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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *ALTERNARIA ALTERNATA* CONCOMITANT WITH LEAF NECROSIS OF *SPINACIA OLERACEAE* L. IN PAKISTAN

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Abstract

Spinach (*Spinacia oleraceae* L.) is an edible yearly grown crop, having worldwide high nutritious value. Numerous studies have revealed that fungal diseases are the major reason for the yield loss in spinach. A study was carried out in fields of Lahore, Depalpur, Okara from August and September 2018 and fungal leaf spot infection was detected in spinach plants. Diseased specimens were collected for isolation and characterization of the causal agent. The identification was processed by morphological depiction and inherently from nucleotide sequencing of amplified ITS1-5.8S-ITS4 region, partial Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and partial elongation factor (EF1/EF2) coding section of rDNA. *Alternaria alternata* was recognized as a leaf spot triggering causal agent of the spinach plant. Subsequently, the pathogenicity tendency of the known pathogen was confirmed by re-extraction of the identical pathogen from preciously injected leaves of spinach. This learning implies the first report of *A. alternata* as a pathogen of *S. oleraceae* and obliges the speedy expansion of management tools.

Keywords: *Alternaria alternata*, Identification, Leaf spots, Pathogenicity, Spinach.

Introduction

Spinach is the best imperative nutritive vegetable consumed raw or cooked. It is a green leafy vegetable widely growing throughout Pakistan. It is estimated that the production of spinach is 30.02 million tons (MT) from approximately 930,791 ha with 250 Kg/ha yield (FAOSTAT, 2015; FAOSTAT, 2020). Pakistan being one of the top ten spinach-producing countries produces 97000 tons with an average yield of 1.5 tons/ha. It has a figured therapeutic use and food value. It contains a great number of vitamins B6, iron, riboflavin, folate, niacin, soluble dietary fibre, omega 3-fatty acid and minerals. It precludes ailments such as osteoporosis, iron deficit results in anaemia, antioxidant, and cancer preventative (Patricia, 2014). Spinach may also reduce age-related eyesight worsening from macular deterioration and cataracts (Agoreyo *et al*., 2011; Sri Lasya, 2022).

The quantity and quality of crop yield is lost by a huge amount due to a variety of pathogens. The present study is restricted to fungal leaf spot diseases. The most important reported causal pathogens of this

disease are *Cladosporium variabile, Stemphylium botrysum, Rhizoctonia solani, Albugo pccidentalis, Peronospora farinose, Colletotrichum spinaciae, Fusarium oxysporum f. sp spinaciae* and *Vertcillium dahlia* (Larsson and Gerhardson, 1992; Correll *et al*., 1994; Koike *et al*., 2001). Initially, leaf spots appear in 2-5 mm in width oval to circular in form, and grey and green in colour. After the disease progression leaf spots expand and become papery in texture and leading to the death of a significant portion of leaves. If the management in the field is not proper, all crops must be ruined 100% (Beckman, 1987; Koike *et al*., 2001).

With an increasing infection potential by pathogenic fungi on Spinach leaves, causing loss of spinach during the growing season throughout the world leads to economic loss. The goal of the contemporary trial was to analyze the leaf spot eliciting organism in spinach. The goal of the study was the isolation, identification, and categorization of the leaf spot pathogen of spinach and the assessment of the pathogenic potential of the identified pathogen.

Materials and Methods

Study and Assemblage of Diseased Samples

To study spinach leaf spots a field survey was led to the investigational field of FAGS (Faculty of Agricultural Sciences) University of the Punjab Lahore, Depalpur as well as Okara field in the months of August and September 2018. Several spinach plants were seemed to be symptomized with leaf necrosis and wilting symptoms. Photography of diseased leaves was done and size, form, colour, appearance of spots was noticed for comparison. Infested leaves presenting leaf spot indications were kept in sanitized polythene bags and brought to the workroom for further research. For further study the samples were stowed at 4 °C. Percentage disease incidence and severity of designated fields was documented for the assessment of spread of infection.

$$\text{Disease Incidence (\%)} = \frac{\text{Number diseased plants}}{\text{total number of plants}} \text{ x } 100$$

$$\text{Disease Severity (\%)} = \frac{\text{Infected area of plant leaves}}{\text{Total area of leaves}} \times 100$$

