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Ascertaining lentil wilt incidence in two districts of Uttarakhand and Uttar Pradesh and categorization of *Fusarium oxysporum* f.sp. *Lentis* isolates based on pathogenic variability

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Abstract
Lentil is an important legume crop of dryland often affected by wilt caused by *Fusarium oxysporum* f.sp. *lentis* posssing serious threat to economic yield in lentil growing regions of the country, for ascertaining status of wilt in two districts of Uttarakhand and Uttar Pradesh survey was done during 2012-13 and 2013-14 in which average wilt incidence varied from 13.13% (Haldwani) to 24.79% (Bindukhatta). On evaluating pathogenic variability among the isolates collected from different locations in Uttar Pradesh and Uttarakhand during the survey, variation was observed in pathogenic ability of the pathogen which ranged from 31.00 to 62.67 per cent, thereafter isolates were categorized into four groups based on wilt incidence where category 'A' had four isolates, category 'B' seven, thirteen and one isolate in category 'C' and 'D' respectively.

Keywords: Lentil, wilt incidence, *Fusarium oxysporum* f. sp. *Lentis*, pathogenic variability, dryland agriculture, disease survey, isolate characterization

Introduction
Among *Rabi* pulses in India, lentil is next to chickpea, being grown on an area of 1.42 m ha with annual production of 1.13 m tonnes and productivity of 797 kg/ha. It is mainly cultivated in UP, Bihar, MP and West Bengal which together contribute more than 80% area and production (Anonymous, 2014). Among biotic stresses fusarial wilt in lentil is considered as major constraint in increasing production as the pathogen can affect all stages of crop till maturity. Present study was undertaken to ascertain level of wilt incidence in lentil growing areas and a survey was conducted in order to get an idea of wilt incidence in selected locations, further pathogen isolated from collected samples were evaluated for pathogenic variability and categorized accordingly.

Material and methods
Survey and Collection of diseased samples
Disease plants showing wilt symptoms were collected from N E B Crop Research Centre, G.B. Pant University of Agriculture and Technology, Pantnagar and from adjoining two districts in Uttarakhand and three districts in Uttar Pradesh during the roving survey conducted in 2012-13 and 2013-14 for disease assessment, samples were brought to laboratory for microscopic examination, isolation and purified for further studies. Per cent mortality was calculated as follows:-

$$\text{Per cent mortality} = \frac{\text{Number of infected plants in 1 square metre area}}{\text{Number of total plants in 1 square metre area}} \times 100$$

Laboratory Experiments
Cleaning and sterilization of glassware
Glassware (petri plates, culturing tubes, funnels, Erlenmeyer flasks, rods) inoculating needles etc were cleaned in chromic acid (Potassium dichromate 60g, concentrated sulphuric acid 60ml and water 100ml) followed by thorough washing in running water. Petri plates were air dried and sterilized in hot air oven at 180ºC temperature for one hour.

Medium preparation
Required amount of peeled potato was cut into fine pieces, boiled in 500ml of distilled water for 20 minutes and filtered through muslin cloth. 20g dextrose and 20g of agar-agar were dissolved in 500ml boiling water. Potato extract was added in boiling mixture and mixed thoroughly by stirring with glass rod. Total volume 1000ml was adjusted by mixing water. After few minutes of boiling, it was transferred to about 200ml in each 500ml capacity flask and plugged with non-absorbent cotton. The pH of the medium was adjusted 7.0±0.2 and autoclaved at 15 psi (121.6ºC) for 20 minutes.
The melted potato dextrose agar (PDA) medium was transferred @ 5 ml per culture tube. While transferring, care was taken that medium should not touch the inner wall of the test tube. Non-absorbent cotton was used for plugging the culture tubes. The culture tubes were sterilized by usual method. After sterilization, it was allowed to solidify in slant position and then stored in refrigeration for further use.

Isolation of the pathogen fungus
The infected plants were cut with the help of sterilized blade into small pieces of 2-3 mm size having half healthy and half diseased tissues. The small pieces were sterilized with sodium hypochlorite (0.1%) solution for 30 seconds and thoroughly washed in sterilized distilled water 3 times. Then the pieces were placed between two layers of sterilized blotter paper to remove excess of water. These pieces were then transferred to slants and Petri plates containing PDA medium inside laminar flow chamber under aseptic conditions, followed by incubation at 27ºC. After 48 hours of incubation, the superficial growth obtained was sub cultured on fresh PDA slants.

