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Assessment of molecular variability in *Macrophomina phaseolina* isolates of chickpea from different locations of India

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Abstract
An experiment was performed on "Assessment of molecular variability in *Macrophomina phaseolina* isolates of chickpea from different locations of India" The results revealed that, Molecular characterization of twenty isolates includes identification of *M. phaseolina* isolates with species specific primers MpKFI (5'- CCGCCAGAGGACTATCAAAC-3') and MpKRI (5' CGTCCGAAGCGAGGTGTATT-3') yielded single amplified product of 350 bp in all the isolates tested and the PCR amplified 18S rDNA region using ITS primers produced a band size of about 600bp. The results was further evident that, phylogenetic tree having the nucleotide sequences of ITS region. Clusters A and B indicating the variability in *M. phaseolina* and existence of two types of pathotype. In nature, new strains might have been arised by mutation, hybridization, differential cytoplasmic inheritance heterokaryosis and by parasexual life cycle. The pathogenic variability is essential tools for breeding disease resistance in a crop improvement programme. From ongoing discussion, it can be concluded that, potential pathogen often blessed with biodiversity within its population and variation in pathogen is desirable trait for its existence in nature. This variability among the pathogens underlies their diverse nature and ability to withstand the host environment.

Keywords: Molecular variability, *Macrophomina phaseolina*, chickpea

1. Introduction
Chickpea (*Cicer arietinum* L.), also known as Gram or Bengal gram, is the second most important pulse crop in the world, India accounting for 60 to 75 per cent of the world's Chickpea production. Chickpea seeds contain high quality easily digestible protein (25%) and carbohydrates (20%) making it an important source of protein for the vegetarians of the country and thus it is also called "Poor man's meat". The global area under chickpea is 14.80 million ha, with production of 14.23 million tonnes and productivity of 962 kg/ha. In India, it is grown in an area of about 10.74 million ha with production of 9.88 million tonnes and productivity of 920 kg/ha.

In Karnataka, it is grown in 0.95 m ha with production of 0.72 million tonnes and productivity of 757 kg/ha (Anon., 2015) [1]. The per hectare production is low in spite of high yielding varieties and new agronomic practices. The reason for low yield is due to incidence of diseases. The crop is known to be affected by number of soil-borne pathogens, some of which may be devastating. Chickpea suffers from about 172 pathogens consisting of fungi, bacteria, viruses and nematodes. Diseases with limited distribution are economically important because of continuous changes in cultural practices, human interventions and climate change. The dry root rot (*M. phaseolina*) is a major constraint in the chickpea production as it is emerging as a potential threat to chickpea cultivation in semi-arid regions due to moisture stress and high temperatures during the flowering to pod filling stage (Sharma *et al*., 2010). The annual yield loss due to this disease alone is 10-20 per cent (Vishwadhar and Chaudhary, 2001) [14].

The dry root rot disease generally appears around flowering and podding time. The disease may also appear at seedling stage, however, the susceptibility of the plant increases with age. The disease generally appears when day temperature is more than 30 0C and soil moisture content of 60 per cent. Drooping of petioles and leaflets is confine to those at the very top of the plant. Sometimes when rest of the plant is dry, the top most leaves are chlorotic. The leaves and stems of affected plants are usually straw colored. The lower portion of the tap root usually remains in the soil when plants are uprooted. The tap root is dark and is devoid of most of its lateral and finer roots.

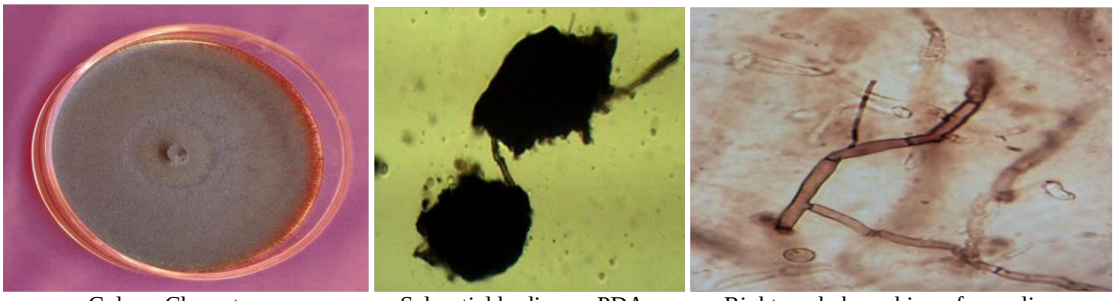
Dark, minute sclerotial bodies can be seen on the roots or inside the wood (Nene *et al.*, 2012) [12]. *M. phaseolina* is primarily seed and soil-borne fungal pathogen. In chickpea, infected seeds and microsclerotia surviving in the soil are the major source of primary inoculum. The pathogen also has wide host range. Since 75 per cent cultivation of chickpea in India is under rainfed, the crop faces severe moisture stress at flowering to Podding stage which predisposes the crop to dry root rot development. The identification of isolates of *M. phaseolina* is usually based on morphological criteria, but due to wide variations in the phenotype of the isolates these criteria are often not reliable. Therefore study of molecular pathogenic variability is essential for breeding disease resistance in crop improvement programme.

