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SURVEILLANCE AND MORPHOLOGICAL CHARACTERIZATION OF FUSARIUM ISOLATES ASSOCIATED WITH LENTIL WILT

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A B S T R A C T

Lentil *(Lens culinaris* Medikus) is an important dietary source of protein in many parts of the world, especially South Asia including Pakistan. The crop is vulnerable to wilt, a serious soil-borne threat incited by the fungus *Fusarium oxysporum* f. sp. *lentis.* In view of the potential threat *Fusarium* wilt can pose to lentils, these studies were considered essential, and consequently this project was initiated for the disease assessment, morphological characterization of recovered isolates the pathogen and determination of their pathogenicity. Nine districts with 28 locations were surveyed during the crop season of year 2012-13, out of which 21 showed 100% disease prevalence. In total, 15 isolates of *F. oxysporum*f. sp. *lentis* were recovered. The length and width of micro-conidia of these isolates ranged from 4.38 to 6.65 µm and 2.31 to 3.2µm, respectively. These were oval shaped for all the isolates except for isolate FOL-6 (2 celled oval) and FOL-10 (oval pyriform). The length and width of macro-conidia ranged from 9.90 to 29.73 µm and 3.02 to 5.06 µm, respectively. The shape of macro-conidia was straight for all the isolates except FOL-6 and FOL-12, which were slightly curved. The mean diameter of chlamydospores ranged from 7.0 to 15.8 µm. During pathogenicity testing of 15 isolates on cv. Masoor-93, the mean disease severity index ranged from 0 (FOL-1, FOL-8 and FOL-11) to 0.72% (FOL-3) and on line ILL 4605 ranged from 0 (FOL-1, FOL-3, FOL-5, FOL-8, FOL-10 and FOL-13) to 0.66% (FOL-2). This line proved to be more resistant than Masoor-93.

Keywords: Lentil, *Fusarium* wilt, Punjab, Pakistan.

INTRODUCTION

Lentil *(Lens culinaris* Medikus) is a high value pulse crop and is an important part of the diet as a source of protein in many parts of the world, especially South Asian including Pakistan. In Pakistan, lentil is cultivated over an area of 19.6 thousand hectares with an annual production of 9.7 thousand tons which is very low as compare to other lentil growing countries (GOP, 2013). The crop is vulnerable to a number of biotic and abiotic factors, which adversely affect seed yield and its quality. Among them, the most significant and serious soil-borne threat is the occurrence of vascular wilt disease incited by the fungus *Fusarium oxysporum* Schlect. ex. Fr. f. sp. *lentis* Vasudeva and Srinivasan (Tuberculariaceae, Hyphales). It is the most significant disease of lentils worldwide and is one of the worst diseases of lentil in Asia (Saxena, 1993). The disease can cause total failure of the crop particularly in a hot spring and dry, warm summer (Agarwal *et al*., 1993). The fungus is seed/ soil-borne and may live in the soil for many years. Chlamydospores are the most likely major fungal structures for extended survival (Erskine and Bayaa, 1996). Several species of *Fusarium* have been isolated from wilted lentil plants (Tosi and Cappelli, 2001). Among these the host range of *F. oxysporum*f. sp. *lentis*is limited to lentil). *F. oxysporum* f. sp. *lentis* has an enormous variability and may produce three types of spores, e.g. micro-conidia, macro-conidia (based on their respective sizes) and chlamydospores. The diverse isolates of *Fusarium* have been categorized on the basis of their nutritional requirements (Khare *et al*., 1975), temperature, sensitivity to chemicals (Agarwal and Khare, 1977), and morphology and aggressiveness (Taheri *et al*., 2010). Various aspects regarding the development of *F. oxysporum* f. sp. *lentis* have been

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investigated by a number of different researchers (Erskine and Bayaa, 1996) who found that an optimum temperature of 22°C for growth of the fungus and little soil moisture with somewhat high soil temperature play key role in symptom development. Disease was promoted by sandy loam compared to clay soil, and mortality of lentil plants increased with increasing pH up to pH 7.5, beyond which disease severity decreased.

Information concerning the latest status of wilt disease *Fusarium oxysporum*, characterization of its isolates is least known. In view of the potential threat fusarium wilt can pose to lentil production, this study was considered essential and initiated with the objectives of assessing disease incidence/severity, investigation of pathogenicity of *Fusarium* isolates from lentil and morphological characterization of *Fusarium* isolates recovered from infected lentil plants collected during crop season of 2012-13.

