

An Independent, Open Access, Peer Reviewed, Non-Profit Journal

## International Journal of Life Sciences

Year 2011

Volume 5, Issue 1



## Research Article

Molecular Characterization of *Macrophomina Phaseolina*, the Incitant of *Coleus Forskohlii* Revealed by Rapd Markers.Chathuri Mudalige<sup>1</sup>, S.T.Girisha<sup>1,\*</sup>, V.B. Raghavendra<sup>2</sup>, M.H.Niranjan<sup>3</sup>, K.Ravikumar<sup>1</sup> & M.G.Sumantha<sup>1</sup><sup>1</sup> Department of Biotechnology, JB campus, Bangalore University, Bangalore. <sup>2</sup> Department of Biotechnology, Manasagongothi, Mysore University, Mysore. <sup>3</sup> Department of Botany, Manasagongothi, Mysore University, Mysore.

## Corresponding author

Email:  
stgirisha@gmail.com

Phone No:

--

Fax No:

--

Editor  
Randeep RakwalDOI  
10.3126/ijls.v5i1.5598

## ABSTRACT

*Coleus forskohlii* belong to family Lamiaceae is one of the commercial plants grown extensively in the country, the chemical found in the *Coleus* which has both medicinal application and gives great economy to the industrial organizations. Unfortunately, these plants are being highly succumbed to serious diseases like wilt and root rot caused by a fungus, hence the growers and industrialists are facing severe problem in safeguarding this crop in the field irrespective of the agro climatic conditions. Root rot disease, is one of the major diseases of *Coleus forskohlii* which, is caused by *Macrophomina phaseolina*, Pathogen variability was studied at both morphological and molecular level using cultural characteristics and Rapid Amplification of Polymorphic DNA (RAPD) analysis respectively. Totally thirty two isolates were isolated from roots of *Coleus forskohlii*. In RAPD 165 bands were obtained out of them 121 bands (73.3%) were polymorphic with a similarity coefficient of 0.48-0.66. Clusters analysis of RAPD data when Unweighted Pair Group Method with Arithmetic Mean (UPGMA) Tree constructed using NTSYS, it showed 6 groups. Among them two were major clusters and 4 were minor clusters with similarity coefficient 0.48-0.66. The pathogenicity of the isolates was tested on *Coleus forskohlii* plants. Analysis of the pathogenicity tests results revealed that the isolates grouped under two major clusters which were different from the one obtained using RAPD data. The results indicate that the data from RAPD analysis and Pathogenicity tests do not correlate with each other.

**Key words:** Molecular diagnosis; Root rot; *Coleus forskohlii*; Pathogenicity; RAPD

## INTRODUCTION

*Coleus* is an important medicinal plant (Virbala shah et al. 1991) where its root rot disease is a major constraint throughout the world (Mihail 1992, Srivastava et al. 2001) the pathogen is soil born, infecting plant through root cause discoloration and foliar chlorosis and the disease cause huge loss (Kamalakkannan et al. 2003). Its sclerotia can survive in soil for 2-15 years even in the absence of host (Young et al. 1983, Baird et al. 2003). The identification of pathogen based on differential hosts is time consuming and may be influenced by environmental factors. Further more in most cases differential diseases reactions do not provide information about the genetic relationship and viability among and with the pathogenic isolates of species hosts.

According to Shyla 1998. root rot and wilt disease of *C. forskohlii* caused by *F. chlamyosporum*

was reported for the first time from Karnataka state, India and it is an important disease occurring in severe form, contrary to this a fungus was isolated aseptically from diseased parts on Potato Dextrose Agar (PDA) medium, the mycelium was initially hyaline and later became grey in color, sclerotia were minute, black, round to oblong or irregular in shape with mycelial attachment, based on the symptoms and mycelial characters, the fungus was identified as *M. Phaseolina* (Dhingra and Sinclair, 1973) simultaneously a field study were undertaken by Boby and Bagyaraj 2003. to study the possibility of controlling this root rot disease caused by *F. chlamyosporum* using three biocontrol agents viz., *Glomus mosseae*, *Pseudomonas fluorescens*, *Trichoderma viride*, singly and in combination.

