# Disease Suppression in *Ganoderma*-infected Oil Palm Seedlings Treated with *Trichoderma harzianum*

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**Abstract**

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Disease suppression in *Ganoderma*-infected oil palm seedlings treated with a conidial suspension of *Trichoderma harzianum* FA 1132 was tested in plant house conditions to determine the effectiveness of the fungus as a biocontrol agent. The highest efficacy of control was achieved by treatment right after artificial infection; the total number of infected plants was reduced to give the lowest disease severity index (DSI) value of 5.0%, compared to the infected and non-treated control that had the highest DSI of 70.0%. After conidia suspension of FA 1132 was applied, the colony forming ability by *Trichoderma* in the soil was dramatically increased, but decreased after some time. Results of the present study are a useful reference basis for further tests in the field and large scale production trials.

**Keywords**: *Ganoderma boninense; Trichoderma harzianum;* oil palm; biological control; basal stem rot

The soilborne fungus *Trichoderma harzianum* isolate FA 1132 is one of the indigenous fungi with potential for use as a biocontrol agent against many soilborne pathogens, especially Basal Stem Rot (BSR) of oil palm, caused by *Ganoderma boninense*. BSR is considered the most serious plant disease in Malaysia (Turner & Bull 1967). Previously, BSR was only found on older plants, but recently the disease was observed on 5-year-old or younger plants. Since the pathogen has caused severe losses of oil palm production, controlling it is an important factor. The available technique of disease control is fungicidal treatment, though often applied ineffectively. *In vitro* studies by Idris *et al.* (2002) claimed that numerous fungicides were strongly inhibitory towards growth of *Ganoderma*. However, the results of fungicidal control of this disease in the field have been inconclusive (Idris *et al.* 2002). This phenomenon is probably due to the fact that *Ganoderma* has various resting stages such as melanised mycelium, basidiospores and pseudosclerotia (Susanto *et al.* 2005) that are more resistant to fungicides. On the other hand, once external symptoms appear the infection is already too severe, and a biocontrol agent cannot control the pathogen during this stage.

Therefore, alternative control measures are focused on the use of biocontrol agents, including *Trichoderma* spp. (Ilias 2000; Sariah *et al.* 2005; Susanto *et al.* 2005). Biocontrol using *Trichoderma* spp. showed a high efficacy in controlling the growth of and infection by *G. boninense* in plant house trials and under field conditions (Abdullah *et al.* 1999; Ilias 2000; Sariah *et al.* 2005; Susanto *et al.* 2005). Abdullah *et al.* (1999) were successful in plant house trials by using iso-

late *T. harzianum* FA 1132, and they showed that the isolate has good antagonist properties against *G. boninense* and is effective in controlling disease development. Strains of *T. harzianum* are currently being commercialised because they exert strong competitive effects for space and nutrients; more importantly they produce toxins against phytopathogenic species, thus making them good bicontrol agents (Zimand *et al.* 1996; Susanto *et al.* 2005; Sharoni *et al.* 2006).

In addition to being a possible biocontrol agent against *G. boninense,* causing BSR, commercially produced *T. harzianum* is also used to prevent development of several pathogenic fungi such as *Pythium graminicola* (*Pythium* root rot; Lo *et al.* 1997), *Rhizoctonia solani* (Brown patch; Lo *et al.* 1997), *Sclerotinia homoeocarpa* (dollar spot; Lo *et al.* 1997), *Alternaria alternata* (Alternaria disease; Roco & Maria Pèrez 2001), *Crinipellis perniciosa* (witches' broom disease; De Marco & Felix 2002), *Botryosphaeria berengeriana* f. sp. *piricola* (apple ring rot; Kexiang Xiaoguang *et al.* 2002), *Serpula lachrymans* (dry rot disease; Phillips-Laing *et al.* 2003) and the root-knot nematode, *Meloidogyne javanica* (Sharon *et al.* 2001).

