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Assessing the cultural variability and pathogenicity of *Macrophomina phaseolina* causing post flowering stalk rot of maize

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Abstract
Macrophomina phaseolina is an important destructive pathogen in maize grown areas of Tamil Nadu and it is also a common pathogen in the tropical and sub-tropical region of the world. Studies have been conducted to assess the disease severity and investigate their morphological characteristics such as colony type, spore shape and proved its pathogenicity. Among the various isolates of *M. phaseolina* isolate MMPES exhibited the per cent root rot disease incidence (100%). Among the various culture media tested, PDA medium supported the maximum mycelial growth of *M. phaseolina* and it grows well at 35 °C with slightly acidic to neutral pH.

Keywords: *Macrophomina phaseolina*, pathogenicity, culture medium, pH, temperature

1. Introduction
Maize is one of the important cereal crops grown well in alluvial and red soil from loamy sand to sandy loam, with a rainfall ranging from 500-1000 mm. During the final growth stages of the crop, it is mainly infected by *M. phaseolina* causing charcoal rot disease. This is the most significant disease producing illness up to 50% yield loss (Chattopadhyay and Kalpanasastry, 2002) [2]. *M. phaseolina* is a soil borne pathogen having sclerotia as resting structures that survives in growing crop, crop residues and farm equipments (Thiyagu *et al*., 2007) [5]. High temperature and low soil moisture favor the severity of the disease. For prolonged studies, the experiments were conducted to isolate different strains of *M. phaseolina* from various regions of Tamil Nadu and prove their pathogenicity. The effect of growth on various media (solid and liquid), temperature and pH influencing the growth of *M. phaseolina* under aseptic conditions was assessed.

2. Materials and Methods
2.1 Assessment of disease severity
A roving survey was conducted in a major maize growing areas of Tamil Nadu and recorded the occurrence and establishment of charcoal rot, this survey was conducted in nine major maize growing districts of Tamil Nadu viz., Madurai, Coimbatore, Dindigul, Erode, Thoothukudi, Villupuram, Ariyalur, Pudukottai and Perambalur. In each village five plots were selected and the disease incidence was calculated.

$$\text{Percent disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \times 100$$

2.2 Isolation of the pathogen
The disease infected samples, shredding of stalk and disintegration of stem were collected from the different agricultural fields of Madurai, Coimbatore, Villupuram, Erode, Perambalur, Dindigul, Ariyalur, Thoothukudi and Pudukottai. For the isolation of the pathogen, the disease infected samples was washed thoroughly under running tap water to remove surface dust, soil, and other contaminants and surface sterilized with sodium hypochlorite (1%). The upper layer of the stem was scraped, sliced into small pieces of about 1cm and placed on Potato Dextrose Agar (PDA) plates and incubated at ±35 °C for 4 days. After 4 days of incubation, uniform growth of fungal mycelia around stem pieces was observed, and it was sub cultured for future use. *M. phaseolina* cultures collected from various locations were kept in separate PDA slants and refrigerated at 4 °C.

2.3 Assessing the morphological characteristics of *M. phaseolina* isolates

To evaluate the morphological characteristics of various *M. phaseolina* isolates, petri dishes were filled with sterilized PDA media and inoculated with actively developing cultures. The plates were incubated at 35 °C. Morphological characters such as margin type, growth pattern, colony color were recorded.

2.4 Assessing the pathogenicity test for *M. phaseolina* isolates

The pathogenicity of the pathogen was demonstrated by soil infestation approach. The pathogen was mass multiplied on sand maize medium. The sand maize medium was prepared by mixing sand and maize grains in the ratio of 19:1 with 50% moisture and autoclaved at 20 Psi for 2 hours. *M. phaseolina* isolates were inoculated in sand maize medium under aseptic conditions and incubated at 35 °C for 15 days.

