

Effect of *Trichoderma* sp. on Anthracnose Disease of Stored Chilli

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ABSTRACT

Chilli is commonly used as spice in Malaysian culinary, principal ingredients in paste (*sambal*) and as the raw material in sauce industry. Anthracnose disease caused by *Colletotrichum capsici* is one of the major causes of economic loss to chilli production especially in Asia. Even a small lesion on chilli might affect the quality, thus the market value of the chilli. Disease symptoms caused by *C. capsici* include brown, circular and sunken lesion with concentric rings of black acervuli. Chemicals have been used to treat the chilli but they might cause environmental pollution, affect human health and lead to pathogen resistance to the chemicals. Therefore, an alternative method to chemical control is required. In this study, *C. capsici* was isolated from a naturally infected chilli fruit (*Capsicum frutescens*), and a species of *Trichoderma* was isolated from the rhizosphere of grasses. Pure cultures of both fungi were established then used in antagonism studies in *in vitro* and *in vivo*. Dual culture of pathogens and *Trichoderma* sp. indicated that *Trichoderma* sp. competed with *C. capsici* for space and nutrients, caused the loss of turgidity of the fungal hyphae, and reduced the fungal growth by producing volatile metabolites. *Trichoderma* sp. decreased disease severity on chilli artificially inoculated fruits up to 64% when *Trichoderma* mycelial plug was used and 55% when culture filtrate was applied. Field trials are recommended to examine the antagonism of *Trichoderma* sp. in real production conditions.

Keywords: Anthracnose, biological control, *Colletotrichum capsici*, *Trichoderma* sp.

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INTRODUCTION

Capsicum frutescens L. or commonly known as bird eye chilli (Williams *et al.*, 1991) belongs to the family Solanaceae and in the Plantae kingdom. Chilli is one of the most important spice crops in the world (Rahman *et al.*, 2013) and in Malaysia it is used as one of the principal ingredients in paste (*sambal*) (Karim *et al.*, 2011). Yet, chilli has been attacked by many diseases, and among them, fungal disease is the most important (Rahman *et al.*, 2013).

Anthracnose or commonly known as ripe fruit rot is one of the major causes of economic loss to chilli production (Than *et al.*, 2008a; Than *et al.*, 2008b), especially in Asia (Sangdee *et al.*, 2011). According to Pandey and Pandey (2003), anthracnose caused yield loss of more than 50% in chilli production in India. Initially, small and circular water-soaked spots will first develop on the skin (Naipagropediarachur, 2012). The infected surface of the fruit will then sunken and dry up (Than *et al.*, 2008a). Anthracnose is caused by *Colletotrichum* spp. (Than *et al.*, 2008b). Kim *et al.* (2014) reported at least five

species, which are *C. gloeosporioides*, *C. acutatum*, *C. coccoides*, *C. dematium* and *C. truncatum* were associated with the anthracnose in chilli.

Chemicals have been used to control the anthracnose of chilli (Benítez *et al.*, 2004). However, resistance to the chemicals has been reported for the pathogen of anthracnose (Benítez *et al.*, 2004). In addition, the extensive use of the chemicals might lead to the pollution of the environment and the health of both growers and consumers. In order to reduce the usage of chemicals on the control of chilli anthracnose, alternative control approaches are needed (Rahman *et al.*, 2011).

Antagonists, also known as biological control agents, are mostly soil microorganisms that can interfere with pest's activities (Chernin & Chet, 2002). There are four mechanisms of antagonists, which are competition, antibiosis, induced resistance and parasitism. *Trichoderma* spp. are one of the popular fungi known for their antagonism against soil pathogen such as *C. truncatum* which causes anthracnose on chilli

plants (Chernin & Chet. 2002; Verma *et al.*, 2007). They are being used widely as biological control agents in many countries (Rahman *et al.*, 2011). Hill *et al.* (2010) reported that selected *Trichoderma* isolates helped to enhance the health of *Acacia mangium* seedlings in Sarawak, Malaysia. Another study by Padder and Sharma (2011) proved that *Trichoderma viride* had the best potential to inhibit mycelial growth and spore germination of *C. lindemuthianum*.

The objectives of this study were: (1) to study the effect of *Trichoderma* sp. on the growth of anthracnose pathogen of chilli on culture medium, and (2) to investigate the efficacy of *Trichoderma* sp. in suppressing development of anthracnose disease on artificially inoculated chilli fruits.

MATERIALS AND METHODS

Infected fruits of *Capsicum frutescens* L. with characteristic symptoms of anthracnose for pathogen isolation and healthy fruits for artificial inoculation were purchased locally.

Pathogen Isolation and Pure Culture Establishment

Naturally infected *Capsicum frutescens* L. fruits showed the characteristic symptoms of anthracnose including sunken lesion on the fruit skin, which contained numerous black acervuli of conidia masses on the surface of the lesion were used for the isolation (Figure 1). The conidia, when observed under a compound

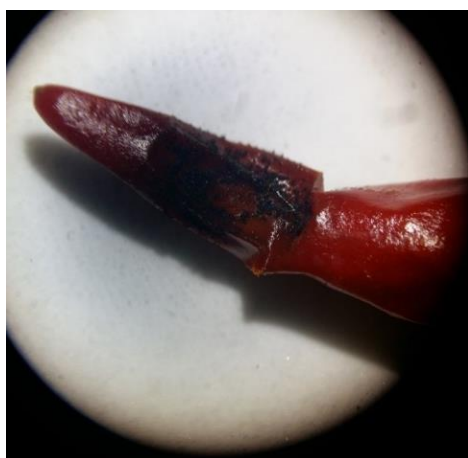


Figure 1. Fruits of bird eye chillies with characteristic symptoms of anthracnose.

microscope at 400× magnification, appeared to be tapering towards each end with acute apex and truncate base.

