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Morpho-cultural and pathogenic variability in *Macrophomina phaseolina* isolates from soybean

Pankaj Kumar Mishra and Mala Kumari

Abstract
Fifteen isolates of *M. phaseolina* were characterized for cultural and morphological characteristics on PDA medium. Two fast growing isolates, seven medium growing isolates and six very slow growing isolates. The hyphal width of isolates was categorized in three groups. First group comprised of eight isolates with hyphal width ranging between 5-6 µm, second group 6-7 µm and third group more than 7 µm. Mycelial growth pattern observed in isolates were fluffy, partial submerged, submerged, and irregular, whereas the colony colour found to be dark black, greyish and light greyish in appearance. Pathogenic variability revealed that, all the isolates were found to be pathogenic and diverse. Aggressiveness study through *in vitro* blotter paper technique and cut stem inoculation technique exposed that variation exists within the isolates.

Keywords: *Macrophomina phaseolina*, morphological character, cultural character aggressiveness

Introduction
Macrophomina phaseolina is soil borne with broad host range infecting more than 500 plants. It causes charcoal rot of soybean *(Glycine max* (L.) infecting soybean at any crop growth stage, but usually, it infects at post flowering stage. The disease cycle of *M. phaseolinia* begins with germination of microsclerotia when temperatures are between 28 °C and 32 °C. Germinated microsclerotia produce germ tubes that develop hyphopodia which, in turn, either penetrate plant epidermal cell walls or through natural openings (Bressano *et al.*, 2010; Dhingra and Sinclair 1978) [7, 11]. During the early stages of infection, hyphae are restricted to intercellular spaces of the root cortex, but subsequently, the fungus uses both mechanical pressure and chemical softening (Ammon *et al.*, 1974) [3] to facilitate its intracellular colonization of xylem in vascular tissues. This fungus provokes disease development in plants by secreting one or more phytotoxins that facilitate host penetration, invasion, and colonization. Several phytotoxic metabolites produced by *M. phaseolina* have been identified in individual isolates (Bhattacharya *et al.* 1992) [5]. These phytotoxins include asperlin, isoasperlin, phomalactone, phaseolinic acid, phomenon, and phaseolinone (Dhar *et al.*, 1982) [10]. Phaseolinone is a non-host-specific toxin that causes wilting of seedlings and formation of necrotic lesions on leaves, similar to those incited by the pathogen (Bhattacharya *et al.*, 1987) [6]. In adult plants, the pathogen causes red to brown lesions on roots and stems, and produces dark mycelia and black microsclerotia. The stem shows longitudinal dark lesions and the plant becomes defoliated and wilted (Abawi and Pastor-Corrales, 1990) [1].
The asexual structures formed by the fungus are pycnidia and microsclerotia. The black, 0.1–1.0 mm sized microsclerotia are formed in soil, infected seeds or host tissues constitute the primary inoculum source of the pathogen (Dhingra and Sinclair, 1978) [11]. Microsclerotia is the heat tolerant structure could withstand a temperature range of 60–65 ºC and could survive up to 15 years under different weather condition (Bega and Smith, 1962; Mihail and Alcorn, 1984) [4, 19].
M. phaseolina isolates exhibits variation in their morphological and pathogenic characters. *Several* attempts have been made to study the variation of *M. phaseolina. Fungus s*howed significant variations morphologically and physiologically (Mihail and Taylor, 1995) [19], pathogenically and genetically (Reyes-Franco *et al*., 2006; Chase and Mihail, 1994; Jana *et al*., 2005) [9, 15, 25]. These variations aid the pathogen to adapt and survive in diverse environments. However for any successful breeding programme, it is very important to know the existence of variability in the population of the pathogen also information on cultural, morphological and pathogenic variability helps in selection of virulent strains for identification of host resistance.

Therefore, present work was aimed to distinguish cultural, morphological and pathogenic characteristics of *M. phaseolina* isolates from soybean.

Materials and Methods

All the experiments were conducted at the soybean experimental field of Department of Plant Pathology, Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.). Soybean seeds were procured from AICRP (All India Coordinated Research Project) on Soybean. IGKV, Raipur for studying the pathogenic variability. For each set of treatment different replications were used in all *in vitro* studies. Petri dish containing potato dextrose agar medium supplemented with streptomycin. Petri disc inoculated with five mm disc of pure culture of *M. phaseolina* and incubated in the 25°C for three days. Observations for the growth and sporulation were recorded at 10 to 15 days after inoculation. Soybean plants showing typical symptoms on stems bearing microsclerotia of *M. phaseolina* and characteristic symptoms of charcoal rot were collected from the infected plants from farmer's field at different locations of Chhattisgarh (Rajnandgaon, Durg, Mungeli, Bemetra, Kabirdham and Raipur). The diseased samples were first packed in paper bags and then in 15×20-cm polyethylene bags, labeled, brought to the lab and stored at 4 °C until processed for identification.

