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Isolation and Characterization of Toxin from *Alternaria helianthi* Inciting Blight in Sunflower

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A B S T R A C T

Alternaria helianthi the causal agent of sunflower leaf blight was isolated from a monoconidial culture derived from blight infected sunflower leaves of MSFH-8.*Alternariahelianthi* was first cultured on PDA and then transferred to Potato Dextrose broth. Toxin was purified from the cultural filtrate by treatment with organic solvents. The identity of the toxin was established by estimating carbohydrate and protein content. Based on the quantity of carbohydrate and protein the toxin was identified as glycoproteinaceous in nature. The toxin inhibited seed germination and affected root and shoot growth.

Introduction

Sunflower (*Helianthus annuus* L.) is a major oilseed crop in India and other countries like Australia, Africa, USA and Yugoslavia. The increase in area and production is concomitant with an increase in the incidence and severity of foliar diseases. Among the major foliar diseases, Alternaria leaf blight caused by *Alternaria helianthi* (Hansf.) Tubaki and Nishihara is an economically important disease and is reported to cause up to 80 per cent losses particularly in *Kharif* season (Balasubrahmanyam and Kolte, 1980; Amaresh 1997). Several toxins produced by microorganisms were reported to be responsible for the induction of diseases in plants. These microorganisms produce toxic metabolites in culture media and in plant tissues which are involved in the disease syndrome (Owens, 1969; Wood *et al.*, 1972). Several species of Alternaria are known to produce different types of toxic metabolites (Otani*et al.*, 1974: Bhaskaran and Kandaswamy 1978). Attempts were made to isolate the toxin produced by *A. helianthi* and study the effect of toxin on seed germination and seedling growth.

Materials and Methods

The pure culture of *A. helianthi* was isolated from a monoconidial culture derived from blight infected sunflower leaves of MSFH-8. *Alternaria helianthi* was first cultured on PDA and then transferred to Potato Dextrose

broth. Potato Dextrose broth was dispensed at the rate of 200 ml per 500 ml conical flask. After sterilising them, one cm disc of the fungus from periphery of seven days old culture which was grown on Potato Dextrose Agar medium was inoculated and incubated at 27 ± 1^0C for 30 days. Mycelial mats were separated from the broth culture by filtration through cheese cloth and finally it was filtered by using Whatman No. 42 filter paper. Further, culture filtrate was centrifuged at 12000 rpm for 10 minutes to remove spores. The supernatant was reduced to $1/5^{th}$ of its original volume by evaporating at 40^0C in hot water bath. Gradually two volumes of acetone was added to the culture filtrate with constant stirring till the precipitation was completed and allowed to stand overnight at 4^0C. The precipitate was separated by centrifugation at 20000 rpm for 10 min at 4^0C and discarded. The acetone was removed from the supernatant liquid by hot water bath (40^0C).

This solution was extracted three times with two parts of water saturated 1-butanal by centrifugation at 20000 rpm for 10 min at 4^0C. The water phase was discarded and the butanol phase were combined and kept on hot water bath (40^0C) till complete dryness.

This solution was extracted twice with 400 ml aliquots of diethyl ether and the ether phases were discarded by centrifugation at 20000 g for 10 min at 4^0C. The water phase was taken to dryness by hot water bath. Completely moisture was removed and dried product was stored in clean and air dried bottle. The toxin was recovered as a brown powder.

Pure toxin of 3 mg was dissolved in 1.0 ml of sterilised distilled water to get 3000ppm concentration. This solution was diluted with distilled water to get 2000, 1000, 500, 200 and 100 ppm of toxin concentrations. To assess the phytotoxicty of toxin, the sunflower seeds were surface sterilized with sodium hypochlorine (2.0% NaOCl) solution and washed in sterile distilled water. Effects of toxin on seed germination and seedling growth was studied by soaking sunflower seeds at different concentration of toxin *viz.*, 100, 200, 500, 1000, 2000 and 3000 ppm for 1 hr and sterile distilled water served as control. One hundred seeds were placed on moist blotters in petri plates and incubated at 27 ± 1^oC for seven days.

