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Activities of chitinase enzymes in the oil palm (Elaeis guineensis Jacq.) in interactions with pathogenic and non-pathogenic fungi

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Abstract

Ganoderma boninense Pat. is a fungal pathogen that causes basal stem rot disease in the oil palm (*Elaeis guineensis* jacq.). Chitinases are important defence enzymes in plants. In this study, the activities of chitinase was analyzed at various time points in roots and leaves of oil palm in the presence of *G. boninense* and *Trichoderma harzianum* (a biocontrol fungus used to combat *G. boninense* infection), either alone or together. At two weeks post infection, *G. boninense* alone treatment showed significant chitinase activity of 35.28 and 30.83 U/mg in the roots and in leaves of oil palms, respectively. In the treatments with *T. harzianum* alone or in combination with *G. boninense* the chitinase activity was significantly increased (compared to control plants) to 15.14 and 18.8 U/mg in leaf tissue, and 26.11 and 22.08 U/mg in root tissue, respectively at two weeks post inoculation. This suggests that the chitinase enzyme activity induced by *T. harzianum* in oil palms may have a role in the defence response against microbial pathogen infections.

Keywords: Oil palm, Basal stems rot, *Ganoderma boninense*, *Trichoderma harzianum*, mycoparasitism, chitinase activity.
Abbreviations: BSR, basal stem rot; PDA, potato dextrose agar; PSA, potato sucrose agar; wpi, weeks post inoculation.

Introduction

The oil palm (*Elaeis guineensis* Jacq.) is one of the world's economic crops. It produces two types of oil: palm oil and kernel oil. There are numerous other uses of the plant. For example, fiber can be used to make pulp, paper, and particle boards; the empty fruit bunches can be used to produce energy and as fertilizer; the trunk can be used to make furniture, particle board, and energy such as biofuels (Ng et al., 2011). The oil palm industry is under threat from a serious fungal disease called basal stem rot (BSR), which is caused by *Ganoderma boninense* (Kandan et al., 2010). Infection by *Ganoderma* spp. begin their attack in the palm roots and gradually spread to the bole of the stem where they cause dry rot, which prevents absorption and transport of nutrients (Sanderson et al., 2000). Infected oil palms gradually lose their ability to produce fruits and eventually collapse. By the time *Ganoderma* fruiting bodies are detectable on the oil palm, about 50% of the internal tissues would have already rotted (Kandan et al., 2010). *G. boninense* also has many forms of resting stages, including resistant mycelium, basidiospores, chlamydospores, and pseudosclerotia, and these are difficult to control (Susanto et al., 2005). The soil-borne fungus, *Trichoderma* sp. has been shown to to be effective in controlling *Ganoderma* infection in oil palm, but only at the early stages of infection of slightly infected palms (Abdullah et al., 2003; Izzati et al., 2008). In the biocontrol process of *Trichoderma* used several mechanisms including mycoparasitism, antibiosis, competition for nutrient and living space, enhanced plant growth hormone, and induced plant defence response had been suggested (Vinale et al., 2007). The use of *T. harzianum* as a biocontrol agent to manage *Ganoderma* infection has potential, but to date it has been shown only to control the disease in some cases and was not able to cure pre-infected palms (Ilias, 2000). Therefore, finding an effective control method for BSR is crucial. Ultimately, the best approach for BSR control is to improve the oil palm's defense system or develop a disease-resistant cultivar. One step towards improving the oil palm's defense system is by elucidating the role of chitinases in the plant-pathogen interaction. Chitin is a linear polymer of β- 1, 4 N-acetylglucosamine (GlcNAc) that is present in the fungal cell wall and chitinases are important defence proteins that degrade chitin to chitin-oligomers (Guan et al., 2008). Chitinases are expressed constitutively at a low level in some plants, and their activity can be induced by wounding, by infection of the tissue with pathogens (Majeau et al., 1990; Roby et al., 1990), or by an abiotic elicitor such as salicylic acid, jasmonic acid, or ethylene (Boller et al., 1983). Chitinases also are expressed during symbiotic processes, such as mycorrhiza or *Glomus* fungal interaction (Volpin et al., 1994), and during plant developmental processes, such as embryogenesis or fruit ripening. The main objective of the present study was to investigate the potential role of chitinase enzyme activity in the oil palm's defense aganist *G. boninense* (strain PER71).

Chitinase activity was also measured in samples treated with *T. harzianum* strain FA1132, which is a biocontrol agent used to combat BSR disease.

