Pak. J. Agri. Sci., Vol. 60(2), 249-264; 2023
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI:10.21162/PAKJAS/23.20
http://www.pakjas.com.pk

Traditional pathology and phylogenetic networking confirm the pathogen causing date palm wilt

Nabeeha Aslam Khan[1], Abdul Rehman[1], Imran Ul Haq[1*] and Iqrar Ahmad Khan[2]

[1]Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan; [2]Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan
*Corresponding author's email: imran_1614@yahoo.com

Phoenix dactylifera L. (Date palm) is one of the leading perennial fruit crops regarding its nutritional profile, medicinal significance, excellent yields, long life, and high adaptability to diverse climatic conditions of semi-arid and arid regions globally. Eleven major palm-growing districts of Punjab, Sindh, and Khyber Pakhtunkhwa were surveyed for wilt disease assessment on date palms. The date palm plantation was highly affected with Fusarium wilt diseases with 55%, the incidence in Khairpur (Sindh province), followed by Bahawalpur at 47.4% (Punjab Province) and 45% in Dera Ismail Khan (KPK province). Disease severity was recorded as 65% in Khairpur, 55% in Bahawalpur, and 50% in Dera Ismail Khan (Sindh, Punjab, and KPK, respectively). Sixty-three Fusarium isolates were obtained from three hundred and eighty (380) diseased samples, and thirty isolates randomly selected out of 63 were studied for morpho-cultural and microscopic characterization. Morpho-cultural characters, i.e., colony pigmentation, growth pattern, conidia (macro and microconidia), shape, apical cell shape, and phalidies studied, coinciding with typical characteristics of *Fusarium oxysporum*. Twenty isolates of the Fusarium population were subjected to the ITS region sequence data-based phylogenetic network. The Neighbor net algorithm computed an unrooted phylogenetic network displayed as four branched genetic network that depicted the division of twenty fungal isolates into four groups. *F. oxysporum* (FMB-FO-PD-011) inoculated onto healthy symptomless detached date palm leaves, seedlings, and suckers under controlled and field conditions showed typical wilt disease symptoms. Hence this study proved that *F. oxysporum* is the potential cause of date palm wilt in Pakistan.

Keywords: Date palm wilt, disease prevalence, phylogenetic network, Neighbor net algorithm, Fusariosis, Koch's postulates, pathogen, *Fusarium oxysporum*

INTRODUCTION

Phoenix dactylifera L. (Date palm) is a monocot perennial palm that belongs to the Palmaceae family, well adapted to the wide geographic range and environmental conditions. It is one of the leading perennial fruit crops regarding its nutritional profile, medicinal significance, excellent yields, long life, and high adaptability to diverse climatic conditions of semi-arid and arid regions globally (Augstburger *et al.*,2002; Hadrami *et al.*, 2011; Sirisena *et al.*, 2015). Dates have been identified as an emerging "healthy, nutritious" food for human health with many functional benefits (Shinwari, 1993; Al-Shahib and Marshall, 2002; Al-Shahib and Marshall, 2003; Ali Mohamed and Khamis, 2004; Al-Farsi *et al.*, 2007; Al-Farsi and Lee, 2008; Elias, 2008; Baliga, 2011; Vayalil, 2012), rich in nutrients, fiber, vitamins, and minerals (Mangan *et al.*, 2016). Dates have a tremendous medicinal significance due to bioactive and functional compounds and prevent several diseases (Gotz *et al*., 1996; Mitscher *et al.*, 1996; Rice-Evans *et al.*, 1997; Uenobe *et al.*, 1997; Offen *et al.*,1998; Morel and Barouki, 1999; Jakus, 2000; Droge, 2002; Javanmardi *et al.*, 2003; Alfaro-Viquez *et al.*, 2018).

It is well adapted to the wide geographic range and environmental conditions, but the yield of the date palm is negatively affected by pathological and physiological factors that ultimately affect the economy (Howard *et al.*, 2001; Anjili *et al.*, 2015). Fungal pathogens pose devastating effects on date palms. Wilt is one of the destructive diseases of various palm species. Infected palms show unusual frond death and ultimately whole; death of date palm may lead to massive loss because date palm economic life is 40-50 years, starts fruit production at the age of 4 to 6 years, and 15 to 20

Khan, N.A., A. Rehman, I.U. Haq and I.A. Khan. Traditional pathology and phylogenetic networking confirm the pathogen causing date palm wilt. Pakistan Journal of Agricultural Sciences 60: 249-264.
[Received 9 Feb 2023; Accepted 20 May 2023; Published 27 Jun 2023]

years old female palm reaches full production age (Nixon and Carpenter, 1978).

Vascular fusariosis, commonly known as Fusarium wilt most devastating disease of date palm worldwide. The Fusarium genus has many widely distributed species causing diseases in numerous essential crops (Zapater and Arrcchea, 1975). Various species of Fusarium can cause wilts, i.e., *F. solani, F. eumartii, F. avenaceum, F. tabacinum, and F. sulphureum,* but *F. oxysporum* is mainly involved in causing wilts in a wide variety of important crops (Beckman, 1987; Okungbowa and Shittu, 2012). The date palm's Fusarium wilt disease has been an emerging threat in Pakistan (Maitlo *et al.*,2009; Abul-Soad *et al.*, 2011) and has become a real threat to current date palm cultivation across the country. Globally different species of Fusarium have been reported as date palm wilt causing agent such as *F. proliferatum, F. oxysporum* f. sp. *albedinis*, *F. solani*, and *F. monliforme* (Mani *et al.*,2005; Alkahtani *et al.*, 2011; Munoz and Wang, 2011; Al-Sadi *et al.*, 2012; Hameed, 2012; Mansoori, 2012; Maitlo *et al.*, 2014; Matlo *et al.*, 2021). External symptoms of Fusarium wilt included; the leaf turns into ash grey, necrosis and chlorosis occur. The premature dropping of leaves, brown strip on rachis, one- sided death of frond and color become brown, and ultimate wilting can be seen on the whole palm, and it becomes dead. The infected frond assumes the shape of an arch that closely resembles a wet feather and eventually hangs down the trunk and dies. This process may take a few days to many weeks after the emergence of the first symptom. (Jansson *et al.*, 2010). The fronds showing external symptoms have reddish- brown discoloration in the water-conducting vessels (xylem). Upon uprooting of seedlings, several infected roots exhibit reddish color. That is why vascular symptoms persist from the roots to the apex of the fronds (Zaid and Wet, 2002; Benzohra *et al.*, 2015).

Nelson *et al.*, 1983; 1994; Summerell *et al*., 2003; Leslie and Summerell, 2006 have provided identification keys for the morphological characterization of the Fusarium genus.

Different scientists have proved the association of the Fusarium genus as a pathogen with date palm using spore suspension and also described the symptoms of wilt (Abdalla *et al.*, 2000; Elliott *et al.*, 2010; Khazaal *et al.*, 2019; Matlo *et al.*, 2019).

The phylogenetic network includes different concepts, as split networks, phylogenetic trees, and reticulate networks. The reticulate networks covering "recombination" "hybridization" networks, and "augmented trees." Recombination networks are related to the graphs used in population studies "ancestor recombination". Split networks by neighbor-net method (Bryant and Moulton 2004) and from character sequences as a median network (Huson and Bryant 2006). In this study phylogenetic networking was used to divide the fungal isolates of one species into different groups on the basis of evolutionary relationship. On the basis of similarity, groups make the study systematic.

Date palms play a substantial role in the trade and economy of the world. In 2019, the worldwide production volume of date fruits reached about 9075,466 tons (1381,43 4 hectares harvested area (FAOSTAT, 2019). It holds a highly significant rank in the horizon of Pakistan agriculture. Pakistan ranks seventh among the top ten countries with 71,494 hundred dollars in export value and 6th annual production globally (FAOSTAT, 2019).

A few reports are available regarding the disease prevalence, etiology, and management of date palm wilt in Pakistan. Considering these perspectives, the research aspects considered were wilt disease assessment on date palms across three provinces of Pakistan, associated fungal characterization based on morpho-cultural characteristics, phylogenetic networking, and Koch's postulates completion for Pathogenicity confirmation.

MATERIALS AND METHODS

Survey and disease documentation: Extensive and investigative surveys of date palm growing regions across Pakistan were conducted in 2018 and 2019. Three provinces

Table 1. Districts of Pakistan surveyed for documentation of wilt disease on date palm.

Provinces	Districts	Locations	GPS Coordinates	No. of samples	Promising research stations/centers/orchards
Punjab Zone-I	Faisalabad	10	31.4504° N, 73.1350°E		PARS, University
	Jhang	10	31.2781° N, 72.3317°E	120	Agriculture Faisalabad.
	Layyah	10	30.9693° N, 70.9428°E		Date palm research station
	Multan	10	30.1575° N, 71.5249°E		Jhang.
Punjab Zone-II	Bahawalpur	10	29.3544° N, 71.691°E		Horticulture research station
	Muzaffargarh	10	30.0736° N, 71.1805°E	120	Bahawalpur.
	Dera Ghazi Khan	10	30.0489° N, 70.6455°E		Khudai farm Muzafargarh
	Rahim yar khan	10	28.4212° N, 70.2989°E		
Sindh	Sukhur	10	27.7244° N, 68.8228°E		Date palm research institute,
	Khairpur	10	27.5256° N, 68.755°E	60	Shah Abdul Latif University
Khyber Pakhtunkhwa	Dera Ismail Khan	10	31.8626° N, 70.9019°E	80	Date Palm Research Center Dera Ismail Khan

were surveyed, i.e., Punjab, Sindh, and Khyber Pakhtunkhwa (KPK). The Punjab province was categorized into two zones, i.e., Punjab Zone-I and Punjab Zone-II; each zone comprised four districts; two districts of Sindh province and one district of KPK were surveyed. Ten locations per district were selected to estimate the wilt disease, including date palm research centers/stations and farmers' orchards (Table 1).

