African Journal of Biotechnology Vol. 5 (6), pp. 497-502, 15 March 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

Molecular characterization of *Fusarium oxysporum* f. sp. *ciceri* causing wilt of chickpea

Bhim Pratap Singh[1]*, Ratul Saikia[2], Mukesh Yadav[1], Rakesh Singh[1], V.S.Chauhan[3] and Dilip K. Arora[1]

[1]National Bureau of Agriculturally Important Microorganisms, Kushmaur, Mau 275101, India.
[2]Division of Biotechnology, Regional Research Laboratory (CSIR), Jorhat 785006, India
[3]Department of Biotechnology, Bundelkhand University, Jhansi, India.

Accepted 16 February, 2006

Thirty isolates of *Fusarium oxysporum* f. sp. *ciceri* were isolated from rhizosphere soil of chickpea from different locations in Northern India. The amount of genetic variation was evaluated by polymerase chain reaction (PCR) amplification with a set of 40 RAPD primers and 2 IGS primers. Less than 10% of the amplified fragments in each case were polymorphic. Genetic similarity between each of the isolates was calculated and results indicate that there was little genetic variability among the isolates collected from the different locations. At the 0.75 similarity index the isolates divides into three groups. Isolates *Foc*-A18, *Foc*-A19, *Foc*-A20 forming a similar group and far different from other isolates.

Key words: *Fusarium oxysporum* f. sp. *ciceri, Fusarium* wilt of chickpea, RAPD, ITS, IGS.

INTRODUCTION

Chickpea (*Cicer arietinum*) is the world's third most important pulse crop after bean and pea, with India accounting for approximately 75% of the world chickpea production (FAO, 1993). Chickpea productivity, however, remained virtually stagnant over recent decades because of its intolerance of the insect pests *Helicoverpa amigera* and because of its susceptibility to diseases such wilt (*Fusarium oxysporum* f. sp. *ciceri*) and charcoal/collar rot caused by *Macrophomina phaseolina*, and blight (*Ascochyta rabie*i). *F. o.* f. sp. *ciceri* is a soil borne, root pathogen colonizing the xylem vessels and blocking them completely to effect wilting (Bateman et al., 1996). The existence of at least 4 races of *F. o.* f. sp. *ciceri* in India, and 7 races from almost all the chickpea growing regions in the world has been reported. These pathogens cause serious diseases in chickpea plants and widespread in India, Iran, Pakisthan, Nepal, Burma, Spain, Maxico, Peru, Syria and USA (Nene et al., 1989). Greater than 80% reduction in yield can occur, if measures are not taken to control these pathogens.

*Corresponding author E-mail: bheem_50@yahoo.co.in, ratulsaikia@yahoo.com.

Accurate and rapid identification of pathogens is necessary for appropriate management of plant diseases. In particular, genetic characterization of pathogenic variants of the plant pathogens prevalent in an area is required for efficient management and increase crop productivity. The *Fusarium* wilt disease can be managed primarily by the use of resistance cultivars (Jalali et al., 1992). This is a large cosmopolitan genus of imperfect fungi and it is of primarily interest because numerous species are important plant pathogens (Austwick, 1982), produce of a wide range of secondary metabolites, and/or cause opportunistic mycoses in humans (Michniewicz et al., 1989; Vesonder et al., 1989). Molecular phylogenetic analyses have helped to clarify ambiguities in traditional classification systems of *Fusarium*. In *Fusarium oxysporum* and *F. redolens*, for example, phylogenetic analyses have revealed that pathogenicity factors have had multiple evolutionary origins (Lobuglio et al., 1993). Genome organization and molecular mechanisms of pathogenicity are still not well understood in many *Fusarium* species (Michniewicz et al., 1999). The advent of pulse field gel electrophoresis has opened a new avenue to study chromosomes of these fungi. Also, using molecular approaches, tolerance against antimicrobial

Table 1. Isolates of *Fusarium oxysporum* f. sp *ciceri* used in this study and their place of collection.

