Efficacy of Five Selected Bacterial Antagonists in Suppressing Foliar Colonization of *Colletotrichum truncatum* on *Capsicum annuum*

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Abstract
Capsicum annum (chilli) leaves serve as a major source of inoculum for the anthracnose causing fungus *Colletotrichum truncatum*. For successful infection, pre-penetration events including strong adherence to the host surface and spore germination play a critical role. As a novel approach, possible suppression of *C. truncatum* leaf infection by antagonistic bacteria was investigated in this study. Abaxial surface of surface sterilized healthy, freshly detached chilli leaves were spot inoculated with 30 µl of *C. truncatum* spore suspension (10^6 spores/ml) followed by 30 µl of cell suspension (10^8 cells/ml) of each F2, F35, F65, F79 and F80 each antagonists separately. The treated leaves were incubated in a humid chamber under dark conditions. One square centimeter of leaf tissues was excised at different time points, viz. 6 Hours after inoculation (HAI), 12 HAI, 36 HAI, 48 HAI, 60 HAI and 72 HAI. Excised leaf tissues were then transferred to a clearing solution (absolute alcohol: acetic acid, 1:2). Tissues were cleared for 24 hours in the clearing solution and then washed with sterilized distilled water. Carefully blot dried tissues were mounted on microscopic slides in lactophenol cotton blue stain. Germinated spore counts and appressorial counts were taken from the tissues excised at different incubation periods. Light microscopic views of untreated control demonstrated the sequence of colonization of *C. truncatum*at the tested time points. In contrast, F2, F65, F79 and F80 treatments completely inhibited the spore germination and subsequent colonization, possibly through hampered spore attachment. However, F35 antagonist significantly reduced the spore germination and limited the colonization process only up to appressoria formation. The results of this study suggest the possibility of using these antagonists in foliar applications in preventing chilli leaf colonization of *C. truncatum*.

Keywords: Bacterial antagonists, Chilli, *Colletotrichumtruncatum*, Inoculum, Leaf colonization
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Introduction

Anthracnose is a highly destructive postharvest disease, which causes high loses in many crops including chilli. It deteriorates the quality of chilli fruits and thereby reduces the commercial value. Therefore, control of this disease owes the economy of chilli crop cultivation.

Chilli anthracnose causal agent, *Colletotrichum truncatum* can infect the chilli plant in many of its growth stages as well as through many of its host's plant parts. Even though chilli fruits are the main target of the pathogen, leaves are equally colonized by this pathogen (Issac, 1992). Ranathunge *et al.* (2012) illustrated the disease cycle of *C. truncatum* indicating that the chilli foliage is a major source of inoculum. They further indicated that, this intramural necrotrophic pathogen can have a quiescent stage on chilli leaves, which could be an effective invasion strategy (Ranathunge *et al.*, 2012).

Therefore, in disease management strategies, it is highly worthwhile to pay attention on the possibility of minimizing foliar infection. Lo *et al.* (1997) mentioned that since many plant pathogens can spread readily through foliar parts, control of these diseases requires both suppression of initial plant infection and reduction of the infection rate. Taking precautions to prevent leaf colonization would be an effective way of reducing the incidence of chilli anthracnose.

In plant disease bio-control, various plant parts and microorganisms possessing antagonistic properties against the pathogen are applied. Bacterial antagonism has become a new paradigm shift in the science of microbial bio control. In this study the effect of five bacterial antagonists were tested for their efficacy in minimizing *C. truncatum* leaf colonization in chilli.

Materials and methods

Bacterial antagonists

Five isolates of *Burkholderia* sp. (Sandani *et al.*, unpublished), isolated from rhizosphere soil of a forest floor and being confirmed for their antagonistic properties against mycelial growth and spore germination of *C. truncatum* (Sandani *et al.*, 2015) were used for this study.

Pathogen

C. truncatum, was isolated from infected chilli fruits collected from Akuressa area in Sri Lanka. Collected chilli fruits were surface sterilized and the pathogen was aseptically isolated. Resulted *C. truncatum* was verified through microscopic observations according to Damm *et al.* (2009). The isolate was maintained on PDA slants at 4º C.

Inoculation of chilli leaves

Healthy, fresh detached chilli (*Capsicum annuum*) leaves were surface sterilized with 20% Sodium hypochlorite and washed three times with sterilized distilled water. The leaves were then air dried in the laminar air flow. Thirty microlitre aliquots of 10^6 spores/ml *C. truncatum* spore suspension and 30 µl of 10^8 cells/ml antagonists' broth cultures were spotted on the same place of the abaxial surface of chilli leaves simultaneously. The inoculated leaves were then incubated in humid chambers at room temperature (27±2 ^{0}C) under dark conditions. The experiment was done in triplicate in a completely randomized design.

Preparation of light microscopic specimens

One square centimeter from inoculated leaf tissues were excised at 6 hours after inoculation (HAI), 12 HAI, 24 HAI, 36 HAI, 48 HAI, 60 HAI and 72 HAI. Excised leaf tissues were introduced to the clearing solution, which contained absolute ethanol: glacial acetic acid in 1:2 proportions. The solution was removed after 24 hours and fresh clearing solution was added again and gently shaken for another 12 hours. Leaf tissues were gently rinsed in sterilized distilled water and blot dried between paper towels. Cleared leaf tissues were mounted on microscopic slides and stained with lactophenol cotton blue.

Microscopic observation

Mounted, leaf tissues were observed under ×400 magnification of light microscope (Carl Zeiss-Axio). Different stages in the process of colonization of *Capsicum annuum* leaf tissue by *C. truncatum* was studied at each time interval. The difference of the colonization stages of *C. truncatum* in the presence of the antagonists was observed compared to the untreated control. Germinated spore and appressorial counts were taken at 6 HAI, 12 HAI and 24 HAI, respectively. Spores in which the germ tubes had lengthened more than the half of the spore length were considered as germinated.

