

## Original Research Article

### **Isolation, Purification, Identification and Pathogenicity test of *Alternaria* leaf spot of Chinese cabbage**

#### **Abstract**

Chinese cabbage (*Brassica chinensis* L.) is an important leafy vegetable crop. It has enough nutritional and medicinal value. Among the disease, *Alternaria* leaf spot is a serious disease hampering successful cultivation to this crop, Keeping in view the serious an experiment on *Alternaria* leaf spot of Chinese cabbage caused by *Alternaria brassicae*, *A. brassicicola* and *Alternaria alternata* was carried out at MES, Vegetable of ANDUA&T, Kumarganj Ayodhya for effetely manage the disease. The disease was first appeared on 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 to 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 observed as colonies pure black amphigenous, effused, radial growth, conidia light brown to black in colour, size of conidia of *A. brassicae*, *A. brassicicola* and *A. alternata* were found 110.16 -162.0  $\mu\text{m}$ , 94.08 – 120.72  $\mu\text{m}$  and 25.44 - 46.32  $\mu\text{m}$ , having septal variation 5-11, 0-8 and 2-6, respectively.

**Keywords:** *Alternaria*, Pathogenicity, Conidia and 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 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]. It 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 choi's with flat smooth leaves and fleshy petioles that don't form a head. The general hypothesis is that Chinese cabbages have been domesticated from non-heading leafy pak choi in China [3]. Compared to the non-heading accessions, the heading types can be transported easily, stored long time and have better cold tolerance and higher yield [4].

It can become popular as principal leafy vegetable crops by growing throughout India. Chinese cabbage is commonly known as white cabbage, flower cabbage, celery cabbage, Pakchoy, michihli and nepa cabbage. Chinese cabbage has somewhat mild sweet test with good source of minerals and vitamins. Per kilogram of Chinese cabbage, it contains 6% dry matter, 10% crude fibre, and numerous minerals such as potassium 2,199 mg, calcium 289 mg, magnesium 146 mg, sodium 111 mg, and vitamin C 316 mg. 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, bacterial and viral. 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 course of survey made for Chinese cabbage at 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 wide spread causing and substantial loss to this crop in addition to other diseases. The first symptom of Alternaria leaf spot of Chinese cabbage appears on 04 November, 2020 as minute yellows pecks on the oldest leaves and stems. The spots are 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 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 percent Mercuric Chloride ( $\text{HgCl}_2$ ) for 30 seconds with the help of sterilized forceps using and washed thoroughly 3 to 4 times with sterilized water to remove the traces of Mercuric Chloride. 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 percent Potato Dextrose Agar medium. Three to four pieces of diseased leaves were placed on per Petri dish at equal distance from each other. Petri dishes were properly marked with glass marking pencil indicating date of isolation and isolate number etc. The Petri dishes were incubated at a temperature of  $24 \pm 1^\circ\text{C}$  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.

### **2.4 Cleaning and sterilization of glassware:**

For all laboratory studies, the sterilized glassware was used. The glassware were boiled for half an hour and then washed with vim powder followed by cleaning 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 gm Potassium Dichromate ( $K_2Cr_2O_7$ ) and 60 ml of concentrated Sulfuric acid ( $H_2SO_4$ ) in one liter of water for a day followed by washing in running water. All The dry glass wares were sterilized at  $160^{\circ}C$  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 the spirit and heating on 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 cultural studies were conducted in aseptic condition under laminar flow cabinet.

### **2.5 Collection of diseased sample:**

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

### **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   | - | 200.00gm |
| Dextrose        | - | 20.00gm  |
| Agar-agar       | - | 20.00gm  |
| 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 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 studied on the potato dextrose agar medium. Each isolated fungi were grown on Potato Dextrose Agar (PDA) 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  $\mu\text{m}$  (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 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.

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

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.

$$\text{Size } (\mu\text{m}) = \text{No. of ocular division occupied by conidia/conidiophores} \times \text{calibration factor of objective.}$$

## **2.9 Pathogenicity test of isolated pathogen:**

The pathogenicity of isolated pathogen was tested following 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 atomizer. Both mechanically injured and uninjured leaves were employed for inoculation purposes. After inoculation the plants were covered with polythene bags for 72hrs to provide sufficient humidity for infection. 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<sup>th</sup> days after inoculation. The causative fungi were re-isolated from the infected leaves of inoculated plants and compared with the original isolated of the pathogens.

