Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

JEB
Journal of Environmental Biology

ISSN: 0254-8704
CODEN: JEBIDP

Molecular characterization of *Fusarium moniliforme* var. subglutinans isolates

Pradeep Kumar[1,4]*, V. K. Gupta[2], A.K. Misra[3] and D. R. Modi[4]

[1]The Department of Biotechnology Engineering, Ben Gurion University of The Negev, Be'er Sheva-84105, Israel
[2]Molecular Glycobiotechnology Group, National University of Ireland Galway-353, Ireland
[3]Discipline of Biochemistry,Central Institute for Subtropical Horticulture, Lucknow-227 017, India
[4]Department of Biotechnology, BBA University, Lucknow- 226 025, India
*Corresponding Author E-mail: pradeepgkp17@yahoo.co.in

Publication Info

Paper received:
21 July 2012

Revised received:
09 January 2013

Accepted:
27 February 2013

Abstract

Molecular characterization of 42 isolates of mango (*Mangifera indica* L.) malformation pathogen *Fusarium moniliforme* var. subglutinans collected from different agro-climatic zones of India were performed using RAPD markers. Twenty RAPD primers of OPA series were selected for screening of which; six primers OPA-1, 3, 7, 8, 9 and 18 were found to be clearly discriminates and differentiated the *F. moniliforme* var. subglutinans isolates and showed reproducible banding patterns. A sum total of 598 bands were generated from these random primers having range from 4.0% to 90.9% of similarity among the 42 isolates of *F. moniliforme* var. subglutinans. The data were used to construct a dendrogram with unweighted pair group method of arithmetical averages (UPGMA) algorithm, using the NTSYS-software. Dendogram revealed more than 90% similarity among all the 42 isolates which shows that all isolates approximately have same genetic identity. Hence, in the current research findings RAPD proved a reproducible and tractable means of differentiating *F. moniliforme* var. subglutinans isolates.

Key words

F. moniliforme, RAPD markers, Malformation, Mango, PCR, Variability

Introduction

Mango (*Mangifera indica* L.) occupies a pre-eminent place amongst fruit crops in India and is acknowledged as 'King of fruits' in the country (Kumar *et al.*, 2011a). Mango malformation is the most serious disease of mango which affects the yield up to 90% (Ploetz *et al.*, 2002) and production of mango in India and other mango growing countries of the world (Crane and Campbell, 1994, Kumar *et al.*, 2011a). This disease is widely spread in flowers and vegetative shoots of mango. In spite of several decades of incessant research, since its recognition, the etiology of this disease remained confusing and no effective control measure is known (Bains and Pant 2003, Kumar *et al.*, 2012), though strong evidence is for its origin of fungal nature (Misra and Singh, 2002, Kumar *et al.* 2011b). It was reported that association of *Fusarium moniliforme* Sheld. with malformation (floral and vegetative) and proved pathogenicity of the fungi on mango plant. Accordingly, Kumar *et al.* (2011b) performed the studies on mass isolation and recovered above 82% *F. moniliforme* var. subglutinans colonies. Cultural and morphological studies give an idea about the variability in the pathogen but do not give definite answer about inter and intra species variability.

Molecular phylogenetic analyses have helped to clarify ambiguity in traditional identification and classification systems of *Fusarium*. Genetic distances among strains have been evaluated through analyses of pathogenicity, vegetative compatibility group (VCG), chromosomal features, restriction fragment length polymorphism (RFLP) and other molecular markers (Bateman *et al.*, 1996). Most studies have focused on individual Formae speciales seeking to characterize the diversity therein, especially as it relates to physiological races. In many cases, it was found that formae specialis are genetically heterogeneous and can sometimes have a polyphyletic origin (O'Donnell *et al.*, 1996). Polymerase chain reaction (PCR) has been widely and

successfully employed for the diversity analysis of important plant-pathogenic fungi including *Fusarium* spp. (Kim *et al.*, 1993, Gupta *et al.*, 2009; Arif *et al.*, 2011).

