World Journal of Agricultural Sciences 12 (2): 91-96, 2016
ISSN 1817-3047

DOI: 10.5829/idosi.wjas.2016.12.2.1905

Morphological and Cultural Characters in Determination of Virulence of *Alternaria helianthi* on Sunflower

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Abstract: Ten isolates of *A. helianthi* were collected from different sunflower growing areas of Tamil Nadu and their pathogenicity was proved under laboratory conditions. The most virulent isolates (I_3) was collected from Narnapuram. Variations among different isolates indicated that, I_3 recorded the maximum conidial length (62.16µm), width (15.60 µm), beak length (24.50 µm) and maximum number of conidial cells (3-8). Among different solid and liquid media tested, Potato dextrose agar medium supported the fungus to attain the maximum growth of all the ten isolates of *A. helianthi.*

Key words: Sunflower · *Alternaria helianthi* · Virulence · Solid and Liquid media

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the important oil seed crops in India and belongs to Asteraceae (Compositae). The loss varies from 11.30 to 73.33% depending on the extent of leaf blight pathogen [1]. *Alternaria* leaf blight causes more than 80% of yield loss in northern Karnataka. The productivity of sunflower declined because of diseases like leaf spot, rust, downy mildew, root rot, collar rot and leaf blight *etc*. Among these disease leaf blight caused by *Alternaria helianthi* is one of the major disease [2] causing severe yield loss under epiphytotic condition [3] *A. helianthi* was blackish ash in colour. The colour development was observed after 8-9 days after incubation.

Growth and sporulation of *A. solani* developed well in Potato Dextrose Agar (PDA) medium [4-6]. The conida of *A. helianthi* was clavate shaped with unbranched conidiophores 3-10 transverse and longitudinal septation with rounded at both ends. Hence, the present investigation was conducted to study the cultural characters and morphological variations on the growth of fungus.

MATERIALS AND METHODS

Isolation of Different Isolates of *A. helianthi*: The diseased sunflower leaf showing the typical symptom of leaf blight were collected fresh from 10 sunflower growing areas of Tamil Nadu. The pathogen isolated from each of the localities formed one isolate of *Alternaria helianthi*. The pathogen was isolated on Potato Dextrose Agar (PDA) medium from diseased specimen showing the typical symptoms. The infected portion of leaf was cut into small bits, surface sterilized in 0.1% mercuric chloride solution for 30 sec washed in repeated changes of sterile distilled water and placed on to sterilized PDA medium poured in Sterilized petridishes. The plates were incubated at room temperature (28 ± 2°C) for five days and observed for the fungal growth the fungus was purified by single spore isolation technique and the purified isolates were maintained on PDA slants for further studies

Small mycelial bits of the pathogen were removed from a seven day old actively growing PDA and placed between the leaves and a thin layer of sterilized moist cotton was placed over the inoculated leaf portion and inoculated plants were incubated inside the moist chamber. The inoculated plants were incubated for 15 days at 28°C inside the glass house.

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Survey on the Collection of Sunflower Leaf Blight Disease in Major Sunflower Growing Areas in Tamil Nadu:

S. No.	Places of collection	Source of Infected parts	Districts
1.	Chittampatti	leaves	Madurai
2.	Arupukottai	leaves	Virudhunagar
3.	Kovilpatti	leaves	Thothukudi
4.	Agricultural college	capitullum	Madurai
5.	Sankarankovil	capitullum	Virudhunagar
6.	Rajapalayam	capitullum	Virudunagar
7.	Narnapuram	capitullum	Tirunelveli
8.	Omalur	Stem	Salem
9.	Lakshmipuram	Stem	Thothukudi
10	Perur	Stem	Coimbatore

Virulence (Pathogencity) of the Isolates: The pathogenicity of the purified isolates from the infected leaves stem, leaves and head of sunflower plants was tested and it was proved by Koch's postulates. *A. helianthi* was tested on the susceptible Sunflower (vr. CO-3) plants. The plants were raised in the glass house in earthen pots (30 cm) which contain 3 kg of uniform pot culture soil containing red soil, sand and farmyard manure at 3:1:1 ratio. Three plants were maintained in each pot. Sunflower plants of 10 days old, the plants were inoculated with conidial suspension (5 x 10^5 spores ml^{-1}) prepared in sterile distilled water from 10 days old culture of the different isolates grown in potato dextrose Agar media. Water congestion was provided to the plants both 24 h prior to and after the inoculation by covering the plants with a moist polythene bag of 100 gauge thickness. The inoculation was done in the cool hours late in the evening. The plants were maintained inside the glass house with constant temperature at 28 ± 2°C, Relative humidity 80 percent. The symptom of the disease was observed on seventh and 15th day after inoculation and the disease severity was recorded. The plants sprayed with sterile distilled water served as control. The symptoms were recorded and compared with the original symptoms. The fungus was reisolated from artificially inoculated leaves and compared with the original isolate. Twenty five leaves for virulence analysis were randomly selected in each replication for each isolate and grades were assigned as per the standard grade chart.

