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CHARACTERIZATION OF *FUSARIUM SOLANI*, CAUSAL AGENT OF PEA WILT AND ITS BIO-MANAGEMENT

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A B S T R A C T

Pea (*Pisum Sativum L.*) is an important winter crop in Pakistan. The quality and production of pea are deteriorated by several biotic and abiotic factors. Among the biotic factors, *Fusarium solani* is one of the major limiting factors in pea production. In current studies, a total of two fields located in Koont Research Farm, PMAS-Arid Agriculture University Rawalpindi, and National Agricultural Research Centre Islamabad (NARC) were surveyed for the determination of Fusarium wilt disease incidence. Total eighteen isolates were collected during the field survey; a pathogenicity test was carried out to confirm the degree of virulence among all pathogens. A total of 03 plant essential oils such as Neem (*Azadirachta indica)*, Eucalyptus (*Eucalyptus globulus Labill*) and Clove (*Syzygium aromaticum (L.)* at 200, 400 and 600 ppm were tested against *F. solani* causing Fusarium wilt of pea. Results revealed that eucalyptus essential oil at all applied concentrations showed the maximum effectiveness against mycelial growth of *F. solani* under *vitro* condition followed by neem and clove essential oil as compared to control. Keeping in view the above mentioned, eucalyptus essential oil can be used as bio fungicide against *Fusarium solani* causing Fusarium wilt of Pea.

Keywords: *Pisum Sativum, Fusarium solani,* Pea Wilt, Bio management.

INTRODUCTION

In Pakistan, Pea (*Pisum Sativum L.*) is an important winter vegetable crop, belongs to the family Leguminosae. It is an annual beat crop of moderate regions of the world; initially grown in the Mediterranean basin (Smartt, 1990; Sardana *et al.*, 2007; Timmerman-Vaughan *et al.*, 2005). Peas are full of nutrition and its grain is the wealthiest protein source i.e. 27.8 percent, vitamins, complex carbohydrates 42.65 percent, minerals, antioxidant compounds, and dietary fibers (Urbano *et al.*, 2003). It is rich with A, B, and C vitamins. Peas are cultivated as green composts and cover-up crops because peas grow rapidly and add nitrogen to the soil (Ingels *et al.*, 1994; Clark *et al*, 2007; Khvostova, 1983). In the world, peas are cultivated on an area of 528.71 thousand hectares. (FAO, 2009). In Pakistan, pea is growing on a10 thousand hectares areas through a total production of 82 thousand metric tons (ÚKZÚZ, 2008). Besides its significant importance, the susceptibility of diseases is one of the main production constraints to the pea crop. The crop is vulnerable to several diseases, which adversely affects seed yield and its quality. Among them, the most significant and serious soil-borne threat is the occurrence of wilt disease incited by several species of *Fusarium* but the most devastating fungus is *F. solani* is the most significant disease of pea worldwide and is one of the devastating diseases of pea in Asia (Joshi *et al.*, 2013). Wilt symptoms become visible in the field in patches at both, the seedling (seedling wilt) and the reproductive (adult wilt) stage. Seedling wilt can be distinguished by abrupt drooping followed by drying of leaves and loss of seedlings. At the

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adult stage, symptoms appear from flowering to the late pod filling stage and are characterized by drooping of the top leaflets of the plant. Roots of such plants are mostly well developed with a minor reduction of lateral roots and generally, no discoloration of vascular structures is seen but roots show reduced proliferation (Stoilova and Chavdarov, 2006). *Fusarium* wilt is a serious disease of pea crop responsible for huge losses each year in Pakistan. A comprehensive study about wilt incidence wilt pathogen species, and non-hazardous management need to be addressed. Therefore, the study was planned keeping in view the national interests to avoid the future losses caused by pea wilt. This study was focused on the following objectives: (1) To investigate the incidence of *Fusarium* wilt in pea producing areas of Punjab (2) Morphological characterization of *Fusarium* isolates recovered from wilted pea plants (3) Management of disease through Plant essential oils.

