wjpmr, 2021,7(10), 173-179

SJIF Impact Factor: 5.922

WORLD JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.wjpmr.com

Research Article

ISSN 2455-3301

WJPMR

IDENTIFICATION AND CHARACTERIZATION OF FUNGAL PATHOGEN CAUSING LEAF SPOT IN MULBERRY LEAVES USING SCANNING ELECTRON MICROSCOPE

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Article Received on 28/06/2021 **Article Revised on 18/07/2021** **Article Accepted on 08/08/2021**

ABSTRACT

Mulberry plants are infected by a variety of diseases. The fungal pathogen causing leaf spot in mulberry leaf was isolated, characterized and identified. The causative agent was identified as *Alternaria alternata* based on morphological features. The conidia of *A. alternata* were clavate and formed single chain. The spores consist both longitudinal and horizontal/transverse septa. The conidium tapers into a narrow and rounded protuberance. The conidia were cylindrical while young and became bottle shaped with branched beaks when matured. A maximum numbers of conidia were produced during 5th and 7th days of incubation at 26 ˚C – 28 ˚C. *A. alternata* conidium germination inoculation and colonization on the mulberry plant studied on scanning electron microscope the length of conidia was 35.25µm and width 4.94 µm

KEYWORDS: *Alternaria alternata, Clavate Conidia, Leaf spot, Morus alba.*

INTRODUCTION

Alternaria alternata is prevalent worldwide and known to cause severe damage to a wide variety of crop plants (Waller and Brayford, 1990; Rotem, 1994). The foliage of mulberry plant is used as an exclusive food for the silkworm, *Bombyx mori.* One of the major foliage diseases of mulberry is leaf spot. It is severe during autumn and spring in most areas, and extending into the monsoon in the hilly regions of India (Govindaiah and Gupta, 2005; Kunoh *et al.*1980). The quality and quantity of mulberry foliage is often hampered by the infestation of fungal pathogens (Philip *et al.* 1994).

The symptoms of the disease occurrence are yellowing margin and burning of leaf lamina resulting in severe defoliation (Govindaiah *et al* .,1990; Gunasekhar *et al*., 1992).Most of the earlier studies are concerned with the occurrence of leaf spots fungal pathogen in mulberry. *Alternaria alternata* (Fr.) Keissler is common saprobe found on many plants and others substrata work wide including pine needles (Lu *et al*., 2000; Grunden *et al*., 2001; Tokumasu and Aoiki, 2002). A thorough study was carried out on isolation and characterization of fungal pathogen *Alternaria alternata* in Uttar Pradesh.

MATERIALS AND METHODS

Field survey

The field surveys were carried out at the mulberry farms of different district of north eastern region of Uttar Pradesh. Infected leaf samples of *Morus alba* were collected between September and November 2010 Lucknow and Lakhimpurkhiri.

Isolation of fungal pathogen

The samples were collected from the infected plants brought to laboratory and kept in polythene bags at low temperature (4 ˚C) incubator for few hours. Selected infected leaves(for leaf spot) were washed by running water for 15-20 min (Yoshida and Shirata, 1999), and surface disinfected by immersing in 1% sodium hypochloride solution for 3 min rinsed with sterilized water dried under aseptic condition for 5 min and cut into small pieces (3-5 mm). The pieces were placed on 2% plate's containg Potato dextrose agar (Hi-media) at pH 5.6±0.2 supplemented with 8 mg/l of Streptomycin sulphate (Baird *et al* 1991).

The plates were incubated at 26 °C – 28 °C for 5-7 days. During the culture period colours of the colony and the presence of the zonation were observed every day,

diameter of colonies were measured until 7th days of culture and their lengths, widths were measured. The number of longitudinal and transverse septa was measured. The fungi were identified based on morphological and culture characteristics by following Booth, 1977and Gerlach and Nirenberg, 1982. The fungi growing from the leaf pieces were subcultured onto Potato dextrose agar (Hi-media).

