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Pathogenicity, Host Range and Influence of Temperature, Humidity and pH Levels on the Growth of *Fusarium oxysporum* f.sp. *lentis*

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ABSTRACT

Background: Lentil is a vital nutritional source of protein in several parts of the world including India. The crop is susceptible to wilt which is a devastating soil-borne disease induced by the fungus *Fusarium oxysporum* f. sp. *lentis*. Insight of the potential threat *Fusarium* wilt can pose to lentils, a present study done on pathogenicity, host range and influence of temperature, humidity and pH levels on the growth of *F. oxysporum* f. sp. *lentis*.

Methods: Ten isolates FOL-01 to FOL-10 of *F. oxysporum* f. sp. *lentis* (*Fol*) were isolated from wilted lentil plants that collected from different major lentil growing parts of Rajasthan. During 2017-18 a pathogenicity test was tested in pot house condition by seed and soil inoculation techniques for all isolates and epidomological factors evaluated *in vitro* conditions.

Result: Results indicated that the *Fol* isolates represent a single race but differ in their aggressiveness on the susceptible cultivar L9-12. Pathogenicity test revealed clearly that *Fol* was associated with wilt symptoms and were pathogenic to lentil plants. A maximum percent disease incidence of 70.00 was showed by isolate FOL-02 in soil inoculation technique. In the morphological and cultural characterization, all the ten isolates showed various character in conidial frequency, colony color and growth pattern. Twenty plant species were tested to know the host range of *Fol*, out of these lentil, chickpea and pea show positive reaction with the pathogen. The influence of temperature, relative humidity and pH on the growth and sporulation of *Fol* was studied under *in vitro* conditions. Maximum mycelial growth and sporulation of the *Fol* were observed at 30°C, 6.0 pH and 60% relative humidity.

Key words: *Fusarium oxysporum* f.sp. *lentis.,* Host range, Humidity, pH, Pathogenicity, Temperature.

INTRODUCTION

Lentil (*Lens culinaris* M.) is a major legume crop after chickpea. This crop has been grown mainly as an efficient source of high-quality protein in human diets (Sen and Kapoor 1975). Lentil crops being affected by a wide range of pathogens (Nelson *et al.*, 1983). In the early stage of the crop cycle, lentil wilt disease can occur as an epidemic however, the wilt damage can take place at any developmental stage of the crop under various climatic conditions such as high temperature, low relative humidity, *etc* (Kataria and Grover 1976). Many fungal pathogens affect the lentil crop and cause severe damage to leaves, stems, roots, and pods as well as reduce the marketability by discoloration of seeds (Buxton *et al.*, 1934). Fusarium wilt disease is caused by the fungus *F. oxysporum* f. sp. *lentis* and is a soil-borne disease (Lindbeck, 2009). It is the most important disease of lentils that causing noteworthy economic losses (Singh *et al.*, 1979). The initial inoculum level is very important for determining the incidence of the disease (Booth, 1985). The disease affects both seedlings and adult stages and appears as spots/ patches in the field. *F. oxysporum* f.sp. *lentis* infection is considered by a sudden drooping of the leaves, followed by dull green leaves with drying and the ultimate death of the seedling. The root system shows brown discoloration of the vascular system (Lindbeck, 2009). The pathogen survives as chlamydospores and dormant mycelium in infected plant debris in the soil that can remain viable for several years (Nene, 1980). Lentil wilt is influenced by many factors such as pathogen virulence, host range and epidemiological factors in general linked with manipulation of the micro-environment. Studies conducted in controlled environment conditions on the effect of temperature on infection of lentil seedlings by *F. oxysporum* f.sp. *lentis* revealed that the disease was severe at high temperatures (20-27.5°C) and decrease in cooler soils (Booth, 1985). Therefore, the present investigation was aimed to examine major three aspects. 1) Pathogenicity test 2) host range of pathogen

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and 3) influence of temperature, relative humidity and pH on the pathogen.

MATERIALS AND METHODS

Isolation of pathogen

Ten samples of *F. oxysporum* f. sp. *lentis* (FOL-01, FOL-02, FOL-03, FOL-04, FOL-05, FOL-06, FOL-07, FOL-08, FOL-09 and FOL-10) were collected from different lentil growing areas of Rajasthan. Infected plants washed carefully to remove the adhering soil particles. The washed plants were cut into small pieces and surfaces sterilized by immersing the root pieces in 1% NaOCl solution for 5 min and then transferred on to Potato Dextrose Agar (PDA) in Petri dishes and incubated at 25±1°C for 7 days. The growing fungi were individually transferred to the PDA medium. Pure cultures of fungi were obtained using a single spore or hyphal tip technique. The fungal isolates were then identified according to (Booth, 1985; Nelson *et al*., 1983). Pure cultures were kept in a refrigerator at 4°C for further studies.

