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**RESEARCH ARTICLE**

# Biochemical characterization of isolates of *Alternaria helianthi* (hansf.) tubaki and nishihara causing sunflower blight

■ **J. RAJENDER, B. PUSHPAVATHI, M. SANTHA LAKSHMI PRASAD AND S. SUMATHI**

## SUMMARY

A pure culture of 25 isolates of *Alternaria helianthi* were collected from IIOR, Rajendranagar, Hyderabad and biochemical nature was tested under *in vitro*. The isolates were characterized based on production of total sugars, total proteins, total free amino acids and phytotoxins. The estimation of all parameters reflected significant variation among all. The isolate Ah-25 produced maximum concentration of total sugar (13.28 mg), while minimum concentration was noticed in Ah-13 (3.10 mg). Similarly, the total proteins content was found highest in the isolate Ah-25 (21.43 mg) and lowest with the isolate Ah-15 (9.53 mg). Among the isolates, the total free amino acids ranged between 5.67 mg (Ah-15) to 21.24 mg (Ah-21). The phytotoxicity of the crude toxin was tested by adopting detached leaf technique at different concentrations. None of the tested isolates have produced symptoms at 50 ppm concentration. However, the typical symptoms of necrotic lesions were observed at 100 ppm with nine isolates (Ah-1, Ah-2, Ah-4, Ah-7, Ah-12, Ah-17, Ah-21, Ah-24 and Ah-25). Among the remaining isolates Ah-3, Ah-5, Ah-6, Ah-9, Ah-10, Ah-11, Ah-16, Ah-18 and Ah-23 showed necrotic symptoms at 200 ppm toxin concentration. Whereas the isolates Ah-8, Ah-13, Ah-14, Ah-15, Ah-19, Ah-20 and Ah-22 resulted in symptom development at 500 ppm concentration. Further, the strains were found to vary in their biochemical composition between all the isolates under the study.

**Key Words :** Sunflower, *Alternaria helianthi*, Phytotoxins

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Sunflower (*Helianthus annuus* L.) is one of the important oilseed crops in the world and it ranks third in area after groundnut, mustard and rapeseed. In India, sunflower is cultivated over an area of 18.1 lakh ha with a production of 11.6 lakh t and productivity of 641 kg $ha^{-1}$ (CMIE, 2009). The major sunflower growing states in India are Karnataka, Maharashtra, Andhra Pradesh, Tamil Nadu and Punjab. Andhra Pradesh is third in position with a cultivated area of 4.3 lakh ha, production of 3.3 lakh t and productivity of 786 kg $ha^{-1}$ (CMIE, 2009). The major sunflower growing districts in Andhra Pradesh are Kurnool, Mahaboobnagar, Anantapur, Nalgonda, Medak and Nizamabad covering Telangana and Rayalseema regions.

Sunflower oil is generally considered as a premium oil compared to most other vegetable oils because of its light colour, bland flavor, high smoke point, high in vitamin E, high level of linoleic acid and absence of linolenic acid *i.e.*, high level cholesterol, a factor which is believed to be related to the incidence of heart diseases. However, the successful cultivation of the crop is constrained by several biotic and abiotic stresses. Among the biotic stresses for successful sunflower production, susceptibility to the Alternaria blight disease caused by *Alternaria helianthi* (Hansf.) Tubaki and Nishihara have been considered as a potentially destructive disease in many parts of the sunflower growing countries (Allen *et al.*, 1983., Morris *et al*., 1983 and Lipps and Herr, 1986). The disease has been reported from different parts of the world including India and is known to cause reduction in plant height, stem girth, flower size, number of seeds per head, seed weight and reduce the seed yield by 27 to 80 per cent, oil yield by 17 to 33 per cent (Mathur *et al*., 1978 and Balasubramanyam and Kolte, 1980). The loss in yield varies from 11.3 to 73.3 per cent depending on the extent of infection (Reddy and Gupta, 1977). The disease also affects seed germination and vigour of seedlings (Kolte *et al.*, 1979). Though several sunflower varieties and hybrids are being released from time to time none of them are showing complete resistance to the disease, indicates the existence of virulent races of the pathogen. Therefore, to detect the most aggressive isolates which have evolved in the recent past, the present studies were conducted on biochemical characterization of *Alternaria helianthi* causing sunflower leaf blight.

