Role of toxin produced by *Alternaria alternata* in leaf blight of anthurium and its detoxification by antagonists

Abstract

A study was conducted on the possible degradation of phytotoxin produced by anthurium leaf blight pathogen *Alternaria alternata* (Fr.) Keissler by antagonistic bacterial and fungal strains. Toxin extracted from *A. Alternata* the causal agent for leaf blight in anthurium produced the typical symptoms such as that of the pathogen detached anthurium leaves and also increased the loss of electrolytes from anthurium leaves. Among the biocontrol agents tested *Pseudomonas fluorescens* strain CFP1 was found to be highly effective in detoxification of the pathogen toxin by utilizing the toxin as a sole carbon source.

Keywords: *Alternaria alternata,* Anthurium, antagonists, toxin and detoxification

Introduction

Many of the fungal pathogens produce low molecular toxic secondary metabolites which are host-specific or host-non specific in nature. The host-specific toxins are considered to the primary determinants in disease establishment (Liakopoulou *et al.*, 1997). Toxins produced by *Alternaria* spp. cause changes in plant cell structures and an increase in electrolyte loss from tissue and invagination of ~~plasmamembrane~~ plasma membrane (Otani *et al.*, 1989). Vidhyasekaran (1997) reported that toxins produced by pathogens cause membrane dysfunction leading to cell death and suppression of defense mechanism of the host. *A. macrospora* was able to produce a toxin affecting the seed germination and plumule elongation in cotton which was of ~~non specific~~non-specific nature (Padmanaban, 1973). Plasma membrane modifications including vesicle formation were reported in *Pyrus pyrifolia* treated with AK-1 a toxin produced by *A. alternata* (Shimizu *et al.*, 2006).

Detoxification or inactivation of the phytotoxin reduced the toxicity of metabolite produced by plant pathogens. This process leads to development of resistant reaction or act 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 (Malathi *et al.*, 2002). In the present paper we report the role of toxin produced by *A. alternata* isolated from anthurium in pathogenesis and also its degradation by biocontrol agents and antagonists.

Materials and Methods

Isolation of toxin

Five-mm-mycelial disc of the fungus grown on PDA was inoculated in 50 ml of sterile Richard's broth and incubated at room temperature (28 ± 2°C) for 21 days. The culture filtrate was separated by filtering it through sterile Whatman No.1 filter paper and used for further extraction of the toxin with different solvents.

Toxin extraction with diethyl ether

The culture filtrate was adjusted to pH 3.0 with 6N HCl and shaken with an equal volume of diethyl ether. The ether phase was again shaken with an equal volume of 10% sodium bicarbonate solution. The aqueous solution was adjusted to pH 3.0 with 6N HCl and again extracted with diethyl ether. The ether extract was evaporated under vacuum at 40°C in a water bath. The yellowish brown residue thus obtained was dissolved in distilled water and used for toxin bioassay (Kohmoto *et al*., 1976).

Toxin extraction with acetone

The fungus was grown on modified Czapek's Dox broth containing 1% glucose at room temperature (28 ± 2°C) for 21 days. Culture filtrate was collected by separating the mycelial mat through Whatman No.1 filter paper and four layers of cheese cloth. Chilled acetone was slowly added to the filtrate at a ratio of 2:1 (v/v) and the mixture was kept overnight at 10°C to allow precipitation. The precipitate was recovered by centrifugation at 15000 rpm for 5 min and dried at 30°C. The ~~whitish grey~~whitish-grey powder obtained was dissolved in distilled water (200 µg ml^{-1}) and used as the toxin source (Gour and Dube, 1984).

Bioassay of toxin produced by *A. alternata*

Detached leaf assay

Anthurium leaves from 90 day-old plants raised in the glasshouse were surface sterilized in 0.1% $HgCl_2$ ~~mercuric chloride~~ for 30 sec and washed with repeated changes of sterile water. They were placed on sterile glass slides kept inside a Petri plate **(**150-mm-dia**)** lined with two layers of moist filter paper. Pinpricks were made using sterile needle on each leaf and 4-mm-dia sterile filter paper disc was placed over the injury. Ten µl of partially purified toxin (acetone or diethyl ether fraction) was applied on the filter paper discs. Filter paper with distilled water served as control. The area of necrosis was measured at regular intervals.

