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Cultural characterization and pathogenicity analysis of *Alternaria mali* (Roberts) causing Leaf spot disease of Apple (*Malus domestica* Borkh.)

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The study focused on the characterization and pathogenicity of *Alternaria mali*, the causal agent of Alternaria leaf spot disease in apple trees. The pathogen was collected from infected trees and subjected to isolation for further analysis. The pathogenicity test was conducted by applying a conidial suspension of 5×10^4 spores/ml to grafted Red Delicious apple trees. Observations were recorded, revealing that symptoms appeared on the 3rd day of inoculation, with a maximum disease incidence of 90.1% and disease severity of 86.58% observed on the 13th day. To understand the nutritional requirements of the pathogen, six different growth media were analysed, including Potato dextrose agar, Oatmeal agar, Czapek's Dox agar, Corn meal agar, Malt extract agar, and Richard's medium. Results indicated that the maximum mycelium growth was observed in potato dextrose agar (88.59 mm), followed by oat meal agar (82.05 mm), while corn meal agar exhibited the minimum growth (60.18 mm).Furthermore, the study investigated the growth characteristics of *Alternaria mali* under different temperature conditions ranging from 5°C to 30°C. Results showed that the maximum mycelium growth (89.16 mm) occurred at 25°C, while the minimum growth (45.97 mm) was observed at 5°C.These findings provide valuable insights into the cultural characteristics, pathogenicity, and optimal growth conditions of *Alternaria mali*, contributing to a better understanding of the disease and aiding in the development of effective management strategies to control Alternaria leaf spot disease in apple orchards..

Keywords : Diseaseincidence, disease severity, growth media, pathogenicity, sporulation

INTRODUCTION

Apple (*Malus domestica* Borkh.), a member of the Rosaceae family, holds great significance as the most important fruit crop worldwide, having been cultivated for centuries. In India, apple cultivation spans an area of approximately 313,000 hectares, with an annual production of 2,437,370 tonnes and a productivity rate of 7.78 tonnes per hectare (Anonymous, 2021). The Himalayan regions, including Jammu and Kashmir, Himachal Pradesh, and Uttarakhand, contribute to 99% of the total apple production.

The remaining 1% is accounted for by North-Eastern states such as Arunachal Pradesh, Sikkim, Nagaland, and Meghalaya. Uttarakhand, with a cultivation area of 10,000 hectares, yields an annual apple production of 64,880 tonnes, resulting in a productivity of 6.4 tonnes per hectare (Anonymous, 2021).

Despite significant expansion in apple cultivation over the past few decades, the rise in production has not kept pace, and productivity remains comparatively low compared to leading apple-growing nations worldwide. Like other horticultural crops, apples are susceptible to various pathogens that affect both the quality and quantity of the fruit (Grove*et al.* 2003). Fungal diseases account for a major portion of the significant crop losses, with common examples including scab, Alternaria leaf blotch, powdery mildew, collar rot, root rot, sooty blotch, and fly speck.

Among these fungal diseases, Alternaria blotch, caused by *Alternaria mali*, has emerged as a significant economic threat to apple cultivation worldwide. *Alternaria mali*, a member of the

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division Ascomycota, class Dothideomycetes, order Pleosporales, family Pleosporaceae, genus Alternaria, and species *A. mali*, was first identified in 1924. It has become a prevalent pathogen in the southeastern United States. While different *Alternaria* spp. can cause leaf blotch and fruit spot on apple trees, *A. mali* (also known as *A. alternata* apple pathotype or *A. alternata*f.sp. *mali*) is the most reported pathogen globally (Johnson*et al.* 2000; Li*et al*. 2013;Gagkaeva and Levitin, 2000).

Alternaria blotch has gained economic importance in several Asian countries, including Japan and India (Shahzad*et al*. 2002)To understand the pathogenicity and growth characteristics of the pathogen under controlled conditions *(in vitro*), the study aimed to investigate the growth of *Alternaria mali* on different media and temperatures.

