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## Control Activity of Potential Antifungal-Producing *Burkholderia* sp. in Suppressing *Ganoderma boninense* Growth in Oil Palm

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### ABSTRACT

Basal Stem Rot (BSR) disease caused by *G. boninense* is one of the most serious diseases in oil palm. Many attempts have been done to prevent or reduce infection of this disease but they have not provided optimum results. *Burkholderia* sp. isolated from rhizosphere and root tissue of symptomless oil palm showed potentials in suppressing *G. boninense* growth *in vitro*. Five isolates of rhizosphere *Burkholderia* sp. and one endophyte *Burkholderia* sp. were used in antagonist test against *G. boninense* growth on PDA media. Antifungal biosynthesis related gene from *Burkholderia* sp. was confirmed by using PCR with pyrrolnitrin (prn), pyoluteorin (plt), phenazine (phz) and DAPG (phl) primers. *Burkholderia* sp. with the highest antagonist activity were applied to oil palm germinated seed for *in vivo* test. Endophyte *Burkholderia* B212 showed the highest antagonist activity against *G. boninense* growth *in vitro* with PIRG 34.38%. The B212 genome was yield an expected PCR product by using prn primers (790 bp). Sequence BLAST result showed the gene was 99% identical with *B. cepacia* partial *prnD* gene, strain ESR63. *In vivo* test of B212 showed that treatment of *Burkholderia* B212 on plant without *G. boninense* infection increased the height and biomass of the plant. However, B212 did not decrease the disease incidence and disease severity on *G. boninense* infected plant. In addition it decreased the plant height and biomass compare to control plant.

**Key words:** Antibiotic-related gene, basal stem rot, biocontrol, endophyte bacteria, dipping-seed

### INTRODUCTION

Oil palm is one of the most important agricultural export crops in Indonesia besides rubber, cocoa, coffee and spices (Stads *et al.*, 2007). The BSR has been a serious threat to the oil palm industry in Indonesia because it shortens the productive life of oil palms and causes serious economic loss. The disease is caused by a white-rot fungi *G. boninense* and in the past few decades has been spreading rapidly, for instance, in North Sumatra, Indonesia, this disease can lead to losses as much as 50% after repeated planting cycles (25 years) (Corley and Tinker, 2003).

The use of fungicides for fungal control did not produce significant results yet (Haas and Defago, 2005). This may be due to the fact that by the time treatment is applied, the palms may already have the disease. Antifungal-producing bacteria is a promising biocontrol agent (BCA) to overcome this disease. The BCA does not necessarily be a cure for the disease but to slowing or even

to stop the disease spread by protecting the plant or enhancing the plant defense. Endophyte BCAs have more value since, it can live inside the plant tissue through the plants lifetime.

Endophyte bacteria is bacteria which live inside the plant tissues without causing apparent harm or symptoms to the host (Munif *et al.*, 2003). Endophyte as the internal plant habitat provide several advantages as BCA. It will be in less competition with other microorganisms, sufficient supply with the nutrients, less exposure to environmental stress factors and better translocation of bacterial metabolites throughout the host plant (Hallmann *et al.*, 1997). Application of endophyte *Pseudomonas aeruginosa* and *B. cepacia* could reduce BSR incidence up to 76% in 8 months oil palm seedling after disease inoculation (Sapak *et al.*, 2008).

Members of the genus *Burkholderia* sp. are known for their ability to suppress soil-borne fungal pathogens by the production of various antibiotic compounds such as pyrrolnitrin and phenazines (Kirner *et al.*, 1998). Other different antibiotics such 2,4-diacetylphloroglucinol (2,4-DAPG) and pyoluteorin has also been found responsible for suppression of soil-borne fungal pathogens (Subagio and Foster, 2003).

Antibiotic-related genes can be detected from BCAs by using Polymerase Chain Reaction (PCR) by using antibiotic specific primers which encode phenazine-1-carboxylic acid, 2,4-DAPG, pyoluteorin and pyrrolnitrin, from *Pseudomonas* and *Bacillus* genome (Zhang *et al.*, 2005).