Isolation of Fungal Pathogen

Malt extract Agar (MEA) medium (2%) was used for the isolation of pathogen. The infected leaf samples were washed thoroughly and diseased parts along with some portion of healthy tissues were cut into approximately 2-3 mm. The diseased parts were superficially sanitized with sodium hypochlorite (1%) for 2 min. and rinsed with autoclaved purified water. Then all bits were dried and 3-4 surface sterilized leaf pieces were transferred aseptically into sterilized malt extract agar containing plates at equal distance from each other and reared at 25-27 °C for 3-4 days. The dishes were scrutinized frequently to observe the growth of emerging mycelia from the infected bites. Fungal colonies grown from the inoculated leaf tissues were transmitted to fresh MEA plates for the purification and left to propagate at room temperature. Petri plates containing pure fungal culture were stowed at 4 °C.

Identification and Characterization of Fungal Pathogen

Initially isolated pathogen was recognized on the cultural appearance and morphological basis, and then the identification was confirmed on the nucleotide sequence analysis basis of Internal Transcribed Spacer (ITS) sequences of rDNA and

partial glyceraldehyde 3 phosphate dehydrogenase (GADPH), and partial elongation factor (EF1/EF2).

Morphological Identification of Pathogens

Pure fungal cultures 4-7 days old grown on MEA at 25 °C were observed for morphological study. The characteristics of colony were observed by naked eye and stereoscopy. Colony characters studied were colour of the colony from front side and back side, presence of progression zone, width of colony, presence of submerged and aerial mycelium abundance attachment and conidia type. Microscopic characterization was done on account of size, conidiophore septation and wall characters, colour, shape, position and number of conidial septa. As an evidence of macro and micro morphological features photographs were taken. Complete description of fungal isolates was prepared based on morphological characteristics.

Genetic Characterization

Fungal mycelia from 2-3 plates of fresh (7 days old) and pure fungal cultures were scratched carefully avoiding agar medium for DNA extraction using nucleon reagent. The coding region of internal transcribed spacer (ITS) sequence, partial Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and partial elongation factor (EF1/EF2) was amplified from the genomic DNA. Commercially accessible 2X Amp Master ^{TM}Taq polymerase (Gene all Biotechnology CO, LTD) was utalized to perform augmentation. 0.8% agarose gel was used to separate the augmented artifact along with 1 Kb DNA marker. Gene products of correct size were sequenced. The results of DNA sequencing were analyzed by Basic Local Alignment Tool (BLAST) for the identification of pathogen constructed on nucleotide homology with conforming strains in GenBank database.

Pathogenicity Test

In vitro Conditions**:** In order to make the suspension, spores from the pure fungus cultures were smashed and put off in 0.9% NaCl under aseptic conditions, then serially diluted to prepare 5×10^5 spore ml^{-1} with help of haemocytometer and used as inoculum.

Detached Leaf Assay**:** Fresh young leaves were reserved from the vigorous plants and kept in sterilized Petri plates. For this method, leaves were taken from the healthy plants and placed in sterilized Petri plates. Each Petri plate was lined with filter paper and 2 ml double distilled water was used to moisten the filter papers. The plant leaves sited in petri plates by touching their petioles to drenched filter paper. The surface leaves were inoculated under aseptic conditions with approximately $5x10^5$ spores ml^{-1} using micropipette and then stowed at 25 °C and repeatedly perceived to scrutinized the expansion of disease. The causal agent was again extracted from the septic leaves to accomplish the Koch's pathogenicity postulates.

In vivo Pathogenicity Testing**:** The pots were filled and leveled with sterilized soil approximately 1kg per pot. Three spinach seeds per hole were disseminated into earthen pots, watered properly and placed into growth room at 24 - 31 °C. Five ml of spore suspension (5×10^5 spores ml^{-1}) was injected in the stem nodes and also sprayed in soil for the confirmation of pathogenicity test. Distilled water was given to the control treatment in same amount. The plants were shielded for 48 h with polythene bags to uphold the suitable humidity for spore propagation and the onset of disease. After that the pots were kept in shadow at optimal temperature (25 – 26 °C) and sprinkled timely. Within 3-4 days of inoculum symptoms were started to appear on plants. Disease incidence and severity were observed and calculated

to confirm and compare the pathogenicity test with the help of disease rating scale as a standard.

