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IDENTIFICATION OF *Trichoderma harzianum* T3.13 AND ITS INTERACTION WITH *Neoscytalidium dimidiatum* U1, A PATHOGENIC FUNGUS ISOLATED FROM DRAGON FRUIT (*Hylocereus polyrhizus*) IN MALAYSIA

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ABSTRACT

Endophytes can produce enzymes which facilitate their initial colonisation of plant tissues and direct interactions with microbial pathogens. In this study, endophytic fungus from the stem of healthy dragon fruit (*Hylocereus* spp.) was successfully identified as *Trichoderma harzianum* T3.13. *T. harzianum* T3.13 was shown to have the ability to produce antagonistic activity against *Neoscytalidium dimidiatum* U1, a pathogen fungus from the stem of unhealthy dragon fruit. The chitinolytic activities of *T. harzianum* T3.13 were 0.194 U/ml in a medium containing 3% (w/v) of colloidal chitin as sole carbon source. Semi-quantitative RT-PCR was used to quantify the expression patterns of the genes during the interaction of *T. harzianum* T3.13 with pathogen *N. dimidiatum* U1 and control pathogen *Colletotrichum gloeosporioides*, respectively. The expression of the exc1 and chit42 genes were observed to be present before and after the interaction occurred in the presence of *N. dimidiatum* U1. However, the expression of the bgn13.1 gene increased after 24 hours up to 96 hours of interaction in the presence of *N. dimidiatum* U1. In the presence of *C. gloeosporioides*, the expression of bgn13.1 and chit42

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gradually decreased during the interaction although the expression of the exc1 gene did not change. The results suggested that the endophytic fungus *T. harzianum* T3.13 has the potential as a good biological control agent against *N. dimidiatum* U1 and *C. gloeosporioides*. Thus, the study provided an insight into cellular and molecular interactions between *T. harzianum* T3.13 and pathogenic fungi.

Keywords: Antagonistic activity, Biocontrol agent, Chitinase, Dragon fruit, Endophytic fungi, Neosytalidium dimidiatum

1. INTRODUCTION

Dragon fruit is a cactus fruit crop that's have a high demand at national and international levels. This fruit has been planted in over 10 countries worldwide (Valencia-Botín *et al*., 2013). Since dragon fruit starts to be farmed in commercial farms, some symptoms of rotting and spots in stems and fruits were being identified (Valencia-Botín *et al*., 2013). Studies on stem soft rot diseases were begun at 1990s in Mexico because of the diseases caused by two types of Enterobacteria. While fungi such as *Botryosphaeria dothidea* Ces. & de Not also identified as causing agent of the spots on stems diseases (Valencia-Botín *et al*., 2013). One more fungal disease become violent as it affects fruits and stems is anthracnose disease. Researcher in United State of America (USA) and Japan revealed their report in etiological study found that the causing agents of anthracnose disease are fungus *Colletotrichum gloesporioides* Penz (Poonpolgul and Kumphai, 2007). In Peninsular Malaysia, red-fleshed dragon fruit (*Hylocereus polyrhizus*) is very popular and widely cultivated (Masyahit *et al*., 2009). However, anthracnose symptoms start to be detected on the stems of dragon fruit in the states of Kedah and Penang in August 2010 and January 2011, respectively (Iskandar Vijaya *et al*., 2015).

Therefore, there is a requirement for eco-friendly biological control agents that can help in undertaking the above problems. There are many types of bacteria and fungi that's involves in biological control activities. Today, *Trichoderma* was identified has a high potential in controlling the plant diseases. *Trichoderma* species can produce different kinds of enzymes which stand as an important role in biological control activities, like cell wall degradation, hypha growth, biotic and abiotic stress tolerance and antagonistic activity against plant pathogens (Hasan, 2014). They can colonize aboveground and belowground plant organs or grow between living cells (endophytes). They can live in soil organic matter as saprophytes and could appear in plant litter and mammalian tissues (Rai and Mehra, 2015). *Trichoderma* species are known for their assembly of cell wall degrading enzymes such as chitinase. Chitinase enzyme has been established massive attention because of their potential use in biological control for phytopathogenic fungus and other phytopathogenic organisms that containing chitin such as

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insects (Souza *et al*., 2003). In these respect, the fungi that's produce chitinase enzyme has intensively studied as biological control agents.

Genes play a major role in the biological control process by regulating signals and leads the secretion of some enzymes that help in the degradation of the pathogens. Increased in the expression of the genes such as chitinase and glucanase gene, helps in enhanced the biological control activity, promoting the plant growth and prevents the plant from pathogen attack (Massart and Jijakli, 2007). In the preliminary study, the endophytic *Trichoderma* from healthy stem dragon fruit and pathogen *Neoscytalidium* from unhealthy stem dragon fruit were isolated (Suhaila, 2014). They are showed an antagonistic activities between each other. Therefore this study has been done to initiate the identification for the genus level for *Trichoderma* and *Neoscytalidium* and observed their interaction activity through scanning electron microscopy. The production of chitinase enzyme by *Trichoderma harzianum* T3.13 has been evaluated too in order to obtain substantial chitinase enzyme activities. Meanwhile, the profile expressions of *T. harzianum* T3.13 genes such as chitinase gene, β-glucanase gene and N-acetylglucosamine gene were also profiled during antagonistic activity against pathogens *Neoscytalidium dimidiatum* U1 and control pathogen *Colletotrichum gloerosporioides*.

1.1 Materials and Methods

1.1.1 Microorganism and Inoculum Preparation

The locally isolated fungi known as endophytic *Trichoderma* sp. and pathogenic *Neosytalidium* sp. were collected from the Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia culture collection. *C. gloeosporioides* as control pathogen was collected from Strategic Research Centre, Malaysian Agricultural and Development Institute (MARDI). *C. gloeosporioides* will be used as a control pathogen in dual culture test and gene expression analysis. Stock cultures were preserved on Potato Dextrose Agar (PDA) (Difco Laboratories, Australia). New subcultures were made every 3 to 4 weeks and after incubations at 30 ± 2°C °C for 7 days, they were stored in the chiller at 4°C. The isolation of endophytic fungi *Trichoderma* sp was obtained from healthy stem cell of dragon fruit while *Neoscytalidium* sp, a plant pathogen was obtained from the unhealthy stem cell of dragon fruit.

