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MYCOPARASITISM ACTIVITY OF *TRICHODERMA HARZIANUM* ASSOCIATED WITH CHITINASE EXPRESSION AGAINST *GANODERMA BONINENSE*

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Abstract

The filamentous fungus *Trichoderma* is an important fungus due to its biocontrol properties. This study was conducted on mycoparasitism activity of *Trichoderma harzianum* T32 against *Ganoderma boninense* upm001 on the basis of Potato Sucrose Agar (PSA) under the scanning electron microscope observation or cell wall degrading enzyme of chitinase gene expression by reverse-transcriptase (RT) PCR. The mycoparasitism process of *Trichoderma harzianum* T32 showed the coiling or killing of the *Ganoderma boninense* upm001 in PSA media. Deformity and shrinkage of *Ganoderma* mycelia was also observed under the scanning microscope in presence of *Trichoderma*. The RT-PCR profile showed that chitinase gene was expressed during the mycoprasitism activity in *T. harzianum* against *G. boninense*. It could be concluded that chitinase gene was an important element in process of biocontrol activity of *Trichoderma* as the gene was expressed against *G. boninense*.

Key words: *Mycoparasitism*, *Ganoderma boninense*, *Trichoderma harzianum*, Reverse-transcriptase PCR, scanning electron microscopy.

Introduction

Trichoderma species are soil borne non phytopathogenic fungus which can be found in every soil and generally, in plant root niche area the density of its population is high. The species of *Trichoderma* is rapidly grow in saprophytic habitat thus easy to grow in media which contain the high density of conidia to enhance their life longevity (Jahan *et al.*, 2013). The *Trichoderma* spp. colonies show different types of color such as dark-green, yellow-green, with wooly flappy or appear as a concentric ring. All these characteristics depend on the fungus. The importance of *Trichoderma* species is that they are used as biocontrol agents (BCAs) against either soil borne or powdery mildew pathogen. The soil borne fungus *Rhizoctonia solani* is one of the most important agent as phytopathogenic fungus for several diseases such as blight of rice, black scurf of potato, belly rot of cucumber. *Trichoderma harzianum* controlled these diseases (Jia *et al*., 2013; Keinath, 1995; Rahman *et al*., 2014). The species of *T. hamatum* or *T. viren* were used successfully to control some phytopathogenic diseases such as basal stem rot of rubber plants, black scurf of potato, belly rot of cucumber, ear rot of corn (Tewari & Sing, 2005; de Silva *et al.*, 2012; Chen *et al*., 2014). The species controls the pathogen growth at the same time it induces some biochemical response in the plant which helps to the plant to be protective from the pathogen (Naher *et al.*, 2014a; Harman, 2011). According to Singh *et al.*, (2011), *Trichoderma* uses some elicitors in their hyphal tips to induce the biochemical response in plants as well as these fungi to give opportunistic effect to the plant such as enhance the photosynthetic process and uptake of soil nutrient. However, in mycoparasitic activity of *Trichoderma* extend the hyphae towards the pathogen and coil around the pathogen to kill it. During this killing process *Trichoderma* secretes hydrolytic enzymes or genes such as glucanase, chitinase or protease that degrade the host cell wall (Mondejar *et al.*, 2011; Verma *et al.*, 2007; Woo *et al.*, 2006. The protease gene was expressed in *Trichoderma* during the mycoparasitism reaction with *Rhizoctonia solani* (Mondejar *et al.*, 2011). *Ganoderma boninense* is a causal pathogen for basal stem rot disease in oil palm (Turner, 1981). The progression of the disease is slow thus when the disease symptoms appear at that time >50% plants are already dead (Kandan *et al*., 2010). The pathogen degrades the lignin component of oil palm stem fiber that affect the oil palm defense system thus plant gradually die. We have reported in our previous studies that *Trichoderma harzianum* suppressed the pathogen growth of *G. boninense* during the inoculation with oil palm plant (Naher *et al.*, 2014b; 2011) while the process of biocontrol mechanism was not observed in the same study. Thus, the aim of this current study was to observe the mycoparsitism process of *T. harzianum* T32 on *G. boninense* upm001 in three different culture media such as potato dextrose agar (PDA), potato sucrose agar (PSA), and malt extract agar (MEA) under the scanning electron microscopy as well as cell wall degrading enzyme of chitinase expression by RT-PCR.