Regard to this field diagnosis and research on disease epidemiology and management inherently require a method of detecting and quantifying the presence of the pathogen responsible. Traditional

methods often require highly skilled diagnosticians, animal-based antibodies, lengthy grow-out or culturing procedures, laboratory equipment and methods unique to each pathogen and tissue fixation and microscopic evaluation or even electron microscopic analysis. Recent advances in molecular biology, however, now permit identification and quantification based on the presence of the pathogen DNA. The amount of pathogen DNA present is directly proportional to the number of pathogen cells present, which in turn is proportional to the mass of mycelium, the number of fungal spores etc. Because DNA is fundamental to all life, and yet the primary structure of the DNA is unique to every species, it is possible to employ a streamlined procedure to isolate the DNA from infected plant specimens and then to utilize a common type of molecular assay (Q-PCR) to determine the precise identity and quantity of pathogen present, Q-PCR.

The utility of DNA markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), variable number tandem repeats (VNTR), and simple sequence repeats (SSR) in detecting genetic variability is well established for many phytopathogenic fungi (Weising et al. 1995, Hantula et al. 1996, Majer et al. 1996, Anderson and Nilsson-Tillgreen, 1997, Bridge et al, 1997, Bridge et al, 1998, Sharma and Tewari, 1998, Sharma et al. 1999, Wo stemeyer and Kreibich, 2002, Sharma et al. 2002, Sharma, 2003). The use of simple sequence repeats (SSR) as primers for the PCR was originally proposed under the names, Microsatellite – primed PCR (MP-PCR; Bus cot et al. 1996), random amplification of microsatellites (RAMS) Hantula et al, 1996, direct amplification of microsatellite region DNA polymerase chain reaction (DAMD-PCR; Longato and Bonfante, 1997) and SSR –PCR (Zietkiewicz et al, 1994). The objective of our study was to use SSR primers for amplification of DNA from the isolates of *F. chlamydosporum* obtained from *Coleus* of different areas to assess their potential for DNA fingerprinting. To the best of our knowledge, these reports of the use of microsatellite markers for molecular discrimination of the root rot pathogen *F. chlamydosporum*.

## MATERIALS AND METHODS

### *Isolation of fungi from infected roots*

The infected plants were collected from Mysore, Bangalore (GKVK), V.C. farm (Mandya), Doddaballapur, Kolar, Chickaballapur,

Chamarajanagar, Coorg and Dhanavanthari Vana (Jnanabharathi campus, Bangalore University).

Infected roots were washed in tap water, and cut into sections with sterilized scalpel. The sections were surface sterilized in 0.1% mercuric chloride and treated with 70% ethyl alcohol to remove the traces of mercuric chloride and rinsed with distilled water. The sterilized roots were placed on potato dextrose agar, Czapek dox Agar and Nutrient Agar. The plated Petri dishes were incubated at room temperature (28 ± 2°C) for seven days and observed daily for fungal development. The developing fungi were identified and pure cultures were prepared and stored in slants for further use and further Koch's postulate is proved.

### *Isolation of fungal DNA and RAPD Assay*

The Genomic DNA of the samples was isolated by using the CTAB method (Murry and Thompson 1980). RAPD assay was carried out in 0.5 ml thin walled PCR tubes. The total reaction volume of 25µl consisted of assay buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 0.5 % SDS, 25mM EDTA), 200µM each dNTPs, 5 pmol of a random primer, 1.5 U Taq polymerase and 50 ng of fungal DNA. The tubes were placed in a PCR thermocycler UNO II (Biometra, Germany) programmed as follows: Initial denaturation for 3 min at 94°C followed by 45 cycles of 1 min at 94 °C, 1 min at 36°C and 2 min at 72 °C, and a final extension step of 10 min at 72 °C. The 10 random decamer primers used were from "Kit A" of Operon Technologies (Alameda, CA, USA) and their sequences are shown in Table 1.

The amplified products were separated by electrophoresis in 1.4 % agarose gel in Tris Borate

Primer	Sequence
OPA-05	5'-CAGGCCCTTC-3'
OPA-07	5'-TGCCGAGCTG-3'
OPA-11	5'-AGTCAGCCAC-3'
OPA-13	5'-AATCGGGCTG-3'
OPA-15	5'-AGGGGTCTTG-3'
OPB-02	5'-GGTCCCTGAC-3'
OPB-03	5'-GAAACGGGTG-3'
OPB-06	5'-GTGACGTAGG-3'
OPB-09	5'-GGGTAACGCC-3'
OPB-12	5'-GTGATCGCAG-3'

Table 1: List of Operon Primers.

