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Biocontrol Agent *Trichoderma harzianum* Strain FA 1132 as An Enhancer of Oil Palm Growth

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ABSTRACT

Agricultural products are mostly and adversely affected by environmental pollution caused by chemical residues of pesticides which are used for plant disease management. Consequently, researchers look for alternative approaches of disease control such as biocontrol agents. The results of this study showed that conidial suspension of the soil borne fungus *Trichoderma harzianum* strain FA 1132 can control *Ganoderma boninense* which causes basal stem rot (BSR) disease in oil palm. The conidial suspension treatment was applied by using *Trichoderma*-incorporated surface mulch. The disease severity index value (DSI) showed that *Ganoderma* infected the root as early as at week 5, with a DSI value of 8.3%, while physical symptoms appeared in leaf at week 8. However, no disease symptom was observed in *T. harzianum* strain FA1132 treated plants and it also markedly increased oil palm root and leaf weights.

Key words: Biocontrol effect, *Trichoderma harzianum* strain FA 1132, *Ganoderma boninense*

INTRODUCTION

The soil-borne fungus, *Trichoderma harzianum*, is known as an early invader of roots which rapidly multiplies in an ecological niche of the roots. It has potential to be a biocontrol agent against many soil-borne pathogens, specifically for *Ganoderma boninense* which causes basal stem rot (BSR) disease of oil palm. The disease and its causal pathogen were first reported in 1931 by Thompson to infected old age oil palm trees (Latiffah *et al*., 2005). In the 1960, the disease was found in younger palms of 10-15 years (Turner, 1981). Currently, *Ganoderma* was reported to cause infection in oil palms as young as 1-2 years of age (Azhar *et al*., 2008).

The available control measures for BSR diseases, such as cultural practices or fungicides, were unsatisfactory due to the fact that *Ganoderma* has various resting stages like melanised mycelium, basiodio-spores and pseudosclerotia (Izzati & Abdullah, 2008). By the time the disease symptoms appear, about more or less 50% of the palm's internal tissues have already rotted. Therefore, fungicides cannot cure such badly infected palms. In order to combat this characteristic, the best approach to control BSR disease is by biological control and the utilization of BSR resistant oil palm plants. It has been established that the biological control of plant pathogens is an alternative approach to decrease the strong dependence of modern

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agriculture on chemical fungicides which cause environmental pollution.

Trichoderma is one of the most exploited fungal biocontrol agents in agriculture for the management of plant diseases caused by a wide spectrum of fungal pathogens (Elad, 2000; Mathivanan *et al*., 2000). *Trichoderma* sp. typically grows toward the hyphae of other fungi, coils about them in a lectin mediated reaction, and degrades the cell walls of the target fungi by the secretion of different lytic enzymes. This process of mycoparasitism limits the growth and activity of plant pathogenic fungi (Almeida *et al*., 2006).

Many in-house trials and field trials have been conducted using various *Trichoderma* spp. which showed a high efficacy for controlling *Ganoderma* infection in oil palms (Abdullah *et al*., 1999; Sariah *et al*., 2005; Susantho *et al*., 2005), and *Trichoderma* spp. are being commercialized for use in the protection and growth enhancement of a number of crops (Samules, 1996). Harman (2004; 2006) stated that *Trichoderma* sp. could control soil-borne pathogens, enhance plant growth and stimulate plant defense mechanism. Therefore, *Trichoderma* sp. has been acknowledged as a potential biocontrol agent for a broad range of plant pathogens in agriculture.

The efficacy of *T. harzianum* strain FA 1132 as a biocontrol agent for BSR disease was already proven by previous studies based on leaf data alone (Izzati, 2008; Sundram, 2008). Therefore, the main aim of this study was to determine whether *T. harzianum* strain FA 1132 only controlled BSR disease or it could also enhance oil palm health. Hence, this study measured both the root and leaf weights separately, determined the chlorophyll concentrations in the treated plants, as well as ascertained the disease severity index (DSI) values of *Ganoderma* infection in root and leaf separately when the palms were inoculated with *T. harzianum* and *G. boninense,* either separately or together.