Conventional methods of identifying pathogenicity and vegetative compatibility analysis of *F. moniliforme* is usually time-consuming and laborious (Jana *et al.*, 2003). RAPD analysis does not require previous detailed knowledge of DNA to be analyzed and have been shown to be reliable technique to distinguish small genomic differences. RAPD has proved successful in generating amplification patterns specific to *F. mangiferae* (Ploetz *et al.*, 2002; Iqbal *et al.*, 2006) a causal agent of mango malformation. These molecular marker based techniques are useful in development of early diagnosis and identification of pathotypes and races (Arif *et al.*, 2011). In the present study, level of genetic diversity was tested among the *F. moniliforme* var. subglutinans isolates, which were collected from severely mango malformation affected regions of India.

Materials and Methods

Pathogencity assay : Roaming surveys were conducted in the major mango growing areas of India and vegetative and floral samples were collected from malformed affected mango plants. After washing in fresh and sterilized water, small pieces from malformed tissue were surface sterilized with 0.1 % mercuric chloride solution, washed thoroughly thrice with sterilized water and dried on sterilized blotting paper. These malformed pieces were subsequently transferred to Petri dishes under aseptic conditions, having 20 ml sterilized potato dextrose agar medium and incubated at 28±2ºC. Pure cultures from each isolate were made using the single spore isolation or hyphal tip isolation technique following standard procedure (Ali and Jackson, 1989).

Pathogencity tests of 42 *F. moniliforme* var. subglutinans isolates were performed on the mango seedlings of Dashehari cultivar (since Dashehari has no known resistance to mango malformation) under field condition. Stem hole inoculation technique was employed in order to reproduce the typical symptoms of malformation (Misra and Gupta 2010). Pure culture of *F. moniliforme* var. subglutinans was inoculated in the month of November in the host tissue and covered with sterile and moist cotton. Prior to inoculation, buds were treated with 20% (H_2O_2) which detoxified mangiferin (the host defense anti Fusarium compound) as described by Chand and Chakrabarti (2003). All the experiments were carried out in polyhouse at a temperature maintained up to 10-19ºC and high relative humidity (> 85 %). The appearance of malformation was recorded in inoculated seedlings and compared with control seedlings of plant.

Genetic analysis : Fresh mycelium of *F. moniliforme* var. subglutinans were collected and used for extraction of genomic DNA following the protocol of Abd-Elsalam *et al.* (2003). The fungal mycelial mat was homogenized using 300 µl of extraction buffer [200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA and 0.5 % of sodium dodecyl sulphate] for 5 min. Then 150 µl of sodium acetate (pH 5.2) and reaction mixture was cooled for 10 min at 20ºC. reaction mixture was centrifuged at 13,000 rpm for 5 min, supernatant transferred in fresh tube and equal volume of isopropanol was added. Precipitated DNA was then subjected centrifugation at 13,000 rpm for 10 min collect pellet and washed with 70% ethanol two times for the removal of excess salt. Dry out the pellet containing DNA and resuspended in Tris-EDTA (10 mM) and stored at 4ºC. DNA was quantified using standard spectroscopic methods.

RAPD-PCR reaction was performed using Eppendorf Master Cycler in 25 µl reaction volume containing 25 ng genomic DNA, 0.4 µl (5 pmole) primer, 1.5 µl dNTPs (25mM), 3µl of 10 X assay buffer with MgCl2 (15mM), 0.5µl (3U μl^{-1}) of Taq DNA polymerase (Bangalore Genei Pvt. Ltd.). The reaction condition employed for the RAPD reaction were as follows: denaturation for 5 min at 94ºC followed by 35 cycles of 1 min each at 94ºC and 35ºC followed by 2 min at 72ºC and final extension for 5 min at 72ºC. PCR products were resolved by horizontal electrophoresis using 1.2% agarose gel with 1% TAE buffer containing ethidium bromide.

Cluster analysis : Based on RAPD data, genetic diversity and variability among 42 *F. moniliforme* var. subglutinans isolates were assessed by using Jaccard's coefficient (1908). The data were subsequently used to construct a dendrogram with Unweighed Pair Group Method of Arithmetical averages (UPGMA) algorithm, as described by Sneath and Sokal (1973) using NTSYS-software (Rohlf, 1998).

