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INCIDENCE, CHARACTERIZATION, AND PATHOGENIC VARIABILITY OF FUSARIUM OXYSPORUM CAUSING CHILI WILT IN PUNJAB, PAKISTAN

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ABSTRACT

The present studies were conducted to determine the incidence of wilt caused by *Fusarium oxysporum* in the major chili-growing districts of Punjab, characterization of *Fusarium* isolates, and assessment of their pathogenic variability. The overall incidence of *Fusarium* wilt in the five major surveyed districts of the Punjab province was 9.2% in the year 2015-16 and 9.3% in the year 2016-17. The maximum disease incidence of 10.04% was recorded in district Multan in the year 2015-16 while it was the minimum (7.06%) in district Sahiwal. A similar trend was observed in the disease incidences during the year 2016-17. The disease incidences varied among different localities of the five districts. In total, 92 isolates were recovered from the chili crop in Punjab. All the recovered purified isolates were morphologically characterized using different parameters like colony color, growth habit, pigmentation, days to fill 9-cm-dish, concentric rings, size and shape of macroconidia and microconidia, phialide, septation in macroconidia, diameter, and formation of chlamydospores and interseptal distance. All the isolates were confirmed as *Fusarium oxysporum*. Based on the pathogenicity test, plants showing more than 75% wilting were rated as highly pathogenic, plants showing 50.1-75% wilting were pathogenic, plants showing 25.1-50% wilting were categorized as moderately pathogenic, and plants showing 0.1-25% wilting were graded as weakly pathogenic. The highly pathogenic and pathogenic isolates were subjected to molecular studies and all the isolates made a band of 550-600 on the gel. All the isolates showed 99-100% similarity with already reported *Fusarium* species. The phylogenetic tree showed a close relationship between all the isolates.

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INTRODUCTION

Chili (*Capsicum annuum* L.) as a spice and condiment is cultivated around the globe and has a significant part in human nutrition. All through the world, chili is used as fresh as well as dried i.e. in powder form. Chili fruit is rich in carbohydrates, lipids, proteins, mineral salts (Fe, Ca, P), fibers, and vitamins such as vitamin A, vitamin B2, vitamin B12, vitamin C, vitamin D3, vitamin E, and vitamin K. Chili has cancer prevention agent against mutagenesis,

hypcholesterolemic and immunosuppressive effects (El-Ghoraba *et al.*, 2013) and also represses platelet agglomeration and bacterial growth (Wahyuni *et al.*, 2013). Worldwide, chili is one of the condiments that earns massive incomes for growers and along these lines contributes to poverty mitigation and enhancement of women's social status (Karungi *et al.*, 2013).

Around the globe, chili is grown on an area of 1.8 million hectares with a total production of 31 million tons

(FAOSTAT, 2015). India is the leading country in chili production contributing one-fourth of the total world's production but export is limited to just 4% owing to its high domestic need. Besides India, China, Peru, Bangladesh, and Pakistan are also major chili producing countries. The chili crop in Pakistan is cultivated on an area of 63 thousand hectares with a total production of 140 thousand tons. The low Average yield (2.2 tons/ha) of chili crop in Pakistan is merely contributing 1.7 percent share of the overall GDP (FAOSTAT, 2017).

Many reasons could contribute to the overall low production of chili crop in Pakistan when compared with countries like China, Jamaica, Morocco, Cape Verde, and other countries with an average yield of 10-20 tons per hectare. Chili crop is susceptible to several fungi, bacteria, nematodes, and viruses (Ashfaq *et al.*, 2014; Riaz *et al.*, 2015; Aslam *et al.*, 2017; Tariq-Khan *et al.*, 2017; Asghar *et al.*, 2020). Fungi and bacteria cause foliar (leaf), fruit, stem, or root diseases. For growers, these ailments cause substantial yield losses which result in huge economic losses. Among fungal diseases, *Fusarium* wilt, damping off caused by *Pythium* spp., and late blight caused by *Phytophthora* spp. are alarming emerging and re-emerging diseases as they are not only limiting overall chili production but also threatening our exports (Saba *et al.*, 2022).

