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Symptomatology and pathogenic variability of *Alternaria carthami* isolates from Maharashtra state infecting safflower crop

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Abstract
The pathogen (*Alternaria carthami*) was isolated successfully from the naturally diseased safflower leaf specimens collected during survey and one each representative isolate from twenty districts of Maharashtra state were obtained, purified and maintained. The pathogenicity test attempted on susceptible safflower cv. Manjira in pot culture under screen house, clearly indicated that all the 20 isolates of *A. carthami* exhibited a wide range of pathogenic variability. However, the aggressive isolates (*viz*., AcHl, AcBl, AcAn, AcJg, etc.) showed least incubation period, highest leaf spot frequency with maximum sized leaf spots. Whereas, moderately virulent / aggressive isolates exhibited moderate incubation period, leaf spot frequency and their size. In less virulent isolates *viz*., AcAm, AcDl, AcNb and AcNs through incubation period was maximum (> 9 days), but leaf spot frequency and their size were of lower minimum. Based on symptomatology, cultural and morphological characteristics, microscopic and pathogenicity test, the test pathogen was identified as *Alternaria carthami*, compared with the descriptions of *Alternaria* spp. (Simmons, 2007) and finally confirmed as *Alternaria carthami*, the incitant of leaf spot / blight in safflower.

Keywords: Symptomatology, pathogenic variability, *Alternaria carthami*, infecting safflower crop

Introduction
Safflower, (*Carthamus tinctorius* L.), is one of the world's oldest important oilseeds crop of the semi-arid regions belonging to the family Asteraceae (Compositae). Safflower is a multipurpose crop grown for its foliage, flowers and seeds (oil) with diversified uses. Safflower is also affected by many biotic and abiotic stresses. Of the biotic agents, fungi cause major diseases, followed by bacteria, viruses and nematodes.

Among fungal diseases, at present leaf spot/blight caused by *Alternaria carthami* (Chowdhury) is widespread and have continued to be the major constraints in the production and productivity of safflower all over the country in general as well as in the state of Maharashtra particularly. The disease (*A. carthami*) has been reported to cause 25 to 60 per cent yield losses all over India (Singh and Prasad, 2005) [18] and 20 to 80 per cent in Maharashtra state (Anonymous, 2010) [1], along with drastic reduction in seed size, seed volume, seed test weight as well as per cent oil content.

The pathogen being seed borne, initially dark necrotic lesions (2-5 mm) appear on the hypocotyls as well as cotyledons. The initial symptoms of leaf blight of safflower incited by *A. carthami* appears right from seedling stage (30 days old) as small isolated light brown to dark brown circular spots (1-2 mm) on the lower leaves and these spots gradually increases in size. As the infection progress, these spots enlarge and coalesce forming bigger spots. In the centre of the spots, there appears a brown dot surrounded by a number of alternating dark concentric rings. The mature spots exhibit shot holes. The disease spreads at alarming rate in susceptible safflower varieties, the entire crop turns brown and dries without producing any seed. In several cases, the symptoms also appears on stem as elongated dark brown to black spots, causing cracking of the affected stem. On floral parts, the symptoms appeared as minute dark brown spots first at the base of involucral bracteoles, which later enlarge, spreads to other parts of the capitulum and unopened capitula shrivel and dry up (Krishna Prasad and Basuchaudhary, 1989) [8].

Materials and Methods

Symptomatology

Visual examination

Visual observations were made for manifestation of the *Alternaria*blight typical symptoms on safflower plants in the safflower crop fields surveyed, collected the disease samples in paper bags and brought to the laboratory for further studies.

Microscopic examination

Fresh leaf samples showing *Alternaria* blight symptoms and collected during survey were brought to the laboratory and washed in tap water to remove extraneous material. Free hand sections of the diseased leaf specimens were cut with sharp blade, also diseased specimens were scrapped and diluted in distilled water. Leaf tissue sections and suspension of scraping were put in a water drop on separate clean glass slides, covered with cover slip, mounted initially under low power objective lens of the compound microscope (make: Labomed Vision 2000) and observed the mycelium, colour of the mycelium and spores, if any. Also temporary mount on glass slide of the pure culture of *A. carthami* was prepared and examined under microscope.

