Morphological characterization and Pathogenicity test of Alternaria leaf spot of Chinese cabbage (*Brassica chinensis* L.)

Abstract

Chinese cabbage (*Brassica chinensis* L.) is an important leafy vegetable crop. It has good nutritional and medicinal value. Among all diseases affecting the plant, Alternaria leaf spot is a serious disease hampering its successful cultivation. Keeping in view this serious implication, an experiment on Alternaria leaf spot of Chinese cabbage caused by *Alternaria brassicae, A. brassicicola* and *A. alternata* was carried out at Main Experimental Station, Vegetable of ANDUA&T, Kumarganj Ayodhya for studying a means to effectively manage the disease. The disease was first appeared in the first week of November as small, yellow to brown spots with a light green to yellow halo. As the disease advances, several lesions coalesced, giving a blighted appearance. Stem and pods were also affected by the disease. The pathogen was readily isolated on 2% Potato Dextrose Agar medium and pathogenicity was proved following Koch's postulates. Cultural and morphological characters were observed as colonies of pure black amphigenous, effused, radial growth, conidia light brown to black in colour. The size of the conidia of *A. brassicae*, *A. brassicicola* and *A. alternata* were found 110.16 -162.0 µm, 94.08 – 120.72 µm and 25.44 - 46.32 µm, having septal variation 5-11, 0-8 and 2-6, respectively.

Keywords: *Alternaria, Pathogenicity, Conidia, PDA medium.*

1. Introduction

Chinese cabbage (*Brassica chinensis* L.), also known as Chinese leaves or celery cabbage, is a winter season crop grown for leafy vegetable & salad. It belongs to the family Brassicaceae. It is characterized by its bright green leaves, a short thick stalk, and a large compact globular head. Chinese cabbage is a special type of cabbage that originated in the Mediterranean region and was brought to China along with the expansion of Buddhism, where it has been in cultivation since the fifth century. Chinese cabbage is produced from a hybrid between the Chinensis and the Turnip varieties from the south and the Turnip variety from the north. On the basis of the tight,

compact heads of interior leaves, two more or less distinct species of Chinese cabbage are grown.

Chinese cabbage is an economical important species that are cultivated as leafy vegetable, oil and fodder crops [1,2]. "Now Chinese cabbage is cultivated in various region of the world, including India, China, Egypt and Europe. The two most important *Brassica rapa* leafy morphotypes are Chinese cabbage with leafy heads and pak choy with flat smooth leaves and fleshy petioles that do not form a head. The general hypothesis is that Chinese cabbages have been domesticated from non-heading leafy pak choy in China" [3]. "Compared to the non-heading accessions, the heading types can be transported easily, stored for a long time and have better cold tolerance and higher yield" [4].

It can become popular as a principal leafy vegetable crop growing throughout India. Chinese cabbage is commonly known as white cabbage, flower cabbage, celery cabbage, pak choy, michihli and nepa cabbage. Chinese cabbage has somewhat mild sweet taste with good source of minerals and vitamins. It contains 6% dry matter, 10% crude fibre, and numerous minerals such as potassium 2199 mg, calcium 289 mg, magnesium 146 mg, sodium 111 mg, and vitamin C 316 mg per kilogram of Chinese cabbage. However, total mean content of nitrates reached the value of 647 mg/kg. [5].

Like other vegetable crops, Chinese cabbage also suffers from various diseases caused by fungus, bacteria and viruses. However, among the fungal disease, Alternaria leaf spot caused by *A. brassicae, A. brassicicola* and *A. alternata* is a serious disease causing heavy losses during all the stages of the plant growth. During the course of survey made for Chinese cabbage at the Main Experimental Station, Vegetable farm of the University, Alternaria leaf spot of Chinese cabbage caused by *A. brassicae, A. brassicicola, and A. alternata,* which is the subject of present investigation, has been found to be widespread causing and substantial loss to this crop in addition to other diseases. The first symptom of Alternaria leaf spot of Chinese cabbage appeared on 4 November 2020 as minute yellows pecks on the oldest leaves and stems. The spots darkened and enlarged into circular, tan to dark brown colour. Alternating light and dark concentric rings give the spots the appearance of a target board; a yellow halo may surround the lesion.

