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DNA sequence analysis, morphology and pathogenicity of *Fusarium oxysporum* f. sp. *lentis* isolates inciting lentil wilt in Pakistan

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Abstract

Fusarium wilt of lentil (*Lens culinaris* Medikus) incited by *F. oxysporum* f. sp. *lentis* causes huge lentil yield losses worldwide. Understanding morpho-molecular and pathogenic variation of *F. oxysporum* isolates is of utmost importance, which has not been previously studied in Pakistan. In this study, extensive two year (2011-12 and 2012-13) wilt surveys of seven districts viz. Chakwal, Jhelum, Gujrat, Sialkot, Layyah, Bhakkar and Khushab of Punjab, Pakistan were conducted, which revealed 100% mean disease prevalence and 25.7% mean incidence. Morphology assessment of recovered 105 isolates showed unique identification characters and variability in morphological measurements that differentiates *F. oxysporum* from other fusaria. Furthermore, sequencing of TEF-1α gene supported the morphological study and confirmed the associated fungi at species level. Phylogenetic analysis grouped all the selected type 31 isolates, obtained from different districts, under a single lineage within *F. oxysporum* species complex. Characterized isolates screened for pathogenicity revealed considerable pathogenic variability on two lentil genotypes (NARC-08-1 and Masoor-93). The inoculation of lentils confirmed the pathogenicity of 30 (96.77%) isolates on NARC-08-1 with 0 to 100% disease severity index and incidence with 19.33 to 100% yield reduction. On Masoor-93, 22 (70.97%) isolates were pathogenic with 0 to 66.66% severity index, 0 to 100% incidence and 6.47 to 53.68% yield reduction. Eight (25.81%) isolates viz. FWC15, FWJ35, FWG1, FWL6, FWL9, FWL12, FWB10 and FWK2 proved the most pathogenic resulting in highly virulent disease reaction (94.07% mean severity index and 100% mean incidence and yield reduction) and complete death of the NARC-08-1 seedlings.

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Introduction

Lentil (*Lens culinaris* Medikus) or masoor is a high value cool season pulse crop and serves as a second major source of dietary proteins (25%) after soybeans in human and animal diet (Rahman *et al.*, 2010). In Pakistan, lentil is the second highly grown winter season legume crop next to chickpea in terms of quality and quantity (Ayub *et al.*, 2001). It is grown on an area of 30.8 thousand hectare annually, out of this, 24 thousand hectare (77.41%) is planted in the Punjab province comprising of Sialkot, Narowal, Gujrat, Rawalpindi, Jhelum, Chakwal and Thal districts where two-third of the area is sown under rain-fed conditions. In Pakistan, about 9.7 thousand tonnes production was recorded during 2012-13, which is much lower than main lentil producing countries, such as, Canada (1.5 million metric tonnes) (Saleem, 2013). The reason to lower productivity includes susceptibility of lentil crop to several biotic and abiotic stresses. Biotic stresses include number of soil- or seed-borne and foliar diseases. Among the soil- or seed-borne borne fungal diseases, the most significant and serious soil-borne threat is the occurrence of vascular wilt disease. It is the most significant disease of lentil worldwide and one of the devastating diseases of lentil in Asia (Erskine *et al.*, 2009). The disease can cause 5-10% losses and may result in total crop loss under conducive conditions (Chaudhary and Amarjit, 2002). In Pakistan, susceptible lentil genotypes have shown 100% yield losses under favorable conditions (Chaudhry *et al.*, 2008). The disease is incited by several species of *Fusarium* but the most devastating fungus is *F. oxysporum* Schlecht. emend. Snyder & Hansen f. sp. *lentis* Vasudeva and Srinivasan (Khare, 1981). Though, isolates of this fungus exhibit significant variability in morphology as well as aggressiveness.

Characterization of associated *Fusarium* pathogens and knowledge of existing variability is very much important and prerequisite for effective management of the disease and therefore, needed to be investigated. Identifications and characterization of *Fusarium* species by morphological characters are highly variable. However, at present various DNA-based molecular techniques are used for identification and determination of phylogeny relationships and study of genetic variability in pathogenic populations of *Fusarium* species (Belabid *et al.*, 2004). Sequence analysis of certain informative regions of DNA is now becoming interesting. In *Fusarium* systematics, several molecular methods based on phylogenetic species concept have been introduced and are now being employed for practical molecular taxonomy of this genus (Geiser *et al.*, 2004). The most commonly used sequences based on DNA sequence analysis for distinguishing among the species of *Fusarium* are portions of the genomic sequences encoding the translocation elongation factor 1-α (TEF) (Wulff *et al.*, 2010). The TEF-1α shows high levels of sequence polymorphism and have been used to design species-specific markers as well as probes for the identification, detection and quantification of pathogenic populations of *Fusarium* (Bogale *et al.*, 2007; Nicolaisen *et al.*, 2009; Arif *et al.*, 2012). These tend to evolve at a rate higher as compared to other markers that are used commonly in fungi at the species and population level such as the ribosomal internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene repeat (O'Donnell *et al.*, 2000).

In order to support the lentil breeding program and enhancing the crop productivity through the development of resistant varieties against devastating wilt disease, assessment of morpho-molecular diversity and pathogenic variability present in *F. oxysporum* isolates is very much essential. Therefore, the study was planned to determine the wilt incidence and prevalence in major lentil producing districts of Punjab, Pakistan and assess the morphology, genetic diversity and pathogenic variability among the currently prevalent isolates of *F. oxysporum* causing lentil wilt in Pakistan.

Materials and methods

Disease assessment and collection of wilted lentil plant samples

Wilt disease surveys during 2011-12 and 2012-13 at two plant growth stages (seedling and reproductive or

adult plant stage) were conducted in seven major lentil growing districts of Punjab, Pakistan viz. Chakwal (32°56'N; 72°53'E), Jhelum (31°20'N; 72°10'E), Gujrat (32°40'N; 74°02'E), Sialkot (32°30'N; 74°31'E), Layyah (23°54'S; 21°55'E), Bhakkar (31°40'N; 71°05'E) and Khushab (32°20'N; 72°20'E). For disease assessment and sampling, 10 spots were randomly selected from each field. The number of total plants and wilted or infected plants in 1 m^2 were counted. These observations were used to calculate the average wilt incidence in each field.

Disease prevalence and incidence were used to assess wilt distribution in surveyed areas and calculated by using the following formulas:

$$\text{Disease Prevalence (\%)} = \frac{\text{Number of infected fields}}{\text{Total number of fields}} \times 100$$

$$\text{Disease Incidence (\%)} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

Wilted samples (25-30 per field) were collected by careful observations of typical wilt disease symptoms (Bowers and Locke, 2000) excluding other lentil root-rot diseased plants. Whole uprooted wilted plant samples were collected and stored in a refrigerator at 5-6°C at Fungal Plant Pathology laboratory of the Department of Plant Pathology, PMAS-Arid Agriculture University Rawalpindi for later isolations and confirmation of associated fungus.

Isolation and purification of isolates

The isolation of the associated *Fusarium* pathogens was done using small surface sterilized infected root pieces of the collected wilted lentil plants on potato dextrose agar (PDA) medium (Bayaa *et al.*, 1994). Each PDA plate was plated using five root pieces and incubated at 25±2°C for 3-4 days. The recovered *Fusarium* pathogens were purified on PDA and malt extract agar (MEA) medium using single spore technique (Choi *et al.*, 1999) and later preserved employing silica gel method (Leslie and Summerell, 2006).

Morphological characterization

The recovered 105 isolates (Table 1) of *Fusarium* were studied morphologically using the identification key of Nelson *et al.* (1983) and Leslie and Summerell (2006). Each isolate was characterized based on morphological characters including colony color, growth habit, pigmentation, days to fill 9 cm dish, concentric rings, size of micro-conidia, shape of micro-conidia, size of macro-conidia, shape of macro-conidia, phialide, shape of apical and basal cells of macro-conidia, septation in macro-conidia, diameter and formation of chlamydospores and interseptal distance. Five random readings were taken for each character studied and resulted mean of readings was calculated. Temporary glass slide mounts of each isolate were made in lactophenol solution and assessed under light microscope (Nikon YS100) at 100X magnification for observation of characters. The morphology study helped in grouping of the isolates into 31 type isolates. All these type isolates were selected for further characterization and confirmation at species level through DNA sequencing. Further, these were tested to characterize their virulence using pathogenicity assay.

Molecular characterization

Molecular study of the isolates was undertaken at the Department of Plant Pathology and Environmental Microbiology, Pennsylvania State University, USA,

Genomic DNA isolation

Fungal growth of each respective isolate was achieved on PDA plates after 7 days of incubation (25°C) in dark and scratched using sterilized surgical blade for transfer to 2 ml microcentrifuge tube. Glass beads (0.1 mm) were used for grinding of mycelia using beat beater and further DNA was extraction for amplification according to Cenis (1992) and Abd-Elsalam *et al.* (2003) with minor modifications. Yielded DNA of each isolate was checked for its quality through agarose gel electrophoresis along with concentration and purity through nanodrop spectrophotometer. This was followed by dissolution of each DNA in TE buffer and storage at -20 °C.