Name of Isolate	Place of Collection
Foc-A20	Allahabad
Foc-A15	Allahabad
Foc-Al9	Allahabad
Foc-A16	Allahabad
Foc-A17	Allahabad
Foc-A18	Allahabad
Foc-A29	Allahabad
Foc-A21	Allahabad
Foc-Al4	Allahabad
Foc-Al2	Allahabad
Foc-Al3	Allahabad
Foc-A22	Allahabad
Foc-Al0	Allahabad
Foc-A09	Allahabad
Foc-Al1	Allahabad
Foc-A07	Allahabad
Foc-A08	Allahabad
Foc-A04	Allahabad
Foc-A05	Allahabad
Foc-A03	Allahabad
Foc-A02	Allahabad
Foc-A01	Allahabad
Foc-A23	Allahabad
Foc-A06	Allahabad
Foc-Jh1	Jhansi21
Foc-Jh2	Jhansi22
Foc-Bho1	Bhopal
Foc-Mu1	Mau
Foc-Mu2	Mau
Foc-Ag01	Agra

compounds, signal transduction systems and some secondary metabolites have been shown to be involved in the pathogenicity of some *Fusarium* species (Khalil et al., 2003). Genetic distances among strains have been evaluated through analyses of pathogenicity, vegetative compatibility group (VCG), chromosomal features, rDNA restriction fragment length polymorphism (RFLP), mtDNA, and other molecular markers (Jacobson and Gordon, 1990; Appel and Gordon, 1995, 1996; O'Donnell et al., 1998; Alves-Santos et al., 1999). Most studies reported to date have focused on an individual *forma specialis,* seeking to characterize the diversity therein, especially as it relates to physiological races. In many cases, it was found that *formae speciales* are genetically heterogeneous, and can sometimes have a polyphyletic origin (O'Donnell et al., 1998). Polymerase chain reaction (PCR) has been widely and successfully employed for the diversity analysis of the important plant-pathogenic fungi including *Fusarium* spp. (Kim et al., 1993). In many cases, primers for these uses were based on DNA sequence polymorphisms existing within highly conserved regions of the nuclear ribosomal DNA, such as the internal transcribed spacer or the intergenic spacer region (Ward et al., 1994; Kabir et al., 1995; Edel et al., 2000).

Molecular biology has brought many powerful new tools to fungal taxonomists including the potential for rapid identification of isolates, methods for rapid determination of virulence or toxicity of strains, and the means to elucidate the relationships among fungal species. Molecular methods have also been used to distinguish between closely related species with few morphological differences and to distinguish strains (or even specific isolates) within a species. Genetic characterization of *F. oxysporum* f. sp *ciceri* isolates causing wilt disease to the chickpea plants is important for the efficient management of Fusarium wilt through use of resistant cultivars in chickpea growing areas. This study will reveal how much diversity exists in the isolates of *F. o.* f. sp *ciceri* and RAPD markers (Jimenej-Gasco et al, 2001) which can be used to unambiguously identify *F. o.* f. sp. *ciceri* isolates. This is the first report for the diversity analysis of *F. o.* f. sp *ciceri* causes wilt disease in chickpea isolated from north India.

MATERIALS AND METHOD

Fungal isolates

Thirty isolates of *F. oxysporum* f. sp *ciceri* used in the present study were isolated from various rhizosphere soils of infected chickpea plants from different areas of Uttar Pradesh, India (Table 1). The isolates collected were identified, purified and preserved in PDA medium and confirmation of isolates by Koch`s pastulation and microscopy observation.

Mycelium production and DNA extraction

DNA extraction was done by the method of Saghai-Maroof et al. (1984) with minor modifications. The fungal cell wall was disrupted by grounded with pestle and mortar in liquid nitrogen. The powdered mycelium is then transferred to an extraction buffer that contains detergent cetyl tri-methyl ammonium bromide (CTAB) and 2-β-mercaptoethanol, EDTA and polyvinyl pyrolidone (PVP). Quantification of DNA was done with spectrophotometer determination. Working concentration of DNA was adjusted to 20 ng/µl and stored at 4°C.

Random amplified polymorphic DNA (RAPD)

The procedure described by Williams et al. (1990) with minor modification was done for carrying out PCR reaction to produce RAPD profiles. Amplification of DNA fragments was carried out by the PCR using 10-mer arbitrary primers. The reaction mixture consisted of 200 µM of dNTP mix (Fermentas), 15 pmol of primer (Metabion), 5 U/µl of *Taq* DNA polymerase (Fermentas) and 25 mM $MgCl_2$. Out of 40 primers tested, 4 primers, OPB-11, OPE-07, OPE-11 and OPA-02, were selected because they showed

Table 2. Sequence of primers and number of fragments of *Fusarium oxysporum* f. sp *ciceri* amplified by RAPD and IGS primers.