Purification and maintenance of the culture

The fungus was purified with hyphal tip method. The pure culture thus obtained was maintained by sub-culturing it regularly on PDA medium and preserved in refrigerator at 4 ºC.

Identification of the fungus

The isolated fungus was identified as *Fusarium oxysporum* f.sp. *Lentis* on the basis of presence of conidial characteristics. Slides were prepared in lactophenol and cotton blue, examined under compound microscope to study the morphological characters of the pathogen.

Pathogenicity tests, Pathogenic variability and reisolation of pathogen

The test pathogen was multiplied on Sand-maize meal medium (Vasudeva and Srinivasan, 1952) [16]. Maize meal 10g, sand 90g and sufficient water to moisten the medium was thoroughly mixed in a tray and placed in 250ml capacity Erlenmeyer flasks and autoclaved. Each flask containing 100g sand maize meal mixture was inoculated with 8.0mm mycelial disc of pure culture of the fungus and incubated at 27±1ºC for 10 days. Flasks were shaken intermittently so as to get a fairly uniform growth of the inoculums on the medium.

Soil inoculation

Colonies of isolates grown on sand maize meal medium was used for artificial inoculation studies. Sand maize meal medium with uniform fungal growth was mixed with autoclaved soil @ 5% w/w soil and filled in 5 kg capacity plastic pots. Inoculated pots were kept for 3 days for the establishment of the pathogen in the soil, ten surface sterilized seeds of a susceptible lentil cultivar L-9-12 were sown in each pot at equal distance. Pots were watered regularly and maintained with appropriate moisture condition. After germination, data was recorded for disease commencing from 7th day of sowing upto 30 days. Reisolation of the causal fungus was confirmed on PDA.

Results and Discussion

Survey for assessing wilt incidence at selected locations of Uttar Pradesh and Uttarakhand

Roving survey was conducted during 2012-13 and 2013-14 in December – January to assess the wilt incidence in two districts of Uttarakhand (Udham Singh Nagar and Nainital) and three districts of Uttar Pradesh (Sultanpur, Faizabad and Ambedkar Nagar). Data pertaining to the survey in 2012-13 are given in Table 1. In Uttarakhand average disease incidence ranged from 13.51% to 22.08%, district U S Nagar had average wilt incidence of 17.22%, where highest incidence was observed in Jaspur (20.09%) followed by Kashipur (19.08%) and lowest in Bajpur (13.70%), while in areas of Nainital district highest incidence was at Bindukhatta (22.08%) followed by Ramnagar (16.95%), lowest in Haldwani (13.51%) and average incidence in district was 16.74 per cent.

Surveyed locations in Uttar Pradesh had wilt incidence from 10.38 to 22.66 per cent and among the districts Ambedkar Nagar district had 16.67 % wilt incidence followed by Sultanpur (15.82%) and lowest in Faizabad (14.99%), while within the Ambedkar Nagar Itauri (18.85%) had highest incidence followed by Jagdishpur (17.07%) and lowest at Tanda (14.68%). In Sultanpur district Dostpur (19.24%) had highest wilt incidence followed by Kurebhar (18.59%) and lowest at Kadipur (10.38%), while in locations of Faizabad district Milkipur (22.66%) had highest wilt incidence followed by Sarai Rasi (15.09%) and lowest at Achhora (10.92%)(Table 1).

During 2013-14 wilt incidence in Udham Singh Nagar district varied from 14.92 to 26.38 per cent and average of 19.65%, in Nainital district it varied in the range of 12.74% to 27.50% with average of 19.92% and lowest at Haldwani (12.74%). In Ambedkar Nagar district of Uttar Pradesh wilt incidence varied from 10.20% in Akbarpur to 25.32% in Itauri, average incidence in district was 16.88%, highest in Itauri (25.32%) while in Faizabad district Milkipur (20.35%) had highest wilt incidence with average wilt incidence of 18.64% and lowest was at Achhora (17.57%).

