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Morphological and molecular characterization of *Macrophomina phaseolina* (Tassi) Goid associated with tuber rot in glory lily

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Abstract
Gloriosa superba L. is an important perennial, medicinal herb widely cultivated in Africa and Southeast Asia. The incidence of tuber rot disease was recorded at up to 25% in major glory lily growing areas of Tamil Nadu. During the survey, symptoms of affected crops along with yellowing, bark shredding and presence of black mustard like sclerotia on rotten tubers were observed. In the present study, the causal agent for tuber rot disease was identified and characterized on the basis of morphological and molecular studies. Morphological examination showed black microsclerotia, round to oblong, irregular in shape with a size of 50 to 90 µm. Furthermore, genomic DNA was extracted from the isolates and PCR amplification was done using universal ITS primers. The sequence analysis confirmed the causal agent associated with tuber rot disease as *Macrophomina phaseolina*.

Keywords: Glory lily, tuber rot, morphological characterization, molecular identification, pathogenicity

Introduction
Gloriosa superba L. is a perennial climber in the Liliaceae family. It's an important medicinal plant, originating from tropical Africa but widely distributed throughout the various parts of South-east Asia, such as India, Burma, Malaysia and Sri Lanka. In India, it grows in areas up to an altitude of 6000 ft. The predominant cultivation areas in the Tamil Nadu regions are Tirupur, Erode, Dindigul, Karur, Ariyalur, Perambalur and Nagapattinam districts (Alice *et al.*, 2012) [1]. It is recognised as Zambia's national flower, as well as the state flower of Tamil Nadu. It is commonly known as climbing-lily, creeping-lily, flame-lily, glory-lily and tiger claw (Pawar *et al*., 2010). It includes alkaloids like colchicines and colchicocides, which are very costly and in high demand by pharma industries. The alkaloids are present in all parts of the plants but are especially rich in seeds. The tubers are used as a tonic, antiperiodic, anti-helminthic, antipyretic, purgative, anti-abortive and also treated against snake bite. Seeds can also be used to relieve rheumatic pain and as muscle relaxants (Pandey *et al.*, 2008) [9].
It is one of the high-value commercial crops and gives better returns than any other cash crop. In India, Tamil Nadu leads in glory lily production. The annual production rates range from 600 to 800 tonnes of dry seeds with a seed productivity of 800 kg/ha (Padmapriya *et al*., 2015). Even though it was high yielding, it was severely infested by tuber rot disease. The tuber was infected by soil-borne pathogens such as *Macrophomina phaseolina* and *Fusarium oxysporum.* During the non-seasonal time, the pathogen spreads from infected tubers of the previous crop to healthy tubers through the soil, which leads to losses in both the field and storage. The yield loss is up to 25 to 100 per cent and has been reported in both the field and storage (Thiribhuvanamala *et al*., 2018) [13]. Tuber rot affects the sprouting or germination of the tuber, including the vigour of the vine and its flowering and fruiting ability. Symptoms of tuber rot were noticed as yellowing, sudden drying of plants, and also rotting of roots after 30 days of planting.
The soil-inhabiting pathogen, *M. phaseolina* (Tassi) Goid, belongs to a basidiomycetous fungus. It is a polyphagous pathogen that causes serious diseases of dry root rot, stalk rot or charcoal rot worldwide, infecting 500 plant species in more than 100 families, including *G. superba* (Gosh *et al.*, 2019). Morphologically, the pathogen has two different stages: the sclerotial stage caused by *M. phaseolina* and the pycnidial stage, which is caused by *Rhizoctonia bataticola*. Morphological characterization of the pathogen was identified by their colony color, mycelium structure, sclerotial size, shape and pycnidial characteristics etc., and this method was used to detect the pathogen at the genus level (Lakhran *et al*., 2018) [6].

Molecular characterization of the pathogen was detected by Polymerase Chain Reaction (PCR), preferably exploiting internal transcribed spacer (ITS) regions, are being used to identify pathogens at the species level (Almomani *et al*., 2013) [2]. They have a diverse host range and a high persistence in soil, making disease control difficult. The present study aimed to investigate the causal agent of tuber rot and its molecular identification. The Koch's postulate of the tuber rot pathogen was also accomplished to confirm the etiology of the pathogen.