Results and Discussion

All 42 isolates of *F. moniliforme* var. subglutinans were found pathogenic to mango seedlings of Dashehari cultivar (Table 1). The minor signs of disease (bud swelling, scale like leaves) appeared approximately 2-3 months after inoculation. Complete symptoms after 6-7 months. Comparison of healthy (control) and malformed plants clearly differentiated the normal and malformed expression. The apex of healthy six-months-old seedling showed normal apical growth while vegetative malformation reflected its unique manifestation on inoculated seedling. The disease was characterized by excessive proliferation of leaves. The gathering of stubby leaves into close folds was similar to incipient bunchy top met under natural normal condition. This finding was in accordance to the earlier reports Iqbal *et al.* (2006) for the pathogencity.

Twenty operan series RAPD primers (OPA1-OPA20) were tested for molecular characterization of 42 isolates of *F. moniliforme* var. subglutinans. The generated fingerprints were evaluated for overall clearness of the banding pattern. Primers OPA1, OPA3, OPA7, OPA8, OPA9 and OPA18 were found to be ideal RAPD markers for characterization of *F. moniliforme* var. subglutinans (Fig. 1 a-c) which showed typical profile and polymorphic bands

Table 1 : Description of mango malformation pathogen *F. moniliforme* var. subglutinans isolates collected from different mango growing regions of India

Place of sample collection	Molecular culture no.	Pathogen isolated	Symptoms	Culture colour	Metabolite colour
Sabour	F1	mFMS	FM	Light pink	Pink
Sabour	F2	FMS	VM	Light pink	Pink
Sabour	F3	FMS	FM	Light pink	Pink
Sabour	F4	FMS	FM	Cream	Light pink yellow margin
Sabour	F5	FMS	VM	Light pink	Light pink
Dholi	F6	FMS	FM	Pink	Light pink
Dholi	F7	FMS	VM	Pink	Pink
Dholi	F8	FMS	VM	Light Pink	Light brown
Pusa	F9	FMS	FM	Light Pink	Light brown
Pusa	F10	FMS	VM	Pink	Light pink
Pusa	F11	FMS	FM	Light Pink	Light yellow
Ranchi	F12	FMS	FM	Light Pink	Light brown
Ranchi	F13	FMS	FM	Light pink	Purple
Ranchi	F14	FMS	FM	Light pink	Purple
Ranchi	F15	FMS	FM	Light Pink	Dark red
Lucknow	F16	FMS	VM	Red	Light brown
Lucknow	F17	FMS	VM	Yellow	Pink
Lucknow	F18	FMS	VM	Pink	Purple
Lucknow	F19	FMS	VM	Pink	Light pink
Lucknow	F20	FMS	VM	Pink	Pink
Lucknow	F21	FMS	VM	Light pink	Pink
Lucknow	F22	FMS	VM	Pink	Pink
Lucknow	F23	FMS	VM	Light yellow	Cream
Chittour	F24	FMS	VM	Pink	Pink with yellow margin
Chittour	F25	FMS	VM	Light pink	Light pink
Chittour	F26	FMS	VM	Light pink	Cream
Chittour	F27	FMS	VM	Light purple	Purple
Chittour	F28	FMS	VM	Light purple	Violet
Lucknow	F29	FMS	VM	Light Pink	Pink
Bangalore	F30	FMS	FM	Light Pink	Purple
Bangalore	F31	FMS	FM	Light pink	Purple
Bangalore	F32	FMS	FM	Purple	Purple
Bangalore	F33	FMS	FM	Light Pink	Dark red
Bangalore	F34	FMS	FM	Red	Light brown
Shaharanpur	F35	FMS	FM	Yellow	Brown
Shaharanpur	F36	FMS	FM	Light pink	Purple
Shaharanpur	F37	FMS	FM	Light pink	Light pink
Shaharanpur	F38	FMS	FM	Light pink yellow	Light pink yellow margin margin
Shaharanpur	F39	FMS	FM	Light Pink	Purple
Lucknow	F40	FMS	VM	Pink	Light pink
Lucknow	F41	FMS	VM	Pink	Pink
Lucknow	F42	FMS	VM	Light Pink	Light brown

mFMS- *Fusarium moniliforme* var. subglutinans; mFM- Floral malformation; mVM- Vegetative malformation

generated with the primers. The number of scorable bands for corresponding primer ranged from 1-5 with an average of 5 bands per individual. Products size ranged from 100 bp to 3000 bp. Similarly, Iqbal *et al.* (2006) and Saharan *et al.* (2006) reported an average of 5 bands per individual for *F. graminearum* isolates and product size ranging between 300-2072 bp.

