



IN VITRO CONTROL OF COLLETOTRICHUM CAPSICI INDUCED CHILLI ANTHRACNOSE BY FUNGICIDES AND BIOCONTROL AGENT

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Abstract

Three fungicides namely carbendazim (0.05%), mancozeb (0.2%) and azoxystrobin (0.1%) and *Pseudomonas* sp. namely Ps 2 and Ps 3 were evaluated *in vitro* by using poisoned food technique and dual culture respectively for their antagonistic activity against *C. capsici*. Among the three fungicides mancozeb (0.2 %) recorded 73.47 % inhibition followed by carbendazim (0.05%) which recorded 64.12% inhibition. The least inhibition (62.21%) was recorded in azoxystrobin (0.1%) treatment. In dual culture assay Ps 2 showed 93.41 % inhibition whereas Ps 3 produced 72.5% inhibition of *C capsici*.

Keywords : Fungicides, *Pseudomonas*, dual culture, anthracnose, *C capsici*

I. INTRODUCTION

Chilli (*Capsicum annuum* L.) is one of the cash crops of India, grown both for the domestic consumption and export purposes. Chilli crop suffers from many diseases like damping off, foot rot, anthracnose, dieback, fruit rot, wilt, leaf spots, powdery mildew etc. Anthracnose caused by *Colletotrichum* spp is a major problem in India and one of the more significant economic constraints to chilli production worldwide, especially in tropical and subtropical regions (Anamika *et al.*, 2014). *Colletotrichum capsici* can survive in and on seed as acervuli and microsclerotia (Pernezy *et al.*, 2003). *Colletotrichum* spp naturally produce micro sclerotia to allow dormancy in the soil during the winter or when subjected to the stressful conditions and these micro sclerotia can survive for many years. *Colletotrichum* spp also produce a series of specialized infection structures such as germ tubes, appressoria, intercellular hyphae and secondary necrotrophic hyphae. Diseased fruits act as a source of inoculum, allowing the disease to spread from plant to plant within the field (Roberts *et al.*, 2001). The symptoms of anthracnose invasion are sunken necrotic lesions on fruits (Weller *et al.*, 2002; Agrios, 2005). Ramachandran *et al.*, (2007) reported that yield losses accounted for more than fifty per cent. Several management strategies are used for the control of *Colletotrichum* diseases such as cultural control, the use of resistant cultivars, biological control, and the use of fungicides (Nayaka *et al.*, 2009). Manandhar *et al.* (1995) found that fungicide spraying is the most common and practical method to control anthracnose. Biocontrol agents are effective alternatives to chemical fungicides due to their safety aspects. Therefore, in the present investigation, inhibition of mycelial growth of *C.capsici* exposed to different concentrations of some fungicides and bioagents were studied *in vitro*. The objectives of the study were to evaluate different fungicides and bioagents under lab conditions to find out the most effective one for final use.

II. MATERIALS AND METHODS

Isolation of *Colletotrichum capsici* from chilli

Infected fruit specimens were collected from Kerala Agriculture University, Vellayani, Thiruvananthapuram. The fruit specimens were washed with tap water, the discolored parts cut into small pieces (5 mm), sterilized with 0.1% NaOCl for two min and rinsed in sterilized water for three times and dried between folds of sterilized filter paper. The sterilized fruit pieces were transferred on sterilized oat meal agar plates and incubated at room temperature for 5 days. Mycelial bits were transferred to sterile petridishes containing oat meal agar medium; later it was purified by hyphal tip method and transferred to Potato dextrose agar (PDA) slants and pure cultures of the pathogens were maintained for further studies. The pathogen was identified on the basis of morphological, cultural and molecular characterization.

Purification of *Colletotrichum capsici* - single spore isolation

Isolated pathogen *Colletotrichum capsici* was purified by single spore isolation (Nene and Thapliyal, 1993). Ten ml of 2% water agar was poured into sterile petridishes and allowed to solidify. Dilute spore suspension was prepared in sterilized distilled water from 7 days old culture and one ml of spore suspension was spread uniformly on agar plate. These plates were incubated at $28 \pm 2^\circ\text{C}$ for 12 hrs. The plates were examined under microscope to locate single isolated and germinated conidium. The growing hyphal tip was cut with the help of cork borer under aseptic conditions and with an inoculation needle it was carefully transferred to PDA slants and incubated at $28 \pm 2^\circ\text{C}$. The evolutionary history of the fungus was inferred by constructing the phylogenetic tree using the sequences by Neighbor-Joining method. The evolutionary relationships were calculated using the Maximum Composite Likelihood method in MEGA4 (Tamura *et al.*, 2004 and 2007). This culture was used for further studies.

