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Developing secondary metabolites, fungal bio-agents and plant extracts for controlling *Colletotrichum capsici* causing anthracnose of chilli

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Abstract
Aiming to develop more efficiency and environmental friendly methods than those are available to control *Colletotrichum capsici,* which causes anthracnose of chilli. The study investigate to appropriate natural management a search for fungal bio-agents, plants and SM of endophytic fungi that able to produce substances active against such pathogen was carried out. In my first experiment three fungal bio-agents were collected from Plant Disease Clinic, PLP, PSTU. Among them the significantly highest inhibition 62.14 % was achieved by *Trichoderma* sp. Thus, the second experiment six plant parts were selected, that were prepared and submitted to *in vitro* assays with that fungus. The best results were obtained with the extracts prepared from Mehgoni seeds (*Switeenia mahagoni*), which inhibited *C. capsici* 83.372% at 40% concentration. In my third experiment, secondary metabolites (SM) of two endophytic fungi were isolated and tested against *C. capsici* and the highest mycelial growth inhibition was found 91.525% by SM of *Pestalotia* sp. Concluding, all the experiments were presented antifungal potential for new bioactive substances to control of *C. capsici,* especially *Trichoderma* sp., Mehgoni seeds and *Pestalotia* sp. highly inhibited the mycelial growth.

Keywords: Chilli, anthracnose, *Colletotrichum capsici,* secondary metabolites, fungal bio-agents

Introduction
Chilli (*Capsicum frutescens*) commonly called bird eye chilli (Williams *et al*., 1991) belongs to the family Solanaceae and within the Plantae kingdom. In Bangladesh, 239 acres of land occupied under cultivation and total production is 158 MT within the year 2019-2020 (BBS, 2020) [4]. It is often cultivated in both the summer and winter seasons. It has many medicinal properties and rich in proteins, lipids, carbohydrates, fibres, mineral salts (Ca, P, and Fe) and in vitamins A, D3, E, C, K, B2 and B12 which is of immense pharmaceutical importance (Saimbhi *et al*., 1977) [13]. This crop suffers from about 83 different fungal, bacterial and viral diseases, of which more than 40 are caused by fungus (Anonymous, 1960) [3]. Among the fungal diseases, anthracnose caused by *Colletotrichum capsici* is most significant one (Rahman *et al*., 2013) [12]. Anthracnose disease has been reported to be a serious constraint in chilli production. The loss is extremely attributable to the post and pre harvest involvement of the pathogen causing a loss of 10–80% of the marketable yield of chilli fruits. The symptom of infected tissue forms a depression and also the fruit shrinks as a result heavy losses ocurred. Chemicals are used to control the *C. capsici* but resistance to the chemicals has been reported for this pathogen (Benitez *et al*., 2004). Additionally, the extensive use of the chemicals might cause the pollution of the environment and therefore the health of both growers and consumers. So, alternative methods are needed for controlling anthracnose of chilli by reducing the usage of chemicals (Rahman *et al*., 2011) [11]. Antagonists are called biological control agents (Chernin & Chet, 2002) [8] having four mechanisms competition, antibiosis, induced resistance and parasitism. On the opposite hands botanicals is now employed in protection of crop and also as environment from pesticidal pollution, which is a global problem (Musyimi and Ogur, 2008; Prakash *et al*., 2008) [9]. Endophytes are newly emerging method involved in plants protection against biotic or abiotic stresses. An endophyte is an endosymbiont that lives within a plant for a minimum of a part of its life cycle without causing apparent disease. Secondary metabolites (SM) from endophytic fungi are low molecular weight substrate that aren't necessary for a cell (organism) to measure, but play a task within the interaction of the cell (organism) with its environment. Thus, the goal of the study whether the anthracnose of chilli could be managed through environment friendly biological, botanicals and secondary metabolites of endophytic fungi.

Materials and Methods
The experiment was conducted in the Plant Disease Clinic (PDC) and Plant Pathology Laboratory of the Department of Plant Pathology, Patuakhali Science and Technology University (PSTU), Patuakhali, Bangladesh during the period from April 2019 to November 2019. Anthracnose affected chilli fruits were collected from

local market in Pirtola Bazar and farmers' field, Dumki, Patuakhali. It was kept in polythene bags and preserved at room temperature (25°C).

Isolation of pathogen

The fungus was isolated from infected fruits of Chilli by Tissue Planting Method (Bhatia, 2015) [7]. The infected plant parts was cut into small pieces (5× 5) mm^2 by using a flame sterilized surgical scissors and surface sterilization was done. Then the pieces was placed onto sterilized Petri dishes (9cm) containing 20 ml PDA medium (added 1 ml lactic acid to prevent bacterial growth) and incubated at room temperature (25±2°C) until the whole plate covered by mycelia. Then pure culture of *C. capsici* was done. Fungal identification was done using morphological characteristics and comparing with established keys (Bernnet and hunter, 1999) [6].