EDTA (90 mM Tris, 90 mM boric acid, 2 mM ethylene diamine tetra acetic acid), stained in Ethidium bromide and visualized by UV fluorescence. The RAPD data from all amplified were recorded by scoring all DNA bands and were compiled in a binary matrix in which 1 indicated the presence and 0 the absence of the marker. 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 Mean (UPGMA) using Tools for Population Genetic Analyses (TFPGA ver 1.3).

### Statistical analysis

The relatedness of isolates was estimated by means of scorable DNA bands amplified from different microsatellite markers. Each band was considered as a binary character of present (coded as 1) and absent (coded as 0). Similarities were determined by Dice coefficient. Cluster analysis was performed with the UN weighted pair group method with an arithmetic average (UPGMA) algorithm by using NTSYS-pc, (ver 2.01; Rohlf 1998). The relationship among different isolates is given graphically in the form of dendrogram.

## RESULTS

### Isolation of causal organism from the infected plant roots

#### Macroscopic Features

It grows rapidly and produce woolly to cottony, flat, spreading colonies. From the front, the color of the colony may be white or cream; from the reverse it is colorless.

#### Microscopic Features

Pycnidiospores of *M. phaseolina* are often seen oozing from ostiole of pycnidia in the form of cirrus which is white and wet in the beginning, becomes drier with age. Spores are 1-celled, hyaline, ellipsoid to abovoid, thin walled, 14- 30 x 5-10 µm. *M. phaseolina* isolates collected from different growing regions were tabulated in Table 2.

### Isolation of fungal DNA

The DNA isolated from the fungus by using two different methods, those are, C-TAB – NaCl method and Genomic DNA purification Kit method. The isolated DNA samples were proceed with Agarose

Isolate No.	Place of origin	Districts
01	Jnanabarathi	Bangalore
02	Jnanabharathi	Bangalore
03	Thimmapura	Kolar
04	Molanki	Kolar
05	Nandi	Chickaballapur
06	Booknalli	Chamarajanagar
07	Karosi	Kolar
08	Doddaballapur	Bangalore
09	Doddaballapur	Bangalore
10	Doddaballapur	Bangalore
11	Doddaballapur	Bangalore
12	GKVK	Bangalore
13	GKVK	Bangalore
14	Hebbal	Bangalore
15	Yelahanka	Bangalore
16	Hirehalli	Mandya
17	Anumanahalli	Mandya
18	V.C. farm	Mandya
19	V.C. farm	Mandya
20	Alur	Hassan
21	Belur	Hassan
22	Yelwal	Mysore
23	Belwadi	Mysore
24	Hampapura	Mysore
25	Banny kuppe	Mysore
26	H.D. Kote	Mysore
27	Arkalgudu	Hassan
28	Somvarpet	Coorg
29	Virajpet	Coorg
30	Gonikoppa	Coorg
31	GKVK	Bangalore
32	GKVK	Bangalore

Table 2. *M. phaseolina* isolates collected across the *Coleus* growing areas in Karnataka.

Gel – Electrophoresis and observed orange color band which is aligning with the 3kb band of the DNA marker under UV- transilluminator. This confirmed the isolation of DNA.

### Fingerprinting of *Macrophomina phaseolina* isolates by RAPD

Scoring of bands was carried out considering the dark and prominent bands. However, bands of lower intensity, but, with high reproducibility were also included in the analysis. Out of the 10 primers screened, a total of 82 bands were scored, 62 bands (75.25%) showed polymorphism using template DNA of 32 *Macrophomina phaseolina* isolates tabulated in table 3.

### Three-Dimensional Scaling

The three Dimensional scaling is one of the multivariate approaches of grouping based on the similarity coefficient values of the component traits of the entities. The 3D scale shows that the various isolates supported the UPGMA results and clarified

