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RESEARCH

MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF GROUNDNUT ROOT ROT DISEASE CAUSED BY *MACROPHOMINA PHASEOLINA* (TASSI.) GOID IN SOUTHERN DISTRICTS OF TAMIL NADU

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Abstract: Out of different solid media tested for the growth of ground root rot pathogen *Macrophomina phaseolina*, Potato Dextrose Agar medium (PDA) supported maximum mycelial growth followed by Peanut leaflet Oatmeal Agar medium (POMA). Among different pH tested, the maximum growth of the pathogen was observed at pH 7. Maximum mycelial growth of the pathogen was recorded at 35°C followed by 30°C. Ground nut root rot pathogen was molecularly characterized and confirmed as *M. phaseolina* by amplifying 530 bp size of ITS region using ITS1 and ITS4 primer pair.

Keywords: Root rot, *Macrophomina*, Groundnut, Pathogen

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important oil seed crop which is cultivated for consumptive use as food and oil. Groundnut production is affected both by biotic and abiotic stresses during different growth stages of crop. Diseases caused by fungi, bacteria, virus and nematode are one of the biotic stresses which hamper the groundnut production (Mayee and Datar, 1986). Soil borne fungi are causing serious yield losses in groundnut (Mathur and Cunfer, 1993). Among the different soil borne fungi, *Macrophomina phaseolina* which causes dry root rot disease is the most devastating pathogen in groundnut.

MATERIALS AND METHODS

Cultural studies of the pathogen

Effect of different culture media on growth of the mycelium and formation of sclerotia

Different solid media *viz*., Oat meal agar, Maize meal agar, Potato Dextrose Agar (PDA), Czapeck's dox medium, Asthana and Howker's medium, Richard's medium, Water agar medium, and Peanut leaflet Oat Meal Agar medium (POMA) were used to test the cultural characteristics of *M. phaseolina*. The above-mentioned solid media were prepared and sterilized by autoclaving at 1.05 Kg cm^{-2} pressure for 15 min. Sterilized PDA media (15ml/plate) were poured into sterilized Petri dishes and allowed to solidify. After solidification of the media, plates poured with different solid media were inoculated with nine mm disc of seven days old culture of *M. phaseolina* and incubated at 27±2°C for one week.

The observations on radial growth of mycelia, color of the colony, colony morphology and spore characters were recorded also production of sclerotia was recorded through the microscopic observation of cultures.

Effect of different pH on the growth of the pathogen

The effect of different pH ranges *viz*., 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 was tested on the growth of *M.phaseolina*. The pH of the medium was adjusted by adding 0.1 N hydrochloric acid or sodium hydroxide and 100 ml of the pH adjusted medium was poured in 250 ml conical flasks and sterilized. Sterilized PDA media with different pH ranges were poured into the sterile Petri dishes and allowed to solidify. The mycelial disc with the size of nine mm from seven days old culture of *M. phaseolina* was cut and transferred aseptically to the middle of the Petri plate and incubated at 28±2°C. Each pH range was replicated thrice. After incubation for seven days, mycelial growth under different pH range was measured.

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Effect of different temperature on the growth of the pathogen

Effect of different temperature on the radial growth of *M. phaseolina* was assessed on PDA medium. A nine mm mycelial disc from seven days old culture of *M. phaseolina* was aseptically transferred to the Petri plates containing sterile PDA media. Inoculated plates were incubated under different temperatures *viz*., 10°C, 15°C, 20°C, 25°C, 30°C and 35°C. Each treatment was replicated thrice and the radial growth of the mycelium was measured.

Molecular identification *M. phaseolina*

Extraction of genomic DNA

Genomic DNA was extracted from *M. phaseolina* isolate GM2 as per the method described by Chakraborty *et al*. (2010). One gram of seven days old fresh mycelia of *Macrophomina phaseolina* was ground using pestle and mortar with CTAB buffer [10mM Tris (pH:8.5)], 250mM EDTA and 0.5% SDS, NaCl 100mM pH 8.0). The mixture was transferred into 2 ml eppendorf tube and incubated at 60°C for one hour. Then the mixture was centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred into a fresh eppendorf tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 15 min. The aqueous phase was then transferred into another fresh eppendorf tube and 50 µl of 100 per cent chilled ethanol was added and centrifuged at 12,000 rpm for 15 min. This process pelletizes the DNA and the pellet was washed with 70% ethanol by centrifugation. The pellet was air dried and suspended with 50 µl of TE buffer (pH 8.0). The quality of isolated DNA was checked by loading 2.5 µl of DNA in 0.8% agarose gel electrophoresis.