However, information on the appropriate time for applying the treatment is limited. On the basis of the importance of early detection for controlling the disease, a study on the appropriate time for treatment with FA 1132 was conducted. We investigated the efficacy of *T. harzianum* FA 1132 as a biocontrol of BSR by using three treatments with conidial suspension: applied immediately after artificial inoculation with *G. boninense,* or treatment was delayed by 2 or 4 weeks. Disease suppression was assessed by calculating the disease severity index (DSI) based on level and degree of external symptoms of the disease.

## MATERIALS AND METHODS

***Sources of fungi and planting materials***. Cultures of *G. boninense* FA 5201, pathogen of basal stem rot of oil palm, and *T. harzianum* FA 1132 (NCBI GeneBank accession number EU443834; Shafiquzzaman *et al.* 2007), acting as a biocontrol agent, were obtained from the Mycology Laboratory, Biology Department, Faculty of Science, University Putra Malaysia (UPM). Isolate FA 5201 originated from a basidiocarp at the base of a BSR-infected oil palm at Banting, Selangor, whereas isolate FA 1132 was originally isolated from oil palm soil at Batang Melaka, Negeri Sembilan. Fifty 5 months-old oil palm seedlings were used for the infection study, that consisted of five experimental variants (two controls and three treatments), each variant with 10 seedlings.

*Trichoderma* ***carrier***. The carrier material for FA 1132 was organic compost; 500 g were placed into heat resistant polypropylene bags (15 × 25 cm) and autoclaved at 121°C, 1.04 kg/cm$^2$ for 45 minutes. The sterile compost was inoculated and mixed with a conidial suspension of FA 1132 obtained from a 7 days-old culture plate. The bags with the inoculated carrier material were incubated in a dark room at 28 ± 1°C for 2 weeks.

***Preparation of wood blocks for artificial inoculation***. Blocks of wood 5 × 5 × 10 cm from the rubber plant (*Hevea brasiliensis*) were autoclaved at 121°C, 15 psi, 45 minutes. About 100 ml per block of hot, liquid malt extract agar (MEA) was poured over the blocks and these were re-sterilised. The cooled blocks containing MEA were inoculated with macerated FA 5201 at a rate of the cultures from one Petri dish per block and incubated for 8–10 weeks. Garden pots of 28.0 × 25.5 × 16.0 cm size were filled to 1/3 with a soil mixture 3:2:1 of peat:clay:sand. The artificial inoculation was conducted by following Khairuddin (1990) with slight modifications. A colonised block was placed in direct contact with three roots of one seedling in a garden pot to avoid root contact with external sources and then covered with soil and the inoculated mulch. Uninoculated control blocks were used as negative control (C1). The seedings of C1 and C2 were covered with uninoculated mulch.

***Experimental layout***. All oil palm seedlings were placed and arranged in a randomised block design under plant house conditions for 20 weeks. Using tap water, the seedlings were watered twice daily. The seedlings were divided into two controls (C1 – non-inoculated plants; C2 – inoculated and untreated plants) and three treatments that differed in the timing of when the inoculated plants were treated with FA 1132 (T1 – treatment immediately after inoculation; T2 – treatment 2 weeks after inoculation; T3 – treatment 4 weeks after inoculation).

***Preparation of conidia suspensions***. Besides to the surface mulch, seedlings were also periodically given a conidial soil drench. Conidia of FA 1132 were harvested from a 7 days-old culture on PDA. The culture plate was flooded with 10 ml distilled

Table 1. The signs and symptoms of plants were scored on a disease scale 0-4 (Abdullah *et al.* 2003; Ilias 2000)

| Disease class | Signs and symptoms of infection |
|---|---|
| 0 | Healthy plants with green leaves without appearance of fungal mycelium on any part of plants |
| 1 | Appearance of white fungal mass on any part of plants, with or without chlorotic leaves |
| 2 | Appearance of basidioma on any part of plants with chlorotic leaves (1–3 leaves) |
| 3 | Formation of basidioma on any part of plants with chlorotic leaves (> 3 leaves) |
| 4 | Formation of well-developed basidioma and the plants dried |

water and the conidia gently dislodged with an L-shaped glass rod. The solution was filtered through a double-layered muslin cloth to remove mycelial debris, and made up to 1 l by adding distilled water. The conidial counts were in the range of 1–9 × $10^7$ conidia/ml. The fresh conidia suspension was poured at 1 l/pot onto variants T1, T2 and T3 with *Trichoderma* carrier mulch, once every 2 weeks. No conidia suspension was applied to plants of variants C1 and C2, although they were watered every day at approximately 1 l/plant.

