

# Molecular Identity of *Fusarium oxysporum* Isolates Collected from Karnataka State

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## ABSTRACT

*Fusarium* species cause wilt, yellowing and root rot in most horticultural crops. *Fusarium oxysporum* generally causes wilt and has many *formae specialis* depending on the host. To know their exact identity, the present study was carried out to differentiate isolates collected from different agro-climatic regions of Karnataka State using molecular methods. *Fusarium* species affecting onion, musk melon, tomato, okra and pea were isolated from infected plants, purified and then multiplied on potato dextrose broth. Isolated DNA of 20-d cultures was amplified by PCR using primers specific to the ITS region. Out of 5 cultures, 4 isolates belonged to one group. The 4 isolates showing the same bands were further differentiated by RFLP analysis using *AluI* to confirm whether they belonged to the same *formae specialis*. Based on PCR-RFLP analysis of amplified product of On-5 and To-3, both isolates were found to be the same; similarly, Mu-2 and Ok-1 were the same while other isolates were different.

**Keywords:** *Fusarium* isolates, ITS, pathogenicity, PCR-RFLP, root rot, vegetable crops

## INTRODUCTION

The genus *Fusarium* is a soil-borne, necrotrophic, plant pathogenic fungus with many species that cause serious plant diseases around the world. *Fusarium oxysporum* causes primarily vascular wilts on many crops, whereas numerous species, especially *F. solani*, cause root and stem rots and rots of seeds. *F. oxysporum* consists of more than 120 *formae specialis* according to the hosts they infect. It is very difficult to manage this pathogen due to its broad host range, extreme variability in pathogenic isolates and prolonged active phase of the disease cycle (Agrios 2005).

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the detection of pathogens at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett 1992). They also occur in multiple copies with up to 200 copies per haploid genome (Bruns *et al.* 1991; Yao *et al.* 1992) arranged in tandem repeats with each repeat consisting of the 18S small subunit, the 5.8S, and the 28S large subunit genes. The nuclear ribosomal DNA repeat includes both highly conserved genes and more variable spacer regions. The spacer regions, including the internal transcribed spacer (ITS) and the intergenic spacer (IGS), have been used to examine relationships among more closely related *Fusarium* taxa by analysis of restriction fragment length polymorphism (RFLP) or sequence analysis (Appel and Gordon 1995; Edel *et al.* 1995; Appel and Gordon 1996; Baayen *et al.* 1998; Lee *et al.* 2000; Zambounis *et al.* 2007). In the broader context, taxon-selective amplification of ITS regions becomes a common approach in molecular identification strategies. Taxon-selective ITS amplification has already been used for detection of the fungal pathogens such as *F. sambucinum* (O'Donnell 1992) and *Verticillium* spp. (Nazar *et al.* 1991).

In the present study, *F. oxysporum* isolates from different agro-climatic regions in India were selected for rapid and precise detection and identification of Karnataka isolates using PCR amplification and RFLP of ribosomal DNA internal transcribed spacer regions.

## MATERIALS AND METHODS

Isolation and purification of *F. oxysporum* cultures was done from fresh infected parts of host such as onion cv. 'Arka Kalyan' (*Fusarium oxysporum* f.sp. *cepe*), musk melon cv. 'Arka Jeet' (*Fusarium oxysporum* f.sp. *niveum*), tomato cv. 'Arka Meghali' (*Fusarium oxysporum* f.sp. *lycopersicum*), okra cv. 'Arka Anamika' (*Fusarium oxysporum* f.sp. *vasinfectum*) and pea cv. 'Arka Ajit' (*Fusarium oxysporum* f.sp. *pisi*) collected from different agro-climatic regions of Karnataka. All these isolates were purified by the hyphal tip method. They were maintained at 15°C on potato dextrose agar (PDA) (Himedia Laboratory Pvt. Ltd., Mumbai, India) in 9 cm Petri dishes for further study.

