

RESEARCH ARTICLE

Bioassay of toxin produced by *Alternaria helianthi* causing leaf blight of sunflower

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ABSTRACT

Sunflower is an important oilseed crop of India. The crop is known to suffer from many diseases among which *Alternaria* leaf blight is one of the most important diseases. *Alternaria helianthi* was isolated from the leaf samples collected during *Khari*. The toxin produced by *Alternaria helianthi* in potato dextrose broth was purified and based on its carbohydrate and protein content, the toxin was identified as glycoprotein. Toxin at 100 ppm concentration didn't affect seed germination while toxin concentration of 200 ppm to 3000 ppm affected seed germination. Minimum seed germination of 24.66 per cent was recorded when seeds were treated with toxin @ 3000 ppm. Minimum length of sunflower shoots (2.53 mm) and root length (2.15 mm) was observed at 3000 ppm. Sunflower seedlings placed in 100 ppm toxin showed curling of leaves and wilting after 24 hr. whereas at 3000 ppm seedlings showed curling of leaves in 5 h and wilted within 12 hr. In detached leaf assay, 100 ppm toxin didn't produce any symptom. However, at concentrations of 500, 1000, 2000 and 3000 ppm, the toxin produced typical necrotic spots without yellow halo on sunflower leaves.

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INTRODUCTION

Karnataka, popularly known as 'Sunflower state of India' occupies first position accounting 54 per cent of total acreage and contributing 35 per cent of the total production of India, with the productivity of 391 kg per ha (Anonymous, 2011). Gulya and Masirevic (1991) listed 80 pathogens occurring on sunflower. Among this *Alternaria* leaf spot caused by *Alternaria helianthi* (Hansf.) Tubaki and Nishihara has been considered as potentially destructive disease in many parts of the sunflower growing countries (Allen *et al.*, 1983; Morris *et al.*, 1983; Lipps and Herr, 1986).

Hansford (1943) first reported the fungus causing leaf spot of sunflower from Uganda. Since then this disease has been reported from almost all sunflower growing countries throughout the world. The severity is known to increase or decrease depending upon the changing environmental conditions during the crop growth period.

The first record of this disease in India was simultaneously reported by Narain and Saksena (1973) and Kolte and Mukhopadhyay (1973) from Uttar Pradesh and subsequently by Anil kumar *et al.* (1974) from Karnataka. It occurred in epidemic form with the disease severity as high as 95-100 per cent during 1988 in Southern India especially in Karnataka destroying a major area of the crop due to heavy unexpected rain during flowering and grain filling stage.

The disease has been known to cause reduction in flower size, number of seeds per head, seed yield per plant, seed weight and also oil content (Balasubramanyam and Kolte, 1980). The loss in yield varies from 11.30 to 73.33 per cent depending on the extent of infection (Reddy and Gupta, 1977). In Northern Karnataka, *Alternaria* leaf spot is known to cause more than 80 per cent of the yield loss under severe epiphytotic conditions (Hiremath *et al.*, 1990; Balasubramanyam and Kolte, 1980; Amaresh, 1997).

Several toxins produced by microorganisms have been reported to be responsible for the induction of diseases in plants. The 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 (Bhaskaran and Kandaswamy, 1978).

The present investigation was directed towards the isolation and bioassay of the toxin produced by *Alternaria helianthi* and its effect on seed germination and plant growth.

MATERIAL AND METHODS

The present investigation was carried out during 2010 - 2011 at the All India Coordinated Research Project on Sunflower, Zonal Agricultural Research Station, GKVK, University of Agricultural Sciences, Bengaluru.

Isolation of the pathogen:

The leaves of sunflower infected with *Alternaria helianthi* showing typical dark brown to black, circular to irregular spots were collected from the field in *Kharif* 2010 and brought to the laboratory for isolation of the causal agent.