Materials and Methods

Preparation of media

Potato dextrose agar (PDA): Potato dextrose agar was prepared according to the manufacture's procedure. 19 g of PDA was poured into a glass conical beaker and then distilled water was top up to 500 ml and the mixture was homogenized by using microwave. The medium was then autoclaved at 121°C for 15 minutes.

Potato sucrose agar (PSA) media: Around 500 g potato was wash vigorously with running tap water and then finally with distilled water. Next, the potato was cut into small slices and then boiled with clean water. The boiled potatoes make as potato broth and sieved the broth using a thin net cloth into a conical beaker. Next, 10.66 g microbiology agar-agar and 10 g of sucrose both were added into the broth. Further, using microwave the broth mixture was homogenized. Then, the media was autoclaved at 121 ℃ for 15 minutes.

***Trichoderm* spp. collection and culture:** *Trichoderma harzianum* strain T32 was obtained from the slant stock culture of the Mycology and plant pathology Laboratory, Department of Biology, Universiti Putra Malaysia (UPM). The fungal mycelium was transferred on potato dextrose agar (PDA) plate at room temperature. After 3 days the culture was transferred on the experimental media of PSA.

***Ganoderma* collection and culture:** *Ganoderma boninense* strain upm001 was collected from the slant culture of plant pathogen laboratory, Department of crop protection, Universiti Putra Malaysia (UPM). The mycelium of *G. boninense* was transferred on fresh potato dextrose agar plate at room temperature and newly growing mycelium was transferred on the experimental media of PSA and in same plate the newly growing *T.harzianum* mycelium was placed to made antagonistic situation.

Mycoparasitism observation by scanning electron microscopy (SEM) analysis: For the analysis of mycoparasitism observation of *Trichoderma harzianum* on *Ganoderma boninense* under scanning electron microscope, the mycelium were cut around 10 cm^2 slices from the contact region in antagonistic plate between *Trichoderma* and *Ganoderma*. The mycelium pieces were transferred into vials containing the solution of 4% Glutraraldehyde. The vials were kept for overnight incubation at 4° C. Next day, Glutraraldehyde solution was discarded by pipetting and washing buffer of 0.1M Sodium Cacodylate was added up to emerged the pieces of fungus and kept for 10 min. This stage was repeated for three times. Then, post fixation was carried out in 1% Osmium Tetroxida for 2 hours incubation at 4° C. After post fixation, again washing was carried out in the same way as stated above. Dehydration by a series of acetone was then carried out in two stages. First series was in 35% acetone and 50% acetone for 10 minutes in each slot and then 75% for overnight. Second series was in 95% acetone for 10 minutes and 100% acetone for 15 minutes for three times. Then, specimens were transferred into specimen basket for critical point drying. Critical point drying was carried out for 30 minutes using CO_2 (Carbondyoxide). The mounting was then done by putting the specimen onto the stub using double sided tape and then coated with gold in sputter coater and observed in SEM (Jeol, japan).

Primer design: To analyze the expression of chitinase gene in *Trichoderma*, the primer has been designed from the available sequence of NCBI (National Center of Biotechnology Institute) that had significant matches (E-value less than 10^{-5}) to chitinases with the accession no is HS574321.1. The forward primer 5′ CAG CTT ACG TCG GTC TTC CA 3′ and reverse primer 5′ CGC TCT CAA AGC CAG TGT TC 3′. The house keeping gene was Alpha tubulin (HS574101). The expected size of both chitinase gene and Alpha tubulin fragments was 100 base pair.