***Disease severity index (DSI)***. The plants were scored for disease class on a scale of 0 to 4 (Table 1; Figure 1). After recording the disease class for each control and treatment, the disease severity index (DSI) was calculated following Abdullah *et al.* (2003) and Ilias (2000). The DSI values were analyzed by using non-parametric technique (Friedman test) using SPSS programme version 11.0. The DSI was calculated every week based on the following formula:

$$\text{Disease severity index (DSI)} = \frac{\Sigma(A \times B) \times 100}{\Sigma B \times 4}$$

where:

A – disease class (0, 1, 2, 3 or 4)

B – number of plants showing that disease class per treatment

***Estimation of the colony forming unit (CFU/g) of*** *Trichoderma* ***in the soils of each control and treatment***. Soil samples from controls and treatments were taken with a 1 cm diameter sterile cork borer. It was pressed into the soil to a depth of 8 cm, and a 10 g soil sample was mixed in 100 ml distilled water by shaking in an orbital shaker at 100 rpm for 10 minutes. Serial dilution of the soil sample was conducted until $10^{-3}$ dilution and 1 ml of the final ($10^{-3}$) diluted soil was pipetted into a Petri dish. Then, 9 ml of Rose Bengal Agar (RBA) was poured onto the diluted soil, gently shaken and left to cool. Cultures were done in three replicates and incubated at 28 ± 1°C. The cfu for each control and treatment were counted and recorded after 5 days. The colonies were cultured on slides and observed the morphological characteristics for species identification.


Figure 1. Samples of plants belonging to each of the disease classes: 0 (healthy plant) to 4 (infected and dead plant)

Table 2. Disease severity index (DSI) of oil palm plants at 12, 14, 16, 18 and 20 weeks after artificial inoculation

| Experiment | Disease severity after week* (%) | | | | |
|---|---|---|---|---|---|
| | 12 | 14 | 16 | 18 | 20 |
| Control 1 (C1)<br>– Negative control, non-infected and no treatment | 0[a] | 0[c] | 0[c] | 0[d] | 0[d] |
| Control 2 (C2)<br>– Positive control, artificially inoculated but untreated | 0[a] | 12.5[a] | 27.5[a] | 62.5[a] | 70.0[a] |
| Treatment 1 (T1)<br>– Artificially inoculated and treated immediately after inoculation | 0[a] | 0[c] | 0[c] | 5.0[d] | 5.0[d] |
| Treatment 2 (T2)<br>– Artificially inoculated and treated 2 weeks after inoculation | 0[a] | 0[c] | 12.5[b] | 17.5[c] | 25.0[c] |
| Treatment 3 (T3)<br>–Artificially inoculated and treated 4 weeks after inoculation | 0[a] | 7.5[b] | 15.0[b] | 37.5[b] | 45.0[b] |

*DSI in each column with different letters is significantly different at $P \leq 0.05$

## RESULTS

### Disease severity index (DSI)

In plant house trials, the conidia suspension of FA 1132 had effects on BSR severity and distribution of the causal fungus. Their application in water reduced the severity of the disease; the oil palm seedlings treated with FA 1132 showed various levels of external signs and symptoms and DSI values. In contrast, non-infected plants of control C1 remained healthy with green leaves. The highest DSI value of 70% at week 20 was shown by the plants of control C2 that were artificially inoculated and non-treated. The best disease control was exhibited by T1 (treatment immediately after inoculation). The DSI was increased after 14 weeks and the DSI values for treatments T1, T2 and T3 at week 20 are 5.0%, 25.0% and 45.0%, respectively, as shown in Table 2. The leaves of infected plants were chlorotic and had a white fungal mass on any part of the plants, then formed basidioma before the plants dried (Figure 2A). The cross-section of an infected plant stem showed a necrotic lesion at the vascular region (Figure 2B).

