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CHARACTERIZATION OF *ALTERNARIA ALTERNATA* ISOLATED AS LEAF SPOT PATHOGEN FROM *SPINACIA OLERACEA* L.

Muhammad Faiq Irfan, Sobiya Shafique, Shazia Shafique[*] and Muhammad Asad Ali

Department of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Lahore 54590, Pakistan.
Corresponding author's email: shazia.iags@pu.edu.pk

ABSTRACT

Spinach (*Spinacia oleracea* L.) is a significant eatable and curative crop grown in many countries but also suffers considerable yield reductions due to the common occurrence of fungal leaf spot diseases. In this regard, a recent field survey was done in the experimental fields of the Punjab University, FAS, Department of Plant Pathology, including Bedian Farms, Ring Road, Lahore, and Defence Market, to determine the incidence of spinach leaf spot disease. In this study, approximately 55-60% of the *S. oleracea* plants showed leaf spot symptoms, with *Alternaria* being isolated and identified as the causative organism through fungal isolation and morphological analysis. *Alternaria* pathogenicity was confirmed in Koch's pathogenicity tests through detached leaf assays and pot experiments. The *Alternaria* pathogen causes economic losses in spinach, so it is crucial to implement timely control measures to safeguard spinach and other vegetables and herbs from its negative consequences.

Keywords: *Spinacia oleracea*, *Alternaria alternata*, Isolation, Pathogenicity, Wilting.

INTRODUCTION

Spinacia oleracea L. is one of the most widely consumed vegetables and is known for the richness of its nutritional value in vitamins, minerals, and antioxidants (Chitwood, 2016; Huda-Faujan *et al*., 2023; Naseem *et al*., 2023). This belongs to the Chenopodioideae subfamily, which has Swiss chard, quinoa, or sugar beet within, nourishing vegetation. It is thought to be of Persian origin and later spread to the Republic of India, Asia, and further to the Mediterranean and Europe (Ladizinsky, 2012). Although spinach is used so extensively in food, there is a lot of potential for its use in medicine (Calub *et al*., 2022; Sami *et al*., 2023). Due to its antibacterial and anti-aging qualities, spinach is touted in many sources as being beneficial for bones, hair, and skin (Diniz do Nascimento *et al*., 2020). Spinach is one of the adaptable vegetables found in the world, whether it is eaten raw or cooked (Wright, 2012; Madison, 2017). It contains a high amount of iron, which prevents disorders such as osteoporosis, which may be caused by a deficiency in iron and leads to anemia (Reinhold, 2020; Ayaz *et al*., 2024). It is also considered to possess an anti-cancer effect, because of its flavonoid contents that serve as antioxidants within the body (Roberts and Moreau, 2016; Ramaiyan *et al*., 2020). It has healthy properties due to polyphenolic ingredients. In general, the healthfulness as well as various health-promoting effects of spinach indicates its role as a vital nutritional element. The only crop grown in Pakistan was about 253,800 hectares of vegetables, most of which had a reasonable percentage exported to other countries (Bosland and Votava, 2012). Among the other essential and economical vegetables, spinach is one of the leafy greens that can be available in large quantities for production. It produces around 250 kg per hectare, and spinach is cultivated on many grounds in almost every part of the world (Thakur, 2017). Global spinach production in 2010 was reported to exceed 20 million tons. China is leading with the greatest share of the total output, accounting for about 90% of the total output (Simko *et al*., 2014; Navarrete, 2016). The most commonly produced crop in the world is spinach, grown on approximately 930,791 hectares, with a production value of 30.02 million tons worldwide (Siddique *et al*., 2021). This vast production has ranked Pakistan as part of the top 10 countries worldwide that produce the highest quantity of spinach, with its production increasing over time to help contribute to the country's economy (Correll *et al*., 2010; Zahra, 2018). The quality of crop yield is affected by the shortages of light, drought stress, insect, and disease development in spinach (Ors and Suarez, 2017; Kabir, 2019; Bhattarai and Shi, 2021). These factors may potentially damage the production and quality of crops (Junaid and Gokce, 2014; Udeigwe *et al*., 2015; Haider *et al*., 2024; Qiao *et al*., 2024). Pests can lead to the transfer of diseases or the destruction of crops, which lowers the crop yield due to common diseases like downy mildew and anthracnose (Parthasarathy *et al*., 2024; Khulbe and Batra, 2024). *Peronospora farinosa f.sp. spiniciae*, is a fungal strain which causes diseases such as downy mildews on leaf and leaf spot diseases. Fungal diseases like *Cercospora beticola* and *Stemphylium botrysum* inflict massive damage on the spinach industry (Rangel *et al*., 2020). It is, therefore, of immense importance to identify as many categories of pathogens and apply appropriate control measures to reduce crop loss considerably (Reddy *et al*., 2024). The present study aimed to isolate the pathogen that causes the leaf spot disease of spinach.