BCAs have been applied in many ways and on many crops species, for instance, seed dipping application on rice seeds to control bacterial blight disease caused by *Xanthomonas oryzae* was able to reduce the disease incidence (Suryadi *et al.*, 2012). Besides microbial pathogen, BCA was also reported able to control plant parasitic nematodes. Munif *et al.* (2013) was reported seed dipping application on tomato seeds able to control *Meloidogyne incognita* penetration and enhanced the plant growth. Oil palm germinated seeds dipping application was also conducted by Dikin *et al.* (2003) to suppress *Schizopyllum commune*, causal agents of brown germ and seed rot in oil palm. In this study, *Burkholderia* sp. control activity against *G. boninense* growth were observed *in vitro* and *in vivo* of oil palm to analyze antifungal encoding gene from *Burkholderia* sp. potential.

## MATERIALS AND METHODS

***Burkholderia* sp. isolation:** *Burkholderia* sp. were isolated from soil near the roots and the roots that were taken from a healthy (symptomless) oil palm tree in an endemic area, in North Sumatera, Indonesia. One gram of soil was diluted in sterile water and poured on Nutrient Agar plates. For the roots, were surface sterilized with 90% alcohol for 1 min, 70% alcohol for 3 min and washed twice with 50 mL sterile distilled water for 30 sec. The roots were crushed aseptically and put on NA in plate. The plate were incubated for 24-36 h. Each of the grown colony were subculture and genome extracted for identification. Purified PCR product of 16S DNA were sent to 1st Base, Singapore.

**Antagonist *in vitro* test:** Antagonist test was observed to determine the Percentage Inhibition of Radial Growth (PIRG) of *G. boninense* (Bivi *et al.*, 2010). Six bacterial isolates of *Burkholderia* sp. were selected to evaluate their efficacy in enhancing growth and inhibit the infection of BSR in oil palm in pre nursery. These *Burkholderia* sp. collection were isolated from rhizosphere and oil palm root tissue. There were five rhizosphere *Burkholderia* sp. (B313, B51a, B52c, B51b, B52a) and one endophyte *Burkholderia* sp. (B212).

*Burkholderia* sp. was streaked into the PDA plate 2.5 cm from the edge of the petri dish. Agar disc cut diameter 5 mm and 5 day old *G. boninense* was placed 2.5 cm from the edge at the opposite

side of the same petri dish. For the control plate, only *G. boninense* was placed in a similar manner without bacteria on a fresh petri dish. The plates were incubated at 28°C for five days. Results shown by measured the radial growth of *G. boninense*. The PIRG was calculated using the equation below (Zaiton *et al.*, 2006):

$$\text{PIRG (\%)} = \frac{R1 - R2}{R1} \times 100$$

Where:

PIRG = Percentage inhibition of radial growth

R1 = Radial growth of *G. boninense* in the absence of bacteria (control)

R2 = Radial growth of *G. boninense* in the presence of *Burkholderia* sp. The three highest PIRG isolates from *in vitro* test were used for *in vivo* test

***In-vivo test:*** *Burkholderia* sp. suspensions were prepared by inoculating 24 h old cultures into Nutrient Broth (NB) and incubated for 20 h and adjusted to 10<sup>8</sup> CFU mL<sup>-1</sup>. During the preparation of mixture, equal volume of the 3 highest PIRG *Burkholderia* sp. were mixed. *Ganoderma boninense* inoculum was prepared on 6×3×15 cm sterilized oil palm fronds. The fronds were sterilized in a heat resistant plastic each before inoculated with *G. boninense*. After sterilization, the fronds were inoculated with a 0.5 cm diameter disc of *G. boninense* mycellia on agar plate. The plastic was sealed and incubated in room temperature for 3 months before used.

