Characterization of antifungal activity of endophytic *Penicillium oxalicum* T 3.3 for anthracnose biocontrol in dragon fruit (*Hylocereus* sp)

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***Abstract*—** *A group of 126 endophytic fungi was isolated from dragon fruit plants in Malaysia. Dual culture and disc diffusion test revealed that seven strains could suppress the growth of Colletotrichum gloeosporioides. Of all the potential strains, strain T3.3 exhibited the best antagonistic activity by producing an inhibition zone of 12±1.0 mm in dual culture test. Disc diffusion test using crude extract produced by this fungus could inhibit the growth of C.gloeosporioides by 33.33±2.89%. The diffused non volatile metabolite produced by this strain suppressed the growth of the pathogen by 97% after 7 days of incubation. Based on morphological characteristics and ITS region sequence, strain T3.3 was identified as Penicillium oxalicum. The carbon utilization profile in Biolog FF Microplate analysis revealed that P.oxalicum T3.3 is a versatile microorganism. P.oxalicum T3.3 was found to produce β-glucanase and chitinase with activity of 3.38 U/mL and 1.19 U/mL respectively. In addition, the ethylacetate extract of P.oxalicum T3.3 could suppress the growth of C.gloeosporioides. Scanning electron microscopy study showed that the release of extracellular antifungal metabolites from the endophytic P.oxalicum T3.3 had caused abnormal hyphal growth of C.gloeosporioides. Treatment with the crude extract from this fungus on dragon fruit could control anthracnose disease in this fruit in vivo. Thus, endophytic Penicillium oxalicum T3.3 is considered as a potential biological control agent of anthracnose disease in dragon fruit.*

Keywords*— *Antifungal, dragon fruit, endophytic fungi, Penicilium oxalicum.

I. INTRODUCTION

Colletotrichum.gloeosporioides is the causal agent of anthracnose disease and an important cosmopolitan fungal pathogen that infects many plant species worldwide (Cannon *et al*., 2012). This pathogen is present in various regions of the world including tropical to subtropical areas and can be destructive if left uncontrolled. In recent years, the occurrence of anthracnose disease in dragon fruit plant has affected fruit yield tremendously. (Masyahit *et al*., 2009). The disease occurs when the weather conditions, such as warm weather and frequent rains as well as inoculum were present. The common symptoms that usually observed in infected plants and fruits are sunken necrotic tissues with concentric rings where ascervuli emerge from it. The infected spot will coalesced to rot (Palmateer and Ploetz, 2006).

This disease is controlled by a combination of cultural management practices and chemical control. The infected plant parts are pruned out and destroyed by burning them. Fungicides such as Benex, Arimo 23, Maneb and Mancozeb were applied to the farm to eradicate the fungal infection. However, the usage of chemical fungicide in the dragon fruit farm gives detrimental effect to the lives of human and had polluted the environment (Crissman *et al*., 1994). Apart from that, the development of fungicide resistance pathogen had complicated the disease management (Pimenta *et al*., 2010).

Under such circumstances, there is a need to search for another alternative control method that is considered safe for human health and the environment. Currently, there is growing interest in using endophytic fungi as biological control agent (BCA). The use of endophytic fungi which reside asymptomatically inside host plant without causing any apparent disease as BCA is beneficial as they occupy the same ecological niche as the pathogens and may induce the defense responses of the hosts against the pathogens (Brum *et al*., 2012). Endophytic fungi also produce an array of secondary metabolites which may be exploited for use in agriculture and industry (Zhang *et al*., 2006).

Many species of endophytic fungi mostly those belonging to the genus *Penicillium* are identified as biocontrol agents with antifungal activities against a number of plant pathogenic fungi (Xu *et al*., 2010; Elsharkawy, 2012; Murali, 2012). *Penicillium sp*. has also been reported to produce lytic enzymes including chitinase and β-glucanase which are involved in

degrading fungal cell walls (Lee *et al*., 2009; Chen *et al*., 2012; Patil *et al*., 2012). Furthermore, *Penicillium sp*. also produce antimicrobial compounds that could control a wide spectrum of microorganisms (Komai *et al*., 2006; Wang *et al*., 2008).

In this study, we reported the screening and isolation of endophytic fungi from healthy dragon fruit plant. The antifungal activity of the potential strain against *C. gloeosporioides* was also characterized. The introduction of the paper should explain the nature of the problem, previous work, purpose, and the contribution of the paper. The contents of each section may be provided to understand easily about the paper.

II. MATERIAL AND METHOD

2.1 Sample collection

Stem and aerial root samples of healthy dragon fruit plants were collected from the dragon fruit field at three different locations in Malaysia including Telong, Kelantan; Mantin; Negeri Sembilan; and Serdang, Selangor from May 2011 to August 2011. The samples were collected using a clean knife and immediately processed for isolation of the endophytic fungi.

2.2 Fungal pathogen

The plant pathogenic fungus *C.gloeosporioides* (Accession number: MARDICG1) was obtained from the Malaysian Agriculture Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. The fungus was maintained on potato dextrose agar (PDA) medium and stored at 4°C.

2.3 Isolation of endophytic fungi

The isolation of endophytic fungi followed the method described by Tan *et al*. (2006) with slight modifications. Malt extract agar (MEA), Potato Dextrose Agar (PDA) and Czapek Dox Agar (CDA) were used as isolation media. The dragon fruit plant part was cut approximately to 4 cm in length with a sterilized blade, cleaned by washing under running tap water several times and dried in a laminar airflow. Then the prepared organ was surface sterilized with 70% (v/v) ethanol for 1 min followed by 2% NaOCl for 1 min and then sequentially rinsed in sterile distilled water three times and dried on sterile filter paper. The surface sterilized organ was cut approximately to about 5 mm in length using a sterile blade and placed onto the isolation medium. All the plates were incubated at 30°C for 7 to 15 days. Individual hyphal tips developed from the stems were transferred onto fresh PDA plates to get pure culture.

