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Effect of Formulated Bioorganic Containing *Burkholderia* GanoEB2 in Suppressing *Ganoderma* Disease in Oil Palm Seedlings

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Abstract

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The ability of *Burkholderia* GanoEB2 formulated in two different bioorganic powders as carriers – bioorganic empty fruit bunch (BEFB) and real strong bioorganic fertilizer (RSBF), and the efficacies of *Burkholderia* GanoEB2 in suppressing *Ganoderma boninense* infection in oil palm seedlings were determined. Results showed that the highest reduction in disease incidence (DI, 18.2%), severity of foliar symptoms (SFS, 26.6%), and disease severity foliar index (DSFI, 27.7%) was achieved by the seedlings treated with RSBF containing *Burkholderia* GanoEB2 compared to the seedlings treated with BEFB containing *Burkholderia* GanoEB2 (36.4% DI, 33.4% SFS, and 31.8% DSFI). Untreated seedlings (as control) had 100.0% DI, 90.4% SFS, and 87.5% DSFI. The disease was reduced by as much as 85.3% for seedlings treated with RSBF and by 70.5% for seedlings treated with BEFB. The formulated bioorganics containing *Burkholderia* GanoEB2 were proven to suppress *Ganoderma* disease in oil palm.

Keywords: *Ganoderma boninense;* bioorganic empty fruit bunch (BEFB); real strong bioorganic fertiliser (RSBF)

Oil palm (*Elaeis guineensis*) is the most important crop plant in Malaysia (MPOB 2013). The oil palm planted area reached 5.23 mil. ha in 2013 (compared to 5.08 mil. ha in 2012, i.e. an increase of 3.0%) (MPOB 2013). To achieve maximum yield and production, the health of oil palms is crucial. Like other crops, oil palm trees are also exposed to various diseases but the most serious disease is basal stem rot (BSR) caused by the fungal pathogen *Ganoderma boninense.* In Malaysia and Indonesia, BSR has been known as the most destructive disease attacking oil palm trees and causing major losses (Idris 2009; Susanto 2009). Recently, BSR has become still more serious attacking still younger oil palms.

Several control strategies have been deployed to reduce BSR disease including soil mounding, removal of the oil palms attacked by the disease, and fungicide treatment. However, this BSR control is only aimed at prolonging the productive life of the already infected palms and delaying the progress of *Ganoderma boninense. In vitro* studies by Idris *et al.* (2002) claimed that numerous fungicides were strongly inhibitory towards *Ganoderma boninense.* However, the results from the nursery studies were inconclusive. This was probably due to the fact that the various resting stages of *Ganoderma boninense* such as melanised mycelium, basidiospores, and pseudosclerotia are more resistant towards the fungicides (Susanto 2009).

Recently, the use of biocontrol agents such as endophytic fungi and endophytic bacteria for suppressing plant disease has gained much attention in pathological research. They have been considered for control

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of various diseases in crop plantations including BSR disease in oil palm tree due to their ability to colonise intracellular and intercellular tissue of plants at a certain time of their life cycle and minimise the chance of such pathogens to colonise the area (Kloepper *et al.* 1999; Hallmann *et al.* 2001), morover without causing any harm to the host or gaining benefit other than residency within the plants tissues (Azevedo *et al.* 2000; Kobayashi & Palumbo 2000). Studies by Abdullah *et al.* (1999), Ilias (2000), Sariah *et al.* (2005), and Susanto *et al.* (2005) proved that endophytic fungus, *Trichoderma* spp., showed high efficacy in controlling the growth of BSR disease in plant house trials and under field condition. Sapak *et al.* (2008) and Bivi *et al.* (2010) proved that endophytic bacteria from the genus *Burkholderia* can reduce the incidence of BSR disease. However, most of the studies focussed just on the use of cell suspension or cell injection which was found to be impractical for a large scale application to control BSR disease in the nursery. Maizatul *et al.* (2012) immobilised the biocontrol *Burkholderia cepacia* into vermiculite powder as a carrier and nutrient supply. The number of viable bacteria cells in the vermiculite powder was 10^8 CFU/g for five months storage.