Pathogenicity tests

The pathogenicity of the isolated fungus *Colletotrichum capsici* causing chilli anthracnose was proved by Koch's postulate both under *in vitro* and *in vivo* conditions.

***In vitro* condition**

Healthy chilli fruits collected from the fields were washed under tap water and then surface sterilized with 70% ethyl alcohol. *C. capsici* was cultured on oat meal agar for 10 days. Then 0.7 cm agar plug containing mycelia of *C. capsici* was placed on pierced area on chili fruit. Fruits inoculated with sterilised water served as control. Inoculated fruits were kept in moistened polythene bags to maintain humidity and incubated at $28 \pm 2^\circ\text{C}$ and observed daily for the disease symptoms (Intana *et al.*, 2007). Pathogen was re isolated from the infected fruits and compared with the original culture.

***In vivo* condition**

Colletotrichum capsici was cultured on oat meal agar for 10 days. Culture suspension was sprayed on healthy chilli plants. The inoculated plants were covered with plastic bags for two days to maintain humidity. The plants were observed for disease development continuously for 30 days (Chandra Nayaka *et al.*, 2009). Pathogen was re isolated from diseased fruits and leaves and compared with the original culture.

Effect of biocontrol agents on mycelial growth of *C. capsici* - dual culture technique The two *Pseudomonas* isolates namely Ps 2 and Ps 3 were tested for their antagonistic activity against the fungal pathogen *Colletotrichum capsici* by dual culture technique (Barhate *et al.*, 2012). The fungal pathogen was grown on PDA plate till it covered the whole surface of the agar. With the help of a sterile cork borer, a disc of fungal growth from this plate was taken and placed at the center of a fresh PDA plate. Twenty four hour old cultures of bacterial strains were then streaked parallel on either side of the fungal disc 2.5 cm away from the fungal disc. The plates were kept for incubation at 30°C for 7 days. Visual

observations on the inhibition of growth of fungal pathogen were recorded after 7 days of incubation in comparison with the PDA plate simultaneously inoculated with only the fungal pathogen. Percentage of reduction in growth was calculated following the formula

$$\% \text{ of inhibition}(I) = \frac{C - T}{C} \times 100$$

Where, C: diameter of fungal growth in control plate

T: diameter of fungal growth in test plate

In vitro* evaluation of fungicides against mycelial growth of *Colletotrichum capsici

The inhibitory effect of different fungicides against mycelial growth of *Colletotrichum capsici* was studied according to the method of Poisoned food technique (Suleiman, 2010). The fungicide used in the study were carbendazim (0.05%), mancozeb (0.2%) and azoxystrobin (0.1%).

In this method 50 ml of distilled water and 50 ml of double strength PDA medium was sterilized separately and added the fungicide in the sterile distilled water and then mixed with 50 ml molten double strength PDA to get desired concentration. Then the poisoned medium was poured into sterilized petriplates and allowed to solidify. The same procedure was repeated for all the fungicides. 5 mm diameter disc of 5 days old *Colletotrichum capsici* culture was placed at the centre of the plates and incubated at $28 \pm 2^\circ\text{C}$ for 48 hrs. Control plates without fungicides were also maintained. Unamended PDA plates inoculated with *Colletotrichum capsici* served as checks. Radial growth of the pathogen was recorded. % inhibition of growth over control was calculated using the formula (Utkhede and Rahe, 1983)

$$\% \text{ of inhibition}(I) = \frac{C - T}{C} \times 100$$

Where,

I = per cent inhibition.

C = growth of *Colletotrichum capsici* in unamended medium.

T = growth of *Colletotrichum capsici* in amended medium.

III. RESULTS AND DISCUSSION

The phytopathogen *Colletotrichum capsici* was isolated from the infected chilli fruits showing typical anthracnose symptoms (Plate 1) collected from Kerala Agriculture University, Vellayani. The cultural and morphological characteristics of the isolate on OMA and PDA was recorded (Plate 2) and the isolate was observed visually under microscope by lactophenol cotton blue staining. Staining of the pathogen showed multinucleate macroconidia curved at both ends (Plate 3). The isolate was fast growing and showed a radial growth of 77.16mm in PDA and 86.67mm in OMA. The isolate produced dark black coloured mycelial growth on PDA and OMA.