## **3. Result and discussion**

### **3.1 Symptoms of Alternaria leaf spot:**

The symptoms of the disease under investigation are described below observed 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 specks 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 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. In 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  $24 \pm 1^\circ\text{C}$ , the growth of fungus fully developed. Pure culture of the *Alternaria* leaf spot of Chinese cabbage obtained 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 Isolation, purification and identification of the pathogen:**

The pathogen was isolated from infected leaves of the Chinese cabbage crop, collected from Main Experimental Station, Vegetable Research Farm of Acharya Narendra Dev University of Agriculture & Technology, Kumarganj, (Ayodhya) Uttar Pradesh. Infected leaves showing the typical symptoms of *Alternaria* leaf spot disease of Chinese cabbage were collected and brought to the laboratory for isolation, purification and identification of the pathogens. The *Alternaria* fungus associated with infected leaves was isolated aseptically using standard procedure on Potato Dextrose Agar (PDA) Medium. The fungus *A.brassicae*, *A. brassicicola* and *A. alternata* were isolated from the infected tissue of Chinese cabbage on 2% Potato Dextrose Agar (PDA) medium by transferring surface sterilized portion of diseased leaves, stem and pods. Subsequently it was purified by single spore techniques. The culture was then maintained on PDA slants for further studies.

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.

### 3.3 Colony Character and mycelial growth:

The fungus was isolated on 2% potato dextrose agar medium for identification and character. The cultural and morphological characters of the fungus were studied and found that the *Alternaria brassicicola* was fast growing fungus and it has amphigenous, effused, radial growth having olivaceous brown to blackish colour with smooth mycelium while *Alternaria brassicae* was medium growing fungus and it has amphigenous, effused, hairy, profuse radial growth having pale-olive colour with smooth mycelium. *Alternaria alternata* was comparatively slow growing fungus and it has amphigenous, effused, cottony growth having gray to green colour with smooth mycelium. (Figure - 2)

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

| S. No. | Colony characters | <i>Alternaria brassicae</i> | <i>Alternaria brassicicola</i> | <i>Alternaria alternata</i> |
|--------|-------------------|-----------------------------|--------------------------------|-----------------------------|
|        |                   |                             |                                |                             |

|   |               |                                     |                                                    |                                      |
|---|---------------|-------------------------------------|----------------------------------------------------|--------------------------------------|
| 1 | <b>Growth</b> | Amphigenous, effused, radial growth | Amphigenous, effused, hairy, profuse radial growth | Amphigenous, effused, cottony growth |
| 2 | <b>Colour</b> | Olivaceous brown to blackish colour | Pale-olive colour                                  | Gray to green colour                 |
| 3 | <b>Shape</b>  | Circular                            | Circular                                           | Circular                             |

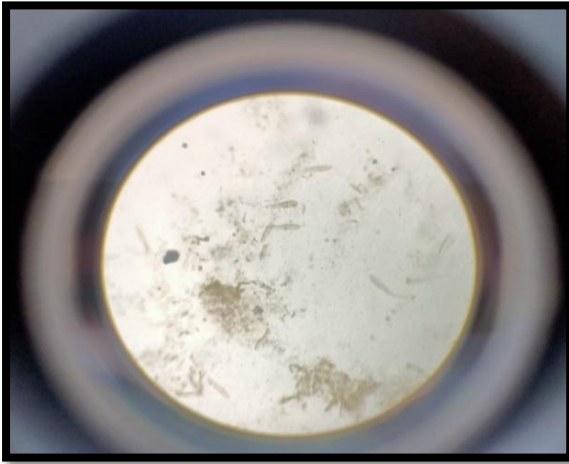
### 3.4 Conidia and conidiophores:

In present investigation the size of conidia of *Alternaria brassicae*, *A. brassicicola* and *A. alternata* were measured 136.08 $\mu$ m, 107.42 $\mu$ m and 37.92 $\mu$ m 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 <i>Alternaria</i> 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 |
| <i>Alternaria brassicae</i>    | 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   |
| <i>Alternaria brassicicola</i> | 107.42                                                  | 94.08 – 120.72 | 30.0         | 25.92 – 34.32 | 16.08            | 10.08 – 24.72 | 5.6                | 0 - 8 | -        | -     | -              | -     |
| <i>Alternaria alternata</i>    | 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  |