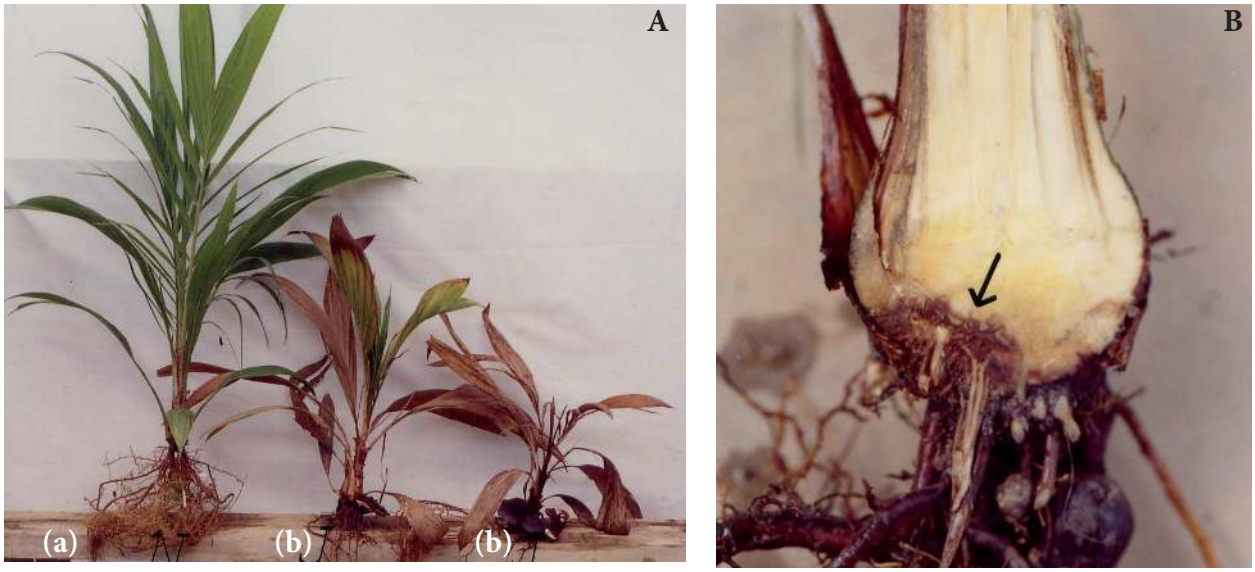


Figure 2. Signs and symptoms of BSR, caused by *Ganoderma boninense*; A – healthy plant (a) and infected plants (b); B – necrotic lesion on infected plant (arrow)