Results

Field investigation and recording of disease symptoms

During the field investigation, the diseased *S. oleraceae* leaves were accumulated from the fields of different areas i.e., Okara, Depalpur, Investigational area of FAGS, and vegetal area of University of the Punjab, Lahore. It was observed that approximately 60% of *S. oleraceae* plants represented leaf necrosis infection. Symptoms detected were blond to tanned circular to asymmetrical abrasions of nearly 2.6 mm to 2.8 mm in extent. These abrasions amalgamated to precede bigger spots of around 30-40% leaf area (Fig. 1).

Fig 1: Infected *Spinacia oleraceae* plants; (A): Infected plants in field; (B): leaf infected by *Alternaria alternata* from abaxial surface; (C): from adaxial surface.

The variation in the percentage of contagion of inspected ranges witnessed the disparity in their disease incidence and disease severity. Okara and Depalpur areas demonstrated the premier disease incidence of 65% and 60%, respectively. Conversely, investigational area of FAGS revealed the lowermost disease incidence of 45% (Fig. 2). The maximal disease severity was recorded in Okara (57.67%) whereas the minimnm severity of 47% was depicted in vegetal area of University of the Punjab, Lahore (Fig 2).

Documentation of Pathogen

Morphological Characterization of Pathogen

For morphological identification; 7 days old pure culture was witnessed under the stereoscope and compound microscope. The fungal colony observed was dark green to brownish in colour from front and dark brown from reverse side on MEA. The size of colony was reaching 5.5-6.5 cm in width after 6 days of gestation. The margins of culture were regular with flattened mycelia. Conidiophores were 55–65 µm x 2.7-3.8 µm in size, pale brown to olive brown, septate and branched. Conidia were long, small oval shaped, light brown, longitudinal and transverse septation, rounded at apex, 27-30 x 4-8 µm with 4-6 transverse septa and 2-3 longisepta. Hyphae were light green and septate. Spore wall was slightly geniculate. In the study of morphology the pathogen was recognized as *Alternaria alternata* (Fig 3).

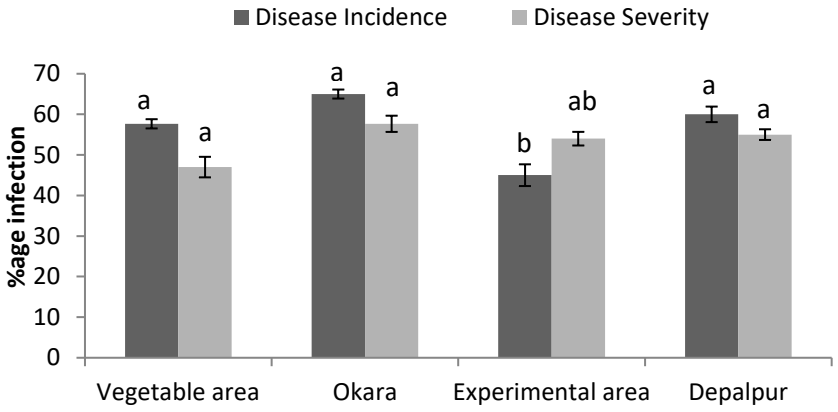

Fig 2: Disease incidence and Disease severity of different areas.

Vertical bras represent standard errors of three replicates. Values with different letters represent significant difference by ANOVA ($p \leq 0.05$) as determined by statistix 8.1 software, LSD test at $p=0.05$.

