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Morphological variability in *Macrophomina phaseolina* isolates of sorghum (*Sorghum bicolor* (L.) and their relatedness using principle component analysis

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Abstract
Fifteen isolates of *Macrophomina phaseolina* were characterised on the basis of different colony and sclerotial characters to study the variability in the population. PCA analysis extracted three main components, No. of sclerotia / microscopic field, colony growth, and dry mycelial weight from the population that described the variability in the population most appropriately. The colony growth highly correlated with the No. of sclerotia / microscopic field and dry mycelial weight. The study has brought out variation in *M. phaseolina*, thus which further helps in identification of physiologic races in the pathogen.

Keywords: *Macrophomina phaseolina*, charcoal rot, variability, PCA and sorghum

Introduction
Sorghum bicolor (L.) Moench commonly called "Jowar" is one of the most important millets of India belongs to the family "Poaceae". It produced annually for different purposes like food, Animal fodder, production of alcohol and bio fuels. Due to its versatile uses, it is cultivated in semi-arid, tropical, sub-tropical and even temperate regions of the world as great millet. The world's sorghum production is 57.50 mt produced from an area of 40.28 m ha with the productivity of 1.43 tonnes ha^{-1} (Anon, 2020) [3]. The major sorghum producing countries are the United States of America, Nigeria, Mexico, China and India. India has an 8% share in world sorghum production after the USA, Nigeria. India ranks third in the area after Sudan and Nigeria. In India, sorghum is cultivated on 4.48 m ha with the production of 4.38 million tonnes and productivity of 995 kg ha^{-1} (Anon, 2020) [3]. It is being grown in two seasons, in the *kharif* season as a rainfed crop while in *rabi* season under residual soil moisture conditions. During the last several years the yield levels of *rabi* sorghum vary dynamically and the cultivating area keeps on decreasing due to increase in susceptibility to biotic and abiotic stresses among the biotic stress the charcoal rot disease is a major constrain, In India, it causes major losses in grain yield (up to 64 per cent) and fodder yield by causing 100 per cent lodging (Mughogho and Pande, 1984) [12].
Charcoal rot caused by *Macrophomina phaseolina* (Tassi) Goid. is a major soil-borne disease (Pun *et al.* 1998) [13]. The disease is well characterized by premature leaf senescence, poor grain filling and crop lodging. Internally, the stem pith of infected plants becomes disintegrated and the separated fibro-vascular bundles and are covered with black coloured microsclerotia and sometimes pycnidia and thus sclerotia allows fungus to survive for prolonged periods in the soil (Baird *et al.* 2003) [6].
The fungus exists in two asexual sub-phases one as a saprophytic phase (*Rhizoctonia bataticola*) that forms microsclerotia and mycelia and another pathogenic phase (*M. phaseolina*) present in host tissues that forms microsclerotia, mycelia and pycnidia. *M. phaseolina* exhibits a high degree of morphological (Mahdizadeh *et al.* 2011) [11], pathogenic physiological (Su *et al.* 2001) [16] and genetic (Babu *et al.* 2007) [5] variability. This immense variation can be partly explained by the presence of heterokaryotic mycelium in isolates (Beas-Fernandez *et al.* 2006) [7]. These challenges have made difficulty in developing effective and sustainable disease management strategies. Thus, it suggests the importance of studying variability in pathogen. Further it may help in designing efficient disease management strategies that could be integrated with other practices in order to manage the pathogen. In this context, the study was conducted to: (i) understand the morphological variability in the pathogen population and (ii) establish the dominating character in describing variability in the population.