2.4 Dual culture test of endophytic fungi against *C.gloeosporioides*

All the pure cultures of the endophytic fungi were tested for their antagonistic activity against *C.gloeosporioides* using dual culture test on PDA plate. Agar discs (10 mm diameter) of pure endophytic fungus and fungal pathogen from 7 day old cultures were put at the periphery of the PDA plate opposed to each other. As for the control, the pathogen was inoculated on PDA with no opposed fungi. All the plates were incubated at 30°C and observed daily for 7 days. Antagonistic activity was observed daily for seven days after incubation. Fungal strains that showed mutual inhibition were chosen and the size of the inhibition zone was measured.

2.5 Cultivation of the potential strains for production of antifungal compounds

The strains that exhibited mutual inhibiton were selected and cultivated for the production of antifungal compounds. An agar plug of the pure culture of the potential strain was inoculated into 100 ml of Richard medium (KNO_3, 1 g/l; glucose, 30 g/; KH_2PO_4, 0.5 g/l; $MgSO_4.7H_2O$, 0.25 g/l; $FeCl_3$, 0.001 g/l) pH 5.5 in a 250 ml of conical flask. The flask was incubated on rotary shaker at 30°C, 150 rpm for 10 days. Each strain was prepared in triplicates.

2.6 Extraction of metabolite

Following fermentation, the mycelia of the fungi were separated from the fermentation broth by passing through a funnel layered with cotton wool. The mycelia were placed in a beaker and an equal volume of ethylacetate was added into the beaker. This mixture was left at room temperature overnight. The solvent fraction was separated and collected from the mixture. As for the fermentation broth, the broth was transferred into a conical flask and an equal volume of ethylacetate was added into the broth. The mixture was stirred using a magnetic stirrer for an hour. After that the solvent phase was separated and collected from the mixture using a separatory funnel. An equal volume of ethylacetate was added for the second time and

the step was repeated twice. All the solvent fractions were combined and dried using a rotary evaporator at 40°C with vacuum pressure until all the solvent was removed.

2.7 Disc diffusion test using the extracted metabolites

Ten mg of the crude extract produced from each of the seven strains was weighed and dissolved in 1000 µl of ethylacetate. Ten µl of the dissolved crude extract was impregnated onto 5 mm sterile filter paper disc (Whatman filter paper No 1) and allowed to dry in a laminar airflow for 30 min. The dried filter paper disc was placed at the center of the PDA plate. An agar plug of *C.gloeosporioides* with diameter of 5 mm from 7 days old culture was placed at a distance of 10 mm opposite the filter paper disc. As for the control, the disc that was impregnated with ethylacetate was used. The test was prepared in triplicate and incubated at 30°C for 2 days. The radial growth of *C.gloeosporioides* towards the disc containing the crude extract (R_2) and that on a control plate (R_1) were measured and percentage of radial growth inhibiton (PIRG) was recorded to this formula: $(R_1-R_2)/R_1 \times 100$.

2.8 Non volatile metabolite test

The agar layer technique (Dennis and Webster, 1971) was used to detect the production of non-volatile metabolites by strain T3.3 and to evaluate their effects against the pathogen. An agar plug (10 mm in diameter) of strain T3.3 was located at the center of the PDA plate which was previously layered with sterile Visking tube and incubated at 30°C. After 7 days, the Visking tube layer containing the fungal growth was removed from the plate. An agar plug (10 mm in diameter) of pathogen was placed at the center of the prepared plate. The plate was incubated at 30°C for 7 days and observed daily. As for the control, strain T3.3 was not inoculated on the layered PDA plate. The radial growth of *C.gloeosporioides* on the plates containing the non volatile metabolite produced by strain T3.3 (R_2) and that on a control plate (R_1) were measured and the PIRG value was calculated according to the formula described previously.

2.9 Identification of potential endophytes using morphological characteristic and ITS sequence

Strain T3.3 which exhibited the highest inhibitory activity was identified macroscopically and microscopically. The morphology of the isolate such as the spore colour, growth of hyphae on PDA and the color of the bottom of the PDA was observed with the naked eyes. The microscopic characteristics of the isolate such as spore arrangement and hyphae structure were observed using a light microscope in order to identify its genus. Strain T3.3 was identified using the ITS method. Seven days old mycelia culture of this strain was used to isolate total genomic DNA. The genomic DNA of the strain was extracted using the Profound and Kestrel Laboratory (PKL) DNeasy Plant Minikit, according to the manufacturer's manual. The DNA was amplified by PCR using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCT TATTGATATGC-3') primers. The PCR was done according to the Fungal ID Kit (PKL) protocol and the conditions were as follows: predenaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, renaturation at 54.5 °C for 20 s and extension at 72 °C for 1 min per-cycle, followed by 35 cycles, and a final extension step at 72 °C for 5 min. DNA sequence homology searches were performed using the online BLAST search engine in GenBank. The phylogenetic tree for the data set was constructed using the MEGA 4.1 programme.

2.10 Biolog FF Microplate analysis

Carbon source utilization assimilation of strain T3.3 was examined by using the Biolog Filamentous Fungi Microplate according to the manufacturer's protocol. The profile was compared with the BIOLOG identification database GN4.01

2.11 Production of cell wall degrading enzymes by strain T3.3

2.11.1 Preparation of dried *C.gloeosporioides* mycelium for β-glucanase production

One cm^2 disc of actively growing mycelium of *C.gloeosporioides* was inoculated in a 250 ml conical flask containing 100 ml potato dextrose broth and incubated at 30 °C for 7 days. The mycelium was then filtered through Whatman No.1 filter paper, washed with distilled water and dried at 60°C overnight. The dried mycelium was ground with mortar and pestle until it turned into fine powder and stored at 4°C until further use.