***Trichoderma* sample collection from antagonistic situation and liquid culture:** For chitinase expression analysis, the sample of *Trichoderma* was collected from three antagonistic situations as just before contact, contact and after contact with *Ganoderma boninense*. A 6 mm diameter *Trichoderma* culture was cut using a sterilized cock borer and transferred in 250 ml of potato dextrose broth for full colony growth. After seven days the culture was collected using whatman filter paper1, foiled it in an aluminum foil and kept in - 80ºC.

RNA extraction: RNA extraction was carried out using Qiagen Plant mini Kit (Qiagen, USA) according to manufacturer instruction. In briefly, 100 mg of mycelium sample was grinned by liquid nitrogen using mortar and pestle. The powder was transferred into a 2ml of microcentrifuge tube. Then, 450 µl of RTL (mentioned in the kit) buffer was added into the same tube and the solution was thoroughly mixed using vortex. A 4 µl of b-mercaptoethanol was then added into the solution. The lysate was transferred to a QIA shredder spin column (Lilac) and placed in a 2 ml collection tube and centrifuged for 2 minutes. Subsequently, carefully transferred the supernatant by flow-through into a new microcentrifuge tube that without disturbing the cell-debris pellet. Then, 0.5 volume of ethanol (96-100%) was added to the cleared lysate and mixed the solution by pipetting. After that, transfer the sample with any precipitate to an RNeasy mini spin column in a 2 ml of collection tube by centrifuged at 10,000 rpm for 15 seconds. After discard the flow-through, in separately 700 µl buffer RW1 and 500 µl buffer RPE was added and both times centrifuged at 10,000 rpm for 15 seconds. After that, 500 µl of RPE buffer was added in to the column and centrifuged at 10,000 rpm for 2 minutes. Finally, RNA was collected the by adding 30 µl DEPC-treated water into the column followed by centrifuged at 10,000 rpm for 1 minute. The purity of RNA extraction was checked using nanodrope spectrometer reading and all the extraction concentration was approximately 206ng/ µl with protein (260/280) ratio was 2.26 and carbohydrate (260/230) ratio was 2.32. Then, the RNA was procced for PCR experiment.