Isolation and identification of pathogen *M. phaseolina*

For isolation of *M. phaseolina*, the samples were cut into small pieces (5-10 mm long), surface sterilized with 1% sodium hypochlorite. The pieces were placed on Potato Dextrose Agar (PDA) medium containing antibiotics to avoid bacterial contamination. Inoculated petridishes were incubated in dark at 25 ± 1 °C for 2-3 days. A small portion of the fastest growing colony of *M. phaseolina* was taken from the periphery of 90 mm diameter petri dish and spread on petridishes containing potato dextrose agar medium. A small portion of the colony having sclerotia was taken into a drop of sterilized water and agitated with a sterilized needle to separate the sclerotia from the mycelia. Sclerotia were then transferred to 90 mm dia. petridishes containing PDA medium. Colonies appearing from single sclerotium were again transferred to PDA medium containing petriplates, incubated and identified. Colony colour of *M. phaseolina* varies in culture from black to brown or gray and becomes dark in color with age. Abundant aerial mycelium is produced in the culture plate with sclerotia imbedded within the hyphae or engrossed in the agar or on the agar surface with smooth precincts. Hyphae are septate, initially hyaline turning to a honey or black color. Numerous dark brown to black colored sclerotia can be seen on the reverse side of the culture plate. Branching occurs at right angle to parent hyphae but branching at acute angles is also common. Microsclerotia are formed from the aggregation of hyphae with 50 to 200 individual cells coupled by a melanin pigment. The microsclerotia of *M. phaseolina* are black in color and their size varies (50-150µm) with the host and the media used (Short and Wyllie, 1978) [27]. Isolates were given to name on the basis of genus and species name of *M. phaseolina* i.e. MP (Table 1). The purified culture (5 mm disc) from each isolate growing on PDA was transferred to 10 ml culture tubes and incubated in dark at 25±1 °C for 6 days, until the surface of PDA was covered with dense sclerotial layer of the fungal culture. The culture tubes were labeled and stored at 4 °C.

Table 1: Description of the rating scale for scoring (Nene *et al.*, 1981) [23].

Rating	Symptoms of charcoal rot
1	No infection on roots
3	Very few small lesions on roots
5	Lesions on roots clear but small, new roots free from infection
7	Lesions on roots many, new roots generally free from lesions
9	Roots infected and completely discoloured

Table 2: Isolates of *Macrophomina phaseolina* collected from different locations of Chhattisgarh

S. No.	Name of district	Block	Name of the isolates
1	Raipur	Dharsiwa	MP1
2	Bemetara	Ranka	MP2
3	Bemetara	Jaibra	MP3
4	Bemetara	Bemetra	MP4
5	Bemetara	Patharra	MP5
6	Bemetara	Saigona	MP6
7	Kawardha	Kawardha	MP7
8	Kawardha	Kawardha	MP8
9	Kawardha	Lohara	MP9
10	Kawardha	Lohara	MP10
11	Rajnandgaon	Gandai	MP11
12	Rajnandgaon	Khairagarh	MP12
13	Rajnandgaon	Salebharii	MP13
14	Durg	Dhamdha	MP14
15	Durg	Dhamdha	MP15

Radial growth

For studying variability in radial growth, the isolates were grown on Potato Dextrose Agar. Fifteen ml of autoclaved PDA was poured in 90 mm diameter petriplates and allowed to solidify. Five-millimeter plug from the actively growing culture of each isolate of the fungus was placed in the centre of PDA plates separately and incubated at 25±1 °C for five days. Each isolate was replicated five times. After stipulated period, the growth of each isolate was measured. On the basis of radial growth, the isolates were categorized as fast (full growth after 72 hr), medium (full growth after 96 hr) and slow (requires more than 96 hr).

Colony colour

The colour of the colony was observed from the bottom side of the culture with the help of the Munsell' soil colour Chart (Munsell' Colour Company, Inc., 1954). The observations were taken on the mycelia growth after 10 days of incubation. Based on the colony pigmentation the cultures were assigned to different groups.