### Variability of *Fusarium* isolates

The cultural character was recorded on day 7 after inoculation of all isolates of *F. oxysporum* on potato dextrose agar media (at 40 g L<sup>-1</sup> was autoclaved at 1 atm for 20 min). Characters such as pigmentation on mycelium, mycelial growth, zonation were recorded by direct observation of culture-grown Petri dishes and sporulation was recorded on PDA by slides of 7-day-old cultures under the microscope. For this purpose five isolates of *F. oxysporum* were taken, representing tomato isolate (To-3) from Kolar District, musk melon isolate (Mu-2) from Hasan District, okra isolate (Ok-1) from Mandya District, pea isolate (Pe-2) from Dharwad District and onion isolate (On-5) from Gulbarga District. All these isolates were tested for their cultural and morphological variations on PDA. For each isolate five Petri dishes were poured with PDA. After solidification of the agar 6 mm culture bits of each isolate were inoculated onto PDA. These inoculated Petri dishes were kept in a BOD incubator (Newtronic Equipment Co., Mumbai, India) at 27 ± 1°C, 65% RH and a 16:8 h photoperiod with a light intensity of 300 µE/m<sup>2</sup>/s for growth. Data was recorded after 3 days from inoculation and radial growth was measured on day 7 on PDA.

Slides were prepared from the culture of all isolates of *F. oxysporum* separately from 7 day-old cultures of PDA and examined under an Acc-Scope 3016 EPI-Fluorescent microscope to record the width of the conidiogenous hyphae.

## Molecular characterization: DNA extraction and PCR amplification

All the above isolates were grown in 100 ml of potato dextrose broth medium (PDA without agar agar) for 9 days in a BOD incubator at  $27 \pm 1^\circ\text{C}$ , 65% RH and a 16:8 h photoperiod. The genomic DNA was extracted and purified using the CTAB buffer method modified from Nicholson *et al.* (1996). The mycelial culture was frozen and ground in liquid nitrogen into fine powder and an aliquot (1 ml) of CTAB (hexadecyl trimethyl ammonium bromide) buffer (CTAB 22 mM, sarkosyl 34 mM, sorbitol 137 mM, EDTA 22 mM, polyvinylpyrrolidone (PVPP) 1%, NaCl 1.2 mM) was added. The resultant mixture was ground into paste in the same mortar. The ground sample was extracted in CTAB buffer for 60 min at  $65^\circ\text{C}$ . One-third volume of 5 M potassium acetate was added along with 1 ml chloroform: isoamyl alcohol (24:1), mixed and held at  $20^\circ\text{C}$  for 20 min. The mixture was then centrifuged at  $1900 \times g$  for 15 min and the aqueous phase was added to two volumes of 95% ethanol. The DNA was precipitated at  $850 \times g$  and the pellet was washed twice with 70% (v/v) ethanol before being dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Extracted DNA was electrophoresed in 0.8% agarose gels at 50 mA for 90 min. The gels were stained for 15 min in ethidium bromide (EtBr;  $0.5 \text{ mg ml}^{-1}$ ) and destained for 15 min in distilled water; alternatively, EtBr was incorporated directly into the gels at a rate of  $0.5 \text{ mg ml}^{-1}$ . The gel was photo graphed using BIO-Vision<sup>++</sup>, transilluminator gel documentation system (Vilber Lourmat Co., Ltd, France) and DNA concentrations were estimated spectrophotometrically using nano drop ND-1000 (JH Bio, Bangalore).