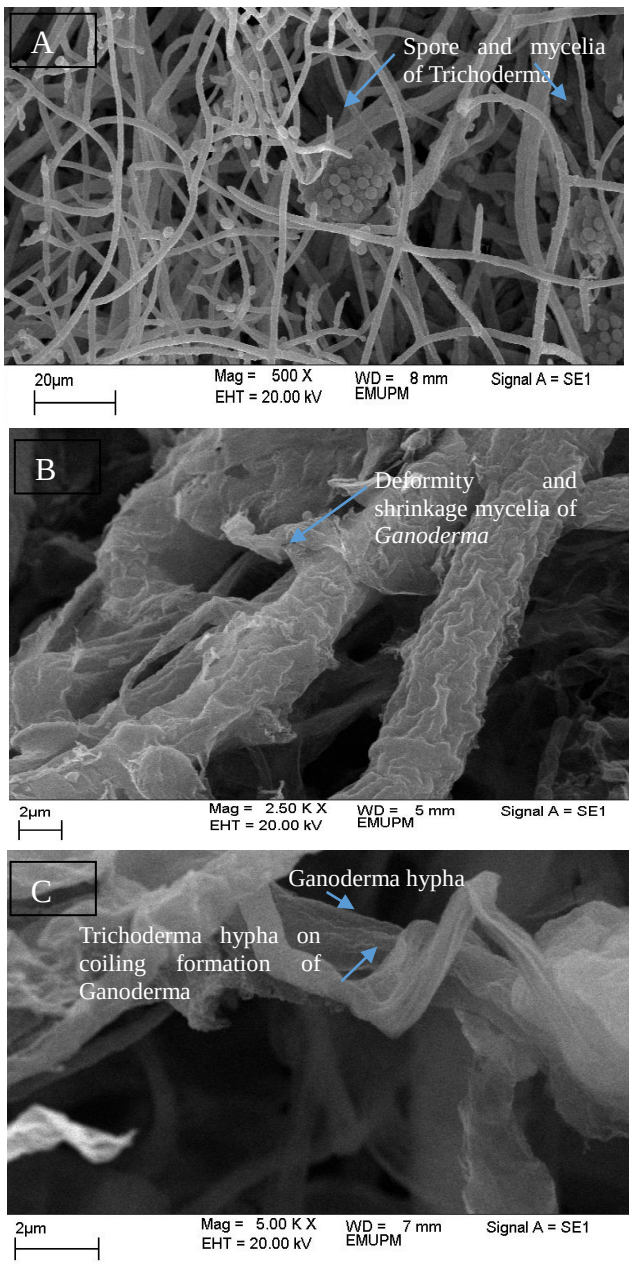

Fig. 1. Observation of mycoparasitism activity of *Trichoderma harzianum* T32 on *G. boninense* upm001under the scanning electron microscope. A, *Trichoderma* spore and mycelia; B, Deformity and shrinkage mycelia of *Ganoderma* in presence of *Trichoderma*; C, mycoparasitism process as coiling formation of *Trichoderma* on *Ganoderma*.

RT-PCR: RT-PCR was performed to observe the chitinase expression during mycoparasitism process in *Trichoderma* against *Ganoderma*. The PCR mixture consisted of 100 ng first strand cDNA, 0.1 µM of each primer, 0.25 µM dNTP, 1X PCR buffer and 0.2 U DyNAzyme II DNA polymerase (Fermentas, USA), in total volume 20 µl. Amplification was performed under the following conditions: 1 cycle at 94 ºC for 3 min followed by 35 cycles at 94 ºC for 1 min with annealing temperature of 60 ºC for 30 sec, 72ºC for 1 min, then 72 ºC for 7 min. The amplicons were checked and visualized on 1.5 % (w/v) agarose gel, run in 1X TAE buffer, and stained with ethidium bromide.