Materials and Methods

Collection, Isolation, purification and maintenance of the isolates

The Charcoal rot affected sorghum plants were collected from parts of Maharashtra, Hyderabad and Karnataka. Further the charcoal rot affected sorghum tissue was washed in running tap water to remove dust particles and an infected portion of the stalk was cut into small pieces of 5 to 7 mm and the bits were surface sterilized with four per cent sodium hypochlorite for one minute followed by three times wash with sterile water and then transferred to Petri dishes containing sterilized Potato Dextrose Agar (PDA) medium amended with streptomycin (250 mg/L) to slow down the bacterial growth, and incubated dishes at 28 ± 1°C for four days to obtain good growth of fungus. The principle growth characters like cultural, morphological and formation of sclerotia were considered for the identification of pure cultures of *M. phaseolina*. These characters were compared as described by Ashby (1927) [4] and Goidanich (1947) [9] and identified as *M. phaseolina* depending on the observed traits. The isolates of *Macrophomina phaseolina* were sub-cultured on PDA slants and kept at 28 ± 1 °C for 15 days and subsequently renewed once after every 30 days. Such isolates were stored in the refrigerator at 4 °C and were revived at monthly intervals for further studies.

Morphological variability in colony traits and sclerotial traits: To study the morphological variability, a five-millimetre disc from the margins of an actively growing five-day-old colony of each isolate was inoculated in the centre of the Petri dish containing PDA and kept for incubation at 28 ± 1^{0}C. Each isolate was replicated three times. The different morphological parameters like mean colony diameter (Fast growing, medium and slow growing), colony colour (black, grayish black, Blackish gray and grayish white), growing pattern of colony (sparse and dense.), colony shape (regular and irregular), texture of colony (cottony and velvety) and dry mycelial weight (high biomass, medium and low biomass) was recorded at nine days after incubation (DAI), and the sclerotial characters such as days taken for sclerotia initiation, the number of sclerotia per microscopic field, shape, size of sclerotia and pattern of production and was recorded. All the 15 isolates were grouped based on the colony traits and sclerotial traits.

Statistical analysis

Data were statistically analysed for principle component analysis and co relation by using SPSS Version 16.0.

Results

Morphological variability for colony characters and distribution of different isolates in the population

All the fifteen isolates were characterised on the basis of the different colony characters (Plate.1) described above into different groups (Table -1). With respect to rate of mycelial growth, the maximum frequency (53.33%) of isolates were of fast growing type, 26.67 per cent isolates were of medium growth type and the 20.00 per cent of isolates were slow growers. In the population of *M. phaseolina*, the maximum frequency (46.67%) of isolates produced the blackish gray colony colour, followed by 33.33 per cent produced the black colony colour and the lowest frequency (13.33 and 6.67%) of isolates were grayish black and grayish white respectively. With respect to mycelial growth pattern, the maximum frequency of isolates (53.33%) showed the dense mycelial growth pattern while the sparse type of mycelial growth pattern was observed with lowest frequency (46.67%). With respect to colony shape, isolates with regular colony shape had maximum frequency of distribution (80%) compared to the isolates with irregular colony shape (20.00%). With respect to texture of colony, 86.67 per cent of isolates showed the cottony texture, whereas the lowest frequency (13.33) of isolates showed the velvety colony texture. With respect to fungal biomass, the maximum frequency (46.67 per cent) of isolates were of low biomass producing type, 33.33 per cent of isolates were of high biomass producing type and the lowest frequency (20.00 per cent) of isolates were of medium biomass producing type.

Table 1: Frequency distribution in population of *M. phaseolina* isolated from different places

Sl. No.	Morphological Traits	Groups	Frequency of distribution
1	Colony growth	A- Fast growing (80.10 - 90.00)	53.33
		B- Medium Growing (70.10-80.00)	26.67
		C- Slow Growing (61.10 -70.00)	20.00
2	Colony Colour	A- Black	33.33
		B- Blackish gray	46.67
		C- Grayish Black	13.33
		D- Grayish white	6.67
3	Mycelial growth pattern	A- Sparse (Feathery)	46.67
		B- Dense	53.33
4	Colony shape	A- Regular	80.00
		B- Irregular	20.00
5	Texture of colony	A- Cottony	86.67
		B- Velvety	13.33
6	Dry mycelial weight (mg)	A- High (260.10- 330.00)	33.33
		B- Medium (190.10-260.00)	20.00
		C- Low (120.01- 190.00)	46.67