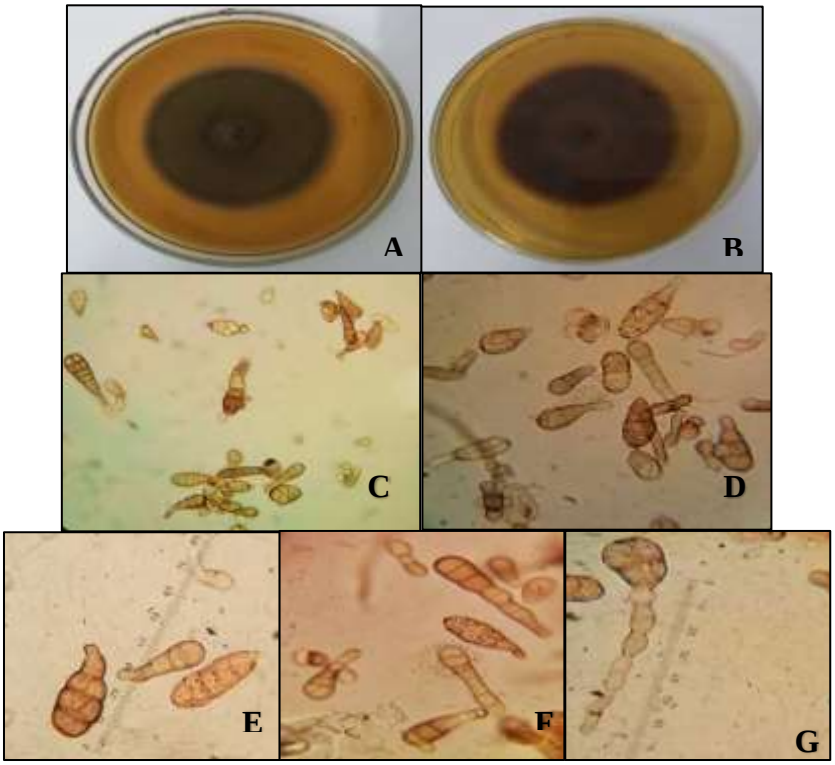

Fig. 3: Cultural and morphological characterization of *Alternaria alternata.* (A): Colony from front side; (B): reverse side; (C): Conidiophore and conidial attachment under stereoscope; (D): under 10X; (E-F): conidial morphology under 10X and 40X, respectively; and (G): conidial morphology under 100X magnification.

Molecular Documentation and Characterization of Pathogen

Genetic characterization of *A. alternata* was carried out to confirm the morphological based identification. For this purpose, high quality DNA was extracted from the pure fungal culture and used as template to amplify ITS1/ITS4 – rDNA, partial GAPDH and Ef1/Ef2 genes, and the resulting PCR products were of 520 bp, 600 bp and 260 bp, respectively and led to nucleotide sequencing (Fig 4-6).

The nucleotide sequencing results were analyzed by BLAST and *A. alternata* isolate of ITS gene showed maximum 99% homology with *A. alternata* (MH920250.1), 98.71% homology with *A. aternata* (MG214857.1) and 98.70% similarity with Sequence ID (MK212914.1) (Fig 4). The intensified ITS nucleotide sequence of *A. aternata* was allocated MN525799 accession ID in GenBank.

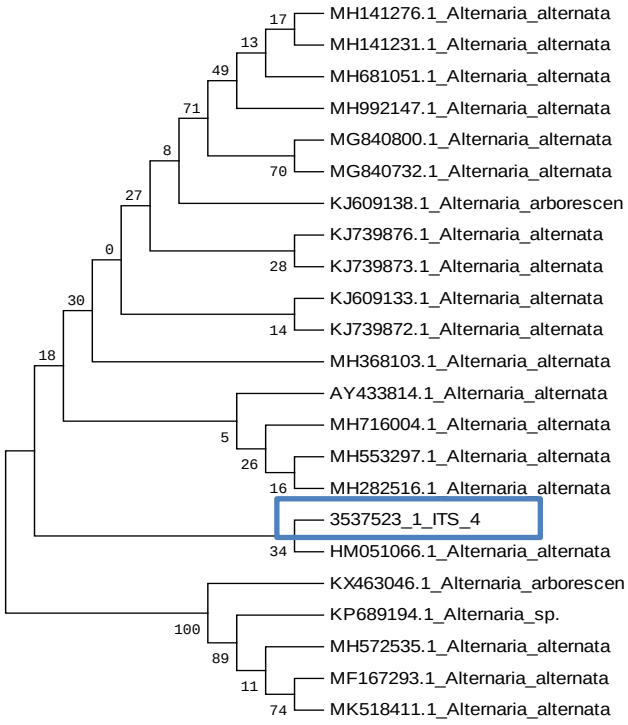

Fig 4: Phylogenetic tree on the basis of Internal Transcribed Spacer region of rDNA (ITS) sequences of *Alternaria alternata*. Evolutionary study was conducted in MEGA 6 (Tumara *et al.*, 2013). Jukes-Cantor model was used to infer evolutionary history (Jukes and Cantor, 1969).