2. Materials and methods

2.1 Study Area

The present investigation was conducted in the laboratory of the Department of Plant Pathology and the field experiment carried out at the Main Experimental Station (MES), Vegetable of Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya, U.P. during 2019-20 and 2020-21.

2.2 Isolation, purification and identification of the pathogen

The diseased leaf samples, showing distinct symptoms were selected for isolation of the pathogen. The selected leaves were washed with fresh sterilized water in order to remove the dust particles and surface contaminants. The washed diseased leaves were cut into small bits, with some healthy portions, with the help of sterilized scalpel and forceps. The cut leaf pieces were surface sterilized with 0.1%$HgCl_2$ for 30 seconds with the help of sterilized forceps and washed thoroughly 3 to 4 times with sterilized water to remove the traces of $HgCl_2$. Excess moisture was removed by placing these pieces in between two folds of sterilized blotting papers under aseptic condition in the inoculation chamber. These pieces were then transferred in Petri-dishes having 2% Potato Dextrose Agar medium. Three to four pieces of diseased leaves were placed per Petri dish at equal distance from each other. Petri dishes were properly marked with glass marking pencil indicating date of isolation, isolate number etc. The Petri dishes were incubated at a temperature of 24$\pm$ 1^{o}C and observed for fungal growth.

2.3 Purification of the isolated fungus

"The purification of culture was done by single spore isolation technique. A dilute spore suspension was poured on plain agar in Petri-dishes and the spores were allowed to settle down on the agar surface. Single spore was selected under the microscope and encircled. They were transferred to Petri dishes containing sterilized Potato Dextrose Agar medium. After proper growth the fungus was transferred into potato dextrose slants and maintained for further studies by sub culturing at intervals". [21]

2.4 Cleaning and sterilization of glassware

Sterilized glassware was used for all laboratory studies. The glassware was boiled for half an hour and then washed with vim powder followed by rinsing in tap water. Whenever required, glassware such as, Petri dishes, test tubes, funnels, glass rods, beakers and conical flasks etc., were kept in the cleaning solution, containing 60 g $K_2Cr_2O_7$ and 60 ml of concentrated H_2SO_4 in one liter of water for a day followed by washing in running water. All the dry glass wares were sterilized at 160^oC for 1.5 hours in hot air oven before use.

"The metallic objects like tips of inoculation needle, forceps and cork borer were sterilized by dipping in spirit and heating in blue flame to red hot before inoculation. Laminar flow cabinet or tissue culture hood was sterilized with ultra violet lamp before use. Sprit was used as general disinfectant for hand and surface of laminar flow. All culture studies were conducted in aseptic condition under laminar flow cabinet". [21]

2.5 Collection of diseased sample

The Chinese cabbage leaves showing characteristic symptoms of the disease were collected aseptically from MES, brought to the Plant Pathology laboratory, ANDUAT, Kumarganj, Ayodhya. The sample was kept in dry paper envelopes especially meant for the purpose and documentation of the details of location, variety, crop stage and date of collection for isolation of pathogen was recorded.

2.6 Preparation of culture media:

The Potato Dextrose Agar medium was used for isolation and purification of fungal culture and rest of *in vitro* experiments. PDA medium consisting following composition was prepared and sterilized using method described by [6].

Peeled potato	-	200.00 g
Dextrose	-	20.00 g
Agar-agar	-	20.00 g
Distilled water	-	1000 ml

The peeled potatoes were cut in 12 mm cubes. Two hundred grams of potato cubes were rinsed in water and boiled for 20 minutes in 500 ml water. Potato broth was filtered through cheese cloth and kept in measuring cylinder. Agar was melted in 500 ml of water by heating and added to the potato broth. Dextrose was added in it. The final volume was made up to 1000 ml by adding distilled water. The pH was adjusted to 7.0. The PDA medium was poured in test tube

for preparation of PDA slant and also in flask. Then these were sterilized at 15 psi for 20 minutes in an autoclave.