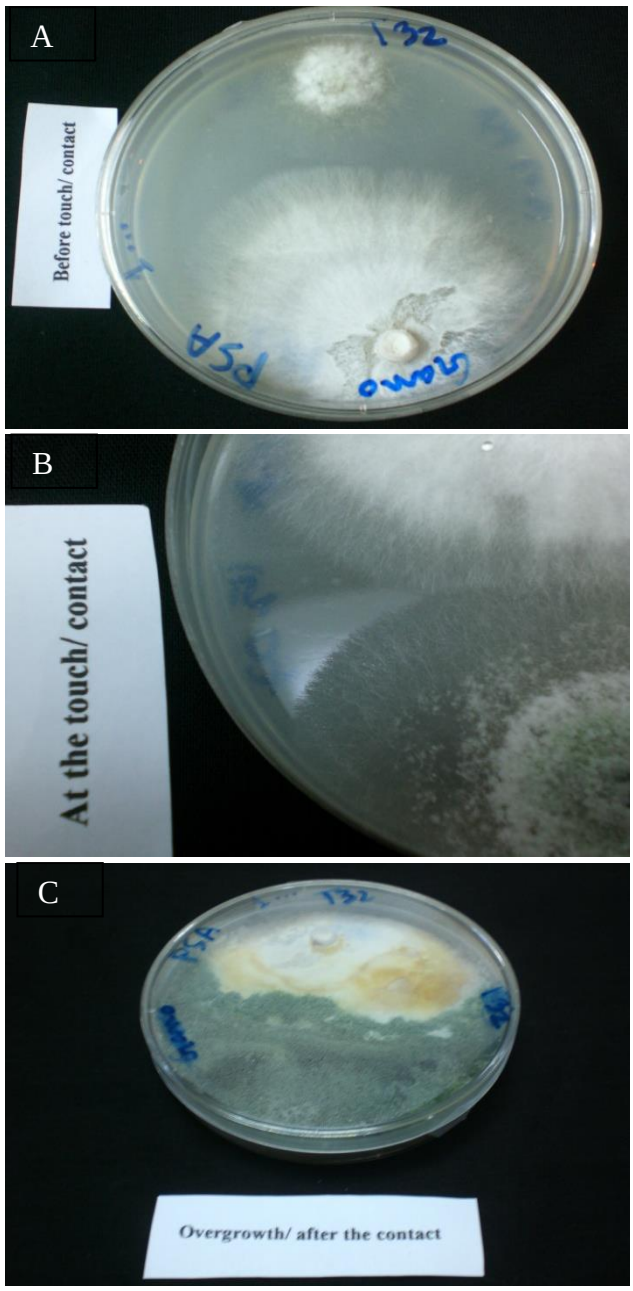

Fig. 2. Antagonistic situation between *Trichoderma hazianum* T32 and *Ganderma boninense* upm001. A, Antagonistic situation of *Trichoderma* just before contact with *Ganderma*; B, at the contact situation of *Trichoderma* with *Ganoderma*; C, Over growth of *Trichoderma* on *Ganoderma*.

Results and Discussion

***In vivo* mycoparasitism observation by scanning electron microscope and chitinase expression by RT-PCR:** In literature it has been documented that *Trichoderma* control the pathogen in different process such as indirectly, by competing for nutrients or food and occupy space, modifying environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis, or directly, through mycoparasitism as means coiling the pathogen (Gomes *et al.*, 2015; Howell, 2003; Vinale *et al.*, 2008). In this study we observed the mycoparasitism process or coiling attachment by scanning light microscope. The coiling step was found only in PSA media while in PDA or MEA media did not appear though

Trichoderma harzianum T32 inhibited *Ganoderma boninense* upm001 growth when cultured in these media. Fig. 1 showed the *Trichoderma* hyphe alone with its spore (1A) *Trichoderma harzianum* hyphe with the spore (1B) Deformity and shrinkage of *Ganoderma* hyphae was found in the presence of *Trichoderma* (1C). The growth and towards coiling process of *Trichoderma* on *Ganoderma* to kill the pathogen as mycoparasitism process of *Trichoderma*. During the mycoparasitis process *Trichoderma* induced some biochemical response like cell wall degrading enzyme such as glucanase or chitinase (Gruber & Seidl-Seiboth, 2012). This study also observed the chitinase expression by reverse-transcriptase PCR (Fig. 3A). The PCR was confirmed with the house keeping gene Alhpa tubulin (Fig. 3B). The gene was expressed in *Trichoderma harzianum* T32 when it was in antagonistic situation with *Ganoderma boninense* upm001 (Fig. 3).

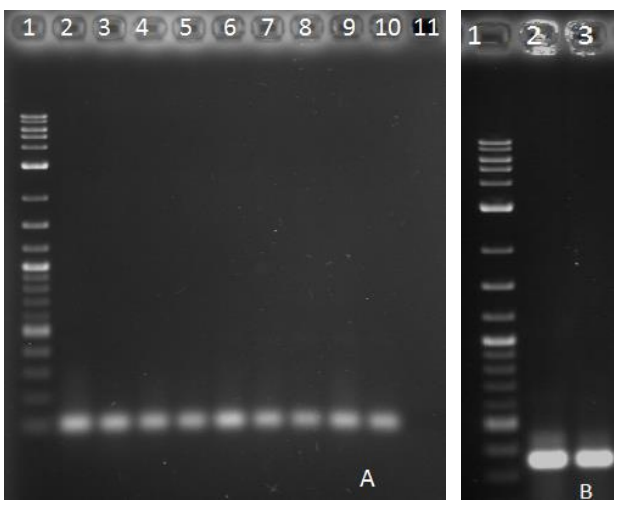

Fig 3: PCR amplification of chitinase in *Trichoderma harzianum* during mycoparasitism with *Ganoderma boninense* (3A) and house keeping gene Alhpa tubulin (3B; lanes 2B and 3B). In Fig. 3A, lane 1 shows the 100 bp marker; lanes 2-4 show the chitinase expression in *Trichoderma* just before contact with *Ganoderma*; lanes 5-7 show the chitinase expression in *Trichoderma* during contact with *Ganoderma*; lanes 8-10 show the chitinase expression in *Trichoderma* after contact with *Ganoderma*; lane 11 shows the negative control for PCR product;

