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Morphological characterization and differential virulence of various Pakistani isolates of *Fusarium oxysporum* f. sp. *tuberosi*

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Abstract

Fusarium wilt of potato caused by *Fusarium oxysporum* is a soil borne fungus that causes huge losses to the potato growers in Pakistan. Limited information is available about this pathogen. Therefore, a survey was conducted to different areas of Punjab to collect the disease samples and they were morphologically identified on the basis of size and shape of microconidia, macroconidia and chlamydospores. Microconidia were oval shaped with no septation whereas macroconidia were straight in shape that were slight curved at apex having 3-5 septations. Pathogenicity test was performed on susceptible potato variety Desiree. Out of 43 isolates, three isolates showed high virulence i.e. W8 (70.80%), S1 (81.75%) and O1 (84.38%). Yield reduction by highly virulent isolates ranged from 59.92 % to 76.26 %. Ten isolates showed moderately virulent response W7 (53.08 %), O3 (55.76 %), TS1 (53.85 %), TS2 (56.14 %), BM3 (53.60 %), FD1 (44.35 %), CT2 (43.00%), O5 (43.0%), N1 (41.81%), and O2 (49.53 %). Yield reduction by moderately virulent isolates ranged from 45.58 to 52.42 %. Thirteen isolates *viz.* FD2, BM1, CT3, N2 , C1, C2, T2, B3, B4 , B1, B2, S5 and W4 yield reduction ranged from 9.64 % to 28.59 %. Three isolates were highly virulent, 5 exhibited moderately virulent and rest of the isolates were avirulent. The recent study will help for efficient management of the pathogen through host plant resistance.

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Introduction

Potato (*Solanum tuberosum*) belonging to family Solanaceae is fourth most important food crop in the world after wheat, maize and rice (Arslanoglu *et al.,* 2011). In Pakistan, it ranks third among food crops and fifth in total production of country (Malik, 1995). It is consumed as a vegetable and considered as a great supplement food source in Pakistan). In Pakistan, the production of potato has increased 6.3% due to increase in area cultivated. Potato is grown over an area of 169.8 thousand hectares with an annual production of 3084.3 tons (FAOSTAT, 2015). Its production shows a strong regional concentration in central and northern plains of Punjab. In Punjab, its cultivation is concentrated mainly in irrigated districts as compared to rainfed districts of province i.e., Okara, Sahiwal, Kausur, Sheikupura, Jhang, Narowal, Lahore, Pakpattan, Gujranwala, T. T. Singh and Khanewal (Ahmad *et al.,* 2004).

Although the economic value of potatoes is clear, potato crops are susceptible to more than 40 pests and diseases that reduced crop yield (Fierse*t al.,* 2012). Among the fungal stresses, black scurf, common scab and wilt are the major soil borne diseases of Pakistan (Bhutta, 2008). Among wilts, *Fusarium* wilt is caused by *Fusarium oxysporum* are important that causes economic losses to potato growers (Turkensteen, 1994). The fungus causes upto100 yield losses world widely (Santos *et al.,* 2002). In Pakistan, the losses due to wilts accounts more than 30 % (Nauman, 1990).

Accurate and rapid identification of pathogen is necessary for appropriate management of diseases. Therefore, the pathogen was first characterized morphologically and various isolates were subjected to pathogenicity test to check the virulence status of various isolates for efficient management of disease.

Materials and methods

Disease survey

During 2011-2012, a survey of different areas of Punjab was conducted to calculate the disease incidence and its distribution (Table 1). The major potato growing areas included were Sahiwal, Okara, Depalpur, Kasur, Sialkot, Gujarawala, Chiniot and Taxila. The survey was carried out at two stages i.e. seedling stage and adult plant stage. Total 9 areas with 14 locations were surveyed by travelling along the road side. From each location, 3 fields were selected and from each field 15 samples were collected systematically by using quadrat on the basis of symptoms. Wilt incidence was calculated using following formula.

$$\text{Disease incidence (\%)} = \frac{\text{Number of Infected Plants}}{\text{Total Number of Plants}} \times 100$$

The symptoms shown were yellowing of the leaves, browning of vascular system which resulted in the stunting, chlorosis and ultimately death of plant. The samples were brought into Fungal Pathology lab, Department of Plant Pathology, PMAS- Arid Agriculture University Rawalpindi and refrigerated at 4 ˚C for isolation of pathogen.