MATERIALS AND METHODS

Survey of Pea Crop Fields:

Two fields from each area of Research Koont Farm, PMAS- Arid Agriculture University Rawalpindi and National Agricultural Research Centre Islamabad (NARC) were surveyed for determination of *Fusarium* wilt disease incidence. Pea plants exhibiting the typical symptoms of *Fusarium* wilt were gathered and samples were placed in sterile paper bags with labelling (date, time and location). These samples were brought to Pir Mehr Ali Shah (PMAS) Arid Agriculture University Rawalpindi (AAUR), at Department of Plant Pathology and were stored at 4°C in fungal plant pathology laboratory for further processing. Incidence percentage calculated by using the following formula:

$$\text{Incidence } \% = \frac{\text{Number of fungal infected pea plants}}{\text{Total number of pea plants examined}} \times 100$$

Isolation of Pathogen from Disease Specimen: From the 15 plants collected per field, five plants with signs of root discoloration were then selected for pathogen identification. Segments of diseased roots were cut and then surface-sterilized with sodium hypochlorite (NaoCl) for 1 mint, dipped thrice on distilled water for 30 sec and plated onto potato dextrose agar (PDA) media**.** Plates were placed in an incubator at 25°C for 7 days until mycelial growth appeared.

Morphological identification: Identification of *Fusarium solani* was carried out on the following parameters; Macroscopic Characteristics**:** Colony color, Pigmentation and Growth rate while Microscopic Characteristic**:** Mean length of macroconidia (µm), Mean width of macroconidia (µm) and Presence of sporodochia Macroconidia septation were examined followed by (James and Nathlie 2001; Cheesbrough 2000).

Preservation on Silica Gel: The pure cultures were preserved on sterilized silica gel for future use following Smith (1983). In this method, tubes were used taking silica gel in it and were then placed in an ice-bath for at least 30 minutes. Pre-cooled 5% skimmed milk was added to mature sporulation cultures grown on agar plates. The spores were scrapped with the help of a sterilized blade. The tubes were removed from the ice-bath. Three-quarters of the silica gel soaked and mixed to make sure the even distribution of spores. The tubes were finally incubated at 25°C for 7-14 days.

Pathogenicity Test: All isolates were subjected to the preliminary pathogenicity test on local pea cultivar. Earthen pots (15 cm) were filled with sterilized soil at 1 kg/pot. The inoculum of each isolate of *F. solani* was thoroughly mixed with the soil. Control pots were prepared using sterilized soil only. Five (15) seeds of local pea cultivar were sown in each pot and grown in the net house. The seed emergence was recorded 21 days after sowing. Observations on the number of plants wilted in each pot were recorded at 30, 45 and 60 days after sowing. The causal agent of wilt incidence was confirmed after re-isolation of the pathogen from the infected root and stems of pea plants. The percent wilt incidence was calculated based on initial plant count and the total number of wilted plants in each pot.

In-vitro screening of Plant essential oils against *F. Solani*

A total of 03 plant oils such as Neem, Euclyptus and Clove were tested against *F. solani* to select the most effective plant essential oils as bio-fungicides (Table 1). After sterilization of PDA media, plant essential oils were added at different concentrations 200, 400 and 600 ppm into 20ml PDA and poured in Petri plates. Mycelial plugs (6mm in diameter) from the edges of each culture were incubated in the centre of each PDA plate (9 cm diameter). Cultures were incubated in the dark at 26°C and 70% RH for 7-10 days. Mycelial growth was measured every day until control plates were completely colonized with mycelium. Replications were considered simultaneously for each concentration of samples. Only PDA was used as a control. All tests were repeated five times.

Table 1. Nomenclature and Sources of Plants Used in Biological Control of *Fusarium solani*.