## MATERIAL AND METHODS

### Pathogen isolates :

About 150 *Alternaria helianthi* isolates collected during survey from major sunflower growing areas of India, were stored under refrigerated conditions in the form of pure culture at IIOR, Rajendranagar, Hyderabad. Of these 25 isolates representing four important sunflower growing states of India, where sunflower is being cultivated over a sizable area, were selected for the present study (Table 1).

### Biochemical variation studies :

For the estimation of total sugars, total proteins and total free amino acids, all the 25 isolates of *A. helianthi* were grown on potato dextrose broth at 25 ± $2^0$C temperatures for 15 days.

### Preparation of crude extracts :

Crude extracts of the pathogen for studying total sugar, total proteins and total free amino acids were prepared by following the method given by Khurana *et al*. (2005).

### Estimation of total sugars (Sadasivan and Manickam, 1996):

Estimation of total sugars was done by adopting Anthrone method.

### Standard curve for sugars estimation :

Glucose containing 1mg per ml was prepared as a stock solution. From this stock solution 10 ml was pipetted into conical flasks and the volume was made upto 100 ml. This contained the glucose @ 100 µg $ml^{-1}$ and was used as working standard. Standard graph was drawn using the absorbance value of standards by plotting concentration of the standard on the x-axis versus absorbance on the y-axis.

### Estimation of total proteins (Sadasivan and Manickam, 1996):

*Standard graph* :

Standard graph was drawn to calculate the amount of protein in the sample using working standard. The amount of protein was expressed in mg $g^{-1}$ sample.

### Estimation of total free amino acids (Sadasivan and Manickam, 1996) :

Amino acid estimation was done by using ninhydrin

reagent method.

*Standard curve* :

Standard curve was drawn using absorbance versus concentration of samples. The concentration of the total free amino acids in the sample was estimated and expressed in milligrams per gram of the sample.

**Isolation of toxin from isolates of *A. helianthi* :**

Isolation of toxin from isolates of *A. helianthi* was done by adopting the procedure given by Maiero *et al*. (1991) with slight modifications.

**Phytotoxicity of toxins :**

The phytotoxicity of toxin isolated from different isolates of *A. helianthi* was tested on sunflower leaves collected from 30 day-old-plants by adopting detached leaf technique (Dater, 1994). Different concentrations (50, 100, 200 and 500 ppm) of crude toxin were prepared by dissolving the toxin powder in sterile distilled water. The toxin thus, obtained was placed on upper surface of detached sunflower leaves randomly at four places. The inoculated leaves were placed in Petriplates lined with 3 layers of moistened blotting papers. The Petriplates along with inoculated leaves were incubated in an incubator at 25 ± 2 $^0$C temperature and observed daily for the development of symptoms. The isolates exhibited symptoms at lower concentration were not further tested for phytotoxicity at higher concentrations.

**Statistical analysis :**

All the experiments were conducted with three replications and the data were statistically analyzed wherever it is necessary. Suitable controls were maintained for all the experiments conducted. Completely Randomized Design (CRD) was used for analyzing the data obtained on total sugars, total proteins and total free amino acids.

## RESULTS AND DISCUSSION

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

**Table 1: List of 25 isolates of *Alternaria helianthi* collected from different locations of South India states / sources of isolates of *Alternaria helianthi***

| Sr. No. | Isolates code | Place of collection | State |
|---|---|---|---|
| 1. | Ah-1 | Dharwad | Karnataka |
| 2. | Ah-2 | Saundatti | Karnataka |
| 3. | Ah-3 | Mudhol | Karnataka |
| 4. | Ah-4 | Lakkundi | Karnataka |
| 5. | Ah-5 | Koppal | Karnataka |
| 6. | Ah-6 | Raichur | Karnataka |
| 7. | Ah-7 | Hirapur | Karnataka |
| 8. | Ah-8 | Bangalore | Karnataka |
| 9. | Ah-9 | Kolar | Karnataka |
| 10. | Ah-10 | Chitradurga | Karnataka |
| 11. | Ah-11 | Coimbatore | Tamil Nadu |
| 12. | Ah-12 | Valaihalli | Tamil Nadu |
| 13. | Ah-13 | Papakkapatti | Tamil Nadu |
| 14. | Ah-14 | Panjapatti | Tamil Nadu |
| 15. | Ah-15 | Irumudipatti-1 | Tamil Nadu |
| 16. | Ah-16 | Irumudipatti-2 | Tamil Nadu |
| 17. | Ah-17 | Latur | Maharashtra |
| 18. | Ah-18 | Solapur | Maharashtra |
| 19. | Ah-19 | Tandul vadi | Maharashtra |
| 20. | Ah-20 | Tuljapur | Maharashtra |
| 21. | Ah-21 | Jalna | Maharashtra |
| 22. | Ah-22 | Patur | Maharashtra |
| 23. | Ah-23 | ICRISAT | Andhra Pradesh |
| 24. | Ah-24 | Rayalpur | Andhra Pradesh |
| 25. | Ah-25 | Kurnool | Andhra Pradesh |