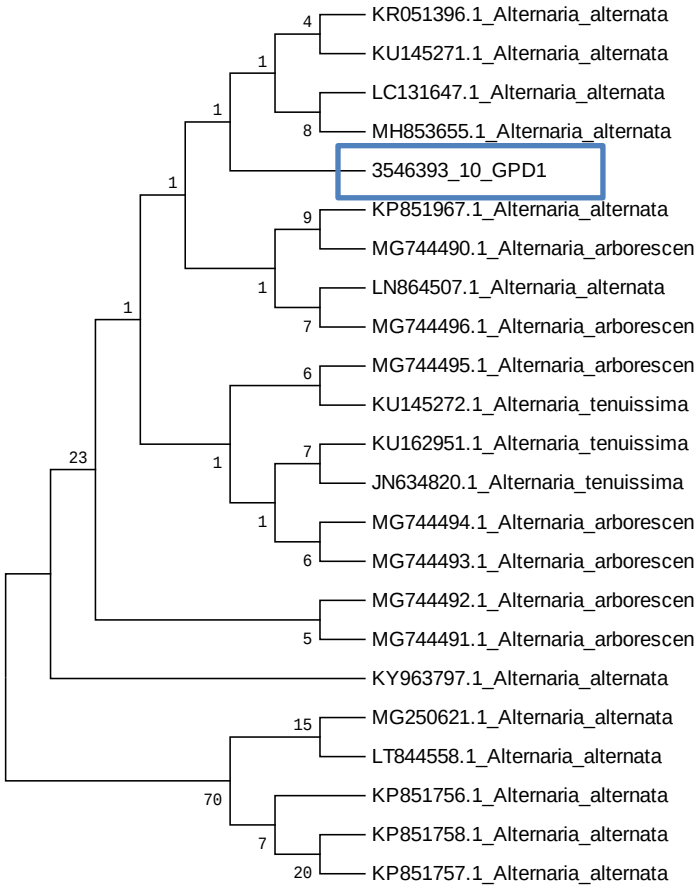

Fig 5: Phylogenetic tree of *Alternaria alternata* on the basis of partial Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) gene sequences. Evolutionary investigation was conducted in MEGA 6 (Tumara *et al.*, 2013). Jukes- Cantor model was used to infer evolutionary history (Jukes and Cantor, 1969).

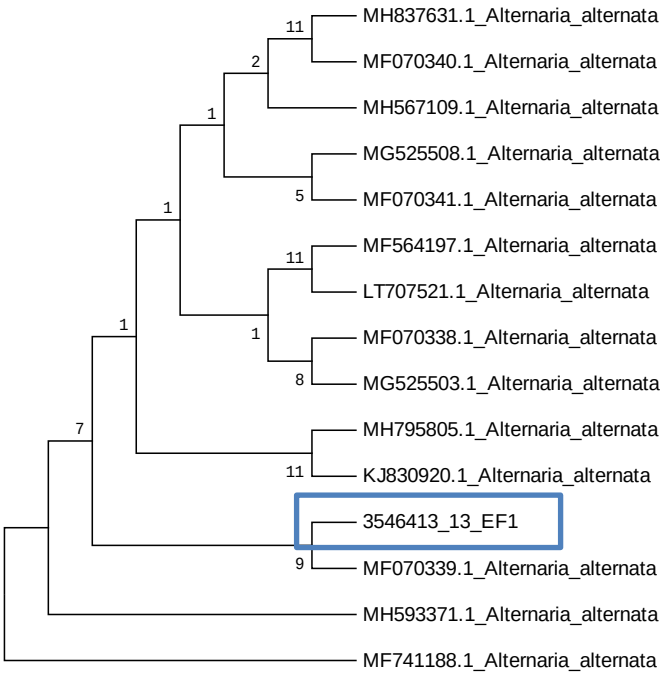

Fig 6: Phylogenetic tree on the basis of Translational Elongation Factor (EF1/EF2) gene sequences of *Alternaria alternata*. Evolutionary study was directed in MEGA 6 (Tumara *et al*., 2013). The Jukes- Cantor model was used to infer evolutionary history (Jukes and Cantor, 1969).