MATERIALS AND METHODS

Sample Collection

A recent field survey was conducted at the experimental locations of the Department of Plant Pathology, FAS at the Punjab University, including Bedian Farms, Ring Road Lahore, and Defence Market, to assess the leaf spot disease on spinach plants. The objective was to gather spinach leaf samples infected with the agent responsible for causing spinach leaf spot disease for identification and characterization (Feng *et al*., 2014; Spawton and du Toit, 2024). Symptoms like yellowing of leaves and watery spores were also noted at the time of sample collection. However, there were also signs of wilting, necrosis, or other signs. Then different diseased leaves were collected and put in sterilized plastic bags and placed in the freezer at 4 °C; until it was time for laboratory examination. The prevalence and extent of the disease in the affected population was also determined. Leaf necrosis and other symptoms were also present. The occurrence and severity of the disease in affected communities were also assessed.

Disease Rating Scale

A disease-scoring system was established; for the disease, to observe the strength of the disease. Disease incidence and severity were assessed through formal observations. The formulas below were used to ascertain the extent of diseases:

Disease Prevalence = (Number of infected fields)/ (Total Number of infected fields) x 100
Disease Incidence = (Number of diseased Plants) / (Total Number of the Plants) x 100
Disease Severity = (Infected part of plant leaf) / (Total part of Plant leaves) x 100

Morphology-Based Fungal Identification

Micro and macroscopic analyses are necessary; to accurately identify pathogens in the culture. The microorganism was grown and recognized on malt extract agar supplemented with 2% MEA at a pH of 6.5. Infected parts of diseased samples were carefully excised using a surgical blade and sterilized with a sodium hypochlorite solution. Subsequently, three to four surface-sterilized leaf segments were aseptically placed onto Petri plates containing agar supplemented with malt extract. The Petri plates were then put in an incubator; set at around 25-27 °C for four to seven days, with the infected specimen surrounds being monitored periodically for the growth of radiating mycelia. After a week of incubation, the newly formed colony was examined using a light microscope to identify its morphological features. A visual examination and microscopic analysis were conducted to clarify the colonial characteristics of both the front and back. Different types of conidia, as well as the growth and size of colonies, were assessed, while fungal structures were examined at magnifications of 4X, 10X, 40X, and 100X.

Pathogenicity Trails

The pathogenicity of a recently discovered fungal pathogen in this study was evaluated; by observing symptoms on spinach plants, with both detached leaf assay and pot experiments. In the detached leaf test, the spinach leaves were excised carefully and positioned in sterilized Petri dishes on moistened filter paper. The petioles of the healthy leaves were positioned directly next to the surface of the Petri plates. The leaves were then inoculated with 1 mL of a suspension containing approximately 5×10^5 spores/mL in a sterile environment. The Petri dishes were incubated at about 25-27 °Celsius, and disease symptoms were monitored periodically. To meet Koch's postulates for pathogenicity; the pathogen was again isolated from the diseased areas. This involved isolating the pathogen from the diseased tissue, culturing it in a sterile environment, and reinoculating it into healthy plants to verify its capacity to cause disease.