Identification of the fungal pathogen A small portion of mycelia was taken aseptically on glass slide with the help of sterilized needle and used for observation under phase contrast microscope. The material was spread over slide by needle and stained with cotton blue (Merck) (for hyaline spores) and mounted with lactophenol (Merck) and studied under phase-contrast microscope (Olympus) at different magnification (40x and 100x.). Measurements of fungi were recorded and photographs were taken using digital camera Olympus. Identification of fungi was carried out by following published literature (Chupp, 1953; Eills; 1971 and 1976; Sutton, 1980; Rinthalinghum, 1980; Ahmed *et al*.1997; Kirk, 2009). Culture sample were sent to the Department of Mycology and Plant Pathology, Agharkar Research institute, G.G .Agarkar Road, Pune for conformation. Literature survey and information on the incidence disease severity and the damage caused due to leaf spot diseases in Uttar Pradesh. Hence detailed information was undertaken with following objectives.

- To survey the incidence of leaf spot diseases of mulberry in north eastern of Uttar Pradesh.
- To isolate and identify the Alternaria Alternata species with leaf spot.

Sample preparation for Scanning Electron Microscope (SEM)

The pathogenic fungal cultured for 48 h were made into a fungal suspension with sterile water and filter o few drops of fixative 2.5 % glutaraldehyde for 2-4 hours at 4 ˚C. and phosphate buffer were added 0.1M (pH 7.2) for 3 times and centrifuged at 3000 to 5000 rpm each of 15 min. at 4 ˚C to collect pellets and discard supernant.1 % osmium tetroxide has used as a post fixation for 2 hours at 4 ˚C and the fungal suspension has washed in 0.1 M phosphate buffer for 3 changes each of 15 min. at 4 ˚C to remove the unreacted fixative and dehydrated in a graded acetone at 30%, 50%, 70%, 90%, 95% and dry acetone for 15 min at 4 ˚C. Fungal suspension were frozen in liquid nitrogen and observed with fungal suspension were mount on to the aluminium stubs with conductive point or adhesive taps (carbon), fungal suspension were dry or put in an dessicator for overnight and sputter coated with pladium and viewed with a scanning electron microscope (SEM) (JEOL 6490 LV) .Operating at 10 KV (Stadtlander *et al*., 2007).

Fig. 1: Leaf spot on infected leaves of Mulberry.

Fig. 2: Leaf spot on infected leaves of Mulberry.

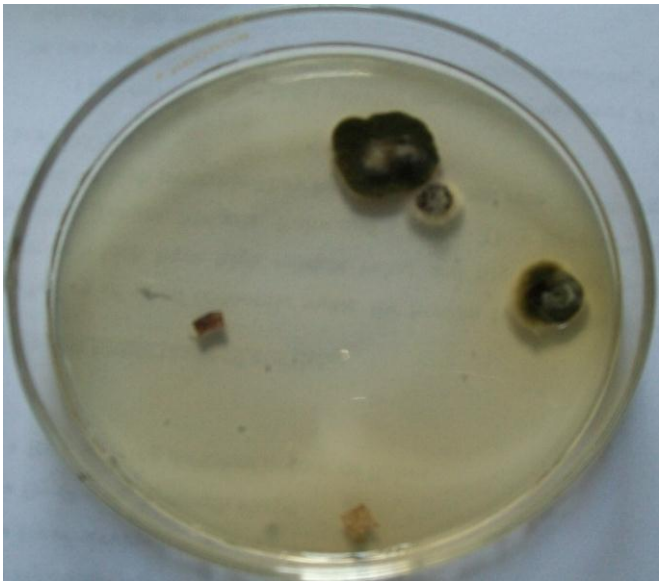


Fig. 3: Culture plate on PDA.

Fig. 4: Subculture plate on PDA.