Plate 1: Diseased sample showing typical anthracnose symptoms



Plate 2: *Colletotrichum capsici* on PDA plate after 14 days of incubation

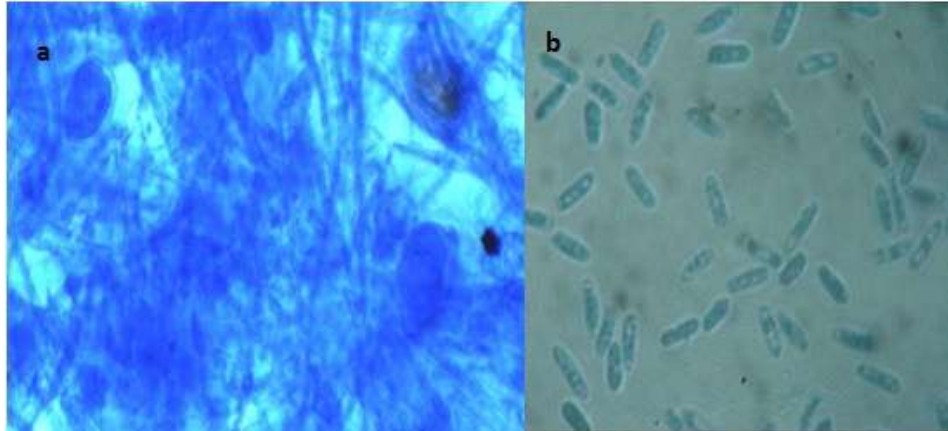


Plate 3: Microscopic observation of *Colletotrichum capsici* (a) appearance of mycelium (b) appearance of macroconidia curved at both ends

Phylogenetic tree construction

The phylogenetic analysis of the fungus was carried out by comparing the nucleotide sequence of the fungus with the sequence of neighbouring representatives. The tree was constructed using the neighbour joining method. From the phylogenetic tree it was evident that the isolated fungus was closely related to *Colletotrichum capsici* JN717227.1 (Figure 1). The *Colletotrichum capsici* JN717227.1 and the selected *Colletotrichum capsici* formed a single clad with 85% boot strap supporting (BSS).

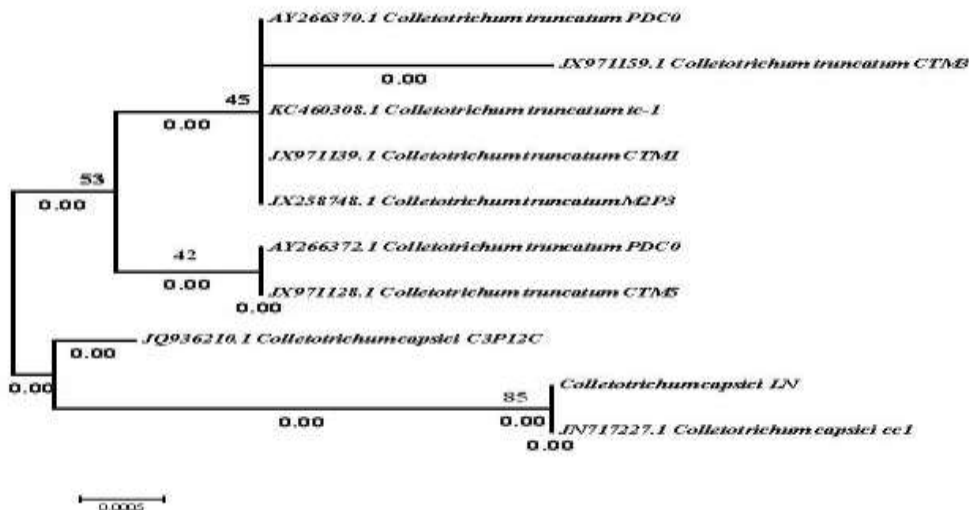


Figure 1: Phylogenetic tree of *Colletotrichum capsici* generated using neighbor joining method
Pathogenicity tests

The pathogenicity of the isolated pathogen was proved following Koch's postulates both under *in vitro* and *in vivo* conditions. Under *in vitro* condition healthy fruits inoculated with the phytopathogen showed mycelial growth and symptoms of anthracnose (Plate 4). The phytopathogen was re isolated from the infected fruits and compared with the original culture and found to be the same. Fruits inoculated with sterile water which served as control did not showed the symptoms of disease.



Plate 4. *In vitro* pathogenicity test proving Koch's postulate

Healthy plants inoculated with the pathogen showed the disease development (Plate 5) under *in vivo* condition. Both the fruits and leaves were infected and showed disease symptoms whereas the control plant remained intact. The fungal culture was re isolated from the infected fruits and leaves and compared with the original culture and found to be the same.