Potato Dextrose Agar (PDA) was used for the isolation of associated pathogen, which followed the method described by Ratanacherdchai *et al.* (2010). The infected fruits were first surface sterilised with 5% of Clorox® bleach (10% sodium hypochlorite) for two minutes and rinsed three times with sterile distilled water. Three sterilised specimens excised from the borderline of infected and healthy area were then placed onto each of five PDA replicate plates, and the plates were incubated for four to seven days at room temperature (approximately 28 °C). Pure culture of the pathogen was obtained using single spore isolation method (Choi *et al.*, 1999). Briefly, a loopful of spore mass of 7-day old culture was transferred into a centrifuge tube which contained 10 ml of sterile distilled water, and the solution was gently shaken to disperse the spore mass. The solution was then poured onto PDA plates and left to stand for 5 minutes. The excessive solution on the plates was then poured off, and the plates were incubated slanting for 12 to 18 hours. Single spores of *Colletotrichum capsici* that germinated between 18 to 24 hours were transferred onto new PDA plates. The isolated *C. capsici* was identified based on the descriptions by Liu *et al.* (2016), based on the macro- and microscopic structures of the fungus.

Pathogenicity Test

Before surface sterilization, healthy fruits of *Capsicum frutescens* L. were washed with running tap water for half an hour to remove any contaminants on the surface of the fruits. The fruits were then surface sterilised with 70% ethanol for five minutes based on the method described by Sangdee *et al.* (2011). The sterilised fruits were then washed with sterile distilled water and left to dry on a sterilised filter paper in a laminar flow. Two wounds per fruit were created on sterilised chilli fruits using sterilised scalpel, and a plug of a 7-day old pure culture of the fungus was transferred and inserted into the wounds. Chilli fruits which served as controls had PDA plugs inserted into the wounds. The inoculated fruits were then

placed in transparent plastic boxes. There were five replicates boxes (five fruits per box) for each treatment and the boxes were kept at room temperature and observed daily. Symptoms at the inoculation site were evaluated one week after the inoculation (Montri *et al.*, 2009) and compared with the symptoms observed from the naturally infected fruits. Re-isolation of the fungus from the artificially inoculated fruits was carried out for comparison with the fungus isolated from the naturally infected fruits. The infected tissues were taken from about 1cm from the point of inoculation and underwent surface sterilization before being transferred onto new PDA plates. The plates were then incubated at room temperature, and the colonies were observed for the spore and colony characteristics.

Isolation of Antagonistic Fungus

Trichoderma sp. was isolated from rhizosphere soil of grasses following the method described by Bharathi *et al.* (2004). Rhizosphere soil samples were collected and diluted in test tubes up to 10^{-6} . Pure culture of *Trichoderma* sp. was established using single spore isolation (Choi *et al.*, 1999), as described earlier for the establishment of *Colletotrichum* pure culture. Identification of the isolated *Trichoderma* sp. was based on the description by Shah *et al.* (2012) by observing the macro- and microscopic structures of the fungus. The pure culture of *Trichoderma* sp. was subsequently used in antagonism test in culture plate with *Colletotrichum* sp. and on artificially inoculated chilli fruits.

Antagonistic Activity of the *Trichoderma* sp. on Culture Plate

Isolated *Trichoderma* sp. was screened for its antagonistic activity against *Colletotrichum* sp. using dual culture technique described by Begum *et al.* (2008). Briefly, an agar disc containing mycelia of 7-day-old *Trichoderma* sp. culture was placed at one end of the agar plate while an agar disc containing mycelia of 7-day-old *Colletotrichum* sp. culture was placed at the other end on the same PDA plate (hereinafter referred to as Treated plate). Plates, which served as controls, had agar disc of *Colletotrichum* sp. mycelia and a disc of PDA placing opposite each other on PDA plates.

There were five replicate plates for each treatment, and the plates were incubated for 15 days at room temperature. The plates were observed daily, and the average growth rate of *Colletotrichum* sp. and *Trichoderma* sp. was computed using the formula described by Rosli (2017) (Eq. 1) and the antagonistic activity of the *Trichoderma* sp. toward *Colletotrichum* sp., expressed as percentage of inhibition of radial growth was determined using the formula described by Begum *et al.* (2008) (Eq. 2) as below.

Average growth rate:

$$\frac{(D2-D1)+(D3-D2)+(D4-D3)+[DN-D(N-1)]}{N-1} \quad \text{Eq. (1)}$$

Where D indicates the average colony radial of *Colletotrichum* sp. and N indicates the number of days after incubation.

Percent inhibition of radial growth:

$$\frac{R1 - R2}{R1} \times 100 \quad \text{Eq. (2)}$$

Where R1 indicates the radial growth of the fungal colony of the control set, and R2 indicates the radial growth of the fungal colony of the treated set.