Polymerase chain reaction (PCR) and DNA sequencing

TEF-1α region was amplified with the primers viz. ef1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and ef2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') (Geiser *et al.*, 2004). Amplification (Williams *et al.*, 1990) was carried out in 50 µl of PCR reaction mixture containing 10X *Taq* Buffer (5 µl), 0.2 mM of dNTPs mix (100 mM of each dNTPs), 25 ng of both primers (1 µl), 0.6 U (0.3 µl) of *Taq* polymerase (New England Biolabs, Ipswich, MA) and 25 ng of template DNA.

The reaction mixture was subjected to PCR and consisted of an initial denaturation step for 4 min at 95°C, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 2 min at 53°C, extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR products were separated electrophoretically through 1% agarose gels. Gels stained with ethidium bromide were visualized under Transilluminator and photographed. The resulting reaction amplicons were purified through ExoSap-IT (USB, Cleveland, USA) according to the manufacturer's instructions and sequencing of the purified amplicons was done from Genomics Core facility available at Pennsylvania State University, USA.

The sequenced data was edited using the software program Sequencher v.4.1.4 (Gene Codes Corp.). Each DNA sequence was blasted against NCBI (http://blast.ncbi.nlm.nih.gov) (Altschul *et al.*, 1997) and FUSARIUM-ID server (http://fusarium.cbio.psu.edu) (Geiser *et al.*, 2004) for species determination.

Phylogenetic analysis

Sequence data was aligned for each tested isolate using the multiple sequence alignment program at MAFFT website (http://mafft.cbrc.jp/alignment/software/macportable.html) (Katoh and Standley, 2013). Phylogenetic tree was constructed and analysis was executed through maximum likelihood-bootstrap (ML-BS) method with 1000 bootstrap replications using MEGA 6 software (Tamura *et al.*, 2013). The sequence types that had the highest sequence similarity to the isolates of the present study and those described by O'Donnell *et al.* (2013) were retrieved from the Genbank database and included in the phylogenetic analysis. *F. beomiforme* and *F. concolor* sequences (NRRL25174 and NRRL13459, respectively) for TEF-1α gene were used as the outgroup for rooting of the tree.

Pathogenicity test

Inoculum preparation and plant inoculation

For *in vitro* pathogenicity assay (Taheri *et al.*, 2010), each *F. oxysporum* isolate was grown in Erlenmeyer flasks (100 ml) containing potato-dextrose broth (50 ml). Mycelial plug (5 mm dia) taken from periphery of pure culture was inoculated in each flask followed by shaking of flasks in a rotary shaker (120 rpm) for 3 days. For inoculation, spore suspension of each isolate was adjusted to 1 x 10^7conidia/ ml through haemocytometer.

For preparation of sterilized potting mixture, prepared formaldehyde (5%) solution from 37% commercial formulation (Merck, Germany) was mixed thoroughly with potting mixture (100 ml/ kg of soil). The mixture was covered properly with polythene sheets. After 2 days, the mixture was exposed to air, turned over and left uncovered for 5 days with to allow escape of formaldehyde fumes. The treated potting mixture was then used for partially filling each pot.

Lentil germplasm viz. line NARC-08-1 and cultivar Masoor-93 was used for the experiment. Lentil seeds were first surface sterilized using sodium hypochlorite (0.5%) for 2 minutes followed by rinsing in sterilized distilled water. After sterilization, seeds were sown in germinator trays filled with sterilized potting mixture composed of sand and farmyard manure (1:1) and watered. After 2 weeks, each seedling was uprooted carefully and dipped into the inoculum of each respective isolate for 10 minutes. After pathogen inoculation, seedlings were transplanted in plastic pots (5 seedlings per pot) filled with sterilized potting mixture (sand/clay/farmyard manure, 1:1:1), maintained in green house at 25±2°C and watered as required. The pots with plants inoculated with sterilized distilled water served as control. The

experiment formed a completely randomized design (CRD) using 3 replications.

Disease assessment

Disease parameters viz. disease severity index, disease incidence and yield reduction were recorded after inoculation. Disease incidence was calculated using the formula as described above. The data on disease severity index was recorded at 2 to 3 days interval after 5th day of inoculation up till maturity. A 0-9 disease rating scale described by Bayaa *et al.* (1995) was followed with minor modification. According to this scale, 0 = no symptoms or infection, 1-3 = yellowing of the basal leaves only, 4-6 = yellowing of 50 percent of the foliage and 7-9 = complete yellowing of the foliage with whole plant or part of the plant wilted and/ or dried. The formula described by Kranz (1998) was then used to calculate the severity index percentage as,

$$\text{Disease severity index (\%)} = \frac{\Sigma (a \times b)}{N.Z} \times 100$$

Where,

Σ (a x b) = Sum of the symptomatic plant and their corresponding scale value.

N = Total number of plants per pot.

Z = Highest scale value.

Based on disease symptoms and the rating scale, the virulence of isolates was further characterized as avirulent (0 scale value), low virulent (1-3 scale range), moderately virulent (4-6) and highly virulent (7-9). To fulfill Koch's postulates, re-isolations of the inoculated fungi were done at the end for confirmations of the associated wilt pathogens.

Statistical analysis of data

The mean data of disease parameters was calculated and subjected to statistical analysis through SPSS statistical software package. Subsequently, means were separated by the least significant difference (LSD) test (5%).

Results and discussion

Disease assessment and recovery of isolates

Extensive lentil wilt surveys were conducted during the years 2011-12 and 2012-13 in the major lentil growing districts of Punjab, Pakistan. All the surveyed districts showed 100% mean disease prevalence and 25.7% mean incidence. Maximum incidence was observed at district Layyah (75.95%) while minimum incidence was recorded in district Sialkot (6.75%). Rest of the districts showed mild incidence of disease viz. Chakwal (17.5%), Jhelum (21.4%), Gujrat (22.92%), Bhakkar (12.9%) and Khushab (22.5%). In a similar study, Chaudhary *et al.* (2010) recorded 0.7-9.3% mean plant mortality and an overall mean mortality of 6.3% due to lentil wilt-root rot incidence in 116 lentil growing districts of India. Likewise, in Syria Bayaa *et al.* (1986) found 2 to 70% wilted plants with a mean of 12% in 27 fields. Similarly, Hamdi and Hassanein (1996) showed 0.5 to 10% proportion of wilted plants in Egypt. Later, Belabid *et al.* (2000) observed high incidence of wilt in North-Western Algeria and found *F. oxysporum* as the major causal agent along with *F. moniliforme* and *F. equiseti* as the minor pathogens.

Varied number of lentil fields were identified in each district at different locations, such as, Chakwal (Bangali Gujar and Piplee), Jhelum (Pindi Gujran, Dhapai, Khaiwal, Morha Skeiha and Chanaal), Gujrat (Jalalpur jatan, Shergarh, Sombre, Naseera, Bhaddar and Lambray), Sialkot (Pasrur road and Chowinda), Layyah (Chowk Azam and Karoor), Bhakkar (Arid Zone Research Institute) and Khushab (Nurpur, Adhikot and Hassan Pur Tiwana). In fields, the disease was observed in patches during both crop stages with maximum recovery of isolates attained at adult plant stage, which might be attributed to the presence of favorable temperature (24-27°C) at reproductive stage than at seedling stage (5-20°C) (Haqqani *et al.*, 2000). The study recovered 105 isolates of *F. oxysporum* from visited locations of seven districts of Punjab, Pakistan as shown in Table 1.

Morphological characterization

Recovered 105 isolates of *Fusarium* were studied morphologically using various characters (Fig. 1 and

Table 2). Colony characters of the isolates showed fluffy growth pattern with white (91 isolates; 86.67%) to pinkish white (14; 13.33%) mycelial color. Violet (22; 20.95%) to dark violet (25; 23.81%) pigmentation was noted in some isolates as illustrated by Leslie and Summerell (2006). Absence of concentric rings was observed after 12 hours light/ dark cycles. Growth rate was observed for distinguishing slow and fast growing isolates (Summerell *et al.*, 2003) and it varied from 7 to 11 days at 25 ºC. Isolate FWG22 took 7 days to fill 9 cm plate and proved to be fastest growing compared to the rest while isolates viz. FWL5, FWL8, FWL9 and FWL10 found slow growing and completed 9 cm growth in 11 days.

Table 1. The details of *Fusarium oxysporum* f. sp. *lentis* isolates used in the present study.

