
First Report on Molecular and Biochemical Variations Among the Populations of *Fusarium oxysporum* Infecting Tobacco in Karnataka, India

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Abstract Genetic diversity in *Fusarium oxysporum* f. sp. *nicotianae* (FON) was investigated and isolated from Hunsur tract of Karnataka, India was studied. The variability of FON isolates were investigated using Random Amplified Polymorphic DNA (RAPD), electrophoretic studies of total protein and isozymes (catalase, cellulase, peroxidase, esterase and protease). Amplification of genomic DNA of 5 isolates with OPA (1- 10) decamer primer series generated 380 polymorphic markers. Based on UPGMA analysis of RAPD, the isolates were delineated to two groups. Group 1 represented isolates 1 FON, 3 FON, 10 FON and 13 FON and group 2 represented the isolate 9 FON which were differing in cultural characteristics (data not shown) indicating the genetic diversity. The isolates were also investigated using electrophoretic studies of total protein and isozymes. The SDS PAGE revealed that, each of the FON isolates was unique in band patterns. The isozyme profiles revealed 78 scorable polymorphic bands and the data was subjected to UPGMA analysis. The isolates were delineated into 2 main groups. Group A contained isolates 3 FON, 1 FON, 13 FON and isolate 9 FON and 10 FON in group B. The spectrophotometric assay revealed that the 13 FON exhibited highest activity of 26.56 units/ml in catalase, 0.09 min/ml in peroxidase and 0.0242 units/ml in protease. Continuous spectrophotometric assay for esterase enzyme revealed highest activity in 13 FON isolate. Results showed that the estimated intraspecific variations were more pronounced with isozyme analysis than with protein markers. Isolate 13 FON was found to be entirely varying in the isozyme band patterns all through. In conclusion, cluster analysis of the DNA RAPD, protein banding patterns by SDS-PAGE, and UPGMA analysis of isozyme banding patterns were found to be efficient and effective tools for finding the genetic variability among the isolates isolated in the same geographical area and environmental conditions.

Keywords: RAPD PCR, SDS PAGE, isozymes zymograms and assay, genetic diversity

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Introduction

Different formae specialis of *Fusarium oxysporum* cause devastating vascular wilt diseases. This fungi attack a diverse group of plants including crops, ornamentals and trees (Nelson *et al.*, 1981). Fusarium wilt is largely confined to transplanted crop and is one of the destructive field diseases. Wilt disease is spreading in an alarming rate in recent years in many economically important crop plants and has become a threat to tobacco cultivation also (Ramakrishnan *et al.*, 2005; Shenoi & Nagarajan 2000; Garner, 1951). It is the disease of priority in Hunsur tract of Karnataka.

Management of wilt by resistance breeding is an economically viable and ecologically desirable strategy. However, this can be exploited by the knowledge of diversity and relatedness among the isolates of the pathogen. Although the taxonomy of plant pathogenic fungi mainly depends on morphological and pathological criteria (Kistler, 1997), molecular and biochemical markers are also useful when these are correlated with the morphological characteristics. Currently, increased attention has been focused on studying polymorphism at the DNA level for genetic characterization. Mycelial differences in isozyme and protein pattern among *Fusarium* spp. and their isolates have been reported by various workers (Kumar *et al.*, 2010; Balali and Iranpoor, 2006; Sharma *et al.*, 2006; Mohammadi *et al.*, 2004; Huss *et al.*, 1996; Matsuyama and Wakimoto, 1977). Electrophoretic analysis of proteins and isozymes can be used as an adjunct to morphological, cultural and pathogenic variability of different isolates of the pathogen (Hall, 1967). Protein and isozyme analysis on PAGE provides a well established and efficient tool for revealing genetic variability in fungal population (El-Kazaz, *et al.*, 2008). This information is important in screening host resistance to Fusarium wilt and monitoring shift in virulence. The assessment of genetic diversity of *F. oxysporum* is required for the development of long-term disease management strategies. Studies on genetic relationship and phylogeny among *Fusarium* species have been conducted at the protein and DNA levels. Molecular markers, apart from elucidation of genetic variability, can also be used to study evolution and monitoring the pathogen variability over time and space. The information regarding genetic variability, protein and isozyme profiles of the wilt pathogen is limited at Hunsur tract of Karnataka, India. In the present study, an attempt was made to find the genetic diversity of *Fusarium oxysporum* f. sp. *nicotianae* (FON) using molecular, total soluble protein profile and the zymograms of Catalase, Cellulase, Esterase, Peroxidase and Protease.

Materials and methods

Isolation and establishment of the pathogen culture

FON strains were isolated from wilt affected tobacco plants collected from Karnataka Light Soil (KLS) region (Johnston and Booth, 1968). Identification was made based on morphological characteristics as given by Booth (1975). Cultures were also sent to Fusarium Research Centre, Penn State University, University Park, PA, USA for confirmation of identification. All cultures were maintained on Potato dextrose agar medium. Cultures obtained by single spore isolation technique (Bayaa *et al.*, 1994) were further subjected to DNA extraction and RAPD studies.

DNA extraction

FON isolates were inoculated to Potato Sucrose Broth (PSB) and was grown for 20 days at 30°C with constant agitation. The mycelia were harvested, pad-dried and kept at -20°C until further use. The frozen mycelia were ground into fine powder in liquid nitrogen using sterile pestle and mortar and DNA was extracted from the mycelial powder of each isolate following the method of Raeder and Broda (1985).

RAPD PCR analysis

RAPD-PCR was performed as described by Kini *et al.* (2002). RAPD primers were obtained from Eurofins Biotechnology, India. Five samples of genomic DNA isolated were subjected to RAPD PCR analysis with 10 OPA series primers.