Fusarium oxysporum Schlecht is associated with wilt disease in chili crop (Mushtaq and Hashmi, 1997). *Fusarium* wilt is one of the destructive diseases of chili that can lead to successful disease development soon after its interaction with the hosts. The pathogen upon colonization solely depends on its host for nutrition and development of its mycelium thus severely disrupting and disturbing the physiological functions. The fungus can directly penetrate the host via hyphae and may also be assisted by the production of special enzymes (Mendgen *et al.*, 1996). *Fusarium* wilt is a warm climate soil-borne parasitic infection that does ideally at temperatures of around 28°C. Temperatures over 34°C or underneath 17°C hinder the disease proliferation. *Fusarium* wilt hinders yields of various crops including several vegetables, fruits, cereals, and fiber crops of agricultural and economic importance (Zitter, 1998). The fungus influences its hosts at any stage and a portion of the manifestations that are displayed are root rots and damping off in young seedlings, marginal yellowing, and sometimes chlorosis on leaves of adult plants (Zitter, 1998). On various occasions, the *Fusarium* wilt pathogen

influences the plants, prompting progressive hindered development of the host plant (Flood, 2006). Inside browning of the vascular tissues is the primary thing that is utilized as a symptomatic measure for *Fusarium* wilt. Moreover, the exudation of sticky red material from the lesion is another normal side effect of *Fusarium* wilt yet as indicated by different audits on the side effects; this might not be mixed up with the oozing due to stem blight (Zitter, 1998). In some uncommon events, *Fusarium* wilt has been related to the unexpected death of host plants.

Despite its nutritional, medicinal, and economic importance, chili remains a neglected crop in developing countries and has rarely been the national priority for sustainable agricultural development. The cultivation of this important crop is still conventional; moreover, the crop production is being limited owing to biotic or biogenic and abiotic stresses (Shahbaz *et al.*, 2015; Ahmad *et al.*, 2017; Tariq-Khan *et al.*, 2020; Aslam and Mukhtar, 2023). Keeping in view the above-stated facts, the objectives of the present study were to determine the incidence of *Fusarium* wilt in the major chili-growing districts of Punjab, characterization of *Fusarium* isolates, and assessment of the pathogenic variability among these isolates originating from different areas of the Punjab province of Pakistan.

MATERIALS AND METHODS

Survey for Incidence

For the assessment of *Fusarium* wilt on chili, major chili growing districts of Punjab viz. Multan, Bahawalpur, Sahiwal, Attock, and Rawalpindi were extensively surveyed. Surveys were conducted at times when the crop was not fully mature. From each district, five localities and from each locality three fields were randomly surveyed. From each field, 50 plants from five different spots were examined randomly for the assessment of disease, and the incidence was recorded as described by Fateh *et al.* (2022).

Collection of Samples

From each field, 10 samples were taken from five random places across a diagonal following the hierarchical sampling strategy (McDonald and Martinez, 1990) from symptomatic plants. The characteristic symptoms included yellowing of leaves, browning of stem and root system, and wilting of the entire chili plant. Samples were also collected from the rhizosphere of infected chili plants at depths of up to 15 cm. All the soil samples from a site were pooled to get the

composite sample. The samples were labeled properly and brought to the laboratory for isolation and further studies on the wilt-causing pathogens involved.

Isolation of *Fusarium* spp. from Plant Material

Infected plant material was cut into small pieces of 1.5 cm lengths. These pieces were then surface sterilized with 10% chlorox for 1-2 minutes. After sterilization, the pieces were rinsed thrice with sterilized distilled water, dried in sterilized blotter paper, and carefully placed on PDA supplemented with Streptomycin (Burgess et al. 1994) under aseptic conditions of laminar airflow. The inoculated media plates were then carefully sealed with parafilm, labeled properly, and incubated at 25°C for 1 week.

Isolation from Soil

The serial dilution method was used to isolate fungi from the soil (Wakesman and Fred, 1922). Soil samples (1g) were taken and added to 9 ml of autoclaved water to obtain 1/10 dilution. A series of 1/100, 1/1000, 1/10,000, and 1/100,000 dilutions were prepared by adding 1 ml of a dilution to 9 ml of sterile distilled water using a fresh sterile pipette. An aliquot of 1 ml from each dilution was plated and spread with a sterile glass rod on PDA and MEA media supplemented with antibiotics to suppress bacterial contamination. The plates were then incubated at room temperature (25°C) for 5 days and growing mycelial colonies were transferred to new petri plates containing PDA.

Purification of Cultures

Pure cultures of *Fusarium* species were prepared by single spore and hyphal tip method by picking a single conidia/tip of the hyphae with the help of a sterilized needle under a stereo-microscope, placed in laminar airflow hood, and was transferred to Petri plates and slants containing PDA. The plates and slants were again incubated at 25°C.

Morphological Identification of *Fusarium* Species

Fresh slides from the purified cultures were prepared and subjected to microscopy for identification. *Fusarium* species were identified based on morphological characteristics like colony color, shape, and pigmentation according to keys (Nelson et al., 1983; Leslie and Summeral, 2006).

