Pak. J. Bot., 54(3): 1139-1145, 2022. DOI: http://dx.doi.org/10.30848/PJB2022-3(21)

ISOLATION AND IDENTIFICATION OF NOVEL LEAF SPOT PATHOGEN *FUSARIUM EQUISETI* FROM *SPINACIA OLERACEA*

SHAZIA SHAFIQUE[1], AYESHA MUBARAK[2], SOBIYA SHAFIQUE[1*], ABRAR HUSSAIN[2], ALINA JAVED[2] AND RUBAB RAFIQUE[2]

[1]*Department of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Lahore 54590, Pakistan*
[2]*Department of Botany, University of Education, Township Campus, Lahore, Pakistan*
[*]*Corresponding author's email: sobiya.iags@pu.edu.pk*

Abstract

In recent past spinach is threatened by various fungal, viral and bacterial diseases which are major cause of loss in crop production. Diseases caused by fungal pathogen, rank amongst the highest cause of spinach yield loss. During the field survey of selected areas of Punjab, spinach samples infected with leaf spot were collected and disease severity was recorded. The fungal pathogen was isolated from diseased spinach samples and based on morphological characterization and analysis of rDNA region of internal transcribed spacer (ITS) sequence, partial elongation factor (EF1/EF2) and Beta tubulin (Bt_2a/Bt_2b), the isolate was identified as *Fusarium equiseti var. bullatum* (Sherb). of *Spinacia oleraceae.* The pathogenicity trails conducted in plates as well as in pots confirmed *Fusarium equiseti,* as the most virulent pathogen of spinach. To our knowledge, this is the first report of isolation of a novel pathogen from *S. oleraceae.*

Key words: *Fusarium equiseti,* Identification, Pathogenicity, Spinach.

Introduction

Spinach (*Spinacia olraceae* L.) is the most important nutritious leafy vegetable eaten raw or cooked among many seasonal economic vegetables (Correll *et al*., 1994; Miano, 2016). It is mainly cultivated in fall, winter or spring season in China, USA, Japan, Turkey, Indonesia and Pakistan. The worldwide average yield of spinach is 250kg/ha (FAOSTAT, 2015). In Pakistan, its production is estimated 96000 tons with 1.5 tons/ha in yield. Spinach cultivation in Pakistan has more consideration as it provides 41 calories by eating 200 gm of spinach. It is an excellent source of vitamin K, flavonoids, carotenes, vitamin C (Miano, 2016). Flavonoids compounds in spinach functions as antioxidant agent which act in body as anticancer agent (Agoreyo *et al*., 2011). Spinach is also rich in iron and used to prevent diseases like osteoporosis, iron deficiency results in anaemia (Patricia, 2014; Miano, 2016). Spinach also reduces age-related eyesight worsening from macular deterioration and cataract (Robinson *et al*., 1983). The literature revealed that spinach is attacked by various fungal diseases (Naiki & Kanoh, 1977; Sunil & Yadav, 2020; Liu *et al*., 2021). The worldwide production of spinach is mainly affected by the pathogen *Fusarium oxysporum f. sp. spinaciae* (Naiki & Kanoh, 1977; Bassi & Goode, 1978; Larsson & Gerhardson, 1992; Correll *et al*., 1994). *Fusarium* spp. and *Cladosporium* spp. can greatly damage the spinach leaves (Dhar *et al*., 2015; Shova *et al*., 2020). Sixteen different fungal diseases of Indian spinach have so far been reported from different parts of the world (Sarker *et al*., 2017). If it is not managed in the field the planting can be 100% destroyed (Beckman, 1987; Shova *et al*., 2020).

Leaf spot diseases caused by fungi have become a major concern in spinach production worldwide. Determining the causal agents of leaf spots on spinach, their prevalence and pathogenicity, and fungicide efficacy against these pathogens is vital for effective disease management. The present study was, therefore, conducted for the detection of pathogen causing leaf spot of spinach followed by its identification using morphological and genetic characterization. In view of this, systematic work was done which included survey for knowing disease severity which enabled to locate the hot spots for disease in addition to know their characteristic symptoms.

Materials and Methods

Study and collection of diseased samples: For the investigation of leaf spot pathogen of spinach a survey was conducted to the fields of IAGS (Institute of Agricultural Sciences) University of the Punjab Lahore, Depalpur and Okara field during August-September 2018. Many spinach plants were found infected with leaf spot disease and collected for the study of pathogen. Photographs of infected leaves were taken and size, shape colour, appearance of spots was noted as reference. Diseased leaves showing leaf spot symptoms were collected in sterilized polythene bags and brought to laboratory for pathogen study and stored at 4°C until processed. The prevalence, incidence and severity of disease of different areas were noted to find out the intensity of disease in different regions of Punjab.