The pot trials will provide insights into the pathogenicity of the newly discovered fungal pathogen, with direct observation of its influence on spinach plants and verification of its ability to cause disease through re-isolation. In this study, different types of spinach seeds were grown in disposable pots to establish nursery conditions. Each type of plant was designated by sowing three seeds in each pot, with four duplicates per type of plant. The pots were watered; and after 20 days of growth, thinning was done so that only one plant of each variety remained in each pot. After completion of the thinning process, every pot was placed in a clean room and treated with 5 mL of a spore suspension containing 5×10^5 spores/mL. The control plants were provided with water; for irrigation only and the spore suspension was not added to these plants. After the application of the spore suspension, the pots were covered with plastic bags for 24 hours to humidify and warm the temperature to 25-28 °C which was favorable for the advancement of the disease. Throughout the experiment, assessments were routinely conducted to determine the presence of any disease symptoms at specific intervals. These observations helped to compare the effect of spore suspension; in producing symptoms of the disease on test plants versus control plants in an orderly manner. These results were of great significance in understanding the pathogenicity and virulence of the fungal pathogen across the different varieties of spinach and in disease processes and control options.

RESULTS

Research carried out in December 2021; on disease-ridden spinach leaves declared that more than 60-70% of *S. oleracea* plants exhibited leaf necrosis, resulting in yellow to brown lesions on the leaves measuring 2.5 to 2.8 mm. Dark brown spots with rings were the leading symptom and covered 30-40% of the leaf area (Fig. 1). Disease prevalence was varied by location. The vegetable area had 87%, then FAS fields with 70%, and finally 52% in Akbari Mandi (Fig. 2). Disease incidence was highest in the Defence vegetable market at 85%, followed by Bedian fields at 66% and Akbari Mandi at 56%, with FAS fields having the lowest at 46.33% (Fig. 3). Disease severity calculations showed Akbari Mandi with the highest at 87% and the vegetable area with the lowest at 40% (Fig. 4).

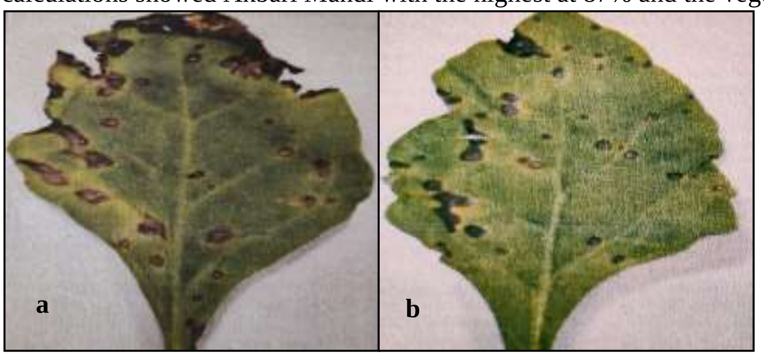


Fig. 1. Diseased plants specimens collected in the field survey. (a): Front side (b): Back side.

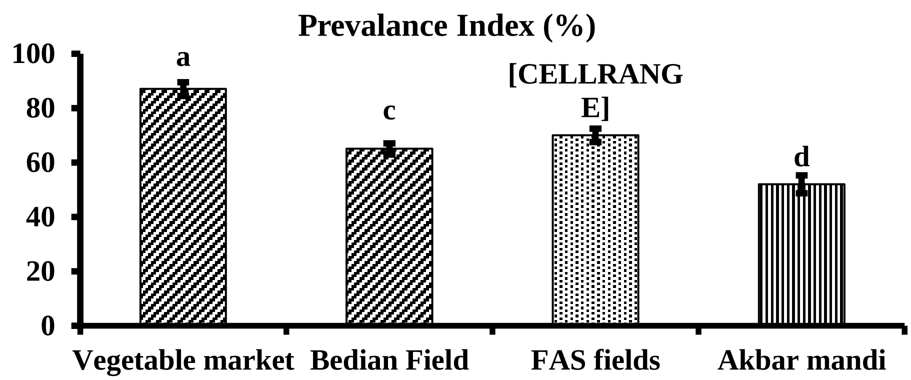


Fig. 2. Disease Prevalence in Different Field Areas.