Pathogenicity and Pathogenic Variability

The pathogenicity of the fungal isolates was performed on the highly susceptible variety (California wonder) of chili. The identified and morphologically characterized 92 isolates of *Fusarium* spp. were taken from 10-day-old

cultures in 100 ml Potato Dextrose Broth (PDB). Each fungal suspension was blended with 500 g sterilized soil mixed with sterilized compost (3:1) in a 30×22×10 cm tray. Chili seeds were surface sterilized with 0.5% aqueous sodium hypochlorite for 5 minutes and rinsed thoroughly with sterile distilled water. The seeds were planted in trays under control conditions. In uninoculated control, seeds were treated similarly but sterilized soil and compost were used. The plants grown were observed for visual symptoms after 50 days of sowing. The data were recorded and analyzed. The fungus was re-isolated from the severely affected plants to ensure Koch's postulates. The purified re-isolated fungus was again confirmed morphologically and microscopically.

Molecular Confirmation

Extraction of DNA

All the purified and morphologically identified isolates were cultured in 250 ml flasks containing 50 ml of PDB. The flasks were placed on a shaker at 120 rpm at 25 °C for 10 days to allow the production of the sufficient mycelial mat. The mycelial mats were removed from the tubes using sterilized forceps and scrapers and were blotted on autoclaved filter paper. The mycelium was then freeze-dried in liquid nitrogen and grounded using a sterile pestle and mortar. DNA was extracted immediately using a standard Proteinase-K method. The final DNA pellets were re-suspended in 10 mM Tris-HCl buffer (pH 8) and were stored at -80°C (Anwar et al., 2022; Ashraf et al., 2022).

Polymerase chain reaction

The internal transcribed spacers region of rDNA was amplified by PCR. The DNA extracted from the mycelium was used as a template for the PCR amplification of the ITS region. For PCR reaction, template DNA, dNTPs, MgCl₂, Taq DNA polymerase, and each of the forward and reverse primers were used to amplify the ITS region of *Fusarium* species.

Gel electrophoresis

Gel electrophoresis was performed to evaluate the quality of the amplified ITS fragment. The PCR products were electrophoresed, using 1.5% agarose gel, and compared with a low mass DNA ladder of 100 bp. The gel was stained with ethidium bromide. The PCR product was mixed with 6× loading dye and was loaded on the agarose gel. The resulting gel image was captured by using the gel documentation unit (Anwar et al., 2022; Ashraf et al., 2022).

RESULTS**Incidences of *Fusarium* wilt in Punjab**

The overall incidence of *Fusarium* wilt in the five major surveyed districts of the Punjab province was 9.2% in the year 2015-16 and 9.3% in the year 2016-17. The maximum disease incidence of 10.04% was recorded in district Multan in the year 2015-16 while

it was the minimum (7.06%) in district Sahiwal. A similar trend was observed in the disease incidences during the year 2016-17. The individual disease incidences in all five districts during both years have been given in Figure 1. The disease incidences varied among different localities of the five districts and are shown in Table 1.

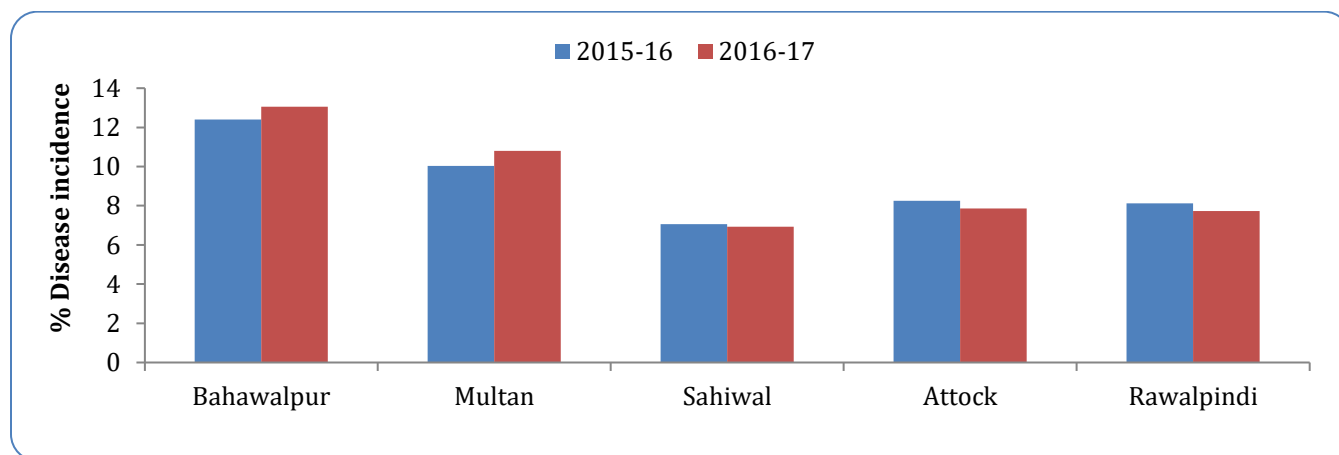


Figure 1. District wise incidence of *Fusarium* wilt in the Punjab province of Pakistan.