2.11.2 Production of β-glucanase and chitinase enzyme

Strain T3.3 was grown on β-glucanase production medium (peptone, 3.0 g/l; $(NH_4)_2SO_4$, 2.0 g/l; yeast extract, 0.5 g/l; KH_2PO_4, 0.5 g/l; $CaCl_2.2H_2O$, 0.3 g/l; $MgSO_4.7H_2O$, 0.3 g/l; Tween 20, 100µl; dried cell wall of *C.gloeosporioides*, 10 g/l;

pH 5.5) and chitin medium ($NaNO_3$, 0.2 g/l; K_2HPO_4, 0.1 g/l; $MgSO_4.7H_2O$, 0.05 g/l; KCl, 0.05 g/l; $FeSO_4.7H_2O$, 0.001 g/l; pH 5.5) supplemented with 10 g/l of colloidal chitin. These media were prepared for β-glucanase and chitinase enzyme production respectively. Two discs (one cm^2) of seven days old culture were inoculated into 100 ml of each medium in 250 ml flask. All the flasks were prepared in triplicate incubated at 30°C, 150 rpm on a rotary shaker for 7 days and sampling was done daily.

2.11.3 β-glucanase and chitinase assays

β-glucanase activity was determined according to the method described by Cao et al., (2009) with some modifications. β-glucanase was assayed by incubating 0.5 ml of 1.0 % (w/v) laminarin in 50 mM acetate buffer (pH 5.5) with 0.5 ml enzyme solution at 50 °C for 30 min. Then 1.5 ml of DNS was added into the mixture and boiled for 15 min. The absorbance was read at 575 nm after the addition of 1 ml of Rochelle salt. Chitinase activity was determined using the colorimetric method described by Patil et al., (2012) with minor modifications. The reaction mixture contained 1 ml of 1% colloidal chitin 0.2 M, pH 5.5 and 1 ml of enzyme solution. The reaction mixture was incubated for 60 min at 50°C. One ml of 1% NaOH was added into the mixture and boiled for one min. The mixture was centrifuged at 7000 rpm for 10 min and 1 ml of the supernatant was collected and transferred to a new test tube. One ml of DNS was added into the supernatant and boiled for 5 min. Then the mixture was cooled at room temperature and the absorbance was read at 575 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute.

2.11.4 Dry cell weight and antifungal activity of strain T3.3

Richard medium was used as the medium to determine the dry cell weight and antifungal activity of strain T3.3. One ml spore suspension of this strain with a concentration of $1x10^6$ per ml was inoculated into 100 ml Richard medium in 250 ml conical flask. Fermentation was carried out at 30°C, 120 rpm for 10 days and sampling was done daily. The biomass produced was used to determine the dry cell weight of this fungus. The culture filtrate collected was processed according to the method described previously. The antifungal activity using the crude extract of isolate T3.3 was calculated following the method described previously.

2.12 Scanning electron microscopy

Zone of interaction from dual culture plate was used as specimen to observe the interaction between strain T3.3 and *C.gloeosporioides*. One cm^2 of the agar plug from the zone of interaction was cut from the dual culture test plate. The specimen was fixed with 4% glutaraldehyde for 12 h at 4°C. Then it was washed with 0.1 M sodium cacodylate buffer for 10 min and this step was repeated three times. The specimen was post fixed with 1% osmium tetroxide for 2 h at 4°C. After post fixation, the specimen was washed again with 0.1 M sodium cacodylate buffer for 10 min three times. The specimen later was dehydrated using a series of alcohol starting from 30%, 50%, 70%, 90% and 100% for 10 min each. The sample was further dehydrated with 100% acetone, 10 min and this step was done twice. The dehydrated specimen was dried in a critical point dryer machine for 30 min .The dried specimen was stuck onto the stub using double sided tape. Finally the specimen was coated with gold in sputter coater for 3 min and it was viewed using a scanning electron microscope LEO 1455 VP SEM attached with EDX.

2.13 Evaluation of strain T3.3 crude extract against *C.gloeosporioides* on detached dragon fruit

The inoculum of the pathogen was prepared by culturing the *C.gloeosporides* on PDA and incubated at 30°C for one week. Ten ml of sterile distilled water was added onto PDA containing the pathogen. The mycelia were harvested by scraping the surface of the plate using a sterile hockey stick. Then the suspension was filtered using sterile cheesecloth to separate the mycelia and conidia. The conidia concentration was calculated using a haemacytometer and adjusted to $1x10^6$ per ml using sterile distilled water. The conidia suspension was stored at 4°C until further use. The fresh and healthy dragon fruits were surface sterilized according to the method described previously. The fruit was artificially wounded using a sterile needle. Ten μl of crude extract with concentration of 100 mg/ml (dissolved in ethanol) was applied onto the wounded area using sterile pipette and allowed to air dry for 30 minutes. Then 10 μl ($1x10^6$ per ml) of fungal pathogen spore suspension was inoculated at the wounded site. As for positive control, the fruit was inoculated with 10 μl conidia of *C.goeosporioides*. The dragon fruit that was inoculated with ethanol was used as negative control. The experiments were prepared in triplicate for each treatment. All the fruits were kept in a moisturized cotton wool layered container at 28°C for 5 days. The disease symptoms and radial lesions were observed and measured daily.

III. RESULTS

3.1 Isolation of endophytic fungi

A group of 126 endophytic fungi were successfully isolated from healthy dragon fruit plants from three different locations in Malaysia. Of that number, 86 endophytic fungi were isolated from stem whereas 40 strains were isolated from aerial roots. Based on morphological identification of the endophtic fungi isolated, *Aspergillus sp., Phoma sp., Monilia sp., Botrytis sp., Trichoderma sp., Penicillium sp*. and *Fusarium sp.* were among the fungal species that resided in dragon fruit plants. Some endophytic fungi did not have reproductive structures and could not be identified.

3.2 Dual culture test and disc diffusion test of potential strains against *C.gloeosporioides*

Based on the interactions observed in the dual culture test plates, seven strains showed mutual inhibition interaction against *C.gloeosporioides*. Further screening of the seven potential strains was carried out by using the disc diffusion test. Referring to table 1, strain T3.3 exhibited the highest antifungal activity against the pathogen tested. The highest inhibition zone against *C.gloeosporioides* was exhibited by strain T3.3 whereas the lowest inhibition zone was shown by strain Ma6. Strain T3.3 produced inhibition zone of 12±1.00 mm (Fig 1). The 10 mg/mL of crude extract of this isolate could inhibit the growth of *C.gloeosporioides* by 33.33±2.89%.