1.1.2 Molecular Identification

The molecular method that were used to identify *Trichoderma* and *Neoscytalidium* at the genus and species levels in this study. Whole-cell DNA was isolated from fresh mycelium as described by Turner *et al*. (1997). A nuclear ribosomal DNA (rDNA) region, containing the internal transcribed spacer (ITS) regions and the 5.8S ribosomal ribonucleic acid (rRNA) gene were

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amplified by PCR using the primer combinations ITS1 (5' TCC GTA GGT GAA CCT GCGG-3'and ITS4 (5' TCC TCC GCT TAT TGA TAT GC-3') (White *et al*., 1990). This method was carried out by sending the pure culture fungi sample to Profound Kestrel Laboratories Sdn. Bhd (Malaysia). The sequence was examined by Basic Local Alignment Search Tool (Blastn) at National Center for Biotechnology Information (NCBI) for identification of *Trichoderma* and *Neoscytalidium* genus and species levels.

1.1.3 Phylogenetic Analysis

The nucleotide sequences of *Trichoderma* and *Neoscytalidium* species were recovered from NCBI for the construction of phylogenetic tree. Multiple sequence alignment were carried out using algorithm method called Muscle (Edgar, 2004a, 2004b). *Trichoderma piluliferum* is out grouped which act as control for phylogenetic tree for *Trichoderma. Neofusicoccum mangiferae* is out grouped which act as control for phylogenetic tree for *Neoscytalidium*. The trees are inferred using the maximum-likelihood (ML) method. The analyses are conducted in Molecular Evolutionary Genetics Analysis (MEGA) software version 6.1 (Tamura *et al*., 2013).

1.1.4 Substrate Preparation and Pretreatment

Ten gram of chitin powder was added into 100 mL concentrated hydrochloric acid (36-38%). The mixture of chitin and concentrated acid were stirred using glass rod for 10 minutes. Then, it was left in the room temperature for another 30 minutes. After 30 minutes, 1 L of 4 °C distilled water was slowly added into the mixture of chitin and hydrochloric acid. The mixture was stirred using magnetic stirred for an hour. The chitin mixture was stored in the fridge at 4 °C overnight. After stored overnight, the mixture has been washed 3 times with tap water. After washing process, the pH is adjusted to approximately 6.5 to 7.0 using 2 M sodium hydroxide (NaOH) (Wiwat *et al*., 1999). To get colloidal chitin from the mixture, sample filtration was done using Whatman filter No.4 (Gomez Ramirez *et al*., 2004). After the above treatment, the loose colloidal chitin was used as a substrate.

1.1.4.1 Preparation of dried mycelium

Erlenmeyer flasks (250 mL) containing 100 mL of potato dextrose broth were incubated with 1 cm^2 discs of potato dextrose agar (PDA) of actively growing of pathogenic *Neosytalidium* sp. The inoculated flasks were incubated at 30 °C for 7 days. The mycelium was then collected by filtration through Whatman No.1 filter paper, washed with distilled water and homogenized in distilled water using a laboratory homogenizer (Heidolph DIAX 900). The suspension was centrifuged three times (6000 x g for 10 min) after washing with distilled water. The mycelium was stored in a lyophilized state and used as a substrate.

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1.1.5 Fermentation

Chitinase enzyme was produced by liquid state fermentation. A series of 250 mL Erlenmeyer flasks with cotton stoppers were autoclaved and used for the production and collection of the enzymes. Mycelia suspension of *T. harzianum* T3.13 was prepared by suspending mycelia discs from 7 days old culture plates in sterile in 100 mL Erlenmeyer flasks containing sterilized potato dextrose broth (PDB). The disc of 5 mm diameter was cut on the mycelia mats of the agar plate using a sterilized cork borer. A total of 5 discs for every 50 mL sterilized PDB were incubate at 30 ± 2˚C in an incubator shaker at 120 rpm for 72 hours (Sandhya *et al*., 2004). A total of 5 mL (5% v/v) mycelia of *T. harzianum* T3.13 was added to 90 mL of selected media in 250 mL Erlenmeyer flasks. The selected media were (in % w/v); Absidia medium (KH_2PO_4 0.70, $MgSO_4.7H_2O$ 0.15, $C_4H_{12}N_2O_6$ 0.20, Na_2HPO_4 0.20, $(NH_4)_2SO_4$ 0.15, colloidal chitin 3.00), Czapek Dok medium ($NaNO_3$ 0.20, KH_2PO_4 0.10, $MgSO_4.7H_2O$ 0.05, KCI 0.05, $FeSO_4.7H_2O$ 0.01, colloidal chitin 1.00), Kawachi medium ($NaNO_3$ 0.20, KH_2PO_4 0.10, $MgSO_4.7H_2O$ 0.05, KCI 0.05, $FeSO_4.7H_2O$ 0.01, pepton 0.50, yeast extract 0.30, colloidal chitin 0.30), Modified Kawachi medium ($NaNO_3$ 0.20, KH_2PO_4 0.10, $MgSO_4.7H_2O$ 0.05, KCI 0.05, $FeSO_4.7H_2O$ 0.01, colloidal chitin 0.30), *Trichoderma* Minimal medium (TMM) (KH_2PO_4 2.00, $MgSO_4.7H_2O$ 0.30, $CaCl_2.6H_2O$ 0.30, colloidal chitin or dried mycelium. The medium were sterilized at 121˚C for 15 minute before transferring the mycelia suspension into the selected culture media. This experiment was run in triplicate. The culture was incubated at 30 ± 2˚C in an incubator shaker at 120 rpm for 8 days. The sampling has been done at 8 days by collecting the culture medium for 5 ml for every type of media. The samples were kept at 4 ℃ for chitinase enzyme assay.