MATERIALS AND METHODS

The Carriers for Trichoderma mulch Preparation

T. harzianum strain FA 1132 was collected from the Mycology and Plant Pathology Laboratory, Department of Biology, Universiti Putra Malaysia (UPM). Tricho-carrier was prepared by using palm pressed mesocarp fibres as organic compost. The whole fibres were washed through running tap water. After that, around 300 g per packet of palm pressed mesocarp fibres was placed into heat resistant polypropylene bags and autoclaved at 121°C, 1.04 kg/cm^2 for 45 min. Then, seven days cultured *T. harzianum* (strain FA 1132) conidial suspension was added into the sterilized palm fibres. Subsequently, the inoculated carrier bags were incubated in a dark chamber, with the temperature of 28 ± 2°C for 2 weeks.

Gano-wood Block Preparation

Gano-wood blocks were prepared by using wood from rubber tree (*Hevea brasiliensi*). The wood was cut into 2.5 x 2.5 x 5 cm^3 sized block each and washed by running tap water before soaking them in distilled water overnight. They were put into plastic bags and autoclaved at 121°C, 15 psi for 45 min. Then, 100 ml of potato sugar agar (PSA) was transferred into the plastic bags containing sterilized block and the blocks were autoclaved once again. Seven day old culture of *Ganoderma boninense* strain PER71, obtained from Malaysian Palm Oil Board (MPOB, Bangi, Malaysia), was cut into pieces and transferred into the cooled PSA containing sterilized block. The blocks were incubated for 10-12 weeks (Abdullah *et al*., 2001).

Experimental Design and Plant Treatment

A glass house trial plant treatment was carried out for 8 weeks using a completely randomized design with three biological replicates. A total of 108 five-months old oil palm seedlings (Dura X Pisifera) were obtained from Sime Darby Seeds & Agricultural Services Sdn. Bhd. (42700

Banting, Selangor, Malaysia). The plants were divided into four treatments: (1) Control oil palm seedlings without any inoculation (C); (2) *Ganoderma* inoculated oil palm seedlings (G); (3) *Trichoderma* inoculated oil palm seedlings (T); and (4) *Trichoderma* and *Ganoderma* together inoculated oil palm seedlings (GT).

Khairuddin's (1990) method was used for the artificial inoculation of *Ganoderma* onto the oil palm seedlings. In brief, a *Ganoderma* colonized wood block was placed in direct contact with the roots of one oil palm plant grown in a garden pot, which was then covered with soil; after that, around 600g Tricho-carrier mulch was placed onto the surface of the soil contained in the pot.

Preparation of Trichoderma Conidial Suspension

In addition to the surface mulch, the *Trichoderma* treated plants were also periodically given *Trichoderma* conidial soil drench. Conidial suspension was prepared from a 7-day old culture plate of *Trichoderma*. The conidia of FA 1132 were briefly harvested in 10 ml distilled water and gently removed with an L-shaped glass rod. The mixture was filtered using Whatman no. 1 filter paper to separate the mycelial debris and was topped up with distilled water to make 1 litre of conidial suspension. The fresh conidial suspension was poured at a rate of 1 litre per plant onto treatments T and GT, with *Trichoderma* carrier mulch, once every 2 weeks.

Trichoderma Colony Forming Unit (cfu) Measurements from Control and Treatment Soils

Samples from the control and treatment soils were recorded by using a cock borer, with a length of 34 cm and a diameter of 3 cm. The sampling was done at 5 cm and 15 cm soil depths for each treatment/ control. Meanwhile, a 10 g soil sample was mixed in 100 ml autoclaved distilled water by shaking it in an orbital shaker at 100 rpm for 15 minutes. Later, this mixture was diluted through serial dilution until 10^{-3} and 1 ml of 10^{-3} diluted soil was transferred onto a Petri plate and 9 ml of Rose Bengal Agar (RBA) was added onto the plate. The plates were gently swirled and incubated at room temperature (28±1°C). The cultures were done in three replicates. The cfu measurements were counted and recorded after 5 days.