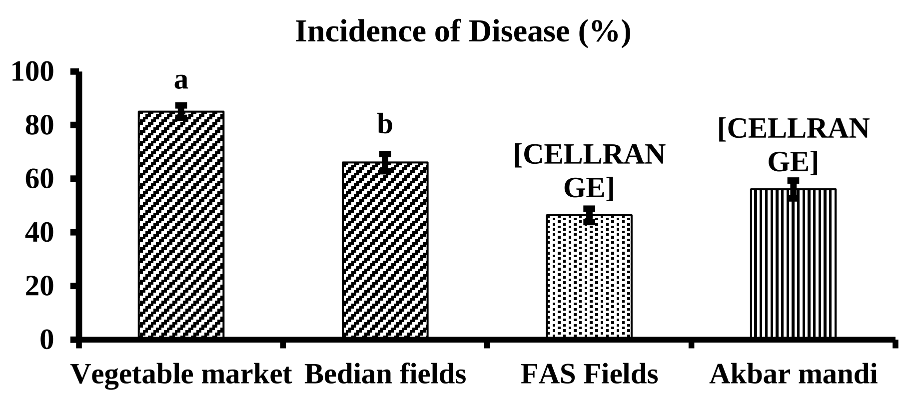


Fig. 3. Disease incidence in Different Field Areas.

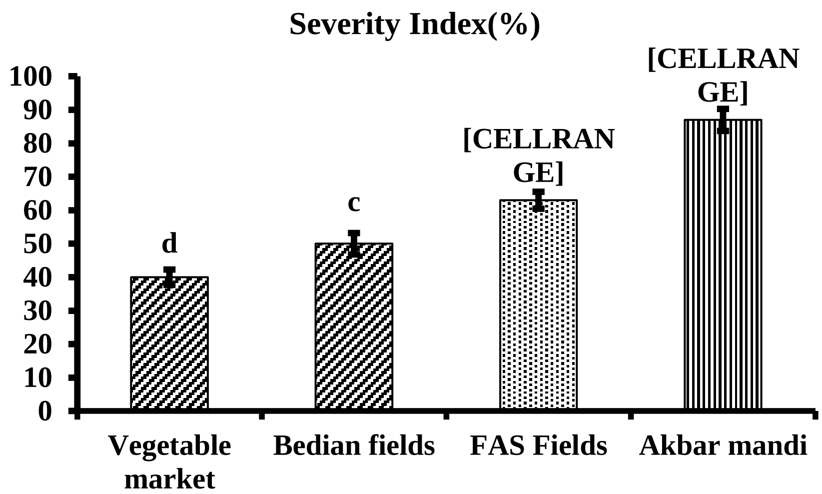


Fig 4. Disease severity in Different Field Areas.

Morphology-Based Fungal Identification

Initially, a thorough analysis of the isolated pathogen's morphology from a pure culture was conducted to determine its identity. Next, purified cultures of isolated pathogens were observed through a microscope at different levels of magnification. The isolated fungi were cultured in pure form on MEA medium plates to characterize their morphology. Colonies grew rapidly, developing into a mature plate in just one week. The purified culture of the isolated pathogen was analyzed by examining its colony shape, colour, fungi growth rate, hyphae spores, and conidia under a microscope, as well as the relationships between these parameters in samples cultured on MEA. The edge of the culture was consistent, displaying flattened mycelium. Conidiophores were light brown to greenish brown, segmented, and divided. The conidia were elongated, small oval-shaped light brown conidia with rounded septation in both longitudinal and transverse directions (Fig 5). Based on the traits analyzed, the pathogen was recognized as *Altenaria alternata*.