During 2012-13 and 2013-14 average wilt incidence in surveyed locations varied from 13.13% (Haldwani) to 24.79% (Bindukhatta), indicating prevalence of wilt pathogen in these locations and under favourable condition for pathogen risk of loss may increase many fold resulting in considerable economic loss to the farmers. Agrawal and Prasad (1997) [1] reported wilt, root rot complex and collar rot as potentially damaging root diseases of lentil in India with wilt as the most widespread disease. Naimuddin and Chaudhary (2009a) [12] reported *F. oxysporum* f.sp. *lentis,* the causal agent of wilt disease of lentil as the most important and widely prevalent pathogen of lentil in all districts of Bundelkhand region surveyed by them, causing plant mortality between 49.76 and 59.17 per cent. During the survey of South east Anatolia region in Turkey for assessing the incidence and severity of diseases in lentil, Bayaa *et al*. (1998) [2] found *Fusarium* wilt as predominant one among fungal diseases.

Table 1: Average disease incidence of lentil wilt at different locations of Uttar Pradesh and Uttarakhand

State	District	Location	Avg. Wilt incidence (%)		Pooled
			2012-13	2013-14	
Uttar Pradesh	Sultanpur	Mayang	15.08	24.24	19.66
		Dostpur	19.24	22.95	21.10
		Kadipur	10.38	16.69	13.54
		Kurebhar	18.59	11.57	15.08
		Mean	15.82	18.86	17.34
	Ambedkar Nagar	Akbarpur	16.08	10.20	13.14
		Itauri	18.85	25.32	22.09
		Jagdishpur	17.07	15.75	16.41
		Tanda	14.68	16.23	15.46
		Mean	16.67	16.88	16.77
	Faizabad	Sarai Rasi	15.09	18.47	16.78

		Milkipur	22.66	20.35	21.51
		Achhora	10.92	17.57	14.25
		Rudauli	11.30	18.18	14.74
		Mean	14.99	18.64	16.82
Uttarakhand	US Nagar	Jaspur	20.09	18.92	19.51
		Kiccha	15.99	17.25	16.62
		Bajpur	13.70	22.03	16.87
		Kashipur	19.08	26.38	22.73
		Mean	17.22	19.65	18.43
	Nainital	Bindukhatta	22.08	27.50	24.79
		Belpadao	14.41	18.17	16.29
		Haldwani	13.51	12.74	13.13
		Ramnagar	16.95	21.27	19.11
		Mean	16.74	19.92	18.33

Collection of diseased samples

Samples of lentil plants showing wilt symptoms were collected from experimental plots and during the survey of lentil growing areas. The samples were brought to the laboratory and examined under the microscope for diagnosis and isolation. Root portion of infected plant were cut, sterilized and placed in the Petri dishes containing solidified PDA medium by usual procedure.

Symptomatology

At pre emergence stage, seeds germinate but seedlings fail to emerge above the soil surface as they die during or immediately after germination. Sometimes seeds may also rot even before germination starts. With the advancement of season when infection occurs at adult stage, the plants show symptoms of true vascular wilt. The leaves turn yellow, the growth of the plant is checked. The top of the infected plant droops and ultimately plant dies. Root symptoms include reduced growth with marked brown discoloration and the root nodules are poorly developed in infected plants. No fungal growth was observed on the root surface but upon splitting the root, the walls of the xylem vessels showed discolouration. Lentil wilt appears in the field in patches at both seedling and adult stage. Seedling wilt is characterized by sudden drooping followed by drying of leaves and the whole seedling and apparently healthy looking roots exhibited reduced proliferation. The plants infected with *F. oxysporum* f.sp. *lentis* produced characteristic symptoms on different plant parts at different stages of plant growth. The disease appeared either in the early stage of crop growth (seedling wilt) or during reproductive growth (adult plant wilt) (Khare, 1981) [9].

General symptoms at the seedling stage include seed rot and sudden drooping more like wilting and damping off (Vasudeva and Srinivasan, 1952; Khare, 1980) [11, 16]. At the adult stage, typical symptoms were observed. Similar symptoms were reported by Vasudeva and Srinivasan (1952) [16], Claudius and Mehrotra (1973) [6], Khare *et al.* (1979) [10]. Later on the leaves turned yellow and drooped from the lower part of the plant upwards and the growth of the plant was checked. Roots of the affected plant turned yellowish brown to dark brown partially or wholly. Similar symptoms have been observed by Shulindin (1950) [14].