This experiment was repeated three times for higher accuracy.

Statistical analysis

The number of germinated spores and the number of appressoria formed in the presence of each antagonist were subjected to ANOVA procedure and the means were compared with the control using Dunnett test in SAS 9.1.3. Software.

Results and Discussion

Adhesion of fungal spores to the plant surface is an essential prerequisite for successful infection by pathogenic fungi (Nicholson and Epstein, 1991).

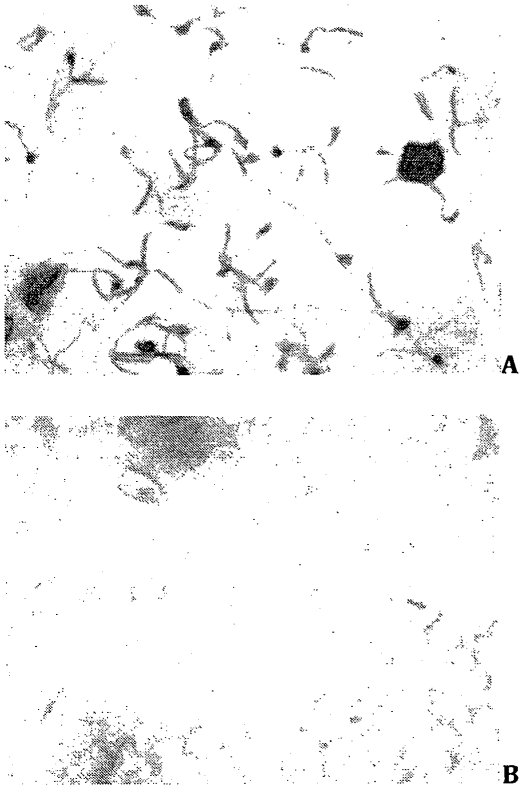

Figure 1: Micrographs of *C. truncatum* colonization events on *C. annum* leaf tissues 24 HAI- A) Control leaf tissue. B) Antagonists treated leaf tissue

In this study, microscopic observation of cleared, excised leaf tissues revealed the negative effect of selected antagonists on *C. truncatum* spore attachment and subsequent colonization on chilli leaves.

In the control experiment, the undisturbed sequence of events of chilli leaf colonization by *C. truncatum* could be observed at 6 HAI, 12 HAI, 24 HAI, 36 HAI, 48 HAI, 60 HAI and 72 HAI in the same way as it was described by Ranathunge *et al.* (2012). In contrast, in F2, F65, F79 and F80 antagonist treatments, attached spores could not be found on the leaf tissues and hence the subsequent colonization events could not be observed (Figure 1).

This confirms the theory of Nicholson and Epstein (1991) regarding the significance of spore attachment prior to colonization and emphasizes the need of further studies on the mechanism of inhibition of spore attachment by these antagonists. However in F35 antagonist treatment, a few attached spores could be seen on the tissue. Nevertheless, the germinated spore count was significantly less than the control (Table 1). The germinated spore count and appressorial count was taken up to 24 HAI (Table 1 and Table 2).

Table 1: Spore germination of *C. truncatum* on *C. annuum* leaf tissue in the presence of antagonists

Time interval	Treatment	Spore germination (%)
6 HAI	F2	0
	F35	0
	F65	0
	F79	0
	F80	0
	Control	98.6
12 HAI	F2	0
	F35	22
	F65	0
	F79	0
	F80	0
	Control	97.2
24 HAI	F2	0
	F35	26
	F65	0
	F79	0
	F80	0
	Control	≈ 100

All the given values were significant at $p<0.05$ level, compared to the control.

Table 2: Average appresorial count of *C. truncatum* on *C. annum* leaf tissue in the presence of antagonists

Time interval	Treatment	Average appressorial count
6 HAI	F2	0
	F35	0
	F65	0
	F79	0
	F80	0
	Control	23
12 HAI	F2	0
	F35	11
	F65	0
	F79	0
	F80	0
	Control	53
24 HAI	F2	0
	F35	19
	F65	0
	F79	0
	F80	0
	Control	72

All the given values were significant at $p<0.05$ level compared to thecontrol.

When considering the average appressorial count of the microscopic view, no appressoria could be seen in F2, F65, F79 and F80 treatments, as those treatments had inhibited the initial spore attachment and thereby no spore was visible on the leaf tissue. In F35 treatment, appressorial count was significantly lower than the control at 12 HAI and 24 HAI (Table 2). At 24 HAI, the appressoria in the control tissue got melanized and appeared in dark brown colour. In contrast, in the F35 treatment, melanization of appresoria was not prominant as in the control. The chilli leaf tissue colonization proceeded only up to appresoria formation in the F35 treatment, whereas in the control tissues appressoria displayed the penetration process after 24 HAI.

In a similar study, Montesinos*et al.* (1996) also reported the efficacy of some antagonistic *Pseudomonas* species in the suppression of *Stemphyliumvesicarium* (causal agent of brown spot of pear) spore germination and disease severity in detached pear leaves.

The findings of this study open a new arena for a successful application of these antagonists in managing leaf infection of *C. truncatum* and thereby preventing the anthracnose disease. As observed by Ranathunge *et al.* (2012) chilli leaves are a major source of inoculum, which also can have a quiescent stage of the pathogen.

Therefore, focusing on the control of colonization of such a necrotrophic fungus as a step in disease management would worth considering as an alternative means of managing *C. truncatum* fruit infection in chilli.

Conclusion

F2, F35, F65, F79 and F80 antagonists have the ability to inhibit the *C. truncatum* spore attachment and colonization in *Capsicum annuum* leaf tissue.

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