PCR analysis was carried out to amplify the internal transcribed spacer (ITS) region in the DNA of the *Fusarium* isolates. The forward primer of *ITSF3* was 5'-GCATCGATGAAGAACGC AGC-3' and the reverse of *ITSR4* was 5'-TCCTCCGCTTATTGA TATGC-3' (White *et al.* 1990). The PCR reaction mixture (20  $\mu\text{l}$ ) contained 0.3 U *Taq* DNA polymerase, 1X assay buffer (10 mM pH 9.0 TRIS-HCl, 50 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 0.01% gelatin), 150  $\mu\text{M}$  of each dNTP, 1  $\mu\text{l}$  of each forward and reverse primer at a final concentration of 0.25  $\mu\text{M}$  and 60 ng template DNA. The reaction mix without template DNA was used as water blank. The PCR reaction profile was composed of 35 cycles, with strand separation at  $94^\circ\text{C}$  for 30 s, annealing at  $55^\circ\text{C}$  for 30 s and extension at  $72^\circ\text{C}$  for 1 min. The program was extended for 10 min at  $72^\circ\text{C}$ . The amplification products were analyzed on 1.0% agarose-EtBr gel.

The amplified product was purified using the GeneElute<sup>TM</sup> PCR Clean up kit (Sigma-Aldrich, Inc., USA) PCR purification kit. The product was later checked for its quality and quantity of the product on a 1.0% agarose-EtBr gel. Approximately 1  $\mu\text{g}$  of PCR amplified product was subjected to RFLP analysis. For this study, restriction enzymes (REs) *AluI*, *EcoRI*, and *HhaI* (New England BioLabs Inc., Chennai, India) at 3 U/ $\mu\text{g}$  of amplified product was digested at  $37^\circ\text{C}$  in a thermo-mixer for 90 min. The RE-digested products were separated on a 1.0% agarose-EtBr gel.

## Pathogenic variability

The pathogenicity test was conducted in order to confirm identification of the disease and its causal agent, under greenhouse conditions in pot experiments with tomato, musk melon, pea, okra and onion. Seedlings of these five vegetables were raised in Protrays<sup>®</sup> (60  $\times$  15 cm) containing soilrite in the nursery and were protected using 80 mesh nylon net to avoid insect attack at the seedling stage. Ten-day old seedlings were transplanted to 45  $\times$  30 cm diameter earthen pots containing autoclaved red loamy soil (volume  $\sim$ 12

kg) and a dose of 120 N: 80 P: 50 K (Nagarjuna Fertilizers Pvt. Ltd., Hyderabad, India) was applied to the soil. Five replications were maintained for each cultivar. Plants 20-25 days old were used for the pathogenicity test by soil inoculation with culture suspension. Nine-day-old *F. oxysporum* isolates (five) were ground separately in 40 ml of sterilized distilled water aseptically. This was filtered with sterilized muslin cloth aseptically and spore concentration was adjusted to  $5 \times 10^5$  spores  $\text{ml}^{-1}$ . Freshly prepared 25 ml of culture suspension was drenched at the root zone of seedlings during 20-25 days after transplantation. The soil inoculation for second time was done during 35-40 days after transplantation.

The observations were recorded periodically at 10-day interval on a per cent disease index starting from 15 days of inoculation. Symptoms expressed were studied and re-isolated from the infected portions of plants. The pathogenicity test as above was repeated twice to ensure reproducibility.

## Statistical procedures

The experiment was conducted in a completely randomized design (CRD). The data was expressed in mm of mycelial growth and hyphal width expressed in  $\mu\text{m}$ . Mean colony sizes and hyphal width in each treatment were submitted to analysis of variance (one-way ANOVA) and CD ( $p=0.05\%$ ) was calculated. All analyses were performed using the SAS (1996) package.

## RESULTS AND DISCUSSION

The results of an *in vitro* assay to determine the variability of *Fusarium* isolates is presented in **Table 1**. The colony characterization of five *F. oxysporum* isolates was recorded 7 days after inoculation. The maximum radius recorded for On-5 isolate on PDA was 52.0 mm followed by 46.6 mm in To-3, 44.3 mm in Pe-2 and 41.7 mm in Mu-2. The minimum radius of 35.1 mm was observed in Ok-1 (**Fig. 1**) on PDA medium on day 7. The isolates showed variability in pigment production on PDA medium. Three isolates i.e., To-3, Mu-2 and Ok-1 were yellow, whereas Pe-2 and On-5 were purple after 7 days' inoculation at  $27 \pm 1^\circ\text{C}$ , 65% RH and a 16:8 h photoperiod (**Table 1**). The observations on mycelial growth patterns revealed that the shape and texture of all the colonies was circular and smooth throughout its vegetative phase. However, zonation was seen in all the isolates except for On-5.