Scanning Electron Microscopy (SEM) was used to observe the interaction between the two fungi after the *Trichoderma* sp. had grown over the *Colletotrichum* sp. on the dual culture plates. Agar plugs from confrontation zone were fixed in phosphate buffer solution (pH 7) for 24 hours as described by Carvalho *et al.* (2014). Phosphate buffer solution was prepared by combining the stock solution of 1 M of dipotassium phosphate (K_2HPO_4) and 1 M of monopotassium phosphate (KH_2PO_4) to 1 l of distilled water. After 24 hours, the samples were rinsed with fresh buffer solution for three times, and the solution was replaced by the lowest concentration of ethanol solution and left for one hour for dehydration purpose. The ethanol concentrations used were 60%, 80% and 100%. Lastly, the samples were placed in 100% ethanol overnight. The samples were then dried with carbon dioxide in a critical point dryer, mounted on aluminium stubs with double-sided tape and coated by gold. The samples were visualized by using a SEM (JEOL, JSM-6390LA).

Action of Volatile Metabolites of *Trichoderma* sp. on *Colletotrichum* sp.

For volatile metabolites test, technique described by Muthukumar *et al.* (2011) was used with modification by sealing the plates together with parafilm. Briefly, agar disc containing mycelia of 7-day-old *Trichoderma* sp. culture and *Colletotrichum* sp. culture were placed separately at the centre of the bottom of each PDA plate, respectively. Next, the lids of the PDA plates which contained *Trichoderma* sp. agar disc were replaced by the bottom of PDA plates which contained *Colletotrichum* sp. agar disk. The two plates were sealed together with parafilm (hereinafter referred to as Treated plate). Plates served as the controls were prepared in the same manner, except that PDA discs were used instead of *Trichoderma* sp. mycelial discs (hereinafter referred to as Control plate). There were five replicate plates for each treatment, and the plates were incubated at room temperature until the mycelia of *Trichoderma* sp. was observed to start growing over the other plate containing *Colletotrichum* sp.. The plates were observed daily, and percentage of inhibition of radial growth of the pathogen was determined. Formula described by Rosli (2017) was used to record the growth of *Colletotrichum* sp. and *Trichoderma* sp.. Formula described by Begum *et al.* (2008) was used to analyse the antagonistic activity of *Trichoderma* sp.

Inhibitory Efficacy of *Trichoderma* sp. against Anthracnose Pathogen Growth and Disease Development on Chilli Fruits

Healthy fruits were first inoculated artificially with *Colletotrichum* sp. then treated with *Trichoderma* sp. based on method described by Kim *et al.* (2014) with modification by creating the wounds using sterilised scalpel. Briefly, healthy fruits were surface sterilised with 70% ethanol for five minutes. The sterilised fruits were then washed with sterile distilled water and left to air dry on a sterilised filter paper in a laminar flow. A plug of 7-day old *Colletotrichum* sp. culture was transferred and inserted into a sterilised fruit after the wound was created on the surface of the fruit using a sterilised scalpel. A second wound was made approximately 1 cm apart from the initial wound, and a plug of 7-day old *Trichoderma*

sp. culture was transferred and inserted into the wound. Chilli fruits, which had *Colletotrichum* sp. culture and a PDA plug inserted into the first and second wound, respectively, served as the controls. Treated chilli fruits were placed separately in plastic containers, three chilli fruits per container, for each treatment, and the containers were arranged in a Completely Randomised Design. There were five replicate containers for each treatment, and the containers were incubated for 15 days at room temperature. Disease incidence and severity were recorded and computed using Eq (3) and Eq. (4).

Percent of fruit rot incidence =

$$\frac{\text{No. of fruit rot}}{\text{Total no. of fruit}} \times 100 \quad \text{Eq. (3)}$$

(Ngullie *et al.*, 2010)

Percent of disease severity =

$$\frac{(R - C)}{R} \times 100 \times 100 \quad \text{Eq. (4)}$$

(Rahman *et al.*, 2011)

Where R indicates the average of lesion radius on chilli fruit in the presence of the antagonist and C indicates the average of lesion radius on chilli fruits without the antagonist (control).

Culture Filtrate Study

Six plugs of *Trichoderma* sp. mycelia were grown in each Schott bottle containing 250 mL sterile potato dextrose broth (PDB), four replicate bottles, on a shaker at 100 rpm for 15 days at room temperature based on method described by Rahman *et al.* (2012). The culture broth was filtered twice, first through a layer of sterilised filter paper (Whatman No. 1) and then a sterilised membrane filter (Whatman, 0.22 µm). Healthy fruits of bird eye chilli were surface sterilised with 70 % ethanol for five minutes. The sterilised fruits were then washed with sterile distilled water and left to air dry on a sterilised filter paper in a laminar flow. Surface sterilised chilli fruits were then dipped in 100 ml of culture filtrate of *Trichoderma* sp. for 24 hours. Chilli fruits which served as controls were dipped in 100 mL of sterile distilled water. After 24 hours, the dipped chilli fruits were left to air dry on a sterilised filter paper in a laminar flow. A plug of *Colletotrichum* sp. culture was transferred and inserted into a wound created on each dipped

fruit using a sterilised scalpel based on method described by Nantawanit *et al.* (2010). Chilli fruits, which served as the controls, had only PDA plug inserted into the wounds. The treated chilli fruits were placed in plastic containers for each treatment, and the containers were arranged in a Completely Randomised Design. There were five replicate containers with three chilli fruits in each container, and the containers were incubated for 15 days at room temperature. Disease incidence and severity were recorded and computed using the formulae described by Nguillie *et al.* (2010) and Rahman *et al.* (2011), respectively.

