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Role of Toxin Produced by *Alternaria alternata* in Leaf Blight of Anthurium and Its Detoxification by Antagonists

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

A study was conducted to investigate the degradation of the phytotoxin produced by the anthurium leaf blight pathogen *Alternaria alternata* (Fr.) Keissler by antagonistic bacterial and fungal strains. Toxin extracted from leaf blight pathogen caused by *A. Alternata* showed the typical symptoms of detached leaves and increased the loss of electrolytes from anthurium leaves. Among the biocontrol agents tested, *Pseudomonas fluorescens* strain CFP1 was found to be highly effective detoxification of the pathogen toxin by utilizing the carbon source.

Keywords: Alternaria alternata; Anthurium; antagonists; toxin; detoxification.

1. INTRODUCTION

Fungal pathogens often produce low molecular toxic secondary metabolites that can be host-specific or host-non specific. The host-specific toxins are responsible for the main factors in disease development [1]. Toxins produced by *Alternaria* spp. cause changes in plant cell structures, leading to an increase in electrolyte loss from tissue and invagination of the plasma membrane [2,3] reported that pathogens produced toxins cause membrane dysfunction leading to cell death and suppression of defense mechanisms in the host. *A. macrospora* produced a toxin affecting the seed germination and plumule elongation in cotton which was non-specific [4]. Plasma membrane modifications including vesicle formation were reported in *Pyrus pyrifolia* treated with AK-1 a toxin produced by *A. alternata* [5].

Detoxification or inactivation of the phytotoxin reduces the toxicity of metabolites produced by plant pathogens. This process leads to the development of a resistant reaction and acts as a defense mechanism in susceptible plants to protect them from pathogen infection. Detoxification of phytotoxin produced by *Colletotrichum falcatum* by *P. fluorescens* strains *viz.*, FP7 and VPT4 caused reduction in electrolyte leakage and loss of symptom expression on susceptible sugarcane leaves [6]. In the present study revealed the role of toxin produced by the pathogen isolated from anthurium crops and also its degradation by biocontrol agents and antagonists.

2. MATERIALS AND METHODS

2.1 Toxin Isolation from Pathogen

The leaf blight pathogen, *Alternaria alternat* was isolated and identification was done based on the morphological and cultural characters. Fungal culture 5 mm mycelial disc grown on PDA medium was inoculated in 50 ml of sterile Richard's broth and incubated at room temperature (28 ± 2ºC) for 21 days. The culture filtrate was separated through sterile Whatman No.1 filter paper and used for further extraction of the toxin from different solvents.

2.2 Toxin Extraction with Diethyl Ether

“6N HCl was added to the fungal culture filtrate and mixed with an equal volume of diethyl ether. The ether phase was mixed again with an equal volume of 10% sodium bicarbonate solution. The aqueous solution was adjusted to pH 3.0 with 6N HCl and extracted with diethyl ether. The ether extract was evaporated under vacuum at 40 °C in a water bath. A yellow-brown residue was obtained, dissolved in distilled water, and used in the toxin bioassay” [7].

2.3 Toxin Extraction with Acetone

“The fungus culture was grown at room temperature (28 ± 2 °C) for 21 days on modified Czapek-Dox broth containing 1% glucose. The culture filtrate was obtained by separating the fungal mycelial mat using Whatman No. 1 filter paper and four layers of cheese cloth. Chilled acetone was added slowly to the fungus culture filtrate in a ratio of 2:1 (v/v) and the mixture was kept at 10 °C overnight to allow precipitation. The precipitate was collected by centrifugation at 15,000 rpm for 5 min and dried at 30 °C. The resulting off-white powder was dissolved in distilled water (200 µg ml-1) and used as a toxin source” [8].