Isolation and identification of fungal pathogen: For isolation purpose, the infected parts of leaf were cut into 2-3 mm pieces along with some healthy leaf tissues, dipped into 1% sodium hypochlorite solution for sterilization and transferred into petri plates containing Malt extract agar. These plates were incubated at 25-27°C for 3-4 days for the purification of isolated fungus.

The morphological studies were carried out using 4-7 days old pure fungal culture. The pathogen was identified on the basis of colony characteristics as colour of culture and growth zone, and the microscopic features included shape, size and number of conidia, conidiophore septation and wall characteristics. For the molecular identification of pathogen, the DNA of pure culture was isolated using revised CTAB method of Shafique *et al*., (2019). The rDNA region of purified total genomic DNA was

amplified with the coding region of internal transcribed spacer (ITS) sequence, partial elongation factor (EF1/EF2), and Beta tubulin (Bt_2a/Bt_2b). The PCR reaction was 30 µL consisting of 2X Amp Master ^{TM}Taq 15 µL; forward primer 1 µL, reverse primer 1 µL. The amplified PCR products were sent for nucleotide sequencing and analysed by BLAST for the identification of pathogens based on nucleotide homology. The phylogenetic tree was conducted to confirm the evolutionary relationship of pathogen (Table 1).

Table 1. Details of primers used for gene amplification for fungal genomic DNA.

Primer	Primer name	sequence (5′-3′)
ITS (Forward)	ITS1	5′- TCC GTA GGT GAA CCT GCG G-3′
ITS (Reverse)	ITS4	5′-TCC TCC GCT TAT TGA TAT GC-3′
EF1 (Forward)	EF1	5'-ATGGGTAAGGA(A/G)GACAAGAC-3'
EF2 (Reverse)	EF2	5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'
Bt_2a (Forward)	Bt_2a	5'–GGTAACCAAATCGGTGCTGCTTTC-3'
Bt_2b (Reverse)	Bt_2b	5'-ACCCTCAGTGTAGTGACCCTTGGC 3'

Pathogenicity test: The pathogenicity test was conducted using detached leaf and pot trials (Akhtar *et al*., 2016). The fresh healthy young leaves were placed on the petri plates lined with moistened filter paper. Approximately 1 mL spore suspension ($5x10^5$ spores mL^{-1}) was aseptically transferred to each petri plate with micropipette and incubated at 25°C. The disease symptoms were appeared after 7 days. Then for the confirmation of Koch's pathogenicity postulates the pathogen was re isolated from the diseased portions. In pot experiment the pots were filled with oven dried sterilized soil then two to three seeds of spinach were sown into the pots and kept at 25-31°C. After 2 weeks for the confirmation of pathogenicity 5 mL of spore suspension was given to the plants and control pots received only water. The pots were regularly observed for the development of disease. The disease ration scale was used to calculate the incidence and severity of disease.

Statistical analysis

Microsoft Excel software was used to analyse the standard errors of all the means replicates of all data analysis. Analysis of variance (ANOVA) at $p \leq 0.05$ and statistix 8.1 was used to analyse all the data recorded during the survey, pot and plate trials. The mean values were compared at probability level 0.05 with LSD test. The Phylogeny of identified pathogens was evaluated using MEGA6 software.

Results

Field survey and recording of disease symptoms: In a survey during August-September 2018, the infected leaves of *S. oleraceae* were collected from the fields of Okara, Depalpur city, vegetable area of University of the Punjab, Lahore and Experimental area of Institute of Agricultural Sciences, University of the Punjab, Lahore. During survey more than 55-60% plants of *S. oleraceae* were found to be infected with leaf necrosis. Symptoms observed were yellow to brown rounded to irregular lesions or spots of approximately 2.6 mm to 2.8mm in size. These spots or lesions joined to form larger spots of about 30-40% leaf area (Fig. 1). Percentage of infection of selected areas was recorded to evaluate the disease prevalence. The highest perceived disease prevalence was 85.67% at vegetable area followed by Depalpur where disease prevalence was recorded up to 72.33%. Whereas the lowest disease prevalence was displayed in Okara (51%) (Fig. 2). In case of disease incidence; number of infected plants, in a field of specific area were recorded. The highest detected disease incidence was 65% in Okara and 60% in Depalpur. The lowest disease incidence (45%) was recorded in Experimental area of Institute of Agricultural Sciences, University of the Punjab, Lahore (Fig. 3). In survey analysis, the disease severity was determined by recording infection per plant in the field. The maximum disease severity was exhibited in Okara i.e., 57.67% and minimum of 47% was revealed in vegetable area (Fig. 4).