Pathogenicity test

Pathogenicity test of different isolates of *F. oxysporum f.* sp. *lentis* (FOL-01 to FOL-10) were tested during Rabi 2017-2018 in cage house condition at Rajasthan Agricultural Research Institute, Durgapura (Jaipur) by seed and soil inoculation techniques suggested by Kataria *et al*., 1976 and soil inoculation technique suggested by Radhakrishnan *et al*., 1985 and Sen *et al*., 1975. Observation of diseased and healthy plants was recorded 7 to 60 days and PDI was calculated by:

$$PDI = \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \times 100$$

Seed inoculation technique

Healthy surface-sterilized lentil seeds (variety L9-12) were taken. The seeds were rolled, on 7 days old sporulating culture of *F. oxysporum* f. sp. *lentis*. Inoculated seeds were sown at 5 cm depth in cemented pots (pre-sterilized and having autoclaved soil) @ of 15 seeds/pot with 4 replication. The un-inoculated healthy seeds served as control. These pots were kept in cage houses and watered regularly.

Soil inoculation technique

The fungus (*F*ol) was grown on sorghum grain medium for 10 days to use as the soil inoculum. Before sowing, pots were sterilized with copper sulfate solution and filled with sterilized soil. These pots were inoculated with fungal inoculum (20gm/pot) before 7 days of sowing. Fifteen healthy and surface sterilized lentil seeds were sown in each pot and replicated four times. Surface sterilized seed sown in un-inoculated sterilized soil served as control. These pots were kept in cage houses and watered regularly as and when required and maintained under identical conditions. Observation on seed germination was recorded 7 days after sowing and post-emergence mortality was recorded up to 60 DAS in both the experiments and finally leading to the death of the plants. The above character of symptoms was taken as a hard stick to confirm the identification of ten isolates of *F. oxysporum* f. sp. *lentis*.

Reisolation and characterization *(*Morphological and Cultural*)*

The fungus was re-isolated from artificially inoculated plants of all isolates and identified isolated ten isolates based on their morphological and cultural characters.

For the morphological identification, from a pure culture of each isolate, temporary slide mounts were made in lactophenol solution and were examined under the light microscope (Nikon YS100) at 100X magnification. The number of observations was taken for the size of macroconidia, the number of conidia per microscopic field belonging to each isolate (Leslie *et al*., 2006). *For the cultural identification,* twenty ml of sterilized PDA was poured aseptically in each Petri-plate after that plates were inoculated with 5 mm dia. mycelial disc obtained 5-day-old cultures of different isolates and then incubated at 25±1°C. One observation regarding mycelium color (Pink, purple, white, and dull white), Pigmentation (pinkish, dark tan, Light buff) were recorded (Leslie *et al*., 2006). Three replications were maintained for each isolate in a completely randomized design (CRD).

Host range of the pathogen

Various crop plant species, belonging to various families were selected for testing host range in the present investigation. The experiment was conducted in cage house conditions. Ten plants were grown in each 30 cm diameter earthen pot (containing sterilized soil) and there were 3 pots or 3 replications for each plant species. The soil was inoculated with 15 days old fungus inoculum. Plants were observed regularly and disease incidence/mortality due to wilt was recorded.

Effect of different temperatures, relative humidity and hydrogen-ion concentrations on growth and sporulation of *Fol in vitro*

Temperatures

20 ml of sterilized PDA was poured into Petri plates. The Petri plates were inoculated aseptically by placing a 5 mm diameter mycelial disc in the center from a 7-day old pure culture of *F. oxysporum* f.sp. *lentis* (highly pathogenic isolate). These Petri plates were incubated at various temperatures *viz.* 20, 25, 30, 35 and 40°C. The colony diameter was recorded after 7 days of incubation and the number of spores per microscopic field was counted through the colony counter. Each treatment was repeated four times.