2.7 Cultural and morphological studies

Cultural and morphological characteristics were done on the potato dextrose agar medium. Each isolated fungi were grown on Potato Dextrose Agar to study their cultural characteristics (like colonial morphology, colony colour, mycelial growth, and morphological characteristics like size, shape and colour of conidia and conidiophores).

2.8 Micrometry for calibration of ocular micrometer for different objectives for measurement of fungal structures

"For calibration of ocular micrometer, a bright-field microscope, an ocular micrometer and a stage micrometer was used. Eye piece of microscope was removed and ocular micrometer glass disc was inserted on the metal diaphragm and eye piece was inserted in the microscope. The stage micrometer glass slide was placed on the microscope stage. Stage micrometer is a special glass slide having one division equals to 10 µm (0.01 mm). Fine graduations of stage micrometer were observed under a sharp focus under low-power and high-power objectives. During observations, the ocular micrometer lines were on the top and the stage micrometer image was on the bottom. The lines of the ocular micrometer parallel with those of the stage micrometer were aligned by fine rotation of the stage micrometer and ocular micrometer and ocular micrometer lines coincide with stage micrometer at the left end and another line coincides in right (completely parallel at both ends) was observed. Number of divisions of ocular and stage micrometers between the two coinciding lines was counted. Five readings were taken with the low power (10x) and high-power (40x) objective. These observations are then used to calculate the calibration factor for the objective lens in use". [21]

$$\text{One ocular Division (µm)} = \frac{Number\ \ of \text{ division on stage micrometer}}{Number\ \ of \text{ division on ocular micrometer}} \times 10$$

After calibration, the ocular micrometer was used to measure the size of conidia and conidiophores, etc. in terms of length and breadth by following formula.

Size (µm) = No. of ocular division occupied by conidia/conidiophores x calibration factor of objective.

2.9 Pathogenicity test of isolated pathogen:

The pathogenicity of isolated pathogen was tested Fallowing Koch's postulate on healthy plants of Chinese cabbage (germplasm Narendra Chinese cabbage-1) which is susceptible to this disease. For this study the plants were inoculated by spraying spore-cum mycelial suspension, prepared in sterilized water with the help of an automizer. Both mechanically injured and uninjured leaves were employed for inoculation purposes. After inoculation the plant were covered with polythene bags for 72hr to provide sufficient humidity for injection. A separate pot of plants was kept as control by spraying only sterile water in place of fungal suspension.

These plants were examined periodically for recording the symptoms and final data on the disease were recorded 10 days after inoculation. The causative fungi were re-isolated from the infected leaves of inoculated plants and compared with the original isolated pathogens.

3. Result and discussion

3.1 Symptoms of Alternaria leaf spot:

The symptoms of the disease under investigation are described below on leaves, stems and pods under natural field conditions. The disease usually appeared during November and reached its peak towards December.

The symptoms of Alternaria leaf spot of Chinese cabbage become visible at the time of seedlings emergence. The symptoms first appeared as minute yellow pecks on the oldest leaves. The spots are darkened and enlarge to circular, tan to dark brown colour. Alternating light and dark concentric rings give the spot appearance of a target broad; a yellow halo may surround the lesion (Figure- 1). As the disease advanced, several lesions coalesced and cover large areas of leaves, giving a blighted appearance, resulting in their eventual death. The lesions on stem were elliptical, oval to oblong, light brown to dark brown, spreading light wires measuring 2-10 mm in diameter. The initial lesions on pods consisted of small, dark brown to black, circular to irregular

spots several lesions coalesced and cover large areas of pods. The mature fruits were readily attacked by the fungus.

The fungus was isolated from infected leaves of Chinese cabbage on PDA medium after ten days of incubation at 24 ± 1°C. The growth of fungus fully developed by this time. Pure culture of the Alternaria leaf spot of Chinese cabbage obtaining by single sporing on liquid agar medium, yielding different *Alternaria* spp. (*A. brassicae*, *A. brassicicola* and *A. alternata*) the detail characterization of fungal colony, conidiophores and conidia was observed.