MATERIALS AND METHODS

Survey and sampling: During the year 2012-13, districts of Punjab province viz. Chakwal, Attock, Gujrat, Sialkot, Narowal, Bhakkar, Layyah, Mianwali and Khushab were surveyed for the estimation of disease prevalence and incidence. Lentil wilt diseased samples were gathered randomly on the basis of symptoms from wilt affected fields and sampling was performed with the help of a quadrate. Samples were placed into polythene bags and samples were appropriate labeled to indicate location, sample number and date of collection. The samples were brought to Fungal Plant Pathology Laboratory of the Department of Plant Pathology, PMAS-AAUR and were placed in a refrigerator set at 4°C for further processing.

Separation, identification and preservation of cultures: The recovery of the linked fungi with the diseased samples was made from the collected wilted lentil plant roots/stem on nutritional artificial media (PDA). The suspected diseased roots of lentils were cut into small pieces (10 mm) and surface sterilization was performed with Clorox (10%). Sterilized pieces were transferred on petri dishes (five pieces per plate) containing sterile PDA. Petri dishes were incubated at 25±2°C for 4 days and fungi were further purified and identified according to the identification keys on genus *Fusarium*(Leslie and Summerell, 2006). The identified isolates of *Fusarium* were then re-cultured on Malt Extract Agar (MEA) medium using single spore technique and preserved through silica gel method (Leslie and Summerell, 2006).

Morphological characterization: From puce culture, temporary slide mounts were made in lacto phenol solution and were examined under light microscope (Nikon YS100) at 100X magnification. Colony color, growing habit, pigmentation, presence/absence of concentric rings, size of micro and macro-conidia, shape of micro and macro-conidia, phialide, shape of apical and basal cells of macro-conidia, septations in macro-conidia, diameter and formation of chlamydospores and inter-septal distance were observed for each test isolate for the morphological characterization of the isolates in question (Leslie and Summerell, 2006).

Pathogenicity determination: The verified and morphologically characterized isolates of *Fusarium* were further tested for their virulence on lentil germplasm i.e. line ILL-4605 and cultivar Masoor-93 under controlled glass house conditions.

Inoculum of each *Fusarium* isolate was prepared by growing the isolates in Erlenmeyer flasks (100 ml) filled with 50 ml potato-dextrose Broth. All flasks were inoculated with a 5 mm diameter plug (mycelium) cut from their pure culture. The inoculated flasks were placed on rotary shaker at 120 rpm for 3 days. The conidial suspensions were adjusted to 1 x 10^7conidia/ml using haemocytometer (14). The seeds of ILL-4605 and cv. Masoor-93 were sterilized and planted in pots (10 seeds/pot) containing sterilized potting mixture (sand/farmyard manure, 1:1;). After 15 days, the seedlings were uprooted cautiously, dipped into the inoculum and were sown in pots. The trial was conducted in completely randomized design (CRD) along with 3 replications. By means of a scale of 1-9 (4) where 1 = no symptoms, 3 = yellowing of the basal leaves only, 5 = yellowing of 50 percent of the foliage, 7 = complete yellowing of the foliage, flaccidity of the top leaves and partial drying and 9 = whole plant or part of the plant wilted and/or dried, the disease severity was recorded starting from the 6th day and continuous for two months. The isolates of *Fusarium* were further characterized/grouped like low virulent, moderately virulent and highly virulent on a 1-3 scale range, 4-6 scale range and with 7-9 scale range, respectively.

RESULTS AND DISCUSSION

Two localities of Khushab district (Hassan Pur Taiwana and Roda) and one locality of Sialkot district (Chowinda) were free from lentil wilt diseases. A disease prevalence of 100% was observed for districts Chakwal, Attock, Gujrat, Bhakkar and Layyah. The minimum mean disease

incidence among surveyed areas was 0% in Chowinda (Sialkot), HasanPur Tiwana and Roda areas of district Khushabwhereas the maximum mean disease incidence was 63.75% in FatehPur (Layyah) followed by 33.33% each in Pipli (Khushab) and Agronomic Research Station, Karor (Layyah). The maximum disease incidence range of 60-70% was also observed in FatehPur (Layyah) followed by Pipli (Chakwal) 30-40%. The minimum range of 5-10% was found on Jand (Attock) as shown in table 1.

Table 1. Lentil wilt Incidence in various districts of Punjab province during crop season of year 2012-2013.