Relative humidity

Five levels of relative humidity *i.e.* 60, 70, 80, 90 and 100 per cent were studies by using concentrate sulphuric acid and sterilized distilled water in different proportions in glass desiccators according to the method suggested by Buxton *et al.* (1934). The composition of the acid solution used was as follows.

Different relative humidity levels

RH (%)	Stock solution (ml)	Distilled water (ml)
60	374.0	396.0
70	348.0	510.3
80	294.0	640.0
90	161.0	712.0
100	0.00	Only distilled water

Petri-plates containing PDA medium were inoculated with a 5 mm disc of 7 days old culture of *Fol*. Inoculated Petri plates were immediately accommodated in glass desiccators and incubated at 25±1°C for 3 days. Observations on mycelial growth were recorded after 3rd day of incubation.

Hydrogen-ion

Effect of different H-ion concentrations on growth and sporulation of *Fol* was studied at different pH levels *viz.,* 6.0, 6.5, 7.0, 7.5 and 8.0 on PDA medium. The pH levels of the media were adjusted by using 0.1 N HCl or 0.1 N NaOH solutions with the help of a pH meter. After adjusting pH, the medium was sterilized in an autoclave (121°C at 15 psi for 15 min). 20 ml of media was poured separately in Petri plates aseptically and transferred 5 mm disc of fungus on media and were incubated at 25±1°C temperature in an incubator. After 7 days of incubation, observations on radial growth and sporulation of fungus were recorded.

RESULTS AND DISCUSSION

Pathogenicity Test

Ten fungal isolates were obtained from different naturally infected lentil plants showing wilt symptoms collected from different locations of Rajasthan. These isolates were identified as *F. oxysporum* f. sp. *lentis*. These isolates were showed various ability to cause wilt symptoms on artificially inoculated lentil plants. The symptoms of wilt consist of dwarfing of plants, yellowing, and drooping of leaves starting from the lower part of the plant and extending upwards. In late infection, the affected plants exhibited wilted, no external rotting but if the transverse cut is given to the root dark brown discoloration of xylem shown in Plate 1. Per cent disease incidence of pathogenicity test of *F. oxysporum* f sp. *lentis* is presented (Table 1). Isolate FOL-02 showed the highest percent disease (70.00) incidence in the soil inoculation technique followed by isolate FOL-08 (68.33 %) in the soil inoculation technique while minimum percent disease incidence was observed in soil inoculation technique in isolate FOL-09 (36.67). Re-isolations from these diseased seedlings yielded the culture of the fungus of each isolate and identical to the original one. The re-isolation culture was again found to produce the disease. A similar finding was reported (Garkoti *et al.* 2013 and Taheri *et al.*, 2010).