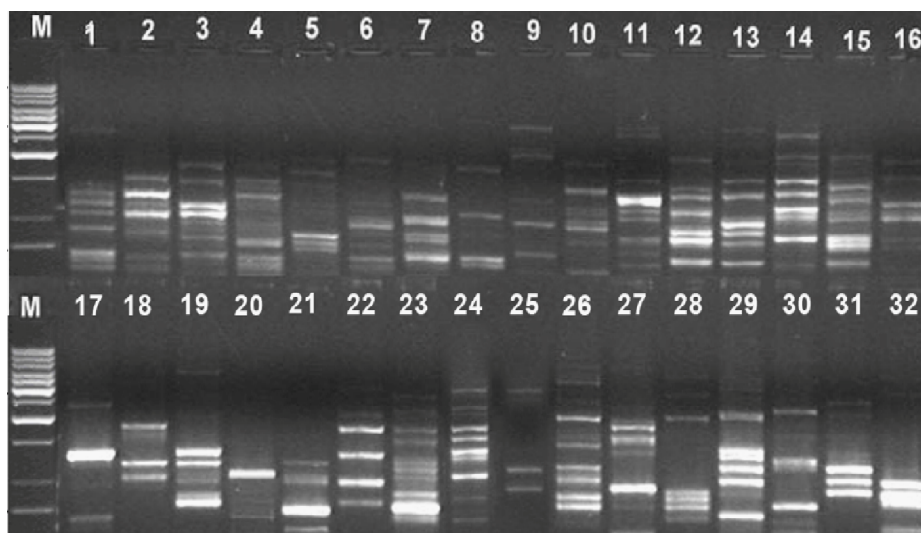


Figure 1: RAPD products from 32 *M. phaseolina* isolates amplified with OPA-11 primer

Lanes 1-32 *M. phaseolina*

relationships among 32 *M. phaseolina* isolates as shown in figure (Fig. 2).

When UPGMA Tree constructed using NTSYS, it showed 6 groups. Among them two were major clusters and 4 were minor clusters with similarity coefficient 0.48-0.66. The major I cluster consisted of 14 isolates, which further constituted four subgroups (I, II, III and IV). Subgroup I consists of four isolates (04, 11, 24 and 25), which were collected from Kolar, Bangalore and Mysore. Sub group II consisting of two isolates (29 and 31) were collected from Coorg and Bangalore. Sub group III consisting of four isolates (26, 30, 07 and 28), which were collected from Kolar, Mysore and Coorg. Sub group IV consisting of four isolates (13, 16, 23 and 32) were collected from Bangalore, Mandya and Mysore. The second major cluster consisting of eight isolates, which further divided into two sub groups (I and II). Sub group I consisting six isolates (01, 09, 08, 18 and 20), which were of Bangalore, Mandya and Hassan. Sub group II consisting of two isolates 03 and 17, were belongs to Kolar and Mandya. Minor cluster I consisting of five

isolates further divided into three sub groups (I, II and III). Sub group I consists of one isolate (02), which were from Bangalore. Sub group II consists of two isolates 08 and 19, which were collected from Bangalore. Sub group III consists of two isolates 14 and 27, belongs to Bangalore and Hassan. Minor cluster II consists of two isolates 12 and 15, which were of Bangalore. Two isolates 06 and 21, collected from Chamarajanagar and Hassan were further grouped under Minor cluster III. Minor cluster IV grouped as separate cluster, which consists only one, isolate 22 collected from Mysore as shown in figure (Fig. 3).

## DISCUSSION

*Coleus* is an important plant possesses many species. *C. forskohlii* are the important species which grow for the various medicinal and industrial purposes. Many industries grow *C. forskohlii* in large scale for the great commercial value of forskolin. Unfortunately these plants are been highly infected

Table 3: Fingerprint pattern generated using the 10 RAPD primers in 32 isolates of *M. phaseolina*

[RAPD primer OPA-13 produced a maximum of 10 bands showing 80% as shown in figure (Fig. 1). Polymorphism followed by 7 bands by OPA-11 with 77.7% and minimum of 5 bands in OPB-09 with 71.4% was observed.]

Sl. No.	RAPD-Primers	Total no. of bands amplified	No. of Polymorphism bands	PIC value (%)
1.	OPA-05	08	06	75.0
2.	OPA-07	08	06	75.0
3.	OPA-11	09	07	77.7
4.	OPA-13	10	08	80.0
5.	OPA-15	07	05	71.5
6.	OPB-02	08	06	75.0
7.	OPB-03	09	07	77.7
8.	OPB-06	08	06	75.0
9.	OPB-09	07	05	71.4
10.	OPB-12	08	06	75.0
	<b>Total</b>	<b>82</b>	<b>62</b>	<b>75.25</b>