Colony texture

Observations for the colony texture were made on the 7th day of growth when the colony growth is at its full. The isolates were designated to different groups based on the nature of the texture of their mycelial growth and appearance of the respective isolates.

Sclerotial characteristics

The slides of various isolates were prepared in lactophenol for morphological studies. For morphological characteristics *viz.*, hyphal width, size of sclerotia and shape of sclerotia were recorded after ten days of incubation (Dhingra and Sinclair, 1978) [11]. For measuring sclerotial size, slides from seven days old pure culture of *M. phaseolina* isolates were prepared and examined under microscope. Size of ten randomly

selected sclerotia was measured using ocular micrometer and their means were found.

Pathogenic variability

***In vitro* blotter paper technique (Nene *et al.*, 1981) [23].**

Five different varieties of soybean such as CG Soya-1, JS 97-52, JS 93-05, JS 335 and RSC-10-46 were used during experiment and screened against *M. phaseolina* by blotter paper technique (Nene *et al*., 1981) [23]. Seeds of all the five varieties were surface sterilized (5 min. in 2.5% sodium hypo chlorite) and sown on sterilized sand. Pure culture of all the isolates of *M. phaseolina* were obtained on potato dextrose agar medium from infected soybean plants collected during field survey. Culture of the fungus was multiplied on 100 ml potato dextrose broth in 250 ml flasks and incubated as stationary culture for 10 days at 25 °C (Fig.1 A & B). Mycelial mats of the flasks were added in the sterilized water at the rate of two flacks in 100 ml, macerated for 5 min (Fig.1 D). Fresh inoculum was used after every ten blotters each containing 10 seedlings. Seven days after sowing, the seedlings were uprooted, and roots were carefully washed in running tap water and then rinsed in sterilized water. Roots of ten seedlings of each test accession were dipped in the inoculums (Fig. 1 D) with an up and down movement for about ten seconds and placed side by side on a paper towel. Each paper towel was then folded, covering the roots and leaving the green tops outside (Fig. 1 E). Control plants were dipped in sterile, distilled water. Paper towels were then kept one over the other in heaps of 5 on a tray and placed in the incubator at 30±2 ºC for 7 days with 12 h artificial light per day for better growth of pathogen. Paper towels were kept moist by adding sterile water as needed. Symptoms were observed on ten seedlings of each variety. Seedlings were examined for the extent root damage using a 0-9 rating scale (Nene *et al*., 1981) [23].

Cut stem inoculation technique (Twizeyimana *et al*., 2012) [30].

Artificial inoculation of the fungus was carried out to test the aggressiveness by cut stem inoculation technique (Twizeyimana *et al.*, 2012) [30]. Adopted soybean varieties CG Soya-1, JS 97-52, JS-335 were used. The stem apex of each six week- old V2-stage soybean plant was cut 2.5cm above the unifoliolate node with a sharp sterilized razor blade (Fig. 2C). The open end of a 10- to 200-µl pipette tip (Fisher Scientific) was pushed into the margin of an actively growing *M. phaseolina* culture growing on potato dextrose agar, and a circular disc of fungal mycelium and agar was cut and removed (Fig. 2 B). The pipette tip containing the agar disc with *M. phaseolina* mycelium was immediately placed over the cut stem and pushed down as far as possible in order to embed the stem into the medium and to secure the tip onto the stem (Fig. 2 D). Three days after inoculation, the pipette tips were removed and discarded. Linear stem necrosis (in cm) caused by individual isolates of *M. phaseolina* on different variety was observed during study to test the aggressiveness (Fig. 2 E).

Results and Discussion

Cultural and morphological variability

Fifteen isolates of *M. phaseolina viz.*, MP1 to MP15 collected from different locations of Chhattisgarh were taken for study. Variability studies among these isolates of *Macrophomina phaseolina* were done on the basis of morphology, mycelial growth pattern and other cultural characteristics. The cultural characteristics *viz*, mycelial growth, hyphal width, type of mycelia growth, colony colour, sclerotial size, number and shape of sclerotia. Variations in cultural and morphological characteristics were observed in all the isolates of *M. phaseolina* and result is represented in table 3.