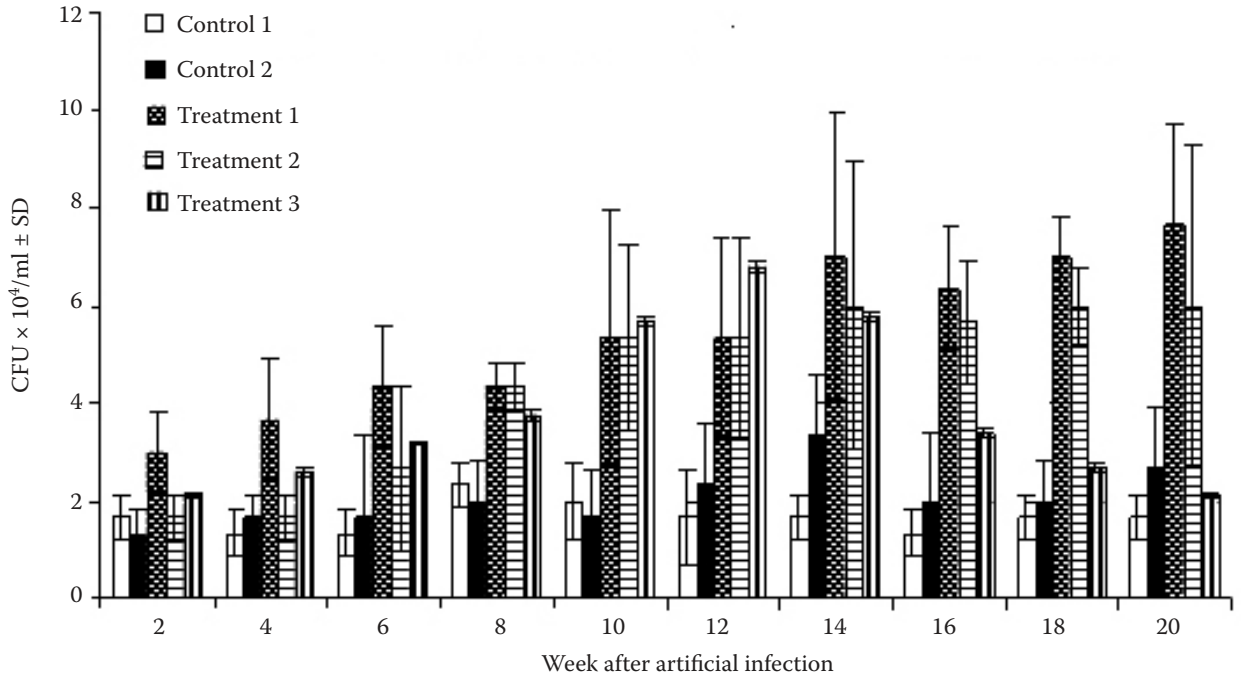


Figure 3. Means of colony forming units (CFU) of soil samples taken every 2 weeks

**Estimating the colony forming unit (CFU) of *Trichoderma* in the soils**

Populations levels of *Trichoderma* CFU in soils increased relative to treated plants after FA 1132 treatment was given as shown in Figure 3. Result showed that the cfu count of *Trichoderma* in the mixture soil (peat, clay and sand) was $1 \times 10^4$/ml. The CFU counts of C1 and C2 were slightly increased until reaching a peak on week 8 and 14,