The results of GAPDH nucleotide BLAST analysis revealed 99.65% similarity with different *A. alternata* isolates Seq. ID (MH567107.1), (KR051394.1), and (MF741817.1) (Fig. 5). Ef1/Ef2 nucleotide sequencing results when analyzed by BLAST showed 100% homology with various *A. alternata* isolates DEF1/EF2 (KY175227.1), (LC 1327711.1) and (KY062988.1) (Fig. 6).

Pathogenicity Analysis

Detached Leaf Assay: The detached leaf method confirmed the pathogenic potential of *A. alternata* on young healthy leaves of 15-day-old spinach plants. According to the disease rating scale, the progressively variable disease severity was recorded after each day. After 15 days of inoculation, the characteristic leaf spot symptoms that were initially observed on the leaves were in accordance with those observed in the field of spinach plants. Photographs were taken at different stages of infection. The pathogen showed varied disease symptoms on the host plant throughout the pathogenicity test.

The pathogen *A. alternata* was observed to be responsible for the lesions and leaf spots symptoms. The appearance of different infection symptoms was very slow as initially very minute yellow spots were started to develop on leaves. The leaf spots became larger with time and converted into dark brown with concentric rings. The pathogen, *A. alternata* was re-isolated from the diseased leaves which inveterate the disease etiology that revealed acute evolution of disease (Fig. 7). A disease development curve was intrigued to display the evolution of disease by

pathogen (Fig. 8). This curve showed the severe symptoms of pathogen and verified the virulent pathogen of spinach and used for further lab experiments.

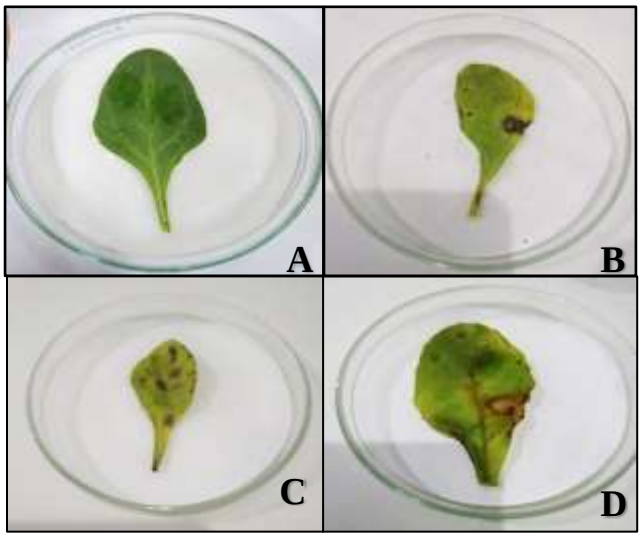

Fig 7: Different stages of development of disease symptoms by *Alternaria alternata* on spinach plant.

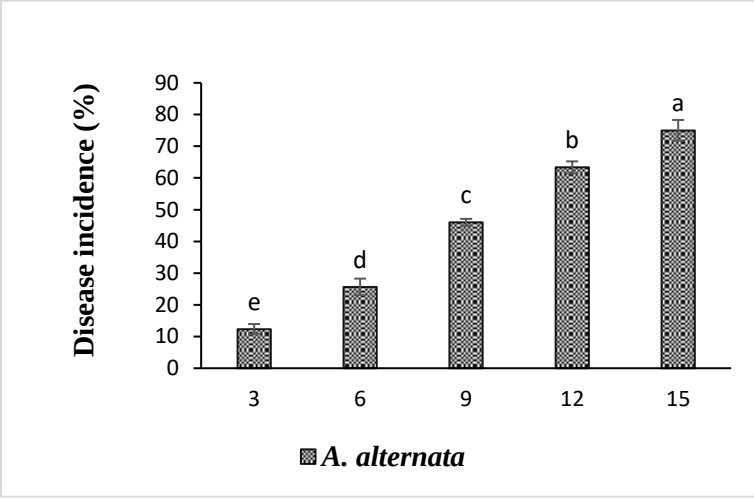

Fig. 8: Analysis of disease progression for *Alternaria alternata* on spinach plants based on detached leaf assay.