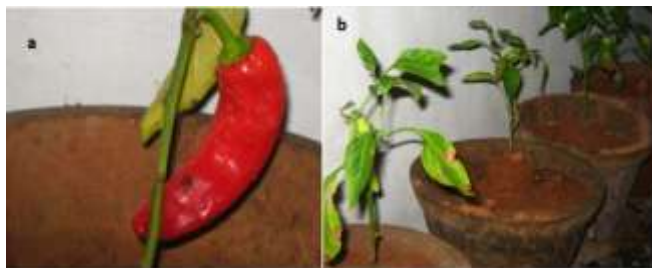


Plate 5: *In vivo* pathogenicity test proving Koch's postulate (a) In fruits (b) In leaves

Thus *Colletotrichum capsici* was confirmed as the species responsible for chilli anthracnose through pathogenicity test. The pathogenicity study showed that the behavior of *Colletotrichum capsici* isolates were homogeneous with regard to disease symptoms. However, variation in virulence or the level of disease within the isolates was observed. Differences in aggressiveness of *C. capsici* isolates have been reported previously by Taylor *et al.* (2007).

Dual culture assay

Antagonistic effect of the bacterial isolates were tested against the chilli anthracnose pathogen *Colletotrichum capsici* by the standard dual culture method. The results revealed that among the isolates PS 2 showed maximum inhibition of 93.41% whereas the other isolate PS 3 showed 72.5% of inhibition against *Colletotrichum capsici* after 7 days of incubation (Plate 6 and 7 T). Similarly in a study carried out by Shilpa and Gokulapalan (2015) *Trichoderma viride* caused 55.5% mycelial growth inhibition of *Colletotrichum capsici* in dual culture. In the same study *Pseudomonas fluorescens* showed 90% radial growth inhibition of *Colletotrichum capsici*. Kaur *et al.*, (2006) noticed 53.0% inhibition of *C.capsici* by *Trichoderma viride*. George *et al.*, (2009) reported the biological control of three *Colletotrichum lindemuthianum* races using *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas fluorescens* WCS365.



Plate 6: Antifungal activity of PS 2 against *Colletotrichum capsici* after 7 days of incubation



Plate 7: Antifungal activity of PS 3 against *Colletotrichum capsici* after 7 days of incubation *In vitro* evaluation of fungicides against *Colletotrichum capsici*

The *in vitro* chemical evaluation of *Colletotrichum capsici* was done by using Poisoned food technique (Table 1). Among the fungicide mancozeb (0.2 %) recorded 73.47 % inhibition. This was followed by carbendazim (0.05%) which recorded 64.12% inhibition. The least inhibition (62.21%) was recorded in azoxystrobin (0.1%) treatment. This is similar to the results of Shilpa and Gokulapalan (2015) who reported 70% inhibition of *Colletotrichum capsici* by mancozeb (0.2%) which was followed by azoxystrobin (0.1%) which recorded 67.50% inhibition and the least inhibition (63.77%) was recorded in chlorothalonil (0.1%). According to Gaikwad *et al.*, (2002) propiconazole was effective in inhibiting 100% radial mycelial growth of *Colletotrichum gloeosporioides* followed by hexaconazole (88.65 %), carbendazim (88.54 %), difenoconazole (77.43 %), mancozeb (69.59 %) and chlorothalonil (67.72%)

Table 1: *In vitro* screening of fungicides against *Colletotrichum capsici* (Percentage of inhibition after 48 hours)

Fungicide	Zone of inhibition (cm)	% of inhibition*
Mancozeb (0.2%)	0.55±0.08	73.47
Carbendazim (0.05%)	0.75±0.04	64.12
Azoxystrobin (0.1%)	0.79±0.12	62.21

*Values are means of three replications ± SD

IV. CONCLUSION

In this study the pathogen responsible for anthracnose of the chilli fruit was isolated and identified as *Colletotrichum capsici* which is the major causative agent of chilli anthracnose. The colony growth rate and characters of the fungal growth was examined which will be helpful in developing control measures against the pathogen. The pathogenicity of the isolate was confirmed by *in vitro* pathogenicity test. The two *Pseudomonas* isolates showed significant level of antifungal activity against *Colletotrichum capsici*. The fungicides selected also showed promising level of suppression of chilli anthracnose. These results provide a boost to chilli industry by reducing the rate of disease incidence in chilli.

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