Wilted date palms were thoroughly observed following the symptoms explained by (Maitlo *et al.* 2009; Abdalla *et al.,* 2010; Abul-Soad, 2011; El-Morsi *et al*., 2012), and data regarding disease incidence and severity was documented by quantitative method. Disease incidence was computed using the following mentioned formula:

$$\text{Disease incidence} = \frac{No.of\ diseased\ plants}{No.of\ diseased\ plants} \times 100$$

Disease severity was estimated using the disease rating scale (El-Morsi *et al*., 2009).

Table 2. Disease Rating Scale used in this study to estimate disease severity on date palms

Class category	Symptom category	Damage (%)
0	healthy	0%
1	mild symptoms	0 to 25%
2	moderate symptoms	26 to 50 %
3	severe symptoms	51 to 75%
4	nearly dead	more than 76%

Sample collection: Plant samples comprising different palm parts, including fronds, rachis, and roots showing characteristic wilt symptoms, as well as asymptomatic healthy tissues, were collected from the locations. For processing, collected samples were brought to Fungal Molecular Biology Laboratory (FMB) Department of Plant Pathology, University of Agriculture, Faisalabad.

Isolation and purification of associated fungi: Samples were thoroughly washed with tap and distilled water for surface sterilization. The plant parts were cut into small bits (1×1 cm) with a sterilized knife soaked in 2% sodium hypochlorite for 1-2 minutes, and two consecutive washings were given with sterilized distilled water, placed on blotter paper in the laminar flow chamber (ESCO LHS-4AG-F8) for drying. About 20ml of Potato dextrose agar medium was dispensed into each petri dish under a laminar flow chamber sterilized to isolate associated fungi from the samples. 3-4 bites of processed samples were transferred onto media containing Petri plates using sterilized forceps and incubated at 28^0C for incubation (ESCO Isotherm low-temperature incubator IFC-110-8). The associated fungi were purified using the single hyphal tip (Narayanasamy, 2001) and the single spore techniques (Choi *et al*., 1999).

Morpho-cultural identification and frequency percentage calculation of associated fungi: Fusarium isolates were frequently isolated from symptomatic and asymptomatic tissues of date palms. Fusarium isolates were subjected to morpho-cultural characterization. All pure cultures were identified by macroscopic features and by observing microscopic key characters of each studied fungus. Pictures were saved to compare with identification keys available in the literature (Webster and Weber, 2007). Morpho-cultural characters include colony pigmentation, growth pattern, conidia (macro and microconidia), shape, and size, apical cell shape, and phalidies (Nelson *et al.,* 1994; Leslie and Summerell, 2006). Fusarium isolates were identified using the available key (Nelson *et al.,* 1983; 1994; Summerell *et al*., 2003; Leslie and Summerell, 2006). The following formula calculated the frequency percentage of associated fungi:

$$\text{Frequency percentage} = \frac{No.of\ isolates\ of\ fungus}{No.of\ total\ fungi} \times 100$$

Phylogenetic network: The phylogenetic network of the Fusarium population was computed using splitsTree4 V4.14.8 software. Five fungal isolates from each of the four populations based on population structure analysis (unpublished data of the Fusarium population genetic diversity) were subjected to genomic characterization based on the internal transcribed spacer (ITS) region. GeneJET Genomic DNA purification kit (Thermo Scientific) was used to isolate fungal DNA. PCR analysis was executed on template DNA of representative fungal isolates using primer pair of ITS region given by White et al. (1990). Sequencing was done from Eurofins Genomics DNA sequencing services, USA. The sequence data of the ITS region of representative fungal isolates was consumed to construct an unrooted phylogenetic network using the Neighbor net algorithm.

Completion of Koch's postulates: One isolate of *Fusarium oxysporum* (FMB-FO-PD-011) was selected (selection was based on a phylogenetic study published (Ul Haq et al., 2022) for the Pathogenicity test. Symptomless healthy plant material was used for trials. Three Pathogenicity trials were conducted a) detached leaf assay, b) seedlings and suckers under controlled conditions, and c) seedlings and suckers under natural conditions. Koch's postulates were followed to assess symptomology, confirmation of Fusarium isolates as the pathogen, and wilt disease severity.

Inoculum preparation: The spore suspension of 10-12 days old actively growing-purified culture-Fusarium isolate (FMB-FO-PD-011 was used to prepare spore suspension. Conidial concentration was adjusted to 10^5/ml, 10^6/ml, 10^7/ml using a hemocytometer. Culture grown on sorghum grains was used for a trial conducted under natural conditions.

Pathogenicity Trial-1: This trial was performed on symptomless healthy leaflets and date palm rachis. Young fronds were detached from the date palm. Leaflets were removed from the rachis, and leaflets and rachis were cut into small pieces (4cm) and thoroughly washed with tap water to remove dust particles. Then surface was sterilized by soaking into 1% sodium hypochlorite for 1.5-2 minutes, then two-time washings were given with sterilized distilled water.

Autoclaved filter papers (soaked with sterilized water) were kept in Petri plates. Carborundum powder was used to make mild injuries on leaflets, and 1-2-mm diameter holes into the rachis were made. Four leaflets and rachis were placed on filter papers in each Petri plate separately with forceps. Leaflets and rachis were inoculated by an eight-day-old culture plug of about 5mm. Petri plates (one per cutting type) containing leaflets and rachis cuttings were inoculated by a PDA plug of the same size to maintain control. Each Petri plate having inoculated planting material was in three replications. Then incubated at 28°C for four weeks. The plates were monitored after regular intervals every week for symptom development. Side-by-side, one another activity was also conducted. One cutting from both processed leaflets and rachis (same as used for the Pathogenicity trial) without inoculation was subjected to a fungal isolation process using a PDA medium to ensure symptomless fronds were Fusarium free or not. This trial was repeated twice. Re-isolation of the fungus was done from the symptomatic area according to the protocol described in the isolation section.

Pathogenicity Trial-2: This trial was conducted according to Abdalla *et al.,* 2000; Khazaal *et al.,* 2019; Matlo *et al.,* 2019. Date palm seeds were disinfected by dipping in 1% sodium hypochlorite (NaOCl) for 10-15 minutes, then soaked in distilled water for 24 hours. Afterward, seeds were placed into pots filled with autoclaved peat moss and sandy loam (1:1). Pots were placed into greenhouse conditions. After emergence, seedlings (6-7 months) were inoculated by spore suspension. Ports were monitored weekly for symptom development and rated the disease progression using a disease rating scale until 50% mortality occurred. The experiment was repeated twice. A culture of *Fusarium oxysporum* (FMB-FO-PD-011) with three conidial concentrations (10^5/ml, 10^6/ml, 10^7/ml) was used for Pathogenicity confirmation. Symptoms began to appear 14 days post-inoculation in seedlings. Data were recorded after 14 days, 21 days, and 28 days.

Inoculation method 1 (rachis): Fifteen seedlings of date palm were mechanically injured by a sterilized sharp knife, and injected the spore suspension (10^5/ml, 10^6/ml, 10^7/ml) was slowly using a hypodermic needle. Wrapped the inoculated area with parafilm. Each suspension concentration treated three seedlings. The control treatment was maintained by injecting sterilized distilled water into seedlings. All seedling pots were covered with plastic bags separately for 24 hours to maintain humidity. Placed the pots under controlled conditions and watered them according to the irrigation requirement of seedlings (Abdalla *et al.,* 2000; Khazaal *et al.,* 2019).

Inoculation method 2 (soil): Fifteen seedlings were used in this inoculation method. The soil of seedlings were inoculated by spore suspension (10^5/ml, 10^6/ml, 10^7/ml) separately near the root zone. Three seedlings per suspension concentration were inoculated. Before inoculation, roots were mechanically injured by making small incisions using a sterilized scalpel to promote infection. In the negative control, seedlings were treated with sterilized distilled water. Ports were covered using plastic bags to maintain humidity. Placed all seedling ports under control conditions (Matlo *et al.,* 2019).

Inoculation Method 3 (rachis+soil): In this method, twelve seedlings out of fifteen were used for inoculation by combining the abovementioned methods (rachis and soil). Three seedlings (rachis and soil) were treated with sterilized distilled water to maintain negative control. Covered the ports with plastic bags for 24 hours to maintain humidity and placed them under control conditions.

Re-isolation: To complete Koch's postulates, the symptomatic parts of seedlings were critically observed, and samples were collected from seedlings having symptomatic and symptomatic tissues to re-isolate the inoculated fungus. Leaflets and rachis showed chlorotic lesions and brown strips were taken for fungus re-isolation. Discolored rachis appeared as reddish-brown streaks on vascular tissues, a characteristic symptom of an infection caused by Fusarium spp. Collected samples were cut into small pieces (1cm) aseptically; surface disinfection was done by rinsing with tap water, soaking in 1% NOCL for 1 minute, and two times rinsed with sterilized distilled water. After drying of moisture, placed the bits on a PDA medium and provided incubation conditions (28°C, alternate light hours) for isolation. Morphological and microscopic characteristics of re-isolated fungus were compared with inoculated fungus in all Pathogenicity trials.

Pathogenicity Trial-3: This trial was conducted according to Elliott *et al.,* 2010. Fifteen suckers were collected from healthy symptomless date palms used in this trial. All collected suckers were potted and left untreated for one month for proper growth and establishment of roots. Before inoculation, part of the leaflets and roots from the same suckers used in this trial were subject to sample processing and a fungal isolation procedure to ensure suckers were Fusarium-free completely. Afterward, suckers were inoculated using Pathogenicity Trial 2; the inoculum formulation was changed. Suckers were observed for the first time after 21 days of inoculation in the case of suckers, and two more readings were taken 28 days and 42 days post-inoculationn Suckers were observed regularly after two weeks intervals for symptom development and progression. The trial was maintained until 50% suckers died.