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

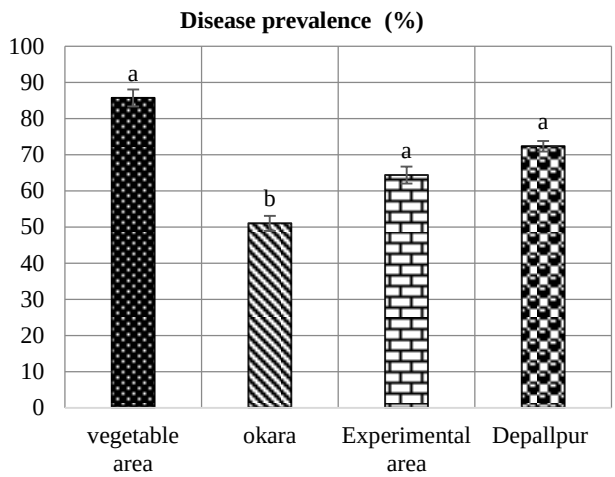


Fig. 2. Disease prevalence of different area.
Vertical bras show standard errors of three replicates. Values with different letters show significant difference by ANOVA as determined by statistix 8.1 software at $p \leq 0.05$.

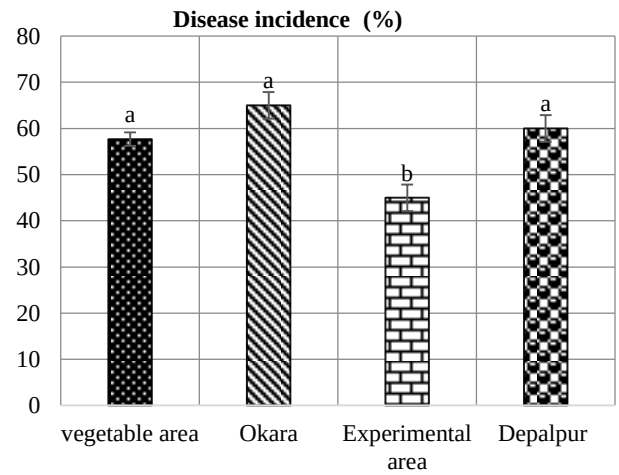


Fig. 3. Disease incidence of different areas.
Vertical bras show standard errors of three replicates. Values with different letters show significant difference by ANOVA as determined by statistix 8.1 software at $p \leq 0.05$.

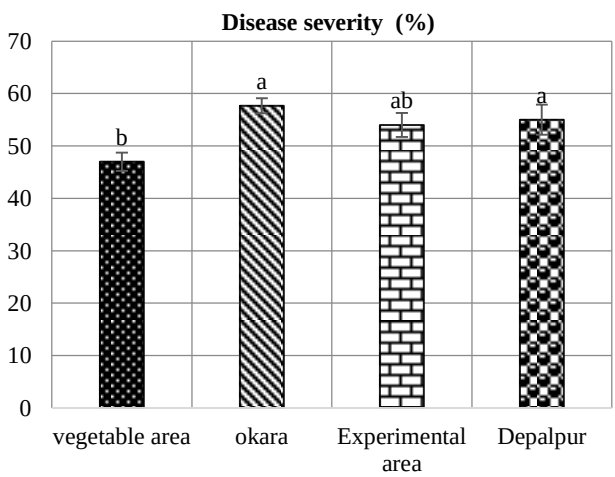


Fig. 4. Disease severity of different areas.
Vertical bras show standard errors of three replicates. Values with different letters show significant difference by ANOVA as determined by statistix 8.1 software at $p \leq 0.05$.

Morphological and molecular characterization of pathogen: To study the morphological features growth of pure fungal culture was observed on MEA medium, after 7 days of incubation at 20 - 25°C. The colour of isolated culture colony was white to creamy with feathery mycelium from front side while it was yellow orange from reverse side. The conidiophores were branched, monophiliads, curved, smooth and septate, oblate to cylindrical, slightly curved, arranged in densely branched clusters 10-15µm x 4-5 µm. Macro conidia were cylindrical, fusiform, 3-5 septate, basal cells, 25-30 µm x 4-4.5 µm in length and 4.5µm-6.7µm in width. On the morphological basis pathogen was identified as *Fusarium equiseti* (Fig. 5).

Further, high quality isolated genomic DNA (10 kb) was used to amplify ITS-rDNA, partial Bt2a/Bt2b and partial Ef1/Ef2 genes for genetic characterization of isolated pathogen. The size of amplified PCR product was 520 bp, 320 bp and 270 bp, respectively (Fig. 6). The nucleotide sequences were analysed by BLAST, the resulting ITS PCR products displayed 99.60% similarity with the *F. equiseti* isolate (Sequence ID KX463031.1) and 99.41% with *F. equiseti* isolate (MH707079.1) (Fig. 7A). The amplified ITS nucleotide sequence of *F. equiseti* was assigned MN524571 accession ID in GenBank.