2.4 Bioassay of Toxin Produced by *A. alternata*

2.4.1 Detached leaf assay

“Anthurium leaves were collected from 90 days old plants grown in a glasshouse. The anthurium leaves were surface-sterilized with 0.1% HgCl2 for 30 s and washed with repeated changes of sterile water. The leaves were placed on sterilized slides and kept in Petri dishes (150 mm diameter) lined with two layers of moist filter paper. Each leaf was made pinpricks using a sterile needle and a 4 mm in diameter sterile filter paper was placed over the lesion. 10 µl of partially purified toxin (acetone or diethyl ether fraction) was applied to the filter paper disc and filter paper with added distilled water was used as a control. The area of necrosis was measured at regular intervals" [9].

2.4.2 Electrolyte leakage

"90-day-old anthurium plants leaves were collected and sliced into small pieces. 100 mg leaf bits was taken and tied with a washed muslin cloth placed in ml of culture filtrate. The leaf tissue was vacuum infiltrated with partially purified toxin for 30 min. After vaccum infiltration, the bags were washed in sterile water and placed in 10 ml of sterile water. Electrical conductivity was measured in a conductivity meter at 15-min intervals up to 30 min and expressed as µ siemens/100 mg of leaf tissue" [10].

2.4.3 Degradation of toxin produced by *A. alternata* by biocontrol agents

Degradation of toxin was determined by the method described [11]. "The pathogen *Alternaria* was cultured in the toxin production medium. The *Alternaria* culture filtrate @ 50 ml/flask was distributed under aseptic conditions. The biocontrol agents *viz*., Pf1, CFP1, BsW1, BsM2, *T. viride* 1 and *T. viride* 2 were inoculated separately in each flask under aseptic conditions and kept for incubation for 6 days for fungal biocontrol agents at room temperature (28 ± 2^{o}C) and 48 h for bacterial biocontrol agents in a rotary shaker at 120 rpm. After incubation, the toxin was filtered through sterile filter paper for fungal biocontrol agents and bacterial filters for the bacterial biocontrol agents. The filtrates were collected in sterile flasks. The Alternaria culture filtrate alone served as control. The fungal toxin degraded by the biocontrol agents was confirmed by the following methods" [9].

2.4.4 Growth and multiplication of biocontrol agents in pathogen toxin

"The bacterial biocontrol agents were multiplied in the toxic filtrate of Alternaria utilizing on carbon source was assessed at 0 and 48 hr of incubation. The growth of fungal biocontrol agents in the toxic filtrate was determined by observing the dry mycelial weight of the fungus grown in the toxin. The approximate e controls of biocontrol agnets *viz*., *P. fluorescens*, *B. subtilis* and *T.viride* in their respective medium were maintained" [9].

2.4.5 Degraded toxin in loss of electrolytes of anthurium leaves

"The bio-degraded toxin and undergraded toxin activities were determined by measuring the electrolytic leakage from anthurium leaves. 90-day-old anthurium plant leaves were collected and sliced into small pieces. 100 mg of leaf bits was tied in a muslin cloth and placed in 3 ml of bio-degraded toxin and undegraded toxin. The leaf tissue was vacuum infiltrated with toxin for 30 min. After infiltration, the bags were washed in repeated changes with sterile water and placed in 10 ml of sterile water. Electrical conductivity was measured at 15-minute intervals up to 30 minutes using a conductivity meter and then expressed as µ siemens/100 mg of leaf tissue" [10].

2.4.6 Extracellular protein of *Bacillus subtilis* in degradation of toxin

The bio-degraded and undegraded toxin filtrates were obtained for the study. Ammonium sulphate (52.3 g/100 ml) was added to the filtrate to 80% saturation incubated overnight at 4^{o}C, and centrifuged at 4^{o}C with 10,000 rpm for 15 min. The protein pellet was dissolved in sodium phosphate buffer pH 7.0 and dialyzed against distilled water overnight at 4^{o}C with continuous agitation. The extracellular protein produced by the bacterial isolates in response to the toxin was analyzed through the protein to SDS-PAGE.