Agarose gel electrophoresis

The gel casting trays were sealed with tape to form a mold. 0.8 gram of agarose was added to the 100 ml of 1X TAE buffer in a 250 ml conical flask and melted until turned to a clear, transparent solution in a microwave oven. When the molten agarose was cooled to 55°C, 3µl of 10 mg/ml ethidium bromide stock was added to Agarose and the solution was mixed gently. Warm agarose solution was poured into gel casting tray to a thickness of 5-10 mm and the comb was placed to make wells to load the DNA samples in the gel. The gel was kept at room temperature for 30 – 45 minutes to solidify completely. Gel along with casting tray was placed into electrophoresis tank filled with 1X TAE buffer. The comb was carefully removed from the gel. The genomic DNA samples were mixed with 1 µl of 6X loading dye. Slowly, the samples were loaded into wells of submerged gel using a micropipette. DNA ladder of 1kb (Bangalore Genei Pvt. Ltd., Bangalore, India) was added to the left side of the gel as a marker. The lid of the gel tank was closed and electrical leads were attached to the tank. A voltage of 5-8 V/cm was applied and allowed to run for half an hour. The gel was examined under a UV light and image was documented using Gel Doc™ apparatus.

ITS sequencing of *Macrophomina phaseolina*

PCR reactions were performed in a total volume of 50 µl containing 25 µl of master using Emerald Amp® GT PCR master mix, 19 µl of de-ionized water, 2 µl of forward primer, 2 µl of reverse primer and 2 µl of template DNA. PCR reaction was done using genomic DNA of *M. phaseolina* as a template. The intermediate 5.8S ribosomal gene along with ITS1 and ITS2 regions were amplified using the ITS1 and ITS4 primers with PCR conditions of initial denaturing at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 sec. annealing at 59ºC for 30 sec and extension at 70ºC for 2 min and final extension at 72ºC for 7 min. The reactions were carried out in Eppendorf tube master cycle gradient PCR machine. The PCR products were resolved by electrophoresis in 1% agarose gel and sent for sequencing at Eurofins Genomics India Pvt. Ltd., Bangalore.

The primers used for amplification of ITS region were,

ITS 1- 5’ TCCGTAGGTGAACCTGCGG 3’ (forward primer)

ITS 4 - 5’ TCCTCCGCTTATTGATATGC 3’ (reverse primer)

RESULTS AND DISCUSSION

Cultural characteristics of *M. phaseolina*

Growth and sclerotial production on different solid media

The effect of different solid media on the mycelial growth of the pathogen was assessed and the growth ranged from 3.80 to 9.00 cm. The maximum growth of the pathogen was recorded in PDA medium (9.00 cm) followed by POMA medium (8.66 cm) and water agar medium (8.35 cm). The least mycelial growth of the pathogen (3.80 cm) was found in Oat meal agar (Table 1; Picture 1). These results were in accordance with work done by Ikkar *et al*. (2012). They studied the growth of *M. phaseolina* isolated from mung bean on different solid media and recorded maximum mycelial growth on PDA medium (89.33 mm).

Effect of different pH

The effect of different pH *viz*., 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 on the mycelial growth of the *M. phaseolina* was assessed. Among the pH tested, pH 7.0 of the medium supported the maximum mycelial growth (8.90 cm) of the pathogen followed by pH 6.0 with the mycelial growth of 8.30 cm. Least mycelial growth (3.16 cm) of the pathogen was recorded in pH 2.0 (Table 2; Picture 2).

Effect of different temperature on the growth of the pathogen

The effect of different temperature *viz*., 10°C, 15°C, 20°C, 25°C, 30°C and 35°C on the growth of dry root rot pathogen was tested under *in vitro*. Among

the different temperatures tested, the maximum mycelial growth (8.85 cm) was observed at 35°C followed by 30°C with 7.85 cm mycelial growth. The least mycelial growth (0.90 cm) was recorded at 10°C (Table 3; Picture 3).

Molecular characterisation of *M. phaseolina*

Identification and confirmation of *M. phaseolina* by molecular method

The ground nut root rot pathogen was further confirmed by molecular technique. Genomic DNA was isolated from ground nut root rot pathogen by CTAB method. Single band of intact genome DNA was visualized on agarose gel. ITS region with the size of 530 bp was amplified from the genomic DNA of GM2 isolate (Picture 4). Purkayastha *et al*. (2007) used ITS1 and ITS4 primers for the amplification of *M. phaseolina* isolates from cluster beans and they obtained ITS amplicons with the size of 580 bp.