Statistical Analysis

All the data were first subjected to a test for Normality. The data were then subjected to Analysis of Variance (ANOVA) if the data were normally distributed or non-parametric test (Wilcoxon signed-rank test and Mann-Whitney U test) if the data were not normally distributed. Tukey test as post-hoc test for ANOVA was used to compare the means between treatments. All statistical analysis was performed using the software SPSS version 24.

RESULTS

Pathogen Isolation and Pure Culture Establishment

Isolation of the causal agent on PDA resulted in two types of colonies. However, a dark grey colour colony with cottony mycelium and concentric rings from the middle of the colony was the most predominant on the culture plate, and therefore was selected to be subcultured for further studies. A pure culture of the predominant fungus had been successfully established, which was then used for pathogenicity test on chilli fruits and preliminary identification.

Figure 2 shows the colony morphology of the pure culture of the microorganism isolated from the naturally infected chilli fruit. As can be seen from the Figure, both upper and reverse view of the colony have dark grey colour with cottony mycelium and concentric rings from the middle of the culture plate. Microscopic observation of the isolated fungus showed setae, which had smooth-walled, tapered to

paler acute apex and slightly swollen at the base and brown in colour (Figure 3A). Hyphae of the isolated fungus were septate and conidia appeared to be a quarter moon shape, tapering towards each end with acute apex and truncate base (Figure 3B).

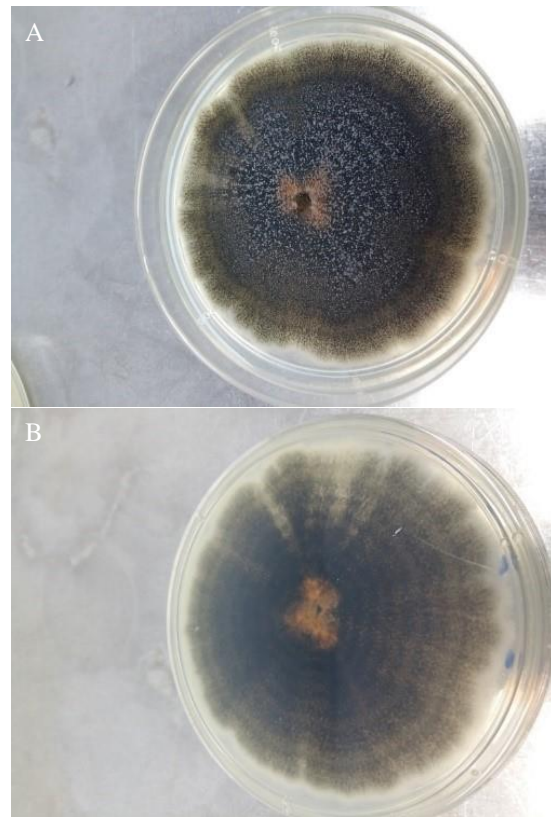


Figure 2. Colony morphology of the pure culture of the isolated fungus on PDA. (A) Upper view, (B) Reverse view.

Pathogenicity Test

All healthy chilli fruits, which were artificially inoculated with the pure culture of the isolated fungus, showed signs of white mycelial growth on the surface of the inoculated chilli fruits from second days after inoculation. Figure 4 shows disease symptoms developed on artificially inoculated and non-inoculated (controls) chilli fruits, respectively, at seven days of incubation. As can be seen from Figure 4, the chilli fruits, which were inoculated with plugs of fresh potato dextrose agar (controls), remained unaffected. However, the wounds on the artificially inoculated chilli fruits, where the mycelial discs were inserted, became black in colour. There were sunken lesions developed and black acervuli were observed on the surface

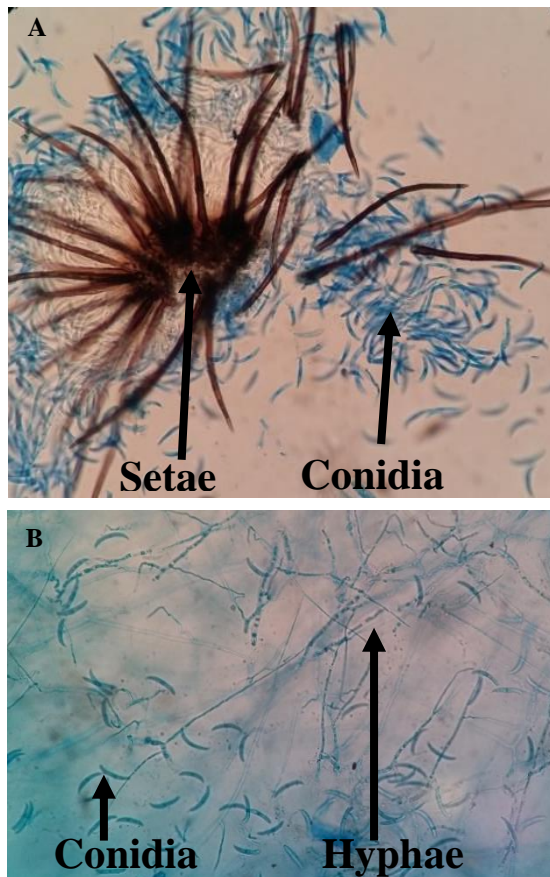


Figure 3. Microscopic structures of *C. capsici* under 400x magnification. (A) Setae and conidia, (B) Hyphae and conidia

of the lesion. These symptoms were similar to those observed from the naturally infected chilli fruits, from which the pathogen was isolated. Re-isolation of the fungus from an area between infected and healthy tissue gave rise to a colony with pale grey in colour and cottony mycelium from the middle of the culture plate from the upper view.