1.1.6 Chitinase enzyme assay

The chitinolytic activity was measured using the amount of the reducing sugar liberated from enzymatic hydrolysis of chitin. N-acetylglucosamine (NAG) was used to form the standard curve using dinitrosalicylic acid (DNS) method (Rojas Avelizapa *et al*., 1999). One unit (U) of enzymatic activity using chitin as a substrate is defined as the liberation of one mM of product (estimated as NAG) per hour. This assay measures the release of NAG, a reducing sugar by the action of chitinase enzyme on chitin substrates (Unit in U/mL). Chitinase activity was assayed using the colorimetric method described by Molano *et al*. (1977) with minor modification. One milliliter (mL) of the enzyme sample and 1 mL of 10 % (w/v) suspension of colloidal chitin in 50 mM sodium acetate buffer (pH 5.2), were incubated at 50˚C for 1 hour. The reaction was stopped by adding 1 mL of 1% NaOH, followed by boiling for 5 min. Sample tubes were then centrifuged at 7000 rpm. 1 mL of supernatant and 1 mL of 1% 3, 5-dinitrosalicylic acid (DNS) were mixed and boiled for 5 min. The absorbance was recorded at 535 nm. The chitinase activity

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was interpolated in a standard curve prepared with a series of dilutions of N-acetyl-D-glucosamine (NAG) and DNS. The chitinase activity in UmL-1 was defined as the amount of the enzyme required to produce 1µmol of NAG in 1 hour (Gomez Ramirez et al., 2004).

1.1.7 Dual culture assay

Plate confrontation assays of *T. harzianum* T3.13 against *N. dimidiatum* U1 and *C. gloeosporioides* were carried out, respectively. Assay were done on agar plates (PDA) covered by a cellulose membrane (Millipore, Bedford, USA) with slightly modification as previously defined (Carsolio *et al*., 1994). Membrane was used to assist the removal of the fungi mycelium from the top of agar plate for RNA extraction process. Agar plugs (0.6 cm) from the corresponding fungi were located at the opposite sides to each other, in 14 cm PDA plates and shielded with sterile cellulose membranes. Sample cultures were incubated at 37 °C for 7 days. Mycelia from both fungi were harvested before and after they had made connection.

1.1.8 Scanning electron microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to study the interaction between *T. harzianum* T3.13 and *N. dimidiatum* U1 during dual culture assay. Specimen preparation has followed the standard critical point dry (CPD) protocol for plant tissues (Pathan *et al*., 2010). The tissues were cut into 1 cm^3 slices. After fixation with 4 % glutaraldehyde (2 days at 4 °C), washing with 0.1 M sodium cacodylate buffer (3 changes of 30 minutes each) and post fixation with 1 % osmium tetroxide (2 hours at 4 °C), the samples were dehydrated through a classified series of acetone (35 %, 50 %, 75 % and 95 % once for 30 to 45 minute at each step), and then dipped in 100 % acetone 3 changes for 1 hour each. The tissues were then transferred to critical point dryer machine for about 30 minute. Samples were mounted on metal base by double tape and the surface was coated with gold to avoid charging. Images were taken by a scanning electron microscope (Leo 1455 Vp SEM attached with Edx) at the voltage of 20 kV.

1.1.9 Analyzing *chit42*, *exc1* and *bgn13.1* gene expression

Mycelia were harvested from dual culture assay before and after the contact between both fungi. The total messenger RNA from *T. harzianum* T3.13 was transcribed to cDNA with random primers. The cDNA was semi-quantified with reverse transcription PCR (RT-PCR), and the expressed for each isolate were normalized with the transcript expression levels of the eukaryotic translation elongation factor 1- alpha (*tef1*) from *T. virens* (Druzhinina *et al*., 2005). The expression levels studied correspond to the genes *exc1* (encoding NAGase activity), *chit4*2 (chitinase activity) and *bgn13.1* (β-1, 3-glucanase activity).

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2. RESULTS AND DISCUSSION

2.1 Identification of the endophytic fungi

The morphology of the endophytic fungi showed the morphology as *Trichoderma* species. The fungi was fast growing and their colonies were initially white, as observed on day 2 to day 3. Then, the colony gradually changed to green after sporulation on day 7. There were no concentric rings observed. Regardless of the formation of spores and observation on the morphological characteristics, this isolate of *Trichoderma* species was also subjected to internal transcript spacer (ITS) gene sequence analysis. The blast result showed 100% similarity to *T. harzianum* from various strains. All the blast results showed 100% for query coverage. The high match of this particular isolate to *T. harzianum* is reflected in the position of the isolate (Sample T3.13) in the dendrogram, which was clustered together with the *T. harzianum* strains (Figure 1). The dendrogram shows that sample T3.13 and *T. harzianum* were highly similar. Sample T3.13 are close to *T. harzianum* strain ICIPE_709 (accession No. KJ619989.1). Hence, this strain was classified as the *Trichoderma harzainum* strain T3.13.