No.	Isolate ID No.	District of Origin	Place of Collection	No.	Isolate ID No.	District of Origin	Place of Collection
1	FWC5	Chakwal	Bangali Gujar	54	FWJ70	Jhelum	Chanaal
2	FWC6	Chakwal	Bangali Gujar	55	FWG1	Gujrat	Jalalpur jatan
3	FWC8	Chakwal	Piplee	56	FWG2	Gujrat	Jalalpur jatan
4	FWC10	Chakwal	Piplee	57	FWG3	Gujrat	Jalalpur jatan
5	FWC11	Chakwal	Piplee	58	FWG4	Gujrat	Shergarh
6	FWC15	Chakwal	Piplee	59	FWG5	Gujrat	Shergarh
7	FWC16	Chakwal	Piplee	60	FWG6	Gujrat	Shergarh
8	FWC21	Chakwal	Piplee	61	FWG7	Gujrat	Shergarh
9	FWC22	Chakwal	Piplee	62	FWG8	Gujrat	Shergarh
10	FWJ1	Jhelum	Pindi Gujran	63	FWG9	Gujrat	Sombre
11	FWJ2	Jhelum	Pindi Gujran	64	FWG10	Gujrat	Naseera
12	FWJ3	Jhelum	Pindi Gujran	65	FWG11	Gujrat	Naseera
13	FWJ4	Jhelum	Pindi Gujran	66	FWG12	Gujrat	Bhaddar
14	FWJ5	Jhelum	Pindi Gujran	67	FWG13	Gujrat	Lambray
15	FWJ6	Jhelum	Pindi Gujran	68	FWG14	Gujrat	Lambray
16	FWJ7	Jhelum	Pindi Gujran	69	FWG15	Gujrat	Lambray
17	FWJ8	Jhelum	Dhapai	70	FWG16	Gujrat	Lambray
18	FWJ9	Jhelum	Dhapai	71	FWG17	Gujrat	Lambray
19	FWJ10	Jhelum	Dhapai	72	FWG18	Gujrat	Lambray
20	FWJ11	Jhelum	Dhapai	73	FWG19	Gujrat	Lambray
21	FWJ12	Jhelum	Dhapai	74	FWG20	Gujrat	Lambray
22	FWJ13	Jhelum	Dhapai	75	FWG21	Gujrat	Lambray
23	FWJ14	Jhelum	Dhapai	76	FWG22	Gujrat	Lambray
24	FWJ15	Jhelum	Dhapai	77	FWG23	Gujrat	Lambray
25	FWJ16	Jhelum	Dhapai	78	FWG24	Gujrat	Lambray
26	FWJ35	Jhelum	Khaiwal	79	FWG25	Gujrat	Lambray
27	FWJ36	Jhelum	Khaiwal	80	FWG26	Gujrat	Lambray
28	FWJ37	Jhelum	Khaiwal	81	FWS1	Sialkot	Pasrur, Field 1
29	FWJ38	Jhelum	Khaiwal	82	FWS2	Sialkot	Pasrur, Field 1
30	FWJ39	Jhelum	Khaiwal	83	FWS3	Sialkot	Pasrur, Field 1
31	FWJ40	Jhelum	Khaiwal	84	FWS4	Sialkot	Pasrur, Field 2
32	FWJ41	Jhelum	Khaiwal	85	FWS5	Sialkot	Pasrur, Field 2
33	FWJ42	Jhelum	Khaiwal	86	FWS6	Sialkot	Pasrur, Field 2
34	FWJ43	Jhelum	Khaiwal	87	FWS7	Sialkot	Pasrur, Field 2
35	FWJ44	Jhelum	Khaiwal	88	FWS8	Sialkot	Chowinda
36	FWJ45	Jhelum	Khaiwal	89	FWS9	Sialkot	Chowinda
37	FWJ46	Jhelum	Khaiwal	90	FWS10	Sialkot	Chowinda
38	FWJ54	Jhelum	Morha Skeiha	91	FWL5	Layyah	Chowk Azam
39	FWJ55	Jhelum	Morha Skeiha	92	FWL6	Layyah	Chowk Azam
40	FWJ56	Jhelum	Morha Skeiha	93	FWL7	Layyah	Chowk Azam
41	FWJ57	Jhelum	Morha Skeiha	94	FWL8	Layyah	Chowk Azam
42	FWJ58	Jhelum	Morha Skeiha	95	FWL9	Layyah	Karoor
43	FWJ59	Jhelum	Morha Skeiha	96	FWL10	Layyah	Karoor
44	FWJ60	Jhelum	Morha Skeiha	97	FWL12	Layyah	Karoor
45	FWJ61	Jhelum	Morha Skeiha	98	FWL13	Layyah	Karoor
46	FWJ62	Jhelum	Chanaal	99	FWL14	Layyah	Karoor
47	FWJ63	Jhelum	Chanaal	100	FWL15	Layyah	Karoor
48	FWJ64	Jhelum	Chanaal	101	FWL16	Layyah	Karoor
49	FWJ65	Jhelum	Chanaal	102	FWB10	Bhakkar	Arid Zone Research Institute
50	FWJ66	Jhelum	Chanaal	103	FWK1	Khushab	Nurpur
51	FWJ67	Jhelum	Chanaal	104	FWK2	Khushab	Adhikot
52	FWJ68	Jhelum	Chanaal	105	FWK3	Khushab	Hassan Pur Tiwana
53	FWJ69	Jhelum	Chanaal				

Microscopic examination of the isolates showed hyaline, branched and septate conidiophores (Nelson *et al.*, 1983; Gupta *et al.*, 1986; Leslie and Summerell, 2006) with interseptal distance measured ranged from 7.8±3.11 (FWC11) to 31.0±10.29 µm (FWG1). Straight, three to four-septate and thin-walled macro-conidia, a character of *F. oxysporum* was noted in the study as proposed by Nelson *et al.* (1983) and Leslie and Summerell (2006). The conidial size varied and ranged from 8.0±1.17 (FWL6) to 29.6±5.18 µm (FWC21) in length and 2.0±0 (FWJ15 and FWL8) to 4.0±0.35 µm (FWC11) in width. Similar variability in size of conidia has been indicated in a study by Booth (1977) and Mandhare *et al.* (2011).

Table 2. Morphological characterization of *Fusarium oxysporum* isolates.

No.	Isolate ID No.	Colony color	Pigmentation	D	Macro-conidia			Micro-conidia			Chlamydospores		Interseptal distance (µm)
					Length (µm)	Width (µm)	S	Length (µm)	Width (µm)	Shape	Diameter (µm)	Formation	
1	FWC5	White	Dark Violet	9	13.8±2.68	2.4±0.22	3-4	4.5±0.71	2±0	2-celled Oval	8.6±0.89	Singly, Pairs	11±4.47
2	FWC6	White	Dark Violet	9	17.8±2.28	3±0	3	5.8±0.84	2.2±0.45	Oval	8.6±0.89	Singly, Pairs	23.6±5.59
3	FWC8	White	-	9	19.8±2.68	3.8±1.30	3	6.9±2.07	2.8±0.27	Oval	7.4±1.67	Singly, Pairs	16.6±6.88
4	FWC10	White	-	8	22.4±5.17	3.3±0.45	3	8.8±1.30	2.9±0.22	Oval	7.8±1.09	Singly, Pairs	10.2±3.49
5	FWC11	White	Violet	9	24.6±3.57	4±0.35	3	5.4±0.89	2.2±0.45	2-celled Oval	11.4±2.79	Singly, Pairs	7.8±3.11
6	FWC15	White	Dark Violet	8	26.2±4.02	3.2±0.45	3	6.8±0.84	2.8±0.27	Oval	14±4	Singly, Pairs	19.4±4.09
7	FWC16	White	Dark Violet	8	25±8.77	3.7±0.83	3	7.8±1.48	2.3±0.27	Oval	7±1.41	Singly, Pairs	18.4±7.30
8	FWC21	White	-	9	29.6±5.18	3.6±0.55	3	8.2±1.48	2.9±0.22	Oval	12±4.74	Singly, Pairs	21.1±2.75
9	FWC22	White	-	9	25±7.681	3.2±0.67	3	6.6±0.89	2.5±0	Oval	12.6±3.37	Singly, Pairs	17.2±5.17
10	FWJ1	White	-	10	18.8±2.28	2.8±0.27	3	6.4±1.14	2.2±0.27	2-celled Oval	15±5.09	Singly, Pairs	18.6±6.23
11	FWJ2	Pinkish White	-	9	18±3.16	2.6±0.42	3	7.1±2.07	2±0	2-celled Oval	10.8±3.03	Singly, Pairs	15.6±6.23
12	FWJ3	Pinkish White	Dark Violet	9	13.2±3.56	2.3±0.27	3	6.8±0.84	2.1±0.22	2-celled Oval	7.8±1.48	Singly, Pairs	10.6±3.36
13	FWJ4	White	Dark Violet	9	15.2±2.28	2.8±0.27	3	5.4±0.55	2.1±0.22	Oval	10.4±1.52	Singly, Pairs	16±2.24
14	FWJ5	Pinkish White	-	9	15±4.79	2.5±0.35	3	4.4±0.55	2.5±0	Oval	9±0.71	Singly, Pairs	16.4±5.73
15	FWJ6	White	Violet	9	17.4±2.79	2.9±0.22	3	5±0.71	2.5±0	Oval	10.2±1.30	Singly, Pairs	14.8±5.02
16	FWJ7	White	-	9	20±1.41	3±0	3	4.6±0.55	2.6±0.22	Oval	10.8±1.30	Singly, Pairs	9.4±2.61
17	FWJ8	White	Violet	8	12.2±1.89	2.5±0	3	5.1±0.22	2.5±0.87	Oval	9.6±2.61	Singly	17.6±3.85
18	FWJ9	White	Violet	8	15.6±7.64	2.7±0.45	3	5.9±0.74	2.65±0.22	Oval	11.2±2.59	Singly	18.2±6.49
19	FWJ10	White	Dark Violet	8	18±3.46	2.6±0.41	3	5.8±0.87	2.5±0.35	Oval	11.2±5.06	Singly	13.6±3.51
20	FWJ11	Pinkish White	-	8	11±1.41	2.4±0.41	3-4	5.3±0.45	2.05±0.59	Oval	11±1	Singly	11±6.48
21	FWJ12	Pinkish White	-	9	14.6±4.34	2.2±0.27	3-4	6.6±0.89	2.75±0.25	Oval	14±3.16	Singly	19.6±3.29
22	FWJ13	White	-	8	17.4±7.33	2.5±0.35	3-4	6.6±0.89	2.3±0.27	Oval	10.4±1.67	Singly	17.2±9.65
23	FWJ14	Pinkish White	Dark Violet	8	19.6±5.13	2.3±0.27	3	7.2±0.84	2±0	Oval	11.4±1.67	Singly, Pairs	14.3±4.12
24	FWJ15	Pinkish White	Dark Violet	8	10.8±2.28	2±0	3	6.6±1.34	2.5±0	Oval	12±4.74	Singly, Pairs	17.6±8.08
25	FWJ16	Pinkish White	-	9	19.4±7.13	2.4±0.42	3	7±0.71	2.1±0.22	Oval	9.4±1.34	Singly, Pairs	18±10.07
26	FWJ35	White	Dark Violet	8	26±8.37	3±0.35	3	6.4±1.14	2.5±0.5	Oval	7.2±1.30	Singly, Pairs	10.6±0.89
27	FWJ36	White	Violet	8	12.6±3.29	2.5±0	3	5.4±1.14	2.3±0.45	Oval	10.4±2.61	Singly, Pairs	12.8±4.55
28	FWJ37	White	Violet	8	17.6±3.21	2.6±0.22	3	5.2±0.84	2.2±0.45	Oval	11.2±3.83	Singly, Pairs	12.6±3.78
29	FWJ38	White	Violet	8	18.3±1.79	2.5±0	3	4.6±1.34	2.3±0.45	Oval	12.4±3.85	Singly, Pairs	12.2±4.71
30	FWJ39	White	Violet	8	20.4±4.84	2.5±0	3	5.8±0.84	2.1±0.22	Oval	11±2.65	Singly, Pairs	14.6±4.77
31	FWJ40	White	-	8	21.8±6.09	2.7±0.45	3	5.4±0.55	2.1±0.22	Oval	14.4±4.39	Singly, Pairs	15.4±5.13
32	FWJ41	White	-	8	16.8±5.93	2.5±0.18	3	5.8±0.45	2.4±0.42	Oval	13.8±4.15	Singly, Pairs	19.6±7.92
33	FWJ42	White	-	8	19.1±4.85	2.5±0	3	5.2±0.84	2.6±0.55	Oval	13±3.61	Singly, Pairs	17.2±6.22
34	FWJ43	White	-	8	20.8±3.03	2.55±0.11	3	5±0.70	2±0	Oval	10.4±1.52	Singly, Pairs	21±4.85
35	FWJ44	White	-	8	19.2±3.96	2.5±0	3	6.2±1.09	2.2±0.27	Oval	14.8±5.76	Singly, Pairs	18.6±8.05
36	FWJ45	White	-	8	22.1±6.47	3±0.35	3	5.4±1.52	2.5±0.5	Oval	9.8±2.49	Singly, Pairs	19.2±5.26
37	FWJ46	White	Violet	8	16.4±4.34	2.6±0.42	3	6.4±1.14	2.7±0.45	Oval	10.4±2.70	Singly, Pairs	13.4±3.13
38	FWJ54	White	-	9	16.1±3.05	3.6±0.55	3	7.4±1.67	3.2±0.45	Oval	13±6.32	Singly, Pairs, Short Chains	16.4±4.39
39	FWJ55	White	Violet	9	17.6±2.70	3±0.35	3	6.6±0.89	2.3±0.45	Oval	8.2±2.05	Singly, Pairs, Short Chains	19.2±10.06
40	FWJ56	White	Violet	9	18.9±3.94	2.8±0.22	3	6.2±1.30	2.6±0.42	Oval	12.8±6.72	Singly, Pairs, Short Chains	20.2±9.86