Materials and Methods

Sample collection and pathogen isolation

The infected tubers of glory lily were collected from different districts of Tamil Nadu *viz*., Dindigul, Tiruppur, and Coimbatore. The infected rotten tuber pieces were immersed in a 1 percent sodium hypochlorite solution for 1 minute before being rinsed with sterile distilled water. The surface-sterilized tuber pieces were placed in a sterilized Petri dish with Potato Dextrose Agar (PDA) medium and incubated at 28 ± 2ºC for five days (Alice *et al*. 2012) [1]. The pure culture of the pathogen was obtained by the single hyphal tip method and maintained the culture on PDA slants. Four isolates of *M. phaseolina* were obtained by the isolation of infected samples from the different growing regions, named MPSRM, MPMLR, MPKAR, and MPKAL.

Morphological characterization

Four isolates of fungus collected during the survey were grown on a PDA medium to study their growth and variability in colony charactersitics. The colony characteristics of each isolate were different from each other. The cultural characteristics of each isolate, like colony colour, appearance, and radial mycelial growth, were recorded. The shape and size of micro sclerotia and micro conidia (pycnidiospore) were measured using a phase-contrast microscope (LEICA DM 2000) at 40X magnification.

Molecular characterization

DNA Extraction

Total genomic DNA was isolated from a five-day-old culture. The mycelium along with conidia was scrapped with the help of a sterilized scalpel and further used for DNA extraction by using the standard CTAB method as described by Moller *et al*. (1992). To extract the DNA, 1 g of scrapped mycelium was ground using a sterile pestle and mortar containing 2ml of 2X CTAB buffer [10mM tris base (pH 8.0), 20 mM EDTA (pH 8.0), 1.4M Nacl, CTAB (2%)] and 750 µl of the mycelial suspension was transferred to sterile 2 ml Eppendorf tubes. Then the mycelial suspension was placed in the water bath at 65ºC for one hr. The suspension was vortexed with an equivalent amount of Phenol-Chloroform-Isoamyl alcohol (25:24:1) mixture and centrifuged at 13,000 rpm for 10 min. Three layers were separated after centrifuging, the upper layer was transferred to a 2 ml sterilized Eppendorf tube. It was mixed with an equal quantity of ice-cold isopropanol, and incubated overnight for DNA precipitation. After incubation, the extract was centrifuged at 13,000 rpm for 15 min and the pellet was washed with ethanol twice. Again, 15 minutes of incubation was given for evaporating excess ethanol. Then the pellet was re-suspended in 30µl of double-distilled water. The quantity and purity of total DNA was measured using Nanodrop at 260 nm. The quality of extracted total DNA was determined through 0.8% agarose gel electrophoresis. It was amplified with primer pairs ITS 1 & ITS 4 (White *et al.*, 1990) [14]

Polymerase Chain Reaction (PCR)

PCR reaction mixture consisted of 5µl of PCR master mix, 1 µl of forwarding primer (ITS 1) and 1 µl of reverse primer (ITS 4), template DNA 1 µl and 2 µl of sterile water. In a thermo cycler, the reaction mixture was amplified at the following conditions. The PCR reaction started with an initial denaturation at 95ºC for 10 minutes, denaturation at 94 ºC for 1 minute, annealing at 56 ºC for 1 minute, extension at 72 ºC for 2 minutes and final extension at 72 ºC for 10 mins was carried out. PCR products were fractionated on 1.2% agarose gel in 0.5 X Tris EDTA (TAE) buffer. On the gel documentation unit, the gel was stained with ethidium bromide and visualized. The DNA sequencing was employed in both forward and reverse directions. To locate the closest analog, the sequence was annotated and analyzed using BLAST.

Pathogenicity test

An equal size of healthy tubers were selected and sliced lengthwise using a sterilized scalpel. The sliced tubers were surface sterilized with 1% sodium hypochlorite for 1 min and rinsed three times with sterile distilled water. The mycelial disc (8mm) of each isolate of *M. phaseolina* was placed on the centre of sterilized tubers. Three replications were maintained for each isolate. Inoculated tubers were incubated at 28±2º C (Oladoye *et al.*, 2016) [8]. The observations were taken based on the rotting lesion visible to the naked eye. The tubers were sliced through the inoculation point, and the scale was used to estimate the degree of rot given by Theron and Holz (1989): a = no apparent symptom (non-virulent), b = 5 to 10 mm^2 of rotten area (hypo virulent), c = 11 to 20 mm^2 of rotten area (Moderately virulent), d = 21 to 30 mm^2 of rotten area (Virulent), e = more than 31 mm^2 of rotten area (Extremely virulent).