Plant	Scientific name	Source
Neem	*Azadirachta indica*	Leaves
Eucalyptus	*Eucalyptus globulus*	Leaves
Clove	*Syzygium aromaticum (L.)*	Seeds

Statistical Analysis

All statistical analysis were carried out using SPSS 16 (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) were performed by ANOVA procedures. Significant differences between means were determined by Duncan's pairwise comparison test at a level of $p<0.05$.

RESULTS AND DISCUSSION

Disease survey and assessment: A systematic survey of pea wilt disease was conducted in lentil growing areas of Punjab *viz*. Research Koont Farm, PMAS- Arid Agriculture University Rawalpindi and National Agricultural Research Centre Islamabad (NARC) respectively. Disease prevalence and incidence were calculated from each surveyed area. During the survey, the disease prevalence was found 100% in all the visited areas. Pea plants with wilt disease symptoms were found in all visited fields but the incidence varied over fields during the study year (Figure 1). An average wilt incidence of 40% was noted in research Koont Farm, PMAS- Arid Agriculture University Rawalpindi while 26% average wilt incidence was observed in National Agricultural Research Centre Islamabad (NARC) respectively. Considerable variation in mean wilt incidence during growth stages of pea may be attributed to high temperature (24-27ºC) at the reproductive stage during the months February and March and disease at seedling stage in November, December and January, where the temperature remained low i.e., 5-20ºC (Haqqani *et al*., 2000).

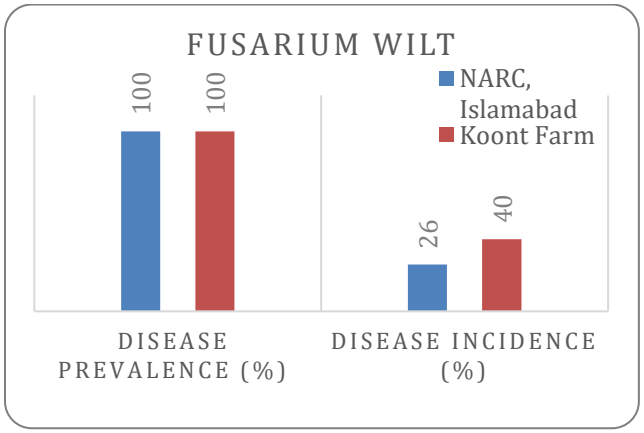

Figure 1. Survey for Disease incidence (%) at two different locations.

Morphological Characterization of *Fusarium Solani*: A total of eighteen isolates were obtained from surveyed location, seven isolates from NARC Islamabad, and eleven were obtained from koont research farm (Table 2)**.** During morphological observation, colony color of all examined isolates varied from pale to brown, White-creamy to white-greyish color with Pale brown, yellowish brown and dark brown pigmentation while some isolates did not developed any pigmentations. The colony diameter of each isolates varied from 7.1 ± 0.2 to 7.4± 0.2 cm respectively (Table 3). During the microscopic observation, the mean length (L) of macroconidias of each isolate was examined from 34.4 µm and 40.1 µm while and width (W) of macroconidias of each isolate were measured from 3.6 to 4.0 µm respectively. During observation of sporodochia varied cream, whitish to blue. The minimum and maximum septations of macroconidia were examined 3 to 5 respectively (Table 4). Macro-conidia is the most important character for the identification of species of *Fusarium* which are differentiated mainly on the basis of the shapes of the macro-conidia they produce (Nelson *et al.*, 1983). Moreover, considerable variability shown in macro-conidial size in the study has also been observed by Mandhare *et al*. (2011)**;** Hafizi *et al.* 2013). The size (length and width) of microconidia measured showed a considerable variation among the isolates. In the same way, the studies of Booth (1977) also showed similar variations in the conidial size of *Fusarium* isolates from chickpea.

Table 2. List of Isolates Collected From Different Pea Growing Areas of Potohar Regions.