The symptoms of Alternaria leaf spot on different parents of Chinese cabbage observed in nature in the present study were quite similar to those described by [7,8,9,10]

Fig. 1. Characteristics symptoms of Alternaria leaf spot of Chinese cabbage

3.2 Colony character and mycelial growth

The fungus was isolated on a 2% potato dextrose agar medium for identification and character. The cultural and morphological characteristics of the fungus were studied and found that the *Alternaria brassicicola* was fast-growing fungus. It has amphigenous, effused, radial growth with olivaceous brown to blackish colour and smooth mycelium while *Alternaria brassicae* was a medium-growing fungus with amphigenous, effused, hairy, profuse radial growth pale-olive colour with smooth mycelium. *Alternaria alternata* was a comparatively slow-growing fungus with amphigenous, effused, cottony growth and gray to green colour with smooth mycelium (Figure - 2). The cultural and morphological characters of the isolated fungus were in agreement with the studies of the other different workers from India and abroad viz. [11,12,13,14,15,16], The cultural and morphological characters of *Alternaria brassicae, A. brassicicola and A. alternata* by above workers were conferred the present finding.

Table 1. Characteristics of colony and mycelium of *Alternaria* spp.

S. No.	Colony characters	*Alternaria brassicae*	*Alternaria brassicicola*	*Alternaria alternata*
1	**Growth**	Amphigenous, effused, radial growth	Amphigenous, effused, hairy, profuse radial growth	Amphigenous, effused, cottony growth
2	**Colour**	Olivaceous brown to blackish colour	Pale-olive colour	Gray to green colour
3	**Shape**	Circular	Circular	Circular

3.3 Conidia and conidiophores:

In the present investigation the size of conidia of *Alternaria brassicae*, *A. brassicicola* and *A. alternata* were measured at 136.08 µm, 107.42 µm and 37.92 µm respectively *A.*

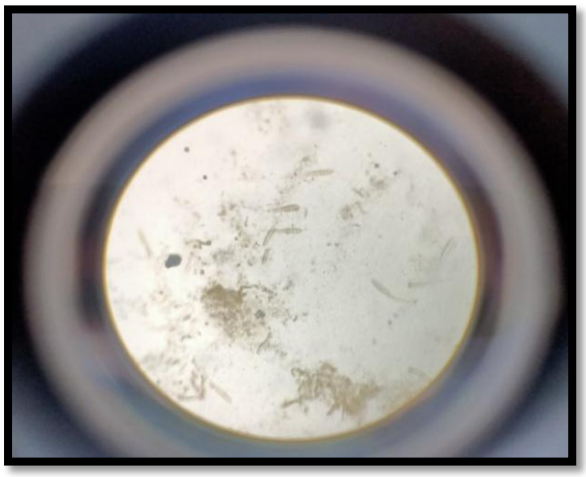
brassicae, *A. brassicicola* and *A. alternata* have variations in number of septa associated with conidiophores which were 5-11, 0-8 and 2-6, respectively.

Table 2. Characteristics of conidia and conidiophores of *Alternaria* spp

Isolates	Morphological characteristics of *Alternaria* spp.											
	Length (µm)		Breadth (µm)		Beak length (µm)		Conidial septation					
	Mean	Range	Mean	Range	Mean	Range	Horizontal		Vertical		Beak septation	
							Mean	Range	Mean	Range	Mean	Range
Alternaria brassicae	136.08	110.16 -162.0	26.01	22.56 - 30.24	49.72	43.44 – 58.52	8.0	5-11	1.4	0-4	2.2	1-3
Alternaria brassicicola	107.42	94.08 – 120.72	30.0	25.92 – 34.32	16.08	10.08 – 24.72	5.6	0 - 8	-	-	-	-
Alternaria alternata	37.92	25.44 - 46.32	12.14	10.08 –13.92	12.33	9.84 – 14.88	3.6	2-6	1.8	1-3	0.6	0 -1