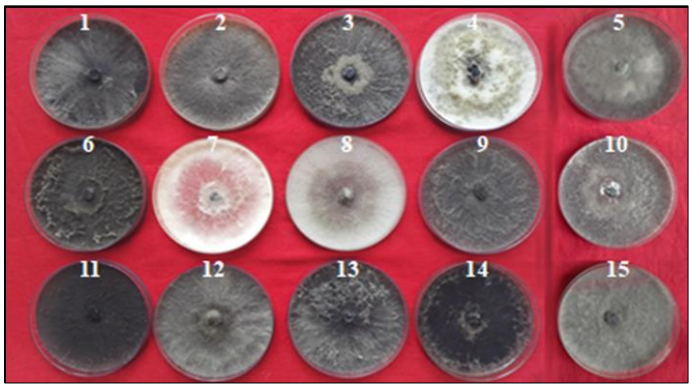

Plate 1: Different phenotypic characters exhibited by the isolates of *Macrophomina phaseolina.* 1-MpSo, 2-MpPa, 3-MpHy, 4-MpGa, 5-MpDh, 6-MpBg, 7-MpGu, 8-MpBv, 9-MpBa, 10-MpVi-1, 11-MpAt, 12-MpHa, 13-MpRa, 14-MpYd and 15-MpVi-2

Morphological variability for sclerotial traits and distribution of different isolates in the population

Morphological variability for different sclerotial traits (Plate 2) and were grouped based on their frequency of distribution (Table 2). The maximum frequency (86.67 per cent) of isolates took 2 days for formation of sclerotia while the least (13.33%) of isolates took 3 days for the formation of sclerotia. With respect to number of sclerotia produced/ microscopic field, the 53.33 per cent of isolates produced highest number (50.10 - 60.00) of sclerotia/ microscopic field, while the 46.67 per cent of isolates produced the lowest number (40.10 - 50.00) of sclerotia. With respect to shape of microsclerotia, the maximum frequency of isolates (53.33%) showed the round shape of sclerotia while the 46.67 of isolates had the oblong shape of sclerotia. With respect to distribution of sclerotia, the maximum frequency of isolates (60.00%) produced the sclerotia in scattered form and the lowest frequency (40.00%) of isolates produced sclerotia in uniform manner.

Table 2: Grouping of *Macrophomina phaseolina i*solates based on morphological traits of sclerotia

Sl. No.	Sclerotial Traits	Groups	Frequency of distribution
1	Number of sclerotia/ microscopic field	A- Highest (50.10 -60.00)	53.33
		B- Lowest (40.10-50.00)	46.67
2	Shape of microsclerotia	A- Round	53.33
		B- Oblong	46.67
3	Distribution of microsclerotia	A- Uniform	40.00
		B- Scattered	60.00
4	Days taken for sclerotial formation	A- 2 days	86.67
		B- 3 DAYS	13.33