Table 1. Incidence of *Fusarium* wilt in individual localities of major chili growing districts of Punjab.

District	Localities	Disease incidence of <i>Fusarium</i> wilt	
		2015-16	2016-17
Bahawalpur	Basti Allah Rakha Saho	12.67 ± 1.3	11.33 ± 2.61
	Chak No 12 BC	12.00 ± 2.26	14.00 ± 2.00
	Hottwali	12.67 ± 1.3	14.67 ± 1.30
	Khanqa Sharif	10.67 ± 1.3	13.33 ± 3.05
	Mubarak Pur	14.00 ± 2.26	12.00 ± 2.26
Multan	Lutaf Abad	10.00 ± 2.26	9.33 ± 2.30
	Qadir Pur Raan	11.33 ± 1.30	11.33 ± 1.30
	Chah Tali Wala	10.00 ± 2.26	10.67 ± 1.30
	8 Kassi	11.33 ± 1.30	13.33 ± 1.30
	5 Kassi	9.33 ± 2.61	9.33 ± 2.30
Sahiwal	Chak Rati Tibi	6.67 ± 1.30	6.67 ± 1.30
	Chak Rang shah	6.67 ± 2.61	7.33 ± 3.45
	Chak 26 EB	7.33 ± 1.30	7.33 ± 1.30
	Chak 19 Sp	6.67 ± 2.61	6.67 ± 2.61
	118-9-L	8.00 ± 2.26	6.67 ± 1.30
Attock	Bahadur Khan	8.00 ± 2.26	6.67 ± 1.30
	Musa	9.33 ± 1.30	8.00 ± 2.26
	Haji Shah	8.00 ± 2.26	9.33 ± 1.30
	Bafahid	7.33 ± 1.30	8.00 ± 2.26
	Hatsal	8.67 ± 1.30	7.33 ± 1.30
Rawalpindi	Rakh ghakhran	8.67 ± 1.15	8.00 ± 2.26
	Bhata	8.00 ± 2.26	9.67 ± 1.72
	Gorakhpur	8.00 ± 2.26	7.33 ± 1.30
	Sood	6.67 ± 1.30	6.67 ± 1.30
	Usman Khatar	9.33 ± 1.30	8.67 ± 1.30

Morphological Characterization of *Fusarium* spp.

In total, 92 isolates were recovered from the chili crop in Punjab. All the recovered purified isolates (Figure 2 a-c) were morphologically characterized using different parameters like colony color, growth habit (Figure 3), pigmentation (Figure 4), days to fill 9-cm-dish, concentric rings, size and shape of macroconidia and microconidia (Figure 5 a-c), phialide (Figure 5 d-e), septation in

macroconidia, diameter, and formation of chlamydoconidia and interseptal distance. For microscopic identification, fresh slides were prepared from pure cultures of each isolate and were visualized under a high-power microscope. Thirty isolates were recovered from Bahawalpur, 13 isolates from Multan, 21 isolates from Rawalpindi, 18 isolates from Sahiwal, and 10 isolates from Attock. All the isolates were confirmed as *F. oxysporum*.

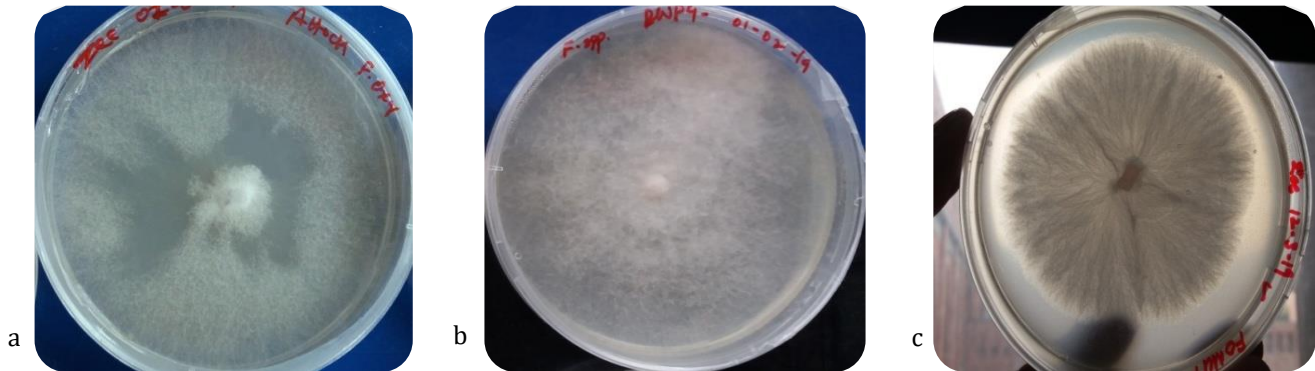


Figure 2. (a-c) Purified cultures of *Fusarium* species on PDA media.