Chlorophyll Determination

The concentrations of chlorophyll a (Chla), chlorophyll b (Chlb) and total chlorophyll were analyzed following the method described by Arnon (1949). N, N-dimethylformamide (DMF), was used for chlorophyll determination by immersing the leaves in the solvents. Leaf samples from the control and treatments were used for the determination of total chlorophyll. The ratio for the extraction was 10% (w/v). After that, the extract was kept in the dark for 48 hours at 4°C prior to spectroscopic examination.

The concentrations of Chlorophyll a and Chlorophyll b were measured using a UV-nanophotometer (IMPLEN, Nanophotometer, Malaysia) at the wavelengths of 663 nm and 645 nm. The concentrations of Chl a, Chl b and total chlorophyll in the leaf tissues were calculated according to the following equations:

$$\text{mg chlorophyll a/ litre} = 0.0127\,(O.D._{663}) - 0.00269\,(O.D._{645})$$

$$\text{mg chlorophyll b/ litre} = 0.0229(O.D._{645}) - 0.00468\,(O.D._{663})$$

Where, O.D is the optical density at that wavelength.

$$\text{Total chlorophyll} = [\text{Chl a}] + [\text{Chl b}]\ \mu g/g.$$

Root and Leaf Weight Determinations

The root and leaf samples from the control and treatments were taken at 2, 5, and 8 weeks of post-inoculation. The seedlings were uprooted and washed with running tap water. The leaves and roots were both dried with paper towels. The control and treated seedling roots and leaves were excised using a pair of clean scissors and their weights were also taken.

Assessment of Disease Signs and Symptoms

Ganoderma infection in the oil palm starts from the root tissues. This study assessed the signs and symptoms of the disease from the root to shoot by destructive sampling at every time point. The signs and symptoms of the disease were examined based on the BSR with some modifications (Izzati & Abdullah, 2008). A disease index value of 0 means that all plants are healthy, while the values between 0 - 100 represent a range of severity. The Mathematical formula of disease severity index (DSI), based on the observation of signs and symptoms of the disease class of the infected plants, gives numerical values ranging from 0 to 4 (Izzati & Abdullah, 2008) as in Table 1.

TABLE 1
Disease signs and symptoms corresponding to the disease class (Izzati & Abdullah, 2008)

Class	Disease sign and symptoms
Class 0	Healthy plant with green leaves and no mycelial development on any part of the plant.
Class 1	Formation of white mass of mycelia on any part of the plant, with or without chlorotic leaves.
Class 2	Appearance of 3 or more chlorotic leaves.
Class 3	Formation of sporophores or basidioma on any part of the plant with chlorotic leaves.
Class 4	Appearance of well-developed basidioma on plants showing at least 50% dried leaves and the plant drying up, is dying or is already dead.

Formula of Disease severity index (DSI) = $$\frac{\sum (A \times B) \times 100}{\sum n \times 4}$$

Where, A= Disease classes (0, 1, 2, 3 and 4).
B= Number of plants showing disease per treatment.
n= total number of plants per treatment, with class 4 represents the highest class of assessment.

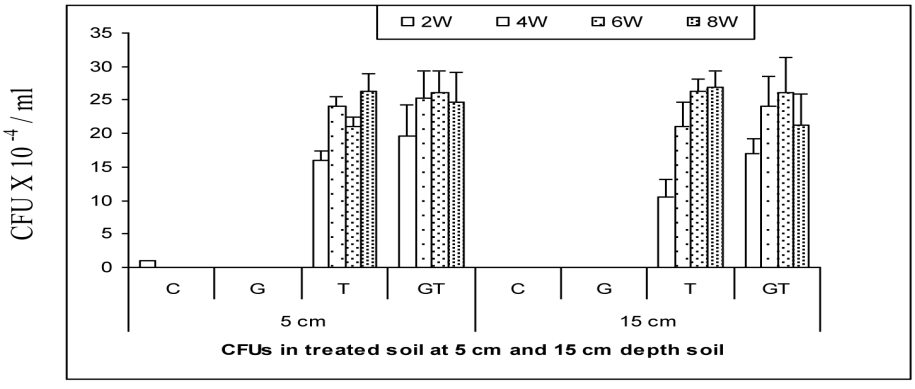
Statistical Analysis

All the data were analyzed using the SPSS software version 17.0. The t-test was carried out on cfu/g between 5 cm and 15 cm of soil depth. The chlorophyll amount, root and leaf weight data were subjected to test for analysis of variances (ANOVA). The mean differences were determined by the Duncan's homogeneity subsets if F tests were significant at 95% probability.