Isolation and Purification of the Pathogen

Pure culture of *Fusarium oxysporum* f.sp. *Lentis* was obtained and maintained for further studies. A total of 26 isolates of *F. oxysporum* f.sp. *Lentis* were isolated from stem of infected plants and were further purified by hyphal tip isolation method.

Purification of culture

Cultures of *F. o.* f.sp. *Lentis* were further purified using hyphal tip method on PDA from 2 days old culture of each isolate which was sub-cultured on freshly poured plates by picking hyphal tip from the periphery then, samples were transferred to fresh agar plates and incubated to develop a pure culture.

The Pathogen

The pathogen isolated from infected roots was identified on the basis of morphological and cultural characteristics. The isolated fungus was identified as *Fusarium oxysporum* f.sp. *Lentis*. The fungus produced whitish mycelium on PDA. The mycelium of the fungus was cottony and white at the beginning but it turned purple within 4-5 days. The mycelium was septate and three types of asexual spores were produced by the pathogen. Micro conidia were thin walled, with no septation, few two celled, ellipsoid to ovate. Macro conidia were hyaline septate sickle shaped with both the ends pointed, Chlamydospores were thick walled, spherical, terminal or intercalary and formed singly or in pairs. The above findings are in accordance with the description of Vasudeva and Srinivasan (1952) [16] and Khare and Joshi (1971) [8].

Mass multiplication of fungus for soil inoculation

The fungus was multiplied on Sand-maize-meal medium (Singh *et al*., 1979) [15]. The medium was autoclaved at 15 psi for 30 minutes. The sterilized medium was aseptically inoculated with pure culture of *F. oxysporum* f.sp. *Lentis.* The inoculated flasks were incubated at 27±1ºC for 15 days and the fungus produces abundant conidia and mycelium on medium that was used for artificial soil inoculation in glasshouse.

Pathogenicity test of isolates

Each isolate was taken and Koch's postulates were confirmed on susceptible lentil cultivar. The pathogenicity test was determined by artificial soil inoculation on susceptible lentil cultivar L-9-12 in plastic pots in the glasshouse. The typical symptoms of disease appeared, which were identical to those recorded and described in naturally infected lentil plants under field conditions. The fungus re-isolated from these infected plants, gave the similar morphological and cultural characteristics as the original one. Thus, Koch's postulates were confirmed. The re-isolation of the pathogen revealed similar morphological and cultural characteristics as described by several workers (Vasudeva and Srinivasan, 1952; Kannaiyan and Nene. 1978; Khare, 1980) [7, 11, 16].

On evaluating pathogenic variability among the isolates of *Fusarium oxysporum* f.sp. *Lentis* collected from different locations in Uttar Pradesh and Uttarakhand, clearly depicts the variation in pathogenic ability of the pathogen which ranged from 31.00 to 62.67 % (Table 2). Isolates from Lucknow (Fol-11), Jhansi (Fol-20) and Sarai Rasi (Fol-22) had caused 31%, 36.33% and 37.67% wilt incidence which were statistically at par. Isolates from Kurebhar (40.33%), Tanda (42.33%), Achhora (43.67%), Akbarpur (43.33%), Belpadao (44.00%), Pantnagar (46.67%) and Jagdishpur (47.33%) had caused wilt incidence in between 40 to 50 per cent where Fol-15 is statistically at par with FOL- 19, 23, 16, 17 additionally Fol-19 is statistically at par with Fol-1 and Fol-18. Fol-14 (50.67%), Fol-10 (51.33%), Fol-3 (52.33%), Fol-5 (53.67%), Fol-8 (55.00%), Fol-24 (53.33%) and Fol-25 (54.33%) were statistically at par. Whereas Fol-2 (59.00%), Fol-4 (59.67%), Fol-6 (60.00%), Fol-9 (59.33%), Fol-12 (62.67%), Fol-13 (58.00%), Fol-17 (59.00%) and Fol-26 (59.33%) had statistically at par wilt incidence. Accordingly isolates categorized into groups based on wilt incidence are given in Table 3. where category 'A' had four isolates, category 'B' seven, thirteen and one isolate in category 'C' and 'D' respectively. Out of 26 isolates evaluated 25 showed pathogenicity while one isolate from Aligarh didn't cause the disease. Naimuddin and Chaudhary (2009b) [13] observed great variation in pathogenic variability of *Fusarium oxysporum* f.sp. *Lentis* isolates which caused mortality in the range from 18.33 to 80.00 per cent. Belabid and Fortas (2002) [4] found differences in aggressiveness of 32 *Fusarium oxysporum* f.sp. *Lentis* isolates on susceptible lines of lentil.