Phylogenetic Investigation

DNA sequences of several isolates from the region of 18S rRNA were retrieved from the NCBI database. Mega 11 software has been used to align by clustalW and cluster the sequences. All sequences were trimmed manually to remove fair comparison. The tree was constructed using the neighbor-joining method in order to derive the evolutionary history. The evolutionary analyses were carried out in MEGA11, which draws the tree to scale, using the branch lengths in the same units as those of the evolutionary distances to construct the phylogenetic tree and calculate the evolutionary distances by using the neighbor-joining method (Tamura *et al*., 2012) [11].

Results and Discussion

The disease severity was assessed by conducting a survey in major glory lily cultivating regions of Tamil Nadu and revealed that maximum disease incidence of tuber rot (25%) was recorded at Kallimandayam followed by a 22% incidence at Kariyampatty of Dindigul district. A minimum tuber rot incidence of 12% was recorded in the Mulanoor village of Dindigul district (Table 1). Alice and Sundaravadhana (2012) [1] reported that the glory lily is susceptible to a number of diseases, including tuber rot caused by *Macrophomina*

phaseolina with disease incidence ranging from 20 to 100% in Tamil Nadu's major producing zone. According Maiti *et al.* (2007) [7] conducted survey on the glory lily in West Bengal's lower Gangetic plains revealed that the leaf blight disease incidence ranged from 65 to 80% and causing yield loss.

The characteristic symptoms that were observed in the field during the survey were yellowing, blackening of the stem, rotting of the tuber with oozing and a foul smell with bark shredding (Figure 1: A). The presence of black dots like sclerotia and also pycnidium was observed on the rotted portion and stem bark. Alice and Sundravadana (2012) [1] observed the symptoms of tuber rot in glory lily caused by *Macrophomina phaseolina*. The infected plants showed sudden yellowing, drooping, wilting of leaves and mycelial growth was seen on rotting tubers. Kamalakannan *et al.* (2006) [4] noticed the symptoms of chlorosis, leaf shredding, blackening of the stem, root rot, basal stem rot, peeling of stem bark and epidermis on the root. The rotting portion of the coleus was found to have minute black sclerotia.

A fungus was isolated under aseptic conditions from infected tuber parts on potato dextrose agar (PDA) medium (Figure 1b). From those, four isolates were isolated and examined for the cultural characteristics of each isolate. The mycelium was fluffy and appressed in nature, and it appeared on the culture plate with abundant tiny black micro sclerotia embedded within the hyphae or immersed in the agar. The hyphae were hyaline, later turning dark brown in colour with septate and they also formed constriction at the point of origin, which is located in branches from the main hyphae. The sclerotia were formed by hyphal aggregation, initially light brown, which became dark black later. They were smooth, round to oblong, reticulate, and irregular in shape. The size of the sclerotia ranged from 50 to 92 µm. Pycnidia were not observed in the culture medium, but an abundant number of minute black pycnidial bodies were seen in the bark shredding region. The pycnidia were flask-shaped and produced a large number of microconidia. They were hyaline, ovoid, and sized at an average of 19.33× 7.96 µm (Table 2). Kamalakannan *et al.* (2006) [4] reported that initially, the mycelium was hyaline, but it eventually turned grey. Sclerotia were small, black, spherical to oblong or irregular in shape, and were attached to hyphae. The fungus was identified as *Macrophomina phaseolina* based on the symptoms and mycelial characteristics. Similar findings were also reported by Lakhran *et al.* (2018) [6] microsclerotia were round, oval, or oblong in shape, initially light brown but darkening as they matured. Pycnidia were rarely observed, dark brown to black, rough, globose, or irregular. Almomani *et al.* (2013) [2] examined the considerable diversity in colour of the mycelium distribution of microsclerotia, and pycnidial formation between isolates on synthetic medium indicated the fungus cultural characteristics.