Markers.	Primers.	Sequences 5`-3`	Amplified fragments	Polymorphic fragments
RAPD	OPB-11	GTAGACCCGT	82	6
	OPE-07	AGATGCAGCC	85	5
	OPE-11	GAGTCTCAGG	80	4
	OPA-02	TGCCGAGCTG	97	7
IGS	CLN12	CTGAACCGCCTCTAAGTCAG	111	7
	IGS2	AATGAGCCATTCGCAGTTC		

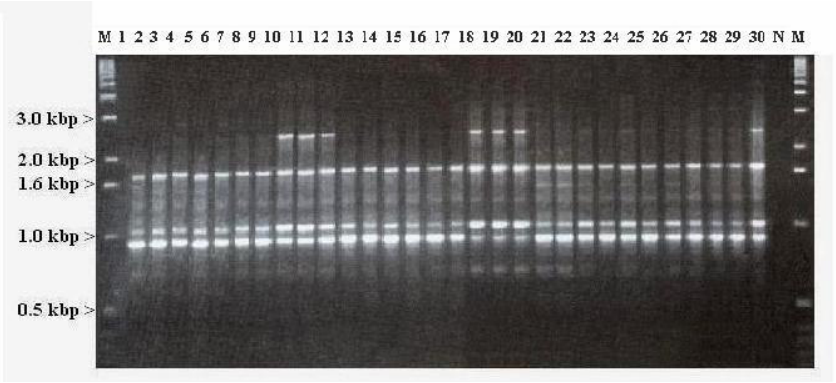

Figure 1. Representative RAPD profile *Fusarium oxysporum* f. sp *ciceri*. Amplification of the genomic DNA was performed using primer OPA-02. Lane 1.1kb marker (M), lane 1 to lane 30 (Fusarium samples), Negative control (N).

polymorphism (Table 2). DNA amplifications were performed in thermocycler with one cycle of initial denaturation 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 2 min, extension at 72°C for 1 min and with a final extension at 72°C for 10 min. Amplified products together with marker (lambda DNA/ *Eco*RI + *Hind*III double digest; Bangalore Genei) were resolved by gel electrophoresis (60 V cm^{-1}) on 1.6% agarose gels in 1X TAE buffer containing 10 mg ml^{-1} ethidium bromide (EB). Gels were photographed by Gel Documentation system (Uvitec).

Table 3. Restriction digests of the IGS fragments of *Fusarium oxysporum* f. sp *ciceri*.

Restriction enzyme	No of Bands obtained
H*ae* III	5
H*ha* I	3
R*sa* I	3

Amplification of intergenic spacer (IGS) region of rDNA

For IGS analysis PCR reactions was performed in 1X PCR reaction buffer containing 20 ng template DNA, 0.25 µmol of each of two opposing primers, 15 mM of each dNTP, 25 mM of $MgCl_2$, 5% of glycerol and 2 U/µl of *Taq* DNA polymerase. DNA amplifications were performed in thermocycler with one cycle of initial denaturation, 94°C for 3 min, followed by 30 cycles of denaturation, 94°C for 40 s, annealing 50°C for 40 s and extension 72°C for 40 s, with a final extension at 72°C for 10 min. Amplified products together with marker (lambda DNA/ *Eco*RI + *Hind*III double digest; Bangalore Genei) were resolved by gel electrophoresis (20 V cm^{-1}) on 2% agarose gels in 1X TAE buffer containing 0.5 mg ml^{-1} ethidium bromide (EB) and gels were photographed.

Amplification products (15 µl of the PCR mix) were digested with 5 units of restriction endonucleases (*HaeIII, HhaI and RsaI*; Table 3) according to the manufacturer's recommendations. Restriction fragments were separated on a 2.5% agarose gel and stained with ethidium bromide. A 1Kb DNA ladder (Bangalore Genei) was used as molecular weight marker. For both RAPD and IGS analysis, PCR amplifications and restrictions were repeated at least three times.

Data analysis

Comparison of each profile for each primer was done on the basis of the presence versus absence (1/0) of RAPD and IGS products of the same length. Bands of the same length were scored as identical. Analyses were based on the simple matching index (Sokal and Michener, 1958), which measures the proportion of common data (either 0 or 1) between the isolates. A dendrogram was derived from the distance matrix by the UPGMA (Sneath and Sokal, 1973) contained in the software package NTSYS 2.02i (Rohlf, 1997).