Collection, isolation and identification of fungal bio-agents

Three fungal bio-agents (*Trichoderma sp*., *Pestalotia sp*. and *Rhizopus* sp.) were collected from the Plant Disease Clinic of the Department of Plant Pathology, PSTU. Pure culture of fungal bio-agents were established through standard procedure as described earlier for the establishment of *Colletotrichum* culture (Figure 1). Seven days old pure culture of three bio-agents with their microscopic views (100X magnification) were showed in figure 1a and 1d *Trichoderma sp*., figure 1b and 1e *Pestalotia sp*. and figure 1c and 1f *Rhizopus* sp. respectively.

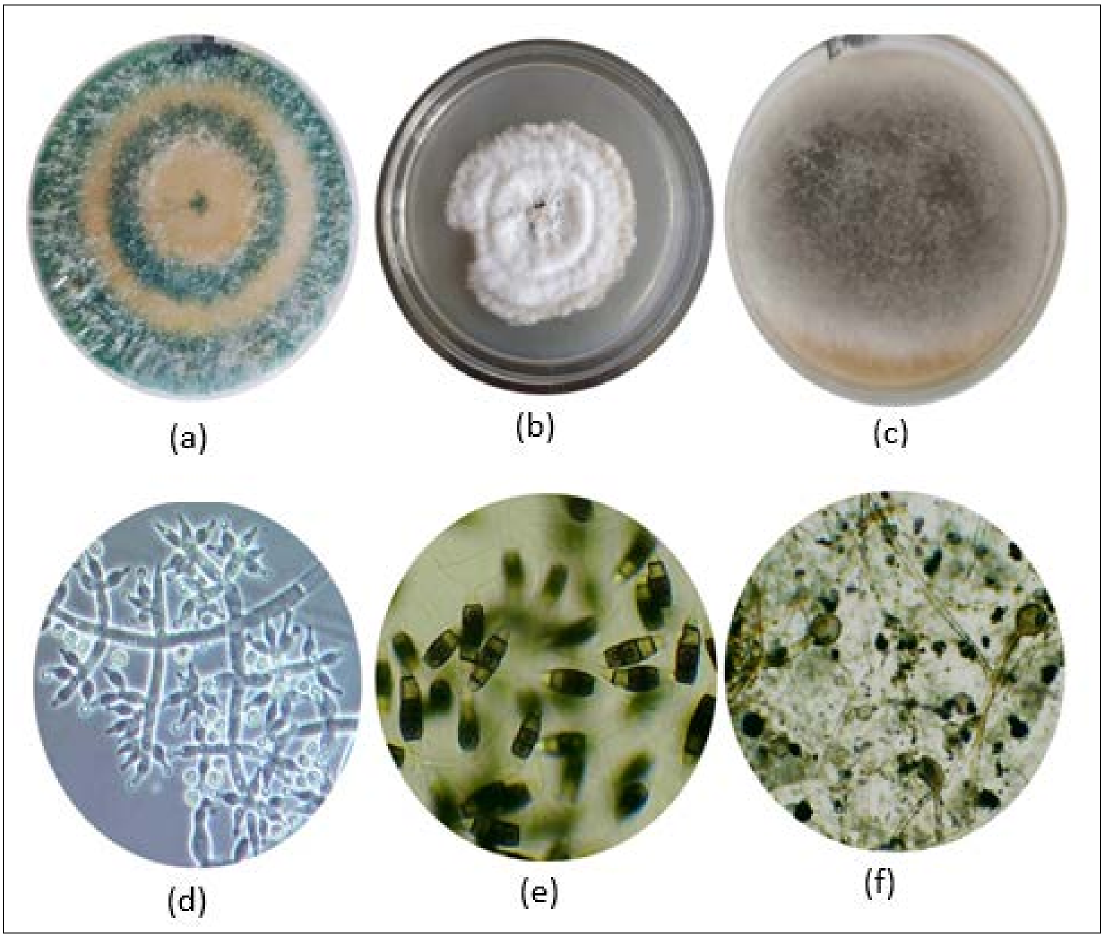

Fig 1: Pure culture and microscopic observation of fungal bio-agents, (a, b and c) Pure culture of three fungal bio-agents, (d, e and f) Microscopic observation of *Trichoderma* sp., *Pestalotia* sp., and *Rhizopus* sp. respectively

Evaluation of fungal bio-agents against *C. capsici*

The antagonistic activity of fungal bio-agents againsts *C.capsici* were determined by dual culture technique (Thahir *et al*., 2010). The sterilized Petriplates was poured into 20 ml of PDA medium and allowed for solidification. Seven days old pure culture of *C. capsici* was taken with the help of sterilized 5 mm mycelial block cutter and placed on the one side of Petriplate. The same procedure was followed and bio-control agents were transferred aseptically to Petriplates on opposite sides with four replicates. Without bio agents served as control when the test fungus was placed at the centre of petri plats. Observation on growth of antagonist and *C. capsici* from centre of disc towards centre of plate was recorded 7 days after inoculation. The percent inhibition of fungal growth was estimated by using the formula given by Alam, *et al*., (2017) [1].

$$Percent\ Control\ Efficacy\ (PCE) = \frac{C - T}{C} \times 100$$

Where,
PCE = Percent Control Efficacy
C = Colony diameter in control and
T = Treatment

Collection and preparation of Plant extracts
Six plant parts namely Onion bulb, Zinger rhizome, Tulsi leaves, Thankuni leaves, Neem leaves and Mehgoni seeds were collected from farmers field and Pirtola bazar, Dumki, Patuakhali. Extracts of collected plant parts were prepared by grinding with mixture-cum grinder of 100 g washed plant parts with 100 ml distilled water and filtered through Whatman No. 1 filter paper using glass funnel and it was made standard plant extracts 100% (Figure 2).