After 15 days of incubation, the multiplied isolates were mixed with soil @ 5% and pots were filled. A susceptible maize variety CO 501 were sown in each pots and inoculated with 9 different isolates, at 3 replications. Uninoculated pots served as control. The plants develop charcoal rot symptoms at 50-60 days after sowing. The pathogen was re-isolated in PDA plates under aseptic conditions and its morphological characters were analysed. The Percent Disease Incidence was estimated as follows.

$$\text{Percent disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \times 100$$

2.5 Assessing the growth of *M. phaseolina* on different solid media

The mycelial growth of the pathogen was assessed on various solid media namely potato dextrose agar (PDA), corn meal agar, wheat meal agar, Czapek-Dox agar and yeast peptone dextrose agar. A fungal disc of *M. phaseolina* was placed in the petri dish and incubated at 35 °C and the mycelial growth were recorded on 3rd, 5th and 7th day of inoculation.

2.6 Assessing the growth of *M. phaseolina* on different liquid media

Mycelial disc of *M. phaseolina* was taken from the 3-4 days old culture and inoculated into each conical flask containing sterilized potato dextrose broth, corn meal broth, wheat meal broth, yeast peptone dextrose broth and Czapek Dox broth and incubated at room temperature (35 °C) for seven days. After seven days, the mycelial mat was filtered through Whatman No. 1 filter paper and oven dried at 60 ºC for 48 h and weighed immediately. The dry weight of mycelium was recorded.

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

PDB with six different pH levels 3, 4, 5, 6, 7 and 8 were taken for the study. A fungal disc of *M. phaseolina* isolates was inoculated into conical flasks containing 100 ml of potato dextrose broth at different pH levels and incubated at 35 °C for 4 days. Mycelial dry weight was recorded.

2.8 Assessing the growth of *M. phaseolina* at different temperatures

The virulent isolate MMPES of *M. phaseolina* were inoculated in the PDA media, and cultures were incubated at four different temperatures *viz*., 25 °C, 30 °C, 35 °C and 40 °C. Three replications were maintained for each temperature. The mycelial growth was measured at 36 h, 48 h, and 72 h of incubation period.

3. Result and Discussion

Nine isolates of *M. phaseolina viz*., MMPMA, MMPCS, MMPDV, MMPES, MMPKM, MMPVP, MMPAS, MMPPK, MMPPA were obtained from diseased plots of the districts Madurai, Coimbatore, Dindigul, Erode, Thoothukudi, Villupuram, Ariyalur, Pudhukottai and Perambalur in Tamil Nadu respectively (Table 1, Fig. 1). During survey, the highest percent disease incidence was observed in the Erode district with 52.33% followed by Dindigul with 50% disease incidence. whereas, least percent of disease incidence was noticed in Pudhukottai district with 30.6%. The nine isolates were distinguished based on their sclerotial and mycelial characters. Sclerotial production was noticed after 6-7 days after inoculation (Table 2, Fig. 2a, 2b) and the same duration was obtained by Lakhran *et al*., (2018) [10] in their study.

Table 1: Assessment of disease survey in different districts of Tamil Nadu

District	Place	Isolate name	Latitude	Longitude	Disease incidence (%)
Madurai	Alanganallur	MMPMA	10.0474◦N	78.0904◦E	43.00
Coimbatore	Singanallur	MMPCS	11.0007◦ N	77.0296 ◦E	32.30
Dindigul	Vagarai	MMPDV	10.5844◦ N	77.5727◦ E	50.00
Erode	Sakthinagar	MMPES	8.7063 ◦N	77.8550◦ E	52.33
Thoothukudi	Murappanadu	MMPKM	11.3186◦N	77.6844 ◦E	37.30
Villupuram	Pappankulam	MMPVP	11.9576◦ N	79.2902 ◦E	34.40
Ariyalur	Salaiyakuruchi	MMPAS	11.1416◦ N	79.0721◦ E	33.00
Pudukottai	Kudumiyanmalai	MMPPK	10.4182 ◦N	78.6583◦ E	30.60
Perambalur	Arumbavur	MMPPA	11.3800◦N	78.7300◦E	44.30

MMP-Maize *Macrophomina phaseolina*.
The fourth letter denotes the district.
The fifth denotes the location where the diseased sample collected.