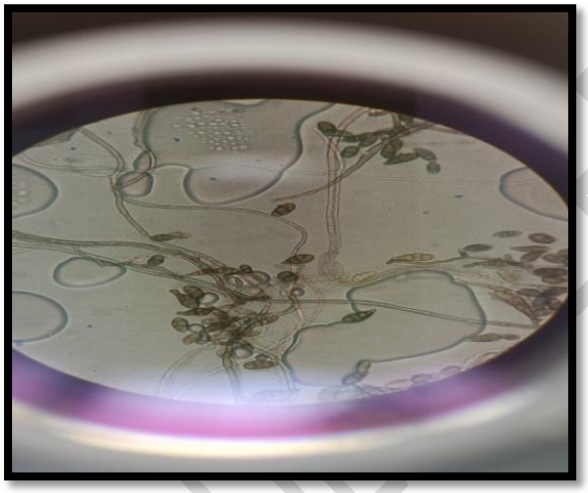
(a) Conidia of *A. brassicae*

Pure culture of *A. brassicae*



(b) Conidia of *A. brassicicola*

Pure culture of *A. brassicicola*



(c) Conidia of *A. alternata*

Pure culture of *A. alternata*

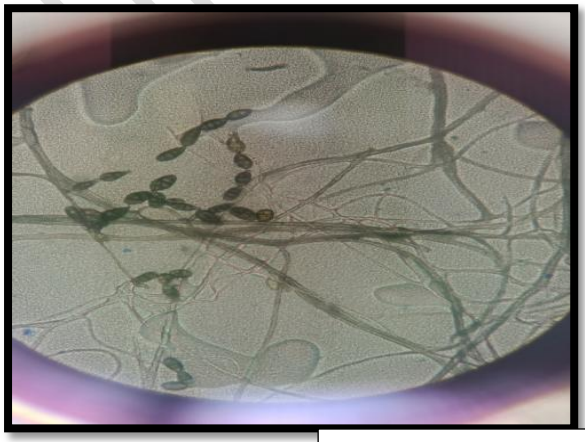


Fig. 2 Different isolates of *Alternaria* spp.

The morphological and cultural characteristics for *A. brassicae, A. brassicicola, A. alternata* observed in the present study are more or less similar to those described by [17,18].

3.4 Pathogenicity test of isolated fungi:

The symptoms appeared on leaves 3-4 days after inoculation. These symptoms were similar to those observed under natural field conditions. The plant sprayed with distilled water, served as a control and remain disease-free from infection. Reisolation from the artificially inoculated leaves produces the same isolates as that of previously of *A. brassicae, a. brassicicola* and *A. alternata* that were isolated from the naturally infected leaves. Thus pathogenicity was proved in accordance with Koch's postulates.

The pathogenicity of this fungus was established on Chinese cabbage and other cruciferous crops by other workers [19,20] also and their findings support the pathogenic nature of the fungus as observed in the present case.

Fig 3. Pathogenicity test

4. Conclusion

Alternaria leaf spot of Chinese cabbage caused by *Alternaria brassicae, A. brassicicola* and *A. alternata* is a very serious disease of Chinese cabbage in recent years. With a view to combating this serious disease for exploiting full yield potential, the studies were taken performed.

(I) first symptom on Alternaria leaf spot of Chinese cabbage disease appears as minute yellows pecks on the oldest leaves, stems and pods. The spots are darkened and enlarged into circular, tan to dark brown colour.

(ii) Morphological characters of the isolated fungus were studied and observed that *Alternaria brassicicola* was fast growing fungus and it has amphigenous, effused, radial growth having olivaceous brown to blackish colour with smooth mycelium. The length and width of conidia of *Alternaria brassicicola* was recorded 107.42×30.0 µm respectively. The horizontal septa of *Alternaria brassicicola* were recorded 0 to 8.

(iii) *Alternaria brassicae* was medium growing fungus and it has amphigenous, effused, hairy, profuse radial growth having pale-olive colour with smooth mycelium. The length and width of conidia of *Alternaria brassicae* was recorded 136.08 µm and 26.01 µm respectively. The horizontal and vertical septa of *Alternaria brassicae* were recorded 5 to 11 and 0 to 4 respectively.

(iv) *Alternaria alternata* was comparatively slow growing fungus and it has amphigenous, effused, cottony growth having gray to green colour with smooth mycelium. The length and width of conidia of *Alternaria alternata* was recorded 37.29×12.14 µm respectively. The horizontal and vertical septa of *Alternaria alternata* were recorded 2 to 6 and 1 to 3 respectively.

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Conidia of *A. alternata*