RESULTS AND DISCUSSION

Different workers have been grouped different fungal population in different regions by using RAPD analysis (Hyun, et al., 1998; Jana et al., 2003, Lanfranco et al.,

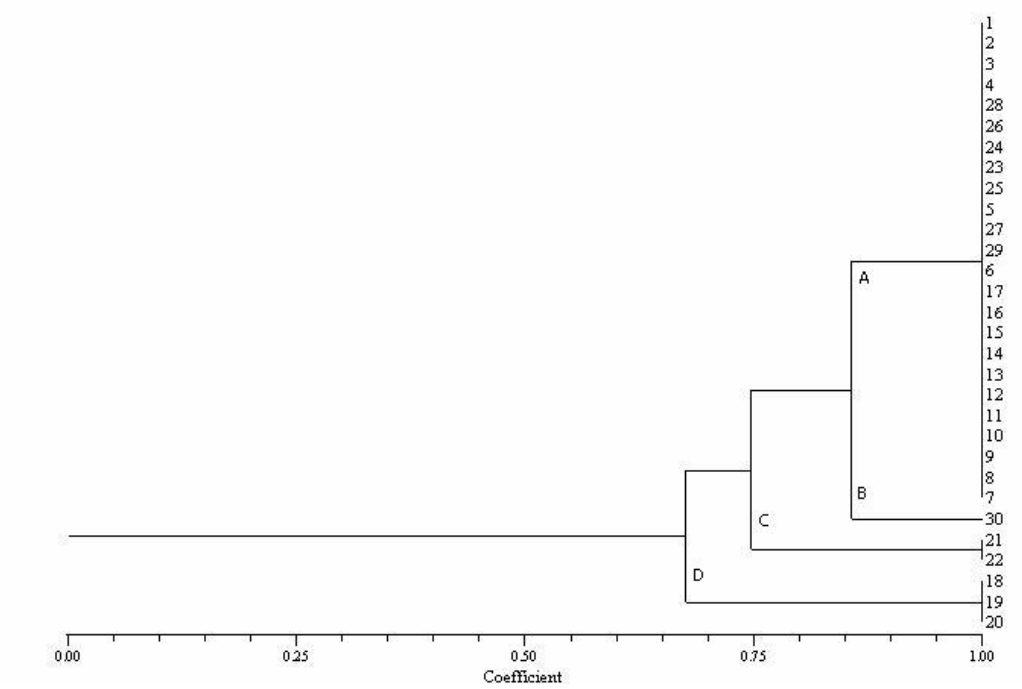

Figure 2. Dendogram of *Fusarium oxysporum* f sp. *ciceri* strains derived from RAPD fingerprints generated byb using four different 10-mer primers.

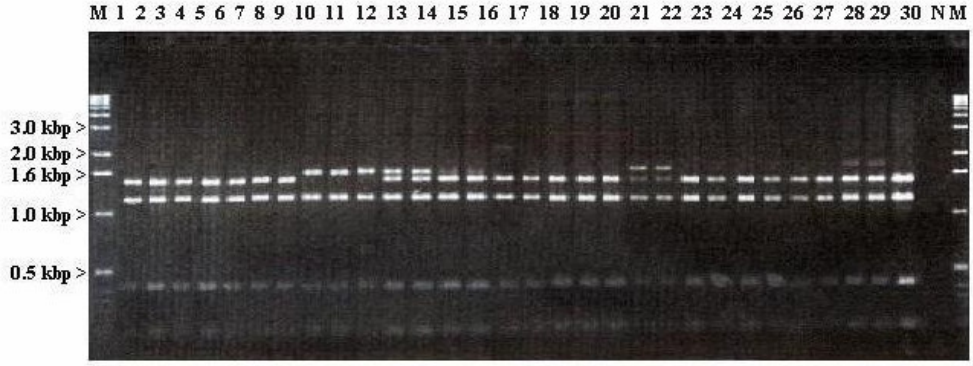

Figure 3. Representative Intergenic spacer (IGS) after restriction with Hae III, lane 1 Marker (1kb), Lane 1 to Lane 30 (*Fusarium oxysporum* f. sp *ciceri*), negative control (N).