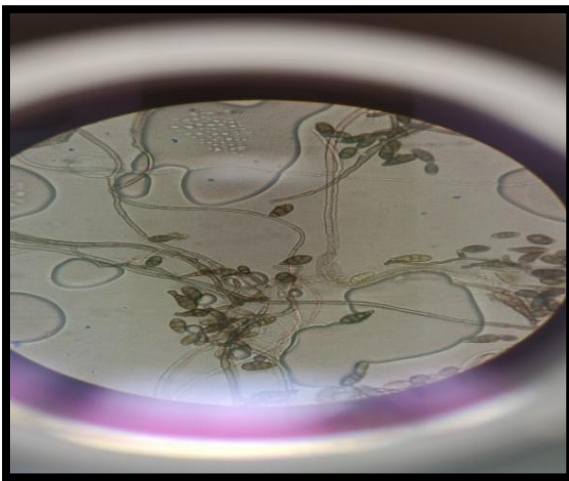
Conidia of *A. brassicae*



Pure culture of *A. brassicae*



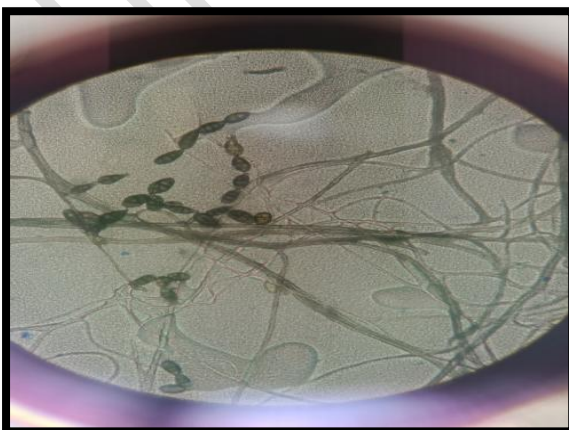
Conidia of *A. brassicicola*



Pure culture of *A. brassicicola*



Conidia of *A. alternata*



Pure culture of *A. alternata*



Fig. 2 Different isolates of *Alternaria* spp.

The morphological and cultural characters of the pathogen observed in the present study are more or less similar to those described by [17,18] and for *A. brassicae*, *A. brassicicola*, *A. alternata* and it was identified as such.

### 3.5 Pathogenicity test of isolated fungi:

The fungus was isolated from the affected tissues of Chinese cabbage on 2% potato dextrose agar medium. The pathogenicity test of the isolated pathogen was done as per procedure described under materials and methods by using mycelial suspension with sterilized atomizer on both injured and injured leaves. The symptoms were appeared on leaves 3-4 days after inoculation. These symptoms were similar to those observed under natural field condition. The plant sprayed with the distilled water, served as control 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 the 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 during recent year with a view to combating this serious disease for exploiting full yield potential the studies were taken off to find out.

1. Isolation, purification and identification of the *Alternaria* spp.

2. Pathogenicity test.

Among leafy vegetable, Chinese cabbage can play an important role in the dietary requirements among vegetarians of the country by growing throughout India. It can play an important role in the supply of vitamins and minerals to under nourished vegetarian population of the country. Chinese cabbage suffers from a number of fungal disease in which *Alternaria* leaf spot is an important disease caused by *Alternaria* spp. different aspect of disease as well as pathogen (*A. brassicae*, *A. brassicicola* and *A. alternata*) where study for the management of the disease.

Fungus *Alternaria brassicae*, *A. brassicicola* and *A. alternata* where isolated from infected Chinese cabbage leaves on potato dextrose agar (PDA) medium.

The information obtained on various aspects in the present study is summarized here briefly.

It was observed that the first symptom on *Alternaria* leaf spot of Chinese cabbage disease appears as minute yellow pecks on the oldest leaves, stems and pods. The spots are 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.

The cultural and 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 while *Alternaria brassicae* was medium growing fungus and it has amphigenous, effused, hairy, profuse radial growth having pale-olive colour with smooth mycelium. *Alternaria alternata* was comparatively slow growing fungus and it has amphigenous, effused, cottony growth having gray to green colour with smooth mycelium.

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