**Biochemical variability among the isolates of *A. helianthi* :**

The present study was under taken to investigate the variation among the isolates of *A. helianthi* on the basis of biochemical characteristics and the results are presented under the following heads.

**Estimation of total sugars :**

Among the tested isolates of *A. helianthi* considerable variation was found for total sugars across the isolates (Fig. 1). Maximum total sugar content was found in Ah-25 (13.28 mg) (Kurnool, A.P) which was statistically superior over all other isolates, followed by Ah-2 (12.38 mg) and Ah-12 (12.23 mg) while, the minimum was noticed in Ah-13 (3.10 mg) from Papakkapatti, Tamil Nadu.

**Table 2 : Phytotoxicity of isolated toxins from *A. helianthi* isolates on sunflower cultivar Morden leaves using detached leaf technique**

| Isolate | Toxin concentration (in ppm) | | | |
|---|---|---|---|---|
| | 50 | 100 | 200 | 500 |
| Ah-1 | - | + | * | * |
| Ah-2 | - | + | * | * |
| Ah-3 | - | - | + | * |
| Ah-4 | - | + | * | * |
| Ah-5 | - | - | + | * |
| Ah-6 | - | - | + | * |
| Ah-7 | - | + | * | * |
| Ah-8 | - | - | - | + |
| Ah-9 | - | - | + | * |
| Ah-10 | - | - | + | * |
| Ah-11 | - | - | + | * |
| Ah-12 | - | + | * | * |
| Ah-13 | - | - | - | + |
| Ah-14 | - | - | - | + |
| Ah-15 | - | - | - | + |
| Ah-16 | - | - | + | * |
| Ah-17 | - | + | * | * |
| Ah-18 | - | - | + | * |
| Ah-19 | - | - | - | + |
| Ah-20 | - | - | - | + |
| Ah-21 | - | + | * | * |
| Ah-22 | - | - | - | + |
| AH-23 | - | - | + | * |
| Ah-24 | - | + | * | * |
| Ah-25 | - | + | * | * |