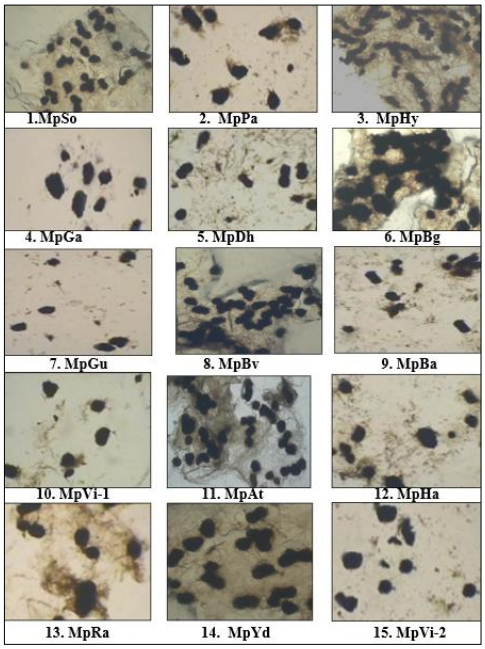

Plate 2: Sclerotial variability exhibited by *Macrophomina phaseolina* isolates. 1-MpSo, 2-MpPa, 3-MpHy, 4-MpGa, 5-MpDh, 6-MpBg, 7-MpGu, 8-MpBv, 9-MpBa, 10-MpVi-1, 11-MpAt, 12-MpHa, 13-MpRa, 14-MpYd and 15-MpVi-2.

Principal component analysis

All the 15 isolates were subjected to Principal Component Analysis (PCA) on the basis of different components (colony growth, colour, shape, growth pattern, texture, days taken for sclerotia production, No. of sclerotia/microscopic field, shape of sclerotia and distribution of microsclerotia) describing the morphological variability in *M. phaseolina* population. No. of Microsclerotia/ microscopic field have the highest proportion (94%) of variable variance followed by colony growth rate have the (91%), while texture of colony had the lowest proportion (49%) of variable variance (Table 3). PCA extracted three main components from the population. Component 1 describes for highest (4.15) Eigen value with variance of 41.53%, followed by component 2 describes 2.21 Eigen value with variance of 22.08 per cent while the lowest (0.12) Eigen value was observed in component 6 with variance of 1.18 per cent. Different components No. of sclerotia / microscopic field, colony growth, and mycelial weight (component 1, 2 and 3, respectively) accounts for Eigen values >1 whereas remaining components accounted for Eigen values<1 (Table 4). Scree plot (Figure 1) graphs the Eigen value against the components described above. Flat line from the third component onwards suggests that each successive component is accounting for smaller and smaller amounts of the total variance.

Table 3: PCA for different morphological components describing variability in *M. phaseolina* population

Variable	Initial	Principle Variable Variance (PVV)
Colony growth	1.00	0.91
Colony colour	1.00	0.63
Growth pattern	1.00	0.82
Colony shape	1.00	0.87
Texture of colony	1.00	0.49
Dry mycelial weight (mg)	1.00	0.90
Days taken for sclerotial formation	1.00	0.50
Number of sclerotia/ microscopic field	1.00	0.94
Shape of microsclerotia	1.00	0.71
Distribution of microsclerotia	1.00	0.71

Table 4: PCA of different morphological variables of *M. phaseolina* population

Component	Eigen values	Variance (%)	Cumulative (%)
1	4.15	41.53	41.53
2	2.21	22.08	63.61
3	1.12	11.22	74.83
4	0.96	9.59	84.42
5	0.75	7.50	91.92
6	0.38	3.77	95.70
7	0.27	2.74	98.44
8	0.12	1.18	99.62
9	0.03	0.33	99.95
10	0.00	0.05	100.00