Composition of the toxin produced by *Alternaria helianthi*

Carbohydrate

One ml of the toxic filtrate of the fungus and partially purified fraction in diethyl ether and acetone extracts were kept in a boiling water bath with 0.5 ml of 2.5 N HCl for 3 h for hydrolysis and cooled to room temperature. The sample was neutralised with sodium carbonate until the effervescence ceased. The volume was made up to 25 ml and centrifuged at 10 000 rpm for 30 min. From that 1 ml of supernatant was taken and 4 ml of anthrone reagent was added and heated for 8 min in boiling water bath. The change in colour of the sample from green to dark green was read at 630 nm. The carbohydrate content in the samples was calculated using a glucose standard graph (Hedge and Hofreiter 1962).

Protein

The protein content of the toxin was estimated by the *Lowryet al.,* (1951) method. Pipette out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of test tubes. Pipette out 0.1ml and 0.2 ml of the sample extract in two other test tubes Make up the volume to 1ml in all the test tubes. A tube with 1ml of water serves as the blank. Add 5ml of reagent C to each tube including the blank. Mix well and allowed to stand for 10 min. Then add 0.5ml of reagent D, mix well and incubate at room temp in the dark for 30 min. Blue colour is developed. Take the readings at 660nm.

Table.1 Effect of purified toxin on seed germination and seedling growth of sunflower

Sl. No.	Toxin concentrations (ppm)	Seed germination		Shoot growth		Root growth	
		Per cent of seed germination	Per cent inhibition of seed germination over control	Shoot growth (mm)	Per cent inhibition of seedling growth over control	Root growth (mm)	Per cent inhibition of seedling growth over control
1.	100	100.00 (89.42)	0.00 (0.00)	11.90 (20.17)	4.03 (11.19)	10.58 (18.98)	0.66 (4.66)
2.	200	96.33 (78.98)	3.66 (11.01)	11.06 (19.43)	11.02 (19.35)	10.37 (18.78)	2.66 (9.27)
3.	500	85.68 (67.80)	14.32 (22.19)	9.33 (17.78)	24.72(29.80)	8.23 (16.66)	22.74 (28.43)
4.	1000	76.33 (60.89)	23.67 (29.10)	7.16 (15.52)	41.91 (40.33)	6.37 (15.20)	35.48 (36.55)
5.	2000	57.32 (49.23)	42.65 (40.76)	5.60 (13.68)	54.76 (47.74)	4.91 (12.79)	53.91 (47.24)
6.	3000	24.66 (29.77)	75.34 (60.22)	2.53 (9.15)	79.57 (63.14)	2.15 (8.43)	79.94 (63.40)
7.	Control	100.00 (89.42)	0.00 (0.00)	12.40 (20.61)	0.00 (0.00)	10.65 (19.05)	0.00 (0.00)

****** Figures in the parentheses indicate arcsine transformed values.

Draw a standard graph and calculate the amount of protein in the sample

Results and Discussion

The toxin was isolated from liquid culture grown on potato dextrose broth. The carbohydrate content of the toxin was 44.00 mg/100mg, whereas the protein was 4.18 mg/100mg. Since the toxin contained both carbohydrate and protein, it was glycoproteinaceous in nature. The glycoproteinaceous nature of the fungal toxin has also been reported to be produced by *Rhizoctonia solani* in rice (Vidhyasekaran *et al.,* 1997) and *Corynespora casiicola* in *Phyllanthus amarus* (Mathiyazhagan *et al.,* 2005).

The present study is in agreement with work of Uma Maheswari and Sankaralingam (2010) who extracted toxin from *Alternaria alternata* and identified that the carbohydrate content of the toxin was greater, ranging from 1975 to 2950 µg/ml, whereas the protein was lower, ranging from 117 to 175 µg/ml. They identified the toxin as glycoprotein nature.

The toxin inhibited germination of sunflower seeds at all the six concentrations tested (Table 1). Per cent of seed germination ranged from 24.66 to 100per cent. Toxin at 100ppm concentration didn't affect seed germination while toxin concentration of 200 ppm to 3000 ppm affected seed germination.

At 200 ppm the seed germination was 96.33 per cent while at 3000 ppm seed germination was 24.66 per cent. Minimum length of sunflower shoot (2.53 mm) and root length (2.15 mm) was observed at 3000 ppm. The present findings are in agreement with Islam and Maric (1980), Robeson and Strobel (1985), Amaresh and Nargund (2005), who reported that the toxin produced by *A. helianthi* inhibited seed germination and root elongation in sunflower.

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