Result and discussion

Selections of time points for the form of G. boninense infections

The chitinase enzyme activities in the oil palm at the early stage of the plant-microbe interaction were investigated in this study. Hence, the first time point was at 2 weeks post inoculation (wpi) because *Ganoderma* is a slow-growing fungus that requires more than 1 week to develop mycelia on the root surface. A previous study had showed that *G. boninense* infected the roots after 3 wpi (Tee, 2008). It was therefore, assumed that early disease development would have occurred by 2 wpi. Thus, the first time point was set at week 2 to check for early disease development, and the plants were sampled at 3 week intervals (i.e., weeks 5 and 8). At 2 wpi plants treated only with *Ganoderma* did not show any visible signs of infection, but the roots were not growing well (Fig 1); this suggested that *Ganoderma* had started to cause internal infection in the roots. The *Ganoderma* infections were visible on the roots at 5 wpi, and at week 8 wpi most of the roots were black in color and some of the basal leaves were necrotic.

Enzyme activity in plants

Chitinases are one of the important pathogenesis-related (PR) proteins that plants use in defense against infection. In this study, exposure to both *G. boninense* and *T. harzianum* induced activity of chitinase in oil palm seedlings. This is because some chitinases are developmentally regulated or induced in specific organs (Busam et al., 1997), chitinase expression was evaluated in both root and leaf tissues. Figure 1 shows the chitinase activity in roots (Fig. 2) and leaves (Fig. 3) of seedlings treated with *G. boninense* and *T. harzianum*, either alone or in combination, and in control oil palm seedlings at 2, 5, and 8 weeks post inoculation (wpi). At 2 wpi, oil palm seedlings treated with *G. boninense* alone showed a dramatic increased (35.28 u/mg) chitinase enzyme activity (Fig. 2) compared to plants in the other two treatments or control plants in both of the tissue types tested. However, at 5 wpi the activity was lower in both tissues and at 8 weeks the chitinase enzyme activity in the root tissue (Fig. 2) was slightly higher in *G. boninense* alone infected plants compared to the others treatments. At this time, however, the activity was not as high as it was at 2 wpi. Hence, this may be characteristic of susceptible plants where the chitinase activity increased at the early stage of infection but decrease later (Mohammadi, 2002). In root tissues of plants treated with *Trichoderma* and *Ganoderma* together, the chitinase activity was higher than that of control plants at 2 and 5 wpi (Fig. 2). In both treatments in leaf tissues, chitinase activity was higher than that of control plants at 2 wpi (Fig 3) and then it gradually decreased during the remaining period of the experiment, although it never was as low as that of control plants. In plants treated with *Trichoderma* alone, chitinase activity was higher in root tissues compared to the control plants at 2 and 5 wpi (Fig 2), while in leaf tissues the chitinase activity was higher than that of the control plants only at 2 wpi (Fig. 3). Both control plants and plants treated with *Trichoderma* alone exhibited chitinase enzyme activity throughout the experiment, which suggests that chitinase plays a role in the basic physiological

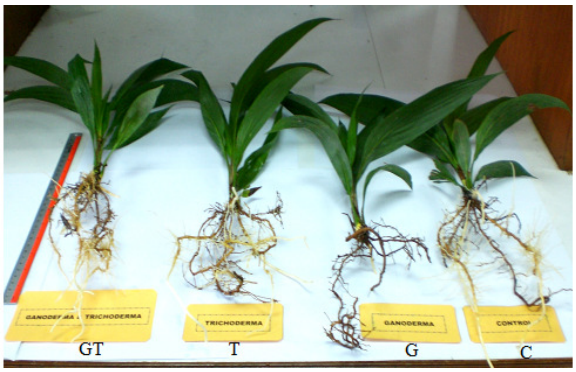

Fig 1. Assessments of treated oil palms at 2 wpi. GT= *Ganoderma+Trichoderma*, T= *Trichoderma* G= *Ganoderma*, and C= Control.

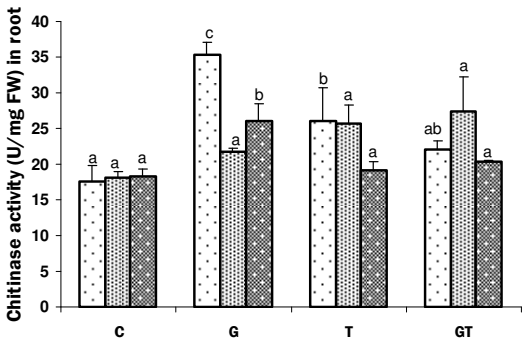

Fig 2. Chitinase activity in root tissues of oil palm seedlings treated with *G. boninense* and *T. harzianum* alone and in combination at 2, 5, and 8 weeks post inoculation (wpi). (Duncan's homogeneity test was used to define ranked class. Means with the same letters are not significantly different. Error bars indicate standard errors. C = Control, G = *Ganoderma*, T = *Trichoderma*, GT = *Ganoderma+Trichoderma*, = 2 weeks, = 5 weeks, = 8 weeks)