In the present study, bacterial *Burkholderia* GanoEB2 identified as *Burkholderia cepacia* isolated from symptomless oil palm root tissues was formulated into bioorganic powder as a carrier. Bioorganic powder was selected owing to its ability to promote plant vigour other than being carrier for the bacteria cells itself. The formulated bioorganic powder containing *Burkholderia* GanoEB2 was tested in the nursery for its efficacy in suppressing *Ganoderma* disease in oil palm seedlings by using two treatments with bioorganic empty fruit bunch (BEFB) and real strong bioorganic fertiliser (RSBF) containing *Burkholderia* GanoEB2. The disease suppression was assessed based on calculation of the disease severity of foliar index (DSFI), severity of foliar symptom (SFS), disease incidence (DI), and dead seedlings (DS) at 2-month intervals within 8 months after inoculation.

MATERIAL AND METHODS

Planting materials and experimental design. This study was conducted at the Malaysian Palm Oil Board (MPOB) Research Station, Bangi, Selangor, Malaysia. Forty-eight four-month-old oil palm seedlings (*dura* × *pisifera,* D×P) were used in this study. The experiment involved seedlings of four variants (12 seedlings per a variant), two controls (C1 and C2), and two treatments (T1 and T2) (Table 1). The seedlings were grown in polybags (12 × 15 cm) containing a 3 : 1 mixture of soil and sand. All oil palm seedlings were placed and arranged in a completely randomized block design (CRBD). The seedlings were watered twice daily – in the morning and afternoon.

Endophytic bacteria Burkholderia GanoEB2 stock culture. The stock culture of *Burkholderia* GanoEB2 was provided by the stock collection of GanoDROP Laboratory, Biological Research Division, MPOB, Kajang, Selangor, Malaysia. The culture was isolated from the symptomless oil palm root tissues and maintained at 28°C in an incubator. Two methods of culture maintenance were used, i.e. nutrient agar (NA) slant added with paraffin oil and glycerol stock.

Preparation of bioorganic formulation containing Burkholderia GanoEB2. Two types of bacterial formulation were developed using two types of bioorganic powder as a carrier: bioorganic empty fruit bunch (BEFB) and real strong bioorganic fertiliser (RSBF). BEFB (Felcra Berhad, Mersing, Johor, Malaysia) contains mulch and is enriched with inorganic elements such as cellulose, glucose, silica copper, calcium, iron, and sodium while RSBF (All Cosmos Industries Sdn. Bhd., Pasir Gudang, Johor, Malaysia) contains nutrients like nitrogen (N), phosphorus (P), kalium (K), magnesium (Mg), boron (B), iron (Fe), zink (Zn), and other trace elements which are combined with plant-based organic matters. These formulations were prepared based on the method described by Nasyaruddin and Idris (2011) with a

Table 1. List of treatments used in the study

Treatment	Seedling description
C1	negative control, seedlings non-infected and non-treated
C2	positive control, seedlings artificially inoculated with *Ganoderma boninense* but untreated
T1	seedlings artificially inoculated with *Ganoderma boninense* and treated with bioorganic empty fruit bunch (BEFB) containing *Burkholderia* GanoEB2
T2	seedlings artificially inoculated with *Ganoderma boninense* and treated with real strong bioorganic fertiliser (RSBF) containing *Burkholderia* GanoEB2

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slight modification. The powder was formulated using vermiculite with the addition of bioorganic fertiliser to promote growth of oil palm other than being carrier itself. The bacterial *Bukholderia* GanoEB2 was grown in an enriched medium at 28°C for 24 hours. About 10^8 CFU/g of bacteria cells were added into vermiculite powder under sterile condition and later mixed with BEFB and RSBF respectively. The powder formulations were then stored at room temperature in a sealed polyethylene bags.