TABLE 1
INHIBITION ZONE AND ANTIFUNGAL ACTIVITIES OF SELECTED ENDOPHYTIC FUNGI ISOLATED FROM DRAGON FRUIT PLANT AGAINST *C.GLOEOSPORIOIDES*

Strain	Origin	Inhibition zone (mm)[a]	PIRG of crude extract (%)[b]
T3.3	Telong, Kelantan	12±1.00	33.33±2.89
Mn10	Mantin, Negeri Sembilan	4±1.00	20.00±5.00
UN9	Telong, Kelantan	6±0.50	26.67±2.89
TPU22	UPM, Selangor	8±0.50	26.67±5.77
Ma6	Telong, Kelantan	3±0.50	13.33±5.77
TPU19	UPM, Selangor	4±0.86	15.00±0.00
Mn21	Mantin, Negeri Sembilan	6±1.00	23.33±5.77

[a]The size of inhibition was measured when the three colonies in the control covered the whole plate.
[b]The PIRG value was measured when the three colonies in the control reached the disc with no crude extract.

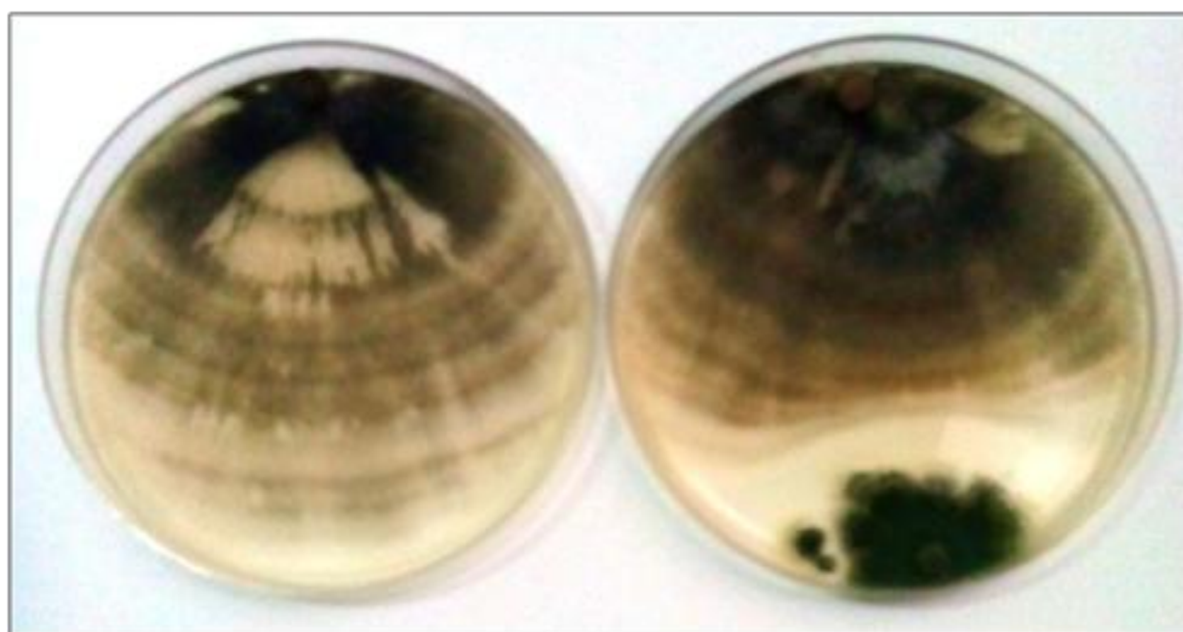

FIG. 1: Growth inhibition of *C.gloeosporioides* by endophytic strain T3.3 cultured on PDA plate at 30°C for 1 week. Left: control; right: in the presence of strain T3.3.

3.3 Non volatile metabolite test of strain T3.3

Strain T3.3 exhibited potential inhibitory activity against C.gloeosporioides. Therefore, this strain was further evaluated using the non volatile metabolite test. The growth of the pathogen on the plate containing the diffused non volatile metabolite of strain T3.3 was suppressed by 97% compared to the growth of the pathogen on the control plate. This test revealed that non volatile metabolites released by this strain contain antifungal metabolites which contribute to its antifungal property. All these characteristics, in addition to its strong inhibition, we recommend strain T3.3 for further evaluation of its biocontrol potential.

3.4 Identification of strain T3.3

Strain T3.3 showed slow and dense growth on PDA plate. The colony size was 32-36 mm in diameter, and the mycelium appeared as white radially plane or sulcate, velutinous within the first three days. After three days of incubation, the white mycelia at the center of the colony sporulated and produced heavy dark green spores. Within seven days, the entire colony appeared as dark green with narrow irregular white margin about 2 mm wide. The reverse side of the colony showed yellow colorations. Under a light microscope, conidiopohores with metulae and phialides were observed and the phialides branched asymmetrically. Conidia were budded from the phialides and were arranged in chains. The conidia were ellipsoidal in shape and the walls were smooth. Based on the phylogenetic tree constructed using ITS sequence (Fig. 2), strain T3.3 was identified as Penicillium oxalicum.

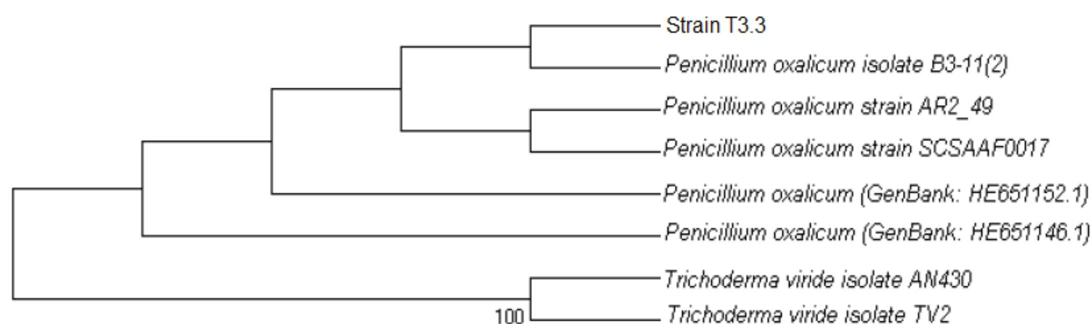

FIG. 2: Neighbour-joining phylogenetic tree showing relationships between strain T3.3 and several other strains of *Penicillium*, based on their ITS DNA sequences. *Trichoderma viride* was used as an out group for reference purposes.