M. phaseolina was further identified and characterized by molecular means. A five-day old mycelial mat of the pathogen was used for DNA extraction by using the CTAB method. The genomic DNA of the virulent isolate (MPKAL) was isolated, purified, and sequenced at Genei Pvt. Ltd. in Bangalore, India. The sequence was compared using the BLAST algorithm in the NCBI database, which showed 100% similarity with *M. phaseolina* accession number MN340005 from *Parthenium hysterophorus*. The sequence was submitted to Genbank and assigned the accession No. MZ234156 for the virulent isolate of *M. phaseolina* (Figure 3). Similar findings were reported by several workers who carried out the molecular characterization of *M. phaseolina* with the help of ITS primers for PCR amplification. Thiribhuvanamala *et al.*, 2020 [12] isolated *M. phaseolina* from infected roots of *Andrographis paniculata* and confirmed it by morphological and molecular characterization using ITS primers. The sequence of the partial 185 rRNA gene and obtained Acc No. MN235943 were compared using the BLAST algorithm in the NCBI database. Kishore Babu *et al.*, 2007 [3] conclude that the molecular identification was performed by using oligonucleotide-specific primes or probes targeting the ITS region. It has been the best way to retrieve the genetic diversity among the *M. phaseolina* isolates.

The etiological nature of a fungus can be proven by performing a pathogenicity test. The pathogen was inoculated into equal-sized sliced tubers under *in-vitro* conditions and incubated at 28 ± 2 ºC. The artificially infected tuber was compared with a control tuber. After incubation, the artificially inoculated tuber revealed clear evidence of infection in the sliced tubers as well as rotting of the tubers. The tubers were cut at the point of inoculation. The prominent lesion was evident in each isolate. The measurement was carried out based on lesion width and depth, MPKAL showed 49 mm^2 followed by 30 mm^2 of the rotten area in MPKAR. Among the four isolates, MPKAL (Kallimandhayam) was classified as highly virulent, followed by MPKAR, which was classified as virulent and MPSRM, MPMLR were as moderately virulent (Figure 1: D, Table 3). Chehri *et al.*, 2014 considered measurements of the lesion's depth and width in the pathogenicity tests. The size of the lesions varied greatly, ranging from 0.0mm^2 to 45mm^2. The virulence of each isolate was classified into five categories based on the size of the lesions: extremely virulent, virulent, moderately virulent, hypovirulent and non-virulent.

Table 1: Survey for the occurrence of tuber rot in major glory lily growing areas

S. No	Isolate Number	District	Village	GPS co-ordinates	Percent disease incidence
1	MPSRM	Coimbatore	Sirumugai	11.3216°N 77.0089°E	20.00
2	MPMLR	Tiruppur	Mulanoor	10.7947°N 77.7111°E	12.00
3	MPKAR	Dindigul	Kariyampatty	10.1662° N 77.8725° E	22.00
4	MPKAL	Dindigul	Kallimandayam	10.5912° N 77.6864° E	25.00

Table 2: Cultural characters of different isolates of *M. phaseolina* under *in vitro* condition

S.no	Cultural characteristics	*M. phaseolina* isolates			
		MPSRM	MPKAL	MPKAR	MPMLR
1.	Colony colour	Greyish Black	Dark Black	Dark black	Black center with grey margin
2.	Colony appearance	Fluffy	Fluffy	Fluffy	Appressed
3.	Radial Mycelial growth	Moderate	Fast	Fast	Moderate
4.	Mycelial growth (mm)	89.50	90.00	90.00	87.00
5.	Branching pattern	Right	Right	Right	Right
6.	Days to cover 9cm petriplate	4	3	3	4
7.	Sclerotial formation (Days after inoculation)	3.00	2.00	2.00	4.00
8.	Sclerotial intensity	Abundant	Abundant	Abundant	Moderate
9.	Shape	Round to oblong	Round to oblong	Round to oblong	Round to irregular
10.	Size of the sclerotia (µm)	50.61	91.20	64.96	75.00
11.	No of sclerotia / microscopic field (10 X)	14.00	22.00	18.00	16.00

Table 3: Pathogenicity test of tuber rot pathogen under *in-vitro* condition

S.no	Isolate Number	Radial mycelial growth (mm)	Measurement (mm)		Virulence scale
			Width	Depth	
1.	MPSRM	76	2.5	5	c
2.	MPMLR	65	4	4	c
3.	MPKAR	80	5	6	d
4.	MPKAL	90	7	7	e

*Virulence scale: a: healthy, no visible symptoms (non-virulent); b: (hypovirulent); c: (moderately virulent); d: (virulent); and e: (highly virulent).