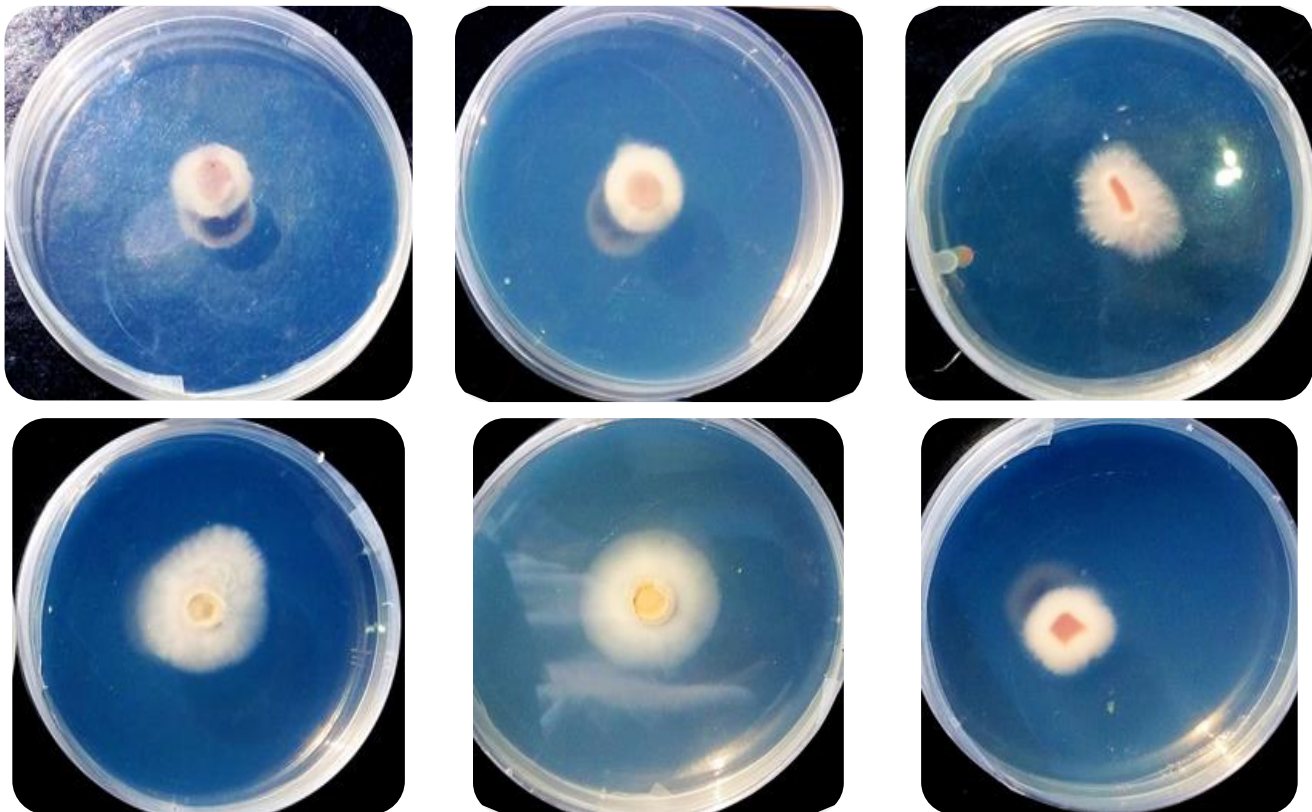


Figure 3. Isolates of *Fusarium* species showing distinct Patterns and color; white to creamy white, brown and pinkish white growth on PDA media