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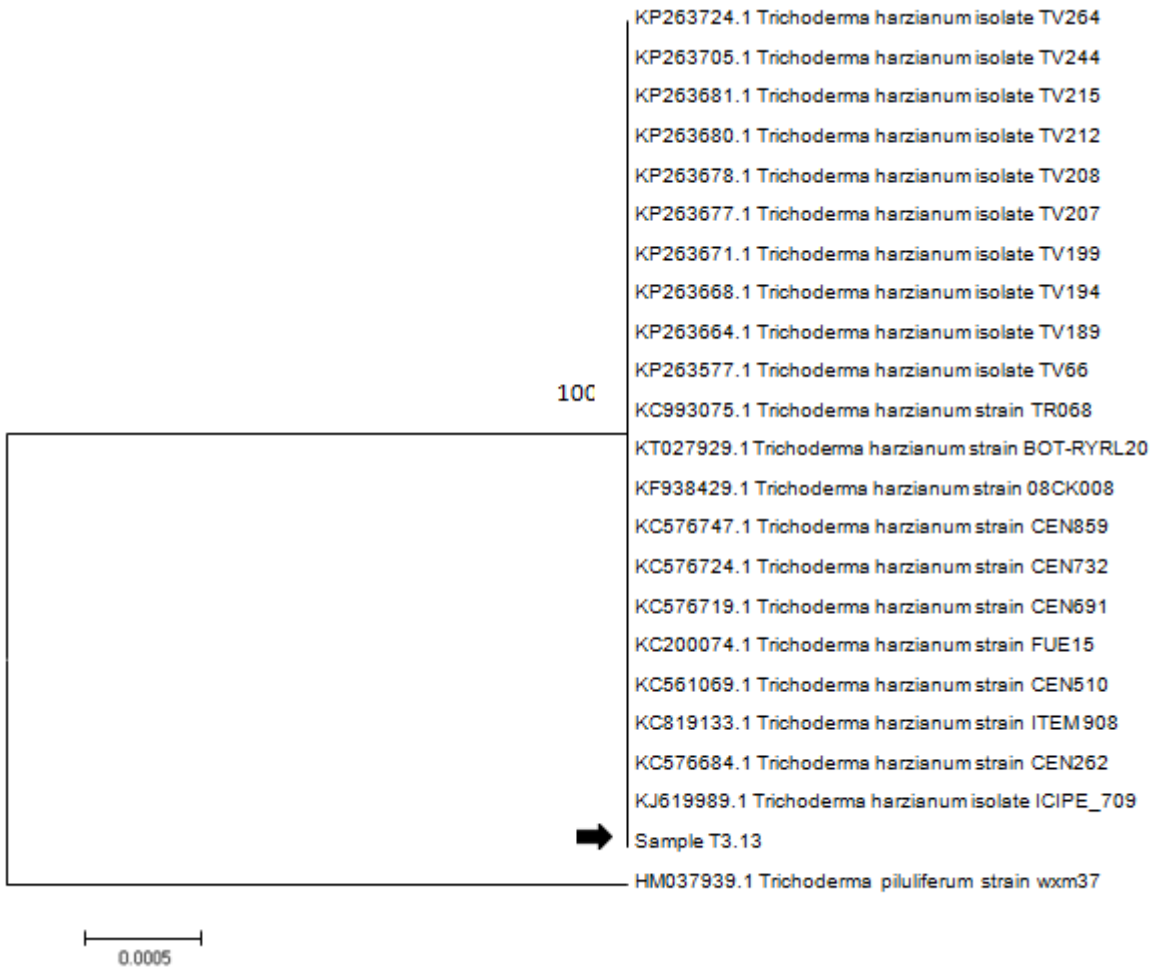

Figure 1: Molecular Phylogenetic analysis of of *T. harzianum* by Maximum Likelihood method. The name of the *T. harzianum* nucleotides sequences used was followed by its gene bank accession number, and *T. piluliferum* is the outgroup.

2.2 Identification of plant pathogenic fungi

The culture of dragon fruit plant pathogenic fungi showed the morphology of fungus primary was identified as *Neoscytalidium* species. At first, the colony characteristics showed effuse, hairy or woolly colony at day 2 to day 3, which was gradually changing to greyish colony at day 4, and dark grey to black pigmentation at day 7. From the dendogram (Figure 2), the local isolate of *N. dimidiatum* (Sample 1) from the unhealthy stem of *H. polyrhizus* in Malaysia (Suhaila, 2014) was clustered into the same subclade with other isolates of *N. dimidiatum* from GenBank. Sample 1 is close to *N. dimidiatum* strain CBS 312.91 (accession No. KJ193679.1) and strain

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HLN5 (accession no. KF612310.1). Based on the results, the sequence variations were not observed among all the isolates of *N. dimidiatum*. Hence, this strain was classified as *Neoscytalidium dimidiatum* strain U1. A previous studies also showed the same morphology characteristics (Crous *et al*., 2006), which was identified as *N. dimidiatum* through ITS sequencing analyses (Mohd *et al*., 2013).