PCR reaction was performed in 25 µl reaction volumes containing 1-1.5 U Taq. DNA polymerase, DNTP mix (0.2mM each of dCTP, dGTP, dATP, dTTP), 1X PCR buffer (10mM TrisHCl, pH 8.5, 50mM MgCl₂, Bangalore Genei, Pvt. Ltd., Bangalore, India), 0.5 µM primer and 25-50ng of genomic DNA. The reaction mixture was vortexed and centrifuged briefly. Amplification was performed in a thermal cycler (JH Bios, USA). Initial denaturation for 3min at 97 °C followed by 45 cycles of 1min at 94 °C, 1min at 36 °C and 2min at 72 °C and final extension step of 10min at 72 °C. All reactions were repeated twice to verify reproducibility. The amplified products were separated by electrophoresis in 1.5% agarose gel in 0.5X TAE buffer at 80volts, in Tris EDTA (90mM Tris, 90mM Boric acid, 2mM EDTA). Gels were stained with ethidium bromide and visualized with UV light and photographed by

using gel documentation system. In the control treatment PCR mixture was supplemented with sterile distilled water instead of genomic DNA.

The RAPD data from all amplifications were recorded by scoring all DNA bands (obtained from OPA1 to OPA10) and were compiled in a binary matrix. The data were converted to distance matrices based on Nei (1978) unbiased minimum distance. The distance matrices were then used to construct a dendrogram by the unweighted pair-group method with arithmetic averages (UPGMA) using tools for population genetic analysis (TFPGA ver.1.3) (Miller, 1997).

Fungal growth and enzyme production

Five isolates were grown on Potato sucrose agar in 250 ml conical flask for 20 days and the mycelial mats were collected on Whatman No. 1 filter papers and washed with sterile deionized distilled water to remove extraneous contaminations. Such mats were blot dried. 0.1g of mycelial mats of each isolate was macerated with 1ml of extraction buffer (100 mM TrisHCl, 1mM EDTA, 10mM KCl, 10mM MgCl₂ supplemented with 4% PVP). The homogenate was centrifuged at 20,000 X g for 10 minutes. The clear supernatant was collected into fresh microfuge tubes and stored at -20⁰ C until use.

Protein determination and SDS PAGE

Protein determinations of all isolates were done according to the method of Lowry (1951). Protein concentration of the mycelial extract of five isolates was adjusted to 1mg/ml with extraction buffer and loaded on to 12 % polyacrylamide gel and SDS-PAGE was carried out at 10-15 mA/cm until the tracking dye reached the separating gel. After electrophoresis gels were carefully removed and immersed overnight in the staining solution containing 0.1 % Coomassie brilliant blue, 40% methanol, 10% acetic acid and 50% of distilled water, at room temperature and destained in a mixture of 40% methanol and 10% acetic acid till the background of the gel was clear (Raghuwanshi and Dake, 2005).

Isozyme Zymograms

Catalase

The gel was washed in distilled water and incubated in the staining solution containing 1% potassium ferric cyanide and ferric chloride for 10-15

minutes. The chromophore band formed slowly. Zymograms were recorded as soon as the achromatic bands were easily visible (Sadasivam & Manickam, 2008).

Cellulase

Samples were electrophoresed in polyacrylamide gels amended with 1% carboxymethylcellulose (CMC). The gel was washed in distilled water and incubated in a staining solution containing 0.4 ml of 1% CMC in sodium citrate buffer (pH 6.2) at 50°C for 30 minutes. The reaction is terminated by adding 3, 5 dinitrosalicylic acid (DNS) and heated for 5-10 minutes in a boiling water bath, then the gels were immersed in 40% Potassium sodium tartarate solution. Zymogram was recorded as soon as the achromatic bands became evident in dark blue background (Teather and Wood, 1982).

Peroxidase

After electrophoresis gel was incubated in the staining solution containing 2.08 g of O- Dianisidine in 18: 100: 80 mL of Glacial acetic acid: 3% H₂O₂: distilled water respectively. Bright blue coloured bands appeared in gel. When the bands were stained sufficiently, the reaction was arrested by immersing the gel in a large volume of 0.67% sodium hydroxide solution for 10 minutes (Reddy and Gasber, 1971).

Esterase

The gel was incubated in a solution (Sodium hydrogen phosphate – 2.8 g; Disodium hydrogen phosphate – 1.1 g; Fast blue RR salt - 0.2g; Alpha naphthyl acetate - 0.03 g; Water - 200 mL) at 37°C for 20-30 minutes in dark. The enzyme reaction was stopped by adding a mixture of methanol: water: acetic acid: ethyl alcohol in the ratio 10: 10: 2: 1 (Smith *et al.*, 1970).

Protease

Gelatin was incorporated by (1%w/w) dissolving in 25 mM phosphate buffer (pH7.2) in the separating gel. After electrophoresis, the gel was incubated at room temperature for 1 hour in 2.5% Triton X-100 in water to remove SDS. The gel was transferred to a bath containing 0.1M glycine – Sodium hydroxide (pH 8.3) and incubated at 37°C for 3-5 minutes. The gel was fixed and stained by immersing in a 0.1% (w/v) solution of Amido black in

methanol: acetic acid: water (30: 10: 60) for 1 hour. The gel was destained in methanol: acetic acid; water, until achromatic bands appeared (Issac and Gokhale, 1982).

Spectrophotometric assay

Catalase

Wave length was set to 240nm in the U. V. visible spectrophotometer (JASCO). The final volume was 3mL approximately. At room temperature, reading was taken against the control cuvette containing enzyme solution as in the experimental cuvette, but containing only phosphate buffer (0.067 M; pH 7.0). 3 mL of H₂O₂ – PO₄ (10%) buffer is mixed with 0.001-0.004 mL sample. Time (t) was recorded to find the decrease in absorbance from 0.45-0.4 (Luck, 1974).