RESULTS AND DISCUSSION

Estimation of T. harzianum Colony Forming Units in Soil

The distribution of the cfu/g soil over 8 weeks is shown in *Fig. 1*. However, the initial load of *Trichoderma* cfu conidial suspension in the surface mulch was 3 x10^{11}, and more or less the same loading was used at the subsequent time points. The result showed that among the treatments T and GT at 5 cm and 15 cm depth soil, the cfu/g values for both were almost similar after 2 weeks. At week 2, the cfu/g at 15 cm depth soil was low in treatment T. On the other hand, it increased dramatically after 2 weeks. The control (C) and *Ganoderma* treated plant (G) soil samples were checked to observe the presence of any *Trichoderma* or other soil fungi. At week 2, only the 5 cm deep control soil had a few colonies, but after that, none was observed in the following samplings. Meanwhile, no significant difference was found when the t-test was carried out on treatments T and GT between 5 cm and 15 cm depth soil cfu/g, the p value was 0.886 and 0.09, respectively. Similar results were also obtained in the oil palm which had been treated with *T. harzianum* strain FA 1132 and the cfu/g decreased at 15 cm depth soil compared to 5 cm depth soil after 14 weeks (Sundram *et al*., 2008). Although the reason for this was not clear, it might involve the timing of the sporulation of the fungi. In this study, the cfu/g did not decrease until the end of the experiment at week 8 and at 15 cm depth soil. Unfortunately, Sundram *et al*. (2008) did no



Legend:
C: Control plant
G: *Ganoderma* infected plant
T: *Trichoderma* treated plant
GT: *Ganoderma* + *Trichoderma* treated plant

Fig. 1: T. harzianum *colony forming units recorded until 8 weeks post inoculation with each sampling taken before the application of* T. harzianum *conidial suspension. Error bar is standard error.*

t report any finding at week 8 of their experiment for us to compare the results of the current study with.

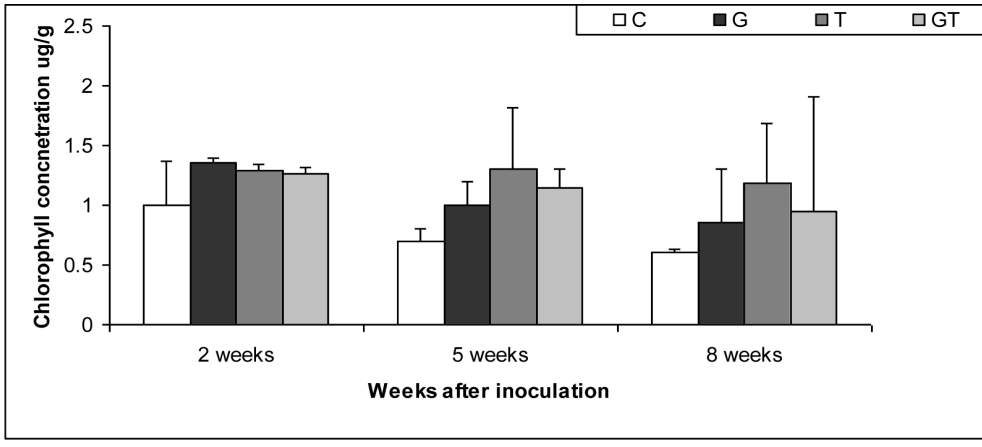
Determination of Chlorophyll Concentration

Chlorophyll concentrations were determined at 2, 5, and 8 weeks of post-inoculation (Figure 2). It is crucial to note that there was no statistically significant difference found between the treatments by the ANOVA test. Even though the concentration of chlorophyll at week 2 was high in *Ganoderma* infected oil palm, it dramatically reduced at the subsequent time points. The concentration of chlorophyll in the *Trichoderma* treated oil palm was slightly reduced after 2 weeks.