On the reverse view, the colony was dark brown colour with concentric rings from the middle of the culture plate. Microscopic structures of the fungus (hyphae and conidia) were similar to those that were isolated from the naturally infected chilli fruits except that the hyphae were much smaller in size. The characteristics of the isolated fungus resembled to those described for *Colletotrichum capsici*, causing anthracnose disease of chilli fruits (Saxena *et al.*, 2016; Shenoy *et al.*, 2007). The fungus, therefore, was preliminarily identified as a *Colletotrichum capsici*, and was confirmed to be the causal agent of the disease.

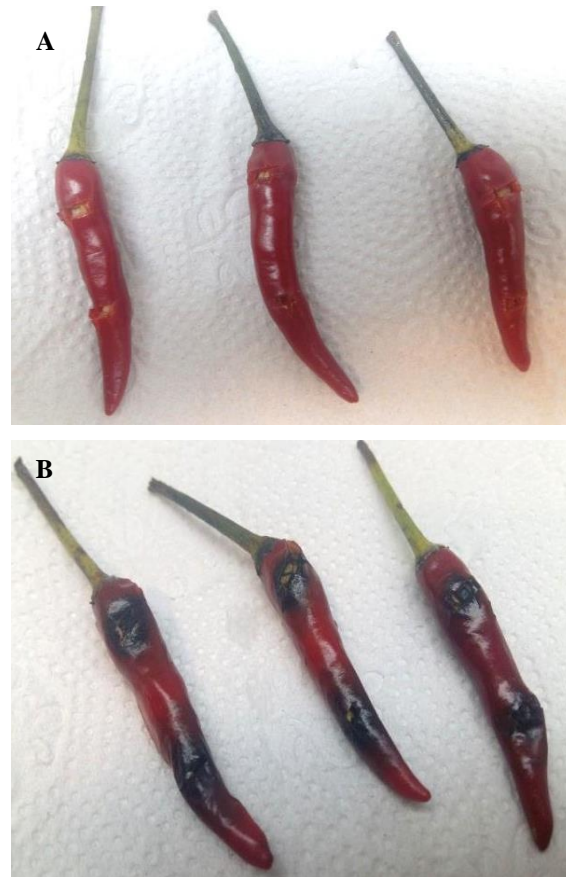


Figure 4. Chilli fruits inoculated with PDA plugs (A; controls) and with mycelial plugs of the isolated fungus (B) on the 7th day of inoculation.

Isolation and Morphology Characteristics of Antagonistic Fungus

A *Trichoderma* sp. was successfully isolated from rhizosphere soil near the root surface of grasses. Pure culture of the isolated fungus showed characteristics of *Trichoderma* sp. based on the description by Shah *et al.* (2012). Colour from upper surface varied from whitish (Figure 5A1) to greenish (Figure 5A2) and lower surface appeared whitish (Figure 5B1) to yellowish. The conidiophore of the isolated species appeared as branches, and its conidia were round in shape (Figure 6).

Antagonistic Activity of the *Trichoderma* sp. on Culture Plate

Dual culture technique was performed to screen for antagonistic activity of *Trichoderma* sp. against *Colletotrichum capsici*. *Trichoderma* sp. grew faster as compared to *C. capsici*. Besides, the colour of *C. capsici* colony

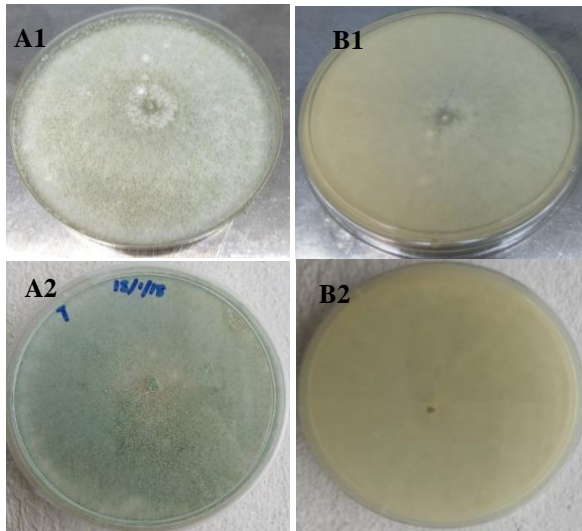
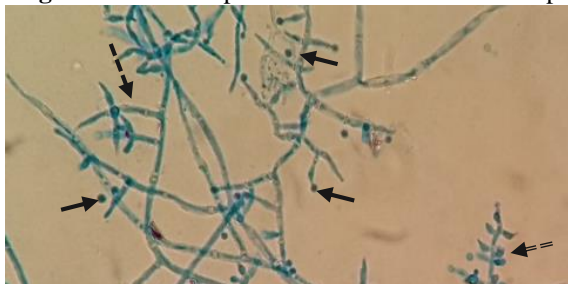


Figure 5. Colony morphology of *Trichoderma* sp. on PDA (A1, 2) Upper view, (B1, 2) Reserve view.

observed in the control plates was lighter in colour as compared to that in the treated plates (Figure 7). On the third day of inoculation, *Trichoderma* sp. started to overgrow the colony of *C. capsici*. Results from the test showed that the average growth rate of *C. capsici* was significantly ($p < 0.05$) slowed down by *Trichoderma* sp.. The average growth rate of *C. capsici* on the control plates and the treated plates was 0.94 cm and 0.85 cm, respectively.