Cellulase

0.45 ml of 1% CMC solution at 55 °C and 0.05ml of enzyme extract were taken in a test tube. The tubes were incubated at 55°C in a water bath for 15 minutes. After incubation tubes were removed from the water bath and 0.5 ml of 3, 5-dinitrosalicylic acid was added. Again the tubes were incubated in water bath for 5 minutes. When the tubes became warm, 1 ml of Rochelle's salt solution was added. Tubes were cooled to room temperature and made up the volume to 5ml by adding 3 ml of distilled water. The absorbance was measured at 540nm using spectrophotometer and plotted on a calibration curve of β-D-Glucose in concentration range of 50-1000µg. Activity is expressed as µg glucose released per minute per mg protein (Denison and Koehn, 1977)

Peroxidase

3.5 mL of 0.1 M phosphate buffer (pH 6.5) was taken in a cuvette. 0.2 mL of enzyme extract and 0.1 mL freshly prepared O-Dianisidine solution (1mg/mL) was added. The assay mixture was brought to 28-30°C and then the cuvette was placed in the spectrophotometer set at 430 nm. Then, 0.2 mL of 0.2 M hydrogen peroxide was added and mixed well. Stop watch was immediately started and the initial absorbance was recorded. The readings were recorded every 30 seconds intervals up to 3 minutes (Putter, 1974; Malik and Singh, 1980).

Esterase

The assay was carried out by modifying the method of Hagerman and Austin, 1986. To achieve a constant starting pH for the reaction, all solutions (pectin, indicator dye, water) were adjusted to pH 7.50 with concentrated (2 N) NaOH just before each trial that was started. This adjustment was made whenever the assay was run or calibrated. 0.5% (w/v) solution of apple pectin (SRL) was prepared in distilled water by heating the mixture to 40 °C while continuously stirring. This solution was used for 1 month when stored at 4 °C. 0.01% (w/v) solution of bromothymol blue was prepared in 0.003 M, pH 7.5, potassium phosphate buffer. The reaction was monitored at 620 nm in a UV-VIS spectrophotometer (Jasco). The temperature was maintained at 25 °C. In a cuvette 2.00 mL of pectin was mixed with 0.15 mL of bromothymol blue and 0.83 mL of water, and the initial absorbance at 620 nm (A_{620}) of the mixture was determined against water. The initial absorbance at 620 nm, measuring about 0.28 remained constant until the enzyme was added, indicating that non-enzymatic hydrolysis. The reaction was started by adding 20 μ l of fungal extract, and the rate of decrease in absorbance at 620 per min was recorded.

Protease

Two vials were taken and marked as test-1 and blank-1. The casein solution (0.65%; pH 7.5) was pipetted into both the vials of 5 mL each. It was equilibrated to 37 °C. Then 1 mL of the enzyme solution (enzyme extract in Sodium acetate buffer, pH 7.5) was added to test-1 vial. Both the reagents and the vials were mixed by swirling and incubated at 37 °C for exactly 10 minutes. Then 5 mL of 10 mM TCA was added to both the vials. 1 mL of enzyme solution was added to only blank-1 vial. Both the vials were swirled and incubated at 37 °C for 30 minutes. The solutions were filtered through Whatman #5 filter paper. The filtrate was used for colour development.

Fresh vials were taken and labeled as test-2 and blank-2 again. 2 mL of test-1 filtrate was pipette into test-2 vial. 2 mL of blank-1 filtrate was pipette into blank-2 vial. 5 mL and 1mL of 500mM sodium carbonate solution and Folin & Ciocalteu's Phenol reagent were added to both the vials respectively. The reagents were mixed thoroughly by swirling and incubated at 37 °C for 30 minutes. The vials were removed and allowed to cool to room temperature. The reagents were filtered again through Whatman #5 filter paper immediately prior to reading. The absorbance was read at 660nm for each of the vials in suitable cuvettes (Anson, 1938; Folin and Ciocalteu, 1929).

Results

Isolation and establishment of the pathogen culture

A total of 5 isolates of *F. oxysporum* f. sp. *nicotianae* were brought into pure culture from root and stem of infected tobacco plants. Based on morphological characteristics the isolates were identified as *F. oxysporum*. Identification was confirmed by Fusarium Research Centre, Penn State University, University Park, PA, USA.

RAPD DNA Finger printing analysis

The primers OPA1 to OPA10 generated a total of 380 scorable markers from the amplification products which were polymorphic. All the primers differentiated isolates isolated from the same geographical area (Fig. 1). Amplified fragments ranged from 100bp to 1Kb. The number of DNA fragments amplified and scored per isolate for individual primer ranged from 2 to 9. Cluster analysis with UPGMA using genetic distances showed that the isolates are divided into 2 main groups (Fig. 2). The isolates isolated from Piriapatna were found to be highly virulent (1 FON, 3 FON, 10 FON and 13 FON) than the isolate (9FON) isolated from Hunsur which was found to be least pathogenic.