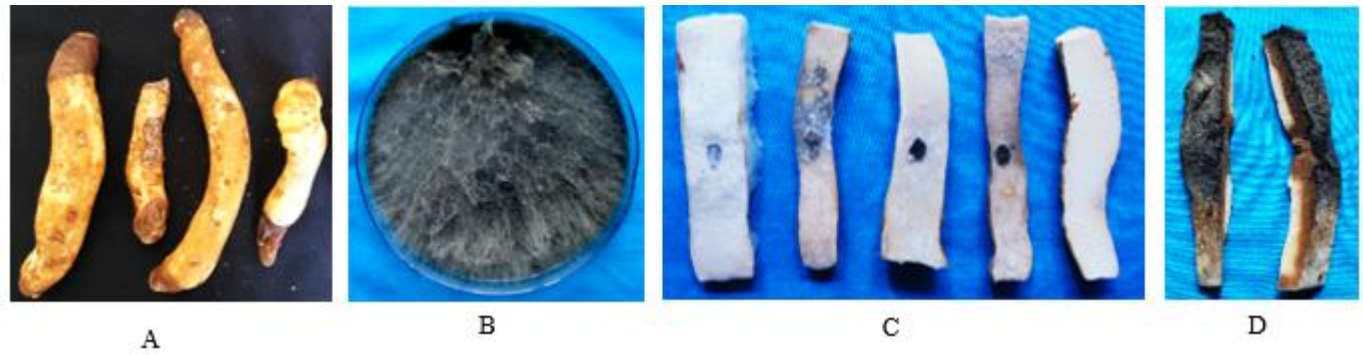

Fig 1: Isolation and pathogenicity A: Tuber rot infected samples; B: Pure culture of *M. phaseolina;* C: *In-vitro* pathogenicity – tuber slice method; D: Cross sectional view of infected tuber

Fig 2: Morphological characterization of *M. phaseolina* E: Brown coloured with septate mycelium; F-G: Formation of micro sclerotia; H: Round to oblong sclerotia formed in hyphal aggregation

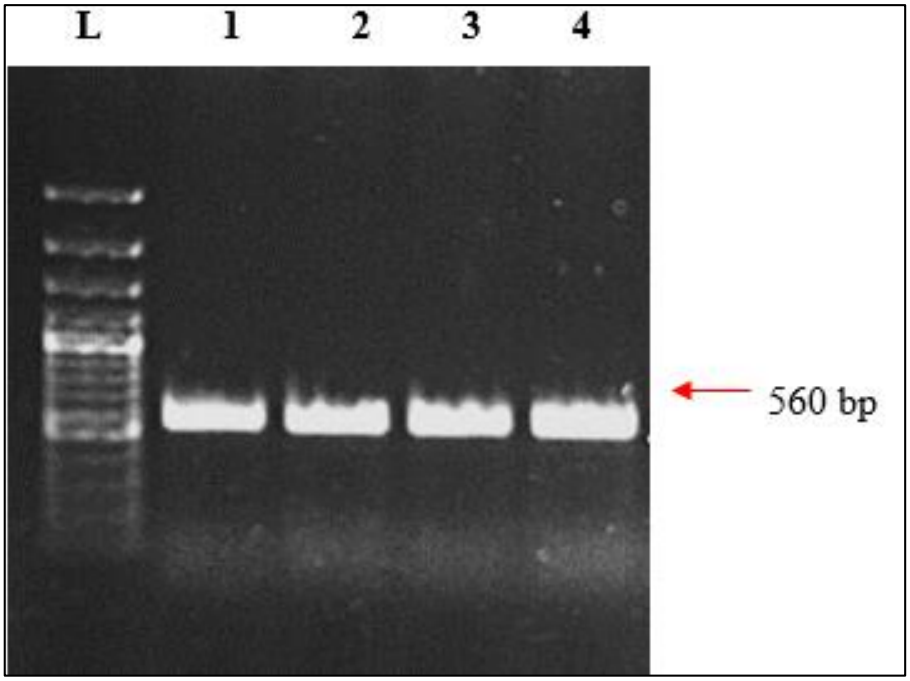

Fig 3: Molecular characterization of *M. phaseolina* using 18S rRNA (L-100 bp ladder, 1-MPSRM, 2- MPMLR, 3- MPKAR, 4- MPKAL)