Isolation of *A. carthami* isolates

Naturally infected safflower leaves specimens showing typical symptoms of *Alternaria*blight and collected during field survey were brought to the laboratory, washed thoroughly with distilled water; blot dried and cut with sharp sterilized blade into small bits (5 mm^2), keeping half healthy and half diseased portion intact. These leaf bits were surface sterilized with 0.1 per cent aqueous mercuric chloride ($HgCl_2$) solution for two minutes, then washed by giving three sequential changes with sterile distilled water in Petri plates to remove traces of mercuric chloride and again blot dried. Later, these leaf bits were inoculated aseptically on autoclaved and cooled Potato dextrose agar (PDA) medium in sterilized Petri plates under Laminar-air-flow cabinet (make: ACS, Banglore). Inoculated plates were incubated in BOD incubator (make: MAC, Delhi) at 28±2 ^{0}C temperature. After a week of incubation, well developed mycelial growth was obtained, which by hyphal tip isolation technique transferred aseptically on the PDA slant in glass test tubes and through frequent sub-culturing, the test isolates were purified and the pure cultures were maintained on PDA slant in glass test tubes. Applying same procedure, a total of 20 isolates of *A. carthami*, one each representing 20 districts and covering various geographic regions and agro-climatic zones of the Maharashtra state surveyed were isolated, purified, multiplied, designated as detailed in the following Table 1 and maintained in refrigerator for further studies.

Table 1: Isolates of *A. carthami*, representing 20 districts of the Maharashtra state

Sr. No.	Districts	*A. carthami* Isolates	Agro-Climatic Zone	Av. Rainfall (mm)
Marathwada Region (08)				
1	Parbhani	AcPb	Assured Rainfall Zone (7)	700-900
2	Nanded	AcNd	Central Vidharbha Zone (8)	900-1150
3	Hingoli	AcHl	Central Vidharbha Zone (8)	900-1150
4	Latur	AcLt	Assured Rainfall Zone (7)	700-900
5	Osmanabad	AcOs	Assured Rainfall Zone (7)	700-900
6	Beed	AcBd	Scarcity Zone (6)	<700
7	Aurangabad	AcAb	Assured Rainfall Zone (7)	700-900
8	Jalna	AcJl	Assured Rainfall Zone (7)	700-900
Vidharbha Region (05)				
9	Buldana	AcBl	Assured Rainfall Zone (7)	700-900
10	Washim	AcWs	Central Vidharbha Zone (8)	900-1150
11	Akola	AcAk	Assured Rainfall Zone (7)	700-900
12	Amaravati	AcAm	Assured Rainfall Zone (7)	700-900
13	Yavatmal	AcYt	Central Vidharbha Zone (8)	900-1150
Khandesh Region (04)				
14	Jalgaon	AcJg	Assured Rainfall Zone (7)	700-900
15	Dhule	AcDl	Scarcity Zone (6)	<700
16	Nandurbar	AcNb	Western Maharashtra Plain Zone (5)	700-1200
17	Nasik	AcNs	Scarcity Zone (6)	<700
Western Maharashtra Region (03)				
18	Ahmadnagar	AcAn	Scarcity Zone (6)	<700
19	Satara	AcSt	Western Maharashtra Plain Zone (5)	700-1200
20	Solapur	AcSl	Scarcity Zone (6)	<700

Pathogenicity assay and pathogenic variability among of *A. carthami* isolates

In order to confirm identification of the disease and its causal agent, the pathogenicity test was attempted in pot culture under screen house conditions. Seeds of safflower cv. Manjira susceptible to *Alternaria*blight (*A. carthami*) were surface sterilized with 0.1 % $HgCl_2$ and sown (@ 10 seeds/pot) in the earthen pots (30 cm dia.) filled with steam sterilized potting mixture of soil: sand: FYM (2: 1: 1). After two week, two healthy growing safflower seedlings per pot were maintained, watered regularly and kept in the screen house for further growth and development. The spore-cum-mycelial suspensions of *A. carthami* test isolates was prepared separately from 10 days pure culture in plates, by flooding with 5-10 ml sterile distilled water. This resultant suspension was suitably diluted with sterile distilled water to get inoculum concentration of 2 x 10^6 spores/ml. Thirty days old seedlings (2 / pot/ isolate / replication) of safflower cv. Manjira growing in earthen pots were artificially spray inoculated the spore-cum-mycelial suspension separately of the test isolates (Giri *et al*., 2013) [5], the experiment was planned with CRD and all the test isolates replicated thrice. Safflower cv. Manjira seedlings grown in earthen pots and sprayed with sterile water (without inoculum) were

maintained as uninoculated suitable control. These pots (both inoculated and uninoculated) were covered with polythene bags during evening hours and kept overnight, watered regularly to create optimum relative humidity and maintained in screen house for development of the disease symptoms.