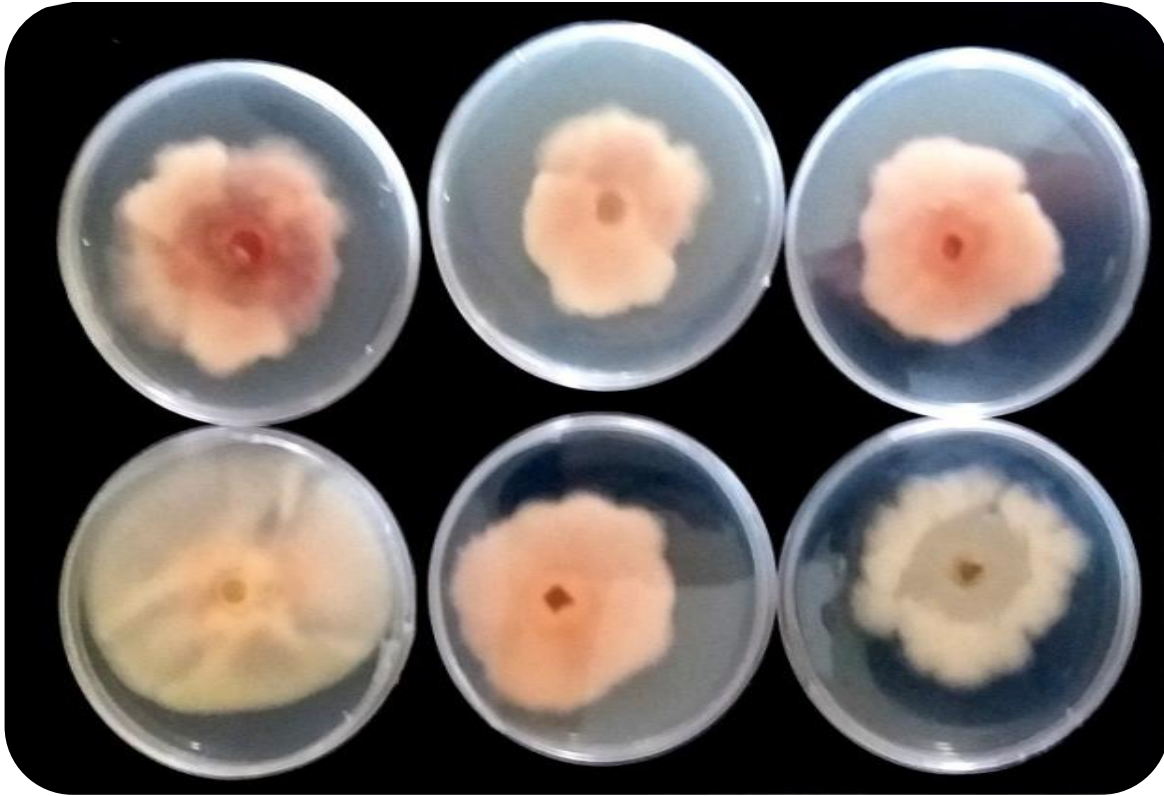


Figure 4. *Fusarium* isolates showing pigmentation.

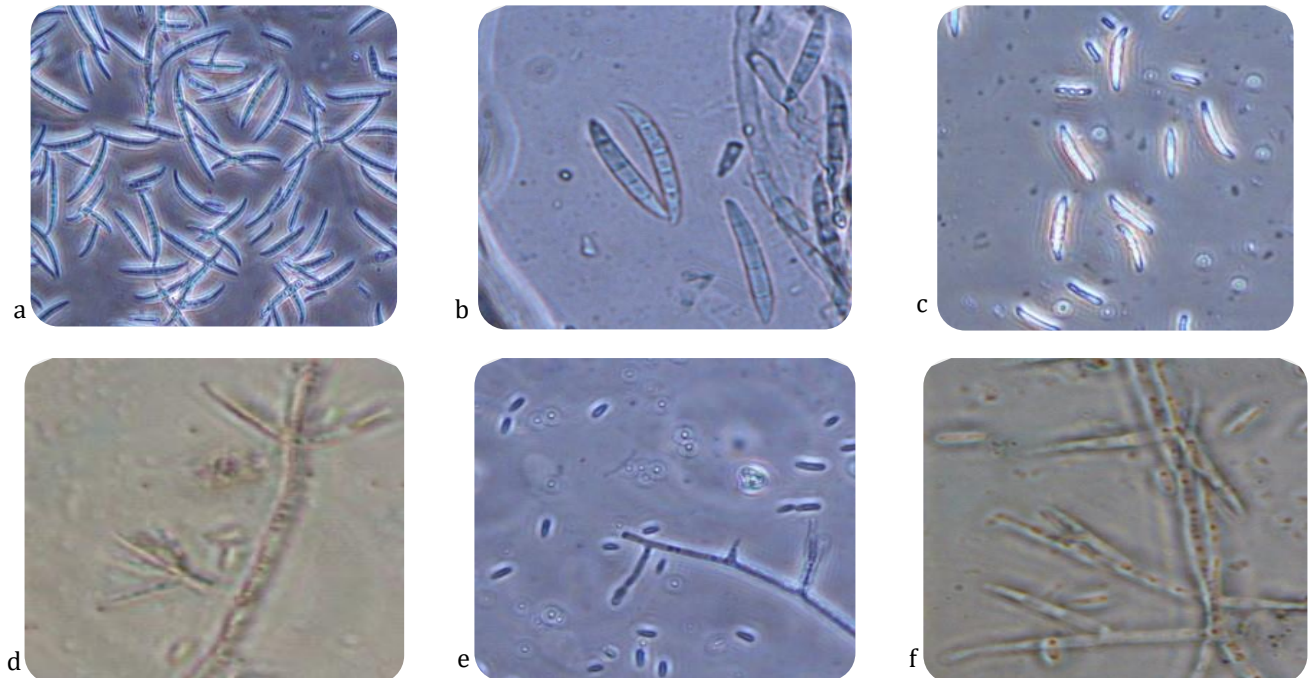


Figure 5. a-c, macrocodia, microconidia and abundance of macroconidia ; d-e, types of phialides