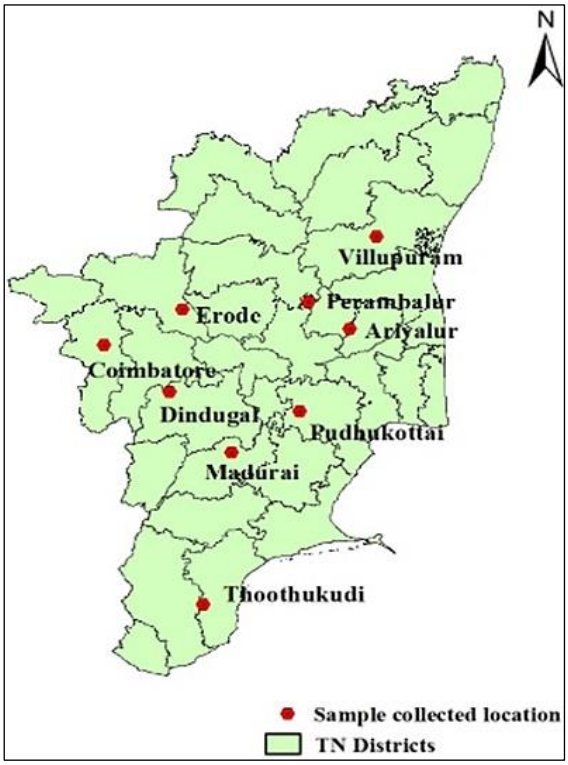


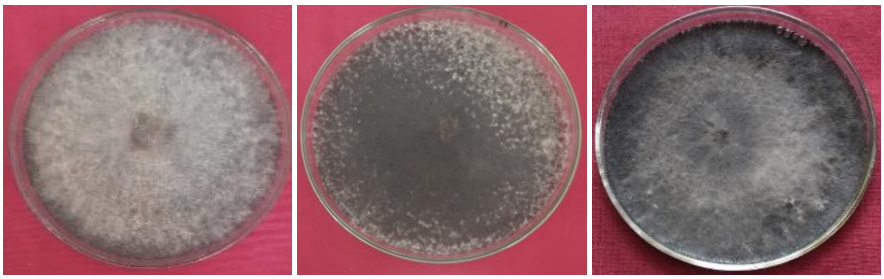
Fig 1: Disease sample collected from different districts of Tamil Nadu and their location

Table 2: Sclerotial formation of *M. phaseolina* isolates

S. No	Isolates	Sclerotial characters
1.	MMPMA	Dark black colour mycelia with dark oblong sclerotia.
2.	MMPCS	Greyish clustered mycelia with scattered sclerotia.
3.	MMPDV	Dark mycelia with large clustered black sclerotia.
4.	MMPES	Clustered mycelia with medium sized sclerotia.
5.	MMPKS	Scattered mycelia with small gathered sclerotia.
6.	MMPVP	Black colour mycelia with pluffy colonies of large circular sclerotia.
7.	MMPAS	Thin hairy mycelia with irregular sized sclerotia.
8.	MMPPK	Clustered mycelia with irregularly shaped huge sclerotia.
9.	MMPPA	Hairy mycelium and black medium sized sclerotia.