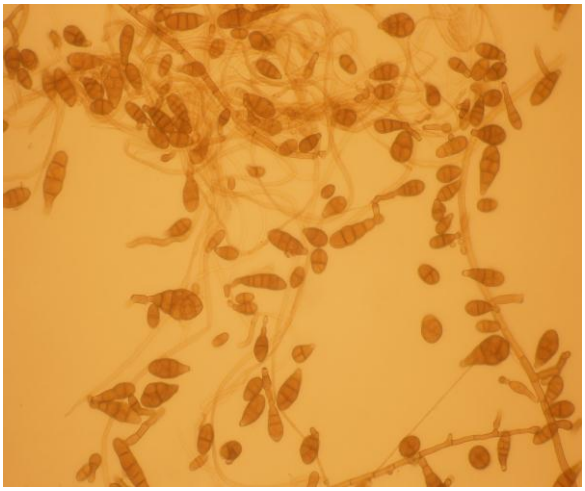


Fig. 5: (40x) Conidia of *Alternaria alternata*

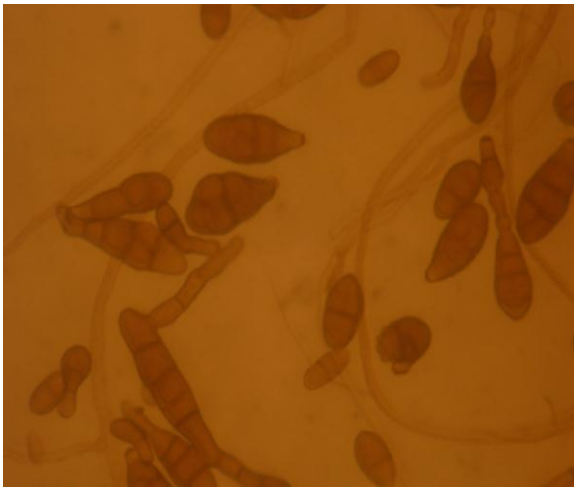


Fig. 6: (40 x) Conidia of *Alternaria alternate.*

Fig. 5 (40x) Conidia of *Alternaria alternate.*

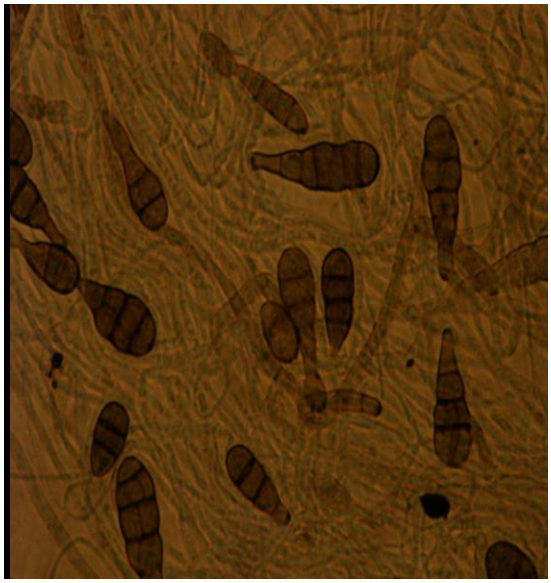
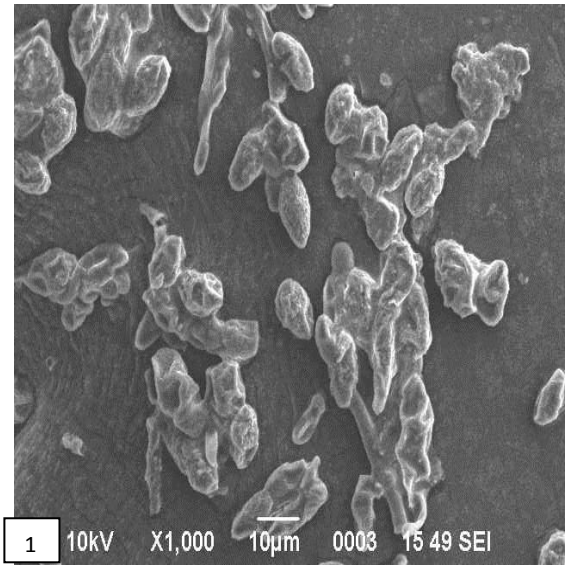