By enhancing our knowledge of the pathogenicity and cultural requirements of *Alternaria mali,* this research contributes to the development of effective management strategies against Alternaria blotch in apple orchards. By employing appropriate preventive measures, such as targeted fungicide applications and environmental controls, farmers can mitigate the impact of this economically significant disease, ensuring better quality and higher yields of apples.

MATERIALS AND METHODS

The investigation was carried out in the Department of Plant Pathology laboratory and Fruit Research block of College of Horticulture, VCSG UUHF, Bharsar, Pauri Garhwal, Uttarakhand during the year 2022-23.

Isolation of the pathogen from diseased Apple tree

A leaf sample showing symptoms of leaf spot disease on apple trees was collected from the Fruit Research block at the College of Horticulture VCSG, UUHF Bharsar, Pauri Garhwal, Uttarakhand. To isolate and identify the pathogen responsible for the disease, the sample underwent a series of steps in the laboratory. First, the leaf was washed with tap water to remove surface contaminants and then air dried. Infected lesions along with healthy tissue were cut into small pieces using sterilized scissors. To eliminate external contaminants, the leaf pieces were surface sterilized by immersing them in a 0.1% sodium hypochlorite solution for 30 seconds. Afterward, the pieces were thoroughly washed in sterilized distilled water to remove any residue of sodium hypochlorite. Aseptically, the leaf pieces were transferred to Petri plates containing sterile Potato Dextrose Agar (PDA) medium, which provides the necessary nutrients for fungal growth. The plates were then incubated at a temperature of 25±2! for one week. Daily observations were made to monitor the mycelial growth of the fungus. After the incubation period, a profuse growth of the fungus was observed. It should be noted that the specific identity of the pathogen causing the leaf spot disease was not mentioned in the given information, and further analysis would be required to accurately identify the isolated fungus, such as microscopic examination or molecular techniques.

Identification of pathogen

Once the fungus had grown profusely in the Petri plates, it was further examined to confirm its identity. The examination involved the use of a compound microscope to observe the morphological characteristics of the pathogen. By studying these morphological features, it was confirmed whether the isolated fungus matched the expected pathogen for the experiment.

Under the compound microscope, various aspects of the pathogen's morphology were observed, including the shape, size, colour, and arrangement of its spores, hyphae, and other structures. These observations were compared to reference materials or existing knowledge about the expected characteristics of the target pathogen. If the morphological features of the isolated fungus closely matched those of the expected pathogen, it would provide a preliminary confirmation that the correct pathogen had been isolated.

Maintenance of Culture

After confirming the identity of the isolated fungus, the next step was to establish a culture for further

studies and long-term preservation. This was achieved through sub-culturing of the fungus in Potato Dextrose Agar (PDA) slants under sterile conditions in a laminar air flow hood. The sub-culturing process involved transferring a portion of the fungal growth from the Petri plates to the slants of PDA medium. This ensured the growth and propagation of the fungus in a controlled environment. The PDA slants were prepared beforehand and sterilized to prevent contamination.

The sub-cultured PDA slants containing the fungus were then incubated at a temperature of 25±2! for approximately 20 days. This provided optimal conditions for the fungus to grow and develop.

After the incubation period, the PDA slants with the grown fungus were preserved for long-term storage. They were placed in a refrigerator set at 4!, which is a common temperature for maintaining fungal cultures. Preserving the cultures in a cold environment helps to slow down their growth and extend their viability.

Periodic sub-culturing was performed to ensure the vitality and purity of the preserved culture. This involved transferring a small portion of the fungus from the preserved slants to fresh PDA media to initiate new cultures. By periodically sub-culturing the fungus, researchers could maintain an active and viable culture for ongoing studies.

The preserved culture served as a valuable resource for future experiments and investigations related to the identified pathogen.