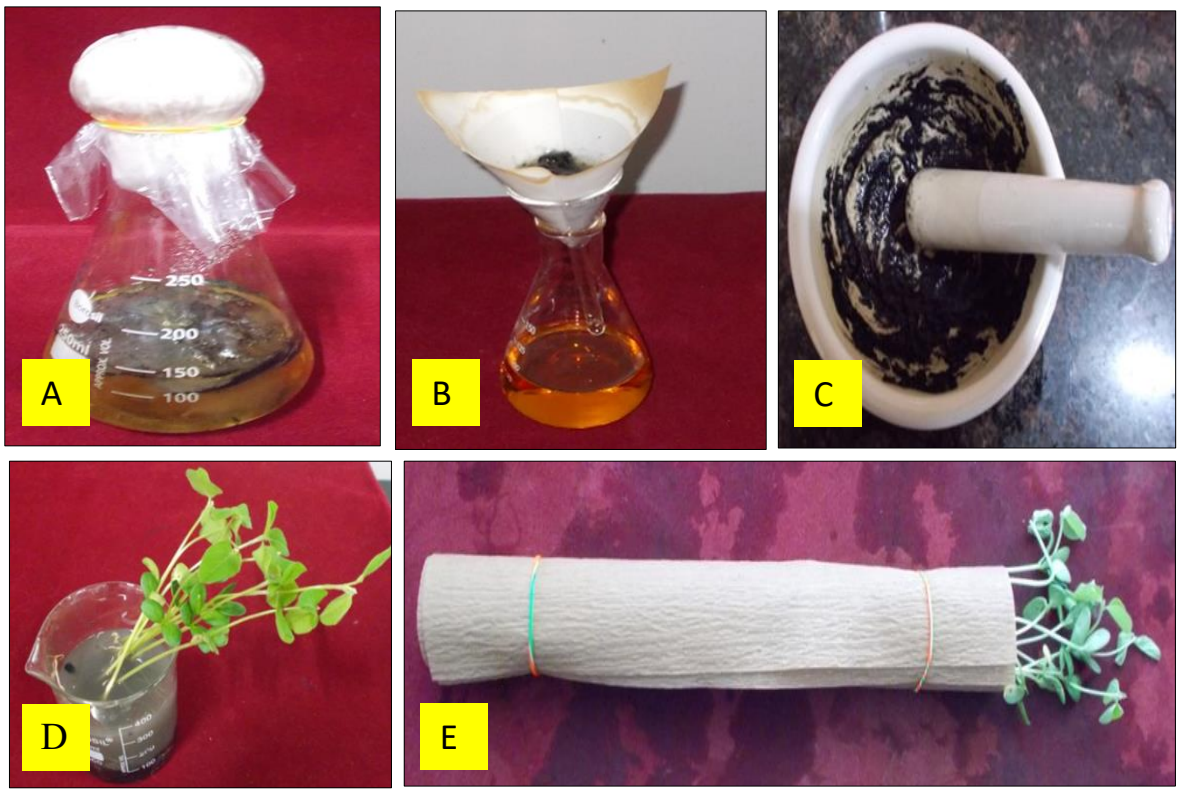

Fig 1: Preparation of *M. phaseolina* inoculum solution for blotter paper technique (A & B) Multiplication of *M. phaseolina* on potato dextrose broth to prepare mycelium mat (C) Filtration of potato dextrose broth (D) Crushing of mycelium mat (E) Dipping of 7 days old soybean seedling in the inoculum solution