Vertical bars indicate standard errors of means of three replicates. Values with different letters indicate significant difference by ANOVA ($p \leq 0.05$) as determined by statistix 8.1 software, LSD test at $p=0.05$.

Pot experiment

The pathogenicity test was also carried out on spinach plant seedlings in pots. After 20 days of sowing spinach plants were sprayed with a spore suspension of the pathogen at the rate of 5 ml/plant using 10^3 conidia/ml from the 7 days old pure culture plates (Fig. 9). The pots were examined repeatedly for the commencement of disease and then after a week, the symptoms appeared were found to be analogous to the primarily assembled diseased specimens. The envisaged symptoms were, yellow abrasions trailed by chlorosis and brown spots followed by wilting of inferior leaflets and finally the necrosis of entire leaves. The results revealed the advancement of disease persuaded the falling of leaves and ultimately the demise of the whole plant. The chlorotic lesions and yellowing of plants were caused by *A. atlternata* (Fig. 9). Through the data analysis of *in vivo* pathogenicity testing, it was concluded that the pathogen showed maximum disease severity in the host plant. The maximum disease severity induced by *A. alternata* was around 82%.

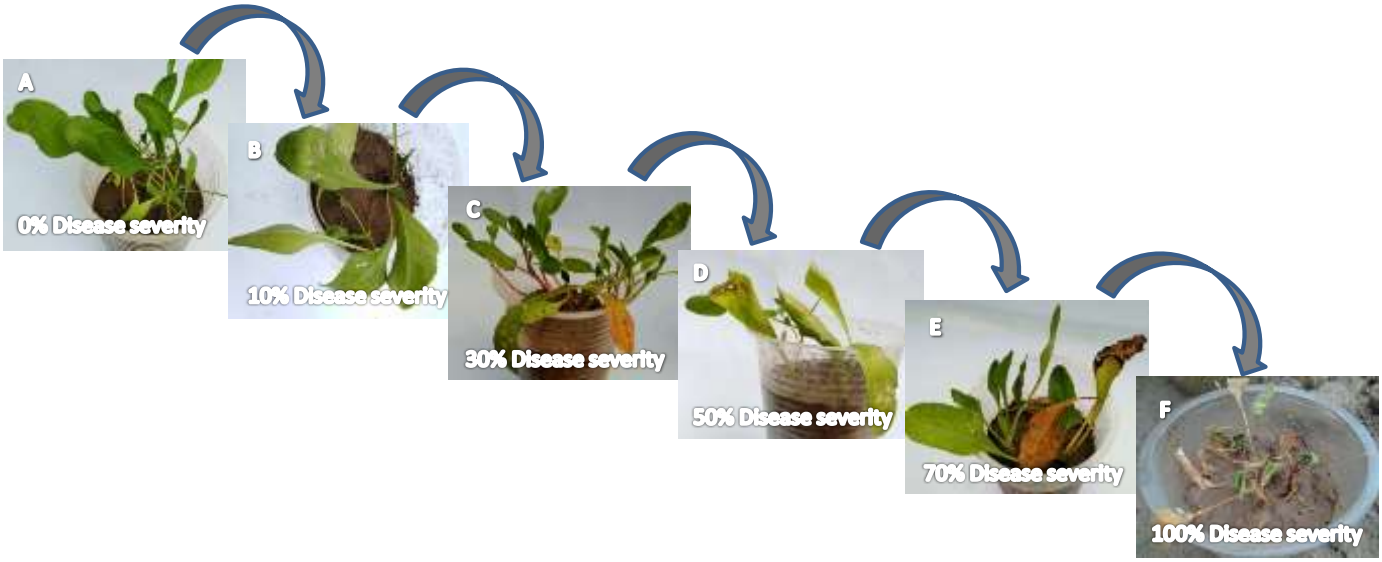

Fig 9: Pictorial representation of disease symptoms development by *Alternaria alternata* in spinach plants.
A: Healthy plant, B: Tiny leaf spots appeared on the leaf, C: Leaf started yellowing, leaf spots appeared on tips and in the middle of the leaves, D: Wilting of leaf started, E: Leaf become necrotic and dropped, F: Death of whole plant.