The leaf spot infected specimen was microscopically examined for confirmation of the fungus. Sections of the diseased leaves were made with the help of a sharp blade on a clean glass slide having a drop of lacto phenol. The slide was then covered with a cover slip and observed under microscope. After confirmation of fungal spores, isolation was done in the laminar air flow chamber under aseptic conditions by following the standard tissue isolation procedure. Infected leaves exhibiting typical *Alternaria* leaf spot symptoms were selected and pathogen was isolated by following standard isolation method. The infected leaf bits along with healthy leaf tissue measuring about 2mm were washed well in running tap water. These bits were surface sterilized with 0.1 per cent mercuric chloride solution for one minute. The bits were then washed thoroughly in sterile distilled water three times to remove the traces of mercuric chloride and then aseptically transferred to sterile Petri plates containing potato dextrose agar (PDA) (3 pieces/dish). These plates were incubated at $27\pm 1^{\circ}$ C. After 7-8 days, the growth of the fungus was observed to know the association of the fungus with the leaf spot. A loopful of fungal culture developed on potato dextrose agar medium in the Petri plates was taken on a glass slide and observed under the microscope for the presence of conidia. From such Petri plates the growing hyphal tip portion was transferred to PDA in Petri plates under aseptic condition and incubated at $27\pm 1^{\circ}$ C for a week. The pure culture of the fungus was made by transferring spores of *A. helianthi* to PDA slants.

Maintenance of culture :

The maintained pure culture was sub-cultured on potato

dextrose agar slants and allowed to grow for one week at $27\pm 1^{\circ}$ C. Such slants were stored in a refrigerator at 4° C and again sub-cultured once in a month during the course of investigation under aseptic conditions to maintain the viability of the pathogen.

Isolation of toxin produced by *Alternaria helianthi* :

The method described by Karr *et al.* (1974); Bhaskaran and Kandaswamy (1978) and Amaresh and Nargund (2005) were followed for the isolation of *A. helianthi* toxin in the laboratory. The pure culture of *A. helianthi* was isolated from a monoconidial culture derived from blight infected sunflower leaves of KBSH 44. *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 sterilizing 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 $27\pm 1^{\circ}$ C 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 1/5th of its original volume by evaporating at 40° C 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° C. The precipitate was separated by centrifugation at 20000 rpm for 10 min at 4° C and discarded. The acetone was removed from the supernatant liquid by hot water bath (40° C). This solution was extracted three times with two parts of water saturated 1-butanol by centrifugation at 20000 rpm for 10 min at 4° C.

The water phase was discarded and the butanol phase were combined and kept on hot water bath (40° C) 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 rpm for 10 min at 4° C. 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.

Preparation of different concentrations of toxin :

Pure toxin of 3000 μ g was dissolved in 1.0 ml of sterilized distilled water to get 3000 ppm concentration. This solution was diluted with distilled water to get 2000, 1000, 500, 200 and 100 ppm of toxin concentrations and used in further studies.

Effect of toxin on sunflower seed germination :

This experiment was conducted as per the procedure followed by Anahosur (1976); Amaresh and Nargund (2005). Three hundred healthy KBSH 44 sunflower seeds were soaked in 100 ml of 100, 200, 500, 1000, 2000 and 3000 ppm of

concentrations of toxin separately for 1 h. They were then placed on sterile Petri plate lined with three layers of moist filter paper. Each treatment was replicated three times containing 100 seeds. Equal numbers of healthy seeds were soaked in the sterilized distilled water which served as control. Observations on germination percentage of sunflower seeds were recorded after seven days. The per cent inhibition of seed germination was calculated by the formula given by Vincent (1947):

$$\text{Per cent inhibition of germination} = \frac{C - T}{C} \times 100$$

where;

C = Number of seeds germinated in control

T = Number of seeds germinated in treatment

Further, observations on root and shoot growth were recorded in each treatment.

Effect of toxin sunflower seedlings :

Effects of toxin at different concentrations viz., 100, 200, 500, 1000, 2000 and 3000 ppm, were tested on sunflower seedlings (KBSH 44). The healthy 25 days old seedlings of sunflower plants were placed in toxin of 10 ml solution. A control was maintained using distilled water. Effect of toxin on seedlings was recorded.

