Original Research Article

Cauliflower Alternaria Leaf Spot Disease Caused by *AlternariaBrassicicola* (Schwein.) Wiltshire: Isolation, Purification, Pathogenicity and *In-Vitro* Studies of Different Culture Media

Abstract

On the farm of the College of Agriculture Chandra Shekhar Azad University of Agriculture and Technology Kanpur, Uttar Pradesh, India, during 2021–22, the cauliflower leaf spot disease (*Brassica oleracae*var. *Botrytis*) caused by *A. brassicicola* (Schwein.) Wiltshire was observed in a moderate to severe form. On potato dextrose agar medium, the pathogenic fungus was purified from cauliflower leaves that had been infected. By inoculating healthy cauliflower seedlings, the isolated fungus' pathogenicity was demonstrated. It was determined that the fungus was *Alternaria spp*. based on common symptoms on foliage, microscopic findings, and culture characteristics of the fungus. Microscopic study in bio-control lab C.S.A.U.A&T Kanpur, identified the pathogenic fungus as*Alternariabrassicicola*(Schwein.) Wiltshire. Eight culture media were tested among that, the potatodextrose agar medium was found most suitable and encouraged maximum radial mycelialgrowth (89.07mm) of *A. brassicicola*. The second best culture medium found was Corn Meal Agarmedium (67.46mm). This was followed by Potato carrot agar medium (54.83 mm), Rose Bengal agar (53.00 mm), Czapex dextrose agar (40.40 mm), Yeast Extract Agar (32.28 mm), and Nutrient Agar (24.28 mm), While minimum (22.16mm) growth was recorded in Starch Agar.

Key words: *Agriculture, Cauliflower, culture, growth, isolation and pathogenicity.*

Introduction

Cauliflower (*Brassica oleracea* var. *botrytis*) is one of the most important winter vegetables grow in India. It is a cool-weather crop and closely related to broccoli and cabbage. Cauliflower, however, has more specific climatic requirements than any of the other cole crops. With the development of heat tolerant varieties, cauliflower is available from September to May in plains of India. The word cauliflower is derived from the Latin words ‘caulis’ meaning stalk and ‘floris’ meaning flower. It is grown for its white tender curd or head. Botanically, the edible part of cauliflower is hypocotyl branches or pre-floral fleshy epical meristem

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Cauliflower grown throughout the world, especially in China, India, USA, Spain, Italy, Mexico and France, due to its higher nutritional value and widespread cultivation.

In India, during 2022cauliflower was grown in an area of about 452.59 thousand hectares,with the production of 8668.22 thousand tonnesand the productivity was about 19.15 MT/ha.

Comment [LCA1]: appointment

In Uttar Pradesh during 2021-22 cauliflower area 22.85 million hectares, production is 1.42 MT/hac and productivity 15-20 ton/hac. **(Horticulture statistics at a glance 2022).** In India, cauliflower is cultivated in almost all the states, mainly Bihar, Uttar Pradesh, Orissa, Assam, Madhya Pradesh, Gujarat and Haryana. Cauliflower plays an important role in the human diet due to its attractive appearance, good taste, and its nutritive value. It is a rich source of protein, carbohydrates, vitamin-B, and C as well as various minerals those are necessary for the human health. Cauliflower is grown for its edible flowering head and consume as a vegetable in curries, soups, and pickles.

Comment [LCA2]:

Materials and Methods

Visualobservations

To track the progression of the disease in a plant population under natural circumstances, visual observations of disease symptoms were made in the field and documented.

Collection of the diseased samples.

Leaves of cauliflower with dark brown spots having characteristic concentric circumferences, often with a yellowish chlorotic halo were collected from farmers field of nearby CSAUA&T, Kanpur and brought to the laboratory for isolation and further studies.

Microscopic study

Spores of *A. brassicicola*were taken from the pure culture and mounted on the clear glass slide. Spores were mixed with thoroughly with lactophenol in order to obtain a uniform spread over which a cover slip was placed. The spores and hyphae of the fungus were observed for spore shape and size and photomicrographs were taken.