RAPD analysis showed highest similarity coefficient greater than 90 % in isolates of F17 (Lucknow) and F34 (Bangalore) followed by similarity greater than 80 % in F3 and F4 (Sabour), F6 (Dholi), F9 and F11 (Pusa), F12, F13 and F14 (Ranchi), F16, F18, F19, F20, F21 and F29 (Lucknow), F33 (Bangalore), F35, F36, F37 and F38 (Shaharanpur) isolates used for molecular characterization. Isolates F17 (Lucknow) and F34 (Bangalore) were genetically most similar (90.90 % similarity) and showed highest similarity among the isolates. This showed that they were genetically similar or may have originated from single lineage. Isolate F36 (Shaharanpur) was genetically

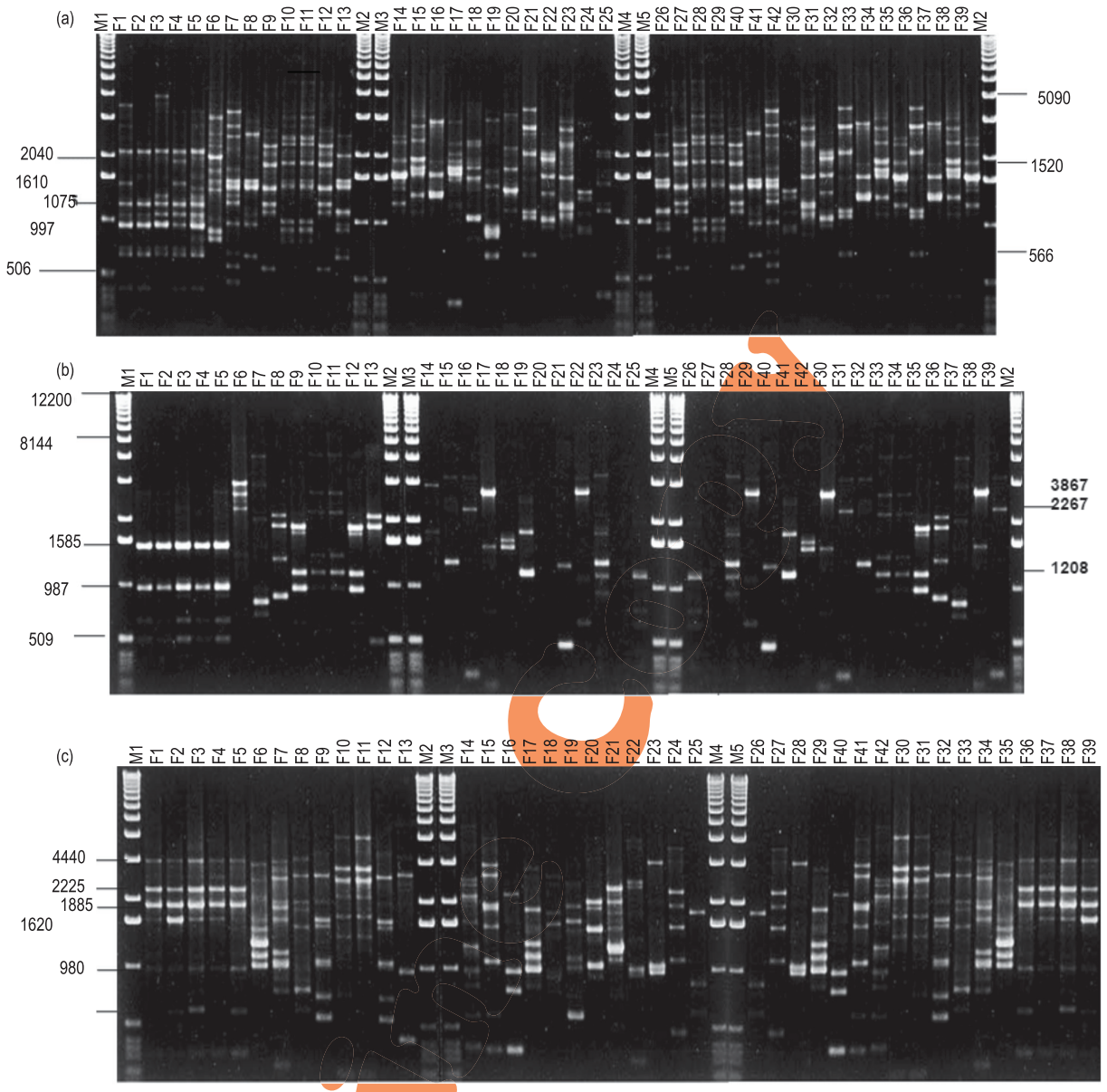

Fig. 1: RAPD based allelic pattern of *F. moniliforme* var. subglutinans obtained from primer a) OPA-01 (b) OPA-03 and (c) OPA-18

similar to F19 (Lucknow). The least genetic similarity was observed among isolates F7, F22, F24, F4, F29, and F3. Coefficients of similarity among *Fusarium* isolates ranged from 4 to 90.9%. These similarity coefficients were used for construction of dendogram to determine the grouping of isolates.

Pertaining to the results, isolate F15 (Ranchi) was out grouped as compared to other isolates that fell in cluster A and B (Fig. 2). Dissimilarity per cent of cluster B in relation to cluster A was recorded to be approximately 5.0 % which is not statistically significant (P=0.05) and hence it may be concluded that all isolates of cluster A were almost similar and nearly same to those with isolates of cluster B. It was also noted that isolates of *F. moniliforme* var. subglutinans collected either from same or different regions showed high similarity values indicating that there existed narrow molecular variation and almost all the isolates were genetically related. Similar to our findings, Iqbal *et al.* (2006) reported a low level of genetic variation in *Fusarium* isolates collected from malformed tissues of different geographical locations in Pakistan based on UPGMA clustering.