3. RESULTS AND DISCUSSION

3.1 Toxin of *A. alternata* in Pathogenesis

Several evidences underscore the importance of toxins in pathogenesis [6,12,13]. Activity and specificity of a toxin could be determined through symptom bioassay on detached leaves as well as by measuring the loss of electrolytes from the host tissue [3,14]. Hence, attempts were made to study the toxin production by the *Alternaia alternata* in pathogenesis.

Among the two methods were used for the extraction of toxin from leaf blight of anthurium caused by *A.alternata*,, the yield of toxin was higher when acetone was used as the solvent. Five days after incubation, the acetone fraction of the toxin caused necrosis on anthurium leaves. The necrotic area increased over time leading to total drying of leaves (Plate 1). No such symptom was observed with diethyl ether fraction and control (Fig. 1).

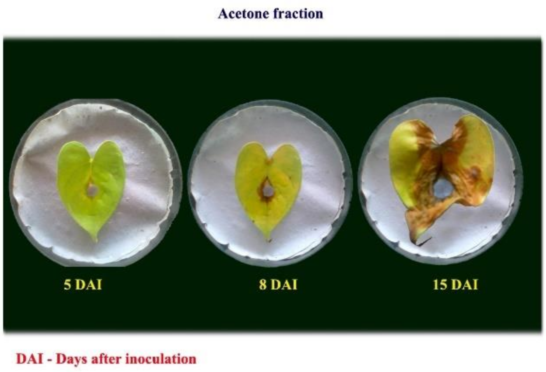

Plate 1. Symptoms bioassay with toxin produced by *Alternaria alternata* – Acetone fraction

Toxins of *Alternaria* have been extracted from culture filtrates with different solvents [1,14,15]. The toxin extracted from *A. alternata* and *A. macrospora* using with diethyl ether produced the typical symptoms as that of the pathogen in cotton leaves, bracts, and bolls [16]. Culture filtrates of *A. alternata* and the toxin fractions induced leakage of electrolytes in anthurium leaves. However, more leakage of electrolytes was observed with solvent fractions than that of culture filtrate. Acetone fraction induced more leakage (1625 µs) than diethyl ether fraction (725 µs) (Table 1).

A bioassay of the toxin produced by *A. macrospora* on leaf blight expression in cotton as well as electrolyte leakage revealed that maximum activity was observed with the partially purified toxin. Moreover, as observed in our study the toxin extracted using acetone produced a maximum area of necrosis with a significant loss of electrolytes [17]. *A. citri* pathogenic to tangerine yielded toxin that visibly affected the host species when applied to leaves causing leakage of electrolytes from the susceptible tissues [18]. Phytotoxins produced by *A. carthami* in safflower were found to suppress the phenyl propanoid metabolism [19].