Therefore, the current study found that the concentration of chlorophyll was not increased at the time points of weeks 2, 5 and 8 in the *Trichoderma harzianum* strain FA 1132 treated plants (T and GT) but the concentration was higher than that of the control (C) plants and *G. boninense* treated plants (G) after 2 weeks. However, the concentration of chlorophyll was increased by *T. harzianum* strain 1295-22, BR16 and *T. virens* strain R42 in cucurbitaceous seedling plants; on the contrary, the concentration in the plants treated with *T. harzianum* strain Zts428 was not increased in the same study (Lo & Lin, 2002). The increase of chlorophyll in plants may depend on fungal strains.

Estimation of Root and Leaf Weights

The ANOVA test showed that the root weights (*Fig. 3*) of the G (*Ganoderma* alone) treated plants were significantly different from C, T and GT plants at 2, 5 and 8 weeks of post-inoculation, while the weight of the leaf (*Fig. 4*) was significantly different at week 8, indicating that *Ganoderma* infected the root within 2 weeks and the disease spread to the basal tissue and leaf within 8 weeks. At 8 weeks of post-inoculation, *Ganoderma* sporophore was observed on the root, while basal tissue and some bottom leaves had already turned yellow from this infection (*Fig. 4* and *Fig. 5*). However, no infection was observed in the plants treated with *Ganoderma* and *Trichoderma* together. Moreover, it was



C = Control, G = *Ganoderma*, T = *Trichoderma*, GT = *Ganoderma* +*Trichoderma*

Fig. 2: Chlorophyll concentrations in the treated leaf samples. Error bar is standard deviations.

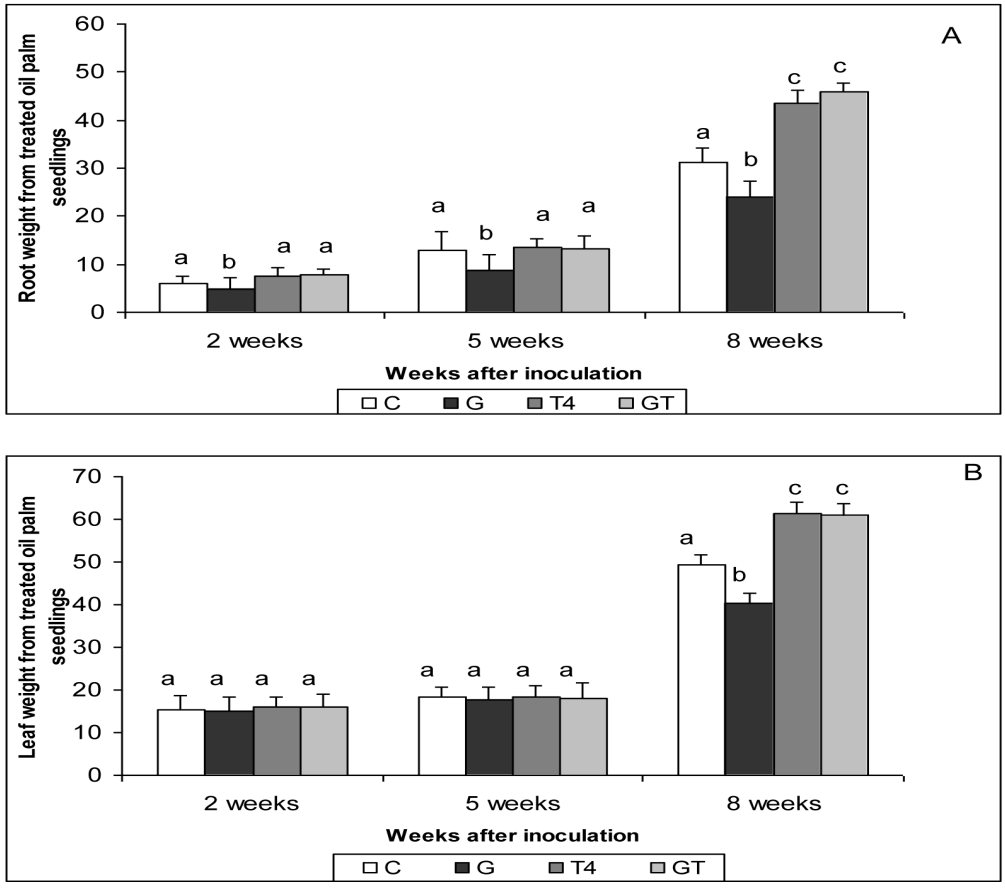
found that in either the *Trichoderma* alone (T) or together with the *Ganoderma* (GT) treated plants, the root and leaf weights increased gradually compared to the control (C) plant and the plants treated with *Ganoderma* alone (G). However, it is also well known that *Trichoderma* species enhances plant growth (Baker, 1991; Kleifeld & Chet, 1992; Sivan *et al*., 1984). This study found that *T. harzaianum* strain FA1132 increased oil palm leaf and root weights compared to that of the control plants (C) and the plants infected with *Ganoderma* alone (G). Meanwhile, the root and leaf weights were significantly different between the treatments. An enhanced root growth by the application of *Trichoderma* sp was also observed in the previous studies on other plants. For example, *T. harzianum* increases plant vigour of bentgrasses and Cron plant's root and shoot growth (Lo *et al*., 1997; Bojrkman *et al*., 1994).