Fig. 5 (100x) Conidia of *Alternaria alternate.*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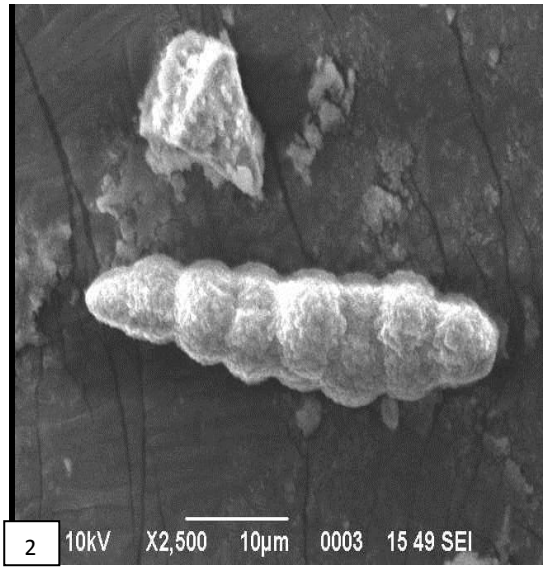
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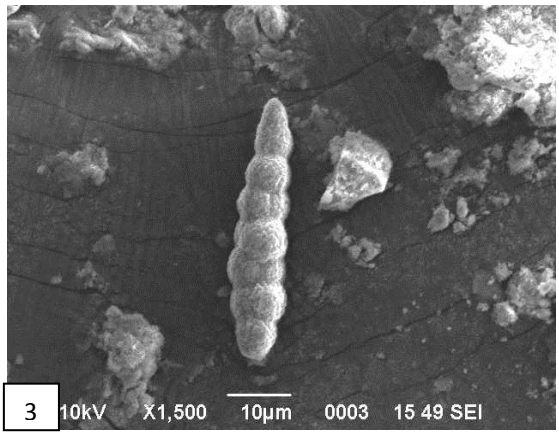
10kV X2,500 10µm 0003 15 49 SEI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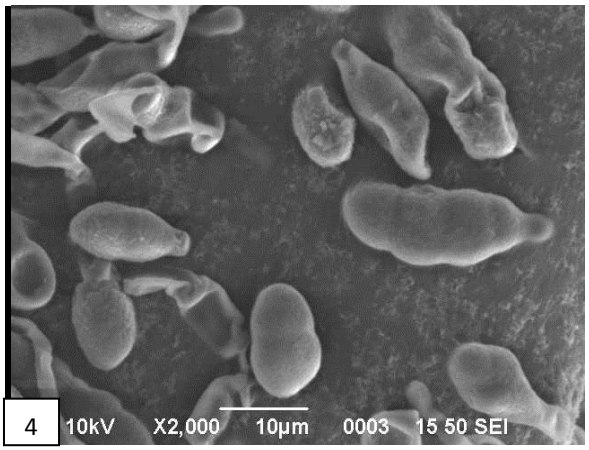
10kV X1,500 10µm 0003 15 49 SEI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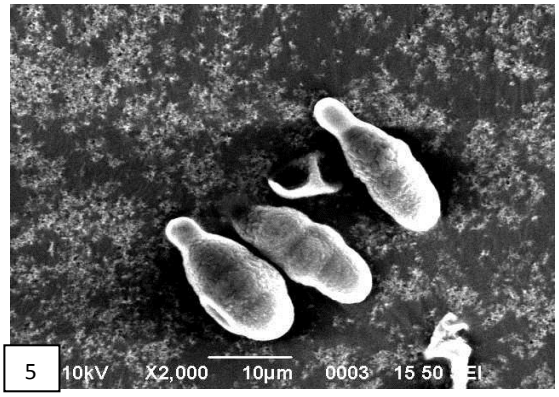
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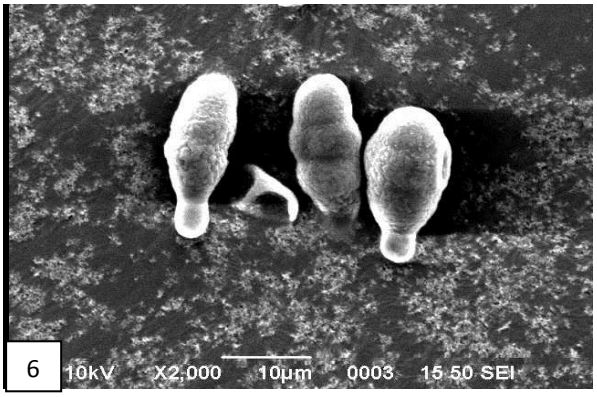
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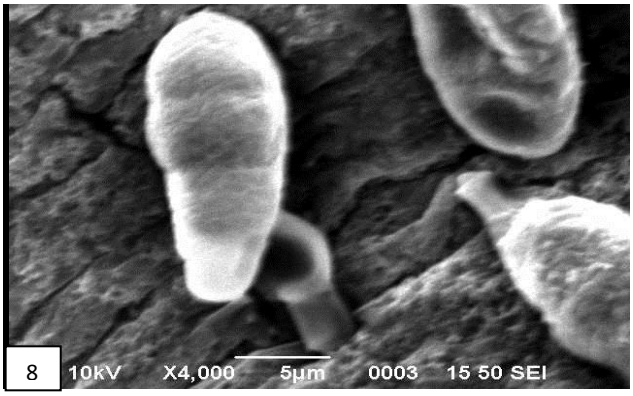
10kV X2,000 10µm 0003 15 50 SEI