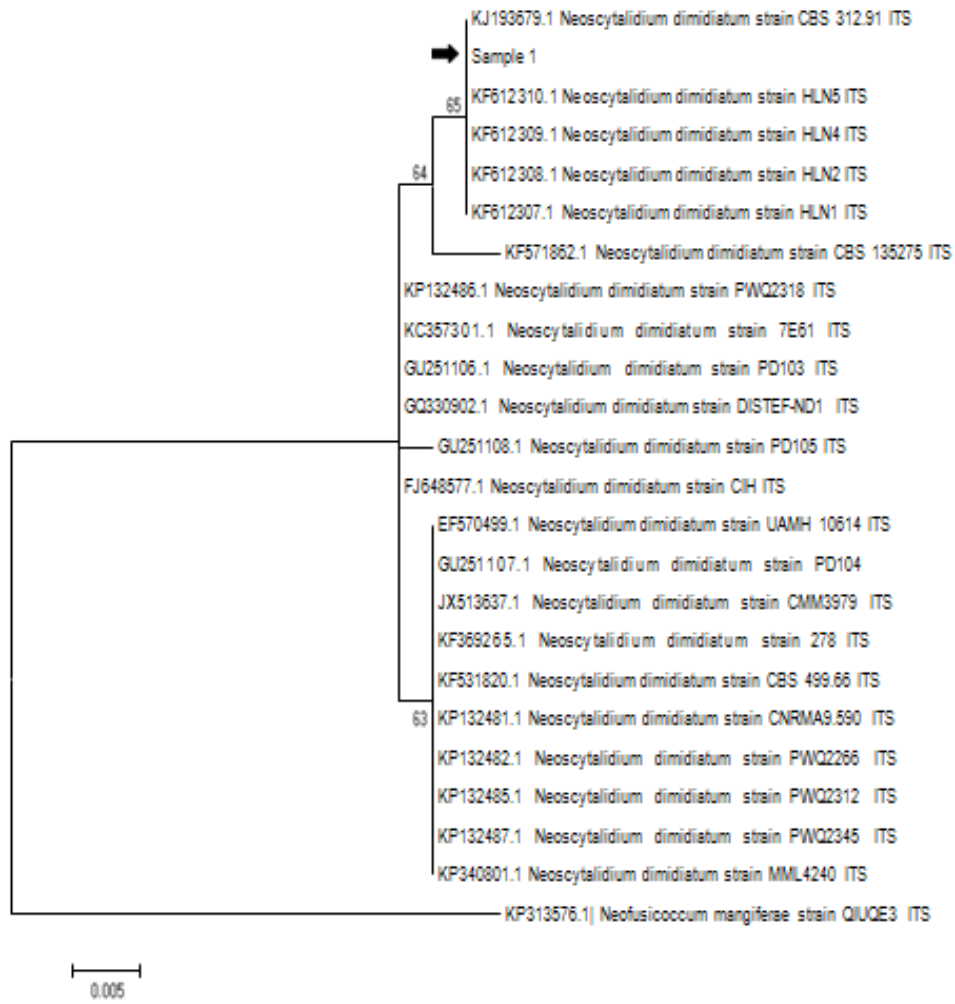

Figure 2. Molecular Phylogenetic analysis of *N. dimidiatum* uisng the Maximum Likelihood method. The name *Neoscytalidium* nucleotides sequences used was followed by its gene bank accession number. An out-group is *Neofusicoccum mangiferae*, a sequence that is more distantly related to the remaining (in-group) sequences than they are to each other.

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2.3 Chitinase enzyme assay activity

Figure 3 shows the chitinase enzyme in the Absidia medium kept increasing from the initial day until day 6, where it reached the peak at about 0.2 U/mL on day 8 of fermentation. The Kawachi medium also showed a similar increasing profile but the medium showed the highest activities at day 7, with 0.15 U/mL on day 8 of fermentation. Third, the Czapeck medium produced the highest chitinase activity with 0.11 U/ml at day 8 of the fermentation. Meanwhile, the modified Kawachi and *Trichoderma* minimal medium did not produce any chitinase activities. The results based on the maximum enzyme activity were produced for each type of medium. The explanation of one unit (U) of enzymatic activity using colloidal chitins as substrates is defined as the liberation of one mM of product (estimated as NAG) per hour (Avelizapa *et al*., 1999). The suitable medium for the highest chitinase production was the Absidia medium. This is because the Absidia medium contains organic nitrogen sources such as ammonium tartrate ($C_4H_{12}N_2O_6$), KH_2PO_4, $MgSO_4.7H_2O$, Na_2HPO_4, inorganic nitrogen source ($(NH_4)_2SO_4$) and colloidal chitin as sole carbon sources.

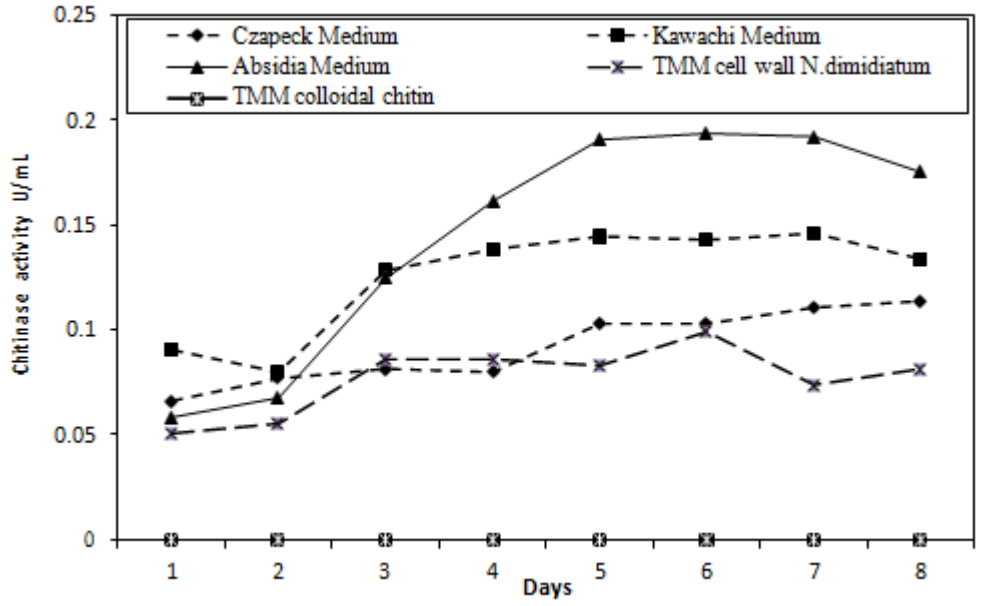

Figure 3: The effect of different types of medium composition on chitinase enzyme production. Values are means of 3 replicates ± SD. Symbols represent: (▲) Absidia Medium; Symbols represent: (■) Kawachi Medium; Symbols represent: (x) TM Medium cell wall *N. dimidiatum*; Symbols represent: (♦) Czapeck Medium; Open symbols represent: TM Medium colloidal chitin.

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Two other media like the modified Kawachi medium and *Trichoderma* minimal medium did not produce chitinase enzyme. The effects of peptone and yeast extract in the Kawachi medium enhanced the production of chitinase enzyme. Yeast extract contains nitrogenous compounds, some growth factors and oligomers of NAG. It also can stimulate cell growth through enhancing the repression of enzyme but unfortunately only in the initial stage of fermentation (Nawani & Kapadnis, 2003). Chitinase enzyme activities were dropped at day two in all medium. The simplest cause of this phenomenon could be the oxygen limitation (Chovanec *et al*., 2005). In this study, mycelia was used as inoculum. The rapid growth during day 1 of fermentation caused limited oxygen to occur (Simkovic *et al*., 2007) and affected the production of enzyme. A time course for chitinase activity in the extracellular fluid of *T. harzianum* T3.13 grown on cell wall of *N. dimidiatum* U1 is shown in Figure 3. The use of cell wall of *N. dimidiatum* U1 instead of chitin for inductions of chitinase would more likely resemble a mycoparasitism situation. Enzyme activity was first detected after 1 day incubation and reached a peak at 6 day of fermentation.