3.5 Biolog FF Microplate analysis of endophytic P.oxalicum T3.3

Biolog FF Microplate was recently introduced as a new method to identify filamentous fungi instead of using morphological and DNA sequence identification. This method had also been used to characterize the metabolism of a certain microorganism (Singh, 2009; Papaspyridi *et al.,* 2011). Based on Biolog FF Microplate analysis, P.oxalicum T3.3 was identified as Penicillium oxalicum Currie and Thom. The Biolog analysis also analyzes fungal growth via turbidimetric analysis.The turbidities of this fungus were significantly high in wells containing sucrose, xylitol, maltose, maltotriose gentibiose and i-erythritol as carbon sources. These groups of carbon sources are mainly carbohydrates and sugar alcohols. The turbidities of *P.oxalicum* T3.3 in wells containing D-cellobiose, D-fructose, α- D-glucose, D-raffinose D-trehalose were considered as moderate. However, there was no growth observed in wells containing N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, L-sorbose, glucuronamide, L-fucose as carbon sources.

3.6 β-glucanase and chitinase production by *P.oxalicum* T3.3

Endophytic P.oxalicum T3.3 was found to produce cell wall degrading enzymes including β-glucanase and chitinase enzymes. The highest β-glucanase and chitinase activities from this fungus were obtained on the third and sixth days of fermentation with activities of 3.38 U/ml and 1.19 U/ml respectively (Fig. 3). Both β-glucanase and chitinase enzymes were hydrolytic enzymes which degrade the cell wall of the phytopathogens and had been reported as a mechanism of suppression of fungal pathogen by some biocontrol agents (El-Katatny *et al*., 2000).

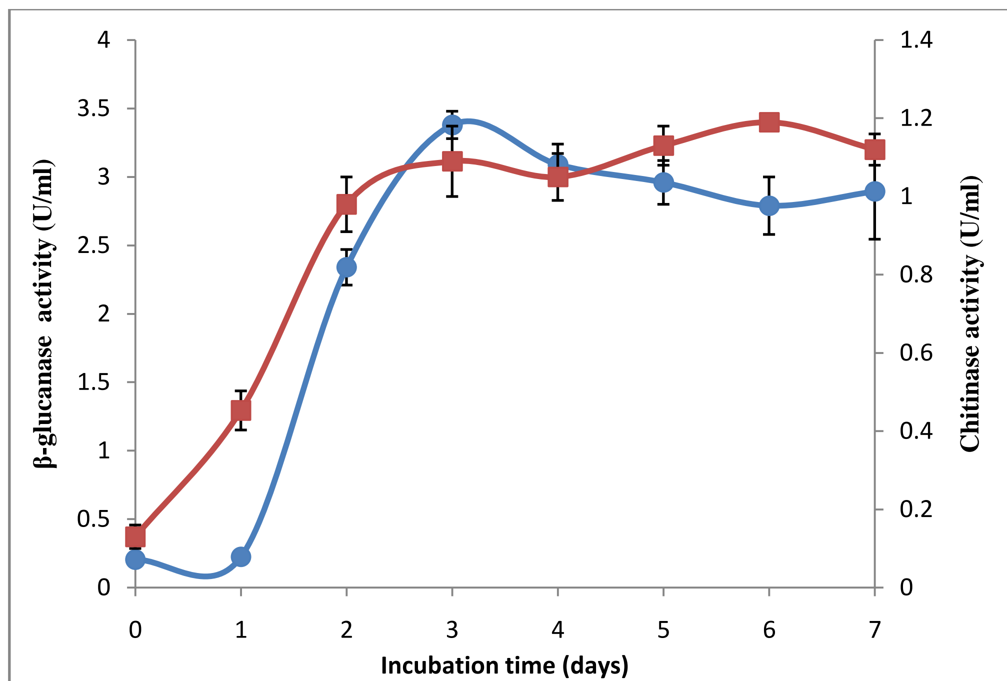

FIG. 3: Time course of β-glucanase and chitinase enzymes by *P.oxalicum* T3.3 in β-glucanase and chitinase production medium respectively at 30°C. Data represent the means from three different flasks. The highest β-glucanase activity was on the third day (3.38 U/ml) and the highest chitinase activity was on the sixth day of fermentation (1.19 U/ml). Error bars represent standard deviation from three replicates. Symbols represent: (●) β-glucanase activity; (■) chitinase activity

3.7 Antifungal activity of the extract of *P.oxalicum* T3.3

P.oxalicum T3.3 showed the presence of white smooth balls not more than one mm in diameter in Richard medium after 24 h of incubation. After two days, the ball - like masses of mycelia grew bigger ranging from two to six mm in diameter and after 10 days of fermentation, the clear medium changed to yellow colour indicating that metabolite was excreted out into the medium. Dark brown crude extract was obtained after extraction of the culture filtrate by ethylacetate. Based on Fig 4, the antifungal activities of the crude extract of *P.oxalicum* T3.3 were not detected from day 0 to day 4. On day 5, antifungal activity was detected and reached a peak on the 9th day of fermentation and decreased on the subsequent day. In this figure, it was shown that the antifungal activity was directly proportional to the biomass produced.