41	FWJ57	White	Violet	9	17.2±4.87	2.9±0.22	3	7.2±1.92	2.7±0.57	Oval	11.2±1.79	Singly, Pairs, Short Chains	14.6±8.05
42	FWJ58	White	Violet	9	18±1.41	2.9±0.22	3	7.8±1.79	2.9±0.65	Oval	7.8±3.70	Singly, Pairs, Short Chains	27.8±8.14
43	FWJ59	White	-	9	17.6±4.93	2.7±0.27	3	7±2.12	2.6±0.55	Oval	14.2±3.77	Singly, Pairs, Short Chains	18±8.37
44	FWJ60	White	-	9	18.9±2.61	2.7±0.27	3	5.6±0.89	2.4±0.42	Oval	10±2.45	Singly, Pairs, Short Chains	19.4±6.99
45	FWJ61	White	-	9	20.8±2.68	2.8±0.27	3	6.6±2.30	2.4±0.65	Oval	9.4±2.61	Singly, Pairs, Short Chains	14.2±6.02
46	FWJ62	White	-	8	15.2±3.42	2.6±0.22	3	6.7±1.48	2.6±0.55	Oval	8.2±3.11	Singly, Pairs, Short Chains	20.2±9.58
47	FWJ63	White	-	8	17.7±5.78	2.7±0.27	3	6.4±1.95	2.4±0.55	Oval	14±4	Singly, Pairs, Short Chains	15±7
48	FWJ64	White	-	8	16.5±1.41	2.5±0	3	5.7±2.22	2.2±0.27	Oval	9.8±2.49	Singly, Pairs, Short Chains	16.2±4.60
49	FWJ65	White	-	8	18.7±4.99	2.8±0.27	3	5.4±1.82	2.3±0.45	Oval	13.8±4.38	Singly, Pairs, Short Chains	15.4±7.60
50	FWJ66	White	-	8	20.4±6.84	2.85±0.42	3	6.8±1.09	2.3±0.45	Oval	10±2.55	Singly, Pairs, Short Chains	18.2±2.86
51	FWJ67	White	-	8	19.4±6.19	2.75±0.43	3	6.8±1.30	2.6±0.42	Oval	13.6±3.78	Singly, Pairs, Short Chains	17±4
52	FWJ68	White	Violet	8	22.4±6.23	2.9±0.41	3	6.4±1.52	2.5±0.35	Oval	11.2±2.39	Singly, Pairs, Short Chains	14.4±6.22
53	FWJ69	White	Violet	8	19±7.48	2.7±0.45	3	6.4±1.52	2.3±0.45	Oval	8.6±2.19	Singly, Pairs, Short Chains	17.6±4.56
54	FWJ70	White	-	8	16.9±3.65	2.5±0	3	6.2±1.30	2.5±0.5	Oval	10.8±2.95	Singly, Pairs, Short Chains	22.6±4.34
55	FWG1	White	Dark Violet	10	16.2±3.03	2.8±0.27	3	7.4±2.30	2.8±0.45	Oval	14.6±5.73	Singly	31±10.29
56	FWG2	White	Dark Violet	9	16.4±2.61	2.7±0.27	3	5.8±0.84	2.4±0.42	Oval	12.6±3.13	Singly	29.8±13.68
57	FWG3	White	Violet	9	20.3±2.77	3.1±0.22	3	6±1.87	2.8±0.45	Oval	13.6±4.34	Singly	15.2±4.09
58	FWG4	Pinkish White	Dark Violet	9	17±2.83	2.7±0.27	3	6±1.22	2.6±0.42	Oval	8.6±0.89	Singly	11.8±2.49
59	FWG5	Pinkish White	Dark Violet	9	20±6.32	2.9±0.42	3	6±1.22	2.9±0.22	Oval	12.2±1.79	Singly	20.8±2.28
60	FWG6	White	Dark Violet	9	16±2.55	2.5±0	3	6.2±1.30	2.5±0.5	Oval	15.6±3.85	Singly	21.2±5.17
61	FWG7	White	Violet	9	17.8±5.59	2.65±0.22	3	5.8±0.84	2.5±0.35	Oval	7.6±1.52	Singly	20.6±7.99
62	FWG8	White	Violet	9	23±5.39	2.75±0.25	3	6±1.22	2.4±0.42	Oval	9.8±2.28	Singly	16.6±4.67
63	FWG9	Pinkish White	Dark Violet	9	14.4±2.61	2.5±0	3	5.8±1.30	3±0	Oval	13.2±6.09	Singly	16.2±6.06
64	FWG10	White	Dark Violet	9	20.4±5.18	3.1±0.55	3	6.2±1.30	2.8±0.27	Oval	10.4±4.56	Singly	24±8.69
65	FWG11	White	Dark Violet	10	18.4±7.27	2.85±0.42	3	6.8±2.17	2.5±0.5	Oval	14±5.48	Singly	21.2±6.46
66	FWG12	White	Dark Violet	10	20.6±5.98	2.9±0.42	3	6.2±1.09	2.7±0.45	Oval	9±1.73	Singly	24±7.38
67	FWG13	White	-	8	19.4±3.13	3.8±0.27	3	5.6±2.07	2.4±0.42	Oval	9.6±3.21	Singly, Pairs, Short Chains	12.2±2.28
68	FWG14	White	-	8	18.6±2.41	3.4±0.42	3	5.8±1.48	2.2±0.27	Oval	11.2±2.78	Singly, Pairs, Short Chains	16±5.05
69	FWG15	White	-	8	19.6±6.02	3.5±0.61	3	6±1.41	2.6±0.22	Oval	7±1.41	Singly, Pairs, Short Chains	14.4±4.34
70	FWG16	White	-	9	20±4.47	3.4±0.42	3	5±1	2.4±0.22	Oval	11.8±6.02	Singly, Pairs, Short Chains	12.2±3.63
71	FWG17	White	-	9	18.8±4.76	3.7±0.67	3	5.4±0.55	2.5±0	Oval	10.2±2.68	Singly, Pairs, Short Chains	16±4.69
72	FWG18	White	-	8	19.2±3.35	3±0.35	3	5.6±1.52	2.3±0.27	Oval	11.6±2.70	Singly, Pairs, Short Chains	15±7
73	FWG19	White	-	8	19±4.36	3.4±0.65	3	6.8±1.64	2.5±0.35	Oval	8.8±1.79	Singly, Pairs, Short Chains	11.6±3.85
74	FWG20	White	-	9	19.08±5.55	3.3±0.57	3	5.6±1.14	2.5±0.36	Oval	15.2±5.02	Singly, Pairs, Short Chains	13.6±4.33
75	FWG21	White	-	8	17.4±6.31	3.1±0.82	3	6.4±1.14	2.8±0.27	Oval	10±1.41	Singly, Pairs, Short Chains	18.6±3.29
76	FWG22	White	-	7	20±6.32	3.2±0.57	3	6.2±1.30	2.55±0.37	Oval	11.2±1.79	Singly, Pairs, Short Chains	13.4±5.64
77	FWG23	White	-	8	17.6±3.91	3±0.61	3	5.4±0.55	2.5±0	Oval	14.2±5.12	Singly, Pairs, Short Chains	19.2±1.30
78	FWG24	White	-	9	19.4±4.88	3.2±0.57	3	5.6±1.14	2.7±0.27	Oval	9.4±0.89	Singly, Pairs, Short Chains	18.8±4.60
79	FWG25	White	-	9	17.6±3.58	2.8±0.45	3	6±1.22	2.6±0.42	Oval	8.8±3.56	Singly, Pairs, Short Chains	14.6±6.07