Sr. No	Locations	No of Isolates	Names of Isolates
1	Research Koont Farm,	11	*FuSo*K, *FuSo*K1, *FuSo*K2, *FuSo*K3, *FuSo*K4, *FuSo*K5, *FuSo*K6, *FuSo*K7, *FuSo*K8, *FuSo*K9, *FuSo*K10
2	NARC, Islamabad	7	*FuSo*Na, *FuSo*Na1, *FuSo*Na2, *FuSo*Na3, *FuSo*Na4, *FuSo*Na5, *FuSo*Na6
Total	2	18	

Table 3. Macroscopic Characteristics of isolated fungi.

No.	Isolate	Colony color	Pigmentation	Growth rate (cm)
1	*FuSo*K	Pale to brown	Pale brown yellowish brown with dark brown zonation	7.4 ± 0.2
2	*FuSo*K1	white-greenish	white-creamy with dark brown zonation	7.1 ± 0.2
3	*FuSo*K2	Pale to brown	Pale brown yellowish brown with dark brown zonation	7.3 ± 0.2
4	*FuSo*K3	White-creamy to greyish	Colorless	7.2 ± 0.2
5	*FuSo*K4	white-greenish	white-creamy with dark brown zonation	7.5 ± 0.2
6	*FuSo*K5	Pale to brown	Pale brown yellowish brown with dark brown zonation	7.4 ± 0.2
7	*FuSo*K6	Pale to brown	Pale brown yellowish brown with dark brown zonation	7.5 ± 0.2
8	*FuSo*K7	Pale to brown	white-creamy with dark brown zonation	7.5 ± 0.2
9	*FuSo*K8	white-greenish	Pale brown yellowish brown with dark brown zonation	7.6 ± 0.2
10	*FuSo*K9	White-creamy to greyish	Pale brown yellowish brown with dark brown zonation	7.2 ± 0.2
11	*FuSo*K10	White-creamy to greyish	white-creamy with dark brown zonation	7.2 ± 0.2
12	*FuSo*Na	white-greenish	pale to dark brown	7.5 ± 0.2
13	*FuSo*Na1	Pale to brown	white-creamy with dark brown zonation	7.2 ± 0.2
14	*FuSo*Na2	white-greenish	Pale brown yellowish brown with dark brown zonation	7.5 ± 0.2
15	*FuSo*Na3	White-creamy to greyish	white-creamy with dark brown zonation	7.6 ± 0.2
16	*FuSo*Na4	Pale to brown	Pale brown yellowish brown with dark brown zonation	7.4 ± 0.2
17	*FuSo*Na5	white-greenish	Pale brown yellowish brown with dark brown zonation	7.1 ± 0.2
18	*FuSo*Na6	Pale to brown	Colorless	7.4 ± 0.2

Table 4. Microscopic Characteristics of isolated fungi.

No.	Isolates	Mean length of Macroconidia (µm)	Mean width of Macroconidia (µm)	Sporodochia	Macroconidial septation
1	*FuSo*K	34.4	3.7	Cream and blue	3–5
2	*FuSo*K1	35.1	3.9	Creamy	3–5
3	*FuSo*K2	36.2	4.0	Bluish	3–7
4	*FuSo*K3	37.4	4.1	Cream	3–7
5	*FuSo*K4	35.8	3.8	Blue	3–5
6	*FuSo*K5	38.6	3.9	Blue	3–7
7	*FuSo*K6	39.5	3.9	Creamy	3–7
8	*FuSo*K7	34.8	3.6	Whitish	3–5
9	*FuSo*K8	39.3	3.9	Blue	3–7
10	*FuSo*K9	36.3	3.5	Cream	3–5
11	*FuSo*K10	37.6	3.8	Bluish	3–5
12	*FuSo*Na	35.8	3.8	Bluish	3–5
13	*FuSo*Na1	37.6	3.9	Cream	3–7
14	*FuSo*Na2	36.6	3.7	Cream	3–5
15	*FuSo*Na3	38.7	3.7	Bluish	3–7
16	*FuSo*Na4	35.5	3.6	Creamy	3–5
17	*FuSo*Na5	38.1	3.8	Whitish	3–7
18	*FuSo*Na6	40.1	4.0	Blue	3–7