processes and during environmental (including microbial) challenge (Punja and Zhang, 1993). Fanta et al. (2003) suggested that a pre-existing defense mechanism could be present in the plant tissues or that chitinase might cooperate with phytoalexins (the antimicrobial compound) to effectively control fungal growth and development. It was also noted that plants treated with *Trichoderma* alone or in combination with *Ganoderma* the activity was higher in root tissues compared to control plants. Thus, it can be suggested that *Trichoderma* induced a local defense response in plants. In conclusion, the results of this study indicate that penetration of *G. boninense* into oil palm roots caused a local defense response in the plant. Chitinase activity was highest when the infection was first developing (2 wpi), but by the time the disease was established the activity levels declined. However, this study only measured total chitinase activity, while there were many chitinolytic enzymes present in plants. Therefore, the measurement of total chitinase activity may not reveal the complete picture. Thus, determination of the activity of other enzymes, including exo or endochitinase activity in oil palms should also be evaluated in future studies. At some time points, chitinase activity was increased in oil palm seedlings treated with a combination of *G. boninense* and *T. harzianum* or *T. harzianum* alone. These results also indicate that induction of chitinases in plants was

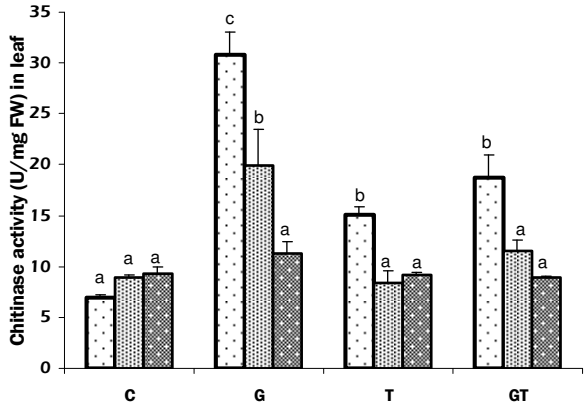

Fig 3. Chitinase activity in root tissues of oil palm seedlings treated with *G. boninense* and *T. harzianum* alone and in combination at 2, 5, and 8 weeks post inoculation (wpi). (Duncan's homogeneity test was used to define ranked class. Means with the same letters are not significantly different. Error bars indicate standard errors. C = Control, G = *Ganoderma*, T = *Trichoderma*, GT = *Ganoderma*+*Trichoderma*, = 2weeks, = 5 weeks, = 8 weeks)

caused by *Trichoderma* which enhanced the defense response against the pathogen. Previously, Ahmed et al. (2000) proposed a similar mechanism for phytoalexin induced by *T. harzianum* in pepper plants infected with *Phytopthora capcisi*.

Materials and methods

Sources of G. boninense strain PER71 and T. harzianum strain FA1132 Cultures

Ganoderma boninense strain PER71 was obtained from the Malaysian Palm Oil Board (Bangi, Malaysia) and *T. harzianum* strain FA 1132 was obtained from the slant stock culture maintained in the Mycology Laboratory, Department of Biology, Universiti Putra Malaysia (UPM). *G. boninense* mycelia were transferred onto potato sucrose agar (PSA) plates at room temperature. The culture plates were observed daily to monitor the growth of mycelium and for any contamination by others fungi or bacteria. After 7 days the mycelium was covered with crust culture (which is characteristic of *Ganoderma*). The *T. harzianum* mycelium was transferred onto potato dextrose agar (PDA) plates at room temperature. The culture plates were fully covered with green conidia within 5–6 days. These cultures were used in the following experiments.

Preparation of inocula for plant treatments

The inocula were prepared as follows. Wood blocks from rubber trees were prepared for Gano-wood block inocula and palm-pressed mesocarp fibers were prepared for *Tricho*-mulch inocula as previously described in Naher et al. (2011). Briefly, 7 days cultures of *G. boninense* were transferred into sterilized wood blocks which contained 100 ml of PSA media and then the blocks were kept in the dark room for 10-12 weeks at room temperature. Blocks that were fully covered with mycelia and brown crust were ready for use as inocula to infect oil palm plants. Tricho-mulch was prepared by packing the fibers into 300 g unit; each unit was placed into a small autoclavable bag (10 cm x 32 cm) and autoclaved at 121°C for 45 min. Then, 6 day cultures of *T. harzianum* were prepared as suspension cultures which were transferred onto the sterilized fibers. All bags were incubated in the dark at room temperature for 15 days. Fibers observed to be fully covered with green conidia were ready for use as inocula for oil palm plants.