Preparation of rubber wood blocks inoculated with Ganoderma boninense. Rubber wood blocks (RWB) were prepared according to Sapak *et al.* (2008) with a slight modification. The RWB measuring 6 × 6 × 6 cm were washed and dried in an oven before being autoclaved twice at 121°C for 20 minutes. Each block was individually put into a heat-resistant polypropylene bag and 70 ml of molten malt extract agar (MEA) were added as supplementary nutrients for *Ganoderma boninense*. The bags with RWB and molten MEA were autoclaved at 121°C for 30 minutes. After sterilisation and cooling, the RWB in the polypropylene bag was rotated to ensure it is well covered by MEA before the letter solidifying. When the agar solidified, five plugs (10 mm) taken from the seven-day-old *Ganoderma boninense* culture were inoculated into the surface of each RWB. The inoculated blocks were then incubated at 28°C for 8 weeks until full colonisation by the *Ganoderma boninense* mycelium.

Inoculation and establishment of the oil palm seedlings. The seedlings were artificially inoculated with *Ganoderma boninense* infected RWB according to Khairuddin (1990) with slight modifications. Uninoculated and untreated seedlings were the negative control (C1). Seedlings in positive control (C2) were challenged with fully colonised RWB placed in contact with the roots. The seedlings in T1 and T2 were initially treated with 30 g of BEFB and RSBF containing *Burkholderia* GanoEB2, respectively. After 2 weeks of treatment, the seedlings were uprooted carefully and inoculated with *Ganoderma boninense* fully colonised RWB. 30 g of formulated BEFB and RSBF containing *Burkholderia* GanoEB2 were applied again before placing the seedling in contact with infected RWB. All the seedlings were watered daily and the formulated BEFB and RSBF containing *Burkholderia* GanoEB2 were applied in 2-month intervals.

Disease assessment. The effect the bioorganic formulations containing *Burkholderia* GanoEB2 on *Ganoderma* disease incidence was evaluated based on quantitative assessment measured as percentage of disease incidence (DI), severity of foliar symptom (SFS), disease severity of foliar index (DSFI), and dead seedlings (DS) at 2-month intervals within 8 months after inoculation.

DI referred to the number of seedlings visually assessed as diseased (chlorosis and necrosis of leaves, with or without production of fruiting body) and it was determined after Campbell and Madden (1990) as follows:

(number of seedlings infected/total number of seedlings assessed) × 100

SFS (%) was assessed according to Sariah and Zakaria (2000):

$$SFS\ (\%) = [(a \times 1) + (b \times 0.5)]/c \times 100$$

where: a – number of desiccated (browned/wilted) leaves; b – number of yellowing leaves; c – total number of leaves; 1 – index for desiccated leaves; 0.5 – index for yellowing leaves

DSFI assessment on external symptoms of *Ganoderma* disease infection was calculated according to Nur Ain Izzati and Abdullah (2008) with slight modifications. DSFI was derived from a mathematical formula based on the observable signs of *n* symptoms of 'disease class' of the infected seedlings which was translated from numerical values, ranging from 0 to 4 (Table 2). DSFI was determined at regular 2-month

Table 2. Signs and symptoms of infection and their corresponding disease class values (Nur Ain Izzati & Abdullah 2008) with modification

Class	Associated signs and symptoms of infection
0	healthy seedlings with green leaves and no necrosis/chlorotic leaves (foliar symptoms) and no white fungal mass (mycelium)/fruiting body
1	appearance of white fungal mass/fruiting body and no necrosis/chlorotic leaves (foliar symptoms)
2	appearance of white fungal mass/fruiting body and necrosis/chlorotic leaves (foliar symptoms) ≤ 25%
3	appearance of white fungal mass/fruiting body and necrosis/chlorotic leaves (foliar symptoms) 26–75%
4	appearance of white fungal mass/fruiting body and necrosis/chlorotic leaves (foliar symptoms) > 75% or seedling dead/dried

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intervals. To derive the DSFI, the disease classes were then calculated with the following formula:

$$DSFI = \frac{\Sigma (A \times B)}{\Sigma B \times 4} \times 100$$

where: A – disease class (0, 1, 2, 3, 4); B – number of seedlings showing that disease class per treatment; number 4 – constant representing the highest class of assessment

At the end of the 8-month experiment, DSFI for internal symptoms of the oil palm seedlings was calculated. All data (DSFI, DI, SFS, and DS) were analysed by the Analysis of Variance (ANOVA) using Minitab v. 16 software with the means compared by the Least Significant Difference (*LSD*) at $P \leq 0.05$.