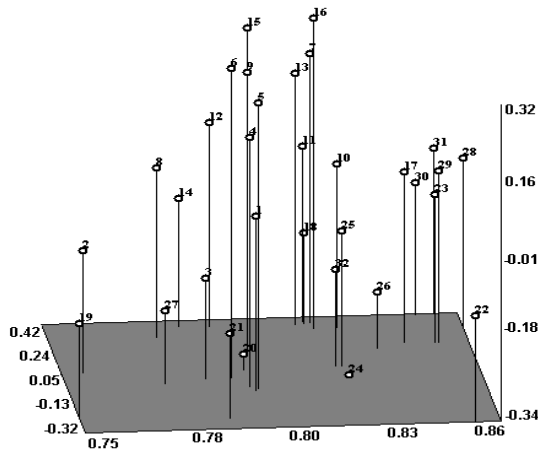
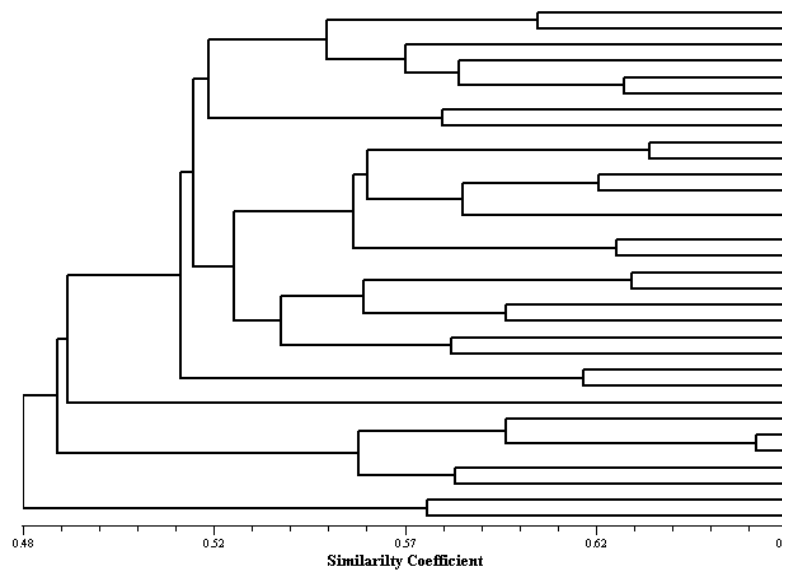


Figure 2 : Three-Dimensional scaling generated by NTSYS of 32 isolates of *M. phaseolina* based on the combination of data obtained with 10 RAPD primers.

Figure 3 : UPGMA dendrogram from RAPD data of 30 isolates of *M. phaseolina* amplified by 10 primers. Distance based on Nei (1978)



due to a severe devastating disease like root rot. In present there are different controversial statement regarding to the causal organism of this disease. According to Boby and Bagyaraj, 2003 root rot disease is caused by *M. phaseolina*. In the present study 32 isolates of *M. phaseolina* used against root rot of *C. forskohlii* disease were characterized based on some morphological and molecular characterization. The experiments showed that the 32 isolates differed in their morphological characters to some extent apart from similarities between each other.

A powerful tool for studying variation and relatedness between different isolates is the random amplified polymorphic DNA (RAPD) which is more convenient than other polymorphic DNA detecting techniques as it is precise, fast, relatively inexpensive, requires small amount of DNA template but not DNA sequence information, without requirement of cloning

or any other form of the molecular characterization of the genome of the species (Bardakci 2001, Powell W 1996). Sequenced Characterized Amplified Regions (SCARs) analysis of RAPD polymorphisms (Bardakci 2001). showed that one cause of RAPD polymorphism and chromosomal rearrangements such as insertions/deletions. Therefore, amplification products from the same alleles in a heterozygote differ in length and will be detected as absences of bands in the RAPD profile (Bardakci 2001).

RAPD technique is used for genetic variation exists among the isolates of *M. phaseolina* obtained from almost nearer geographical regions. Few isolates *Mp 9*, *Mp 12* and *Mp 25* were similar in RAPD analysis. But rest of the isolates did not show much variation. This indicated that the results from morphological characteristics and RAPD analysis did not correlate with each other. The results of the present study are in line with several similar studies on morphological

variation in *Drechslera teres*, *Pochonia chlamydosporia* using RAPD analysis (Morton et al. 2003; Frazzon et al. 2002) and pathogenic variation on *P. brasiliensis* (Teresa R Motta et al. 2002). But the results from two analyses could not be successfully correlated with each other.