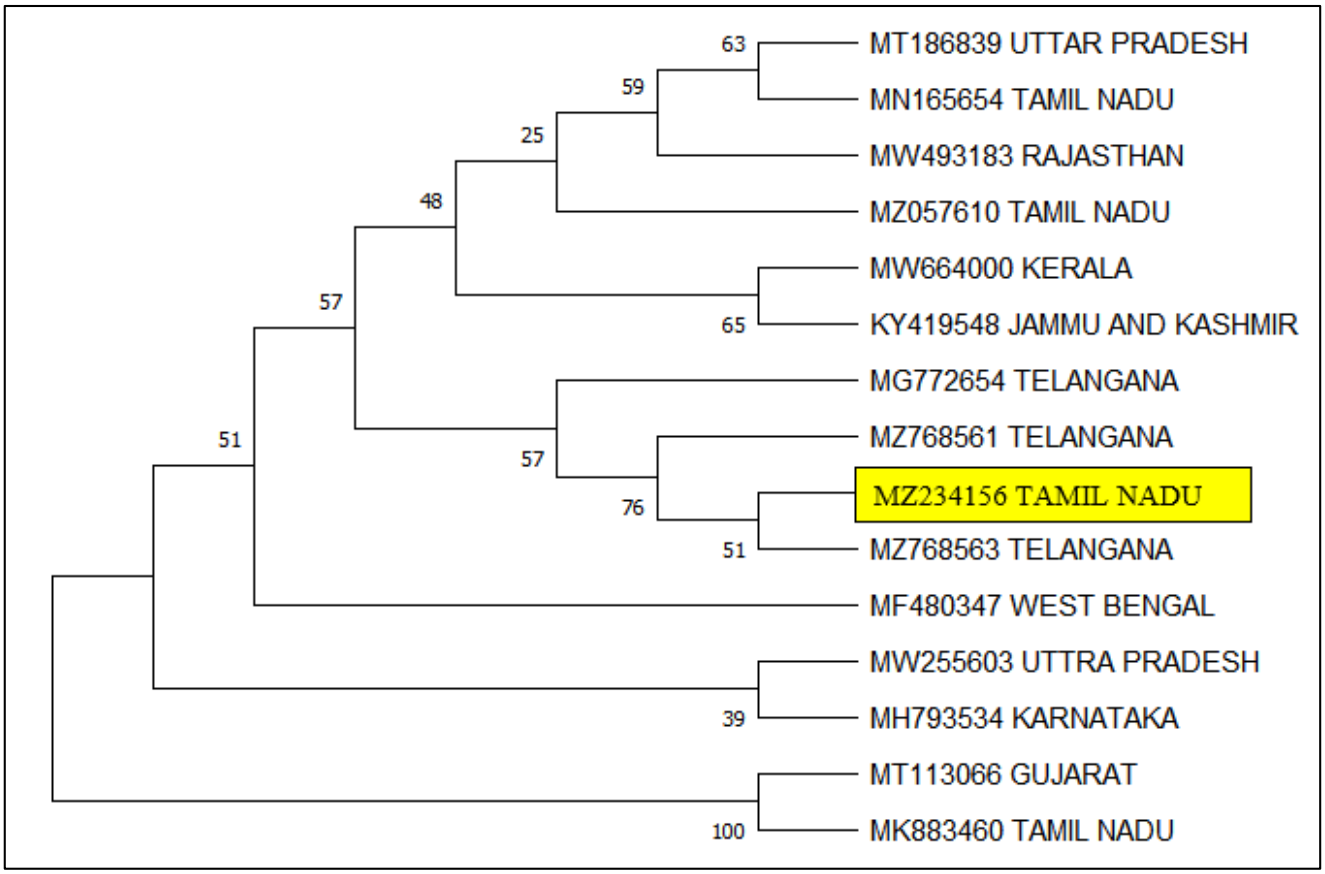

Fig 4: Phylogenetic tree constructed with ITS sequence of 15 isolates of *M. phaseolina* using Neighbour joining tree method. DNA sequence for various isolates were retrived from NCBI database and aligned by using clustalW. The evolutionary distance were computed using the kimura-2-parameter method and the rate of variation among the sites were modeled with a gamma distribution. Numbers above the branches indicate bootstrap values and bar indicates the number of nucleotide substitution per site. Accession number of the *M. phaseolina* isolates followed by origin and the virulent isolate (MPKAL) were highlighted

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