Plant treatments

Five-month-old oil palm seedlings (Dura x Pisifera) obtained from Sime Darby Seeds & Agricultural Services Sdn Bhd (Banting, Selangor, Malaysia) was used in this experiment. Before planting, the seedlings were carefully uprooted from the sand beds and the roots were washed with tap water. A total of 108 plants were treated and all inocula were applied at the beginning of the experiment. A completely randomized design (CRD) with four treatments (with three sampling time frames at 2, 5, and 8 weeks post inoculation) and three biological replicates was used. The four treatments were applied as follows. The artificial inoculation of oil palms using *Ganoderma* was conducted as previously described in Naher et al. (2011). For *Ganoderma* inoculated treatments, a Gano-wood block was attached to the roots of the oil palm seedling to ensure close contact between the mycelia and the oil palm roots (n = 27) and for *Trichoderma*-inoculated treatments, Tricho-mulch was placed on the surface of the soil (n = 27). For the *Ganoderma* + *Trichoderma* treatments, seedlings received both inocula (n = 27). A total of 27 plants were used as control treatments (i.e., without *Ganoderma* or *Trichoderma*). All oil palm seedlings were watered daily. Plants were regularly checked for the presence of pests, and water was sprayed onto the lower surface of oil palm leaves twice a week to wash off any unobservable plant mites or other pests.

Sample collection

Sampling was conducted at 2, 5, and 8 weeks post inoculation (wpi). At each time point nine seedlings were uprooted and washed with running tap water, and the disease severity was recorded. Control and treated roots and leaves were excised using a clean pair of scissors, dried with paper towels, and then weighed, and wrapped in aluminum foil (0.1 g/pack) for enzyme analysis.

Protein extraction

Crude protein was extracted from treated and untreated leaf tissues. For each sample, 100 mg of tissue was homogenized in 2 ml of extraction buffer [consisted of 0.1M citric acid, 0.1% mercaptoethanol, and 0.1 M sodium borate buffer (pH 5.2)] in the ratio of 1:2 (tissue: buffer, w/v), using an ice-chilled mortar and pestle. The homogenates were clarified by centrifugation at 7,000 *g* for 20 min at room temperature, and the supernatants were assayed.

Chitinase enzyme activity assay

A modified version of Tonon et al.'s (1998) method was used to evaluate chitinase activity. Chitinase activity was determined using chitin (Sigma Aldrich, USA) as the substrate (Trudel and Asselin, 1989; Pan et al., 1991). A 300 µl aliquot of the crude extract from each sample was transferred to a centrifuge tube containing 200 µl of chitin [0.5% (w/v) dissolved in 0.05 M sodium acetate buffer, pH 5.5] and the solution was mixed well. The reaction mixture was incubated at 37 ºC for 2 h on a rotary shaker. The enzyme reaction was terminated by centrifuging for 10 min at 7,000 *g*. Next, 250 µl of supernatant were transferred into a

new tube, to which 100 µl of 0.2 M sodium borate buffer (pH 9.1) were added. The mixture was heated in a water bath (100 ºC) for 7 min and then cooled under tap water. When the mixture reached room temperature, 1 ml of Erlich reagent [1 g *p*-dimethylaminobenzaldehyde dissolved in 100 ml glacial acetic acid that contained 1.25 % (v/v) of 10 N HCl] was added, followed by incubation for 30 min at room temperature. The chitinase activity was determined using a spectrophotometer (Thermo Spectronic, USA) at 585 nm wavelength (Reissing et al., 1955). The total chitinase activity was calculated using the equation, Y= 0.0024x+ 0.011 (where, Y = sample OD reading, 0.0024 = rate of change of standard curve, x= total chitinase activity, and 0.011 = intercept of curve line), which was generated from the standard curve obtained from the simpe linear regression model using standard chitinase enzyme from *Trichoderma viride* (Research Biolabs, USA) [30]. Chitinase activity was expressed in units of chitinase activity per milligram fresh weight per hour (Tonon et al., 1998).

Statistical analysis

The enzyme activity data for the four treatments were subjected to analysis of variances (ANOVA). The mean differences among the treatments were determined by multiple comparisons test and were ranked by the Duncan's homogeneity subsets if F tests were significant at the 95% probability level.

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