2. Materials and Methods

A. Hyphal tip isolation: Totally twenty chickpea plant samples showing typical symptom of dry root rot were collected from different states *viz*., Karnataka, Andhra Pradesh, Telangana, Tamil Nadu, Maharashtra, Madhya Pradesh, Punjab, Uttar Pradesh, Bihar, Uttarakhand, West Bengal and Himachal Pradesh. The isolation of the fungus was done by following standard tissue isolation method under aseptic condition. The infected tissues of the root were cut into small bits of 1-2 mm size and surface sterilized in 1:1000 sodium hypochlorite solution for a minute and then washed thrice in sterile distilled water to remove the traces of sodium hypochlorite before transferring them to sterile potato dextrose agar [PDA] slants under aseptic conditions and incubated at 27±1 °C. Hyphal tip isolation was done on two per cent water agar plates. Dilute mycelial suspensions (10^{-6} ml^{-1}) of the fungus were prepared in sterile distilled water. One ml of such (10^{-6} ml^{-1}) suspension was spread uniformly on water agar plates and observed for mycelial growth initiation under microscope such part was marked with marker and that mycelial bit was transferred on PDA plates under aseptic conditions. The PDA plates were incubated at a temperature of 27±1 °C and observed for fungal growth. This method was followed for maintaining pure culture of the fungus. Such plates were stored in refrigerator at 4 °C and maintained by sub culturing at 20 to 25 days interval. The morphological characters (sclerotial bodies, right angle branching of mycelia on PDA) and cultural characters (growth pattern, mycelial colour) were the principal characters considered for identification of pure culture isolates of *Macrophomina phaseolina*. The pure culture of the pathogen was maintained on PDA slants (Plate 1). The isolates collected were designated as KAMP1, KAMP2, KAMP3, KAMP4, KAMP5, APMP6, APMP7, TEMP8, TEMP9, TNMP10, TNMP11, MHMP12, MPMP13, PUMP14, UPMP15, UPMP16, BHMP17, UKMP18, WBMP19, HPMP20 (Table 1 and Plate 2).

Table 1: List of *M. phaseolina* isolates collected from different geographical locations of India

Sl. No.	State	District	Place	Designation of isolates
1.	Karnataka	Bengaluru north	GKVK	KAMP-1
2.	Karnataka	Chitradurga	Hiriyur	KAMP-2
3.	Karnataka	Hubli-Dharwad	Dharwad	KAMP-3
4.	Karnataka	Gulbarga	Jevargi	KAMP-4
5.	Karnataka	Raichur	Sarjapur	KAMP-5
6.	Andhra Pradesh	Karnool	Kurnool	APMP-6
7.	Andhra Pradesh	Karnool	Nandyal	APMP-7
8.	Telangana	Ranga Reddy	ICRISAT	TEMP-8
9.	Telangana	Warangal	RARS, Warangal	TEMP-9
10.	Tamil Nadu	Coimbatore	TNAU, (Pappanaicken Pudur)	TNMP-10
11.	Tamil Nadu	Coimbatore	Periyanayakkanpalayam	TNMP-11
12.	Maharashtra	Jalna	Badnapur	MHMP-12
13.	Madhya Pradesh	Ujjain	Rajwas	MPMP-13
14.	Punjab	Ludhiana	PAU, Ludhiana	PUMP-14
15.	Uttar Pradesh	Kanpur	IIPR, Kanpur	UPMP-15
16.	Uttar Pradesh	Shahjahanpur	Sujalpur	UPMP-16
17.	Bihar	Patna	Mokama	BHMP-17
18.	Uttarakhand	Udham Singh Nagar	CRC, Pantnagar	UKMP-18
19.	West Bengal	Burdwan	Durgapur	WBMP-19
20.	Himachal Pradesh	Sirmour	Dhaulakaun	HPMP-20