Figure 6. Microscopic structure of *Trichoderma* sp.



under 400x magnification. Conidiophore (black dashed arrow) and conidia (black arrow).

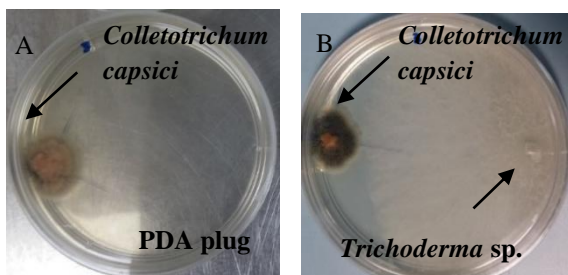


Figure 7. Colour of *Colletotrichum capsici* colony in dual culture plate on second day of incubation. (A) Control plate, (B) Treated plate.

The highest percent inhibition of radial growth of *C. capsici* recorded was on the third day of incubation (30.87%) followed by 26.83% on the first day, 18.81% on the second day and 14.80% on the fourth day. In all plates, *Trichoderma* sp. completely overgrew the colony of *C. capsici* in fourth day of incubation (Figure 8).

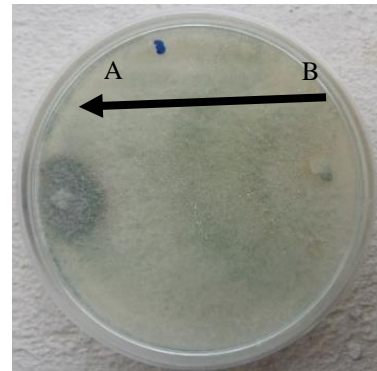


Figure 8. Growth of *Trichoderma* sp. over *Colletotrichum capsici* on a dual culture plate on 14th day of incubation. (A) *Colletotrichum capsici* colony, (B) *Trichoderma* sp. colony.

Morphology of the hyphae of *C. capsici* at the confrontation zone showed a deformed shape when observed under SEM (Figure 9). The hyphae of *C. capsici* were less turgid and seemed lysed by *Trichoderma* sp. (Figure 9b).

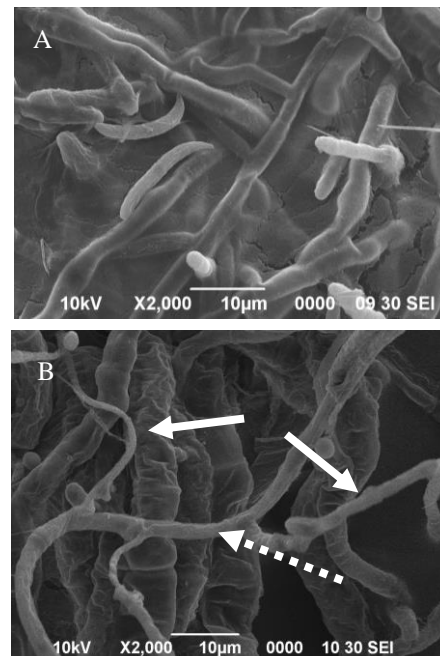
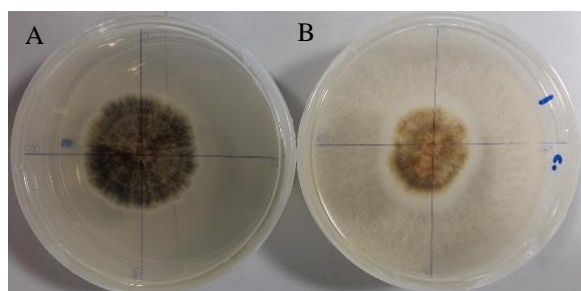


Figure 9. Change in hyphal morphology of *Colletotrichum capsici* (white arrow) when in contact with hyphae of *Trichoderma* sp. (dashed arrow) 14 days after incubation (B) under SEM with 2000x magnification as compared with control set (A).

Action of Volatile Metabolites of *Trichoderma* sp. on *Colletotrichum capsica*

Trichoderma sp. grew faster as compared to *Colletotrichum capsici*. The colour of the *C. capsici* colony observed in the Control plates was lighter as compared to that in the Treated plates (Figure 10). Besides, the growth of the colony in the Control plates was more even as that compared to the Treated plate. On the sixth day of incubation, *Trichoderma* sp. started to grow over toward the colony of *C. capsici* plated on top of the *Trichoderma* sp. plate. The result of the volatile metabolites effect of *Trichoderma* sp. showed that *Trichoderma* sp. did not significantly ($Z(6) = 1.59$, $p = 0.26$) inhibited radial growth of *C. capsici* after seven days of inoculation on PDA medium. However, growth rate of *C. capsici* in the Control plates and the Treated plates was significantly different ($p < 0.05$). The average growth rate of *C. capsici* in the Control and Treated plates was 1.08cm and 0.93cm, respectively. In all plates, *Trichoderma* sp. completely grew over onto the plate containing the colony of *C. capsici* in 14th days of incubation.