The Beta-Tubulin nucleotide sequencing results showed 99% homology with *F. equiseti* isolate (Sequence ID JX241676.1) and 98.26% similarity with *F. equiseti* isolate (MF662650.1). The results of BLAST analysis of EF1/EF2 displayed 99.59% homology with *F. equiseti* isolate (Sequence ID KT213277.1) and 99.59% homology with *F. equiseti* isolate (HM805100.1). The evolutionary study was directed in MEGA 6 (Tumara *et al*., 2013). The Jukes- Cantor model was used to infer evolutionary history (Jukes & Cantor, 1969) (Fig. 7B-C).

Pathogenicity analysis: Pathogenicity assays were performed in laboratory as well as in pots to test the pathogenic potential of *F. equiseti* on the host plant for the verification of Koch's postulates. One mL of inoculum containing $5x10^5$ spores mL^{-1} of *F. equiseti* was given to the detached leaf separately, as well as *In vivo* to the host plants (5 mL of spore suspension/Plant) and checked for the manifestation of disease.

The symptoms observed due to *F. equiseti* were wilting of plants. At the very first stage yellowing of midrib and leaf tissues was started that progressed to the chlorosis of leaves followed by browning and necrosis which led to the death of plants (Fig. 8). To check the progression rate of disease of pathogen, disease progression curve was plotted (Fig. 9). Disease progress curve depicted that the pathogen exhibited sever symptoms and proved virulent pathogen of spinach as the severity went on increasing with the time scale and the total plant part was collapsed within 15 days.

In pot trials, spinach plants were sprayed with spore suspension from 7 days old pure culture plates after 20 days of sowing. The pots were monitored regularly for the onset of disease and after 7 to 10 days of inoculation the observed symptoms were found to be similar with the initially collected infected samples. The visualized symptoms were, yellowing followed by chlorosis with brown lesions and wilting in lower leaflets and later on, necrosis of whole leaves on both sides. It was observed that progression of disease due to *F. equiseti* induced dropping of leaves and eventually the death of whole plant was recorded (Table 2).

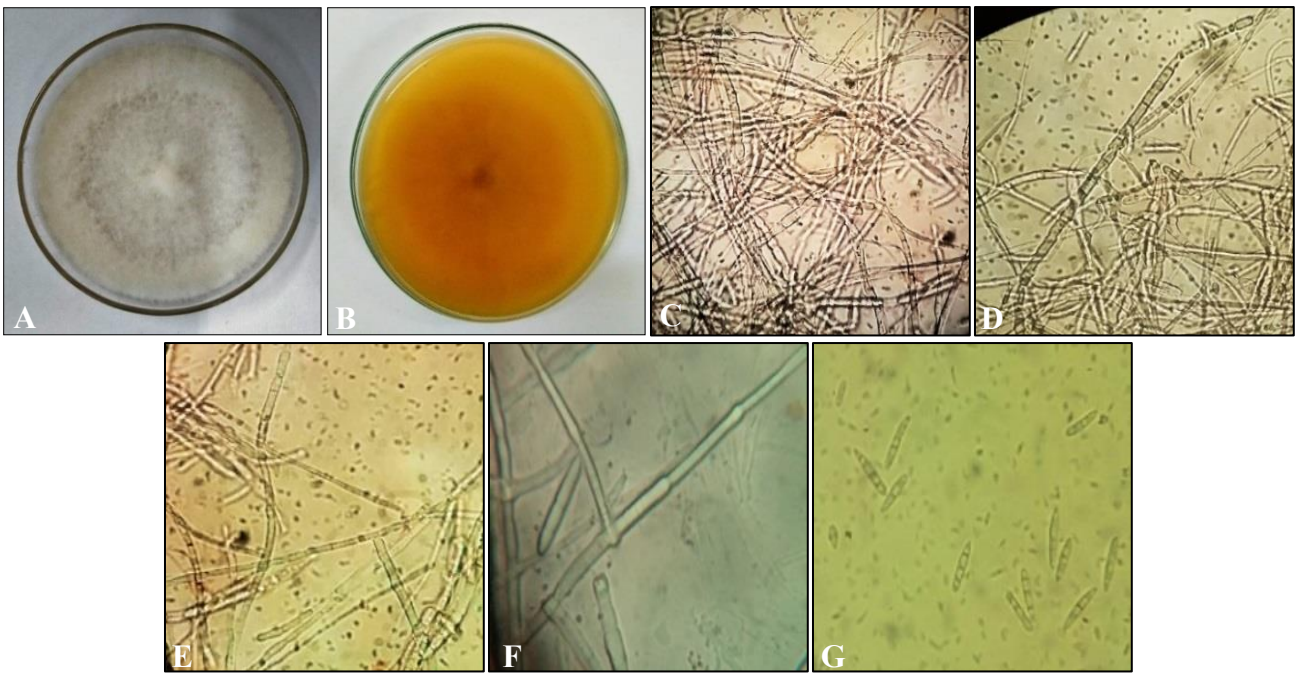
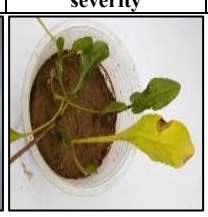