Sequence analysis of ITS region

Amplified PCR product of *M. phaseolina* ITS region was sequenced. The full length of ITS sequence obtained for *M. phaseolina* was BLAST searched in nucleotide database of National Centre of Biotechnology Information (NCBI). The output data showed matching sequences of *M. phaseolina* already in the database, so the virulent isolate GM2 used in the entire study was confirmed as *Macrophomina phaseolina* and published in the database with the accession number MW487059 (Figure 1).

Table 1. Effect of different culture media on growth of *M. phaseolina* and formation of sclerotia

Treatments	Solid media	*Mycelial growth of the pathogen (cm)	Sclerotial production
T1	Oat Meal Agar	3.80^{h}	++
T2	Maize Meal Agar	4.33^{g}	++
T3	Potato Dextrose Agar	9.00^{a}	++++
T4	Czapeck's dox Agar	6.53^{e}	+
T5	Richard's Agar	7.26^{d}	+++
T6	Water agar	8.35^{c}	+++
T7	Asthana & Howker's medium	6.26^{f}	++
T8	Peanut leaflet Oatmeal Agar	8.66^{b}	++++
SE(d)		**0.12**	**-**
CD(p=0.05)		**0.25**	**-**

***Means of three replications**

Values in the column which are followed by different alphabets are significantly different from each other

Table 2. Effect of different pH on the growth of *M. phaseolina*

Treatment No.	pH	*Mycelial growth of the fungus (cm)
T1	7	8.90^{a}
T2	6	8.30^{b}
T3	5	7.53^{c}
T4	4	7.00^{d}
T5	3	6.40^{e}
T6	2	3.16^{f}
SE(d)		**0.16**
CD(p=0.05)		**0.35**

***Means of three replications**

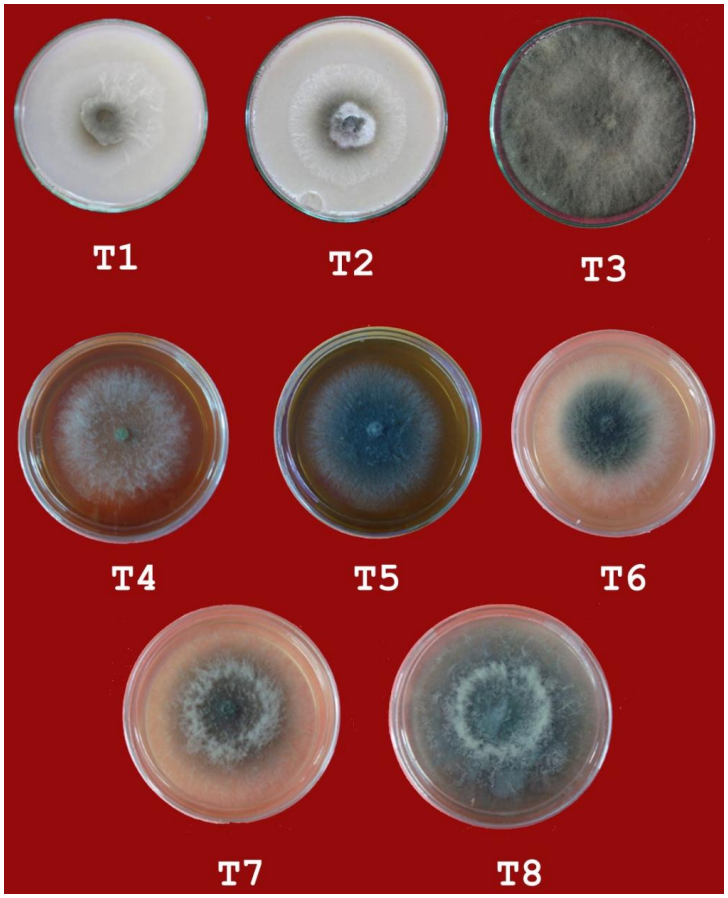
Values in the column which are followed by different alphabets are significantly different from each other

Table 3. Effect of different temperature on the growth of *M. phaseolina*

Treatment No.	Temperature(°C)	*Mycelial growth of the pathogen (cm)
T1	10	0.90[f]
T2	15	2.27[e]
T3	20	3.48[d]
T4	25	6.56[c]
T5	30	7.85[b]
T6	35	8.85[a]
SE(d)		**0.10**
CD(p=0.05)		**0.23**