2.4 Scanning Electron Microscopy (SEM) Analysis

Figure 4 shows the *T. harzianum* T3.13 hyphae tangled with the hyphae of *N. dimidiatum* U1. Mycoparasitism is a complex process in which the *Trichoderma* species grows chemotropically to its host and attaches to and coils around the host hyphae before penetrating them (Carsolio *et al*., 1999). Fractional degradation of the host cell wall is usually observed in later stages of the parasitic process. The effects of chitinase, the degrading enzymes on the host have been observed by using enzyme assay approaches. An electron microscope observation (Figure 4), have led to the hypothesis that during the interaction of *T. harzianum* T3.13, the *N. dimidiatum* U1 cell walls are enzymatically digested by the *T. harzianum* T3.13. In order to be able to degrade phytopathogen cell walls, the antagonistic *Trichoderma* induces the production of extracellular hydrolytic enzymes. These enzymes are responsible for the direct attack against the pathogen and have been reported mainly in the isolates of *T. harzianum* (Lorito *et al*., 1998).

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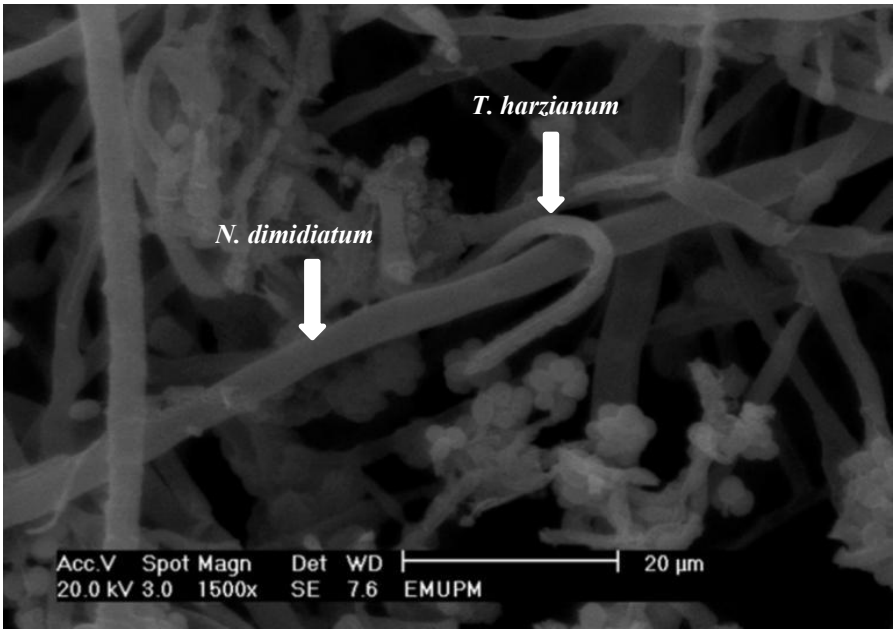

Figure 4: Scanning Electron Microscopy (SEM) Analysis of mycoparatism activities between *T. harzianum* T3.13 with *N. dimidiatum* U1.

2.5 Dual culture test

The antagonism of *T. harzianum* T3.13 against pathogens was investigated with dual culture tests. The mycoparasitism of *T. harzianum* T3.13 was observed by the interaction of hyphae from the interaction zone between *T. harzianum* T3.13 and test pathogens. *T. harzianum* T3.13 produced inhibited radial growth by establishing a clear inhibition zone in the dual culture tests against the control pathogen *C. gloeosporioides* (Figure 5B), ranging from 10 to 12 mm. The pathogen *C. gloeosporioides* stopped growing before any direct contact was made, presumably in response to diffusible inhibitors that were released by *T. harzianum* T3.13. This strain was suspected to inhibit the development of *C. gloeosporioides* by using antibiosis mechanism. *Trichoderma* are known to inhibit the growth of pathogen by producing a number of antibiotics such as trichodermol trichodermin, harzianum A, trichotoxin, and harzianolide (Dennis and Webster, 1971). These compounds are possibly responsible for the inhibition of control pathogen *C. gloeosporioides* in this study, and have also been previously described in the study involving the biocontrol of fungal phytopathogens such as *Colletotrichum* sp. by Svetlana Živkovic *et al*. (2010). However, *T. harzianum* T3.13 showed inhibition against *N. dimidiatum* U1 by overgrowing the isolated pathogen *N. dimidiatum* U1 (Figure 5A). *T. harzianum* T3.13 stopped the growth of *N.dimidiatum* U1 prior to contact, and this isolate was considered as highly

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antagonistic. From the observations and scanning electron analysis in Figure 4, the result showed that *T. harzianum* T3.13 inhibited the growth of *N. dimidiatum* U1 by mycoparasitic mechanism. The isolate of pathogen *N. dimidiatum* U1 and control pathogen *C. gloeosporioides* showed significant differences in the mycelial growth in the dual culture assay.