Isolation, purification and preservation of Fusarium oxysporum f. sp. tuberosi

Total 675 samples showing characteristic symptoms of wilt were processed further for isolation of pathogen on Potato Dextrose Agar (PDA) diseased roots were cut into small pieces (5 cm) and surface sterilized by dipping in 10% sodium hypochorite solution and blot dry.

The sterilized pieces were then placed on potato dextrose agar medium in petri dishes and incubated at 25 ° C for 4-5 days in dark. After 4 days, mycelia of pathogen were grown on the cut root samples. The cultures were purified by taking hyphal tips of growing mycelia from the colonies (Nelson *et al.,* 1983) and transferring it to new PDA plates. The Pure cultures were re-cultured on Malt Extract Medium for short period preservation whereas long term preservation was done on silica gel following the procedure of Perkins (1962) with some modifications.

Morphological characterization

After preservation, isolates were morphologically characterized by using Laboratory manual of

Leslisand Summerell (2006) and Toussoun and Nelson (1976). Five mm mycelial disc was taken from the periphery of the each isolate by using sterilized cork borer and placed in the center of already poured PDA medium plates. The plates were then incubated at 25 ˚C. For observation of characters of isolate, temporary glass slide mount with each isolate was made in lactophenol blue solution and studied under light microscope at 100X magnification.

Morphological characters studied were colony color, growth habit, size and shape of micro-conidia, size and shape of macro-conidia, shape of apical cells of macroconidia, septation in macroconidia, and diameter of chlamydospore. Five readings of each parameter were taken.

Pathogenicity test

Inoculum preparation

Isolates were tested for their pathogenicity on available potato germplasm collected from National Agriculture Research Centre (NARC), Islamabad. For inoculum preparation, mycelium was transferred to 150 ml of potato Dextrose Broth (PDL) and incubated at 25° C for 5 days in rotary shaker (120 rpm).

The liquid culture was filtered and conidial suspension was adjusted to 10^7 spores per ml by a haemocytometer.

Pathogenicity test was conducted in greenhouse on susceptible genotype of potato (*i.e.,* Desiree) by using fungal isolates by the method of Ayed *et al.,* 2006. One sprouted tuber of almost same size was planted in each of plastic pots containing sterilized potting mixture (sand/clay/farmyard manure, 1:1:1) and kept at 23 ± 2°C.

The potting mixture was sterilized following procedure of Naz *et al.* (2008). Two weeks after emergence plant inoculation was done by irrigation with 100 mL^{-1} conidial suspension. Non-inoculated control plants were inoculated similarly with 100 mL sterile distilled water. The experiment was conducted using completely randomized design (CRD) with 3 replications.

Disease parameters

After inoculation, data of following three parameters *viz.* percent disease severity index and yield reduction were recorded.

The disease severity was recorded 27 days after inoculation and continued till maturity using modified scale of Silva and Bettiol, (2005) as 1 = no symptoms, 2 = 1-20 % yellowing and wilting, 3 = 21-40% yellowing and wilting, 4 = 41-60 % yellowing and wilting, 5 = 61-80 % yellowing and wilting, 6 = 100 % yellowing and Plant die. The virulence of pathogen was categorized on the basis of disease symptoms and rating scale as DSI = 1 (Avirulent), DSI ≤3 (Low virulent), DSI > 3-4 (Moderately virulent) and DSI = 6 (Aggressive / Highly Virulent). The severity index was calculated by formula of Naz *et al.* (2008).