Pathogenicity and Pathogenic Variability

The pathogenicity measuring scale devised by Patra and Biswas (2016) was used to assess the pathogenic

variability. The results were obtained after 50 days of sowing under controlled conditions. Disease grading was done following the scale of Patra and Biswas (2016) i.e.

plants showing more than 75% wilting were rated as highly pathogenic, plants showing 50.1-75% wilting were pathogenic, plants showing 25.1-50% wilting were categorized as moderately pathogenic and the plants showing 0.1-25% wilting were graded as weakly pathogenic (Table 2).

Molecular Confirmation

Only highly pathogenic and pathogenic isolates were further subjected to molecular studies i.e. isolation of their DNA through the Proteinase-K method, amplifying their Internal Transcribed Spacer (ITS) region. After amplification, they were subjected to gel electrophoresis

to confirm the success of PCR. All the isolates made a band of 550-600 on the gel. The obtained sequences were BLAST on NCBI. All the isolates showed 99-100% similarity with already reported *Fusarium* species. ITS1 sequences were 99% similar to previously reported sequences. The accession numbers for the isolates FOB-12, FOB-29, FOM-12, FOR-21, and FOS-09 were obtained from the gene bank which are given in Table 3. Phylogenetic analysis of *F. oxysporum* isolates was done by using MEGA-X Software. The phylogenetic tree showed a close relationship between all the isolates as shown in Figure 5.

Table 2. Pathogenicity and pathogenic variability among *Fusarium* isolates.

Sr. No.	Category	No. of isolates	Isolates
1	Highly pathogenic	2	FOS09, FOB12
2	Pathogenic	3	FOB29, FOM12, FOR21,
3	Moderately pathogenic	36	FOB2, FOB3, FOB10, FOB14, FOB15, FOB16, FOB18, FOB19, FOB20, FOB26, FOB27, FOM3, FOM4, FOM5, FOM10, FOM13, FOR2, FOR3, FOR4, FOR5, FOR8, FOR9, FOR10, FOR11, FOR12, FOS5, FOS6, FOS7, FOS10, FOS11, FOS12, FOS13, FOS14, FOS15, FOA5, FOA6
4	Weakly pathogenic	25	FOB4, FOB5, FOB6, FOB8, FOB9, FOM6, FOM7, FOM8, FOM9, FOR14, FOR15, FOR16, FOR17, FOR18, FOR19, FOR20, FOS1, FOS2, FOS3, FOS4, FOA7, FOA8, FOA9, FOA10, FOS16,

Table 3. Isolates along with their accession numbers.

S. No.	Isolate ID	Accession Number	Species
1	FOB-12	MN262091	<i>Fusarium oxysporum</i>
2	FOB-29	MN272278	<i>Fusarium proliferatum</i>
3	FOM-12	MN272279	<i>Fusarium proliferatum</i>
4	FOR-21	MN272281	<i>Fusarium proliferatum</i>
5	FOS-09	MN272280	<i>Fusarium oxysporum</i>

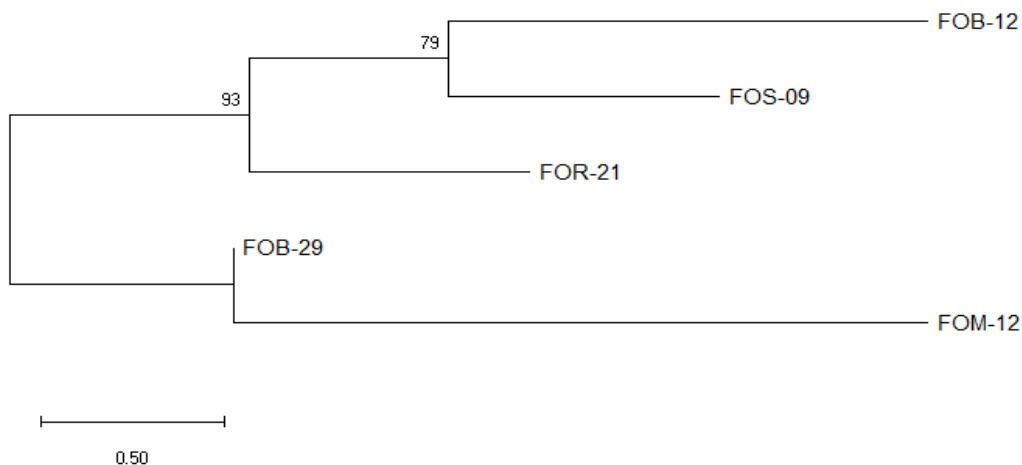


Figure 5. Phylogenetic analysis among recovered pathogenic isolates.

