamily Cocosoideae [1]. It is a major crop that grows in the tropical areas mainly in Southeast Asia. Palm oil is used worldwide for processing food, cosmetics, pharmaceuticals, biodiesel and in oleo chemical industry [1–3]. Oil palm industry contributes to the Malaysian economy and in the development of the country's rural areas [4]. In Malaysia, the cultivation of oil palm has increased from 1.5 million hectares (ha) in the year 1985 to 5.74 million ha in 2016 [5].

However, oil palm has been subjected to numerous devastating diseases such as basal stem rot (BSR) [6], vascular wilt [7] spear rot [1] sudden wither and red ring [8]. Several attempts have been made to control BSR using various control methods such as cultural techniques, mechanical and chemical control. Nevertheless, all these techniques have failed to control the disease successfully. This problem may be due to the characteristic of the *G. boninense,* one of the species that causes the BSR disease which is classified as a soil borne pathogen, thus causing fungicide to be ineffective due to the degradation in the soil before they could reach their target [9]. In addition, *G. boninense* has many forms of resting stages including resistance mycelium, basidiospores, chlamydospores, and pseudosclerotia. In order to combat these characteristics, the best approach to control BSR disease could be biological control and the utilisation of the resistant palms. Though, breeding for resistance is a long term procedure, conversely biological control could be developed over a short time scale.

Some endophytic bacteria have been classified as plant growth-promoting bacteria (PGPB). *Pseudomonas aeruginosa* has been shown to improve plant growth and classified as a PGPB. It is known to synthesise 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 [10–12]. A study conducted by Zaiton et al. [13] reported that *P. aeruginosa* significantly increased the seedling plant growth and root mass compared to *Bukholderia cepacia. P. aeruginosa* is more effective in controlling *Ganoderma* compared to *B. cepacia*. In addition, this study was supported by *in vitro* activities conducted by Bivi et al. [14], which suggested that *P. aeruginosa* could play a role as an BCA against *G. boninense.* Besides that, the most common biocontrol agents of oil palm rhizosphere are *Trichoderma* spp. [9].

Most of the studies on biological control of plant pathogens dealt

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with single BCA applied as an antagonist to a single pathogen. However, application of solitary BCAs may not be effective in all soils, as environmental requirements of differing microorganisms will vary. A combination of biocontrol agents may offer advantages over a single agent in suppressing pathogens [15–17]. To improve biocontrol efficacies achieved through the use of a single BCA, there has been increasing interest recently among researchers in using the mixtures of BCAs. In addition, in most cases of naturally occurring biological control probably resulted from mixed antagonist population rather than high numbers of single antagonist. Mixtures of antagonists are more stable and have a wider spectrum of activity as well as the ability to enhance efficacy and reliability of biological control [18]. Moreover, a range of biocontrol mechanisms may operate in mixed BCA populations [19–22]. Therefore, this present study was conducted to screen the selected biocontrol agents against *G. boninense in vitro.*

2. Materials and method

2.1. Stock culture

G. boninense (UPM13) and *T. asperellum* (UPM29) culture were obtained from the culture collection of the Department of Plant Protection, Faculty of Agriculture, University Putra Malaysia; Selangor, Malaysia. *P. aeruginosa* (UPMP3) culture was purchased from the Institute of Bioscience (IBS) University Putra Malaysia, Selangor, Malaysia. All fungus and bacteria cultures were maintained on Potato Dextrose Agar (PDA) (Difco ™) and Nutrient Agar (NA) (Difco ™) respectively, until further use.

2.2. Preliminary in vitro screening for antagonistic activity against Ganoderma boninense (UPM13)

2.2.1. Dual culture test

Both BCAs were subjected to preliminary screening for antagonistic activity against *G. boninense via* dual culture technique [23] based on the percentage inhibition of radial growth (PIRG).

A 5 mm diameter mycelia agar disc was cut from the margin of 10-days-old culture of *G. boninense* and placed 2 cm from periphery of a 9 cm diameter Petri dish containing PDA medium and incubated for 48 h. Another 5 mm mycelia agar disc from 7-day-old culture of *T. asperellum* was placed 3 cm away from the *G. boninense* disc on the same plate, whereas a loopful of *P. aeruginosa* from 48 h NA culture with the concentration of 10^8 CFU/ml was then taken and streaked 3 cm away from the *G. boninense* disc on the same plate. Plates inoculated with *G. boninense* agar plugs alone were used as the control. All test pairings were incubated at 28 ± 2 °C for 7 days. Antagonistic activity of the *T. asperellum and P. aeruginosa* was assessed after 7 days of incubation by measuring the radius of the *G. boninense* colony using the following formula:

$$\text{Percent inhibition of radial growth(PIRG)} = \frac{\mathbf{r1\text{-}r2}}{\mathbf{r1}} \times \mathbf{100}$$

Where r1 is the radial growth of fungal colony in the control plate, while r2 represents the radial growth of fungal colony in the dual culture plate.