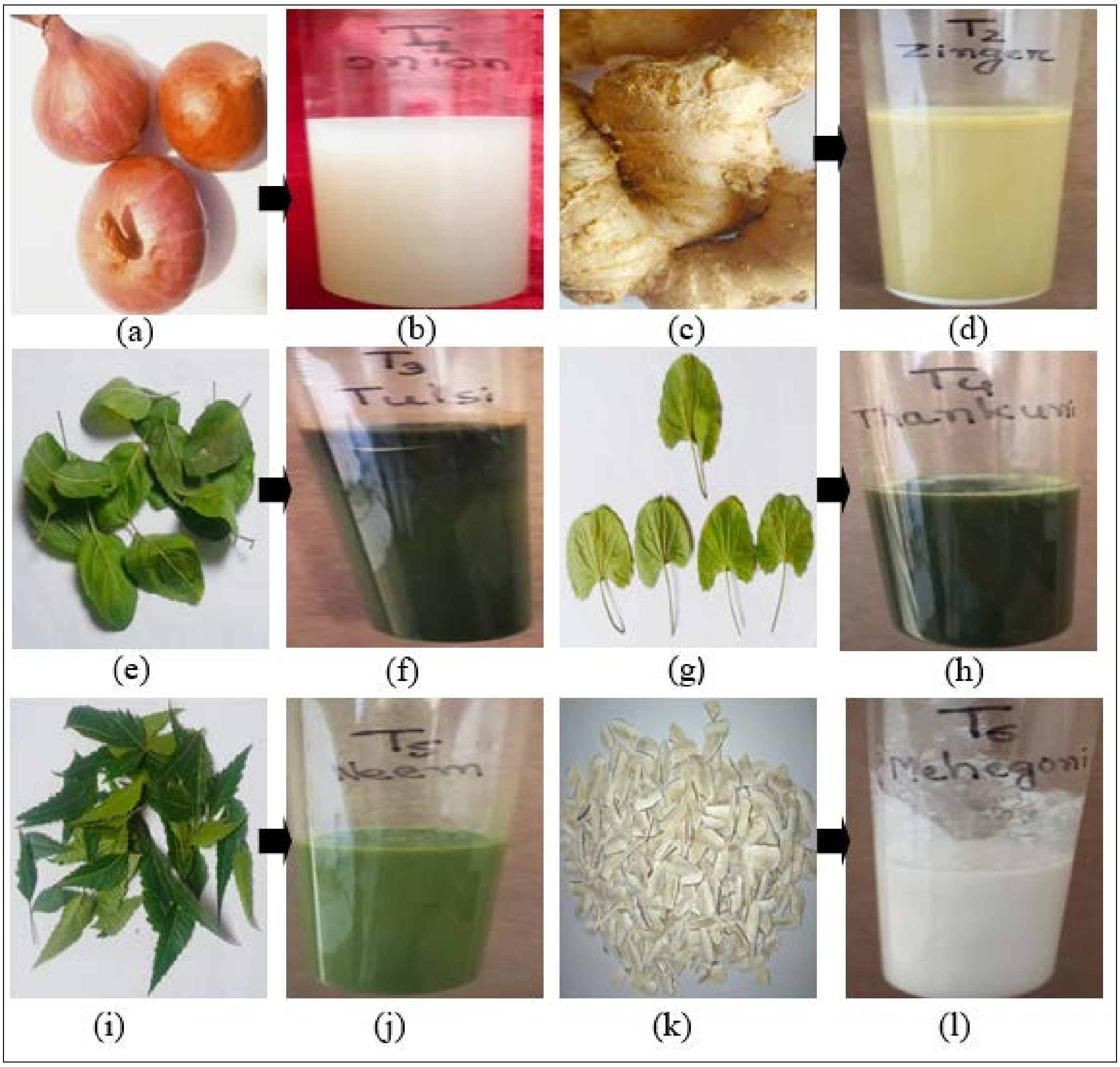

Fig 2: Plant parts used and its extracts. (a and b) Onion bulb and extracts; (c and d) Zinger rhizome and extracts; (e and f) Tulsi leaves and extracts; (g and h) Thankuni leaves and extracts; (i and j) Neem leaves and extracts; (k and l) Mehgoni seeds and extracts

Test of plant extracts against *C. capsici*
Six crude plant extracts were used for their potentiality against *C. capsici* using the Poisoned Food Technique (Schmitz, 1930). Each of 10 ml botanical extracts were mixed thoroughly with 100 ml PDA medium (after autoclaved and kept for 40ºC) in conical flasks and poured aseptically into 5 petri plates (9cm) equally. After solidification, all the plates were inoculated with 5 mm mycelial disc of the test fungus inversely on the center of the petri plates and incubated at 25±2°C. PDA medium without botanical extract served as control. Each treatment was five replicates. The cross wise diameters of fungal colonies were measured by taking average of the two diameters when the control plates were fully covered with mycelial growth of the test fungus. The percent inhibition of fungal growth was estimated by using the formula given by Alam (2017) [1] as described earlier.