From the artificially inoculated and *Alternaria* blight diseases safflower leaves the test isolates were reisolated separately on PDA medium and incubated at 28±2 ^{0}C. After a week of incubation the cultural and morphological characteristics developed of the test isolates were observed and compared with the characteristics (cultural and morphological) of the original test isolates obtained from naturally *Alternaria* blight diseased safflower foliage. To satisfy Koch's postulates, symptoms developed on artificial inoculated safflower leaves were compared with original symptoms on naturally diseased plants.

Observations on incubation period (days to expression of initial symptoms), number of lesions / plant and diameter of the lesions were recorded.

Identification of the pathogen

On the basis of *Alternaria* blight disease symptoms expressed (both on naturally and artificially diseased) on safflower plants, pathogenicity test and cultural and morphological characteristics the test pathogen was identified and further confirmed by comparing with the descriptions of *Alternaria* spp. given by Simmons (2007) [16].

Results and Discussion

Symptomatology

Visual observations

During field survey study (Plate1), various field experiments (Plate 2) and pathogenicity test (Plate3), the typical symptoms induced by *A. carthami* on safflower plants were observed critically. Initially symptoms appeared seedlings (30 days old) were small isolated light brown to dark brown circular spots (1-2 mm dia.) on the lower leaves, which gradually spread to upper leaves. As the infection progressed, these spots enlarged and coalesced forming large sized spots and or leaf blight. In the centre of such spots, brown dot surrounded by the number of dark alternating concentric rings also appeared in some cases. In matured spots, shot holes usually appeared in the infected area.

Under field conditions, the disease appeared usually in the month of November and attained to its maximum proportion by mid of February. The disease spread was very fast in the susceptible safflower varieties, such severely affected plants turned brown and dried without producing any seed. In other safflower cultivars, the disease symptoms developed on the leaves occupying lower half portion of the stem and also appeared on the upper leaves as small scattered spots. In several cases, the symptoms also appeared on stem as elongated dark brown to black discolorations, causing cracking of such stems. On floral parts, the symptoms observed as minute dark brown spots, first at base of the involucral bracteoles, which later enlarged and spread to other parts of the capitulum (Plate2) and such diseased capitula remained unopened, shriveled and dry up.

Microscopic examinations

Microscopic examinations of the temporary mounts prepared from *Alternaria* affected safflower leaf tissues and pure culture of *A. carthami* (Plate 4) revealed presence of olivaceous brown colouredseptate mycelium. Conidiophores arose singly from the mycelium present in dead centers of the spots. Conidia were usually solitary, obclavate, oblong to ellipsoid, tapering to a beak. Conidia were muriform, pale to brown and smooth with varied number of horizontal and vertical septa.

Similar symptoms of safflower *Alternaria* blight observed in present studies were also reported earlier by many workers. Chowdhury (1944) [2] reported that *A. carthami*, initially caused minute brown spots with concentric rings, which later enlarged and coalesced. He also reported spots on stem and petiole. Krishna Prasad and Basuchaudhary (1989) [8] reported that *A. carthami* induced small, scattered irregular shaped leaf spot surrounded by yellowed halo without target board appearance and light brown dot in the centre of the spot. Similar symptoms produced by *A. carthami* observed in present study were reported in safflower earlier by several workers (Prabhakar *et al.*, 2012; Taware *et al.*, 2014 and Gholve *et al.*, 2015) [11, 19, 3].

Isolation of *A. carthami* isolates

Applying tissue isolation technique, the 20 isolates of *A. carthami* were isolated aseptically from the safflower leaf samples naturally infected by *Alternaria* blight disease, using Potato dextrose agar (PDA) medium and incubated at 28 ± 2 ^{0}C. After a week of incubation, the test pathogen produced initially white, cottony, profused aerial mycelium, which gradually turned greenish grey in colour. Aged culture appeared completely black with no aerial mycelium. The pathogen is characterized by dark, pluriseptate hyphae. Conidiophores were erect, simple, 0 to 5 septate, brown to olivaceous, geniculate at the apex and sometimes laterally, occasionally showing a spherical swelling at the base. Conidia were light brown of variable shapes in chains of least two and consisting of 3 to 11 cells, with varied number of horizontal and vertical septa (Plate 4). Pure cultures of all 20 test isolates were obtained and maintained on PDA slant in test tubes, which was preserved in refrigerator for further studies.