Other reports on the use of the RAPD have suggested that even though it can be used successfully to characterize isolate variability among pathogens, the result quite often may not correlate with the morphological and genetic variation studies. More detailed analysis using a large number of isolates from different geographical regions and more number of primers will give a better understanding of the differentiation.

In the present study, a cluster analysis with UPGMA, the dendrogram is constructed. The isolates were divided into two major clusters and first major cluster was subdivided into three sub minor cluster.

### Acknowledgements

The authors are thankful to Bangalore University for providing infrastructure and financial assistance.

### REFERENCES

- Anderson, T.H. & Nilsson-Till green, T. 1997. A fungal mini-satellite. *Nature* 386: 771. PMID:9126733
- Bardakci, F. & Skibinski, D.O.F. 1999. A polymorphic SCAR-RAPD marker between species of tilapia (Pisces: Cichlidae). *Animal Genetics* 30: 78–79. <http://dx.doi.org/10.1046/j.1365-2052.1999.00323-18.x> PMID:10050307
- Baird, R.E.; Watson, C.E. & Scruggs, M. 2003. Relative longevity of *Macrophomina phaseolina* and associated mycobiota on residual soybean roots in soil. *Plant Disease* 87: 563–566. <http://dx.doi.org/10.1094/PDIS.2003.87.5.563>
- Boby, V.U. & Bagyaraj, D.J. 2003. Biological control of root-rot of *Coleus forskohlii* Briq. using microbial inoculants. *World Journal of Microbiology and Biotechnology* 19:175–180. <http://dx.doi.org/10.1023/A:1023238908028>
- Bridge, P.D.; Couteaudier, Y. & Clarkson, J. 1998. Molecular Variability of Fungal Pathogens. Eds. CAB International., Wallingford.
- Bridge, P.D.; Pearce, A. & Rutherford, M.A. 1997. VNTR derived oligonucleotides as PCR primers for population studies in filamentous fungi. *Letters in Applied Microbiology* 24: 426–430. <http://dx.doi.org/10.1046/j.1472-765X.1997.00060.x>
- Buscot, F.; Wipe, D.; Di-Battista, C.; Munch, J.C.; Botton, B. & Martin, F. 1996. DNA polymorphism in morels: PCR/RFLP analysis of the ribosomal DNA spacers and microsatellite-primed PCR. *Mycological Research* 100: 63–71. [http://dx.doi.org/10.1016/S0953-7562\(96\)80101-8](http://dx.doi.org/10.1016/S0953-7562(96)80101-8)
- Dhingra, O.D. & Sinclair, J.B. 1973. Location of *Macrophomina phaseolina* on soybean plants related to culture characteristics and virulence. *Journal of Phytopathology* 63:934–936. <http://dx.doi.org/10.1094/Phyto-63-934>
- Frazzon, A.P.G.; Matsumura, A.T.S. & Van Der Sand, S.T. 2002. Morphological characterization and genetic analysis of *Drechslera teres* isolates. *Genetics and Molecular Biology* 25: 235–241. <http://dx.doi.org/10.1590/S1415-47572002000200019>
- Hantula, J.; Dusabenyagasani, M. & Hamelin, R.C. 1996. Random amplified microsatellites (RAMS) a novel method for characterizing genetic variation within fungi. *European Journal of Forest Pathology* 26: 159–166. <http://dx.doi.org/10.1111/j.1439-0329.1996.tb00720.x>
- Kamalakannan, A.; Mohan, L.; Valluvaparisadan, V.; Mareeswari, P. & Karupiah, P. 2003. First report of *Macrophomina* root rot (*Macrophomina phaseolina*) on medicinal *Coleus* (*Coleus forskohlii*) in India. *Journal of Plant Pathology* 55(2): 302 – 302.
- Longato, S. & Bonfante, P. 1997. Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. *Mycological Research* 101: 425–432. <http://dx.doi.org/10.1017/S0953756296002766>
- Majer, D.; Mithen, R.; Lewis, B.G.; Vos, P. & Oliver, R.P. 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* 100: 1107–1111. [http://dx.doi.org/10.1016/S0953-7562\(96\)80222-X](http://dx.doi.org/10.1016/S0953-7562(96)80222-X)
- Mihail, J.D. 1992. *Macrophomina*. In *Methods for Research on Soil-borne Phytopathogenic Fungi* (L. S. Singleton, J. D. Mihail and C. M. Rush, Eds), pp. 134–134. American Phytopathological Society Press, St Paul, MN.
- Morton Oliver, C.; Tim, H.; Mauchlinea, R.; Kerrya & Penny, R. 2003. PCR-based DNA fingerprinting indicates host-related genetic variation in the nematophagous fungus *Pochonia chlamydosporia*. *Mycological Research* 107: 198–205. <http://dx.doi.org/10.1017/S0953756203007251> PMID:12747331
- Murray, M.G. & Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Research* 8: 4321–4325. <http://dx.doi.org/10.1093/nar/8.19.4321> PMID:7433111 PMCID:324241
- Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583–590. PMID:17248844 PMCID:1213855
- Powell, W.; Morgante, M.; Andre, C. Hanafey, M.; Vogel, J.; Tingey, S. & Rafalski, A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding* 2: 225–238. <http://dx.doi.org/10.1007/BF00564200>
- Rohlf, F.J. 1998. NTSYS-pc Numerical Taxonomy and Multivariate Analysis System. Version 2.01. Exeter Software, Setauket, New York.
- Sharma, T.R. & Tewari, J.P. 1998. RAPD analysis of three *Alternaria* species pathogenic to crucifers. *Mycological Research* 120: 807–814. <http://dx.doi.org/10.1017/S0953756297005479>