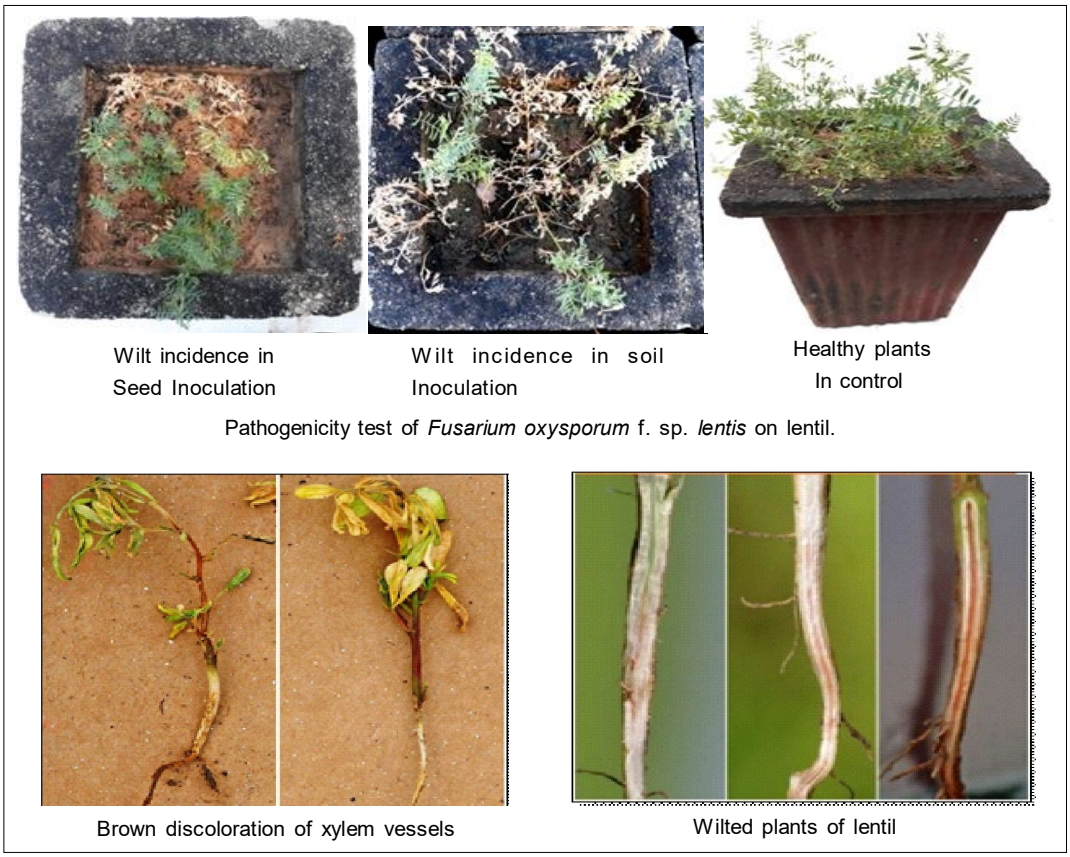

Plate 1: Pathogenicity test and symptoms of lentil wilt disease.

Morphological and cultural characterization

Conidial size and frequency of macroconidia of 10 isolates of *Fol* were studied. The macroconidia measuring >15µ were grouped into large and conidia measuring <15 µ were grouped in small. Large conidia, dull-white color with fluffy growth pattern were observed in four isolates FOL-03, 04, 07, 09 and 10 while, small conidia were observed in five isolates FOL-01, 02, 05, 06 and 08. Macro and micro conidial frequency of ten isolates were categorized as high and low. High frequency, was observed in five isolates FOL-01, 02, 04, 05 and 08 while, five isolates 03, 06, 07, 09 and 10 were of low conidial frequency. Two isolates FOL-03 and FOL-06 showed pinkish color with an appressed growth pattern and four isolates 04, 07, 09 and FOL-10 were milky white with partially appressed colony growth pattern. In color pigmentation, out of ten isolates, four isolates FOL-01, 02, 08 and 05 were dark tan, four isolates FOL-04, 07, 09 and 10 were purple (Table 2). The length and width of micro-conidia ranged were found from 4.38 to 6.65 µm and 2.31 to 3.2 µm of *F. oxysporum* f. sp. *lentis* isolates, respectively (Altaf *et al.*, 2014). These results were also similar to findings (Rafique *et al.,* 2015; Hiremani *et al.,* 2016; Dubey Khushboo *et al.,* 2018).

Host range of the *Fol*

Twenty various plant species including crop plants and spices plants belonging to different families were tested for their screening against *Fol* of lentil. Three species showed a positive reaction *to F. oxysporum* f. sp. *lentis,* and the remaining all species were found to be free from infection (Table 3). Host range studies indicated that the pathogen has a narrow host range and it was incapable of infecting all the tested host plants except lentil, chickpea, and pea. Our observations confirm the findings of (Armstrong *et al.*, 1981) plant pathogenic forms of *F. oxysporum* are divided into *formae speciales* based on the hosts they attack. Padwick (1941) reported that the fungus could infect lentil as well as chickpea, but the chickpea isolate did not infect lentil.

Table 1: Pathogenicity test of the isolates of *Fusarium oxysporun* f. sp. *lentis* on lentil.

Isolates	*Germination %		**PDI	
	Seed inoculation	Soil inoculation	Seed inoculation	Soil inoculation
FOL-01	86.68(68.91)	83.31(66.01)	58.34(49.80)	63.30(52.75)
FOL-02	80.00(63.59)	76.67(61.17)	65.00(53.79)	70.00(56.82)
FOL-03	91.67(75.87)	86.68(68.91)	53.00(46.92)	56.60(48.84)
FOL-04	93.32(77.16)	90(71.81)	50.00(45.00)	55.00(47.88)
FOL-05	85.00(67.62)	80(63.59)	60.00(50.80)	63.34(52.75)
FOL-06	90.00(71.80)	86.67(68.91)	56.67(48.84)	61.00(50.80)
FOL-07	95.00(78.77)	90.01(71.82)	41.60(40.20)	47.67(43.08)
FOL-08	81.67(65.20)	76.66(61.17)	63.33(52.75)	68.33(55.83)
FOL-09	96.67(82.51)	89(70.83)	33.34(35.16)	36.67(37.25)
FOL-10	93.36(77.18)	86.66(69.39)	45.00(42.13	50.00(45.00)
Check	98.33(86.25)	98.33(86.26)	0.00(0.00)	0.00(0.00)
CV%	6.41	6.09	9.05	8.05
SEM	2.89	2.62	2.38	2.30
CD at 5%	8.35	7.56	6.91	6.66

*Average of four replications; **Per cent disease incidence.