Nonetheless, the mechanism of plant growth induction by *Trichoderma* spp. is not clear. There may be a few factors involved for its beneficial effects on plant growth, such as: (1) controlling deleterious microorganisms, (2) stimulating plant growth factors like plant hormones, (3) increasing nutrient up take or enhancing availability of the necessary nutrients, and (4) reducing the concentrations of soil substances which are inhibitory to plant growth (Kleifeld & Chet, 1992; Wang, 2000; Windham, 1986; Lo & Lin, 2002).

Disease Severity Index

Treatment G (*Ganoderma* alone) showed disease symptoms as early as week 5 (*Fig. 6*) in the root, with a DSI value of 8.3%. Nevertheless, no disease symptoms were observed in control (C), *Trichoderma* alone (T), and *Ganoderma* + *Trichoderma* (GT) at any time point in either tissue (*Fig. 4*) at all time points in both tissues (Table 2).

Trichoderma spp are well established as biocontrol agents for several plant diseases (Harman *et al*., 1989; Lo *et al*., 1997; Papavizas, 1985). This study aimed to study the disease development and the control of the disease at its early stage of infection. The researchers found that *T. harzianum* strain FA1132 could control *G. boninense* as well as increase oil palm root (A) and leaf (B) growth (Figure 3). Some previous studies have proven *Trichoderma* spp to have high efficacy for controlling *G. boninense* infection in oil palms (see Abdullah *et al*., 1999; Sariah *et al*., 2005; Susantho *et*



Legend:
C: Control plant
G: *Ganoderma* infected plant
T: *Trichoderma* treated plant
GT: *Ganoderma+Trichoderma* treated plant

Fig. 3: Weight of oil palm seedlings roots (A) and leaves (B) after the treatments. Duncan's homogeneity test was used to define ranked classes. Means with the same letter are not significantly different. Error bars represent standard deviations.

al., 2005). Meanwhile, *T. hazrianum* strain FA1132 was found to be an efficient biocontrol agent of *Ganoderma* for BSR disease of oil palm (Abdullah *et al*., 2003; Izzati *et al*., 2008; Sundram *et al*., 2008). However, previous studies only observed the leaf portion. Thus, this study estimated the DSI values in the root and leaf separately by destructive sampling because by the time the external disease symptoms appeared, most of the internal tissues had already died. The authors observed a DSI value of 8.3% in the root at week 5, whereas no disease symptom was recorded in the leaf at week 5. In the leaf, the disease symptoms were first observed at week 8 with a DSI value of 11.11%, whereas at week 8, most roots had already been damaged with a DSI value 16.66%. Sundram *et al.* (2008) and Izzati (2008) only recorded the disease symptoms at weeks 12 and 14 and obtained the DSI values of 16.67% and 12.5%, respectively, in the leaf tissues alone. Later, their time periods were compared to the