Pathogenicity test and pathogenic variability

Pathogenicity test (Plate 3) of *A. carthami* (20) was conducted in screen house using *Alternaria* blight susceptible safflower cv. Manjira and results obtained on pathogenic traits *viz*., incubation period, number of spots and size of the spots are presented in Table 2.

Symptoms

All 20 test isolates of *A. carthami* were found pathogenic to safflower. The symptoms induced under pathogenicity test were identical to those symptoms (Plate 1) observed on naturally diseased safflower crop foliage during survey.

Incubation period

Results (Table 2) revealed that among various pathogenic traits of *A. carthami*, the incubation period was varied in the susceptible safflower cv. Manjira. On the basis of incubation period (day to expression of first symptom), the test isolates were categorized in three groups *viz*., A (highly aggressive with minimum of 5-6 days incubation period), B (moderately aggressive with moderate of 7-8 days incubation period) and C (less aggressive with 9 or more days incubation period). Accordingly, in group A the isolates (04) included were AcHl, AcAn, AcBl and AcJg; in group B the isolates (12) included were AcPb, AcNd, AcSt, AcOs, AcAb, AcWs, AcAk, AcLt, AcBd, AcJl, AcYt and AcSl and in group C the isolates (04) included were *viz*., AcAm, AcNb, AcDl and AcNs.

Frequency of leaf spots

There were significant variations in the frequency of leaf spots (average number of spots / plant) on safflower cv. Manjira (Table 2) and it was ranged from 5.00 (AcDl) to 22.00 (AcHl). On the basis of frequency of the leaf spots, the test isolates were categorized in to three groups *viz*., A (highly virulent = leaf spot frequency in the range of 22.00 to 15.67 / plant), B (moderately virulent = leaf spot frequently in the range of 10.50 to 14.00 / plant) and C (less virulent = leaf spot frequency in the range of 5.00 to 13.33 / plant). In group A of highly virulent isolates the leaf spot frequency was ranged from 15.67 (AcNd) to 22.00 (AcHl) per plant; however, it was significantly highest with the isolate AcHl (22.00), followed by AcBl (21.33), AcAn (19.67), AcJg (18.50), AcPb (16.33) and AcNd (15.67). In group B of moderately virulent isolates, the leaf spot frequency was ranged from 10.50 (AcSt) to 14.00 (AcWs); however, it was significantly maximum with the isolate AcWs (14.00), followed by AcAb (13.33), AcLt (12.50), AcAk (11.00), AcOs (10.67) and AcSt (10.50), later two isolates were at par. In group C of less virulent isolates, the leaf spot frequency was ranged from 5.00 (AcDl) to 13.33 (AcAb) per plant; however, it was significantly maximum with the isolate AcLt (12.50), followed by AcAk (11.00), AcOs (10.67), AcSt (10.50), AcBd (9.67) and AcYt (9.00).

Table 2: Pathogenic variability among *A. carthami* isolates

Sr. No.	Isolates / Districts	Av. incubation period (days)*	Av. no. of spots*	Av. size of spot (mm^2)*
1	AcPb (Parbhani)	7	16.33	14.28
2	AcNd (Nanded)	7	15.67	13.88
3	AcHl (Hingoli)	5	22.00	17.14
4	AcSt (Satara)	7	10.50	09.07
5	AcOs (Osmanabad)	7	10.67	09.52
6	AcBd (Beed)	8	09.67	08.64
7	AcAb (Aurangabad)	7	13.33	11.90
8	AcJl (Jalna)	8	08.50	07.16
9	AcYt (Yavatmal)	8	09.00	07.79
10	AcWs (Washim)	7	14.00	12.96
11	AcAk (Akola)	7	11.00	10.43
12	AcLt (Latur)	7	12.50	11.10
13	AcAn (Ahmadnagar)	5	19.67	15.82
14	AcDl (Dhule)	9	05.00	02.80
15	AcBl (Buldana)	5	21.33	16.54
16	AcAm (Amravati)	9	06.33	04.77
17	AcNb (Nandurbar)	9	05.50	03.18
18	AcNs (Nasik)	9	07.00	05.37
19	AcSl(Solapur)	8	07.67	06.72
20	AcJg(Jalgaon)	6	18.50	15.11
	SE±	0.28	0.15	0.13
	CD (P = 0.01)	0.82	0.41	0.34

* : Mean of three replications, Av. : Average, No. : Number

Size of leaf spots

Size / diameter of the spots induced by *A. carthami* isolates on foliage of safflower cv. Manjira was also found to be varied significantly among the test isolates and it was ranged from 2.80 to 17.14 mm^2.