Table 2: Morphological and cultural characters (microscopic) of different isolates of *Fusarium oxysporum* f. sp. *lentis*.

Name of isolates	Frequency of conidia macro/micro	Macro conidial size	Colony color	Growth pattern
FOL-01	High	Small	Dull white	Fluffy
FOL-02	High	Small	Dull white	Fluffy
FOL-03	Low	Large	Pinkish	Appressed
FOL-04	High	Large	Milky white	Partial appressed
FOL-05	High	Small	Dull white	Fluffy
FOL-06	Low	Small	Pinkish	Appressed
FOL-07	Low	Large	Milky white	Partial appressed
FOL-08	High	Small	Dull white	Fluffy
FOL-09	Low	Large	Milky white	Partial appressed
FOL-10	Low	Large	Milky white	Partial appressed

Effect of temperature levels

Effect of different temperature levels *viz.,* 20, 25, 30, 35 and 40°C on radial growth and sporulation of *Fol* was studied and observations were presented in (Table 4 and Plate 2). *Fol* showed maximum growth (90.00 mm) and sporulation at 30°C followed by 25°C temperature. Minimum growth (36.10 mm) and sporulation were recorded at 40°C temperature. The results indicate that a slight increase or decrease in the temperature from 30°C the growth and sporulation of fungus adversely affected. The present results agreed with the finding of Harichand *et al.* (2009); Landa *et al.* (2006).

Effect of relative humidity levels

It was observed (Table 5 and Plate 3) that all the five humidity levels (60 to 100 %) induced the growth of *Fol*. Significantly best mycelial growth (90.00 mm) was recorded at 60 percent relative humidity followed by growth (86.75 mm) at 70 percent. A significant decrease in mycelium growth was observed at 80, 90, and 100 percent humidity levels. Minimum mycelium growth (60.75 mm) was observed at 100

Table 3: List of plant species used for host range studies under artificial inoculation with *F. oxysporum* f. sp. *lentis*

Botanical name	Common	Family name	Disease production
Vigna mungo	Black gram	Leguminosae	-
Cicer arietinum	Chickpea	Leguminosae	+
Lens escuentus	Lentil	Leguminosae	+
Cyamposis tetragonoloba	Cluster bean	Leguminosae	-
Vigna unguiculata	Cowpea	Leguminosae	-
Arachis hypogaea	Groundnut	Leguminosae	-
Linum usitatissimum	Linseed	Linaceae	-
Vigna aconitifolia	Moth bean	Leguminosae	-
Vigna radiata	Mung bean	Leguminosae	-
Pisum sativum	Pea	Leguminosae	+
Pennisetum typhoides	Pearlmillet	Gramineae	-
Phaseolus vugaris	Rajmash	Leguminosae	-
Cajanus cajan	Red gram	Leguminosae	-
Brassica juncea	Indian mustard	Brassicaceae	-
Spinacea oleracea	Spinach	Chenopodiaceae	-
Hordeum vulgare	Barley	Graminae/poaceae	-
Triticum aestivum	Wheat	Graminae/poaceae	-
Trigonella foenum-graecum	Fenugreek	Leguminosae	-
Coriandrum sativum	Coriander	Umbelliferae	-
Foeniculum vulgare	Fennel	Umbelliferae	-