Disease severity index

$$DSI\,(\%) = \frac{1(n1) + 2(n2) + 3(n3) + 4(n4) + 5(n5) + 6(n6)}{N \times 6} \times 100$$

Where

n_1 = Number of infected leaves scored as 1

n_2 = Number of infected leaves scored as 2

n_3 = Number of infected leaves scored as 3

n_4 = Number of infected leaves scored as 4

n_5 = Number of infected leaves scored as 5

n_6 = Number of infected leaves scored as 6

N = Total number of leaves

6 = Higest scale value

Plant height was recorded two months after inoculation. Pathogen isolation was done at the end of bioassay.

$$\text{Yield reduction (\%)} = \frac{\text{Weight of healthy potato tubers} - \text{Weight of diseased potato tubers}}{\text{Weight of healthy potato tubers}} \times 100$$

Data was taken using completely randomized design (CRD) with three replications and analysed

statistically using software Statistix 8.1. Mean comparison was done using LSD test.

Results and discussion

Disease survey

A survey of different potato growing areas of Punjab was conducted to collect the diseased samples of potato during year 2011-2012. Total 14 locations and 42 fields were surveyed from which the diseased samples were collected on the basis of symptoms. Highest disease incidence was recorded in Okara (24 %), Depalpur (23 %) and Faisalabad (20 %) whereas the average wilt incidence in Chiniot (19 %), Sahiwal (15.08 %), Daska (11%), Taxila (10.25%) and Sialkot (9.3 %) was noticed (Table 1).

Table 1. Areas surveyed along with their collection sites and recovered isolates.

Sr. No.	Areas surveyed	Location	Isolates	Disease incidence (%)
1	Sahiwal	Punjab seed Corporation Farm	S1, S2,S3	24
		100/9L	S4, S5	11.5
		112/6R	S6	10
2	Okara	Sub-urb areas	O1, O2, O3	24
3	Depalpur	Sub-urb areas	O4,O5	23
4	Faisalabad	Chak Jumara	FD5, FD6	20
5	Sialkot	Nanderpur	N1, N2	9.5
		Tebinehr shah di	TS1, TS2	10
		ChakTiyar	CT1, CT2, CT3	8.25
		Bara majra	BM1, BM2, BM3	9.50
6	Daska	Bhopalwala	B1, B2,B3,B4,B5,B6	11
7	Wazirabad	Johara	W1, W2, W3, W4,W5,W6, W7,W8	10.50
8	Chiniot	Haji and sons Farms	C1, C2, C3	19
9	Taxila	Taxila Fields	T1,T2,T3	10.25

The survey of different areas of Punjab showed that disease is prevailing in all potato production areas. As wilt incidence vary from one area to another area, this is due to the cultivation of different potato varieties and environmental conditions of that areas (Sayed *et al.,* 2008). Soil-borne fungal diseases have been reported as second major cause of loss of potato crop (PARC, 1998). Wilt diseases caused by *Fusarium avenacea rum, F. oxysporum, F. solani. Verticillum alboat rum* and *V. dahlia* results in losses up to 30 % in Pakistan. The areas which showed minimum wilt incidence must be addressed properly so that the crop must be saved from further losses.

Shape and size of micro and macro-conidia

The size of micro and macro-conidia was measured randomly 5 times using ocular micrometer under light microscope at 100X magnification. While studying morphological variations among micro and macroconidia and chlymydospore, the mean length and width of microconidia ranged from 4.10 x 3.62 to 5.12 x 10.28 µm. Most of micro-conidia were oval shaped with no septation while 3 isolates (S2, O6 and C3) were having oval and slightly curved spores (Table 2).

Macroconidia produced in sporodochia were observed after 15-20 days of incubation in pure cultures. The mean length and width of macroconidia was ranged from 3.72 x 11.36 to 6.3 x 30.2 µm. Most of macroconidia were straight in shape with 3-5 septations whereas 13 isolates (W2, W3, W6, S2, B1, B3, T1, O2, C2, C3, CT1, FD1 and BM3) were slightly curved at apex. The mean diameter of chlamydospore was ranged from 7.4 to 13.6 µm while formation of the chlamydospore was singly in most of the isolates.