(A) Control plate, (B) Treated plate.

Figure 10. Colour of *Colletotrichum capsici* colony in volatile test on fourth day of incubation.

Table 1: Effect of *Trichoderma* sp. volatile metabolites on radial growth of *Colletotrichum capsica*.

Day	Percent inhibition of mean radial growth (%)
1	8.33 ± 6.134
2	25.95 ± 3.983
3	20.72 ± 4.467
4	17.50 ± 3.340
5	17.01 ± 3.086
6	11.57 ± 1.765
7	13.02 ± 4.091

± standard deviation

Inhibitory Efficacy of *Trichoderma* against Anthracnose Pathogen Growth and Disease Development on Chilli Fruits

The results showed that *Trichoderma* sp. significantly ($p < 0.05$) inhibited growth and development of *Colletotrichum capsica* (Table 2). All the fruits, artificially inoculated with plugs of *C. capsici* regardless of presence of *Trichoderma* sp., showed symptoms of anthracnose. The disease incidence was therefore 100%. The symptoms observed on the fruits in this experiment were the same as those observed in the pathogenicity test (Figure 11). However, mycelia of *Trichoderma* sp. were observed to cover all over the wounded chilli fruits (Figure 11B).



Figure 11. Diseases symptoms on chilli fruits artificially inoculated with *Colletotrichum capsici* (A) treated with PDA plugs and (B) treated with *Trichoderma* sp. plugs on fifth day after inoculation.

Table 2: Effect of *Trichoderma* sp. on disease severity caused by *Colletotrichum capsica*.

Day	Percent of disease severity (%)
2	14.11 ± 7.926
3	19.21 ± 6.036
4	64.35 ± 22.082
5	50.09 ± 19.527

± standard deviation

Culture Filtrate Study

The results indicated that the culture filtrates of *Trichoderma* sp. significantly inhibited the growth of *Colletotrichum capsici* and the development of anthracnose disease on the artificially inoculated chilli fruits (Table 3). There was 100% of disease incidence, and the symptoms that were developed on the fruits in this experiment were the same as those observed in the pathogenicity test (Figure 12).

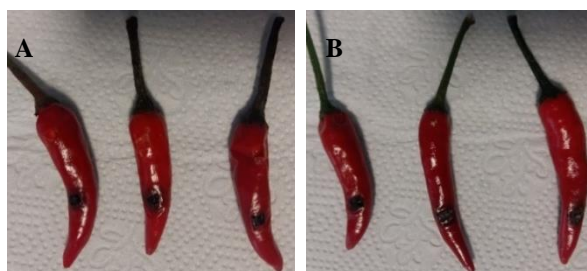


Figure 12. Culture filtrate of *Trichoderma* sp. on wounded chilli fruits with *Colletotrichum capsici*. (A) Control treatment, (B) Treated treatment.

DISCUSSION

Anthracnose is one of the major causes of economic loss to chilli production (Than *et al.*, 2008a; Than *et al.*, 2008b). Chemicals have been used to control the anthracnose of chilli but resistance has been developed in the pathogen of anthracnose (Benítez *et al.*, 2004). In order to reduce the usage of the chemicals on the control of chilli anthracnose pathogen, alternative control approaches are needed (Rahman *et al.*, 2011). Antagonists, soil microorganisms that can interfere with pest's activities such as *Trichoderma* spp. are one of the popular fungi known for their antagonism (Chernin & Chet, 2002; Verma *et al.*, 2007).

The pathogen associated with the naturally infected chilli was successfully isolated out and proved to be pathogenic. The pathogen was preliminarily identified as *Colletotrichum capsici* since it had similar characteristics of dark grey colony to that described for *C. capsici* by Liu *et al.* (2016), of septate hyphae, the quarter moon shape of the conidia, and the presence of setae as those described for *C. capsici* by Shenoy *et al.* (2007). However, it is worthwhile to have a molecular analysis to confirm the species.

Table 3: Effect of *Trichoderma* sp. culture filtrate on disease severity caused by *Colletotrichum capsici*

Day	Percent of disease severity (%)
2	18.51 ± 4.383
3	37.14 ± 5.389
4	31.70 ± 11.612
5	55.00 ± 13.845

The antagonist that successfully isolated out was identified as a *Trichoderma* sp.. The green colony characteristics observed in the present study were in agreement with those described in the studies by Shah *et al.* (2012). The branching conidiophore and round and green colour conidia of the isolated *Trichoderma* sp. in the present study were similar to those described in the study by Armando *et al.* (2017).

In the present study, *Trichoderma* sp. reduced the mycelia growth of *Colletotrichum capsici* in dual culture assay. The mechanism observed in the dual culture assay by *Trichoderma* sp. might be the competition for space and nutrients between the pathogen and the antagonist (Amin *et al.*, 2010b) and lysis (Begum *et al.*, 2008). Growth of *Trichoderma* sp. over *C. capsici* was also observed in the study by Amin *et al.* (2010a) and Sawant (2014). In addition, Intana *et al.* (2007), when examined the efficacy of three mutant and two wild type strain of *T. harzianum* in inhibiting mycelia growth of *C. capsici*, also reported that all the strains of *Trichoderma* were able to inhibit and overgrow the colony of *C. capsici*. A similar study by Begum and Nath (2015) indicated that *T. harzianum* isolate Th-2 was effective on inhibiting the mycelia growth of all isolates of *C. capsici* where the highest percent that been observed was 100% inhibition. However, in the present study, the inhibition of mycelial growth of *C. capsici* by the *Trichoderma* sp. was only 30.87%.