Bioassay of toxin by detached leaf assay :

One drop of toxin of at different concentration viz., 100, 200, 500, 1000, 2000 and 3000 ppm was spot inoculated on the third or fourth leaves from the top collected from 30 days old plants of KBSH 44 in pots. Appropriate controls with distilled water were also maintained. Each treatment was replicated three times. These inoculated leaves were placed in Petri plate (150 mm) lined with two layers of moistened blotting papers. Effects of toxin, on this leaf were recorded after 72 h. after inoculation.

Statistical analysis :

Statistical analysis was carried out as per the standard

procedure. Data in percentage were transformed to arc sine and square root values and analysis was done using M-Stat C.

RESULTS AND DISCUSSION

The results of the present investigation as well as relevant discussion have been summarized under following heads :

Isolation and identification of the pathogen :

Isolation of the fungus from leaf spot areas of infected plants collected from the field during the study revealed the presence of the pathogen *Alternaria helianthi*. The conidia were cylindrical to long ellipsoidal straight or curved slightly pale yellow to pale brown 2-10 septate (avg. 5 septa), usually with transverse or occasionally with longitudinal septa and some what rounded at both the ends.

Isolation of toxin produced by *Alternaria helianthi* :

The pure culture of *A. helianthi* isolated from a monoconidial culture from sunflower leaves of KBSH 44 was cultured on Potato dextrose agar medium and was further, grown on Potato dextrose broth. Crude toxin was purified from culture filtrate and culture filtrate was centrifuged at 12000 rpm for 10 minutes to remove spores. The supernatant was reduced to 1/5th of its original volume by evaporating at 40°C 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°C. The precipitate was separated by centrifugation at 20000 rpm for 10 min at 4°C and discarded. The acetone was removed from the supernatant liquid by hot water bath (40°C). This solution was extracted three times with two parts of water saturated 1-butanol by centrifugation at 20000 rpm for 10 min at 4°C. The water phase was discarded and the butanol phase were combined and kept on hot water bath (40°C) till complete dryness. This solution was extracted twice with 400 ml aliquots of diethyl ether and the ether phases were discarded by

Table 1 : Effect of toxin on seed germination

Sr. No.	Toxin concentration (ppm)	Per cent of seed germination	Per cent inhibition of seed germination over control
1.	100	100.00 (89.42)**	0.00 (0.00)
2.	200	96.33 (78.98)	3.66 (11.01)
3.	500	85.68 (67.80)	14.32 (22.19)
4.	1000	76.33 (60.89)	23.67 (29.10)
5.	2000	57.32 (49.23)	42.65 (40.76)
6.	3000	24.66 (29.77)	75.34 (60.22)
7.	Control	100.00 (89.42)	0.00 (0.00)
S.Em±			0.86
CD@1%			2.61

** Figures in the parentheses indicate arcsine values

centrifugation at 20000 rpm for 10 min at 4°C. 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. From 1000 ml of culture filtrate, four gram of brown colour toxin powder was obtained.

Composition of toxin produced by *A. helianthi* :

The toxin was known to contain carbohydrate and protein. The carbohydrate content of the toxin was (44.00 mg/100mg of toxin) greater than protein (4.18 mg) content. Since, the toxin contained both carbohydrate and protein, it was glycoproteinaceous in nature.