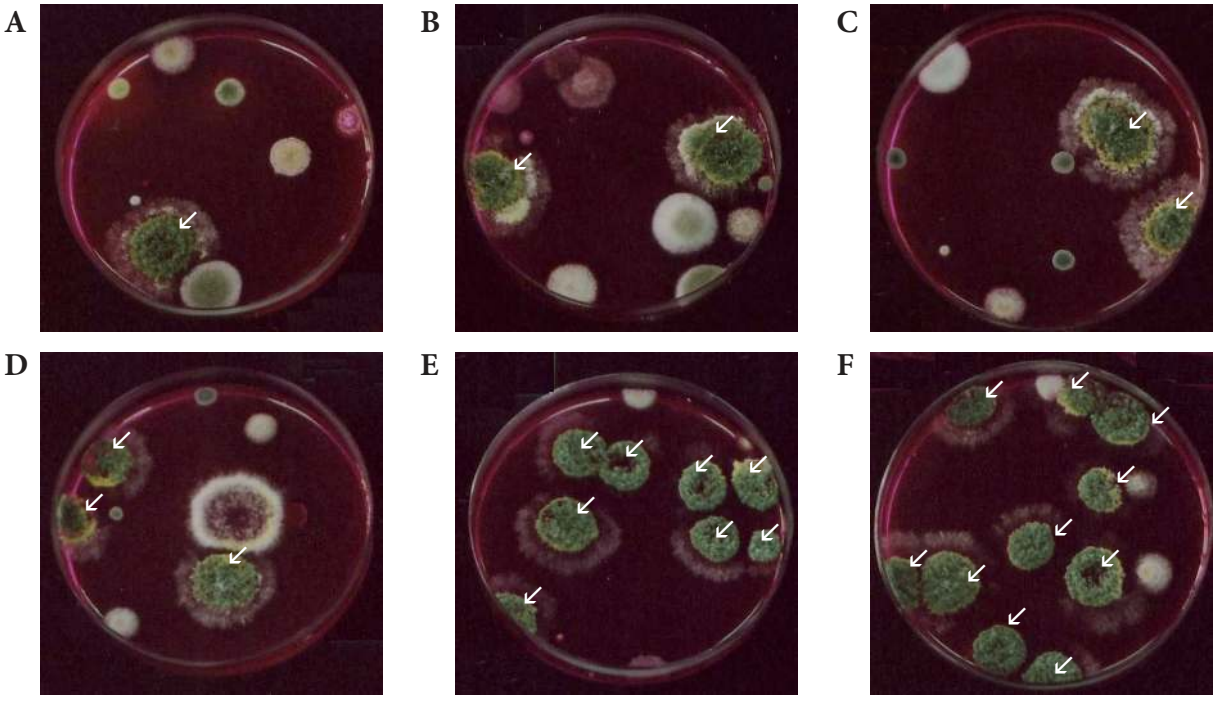


Figure 4. Comparison of the CFU of *Trichoderma* (arrow) on RBA in untreated and treated soils after week 14; A–C: Control 1 (C1); D–F: Treatment 1 (T1)

respectively; then they slightly decreased. For the three treatments, the CFU counts dramatically increased by the week until week 14, and then slightly decreased. The CFU for T2 and T3 was decreased after week 14 and 12, respectively. Figure 4 (A–F) showed the CFU of *Trichoderma* at week 14 for variants C1 and T1.

## DISCUSSION

Artificial infection of plants by contact with the inoculum block carrying *G. boninense* FA 5201 is an effective strategy for inducing the infection, and *T. harzianum* isolate FA 1132 was able to suppress BSR disease development in the plant house trials. A similar outcome of trials on the efficacy of isolate FA 1132 was reported by Ilias (2000) and Abdullah *et al.* (2003). Isolate FA 1132 inhibited the *G. boninense* isolate FA 5201 apparently by direct antagonism, with minor inhibition by antibiosis. *Trichoderma* spp. are known to be mycoparasites on a range of fungi, and this species is an active parasite of hyphae (Elad *et al.* 1983).

The BSR disease severity and disease development were not consistent for all treatments. The DSI values at week 20 were high (45.0%) if the first treatment was given 4 weeks after inoculation compared to treatment given right after inoculation (DSI value at 5.0%). This study concluded that early interaction between isolates FA 1132 and FA 5201 (pathogen) is significant when developing and applying an effective biocontrol agent. Ilias (2000) used isolate FA 1132, but terminated the test earlier, at week 10, with a DSI value of 40.0% after treatments. In this study, the treatments with FA 1132 were given repeatedly and consistently conidial every 2 weeks after artificial inoculation with FA 5201 up to the end of the experiment; the DSI at week 20 were 5.0% in variant T1 (first treatment immediately after inoculation), 25.0% in variant T2 (first treatment 2 weeks after inoculation) and 45.0% in variant T3 (first treatment 4 weeks after inoculation).

The ideal of biocontrol strategy attempts to introduce or promote the activity of biocontrol agents only when and where they are needed or are most effective, and minimises wasteful application of inoculum on non-target habitats. For effective delivery, we need to consider the plant-pathogen-antagonists interaction in terms of time. Therefore, this study was conducted to ensure the efficacy of the *Trichoderma* treatment on the basis of its timing. Based on our collective results, a possible strategy for effective control of BSR begins with an application of a conidial suspension of the bioagent as early as on 5 months-old seedlings.

Different mechanisms have been suggested as being responsible for the effects of biocontrol agents; they include competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of an inhibitory compound (Haram *et al.* 1996; Zimand *et al.* 1996). Thus, the timing of when to apply the treatments is important due to the probability of changes brought about by the biocontrol agent, e.g. by the latter's dominating requirements for space and nutrients.

The proliferation of the population of a fungal biocontrol agent in the soil will affect the success of it in the field. Chet (1987) estimated the natural populations of *Trichoderma* from soil samples and the minimal *Trichoderma* G that should be sustained in the soil for it to be effective was $10^6$ CFU/g of soil. Dissimilar results were observed in the present study, the CFU counts being lower than in the literature, ranging from $1.0 \times 10^4$ to $7.67 \times 10^4$ CFU/g. Thus, the quantity of colonies is not the only factor involved in the effectiveness of a biological control agent, but the species of the pathogen will also affect the activity of the bioagent. Some literature reported that *T. harzianum* is more effective as an antagonistic agent against *G. boninense* than *T. longibrachiatum* and *T. virens* (Abdullah *et al.* 1999; Ilias 2000).

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