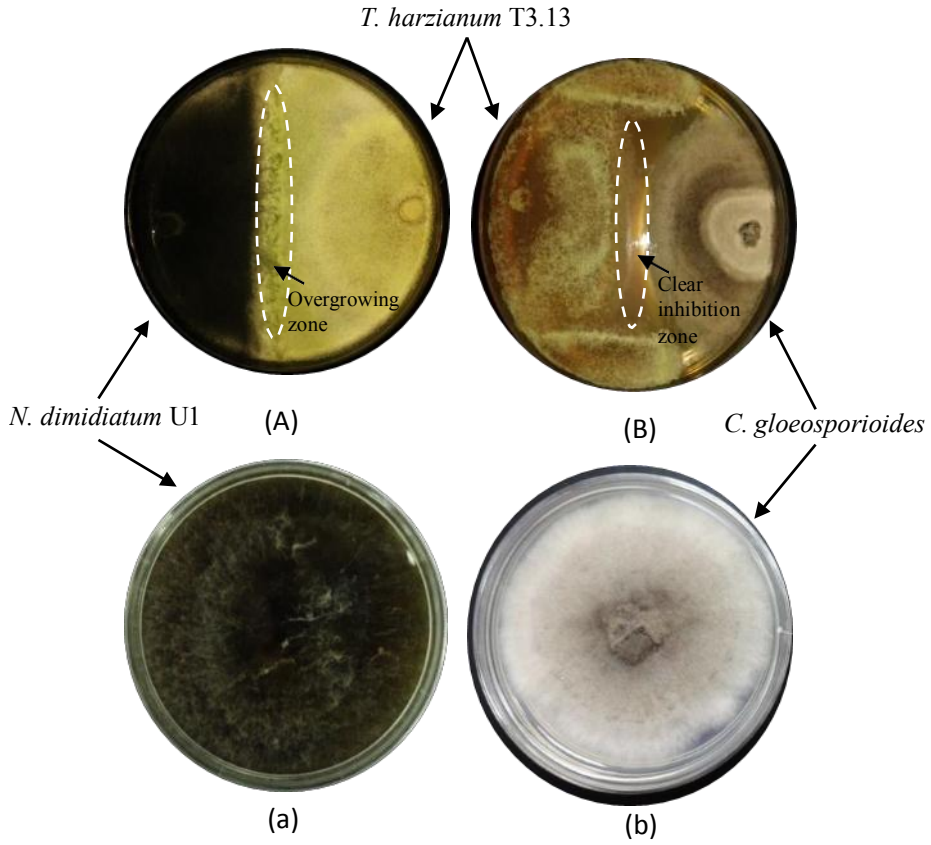

Figure 5: Mycoparasitism of *T. harzianum* T3.13 to phytopathogenic fungus at 5 days. (A) Confrontation of *T. harzianum* T3.13 with *N. dimidiatum* U1 (B) Confrontation of *T. harzianum* T3.13 with *C. gloeosporioides*; (a), (b) are the corresponding control for (A), (B), respectively

2.6 Expression analyses of *exc1*, *chit42*, *bgn13.1* of *T. harzianum* T3.13

Direct confrontation assays are great tool to learning the phenomenon of mycoparasitism by antagonists (Viterbo *et al*., 2002; Sanz *et al*., 2005). In order to inspect the possible effects of the *exc1, chit42* and *bgn13.1* genes on mycoparasitism, the expressions during the confrontation of *T. harzianum* T3.13, grown under simulated antagonism against *N. dimidiatum* U1 and *C. gloeosporioides*, were analysed. *Exc1* was expressed during the confrontation between *T.*

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harzianum T3.13 and *N. dimidiatum* U1, and its expression pattern seemed to be consistent over the time points. The same pattern was observed in the case of *chit42* gene (Figure 6). However, the expression of *bgn13.1* was different from these two genes. The expression of the *bgn13.1* gene was increased after 24 hours of the interaction (Lane 3), and the expression of *bgn13.1* was increased until the experiment ended (96 hours of confrontation). This expression could be related to mycelia growth since these enzymes are involved in morphogenetic processes, such as spore germination and hyphal elongation and branching (Martin *et al*., 2007).

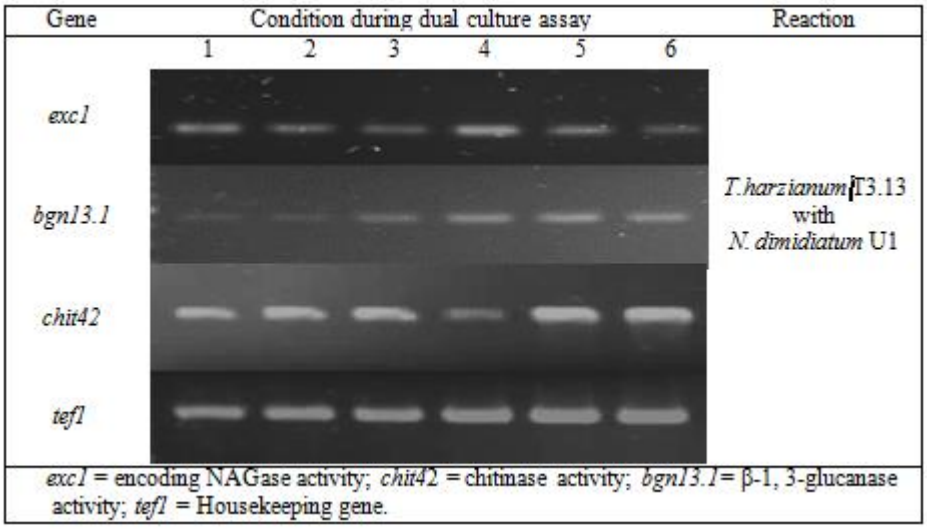

Figure 6: Expression patterns of *bgn13*.1, *chit42* and *exc1* gene during confrontation between *T. harzianum* T3.13 and *N. dimidiatum* U1. The comparative expression analysis of *bgn13.1, chit42 and exc1* genes from the plate confrontation assays between *N. dimidiatum* U1 and *T. harzianum* T3.13. Lane 1: i (the hyphae of both fungi when they were distanced 8cm apart prior contact), Lane 2: ii (a 2 cm strip of both fungi at the zone of interaction after 12 hours of interaction), Lane 3: iii (after 24 hours of interaction), Lane 4: iv (after 48 hours of interaction), Lane 5: v. (after 72 hours of interaction), Lane 6: vi. (After 96 hours of interaction).