The plant material was selected as the most *G. boninense* susceptible progeny. Two different progenies were used in this study. Briefly, oil palm germinated seeds were treated with bacterial suspension (10<sup>8</sup> CFU mL<sup>-1</sup>), dipped for 20 min (seed bacterization) and air dried for 10 min before planted.

Oil palm germinated seeds were planted in polybags (15×20 cm) regarding to the Standard Operation Prosedure (SOP) in prenursery of oil palm plantation. There were four treatments in this study. Treatment A and C were not inoculated with *G. boninense* and treatments C and D were inoculated with *G. boninense*. Treatment B and C were using seedling treated with *Burkholderia* sp. Treatments with *G. boninense*, the seedlings were placed in contact with radricula. The pots were placed under shead, watered daily and no supplementary organic fertilizer was applied for 3 months (prenursery). A destructive observation was conducted after 3 months.

The infection of *G. boninense* on plant can be scored by observation on signs and symptom on the treatment plants using Disease Severity Index (DSI) (Abdullah *et al.*, 2003). The DSI was observed from the external symtomp from foliar and the roots (destructive method).

The score can be calculated by the equation of Mohd Zainudin and Abdullah (2008):

$$\text{DSI (\%)} = \frac{\sum(A \times B)}{\sum(B) \times 4} \times 100$$

Where:

A = Disease class (0, 1, 2, 3 or 4)

B = Number of plants showing that disease class per treatment

Table 1: Polymerase chain reaction primers and expected amplification products from genes encoding enzymes involved in the biosynthesis of several antibiotics (Zhang *et al.*, 2005)

Primer	Antibiotic	Sequence	Sequence size (bp)
Phl2a	2,4-DAPG	GAGGACGTCGAAGACCACCA	745
Phl2b		ACCGCAGCATCGTGTATGAG	
PHZ1	Phenazine	GGCCAGATGGTCAACGG	1400
PHZ2		CGGCTGGCGGCGTATAT	
PRND1	Pyrrolnitrin	GGGGCGGGCCGTGGTATGGA	790
PRND2		YCCCGCSGCCTGYCTGGTCTG	
PLTC1	Pyoluteorin	AACAGATCGCCCCGGTACAGAACG	438
PLTC2		AGGCCCGGACACTCAAGAAACTCG	

**Detection of antifungal gene:** Antifungal gene detection was conducted by using PCR. *Burkholderia* sp. was prepared in liquid medium for DNA isolation by using GeneJET Genome DNA Purification Kit from Thermo Scientific. Specific primers for DAPG (Phl2a-Phl2b), phenazine (PHZ1-PHZ2), pyrrolnitrin (PRND1-PRND2) and pyoluteorin (PLTC1-PLTC2) were used for detection according to Zhang *et al.* (2005) (Table 1).

PCR product was gel extracted from agarose by using Gene JET Gel Extraction Kit (Fermentas), according to manufacturer's instruction. After purification, the PCR products were sent to 1st Base, Singapore, to be sequenced. The antifungal genes sequences were aligned by using BioEdit software and searched for sequence similarity to other sequences which are available in the NCBI database at <http://www.ncbi.nlm.nih.gov/> using Basic Local Alignment Search Tool (BLAST) algorithm. Multiple sequence alignments were performed on the selected closely related sequence accessions available using CLUSTAL W software in Mega 5.

**Statistical analysis:** The treatment was repeated into 13 replicates. This research used factorial design with one factor and the environmental design is complete randomized design. Statistic analysis was done by using general method linear model univariate. If there was a significant difference further analysis will be analyzed with a value is 5% by using SAS.

## RESULTS

**Antagonist *in vitro* test:** All of the *Burkholderia* sp. isolates showed an inhibition activity against *G. boninense* growth *in vitro* (Table 2). Scores followed by the same letter indicated that they were insignificantly different scores. The highest inhibition activity was shown by isolate B212 with percentage PIRG was 34.38% but not significantly different with B52a and B52c which were both 27.50%. *Burkholderia* B313 showed the lowest activity (23.75%) and significantly different with B212 but still has an antagonist activity against *G. boninense* growth *in vitro*.