Effect of toxin on sunflower seed germination :

The toxin of *A. helianthi* inhibited the germination of sunflower seeds at all six concentrations tested (Table 1). The per cent germination of sunflower seeds varied significantly at different concentrations of the toxin tested. At concentrations of 100 and 200 ppm, cent per cent and 96.33 per cent seed germination was noticed. At 500 ppm, 85.63 per cent and at 1000 ppm, 76.33 per cent seeds germination was noticed. Only 57.32 per cent of sunflower seeds germinated at 2000 ppm, which reduced to 24.66 per cent in 3000 ppm

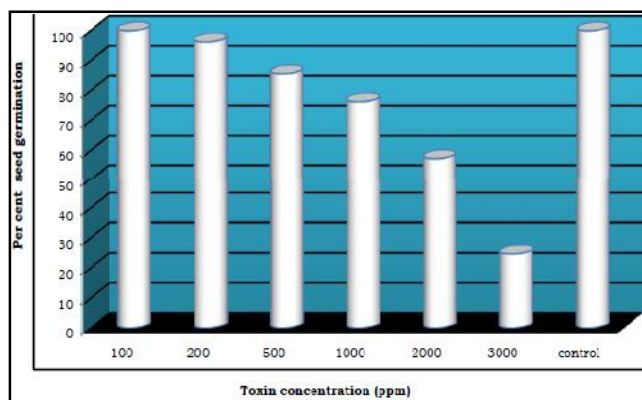


Fig. 1 : Effect of toxin on sunflower seed germination

concentration (Fig 1).

Seedling growth at different concentrations of toxin was found to be significant (Table 2 and 3) and (Fig. 2 and 3). Maximum length of shoot (11.90 mm) and root length (10.58 mm) was noticed at concentration 100 ppm concentration. At 2000 ppm concentration, shoot (5.60 mm) and root length (4.91mm) was observed. Minimum length of sunflower shoot (2.53 mm) and root length (2.15 mm) was observed at 3000 ppm concentration (Fig. 4 and 5).

Sr. No.	Toxin concentrations (ppm)	Root growth (mm)	Per cent inhibition of seedling growth over control
1.	100	10.58 (18.98)**	0.66 (4.66)**
2.	200	10.37 (18.78)	2.66 (9.27)
3.	500	8.23 (16.66)	22.74 (28.43)
4.	1000	6.37 (15.20)	35.48 (36.55)
5.	2000	4.91 (12.79)	53.91 (47.24)
6.	3000	(2.15 (8.43)	79.94 (63.40)
7.	Control	10.65 (19.05)	0.00 (0.00)
S.Em ±			0.18
CD@1%			0.55

** Figures in the parentheses indicate arcsine values

Sr. No.	Toxin concentrations (ppm)	Shoot growth (mm)	Per cent inhibition of seedling growth over control
1.	100	11.90 (20.17)	4.03 (11.19)**
2.	200	11.06 (19.43)	11.02 (19.35)
3.	500	9.33 (17.78)	24.72 (29.80)
4.	1000	7.16 (15.52)	41.91 (40.33)
5.	2000	5.60 (13.68)	54.76 (47.74)
6.	3000	2.53 (9.15)	79.57 (63.14)
7.	Control	12.40 (20.61)	0.00 (0.00)
S.Em±			0.16
CD@1%			0.50