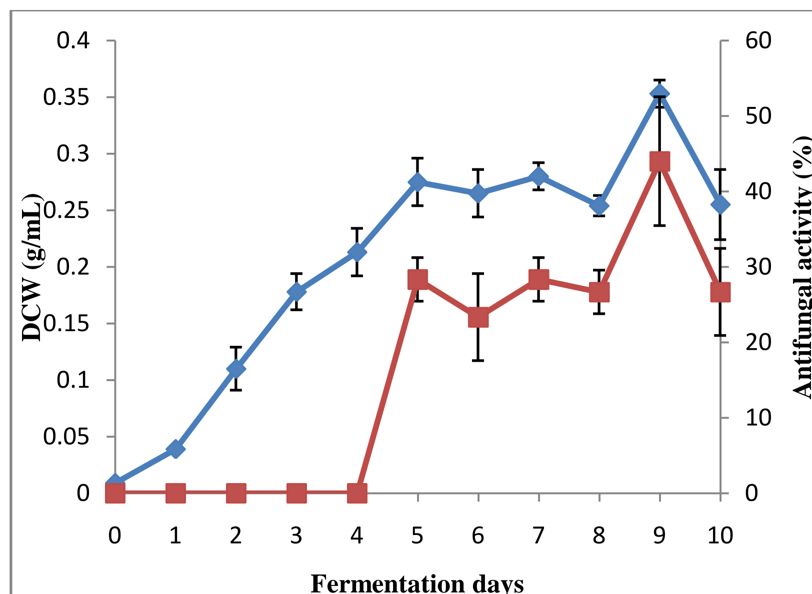

FIG. 4: Dry cell weight and antifungal activity of endophytic *P.oxalicum* T3.3 grown in Richard medium at 30°C, pH 5.5. Error bars represent standard deviation from three replicates. Symbols represent: (●) Dry cell weight; (■) antifungal activity.

3.8 Scanning electron microscopy

The SEM images revealed no direct contact between the hyphae of endophytic *P.oxalicum* T3.3 and *C.gloeosporioides*. The hyphae of *C.gloeosporioides* showed normal and rigid structure in the absence of *P.oxalicum* T3.3 (Fig. 5a). Compared to the pathogen hyphae in the control plate, there were severe morphological alterations in hyphae of this pathogen in the presence of *P.oxalicum* T3.3. The mycelial cell wall of this pathogen showed abnormal growth and became disintegrated resulting in growth inhibition of the fungal pathogen (Fig. 5b). The metabolites secreted by the endophytic *P.oxalicum* T3.3 in the medium had evoked abnormal growth of *C.gloeosporioides* where the growth of the hyphae became irregular, such as shriveling of the pathogen hyphae (Fig. 5c), necrosis of the hyphal cell wall (Fig. 5d) and leakage in the hyphae of *C.gloeosporioides* (Fig. 5e).