Electrolyte leakage

Anthurium leaves from 90-day-old plants were sliced into small pieces. One hundred mg of leaf bits was tied in a washed muslin cloth and placed in 3 ml of culture filtrate and partially purified toxin. The leaf tissue was vacuum infiltrated with toxin for 30 min. After infiltration, the bags were washed in repeated changes of sterile water and placed in 10 ml of sterile water. Electrical conductivity was measured at 15-min intervals up to 30 min in a conductivity meter and expressed as µ siemens/100 mg of leaf tissue (Vidhyasekaran *et al*., 1986).

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

Degradation of toxin was determined following the method of Umamaheswari and Sankaralingam (2010). *A. alternata* was cultured on toxin production medium. The culture filtrate of Alternaria was distributed @ 50 ml/flask under aseptic condition. The biocontrol agents *viz*., Pf1, CFP1, BsW1, BsM2, *T. viride* 1 and *T. viride* 2 were inoculated separately and aseptically in each flask and incubated for 48 h in case of bacterial biocontrol agents and 6 days for *T.viride* at room temperature (28 ± 2^{o}C) in a shaker at 120 rpm. After incubation the toxin was filtered through sterile filter paper for *T. viride* and through bacterial filters for the bacterial antagonists. The filtrates were collected in sterile flasks. The culture filtrate of Alternaria without the biocontrol agent (i.e.) undegraded toxin served as control. The ability of the biocontrol agents to degrade the toxin was confirmed by the following methods.

Growth and multiplication of biocontrol agents in pathogen toxin

The ability of the bacterial biocontrol agents to multiply in the toxic filtrate of Alternaria utilizing it at a carbon source was assessed at 0 and 48 hr of incubation. The growth of *T. viride* in toxic filtrate was determined by recording the dry mycelial weight of the fungus grown in the toxin. Appropriate controls having *P. fluorescens, B. subtilis* and *T.viride* in their respective medium were maintained.

Degraded toxin in loss of electrolytes of anthurium leaves

The activities of bio-degraded toxin and undegraded toxin were determined by measuring the electrolytic leakage from anthurium leaves. Anthurium leaves from 90-day-old plants were sliced into small pieces. One hundred mg of leaf bits was tied in a washed 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 of sterile water and placed in 10 ml of sterile water. Electrical conductivity was measured at 15-min intervals up to 30 min in a conductivity meter and expressed as µ siemens/100 mg of leaf tissue (Vidhyasekaran *et al*., 1986).

Extracellular protein of *Bacillus subtilis* in degradation of toxin

The degraded and undegraded toxin filtrates obtained as detailed in 3.18 were used for the study. Ammonium sulphate was added to the filtrate to 80% saturation (52.3 g/100 ml), incubated overnight at 4^{o}C and centrifuged at 10,000 rpm for 15 min at 4^{o}C. The protein pellet was dissolved in sodium phosphate buffer pH 7.0 and dialysed 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 analysed by subjecting the protein to SDS-PAGE.

Results and discussion

Toxin of *A. alternata* in ~~pathogenisis~~pathogenesis

Several evidences underscore the importance of toxins in ~~pathogenisis~~ pathogenesis (Bains and Tewari., 1985; Kohmoto *et al*., 1992; Malathi *et al*., 2002). 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

(Vidhyasekaran *et al.*, 1997; Sriram, 1997). Hence, attempts were made to study the role of toxin produced by *A. alternata* in ~~pathogenisis~~pathogenesis.

Among the two methods used for the extraction of toxin from *A. alternata* that causes leaf blight of anthurium, the yield of toxin was more when acetone was used as the solvent. Five days after incubation, the acetone fraction of 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). Toxins of *Alternaria* have been extracted from culture filtrates with different solvents (Liakopoulou *et al*., 1997; Sriram, 1997; Umamaheswari, 2005). The toxin of *A. alternata* and *A. macrospora* when extracted with diethyl ether produced the typical symptoms as that of the pathogen in cotton leaves, bracts and bolls (Krishnamohan, 1986). 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). In cotton, bioassay of the toxin produced by *A. macrospora* on leaf blight expression 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 maximum area of necrosis with significant loss of electrolytes (Vijayasamundeeswari, 2006). *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 (Kohmoto *et al.*, 1979). Phytotoxins produced by *A. carthami* in safflower were found to suppress the phenyl propanoid metabolism (Tietjen and Matern, 1984).

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, bacterial biocontrol agents were tested for their ability to multiply in the toxin of *A. alternata* (Fig 2.). The results showed that the initial population of the isolates in both the degraded and undegraded toxin were minimum. 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 for all the isolates in 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 as against 278 and 256 mg in culture broth

without toxin (Table 2). The ability 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 ability 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* (Kneusel *et al.*, 1994). In sugarcane, Malathi *et al.* (2002) 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 (Umamaheswari, 2005). Vijayasamundeeswari (2006) 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.