Districts	Area/ Location	Farms visited	Disease Prevalence (%)	Disease incidence (%)	
				Range	Mean
Chakwal	Sehgalabad	5	100	10-25	16.40
	Dhudial	4	100	15-30	21.25
	Piplee	3	100	30-40	33.33
Attock	Tanazya Dam	5	100	5-15	9.80
	Khaur	3	100	15-20	18.33
	Jand	3	100	5-20	11.00
	ThattiRehmo	3	100	10-20	13.33
Gujrat	DaulatPur	3	100	10-15	13.33
	JalalpurJattan	5	100	10-25	14.00
	Bhagowal	2	100	10-15	12.5
Sialkot	Chowinda	4	0	0-0	0
	Pasrur Road	2	100	20-25	22.5
Narowal	Dongian	3	100	10-15	12.5
	Behble	4	80	0-50	25.00
	Zafarwal Road	5	100	10-15	12.00
Mianwali	Harnoli	5	100	15-30	22.00
	Piplaan	4	80	0-40	21.25
	Chashma	4	60	0-50	23.75
Bhakkar	Darya Khan	5	100	5-30	16.00
	ARI	5	100	15-30	22.00
	Mankera	4	100	10-15	13.00
Layyah	FatehPur	4	100	60-70	63.75
	ChokAzam	5	100	25-50	34.00
	ARS, Karor	3	100	25-50	33.33
Khushab	AdhiKot	6	35	0-48	26.83
	NurPur	5	100	10-15	12.00
	Hassan PurTiwana	4	0	0-0	0
	Roda	5	0	0-0	0

In total, 15 isolates of *Fusarium*were recovered which are coded from FOL-1 to 15 and all were *F. oxysporum*f. sp. *lentis.* Among various districts, 3 isolates were recovered from each of Chakwal and Bhakkar. Two isolates each were recovered from three districts (Gujrat, Narowal and Layyah) whereas only one isolate each was recovered from the remaining districts of Attock, Sialkot and Mianwali (Table 2). Agrios (2005) reported that most of them belong to *F. oxysporum.* Also, Khare *et. al.* (1979) documented several *Fusarium* spp. from wilted lentils but in our case all belongs to *F. oxysporum.* Colony morphology, pigmentation etc. cannot be used in differentiating species and are helpful in establishing the cultivars only after substantial acquaintance with the genus. Yet, these characteristics can be the starting point towards species identification.

Most of these isolate exhibited white colonies except FOL-7 (whitish colony), FOL-9 (creamy white colony), FOL-10 and FOL-11 (pink colony). Similarly, except isolates FOL-10 and FOL-11 with flat growth habit and isolates FOL-12 and FOL-13 with compact growth habit, all the isolates showed fluffy growth habit. In terms of

pigmentation, pink and peach pigmentation were observed in FOL-5 and FOL-9 respectively while rest isolates showed no pigmentation at all. Only FOL-5 and FOL-6 showed concentric rings. Nine isolates (FOL-1, FOL-2, FOL-3, FOL-7, FOL-8, FOL-9, FOL-11, FOL-12 and FOL-13) took eight days and five (FOL-4, FOL-5, FOL-6, FOL-14 and FOL-15) took nine days to fill 9 cm plate. The length of micro-conidia ranged from 4.37 (FOL-6) to 6.65 µm (FOL-12) whereas the width of micro-conidia ranged from 2.31 (FOL-11) to 3.82 µm (FOL-8). These micro-conidia were oval shaped for all the isolates except for FOL-6 (2 celled oval) and FOL-10 (oval pyriform) (Table 2 and Fig 1).