Fig. 5. Cultural and morphological characterization of *Fusarium equiseti*. (A): Colony from front side; (B): reverse side; (C): Conidiophore and codial attachment under stereoscope; (D): under 10X; (E-F): conidiophore and conidial morphology under 10X and 40X, respectively; and (G): conidia under 100X magnification.

Table 2. Pictorial representation of disease rating scale of symptoms development by *Fusarium equiesti* in spinach plants.

Scale	0% Disease severity	10% Disease severity	30% Disease severity	50% Disease severity	70% Disease severity	100% Disease severity
Symptoms						
	Healthy plant	Tiny leaf spots appeared on the leaf	Leaf started yellowing, leaf spots appeared on tips and in the middle of the leaves	Leaf spot became enlarged	Leaf become necrotic	Death of plant

Present study concludes the report of novel isolation of leaf spot pathogen from Spinach. This study emphasizes the need of management of this pathogen which is responsible for yield loss of this important vegetable crop

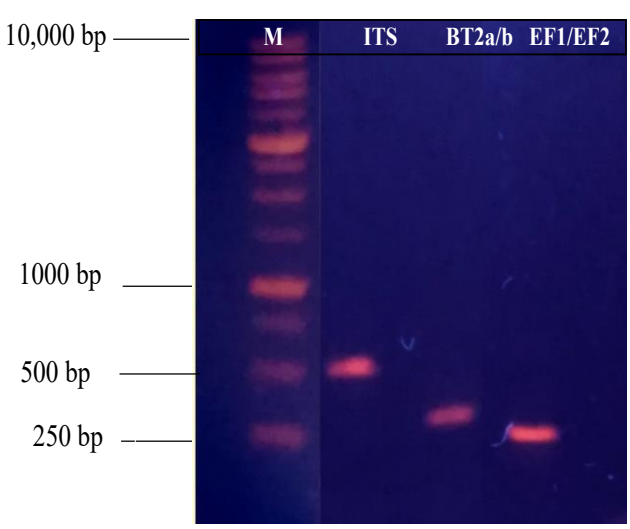


Fig. 6. Agarose gel electrophoresis of total genomic DNA of *Fusarium equiseti* amplified with different primers. M: DNA size marker.

Discussion

S. oleraceae is the most important nutrition rich vegetable eaten as salad and cooked worldwide (Sheetal *et al*., 2006). The major threat to spinach is the fungal diseases that cause a significant loss in yield (Larsson & Gerhardson, 1992; Correll *et al*., 1994). Among these diseases fungal leaf spot is the most active in causing severe infection. For the control of leaf spot fungi of spinach it is necessary to accurately identify the fungi associated with leaf spot disease. This study accordingly emphasized the precise identification of the agent causing leaf spots. Presently, during the field survey of different areas of Punjab the disease prevalence, incidence and disease severity of leaf spot disease was determined. The similar work was performed by Shazia *et al*., (2003) in which during the survey disease ridden plants were observed in rice and wheat fields of four different regions.