Collection and isolation of endophytic fungi

Healthy keora (*Sonneratia apetala)* leaves were collected from Sundarban (Figure 3a). The leaves were washed and Surface sterilization was done by standard procedure. Then leaves were cut aseptically to expose the interior surface to the PDA medium. Five (5) segments were placed in each petri dish containing PDA (Figure 3b). After 7 days of incubation the endophytic fungi were transferred aseptically to the new plate for pure culture. Preparing clean glass slide it was observed under microscope for identification (Figure 3). Two endophytic fungi were identified through microscope with 100X magnification namely *Pestalotia* sp. (Figure 3d) and unknown fungi (Figure 3f).

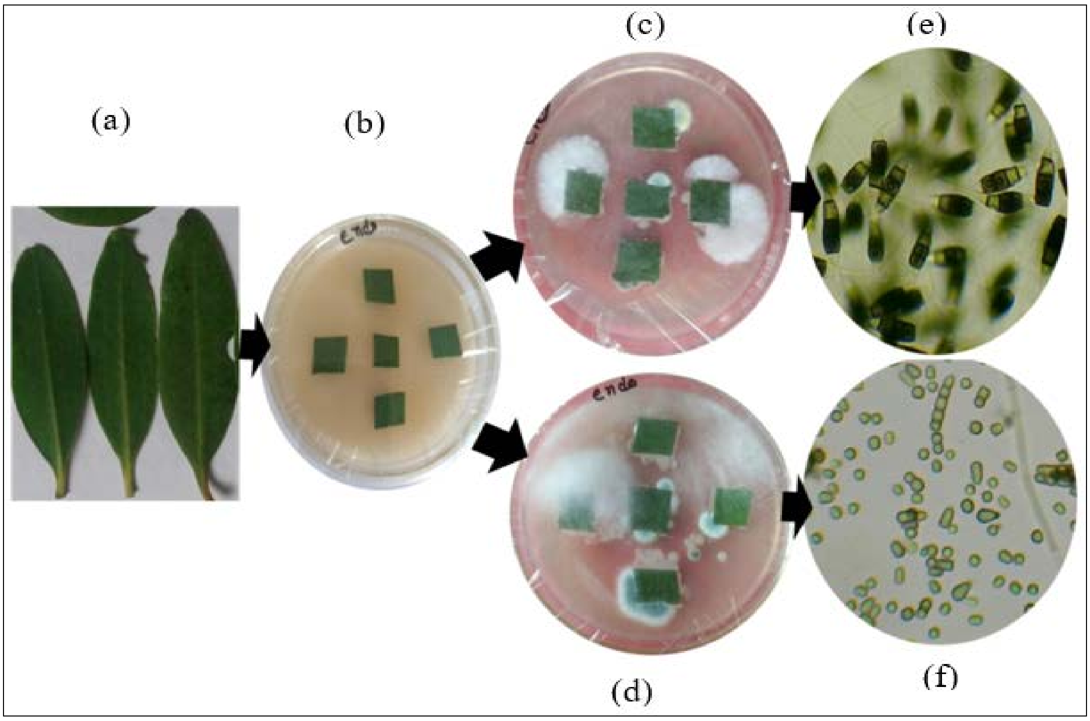

Fig 3: Fresh leaves of Keora (*Sonneratia apetala)* and isolation of endophytic fungi, *(*a) Keora leaves, (b) Cut pieces placed on PDA medium containing Petriplate, (c and d) Isolation of endophytic fungi, (e) *Pestalotia* sp., f) unknown fungi

Extraction of secondary metabolites

Two fifty (250) ml PDB medium was prepared and poured into 500 ml conical flask. Five (5) mm mycelial block of 7 days old pure culture of endophytic fungi was placed into the flask and incubated at 25ºC (Figure 4). Ten pieces of each endophytic fungi pure culture were placed separately. After 28 days of incubation, PDB was sieved by filter paper and Secondary metabolites SM1 (Figure 4a) and SM2 (Figure 4b) were extracted.