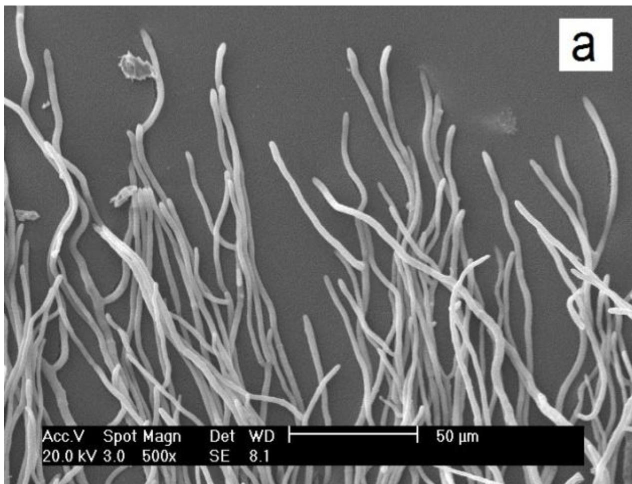

FIG. 5 (a): intact and healthy *C.gloeosporioides* hyphae from control plate

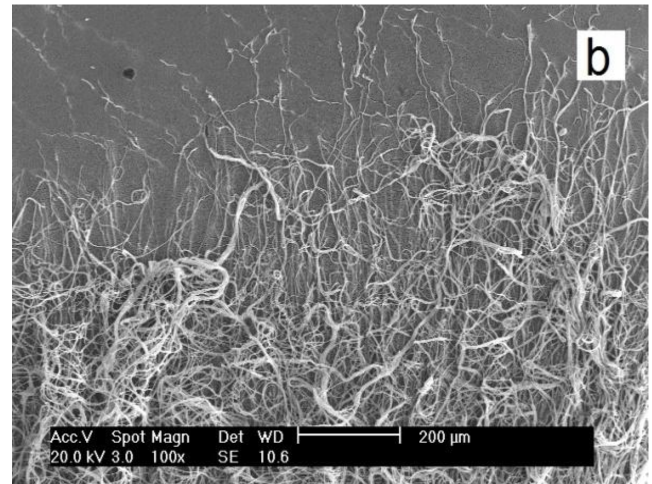

FIG. 5(b): abnormal growth of *C.gloeosporioides*

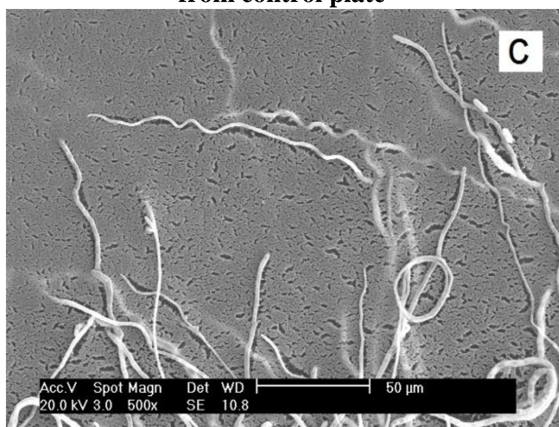

FIG. 5(C) distorted hyphal growth

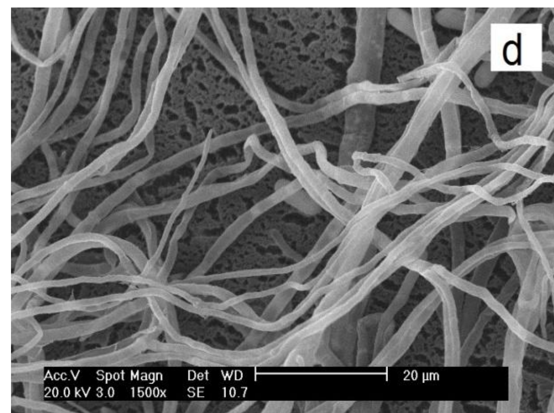

FIG. 5(d) shriveled *C.gloeosporioides* hyphae

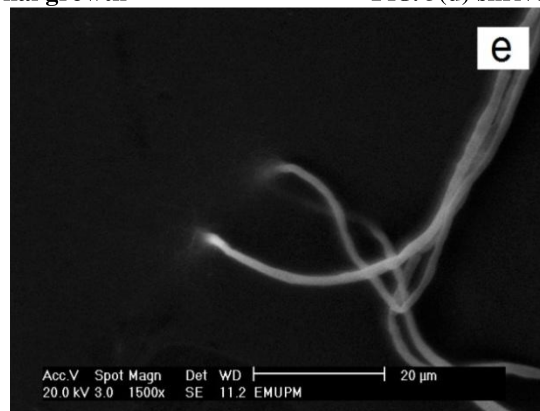

FIG. 5(e) Incomplete cell wall formation causing cytoplasm leakage from the hyphae.

3.9 Evaluation of P.oxalicum T3.3 crude extract against *C.gloeosporioides* on detached dragon fruit

The crude extract of endophytic *P.oxalicum* T3.3 was tested in vivo to evaluate its activity against anthracnose disease. Based on this work, no lesion was observed on dragon fruits from negative control (Fig. 6). Anthracnose symptoms were observed on dragon fruits from the positive control after 2 days of incubation at 28°C and it was similar to the symptoms reported by Masyahit *et al.,* (2009). Sunken necrotic lesions with radius 13.5±0.22 mm were observed on the fruit from positive control after 5 days of incubation. The dragon fruit that was treated with crude extract of *P.oxalicum* T3.3 did not exhibit serious disease symptom. Necrotic lesions with radius of 1.00±0.03 mm were observed around the inoculation site on day 5. Thus, treatment with 100 mg/mL of crude extract could reduce the lesions caused by the pathogen on the fruit.

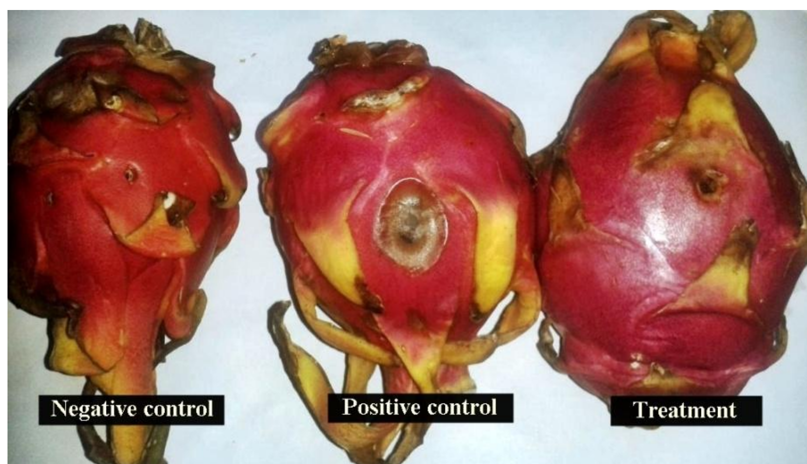

FIG. 6: Anthracnose disease suppression by 100 mg/mL of crude extract of *P.oxalicum* T3.3 on dragon fruit at 28°C on day 5. Negative control: dragon fruit treated with ethanol. Positive control: dragon fruit inoculated with *C.gloeosporioides*. Treatment: Dragon fruit inoculated with 10 µl of 100 mg/ml crude extract from *P.oxalicum* T3.3 prior to *C.gloeosporioides* inoculation

IV. DISCUSSION

Endophytic fungi are the most unexplored group of organisms and have remained uncharacterized. Out of the 1.5 million plant species worldwide, only less than 5% of endophytic microorganisms from plants have been figured out (Gilbert, 2002).To our knowledge, this is the first report regarding to the occurrence of endophytic fungi in dragon fruit plant. In this study, endophytic *Penicillium oxalicum* T3.3 has been identified as one of the endophytic fungi that are capable of living inside the dragon fruit plant. *Endophytic Penicillium* has been isolated from different host plants such as *Scoparia dulcis Linn*, *Hevea brasiliensis* , *Azadirachta indica A. Juss* (Mahesh *et al.,* 2005; Mathew *et a*l., 2010; Saithong *et al*., 2010; Gazis and Chaverri, 2010Tenguria and Khan, 2011). Different strains of *Penicillium* have also been isolated from coffee plants (Vega *et al*., 2006). *P.oxalicum* has also been frequently isolated from the soil and from various organic materials undergoing slow deterioration (Santamarina *et al*., 2002; Kurakake *et al*., 2006; Li*, et al*., 2007; Pandey *et al*., 2008).

Our findings show that endophytic *P. oxalicum* T3.3 demonstrated antagonistic activity towards *C.gloeosporioides*. The large inhibition zone produced by the endophytic *P.oxalicum* T3.3 from among the other seven potential strains indicated that this fungus contains the most potent antifungal activity against the pathogen tested. *P. oxalicum* has been reported to produce inhibition zone against a wide range of pathogenic fungi during dual culture test (Yang *et al.,* 2008, Paul *et al*., 2012). Inhibition zones were also produced by several *Penicillium* species towards *Rhizoctonia solani* (Nicoletti *et al.,* 2004). In contrast, Sempere and Santamarina (2008) reported that *P.oxalicum* overgrew *Nigrospora oryzae* and suggested that competition for space and nutrients are the mechanisms that present *P. oxalicum* as a biocontrol agent. The non volatile metabolite test revealed that this fungus also produced other antifungal metabolites that have not been extracted by ethylacetate. Hydrolytic enzymes such as β-glucanase, chitinase and protease might have contributed to the inhibitory activity of this strain as well since there has been a study which reported that endophytes directly suppress pathogens by producing either antibiotics or lytic enzymes (El-Katatny *et al*., 2006).