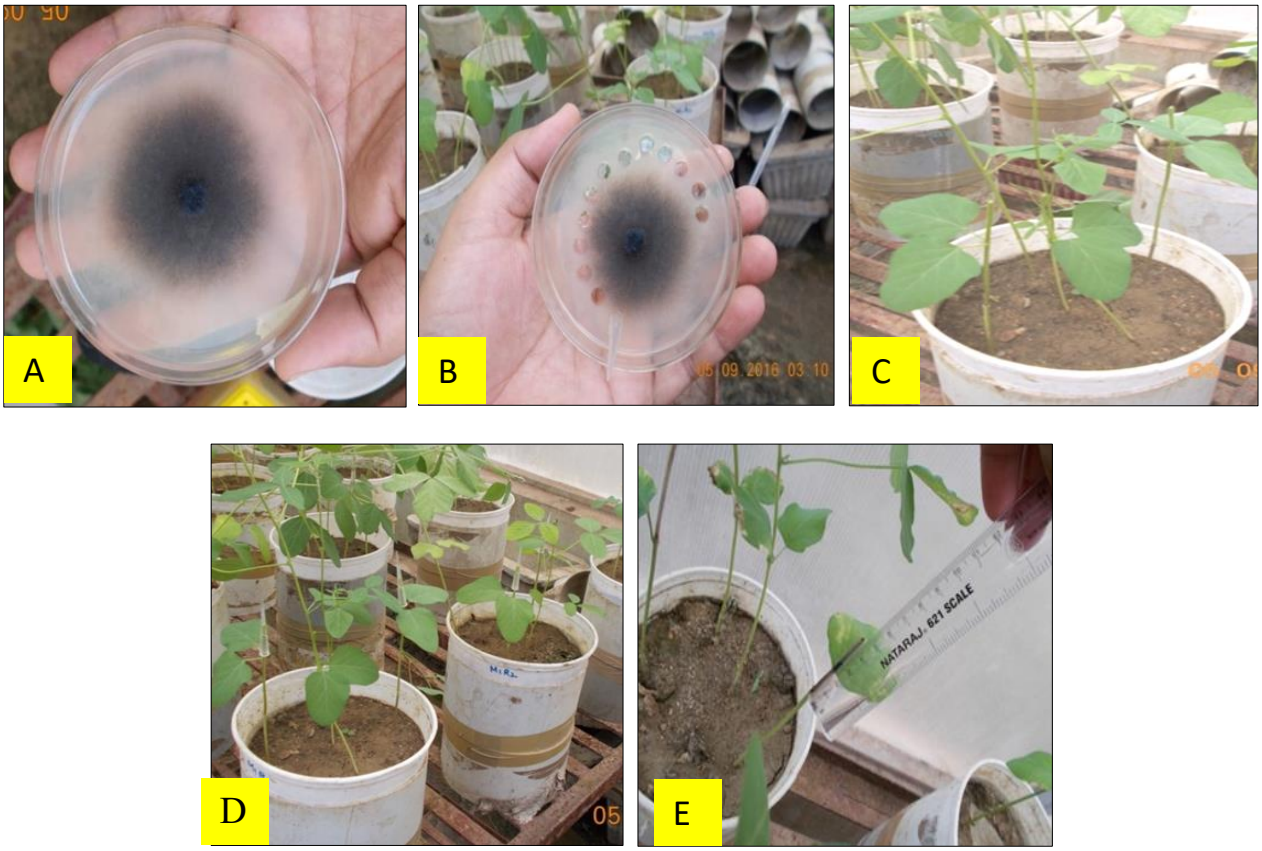

Fig 2: Steps performed during cut stem inoculation technique (A) Pure culture of *M. phaseolinae* (B) Pipette tip loaded with fungal inoculum (C) Cutting of 45 days old healthy soybean plant, 2.5cm above unifoliolate node (D) Transfer of pipette to the cut portion of soybean plant (E) Measurement of necrosis lesion in cm

Table 3: Cultural and morphological characteristics of various isolates of *M. phaseolina*

S. No.	Isolates	Location	Mycelial growth (mm)				Hyphal width (µm)*	Type of mycelial growth	Colony colour
			24 hr	48 hr	72 hr	96 hr			
1	MP1	Dharsiwa	37.33	58.33	90.00	90.00	5.73	Submerged	Greyish
2	MP2	Ranka	12.33	39.00	59.00	75.67	6.20	Partial submerged	Dark Black
3	MP3	Jaibra	18.33	51.67	76.67	90.00	5.70	Submerged	Light greyish
4	MP4	Bemetra	16.67	47.67	69.67	90.00	7.27	Fluffy	Dark Black
5	MP5	Patharra	21.00	52.33	71.67	90.00	7.53	Submerged	Dark Black
6	MP6	Saigona	23.33	43.33	63.33	90.00	5.83	Partial Submerged	Dark Black
7	MP7	Chimagondi	19.67	44.67	61.67	73.33	6.50	Submerged	Greyish
8	MP8	Maharajpur	32.33	54.00	77.67	90.00	7.63	Submerged	Dark Black
9	MP9	Lohara	11.33	29.67	40.67	65.67	5.97	Irregular	Dark Black
10	MP10	Udiya Khurd	18.67	43.00	60.67	74.00	6.50	Fluffy	Greyish
11	MP11	Gandai	19.00	44.67	62.00	72.33	6.67	Fluffy	Greyish
12	MP12	Kanhar	22.00	49.33	72.00	90.00	5.60	Partial submerged	Dark Black
13	MP13	Bhulatola	22.67	49.33	71.00	90.00	5.50	Fluffy	Greyish
14	MP14	Pendri	34.00	62.00	90.00	90.00	5.60	Submerged	Dark Black
15	MP15	Dhamdha	14.33	42.33	61.00	72.33	5.67	Partial Submerged	Light Greyish
	S.Em (±)		2.727	1.288	1.774	0.993	0.162		
	CD (5%)		7.876	3.720	5.125	2.867	0.467	*Average of 10 hyphae	