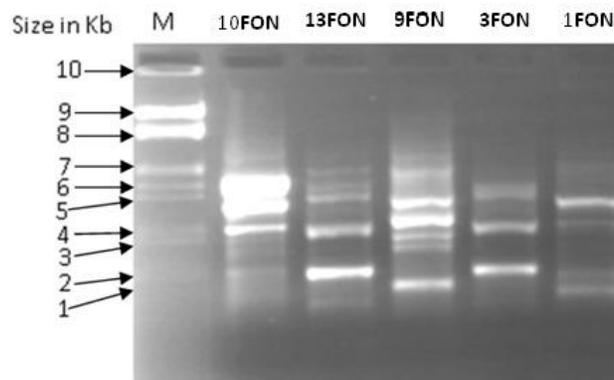


Fig. 1. DNA finger printing profile of 5 isolates of *F. oxysporum* f. sp. *nicotianae* obtained from Random Amplified Polymorphic DNA (RAPD) marker. RAPD profiles obtained with primer OPA1. Lane M: DNA marker; 1-5 : *F. oxysporum* f. sp. *nicotianae* DNA

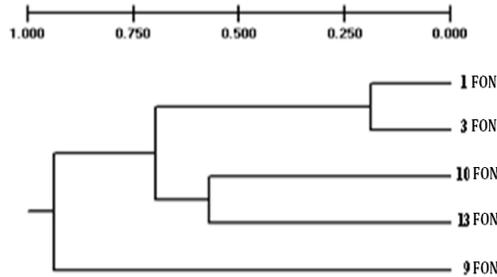


Fig. 2. Dendrogram of 5 isolates of *F. oxysporum* f. sp. *nicotianae* revealed by UPGMA cluster analysis of genetic similarities based on RAPD data.

SDS PAGE

Results showed that each of *F. oxysporum* strains had their own unique protein profiles (Fig. 3). The molecular weight of all protein bands ranged from 14.3 to 116.88 kDa (Table. 1). The protein profile of different isolates showed common banding protein band of 97.4 kDa and 14.4 kDa in all 5 isolates of *F.oxysporum*. 13 FON and 1 FON isolates were distinct in having bands of 116.88 kDa. 13 FON, 3 FON and 10 FON showed similar protein banding pattern at 82.79 and 64.0 kDa. Isolate 3 FON was unique having protein band of 56 kDa. Isolate 13 FON had two distinct protein bands of 109.57 and 20.3 kDa. Protein band of 39.41kDa was present only in isolates 13 FON and 10 FON. Isolates 9 FON and 3 FON exhibited similar banding patterns of 90.09 kDa. The present study revealed that the isolate 13 FON was found to be unique in having two additional bands of molecular weight 109.57 and 20.3 kDa which were absent in others.

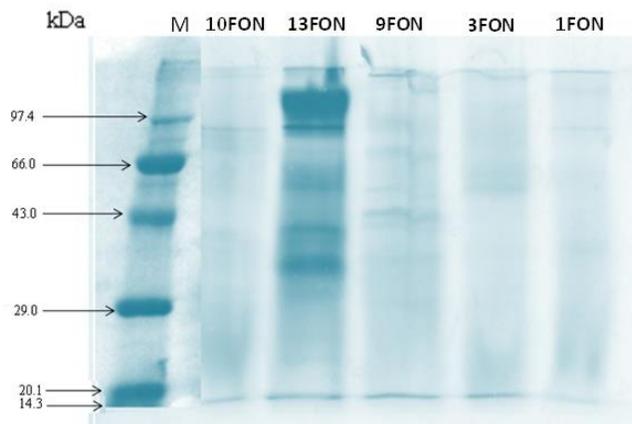


Fig. 3. The SDS-PAGE protein profile showing variability in five isolates of *F. oxysporum* f. sp. *nicotianae*

Table 1. Rm values of protein banding pattern of mycelial extract of the 5 isolates of *F. oxysporum* f. sp. *nicotianae*

Bands	Standard markers	10 FON	13 FON	9 FON	3 FON	1 FON	Molecular wt in kDa
1	-	-	0.004	-	-	0.004	116.88
2	-	-	0.1	-	-	-	109.57
3	0.2	0.2	0.2	0.2	0.2	0.2	97.4
4	-	0.26	-	0.26	-	-	90.09
5	-	-	0.32	0.32	0.32	-	82.79
6	0.34	-	-	-	-	-	66.0
7	-	-	0.36	0.36	0.36	-	64.0
8	-	-	-	0.44	-	-	56.0
9	-	0.46	0.46	0.46	-	-	54.0
10	0.52	-	-	-	-	-	43.0
11	-	-	0.56	-	0.56	-	39.41
12	-	-	0.6	0.6	-	-	35.33
13	-	-	0.72	0.72	-	-	31.08
14	0.8	-	-	-	-	-	29.0
15	-	-	0.86	-	-	-	20.3
16	0.94	-	-	-	-	-	20.1
17	0.98	0.98	0.98	0.98	0.98	0.98	14.3

Zymograms

Catalase

Catalase zymogram clearly separated FON isolates with a few exceptions (Fig. 4). In catalase isozyme profile isolate 13 FON exhibited one thick band of Rm 0.21 and remaining thin bands at Rm values 0.31, 0.38, 0.5, 0.55, 0.6 and 0.78. All the remaining isolates except 13 FON, exhibited common band at Rm value 0.66 (Table. 2).

Table 2. Rm values of Catalase zymogram of 5 isolates of *F. oxysporum* f. sp. *nicotianae*

Catalase				
10 FON	13 FON	9 FON	3 FON	1 FON
0.28	0.216	0.66	0.66	0.66
0.66	0.31	--	--	--
--	0.38	--	--	--
--	0.5	--	--	--
--	0.55	--	--	--
--	0.6	--	--	--
--	0.78	--	--	--

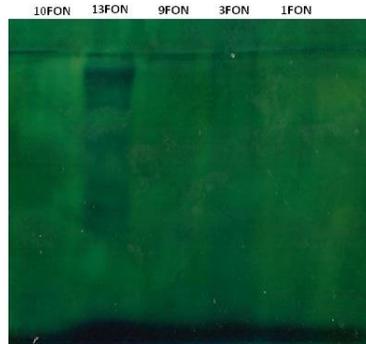


Fig. 4. Catalase zymogram of all five *F. oxysporum f. sp. nicotianae* isolates

Cellulase

Cellulase zymogram of all 5 isolates showed variation (Fig. 5). The band at Rm 0.41 was seen to be present in isolate 13 FON which was not common to all. And a common band at Rm 0.5 was seen in 13 FON and 1 FON. Except these two isolates, no bands were seen in the other isolates (Table. 3).