80	FWG26	White	-	8	19.4±6.69	3.1±0.82	3	5.4±1.52	2.55±0.37	Oval	13±4.12	Singly, Pairs, Short Chains	15±5.43
81	FWS1	White	-	9	20.6±5.08	2.9±0.22	3	8±1.58	2.9±0.22	Oval	9.4±1.14	Singly, Pairs, Short Chains	12.2±4.38
82	FWS2	White	-	9	18.2±4.82	2.5±0	3	4.8±0.91	2.5±0	Oval	12±0.71	Singly, Pairs, Short Chains	15±6.59
83	FWS3	White	-	9	19.2±8.44	2.7±0.27	3	5.2±0.45	2.5±0	Oval	8.4±1.67	Singly, Pairs, Short Chains	12±4.64
84	FWS4	White	-	9	15.2±6.30	2.6±0.22	3	6.2±1.30	2.6±0.42	Oval	8.6±0.89	Singly, Pairs, Short Chains	22±8
85	FWS5	White	-	9	18.7±1.20	2.5±0	3	5.3±0.67	2.5±0	Oval	14±5.70	Singly, Pairs, Short Chains	21.2±5.40
86	FWS6	White	-	9	17.2±3.27	2.5±0	3	4.8±0.91	2.4±0.22	Oval	7.6±1.82	Singly, Pairs, Short Chains	17.4±12.24
87	FWS7	White	-	9	18.8±5.22	2.6±0.22	3	6.4±0.55	2.7±0.27	Oval	9.8±3.19	Singly, Pairs, Short Chains	13.6±2.61
88	FWS8	White	-	9	18.5±3.04	2.55±0.11	3	4.8±0.84	2.45±0.27	Oval	8.6±0.89	Singly, Pairs, Short Chains	10.6±3.29
89	FWS9	White	-	9	21±3.46	2.6±0.22	3	6±1.73	2.6±0.22	Oval	9.2±1.30	Singly, Pairs, Short Chains	17.2±4.87
90	FWS10	White	-	9	16±4.85	2.5±0	3	5.2±0.84	2.5±0	Oval	13±4.47	Singly, Pairs, Short Chains	8.1±1.95
91	FWL5	White	Dark Violet	11	10.2±1.44	2.2±0.27	3	4±0.94	1.75±0.5	Oval	9.2±0.45	Singly, Short Chains	11.9±5.44
92	FWL6	White	Dark Violet	8	8±1.17	2.5±0.35	3	5±0	2.4±0.22	Oval	7±1	Singly, Pairs, Short Chains	10.2±6.87
93	FWL7	Pinkish White	-	8	16.2±3.63	3±0	3	5.8±1.09	2.1±0.22	Oval	10.6±3.13	Singly, Pairs, Short Chains	9.6±3.51
94	FWL8	Pinkish White	-	11	10±1.06	2±0.35	3	4±0	1.75±0	Oval	8.8±0.84	Singly, Pairs, Short Chains	15.4±9.53
95	FWL9	Pinkish White	-	11	10±1.66	2.2±0.31	3	4±0.94	1.75±0.5	Oval	8.8±0.84	Singly	11.6±6.73
96	FWL10	White	-	11	19±5.39	3±0.35	3	6±1.87	2±0	Oval	7.8±1.48	Singly	10.6±6.99
97	FWL12	White	-	8	13±2.98	2.4±0.42	3-4	4.2±0.84	1.95±0.45	Oval	9±0.71	Singly	15.6±8.56
98	FWL13	White	Violet	8	13.4±1.95	3±0	3-4	5.4±0.55	2.8±0.27	Oval	10.2±1.30	Singly	10±3.54
99	FWL14	White	Violet	8	14±1.87	2.9±0.22	3-4	5.6±0.89	2.9±0.22	Oval	10.6±1.67	Singly	15±5.19
100	FWL15	White	Violet	8	16.8±3.63	3±0	3-4	5.4±0.55	2.7±0.27	Oval	11.4±0.89	Singly	16.2±5.67
101	FWL16	White	Violet	8	17±2	3±0.35	3-4	6±1	2.8±0.27	Oval	9.6±0.89	Singly	16.1±6.43
102	FWB10	White	Dark Violet	10	19±2.65	3±0	3	6.6±0.89	2.9±0.42	Oval	8.8±1.09	Singly	14.2±7.53
103	FWK1	White	Dark Violet	8	18.8±4.55	2.6±0.22	3	5.8±1.30	2.5±0	Oval	12±5.48	Singly, Pairs, Short Chains	16.2±6.09
104	FWK2	White	Dark Violet	8	19.7±1.30	2.55±0.11	3	6±1	2.5±0.5	Oval	13.8±3.03	Singly, Pairs, Short Chains	18.6±2.71
105	FWK3	White	Dark Violet	8	15.8±3.63	2.6±0.22	3	6.4±0.89	2.4±0.42	Oval	14±5.15	Singly, Pairs, Short Chains	12.6±3.97

Data based on mean of five readings per morphological character, ± = Standard deviation, - = Absence, D= Days to fill 9 cm plate, S= Septation.

The cells at the end of macro-conidia serves an important identification character of *Fusarium* species and pointed apical and foot-shaped basal cell were observed among the isolates (Toussoun and Nelson, 1976; Nelson *et al.*, 1983; Leslie and Summerell, 2006). Single-celled oval micro-conidia were noted and in some (5 isolates; 4.77%) viz. FWC5, FWC11, FWJ1, FWJ2 and FWJ3, 2-celled oval conidia produced in false heads were also seen (Burgess *et al.*, 1989). Micro-conidia size ranged from 4.0±0.94 (FWL5, FWL8 and FWL9) to 8.8±1.30 µm (FWC10) in length and 1.75±0.5 (FWL5, FWL8 and FWL9) to 3.2±0.45 µm (FWJ54) in width. Conidiogenous cells were found short and plump monophialides. This unique character observed in the study helped differentiating *F. oxysporum* from other species such as, *F. solani* with long and slender monophialides (Seifert, 2001), *F. commune* with long monophialides and polyphialides (Skovgaard *et al.*, 2003).

Smooth and rough-walled chlamydospores formed singly, in pairs and short chains were seen in 3-4 weeks old cultures (Leslie and Summerell, 2006). These were either produced terminally or intercalary. The diameter of chlamydospores ranged from 7.0±1.41 (FWC16, FWG15 and FWL6) to 15.6±3.85 µm (FWG6).

The isolates showed variations in most of the morphological characters studied such as pigmentation, days to fill 9 cm plate, microscopic measurements including micro-conidia, macro-conidia, chlamydospore, interseptal distance and therefore grouped accordingly. Of the 105 isolates characterized morphologically, 31 isolates were selected as type isolates for molecular analysis and pathogenicity testing.

Table 3. Mean wilt severity index, incidence and yield reduction caused by *Fusarium oxysporum* f. sp. *lentis* isolates on lentils.