Isolation

For isolation of pathogen, small pieces of the leaves were cut from the diseased portion along with some healthy tissues and surface sterilized with 1% sodium hypochlorite solution for one minute followedby three consecutive washings with sterilized distilled water. Thesurface sterilized pieces were transferred to Petri plates containingPotato Dextrose Agar (PDA) and

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incubated at 25+1°C in BODincubator. After seven days of incubation, the fungal growth transferredaseptically to PDA slants and purified following single spore technique.

Comment [LCA3]: meaning

Pathogenicity test

The pathogenicity was tested by following Koch's postulates. Cauliflower leaves were artificially inoculated with *A. brassicicola*by pin prick method (Pattanamahakul and Strange, 1999). To harvest conidia of the fungus, sterile distilled water was added to 10 days old culture growing on PDA Petri plates. The fungal spores were gently scrapped with the help of sterilized slide and conidial suspension was filtered through sterilized muslin cloth. The concentration of conidial suspension was adjusted to $4x10^5$ spores/ml with the help of haemocytometer. Subsequently, conidial suspension was sprayed on 35 days old cauliflower seedlings grown in pots having dimensions 7×8.5cm. Before inoculation the leaves were surface sterilized with 70 per cent ethanol with the help of a cotton swab. Then gentle pricking was done with the help of sterilized needle. After inoculum spraying, plants were covered with perforated and moistened transparent plastic cover at a temperature ranging from 20-25°C. Inoculated plants were labeled and kept under humid conditions to maintain proper moisture for disease development. High relative humidity was maintained with help of water spraying inside the polythene bags after every 12 hours. Leaves were observed for symptom development at regular intervals after inoculation. In case of control plants, sterile distilled water was sprayed.

Effectofculturemediaongrowthandsporulation

Alternariabrassicicola isolates were grown on eight different media viz. Potato dextrose agar (PDA), Corn meal agar (CMA), Czapek's (dox) agar (CZA), Starch agar (SA), Nutrient agar (NA), Yeast extract agar (YEA), Potato carrot agar (PCA), and Rose Bengal agar (RBA) at 25 ± 2 °C to identify the medium on which fungus grow faster than other.

I. Potato dextrose agar (PDA)

Potato	: 200 g
Dextrose	: 20 g
Agar-agar	: 20 g
Distilled water	: 1000 ml

II. Corn meal agar (CMA)

Corn meal	: 20 g
Agar-agar	: 18 g

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Distilled water	: 1000 ml

III. Czapek's dox medium

Sucrose	: 30.00 g
Sodium nitrate ($NaNO_3$)	: 2.00 g
Potassium dihydrogen phosphate (KH_2PO_4)	: 1.00 g
Magnesium sulphate ($MgSO_4$ 7H_2O)	: 0.50 g
Ferric chloride ($FeCl_3$ 6H_2O)	: 0.01 g
Potassium chloride (KCl)	: 0.50 g
Agar - agar	: 20.00 g
Distilled water	: 1000 ml

IV. Starch's agar

Soluble starch	: 2.00 g
Beef extract	: 3.00 g
Bacto peptone	: 5.00 g
Agar agar	: 5.00 g
Distilled water	: 1000 ml.

V. Potato carrot agar (PCA)

Carrot	: 200. g
Potato	: 250. g
Agar	: 15. g
Distilled water	: 1000 ml.

VI. Rose Bengal agar (RBA)

Soytone	: 5 g
Dextrose	: 10 g
KH_2PO_4	: 1 g
Magnesium Sulfate	: 0.5 g
Rose Bengal	: 0.05 g
Agar	: 15 g
Distilled water	: 1000 ml

VII. Yeast extract agar (YEA)

Soluble starch	: 10.00 g

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Yeast extract	: 1.00 g
Agar agar	: 20.00 g
Distilled water	: 1000 ml

VIII. Nutrient agar (NA)

Yeast extract/Beef extract	: 3 g
Peptone	: 5 g
NaCl	: 5g
Agar	: 15 g
Distilled water	: 1000 ml

In a beaker, quantity (grams) of the dehydrated powder or lab-prepared media is added to 1000 milliliters of distilled or deionized water. Suspension is then heated to boiling to dissolve the medium completely. dissolved medium is then autoclaved at 15 lbs pressure (121°C) for 15 minutes. Once the autoclaving process is complete, the beaker is taken out and cooled to a temperature of about 40-45°C. If enrichment is desired, the addition of blood or biological fluids can be done after the autoclaving process. media is then poured into sterile Petri plates under sterile conditions. Once the media solidifies, the plates can be placed in the hot air oven at a lower heat setting for a few minutes to remove any moisture present on the plates before use.