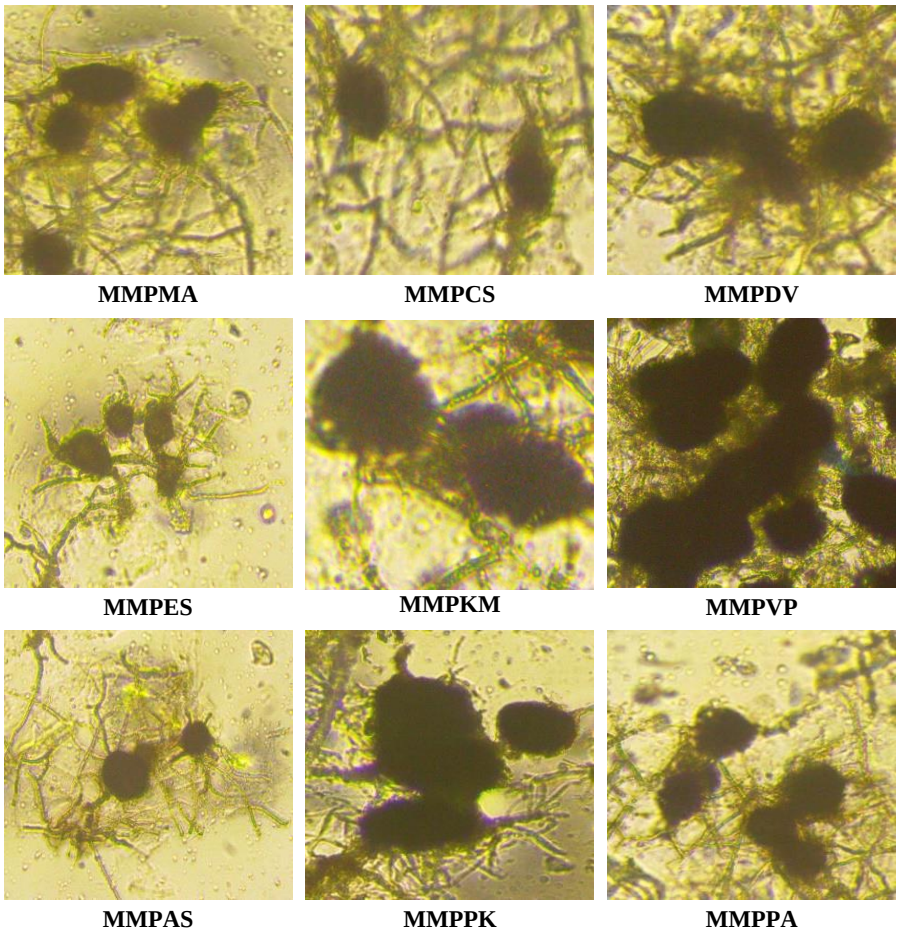
MMPMA **MMPCS** **MMPDV**

MMPES **MMPKM** **MMPPVP**



MMPAS **MMPPK** **MMPPA**

Fig 2a): Cultural variability of *Macrophomina phaseolina*



MMPMA **MMPCS** **MMPDV**

MMPES **MMPKM** **MMPVP**

MMPAS **MMPPK** **MMPPA**

Fig 2b): Sclerotial formation of various isolates of *M. phaseolina*

The pathogenicity test for nine isolates of *M. phaseolina* was carried out using the soil infestation method and results revealed that *M. phaseolina* isolate MMPES has the highest incidence of 100 percent followed by the isolates MMPCS and MMPAS which are on par with each other showed 75% disease incidence whereas, least incidence of 50% was observed in the isolates MMPKM and MMPPK in pot culture. Hence, *M. phaseolina* isolate MMPES was the most virulent of the nine isolates examined and it showed well established symptoms (Table 3, Fig. 3). Akhtar *et al*. (2011) [1] conducted a pathogenicity test on seedlings and recorded the pathogen's virulence and Thirunarayanan *et al*., (2020) [9] obtains the similar results.

Table 3: Pathogenicity test for various *M. phaseolina* isolates

S. No.	Isolates	Disease incidence%
1.	MMPMA	58.33 (50.00)^{cd}
2.	MMPCS	75.00 (60.00)^{b}
3.	MMPDV	66.66 (55.00)^{bc}
4.	MMPES	100 (87.13)^{a}
5.	MMPKM	50

		(45.00)[d]
6.	MMPVP	66.66 (55.55)[bc]
7.	MMPAS	75 (60.00)[b]
8.	MMPPK	50 (45.00)[d]
9.	MMPPA	58.33 (50.00)[cd]
10.	Uninoculated (control)	0 (2.833)[e]
	CD (0.05%)	9.32

Mean of three replications.
The values in the parentheses are arc sine transformed values.
The data´s are computed using DMRT analysis.