RESULTS

The external symptoms of BSR infection were observed 4 months after *Ganoderma boninense* inoculation. The symptoms started with yellowing at the lower frond followed by necrosis, the leaves then started to desiccate from the oldest to the youngest ones. As the disease progressed, young oil palm seedlings appeared pale with retarded growth and the leaves spear remained unopen. Formation of white mycelia at the basal stem part were observed after 5 months of infection and later developed into the fruiting body and caused the death of seedlings (Figure 1).

In this experiment, both formulations of BEFB and RSBF containing *Burkholderia* GanoEB2 had effects on the BSR severity caused by fungus *Ganoderma boninense*. After 8 months of inoculation, non-infected and non-treated seedlings (C1) remained healthy with green leaves (0.0% DSFI). Seedlings inoculated with *Ganoderma boninense* but untreated (C2) showed the highest DSFI value of 87.5%. Seedlings artificially inoculated with *Ganoderma boninense* and treated with 10 g real strong bioorganic fertiliser (RSBF) containing *Burkholderia* GanoEB2 (T2) exhibited the best control measure of the disease with the lowest DSFI value of 27.7% compared to T1 (seedlings artificially inoculated with *Ganoderma boninense* and treated with 30 g bioorganic empty fruit bunch (BEFB) containing *Burkholderia* GanoEB2) with DSFI value of 31.8% (Table 3).

After 8 months of inoculation, results showed that the highest reduction in DI (Table 3) and SFS (Table 4) was achieved by seedlings in T2 (18.2% of DI;