Eight media were evaluated in the present study. Media were prepared with given composition. The initial pH of each medium was adjusted to 6.5 prior to autoclaving. The medium was prepared with given composition and dispended in conical flask.

The flasks were plugged with non-absorbent cotton plugs and sterilized in an autoclave at 15 lbs. psi for 20 minutes. Petri plates were sterilized in hot air oven at 160^0C for 1 hour. Such sterilized Petri plates were poured with 20 ml of molten medium and allowed to solidify. Five millimeter diameter disc of the test fungus was cut with the help of incinerated cork borer and inoculated at the center of Petri plates. The inoculated plates were then incubated at room temperature (27 ± 2^0C) for 7 days. The compositions of all the media used were obtained from Ainsworth and Bisby's Dictionary of the fungi as mentioned upper. Each treatment was replicated thrice. The measurement of the colony diameter was taken when the maximum (90mm) growth was achieved in any one of the media tested. The cultural characters such as colony diameter, colony colour and degree of sporulation were recorded.

Statisticalanalysis

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In the present investigation, lab experiment was conducted in complete randomize design. The data obtained from all the experiments were statically analyzed following the standard procedures (Gomez and Gomez 1984)

Results and Discussion

Visualobservation

Visualobservationsofdiseasesymptomswererecordedinthefieldall the aerial parts of the plants *viz*., stem, leaves, fruits, pods and heads. Generally, in the beginning the spots remain small and circular or elliptical in shape with colour of spots varying from species to species. These may be pale, brown, olivaceous brown, grayish or black *etc*. and typically surrounded by halo-chlorotic tissues. Lateron these spot enlarged into gray to black lesionsof0.5to1cmdiameter.Later on, the spots gradually increase in size (varying with species) in a concentric manner and often coalesce, leading to leaf spot appearance.(PLATE-I)

Microscopicexamination

Diseased leaves samples, exhibiting the characteristic Alternaria leaf spot of cauliflower were examined visually followed by microscopic examination of sections of infected tissues. The pure culture isolates obtained from the diseased specimens of leaves were identified as *Alternariabrassicicola*(Schw.) Wiltsh. On the basis of the symptoms observed, macroscopic and microscopic characters of the test fungus. As per the present morphological studies the mycelium of the fungal pathogen was septate, olive grey to greyish black in colour, conidiophores were olivaceous, septate and branched, conidia were dark cylindrical to oblong, muriform and produced in chains of 8-10. The conidia were devoid of any prominent beak and found to have 5-8 transverse and 0-4 longitudinal septation. The identification of the fungus was further reconfirmed as *Alternariabrassicicola*(Schw.) Wiltsh. (PLATE-II)

Comment [LCA4]: there are no Plate II

Isolation

On potato dextrose agar medium, the pathogen was successfully isolated from diseased tissue displaying well-developed lesions together with healthy fraction that was brought to the lab from naturally infected cauliflower plants. The inoculation plates were incubated in the BOD incubator for 5 to 7 days at a temperature of 25 to 20C. The fungus culture was transplanted to PDA in Petri plates and multiplied in the lab after being isolated from diseased tissue.

Purificationoffungalculture

After seven days of incubation, the test fungus generated greenish-gray to black-colored, fluffy,

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lanose to loose cottony growth on potato dextrose agar medium. The pure culture's slants were sealed with paraffin wax and kept in the lab's refrigerator for later use.

Pathogenicitytest

The pure culture of *Alternariabrassicicola*obtained by single spore isolationmethod was used for pathogenicity test. The test was carried out as described in materialand methods by spore suspension spray inoculation on the foliage of 35 days oldsusceptible Pusa snowballvariety of cauliflower The symptoms appeared on inoculated leaves asbrown, circular or oval necrotic spots with concentric rings and surrounded by yellowhalo after twenty days after inoculation (Plate 2 and 5). The fungus was re-isolated andpurified culture from these artificially infected leaves was compared for similarity to thatof original culture. The plants which were not inoculated with the fungal sporesuspension did not show any symptoms of the disease. Similar technique was followed by (Humpherson-Jones, 1992; Paul and Rawlinson, 1992; Yu, 1992; Howard *et al.*, 1994; Verma and Saharan, 1994; Poapst*et al.*, 1979). The re-isolated *A. brassicicola*exhibited similar characters as in the originally isolated culture.