(a) (b)

Fig 4: Extraction of secondary metabolites from two endophytic fungi (a) *Pestalotia* sp. (SM1), (b) unknown (SM2)

Screening of secondary metabolites against *C. capsici*

Extracted secondary metabolites were mixed with PDA medium by Poisoned Food Technique (Schmitz, 1930). Each of 10 ml SM were mixed thoroughly with 100 ml PDA medium in conical flasks and poured aseptically into 5 petri plates (9cm) equally for solidify. Then all the plates were inoculated aseptically with 5 mm mycelial disc of the test fungus inversely on the center of the petri plates and incubated at 25±2°C. The Average Linear Growth Rate (ALGR) was measured by the following formula given by Alam (2016) [2]:

$$ALGR = \frac{C_8 - C_0}{C_8} \times 100$$

Where,
ALGR= Average Linear Growth Rate
C_8 = after 8 days colony growth
C_0 = Initial Colony growth

Data analysis

The experiment was conducted with CRD (Completely Randomized Design) method with 5 replicates. Data were analyzed by using Minitab software version 17 and means were compared Tukey's method at 95% confidence level.

Results and Discussion

Isolation and identification of pathogen

From the collected samples *C. capsici* were isolated based on morphological characters such as colony color was white (Figure 5a), sickle or curve shape hyaline conidia, existence of acervuli with numerous short conidiophores and needle like black color setae (Figure 5b), which was identified as *Colletotrichum capsici* through standard fungal identification keys.

(a) (b)

Fig 5: Pure culture and microscopic view of *Colletotrichum capsici* (a) Colony and (b) Setae, conidiophores and conidia of *C. capsici*

Efficacy of fungal bio-agents against *C. capsici*

Among the three fungal bio- agents were evaluated against *Colletotrichum capsici.* Significantly ($p<0.05$) the highest mycelial growth inhibition was found by *Trichoderma* sp. at 62.14% followed by the *Rhizopus* sp. and *Pestalotia* sp. at 11.80% and 27.75% growth inhibition respectively which was statistically similar (Figure 6).

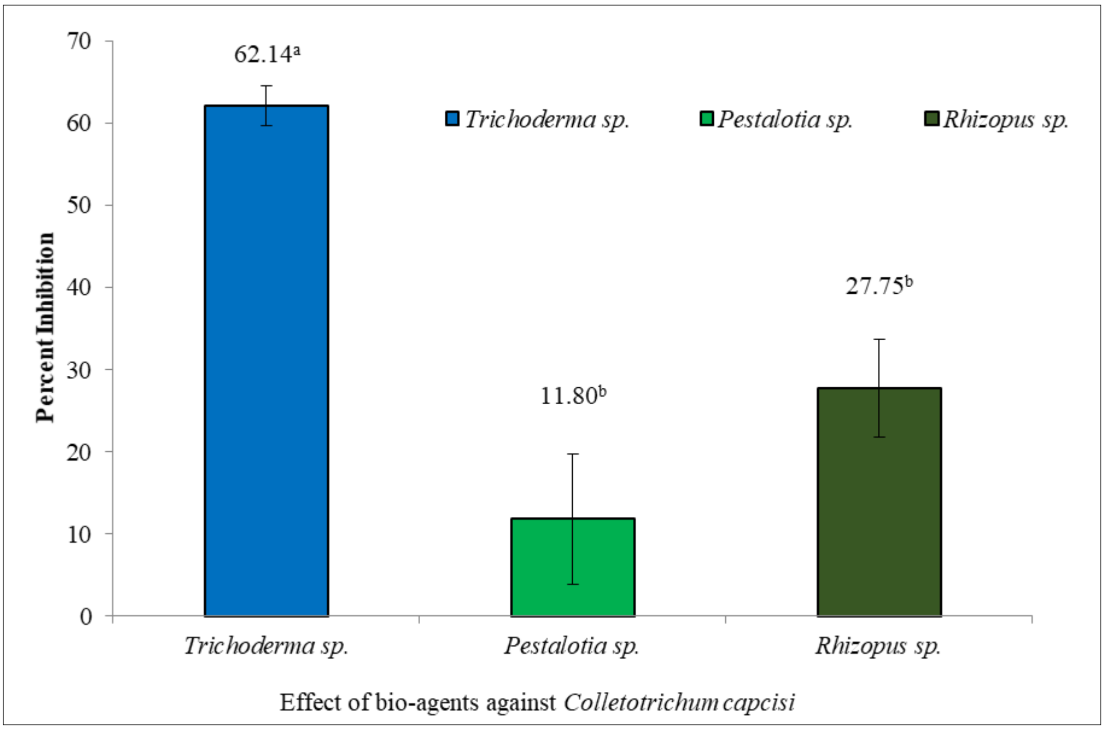

Fig 6: Graph showing the effect of selected bio agents on *Colletotrichum capsici*

The bio-agent *Trichoderma* sp. was fast growing (Figure 7b) followed by Both *Pestalotia* sp. and *Rhizopus* sp. But in figure 5c and 5d were medium growing which significantly slow growth from *Trichoderma* sp. and there is no statistical difference between them.