Table 2: Variation of *Fusarium oxysporum* f.sp. *Lentis* isolates based on disease incidence

Isolate	Location	Wilt incidence %	Isolate	Location	Wilt incidence %
Fol-1	Pantnagar	46.67 (43.07)	Fol-14	Kadipur	50.67(45.05)
Fol-2	Jaspur	59.00 (50.19)	Fol-15	Kurebhar	40.33(39.91)
Fol-3	Kiccha	52.33 (46.34)	Fol-16	Akbarpur	43.33(41.20)
Fol-4	Bajpur	59.67(50.58)	Fol-17	Itauri	59.00(50.15)
Fol-5	Kashipur	53.67(47.10)	Fol-18	Jagdishpur	47.33(43.47)
Fol-6	Bindukhatta	60.00(50.51)	Fol-19	Tanda	42.33(40.59)
Fol-7	Belpadao	44.00(41.55)	Fol-20	Jhansi	36.33(37.06)
Fol-8	Haldwani	55.00(47.92)	Fol-21	Aligarh	0.00(0.00)
Fol-9	Ramnagar	59.33(50.50)	Fol-22	Sarai rasi	37.67(37.85)
Fol-10	Milkipur	51.33(45.92)	Fol-23	Achhora	43.67(41.26)
Fol-11	Lucknow	31.00(33.83)	Fol-24	Rudauli	53.33(46.91)
Fol-12	Mayang	62.67(52.91)	Fol-25	Kanpur	54.33(47.40)
Fol-13	Dostpur	58.00(49.90)	Fol-26	Ghazipur	59.33(50.10)

*Figures in parentheses are angular transformed values

Table 3: Categorization of isolates into groups based on wilt incidence

Group	Wilt Incidence (%)	Isolate	
		Uttarakhand	Uttar Pradesh
A	31-40	-	Fol-11, Fol-15, Fol-20, Fol-22
B	41-50	Fol-1, Fol-7	Fol-14, Fol-16, Fol-18, Fol-19, Fol-23
C	51-60	Fol-2, Fol-3, Fol-4, Fol-5, Fol-6, Fol-8, Fol-9	Fol-10, Fol-13, Fol-17, Fol-24, Fol-25, Fol-26
D	61-70	Fol-12	-

Variability

Variability plays vital role in survival and perpetuation of an organism, making it more versatile and adaptable to various environmental conditions. Khare and Joshi (1971) [8] grouped the isolates of wilt organism as L1, L 2, L3, L4, L5, L6, L7 and L8 the isolates were differentiated on the basis of mycelial colour, substrate pigmentation, aerial mycelium, size and septation of macro-conidia and micro- conidia and presence or absence of chlamydospores. They found that isolate L6 was more virulent among the tested isolates. Claudius and Mehrotra (1973) [6] observed variation in virulence of 14 isolates of *Fusarium oxysporum* f.sp. *Lentis* on two lentil varieties. Kannaiyan and Nene (1978) [7] also reported variation in morphology, cultural and pathogenic characters of different isolates of *Fusarium oxysporum* f.sp. *Lentis*. Chaudhary (2008) [5] found variation in pathogenicity of 30 isolates studied by them. Naimuddin and Chaudhary (2009b) [13] studied 102 *F. o* f.sp. *Lentis* isolates from different regions of Uttar Pradesh and found vast degree of variation in pathogenicity of isolates. Belabid *et al*. (2004) [3] studied virulence and vegetative compatibility of 32 Algerian isolates of *F. oxysporum* f.sp. *Lentis* and grouped them as a single race, though these isolates differed in their aggressiveness on susceptible lines.

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