Grades	Leaf Area Blighted	Reactions
0	Healthy	Immune
1	1-5	Resistant
3	6-10	Moderately resistant
5	11-25	Moderately susceptible
7	26- 50	Suceptible
9	More than 50	Highly susceptible

The per cent disease index (PDI) was worked out by using the following formula [7].

$$PDI = \frac{\text{Total Sum of Numerical Rating X}}{\text{Total No of Leaves Observed X}} \frac{100}{\text{Max Disease Grade}}$$

The most virulent isolate was selected based on the disease intensity (PDI) and it was used throughout the study. Inoculation were repeated and obtained the same results.

Morphological Characters of Conidia of A. *helianthi:* A nine mm culture disc of the pathogen was removed from a 15 day old culture by using a sterilized cork borer and placed at the center of sterilized Petridishes containing 20 ml of PDA medium under aseptic conditions. Fifteen days after incubation at room temperature (28 ± 2°C) the mycelial growth and morphological characters of the isolates were observed.

The morphological characters *viz.,* growth, colour, septation of the mycelium, conidia, size (length and width) and shape of the conidia were observed. The measurement of 100 spores was observed under the 100X magnification microscope in respect of each isolate by using ocular and stage micrometers. The mean values of these measurements were calculated [8].

Growth of *Alternaria helianthi* Isolates on Solid Medium: The following six solid media *viz.,* Potato dextrose media, Sunflower extract media, Oat meal media, Czepek's dox media, Modified Czepek's dox media and Richard's media for the growth of different isolates of *A. helianthi.* The medium was sterilized in an autoclave at 1.4 kg / cm^2 pressure for 20 min and then 20ml of warm medium was poured in sterilized Petridishes (10 cm) under aseptic conditions and allowed to solidify. The isolates were inoculated at the centre of the plate by placing a 15 day old nine mm PDA culture disc. The plates were then incubated at room temperature (28 ± 2°C) for 20 days. Three replications were maintained for each isolate in each medium. Five Petri Plates were used for one replication. The radial growth of the mycelium was measured after incubation. The experiments were repeated and obtained the same results.

Growth of *A. hel*ianthi Isolates on Different Liquid Broth: Six liquid broth *viz.,* Potato dextrose broth, Sunflower extract broth, Oat meal broth, Czepek's dox broth, Modified Czepek's dox broth and Richard's broth were prepared. Then 100 ml of the respective broth was distributed in 250 ml Erlenmeyer flasks, autoclaved at 1.4 kg / cm^2 for 20 min and cooled at room temperature [9].

Each flask was inoculated separately with a 15 day old nine mm disc of the respective isolate of *A. helianthi* under aseptic condition. The flasks were incubated at room temperature 28 ± 2°C for 20 days. Three replications were maintained for each isolate. The mycelial mat was filtered through what man No. 1 filter paper and then dried in the hot air oven at 60°C for 24 h till a constant weight was obtained. Five conical flask were used for one replication the mycelial dry weight was calculated. The experiments were repeated and obtained the same results.

Statistical Analysis: The data were statistically analyzed by using AGRES software package developed by Tamil Nadu Agricultural University, Coimbatore. The mean values were compared by using Least Square Design (LSD).

RESULTS AND DISCUSSION

Virulence of *Alternaria helianthi* Isolates on Sunflower: The present study revealed that among the 10 isolates, isolate I_3 collected from Naranapuram was the most aggressive (70.22 %) while I_7 collected from Kovilpatti was the least virulent (19.11%). The geographical distances did not have any relation to the virulence of the isolates [10]. The pathogenicity index of the isolates of *A. helianthi* from sunflower was not related to climatic area and great variability in pathogenicity in all the climatic areas suggested good adoption to the host by the pathogen [11]. Significant difference in the pathogenicity of different isolates of *A. helianthi* from sunflower was recorded by [12, 13, 14]. The virulence of *A. helianthi* isolates was highly variable [15]. Therefore, the difference in the *Alternaria* blight incidence caused by different isolates of *A. helianthi* could be well attributed to the highly variable nature of their virulence prevalent in the respective areas. Naranapuram from where the most virulent isolates (I_3) were collected is a conventional sunflower growing belt where the continuous sunflower cultivation might have contributed the existence of the virulent pathotype (Table 1).