No.	Isolate ID	GenBank Accession No.	District of Origin	NARC-08-1				Masoor-93			
				Disease Severity* (%)	Disease Incidence (%)	Yield Reduction (%)	DR	Disease Severity* (%)	Disease Incidence (%)	Yield Reduction (%)	DR
1	FWC5	KR139797	Chakwal	53.3 ^{f}	100 ^{a}	51.71 ^{c}	M	3.7 ^{j}	26.67 ^{cd}	26.81 ^{ghij}	L
2	FWC6	KR139798	Chakwal	54.81 ^{f}	100 ^{a}	47.69 ^{cd}	M	11.11 ^{i}	100 ^{a}	26.31 ^{hij}	L
3	FWC8	KR139799	Chakwal	55.55 ^{ef}	100 ^{a}	47.26 ^{cd}	M	0 ^{k}	0 ^{f}	6.47 ^{l}	A
4	FWC10	KR139800	Chakwal	0 ^{h}	0 ^{b}	19.33 ^{f}	A	0 ^{k}	0 ^{f}	21.62 ^{ijk}	A
5	FWC11	KR139801	Chakwal	60 ^{d}	100 ^{a}	42.62 ^{de}	M	1.48 ^{jk}	13.33 ^{e}	34.67 ^{efg}	L
6	FWC15	KR139802	Chakwal	88.14 ^{b}	100 ^{a}	100 ^{a}	H	65.92 ^{ab}	100 ^{a}	47.12 ^{ab}	M
7	FWC21	KR139803	Chakwal	45.18 ^{g}	100 ^{a}	37.66 ^{e}	M	0 ^{k}	0 ^{f}	19.88 ^{jk}	A
8	FWC22	KR139804	Chakwal	54.07 ^{f}	100 ^{a}	49.90 ^{cd}	M	57.03 ^{c}	86.7 ^{b}	39.63 ^{bcde}	M
9	FWJ2	KR139805	Jhelum	60 ^{d}	100 ^{a}	48.63 ^{cd}	M	16.29 ^{h}	100 ^{a}	36.68 ^{def}	L
10	FWJ4	KR139806	Jhelum	63.7 ^{c}	100 ^{a}	52.95 ^{c}	M	65.92 ^{ab}	100 ^{a}	37.97 ^{cdef}	M
11	FWJ8	KR139807	Jhelum	64.44 ^{c}	100 ^{a}	49.12 ^{cd}	M	45.92 ^{fg}	100 ^{a}	40.56 ^{bcde}	M
12	FWJ11	KR139808	Jhelum	66.66 ^{c}	100 ^{a}	45.21 ^{cde}	M	0 ^{k}	0 ^{f}	22.86 ^{hijk}	A
13	FWJ14	KR139809	Jhelum	45.18 ^{g}	100 ^{a}	41.61 ^{de}	M	48.14 ^{ef}	100 ^{a}	47.00 ^{ab}	M
14	FWJ15	KR139810	Jhelum	45.92 ^{g}	100 ^{a}	49.09 ^{cd}	M	0 ^{k}	0 ^{f}	23.64 ^{hijk}	A
15	FWJ16	KR139811	Jhelum	58.51 ^{de}	100 ^{a}	49.67 ^{cd}	M	0 ^{k}	0 ^{f}	27.70 ^{ghij}	A
16	FWJ35	KR139812	Jhelum	100 ^{a}	100 ^{a}	100 ^{a}	H	49.63 ^{e}	100 ^{a}	46.51 ^{ab}	M
17	FWG1	KR139813	Gujrat	89.62 ^{b}	100 ^{a}	100 ^{a}	H	57.77 ^{c}	100 ^{a}	46.87 ^{ab}	M
18	FWG13	KR139814	Gujrat	46.66 ^{g}	100 ^{a}	49.07 ^{cd}	M	12.59 ^{i}	33.33 ^{c}	30.15 ^{fgh}	L
19	FWS1	KR139815	Sialkot	44.44 ^{g}	100 ^{a}	48.23 ^{cd}	M	2.22 ^{jk}	20 ^{de}	28.11 ^{ghi}	L
20	FWS3	KR139816	Sialkot	66.66 ^{c}	100 ^{a}	66.32 ^{b}	M	2.22 ^{jk}	20 ^{de}	25.66 ^{hij}	L
21	FWS5	KR139817	Sialkot	53.33 ^{f}	100 ^{a}	52.93 ^{c}	M	48.89 ^{e}	86.7 ^{b}	45.09 ^{bc}	M
22	FWS7	KR139818	Sialkot	66.66 ^{c}	100 ^{a}	42.65 ^{de}	M	44.44 ^{g}	100 ^{a}	45.29 ^{bc}	M
23	FWS9	KR139819	Sialkot	59.26 ^{d}	100 ^{a}	52.24 ^{c}	M	0 ^{k}	0 ^{f}	17.13 ^{k}	A
24	FWL5	KR139820	Layyah	65.18 ^{c}	100 ^{a}	67.74 ^{b}	M	0 ^{k}	0 ^{f}	16.47 ^{k}	A
25	FWL6	KR139821	Layyah	100 ^{a}	100 ^{a}	100 ^{a}	H	44.44 ^{g}	100 ^{a}	43.12 ^{bcd}	M
26	FWL7	KR139822	Layyah	55.55 ^{ef}	100 ^{a}	47.46 ^{cd}	M	0 ^{k}	0 ^{f}	23.52 ^{hijk}	A
27	FWL9	KR139823	Layyah	100 ^{a}	100 ^{a}	100 ^{a}	H	63.7 ^{b}	100 ^{a}	46.54 ^{ab}	M
28	FWL12	KP297995	Layyah	100 ^{a}	100 ^{a}	100 ^{a}	H	66.66 ^{a}	100	53.68 ^{a}	M
29	FWB10	KR139824	Bhakkar	100 ^{a}	100 ^{a}	100 ^{a}	H	54.07 ^{d}	100 ^{a}	44.71 ^{bcd}	M
30	FWK1	KR139825	Khushab	60 ^{d}	100 ^{a}	53.24 ^{c}	M	1.48 ^{jk}	20 ^{de}	30.69 ^{fgh}	L
31	FWK2	KR139826	Khushab	89.62 ^{b}	100 ^{a}	100 ^{a}	H	44.44 ^{g}	100 ^{a}	46.77 ^{ab}	M
32	Control	-	-	0 ^{h}	0 ^{b}	0 ^{g}	A	0 ^{k}	0 ^{f}	0 ^{l}	A
LSD Value at α = 0.05				3.0	0	8.98	-	2.96	9.42	8.18	-

Data based on mean of three replications, At α=0.05 level of significance means sharing same letters are non-significant, DR = Disease.

Reaction, H= Highly, M= Moderately, L= Low Virulent and A= Avirulent.

Sequencing of TEF-1α and phylogenetic analysis

Use of morphological characters for identification of species under the genus *Fusarium* is often considered time consuming and needs vast experience to differentiate among closely related species (Baayen *et al.*, 2000). Therefore, molecular techniques based on PCR offer a rapid and consistent mean for detection, identification and differentiation of morpho-molecularly close *Fusarium* species. They have been proved to be sensitive and specific for diagnosis of several fungal pathogens (Martin *et al.*, 2000; Boonham *et al.*, 2008). Vascular wilt caused by *F.*

oxysporum f. sp. *lentis* is a devastating pathogen that causes huge yield losses in lentil crop. Early detection of this pathogen is very much essential for effective disease management (Haware and Nene, 1982). In this study, morphological study of the isolates was followed by DNA sequencing and phylogenetics based on the amplification of TEF-1α nuclear gene region for species identity.

Sequencing of TEF-1α nuclear gene region was analyzed for each selected type isolate under study for species identification and phylogenetic analysis.

The sequenced data of each isolate has been deposited in Genbank database under accessions KP297995 and KR139797 to KR139826.

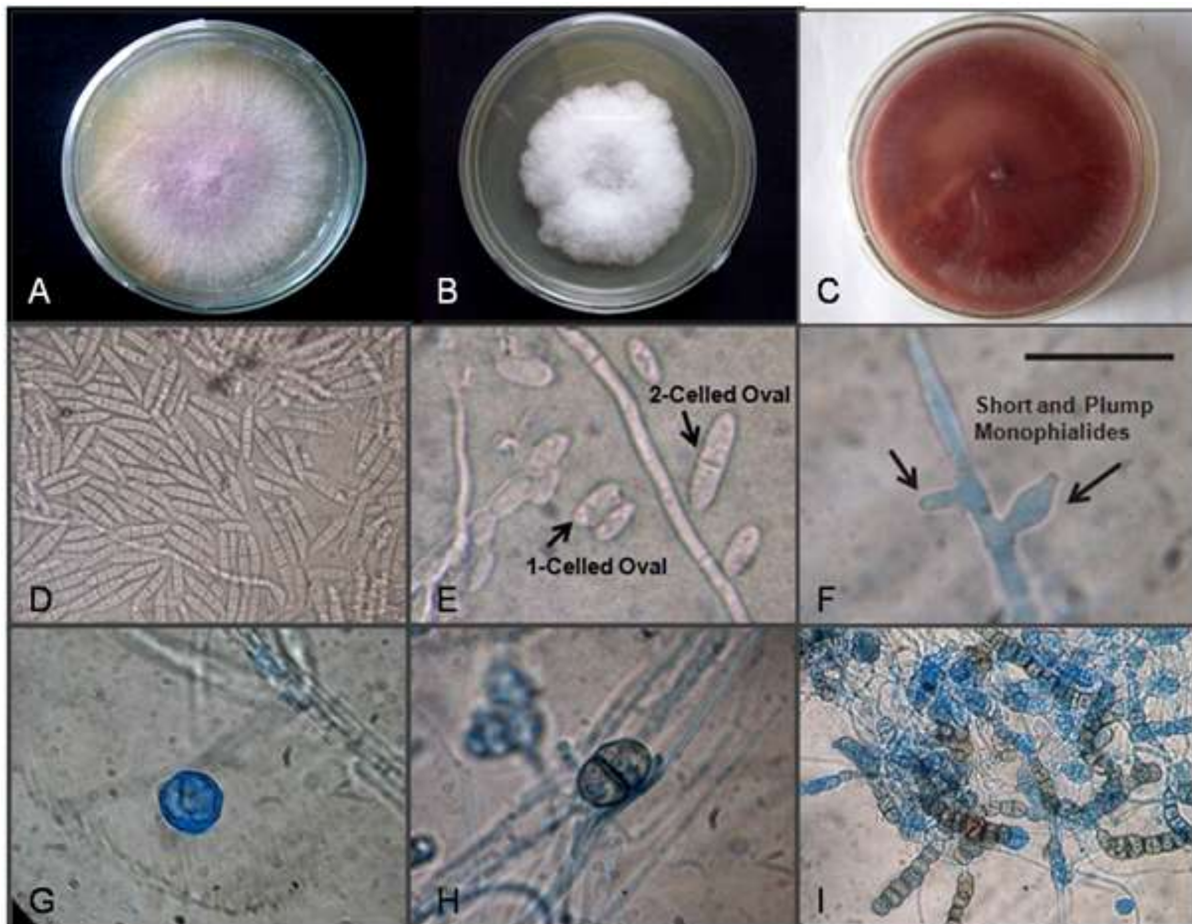

Fig. 1. *Fusarium oxysporum.* A-C: Colony morphology on potato dextrose agar medium, A: Pinkish colony color, B: Fluffy white growth, C: Dark violet pigmentation on the under surface of plate; D: Macro-conidia; E: Micro-conidia of single-celled and 2-celled oval shape; F: Conidiogenous cells; G–I: Formation of Chlamydospores, G: Singly, H: Pairs, I: Short chains; D - I, scale bar = 25 µm.