Pathogenicity test of the pathogen

The isolate of *A. mali* was multiplied in PDA and pure culture was maintained. The pathogenicity test was conducted by artificial inoculation, according to Koch's postulates. Grafted Red Delicious in the Fruit Block Department were sprayed with spore suspension containing approximately 5×10^4 spores/ml of frequently intercepted fungi viz., *Alternaria mali.* After inoculation, the plants were immediately covered with the polythene bags individually for 48 h and incubated in a net house under semi controlled conditions thereafter. Similar set of apple plants sprayed with distilled water served as control. Observations were recorded on disease appearance, incubation period, incidence, and severity of the disease.

Cultural Studies

Growth on different media

To study the growth characteristics of the fungus, several different solid media were used, including Potato Dextrose Agar (PDA), Czapek's Dox Agar, Oatmeal Agar, Malt Extract Agar, Corn Meal Agar, and Richard's Agar Medium. Before their use, all the media were sterilized by autoclaving at 121! at 15 psi (pounds per square inch) for 15 minutes. This process ensured the elimination of any contaminants and provided a sterile environment for the fungal growth. For each medium, 20 ml was poured into 90 mm diameter Petri plates, which served as the growth substrate for the fungus. Each treatment, representing a specific medium, was replicated four times to ensure reliability and accuracy of the observations. To initiate fungal growth on each plate, a 5 mm disc taken from a previous fungal culture was inoculated onto the centre of the plate. This ensured consistent starting conditions for each treatment. The Petri plates were then incubated at a temperature of 25±1!, which provided an optimal environment for fungal growth. The incubation period allowed sufficient time for the fungus to grow and develop on the respective media.

On the 7th day of inoculation, the fungal colonies were measured. This involved assessing the diameter of the fungal growth on each plate. By measuring the colonies, researchers could quantify and compare the growth rate and overall size of the fungus in different media.

By conducting the experiment with multiple media and replicates, the researchers could assess how the growth of the fungus varied under different nutrient conditions provided by the various media. This information could provide insights into the preferred growth requirements and characteristics of the pathogenic fungus being studied.

Growth at different temperatures

Effect of temperature on mycelium growth of *Alternaria mali* was observed at various temperatures. Twenty ml of sterilized PDA medium was poured in each sterilized Petri plate. Inoculation was done by 5 mm disc of 7th days old culture of *Alternaria mali* with the help of sterilized cork borer and four replicates of Petri plates were incubated at 6 different levels of temperature *viz.,* 5, 10, 15, 20, 25 and 30! for 7 days. Observations on mycelial growth were recorded on 7th days of incubation.

RESULTS AND DISCUSSION

Isolation and identification of pathogen

The process of isolation successfully led to the identification of the pathogen as *Alternaria mali* from the collected samples of infected plants. Microscopic identification and the formation of conidia were crucial in confirming the pathogen's identity. The morphological characteristics observed in this study were consistent with previous reports.Under a compound microscope, the fungal hyphae and spores were carefully examined. A pure culture of *Alternaria mali* was obtained from the cottony growth, which exhibited abundant aerial mycelium. Initially, the fungus colony appeared white with an irregular margin, gradually turning grey over time. In the later stages of growth, the culture became completely black, devoid of any aerial mycelium.

Conidiophores were observed to be hyaline to golden brown, possessing 2-11 septa, with lengths ranging from 52.8 to 180.5 µm and widths from 9.9 to 20.3 µm. The conidia displayed typical characteristics, being muriform (having several cells arranged in a brick-like pattern), dark brown, and thick-walled. They formed long chains, usually numbering between 9 and 15 conidia per chain. Some conidia were found to be short and rudimentary, with dark brown beaks measuring 17.07 µm in length and 8.09 µm in width. The conidia exhibited 6-8 transverse septa and 0-3 longitudinal septa.