DISCUSSION

In the present study, 100% disease prevalence was found in all the visited districts during 2015-16 and 2016-17. Chili plants with wilt symptoms were found in all the visited fields but the incidence varied from field to field and from district to district during both the years. The overall incidence of disease in the Punjab province was found to be 9.5% and the prevalence was 100%. Little variations in disease incidence were recorded during both years. During both the years, the maximum disease incidence was recorded in district Bahawalpur (Sandy Deserts) followed by Multan (Northern Irrigated Plain), Attock (Barani Areas), Rawalpindi (Barani Areas) and Sahiwal (Northern Irrigated Plain). Soil plays a crucial role for growth, survival, multiplication and dissemination of soil borne microorganisms because it provides a habitat for their growth and development (Chuankun *et al.*, 2004). The ability of pathogen to cause disease is abruptly affected by different types of soils (Ozer *et al.* 2009) like sandy, clay and sandy loam and loam soils. These soils possess different electric conductivity, pH (alkaline and acid character), organic manure, soil texture (ratio of sand, silt and clay particles) and inhibitory volatile fungistatic compounds which consequently alters the activity of soil borne plant pathogens (Ma *et al.*, 2001). Conduciveness or suppressiveness is the ability of a land or soil to enhance or inhibit soil-borne disease. The soils in which disease impact is low despite the presence of susceptible host, pathogen and conducive environment are suppressive soils (Baker and Cook, 1974). This is either due to triggering plant's defense mechanism, inhibition of the pathogen's growth or virulence, increasing the population and activity of antagonistic microorganism, or the collective effect of all these mechanisms.

Environmental conditions like temperature, spore density and water potential also influence the germination of *Fusarium* conidia (Stakheev *et al.* 2011). *F. oxysporum* is a typical soil borne fungus and survives for long period of time in the soil in the form of chlamydospores (Garret, 1960). *Fusarium* spp. produces different types of spores i.e. macroconidia, microconidia and chlamydospores (Nelson *et al.*, 1981) which act as asexual spores and help in the survival of the fungus. Spore production is triggered by the factor like nutrient sources, lights, metals, lipid signals and the chemistry of the plant host (Brodhagen and Keller, 2006). The optimal growth of the genus *Fusarium* occurs between

25-28°C while the maximum growth is generally obtained at a temperature 28°C. The growth is inhibited above 33°C and is not favored below 17°C (Cook and Baker, 1983). High temperature and high moisture has an important role in disease development (Sanogo, 2003). The significance of *Fusarium* wilt also varies with host susceptibility, pathogen virulence, soil type, and environmental conditions (Goldberg, 2010). The fungus penetrates the plant through root tips and can remain viable in the soil for up to 30 years (Thangavelu *et al.*, 2003; Sally *et al.*, 2006). The mycelium grows in the xylem vessels and hinders the supply of water causing the plants to wilt (Stephen *et al.*, 2003). There is often an association of *Fusarium* wilt and nematode colonization where the nematodes provide entry route for the fungus (Mukhtar and Kayani, 2019, 2020; Haq *et al.*, 2022; Shahid *et al.*, 2022). Enzymes may also facilitate *Fusarium* penetration into the plant host (Babalola, 2010).

Depending on the species, *Fusarium* can be dispersed by one of several means including the movement of contaminated seed, corms and bulbs; water-borne and wind-borne soil; and in infected cuttings and transplants. This pathogen spreads in two basic ways: it spreads short distances by water splash, and by planting equipment, and long distances by infected transplants and seeds. Spores are disseminated by the wind, in ground water, or by movement of the contaminated soil, stake, or equipment (Jaywant, 2016).

CONCLUSION

It is concluded that *Fusarium* species are actively involved in the development of wilt disease and widely prevalent in the major chili growing districts of the Punjab province of Pakistan demanding strict management strategies (Mateen *et al.*, 2022; Mukhtar *et al.*, 2023). Biocontrol is by far the best and effective control strategy when dealing with the soil borne pathogens such as *Fusarium* species. This method has many advantages such as being ecofriendly, cost effectiveness and extended plant protection.

AUTHORS' CONTRIBUTION

MZ and TM designed the study, conducted surveys and performed the experiments, MZ collected the data, MIH and MJA provided technical assistance, TM supervised the work, and MZ and TM wrote and proofread the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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