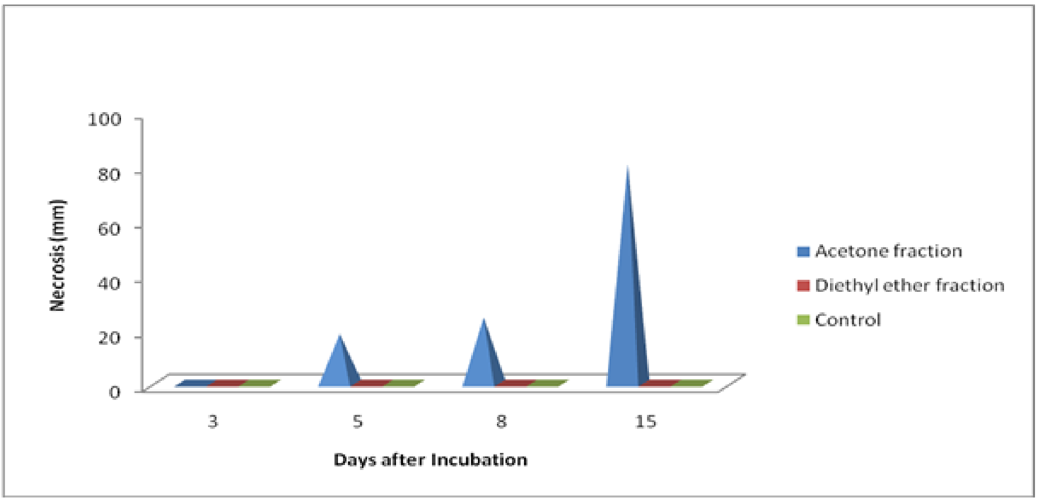

Fig. 1. Activity of toxin produced by A. alternate in symptom bioassay

Table 1. Activity of toxin produced by *A. alternata* in loss of electrolytes

Sl. No.	Treatments	Loss of electrolytes (µs)
1.	Culture filtrate (Richard's broth)	485^{d}
2.	Diethyl ether fraction	725^{c}
3.	Culture filtrate (Modified Czapek's Dox broth)	763^{b}
4.	Acetone fraction	1625^{a}
5.	Sterile water	63^{e}

**Mean of 3 replications*

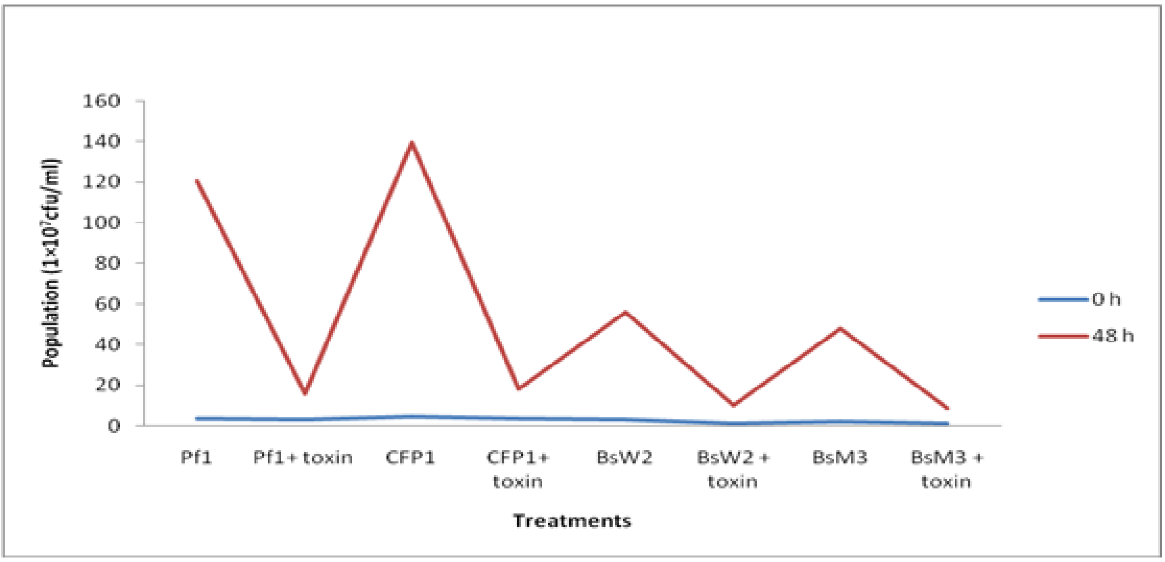

Fig. 2. Multiplication of bacterial biocontrol agents in toxin of *A. alternata*

3.2 Biodegradation of Toxin Produced by *A. alternata*

The toxins produced by plant pathogens may be sensitive to biological degradation leading to loss of activity. In this study, the efficacy of bacterial biocontrol agents were tested to multiply in the toxin of *A. alternata* (Fig. 2.).