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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]

Means in columns followed by the same letter are not significantly different ($p<0.05$) according to DMRT.

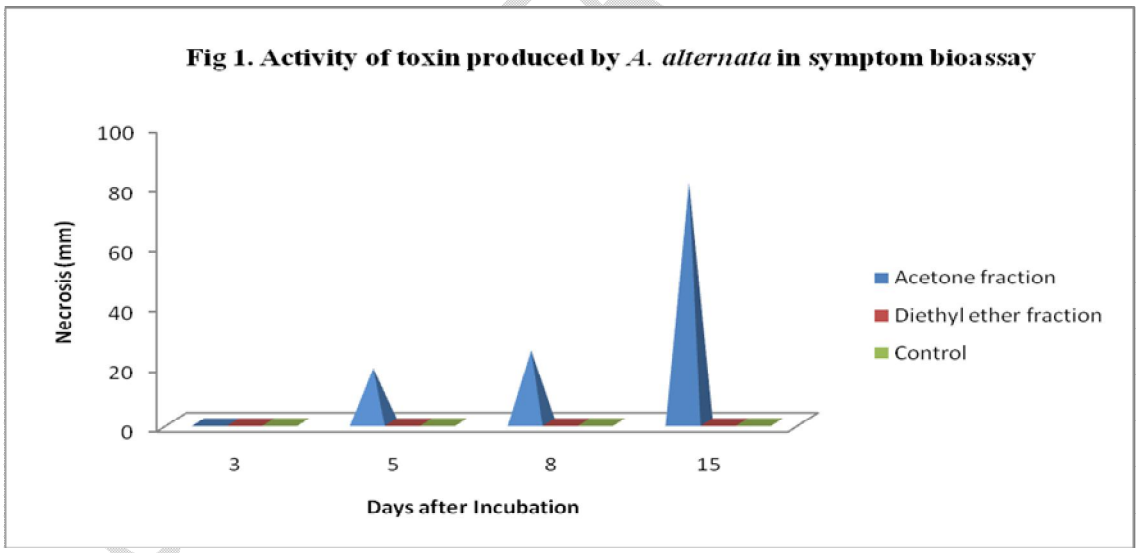
Table 2. Growth of *T. viride* in toxin of *A. alternata*

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]

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}

Means in columns followed by the same letter are not significantly different ($P < 0.05$) according to DMRT.



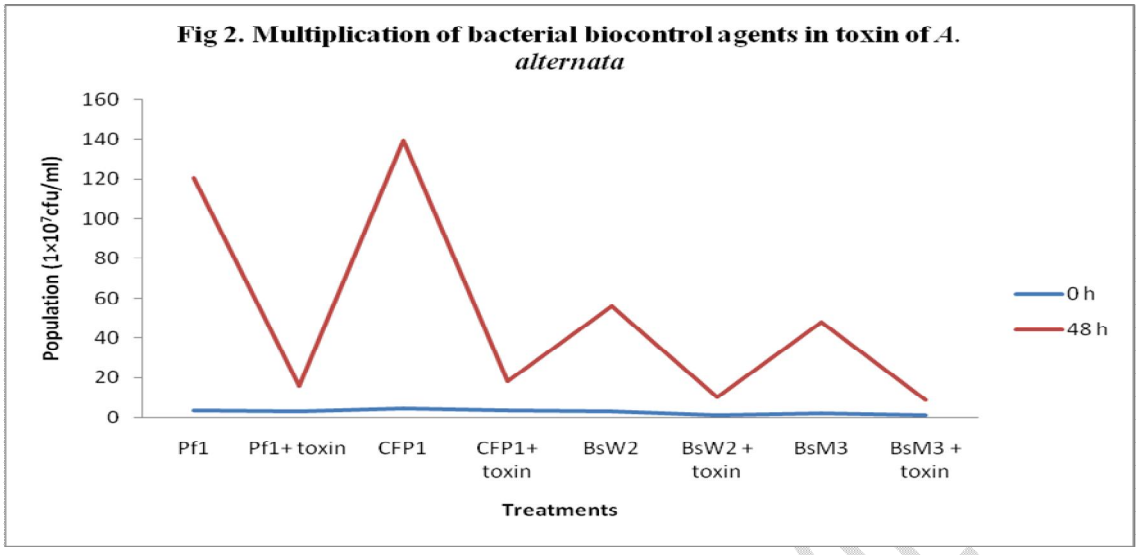


Plate 1: Symptoms bioassay with toxin produced by *Alternaria alternata* – Acetone fraction