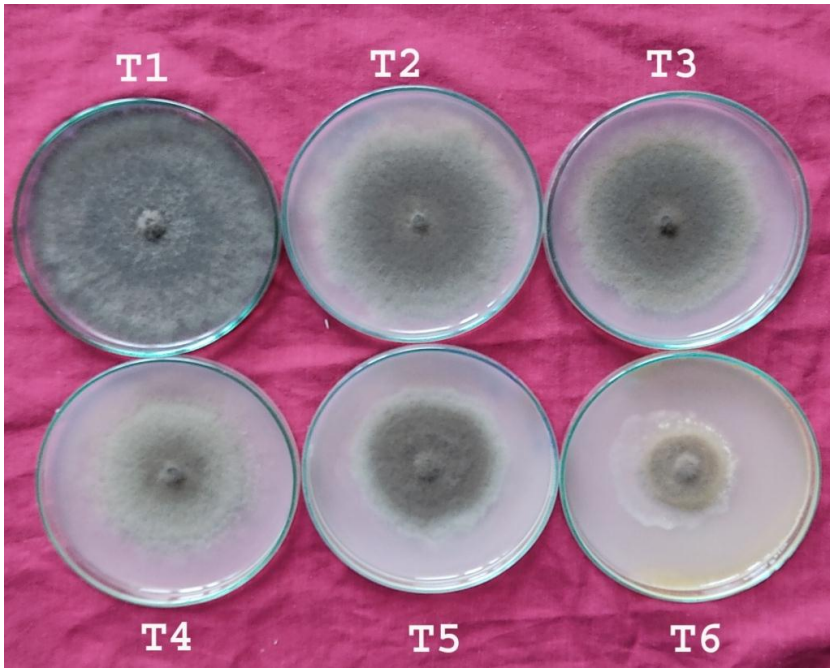
***Means of three replications**

Values in the column which are followed by different alphabets are significantly different from each other



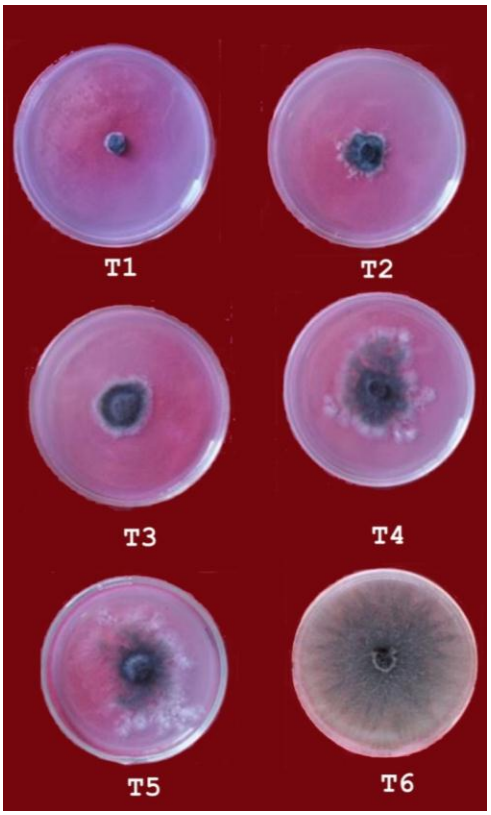
T1 – Oat Meal Agar
T2 – Maize Meal Agar
T3 – Potato Dextrose Agar
T4 – Czapeck's dox Agar
T5 – Richard's Agar
T6 – Water Agar
T7 – Asthana & Howker's medium
T8 – Peanut leaflet Oat Meal Agar

Picture 1. Effect of different solid media on growth of the mycelium and formation of sclerotia