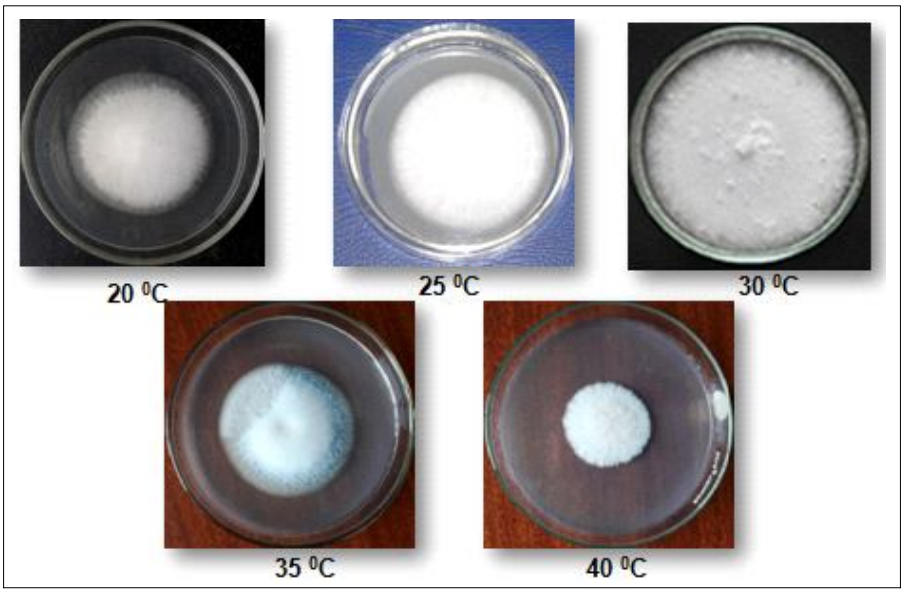

Plate 2: Growth of *Fusarium oxysporum* f. sp. *lentis* on different temperatures.

Table 4: Effect of different temperatures on growth and sporulation of *F. oxysporum* f. sp. *lentis* in *in vitro*.

Temperature	Average colony diameter (mm)*	Sporulation**
20	69.34 (56.38)	++
25	77.42 (61.63)	+++
30	90.00 (71.66)	++++
35	62.98 (52.52)	++
40	36.10 (36.93)	+
S.Em.±	0.71	
C.D. at 5%	2.18	
C.V. %	2.11	

*Mean of four replications.
Figures in parentheses are angular transformed values.
**Categories of sporulation.
+ : Scanty; ++ : Moderate; +++ : Good; ++++ : Abundant.

Table 5: Effect of different relative humidity on mycelial growth of *F. oxy.* f.sp. *lentis* in *in vitro*.

Relative humidity (%)	Mycelial growth(mm)*	Sporulation**
60	90.00 (71.75)	++++
70	86.75 (68.68)	+++
80	80.65 (63.91)	++
90	74.50 (59.68)	+
100	60.75 (51.21)	-
SEm±	0.91	
C.D. at 5%	2.80	
C.V. %	2.32	

*Average of four replications.
Figures in parentheses are angular transformed values.
**Categories of sporulation.
+ : Scanty; ++ : Moderate; +++ : Good; ++++ : Abundant.

percent relative humidity level. It can be concluded that low humidity favored the growth of *F. oxysporum* f. sp. *lentis*. The present results agreed with the finding of (Khilare *et al.*, 2012).

Effect of various pH levels

Growth of the test fungus was obtained at all the pH levels tested but it was maximum (90.00 mm) at pH 6.0 after 7 days of inoculation and also pH 6.5 (79.50 mm), pH 7.0 (67.00 mm) were found favorable. Growth of the test fungus decreased when increasing pH levels from 6.0 levels. Maximum sporulation was observed at pH 6.0 followed by pH 6.5 while minimum sporulation was recorded at pH 8.0 (Table 6 and Plate 4). The results of the present study are in agreement with those achieved by Khilare *et al.*, 2012). Gangadhara *et al.* (2010) studied the effect of pH levels on the growth of *F. oxysporum* f. sp. *vanilla* isolates.

Table 6: Effect of different pH level on growth and sporulation of *F. oxysporum* f. sp. *lentis* in *in vitro*.

pH	Average colony diameter (mm)*	Sporulation**
6.0	90.00 (71.75)	++++
6.5	79.50 (63.08)	++++
7.0	67.00 (54.94)	+++
7.5	30.75 (33.68)	++
8.0	25.00(30.00)	+
S.Em.±	0.90	
C.D. at 5%	2.77	
C.V. %	3.08	

*Mean of four replications.
Figures in parentheses are angular transformed values.
**Categories of sporulation.
+ : Scanty; ++ : Moderate; +++ : Good; ++++ : Abundant.