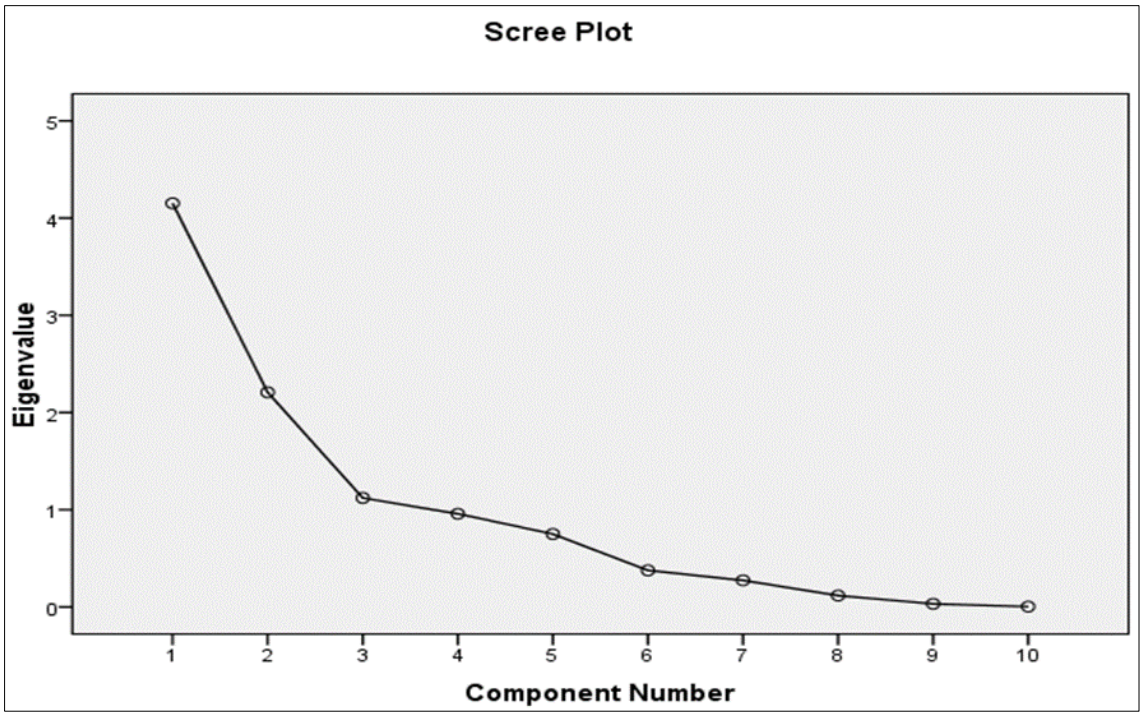

Fig 1: Scree plot showing the Eigen value against the component numbers

Correlation between different morphological variables of *M. phaseolina* population: Growth of colony was significantly ($p \leq 0.05$) and strongly correlated to number of sclerotia/ microscopic field (r=0.98) and dry mycelial weight (r=0.95). A negative correlation was also observed among the variables. Significantly ($p \leq 0.05$) negative (weak) correlation was observed between colony growth pattern and shape of colony with r value of -0.54 (Table 5).

Table 5: Correlation matrix for different morphological variables of *M. phaseolina* population

	Colony growth	Colony colour	Growth pattern	Colony shape	Texture of colony	Dry mycelial weight	Days taken for sclerotia formation	Number of sclerotia/ microscopic field	Shape of sclerotia	Distribution of sclerotia
Colony growth	1.00	-0.39	-0.21	-0.22	0.40	0.95*	-0.23	0.98*	0.07	-0.69*
Colony colour		1.00	-0.07	0.63*	-0.20	-0.31	0.26	-0.37	0.07	0.41
Growth pattern			1.00	-0.54*	-0.42	-0.26	-0.42	-0.20	0.07	0.33
Colony shape				1.00	0.29	-0.18	0.29	-0.22	0.20	0.07
Texture of colony					1.00	0.47	-0.15	0.45	0.03	-0.48
Dry mycelial weight						1.00	-0.13	0.98*	0.11	-0.68*
Days taken for sclerotia formation							1.00	-0.23	0.03	-0.08
Number of sclerotia/ microscopic field								1.00	0.08	-0.70*
Shape of sclerotia									1.00	-0.06
Distribution of sclerotia										1.00

*Significance at 0.05

Discussion

A potential pathogen, such as *M. phaseolina* is blessed with diversity as a desirable trait for its existence in its natural environment. Variability within the pathogen population emphasises their diverse nature and their ability to withstand the host environment. Such broad ranges of variability also guarantee selection among the population and finally the adaptability of the fungus to different environmental conditions (Mc Donald 1997).