The size of conidiogenous hyphae varied in all the isolates. The maximum diameter of 6.9  $\mu\text{m}$  was observed in On-5 followed by 6.5  $\mu\text{m}$  in Ok-1, 6.3  $\mu\text{m}$  in Pe-2, 5.1  $\mu\text{m}$  in Mu-2 and the minimum diameter of 4.7  $\mu\text{m}$  in To-3 (**Table 1**). Eleven tomato isolates from India were examined for cultural, morphological and pathogenic variations of *Alternaria solani* by Kumar *et al.* (2008). The thickness of conidiogenous hyphae varied between 1.17 and 9.56  $\mu\text{m}$  in all their isolates, Most of which showed smooth mycelial growth with circular and irregular margin and without concentric zonation and sporulation as we also describe in this study.

## PCR amplification

To understand the relationship between the isolates of *F. oxysporum* genomic DNA was extracted (**Fig. 2A**) and amplified for comparison with ITS primer pairs, ITS 3/4. PCR amplification of *F. oxysporum* f.sp. *cepe*, *F. oxysporum* f.sp. *niveum*, *F. oxysporum* f.sp. *lycopersicum*, *F. oxysporum* f.sp. *vasinfectum* with ITS primers 3/4 yielded an estimated 715

**Table 1** Cultural, morphological and pathogenic variability of different isolates of *F. oxysporum*.

Isolate	Pigmentation	Diameter of conidiogenous hyphae ( $\mu\text{m}$ )	Sporulation on PDA	Shape of colony (circular)	Texture of colony (smooth)	Zonation
To-3	Yellow	4.7	No	+	+	+
Mu-2	Yellow	5.1	No	+	+	+
Pe-2	Purple	6.3	No	+	+	+
Ok-1	Yellow	6.5	No	+	+	+
On-5	Purple	6.9	No	+	+	-

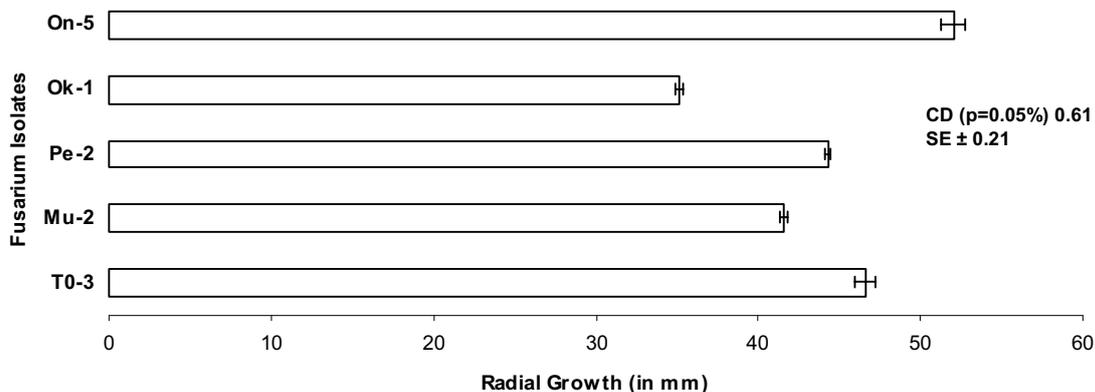


Fig. 1 Radial growth of *Fusarium oxysporum* isolates on potato dextrose agar medium at day 7.