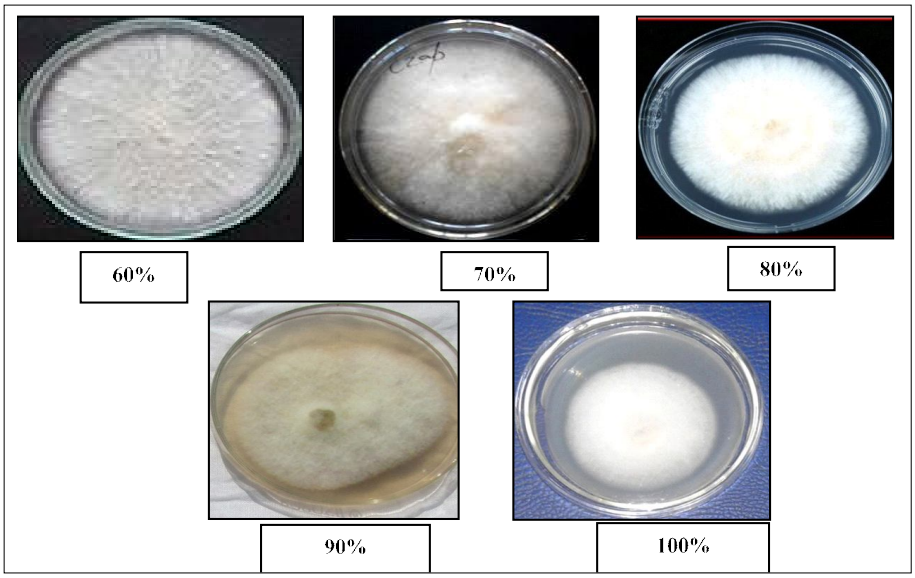

Plate 3: Growth of *Fusarium oxysporum* f. sp. *Lentis* on different RH levels.

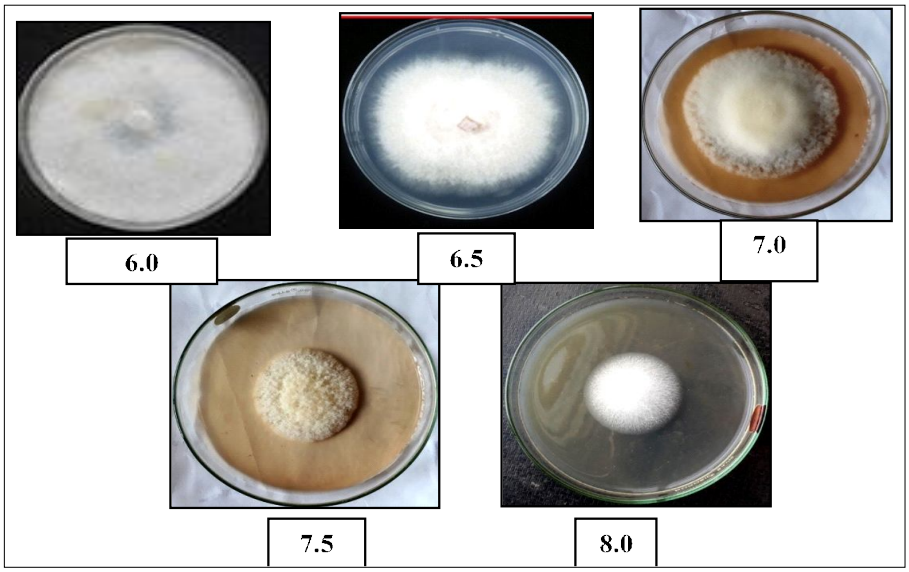

Plate 4: Growth of *Fusarium oxysporum* f. sp. *lentis* on different pH levels.

CONCLUSION

Ten isolates of *F. oxysporum* f. sp. *lentis* were pathogenic on lentil susceptible variety L9-12, tested by seed and soil inoculation method. The highest percent disease incidence was observed in soil inoculation technique in isolate FOL-02. In the morphological and cultural characterization, all the ten isolates showed various character in conidial frequency, colony color and growth pattern. Out of 20 tested plant species for their reaction to *F. oxysporum* f. sp. *lentis* only three *viz*., lentil, chickpea and pea were shown positive reaction. Virulent isolates FOL-02 of *F.oxysporum* f. sp. *lentis* was used for epidemiological studies, maximum mycelial growth and sporulation of the *F. oxysporum* f.sp. *lentis* was observed at 30°C, 6.0 pH and 60% relative humidity.

Conflict of interest

All authors declared that there is no conflict of interest.

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