Discussion

Spinach is the supreme imperative nutrition-rich vegetable consumed as salad and cooked. It is used as food worldwide due to its health benefits and dietary content like carotenes, vitamin C, and minerals such as calcium and iron (Sheetal *et al*., 2006). It has been growing throughout Pakistan and other countries due to its therapeutic effects (Patricia, 2014; Tehseen, 2016). Fungal diseases are considered as a chief menace to the significant loss in yield of spinach (Bassi and Goode, 1978; Larsson and Gerhardson, 1992; Correll *et al*., 1994). Amongst these diseases, fungal leaf spot is more dynamic in instigating severe infection. Mostly these diseases are vigorous during

spring or early fall, but a few of them need significant control measures. Fungicides were considered the sole method of subsiding leaf spot diseases (Reshu and Khan, 2012). But during the last couple of decades, in order to control plant diseases innovative concerns have been introduced towards the correct identification of pathogen to suggest their proper management.

Currently, a field survey of different Punjab areas was conducted for the collection of infected samples, and disease incidence and disease severity of infected spinach plants in specific areas were determined. Survey analysis revealed the highest disease incidence and severity in district Okara. An analogous study was executed by Shazia *et al*. (2003) in which foliar disease prevalence, incidence, and severity were determined in a survey in rice and wheat fields of four districts of Punjab i.e., Sheikhupura, Sialkot, Narowal, and Gujranwala.

Afterwards, the infected samples from different areas with different disease symptoms were inoculated on MEA medium for the isolation and identification of pathogens. At the species level, morphological identification is still considered as the steadiest technique, but sometimes misidentification may occur (Anderson *et al*., 2006). Innumerable molecular studies i.e., analysis of ribosomal DNA (rDNA) sequences subsidize to find out the molecular phylogenetic relationship between the groups of fungi (Mirhendi and Rezaei, 2007). Currently, the isolated pathogen was identified by reviewing its morphology followed by fungal genomic analysis with universal primers which was later confirmed by BLAST analysis. The *Alternaria alternata* was identified as isolated pathogen causing leaf spot disease of spinach. Usage of nucleotide sequences of ITS in amalgamation with any gene coding primers such as Beta Tubulin, GAPDH, and Elongation factor (EF1) has been assumed as a reliable method for the identification of fungal species (Schoch *et al*., 2012). Recently the same study was conducted by Shafique *et al*. (2019) who isolated and identified *Cladosporium cladosporioides* giving a comprehensive depiction of macro and microscopic features followed by identification using rDNA spacer sequence and confirmed that *Cladosporium cladosporioides* was the causal agent of leaf spot of *Sonchus oleraceus.* Earlier in the similar kind of study, *Alternaria ochroleuca* was identified as a leaf spot causing agent of money plant on the basis of macro and microscopic characterization followed by identification using rDNA spacer sequence of amplified ITS1-ITS4 region of rDNA (Shafique *et al*., 2017).

In the current research work, the detached leaf method and pot trials were used to perform the pathogenicity test for the evaluation of the pathogenic potential of the pathogen by observing induced diseased symptoms in spinach. Visually it was verified that the symptoms produced by *Alternaria alternata* were drastic and comparable to the symptoms of earlier collected samples. Recently the same experiment was done by Shafique *et al*. (2018) on the Dracaena Red Edge (*Dracaena mariginata*) and Sow thistle (*Sonchus oleraceus*) plants to confirm the pathogenic potential of *Alternaria arborescens* and *Phyllosticta aristolochiicola* using detached leaf assay and Mahmood (2010) used pot trials to evaluate the pathogenic potential of *Alternaria alternata* in tomato plants.

Conclusion

Thus the current work reports the identification and screening of *Alternaria alternata* from *S. oleraceae* samples. Precise identification of the pathogen causing leaf spots in *S. oleraceae* is

imperative for the management of the most devastating pathogen and preparing defense materials against them.

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Conflict of interests

The authors do not have any conflict and relevant financial or non-financial interests to disclose among themselves that are relevant to the content of this article

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