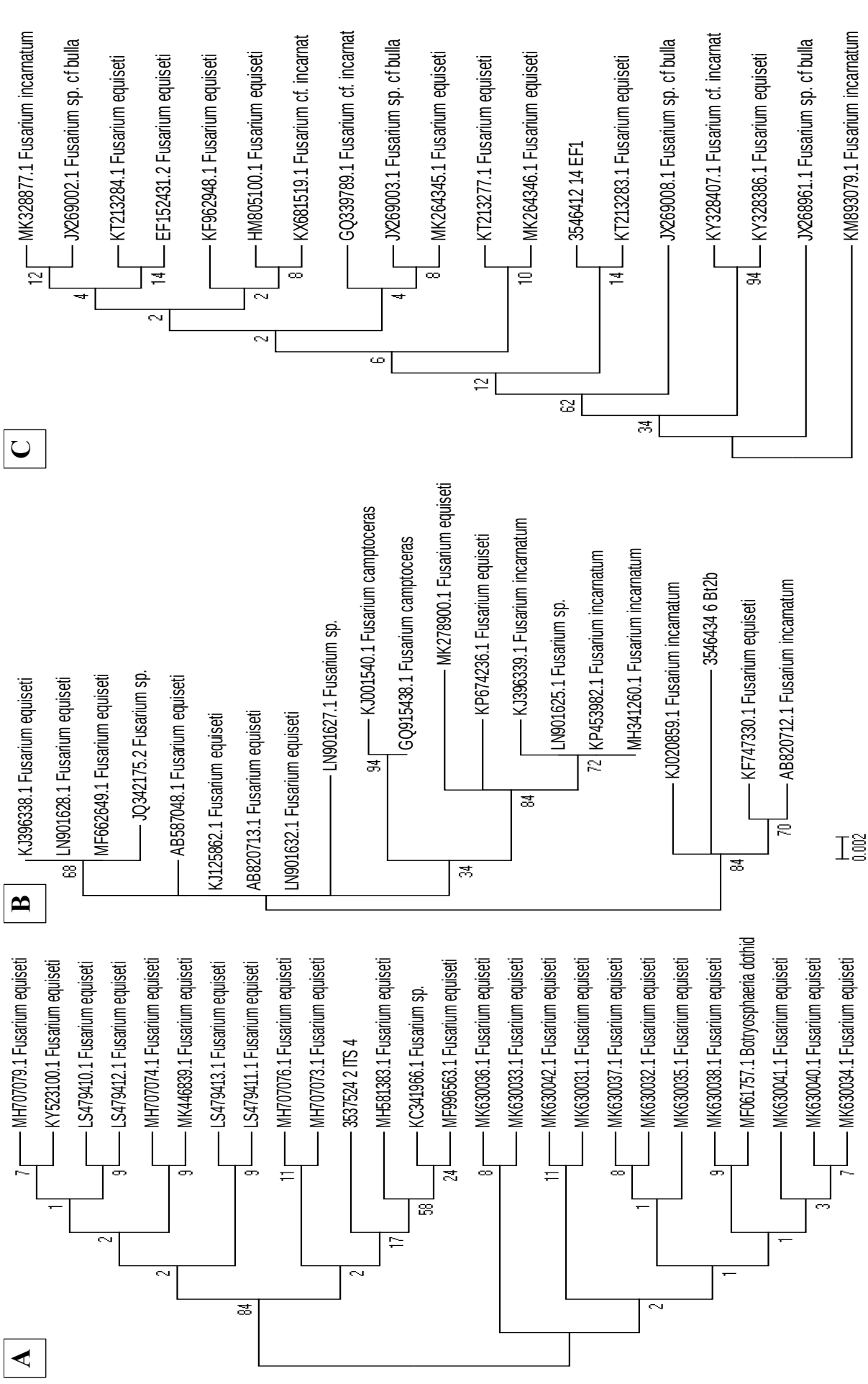


Fig. 7. Phylogenetic Tree of *Fusarium equiseti* for evolutionary analysis with maximum likelihood method. (A): Internal Transcribed Spacer region of rDNA (ITS), (B): Beta tubulin region of rDNA and (C) EF1/EF2 gene sequences region of rDNA.

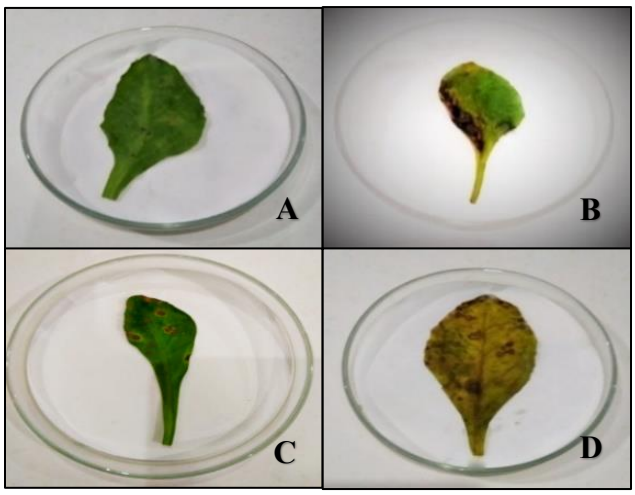


Fig. 8. Different stages of development of disease symptoms by *Fusarium equiseti* on spinach plant.

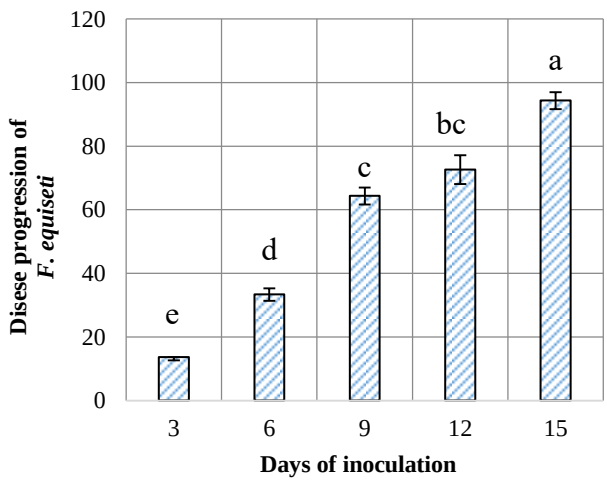


Fig. 9. Analysis of disease progression of *Fusarium equiseti* on spinach plants based on detached leaf assay.
Vertical bars indicate standard errors of means of three replicates. Values with different letters show significant difference as determined by statistix 8.1 software, at $p \leq 0.05$.