1995). Out of the 40 primers initially tested four (OPB-11, OPE-07, OPE-11 and OPA-02) consistently generated reproducible RAPD patterns; therefore they were used for a comparative analysis of all the isolates. For RAPD a total of 97 bands were scored, of which 7 were polymorphic by using a specific primer OPA-02 (Figure 1). For IGS, a total of 111 bands were scored and 9 were polymorphic using a restriction digestion of the IGS amplicon with *Hae*III restriction enzyme (Figure 3). The number of amplified products varied between 4 and 7 and the size of the products ranged from 0.6 – 2.9 kb and from 0.4 –1.5 kb for RAPD (Figure 1) and IGS, respectively. The RAPD profiles showed that whole isolates form four groups (A, B, C and D). Group A isolates were from the same climatic area of the North India (Allhabad, UP), 30 (Ag 30) form a separate group "B" which were taken from Agra (UP); Sl. No. Foc-Mau1 and Foc-Mau2 (Group C) of Mau (UP), and finally three isolates, Foc-Bho1 (collected from Bhopal, MP) and Foc-Jh1 and Foc-Jh2 (collected from Jhasi, U.P) formed a similar group (D). The variable banding patterns four primer are summarized by cluster analysis (Figure 3). The isolates were first separated into two clusters based on the similarity value 0.65, and one clusters was

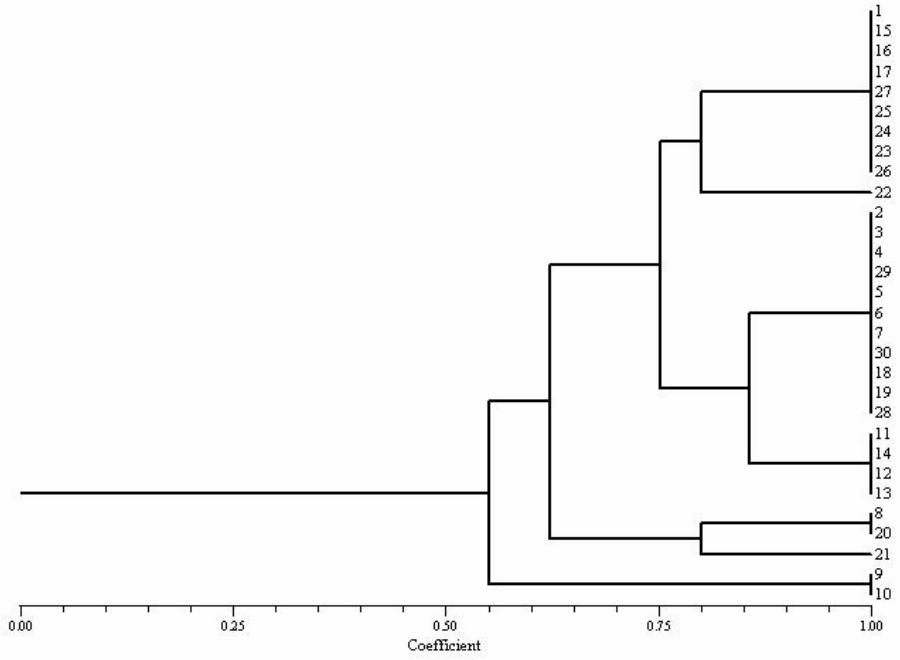

Figure 4. UPGMA dendogram of IGS region after restriction with endonuclease Hae III.

separated into two clusters with similarity value of 0.75. Of these two clusters, one is separated again into cluster at similarity value 0.85. IGS analysis shows that the isolates No 9 and 10 were far away from other isolates and the pattern shows more diversification among the isolates collected from different parts of India. IGS product after digestion with restriction enzyme *Hae*III results also divides the isolates into four clusters (Figure 4).

ACKNOWLEDGEMENTS

The authors are grateful to the Indian Council of Agriculture Research (ICAR), Govt. of India for providing financial assistance for this project.

REFERENCES

Armstrong GM, Armstrong JK (1981). Formae speciales and races in *Fusarium oxysporum* causing wilt diseases Pp. 391–399. *In* P. Nelson TA, Tousson, Cook RJ, eds. *Fusarium*: diseases, biology, and taxonomy. Pennsylvania State University Park, University Park, Pa.

Austwick PKC (1982). *Fusarium* infections in man and animals. In MO Moss and JE Smith, ed. The applied mycology of *Fusarium*. Cambridge: Cambridge University Press. pp. 129-140.

Bateman GL, Kwasna H, Ward E (1996). Reletionship among Fusarium spp. estimated by comparing restriction fragment length polymorphism in polymerase chain reaction amplified nuclear DNA. Can. J. Microbiol. 42: 1232-1240.