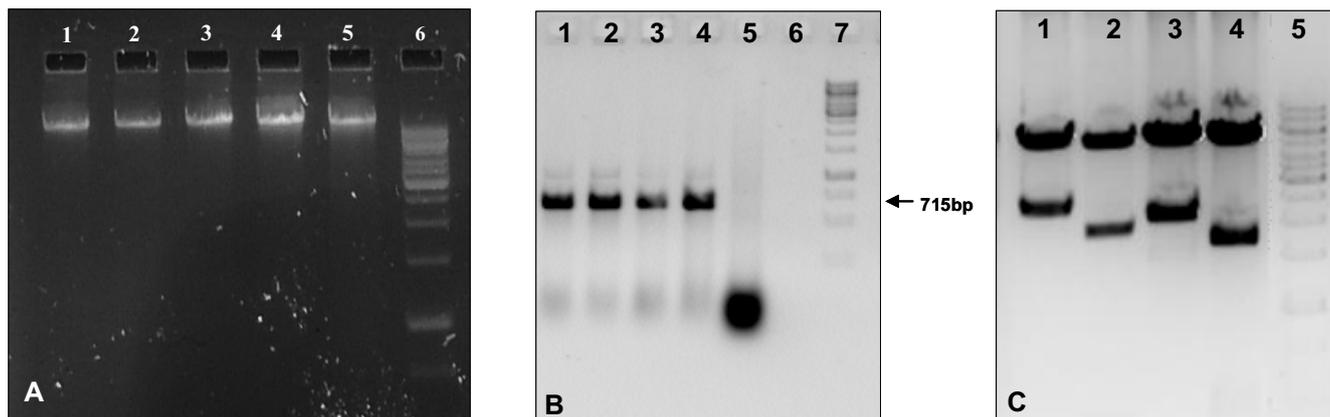


Fig. 2 (A) Genomic DNA of 1) *Fusarium oxysporum* f.sp. *cepe*, 2) *F. oxysporum* f.sp. *niveum*, 3) *F. oxysporum* f.sp. *lycopersicum*, 4) *F. oxysporum* f.sp. *vasinfectum*, 5) *F. oxysporum* f.sp. *pisi*, 6) 1 kb ladder; (B) PCR amplification of *Fusarium* isolates 1) *F. oxysporum* f.sp. *cepe*, 2) *F. oxysporum* f.sp. *niveum*, 3) *F. oxysporum* f.sp. *lycopersicum*, 4) *F. oxysporum* f.sp. *vasinfectum*, 5) *F. oxysporum* f.sp. *pisi*, 6) water blank 7) 1 kb ladder; (C) 1) *Fusarium oxysporum* f.sp. *cepe*, 2) *F. oxysporum* f.sp. *niveum*, 3) *F. oxysporum* f.sp. *lycopersicum*, 4) *F. oxysporum* f.sp. *vasinfectum*, 5) Low molecular weight DNA marker.

bp product (Fig. 2B). However, no such amplification was seen in *F. oxysporum* f.sp. *pisi* and the water blank (reaction mixture with out template). The amplified product was digested with *AluI*, *EcoRI*, and *HhaI*. *EcoRI* and *HhaI* did not digest the 715 bp product whereas *AluI* digested it into 496 and 219 bp fragments in *F. oxysporum* f.sp. *cepe* and *F. oxysporum* f.sp. *lycopersicum* forming one group. Similarly, 470 and 245 bp fragments were observed in *F. oxysporum* f.sp. *niveum* and *F. oxysporum* f.sp. *vasinfectum* (Fig. 2C) forming another group.