The primers ef1 and ef2 amplified a single band of size 700bp in all the isolates (Fig. 2) as illustrated by Geiser *et al.* (2004). The database blast results showed 99 to 100% similarity of the sequences with *F. oxysporum*, which was also revealed through phylogenetic analysis. The phylogenetic tree inferred from the partial TEF-1α sequence data of each isolate is presented in fig. 3. ML-BS analysis of the TEF-1α provided support for the recognition of morphologically identified isolates within the *F. oxysporum* species complex clade. All the isolates nested within the clade forming a monophyletic group and the sequence data separated the isolates into a strongly-supported lineage (BS = 100%). The earliest diverging lineage comprising of *Gibberella fujikuroi* species complex was not supported by bootstrapping but showed strong (BS = 100%) support with the *F. oxysporum* species complex lineage.

The 31 Pakistani isolates studied were obtained from different locations of the lentil growing region of the Punjab. The isolates grouped under a single lineage but within the lineage grouped separately with specific type strains. Within the lineage, the isolates showed varied bootstrap support with the type strains, such as, isolates FWJ2 and FWJ4 from district Jhelum resolved with the type strain MUCL14162 with strong support (BS = 86%) and NRRL 25603 (BS = 97%).

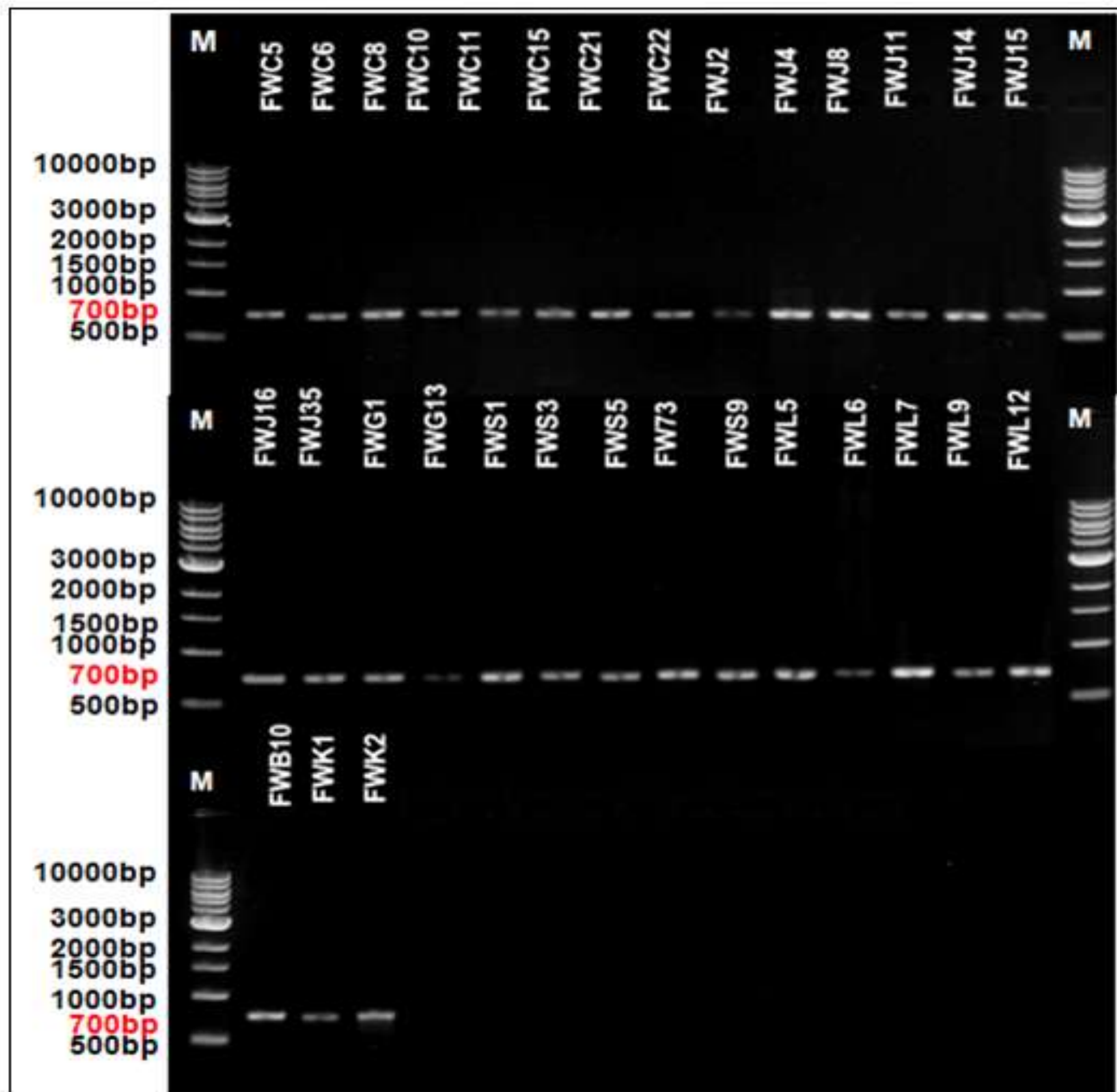

Fig. 2. PCR amplification products (700bp) of genomic DNA of 31 *Fusarium* oxysporum f. sp. *lentis* isolates at 53°C annealing temperature using primers ef1 and ef2. Lane M= 1kb DNA ladder (New England Biolabs, Ipswich, MA).

The rest of the isolates also resided under strongly support bootstrapping (BS = 90%). Isolates FWL6 from district Layyah resided close to the strains NRRL32154 and NRRL32156 with no support value. The next group with low support (BS = 52%) included six isolates obtained from district Chakwal viz. FWC8 and FWC10 and one isolate FWB10 from district Bhakkar. These formed grouping with the strains viz. NRRL43668, NRRL53121, NRRL52787, NRRL34936, NRRL52785 and NRRL32153 with low to strong bootstrapping support (BS = 57 - 77%). The next group within the lineage showed low support (BS = 56%), where two Chakwal isolates viz. FWC15 and FWC21 resided alone but with strong support (BS =

92%) and rest of the isolates grouped with the type strains. This group also resolved with low bootstrapping support (BS = 61%). Isolates from district Khushab (FWK1 and FWK2), Layyah (FWL5, FWL7, FWL12 and FWL9), Sialkot (FWS7, FWS1, FWS3, FWS9 and FWS5), Gujrat (FWG13 and FWG1) and Jhelum (FWJ16, FWJ14, FWJ15, FWJ35, FWJ11 and FWJ8) resided with the strains (10-110, NRRL52736, NRRL25387, JG22-5, NRRL26871, NRRL32158 and FO-02911) with low support (BS = 52 - 66%). The study did not result in the separation of the isolates according to their pathogenicity. This confirmed that pathogenicity of the tested isolates does not necessarily correlate with the phylogenetic grouping. The use of phylogenetic analysis in addition to morphological characterization greatly helped in the confirmation of recovered wilt pathogens at species level. This was supported by the concept given by Aoki *et al.* (2003) who suggested that phylogenetic techniques help identify new species, which is usually difficult and often impossible by using conventional morphological characters.