Pathogenicity test: The pathogenicity test of *Fusarium solani* against local pea cultivar showed different degrees of virulence. Isolates (*FuSo*Na3) showed maximum morality against pea cultivar (71%) while the isolate *FuSok* showed minimum morality 39% respectively. In control sets, where plants inoculated with sterile distilled water remained healthy (Table 5). The results of the experiment showed a significant difference in the response of the isolates tested on germplasm. The variation recorded towards the disease reaction of the germplasm suggested that in the availability of the same environmental conditions and amount of pathogen inoculum, the genetic makeup of the plants also plays role in resistance reaction of the plants towards the inoculated pathogens (Cheri *et al.*, 2011).

Table 5. Pathogenicity test of *Fusarium solani* against local cultivar of pea.

No.	Isolate	% Wilted plant (mortality)	No.	Isolate	% Wilted plant (mortality)
1	*FuSo*Na3	71.000a	10	*FuSo*Na4	54.667ef
2	*FuSo*K5	70.667ab	11	*FuSo*K1	53.667fg
3	*FuSo*Na6	70.000abc	12	*FuSo*K8	53.667fg
4	*FuSo*K9	67.667bc	13	*FuSoK*10	51.333gh
5	*FuSo*Na2	67.333c	14	*FuSo*K2	48.667hi
6	*FuSo*Na1	60.000d	15	*FuSo*K4	45.667i
7	*FuSo*Na5	57.667de	16	*FuSo*Na	42.000j
8	*FuSo*K3	56.000ef	17	*FuSo*K6	41.000j
9	*FuSo*K7	55.333ef	18	*FuSo*K	39.333j
19	Control		0.000k		

***In- vitro* evaluation of plant essential oils against *F. solani* after 24 hours:** According to the results showed that eucalyptus EO was found significantly most effective to inhibit the growth of *F. solani* (0.5, 0.3 and 0.2 cm) at 200, 400 and 600 ppm. While Neem was 2nd most effective followed by clove EO at 200, 400 and 600 ppm respectively after 24 hours of incubation at 25 C Table 6.

Table 6. *In-vitro* evaluation of different plant essential oil against *F. solani* after 24 hrs

Essential oils	Concentrations (ppm)			Means
	200 Colony diameter (cm)	400 Colony diameter (cm)	600 Colony diameter (cm)	(cm)
Neem	0.70 h	0.50 i	0.30 k	0.50 c
Eucalyptus	0.50 e	0.30 f	0.20 j	0.33 d
Clove	1.10 c	1.00 f	0.80 k	1.2 b
Control	1.40 a	1.30 b	1.50 a	1.4 a
Means	0.92 a	0.775 b	0.7 b	

Alpha = 0.05, LSD value (F) = 0.0852, LSD value (C) = 0.0461, LSD value (FxC) = 0.266

***In-vitro* evaluation of different essential oils against *F. solani* after 48 hours:** The results indicated that there was a difference among the different essential oils in inhibiting the mycelial growth of *F. solani*. According to, eucalyptus EO was found significantly most effective to inhibit the growth of *F. solani* that showed (1, 0.80 and 0.60 cm) at 200, 400 and 600 ppm. While neem was found 2nd most effective 1.4, 1.2 and 1.1 cm followed by clove EO (2.4, 2.1 and 1.6 cm) at 200,400,600 ppm. Whereas in control, results revealed that maximum growth of *F. solani* (5.4 to 5.2 cm) respectievely after 48 hrs of incubation at 25 °C (Table 7).