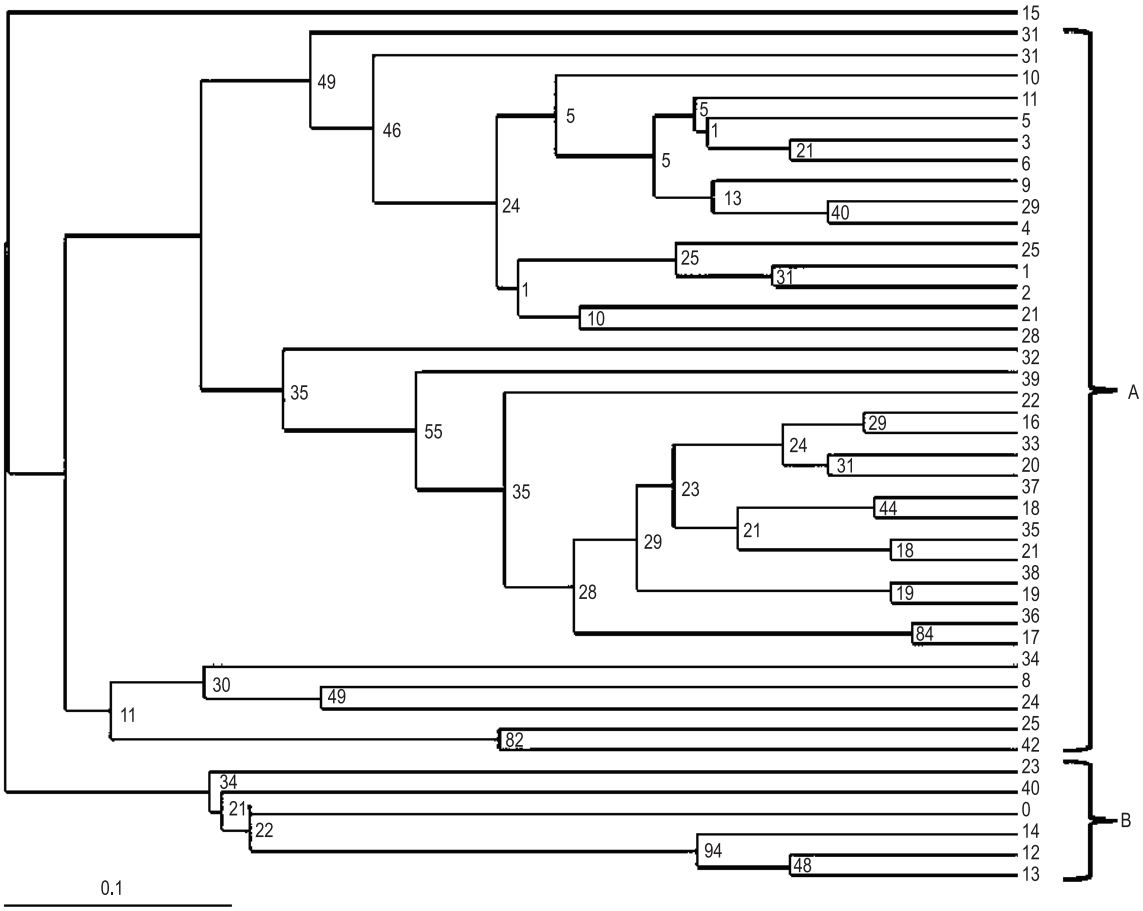

Fig. 2 : Dendogram constructed using an UPGMA from Jaccard's similarity matrix data from RAPD data

Similarly, Freeman and Maymon (2000) and Sabir (2006) reported that low level variability among *Fusarium* isolates collected from different geographical locations. Arif *et al.* (2011) also reported variability among the *F. moniliforme* var. *subglutinans* isolates of mango malformation by using RAPD but this study was limited to assessment of genetic diversity of *Fusarium moniliforme* isolates of Pantnagar, India. However, present study represented the collection of isolates from severely affected area of mango malformation across India followed by pathological and molecular characterization. This variation in isolates may be because they belong to different geographic regions of India where the climate and farm practices and/or crop rotations vary greatly. There are different reports suggesting the existence of genotypic diversity among the isolates of Fusarium (Saharan et al., 2006). Moreover, because of this low level variability there was no possibility of different races among the isolates even though they were from different agro-climatic regions.