Fig 3: Pathogenicity test of *Macrophomina phaseolina*

3.1 Effect of different solid and liquid media on the mycelial growth of *M. phaseolina*

The growth of the pathogen was tested on six different solid media and it was observed that the mycelial growth of the virulent isolate MMPES ranged from 7.5 cm to 8.8 cm on 7th day after inoculation. The results revealed that PDA media supported the highest mycelial growth of 8.8 cm closely followed by corn meal agar which showed mycelial growth of 8.2 cm. whereas, the least mycelial growth was observed in Czapek Dox and oat meal agar which are statistically on par with each other (Table 4, Fig. 4).

Similarly, among the six liquid media examined, PDB yielded the highest biomass of 1.06g of dry weight followed by corn meal broth 0.41g and least in case of Czapek Dox broth with 0.06g of mycelial dry weight (Table 5).

The results of the present study are in agreement with the findings of Mohanapriya *et al*. (2017) [8], Tandel *et al*. (2012) [4] and Sukanya *et al*. (2016) [3] who reported that potato dextrose agar is the best solid medium and potato dextrose broth is the best liquid media for the growth of *M. phaseolina*.

Table 4: Effect of different solid media on the mycelial growth of *M. phaseolina* isolate MMPES

S. No.	Media	Mycelial growth (cm) in 3rd day	Mycelial growth (cm) in 5th day	Mycelial growth (cm) in 7th day
1.	Potato Dextrose Agar	7.6[a]	8.1[a]	8.8[a]
2.	Corn meal agar	7.1[b]	7.6[bc]	8.2[b]
3.	Wheat meal agar	7.0[b]	7.3[bc]	7.9[c]
4.	Oats meal agar	6.8[b]	7.5[b]	7.8[d]
5.	Yeast peptone dextrose agar	6.3[c]	7.4[bc]	8.0[bc]
6.	Czapek-Dox agar	6.1[c]	7.2[c]	7.5[d]
	CD (0.05%)	0.461	0.363	0.278

Mean of three replications
The data´s are computed using DMRT analysis.

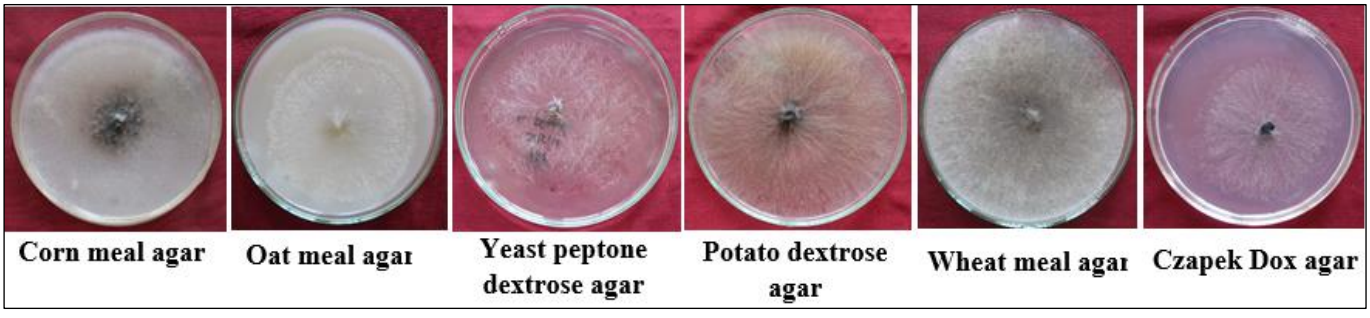


Fig 4: Effect of different solid media on the mycelial growth of *M. phaseolina* isolate MMPES

Table 5: Effect of different liquid media on the mycelial growth of *M. phaseolina* isolate MMPES

S. No.	Media	Mycelial dry weight(g)
1.	Potato Dextrose Broth	1.06[a]
2.	Corn meal broth	0.41[b]
3.	Wheat meal broth	0.13[b]
4.	Oats meal broth	0.13[b]
5.	Yeast peptone dextrose broth	0.27[c]
6.	Czapek Dox broth	0.06[c]
	CD(0.05%)	0.029

Mean of three replications
The data´s are computed using DMRT analysis.