Based on the colony's appearance, as well as the morphological features of the conidia and

Table 1. Pathogenicity test of *Alternaria mali* on "Apple cultivar Red Delicious

Incubation days	Disease incidence (%)	Disease severity (%)
3	12.4	9.3
5	28.2	20.10
7	42.6	30.78
9	60.7	47.28
11	78.4	66.30
13	90.1	86.58
Mean	52.06	43.39

Table 2: Effect of different media on mycelium growth (mm) of the pathogen

Media	Growth at 7th day± S.E.(m)
T_1 (Potato dextrose agar)	88.59±0.23
T_2 (Oatmeal agar)	82.05±0.33
T_3 (Czapeck's Dox agar)	70.53±0.46
T_4 (Corn meal agar)	60.18±0.07
T_5 (Malt extract agar)	77.65±0.50
T_6 (Richard's medium)	78.01±0.30
S.E.(d)	0.49
C.D. (0.05)	1.04

Table 3: Effect of different temperature regimes on the vegetative growth of *Alternaria mali*

Temperature (?)	Growth at 7^{th}day± S.E.(m)
T_1 (5)	45.97±0.30
T_2 (10)	57.29±0.50
T_3 (15)	71.11±0.23
T_4 (20)	78.10±0.12
T_5 (25)	89.16±0.22
T_5 (2 T_6 (30)	80 80.53±0.30
S.E.(d)	0.43
C.D. (0.05)	0.91

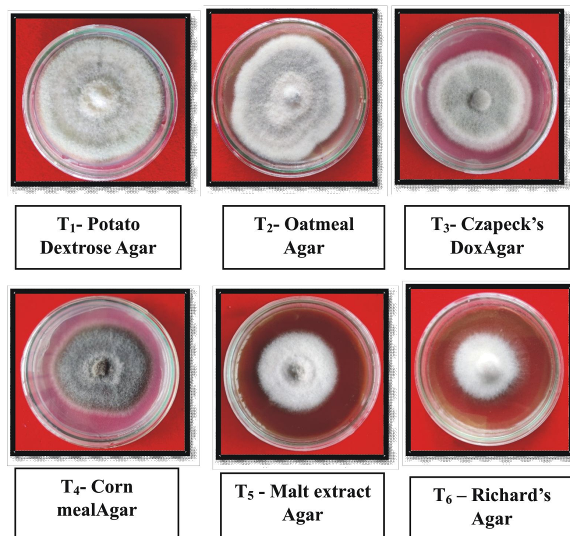

Fig.1: Mycelial growth (mm) of pathogen on different media

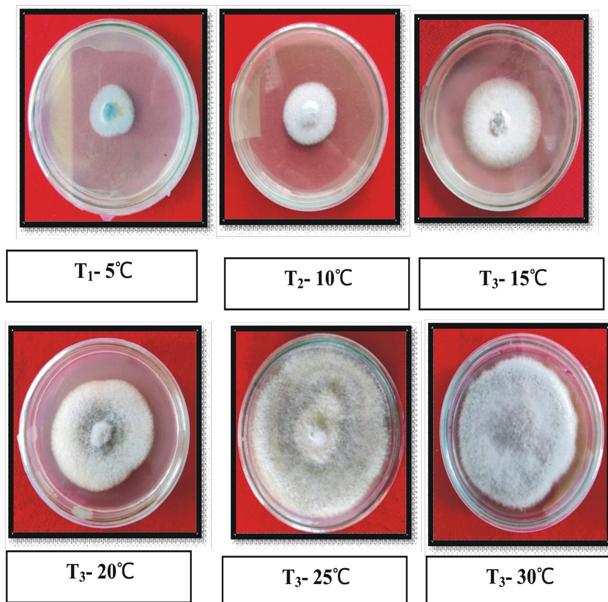

Fig. 2. Mycelial growth (mm) of pathogen on different temperature

conidiophores, the fungus was confidently identified as *Alternaria mali* Roberts. The confirmation of the pathogen's identity is crucial for understanding its biology, behavior, and potential control strategies in the context of the leaf spot disease it causes on apple trees.