Based on average size (mm^2) of the leaf spots, the test isolates were categorized into three groups *viz*., A (with maximum average leaf spot size in the range 12.96 to 17.14 mm^2), B (with medium average leaf spot size in the range of 7.16 to 11.90 mm^2) and C (with small average leaf spot size in the range of 2.80 to 6.72 mm^2). In group A, those isolates which induced large sized spots (12.96 to 17.14 mm^2) were included and significantly large sized leaf spots were induced by the isolates AcHl (17.14 mm^2), followed by the isolates AcBl (16.54 mm^2), AcAn (15.82 mm^2), AcJg (15.11 mm^2), AcPb (14.28 mm^2), AcNd (13.88 mm^2) and AcWs (12.96 mm^2). In group B of medium sized leaf spots, maximum sized spots were induced with the isolate AcAb (11.90 mm^2), followed by the isolates AcLt (11.10 mm^2), AcAk (10.43 mm^2), AcOs (9.52 mm^2), AcSt (9.07 mm^2), AcBd (8.64 mm^2), AcYt (7.79 mm^2) and AcJl (7.16 mm^2). In group C of small sized leaf spot, maximum sized spots were induced with the isolate AcSl (6.72 mm^2), followed by AcNs (5.37 mm^2) and AcAm (4.77 mm^2); whereas significantly smallest sized leaf spots were induced by the isolates AcDl (2.80 mm^2) and AcNb (3.18 mm^2).

Thus, from this ongoing results it was concluded that all 20 isolates of *A. carthami* representing four geographic regions of the Maharashtra state exhibited a wide range of pathogenic variability. However, the aggressive isolates (*viz*., AcHl, AcBl, AcAn, AcJg, AvNd, etc.) showed highly virulent least incubation period, highest leaf spot frequency and maximum sized leaf spots. Whereas, moderately virulent / aggressive isolates exhibited each moderate incubation period, leaf spot frequency and their size. In less virulent isolates *viz*., AcAm, AcDl, AcNb and AcNs through incubation period was maximum (> 9 days), but leaf spot frequency and their size were of lower degree.

Pathogenic association of *A. carthami* with safflower causing *Alternaria* leaf spot / blight was reported earlier by several workers under controlled conditions of screen house by inoculating spore cum mycelial suspension (*A. carthami*) on susceptible safflower cultivars (Relekar *et al*., 2010; Ranaware *et al.*, 2010; Gholve *et al*., 2015) [14, 13, 3].

The cultural and morphological characteristics of *A. carthami* found in present study are in consonance with the earlier reported (Taware *et al.*, 2014) [19]. Gholve *et al.* (2015) [3] reported that *A. carthami* produced initially white, cottony profused aerial mycelium, which gradually turned greenish grey in colour. Aged culture completely black with no aerial mycelium on Potato dextrose agar Microscopic characteristics such asbrownish black septate mycelium and dark brown beaked conidia with transverse and longitudinal septation of

A. carthami observed during present study were also reported earlier by several workers (Ranaware *et al.*, 2010; Prasad *et al.*, 2012; and Gholve *et al.*, 2015) [13, 12, 3].

Pathogenic diversity among isolates of the *Alternaria* spp. infecting various oilseeds and vegetable crops was reported in past. Meena *et al*. (2011) [9] reported pathogenic variability in *A. brassicae* isolates in respect of disease severity on host differentials and lesion size. Virulent isolates produced large sized lesions with higher per cent disease severity. Mehta (2012), Sigh *et al*. (2013) [10], Sharma *et al*. (2013) [15] and Giri *et al*. (2014) [4] were of similar opinion in respect of pathogenic variability in *A. brassicae* causing leaf blight of rapeseed-mustard. Kolte *et al*. (2005) [7] reported pathogenic variability in leaf spots produced. Virulent isolates produced large spots with dark margins. Jadhav *et al*. (2011) [6] reported existence of pathogenic variability in *A. macrospora* isolates from cotton. They found that in virulent isolates the incubation period very short (8-9 days), large lesion size and maximum number of lesions per unit leaf area; whereas, the less virulent isolates required maximum incubation period (8-13 days), small sized lesion and their minimum frequency.