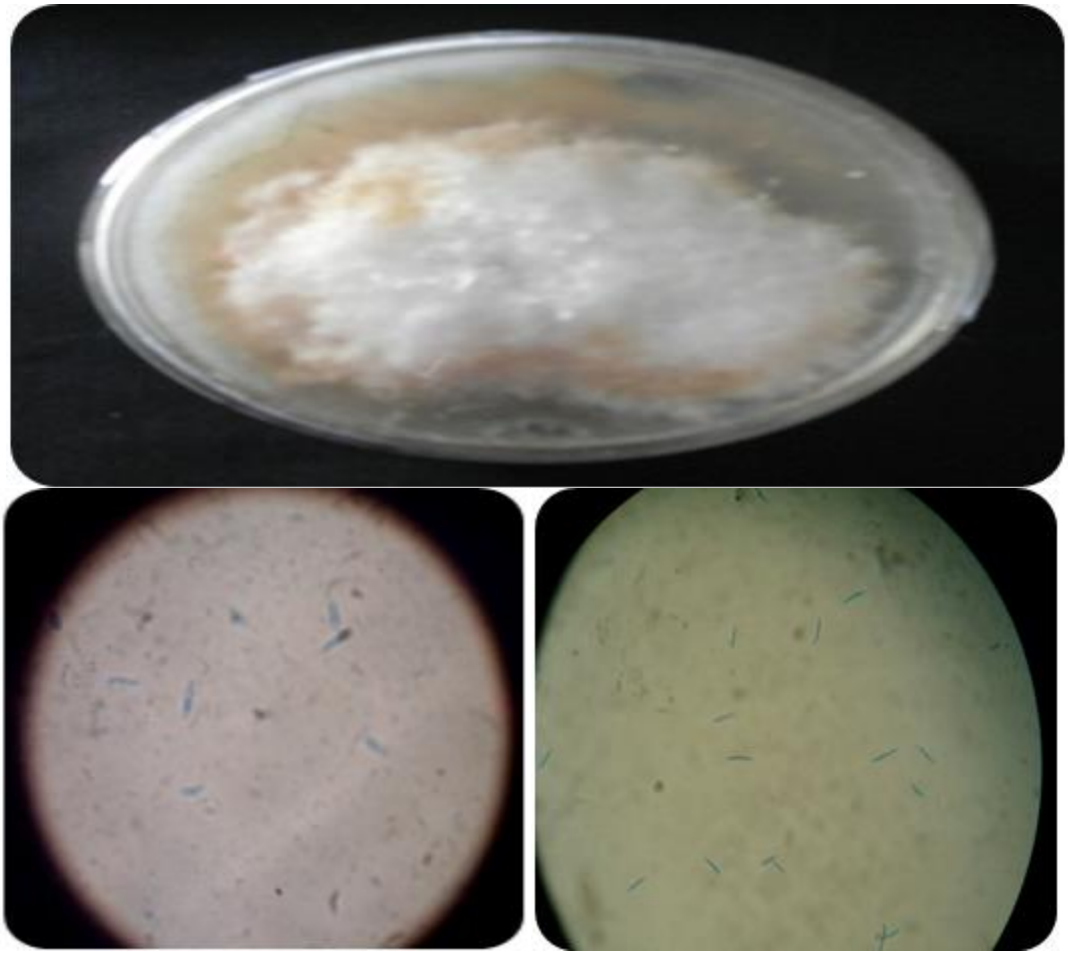

Figure 1. Morphological Identification of *Fusarium* isolates.

Species of *Fusarium* are distinguished chiefly based on the shapes of macro-conidia. Their basal cells have a diagnostic hook or notch depending on the species (Toussoun and Nelson, 1976). Macro-conidia also showed great variation in terms of size, shape and septation. The length of macro-conidia ranged from 9.90 (FOL-10) to 29.73 µm (FOL-9). The width of macro - conidia ranged from 3.02 (FOL-13 of KalurKot, Bhakkar) to 5.06 µm (FOL-8). The shape of macro-conidia was observed to be straight for all the isolates except FOL-6 and FOL-12 which were slightly curved. There was a minimum of one septum observed for FOL-2 and a maximum of 5 septa observed for at least seven isolates. Chlamydospores of these isolates were also variable in their diameter and formation. The mean diameter of chlamydospores ranged from 7.0 for FOL-11 to 15.8 µm for FOL-8. The formation of these chlamydospores was either singly or mixed (singly, pairs and clusters). Single chlamydospores were observed for 10 isolates (FOL-1 to FOL-4 and FOL-10 to FOL-15). Mixed pattern was observed for remaining 5 isolates (FOL-5 to FOL-9). According to Toussoun and Nelson (1976) species of *Fusarium* are distinguished chiefly on the basis of shapes of macro-conidia. In cultivar Masoor-93, the mean severity index ranged from 0 to 72% and FOL-3 proved to be most virulent on Masoor-93.