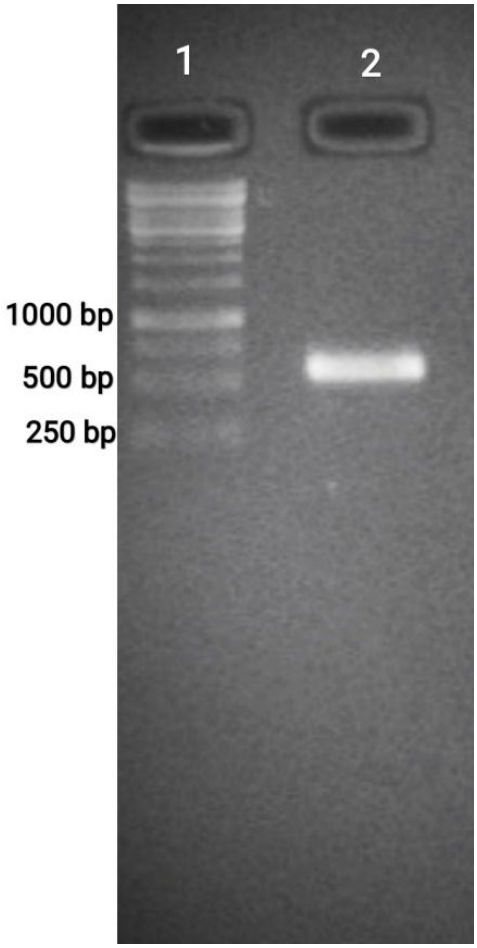
T1 – pH 7
T2 – pH 6
T3 – pH 5
T4 – pH 4
T5 – pH 3
T6 – pH 2

Picture 2. Effect of different pH on the mycelial growth of *M. phaseolina*



T1 – 10°C
T2 – 20°C
T3 – 30°C
T4 – 15°C
T5 – 25°C
T6 – 35°C

Picture 3. Effect of different temperature on the growth of *M. phaseolina*



Lane 1: 1kb ladder Lane 1: Ladder
Lane 2: *M. phaseolina* (GM2 isolate)
Picture 4. PCR amplification of ITS region of *M. phaseolina*

Description	Common Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Macrophomina phaseolina isolate Mp5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Macrophomina ...	1254	1827	77%	0.0	96.48%	763	MT852966.1
Macrophomina phaseolina isolate Mp7 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Macrophomina ...	1247	1813	77%	0.0	96.34%	761	MT852968.1
Macrophomina phaseolina isolate Mp12 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Macrophomina ...	1242	1807	77%	0.0	96.21%	761	MT852941.1
Macrophomina phaseolina isolate Mp9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Macrophomina ...	1240	1803	77%	0.0	96.21%	760	MT852057.1
Macrophomina phaseolina isolate HB28 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Macrophomina ...	1238	1534	73%	0.0	94.90%	898	MT119461.1
Macrophomina phaseolina strain MP1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an...	Macrophomina ...	1232	1651	78%	0.0	92.17%	1331	MG372013.1
Macrophomina phaseolina isolate Mp8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Macrophomina ...	1230	1796	77%	0.0	95.96%	761	MT852965.1
Macrophomina phaseolina isolate Mp1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Macrophomina ...	1227	1779	77%	0.0	96.07%	751	MT852964.1
Macrophomina phaseolina isolate HB51 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Macrophomina ...	1223	1514	76%	0.0	96.04%	752	MT127397.1
Macrophomina phaseolina isolate Mp4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene a...	Macrophomina ...	1221	1774	77%	0.0	95.94%	751	MW145362.1
Macrophomina phaseolina isolate Mp11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Macrophomina ...	1214	1779	77%	0.0	95.57%	761	MT852967.1
Macrophomina phaseolina isolate Mp10 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Macrophomina ...	1214	1779	77%	0.0	95.57%	761	MT852058.1
Macrophomina phaseolina strain MML4004 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge...	Macrophomina ...	1212	1892	77%	0.0	92.92%	841	JX535007.1
Macrophomina phaseolina isolate HB27 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene...	Macrophomina ...	1210	1479	71%	0.0	94.78%	784	MT127381.1

Figure 1. NCBI BLAST analysis for ITS region of *M. phaseolina*

CONCLUSION

Among all the different solid media tested for the growth of *M. phaseolina*, PDA medium supported the maximum mycelial growth (9.00 cm) whereas, Oatmeal agar medium allowed minimum mycelial growth (3.80 cm). Maximum mycelial growth of the pathogen was recorded at temperature 35°C (8.85 cm) followed by 30°C (7.85 cm). Among the pH tested, pH 7.0 of the medium supported the maximum mycelial growth (8.90 cm) of the pathogen. The dry root rot pathogen was conformed as *M. phaseolina* through PCR and nucleotide sequencing.

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AUTHORS CONTRIBUTION

NR, KE carried out the implementation and development of the work. JS corrected the work and analyzed the data. All authors read and approved the final manuscript. All the authors read and approved the final manuscript.

COMPETING INTEREST

The authors declare that they have no competing interests.

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