The accurate identification of causal agent is prerequisite in order to control fungal diseases. The morphological identification of pathogens at species level is the most reliable conventional method but sometime misidentifications may occur (Anderson *et al*., 2006). Various molecular nucleotide studies like the investigation of ribosomal DNA (rDNA) sequences contribute to find out the molecular phylogenetic relationship between the groups of fungi (White *et al*., 1990; Mirhendi *et al*., 2007). To determine the presence of pathogen, researchers need authentic tools which can cater to the increasing need of finding faster, accurate analytical techniques for discovering agents. Therefore, molecular data i.e., the sequencing of genomic DNA and confirmation by phylogenetic tree or by using mitochondrial small subunit rDNA sequence method in combination with morphology are used for identification of fungi (Kretzer *et al*., 1996; Mirhendi *et al*., 2007; Porras-Alfaro *et al*., 2014; Javaid *et al*., 2018). In present study the isolated pathogen was identified while studying its morphology on the basis of growth pattern, conidiophore and conidial size, shape and colour etc. To confirm the identification at molecular level; the total fungal genomic DNA was used as template with universal primers i.e., ITS1/ITS4, Bt_2a/Bt_2b, and EF1/EF2. The *F. equiseti* was identified and confirmed as the novel leaf spot pathogen of spinach. The phylogeny and evolutionary history of the identified pathogen was confirmed by constructed phylogenetic trees. Using nucleotide sequences of ITS in combination with any gene coding primers such as Bt_2a/Bt_2b, and EF1/EF2 has been considered an authentic way for the proof of identity of fungal species (Schoch *et al*., 2012; Akhtar *et al*., 2016). In the contemporary lines Bashir *et al*., (2014) isolated and identified *Alternaria metachromatica* on the basis of ample description of macro and microscopic characters followed by identification using rDNA spacer sequence and revealed that *Alternaria metachromatica* was the causal agent of leaf spot of tomato. Recently in another study, Asghar (2017) evaluated the pathogenic potential of *Alternaia alternata* and *Cladosporium oxysporum* by applying Koch's postulates using leaf detached method and pot trials and found a sharp progressive disease curve with 99% and 97% of infected area, respectively.

Conclusions

The present study concludes the novel illustration of *F. equiseti* as a leaf spot pathogen of *S. oleraceae*. The study will contribute to distinguish the actual biological agent causing damage to crop and will assist to suggest an authentic control measure for the pathogen in broad spectrum rather than use of fungicides and chemicals.

Acknowledgements

This work was supported by the facilities available in fungal biotechnology research laboratory in Institute of Agricultural Sciences, University of the Punjab, Lahore.

References

Agoreyo, B.A., O. Akpirorah, O.A. Orukpe and O.R. Osaweren. 2011. The effect of various drying methods on the nutritional composition of *Musa Paradisaca*, *Dioscorea rotundata* & *Colocasia esculenta*. *Asian J. Biol. Chem*., 6(6): 548-464.

Akhtar, N., A. Naseer, S. Shafique, S. Shafique, K. Hanif and R. Hafeez. 2016. First report of *Phyllosticta aristolochiicola* causing leaf spot of *Sonchus oleraceus* in Pakistan. *Plant Dis*., 100: 1503.

Anderson, B., G.J. Soneds, I. Jorring, P. Skoubou and L.H. Pedersen. 2006. Real time PCR quantification of the AM-toxin gene & HPLC quantification of toxigenic metabolites form Alternaria species from apples. *Inc. Agri. Bio*., 4: 638-642.

Asghar, Q.A. 2017. Characterization and *In vitro* Control of leaf spot causing pathogens of cabbage. In thesis: Institute of Agricultural Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan, pp. 1-2.

Bashir, U., S. Mushtaq and N. Akhtar. 2014. First report of *Alternaria metachromatica* from Pakistan causing leaf spot of tomato. *Pak. J. Agri. Sci*., 51(2): 305-308.

Bassi, A.J. and M.J. Goode. 1978. *Fusarium oxysporum* f. sp. spinaciae seedborne in spinach. *Plant Dis. Rep*., 62: 203-205.

Beckman, C.H. 1987. The nature of wilt diseases of plants. American Phytopathological Society Press, St. Paul, 21(3): 175-183.