Method 1 (stem): Suckers were inoculated by making the shallow cut of the slit 2cm lengthwise in the adaxial surface and injected spore suspension 10^5/ml, 10^6/ml, 10^7/ml of 20ml volume separately using a hypodermic needle or syringe (Elliott *et al.,* 2010). Four suckers were used per treatment (volume of spore suspension). The inoculated area was wrapped with parafilm to prevent moisture loss. Suckers were covered with plastic bags for 24 hours and kept under natural conditions.

Method 2 (soil): The soil of suckers was inoculated by direction incorporation of infected powder of sorghum grain (2g, 4g, 6g) instead of spore suspension. Roots were mechanically injured using a sterilized scalpel to promote the entry of fungus into suckers. Four replications per inoculum level were maintained, and one sucker was incorporated with sterilized sorghum grains powder (non-infected grains) as the negative control. To maintain humidity, cover the suckers with plastic bags for 24 hours. Kept under natural conditions.

Method 3 (stem+soil): Twelve suckers were inoculated by combining methods 1 and 2 of inoculation in Pathogenicity Trial 2. In this method, suckers received a double quantity of inoculum (10^5/ml +2g, 10^6/ml +4g, 10^7/ml+6g). Re-isolation of the fungus was done according to the protocol described in Pathogenicity Trial 2. Negative control on three suckers was maintained like methods 1 and 2 (Pathogenicity trial 2).

Disease assessment: Disease severity was assessed using a rating scale (Table 3) (Khazaal *et al.*, 2019). All seedlings and offshoots were rated from 0 to 4 classes based on symptoms appearance. The disease severity index (DSI) was computed using the formula:

$$DSI = \frac{\Sigma \text{ (seedlings per class} \times \text{class score)}}{\text{Total numbe rof seedlings}}$$

Table 3. Disease Rating Scale used for estimation of the disease severity index

Class score	Symptom Category	Lesion size
0	No visible symptoms	-
1	Small lesion	5 to 10 mm
2	Medium lesion	10 to 30 mm
3	Large lesion	30 to 70 mm
4	The whole leaf blighted	-

RESULTS

Survey and disease documentation: Major date palm growing eleven districts (10 locations per district) of Punjab, Sindh, and Khyber Pakhtunkhwa, including research centers/stations, local orchards/nurseries, and random plant/cultivation, were visited to assess the incidence of wilt disease. The highest disease incidence was recorded in Khairpur (55%), followed by Sukkar (52.3%), Bahawalpur (47.4%), Rahim Yar Khan (45.1%), Dera Ghazi Khan (45%), Dera Ismail Khan (45%), Muzaffargarh (35%), Multan (33.3%), Layyah (32.7%), Jhang (30.5%) and Faisalabad (20.2%) (Figure 1). Disease severity was calculated using a rating scale (El-Morsi *et al*., 2009). The disease severity (DS) was recorded as 65% in Khairpur, 60.7% in Sukkar, 55% in Bahawalpur, 50% in Dera Ismail Khan, 48.5% in Rahim Yar Khan, 40.8% in Muzaffargarh, 40% in Dera Ghazi Khan, 37% in Multan, 35% in Layyah and Jhang and 27% in Faisalabad (Figure 2).

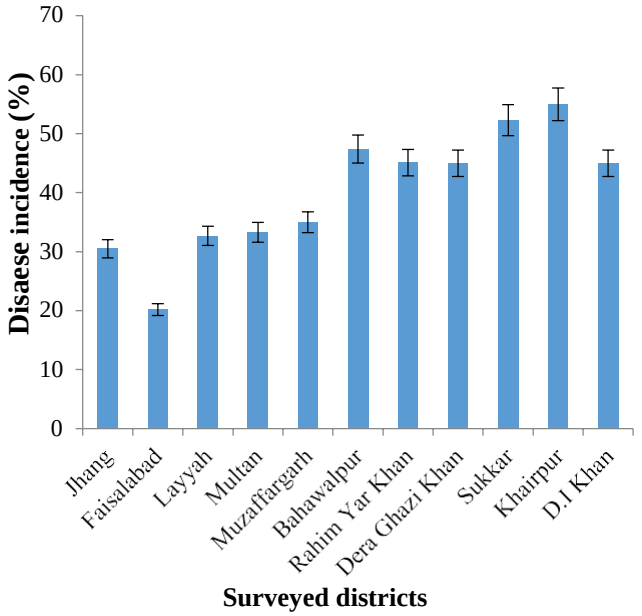

Figure 1. Graphical representation of disease incidence % in different districts of Punjab, Sindh, and Khyber Pakhtunkhwa

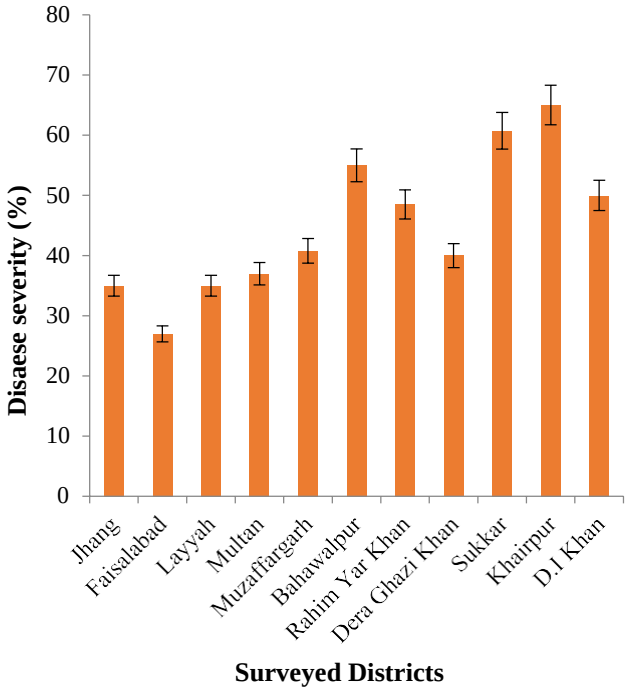

Figure 2. Graphical representation of Disease Severity % in different districts of Punjab, Sindh, and Khyber Pakhtunkhwa

Sindh is the leading province regarding disease incidence at 53.65%, followed by Khyber Pakhtunkhwa at 45% and Punjab at 36.15. The leading pattern of disease severity %

(Sindh 62.85%, Khyber Pakhtunkhwa 50%, and Punjab 39.78%) across provinces was the same as disease incidence.

***Wilt symptoms*:** A wide range of symptoms was carefully observed on wilted date palms. The typical wilt symptoms were seen on rachis, leaflets, vascular bundles, and roots (Maitlo *et al.*, 2009; Abdalla *et al.*, 2010; Abul-Soad, 2011; El-Morsi *et al*., 2012).

Yellowing and drying of the leaves: Chlorosis and necrotic patches were observed on leaflets. The affected leaves showed pale green color instead of standard green; yellowing progressed entirely on the side of the frond, leading to one-sided death (a typical symptom of Fusarium wilt). Then one-sided yellowing turned brown, and unhealthy fronds looked wilted (figure 3, 4).

Discoloration of rachis: The yellow stripes observed on the rachis, dark brown spots, and broader stripes on half (dorsal side) portion of the rachis of infected fronds were also seen with disease progression, and these stripes were seen as lengthwise covering the whole rachis (Figure 5, 6).

Streaks on vascular bundles and roots: Longitudinal streaks on vascular bundles were also observed, progressing to the maximum portion of tissues; the roots of infected offshoots were red (figure 7).

Death of the entire palm: Thoroughly wilted fronds attained an arch shape and hung down the palm. Symptoms appearance (discoloration and wilting) starts at the top of fronds and then gradually progress downwards as Fusarium blocks the water-conducting vassals, ultimately wilting was seen on the date palm (FigurRange of severity was noticedas some trees showed few symptomatic fronds, and some date palms were completely wilted. e 8, 9). Some visited locations of date palms exhibit severe symptoms; maximum fronds of a tree were entirely wilted, and ultimately whole wilted date palms were ay wilted.

Figure 3. a and b showed one-sided yellowing of leaves, c-green color turns into brown, starts from tip progress towards rachis, d-browningprogresses on a maximum portion of the frond

Figure 4. a-One-sided yellow and brown discoloration, b-One-sided complete death of frond, c-entire frond dead from both sides

Figure 5. Yellow stripe on rachis, b, and c represents vertical dark brown stripes on rachis

Figure 6. a and b Green color of the rachis turned into dark brown, showing infection

Figure 7.Longitudinal streaks on vascular bundles indicating severe systematic infection persist in the whole palm

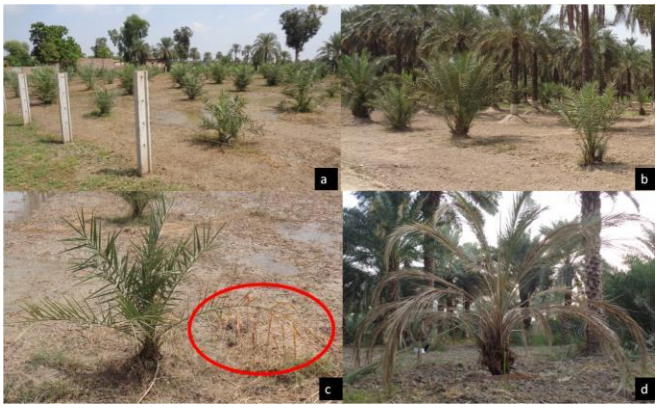

Figure 8. a, b, c, d represents wilt symptoms on offshoots in the field

Figure 9. a and b showing wilted fronds become arch-shaped and hanging down the palm

Isolation and frequency calculation of associated fungi: Across three provinces (Punjab Sindh and KPK) of Pakistan, three hundred and eighty (380) collected diseased samples were subjected to an isolation procedure. Purification of isolated fungi was successfully done using the single hyphal tip, and single spore techniques yielded a range of fungi, including Fusarium spp., Aspergillus spp., Alternaria spp., Rhizopus spp., and *Curvularia spp*. These fungi were identified based on the growth pattern, pigmentation, and spore characteristics**.**

A table shows Fungi's frequency (%) associated with collected samples from all visited districts (Table 4). Fusarium spp. (82.6%) was frequently isolated fungus followed by Aspergillus spp. (10.81%), Rhizopus spp. (10.04%), Alternaria spp. (3.61%) and Curvularia spp. (3.508%) (Figure 10).