(-) No symptoms, (+) Presence of necrotic lesion,
Note: (*) = Not tested

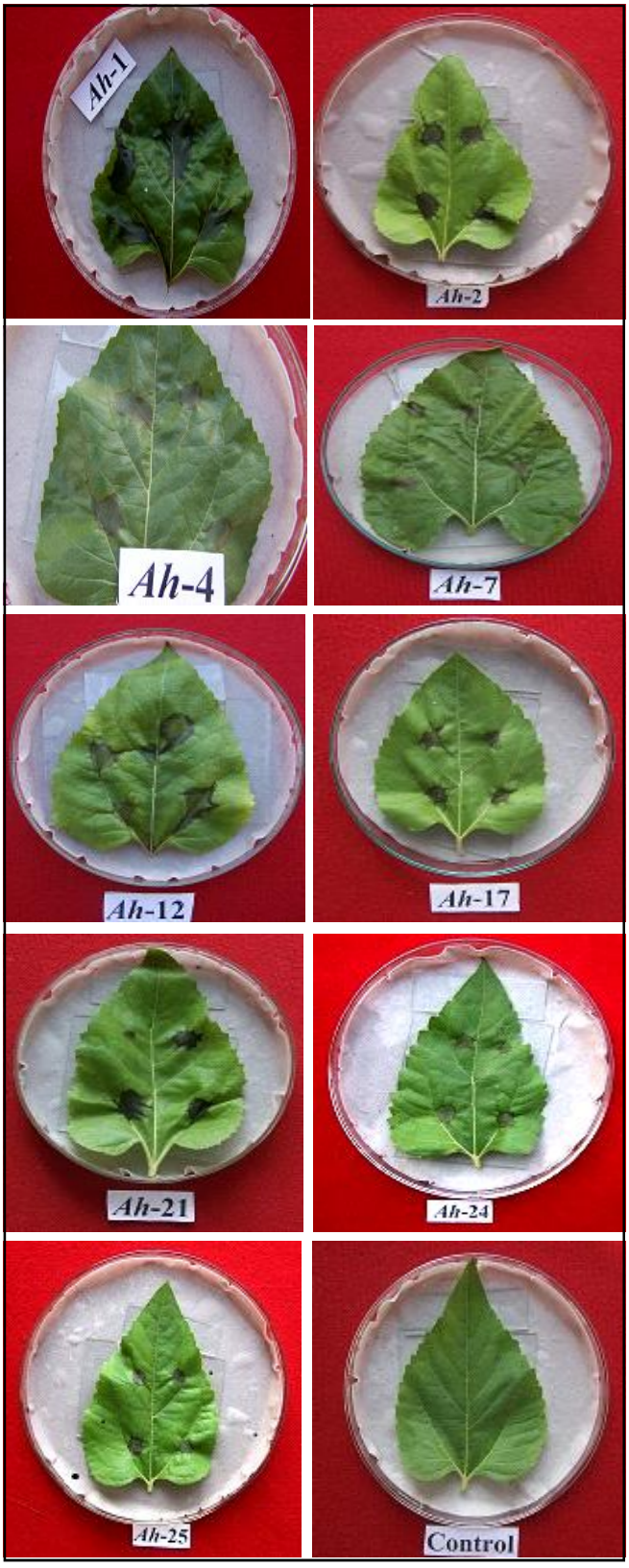


**Fig. 1: Phytotoxicity of crude toxin of nine isolates of *Alternaria helianthi* on sunflower leaves at 100 ppm concentration**

**Estimation of total proteins :**

Protein content of different isolates of *A. helianthi* also showed significant differences between the isolates (Fig. 2). The results indicated that, the isolate Ah-25 (21.43 mg) contained highest quantity of proteins followed by Ah-21 (21.35 mg) which were statistically significant over other isolates, while the isolate Ah-15 (9.53 mg) had lowest quantity of proteins followed by Ah-20 (9.58 mg).

**Estimation of total free amino acids :**

The results pertained to estimation of total free amino acids in 25 isolates of *A. helianthi* are presented in Fig. 2. The data indicated that, across the isolates of *A. helianthi*, the isolate Ah-21 (21.24 mg) showed maximum quantity of free amino acids which was statistically superior over others, followed by Ah-2 (19.74 mg) and Ah-17 (19.27 mg) (Fig.2). The isolates Ah-25 (18.57 mg), Ah-7 (18.22 mg) and Ah-12 (18.12 mg) were at par to each other for free amino acids and differed significantly with rest of the isolates. The minimum quantity of free amino acids was recorded by the isolate Ah-15 (5.67 mg) followed by Ah-20 (5.77 mg), however, these two isolates were collected from two different states (Tamil Nadu and Maharashtra).

In general, majority of the high pathogenic group isolates (Ah-2, Ah-7, Ah-12, Ah-21 and Ah-25) showed more amount of total sugars, proteins and free amino acids when compared to low pathogenic group isolates. The present results are in accordance with the earlier findings of Vishwanath and Kolte (1997). Therefore, it appears that the maximum content of carbohydrates, proteins and free amino acid is an indicative of high virulence nature of pathogen.

Though the taxonomy of fungi based on morphology has been universally accepted as a sound approach in some genera or species it becomes difficult because, morphological characteristics are not distinct enough to differentiate. In such cases, differences in biomolecule composition among different species of the genus have been used as supplementary information to differentiate distinct isolates of fungal pathogens (Vishwanath and Kolte, 1997).

**Phytotoxicity of toxin isolated from *A. helianthi* isolates :**

It is evident from the results presented in Table 2 that none of the 25 isolates did not produce the symptoms at 50 ppm concentration. But the isolates isolates Ah-1, Ah-2, Ah-4, Ah-7, Ah-12, Ah-17, Ah-21, Ah-24 and Ah-25 showed necrotic symptoms (Table 2 and Fig. 1) with 100 ppm toxin on second day of application. However, the symptoms were more prominent in case of isolates Ah-2, Ah-17 and Ah-21 when compared to other isolates. As these nine isolates were exhibiting phytotoxicity at