Comment [LCA5]: chronological order

Identification of the pathogen

The reisolated pure fungal culture were identified in the Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology Kanpur by comparing its morphological characters with the information available on the standard websites for fungal identification as well as in the reviewed literature. In present investigation, identification of causal organism was further confirmed as *A. brassicicola*. Earliar Ellis (1968) first time described *A. brassicicola*on cauliflower leaves producing dark brown to almost black circular 1-10 mm diameter zonate spots.

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Plate 1: General view of fieldsymptomsof leafspotin cauliflower

Plate.2: Pure Cultureof*Alternariabrassicicola*anditsspores

Effectofculturemediaongrowthandsporulation of *Alternariabrassicicola*(Schwein.) Wiltshire.

Radial growth of the pathogen

In order to find out the most effective culture medium for the growth of *A. brassicicola*. eight culture media were evaluated against *A. brassicicola*under *in vitro* condition and the data are summarized in **Table No. 1** Which reveals that Potato dextrose agar medium was significantly superior over other tested media at 3, 5 and 7 days after inoculation.**(Fig-1)**

Seven days after inoculation maximum mycelial growth was recorded in Potato dextrose agar medium (89.07mm) followed by Corn meal agar (67.46mm), Potato carrot agar medium (54.83 mm), Rose Bengal agar (53.00 mm), Czapex dextrose agar (40.40 mm), Yeast Extract Agar (32.28 mm), and Nutrient Agar (24.28 mm), While minimum (22.16mm) growth was recorded in Starch Agar (**Plate-3).**

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The mycelial growth of *A. brassicicola*fully covered the plate on Potato dextrose agar medium at 7 days after inoculation and it was significantly superior over the remaining tested media. The next effective medium was Corn meal agar medium which was significantly superior over eight media. Excellent sporulation more than 20 spores/microscopic field) was observed on Carrot potato dextrose agar medium, Corn Meal Agar medium, Rose Bengal agar medium, and potato dextrose agar medium, where as good sporulation was found in Yeast Extract Agar medium and Czapex dextrose medium. Poor sporulation was found in 2 culture media viz., Nutrient Agar and starch agar medium **(Table-1).**