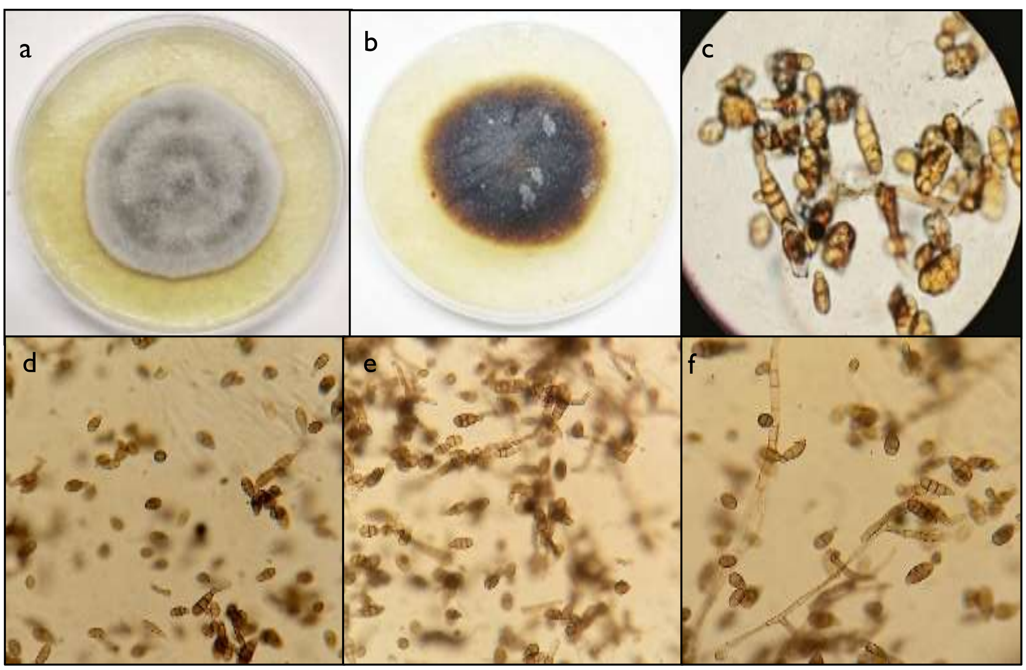


Fig 5. Description of the Culture and Morphology of *Alternaria alternata*. (a): View of the colony from the front; (b): View of the colony from the back; (c): Conidiophore and conidial attachment at 10X magnification; (d): Morphology of conidial heads at 40X magnification; (e-f): Conidial morphology at 100X magnification.

Pathogenicity Analysis

In-Vitro Pathogenicity

The isolated pathogen's use on the test host plant to validate Koch's postulates for pathogenicity confirmed the pathogen's identity and impact. The pathogenic potential of the identified pathogen was confirmed by intentionally infecting host plants and conducting a detached leaf test in the laboratory. A disease rating system was used to assess the severity of the disease, as shown in Table 1.

Table 1. Disease Rating Scale.

Infected Leaf Area (%)	Days after Inoculation	Status
0-20	1-2	Strongly resistant
21-40	3-4	Resistant
41-60	5-6	Moderately susceptible
61-80	7-8	Susceptible
81-100	9-10	Highly susceptible

A pathogenicity assessment was carried out on different spinach plants to determine the virulence of the pure fungal pathogen. Leaf yellowing was noticeable 48 hours after applying the *Alternaria alternata* suspension. Symptoms evolved from dark black spots to widespread necrosis. Progress of the disease on Petri dishes was fast, with 90% of the Multani variety's leaf area being impacted after 12 days (Fig. 6). Evaluation of the disease progressive curve on the spinach representative varieties revealed that the Multani variety was the most vulnerable, whereas Desi variety displayed the greatest immunity against *Alternaria alternata* (Fig 7).

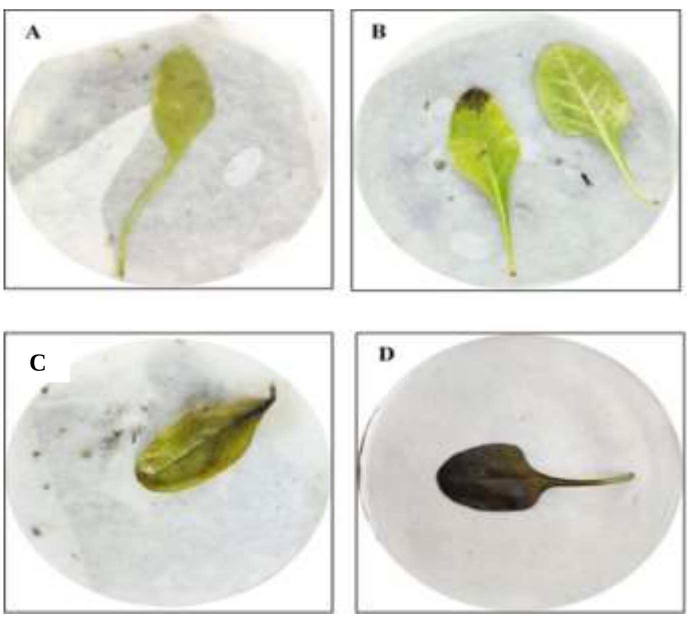


Fig. 6. Detached leaf pathogenicity assay by *Alternaria alternata* on spinach plants. a: Control Leaves; b-d: different stages of symptoms observed on leaves.