2.2.2. Scanning electron microscopy

The samples of dual culture plate and pure culture of each bacterial and fungal culture were observed using a scanning electron microscope (SEM) (JEOL JSM 6400, Japan) with three replicates of each sample. The samples were cut into a number of 1 cm^3 slices and placed into separate vials, which were then fixed in 4% glutaraldehyde for 12–24 h at 4 °C. Then, the samples were washed with 0.1 M sodium cacodylate buffer with 3 changes of 10 min each and post-fixed in 1% osmium tetroxide for 2 h at 4 °C. Next, the samples were again washed with 0.1 M sodium cacodylate buffer with 3 changes of 10 min each. Next, the samples were dehydrated in a serial dilution of acetone (35, 50, 75, and 95%) for 10 min each and in 100% with 3 changes of 15 min each. Finally, the samples were dried in a Critical Point Drying (CPD) (Baltec, Japan) apparatus. The dried discs were mounted on aluminium stubs and coated with gold using a Polaron Sputter Coater (Baltec, Japan)and viewed under SEM [24].

2.2.3. Mycelium growth test

2.2.3.1. Preparation of bacterial suspension. 25 ml of Nutrient Broth (NB) was prepared for each 150 ml conical flask. The NB in the conical flask was then autoclaved and allowed to cool. After that, one loop of *P. aeruginosa* was transferred into each conical flask and shaken for 48 h at 160 rpm. The bacterial suspensions were then adjusted to 10^8 CFU/ml using a spectrophotometer at 600 nm wavelength with optical density (OD) 0.6.

2.2.3.2. Preparation of fungal suspension. 25 ml of Potato Dextrose Broth (PDB) was prepared for each 150 ml conical flask. Then, the PDB in the conical flask was autoclaved and allowed to cool. After that, three plugs of *T. asperellum* were transferred into each conical flask and shaken for 24 h at 160 rpm. The conidia counts were fixed in the range of 1–9 10^6 conidia/ml.

2.2.3.3. Mycelia growth test. A plug of 7 day-old culture of *G. boninense* was dipped into *P. aeruginosa* and *T. asperellum* suspension for 30 min and then air dried in the laminar airflow chamber. Then, the treated mycelia plug was put onto Petri dishes containing PDA medium. *G. boninense* plug was dipped in sterile distilled water and used as control. The PDA plates were incubated at 28 ± 2 °C for 7 days. After 7 days incubation, hyphal strands at the end of the fungal colony were removed and examined under compound microscope (Olympus) for abnormalities, if any.

2.2.4. Culture filtrate test

Culture filtrate tests were performed to detect the production of non-volatile and diffusible inhibitors produced by all isolates in standing liquid culture either as antibiotics, enzymes or in the form of inhibitors.

T. asperellum and *P. aeruginosa* were inoculated in 250 ml PDB and NB, respectively, and incubated at 28 ± 2 °C in the dark for 7 days. Then, the cultures were centrifuged at 10,000 rpm for five minutes, while the supernatants were collected with the pellets discarded. Then, the supernatants were filtered through 0.25 µm membrane filters in sterile conditions and the filtrate was incorporated into sterilised double strength PDA (50 °C) in the ratio 2:1. After that, 20 ml of the amended agar was poured into each petri plate and allowed to solidify. Then, 5 mm disk of a 7-day old pure culture of *G. boninense* was inoculated in the middle of the plates. Non-amended PDA was used as the control. The plates were incubated at 28 ± 2 °C for 7 days and the mycelial growth of the pathogen was then measured [25].

2.3. In vitro screening for enzyme activity by biological control agents against Ganoderma boninense (UPM13)

2.3.1. Chitinase production

2.3.1.1. Preparation of colloidal chitin. Colloidal chitin was prepared using commercial crude chitin (crab shell) adopting the method by Roberts and Sclitvennikoff [26] with slight modifications and supplemented in the chitinase assay medium as a sole carbon source. Acid hydrolysis of chitin was done in concentrated hydrochloric acid (HCl) by constant stirring using a magnetic stirrer at 4 °C overnight followed by the extraction of colloidal chitin in 2000 ml of ice-cold 95% ethanol neutralisation kept at 26 °C overnight. Then, it was centrifuged at 3000 rpm for 20 min under 4 °C. Next, pellet was washed with sterile distilled water by centrifugation at 3000 rpm for 5 min at 4 °C until the smell of alcohol was completely removed. The colloidal chitin obtained