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

M. phaseolina isolate MMPES inoculated in the PDB with different pH levels of 3, 4, 5, 6, 7 and 8 and the results showed that pH 6 and 7 are statistically similar by yielding maximum mycelial dry weight of 0.61g and 0.60g respectively after 7th day of inoculation (Table 6, Fig. 5). Premalatha *et al*. (2020) [7] reported that pH 7 shows better result.

Table 6: Effect of pH on the mycelial growth of *M. phaseolina* on PDB isolate MMPES

S. No.	Different pH	Mycelial dry weight after 7th of inoculation (g)
1.	3	0.46[d]
2.	4	0.51[c]
3.	5	0.54[bc]
4.	6	0.61[a]
5.	7	0.60[a]
6.	8	0.56[d]
	CD (0.05%)	0.040

Mean of three replications
The data´s are computed using DMRT analysis.

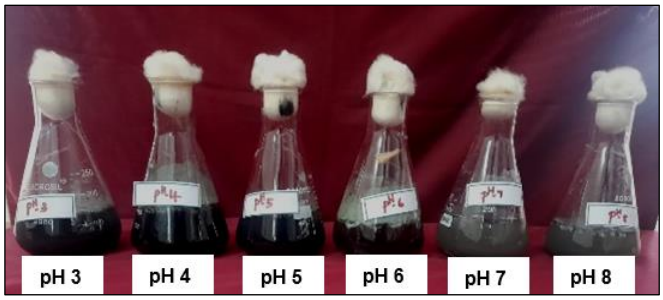


Fig 5: Effect of pH on the mycelial growth of *M. phaseolina* isolate MMPES in PDB

3.3 Effect of temperature on the mycelial growth of *M. phaseolina*

The virulent isolate MMPES of *M. phaseolina* incubated at different temperatures of 25 °C, 30 °C, 35 °C and 40 °C and the results revealed that highest mycelial growth was recorded at 35 °C of incubation closely followed by 30 °C of incubation. However, least mycelial development was observed at 40 °C compared to all other temperatures.

Temperature affects pathogen proliferation as well as illness progression. At high temperatures, *M. phaseolina* flourished successfully. The results of this study showed that a high temperature of 35 °C promotes mycelial growth and disease development whereas, a gradual increase in temperature at 40 °C inhibits mycelial growth. Similar results were obtained by Csöndes *et al*. (2012) [6] and Sukanya *et al*. (2016) [3] who reported that better and quicker development of *M. phaseolina* was observed at 35 °C. whereas, minimum mycelial growth was observed at 40 °C of incubation (Table 7, Fig. 6).

Table 7: Effect of temperature on the mycelial growth of *M. phaseolina* isolate MMPES

S. No	Temperature	Mycelial growth		
		36h after inoculation	48h after inoculation	72h after inoculation
1	25 °C	1.9[c]	4.0[c]	5.9[c]
2	30 °C	2.9[b]	5.2[a]	7.6[b]
3	35 °C	2.6[a]	5.5[a]	7.5[a]
4	40 °C	1.7[d]	4.5[b]	6.1[d]
	CD (0.05%)	0.203	0.388	0.282

Mean of three replications
The data´s are computed using DMRT analysis.

Temperature	36 h after inoculation	48 h after inoculation	72 h after inoculation
25 °C			
30 °C			
35 °C			

Fig 6: Effect of temperature on the mycelial growth of *M. phaseolina* isolate MMPES

3. Conclusion

Among the nine districts of Tamil Nadu surveyed for *M. phaseolina*, Erode district (MMPES) shows maximum disease incidence. The identified virulent isolate MMPES is cultured in different media and they showed the maximum growth in PDA and PDB in 35 °C at pH 7.

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