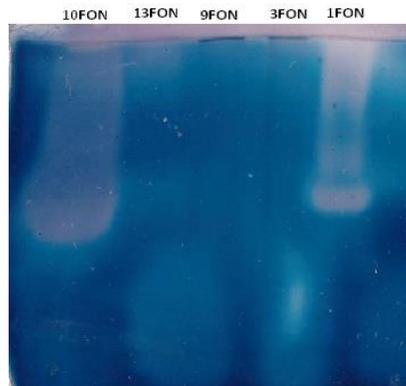


Fig. 5. Cellulase zymogram of all five *F. oxysporum f. sp. nicotianae* isolates

Table 3. Rm values of Cellulase zymogram of 5 isolates of *F. oxysporum f. sp. Nicotianae*

Cellulase				
10 FON	13 FON	9 FON	3 FON	1 FON
0.5	0.41	--	--	0.5
--	0.5	--	--	--

Peroxidase

Peroxidase zymogram exhibited polymorphism in all isolates (Fig. 6). Rm values ranged from 0.33 to 0.51 among them (Table. 4). A single band of Rm 0.51 was different in 10 FON. A common band of Rm 0.33 was observed in 13 FON and 9 FON.



Fig. 6. Peroxidase zymogram of all five *F. oxysporum* f. sp. *nicotianae* isolates

Table 4. Rm values of Peroxidase zymogram of 5 isolates of *F. oxysporum* f. sp. *nicotianae*

Peroxidase				
10 FON	13 FON	9 FON	3 FON	1 FON
0.41	0.33	0.33	0.36	0.36
0.51	0.41	0.4	0.4	0.4

Esterase

Polymorphism was seen in esterase zymogram to some extent (Fig. 7). A common band at Rm 0.4 was observed to be seen in all the isolates except in 10 FON, and Rm 0.55 were common to isolates 13 FON and 3 FON, 0.63 were common to 3 FON and 1 FON; 0.66 was again found in 13 FON and 10 FON which was not seen to be present in others and Rm 0.7 was found to be common to 9 FON, 3 FON and 1 FON isolates (Table. 5).

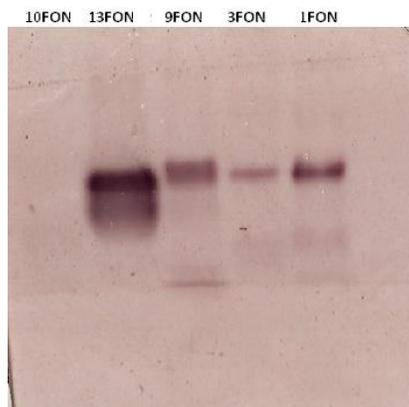


Fig. 7. Esterase zymogram of all five *F. oxysporum f. sp. nicotianae* isolates

Table 5. Rm values of Esterase zymogram of 5 isolates of *F. oxysporum f. sp. nicotianae*

Esterase				
10 FON	13 FON	9 FON	3 FON	1 FON
0.66	0.4	0.4	0.4	0.4
--	0.55	0.55	0.63	0.63
--	0.66	0.7	0.7	0.7

Protease

Protease zymogram of the isolates exhibited polymorphism (Fig. 8) and the Rm values ranged from 0.11 to 0.65, but isolate 3 FON did not show any of the bands (Table. 6).



Fig. 8. Protease zymogram of all five *F. oxysporum f. sp. nicotianae* isolates

Table 6. Rm values of protease zymogram of 5 isolates of *F. oxysporum* f. sp. *nicotianae*

Protease				
10 FON	13 FON	9 FON	3 FON	1 FON
0.11	0.43	0.1	--	0.31
0.16	0.41	0.23	--	--
--	0.65	--	--	--

Isozyme Cluster analysis

A cluster analysis with UPGMA using genetic distances showed that all the 5 isolates formed two main groups, A and B (Fig. 9). Three isolates out of five clustered in A, the isolates were further divided into sub-cluster A1 and A2. Within the sub-cluster A1 the isolates were subdivided into I and II clusters. The isolate in I cluster (3 FON) and the isolate in II cluster (1 FON) were found to be similar in their zymograms and enzyme activity. Isolate 13 FON formed separate cluster of A2 and found to be unique in all the zymograms and enzyme activity. Isolates in the cluster B was further divided into B1 and B2. Isolate 9 FON formed in B1 and isolate 10 FON in cluster B2 exhibited the similar zymogram pattern and enzyme activity.

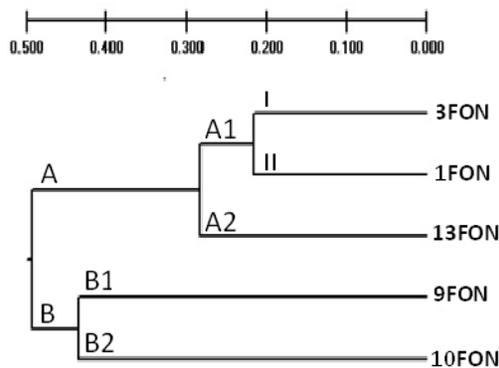


Fig. 9. Dendrogram of all Zymograms of all the five isolates of *F. oxysporum* f. sp. *nicotianae*

Spectrophotometric assay

All isolates showed enzyme activity and the amount of activity varied from isolate to isolate (Table 7). 13 FON exhibited highest catalase activity of 26.56 units/ ml compared to all the others. Exception was observed with respect to 13 FON and 3 FON where cellulase activity was not seen. Cellulase activity was seen only in 10 FON, 9 FON and 1 FON. Among the isolates 10