Evaluation of Pathogenicity Conducted In-Vivo Conditions

During the pathogenicity analysis in pot trials, seedlings were started by sowing seeds in plastic pots. Next, a subsequent test determined the pathogenicity by using a 5 mL spore solution of the pathogen to spray on 20-day-old spinach plants of chosen strains. Regular assessments were carried out to monitor the appearance of disease symptoms induced by *Alternaria alternata* in all varieties. The symptoms seen in the plants were very similar to those found in disease samples collected earlier, such as yellowing of the leaves, the development of dark brown lesions, and eventual plant death due to necrosis (Fig. 8). Examination of data gathered from the pot experiments showed that *Alternaria alternata* caused the greatest death rates in every spinach variety tested. In Multan, Centrus 1232-B, Centrus 1232-A, and Desi varieties, infection rates were 25%, 22%, 20%, and 13%, within three days of being inoculated. On day 15 after inoculation, the severity of the disease was 90% in the Multan variety, 60% in

Centrus 1232-B, 50% in Centrus 1232-A, and 30% in the Desi variety. The Desi type demonstrated the most resistance, while the Multan type displayed the highest vulnerability compared to all other varieties tested (Fig. 9).

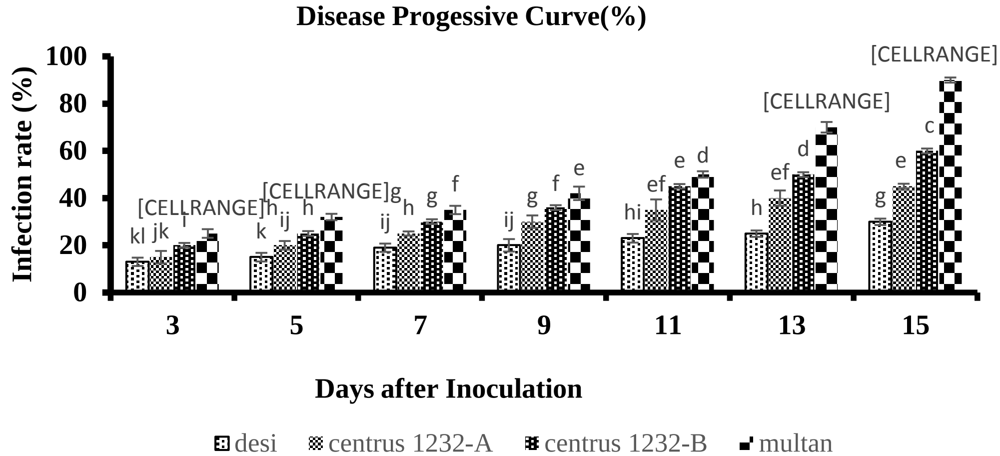


Fig. 7. Disease progression curve of *Alternaria alternata* on representative varieties of spinach plants based on detached leaf assay.

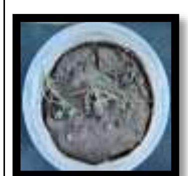
0% Disease Severity	20% Disease Severity	40% Disease Severity	60% Disease Severity	80% Disease Severity	100% Disease Severity
					
Healthy Plant	Small spots looked on the surface of leaf	Yellowing of leaves begin and leaf spots develop in the center of leave.	Wilting of the whole Plant	The leaf becomes necrotic and dropped	Death of the whole Plant

Fig.8. Pictorial representation of disease rating scale of *Alternaria alternata* based on symptom development.