Colony Character | Sclerotial bodies on PDA | Right angle branching of mycelium

Plate 1: Morphological characters of *M. phaseolina*

Plate 2: *M. phaseolina* isolates collected from different locations of India

B. Isolation of DNA from *M. phaseolina* isolates: The genomic DNA was extracted from *M. phaseolina* isolates by following CTAB extraction method (Doyle and Doyle, 1987). Each fungal culture was inoculated in potato dextrose broth (PDB) and grown at a temperature of 30 °C for 7 days with sufficient shaking and aeration. Approximately two grams of mycelium was harvested, frozen in liquid nitrogen and crushed in liquid nitrogen in a pre-cooled pestle and mortar to make fine powder. The grinded powder was transferred to a 2 ml sterile polypropylene tube containing 1 ml of extraction buffer (2% w/v CTAB, 1.4 M NaCl, 0.1 M Tris HCl, pH 8.0, 0.03% β-Mercapto ethanol, 0.1% PVP) pre-warmed to 65 °C. The samples were incubated at 65 °C for 60 minutes with intermittent mixing of tubes at every 15 minutes. After bringing back tubes to room temperature, equal volume (1 ml) of chloroform: Isoamyl alcohol (CIA) (24:1 v/v) was added to each tube containing sample and extraction buffer then mixed by inverting (no vortex). Tubes were then centrifuged at 8500 rpm for 30 minutes. Upper aqueous phase was carefully transferred to new sterile tube (CIA step was repeated once again if the upper layer did not look clear). Then equal volume of pre-chilled isopropanol was added and mixed gently. Tubes were kept for 1-2 hr at -20 °C / overnight and centrifuged at 8500 rpm for 15 minutes to pellet the DNA. The supernatant was decanted and the pellet was washed with 1 ml of 70 per cent ethanol and then air dried completely for 1-2 hrs (there should not be any traces of alcohol). The DNA pellet was suspended in 200-400 µl of $T_{10}E_1$ (10 mM Tris-HCl; 1 mM EDTA maintained at pH 8) in sterile centrifuge tube. When DNA was fully dissolved, 1 µl of RNase (stock 10 mg/ml) per 100 µl volume of sample was added (Final RNase concentration 100 µg/ml) and incubated at 37 °C for one hour or at room temperature for overnight. Finally the samples were stored at -20 °C.

C. DNA quality, quantity estimation and: To assess the quality of DNA, samples were run on 1 per cent agarose gel in 1x TAE buffer (For 250 ml 50x TAE – 60.5 gm of Tris base, 14.25 ml glacial acetic acid and 25 ml of 0.5M EDTA) and stained with 5 µl ethidium bromide (10 mg/ml stock) per 100 ml of gel and checked for shearing of DNA and contamination of RNA.

D. Methodology for PCR (Polymerase Chain Reaction): The following materials were used for PCR (1) Template: Purified genomic DNA extracts (20-30 ηg/µl) of isolates were used as template DNA. (2) Buffers: 10x assay buffer from M/s Bengaluru Black biotech Pvt. Ltd. Bengaluru, India was used. (3) dNTP: Individual dNTPs, *viz*., dATP, dGTP, dCTP and dTTP were obtained from M/s Bengaluru Black biotech Pvt. Ltd., Bengaluru. (4) Thermal cycler: BIO-RAD thermal cycler was used for cyclic amplification of DNA. (5) Taq: Taq DNA polymerase (3 U/µl) was obtained from M/s Bengaluru Genei Pvt. Ltd., Bengaluru. (6) Primers: Primers were synthesized from Sigma Pvt. Ltd., Bengaluru, India.

E. Molecular identification of *M. phaseolina* isolates: Molecular identification of isolates was done by using species specific primers MpKFI (5'-CCGCCAGAGGACTATCAAAC-3') and MpKRI (5'-TCCGAAGCGAGGTGTATT-3') in a PCR (Babu *et al*., 2007) [3]. Reactions were carried out in a final volume of 15 µl containing 1.2 µl of template DNA (30 ng/µl), 0.3µl of Taq polymerase (3 U/µl), 1.5 µl Taq buffer containing $Mgcl_2$ (10x), 0.5 µl dNTP's (2.5 mM) each, 0.6 µl (10 pmol) each of forward and reverse primers and 10.3 µl of nuclease free water.

F. Amplification conditions (PCR protocol): The following amplication protocol were followed for PCR protocol *viz.*, Initial denaturation (95 °C for 5 min upto 40 cycles), Denaturation (94 °C for 45 sec), Annealing (58 °C for 1 min), Extension (72 °C for 2 min), final extension (72 °C for 8 min) and final hold (4 ºC), respectively.

G. ITS region amplification: The ITS regions and the 5.8S rDNA of 20 *M. phaseolina* isolates were amplified with a set of primers nITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-CTCCGCTTATTGATATGC-3'). The PCR- amplification reaction was optimized with various

concentrations of $MgCl_2$, dNTPs, primer and template DNA. The amplification was carried out in a BIO-RAD thermal cycler in a 30 µl PCR reaction consisting of 3µl PCR buffer with $MgCl_2$ (10X), 0.6 µl of (3 U/µl) Taq polymerase (Bengaluru Genei, India), 1 µl (2.5 mM) dNTPs each, 1 µl (10 pmol) of each primer and 2.4 µl (30-50 ηg) of genomic DNA. Initial denaturation of 95 °C for 5 min was followed by 40 cycles of 94 °C for 45 sec, 58 °C for 1 min (primer annealing), 72 °C for 2 min (primer extension). A final extension of 72 °C for 8 min was incorporated into the program, followed by cooling to 4 °C until recovery of the samples. PCR product (5 µl) was mixed with loading buffer (2 µ1) containing 0.25 per cent bromophenol blue, 40% w/v sucrose in water and then loaded in 1.5 per cent Agarose gel with 0.1 per cent ethidium bromide for examination with horizontal electrophoresis. Nucleotide base pairs of the amplicons were determined on the basis of its migration and confirmation relative to the molecular size marker of 1000 kb ladder.