FON and 1 FON showed activity of 0.074 $\mu\text{m}/\text{min}/\text{mL}$ and 9 FON exhibited 0.037 $\mu\text{m}/\text{min}/\text{mL}$. Peroxidase activity ranged from 0.01 to 0.09/min/mg protein. Isolate 13 FON was found to be highest in its activity of 0.09/min/mg of protein and 3 FON being the least of 0.01/ min/mg protein among all the other isolates. The highest esterase activity was exhibited by the isolate 13 FON when compared to all the other isolates and the least activity was observed in 9 FON (Fig. 10). The Protease activity was found to be different ranging from 0.00302 to 0.0242 units/ ml. Activity was seen to be highest in 13 FON of 0.0242 units/ mL 9 FON had least proteolytic activity compared to 3 FON.

Table 7. Spectrophotometric assay of all the zymograms of *F. oxysporum* f. sp. *Nicotianae*

FON Isolates	CATALASE units/ ml	CELLULASE $\mu\text{m}/\text{min}/\text{ml}$	PEROXIDASE min/mg protein	PROTEASE units/ml
10 FON	6.20	0.074	0.08	0.0151
13 FON	26.56	----	0.09	0.0242
9 FON	8.5	0.037	0.04	0.00302
3 FON	15.17	-----	0.01	0.0181
1 FON	0.0151	0.074	0.08	0.0151

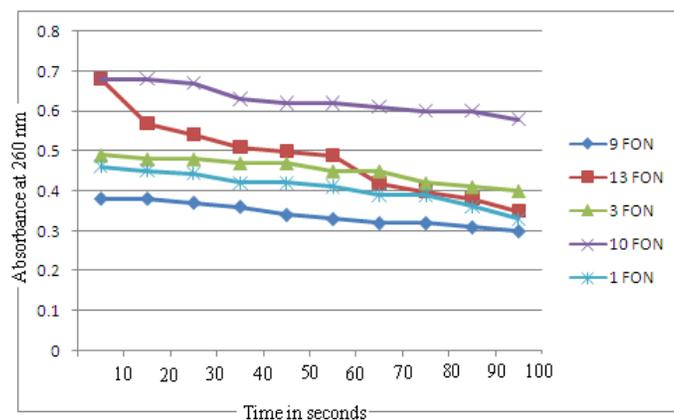


Fig. 10. Continuous Spectrophotometric assay of all the zymograms of *F. oxysporum* f. sp. *nicotianae*

Discussion

RAPD studies suggested that considerable genetic variation existed among the isolates of FON isolated from different regions of Karnataka. In concurrence with the present study Kini *et al.* (2002) were able to differentiate 28 isolates of *F. moniliformae* using RAPD. Similar observations were made by

Crowhurst *et al.* (1991) for *F. solani* f. sp. *cucurbitae* and several other formae specialis of *F. oxysporum* (Assigbetse *et al.*, 1994; Grajal- Martin *et al.*, 1993; Manulis *et al.*, 1994). The ability of RAPD analysis to distinguish different isolates of FON will facilitate further investigations of this pathogen with respect to genetic variability.

In the present study, the SDS PAGE also showed variation among the isolates and this may be a supporting data for the presence of high genetic diversity. Similar studies were also reported by Ibrahim *et al.* (2003). This is the first report on the diversity of FON based SDS PAGE. Such variations based on SDS PAGE in the case of another formae specialis *viz.*, *F. oxysporum cubense* is reported by Kumar *et al.*, 2010. Moreover, in some cases the results obtained by SDS-PAGE of whole-cell proteins can discriminate at the same level as DNA fingerprinting (Priest and Austin, 1993; Brown, 1996). The present study showed that five isolates of FON collected from different locations of Karnataka were composed of heterogenous group. Similar type of work was carried out by Bielenin *et al.* (1988) and Mills *et al.* (1991) in *Phytophthora cryptogea*.

During the present investigation, the genetic and biochemical characteristics of the FON isolates colonizing tobacco were varying to a greater extent. This was revealed through the variations in the polymorphic bands of all the isozymes tested. Our study is in concurrent with the earlier workers where they also have found out significant variation in isozyme patterns of various *Fusarium* species. The use of isozyme markers for estimating the extent of diversity within a fungal population is well documented (Michales *et al.*, 1986; McDonald and McDermott, 1993). Similarly Ye and Wu (1985) showed the variations in the isozyme pattern that were distinct for 24 *F. graminearum* isolates. Such studies have also been focused on the biochemical capabilities of the *F. oxysporum* in secreting the cell wall degrading enzymes like catalase, protease and peroxidase (Yoshida *et al.*, 1989; Kono *et al.*, 1995; Barata *et al.*, 2002; di Pietro *et al.*, 2001; Rypniewski *et al.*, 1993). Variation among the isolates from restricted regions was observed by Burdon (1993) in *F. oxysporum cubense*. The isozyme patterns may provide additional information about the genetic structure of FON isolates. The polymorphic isozyme systems could serve as an indicator of genetic variability, identification and characterization of the pathogen.