Wilson *et al.* (2004) grouped and differentiated *F. sporotrichioides* and *F. langsethiae* isolates in a phylogenetic tree using ITS sequence dissimilarity. A robust and repeatable PCR-assay was developed for the detection and differentiation of both species. These assays were able to detect both species in samples of grain taken from the field. In another study Kamel *et al.* (2003) developed two taxon-selective primers for rapid identification of *Fusarium* spp. cultures by PCR amplification of the ITS regions. Application of PCR assays is highly selective and sensitive in detecting *Fusarium* species for *in vivo* detection in infected host tissue. These results correlate with the report in which PCR amplification of the ribosomal IGS regions combined with digestion with three restriction enzymes (*AluI*, *HaeIII*, *RsaI*) resulted in three unique restriction profiles for Australian *F. oxysporum* f. sp. *vasinfectum* isolates, which could be distinguished from other *formae speciales* of *F. oxysporum* (Zambounis *et al.* 2007).

### Pathogenic variability

The biodiversity is noticeable particularly among facultative pathogens such as *F. oxysporum*. The results of several groups indicate that the pathogenic abilities of these isolates depend not only on their genomes but also on environmen-

tal factors (Alabouvette *et al.* 1992; Wagner 1997). In the present study, under controlled environmental conditions pot experiments were conducted and observations on disease symptoms were recorded 40 days after transplantation. All the isolates successfully expressed wilting symptoms (Synder and Toussoun 1965; Alexopoulos and Minis 1979) in their respective host plants. Initially, symptoms of leaf yellowing were observed in the host plants up to 25 days after inoculation (DAI). The disease progressed gradually and all the host plants completely wilted at about 45 DAI and died. In a similar study, three *F. oxysporum* isolates from different soil environments were tested for their pathogenicity to tomato cv. 'Rumba' seedlings (Wagner *et al.* 2007) and pathogenicity was determined using the symptoms of wilt. In another study by Vakalounakis *et al.* (2004) 34 Chinese *F. oxysporum* isolates were obtained from cucumber plants showing *Fusarium* wilt (*F. oxysporum* f. sp. *cucumerinum*) symptoms, and were characterized by pathogenicity, vegetative compatibility, and RAPD. The finding of Pandey and Dwivedi (2008) is in agreement with our present findings of pathogenicity test. Their test results confirmed the causal agent of the guava wilt i.e. *F. oxysporum* f. sp. *psidii* in pot experiments. Overall, based on PCR-RFLP analysis the molecular methods we describe will provide a useful and rapid tool for the identification of economically important species within this plant pathogenic genus.