** Figures in the parentheses indicate arcsine values

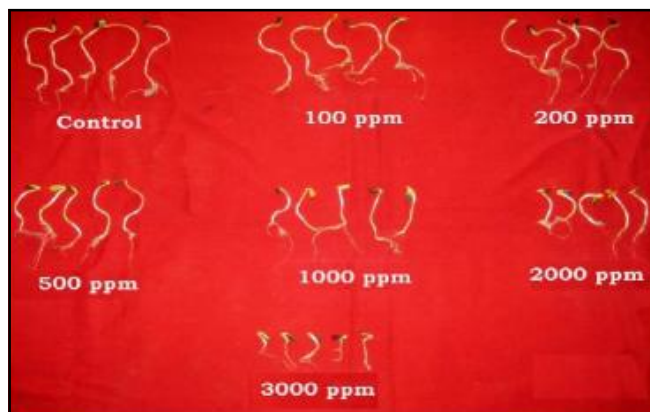


Fig. 2 : Effect of toxin on shoot and root growth

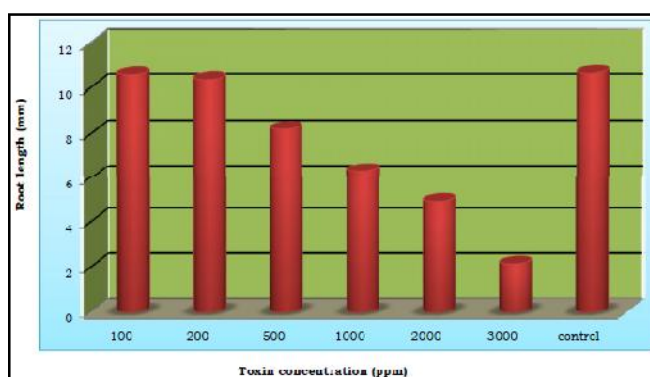


Fig. 3 : Effect of toxin on sunflower root growth

Effect of toxin on sunflower seedlings :

Effect of toxin at different concentrations viz., 100, 200, 500, 1000, 2000 and 3000 ppm tested on sunflower seedling showed that at concentrations of 500 and 200 ppm, curling of leaves and wilting was prolonged for more than 24 hr and seedlings wilted within 36h. The sunflower seedlings which were placed in concentration viz., 500, 1000, 2000 and 3000 ppm showed curling of leaves, within 5 -15 hr and wilted within 12 - 18 hr (Table 4 and Fig. 4).

Bioassay of toxin on sunflower by detached leaf method :

The effect of different concentrations of toxin viz., 100,



Fig. 4 : Effect of toxin on sunflower seedlings

200, 500, 1000, 2000 and 3000 ppm was tested by detached leaf assay, which revealed that at concentrations of 100 and 200 ppm of the purified toxin was not capable of producing symptoms. However, at concentrations of 500, 1000, 2000 and 3000 ppm the toxin produced typical necrotic spots without yellow halo on sunflower leaves. No symptoms were developed on leaves inoculated with sterile distilled water. Thus, the role of toxin in the pathogenesis was confirmed by the production of necrotic spots on inoculation of toxin to leaves (Fig. 5).

Isolation of the pathogen:

The colonies of *Alternaria helianthi* on PDA were dark green coloured, with olivaceous green mycelium, conidiophores were septate, smooth and branched. The fungus produced cylindrical to long ellipsoidal conidia. This showed that this fungus is similar to the one described by Narasimha Rao and Rajagopalan (1977) who reported the morphology of *Alternaria helianthi*, as mycelial colonies on Potato dextrose agar were dark, profusely branched and frequently septate. The conidiophores were cylindrical, often branched, septate and difficult to be distinguished from the mycelium. Conidia were golden yellow or dark brown, ellipsoidal having tapered apex. Tubaki and Nishihara (1969) described the morphology of the pathogen. Mycelium was olivaceous green, septate, smooth and branched. The

Table 4 : Duration taken for symptom expression in sunflower seedlings by using toxin

Sr. No.	Toxin concentration (ppm)	Time taken (hr)	
		Curing of leaves	Wilting
1.	100	24	>24
2.	200	20	24
3.	500	15	18
4.	1000	10	15
5.	2000	7	12
6.	3000	5	<12
7.	Control	-	-



Fig. 5 : Bioassay of toxin on detached leaves

conidiophores were cylindrical, scattered or gregarious, pale grey yellow upto five septa, geniculate, single or branched.

Toxin studies :

Many plant pathogenic fungi produce toxic metabolites in culture media and in plant tissues which take part in the pathogenesis and symptom expression (Wood *et al.*, 1972). *Alternaria helianthi* produced a toxin *in vitro* and in plant tissues and was reported by early workers (Bhaskaran and Kandaswamy, 1978; Islam and Maric, 1980; Robeson and Strobel, 1982, and Amaresh and Nargund, 2005). *Alternaria helianthi* toxin was isolated from standard Potato dextrose broth as pure toxin and recovered as brown powder. The toxin was known to contain carbohydrate and protein. The carbohydrate content of the toxin (44.00 mg/100mg of toxin) was greater than protein (4.18 mg) content. Since, the toxin contained both carbohydrate and protein, it was glycoproteinaceous in nature. The present study is in