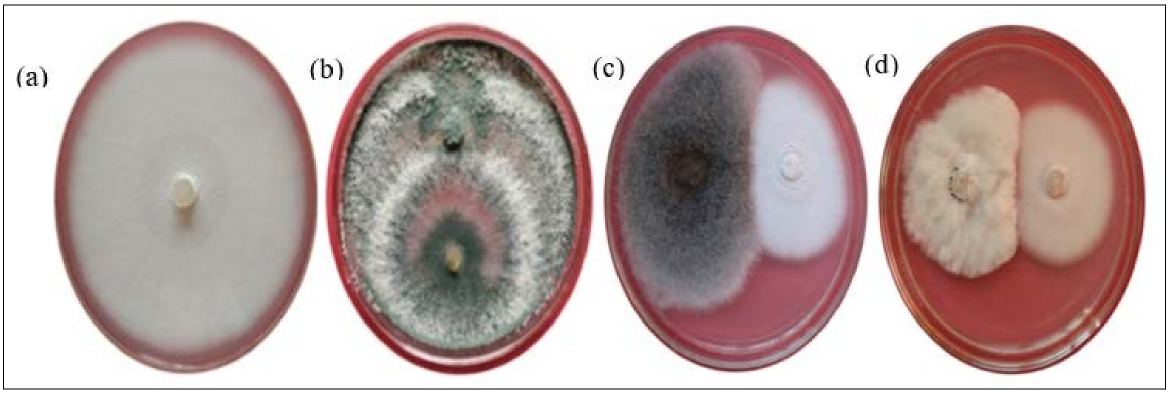

Fig 7: Fungal bio-agents inhibit the mycelial growth of *Colletotrichum capsici* (a) Control-full growth of mycelium (b) *Trichoderma* sp., (c) *Pestalotia* sp. and (d) *Rhizopus* sp.

Test of selected plant extracts against *C.capsici*

Effect of the plant extracts in controlling *C. capsici* were evaluated to *in vitro* condition. After seven days of inoculation, significantly highest percent inhibition 43.414% was achieved by Mehgoni seeds and the lowest percent inhibition at 2.238 % was recorded by Onion bulb followed by Neem leaves 29.97%, Zinger rhizome 24.36%, Tulsi leaves 7.828% and Thankuni leaves 5.587% respectively (Table 1).

Table 1: Evaluation of six plant extracts against *C. capsici*

Sl No.	Plant Extracts	Mycelial growth (mm/day) Mean ± SD	Inhibition (%)
1	Onion Bulb	87.25 ± 0.902	2.238^{e}
2	Zinger Rhizome	67.50 ± 2.52	24.36^{c}
3	Tulsi Leaves	82.25 ± 1.969	7.828^{d}
4	Thankuni Leaves	84.25 ± 1.992	5.587^{de}
5	Neem Leaves	62.50 ± 2.21	29.97^{b}
6	Mehgoni Seeds	50.50 ± 1.494	43.414^{a}

Note: Different letters of the same column are different significantly. Data were compared at 5% level of significance.

In case of six plant extracts, onion bulb, tulsi leaves and thankuni leaves extracts treated plates were showed minimum inhibition because of maximum mycelial growth (Figure 8b, 8d and 8e). Zinger rhizome and neem leaves extract mixing plates were showed medium inhibition (Figure 8c and 8f). Only mehgoni Seeds extract poisoning plate was observed highest inhibition because mycelial growth was 50.50mm (Figure 8g).

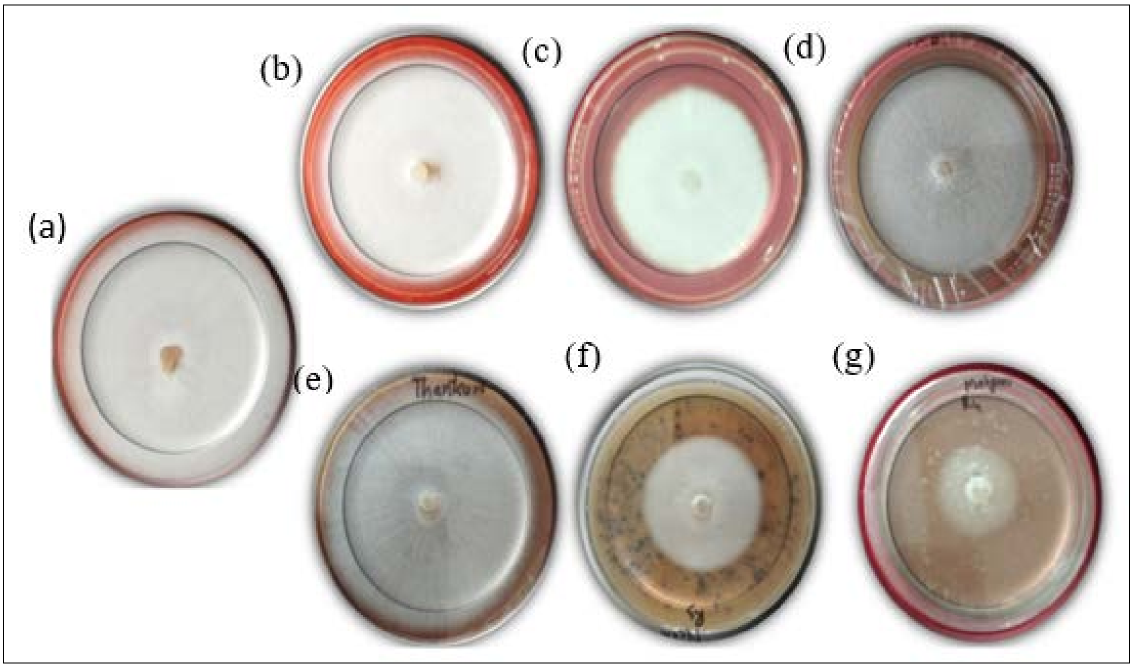

Fig 8: Different plant extracts inhibits mycelial growth of *C. capsici* a) Control-full growth of mycelium b) Onion, c) zinger, d) Tulsi, e) Thankuni, f) Neem and g) Mehgoni treated medium restricted the mycelial growth respectively