***In vivo* test:** The necrosis and chlorosis foliar was not seen in all treatments, though the height of the plants between treatment was seem different (Fig. 1a). The DSI after 3 months only showed in treatment C and D (25%) (Table 3). Destructive observation showed that all plants in treatment C and D were infected with *G. boninense*. Treatment C and D which were inoculated with *G. boninense*, showed a brown-blackening roots especially on the parts which colonized with the *G. boninense* (Fig. 1b). The roots in treatment A and C, without *G. boninense*, were cream-brown colored.

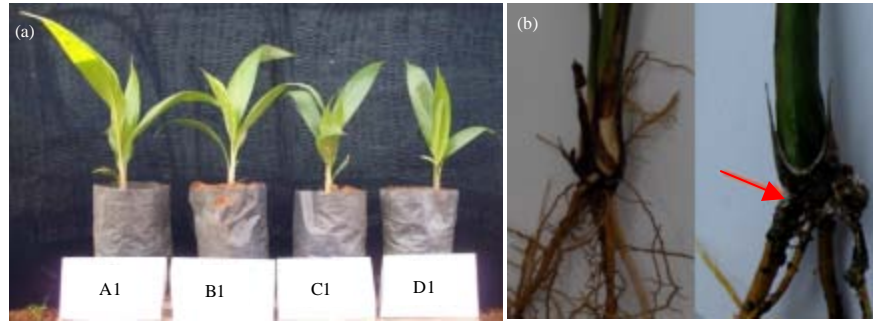


Fig. 1(a-b): (a) Plants treatment left to right (A-D) and (b) Healthy root with no appearance of fungal mycellia (left). Appearance of fungal mycellia (red arrow)

Table 2: Percentage of inhibition ratio from *Burkholderia* sp. against *Ganoderma boninense* growth *in vitro*

Isolates	PIRG (%)
B212	34.38 <sup>a</sup>
B52a	27.50 <sup>ab</sup>
B51a	25.00 <sup>b</sup>
B52c	27.50 <sup>ab</sup>
B251a	25.63 <sup>b</sup>
B313	23.75 <sup>b</sup>

Table 3: Disease Severity Index (DSI) of treated plant with *Ganoderma boninense*

Treatments	DSI (%)
Un-inoculated seed (A)	0
Un-inoculated seed+ <i>Burkholderia consortia</i> (B)	0
Inoculated seed+ <i>Burkholderia consortia</i> (C)	25
Inoculated seed (D)	25

Progeny give a significant different mostly to the shoot length and dry weight (Table 4). Total number of roots between progeny also showed a significant difference. However, there is no correlation between progenies and treatments though in some parameter that were observed was show a significant difference.

*Burkholderia* sp. consortia application on inoculated seeds showed significantly lowest in shoot growth compare to control and other treatments. In all parameters that were measured, the treatment decrease the plant growth and significantly decrease shoot length, shoot dry weight, total number of shoot and root (Table 5). However, *Burkholderia* sp. consortia application on un-inoculated seeds showed the highest in shoot and root growth compare other treatments. The shoot length and dry weight was slightly different compare to control. The root length did not showed a significant different among all the treatment.

**Antifungal gene detection:** Primers PRND1 and PRND2 amplified the predicted 790 bp fragment from DNA of B212, B313, B51a and B52c. Primers PLTC1 and PLTC2 amplified the predicted 438 bp from DNA of *Burkholderia* B313, B51a and B52c (Fig. 2). Primers for phenazine and DAPG did not yield a PCR product from all isolates. This may be concluded that these isolates do not contain phenazine and DAPG biosynthesis gene.

Table 4: Effect of different progeny on root and leaf total number, length and dry weight after treatment with *Ganoderma boninense* and *Burkholderia* sp.