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## REFERENCES

- Agrios G** (2005) *Plant Pathology* (5<sup>th</sup> Edn), Academic Press, London, 952 pp
- Alabouvette C, Eparvier A, Couteaudier Y, Steinberg C** (1992) Methods to be used to study competitive interactions between pathogenic and nonpathogenic *Fusarium* spp. In the rhizosphere and at the root surface. *IOBC/OILB Bulletin* **15**, 1-7
- Alexopoulos CJ, Mims CW** (1979) *Introductory Mycology* (3<sup>rd</sup> Edn), John Wiley and Sons, Inc. New York, 613 pp
- Appel DJ, Gordon TR** (1995) Intraspecific variation within populations of *Fusarium oxysporum* based on RFLP analysis of the intergenic spacer region of the rDNA. *Experimental Mycology* **19**, 120-128
- Appel DJ, Gordon TR** (1996) Relationships among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. *Molecular Plant-Microbe Interactions* **9**, 125-138
- Baayen RP, Förch MG, Waalwijk C, Bonants PJM, Löffler HJM, Roebroek EJA** (1998) Pathogenic, genetic and molecular characterisation of *Fusarium oxysporum* f.sp. *lilii*. *European Journal of Plant Pathology* **104**, 887-894
- Bruns TD, White TJ, Talyor JW** (1991) Fungal molecular systematics. *Annual Review of Ecology and Systematics* **22**, 525-564
- Edel V, Steinberg C, Avelange I, Laguerre G, Alabouvette C** (1995) Comparison of three molecular methods for the characterization of *Fusarium oxysporum* strains. *Phytopathology* **85**, 579-585
- Hibbett DS** (1992) Ribosomal RNA and fungal systematics. *Transactions of the Mycological Society* **33**, 533-556
- Kamel A Abd Elsalam, Ibrahim N Aly, Mohamed A Abdel Satar, Mohamed S Khalil, Joseph A Verreet** (2003) PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data. *African Journal of Biotechnology* **2**, 82-85
- Kumar V, Haldar S, Pandey KK, Singh RP, Singh AK, Singh PC** (2008) Cultural, morphological, pathogenic and molecular variability amongst tomato isolates of *Alternaria solani* in India. *World Journal of Microbiology and Biotechnology* **24**, 1003-1009
- Lee YM, Choi YK, Min BR** (2000) PCR-RFLP and sequence analysis of the rDNA ITS region in the *Fusarium* spp. *The Journal of Microbiology* **38**, 66-73
- Manicom BQ, Bar Joseph M, Rosner A, Vigodsky Haas H, Kotze JM** (1987) Potential application of random DNA probes and restriction fragment length polymorphisms in the taxonomy of *Fusaria*. *Phytopathology* **77**, 669-672
- Nazar RN, Hu X, Schmidt J, Culham D, Robb J** (1991) Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogens. *Physiology and Molecular Plant Pathology* **39**, 1-11
- Nicholson P, Lees AK, Maurin N, Parry DW, Rezanoor HN** (1996) Development of a PCR assay to identify and quantify *Microdochium nivale* var. *nivale* and *Microdochium nivale* var. *majus* in wheat. *Physiology and Molecular Plant Pathology* **48**, 257-271
- O'Donnell K** (1992) Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Current Genetics* **22**, 213-220
- Pandey RR, Dwivedi S** (2008) *Fusarium oxysporum* f. sp. *psidii* as a pathogen causing wilt of guava in Varanasi District, India. *Journal of Phytopathology* **114** (3), 243-248
- Smith IM, Dunez J, Philips DH, Lelliott RA, Archer SA** (1988) *European Hand Book of Plant Diseases*, Blackwell, Oxford, UK, 583 pp
- Synder WC, Toussoun TA** (1965) Current status of taxonomy in *Fusarium* species and their perfect stages. *Phytopathology* **55**, 833-837
- Vakalounakis DJ, Wang Z, Fragkiadakis GA, Skaracis GN, Li DB** (2004) Characterization of *Fusarium oxysporum* isolates obtained from cucumber in China by pathogenicity, VCG, and RAPD. *Plant Disease* **88**, 645-649
- Wagner A, Jamiolkowska A, Michalek W** (2007) Pathogenicity of *Fusarium oxysporum* from different soil environments and its effect on photosynthetic activity of tomato plants. *Electronic Journal of Polish Agricultural Universities* **10** (1), 29-34
- Wagner A** (1997) Pathogenicity of some isolates of *Fusarium oxysporum* Schlecht. to lentil (*Lens culinaris* L.). *Annales ANPP* **2**, 701-705
- White TJ, Bruns T, Lee S, Taylor JW** (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds) *PCR Protocols: A Guide to Methods and Applications*, Academic Press, Inc., New York, pp 315-322
- Wilson A, Simpson D, Chandler E, Jennings P, Nicholson P** (2004) Development of PCR assays for the detection and differentiation of *Fusarium sporotrichioides* and *Fusarium langsethiae*. *FEMS Microbiology Letters* **233**, 69-76
- Yao C, Frederiksen RA, Magill CW** (1992) Length heterogeneity in ITS2 and the methylation status of CCGG and GCGC sites in the rRNA genes of the genus *Peronosclerospora*. *Current Genetics* **22**, 415-420
- Zambounis AG, Paplomatas E, Tsafaris AS** (2007) Intergenic spacer-RFLP analysis and direct quantification of Australian *Fusarium oxysporum* f.sp. *vasinfectum* isolates from soil and infected cotton tissues. *Plant Disease* **91**, 1564-1573