Bunting TE, Plumley KA, Clarke BB, Hillman BI (1996). Identification of *Magnaporthe poae* by PCR and examination of its relationship to other fungi by of their nuclear rDNA ITS1 regions. Phytopathology 86: 398-404.

Edel V, Steinberg C, Gautheron N, Recorbet G, Alabouvette C (2000). Genetic diversity of *Fusarium oxysporum* populations isolated from different soils in France. FEMS Microbiol. Ecol. 36: 61-71.

Gordon TR, Martyn RD (1997). The evolutionary biology of *Fusarium oxysporum.* Ann Rev. Phytopathol 35: 111-128.

FAO (1993). FAO Yearbook Production. Rome, Italy: FAO 46: 105-115.

Henson JM, French R (1993). The polymerase chain reaction and plant diseses diagnosis. Ann. Rev.Phytophathol.31: 81-109.

Hyun JW, Clark CA (1998). Analysis of *Fusarium lateritium* using RAPD and rDNA RFLP techniques. Mycolo. Res. 102: 1259-1264.

Jalali BL, Chand H (1992). Chickpea wilt. pp. 420-444 in: Plant diseases of International Importance. Vol.1. Diseases of cereals and pulses. U.S. singh, Mukhopadhayay AN, Kumar J, Chaube HS, eds. Prentice Hall, Englewood Cliffs, NJ.

Jana TK, Sharma TR, Prasad D, Arora DK (2003). Molecular characterization of *Macrophomina phaseolina* and *Fusarium* species by single primer RAPD technique. Microbiolog. Res. 158: 264-274.

Jimenej-Gasco MM, Perez-Artes E, Jimenez-Diaz RM (2001). Identification of pathogenic races 0, 1B/C, 5 and 6 of Fusarium oxysporum f. sp. Ciceris with random amplified polymorphic DNA (RAPD). Eur. J . Plant Pathol. 107: 237-248.

Khalil MS, Abdel-Sattar MA, Aly IN, Abd-Elsalam KA, Verreet JA (2003) Genetic affinity of *Fusarium* spp. and their correlation with origin and pathogenicity. Afr. J. Biotechnol. 2: 109-113.

Kim DH, Martyn RD, Magill CW (1993). Mitochondrial DNA-relatedness among formae specials of *Fusarium oxysporum*min the Cucurbitaceae. Phytopathology 83: 91-97.

Lanfranco L, Wyss P, Marzachi C, Bontante P (1995). Generation of RAPd PCR primers for the identification of the isolate of *Glomus mosseae,* an arbuscular mycorrhiza fungus. Mol. Ecol. 4: 61-68.

Lobuglio KF, Pitt JI, Taylor JW (1993). Phylogenetic analysis of two ribosomal DNA regions indicates multiple independent losses of a sexual *Talaromyces* state among asexual *Penicillium* species in subgenus *Biverticillium.* Mycologia 85: 592-604.

Martin R, James D, Levesque CA (2000). Impacts of molecular diagnostic technologies on plant disease management. Ann. Rev. Phytophatol. 38: 207-239.

Michniewicz M (1989). Growth regulators formed by Fusaria: Their significance for the fungus growth, sporulation and pathogenicity

towards the host plant. In: J Chelkowski, (ed). Fusarium, Mycotoxins, Taxonomy and Pathogenicity. Amsterdam: Elsevier Scientific Publishers. pp. 227-241.
O'Donnell K (1996). Progress towards a phylogenetic classification of *Fusarium*. Sydowia 48: 57-70.
Rohlf FJ (1997). NTSY Spc, version 2.02. Exeter Software. Applied Biostatistics Inc.
Sokal RR, Michener CD (1958). A statistical method for evaluating systematic relationships. Univ. Kans. Sci. Bull. 38: 1409-1438.
Sneath PHA , Sokal RR (1973). Numerical Taxonomy. W.H. Freeman and company, San Francisco. p. 573
Saghai -Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984). Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81: 8014-8018.
Ward E (1994). Use of polymerase chain reaction for identifying plant pathogens. J.P.Blakemen and B.Williamson, eds. CAB International. Oxon, UK.
Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6631-6635.
Vesonder RF, Golinski P (1989). Metabolite of *Fusarium*. In: J Chelkowski, ed. *Fusarium*, Mycotoxins, Taxonomy and Pathogenicity. Amsterdam: Elsevier Scientific Publishers. pp. 1-39.