Progeny	Length (cm)		Dry weight (g)		Total number of	
	Shoot	Root	Shoot	Root	Shoot	Root
1	24.794 <sup>a*</sup>	28.304 <sup>a</sup>	1.162 <sup>a</sup>	0.989 <sup>a</sup>	5.130 <sup>a</sup>	4.413 <sup>a</sup>
2	22.789 <sup>b</sup>	29.145 <sup>a</sup>	1.011 <sup>b</sup>	0.916 <sup>a</sup>	4.872 <sup>a</sup>	3.829 <sup>b</sup>

\*Means within a column with the same letter are not significantly different at p<0.05 using DMRT

Table 5: Effect of dipping treatment of antagonistic bacteria on seedling of oil palm inoculated with *Ganoderma boninense* in pre-nursery at 3 months after planting

Treatment	Length (cm)		Dry weight (g)		Total number of	
	Shoot	Root	Shoot	Root	Shoot	Root
Un-inoculated seed (A)	24.671 <sup>ab*</sup>	29.109 <sup>a</sup>	1.207 <sup>ab</sup>	0.831 <sup>b</sup>	5.217 <sup>a</sup>	4.609 <sup>a</sup>
Un-inoculated seed+ <i>Burkholderia consortia</i> (B)	26.826 <sup>a</sup>	30.174 <sup>a</sup>	1.340 <sup>a</sup>	0.931 <sup>b</sup>	5.348 <sup>a</sup>	4.783 <sup>a</sup>
Inoculated seed+ <i>Burkholderia consortia</i> (C)	20.222 <sup>c</sup>	26.791 <sup>a</sup>	0.697 <sup>c</sup>	0.797 <sup>b</sup>	4.522 <sup>b</sup>	3.522 <sup>b</sup>
Inoculated seed (D)	23.333 <sup>b</sup>	28.838 <sup>a</sup>	1.083 <sup>b</sup>	1.238 <sup>a</sup>	4.917 <sup>ab</sup>	3.583 <sup>b</sup>

\*Means within a column with the same letter are not significantly different at p<0.05 using DMRT

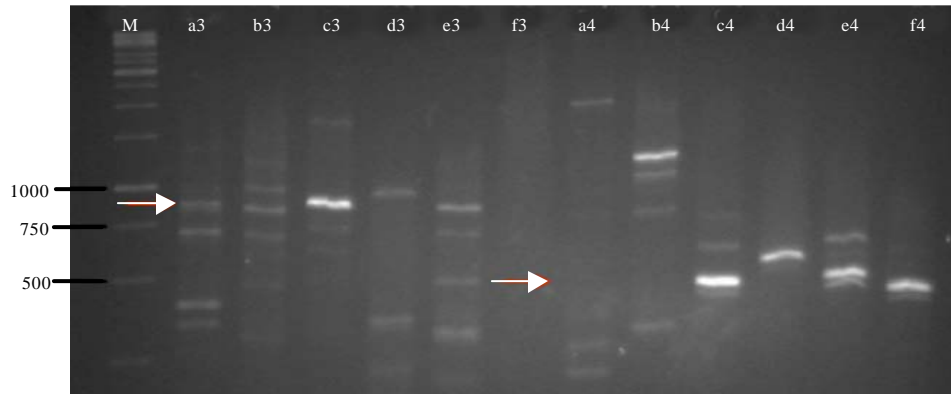


Fig. 2: PCR product of pyrrolnitrin and pyoluteorin gene amplification. M: Marker, 3. prn primer, 4. plt primer, a: B212, b: B313, c: B51a , d: B52c, e: B51b, f: B52a. Red arrows showed the expected band size