Morphological Characters of Conidia of *A. helianthi:* To study the variability among the isolates of *A. helianthi* culture and morphological characters of different isolate were studied and the results were furnished in Table 2.

Conidial Length of *A. helianthi:* The conidial length varied among the isolate from 38.62 µm to 62.16 µm. The maximum conidial length was observed in isolate I_3 of *A. helianthi* had (62.16 µm) followed by I_1 (61.15 µm). While the minimum conidial length was found in I_7 (38.62 µm). The morphology of *Alternaria alternate*, according to him the colonies were usually black or olivaceous black and some times grey. Conidiophores produced singly or in small groups, simple or branched, straight or flexuous, sometimes geniculate, pale to mid olivaceous or golden brown, smooth, up to 50 µm long, 3-6 µm thick, with one or several conidial scars (16).

Conidial Width of *A. helianthi:* The conidial width of *A. helianthi varied* from 10.00 µm to 15.60 µm. While it was maximum in I_3 isolate of *A. helianthi* (15.60 µm) followed by I_1 (13.86 µm). The remaining isolates conidial width ranges between 10.45 µm – 13.35 µm. The conidia of *A. helianthi* found to be solitary, non-beaked, born on simple unbranched conidiophores, cylinderic to elongate elliptic, yellowish brown septate with 3-10 transverse or occasionally longitudinal septa, constricted at septa, rounded at both ends and 40 - 120µm X15-28µm (average 100.6 X 25.5µm) in size [17]. The shape and size of conidia were similar to those of *A. helianthi* [18].

Conidial Cells of *A. helianthi:* The maximum number of conidial cells observed in I_3 isolate of *A. helianthi* had (3-8 cells) followed by I_1 isolate (3-7 cells). The remaining isolates cells ranges between 2-8 to 3-4 cells while it was minimum in I_7 isolate.

Effect of Different Solid Media on the Growth of *Alternaria helianthi* Isolates: The growth characters of *A. helianthi* were studied on six solid media and broth and tested PDA recorded significantly the maximum mycelial growth (7.64 cm) followed by sunflower leaf extract agar (6.95 cm) [19, 20, 21]. The Colonies of *A. helianthi* on PDA light brown to olive brown, velvety, slowly growing 15-20mm (in darkness) or 35mm (under light) in dia after 20 days at 25°C sporulation was abundant [22].

Effect of Different Liquid Media on the Growth of *Alternaria helianthi* Isolates: In liquid medium maximum mycelial dry weight of *A. helianthi* (2.59 g) was found in Richard's broth followed by Potato dextrose broth was the best broth suited for the growth of *A. helianthi*. Potato dextrose broth supported the maximum mycelial growth of *A. tenuisima* followed by Richard's, oat meal and Czapeks'dox media [23]. Richard' broth was the best for the growth of *A. tenuis causing* fruit rot of chilly [24]. Richard's broth was the best for the growth of *A. alternata* on onion [25] (Table 3).

Table 1: Virulence of different isolates of *A.helianthi* inoculated in sunflower vr Co3

S. No.	Isolate	Place of collection	Source of pathogen	District	Mean Disease grade 15 (DAI)	Percent disease index (PDI)
1.	I_1	Chittampatti	leaves	Madurai	5.68	63.11(52.60)
2.	I_2	Arupukottai	leaves	Virudhunagar	3.56	39.56(38.97)
3.	I_3	Narnapuram	leaves	Tirunelveli	6.32	70.22 (56.91)
4.	I_4	Agricultural college	capitullum	Madurai	2.56	28.44(32.23)
5.	I_5	Sankarankovil	capitullum	Virudhunagar	2.12	23.56(29.03)
6.	I_6	Rajapalayam	capitullum	Virudunagar	1.92	21.33(27.50)
7.	I_7	Kovilpatti	capitullum	Tuticorin	1.72	19.11(25.92)
8.	I_8	Omalur	Stem	Dharmapuri	2.72	30.22(33.22)
9.	I_9	Lakshmipuram	Stem	Tuticorin	4.72	52.44(33.35)
10.	I_{10}	Perur	Stem	Coimbatore	3.72	41.33(40.01)
CD(P=0.05)					1.20	

CD = Critical difference at 0.05%, Mean of three replications (Figures in the parentheses are arc sine transformed values) and DAI = Days after inoculation.