Table2. Morphological Characterization of *F. oxysporum* f. sp. *Lentis.*

Sr. no.	Districts	Area	Isolate	Micro-conidia		Macro-conidia			Chlamydospores	
				Size (µm) Mean	Shape	Size (µm)	Shape	Septation	Dia(µm) Mean	Formation
1	Chakwal	Dhudial	FOL-1	5.46×3.13	Oval	11.59×3.36	Straight	3-5	8.4	Singly
		BARI	FOL-2	5.69×3.00	"	24.80×3.84	"	1	8.2	"
		Sehgalabad	FOL-3	6.40×2.83	"	22.88×4.50	"	2-3	13.2	"
2	Attock	Khaur	FOL-4	5.88×2.91	"	13.63×4.00	"	3-5	12.2	"
3	Gujrat	Daulatpur	FOL-5	4.38×2.75	"	11.26×3.75	"	3-5	9.2	Singly,Pairs, Clusters
		Bhagowal	FOL-6	4.37×2.71	2-celled Oval	14.64×4.69	Slightly Curved	3-4	13.8	"
4	Sialkot	Pasrur Road	FOL-7	5.59×2.87	Oval	15.90×4.68	Straight	3-5	8.8	"
5	Narowal	Zafarwal	FOL-8	6.10×3.82	"	18.58×5.06	"	3-5	15.8	"
		Dhongian	FOL-9	5.36×2.97	"	29.73×4.66	"	3	10.4	"
6	Mianwali	Chashma	FOL-10	5.88×2.94	Oval Pyriform	9.90×4.59	"	3	8.4	Singly
7	Bhakkar	ARI	FOL-11	4.55×2.31	Oval	13.40×3.65	"	3-5	7.0	"
		Darya Khan	FOL-12	6.65×2.54	"	11.42×3.80	Slightly Curved	3-5	8.2	"
		KalurKot	FOL-13	6.20×3.75	"	16.14×3.02	Straight	3-4	14.2	"
8	Layyah	ChokAzam	FOL-14	6.29×2.53	"	22.00×4.10	"	1-3	8.0	"
9		Chobara	FOL-15	6.30×2.85	"	12.16×4.48	"	1-3	8.0	"

Table 3. Mean severity index of of two genotypes.

Isolates	Mean Severity Index (MSI)		Isolates	Mean Severity Index (MSI)	
	ILL4605	Masoor-93		ILL4605	Masoor-93
FOL-1	0.00	0.00	FOL-9	0.42	0.36
FOL-2	0.66	0.09	FOL-10	0.00	0.09
FOL-3	0.00	0.72	FOL-11	0.41	0.00
FOL-4	0.45	0.25	FOL-12	0.13	0.19
FOL-5	0.00	0.45	FOL-13	0.00	0.17
FOL-6	0.09	0.533	FOL-14	0.48	0.25
FOL-7	0.19	0.18	FOL-15	0.27	0.25
FOL-8	0.00	0.00			

The second most virulent isolate was FOL-6 (53.3%) followed by FOL-5 (45%), FOL-9 (36%), FOL-4 and FOL-14 (25%), FOL-12 (19%), FOL-7 (18%),FOL-13 (17%) and FOL-2 and FOL-10 (9%) respectively (Table3). In case of ILL4605,the mean severity index ranged from 0 to 66% and FOL-2 proved to be most virulent followed by FOL-14 (48%), FOL-4 (45%), FOL-9 (42%), FOL-11 (41%), FOL-7 (19%), FOL-12 (13%) and FOL-6 (9%) respectively (Table 3).In comparison, ILL4605proved to be more resistant than Masoor-93 towards the retrieved isolates of *F. oxysporum*f. sp. *lentis* collected from wilt affected areas during this study. Stoilova and Chavdarov (2006) screened 32 lentil genotypes of diverse geographical origin for reaction to *F. oxysporum* f. sp. *lentis* during 2003-2004 under greenhouse conditions. The authors reported three accessions viz. 91-001, 91-028 and 98-001 susceptible with 45 and 50% of wilted plants. The results of this study match with Belabid *et. al*. (2004) who reported a single race *of F. oxysporum*f. sp. *lentis* in Algeria, but pathogenicity results showed a range of aggressiveness on susceptible lines as was observed in this study. Bhalla *et. al*., (1984) reported a severe root rot of lentil in 2 consecutive years in Ottawa caused by *F. oxysporum.* Fusarium wilt is prevalent and widely distributed in all lentil growing areas of Punjab and this disease must not be ignored as presence of wide-spread inocula can cause severe epidemics under suitable environment. The pathogen exhibiting considerable variability in their morphological characteristics and the isolates are variable in their ability to cause Fusarium wilt on lentil germplasm. Resistant or tolerant lentil varieties and proper management of Fusarium wilt may play a vital role in reducing yield losses and thus may improve farmer's income.

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