7
10kV X1,500 10µm 0003


8
10kV X4,000 5µm 0003 15 50 SEI

Scanning electron microscope of conidia and pre penetration structure of *Alternaria alternata* on different magnification (Fig.1&8).

Table 1: Metreological data for September 2010 to November 2010 cultivars of different mulberry varieties in this study area of north eastern Uttar Pradesh.

Place	Months	Temperature(˚C)		Humidity (%)		Rainfall(ml)
		Max average	Min average	Max average	Min average	Average
Lucknow	September2010	30	26	85	86	7
	October 2010	27.58	18.16	73.12	63.93.	78
	November2010	25	8	77	43	0
Place	**Months**	**Temperature(˚C)**		**Humidity (%)**		**Rainfall(ml)**
Lakhimpurkhiri	September 2010	28.45	24.50	76	79	8.4
	October 2010	25.24	17.32	69.42	60.45	68
	November 2010	23.56	7.92	73.57	61.23	4.5

Table 02: Colony characteristics on Potato dextrose agar plates.

	Day	Diameter of colony (mm)	Colour
Culture characteristics on PDA plates	1st	20.45	greenish
	2nd	21.52	greenish
	3rd	22.35	greenish
	4th	36.38	greenish
	5th	44.84	greenish
	6th	54.57	greenish
	7th	56.21	greenish

Table 03: Colony characteristics on Water agar plates.

	Day	Diameter of colony (mm)	Colour
Culture characteristics on water agar plates	1st	18.72	greenish
	2nd	20.11	greenish
	3rd	21.12	greenish
	4th	33.21	greenish
	5th	41.51	greenish
	6th	51.32	greenish
	7th	53.34	greenish

RESULTS

Infected leaves were collected based on various symptoms observed that is small circular light brown spots developed on the leaves which increased in size and turned dark brown. These spots later on become shot holes. Severely affected leaves become yellowish and fall prematurely (Fig.1&2). The fungal pathogen was isolated from the lesion brown leaf spot of the infected mulberry leaves. The initial symptoms of infection are the occurrence of patches. Colonies size reaches 2.5 to 5.85cm on following incubation period at 26 ˚C – 28 ˚C for 7 days on Potato dextrose agar and Water agar (Fig.3&4). The colonies surface was greenish black with a light border, while the reverse phase is typically brown to black in colour. The fungus produces abundant branched. Microscopically studied, septate, brownish hyphae bearing simple large brown conidia with transverse septations have been identified using cotton blue and Lactophenol preparations. The mature conidia were with short beak and wide ellipsoid to ovoid in shape with short chain. The conidia were clavate (Shaped like a bowling pin) and single file chains (Fig.5 and 6). The spores have both longitudinal and horizontal septae. Each conidium tapers into a narrow rounded protuberance. The conidiophores of *A. alternate* produced conidia as chain. Conidiophore arising from single septate is brown solitary, straight and clavate. The number of longitudinal and transverse septa was 1-3 and 2-8 respectively per conidium. SEM studied we that the conidia length was 35.25µm and average width 4.94 µm of different magnification(Fig.1&8). Incidence of diseases including brown leaf spot caused by *A. alternata* was encountered in the north eastern region of Uttar Pradesh. The highest incidence of leaf spot was found during warmer, high rainfall, high humidity area of the north eastern region of Uttar Pradesh show in **Table 1**. This would agree with the observation that most infection seems when it rains during (November-December). The fungus has been identified to be *Alternaria alternate.*