The current research findings clearly show that RAPD markers can efficiently be used as a marker for the characterization and variability analysis among *F. moniliforme* var. subglutinans isolates causing mango malformation from different geographical regions of India. Hence, this study further helps in ameliorating the disease using proper management practices which can be implemented efficiently.

Acknowledgments

Authors are thankful to the Director, CISH (ICAR) Rehmankhera, Lucknow, U.P., India for providing necessary research facilities and fund during the course of investigations. First author (PK) also pay sincere thank to Dr. S. Saxena (Head), Department of Biotechnology, B. B. A. University, Lucknow and Dr. B. K. Pandey (Principal Scientist), Dr. Muthu Kumar M. (Scientist) CISH, Lucknow, India for their support and critical suggestions.

References

Abd-Elsalam, K.A., F. Schnieder and J.R. Guo: A modified DNA extraction mini-preparation protocol for *Fusarium isolates*. *J. Rap. Meth. Autom. Microb.,* **11**, 75 (2003).

Ali, N.A. and R.M. Jackson: Stimulation of germination of spores of some ectomycorrhizal fungi by other micro-organisms. *Mycol. Res.,* **93**, 182-186 (1989).

Arif, M., D.R. Pani, N.W. Zaidi and U.S. Singh: PCR-based identification and characterization of *Fusarium* sp. associated with mango malformation. *Biotechnol. Res. Int.,* **2011**, 1-6 (2011).

Bains, G. and R.C. Pant: Mango malformation: Etiology and preventive measures. *Physiol. Mol. Biol. Plant.,* **9**, 41-61 (2003).