H. ITS region sequencing: The ITS region amplified PCR product produced bands (~550-600 bp) of 20 isolates on agarose gel were sent to sequencing to Genotypic Technology Pvt. Ltd., Bengaluru. The sequenced PCR product was aligned with ex-type strain sequences from NCBI Gene Bank and established fungal taxonomy for identification and variability studies. Sequences were aligned following the Clustal W algorithm included in the Megalign module (DNASTAR Inc.) Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. Both values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for the pair wise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package version 7 (Kumar *et al*., 2015) [8]. Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic inference was performed by the neighbour joining method. Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained (Felsenstein, 1985) [6]. An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny.

3. Results

The total genomic DNA of twenty *M. phaseolina* isolates were amplified using species specific primers MpKFI (5'-CCGCCAGAGGACTATCAAAC-3') and MpKRI (5'-CGTCCGAAGCGAGGTGTATT-3') by using PCR. The primers yielded single amplified product of 350 bp in all the isolates tested. The phylogenetic tree was constructed by using nucleotide sequences from ITS region of 20 different *M. phaseolina* isolates along with 15 sequences of *M. phaseolina* sequences obtained from NCBI GenBank data base which generated a phylogenetic tree with clusters A and B indicating the variability in *M. phaseolina* and existence of two types of pathotype (Fig. 1). All the isolates showed about 65.28 to 100 per cent nucleotide similarity with already deposited *M. phaseolina* sequences in NCBI data base. Among the 20 isolates used in the present study the highest similarity (100%) was observed between most of the isolates (Table 2). ITS region of rDNA was amplified using ITS-1 and ITS-4 primers for sequencing of its 18S rDNA region. The PCR amplified 18S rDNA region using ITS primers produced a band size of about 600bp (Plate 4). The sequence obtained upon sequencing of the ITS region from 20 different isolates were subjected to homology study using Mega 7 software along with sequences downloaded from NCBI database. All the isolates showed about 65.28 to 100 per cent nucleotide similarity with already deposited *M. phaseolina* sequences in NCBI data base. The ITS sequence of all the isolates showed about 97 to 100 per cent similarity with already deposited *M. phaseolina* sequences in NCBI data base, the lowest similarity (32 to 37%) was observed between the isolates and other genus plant pathogens.

4. Discussion

This amplification of specific band confirmed that all the twenty isolates belonged to *M. phaseolina* (Plate 3). The results are in conformity with Mahdizadeh *et al*. (2011) [9] who identified fifty two *M. phaseolina* isolates that were recovered from 24 host plant species through the 14 Iranian provinces using species-specific primers MpKFI and MpKRI. Babu *et al*. (2010) [2] designed two species-specific primers MpKFI and MpKRI from the conserved region, adjacent to ribosomal 5.8 S gene. The designed primers yielded single amplified product of 350 bp with all the *M. phaseolina* isolates tested. Similarly twenty isolates of *M. phaseolina* were identified in chickpea by using species specific primes MpKFI and MpKRI (Manjunatha, 2014) [10]. The sequence obtained upon sequencing of the ITS region from 20 different isolates were subjected to homology study using Mega 7 software along with sequences downloaded from NCBI database. The homology studies revealed that the partial sequence of ITS1-5.8S-ITS4 rRNA gene was as variable as rDNA regions. The sequence information was then analyzed through BLASTn program which indicated that the sequences contain the genetic information of internal transcribed spacer region of rDNA gene of *M. phaseolina* with nearly 96 - 100 per cent similarity. The identified *M. phaseolina* rDNA gene sequences obtained from NCBI GenBank of various host plants were selected for comparison with the rDNA gene sequence of *M. phaseolina* isolate of chickpea. The sequence alignment of the isolate of *M. phaseolina* shows variation in this gene. The available sequences of *M. phaseolina* from NCBI were used in the pair wise and multiple sequence alignment using Bioedit software for determining the conserved regions of rDNA gene. Multiple sequence alignment revealed that there were quite a number of gaps introduced in the alignment within the ITS region which were closely related. Multiple and pair wise sequence alignment were generated and used to calculate evolutionary distances and per cent homology of sequence and the phylogenetic tree was constructed. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 per cent bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the

branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of number of base substitutions per site. The analysis involved 35 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 509 positions in the final dataset. An evolutionary analysis was conducted in MEGA7.