Morphological variability and distribution of the isolates in the population

In the present investigation, the isolates of *M. phaseolina* exhibited several morphological variations in the colony and sclerotial traits. The isolates were characterised for both colony and sclerotial traits. *M. phaseolina* isolates with fast growing type, blackish gray colour, dense growth pattern, regular colony shape, cottony texture, medium dry mycelial weight, took 2 days for sclerotia formation with high No. of sclerotia having round shape and were distributed in scattered form were isolated most frequently from the sorghum infected stalks. Similar variation with respect to morphological traits like colony colour, colony shape, growth pattern and texture of colony were made by Aboshosha *et al.* (2007) [1], and Tanjin *et al*. (2017) [18] and reported that the blackish grey colour, dense mycelial growth pattern, regular colony shape and the cottony texture of mycelia being the most frequent traits among the isolates. The similar variations in sclerotial traits were reported by Leyva-Mir *et al*. (2015), the isolates exhibited low to abundant sclerotial population, varied sclerotia size and shape (oblong to round). Almomani *et al*. (2013) [2], reported that there was presence of heterogeneity for sclerotial distribution varied from scattered to regular.

PCA in the present study extracted three main components, No. of sclerotia / microscopic field, colony growth, and mycelial weight of isolates can describe the variability in *M. phaseolina* on morphological basis similarly, Kaur *et al*. (2013) [10] analysed the main components responsible for variability in the population through principle component analysis were microsclerotia colour and texture. Further, quoted that the microsclerotia was the one of the main principle component for variation among the isolates and isolates with the production of microsclerotia were more aggressive as compared to isolates without microsclerotia.

High levels of morphological variability of *M. phaseolina* across geographical regions suggest that this species may be divided into subgroups (Beas Fernandez *et al*. 2006; Aboshosha *et al*. 2007) [7, 1]. However, grouping of isolates by formae specialis, subspecies, or physiological race has never been reported. Exhibition of morphological variability by *M. phaseolina* reflects its heterokaryotic behaviour. Phenomenon of heterokaryotic was observed in *Macrophomina* during hyphal fusion, mitotic crossing-over and recombination (Punithalingam 1983) [14]. Therefore, in the absence of sexual reproduction parasexual mechanism might be one of the causes for the morphological and cultural variability in *M. phaseolina* (Saleh *et al*. 2010) [15].

In the present study the growth rate and the no of sclerotia and dry mycelial weight are highly correlated. Similarly, Dhingra and Sinclair (1973) [8] reported a positive relationship between growth rate and pathogenicity among isolates of *M. phaseolina* from soybean. Subramanian *et al*. (2011) [17] observed that the degree of sclerotia production and growth rate of pathogen was directly correlated to pathogenicity of the isolates. Isolate of *M. phaseolina* producing high microsclerotia was also reported to produce higher amount of different hydrolytic enzymes, such as cellulases, hemicellulose and amylase which further involved in pathogenesis in plant.

Conclusion

The present investigation was undertaken to study the variability in *M. phaseolina.* Fifteen isolates of *Macrophomin*a were characterised on the basis of colony and sclerotial *in vitro*. Isolates with fast growing type, blackish gray colour, dense growth pattern, regular colony shape, cottony texture, medium dry mycelial weight, formation of sclerotia in 2 days with high No. of sclerotia having round shape and were distributed in scattered form were isolated most frequently from the sorghum infected stalks. Principle component analysis extracted three main components *i.e.*, No. of sclerotia / microscopic field, colony growth, and mycelial weight for variability in the pathogen population. The correlation indicates that growth rate of pathogen was strongly co-related with No. of sclerotia / microscopic field, colony growth, and mycelial weight. The study has brought out pathogenic variation in *M. phaseolina.* Thus, for better understanding of host pathogen relationship further studies is required to identify physiologic races for well defined resistance breeding programme. Characterisation of the pathogen on various aspects, such as morphology and genetic makeup will provide ample opportunities to understand the

population genetics and fitness parameters. Variability in the population of pathogen suggests high adaptability which enables the pathogen to withstand adverse environmental conditions. Hence, the information can be utilised for further research.

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