Pathogenicity test

The pathogenicity test conducted on the Red Delicious apple cultivar confirmed the ability of the isolated fungi to cause disease. Typical disease symptoms were observed on the injured leaves within 4 to 7 days of inoculation, indicating that the isolated fungi were responsible for the leaf spot disease. However, no symptoms developed on the uninjured leaves, even after 15 days of inoculation. To fulfil Koch's postulates, re-isolation of the fungi from the infected leaves was performed, and it yielded fungi identical to the ones initially inoculated. This confirmed that the isolated fungi were indeed the causative agents of the disease. The pathogenic cultures were maintained by sub-culturing at monthly intervals and stored in a refrigerator for further studies, ensuring a continuous supply of viable cultures for future research. In the case of *Alternaria mali*, symptoms appeared on the younger leaves of the plants within 3 days of inoculation, while the older mature leaves were the last to get infected, showing symptoms after a longer duration (Kumar, 2004). These observations were consistent with the descriptions in the literature, where light brown spots gradually enlarged and developed into deep brown to purple-colored lesions within a similar timeframe (Chauhan, 2018).

Table 1 presents the data on disease incidence and severity. It showed that the maximum disease incidence (90.1%) and severity (86.58%) were recorded after 13 days of inoculation. The data indicated that the disease incidence increased from 12.4% at 3 days of inoculation to 90.1% at 13 days. Similarly, disease severity increased from 9.3% at 3 days to 86.58% at 13 days.

These findings provide valuable insights into the pathogenicity and progression of the leaf spot disease caused by the isolated fungi, particularly *Alternaria mali*, on Red Delicious apple leaves. The data presented in Table 1 demonstrate the increasing impact of the disease over time, with a significant rise in both disease incidence and severity as the infection progresses.

Cultural characters

Mycelial growth of pathogen on different media

The results in Table 2, Fig.1, revealed that all the six media supported vegetative growth of the fungus; however, maximum growth of *Alternaria mali* was found on Potato dextrose agar medium

(88.59 mm) after 7 days of incubation. The next best medium was Oatmeal agar (82.05mm) followed by Richard's medium and malt extract medium which gave the similar growth of the fungus (78.01mm and 77.65mm), Czapeck's Dox agar (70.53mm), Corn meal agar medium supported the minimum growth (60.18 mm) of this fungus.

Similar results were found in consonance with the findings, maximum mycelium growth of Alternaria blight of cotton was recorded in PDA (88.18 mm)(Jagtap*et al.* 2012). Also, *Alternaria* spp. showed maximum growth rate on potato dextrose agar (81.52 mm) at 8th day of inoculation(Chauhan *et al.* 2018) and maximum mean radial growth of *A. macrospora*was observed on PDA (89.87 mm) (Rajesha *et al.* 2020).

Mycelial growth of pathogen at different temperature

To determine the optimum temperature required for vegetative growth of the fungus, six different temperatures, ranging from 5! to 30°C were included in the studies. The data recorded in each case are presented in Table 3. which revealed that the fungus could grow on a wide range of temperature ranging from 5 to 30°C.

Maximum diametric growth was however, observed at 25! (89.16 mm) followed by 30°C (80.53 mm). Minimum fungal growth (45.97 mm) was observed at 5! indicating thereby that very low temperature is not suitable for the growth of this fungal pathogen. The fungal growth at 10! (57.29mm) followed by 15! (71.11mm) (Fig.2).

These results agree with the findings of followings workers. *Alternaria solani*showedthe most favourable temperature for growth of the pathogen was 25°C with maximum average radial growth (48.65 mm) after 9 days of inoculation (Kumar *et al.* 2015). The maximum mycelium growth of *Alternaria* sp. was recorded at 25°C (87.47 mm) (Bais*et al.* 2019).

CONCLUSION

Based on isolation and identification the pathogen was confirmed as *Alternaria mali*, which on examination showed cottony growth with profuse aerial mycelium. The pathogenicity test revealed that the pathogen produces symptoms within 3 to 13 days of inoculation. Potato dextrose agar medium favoured for the maximum mycelium growth of the pathogen. The excellent mycelium growth was seen at 25°C temperature which was found to be an ideal temperature for the pathogen to proliferate.

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DECLARATIONS

Conflict of interest. Authors declare no conflict of interest.

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