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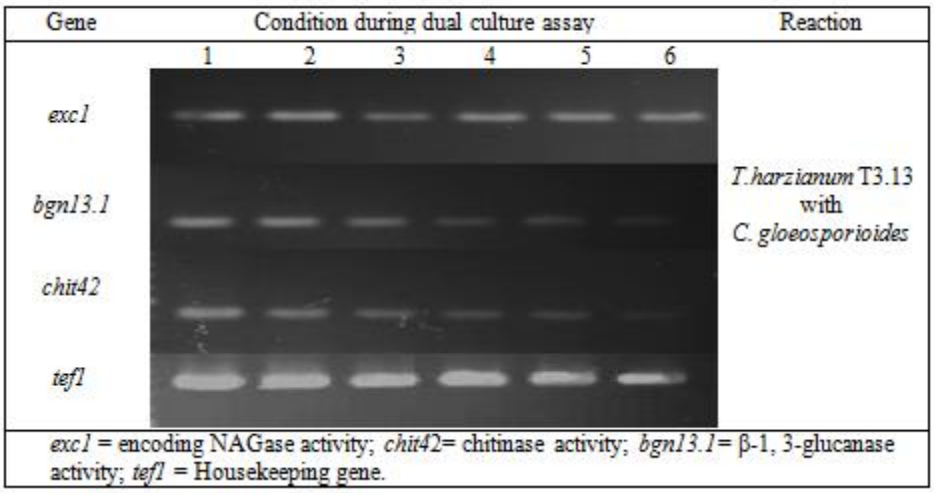

Figure 7: Expression pattern of *bgn13.1*, *chit42* and *exc1* gene during the confrontation between *T. harzianum* T3.13 and *C. gloeosporioides*. Comparative expression analysis of *bgn13.1, chit42 and exc1* genes from the plate confrontation assays between *C. gloeosporioides* and *T. harzianum* T3.13. Lane 1: i (the hyphae of both fungi when they were distanced 8cm apart prior contact), Lane 2: ii (a 2 cm strip of both fungi at the zone of interaction after 12 hours of interaction), Lane 3: iii (after 24 hours of interaction), Lane 4: iv (after 48 hours of interaction), Lane 5: v. (after 72 hours of interaction), and Lane 6: vi. (After 96 hours of interaction).

In the confrontation between *T. harzianum* T3.13 and *C. gloeosporioides*, the expression patterns of the three genes were also found to vary (Figure 7). Two of the genes (*bgn13.1* and *chit42*) exhibited a gradual decrease in expression during the confrontation. However, the *exc1* gene demonstrated no change in its expression pattern. This study confirmed that the isolated endophytic *T. harzianum* T3.13 contained the genes encoding for NAGases (*exc1*), chitinases (*chit42*) and β-glucanases (*bgn13.1*). RT-PCR revealed that the *bgn13.1, chit42 and exc1* gene in *T. harzianum* T3.13 can be detected even before physical contact (Figure 7, lane 1). The expression patterns of *chit42*, *exc1* and *bgn13.1* genes of *T. harzianum* T3.13 showed different behaviours during their confrontation with *N. dimidiatum* U1 and *C. gloeosporioides*. These differences could suggest that the fungus used different modes of action when in confrontation with different fungus. The mechanisms involved in the control of phytopathogens by *Trichoderma* species included antibiosis and mycoparasitism (Hjeljord and Tronsmo, 1998).

When *T. harzianum* T3.13 was confronting the control pathogen *C. gloeosporioides*, it produced a clear zone that stopped the growth of the pathogen (Figure 5B). Studies on *T. harzianum* by Calistru *et al*. (1997) suggested that the existence of the inhibition zone in dual culture without

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the hyphal contact or overgrowth due to the ability of *T. harzianum* to discharge an inhibitory enzymes or antimicrobial substances. The clear zone might contain hydrolytic enzymes or secondary metabolites such as antibiotic, which could inhibit the growth of pathogen. The mode of mechanism of antagonism involved in controlling phytopathogens by *Trichoderma* species might consist antibiotics (Howell, 2003).

However, when *T. harzianum* T3.13 confronted *N. dimidiatum* U1, it overtook the pathogen by overgrowing and killing the pathogen before utilising the carbon from dead pathogen. In this process, *Trichoderma* secretes hydrolytic enzymes that hydrolyse the cell wall of the host fungus (Woo *et al*., 2006; Verma *et al*., 2007). The main mechanism of antagonism of *T. harzianum* against pathogenic fungi is the extracellular secretion of chitinases, β-1, 3-glucanases and proteases (López-Mondéjar *et al*., 2011). In the chitinolytic system of *Trichoderma*, there are endochitinases (*CHIT42* and *CHIT33*) (Gokul *et al*., 2000) and N-acetyl-β-D-glucosamine (*EXC1* and *EXC2*) (Seidl *et al*., 2006). Studies found that these multiple chitinases have showed the synergistic action on degradation of pathogen cell wall (De La Cruz *et al*., 1992). The expression and secretion of chitinase and β-1, 3-glucanase enzymes can result in the suppression of plant pathogen activities directly. Gene expression in the host *Trichoderma* interaction area during in vitro confrontation assays has also been reported for endochitinase produced by *T. asperellum* (Viterbo *et al*., 2002) and α-1, 3-glucanase (Sanz *et al*., 2005). The authors also showed the expression of lytic enzyme such as endochitinase (*chit36Y*) and α-1, 3-glucanase (*agn13*.2) when *T. asperellum* grew under simulated antagonism against *B. cinerea* and *R. solani*, respectively.

3. CONCLUSION

The potential for biological control agent for diseases in dragon fruit plant has been identified as *T. harzianum* T3.13 and *N. dimidiatum* U1 as a pathogen isolate. Results from the chitinase assay and SEM suggested that cell wall degrading enzymes contribute to inhibition of fungal growth. Our data showed that the expression of *bgn13.1, chit42 and exc1* gene implicated in the mycoparasitism against *N. dimidiatum* U1 and *C. gloeosporioides*. It has been demonstrated that the isolate *T. harzianum* T3.13 possess promising mycoparasitic activity against *N. dimidiatum* U1 and could be used in agricultural situations to reduce infection by this pathogen.

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