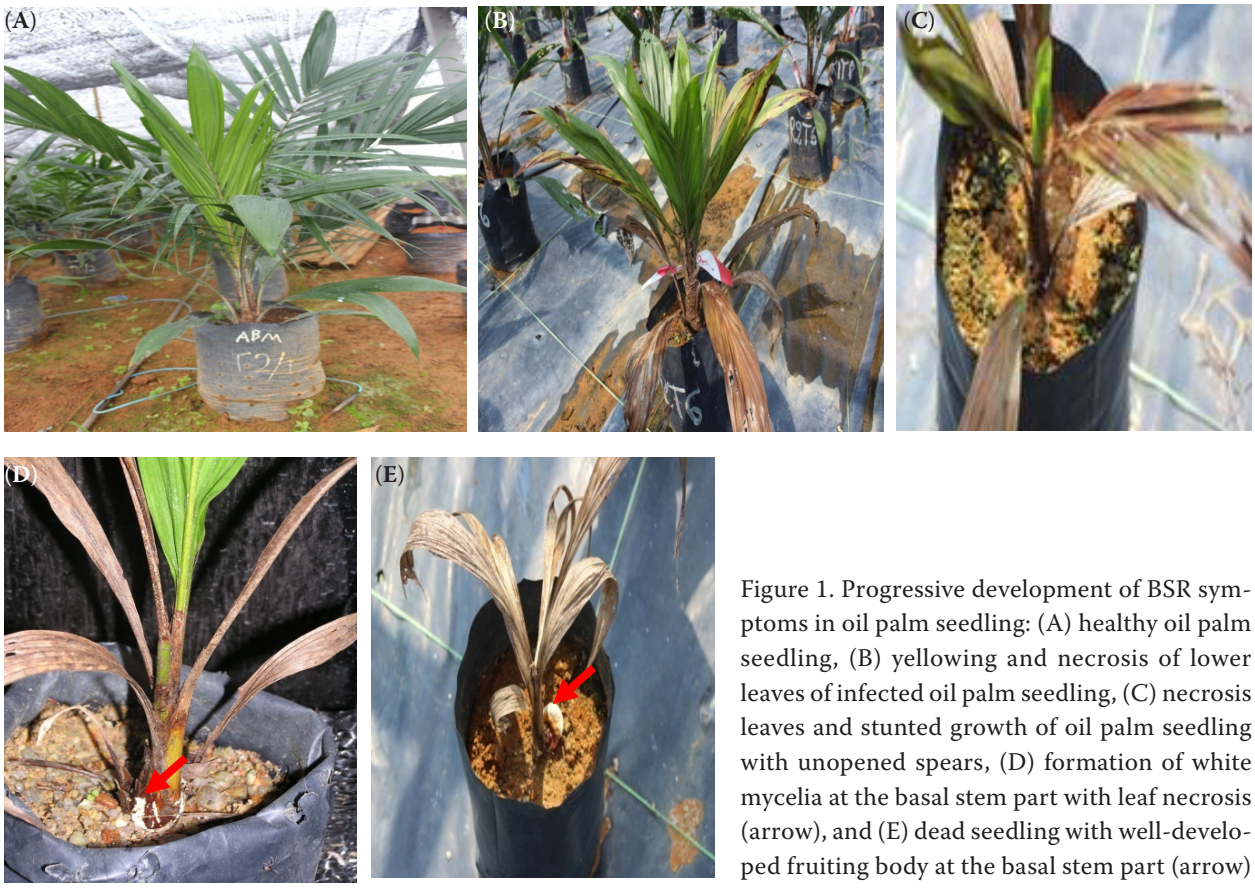

Figure 1. Progressive development of BSR symptoms in oil palm seedling: (A) healthy oil palm seedling, (B) yellowing and necrosis of lower leaves of infected oil palm seedling, (C) necrosis leaves and stunted growth of oil palm seedling with unopened spears, (D) formation of white mycelia at the basal stem part with leaf necrosis (arrow), and (E) dead seedling with well-developed fruiting body at the basal stem part (arrow)

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Table 3. Disease severity of foliar index (SDFI) and sisease incidence (DI) of oil palm seedlings after *Ganoderma boninense* artificial inoculation

Treatments	DSFI after months (%)				DI after months (%)			
	2	4	6	8	2	4	6	8
C1	0[a]	0[c]	0[c]	0[c]	0[a]	0[b]	0[b]	0[c]
C2	0[a]	8.3[a]	20.8[a]	87.5[a]	0[a]	41.7[a]	66.7[a]	100.0[a]
T1	0[a]	2.3[b,c]	4.6[b]	31.8[b]	0[a]	9.1[b]	18.2[b]	36.4[b]
T2	0[a]	0[c]	4.6[b]	27.7[b]	0[a]	0[b]	18.2[b]	18.2[b,c]

In each column with different letters is significantly different at $P \leq 0.05$; for treatment see Table 1

Table 4. Severity of foliar symptoms (SFS) of oil palm seedlings and dead seedlings (DS) of oil palm after *Ganoderma boninense* artificial inoculation

Treatments	SFS after months (%)				DS after months (%)			
	2	4	6	8	2	4	6	8
C1	0[a]	0[c]	0.4[c]	1.3[c]	0[a]	0[a]	0[a]	0[b]
C2	1.4[a]	15.9[a]	20.9[a]	90.4[a]	0[a]	0[a]	16.7[a]	75.0[a]
T1	0[a]	8.2[b]	11.5[b]	33.4[b]	0[a]	0[a]	8.3[a]	25.0[b]
T2	1.2[a]	6.3[b]	17.0[a]	26.6[b]	0[a]	0[a]	0[a]	16.7[b]

In each column with different letters is significantly different at $P \leq 0.05$; for treatment see Table 1

26.6% of SFS) compared to T1 (36.4% of DI; 33.4% of SFS), and C2 (100% of DI; 90.4% of SFS). The negative control treatment (C1) remained healthy with no dead seedlings recorded. The positive control treatment (C2) showed the highest percentage of dead seedlings at 75.0% followed by T1 at 25.0% and T2 at 16.7%, respectively (Table 4).