DISCUSSION

In this report I discussed the symptomatology of *Alternaria alternata* on mulberry plant at Gonda and Lucknow in north eastern of Uttar Pradesh. As a species of the genus *Alternaria, Alternaria Alternata* belongs to the family of black pigmented molds Dematiaeceae *Alternaria Alternata* has wide host range, causing leaf spots on many plants parts (King and Schade.,1984). *Alternaria Alternata* are common saprophytes on trees and shrubs, this study clearly demonstrated that *A. Alternata* is a primary pathogen in lilac (Syringa sp.), causing a leaf blight that affects different Syringa species (Mmbaga *et al*., 2011). *Alternaria Alternata* is a pathogen of *Amaranthus cruentus* and *A.paniculatus* in Poland with low host specificity (Pusz.,2009).Based on pathogenicity morphology and rDNA spacer sequences the pathogen was identified as Alternaria Alternata (Fr.)Keissler.This report is the first of Alternaria leaf blight of money plant(Sankar *et al* .,2011).A leaf spot disease was observed on Aloe vera plants as small, circular to oval dark brown necrotic sunken spots on the leaves. the pathogen was identified as Alternaria Alternata on the basis of morphological and cultural characteristics(Bajwa *et al*., 2010).It can be recommended to the farmers for the efficient management of Alternaria Alternata leaf blight of chrysanthemum(Kumar *et al*., 2011).The colonies was greyish white t the beginning which later darkened and became greenish black with a light border. Microscopically septate brown hyphae bearing sample large brown conidia. The fungus has been identified to be *Alternaria Alternata* (Allah Abd.,2008). *Alternaria Alternata* fungi brown spot of tangering hybrids and Alternaria black rot of the novel orange fruit *Alternaria Alternata* a conidium germination inoculation and colonization on the plant surface studied using electron microscope.(Dehpour *et al*.,2007).The number of air borne spores significantly decreased 6m from the infection source. Periodically trapping of air borne of *Alternaria Alternata* in a cotton growing region for two years revealed that their air dispersal is local probably at the field level. *A. Alternata* air borne spores were also trapped in rather low number regardless of the presence of infected cotton plants (Bashan *et al*.,1990). *Alternaria Alternata* were isolated and identify from root, foliage and soil of both wheat and rice crops and their aggressiveness was studied using aggressiveness analysis ,isolates of were genetically characterized using RAPD's (Iram and Ahmad,2005). Several diketopiperazines have been isolated from liquid cultures of). *Alternaria Alternata* the causal agent of black blight of spotted knapweed (Stierle *et al* .,1988).Conidia of *Alternaria Alternata* were small and septate ranging from 7 to 30µm invivo with long filiform beaks Germinated conidia were not dislodged during SEM preparation and so it was concluded that they adhered strongly to the leaf surface (Dehpour *et al*.,2007).

CONCLUSION

It has been conducted from research that one of the important factors which disappearing small circular light brown spots developed on the leaves which increased in size and turned dark brown. These spots later on become shot holes. Severely affected leaves become yellowish and fall prematurely on leaf spot of Mulberry plants could be influence of *Alternaria alternata*.

ACKNOWLEDGEMENTS

We are grateful Dr. Sanjay K.Singh, Scientist, Mycology and Plant Pathology group, Agharkar Research Institute, Pune for helping in identification .Harendra kumar is a recipient of University Research Fellowship.

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