Discussion

The fungus *Trichoderma* is a good biocontrol agent for managing plant pathogen. In biocontrol program in many studies used the species of *Trichoderma* group fungus. *Trichoderma harzianum* strain T32 was used in this study as a biocontrol agent against a devastating fungus *Ganoderma boninense* caused basal stem rot disease in oil palm (Naher, 2014). During the antagonistic activity in *Trichoderma* showed the mycoparasitism process to kill the pathogen. Mycoparasitism is complex process that need several steps with that coiling and expression of cell wall degrading enzymes or gene in *Trichoderma* are the main part to kill the pathogen. In the process of coiling this study found that *T. harzianum* T32 grow on *G. boninense* upm001 and produced coil formation of *Trichoderma* around the *Ganoderma* (Fig 1C). The coiling process was found in *Trichoderma* sp. on *Rhizoctonia solani* (Brotman *et al*., 2010). In study of Lu *et al.,* (2004) observed the mycoparasitism coiling process of *T. atroviride* on *Pythium ultimum* and *R. solani* under the confocal scanning laser microscopy and fluorescence. Deformity and shrinkage of *Ganoderma* hyphae were observed when *Ganoderma* was treated with the beneficial endophytic bacteria (Rahamath *et al*., 2010). The deformity and shrinkage of mycelia of *Ganoderma* in antagonistic situation with *Trichoderma* was observed (Fig. 1B). No reports were found on mycoparasitism activity of *Trichoderma harzianum* on *Ganoderma boninense*. Hence, the findings of this study are the first report of mycoparasitism activity of *Trichoderma harzianum* on *Ganoderma boninense*. During the coiling step expression or induced of cell wall degrading enzyme or genes such as chitinase, glucanase or protease in *Trichoderma* is another mechanism to suppress the pathogenic fungal growth (Mondejar *et al*., 2011). This study was conducted on chitinase gene expression in *T. harzianum* T32 during mycoparasitism process on *G. boninense* upm001. The chitinage gene expression was analyzed in *T. harzianum* T32 against *G. boninense* upm001 in three different situations such as just before contact, contact and after contact with *Ganoderma boninense* upm001 (Fig. 2a, 2b and 2c). The chitinase was expressed in all antagonistic situations (Fig. 3A) in *T. harzianum* against *G. boninense* whereas the gel image intensity was different based on the situation. Chitinase enzyme activity was highest in *T. asperellum* against *Rhizoctonia solani* while chitinase enzyme activity was higher in *T. harzianum* against *Sclerotinia sclerotiorum* (Qualhato *et al.,* 2013). Mondejar *et al*., (2011) measured the chitinase expression by real-time PCR and found that chitinase chit42 expression was increased in *Trichoderma harzianum* strain T30 among the five strains against *Fusarium oxysporum*. In conclusion, this present study only observed the mycoparasitism process under scanning electron microscope which found the deformity and shrinkage of *Ganoderma* mycelia in the presence of *Trichoderma* and might be chitinase gene also involved in mycoparasitism process in *Trichoderma harzianum* as chitinase gene was expressed. This study did not measure either chitinase gene expression by real-time or enzyme activity by spectrometer reading thus it might be not suitable to compare the expression analysis. Thus further study will be required to determine the expression analysis of chitinase gene in between *Trichoderma harzianum* and *Ganoderma boninense*.

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