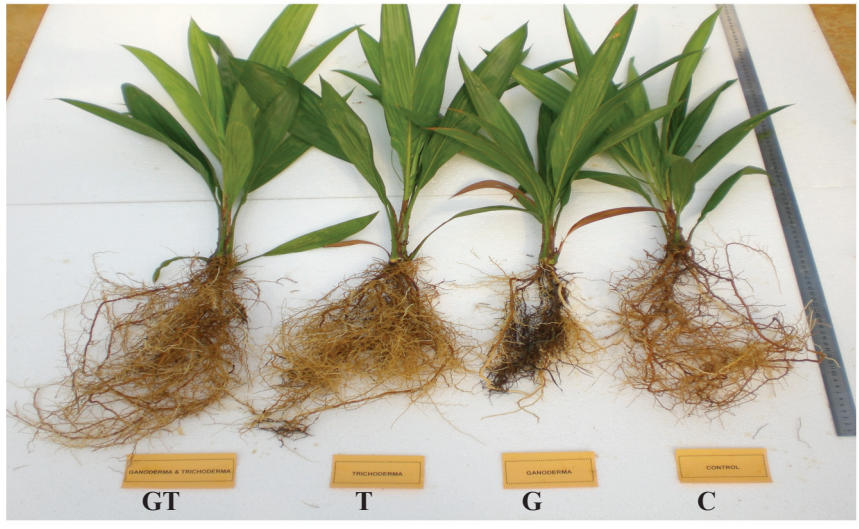


Fig. 4: Treated plants after post-inoculation. C - control plant, G -Ganoderma *infected plant, T* - Trichoderma *inoculated plant and GT* - Ganoderma + Trichoderma *together treated plant.*

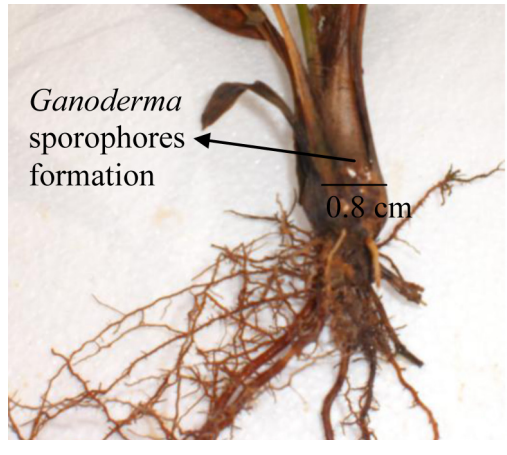


Fig. 5: Ganoderma *sporophores formation on plants. Scale bar is an equal amount to 0.8 cm.*

time points used in this study which aimed at the earlier stages of BSR disease. There might be differences in the pathogen growth and environmental effects for disease development (George, 2005). As mentioned earlier, most of the tissues were already internally infected by *G. boninense* when the disease symptoms appeared physically. However, information on early plant infection is still lacking. Therefore, to deal with this lack of information, this study observed *G. boninense* infection by destructive sampling and found that within one month, the plant roots were already infected but it was only at two months that the physical symptoms appeared in the leaf. Nevertheless, no disease symptom was observed in the leaf and root tissues until the end of the experiments in the current study, i.e. when the plants had simultaneously been infected with

TABLE 1
Disease Severity Index (DSI) values over 8 weeks of inoculation

Treatments	Disease severity index (%)					
	2 weeks		5 weeks		8 weeks	
	Root	Leaf	Root	Leaf	Root	Leaf
C	0.00	0.00	0.00	0.00	0.00	0.00
G	0.00	0.00	8.3	0.00	16.66	11.11
T	0.00	0.00	0.00	0.00	0.00	0.00
GT	0.00	0.00	0.00	0.00	0.00	0.00

G. boninense and *T. harzianum*. The treatments with *T. hazrianum* strain FA1132 conidial suspension were given at every 2-week intervals up to the end of the experiment. Therefore, the treatment with *T. harzianum* is a good approach for controlling *G. boninense* infection.

CONCLUSIONS

In this study, *G. boninense* was found to infect the roots within 2 weeks but the disease only appeared in the leaves at week 8. It was observed that *T. harzianum* (strain FA1132) was not only efficient for controlling the BSR disease but it also enhanced chlorophyll concentration, as well as the weights of root and leaf. Therefore, *T. harzianum* mulch and its conidial suspension can be used as fertilizers to increase oil palm health and protect the plant from BSR disease.

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