Correll, J.C., T.E. Morelock, M.C. Black, S.T. Koike, L.P. Brandenberger and F.J. Dainello. 1994. Economically important diseases of spinach. *Plant Dis*., 78: 653-660.

Dhar, D., M. Ghosal and A.D.K. Mitra. 2015. Effect of *Fusarium* spp. and *Cladosporium* spp. infection on *Basella alba* leaves. *J.C.B.P.S., Section B5.* (2): 1740-1748.

FAOSTA., 2015. FAOSATAT online database.

Javaid, A., G. Shahzad, N. Akhtar and D. Ahmed. 2018. First report of Alternaria leaf spots of broccoli in Pakistan and management of pathogen by leaf extract *Syzigium cumini*. *Pak. J. Bot*., 5(4): 1607-1614.

Kretzer, A., Y. Li, T. Szaro and T.D. Bruns. 1996. Internal transcribed spacer sequences from 38 recognized species of Suillus sensu lato: Phylogenetic and taxonomic implications. *Mycol*., 88: 776-785.

Larsson, M. and B. Gerhardson. 1992. Disease progression & yield losses from root diseases caused by soil borne pathogens of spinach. *Phytopathol*., 82: 403-406.

Liu, B.L., K. Stein, K. Cochran, L.J.D. Toit, C. Feng, and J.C. Correll. 2021. Three new fungal leaf spot diseases of spinach in the united states and the evaluation of fungicide efficacy for disease management. *Plant Dis*., 105 (2): doi.org/10.1094/PDIS-04-20-0918-RE.

Miano, T.F. 2016. Nutritional Value of *Spinacia oleraecea* Spinach-An Overview. *Int. J. Life Sci. Rev.,* 2(12): 172-174.

Mirhendi, H., K. Diba, A. Rezaei, N. jalalizand, L. Hosseinpur and H. Khodadadi. 2007. Colony PCR is a rapid and sensitive method for DNA amplification in yeasts. *Iran. J. Pub. Heal*., 36: 40-44.

Naiki, T. and M. Kanoh. 1977. On Fusarium wilt of spinach & its causal fungus. *Ann. Phytopathol. Soc. Japan*, 43: 297-300.

Patricia, O. 2014. Proximate composition & nutritive value of leafy vegetables consumed in northern côte d'ivoire. Europ. *Sci. J*., 10(6): 1857.

Porras-Alfaro, A., K.L. Liu, C.R. Kuske and G. Xiec. 2014. From genus to phylum: large subunit and internal transcribed spacer rRNA operon regions show similar classification accuracies influenced by database composition. *App. Env. Microbio*., 80: 829-840.

Robinson, S.P., W.J.S. Downton and J.A. Millhouse. 1983. Photosynthesis & Ion Content of Leaves & Isolated Chloroplasts of Salt-Stressed Spinach. *Plant Physiol*., 73: 238-242.

Sarker, S., A.H.M.M. Haque, M. Islam, S. Dey and A. Biswas. 2017. Evaluation of different botanicals and biological agents to control Cercospora leaf spot of Indian Spinach in field condition. *J. App. Life Sci. Intl*., 13(3): 1-8.

Schoch, L.C., A.K. Seifert, H. Sabine, R. Vincent, L.S. John, C.L. ré and W. Chen. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi & Fungal Barcoding Consortium. 109(16): 6241-6246.

Shafique, S., S. Shafique, M. Zameer and M. Asif. 2019. Plant defense system activated in chili plants by using extracts from *Eucalyptus citriodora. Biocont. Sci*., 24: 137-144.

Shazia, I., M. Ashraf and A. Iftikhar. 2003. Prevalence, incidence & severity of soil-borne diseases & fungi of wheat in rice-wheat cropping system of Punjab Province of Pakistan during the Cropping Season 1999. *Pak. J. Biol. Sci*., 6: 36-40.

Sheetal, G., L. Jyothi and P. Jamuna. 2006. *In vitro* bioavailability of calcium & iron from selected green leafy vegetables. *J. Sci. Food Agri*., 86(13): 2147-2152.

Shova, N.J., S. Shamsi and M.A. Bashar. 2020. Prevalence and pathogenic potentiality of fungi associated with leaf spot of *Basella alba* and *B. rubra. Dhaka Univ. J. Biol. Sci*., 29(2): 183-189.

Sunil, P. and L.P. Yadav. 2020. Isolation of fungal pathogens from seeds of selected vegetables and study of their impact on germination and seedling growth. *Ind. J. Sci. Res.*, 11(1): 139.

White, T.J., T. Burns, S. Lee and J. Taylor. 1990. Amplification & direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocol guide to methods & applications, Innis, M.A., Gelf &, D.H., Sninsky, J.J., White, T. J (ed). Academic Press, San Diego, California. 315-322.

(Received for publication 23 September 2020)