Table 2: Morphological characters of different isolates *A.helianthi* conidia

Isolate	Mycelial characters	Conidial Characters	Length*(µm)	Width*(µm)	No. of cells*
I_1	Mycelium light brown with septate hyphae	Conidia septate,clavate formed singly at the tip of conidiophore the number of spores varied from two to eight.	61.15	13.86	3-7
I_2	Mycelium fulginaceous colour Hyphae septate and branched irregularly, changing dark brown at later age	Conidiophores straight light brown conidia at the blunt tip conidia light brown multiseptate, muriform shaped	52.12	12.12	3-4
I_3	Mycelium fulginaceous colour	Conidia septate, obclavate and borne at the tip of conidiophore the number of spore varied from one to eight.	62.16	15.60	3-8
I_4	Mycelium olivaceous brown and hyphae septate, bearing conidia at the tip	Conidia obclavate with round base, septate, light brown.	54.00	10.45	3-4
I_5	Mycelium olivaceous brown	Conidiophore light brown, simple 2-3 septate: conidia light brown to dark brown in colour: muriform with 1-6 transverse septa and 0-2 longitudinal septa, obclavate to oval in shape	58.50	11.32	3-7
I_6	Mycelium fulginaceous colour	Muriform	50.50	13.35	3-7
I_7	Mycelium olivaceous brown	Muriform	38.62	10.00	3-7
I_8	Dark brown	Muriform conidia	48.22	12.00	2-8
I_9	Dark brown	Muriform	56.30	11.35	3-7
I_{10}	Dark brown	Muriform	40.32	10.99	2-6

*Mean of ten conidia in 100 X microscopic field.

Table 3: Growth of *A. helianthi* isolates on different solid media

	Diameter of mycelial growth (cm)						
Isolates	Oats agar	Richard's agar	Potato dextrose agar	Czapek's dox agar	Sunflower leaf extract agar	Modified czapek's dox agar	Mean
I_1	6.33	5.13	7.97	5.53	7.43	4.47	6.14
I_2	6.23	4.90	7.83	5.33	7.17	4.33	5.96
I_3	6.73	5.43	8.50	6.53	7.80	8.03	7.17
I_4	6.10	4.90	7.67	5.23	6.93	4.37	5.86
I_5	5.70	4.70	7.50	5.00	6.77	4.27	5.65
I_6	6.00	4.67	7.80	4.87	6.53	4.20	5.67
I_7	6.00	4.80	7.63	3.10	6.60	4.20	5.38
I_8	6.00	4.83	7.23	4.80	6.40	3.96	5.53
I_9	6.10	4.37	7.33	4.86	6.38	4.10	5.52
I_{10}	6.40	4.90	7.00	5.09	7.53	4.53	5.90
Mean	6.15	4.86	7.64	5.03	6.95	4.64	
CD (P=0.05)			Isolate		: 0.59		
			Media		: 0.76		
			Isolate x Media		:1.87		

CD = Critical difference at 0.05% and mean of three replications (Figures in the parentheses are arc sine transformed values).

Table 4: Growth of *A. helianthi* isolates on different liquid broth

	Mycelial dry weight (g)						
Isolates	Oats agar	Richard's agar	Potato dextrose agar	Czapek's dox agar	Sunflower leaf extract agar	Modified czapek's dox agar	Mean
I_1	2.36	2.83	2.49	2.45	2.78	1.35	2.38
I_2	2.43	2.59	2.17	2.44	2.37	1.18	2.20
I_3	2.73	2.94	2.50	2.63	2.92	2.86	2.76
I_4	2.45	2.71	2.32	2.43	2.56	1.47	2.32
I_5	2.50	2.58	2.28	2.22	2.52	1.32	2.24
I_6	2.57	2.83	2.13	2.37	2.15	1.98	2.34
I_7	2.25	2.26	2.15	2.14	2.26	1.53	2.10
I_8	2.15	2.33	2.30	2.32	2.25	1.93	2.21
I_9	2.19	2.30	2.02	2.12	2.27	1.94	2.14
I_{10}	2.24	2.53	2.14	2.21	2.32	2.15	2.44
Mean	2.39	2.59	2.25	2.33	2.44	2.33	
	CD (P=0.05)		Isolate		: 0.10		
			Media		: 0.13		
			Isolate x Media		: 0.33		

CD = Critical difference at 0.05% and mean of three replications (Figures in the parentheses are arc sine transformed values)

CONCLUSIONS

The present investigation revealed that the isolates of *A. helianthi* exhibited high variability in morphological, cultural character and pathogenicity, which could be useful for culturing the fungus in the artificial conditions and furnish the essential elements and compounds in the medium which are required for the growth of *A. helianthi*. The morphological characters of different isolates and Pathogenicity taken for the study which would be useful for the development of race specific resistant varieties for the controlling of leaf blight disease in sunflower.

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