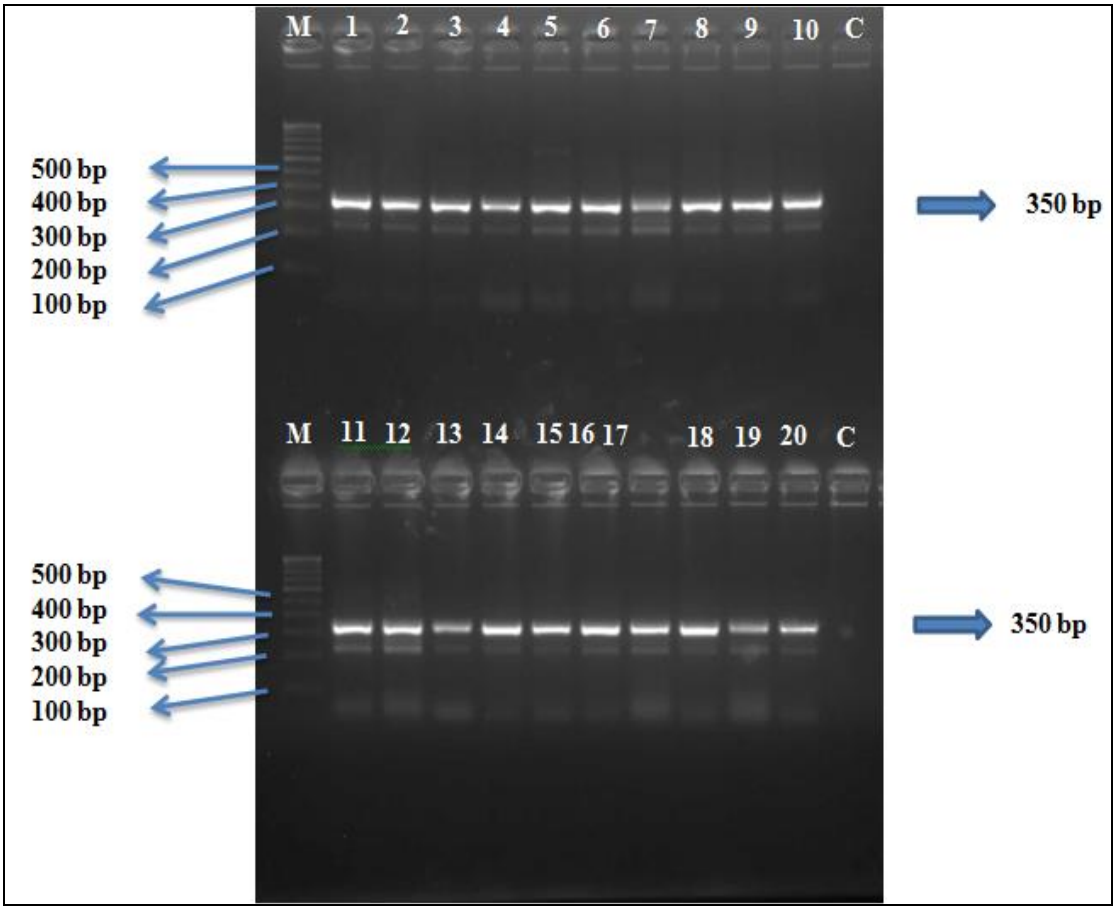


Plate 3: PCR amplification of *M. phaseolina* with species specific primers MpKF1 and MpKR1 (M: Marker 1000 bp,1-20: *M. phaseolina* samples, C: Control)