Effect of different concentrations on growth inhibition of *C. capsici*

In case of concentration, the highest percentage inhibition 83.372% was observed at 40% concentration and the inhibition percentage 68.455%, 61.003%, 57.853% and 53.267% was observed at 35%, 30%, 25% and 20% concentration which were statistically similar. The lowest percent inhibition 41.813% was observed at 15% concentration. With the increase of concentrations, the mycelial growth inhibitions were increased significantly (Figure 9).

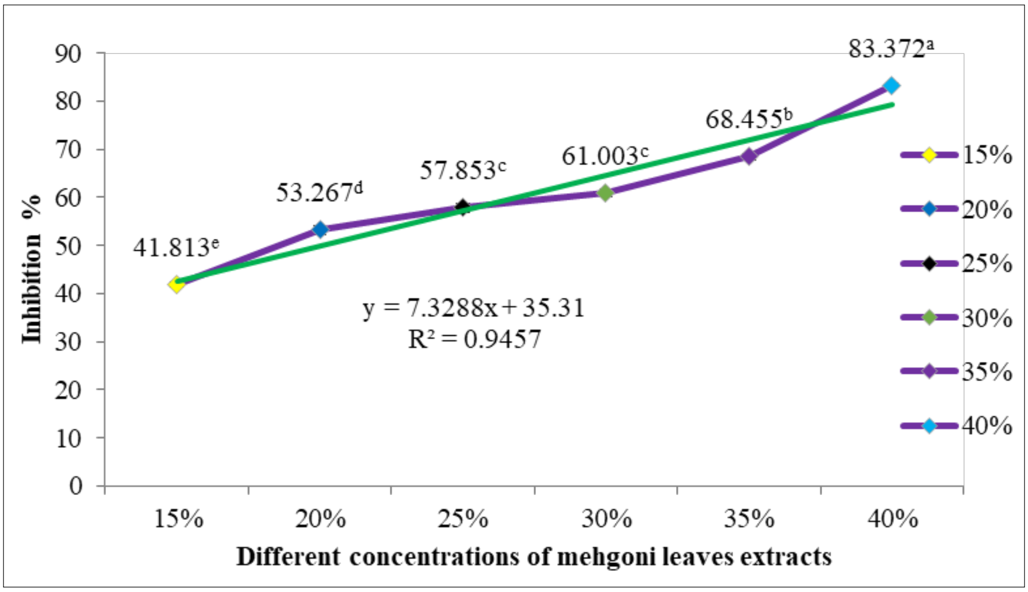

Fig 9: Graph showing the effect of different concentration on *C. Capsici*

Figure 10 indicates that the highest inhibition percentage was found in 40% concentration (Figure 10g) which was the highest concentration used in this study. Decreases concentration inhibition % was gradually deceases (Figure 10b-f).