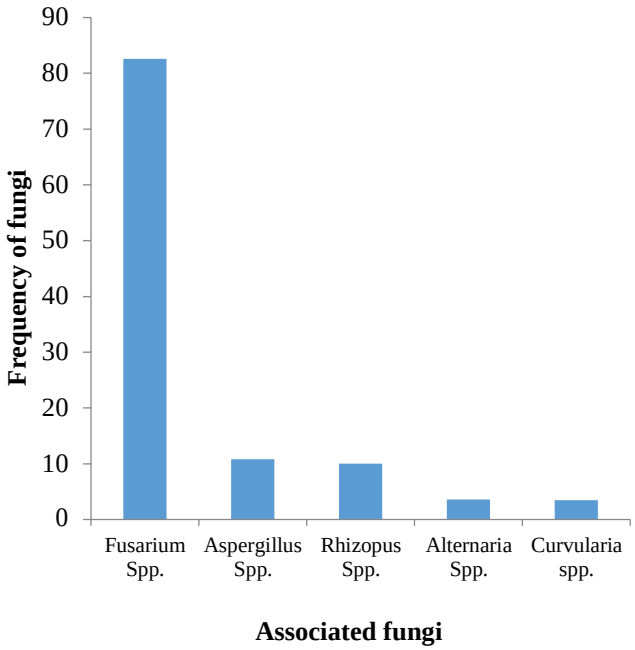

Figure 10. Frequency (%) of fungi associated with Palm.

Morpho-cultural characterization: Most isolates exhibited abundant floccose aerial mycelium of creamy White to peach white and pale-violet coloration from the inverse of colony growth (figure 11). Conidia (microconidia, microconidia) were thin-walled hyaline. Microconidia were 3.9-13.6µm in length and 1.6-4.0 µm in width with elliptical, obovoid, oval, or reniform in shape, and have no or single septations. Macro conidia were 31.6-45.3 × 2.8-5.4 in size, fusiform with tapered ends; apical cells were straight to slightly curved most of them were narrower with 3-7 septations. Branched Phialides were also observed in microscopic studies.

Table 4. Frequency (%) of Fungi associated with collected samples from all visited districts

Districts name	Frequency Percentage of fungal Isolates				
	Fusarium Spp.	Aspergillus Spp.	Rhizopus Spp.	Alternaria Spp.	Curvularia spp.
Jhang	82.4	8.44	9.88	-	2.20
Faisalabad	84.3	12.88	-	-	5.85
Dera Ghazi Khan	82.2	9.25	9.88	3.00	-
Layyah	84.7	11.00	-	3.33	3.83
Muzaffargarh	81.6	10.25	9.55	-	2.66
Bahawalpur	85.2	10.80	-	5.00	-
Rahim Yar Khan	84.8	9.75	9.25	-	-
D.I Khan	82.7	10.28	8.88	-	3.00
Sukkar	70.3	20.20	13.57	-	-
Khairpur	89.6	6.60	-	4.16	-
Multan	80.8	9.50	9.33	2.60	-
Average	82.6	10.81%	10.04%	3.61%	3.51%

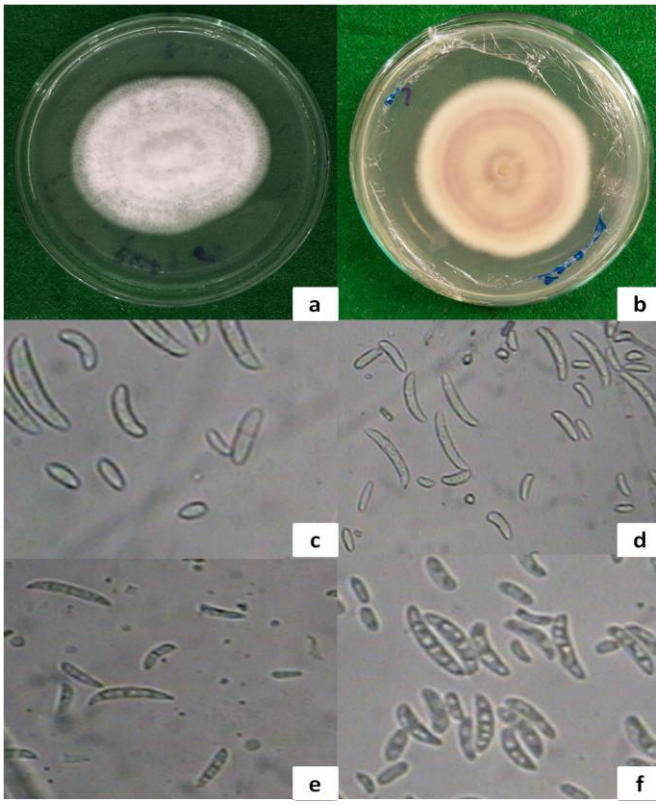

Figure 11. a) Front view of fungal growth, b) Back view of fungal growth, c) oval, curved, bicelled microconidia, b,c,d) septated crescent-shaped macroconidia.

Phylogenetic network: The Fusarium community sampled from wilted tissues of date palm was divided into four populations (based on the population genetic structure unpublished data of genetic diversity analysis). Five fungal isolates were selected from each population and characterized for the ITS region sequence (Table 5) data-based phylogenetic network. The Neighbor net algorithm computed an unrooted phylogenetic network displayed as four branched genetic network that depicted the division of twenty fungal isolates into four groups (Fig. 12).

***Symptom development*:** Wilt symptoms appeared gradually, and typical Fusarium wilt symptoms were observed throughout the Pathogenicity trials.

***Pathogenicity trial 1*:** Inoculated pieces of leaves showed lesions after one week of inoculation. Inoculated rachis also showed discoloration symptoms after two weeks of inoculation (Figure 13). Three weeks post-inoculation, the leaves were completely discoloured, and the rachis also became brown to black coloured (Figure 14). Then re-isolation was done, and recovered isolates exhibited the same microscopic characteristics.

Table 5. A list of Isolates of *Fusarium oxysporum* and Genebank Accession numbers of sequences of ITS

Sr.	Isolate name	GenBank accession numbers ITS
1	FMB-FO-PD-005	OM319651.1
2	FMB-FO-PD-011	OM327774.1
3	FMB-FO-PD-017	OM391976.1
4	FMB-FO-PD-020	OM391977.1
5	FMB-FO-PD-045	OM391978.1
6	FMB-FO-PD-041	OM391979.1
7	FMB-FO-PD-061	OM391980.1
8	FMB-FO-PD-056	OM391981.1
9	FMB-FO-PD-052	OM391982.1
10	FMB-FO-PD-001	OM391983.1
11	FMB-FO-PD-032	OM391984.1
12	FMB-FO-PD-044	OM391985.1
13	FMB-FO-PD-034	OM391986.1
14	FMB-FO-PD-037	OM391987.1
15	FMB-FO-PD-059	OM391988.1
16	FMB-FO-PD-022	OM391989.1
17	FMB-FO-PD-029	OM391990.1
18	FMB-FO-PD-060	OM391991.1
19	FMB-FO-PD-027	OM391992.1
20	FMB-FO-PD-003	OM391993.1

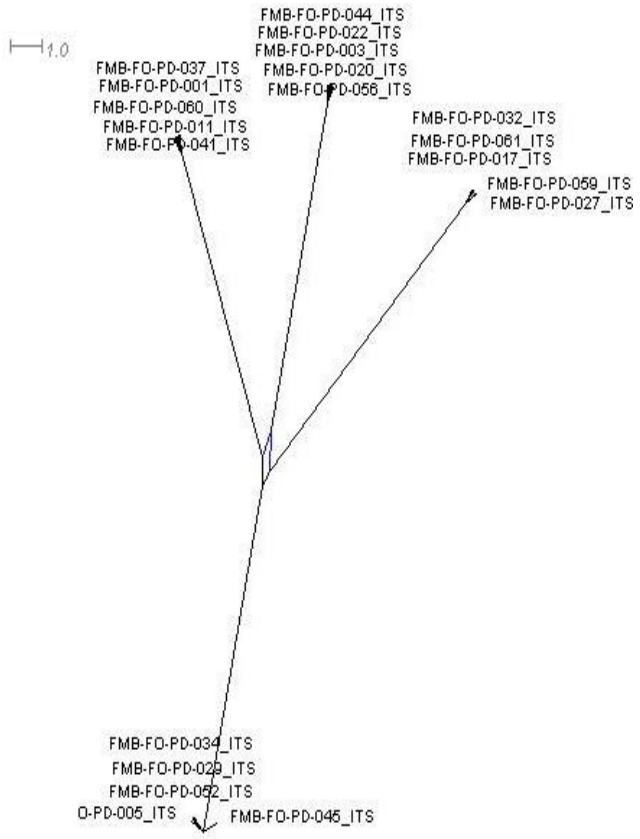

Figure 12. Phylogenetic network based on Neighbor net algorithm displayed division of twenty fungal isolates into four distinct clades.

***Pathogenicity Confirmation*:** One isolate was randomly selected (based on a phylogenetic study published (Haq et al., 2022) to prove pathogenicity.