Previous study reported inhibitory mechanisms by *Trichoderma virens* and *Trichoderma harzianum* to *C. truncatum* through competition, parasitism and antibiosis (Begum *et al.*, 2008). However, in this study, coiling and penetration of *Trichoderma* sp. were not observed in the SEM analysis. The *Trichoderma* sp. grew over the colony of *C. capsici* and at the point where the two fungi encountered, mycelia of *C. capsici* was found to

change in turgidity. This change in shape might be attributed to the ability of *Trichoderma* sp. to secrete enzymes such as chitinase and glucanase which were reported in the study by Alka *et al.* (2017). According to Cuervo-Parra *et al.* (2011), deformation and disorganization of cell wall structure of *Moniliophthora rorer* which became rough was due to antifungal substances secretion of *Trichoderma* such as enzymes and antibiotics. In addition, the change in the shape of hyphae of *C. capsici* by *Trichoderma* sp. which became rough in structure in this study might also be due to lysis (Shahbazi *et al.*, 2014). A study conducted by Palaniyandi *et al.* (2013) also recorded the occurrence of lysis on the fungal mycelia of *C. coccodes* by *Streptomyces phaeopurpureus*.

In this study, reduced growth of *C. capsici* in the volatile metabolite experiment illustrated the ability of the *Trichoderma* sp. to produce volatile metabolites. This mechanism has been reported for the *T. virens* and *T. harzianum* by Amin *et al.* (2010b) to control the mycelial growth of *C. capsici* by more than 50% and the radial growth was 12.73 mm and 13.41 mm respectively. Waterhouse (1968) also reported that metabolites produced by *Trichoderma* sp. was effective against *C. gloeosporioides*. Besides, *Trichoderma* sp. produced volatile metabolites which suppressed the growth of *Pythium aphanidermatum* (Muthukumar *et al.*, 2011). Well known volatiles produced by *Trichoderma* spp. are trichodermin and trichodermol which are able to degrade cell wall of pathogens (Elad, 2000).

In vivo study was implemented to examine or verify whether the isolated *Trichoderma* would be effective in natural condition. In the presence of *Trichoderma* sp., disease severity caused by *C. capsici* was reduced in the present study. Similarly, in the study by Vasanthakumari and Shivanna (2014), *T. harzianum* decreased incidence and severity of the disease caused by *C. graminicola* in sorghum. The growth of the *Trichoderma* sp. on the surface of the wounded chilli fruits in this study might be due to the ability of *Trichoderma* sp. to colonize on the fruit surface (Ippolito & Nigro, 2000) and niche overlap between competitors is required to perform successful colonization (Kinkel & Lindow, 1997). However, the presence of the

Trichoderma mycelia on chilli fruits would affect the visual appearance of the fruits, which in turn would reduce its acceptability by consumers. Therefore, culture filtrate of *Trichoderma* sp. was used in a subsequent *in vivo* experiment.

Significant suppression in the growth of *C. capsici* and the development of anthracnose disease on the artificially inoculated chilli fruits by the culture filtrates of *Trichoderma* sp. suggested that the isolated *Trichoderma* sp. produced substances which might have antifungal effect. Shi *et al.* (2012) reported the production of antimicrobial peptides by *T. pseudokoningii* against a number of plant fungal pathogens while Vinale *et al.* (2014) discussed various secondary metabolites produced by *Trichoderma* genus, which are toxic to phytopathogens. *Trichoderma* spp. were also reported to produce many cell wall degrading enzyme such as xylanase and chitinase (Pandey *et al.*, 2015). The finding in the present study agree with the study conducted by Rahman *et al.* (2012) who also found that application of culture filtrate of *T. harzianum* significantly decreased the disease severity caused by *C. capsici*. In addition, Rahman *et al.* (2013) reported that 30 day old culture filtrates of all *Trichoderma* strains in their study significantly reduced percentage of anthracnose disease severity on chilli fruits. According to Padder and Sharma (2011), *Trichoderma viride* has the best potential to inhibit mycelia growth and germination of spore of *Colletotrichum lindemuthianum*. Finally, higher percent inhibition (64%) of *Trichoderma* sp. toward *C. capsici* when *Trichoderma* plugs were used compared to 55% inhibition when culture filtrate was applied in this study indicated that, apart from antifungal effects, competition for space and nutrients could be another antagonism mechanism employed by *Trichoderma* sp. to inhibit the growth of *C. capsici* and the anthracnose development on the artificially infected fruits.

CONCLUSION

The isolated *Trichoderma* sp. has the potential as a biological control agent for *Colletotrichum capsici*, the pathogen of chilli anthracnose. It inhibited the growth of the pathogen on culture and reduced the disease severity on the chilli

fruits. For post-harvest treatment, application of a culture filtrate of the *Trichoderma* sp. would be more appropriate to avoid the growth of the antagonist on the fruit surface, which will reduce the product appearance, thus market quality of the chilli fruits. However, in the field, the antagonist might be used in either form, with and without mycelia. In this study, the antagonistic ability of the isolated *Trichoderma* sp. was only tested in the laboratory or a controlled environment, so it would be worthwhile to examine the effect of the isolated *Trichoderma* sp. in the field conditions.

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