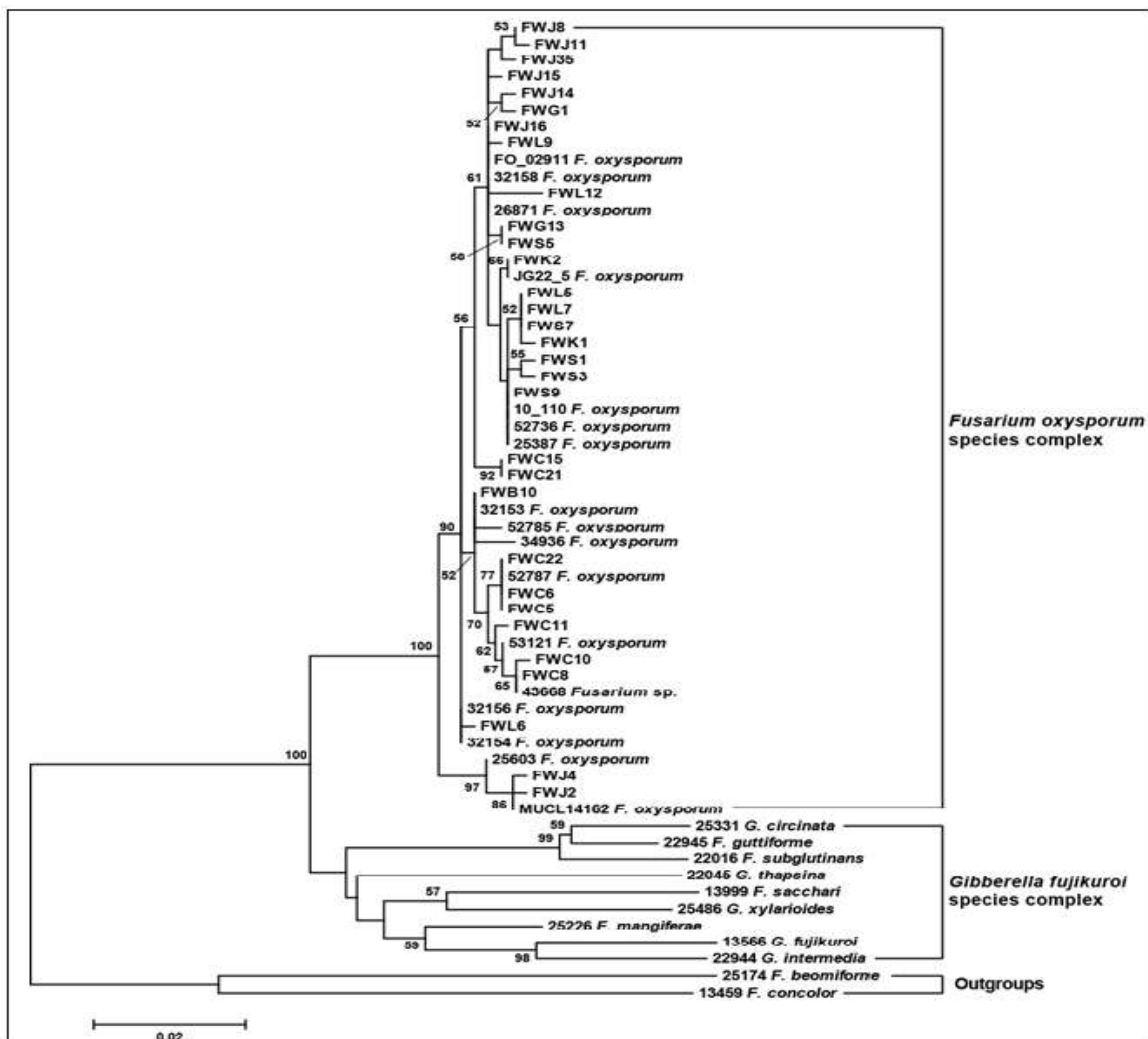

Fig. 3. Maximum likelihood phylogenetic tree constructed with partial TEF-1α gene sequences of *Fusarium oxyporum* isolates from lentil. Sequences of the following strains represent sequence type group in the tree: NRRL22944, NRRL13566, NRRL25226, NRRL25486, NRRL13999, NRRL22045, NRRL22016, NRRL22945, NRRL25331, MUCL14162, NRRL25603, NRRL32154, NRRL32156, NRRL43668, NRRL53121, NRRL52787, NRRL34936, NRRL52785, NRRL32153, NRRL25387, NRRL52736, 10-110, JG22-5, NRRL26871, NRRL32158 and FO-02911. *F. beomiforme* (NRRL25174) and *F. concolor* (NRRL13459) sequences were used as outgroup taxa to root the tree.

The results suggested that TEF-1α gene proved an ideal target for identification of species and thus, can be efficiently used for the detection and identification of *F. oxysporum* isolates. As, numerous studies have indicated that TEF-1α gene is suitable for differentiation of *Fusarium* species (Baayen *et al*., 2000; Jimenez-Gasco *et al*., 2002).

Pathogenicity test

Characterization and identification of pathogenic variability in *F. oxysporum* f. sp. *lentis* isolates is imperative for efficient management of lentil wilt through host plant resistance. In present study, 31 isolates of *F. oxysporum* f. sp. *lentis* were studied through pathogenicity assay to identify pathogenic variation using two lentil germplasm. The inoculation of lentils confirmed the pathogenicity of 30 (96.77%) isolates of *F. oxysporum* on line NARC-08-1 (Table 3). Almost similar results were also achieved by Taheri *et al.* (2010) who found 27 (81.82%) pathogenic isolates out of tested 33 isolates. In case of cv. Masoor-93, 22 (70.97%) isolates confirmed their pathogenicity. Typical wilt disease symptoms illustrated by Bowers and Locke (2000) were used for the pathogenic characterization of the isolates and identification of the highly virulent isolates based on 0-9 disease rating scale (Bayaa *et al*., 1995). Symptoms induced by inoculated isolates of *F. oxysporum* on lentil germplasm included plants drooping, yellowing of lower leaves and ultimately drying and death of the plants in the later stage. Internal discoloration of xylem vessels was also seen. In contrast, no disease symptoms were observed on control plants. The incubation period ranged from 20 to 25 days in case of line NARC-08-1 while 30-40 days in Masoor-93.

Data on disease parameters showed difference in virulence level of tested *F. oxysporum* isolates as observed by Belabid and Fortas (2002) and Belabid *et al.* (2004). Disease incidence ranged from 0 to 100% in both germplasm. Disease severity index varied among the two different lentil germplasm i.e. 0 to 100% in NARC-08-1 and 0 to 66.66% in Masoor-93. Yield reduction ranged from 19.33 to 100% (NARC-08-1) and 6.47 to 53.68% (Masoor-93). The control plants resulted in 0% infection and yield reduction. The data indicated that the wilt disease is greatly involved in the reduction of plant yield and this yield reduction differed in the two tested germplasm. The result was supported by the concept given by Khare *et al.* (1979) who proposed that yield losses depend on the crop variety.

The inoculations not only helped in identifying and confirming the pathogenicity of the tested isolates but also revealed difference in severity of wilt symptoms that helped in the characterization of pathogens virulence using modified 0-9 disease rating scale as described by Baaya *et al.* (1995). Based on disease reaction on lentil line NARC-08-1, isolates were characterized as highly virulent (7-9 scale range), moderately virulent (4-6) and avirulent (0). The eight (25.81%) isolates viz. FWC15, FWJ35, FWG1, FWL6, FWL9, FWL12, FWB10 and FWK2 were found to be the most pathogenic and produced highly virulent disease reaction (88.14 to 100% disease severity index and 100% disease incidence and yield reduction) on line NARC-08-1 that caused the death of the seedlings within 25 days after inoculation. The moderately virulent 22 (70.97%) isolates included FWC5, FWC6, FWC8, FWC11, FWC21, FWC22, FWJ2, FWJ4, FWJ8, FWJ11, FWJ14, FWJ15, FWJ16, FWG13, FWS1, FWS3, FWS5, FWS7, FWS9, FWL5, FWL7 and FWK1, which caused moderate infection (44.44 to 66.66% severity index and 100% incidence) and infected plants produced seeds that were found mostly shriveled (37.66 to 67.74% yield reduction). None of the isolate fell under low virulent category and only one isolate (FWC10) was found avirulent (0% infection and 19.33% yield reduction). In case of cv. Masoor-93, isolates were characterized as moderately virulent to avirulent, where 14 (45.16%) isolates viz. FWC15, FWC22, FWJ4, FWJ8, FWJ14, FWJ35, FWG1, FWS5, FWS7, FWL6, FWL9, FWL12, FWB10 and FWK2 were found moderately virulent with 44.44 to 66.66% severity index, 87.7 to 100% incidence and 37.97 to 53.68% reduction in yield, 8 (25.81%) isolates viz. FWC5, FWC6, FWC11, FWJ2, FWG13, FWS1, FWS3 and FWK1 were low virulent (1.48 to

16.29% severity index, 13.33 to 100% incidence and 25.66 to 36.68% yield reduction) while rest of the 9 (29.03%) isolates were avirulent (0% infection and 6.47 to 27.70% yield reduction). The results showed statistically significant difference ($p = 0.05$) compared to the control plants. The variation recorded towards the disease reaction of the two different germplasm suggested that in the availability of same environmental conditions and amount of pathogen inoculum, the genetic makeup of the plants also plays significant role in resistance reaction of the plants towards the inoculated pathogens (Mohammadi *et al.*, 2012). The wide range of pathogenic variability reported in this study was also reported by Naimuddin and Chaudhary (2009). Re-isolated *F. oxysporum* isolates from wilted plants were identified and confirmed as being the same as that were initially used for inoculations. The study greatly helped in the identification of the pathogenic isolates of *F. oxysporum* f. sp. *lentis* prevalent in the country.

Conclusion

The present study reports wide distribution and incidence of lentil wilt disease in the major lentil producing region of Punjab, Pakistan and reveals prevalence of morphologically and genetically diverse isolates of *Fusarium oxysporum* f. sp. *lentis* possessing great pathogenic variability. The use of TEF-1α based DNA sequencing along with morphological and pathogenic data for characterization of the isolates greatly enhanced the understanding of the variability within this important fungus. This could ultimately benefit for the management of wilt disease through host plant resistance.

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