***Pathogenicity trial 2*:** Symptoms began to appear 14 days post-inoculation in seedlings. Seedlings showed chlorosis (yellowing) necrotic patches; browning progressed to the maximum portion of the leaves. Lesion size depended upon the inoculation method and conidial concentration. Inoculated seedlings showed symptoms (Figure 15). After 14 days of inoculation, seedlings exhibited larger-sized lesions in inoculation method 3 at 4.9 cm (10^7/ml) followed by 3.4 cm (10^6/ml) and 2.1 cm (10^5/ml) (Figure 16). After 21 days of inoculation, lesion size was recorded as 4.4-5.8 cm (10^7/ml) followed by 2.8-4.6 cm (10^6/ml) and 2.1-3 cm (10^5/ml). The seedlings were inoculated with conidial concentration 10^7/ml showed lesions of 6-7.5cm followed by 4.9cm-6.8cm (10^6/ml), and seedlings inoculated with 10^5/ml (4-5.1 cm) 28 days post-inoculation (Figure 17). Among all inoculation methods, maximum lesion sizes were obtained in seedlings inoculated by method 3 (rachis+soil) and seedlings subjected to 10^7/ml followed by 10^6/ml and 10^5/ml (figure 18). Negative control seedlings remained healthy. The whole plant became wilted after 42 days of inoculation. The maximum disease severity percentage was 95 observed in plants inoculated by method three and a conidial concentration 10^7/ml (Figure 19).

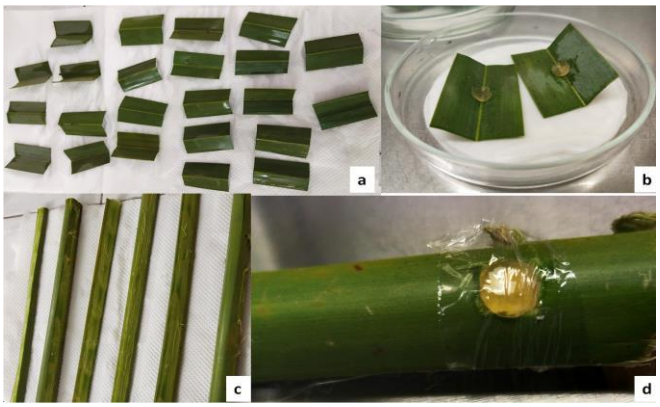

Figure 13. a- Health pieces of leaves, b inoculated leaves, c-healthy rachis, d-inoculated rachis

Figure 14. a-lession on the leaf, b completely discoloured c-discolured vascular bundles, d complete discoloration

Figure 15. a-Healthy seedling, the b-normal green colour changed into brown, c-complete wilting, d-negative control

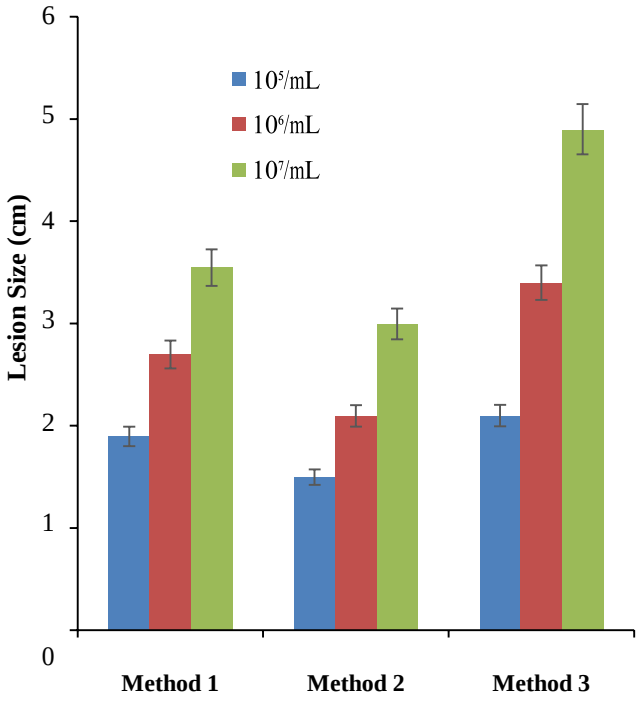

Figure 16. Graphical representation of lesions development on seedlings of date palm inoculated with *Fusarium* isolate (FMB-FO- PD-011) by different methods post 14 days.

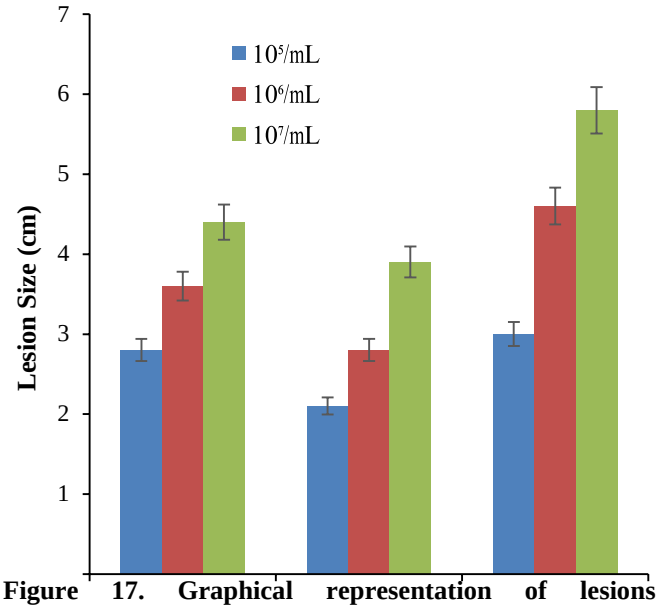

Figure 17. Graphical representation of lesions development on seedlings of date palm inoculated with *Fusarium* isolate (FMB-FO-PD-011) by different methods post 21 days of inoculation

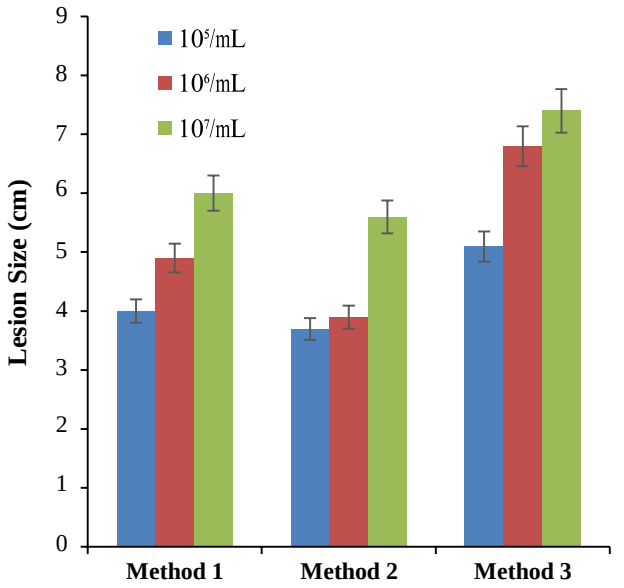

Figure 18. Graphical representation of lesions development on seedlings of date palm

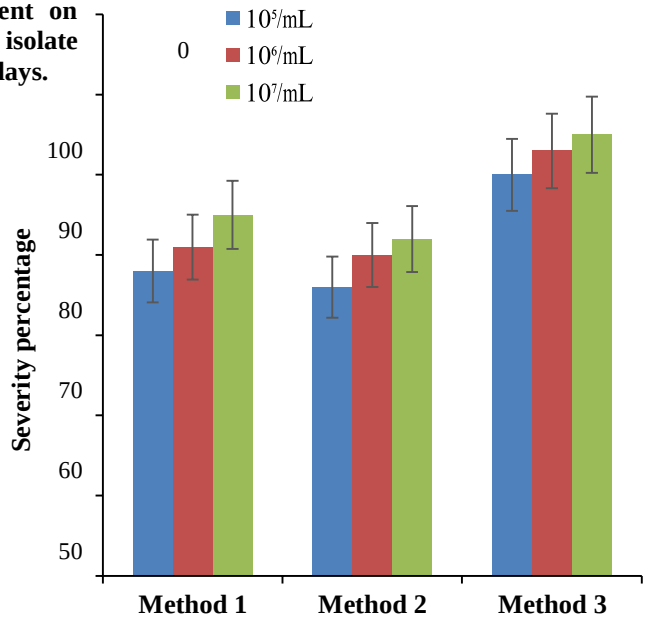

Figure 19. Graphical representation of disease severity % on seedlings of date palm inoculated with *Fusarium* isolate (FMB-FO-PD-011) by different methods post 28 days of inoculation.

Pathogenicity trial 3: *Fusarium oxysporum* (FMB-FO-PD-011) inoculated onto healthy symptomless suckers under field conditions showed typical wilt disease symptoms. Typical

symptoms were observed, including yellowing starting from the tip of the leaflet, one-sided complete yellowing of leaves, vascular discoloration, and ultimately wilting of the whole sucker (Figure 20). Maximum lesion length was obtained in suckers inoculated by method 3 (stem+soil) compared to method 1 and method 2. Conidial suspension 10^7/ml produced larger lesions among all conidial concentrations (Figures 21, 22, 23). After 21 days of inoculation, lesion size ranged from 11.3-19.3 cm (method 3), 5.9-12.2 (method 2), and 7.1-14.6 cm (method 1). After 28 days of inoculation, lesion size was 16.1-25.5 cm (method 3), 9.6-17.3 cm (method 2), and 11.3-20.1 cm (method 1). After 42 days of inoculation, suckers inoculated by method 3 exhibited 23.3-36.2 cm, method 2 ranged in 12.9-23.7 cm, and method 1, lesion size followed in the range of 19.2-28.9 cm. Negative control remained healthy.

Re-isolation: Symptomatic tissues of inoculated plants upon isolation (procedure discussed in isolation section) yielded Fusarium oxysporum with the same characteristics of colony growth pattern and pigmentation (Figure 23). Then compared, microscopic characteristics with initial studies, which were also the same. This identification proved that inoculated *Fusarium oxysporum* is a real etiology of wilt in date palms.