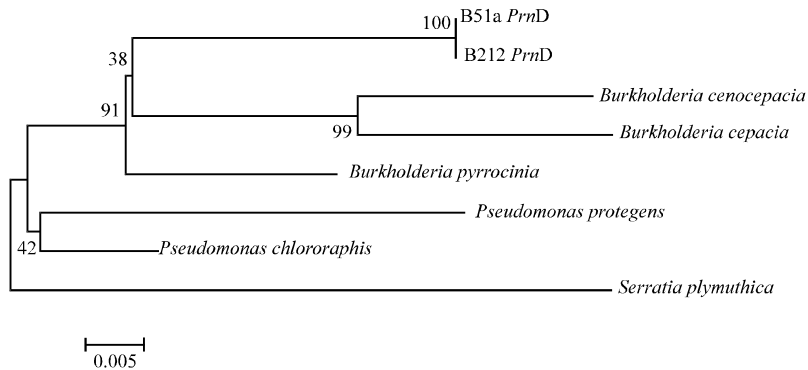


Fig. 3: Phylogenetic tree of PrnD gene from B212 and B51a

The PCR product were BLAST and showed a 99% identity with *B. cepacia* partial *prnD* gene, strain ESR63 (Fig. 3). It is confirmed B212 has a potency to produce pyrrolnitrin and assumed pyrrolnitrin is the responsible compound in inhibiting *Ganoderma* growth *in vitro*.

## DISCUSSION

*Ganoderma boninense* has been the most threatening disease in oil palm plantation. *Ganoderma boninense* has the ability to degrade lignin in the plant and decay the lower stem and sometimes the root system, leading to severe symptoms such as flattening of the crown and unopened spear leaves (Cooper *et al.*, 2011). Many attempts have been made to control this disease including using BCAs such as bacteria.

Bacteria have various mechanism of antagonistic such as synthesizing antibiotic compounds, production of hydrolytic enzymes, siderophore production, competition for substrates and also induction of systemic resistance in the host plant will increase the plant resistance to a broad spectrum of pathogens (Kloepper and Ryu, 2006). Many soil-borne plant diseases caused by fungi and oomycetes can be controlled by strains of the genus *Burkholderia* (Kirner *et al.*, 1998).

In this study endophyte *Burkholderia* B212 showed the highest antagonist activity against *G. boninense* growth (Table 2). Several strains of *Pseudomonas* and *Burkholderia* species can produced a broad-spectrum of antibiotics which play an important role in the suppression of multiple plant pathogenic fungi (De Souza and Raaijmakers, 2003).

*In vivo* test showed a different result from the *in vitro* test. In comparison to Mohd Zainudin and Abdullah (2008) and Sapak *et al.* (2008), the external symptom of *G. boninense* infection in oil palm appeared after 4 months after planting even though the disease has been invested from seedling or after 3 months planting. However, external symptom may also occur after 2 months planting with the disease.

This differences may be caused by many factors such as pathogenicity level of *G. boninense*, inoculum source of *G. boninense*, plant tolerance and climate. White-rot fungi has been classified to the ligninolytic enzymes they express and able developed unspecific ligninolytic systems consisting of peroxidases and laccases which employ an oxidative process to degrade the wood cells (Paterson, 2007). It can be assumed that these enzymes activity can be related to *G. boninense* pathogenicity level. There are two type of *G. boninense* inoculum source used in *G. boninense* research, wood block and oil palm fronds. Oil palm fronds are more easy to get since it is abundance in oil palm plantation. Different climate may also effect lignin degradation which lignin degradation is less efficient at 37°C compare to 25°C (Paterson *et al.*, 2008).

Application method of *Burkholderia* sp. may also effect the result of infection. *Burkholderia* sp. has been reported that its antifungal producing is related to quorum-sensing which is mean that the antifungal activity depends on *Burkholderia* sp. biomass (Chapalain *et al.*, 2013). *Ganoderma boninense* infection were found in all plants which were treated with the disease including the *Burkholderia* sp. consortia treatment. *Burkholderia* sp. consortia were expected to inhibit the disease infection but the result showed on the contrary. It is very different to other report which is using soil drenched application and the result showed that application with *Burkholderia* and *Pseudomonas* able to reduce *G. boninense* incidence (Sapak *et al.*, 2008). Different method of application and number of *Burkholderia* sp. applied may come to a different results.