Sharma, T.R. 2003. Molecular diagnosis and application of DNA markers in the management of fungal and bacterial plant diseases. *Indian Journal of Biotechnology* 2: 99–109.

Sharma, T.R.; Chauhan, R.S.; Singh, B.M.; Paul, R.; Sagar, V. & Rathour, R. 2002. RAPD and pathotype analysis of *Magnaporthe grisea* population from North-Western Himalayan Region of India. *Journal of Phytopathology* 150: 649–656. <http://dx.doi.org/10.1046/j.1439-0434.2002.00812.x>

Sharma, T.R.; Prachi, & Singh, B.M. 1999. Application of polymerase chain reaction in phytopathogenic microbes. *Indian Journal of Microbiology* 39: 79–91.

Shyla, M. 1998. Etiology and management of root rot of *Coleus forskohlii*. MSc Thesis University of Agricultural Science, Bangalore, India.

Srivastava, A.K.; Singh, T.; Jana, T.K. & Arora, D.K. 2001. Microbial colonization of *Macrophomina phaseolina* and suppression of charcoal rot of chickpea. In *Microbes and Plants* (A.Sinha, ed.): pp. 269–319. Vedams eBooks, New Delhi.

Teresa, R.M.; Moreira-Filho, C.A.; Mendes, R.P.; Souza, L.R.; Sugizak, M.F.; Baueb, S.; Calich, V.L.G. & Vaz, C.A.C. 2002. Evaluation of DNA polymorphisms amplified by arbitrary primers (RAPD) as genetically associated elements to differentiate virulent and non-virulent *Paracoccidioides brasiliensis* isolates. *FEMS Immunology & Medical Microbiology* 33: 151–157. <http://dx.doi.org/10.1111/j.1574-695X.2002.tb00585.x>

Shah, V. & Kalakoti, B.S. 1991. Development of *Coleus forskohlii* as a medicinal crop, Research centre, FAO Corporate Document Repository. Hoechts Marion Roussel Limited, Mulund, Bombay, India.

Weising, K.; Nybom, H.; Wol, K. & Meyer, W. 1995. DNA Fingerprinting in Plants and Fungi. CRC Press., Boca Raton.

Wöstemeyer, J. & Kreibich, A. 2002. Repetitive DNA elements in fungi (mycota): impact on genomic architecture and evolution. *Current Genetics* 41: 189–198.

Young, D.L.; Gilbertson, R.L. & Alcorn, S.M. 1983. A new record for longevity of *Macrophomina phaseolina* sclerotia. *Mycologia* 74: 504–505. <http://dx.doi.org/10.2307/3792973>

Zietkiewicz, E.; Rafalski, A. & Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR-) anchored polymerase chain reaction amplification. *Genomics* 20:176-83. <http://dx.doi.org/10.1006/geno.1994.1151> PMID:8020964

© International Journal of Life Sciences