Bateman, G.L., H. Kwasna and E. Ward: Relationship among *Fusarium* spp. estimated by comparing restriction fragment length polymorphism in polymerase chain reaction amplified nuclear DNA. *Can. J. Microb.,* **42**, 1232 (1996).

Chand, D. and D.K. Chakrabarti: Techniques to produce malformation in mango (*Mangifera indica* L.). *J. Mycol. Plant Pathol.,* **33**, 431-438 (2003).

Crane, J.H. and C.W. Campbell: Fact sheet HS-2, Horticultural Science Department, Florida cooperative extention service, Institute of Food and Agricultural Sciences, University of Florida, USA (1994).

Freeman, S. and M. Maymon: Reliable detection of the fungal pathogen *Fusarium oxisporum* f. sp. albedinis, causal agent of bayound disease of date palm using molecular technique. *Phytopara.,* **28**, 341-384 (2000).

Gupta, V.K., A.K. Misra, A. Gaur, R. Pandey and U.K. Chauhan: Studies of genetic polymorphism in the isolates of *Fusarium solani*. *Aust. J. Crop Sci.,* **3**, 101-106 (2009).

Iqbal, Z., U.M. Rahman, A.A. Dasti, A. Saleem and Y. Zafar: RAPD of *Fusarium* isolates causing mango malformation disease in Pakistan. *World J. Microb. Biotechnol.,* **22**, 1161-1167 (2006).

Jana, T.K., T.R Sharma, D. Prasad and D.K. Arora: Molecular characterization of *Macrophomina phaseolina* and *Fusarium species* by single primer RAPD technique. *Microbiol. Res.*, **158**, 264-274 (2003).

Jaccard, P.: Etude comparative dela distribution florale dans une portion des Alpes et des Jura. *Bulletin Vaudoise Des Sci. Natu.,* **44**, 223 (1908).

Kim, D.H., R.D. Martyn and C.W. Magill: Mitochondrial DNA-relatedness among formae specials of *Fusarium oxysporumm* in the Cucurbitaceae. *Phytopathology.,* **83**, 91-97 (1993).

Kumar, P., A.K. Misra, D.R. Modi and V.K. Gupta: Biocontrol potential of *Trichoderma* species against mango malformation pathogens. *Arch. Phyto. Plant Prot.,* **45**, 1237-1245 (2012).

Kumar, P., A.K. Misra and D.R. Modi: Current status of mango malformation in India. *Asian J Plant Sci.,* **10**, 01-23 (2011a).

Kumar, P., A.K. Misra, A.K. Srivastava and D.R. Modi: Mapping of *F. moniliforme* var. subglutinans from normal and malformed panicles and seedlings of mango by recovery method. *Plant. Archives.,* **11**, 567-569 (2011b).

Misra, A.K. and V.K. Gupta: Pathogenecity of *Fusarium* spp. isolates of guava wilt. *J. Mycol. Plant Pathol.,* **40**, 72-77 (2010).

Misra, A.K. and V.K. Singh: *Fusarium subglutinans* (*F. moniliforme* var. subglutinans) in relation to mango malformation. *Ind. J. Plant Pathol.*, **20**, 81-83 (2002).

O'Donnell, K.: Progress towards a phylogenetic classification of *Fusarium*., Sydowia, 48, 57-70 (1996).

Ploetz, R., Q.I. Zheng, A. Vazquez and M.A. Abdel Sattar: Current status and impact of mango malformation in Egypt. *Int. J. Pest Manag.,* **48**, 279-285 (2002).

Rohlf, F.J.: NTSYS-pc. Numerical taxonomy and multivariate analysis system, Version 2.0 –Applied Biostatistics, New York (1998).

Sabir, J.S.M.: Genotype identification for some *Fusarium sambusinum* strain isolated from wheat in upper Egypt. *World J. Agricul. Sci.,* **2**, 6-10 (2006).

Saharan, M.S., A. Naef, J. Kumar and R. Tiwari: Characterization of variability among isolates of *Fusarium graminearum* associated with head scab of wheat using DNA markers. *Cur. Sci.,* **92**, 230-235 (2006).

Sneath, P.H.A. and R.R. Sokal: Numerical taxonomy. Freeman, San Francisco, p. 15 (1973).