Present investigation revealed the correlation between the zymogram banding pattern and enzyme activity. Our pathogenicity studies (data not shown) have shown that 13 FON is the most virulent pathogen and the present study has revealed intense zymogram banding pattern and highest activity of esterase by the same isolate. MacHardy and Beckman (1981) have also reported the role

of esterase enzyme production and activity in the pathogenicity of *Fusarium* species. 13 FON being the highest virulent fungus showed distinct zymogram banding pattern as well as highest enzyme activity in case of catalase, peroxidase, protease and esterase. However, the same isolate even though did not show any banding pattern for cellulase, showed highest cellulase activity. This result shows the importance of checking the activity of various isozymes which may have an important role in pathogenicity. Importance of cellulase enzyme during wilt induction and further enhancement of disease was explained by Husain and Dimond (1960). According to them *Fusarium* produces C1 and Cx cellulase enzymes. They also reported that, the action of cellulase produced by the pathogen firstly involves in wilt induction, secondly, hydrolytic products produced by cellulase activity provide the carbohydrates which enhances the continued development of the pathogen on the host and thirdly, it is involved in the escape of pathogen through vascular tissue at advanced stages of disease development when the host is in dying condition. Thus activity of cellulase appears to be critical for a virulent pathogen. Thus the present work has shown for the first time the importance of checking the activity of isozymes in addition to the study of zymogram.

The isozymes namely protease, peroxidase and catalase were also found to be variable and their activity probably helps in various steps for successful infection. Thus the present study has reported for the first time the extent of genetic and biochemical variations existing in a devastating pathogen like FON infecting tobacco in Karnataka, India. The data will be very useful in resistance breeding of the host plant against the wilt pathogen.

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References

- Anson, M. L. (1938). The Estimation of pepsin, trypsin, papain and cathepsin with haemoglobin. *The Journal of General Physiology* 22:79-89.
- Assigbetse, K. B., Fernandez, D. Dubois, M. P. and Geiger, J. P. (1994). Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. *Phytopathology* 84:622-626.
- Balali, G. R. and Iranpoor, M. (2006). Identification and genetic variation of *Fusarium* species in Isfahan, Iran, using pectic zymogram technique. *Iranian Journal of Science and Technology* 30:91-102.

- Barata, R. A., Andrade, M. H. G. Rodrigues, R. D. and Castro, I. M. (2002). Purification and characterization of an extracellular trypsin-like protease of *Fusarium oxysporum* var. *lini*. Journal of Bioscience and Bioengineering 94:304-308.
- Bayaa, B., Erskine, W. and Abbas, A. (1994). Evaluating different methods for screening lentil germplasm for resistance to lentil wilt caused by *Fusarium oxysporum* f. sp. *lentis*. Arab Journal of Plant Protection 12:83-91.
- Bielenin, A., Jeffers, S. N. Wilcox W. F. and Jones, A. L. (1988). Separation by protein electrophoresis of six species of *Phytophthora* associated with deciduous fruit crops. Phytopathology 78:1402-1408.
- Brown, J. K. M. (1996). The choice of molecular marker methods for population genetic studies of plant pathogens. New Phytologist 133:183-185.
- Burdon, J. J. (1993). The structure of pathogen populations in natural plant communities. Annual Review of Phytopathology 31:305-323.
- Crowhurst, R. N., Hawthorne, B. T., Rikkerink, E. H. A. and Templeton, M. D. (1991). Differentiation of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. Current Genetics 20:391-396.
- Denison, D. A. and Koehn, R. D. (1977). Cellulase activity of *Poronia oedipus*. Mycologia 69:592-603.
- di Pietro, A., Huertas-Gonzalez, M. D. Gutierrez-Corona, J. F. Martinez-Cadena, G. Meglecz E. and Roncero, M. I. G. (2001). Molecular characterization of a suitable from the vascular wilt fungus *Fusarium oxysporum*. Molecular Plant Microbe Interactions 14:653-662.
- Folin, O. and Ciocalteau, V. (1929). On tyrosine and tryptophane determinations in proteins. Journal of Biological Chemistry 73:627-650.
- Garner, W. W. (1951). The Production of Tobacco. New York, USA: The Blakiston Company 520 pp.
- Grajal-Martin, M. J., Simon, C. J. and Muehlbauer, F. J. (1993). Use of random amplified polymorphic DNA (RAPD) to characterize race 2 of *Fusarium oxysporum* f.sp. *pisi*. Phytopathology 83:612-614.
- Hagerman, A. E. and Austin, P. J. (1986). Continuous spectrophotometric assay for plant pectin methyl esterase. Journal of Agricultural and Food Chemistry 34:440-444.
- Hall, R. (1967). Proteins and Catalase isozymes from *Fusarium solani* and their taxonomic significance. Australian Journal of Biological Science 20:419-428.
- Husain, A. and Dimond, A. E. (1960). Role of cellulolytic enzymes in pathogenesis by *Fusarium oxysporum* f. sp. *lycopercici*. Phytopathology 50:329-331.
- Huss, M. J., Campbell, C. L. Jennings, D. B. and Leslie, J. F. (1996). Isozyme variation among biological species in the *Gibberella fujikuroi* species complex (*Fusarium* section *Liseola*). Applied and Environmental Microbiology 62:3750-3756.
- Ibrahim, S. M. (2003). Biochemical and molecular genetic fingerprints of some *Cruciferae* plants. (Master's thesis). Faculty of Education, Ain Shams University, Cairo, Egypt.
- Issac, S. and Gokhale, A. V. (1982). Autolysis: a tool for protoplast production from *Aspergillus nidulans*. Transactions of the British Mycological Society 78:389-394.
- Johnston, A. and Booth, C. (1968). Plant Pathologist's Pocketbook. Commonwealth Mycological Institute, Oxford & IBH Publishing Co. 439 pp.
- Kini, K. R., Leth, V. and Mathur, S. B. (2002). Genetic variation in *Fusarium moniliformae* isolated from seeds of different host species from Burkinafaso on Random Amplified Polymorphic DNA analysis. Journal of Phytopathology 150:209-212.
- Kistler, H. C. (1997). Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. Phytopathology 87:474-479.