Table 2. Growth of *T. viride* in toxin of *A. alternata*

Sl. No.	Treatments	Dry mycelial weight (mg)
1.	Tv1	278^{a}
2.	Tv1 + toxin	43^{c}
3.	TV2	256^{b}
4.	Tv2 + Toxin	23^{d}

The results showed the isolates in both the degraded toxin and undegraded toxin that the initial population was minimal. There was an increase in the population of CFP1 from 3.7 x 10^{7} to 18.0 x 10^{7} cfu^{-ml} between 0 and 48 h of inoculation in the toxin indicating the isolate could multiply in the toxin by degrading it. However, the multiplication was higher in all the isolates for their respective medium. Though *T. viride* isolates could grow on the toxin, their growth was negligible as they recorded dry mycelial weight of 43 and 25 mg when compared to culture broth without toxin of 278 and 256 mg respectively (Table 2). Similar results of the combined inoculation of *Pseudomonas* and *Alternaria* in wheat grains might reduce the toxin production by *Alternaria* and affect the disease development. *Pseudomonas simiae* had the potential to degrade various kinds of toxins produced by Alternaria in wheat [20]. Rajesha et al. [21] reported that in the six bacterial isolates tested, electrolytic leakage was observed least in *B. amyloliquefaciens* isolate EBs2 (108 μs)

Table 3. Efficacy of biocontrol agents in degradation of toxin produced by *A. alternata*

Sl. No.	Treatments	Electrolytic leakage (μs)		
		0 min	15 min	30 min
1.	Pf1 + toxin	578^{d}	620^{d}	636^{d}
2.	CFP1 + toxin	486^{e}	514^{e}	610^{d}
3.	BsW2 + toxin	632^{c}	657^{c}	681^{c}
4.	BsM3 + toxin	613^{c}	636^{c}	679^{c}
5.	Tv1 + toxin	652^{b}	725^{b}	791^{a}
6.	Tv2 + toxin	630^{bc}	704^{b}	756^{b}
7.	Toxin	683^{a}	743^{a}	816^{a}
8.	Sterile water	63^{f}	72^{f}	79^{e}

**Mean of 3 replications*

compared to all other five isolates and differed significantly with EBs6 (129 µs) in comparison with toxin alone (171 µs). This study clearly showed the potential of *B. amyloliquefaciens* isolate EBs2 in degradation of toxin and thereby reducing symptom development.

The effect of biocontrol agents to degrade toxin of *A. alternata* was assessed through electrolyte leakage studies (Table 3). The results showed that maximum loss in electrolytes was recorded in the undegraded toxin of *A. alternata* (816 µs) at 30 min. The efficacy of the biocontrol agents to degrade toxin varied with the isolates.

Loss of electrolyte leakage was less in degraded toxin compared to undegraded toxin at all the time intervals. Among the isolates, the electrolyte leakage was 610 µs at 30 min with the CFP1degraded toxin when compared to other isolates. The results were further confirmed through the analysis of extracellular proteins produced by the isolates in degraded toxins. Discrete bands produced by CFP1 and BsW2 were noticed in toxins of *A. alternata* at 12.97 kDa and 34.96 kDa. The results of present study are in line with the earlier reports of toxin degradation. The esterases of *B. subtilis* were capable of detoxifying brefeldin of *A. carthami* [22]. In sugarcane, [6] reported that the phytotoxin produced by *C. falcatum* was degraded by *P. fluorescens* resulting in reduced leakage of electrolytes and loss of symptom expression. As noticed in our study detoxification of phytotoxin produced by *A. alternata* by *B.subtilis* BsW1 caused reduction in electrolyte leakage in watermelon [15]. Krishnamohan et al. [17] observed that the loss of electrolyte leakage in cotton was less in *P. fluorescens* isolate K4 degraded toxin of *A. macrospora.* Isolates of *B. subtilis* DGL9 and *P. fluorescens* K4 produced prominent protein bands with molecular weight of 65 kDa and 45 kDa in the toxin of *R. solani* and *A. macrospora* respectively which were absent in the undegraded toxins.

4. CONCLUSION

The present study revealed the role of toxin produced by the pathogen isolated from anthurium crops and also its degradation by biocontrol agents and antagonists. The study found that the biocontrol agents might be able to manage leaf blight disease in field conditions due to its significant toxin degrading ability.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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