Chlamydospores characters

Chlamydospore is also important character to identify

Fusarium oxysporum species. It is thick walled cell that is produced on sporodocial macroconidia in culture after 25-30 days of incubation singly, in pairs, in short chains and in clusters. Almost all the isolates produced chlamydospore. The mean diameter of chlamydospore ranged from 7.4 to 13.6 µm while formation of the chlamydospore was single in most of the isolates.

Same characters were studied by Chehri *et al.* (2011). They proved that microconidia were oval shape with no septation and macro-conidia were straight or curved with 3-5 septations. Altaf *et al.* (2014) proved that shape of micro-conidia were two-celled oval and oval-pyriform. Amini and Sidovich (2010) reported white cottony to pink often with purple color of mycelium. Microconidia were oval in shape whereas macro-conidia were thin walled with 3-5 septations and pointed at both ends. Chlamydospore was smooth and rough walled and no sexual stage was observed in isolates (Table 2).

Table 2. Morphological variation in micro-conidia, macro-conidia and chlamydospores of *Fusarium oxysporum* f. sp. *tuberosi.*

Sr. No.	Isolate (s)	Micro-conidia		Macro-conidia					Chlamydospore	
		Size Mean	Shape	Size	Shape	Apical	Foot	Septation	Diameter (um)	Formation
1	W1	6.7×2.75	Oval	12.94x5.58	Straight	curved	foot	3-5	11.6	Singly, short chains
2	W2	6.42×2.90	Oval	17.82x3.72	Slightly curved	,,	,,	,,	11.0	,,
3	W3	5.50×3.00	Oval	24.69x4.68	Slightly curved	,,	,,	,,	9.0	Singly
4	W4	5.72×2.75	Oval	20.86x5.14	Straight	,,	,,	3	11.4	,,
5	W5	6.31×2.93	Oval	22.50x5.12	-	,,	,,	,,	11.2	Singly, short chain, clusters
6	W6	6.24×2.83	Oval	21.20x4.90	Slightly curved	,,	,,	,,	12.8	,,
7	W7	10.28×5.12	Oval	30.2x6.38	Straight	,,	,,	,,	10	Singly
8	W8	6.68×3.57	Oval	20.82x5.20	-	,,	,,	,,	14	,,
9	S1	5.78×2.95	Oval-	19.70x4.85	-	,,	,,	3-5	12	Singly, short chain, clusters
10	S2	6.55×2.31	Oval, slightly curved	27.73x4.66	Slightly curved	,,	,,	3	9	Singly
11	S3	4.70×3.13	Oval	13.49x3.26	Straight	,,	,,	3-5	8	,,
12	S4	5.80×4.04	Oval	23.40x4.94	-	,,	,,	3	12	Singly, short chains, clusters
13	S5	5.75×1.99	Oval	16.80x3.75	-	,,	,,	3	7	Single
14	S6	5.29×3.02	oval	11.36x3.72	Straight	,,	,,	3-4	8	Single
15	B1	4.10×3.62	Oval	30.00x5.16	Slightly curved	,,	‘’	3	8	Single
16	B2	5.32×3.04	Oval	13.10x5.60	Straight	,,	,,	3-5	11.2	Singly, short chains
17	B3	5.76×3.13	Oval	19.10x4.30	Slightly curved	,,	,,	,,	11.2	,,
18	B4	4.47×2.30	Oval	22.70x4.80	Slightly curved	,,	,,	,,	9.6	Singly
19	B5	4.91×2.40	Oval	26.30x5.20	Straight	,,	,,	3	11.4	,,
20	B6	5.01×3.03	Oval	27.90x5.20	-	,,	,,	,,	11.2	Singly, short chain, clusters
21	T1	5.14×2.94	Oval	15.90x5.20	Slightly	,,	,,	,,	12	,,