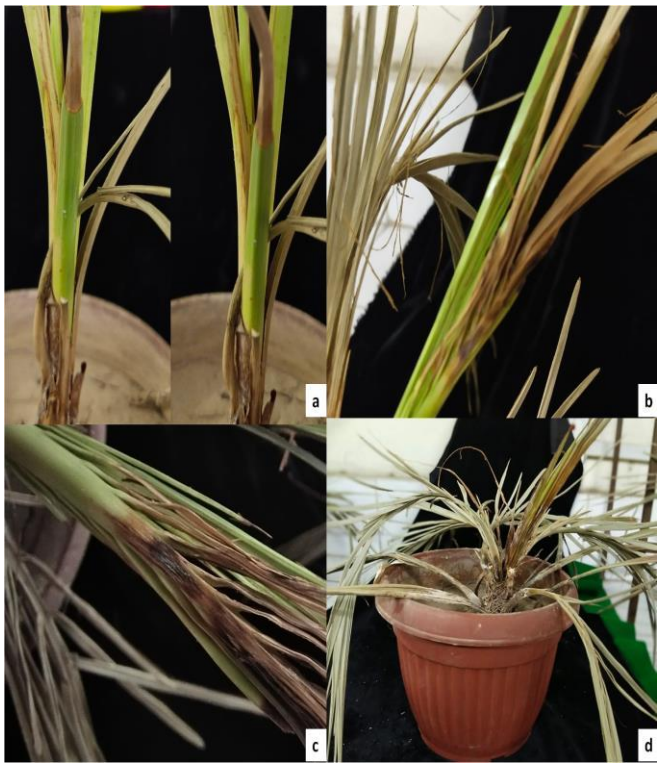

Figure 20. a-discoloration on rachis, b-one-sided death of leaf, c-severe discoloration, d- complete wilted plant

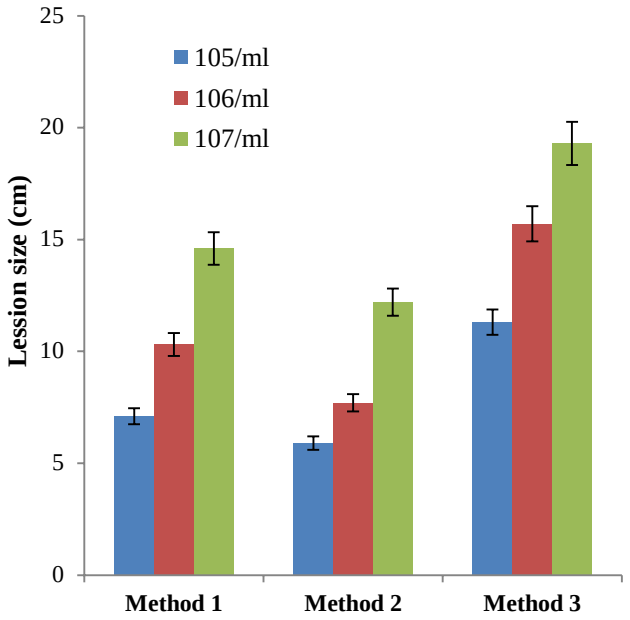

Figure 21. Graphical representation of lesions development on suckers of date palm inoculated with *Fusarium* isolate (FMB-FO-PD-011) by different methods post 21 days of inoculation.

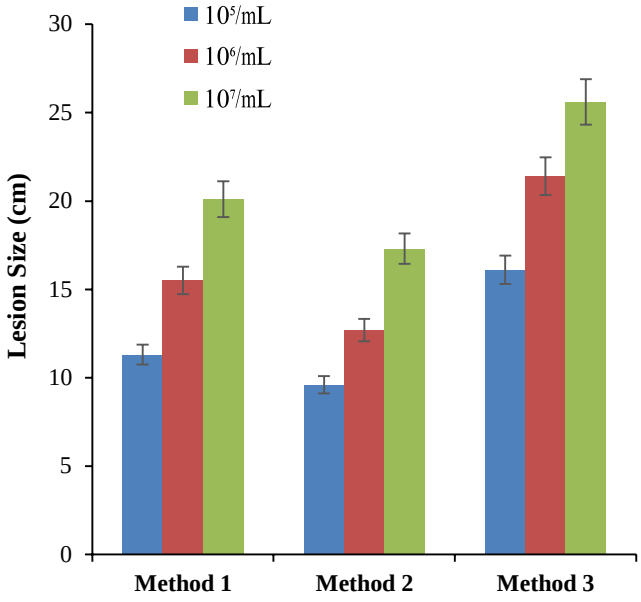

Figure 22. Graphical representation of lesions development on suckers of date palm inoculated with *Fusarium* isolate (FMB-FO-PD-011) by different methods post 28 days of inoculation.

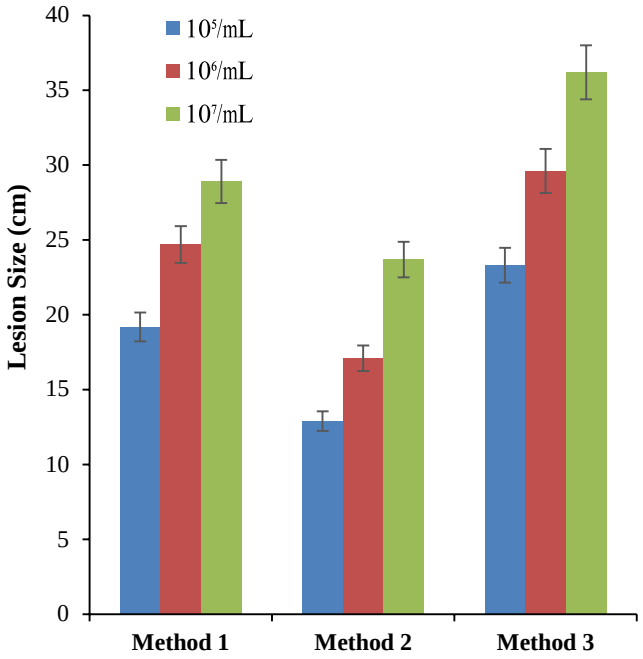

Figure 23. Graphical representation of lesions development on suckers of date palm inoculated with *Fusarium* isolate (FMB-FO-PD-011) by different methods post 42 days of inoculation.

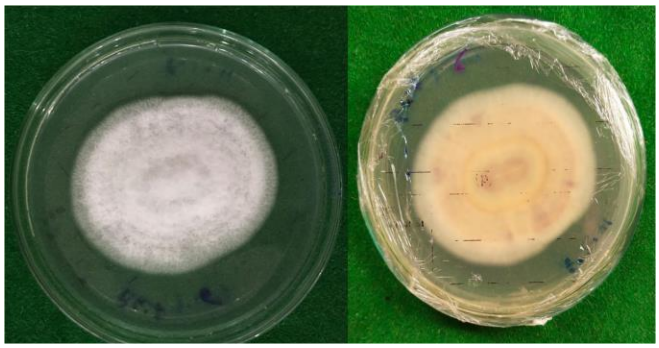

Figure 24. *Fusarium oxysporum* (FMB-FO-PD-011) upon re-isolation

DISCUSSION

The "*Phoenix dactylifera*" is a dioecious (separate male and female palms), monocot perennial palm that belongs to the Palmaceae family (Dowson, 1982; Sudhersan and El-Nil, 1999; Zaid and Arias-Jimenez, 2002) and leading perennial fruit crop of arid and semi-arid regions worldwide (Barrow, 1998; Sirisena *et al.*, 2015).

The date palm's Fusarium wilt disease has been an emerging threat in Pakistan (Maitlo *et al.*, 2009; Abul-Soad, 2011). Therefore, considering the significance of the issue, this research study was designed to assess the wilt disease on date palms across the three provinces and to determine the etiology of the disease in Pakistan using pathological aspects.

The major date palm growing regions were surveyed in 2018 and 2019, and wilt was documented with the maximum incidence in Sindh province at 53.65%, followed by Khyber Pakhtunkhwa at 45% and Punjab at 36.15. The data on disease prevalence depicted that the situation is even worse, as Maitlo *et al*. (2009) reported. They reported only 50.17% incidence in Sindh, but no data were available on the date palm wilt from Punjab and KPK provinces. This study revealed that disease is now prevailing in all the areas where the date palm has been growing across the country. The characteristic symptoms observed include chlorosis, one-sided yellowing, fronds killing, brown-colored stripes on the dorsal side of rachis, strikes on vascular bundles, and red discoloration on the roots, and these wilt disease symptoms were very similar to the typical symptoms of Fusarium wilt described by the other pathologists from various parts of the world (Maitlo *et al.* 2009; Abdalla *et al.* 2010; Abul-Soad, 2011; El-Morsi *et al.* 2012 and Naheed *et al.* 2023)

Various species of *Fusarium* can cause wilts, i.e., *F. solani*, *F. eumartii*, *F. avenaceum*, *F. tabacinum*, and *F. sulphureum*, but *F. oxysporum* is mainly involved in causing wilts in a wide variety of important crops (Beckman 1987; Okungbowa and Shittu, 2012). This fungus comprises several important pathogenic and nonpathogenic species (Shishido *et al.*, 2005). The same pattern of Fusarium association (82.6%) was observed while isolating the fungi from the wilted plant tissues and proved as predominating fungus associated with date palm wilt. In this study, most isolates exhibited abundant floccose aerial mycelium of white to creamy White on PDA medium and yellowish to peach white and pale-violet coloration from the inverse of colony growth (figure). Conidia (microconidia, microconidia) were thin-walled hyaline. Microconidia were 3.9-13.6µm in length and 1.6-4.0 µm in width with elliptical, obovoid, oval, or reniform in shape, and have no or single septation. Macro conidia were 31.6-45.3 × 2.8-5.4 in size, fusiform with tapered ends; apical cells were straight to slightly curved most of them were narrower with 3-7 septations. The morphological characterization depicted typical characters of *F oxysporum*. The results coincided with Hafizi *et al*. (2013); Ambreen *et al.* (2022).