The Biolog FF Microplate analysis demonstrated that *P.oxalicum* T3.3 is a versatile microorganism as it able to utilize a wide range of carbon sources including monosaccharides, disaccharides, polysaccharides and sugar alcohols. Papaspyridi *et al*., (2011) suggested that the substrate assimilation fingerprint obtained from the Biolog FF Microplate analysis is useful in selecting components for media optimization of maximum biomass production.

Biological control agents produced cell wall degrading enzymes such as chitinase, glucanase, cellulases and proteases and antifungal secondary metabolites that are involved in controlling the growth of the pathogen. In this study, endophytic *P.oxalicum* T3.3 was found to produce cell wall degrading enzymes including chitinase and β-glucanase. This is in agreement with Sempere and Santamarina (2008) who suggested that *P.oxalicum* secretes chitinases and β-glucanases to degrade and penetrate into the conidiophores and spores of *N. oryzae*. These enzymes are involved in degrading the cell wall of fungal pathogen due to fact that the cell walls of the fungi are constituted primarily by β-glucan and chitin (Cao *et al*., 2009). The highest β-glucanase and chitinase activities from endophytic P.oxalicum were obtained on the third and sixth days of fermentation respectively. El – Katatny *et al.,* (2000) reported that the maximum β-glucanase and chitinase levels from *Trichoderma harzianum* were on the fourth and seventh day respectively. As reported in another study, *P.oxalicum* produced β-glucanase with the activity of 3.2 U/mL on day five (Doughari, 2011). *P.oxalicum* also produced β-glucanase enzyme during its autolysis occuring after 28 days of fermentation (Copa-Patino *et al.,* 1990). Chitinase production in *P.oxalicum* had been previously reported in other studies (Rodriguez *et al*., 1993, Rodriguez *et al*., 1995)

Along with β-glucanase and chitinase, *P.oxalicum* T3.3 also produced antifungal compounds which contributed to its antifungal activity. This is in accordance with Yang *et al*., (2008) who reported that the ethylacetate extract of *P.oxalicum* strain PY-1 contained two active compounds which suppressed the hyphal growth of *Sclerotinia sclerotiorum*. Pandey *et al.*, (1993) suggested that *P. oxalicum* produced volatile substances which inhibited the growth of *C.gloeospoiroides* in guava. *Penicillium* genus has been reported to produce antifungal compounds. For example, endophytic *Penicillium sp.* associated with *Hopea hainanensis* produced antifungal compounds that showed antifungal activity against *Candida albicans* and *Aspergillus niger* (Wang *et al.,* 2008). *Penicillium simplicissimum* produced altenusin and dehydroaltenusin that exhibited antifungal activities against *Aspergillus fumigatus*, *A.niger, C. albicans* and *Candida neoformans* (Komai *et al*., 2006).

Under SEM imaging, the hyphal morphology of *C.gloeosporioides* was greatly affected by the metabolite released by *P.oxalicum* T3.3. There is no direct contact observed between the hyphae of *P.oxalicum* and *C.gloeosporioides*. This finding suggests that the release of extracellular metabolites containing both the cell wall degrading enzymes and antifungal compounds from endophytic *P.oxalicum* may be related to abnormal and distorted hyphal growth of the *C.gloeosporioides*. Sempere and Santamarina (2008) reported that *P.oxalicum* showed direct contact with *Nigrospora oryzae*. By Cryo-SEM microscopic investigations, they revealed that P. oxalicum coiled and penetrated around spores and conidiophores of N. oryzae, causing deformation, morphological changes and disintegration of the spores and conidiophores walls. P.oxalicum also mycoparasitised and penetrated into *Alternaria alternata (*Sempere and Santamarina, 2010). The disintegration of conidium of *A. alternata* by the action of *P. oxalicum* revealed by SEM images suggested that *P. oxalicum* produced antifungal components and extracellular metabolites like the cell wall degrading enzymes chitinases, β-glucanases and proteases. Thus, it was proven in this study that the abnormal growth, swelling, necrosis and hyphal leakage of *C.gloeosporioides* were mainly associated with the cell wall degrading enzymes; β-glucanase and chitinase and antifungal compounds of *P.oxalicum* T3.3.

Crude extract of *P.oxalicum* T3.3 could control the development of anthracnose disease on dragon fruit. This result clearly indicated that crude extract of this fungus could provide protection to dragon fruit from infection by *C.gloeosporioides* and could reduce disease severity. This is in agreement with Palaniyandi *et al*., (2011) who reported that the crude culture filtrate extract produced by *Streptomyces sp*. MJM5763 at 100 µg/ml suppressed anthracnose disease in detached yam leaves. The crude culture filtrate extract of *Streptomyces sp.* MJM5763 treatment was most effective to reduce anthracnose severity and incidence in yam.

The role of *P.oxalicum* in biological control had been well documented and commercial formulations had been developed (Larena *et al*., 2003; Sabuquillo *et al*., 2005). *P.oxalicum* conferred various degrees of protection on different plant species against plant pathogenic fungi. For example, *P.oxalicum* was successfully applied as a biocontrol agent against *Fusarium oxysporum f. sp. lycopersici* (De Cal and Malgarejo, 2001). The application of conidial suspension of *P.oxalicum* to tomato seedlings resulted in a significant reduction of fusarium wilt incidence. *Penicillium oxalicum* was able to suppress wilt caused by *Fusarium oxysporum f. sp. melonis* and *F. oxysporum f. sp. niveum* on melon and watermelon (De Cal *et al*., 2009). A significant reduction of powdery mildew disease also been observed in *P.oxalicum*-treated strawberry cultivars (De Cal *et al*., 2008). *P.oxalicum* also was reported as a biocontrol agent against other crop diseases as well (Larena *et al*., 2001).

V. CONCLUSION

In brief, endophytic *P.oxalicum* T3.3 has been demonstrated to be a potential BCA against anthracnose disease in dragon fruit plant by producing β-glucanase, chitinase and antifungal compounds involved in controlling the growth of the pathogen

tested. Therefore, the discovery of endophytic *Penicillium oxalicum* T3.3 in this study is significant as this fungus is a potential alternative to fungicides to control anthracnose disease in dragon fruit plant.

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