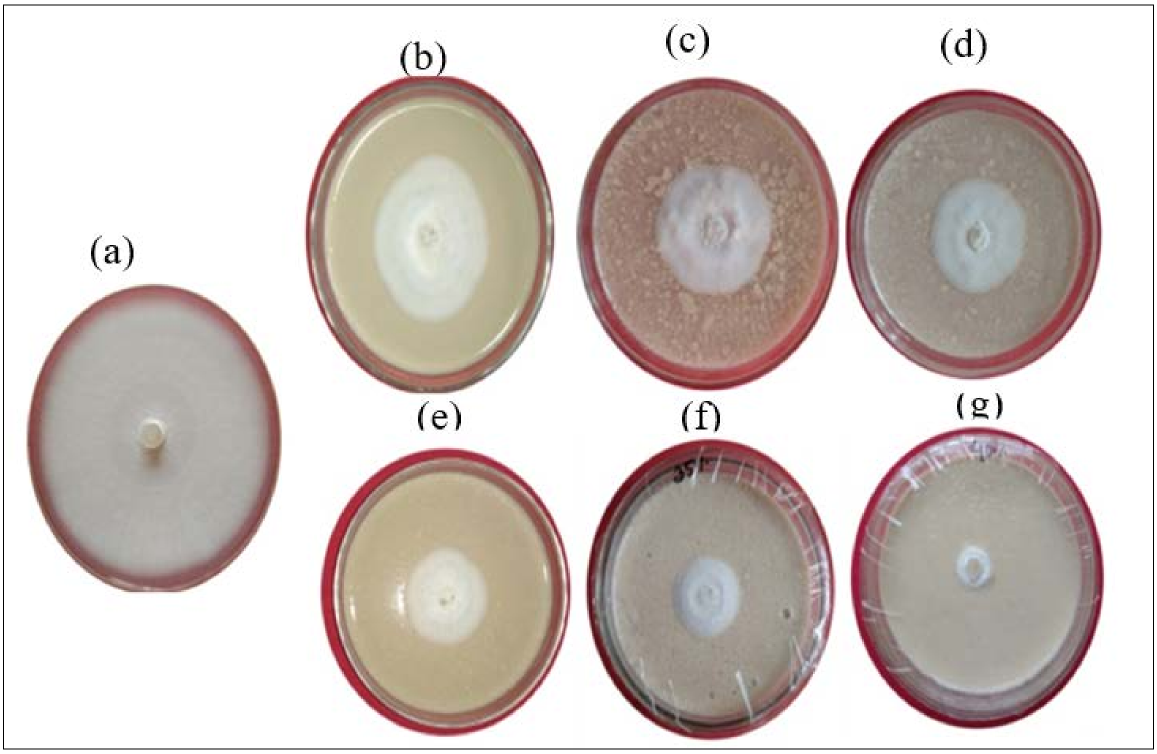

Fig 10: Different concentration of Mehgoni seeds inhibits mycelial growth of *Colletotrichum capsici* a) Control-full growth of mycelium b) 15ml, c) 20ml, d) 25ml, e) 30ml, f) 35ml and g) 40ml arrested the mycelial growth respectively

Effect of secondary metabolites against *C. capsici*

Secondary metabolites of two endophytic fungi significantly influenced negatively to *C.capsici* at different mean mycelial growth with standard deviation. The highest percent inhibition of mycelial growth of *C. capsici* was 91.525 % by SM of *Pestalotia* sp. and the lowest percent inhibition 81.359 % by SM of unknown endophytic fungi was observed (Table 2 and Figure 11).

Table 2: Antifungal activity of SM at 10 % concentration against *C.capsici*

Sl No.	SM of endophytic fungi	Mycelial growth (mm/day) Mean ± SD	Inhibition (%)
1	*Pestalotia* sp. (SM1)	7.50 ± 0.655	91.525[a]
2	Unknown (SM2)	16.50 ± 1.409	81.359[b]

Note. Different letters of the same column are different significantly. Data were compared at 5% level of significant

SM1 treated plate significantly inhibit the mean mycelial growth 7.50 mm (Figure 11a) of *C.capsici* as compared to SM2 16.50 mm (Figure 11b).

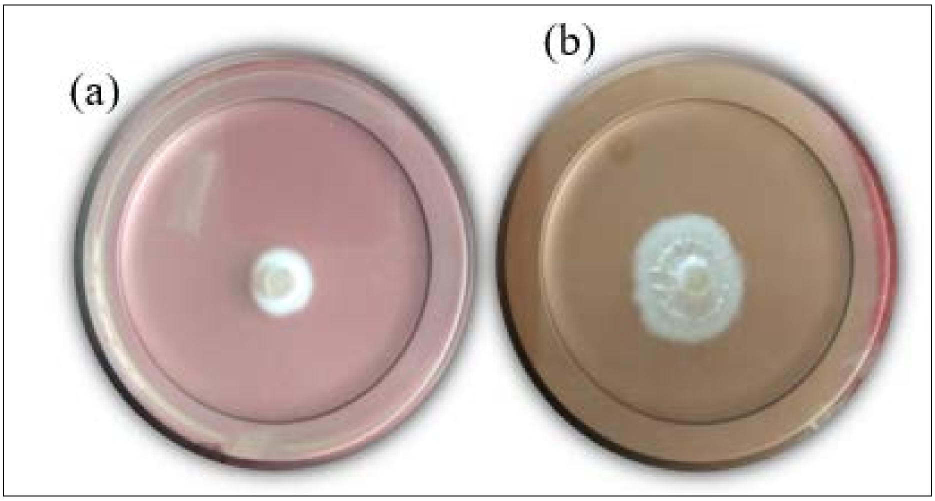

Fig 11: Mycelial growth inhibition at 10% concentration of SM, (a) SM of *Pestalotia* sp. treated medium, (b) SM of unknown endophytic fungi treated medium

Conclusion

In the present study, among the three fungal bio-agents the best performance was achieved by *Trichoderma* sp. followed by *Rhizopus* sp. and *Pestalotia* sp. over control. The study investigate six plant extracts were evaluated for their antifungal effect against *Colletotrichum capsici* and revealed that Mehgoni seeds extract gave the highest anti fungal activity with 40% concentration. SM of two endophytic fungi were used, *Pestalotia* sp. producing SM was highly performed against the pathogen. In conclusion the mycelial growth of tested fungi was highly affected *Trichoderma* sp. as bioagent, Mehgoni seeds extract as botanicals and SM of *Pestalotia* sp. may use in controlling anthracnose of Chilli.

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