DISCUSSION

Bacterial *Burkholderia* GanoEB2 in BEFB or RSBF formulations was able to suppress BSR disease development in oil palm seedlings. Maizatul and Idris (2009) reported that *Burkholderia* isolated from oil palm was capable of suppressing *G. boninense in vitro* and in a nursery. A similar trial on the efficacy of *B. cepacia* (B3) in the form of bacterial suspension was reported by Sapak *et al.* (2006). The efficacy of using endophytic biological control agent in the field is determined largely by its formulation, shelf life, and delivery techniques (Stephens & Rask 2000; Khavanzi *et al.* 2007; Albareda *et al.* 2008). Successful powder formulation of *Burkholderia* GanoEB2 using vermiculite as carrier was developed by Nasyaruddin and Idris (2011). In this study, bacterial *Burkholderia* GanoEB2 was incorporated into bioorganic empty fruit bunch (BEFB) and real strong bioorganic fertilizer (RSBF) powder formulation as carrier and the quality of the viable cells after remain 10^8 CFU/g after 5^{th} and 6^{th} months of storage, respectively (Ili Nadhrah 2014). Integrating endophytic bacteria *Burkholderia* GanoEB2 into BEFB or RSBF can increase the soil pH and thus the efficiency of nutrient uptake by the crop and enhance the retention of nutrients in the soil in the long term to improve the soil quality. Bioorganic fertiliser is a substance containing living microorganisms which, when applied to seed, plant surfaces, or soil, colonises the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey 2003).

Table 5. Effect of bioorganic *Burkholderia* GanoEB2 on BSR development in oil palm seedlings after 8 months of inoculation

Treatments	AUDPC (units)	DR (%)
C1	0.0	–
C2	308.3	–
T1	90.9	70.5
T2	45.5	85.3

AUDPC – area under disease progressive curve; DR – disease reduction; for treatment see Table 1

Effectiveness of the formulated BEFB and RSBF containing *Burkholderia* GanoEB2 in controlling BSR disease in oil palm was evaluated based on

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quantitative assessments measured as percentage of disease incidence (DI), severity of foliar symptom (SFS), disease severity of foliar index (DSFI), and dead seedlings (DS) at a two-month interval. Percentage of disease reduction (DR) was calculated based on area under disease progressive curve (AUDPC).

DI was assessed based on foliar symptom and the appearance of white mycelia, white button or fruiting body in the seedling. Disease symptoms among the treated plants first appeared after 4 months of *G. boninense* inoculation. Lower DI values indicate the suppression of the disease by the seedlings. In this study, non-infected and untreated seedlings (C1) remained healthy with green leaves throughout the experiment. Four months after inoculation, C2 seedlings that were artificially inoculated with *G. boninense* and untreated, showed the highest DI (41.7%) and were significantly different if compared to seedlings inoculated with *G. boninense* and treated with BEFB containing *Burkholderia* GanoEB2 (T1) and seedlings inoculated with *G. boninense* and treated with RSBF containing *Burkholderia* GanoEB2 (T2) (9.1 and 0.0%, respectively) (Table 3). White mycelia of *Ganoderma boninense* appeared six months after inoculation and later, during the the 7th month, developed into fruiting body. At the end of experiment (8th month), as expected, seedlings in C2 recorded the highest value of DI, all of them were infected with *Ganoderma boninense,* and were significantly different from those in T1 and T2. T1 and T2 seedlings did not show any significant difference from the onset of *Ganoderma boninense* infection, however T2 showed the lowest DI values and could suppress the infection better than T1. This result suggests that T2 seedlings produced a good level of disease suppression as compared to T1. Both formulated BEFB containing *Burkholderia* GanoEB2 and RSBF containing *Burkholderia* GanoEB2 are capable of suppressing the BSR infection because the DI value was significantly reduced. Disease suppression is mainly due to induction of the host's defense mechanism, such as the formation of structural barriers, like lignified cell walls, to keep out the pathogen and the production of antifungal metabolites to slow down the infection progress and improve growth and plant vigour (Hammerschmidt & Kuc 1995). Sapak *et al.* (2006) in their studies proved that bacterial endophytes *Pseudomonas* and *Burkholderia* play a significant role in inhibiting the penetration of *Ganoderma boninense* into the vascular system. They were found histologically concentrated in the vascular system of roots taken from symptomless palm.