Table 7. *In-vitro* effect of different essential oils against *F. solani* after 48 hours.

Fungicides	Concentrations (ppm)			Means
	200 Colony diameter (cm)	400 Colony diameter (cm)	600 Colony diameter (cm)	(cm)
Eucalyptus	1.00 h	0.80 j	0.60 k	0.80 d
Neem	1.40 f	1.20 g	1.1 i	1.23 c
Clove	2.40 c	2.10 d	1.60 e	2.03 b
Control	5.4 a	5.2 b	5.4 a	5.33 a
Means	2.55a	2.37 b	2.25 c	

Alpha = 0.05, LSD value (F) = 0.0172, LSD value (C) = 0.0133, LSD value (FxC) = 0.0378

***In-vitro* effect of different plant essential oils against *F. solani* after 72 hours:** The results revealed in Table 8 indicated that eucalyptus EO was found significantly most effective in inhibiting the growth of *F.solani* that showed 1.4, 1.2 and 1.0 cm at 200, 400 and 600 ppm. While Neem was 2nd most effective followed by clove EO at 200, 400 and 600 ppm respectively. All the concentrations reduced the growth of *F. solani* but 600 ppm of eucalyptus EO gave maximum control over mycelial growth of *F. solani* after 72 hrs of incubation at 25 C (Table 8).

Table 8. *In-vitro* effect of different essential oils against *F. solani* after 72 hrs.

Oils	Concentrations (ppm)			Means (cm)
	200 Colony diameter (cm)	400 Colony diameter (cm)	600 Colony diameter (cm)	
Eucalyptus	1.4 fg	1.2 h	1.0 i	1.33 d
Neem	2.1 e	1.7 f	1.5 g	1.76 c
Clove	3.7 b	3.3 c	2.8 d	3.26 b
Control	7.6 a	7.5 a	7.6 a	7.56 a
Means	3.75 a	3.45 b	3.25 c	

Alpha = 0.05, LSD value (F) = 0.0717, LSD value (C) = 0.0621, LSD value (FxC) = 0.1242

In This study, three plant essential oils *viz.* Neem, Eucalyptus and clove EOs were evaluated at 200, 400 and 600 ppm concentrations against *F. solani* and found that eucalyptus essential oil has considerable effect to inhibit the mycelial growth of *F. solani* at all applied concentrations. Similar results about inhibitory activity of eucalyptus EO were also conducted by other scientists, (Hatamleh *et al*., 2014). According to our result eucalyptus essential oil inhibit mycelial growth (1.4, 1.2 and 1 cm) of Fusarium *solani* at 200, 400 and 600 ppm concentrations which is agreement with the results obtained by Hur *et al*. (2000) and Katooli *et al*. (2011) showed that Eucalyptus EO inhibited the mycelial growth of two fungal pathogens such as *C. gloeosporioides* and *R. solani*. Similar result is also demonstrated by Hatamleh *et al*. (2014) that eucalyptus EO had significantly inhibited the mycelial growth of three *Fusarium* species respectively. The maximum efficacy of this essential oil This high antifungal activity of eucalyptus oil is due to presence of several antifungal compounds such as 1,8-cineole, citronellol (Su *et al*., 2006), citronellal (Batish *et al*., 2006) and ρ-cymene (Su *et al*., 2006) respectively.

CONCLUSION

The use of essential oils as natural fungicides is of immense significance because of the environmental and toxicological implications of the indiscriminate use of synthetic fungicides and reducing the problem of increasing fungi resistance.

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Asfand Iqbal	:	Designed experiment, conducted research and wrote the manuscript
Gulshan Irshad	:	Major supervisor
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Salman Ghuffar	:	Helped manuscript writeup
Rabia Khursheed	:	Helped in interpretation of result
Aamir Bashir	:	Helped in interpretation of result
Muhammad Zeeshan	:	Helped in statistical analysis
Najam ul Hassan	:	Helped in identification of disease