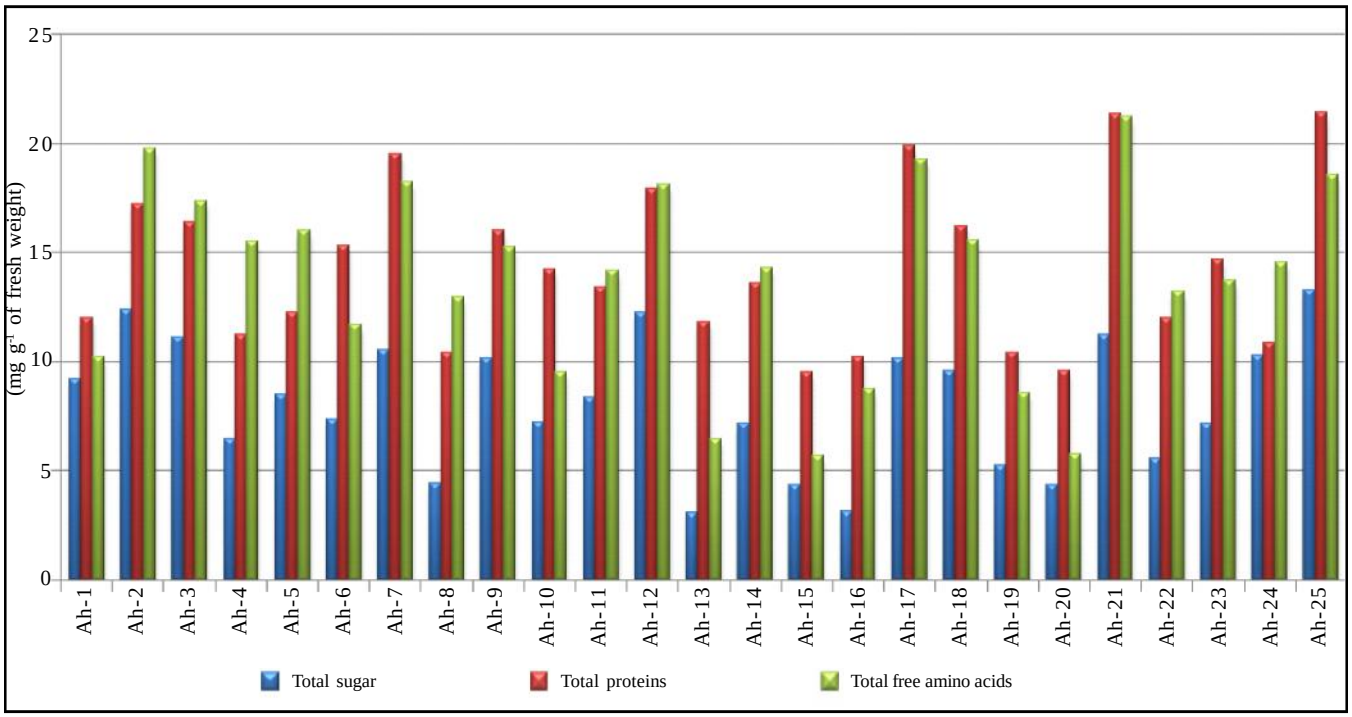


**Fig. 2: Amount of total sugars, proteins and free amino acids of 25 isolates of *A. helianthi***

100 ppm concentration these were not tested at 200 and 500 ppm concentration. At 200 ppm concentration of toxin, the isolate Ah-3, Ah-5, Ah-6, Ah-9, Ah-10, Ah-11, Ah-16, Ah-18 and Ah-23 exhibited necrotic lesions (Table 1), while same lesions were observed with the isolates Ah-8, Ah-13, Ah-14, Ah-15, Ah-19, Ah-20 and Ah-22 at 500 ppm (Table 1).

The present study revealed the presence of active toxic substance in culture filtrates of all the isolates. Variability among the 25 isolates of *A. helianthi* was confirmed by producing the necrotic lesions at different concentration of isolated toxin. The role of crude toxin in the pathogenesis was proved by reproducing typical leaf spot symptoms without any yellow halo. These results are in agreement with Amaresh and Nargund (1999) who recovered the toxin from *A. helianthi* isolates under *in vitro* conditions and confirmed the phytotoxicity of toxin on sunflower leaves by reproducing typical leaf spot symptoms. They also tested its toxic effect on germination of sunflower and sorghum seeds. The phytotoxic effect of toxic metabolites produced by different *A. helianthi* and *A. solani* isolates were also reported by several other earlier researchers on sunflower (Bhaskaran and Kandaswamy, 1978 and Robeson and Strobel, 1985) and tomato (Maiero *et al*., 1991) crops.

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