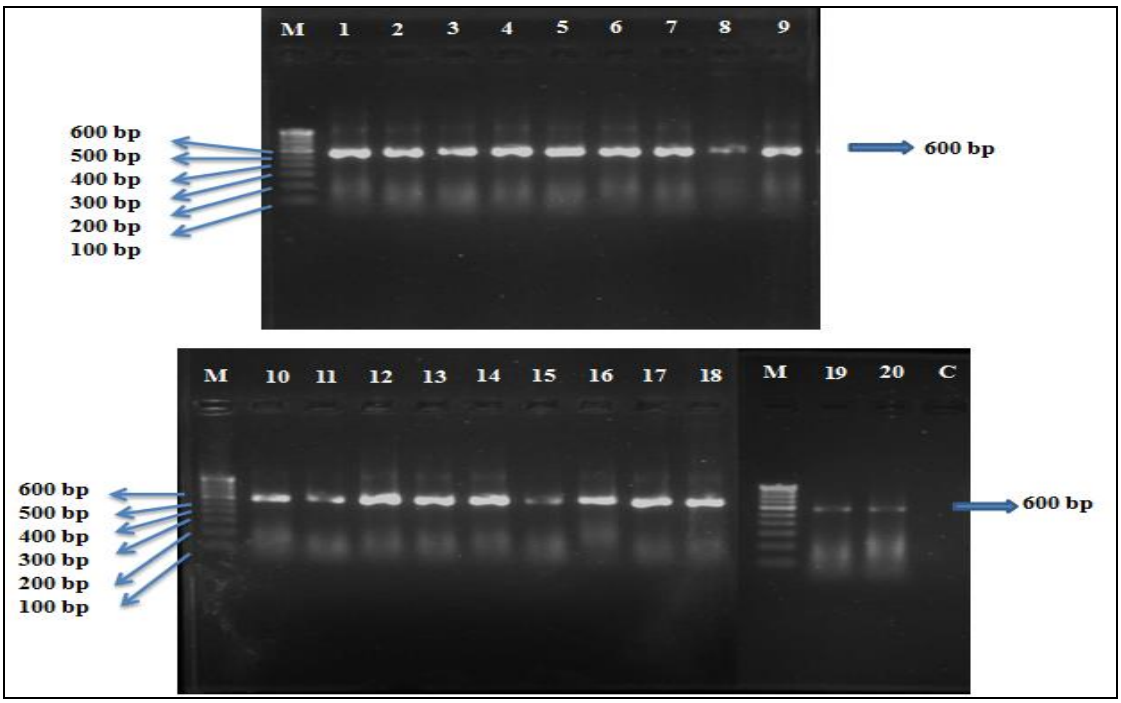


Plate 4: PCR amplification of ITS region of *M. phaseolina* with primers ITS1and ITS4 (M: Marker 1000 bp, n1-20: *M. phaseolin* samples, C: Control)

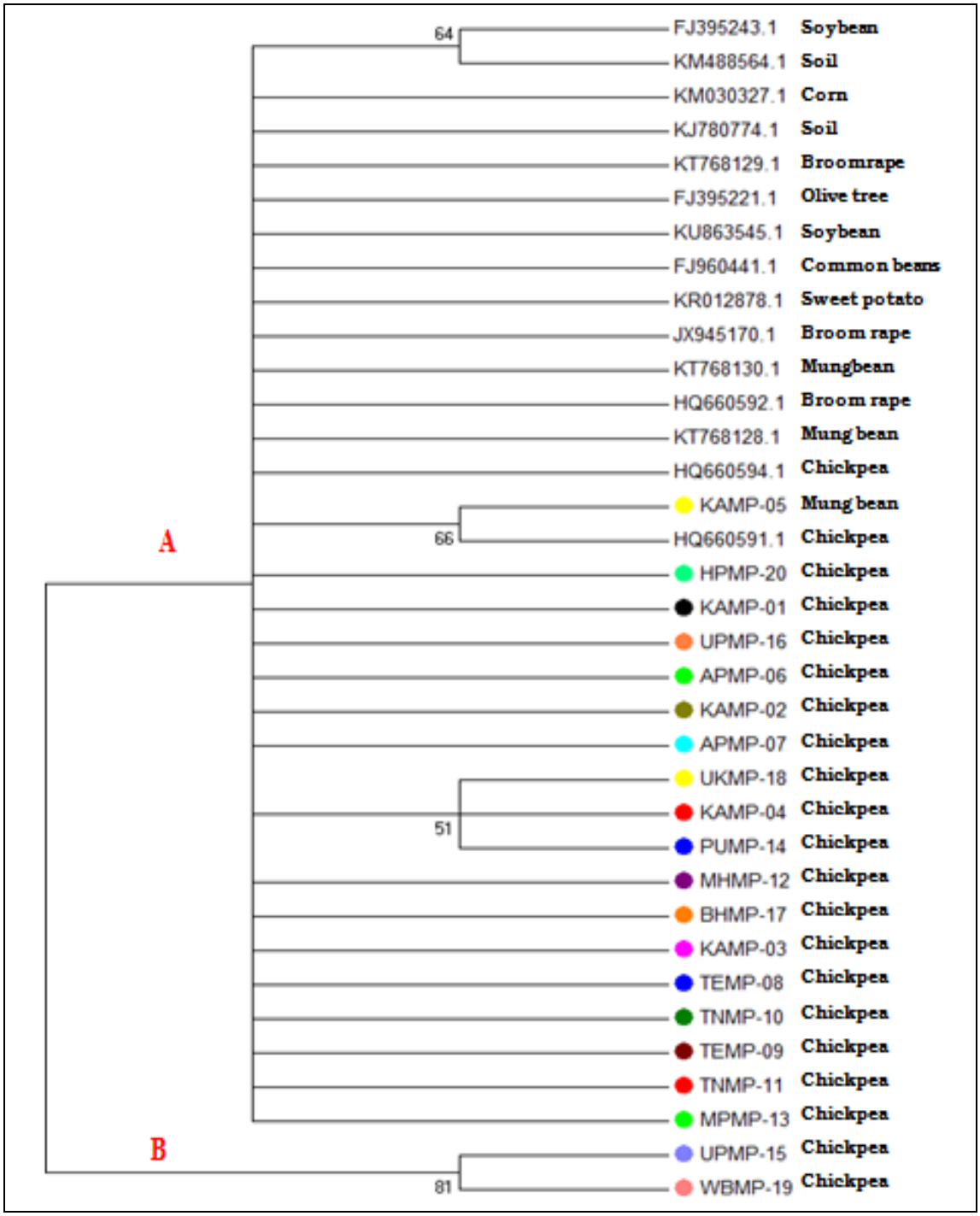


Fig 1: Phylogenetic tree showing relationship among the *M. phaseolina* isolates based on ITS sequences

Table 2: Per cent similarity matrix for ITS region sequences of *M. phaseolina* with other reference sequences from Gene Bank