					curved					
22	T2	8.00×5.10	Oval	30.40x6.30	Straight	,,	,,	,,	12.2	Singly
23	T3	6.24×3.80	Oval	21.00x5.10	-	,,	,,	,,	12	,,
24	O1	5.60×2.30	Oval	22.80x5.00	-	,,	,,	3-5	9.4	Singly, short chain, clusters
25	O2	6.00×2.70	Oval, slightly curved	27.50x5.00	Slightly curved	,,	,,	3	8.8	singly
26	O3	4.10×3.07	Oval	15.00x4.20	Straight	,,	,,	3	8.8	Singly
27	O4	5.90×3.20	Oval	22.70x5.10	-	,,	,,	3	8.8	Singly
28	O5	5.10×3.15	Oval	17.10x4.20	-	,,	,,	3	8.8	Singly
29	C1	5.89×2.33	oval	11.60x3.70	Straight	,,	,,	3	8.8	Singly
30	C2	5.20×3.49	Oval	28.40x5.10	Slightly curved	,,	,,	3	8.8	Singly
31	C3	6.42×3.02	Oval, slightly curved	12.80x4.90	Slightly curved	,,	,,	3	8.8	Singly
32	N1	6.86×3.00	Oval	18.00x6.40	Straight	,,	,,	3	8.8	Singly
33	N2	5.76×3.40	Oval	25.80x5.00	-	,,	,,	3	8.8	Singly
34	TS1	5.40×2.84	Oval	24.50x5.30	-	,,	,,	3	8.8	Singly
35	TS2	6.20×3.18	Oval	26.72x5.00	Straight	,,	,,	3	8.8	Singly
36	CT1	6.14×2.85	Oval	24.50x5.00	Slightly curved	,,	,,	3	8.8	Singly
37	CT2	9.48×5.22	Oval	23.50x5.60	Straight	,,	,,	3	8.8	Singly
38	CT3	6.08×3.75	Oval	20.70x5.30	Slightly curved	,,	,,	3	8.8	Singly
39	FD1	5.60×3.19	Oval	20.80x5.30	Slightly curved	,,	,,		12.2	Singly
40	FD2	6.70×2.40	Oval	24.90x4.20	Straight	,,	,,	3	8.4	,,
41	BM1	4.70×2.90	Oval	14.60x3.80	-	,,	,,		9.4	Singly, short chain, clusters
42	BM2	6.00×3.90	Oval	20.60x4.50	Slightly curved	,,	,,		7.8	,,
43	BM3	5.10×2.70	Oval	14.20x4.30	Straight	,,	,,		11.2	Singly

Pathogenicity test

To find out virulence of the 43 morphologically characterized isolates, pathogenicity test was performed on susceptible variety "Desiree". Three isolates were highly virulent i.e. W8 (70.80%), S1 (81.75%) and O1 (84.38%). The yield reduction by highly virulent isolates ranged from 59.92 % to 76.26 %. Ten isolates showed moderately virulent response W7 (53.08 %), O3 (55.76 %), TS1 (53.85 %), TS2 (56.14 %), BM3 (53.60 %), FD1 (44.35 %), CT2 (43.00%), O5 (43.0%), N1 (41.81%), and O2 (49.53 %). The yield reduction by moderately virulent isolates ranged from 520.42 % to 45.58 %. Thirteen isolates viz. FD2 (12.75 %), BM1 (14.72 %), CT3 (13.56 %), N2 (14.32 %), C1 (13.36 %), C2 (13.16 %), T2(13.27 %), B3 (25.76 %), B4 (25.87 %), B1 (10.45 %), B2 (19.25 %), S5 (15.23 %) and W4 (15.77 %) with yield reduction ranged from 9.64 % to 28.59 %. Whereas the rest of isolates proved non- pathogenic with yield reduction of 6.72 % to 8.32%.Manici and Cerato (1994) studied pathogenicity of eight isolates of *F. oxysporum* f. sp. *tuberosi*. Green house results showed that all isolates infect vascular tissues of the plant but no plant wilt. This showed that under unfavorable conditions, pathogen did not produce symptoms.