agreement with work of Uma Maheswari and Sankaralingam (2010) who extracted toxin from *Alternaria alternata* and identified that carbohydrate content of the toxin was greater, ranging from 1975 to 2950 ig/ml, whereas the protein was lower, ranging from 117 to 175 ig/ml. Since, the toxin contained both carbohydrate and protein, it was glycoproteinaceous in nature. Similar observations were made by Mathiyazhagan *et al.* (2005). They reported for the first time on *Phyllanthus amarus*, *Corynespora. cassiicola* producing glyco-proteinaceous toxins responsible for the symptoms similarly produced by the pathogen. A preliminary indication of toxin production by any fungus *in vitro* has been provided by a number of bioassay methods *viz.*, bioassay methods of plant cutting (Rai and Strobel, 1969), seed germination bioassay (Anahosur, 1976) and root and shoot elongation bioassay (Anahosur, 1976). In the present study, purified toxin obtained from *A. helianthi* was subjected to all the above bioassay methods to identify the characters of toxin.

On sunflower seedlings, toxin concentrations at 100 and 200 ppm, showed curling of leaves and wilting was prolonged for more than 24 hr and seedlings wilted within 36 hr. The sunflower seedlings which were placed in concentrations 500, 1000, 2000 and 3000 ppm showed curling of leaves, within 5 -15 hr and wilted within 12 - 18 hr. These results are in agreement with observation of Bhaskaran and Kandaswamy (1978), Patil, (1989) and Amaresh and Nargund (2005), who have reported toxicity of culture filtrate of *A. helianthi*. Mahabaleswarappa (1981) also made similar observation while working with *Alternaria carthami* on safflower. Kumar *et al.* (1991) also showed toxin production by using detach leaf bioassay technique method. At concentrations of 100 and 200 ppm, cent per cent and 96.33 per cent seed germination was noticed. At 500 ppm, 85.63 per cent and at 1000 ppm, 76.33 per cent seeds germination was noticed. Only 57.32 per cent of sunflower seeds germinated at 2000 ppm. which reduced to 24.66 per cent in 3000 ppm concentration. Maximum inhibition of sunflower shoot (2.53 mm) and root length (2.15 mm) was observed in 3000 ppm toxin concentration. The present findings are in agreement with Islam and Maric (1980), Robeson and Strobel (1982), Amaresh and Nargund (2005), who reported that the toxin produced by *A. helianthi* inhibited seed germination and root elongation in sunflower. At concentrations of 100 and 200 ppm, toxin was not capable of producing symptoms. However, at concentrations of 500, 1000, 2000 and 3000 ppm the toxin produced typical necrotic spots without yellow halo on sunflower leaves. Thus, the role of toxin in the pathogenesis was confirmed by the production of necrotic spots on inoculation of toxin to the leaves. No symptoms were developed on leaves inoculated with sterile distilled water. Leaf blight severity varied from 2 to 60 per cent in different plants in glass house seven days after inoculation of toxin.

No symptoms were observed when the plants were sprayed with 100 ppm concentration. At concentrations at 200 and 500 ppm the severity of leaf blight was 2.16 per cent and 8.32 per cent, respectively. The severity of leaf blight was maximum (53.24 %) in plants sprayed with 3000 ppm toxin. These results are in agreement with Bhaskaran and Kandaswamy (1978) who reported toxin production of *Alternaria helianthi* *in vitro* and toxin produced typical symptoms of the disease when inoculated on the leaves of *Helianthus annuus* except the yellow halo around necrotic spot. Similar results were obtained by Islam and Maric (1980); Robeson and Strobel (1982) and Amaresh and Nargund, (2005). Uma Maheswari and Sankaralingam (2010) extracted toxin from *Alternaria alternata*, the causal agent for leaf blight in watermelon (*Citrullus lanatus*) and the toxin produced typical symptoms such as that of the pathogen on detached watermelon leaves.

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