Sequence	UPMP-15	WBMP-19	KM030327.1	FJ395221.1	FJ395243.1	KAMP-03	HPMP-20	KAMP-01	TNMP-11	JX945170.1	KU863545.1	KT768129.1	KT768128.1	KT768130.1	MPMP-13
UPMP-15	ID														
WBMP-19	91.17	ID													
KM030327.1	91.49	94.50	ID												
FJ395221.1	92.88	94.79	99.35	ID											
FJ395243.1	92.71	94.62	99.25	99.93	ID										
KAMP-03	89.41	90.42	93.92	95.27	95.10	ID									
HPMP-20	90.67	93.33	97.87	97.40	97.22	92.94	ID								
KAMP-01	91.60	93.61	97.50	98.42	98.25	94.00	96.30	ID							
TNMP-11	93.17	94.67	98.60	99.69	99.54	95.13	97.17	98.15	ID						
JX945170.1	93.17	95.00	98.92	100.00	99.85	95.50	97.50	98.50	99.71	ID					
KU863545.1	93.17	95.00	98.91	100.00	99.85	95.46	97.50	98.49	99.71	100.00	ID				
KT768129.1	93.17	95.00	98.91	100.00	99.85	95.46	97.50	98.49	99.71	100.00	100.00	ID			
KT768128.1	93.17	95.00	98.91	100.00	99.85	95.46	97.50	98.49	99.71	100.00	100.00	100.00	ID		

KT768130.1	93.17	95.00	98.91	100.00	99.85	95.46	97.50	98.49	99.71	100.00	100.00	100.00	100.00	ID	
MPMP-13	81.33	82.83	69.63	69.37	69.29	81.80	86.00	84.64	81.89	81.20	81.89	81.89	81.89	81.89	ID
UPMP-16	85.12	88.80	81.71	81.97	81.83	87.46	90.47	91.14	85.99	85.61	86.30	86.30	86.30	86.30	68.66
KAMP-02	86.59	90.07	92.69	91.14	90.97	89.63	94.43	93.32	91.14	91.14	91.14	91.14	91.14	91.14	87.59
BHMP-17	88.95	90.94	97.67	98.48	98.30	91.70	93.66	94.40	95.65	96.03	96.01	96.01	96.01	96.01	69.54
PUMP-14	86.50	88.50	67.26	65.43	65.28	88.74	91.83	91.93	87.12	87.35	87.42	87.42	87.42	87.42	80.43
MHMP-12	88.40	90.92	69.96	67.76	67.69	91.02	93.61	93.90	88.13	88.16	88.13	88.13	88.13	88.13	80.02
APMP-07	85.12	87.46	72.93	72.84	72.64	86.96	90.97	90.30	87.00	86.43	87.31	87.31	87.31	87.31	71.27
UKMP-18	89.11	91.96	70.82	68.21	68.06	91.60	94.64	94.79	90.91	90.98	91.22	91.22	91.22	91.22	81.11
KAMP-04	87.12	89.80	69.45	67.86	67.70	90.07	91.97	93.10	88.28	88.08	88.58	88.58	88.58	88.58	77.55
TNMP-10	90.47	91.30	69.48	69.12	68.96	92.41	94.31	95.28	90.56	90.43	90.56	90.56	90.56	90.56	77.31
TEMP-08	91.18	91.52	97.23	97.83	97.65	92.75	94.46	94.82	96.19	96.37	96.54	96.54	96.54	96.54	86.40
APMP-06	89.63	91.14	70.03	68.71	68.55	92.24	94.15	95.28	90.21	90.14	90.52	90.52	90.52	90.52	78.12
KJ780774.1	89.83	92.24	98.71	98.20	98.02	92.08	95.00	95.18	96.55	96.73	96.90	96.90	96.90	96.90	86.45
TEMP-09	90.30	92.14	73.27	74.16	73.97	93.25	95.15	96.29	90.43	90.06	90.43	90.43	90.43	90.43	77.11
KAMP-05	90.78	92.73	98.48	99.44	99.26	93.10	95.92	96.28	97.70	97.88	98.05	98.05	98.05	98.05	86.44
KM488564.1	90.16	92.06	97.61	98.56	98.74	92.41	95.16	95.52	96.89	97.07	97.24	97.24	97.24	97.24	86.36
KR012878.1	91.35	93.25	98.89	99.82	99.64	93.61	96.37	96.72	98.10	98.27	98.44	98.44	98.44	98.44	86.00
FJ960441.1	91.18	93.08	98.71	99.64	99.46	93.44	96.19	96.55	97.92	98.10	98.27	98.27	98.27	98.27	87.44
HQ660594.1	91.18	92.91	98.71	99.64	99.46	93.26	96.02	96.37	97.75	97.93	98.10	98.10	98.10	98.10	87.61
HQ660592.1	91.00	92.91	98.71	99.64	99.46	93.26	96.02	96.37	97.75	97.93	98.10	98.10	98.10	98.10	87.61
HQ660591.1	91.00	92.91	98.71	99.64	99.46	93.26	96.02	96.37	97.75	97.93	98.10	98.10	98.10	98.10	87.61