The mainly reported Fusarium spp causing wilt on date palm in the literature are *F. oxysporum, F. solani*, *F. Proliferatum* (Louvet and Toutain, 1981; Abdelhafez *et al.*, 1990; Khodair *et al.*, 1991; El-Abyad *et al.*,1993; Abbas and Mandeel, 1995; Mandeel *et al.*, 1995; Sangalang *et al.*, 1995; Mandeel *et al.*,2005; Maitlo *et al.*,2009; Cohen *et al.*, 2010; Jansson *et al.*, 2010; El-Morsi *et al.*,2012).

In the presented study, generated a networking based on a neighbor net algorithm to assess genetic variation within the *Fusarium oxysporum* population. Neighbor net algorithm

divided isolates of *Fusarium oxysporum* into four clades Bryant and Moulton (2004); Dara *et al*. (2023)

Fusarium oxysporum, frequently isolated from symptomatic date palm tissues, was used for pathogenicity confirmation, and it fulfilled all steps of Koch's postulates. The study proved *F. oxysporum*, a pathogen causing wilt on date palm in Pakistan, and endorsed the findings of Louvet and Toutain (1981); Plyler *et al*. (2000); Hernandez-Hernandez *et al.* (2010); El-Morsi *et al*. (2012), who also reported the same pathogen of this disease on date palms. However, the Etiology established in this study contradicts Maitlo *et al*. (2009); Matlo *et al*. (2021), who reported *F. solani* as a potential pathogen.

Conclusion: It is concluded that wilt disease prevails in date palms grown across the country, however the severity of the disease varies at different location of the surveyed provinces. The results depicted that Sindh province was the leading province regarding the incidence and severity of Fusarium wilt disease in date palms, followed by Punjab and KPK. Fusarium isolates were frequently obtained from symptomatic tissues of wilt affected date palms and subjected to Morpho-cultural characterization, which revealed that Fusarium isolates exhibited typical characteristics of *Fusarium oxysporum*, that fulfilled Koch's postulates and proved a potential pathogen for date palm wilt in Pakistan.

***Conflict of interest*:** All authors declare no conflict of interest.

***Authors' Contribution Statements*:** Abdul Rehman, Imran ul haq, and Iqrar Ahmad Khan planned the research, designed the experiment, and helped interpret the data. Nabeeha Aslam Khan conducted the experiments, recorded the data, and prepared the draft.

Acknowledgment: This study was funded by the Punjab Agricultural Research Board project (PARB Project # 802), "Evaluation and management of indigenous date palm genetic resources for economic traits, *Fusarium* wilt, and red palm weevil" to conduct research work.

REFERENCES

Abbas, J. and Q. Mandeel. 1995. Survey of Fusarium species in an arid environment of Bahrain III. Abundance and seasonal changes of Fusarium species in the terrestrial habitats of Bahrain Island. Arab Gulf Journal of Scientific Research 13:637-651.

Abdalla, M., A. Al-Rokibah, A. Morettiand and G. Mule. 2000. Pathogenicity of toxigenic Fusarium proliferatum from date palm in Saudi Arabia. Plant Disease 84:321-324.

Abdelhafez, A., M. Mazen and A. Galal. 1990. Glycophilic and cellulose-decomposing fungi from soils of Sinai Peninsula, Egypt. Arab Gulf Journal of Scientific Research 8:153-168.

Abdullah, S. K., L. Lorca and H. Jansson. 2010. Diseases of date palms (*Phoenix dactylifera* L.). Basra Journal for Date Palm Research 9:1-44.

Abul-Soad, A. A., W. A. Maitlo, G. S. Markhand and S. M. Mahdi. 2011. Date palm wilt disease (sudden decline syndrome) in Pakistan, symptoms and remedy, Bless Tree. 3:38-43.

Alfaro V., Emilia, F. Brent, G. Roling, Christian, J. K. Charlene, D. R. Jess Reed and M. L. Ricketts. 2018. An extract from date palm fruit (*Phoenix dactylifera*) acts as a co-agonist ligand for the nuclear receptor FXR and differentially modulates FXR target-gene expression in vitro. Public Library of Science (PLOS ONE) 13:190-210.

Al-Farsi, M., C. Alasalvar, M. Al-Abid, K. Al-Shoaily, M. Al-Amry and F. Al-Rawahy. 2007. Compositional and functional characteristics of dates, syrups, and their by-products. Food Chemistry 104:943-947.

Al-Farsi, M. Ali. and C.Y. Lee. 2008. Nutritional and functional properties of dates: a review. Critical Reviews in Food Science and Nutrition 48:877-887.

Ali-Mohamed, A.Y. and AS Khamis. 2004. Mineral ion content of the seeds of six cultivars of Bahraini date palm (*Phoenix dactylifera*). Journal of Agricultural and Food Chemistry 52:6522-6525.

Alkahtani, M., M. El-Naggar, E. Omer, M. Abedl-Kareem and M. Ammar. 2011. Effect of toxic Fusarium moniliforme on some biochemical component of some date palm cultivars. American Journal of Food Technology 6:730-741.

Al-Sadi, A., A. Al-Jabri, S. Al-Mazroui and I. Al-Mahmooli. 2012. Characterization and pathogenicity of fungi and oomycetes associated with root diseases of date palms in Oman. Journal of Crop Protection 37:1-6.

Al-Shahib, W. and R.J. Marshall. 2002. Dietary fibre content of dates from 13 varieties of date palm *Phoenix dactylifera* L. International Journal of Food Science & Technology 37:719-721.

Al-Shahib, W.and R.J. Marshall. 2003. The fruit of the date palm: its possible use as the best food for the future? International Journal of Food Science & Nutrition 54:247-259.

Ambreen, K., A. Faraz, N.A. Khan and N.A. Khan. 2022. Molecular identification of Fusarium spp causing wilt on Araucaria araucana and its management. Phytopathogenomics and Disease Control 1:67-72

Anjili, S., F. Channyaand I. Chimbekujwo. 2015. Fungi Associated with Post-harvest Spoilage of Date Palm (*Phoenix dactylifera* L.) in Yola, Adamawa State. International Journal of Research 14:…?

Augstburger, F., J. Berger, U. Censkowsky, P. Heid, J. Milz and C. Streit. 2002. Date Palm, 1st Ed. Naturland, Germany.

Baliga, M.S., B.R.V. Baliga, S.M. Kandathil, H.P. Bhat and P.K. Vayalil. 2011. A review of the chemistry and pharmacology of the date fruits (*Phoenix dactylifera* L.). International Food Research Journal 44:1812-1822.

Barrow, S.C. 1998. A monograph of phoenix L.(palmae: Coryphoideae). Roy. Bot. Gard. Springer 53:513-575.

Beckman, C. 1987. The nature of wilt diseases. American Phytopathological Society press.

Benzohra, I.E., M. Megateliand R. Berdja. 2015. Bayoud disease of date palm in Algeria: History, epidemiology and integrated disease management. African journal of Biotechnology 14:542-550.

Bryant, D., and V. Moulton. 2004. NeighborNet: an agglomerative algorithm for the construction of planar phylogenetic networks. Molecular Biology and Evolution 21:255–265.

Choi, Y.-W., KD. Hyde and W. Ho. 1999. Single spore isolation of fungi, Fungal Diversity.

Cohen, Y., S. Freeman, A. Zveibil, R.B. Zvi, Y. Nakache, S. Biton and V. Soroker. 2010. Reevaluation of factors affecting bunch drop in date palm. Journal of the American Society for Horticultural Science 45:887-893. Dara, M.Z.N., M.K. Sarwar and A. Nisar. 2023. Molecular probing of wilt etiology in *Thuja orientalis*, and pathogen confirmation through Koch's Postulates. Phytopathogenomics and Disease Control 1:79-83.

Dowson, V.H.W. 1982. Date production and protection with special reference to North Africa and the Near East. FAO Technical Bulletin Rome, Italy. No. 634.62.

Droge, W. 2002. Free radicals in the physiological control of cell function. Physiological Reviews journal published by the American Physiological Society 82: 47- 95.

El-Abyad, M., F. Migahed and E.M.U. El-Morsy. 1993. Egyptian mangrove mycoflora. Journal of Environmental Science (Mansora University) 5:25-47.

Elias, Y. M. 2008. Date - A Wonder Fruit of Arabia. Retrieved April 1, 2009, from http://ezinearticles.com/?Date---A-Wonder-Fruit-of-Arabia&id=1527461.

Elliott, M., E. Des Jardin, K. O'Donnell, D. Geiser, N. Harrison and T. Broschat. 2010. Fusarium oxysporum f. sp. palmarum, a novel forma specialis causing a lethal disease of Syagrus romanzoffiana and Washingtonia robusta in Florida. Plant Disease 94:31-38.

El-Morsi, M. E. A., M. A. M. Kamhawy and M. A. A. Sallam. 2009. Effectiveness of some organic compounds in controlling pathogenic fungi associated with roots of date palm offshoots in New Valley Governorate, Egypt. Assiut Journal of Agricultural Sciences 28:137-50.

El-Morsi, M., M. A. Rehab and J. A. Elmalek. 2012. Pathological Studies on Fungal Diseases of Date Palm in New Valley Governorate. Egypt. Journal of plant pathology 40:59-72.

FAOSTAT. 2019. FAO Statistical Database. Food and agricultural organization of the United Nation. Available online with updates at http://www.fao.org/faostat/en/#compare.

Gotz, J. C. I., H. W. Va Kan, I. Verspaget, C. B. Lamers Biemond and R. A. Veenendaal. 1996. Gastric mucosal superoxide dismutases in Helicobacter pylori infection. Gut 38:502-506.

Hadrami, A.E., F. Daayf and I. El Hadrami. 2011. Secondary metabolites of date palm. In: Jain S., Al-Khayri J. and J. D., (eds.), Date palm biotechnology. Springer, Dordrecht, pp. 653-674.