Ayed *et al.* (2006) studied pathogenicity of 13 isolates of *F. oxysporum* f. sp. *tuberosi*. He concluded that all isolates produced typical symptoms of *Fusarium* wilt. The symptoms were appeared at 2-22 ° C within 27 days of inoculation. Inoculated plants collapse wilted

three months after planting because of browning and clogging of vascular tissues. Syad *et al.* (2008) carried out pathogenicity test against 23 isolate of *Fusarium oxysporum,* eight isolates of *Fusarium solani* and four isolates of *Rhizoctonia solani* and proved that isolates varied in their virulence behavior.

The symptoms appeared after 40 days of inoculation. Symptoms appeared first on lower leaves, then made progress upward. Out of 23 isolates, 14 isolates of *F. oxysporum* proved highly virulent, 1 isolate of *F. solani* and 3 isolates of *R. solani.* Amini and Sidovich (2010) conducted pathogenicity test against *Fusarium* isolates and recorded that disease symptoms appeared two weeks after inoculation. The same fungus was re-isolated from diseased tissues of the plant. Zaheer *et al.* (2012) studied pathogenic property of three *Fusarium* isolates after inoculating on potato plant and proved that FCBP-434 was most pathogenic isolate.

Table 3. MDSI (%) and yield reduction (%) in "Desiree" as shown in Pathogencity test.

Sr.No.	Isolate	Mean diseases Severity index (%)	Virulence level	Yield reduction (%)
1	W1	0.000k	AV	6.72fg
2	W2	0.000k	AV	7.72fg
3	W3	53.500cd	MV	62.64ab
4	W4	15.777ij	LV	14.006fg
5	W5	0.000k	AV	6.72fg
6	W6	0.000k	AV	7.5fg
7	W7	53.083cd	MV	55.83abcd
8	W8	70.807b	HV	61.86ab
9	S1	81.750a	HV	59.92abc
10	S2	0.000k	AV	6.74fg
11	S3	0.000k	AV	6.74fg
12	S4	0.000k	AV	7.74fg
13	S5	15.233j	LV	28.59cdef
14	S6	26.240g	LV	13.61ef
15	B1	10.453j	LV	6.61fg
16	B2	19.250hi	LV	13.812ef
17	B3	25.760g	LV	14.006ef
18	B4	25.873g	LV	14.20ef
19	B5	0.000k	AV	7.42fg
20	B6	0.000k	AV	8.32fg
21	T1	15.183ij	LV	11.88f
22	T2	13.273j	LV	9.64fg
23	T3	24.833gh	LV	19.06def
24	O1	84.380a	HV	76.26a
25	O2	49.533de	MV	20.427def
26	O3	55.767c	MV	37.158bc
27	O4	13.223j	LV	13.81ef
28	O5	43.000f	MV	23.15cdef
29	C1	13.367j	LV	15.95ef
30	C2	13.167j	LV	12.061ef

31	C3	81.870a	HV	64.39ab
32	N1	41.817f	MV	34.82bcdef
33	N2	14.323ij	LV	12.83ef
34	TS1	53.857cd	MV	50.58abcd
35	TS2	56.140c	MV	29.76bcdef
36	CT1	0.000k	AV	6.52fg
37	CT2	43.000f	MV	49.22bc
38	CT3	13.567ij	LV	8.55fg
39	FD1	44.350ef	MV	28.19bcdef
40	FD2	12.750j	LV	12.64ef
41	BM1	14.727ij	LV	11.86fg
42	BM2	0.000k	AV	8.52fg
43	BM3	53.6000gh	MV	45.58abcd
44	Control	0.000k		0.000g
	LSD	5.69		38.57

LSD= 0.05 (Means followed by same letters are non-significant) (HV= Highly Virulent, MV= Moderately Virulent, LV= Low virulent, AV= Avirulent).

Conclusion

Fusarium wilt of potato caused by *Fusarium oxysporum* is a soil borne fungus that causes huge losses to the potato growers in Pakistan. The present study reports the distribution of *Fusarium* wilt in major potato growing areas of the Punjab. Forty three isolates were recovered from infected samples.

Virulence of isolates was checked through pathogenicity test and three isolates showed highly virulence response that will be taken for management of disease through novel methods including host resistance, biological control, fungicides and use of biofumigant crops.

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