Sequences	UPMP-16	KAMP-02	BHMP-17	PUMP-14	MHMP-12	APMP-07	UKMP-18	KAMP-04	TNMP-10	TEMP-08	APMP-06	KJ780774.1	TEMP-09	KAMP-05
UPMP-16	ID													
KAMP-02	87.48	ID												
UPMP-17	70.75	95.82	ID											
PUMP-14	80.70	89.27	92.93	ID										
MHMP-12	79.77	92.49	93.60	77.59	ID									
APMP-07	71.29	91.77	73.28	76.26	77.53	ID								
UKMP-18	81.55	92.21	93.45	79.80	82.52	78.02	ID							
KAMP-04	78.80	91.14	91.88	77.03	80.19	77.42	80.67	ID						
TNMP-10	81.13	90.78	94.91	75.92	82.12	78.72	80.54	83.04	ID					
TEMP-08	90.02	94.95	94.39	92.21	93.74	92.25	93.46	95.88	95.52	ID				
APMP-06	82.83	91.62	95.64	76.07	78.14	80.07	81.16	78.62	82.31	96.03	ID			
KJ780774.1	90.43	95.14	94.41	92.41	93.93	91.97	93.85	96.07	95.17	96.73	95.52	ID		
TEMP-09	80.97	90.64	95.64	79.00	80.71	78.03	84.47	79.68	82.73	96.21	86.83	96.38	ID	
KAMP-05	89.75	95.93	92.08	94.68	94.47	91.76	94.98	96.97	97.17	97.72	97.70	97.55	98.06	ID
KM488564.1	89.27	95.32	92.55	93.44	93.75	90.76	93.76	95.88	96.04	97.08	96.73	96.92	97.07	98.82
KR012878.1	89.59	96.39	92.72	93.77	94.96	91.74	94.09	96.70	97.07	98.11	97.59	98.11	98.10	99.66
FJ960441.1	91.74	96.21	95.48	93.60	94.78	93.63	94.84	97.25	96.90	97.94	97.41	98.11	97.93	99.47
HQ660594.1	91.57	96.21	95.66	93.43	94.61	93.46	94.66	96.91	96.72	97.60	97.24	97.76	97.76	99.12
HQ660592.1	91.57	96.21	95.66	93.43	94.61	93.46	94.66	96.91	96.72	97.60	97.24	97.76	97.76	99.12
HQ660591.1	91.57	96.21	95.66	93.43	94.61	93.46	94.66	97.25	96.72	97.60	97.24	97.76	97.76	99.47

Sequences	KM488564.1	KR012878.1	FJ960441.1	HQ660594.1	HQ660592.1	HQ660591.1
KM488564.1	ID					
KR012878.1	98.35	ID				
FJ960441.1	98.80	99.83	ID			
HQ660594.1	98.46	99.49	99.31	ID		
HQ660592.1	98.46	99.49	99.31	99.66	ID	
HQ660591.1	98.46	99.49	99.31	99.66	99.66	ID

All the isolates showed about 65.28 to 100 per cent nucleotide similarity with already deposited *M. phaseolina* sequences in NCBI data base. Similar results were recorded by Manjunatha (2014) [10] using the primers ITS-1 and ITS-4, a single band of 600bp was generated in the amplified pattern of all the isolates.

The ITS sequence of all the isolates showed about 97 to 100 per cent similarity with already deposited *M. phaseolina* sequences in NCBI data base, the lowest similarity (32 to 37%) was observed between the isolates and other genus plant pathogens.

Monga *et al*. (2007) [11] made studies on 25 isolates of *R. bataticola* and obtained similar results. All the isolates belonging to one geographical location had not come under a single group, reflecting the fact that the variation was independent of geographical nearness of agroclimatic zone. These findings indicated a clear variation among the isolates and strongly support the possibility of existence of pathotypes in *M. phaseolina*. Taxon-selective amplification of ITS regions was a common approach in molecular identification strategies. ITS regions were used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan *et al*., 1995) [4]. These rDNA was highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett, 1992) [7].

5. Conclusion

Molecular characterization of twenty isolates includes

identification of *M. phaseolina* isolates with species specific primers MpKFI (5'- CCGCCAGAGGACTATCAAAC-3') and MpKRI (5' CGTCCGAAGCGAGGTGTATT-3') yielded single amplified product of 350 bp in all the isolates tested and the PCR amplified 18S rDNA region using ITS primers produced a band size of about 600bp. The phylogenetic tree obtained by comparing the nucleotide sequences of ITS region resulted in clusters A and B indicating the variability in *M. phaseolina* and existence of two types of Pathotype. In nature, new strains may arise by mutation, hybridization, differential cytoplasmic inheritance heterokaryosis and by parasexual life cycle. Study of pathogenic variability is essential for breeding disease resistance in a crop improvement programme. A potential pathogen is often blessed with biodiversity within its population. Basically, variation in pathogen is desirable trait for its existence in nature. This variability among the pathogens underlies their diverse nature and ability to withstand the host environment.

6. References

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