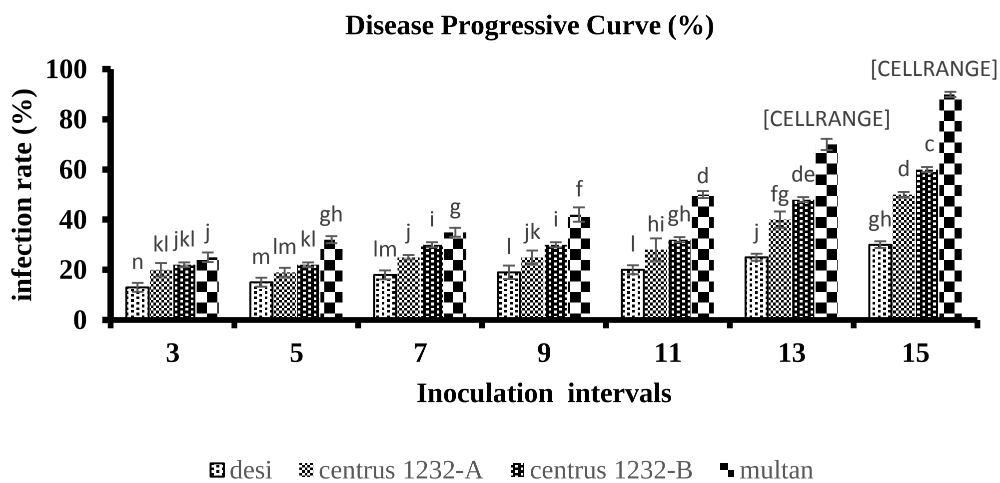


Fig. 9. Disease progression curve of *Alternaria alternata* on selected varieties of spinach varieties in pot experiments.

DISCUSSION

Spinach is a highly affordable crop extensively grown worldwide and often utilized as a key ingredient for cooking and preparing chutneys and salads (Prasoona *et al*., 2020; Cooray, 2021). Many diseases of the fungal group adversely affect the productivity of the spinach crops; among the most common is *Alternaria* leaf spot disease caused by *Alternaria alternata* (Kakar *et al*., 2020; Kumar, 2022). The identification of the pathogen responsible for the causes of the leaf spot disease in spinach crops is fundamental to the management of the *Alternaria* leaf spot fungi (Kirarei, 2019; Shafique *et al*., 2023). The disease spreads so rapidly in Punjab, Pakistan, that often growers experience critical losses in their crop harvesting (Abid *et al*., 2016; Abbas *et al*., 2019). Currently, a survey was carried out in the experimental zones of Punjab University's FAS Department, encompassing Bedian Farms, Ring Road, Lahore, and the Defence Market, for evaluating the occurrence of leaf spot disease in spinach to examine the prevalence, incidence and intensity of leafspot disease. Shafique *et al*. (2022) has conducted a similar study where the researchers aimed to investigate the prevalence, incidence, and intensity of spinach leaf spot disease in four different areas under their study. Accurate identification of the causative agent is the right approach to effective management of fungal diseases (Ahmed *et al*., 2024; Thambugala *et al*., 2024). The most accurate traditional method of delineation of disease based on their morphology is at the species level, but sometimes, mistakes can occur (Aththanayaka *et al*., 2024; Pham *et al*., 2024). The researchers in this study identified the isolated pathogen by the morphology, including growth structure conidiophore and conidial size, shape, and color, among others characterizations (Ogunsanya *et al*., 2024; Reiabi and Salariyan, 2024). The detaching leaf method and pot trials were conducted in an experiment to measure the pathogen virulence as a function of disease symptomatology in spinach plants. Inspecting closely, it seemed that the *Alternaria alternata* disease symptoms were very severe. Similar study was conducted by Shafique *et al*. (2018), used detached leaf assay method to confirm the pathogenicity of *Alternaria arborescens* and *Phyllosticta ristolochiicola* in *Dracaena marginata* and *Sonchus oleraceus* plants. Mahmood (2010) experimented on pot trials for testing the disease-causing ability of *Alternaria alternata* in tomato plants. The outcome of this experiment revealed that Multani was the most susceptible variety to spinach leaf spot out of all the varieties tested, and Desi had the highest resistance. Additionally, *Alternaria alternata* was considered one of the most aggressive pathogens causing spinach leaf spot disease in both experimental tests. In 2020, Altino and his colleagues analyzed the *Fusarium oxysporum f. sp. capsici.* 64 of the tested 129 isolates were pathogenic on the 'Atalante F1' pepper, and were identified as *F. oxysporum f. sp. capsici (Foc),* by satisfying Koch's postulates (Altino *et al.*, 2020).

CONCLUSION

Alternaria alternata is the main pathogen causing the disease in spinach. This disease causes severe yield loss in the spinach crop. Further molecular approach is necessary for accurate identification and proper management of the pathogen.

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