

RESEARCH ARTICLE

Isolation of toxin produced by *Alternaria helianthi* causing blight in sunflower

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ABSTRACT

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 further grown on Potato dextrose broth. Crude toxin was purified from 1000 ml of culture filtrate. The toxin was recovered as a brown powder. The toxin contained both carbohydrate and protein and was glycoproteinaceous in nature.

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INTRODUCTION

Sunflower (*Helianthus annuus* L.), a member of Asteraceae, is a major edible oilseed crop of importance after soybean and groundnut at the global level. Sunflower oil is a combination of mono-unsaturated and poly-unsaturated fatty acids with low saturated fat levels. The versatility of this oil is recognized by cooks all over the world. It supplies more vitamin E than any other vegetable oil. Sunflower oil is odourless, which makes it useful in fragrance, aromatherapy and massage oil products. Sunflower oil is almost colourless making it ideal for use in cosmetics and other coloured products. Gulya and Masirevic (1991) listed 80 pathogens occurring on sunflower. Among these, *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).

The fungus produces a toxic metabolite, presumably a phenolic compound, both in culture and in infected leaf tissues. Necrotic spots on leaves were produced when the toxin was

sprayed on leaves but spots were not characteristic of those naturally produced by the pathogen. 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 were involved in the disease syndrome (Owens, 1969; Wood *et al.*, 1972). Several species of *Alternaria* were known to produce different types of toxic metabolites Bhaskaran and Kandaswamy, 1978).

Therefore, the present investigation was directed towards the isolation and composition of the toxin produced by *Alternaria helianthi*.

MATERIALS AND METHODS

Isolation of the pathogen :

Media preparation :

Potato dextrose agar medium was used for isolation of the pathogen and also in most of the experimental studies. The composition of Potato dextrose agar medium used was as follows :

Potato	:	200 g
Dextrose	:	20 g
Agar agar	:	20 g
Distilled water	:	upto 1000 ml
pH	:	7.2

Two hundred grams of peeled potatoes were cut into small pieces and boiled in distilled water and then the extract was collected by filtering through muslin cloth and 20 grams of dextrose was dissolved in the extract. Then 20 grams of agar agar was melted in half litre of distilled water separately. Both the solutions were mixed and final volume was made to 1000 ml with distilled water, later it was sterilized in an autoclave at 121°C at 15 lbs pressure for 20 minutes and preserved for further use.

Isolation of the pathogen :

The leaves of sunflower infected by *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 tissue isolation method. The infected leaf bits along with healthy leaf tissues measuring about 2 mm 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±10°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±10°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±1°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 Arthur *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 flasks. 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±10°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 40°C. The precipitate was separated by centrifugation at 20000 rpm for 10 min at 40°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 40°C. The water phase was discarded and the butanol phase was 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 40°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.

Composition of toxin produced by *Alternaria helianthi* :

Carbohydrate :

This experiment was conducted as per the procedure followed by Hedge and Hofreiter (1962). The carbohydrate content of the toxin was estimated by the Anthrone method.

Material srequired :

2.5 N-HCL, Anthrone Reagent: Dissolve 200mg anthrone in 100ml of ice cold 95 per cent H₂SO₄. Prepare fresh before use. Standard Glucose: Stock- Dissolve 100 mg in 100 ml water. Working standard- 10 ml of stock diluted to 100 ml with distilled water. Store refrigerated after adding a few drops of toluene.

Procedure :

Weigh 100 mg of the toxin sample into a boiling tube. Hydrolyse by keeping it in a boiling water bath for three hours with 5ml of 2.5 N-HCL and cool to room temperature. Neutralise it with solid sodium carbonate until the effervescence ceases. Make up the volume to 100ml and centrifuge. Collect the supernatant and take 0.5 and 1ml aliquots for analysis. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard. '0' serves as blank. Make up the volume to 1ml in all the tubes including the sample tubes by adding distilled water. Then add 4ml of Anthrone reagent. Heat for eight minutes in a boiling water bath. Cool rapidly and read the green to dark green colour at 630 nm. Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph calculate the amount of carbohydrate present in the sample tube.

Calculation :

$$\text{Amount of carbohydrate present in 100 mg of the sample} = \frac{\text{Mg of glucose}}{\text{Aliquot of test sample}} \times 100$$

Protein :

This experiment was conducted as per the procedure followed by Lowry *et al.* (1951). The protein content of the toxin was estimated by the Lowry's method.

Materials required :

2 per cent Sodium carbonate in 0.1N Sodium hydroxide (Reagent A).- 0.5 per cent copper sulphate (CuSO₄.5H₂O) in 1 per cent potassium sodiumtartrate (Reagent B). Alkaline copper solution: Mix 50 ml of A and 1ml B prior to use (Reagent C). Folin-Ciocalteau reagent (Reagent D). Protein solution

(Stock Standard)- weigh accurately 50 mg of bovineserum albumin(Fraction V) and dissolve in distilled water and make up to 50 ml on a standard flask. Working standard- Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask.

Procedure :

Extraction of protein from sample :

Weigh 100 mg of toxin and add 10 ml of phosphate buffer pH7.0.

Estimation of protein :

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 allow 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 was developed. Take the readings at 660 nm. Draw a standard graph and calculate the amount of protein in the sample

Calculation :

Express the amount of protein mg/g or 100 g sample.

RESULTS AND DISCUSSION

The results of the present study as well as relevant discussions have been presented under following sub 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 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 1000 ml of culture filtrate. The 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. From 1000 ml of culture filtrate, four gram of brown colour toxin powder was obtained.

Composition of toxin produced by *Alternaria 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.

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* produces a toxin *in vitro* and in plant tissues was reported by early workers (Bhaskaran and Kandaswamy (1978), Islam and Maric (1980), Robeson and Strobel (1982), and Amaresh and Nargund (2005).

Since, the toxin contained both carbohydrate and protein, it was glycoproteinaceous in nature. The present study is in agreement with work of 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 µg/ml, whereas the protein was lower, ranging from 117 to 175 µg/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 glycoproteinaceous toxins responsible for the symptoms similarly produced by the pathogen.

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