Disease development was also measured based on the percentage of severity of foliar symptom (SFS). SFS was calculated based on the number of desiccated leaves and yellowing leaves of oil palm seedlings. Low severity of the foliar symptoms indicated slow progress of the *Ganoderma boninense* infection in oil palm and expressed a partial resistance towards the disease. The seedlings started to display severity of the foliar symptom 4 months after inoculation (Table 4). Negative control seedlings (C1) remained healthy till the end of the experiment. A month after inoculation, T1 and T2 showed higher reduction of SFS percentage relative to the positive control (C2). This explained that, in the affected oil palm seedlings, the pathogen produced dry rot of internal tissues at the roots and the base of the stem part. This severely restricted the supply of water and nutrients to the palm and later caused yellowing and wilted leaves.

As shown in Table 4, the percentage of dead seedlings was not statistically different between the two treatments, however T2 recorded lower percentage of dead seedling compared to T1 showing that RSBF containing *Burkholderia* GanoEB2 formulation was more efficient in reducing the infection.

The disease severity of foliar index (DSFI) was scored on a scale of 0 to 4. The application of BEFB and RSBF containing *Burkholderia* GanoEB2 affected BSR severity and the DSFI was reduced compared to the untreated seedlings infected with *Ganoderma boninense* (C2). After 8 months of inoculation, the highest DSFI value was shown by the plants in C2, followed by T1 and T2. No significant difference was shown between the two treatments, the results however showed that T2 was more effective in slowing down the disease severity in oil palm seedlings.

Bacterial *Burkholderia* GanoEB2 inhibited *Ganoderma boninense* by colonising the intracellular and intercellular tissue of plant and minimised the chances of the pathogens for both nutrient and niches without causing any harm to the host plant (Kloepper *et al.* 1999; Hallmann *et al.* 2001). Significant disease suppression was also reported for wheat plants endophytically colonised with *Bacillus subtilis* (Liu *et al.* 2009). Another report stated that banana plants pre-inoculated with endophytic *Pseudomonas* and *Burkholderia* were able to suppress fungal disease caused by *Fusarium oxysporum* (Fishal *et al.* 2010).

Disease development was also assessed using the AUDPC calculated based on the DI, SFS or DSI values. Lower AUDPC values indicate less disease

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symptoms progress, and are therefore indicative of increased disease resistance. The ability of *Burkholderia* GanoEB2 in bioorganic formulations to reduce *Ganoderma boninense* infection was expressed as the percentage disease reduction (% DR) derived from AUDPC values. C2 showed the highest AUDPC value of 308.3, significantly different as compared to both treatments. T1 and T2 had lower AUDPC values of 90.9 and 45.5, respectively but there was no significant difference between them. The lowest AUDPC corresponded to the effectiveness of the endophytic bacteria *Burkholderia* GanoEB2 in reducing the disease. Thus, RSBF containing *Burkholderia* GanoEB2 (T2) was the most effective in suppressing the disease followed by BEFB containing *Burkholderia* GanoEB2 (T2) based on the % DR. The disease was reduced up to 85.3% DR in T2 compared to T1 (70.5% DR).

The present study showed that both BEFB and RSBF containing *Burkholderia* GanoEB2 showed high potential in reducing the *Ganoderma boninense* infection in oil palm seedlings. The lower the DI%, SFS%, DSI%, DS%, and the higher the DR%, suggested that the oil palm seedlings are able to resist the disease and reduce the incidence of *Ganoderma boninense.*

Early interaction between biocontrol agent and pathogen and repeated application of effective biocontrol agent can significantly reduce the BSR disease incidence. Sapak *et al.* (2006) in their studies applied the *Burkholderia cepacia* suspension twice before being challenged with *Ganoderma boninense,* and after 8 months of inoculation, *Burkholderia cepacia* recorded the DI value of 60.0%. In this study, two treatments (BEFB and RSBF containing *Burkholderia* GanoEB2) were applied repeatedly and consistently in regular two-month intervals up to the end of the experiment. The results showed that the DI value decreased to 36.4 and 18.2% for T1 and T2, respectively. The results suggested that the timing and frequency of application of bioorganic formulations are important for effective strategy of BSR disease control. Although promising results were obtained from using BEFB and RSBF containing *Burkholderia* GanoEB2 to control BSR disease in the field, future studies should determine the required population density of bacterial endophytes in application doses to minimise wasteful application and maximise the effect of the product in suppressing the BSR disease in seedlings.

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