Identification of the pathogen

Based on symptomatology, cultural and morphological characteristics and pathogenicity test, the test pathogen was identified as *Alternaria carthami*. Further, cultural and morphological characteristics of the test isolate were compared with the descriptions of *Alternaria* spp. given by Simmons (2007) [16] and finally confirmed as *Alternaria carthami*, the incitant of leaf spot / blight in safflower.

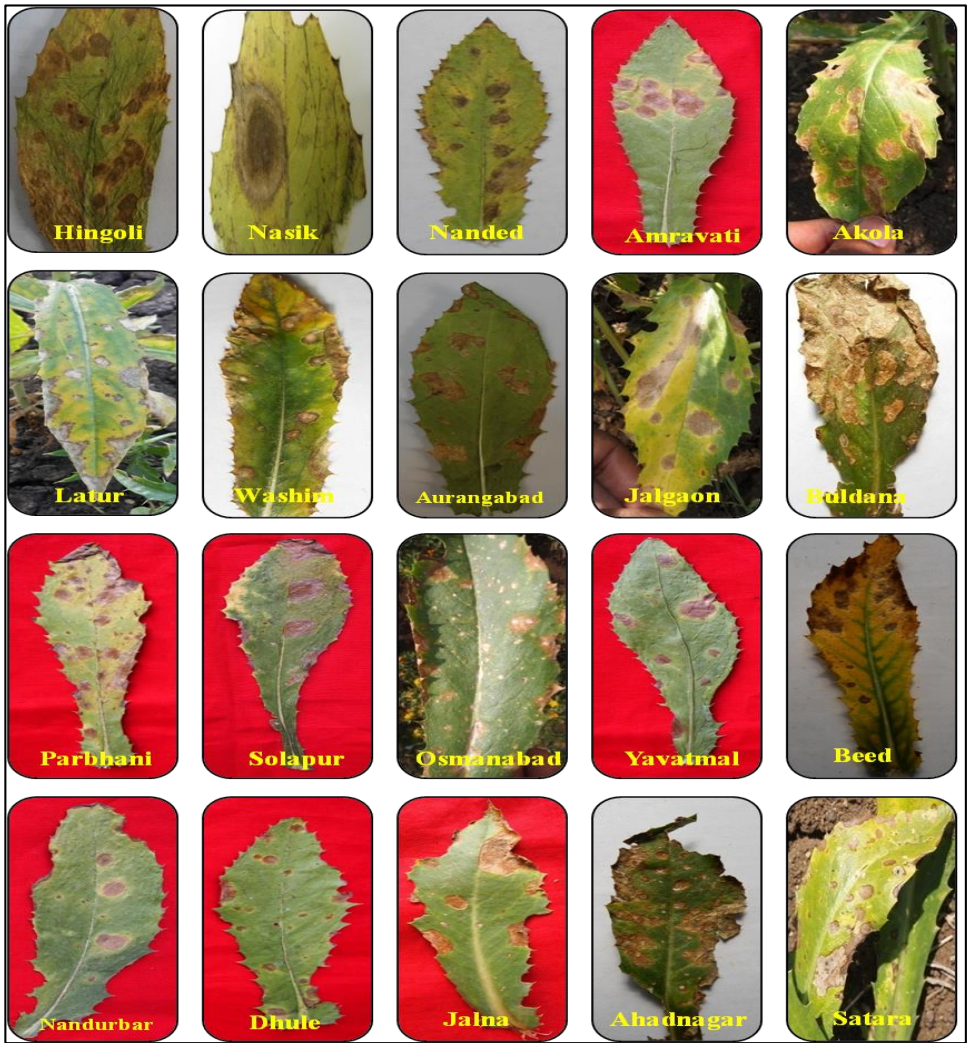

Plate 1: *Alternaria* blight diseased safflower leaf samples collected during field survey

Plate 2: Typical symptoms of *Alternaria* blight on safflower crop experiment field

Plate 3: Pathogenicity test and pathogenic variability of *A. carthami* isolateson safflower Cv. Manjira

Plate 4: *A. carthami* Pure culture, Microphotographs of its Mycelium and Conidia

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