Hafizi, R., B. Salleh and Z. Latiffah. 2013. Morphological and molecular characterization of Fusarium. solani and F. oxysporum associated with crown disease of oil palm. Brazilian Journal of Microbiology 44:959-968.

Hameed, M.A. 2012. Inflorescence rot disease of date palm caused by Fusarium proliferatum in Southern Iraq. African journal of Biotechnology 11:8616-8621.

Haq, I.U.; Ijaz, S. Khan, N.A. Khan, I.A.; Ali, H.M.; Moya-Elizondo, E.A. Integrative Pathogenicity Assay and Operational Taxonomy-Based Detection of New Forma Specialis of *Fusarium oxysporum* Causing Datepalm Wilt. *Plants* **2022**, *11*, 2643. https://doi.org/10.3390/plants11192643

Hernandez-Hernandez, J., A. ESPiNo, J.M. RoDRÍGuEZ-RoDRÍGuEZ, A. Pérez-Sierra, M. Leon, P. Abad-Campos and J. Armengol. 2010. Survey of diseases caused by Fusarium spp. on palm trees in the Canary Islands. Phytopathologia Mediterranea 49:84-88.

Howard, F., D. Moore, R. Giblin-Davisand R. Abad. 2001. Insects on Palms CABI International, Wallingford, Oxon, UK.

Huson, D. H., and Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Molecular biology and evolution 23*:254-267.

Jakus, V. 2000. The role of free radicals, oxidative stress and antioxidant systems in diabetic vascular disease. Bratislavske lekarske listy 101:541-551.

Jansson, H., L. Lorca and S. K Abdullah. 2010. Diseases of date palms (*Phoenix dactylifera* L.). Basra Journal for Date Palm Research 9:1-44.

Javanmardi, J., C. Stushnoff, E. Locke, and J. M. Vivanco. 2003. Antioxidant activity and total phenolic content of Iranian Ocimum accessions. Journal of Food chemistry 83:547-550.

Khazaal, F. A., M. K. Ameen and H. A. Ali. 2019. Pathogenic fungi accompanied with sudden decline syndrome (wilting disease) of date palm tree (*Phoenix dactylifera* L.). Basrah journal of science 37: 376-397.

Khodair, A., A. Ramadani and A. Aggab. 1991. Occurrence and density of alkalophilic bacteria and fungi in saline

soils of Makkah District, Saudi Arabia. Arab Gulf Journal of Scientific Research 9:119-132.

Leslie, J. and B. Summerell. 2006. The Fusarium Laboratory Manual Blackwell Publishing, Ames, Iowa.

Louvet, J. and G. Toutain. 1981. Bayoud, Fusarium wilt of date palm. In: P.E. Nelson, T. A. Toussoun and R. J. Cook, (eds.), Fusarium: Diseases, Biology and Taxonomy. Pennsylvania State University Press, London, UK, pp. 13-20.

Maitlo, W., G. Markhand, A. A. Soad, M. Pathan and A. Lodhi. 2009. Comprehensive study on pathogen causing sudden decline disease of date palm (*Phoenix dactylifera* L.). In Proceeding of the first International Dates Seminar, Date Palm Research Institute, Shah Abdul Latif University Khairpur. pp. 71-76.

Maitlo, W.A., G.S. Markhand, A.A. Abul-Soad, A.M. Lodhi and M.A. Jatoi. 2014. Fungi associated with sudden decline disease of date palm (*Phoenix dactylifera* L.) and its incidence at Khairpur, Pakistan. Pakistan Journal of Phytopathology 26:67-73.

Maitlo, W.A., G. H. Jatoi, G. H. S. Channa, N. W. Awan, M. A. Abro , M. A. Bhagat, M. H. Memon, M. Chandio, A. Siddiqua. 2021. Pathogenicity test of different fungi on date palm causing sudden decline disease of date palm (*Phoenix dactylifera* L.) in Khairpur, Pakistan. International Journal of Biosciences 15:89-96.

Mandeel, Q., N. Ayub and J. Gul. 2005. Survey of Fusarium species in an arid environment of Bahrain. VI. Biodiversity of the genus Fusarium in root-soil ecosystem of halophytic date palm (*Phoenix dactylifera*) community. Cryptogamie, Mycologie 26:365.

Mandeel, Q.A., J.A. Abbas and A.M. Saeed. 1995. Survey of Fusarium species in arid environment of Bahrain II. Spectrum of species on five isolation media. Sydowia 47:223-239.

Mangan, T., A.S. Jamro, M.I. Kumbhar and A.G. Kalwar. 2016. Profit earned by date palm farmers and contractors in district Khairpur, Sindh, Pakistan. Science International 28:3.

Mani A, Handoo ZA, Livingston S. 2005. Plant-parasitic nematodes associated with date palm trees (*Phoenix dactylifera* L.) in the Sultanate of Oman. Nematropica 35:135-144.

Mansoori, B. 2012. Fusarium proliferatuminduces gum in xylem vessels as the cause of Date bunch fading in Iran. Journal of Agricultural Science and Technology 14:1133-1140.

Metlo, W.A., G. S. Markhand, Z. A. Chandio, Q. UA. Shaikh, L. Bux and W.A. Jatoi.2021. Occurrence of sudden decline disease of date palm (*Phoenix dactylifera* l.) in khairpur, Pakistan. Pakistan Journal of Phytopathology 33:75-81.

Mitscher, L. A., H. Telikepalli, E. McGhee and D. M. Shankel. 1996. Natural antimutagenic agents. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 350:143-152.

Morel, Y. and R. Barouki. 1999. Repression of gene expression by oxidative stress. Biochemical Journal 342:481-496.

Munoz, A. and S. Wang. 2011. Detection of Fusarium oxysporum f. Sp canariensis and F. proliferatum from palms in southern Nevada. In Phytopathology 101:124-125.

Naheed, T., S.F. Kazmi, H. Kanwal, U. Javed, S. Shahid, S. Fardus and A. Ilyas. 2023. The ethnobotanical study of native plants of district Layyah. Phytopathogenomics and Disease Control 2:15-22.

Narayanasamy, P. 2001. Plant pathogen detection and disease diagnosis. CRC press.

Nelson, P. E., T. A. Toussoun and W.F.O. Marasas. 1983. Fusarium species: an illustrated manual for identification.

Nelson, P.E., M.C. Dignani and E.J. Anaissie. 1994. Taxonomy, biology, and clinical aspects of Fusarium species. Clinical Microbiology Reviews 7:479-504.

Nixon, R. and J. Carpenter. 1978. Growing Dates in the United States. Department of Agriculture, Science and Education Administration, Washington, USA.

Offen, D., P. M. Beart, N. S. Cheung, C. J. Pascoe, A. Hochman, S. Gorodin, E. Melamed, R. Bernard and O. Bernard. 1998. Transgenic mice expressing human Bcl-2 in their neurons are resistant to 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. Proceedings of the National Academy of Sciences, USA 95:5789-5794.

Okungbowa, F. and H. Shittu. 2012. Fusarium wilts: An overview. Environmental Research Journal 6:83-102.

Plyler, T., G. Simone, D. Fernandez and H. Kistler. 2000. Genetic diversity among isolates of Fusarium oxysporum f. sp. canariensis. Journal of Plant pathology 49:155.

Rice-Evans, C. A., N. J. Miller and G. Paganga. 1997. Antioxidant properties of phenolic compounds. Trends Plant Sciences 2:152-159.

Sangalang, A., L. Burgess, D. Backhouse, J. Duff and M. Wurst. 1995. Mycogeography of Fusarium species in soils from tropical, arid and Mediterranean regions of Australia. Mycological Research 99:523-528.

Shinwari, M. A. 1993. Date palm. In: Macrae. R., R. K. Robinson and M. J. Sadler. (Eds.) Encyclopaedia of Food Science, Food Technology & Nutrition, London. Academic Press 2:1300-1305.

Shishido, M., C. Miwa, T. Usami, Y. Amemiya and K.B. Johnson. 2005. Biological control efficiency of Fusarium wilt of tomato by nonpathogenic Fusarium oxysporum Fo-B2 in different environments. Phytopathology 95:1072-1080.

Sirisena, S., K. Ng and S. Ajlouni. 2015. The emerging Australian date palm industry: Date fruit nutritional and

bioactive compounds and valuable processing by products. Comprehensive Reviews in Food Science and Food Safety 14:813-823.

Sudhersan, C. and M.A.J.P.-L.-. El-Nil. 1999. Occurrence of hermaphroditism in the male date palm. Palms 43:18-19. Summerell, B. A., B. Salleh, and J. F. Leslie. 2003. A utilitarian approach to *Fusarium* identification. Plant Disease 87:117-128.

Uenobe, F., S.-i. Nakamura and M. Miyazawa. 1997. Antimutagenic effect of resveratrol against Trp-P-1. Mutation Research Fundamental and Molecular Mechanisms of Mutagenesis 373:197-200.

Webster, J. and R.W.S. Weber, 2007. Introduction to fungi Third edition Cambridge University Press. Cambridge, UK.

Vayalil, P.K. 2012. Date fruits (*Phoenix dactylifera* Linn): an emerging medicinal food. Critical Reviews in Food Science and Nutrition 52:249-271.

Zaid, A. and E.J. Arias-Jiménez. 2002. Date palm cultivation FAO plant and protection paper. Food and Agricultural Organization of The United Nations, Rome.

Zaid, A. and P. de Wet. 2002. Pollination and bunch management. In: A. Zaid, (ed.), Date palm cultivation No. 156. FAO, plant production and protection, Rome, Italy.

Zapater, R. C. and A. Arrechea. 1975. Mycotic keratitis by Fusarium. A review and report of two cases. Ophthalmologica. Journal International d'ophtalmologie. International Journal of Ophthalmology, Zeitschrift fur Augenheilkunde 170:1-12.