*Burkholderia* sp. consortia application on inoculated seeds was expected to have the highest plant growth compare to plants without *Burkholderia* sp. and control. However, the treatment



showed the lowest plant growth compare to all other treatments. *Burkholderia* sp. as endophyte has the ability to penetrate the plant by using cellulase enzyme (Reinhold-Hurek *et al.*, 2006). It can be assumed that the *Burkholderia* sp. has a potency to open a way for *G. boninense* to infect the plants. Biomass of *Burkholderia* sp. held an important key in suppress *G. boninense* infection, since, the antifungal compound is regulated by quorum sensing. This phenomena could explain the infected plants without *Burkholderia* sp. consortia application is higher than the infected plants with *Burkholderia* sp. consortia.

If compared with the shoot and root ratio (S/R) among the treatment, infected plants with *Burkholderia* sp. consortia shown the lowest number. Which means that in this treatment, the root has the higher biomass compare to the shoot. *Burkholderia* sp. consortia application gave a more affect to the roots than the shoots on infected plants. *Burkholderia* sp. also could acted as PGPR which can lead an indirect biocontrol agent, since it could enhanced plant growth. *Burkholderia* has reported as a plant growth promoting rhizobacteria (PGPR) (Compant *et al.*, 2008).

*Burkholderia* sp. consortia application on inoculated seed was significantly higher on root dry weight compare to other treatment. *Ganoderma boninense* as pathogen could induced plant defense by forming lignin as its first defence system. Lignification or cell thickness is the form of plant defense against pathogen (Xu *et al.*, 2011) and could increase root biomass.

Total number of *Burkholderia* applied also play an important role in proper activity of this biocontrol agent. Schmidt *et al.* (2009) has reported that some of antifungal such as pyrrolnitrin is regulated by quorum sensing. Quorum sensing is a mechanism to regulate the production of antimicrobial compounds by population-density-dependent (Liu *et al.*, 2007). Different results between *in vitro* and *in vivo* test may be caused by many factors such as plant ages by the time artificially inoculated, concentration of microbes applied and application technique.

Many research showed that *Burkholderia* could decrease *G. boninense* infection on oil palm but yet a correct application technique of this bacteria also important to get the best result. Antifungal compound is a secondary metabolite which is produce by bacteria in their stationary phase. Further research need to be conducted for the proper technique application of this *Burkholderia* for its optimum action.

Pyrrolnitrin encoded gene was amplified and showed that *Burkholderia* B212 has a potential in producing antifungal agent such as pyrrolnitrin. Pyrrolnitrin has been implicated as an important mechanism of biological control of fungal plant pathogens by several *Pseudomonas* strains (Hasan and Turner, 1998). Pyrollnitrin is a chlorinated phenylpyrrole antibiotic that was first isolated from *Burkholderia pyrrocinia* (Kloepper and Ryu, 2006) and later from other microorganisms, including *Pseudomonas fluorescens*, *P. chlororaphis*, *P. aureofaciens*, *B. cepacia*, *Enterobacter agglomerans*, *Myxococcus fulvus* and *Serratia* species (Hammer *et al.*, 1999).

Pyrrolnitrin is synthesize by four protein encoded by 4 gene, *prna*, *prnb*, *prnc* and *prnd*. The *Prnd* gene was the final protein to form an active pyrrolnitrin compound. The *prnD* catalyzes the oxidation of the amino group of aminopyrrolnitrin to a nitro group to form pyrrolnitrin (Kirner *et al.*, 1998). In some strains of *Burkholderia* species pyrrolnitrin biosynthesis was shown to be regulated by quorum sensing (Schmidt *et al.*, 2009).

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