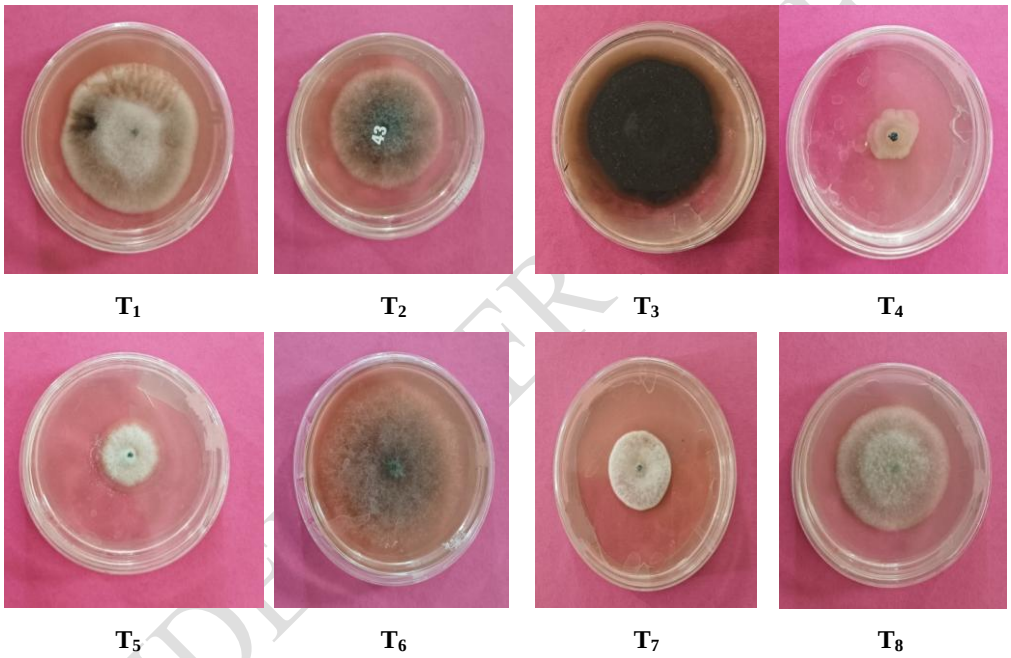

Plate.3: Effect of different culture media on growth of *Alternariabrassicicola*

Colour of the culture

Among all media tested for evaluation, colour of culture did not differ from each other.

Conclusion

It can be conclude that Alternaria leaf spot of cauliflower, which is brought on by *Alternariabrassicicola*(Schwein.) Wiltshire, is a significant disease of cauliflower in the Kanpur region. One of the biotic factors limiting cauliflower output and productivity is Alternaria leaf spot, which is brought on by *Alternariabrassicicola* (Schwein.) Wiltshire.

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Table.1: Effect of culture media on growth and sporulation of*Alternariabrassicicola*

Treatments	Culture media	Average mycelial growth (mm)			Sporulation
		3 DAS	5 DAS	7 DAS	
T_1	Czapek's Dox Agar	11.66	22.00	40.40	++
T_2	Potato Carrot Agar	13.33	28.66	54.83	+++
T_3	Potato Dextrose Agar (PDA)	31.00	62.99	89.07	+++
T_4	Starch Agar	12.66	16.33	22.16	+
T_5	Nutriant Agar	14.00	19.66	24.48	+
T_6	Corn Meal Agar	18.33	51.00	67.46	+++
T_7	Yeast Extract Agar	12.00	22.33	32.28	++
T_8	Rose Bengal Agar	25.33	36.66	53.00	+++
	C.V (%)	**2.41**	**5.86**	**6.01**	
	S.Em.±	**0.66**	**1.10**	**0.60**	
	C.D. at 5 %	**2.02**	**3.32**	**1.86**	

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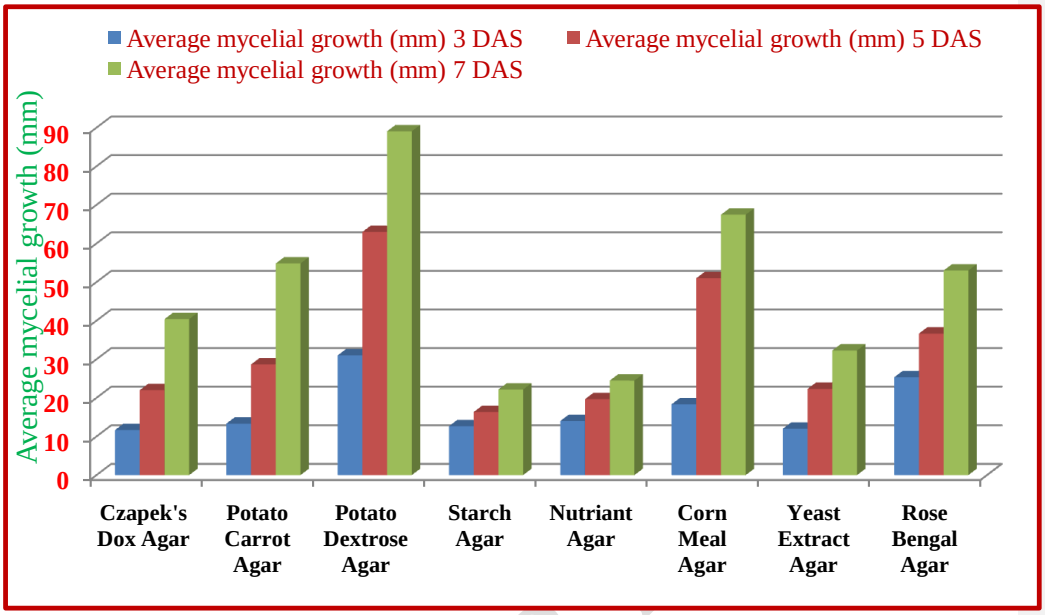

Fig.1: Effect of culture media on growth of*Alternariabrassicicola*.

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