- Kono, Y., Yamamoto, H., Takechi, M. and Komada, H. (1995). Alterations in superoxide dismutase and catalase in *Fusarium oxysporum* during starvation-induced differentiation. *Biochimica et Biophysica Acta* 1268:35-40.
- Kumar, B. H., Shanker, U. A. C. Nayaka, C. S. R. Kini, K. Shetty, H. S. and Prakash, H. S. (2010). Biochemical characterization of *Fusarium oxysporum* f.sp. *cubense* isolates from India. *African Journal of Biotechnology* 9:523-530
- Luck, H. (1974). Catalase. *Methods in Enzyme Analysis*. New York and London: Academic press pp. 885-894.
- Malik, C. P. and Singh, M. B. (1980). *Plant Enzymology and Histoenzymology*. New Delhi: Kalyani Publishers 53 pp.
- Manulis, S., Valinsky, L. Lichter, A. and Gabriel, D. W. (1994). Sensitive and specific detection of *Xanthomonas campestris* pv. *pelargonii* with DNA primers and probes identified by random amplified polymorphic DNA analysis. *Applied and Environmental Microbiology* 60:4094-4099.
- Matsuyama, N. and Wakimoto, S. (1977). A comparison of esterase and catalase zymograms of *Fusarium* species with special reference to the classification of a causal fungus of *Fusarium* leaf spot of rice. *Annals of the Phytopathological Society of Japan* 43:462-470.
- McDonald, B. A. and McDermott, J. M. (1993). Population genetics of plant pathogen fungi. *Bioscience* 43:311-319.
- MacHardy, W. E. and Beckman, C. H. (1981). Vascular wilt Fusaria: Infections and pathogenesis. *Fusarium: Diseases, Biology and Taxonomy*. University Park and London: The Pennsylvania State University Press. pp. 365-390.
- Micales, J. A., Bonde, M. R. and Peterson, G. L. (1986). The use of isozyme analysis in fungal taxonomy and genetics. *Mycotaxon* 27:405-449.
- Miller, M. (1997). Tools for population genetic analysis (TFPGA) 1.3: A Windows programme for the analysis of Allozyme and molecular population genetic data computer software distributed by the author.
- Mills, P. K., Beeson, W. L. Phillips R. L. and Fraser, G. E. (1991) Bladder cancer in a low risk population results from the Adventist Health Study. *American Journal of Epidemiology* 133:230-239.
- Mohammadi, M., Aminipour, M. and Banihashimi, Z. (2004). Isozyme analysis and soluble mycelial protein pattern in Iranian isolates of several formae speciales of *Fusarium oxysporum*. *Journal of Phytopathology* 152:267-276.
- Nelson, P. E., Tousson, T. A. and Cook, R. J. (1981). *Fusarium: Diseases, Biology, and Taxonomy*. University Park, PA, USA: The Pennsylvania University Press.
- Priest, F. G and Austin, B. (1993). *Modern bacterial taxonomy* Second edition. Chapman and Hall, London.
- Putter, J. (1974). *Methods of Enzymatic Analysis* 2. New York: Academic Press. 685 pp.
- Raeder, U. and Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* 1:17-20.
- Raghuwanshi, K. S. and Dake, G. N. (2005). Variability in PAGE protein pattern of safflower wilt pathogen *Fusarium oxysporum* f.sp. *carthami*. *Journal of Mycology and Plant Pathology* 35:122-124.
- Ramakrishnan, S., Sheno M. M. and Sreenivas, S. S. (2005). Preliminary studies on interaction between root-knot nematodes and *Fusarium* wilt disease in FCV tobacco. *Proceedings of the National Conference on Tobacco, Andhra Pradesh, India*. 86 pp.

- Reddy, M. M., and Gasber, E. O. (1971). Genetic studies of variant enzyme. III. Comparative Electrophoretic studies of esterases and peroxidases for species, hybrids and amphidiploid in the genus *Nicotiana*. *Botanical Gazette* 132:158-166.
- Rypniewski, W. R., Hastrup, S. Betzel, C. Dauter, M. Dauter, Z. Papendorf, G. Branner, S. and Willson, K. S. (1993). The sequence and x-ray structure of the trypsin from *Fusarium oxysporum*. *Protein Engineering* 6:341-348.
- Sadasivam, S and Manickam, A. (2008). *Biochemical Methods*. New Delhi: New Age International Publishers Limited. 270 pp.
- Sharma, P., Sharma, K. D. Sharma, R. and Plaha, P. (2006). Genetic variability in pea wilt pathogen *Fusarium oxysporum* f. sp. *Pisi* in North Western Himalayas. *Indian Journal of Biotechnology* 5:298-302.
- Shenoi, M. M and Nagarajan, K. (2000). Diseases of FCV tobacco and their management in Karnataka light soil region. Central Tobacco Research Institute, Rajamundry, (Indian Council of Agricultural Research), CTRI Research Station, Hunsur, Karnataka, India. 31 pp.
- Smith, H. H., Hamill, D. E. Weaver, E. A. and Thompson, K. H. (1970). Multiple molecular forms of peroxidases and esterases among *Nicotiana* species and amphiploids. *Journal of Heredity* 61:203-212.
- Teather, R. M. and Wood, P. J. (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied Environmental Microbiology* 43:777-780.
- Ye, H. Z. and Wu, G. Q. (1985). Polyacrylamide studies of the esterase pattern of *Fusarium* sp. Causing head blight of wheat and and barley. *Acta Phytopathol Sinica* 15:87-94.
- Yoshida, H., Iizuka, M. Norioka, N. Norioka, S. and Sakiyama, F. (1998). Cloning and sequencing of cDNA encoding ribonuclease F1 from *Fusarium moniliformae*. *Journal of Biochemistry and Molecular Biology* 45:555-560.

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