Mycelial growth: The data in table 3 indicates that significant variations were observed in mycelial growth of *M. phaseolina* isolates collected from different location of Chhattisgarh. Isolates of *M. phaseolina* fungus categorized in to three classes on the basis of complete radial mycelium growth. Isolates wise completion of radial growth was recorded at the interval of 72 h, 96 h and more than 96 h and then classified into 3 groups: fast (72 h), medium (96 h), slow growing (>96 h). Two fast growing isolates MP1 and MP14 were completed their radial growth within (72 h) of inoculation. Seven medium growing isolates *viz.*, MP3, MP4, MP5, MP6, MP8, MP12 and MP13 were completed their radial growth within (96 hr) of inoculation. Six isolates namely MP2, MP7, MP9, MP10, MP11 and MP15 were growing very slow and didn't complete the full radial growth even after (96 hr) of inoculation considered as slow growing. On the basis of growth pattern, Iqbal and Mukhtar (2014) [14] reported the significant variation among the isolates of *Macrophomina phaseolina* on the basis of mycelia growth pattern and categorized the isolates in to three group fast growing, medium growing and slow growing. Gupta *et al.* (2012) [12] also categorized the isolates of *R. bataticola* with respect to colony diameter (growth rate) at 4th day of incubation as, slow growing and fast growing.

Hyphal width

Based on the hyphal width of isolates, the pathogen was categorized in three groups. First group comprised of eight isolates namely MP1, MP3, MP6, MP9, MP12, MP13, MP14 and MP15 with hyphal width ranging between (5-6 µm). Second group isolates having hyphal width ranging (6-7 µm), MP2, MP7, MP10 and MP11. Third group consists of only three isolates *viz.*, MP4, MP5 and MP8 with hyphal width more than 7µm. However, maximum and minimum hyphal width was observed in MP8 (Maharajpur) and MP13 (Bhulatola) respectively. The results were in agreement with the findings of Gupta *et al.* (2012) [12]. They observed a wide range of hyphal diameter and classified in three groups very thin (5.2-6.5 µm), thin (6.6-7.9µm) and thick (8.0-9.3µm). These observations coincided with the observations of Sobti and Sharma (1992) [28] who reported that the width of hyphae varied from (F6-8.48µm). Jharia and Khare (1985) [16] observed the hyphal width and found variation among the isolates of root 4.5 µm, soil (3.5µm) and seed (3.0µm).

Type of mycelial growth

Based on mycelial growth pattern on PDA after seven days of incubation, all the fifteen isolates were placed in four groups *viz.*, fluffy, partial submerged, submerged, and irregular. The first group includes, MP4, MP10, MP11 and MP13 isolates having fluffy mycelial growth. Whereas, MP2, MP6, MP12 and MP15 isolates formed in second group partially submerged. Submerged mycelial growth was identified in six isolates of *M. phaseolina*, *viz.*, MP1, MP3, MP5, MP7, MP8 and MP14. Only single isolate MP9 collected from Lohara fall was kept in the fourth group having irregular mycelium growth. In agreement to present finding Kanchan and Biswas (2009) [17] evaluated morphological variability of *R. bataticola*, revealed that the nature of mycelium and colony varied from fluffy dark brown to partially fluffy mycelium colony with smooth margin. In accordance to present investigation Gupta *et al.* (2012) [12] also reported mycelial growth pattern of fourty *R. bataticola* isolates collected from Chhattisgarh, Madhya Pradesh and Maharastra, showed significant variation in the type of mycelial growth (submerged, partially submerged and fluffy). Isolates collected from Chhattishgarh showed significant variation submerged, partial submerged and fluffy confirmed the existence of diversity within the state. Varma and Pathe (2013) [31] also categorized the *R. bataticola* isolates on the basis of mycelial growth pattern into two broad group fluffy and submerged.

Colony colour

Based on the observation on the the colony colour all the fifteen isolates were classified into three groups *viz.*, dark black, greyish and and light grayish in appearance. Eight isolates *viz.*, MP2, MP4, MP5, MP6, MP8, MP9, MP12 and MP14 produced dark black colony colour. Out of fifteen isolates five isolates produced greyish colony *i.e.* MP1, MP7, MP10, MP11 and MP13. Rest two isolates MP3 and MP15 produced light greyish colony. The results were in agreement with Mandal *et al.* (1998) [18] in which they found that the colony colour of *R. bataticola* varied from light to dark brown appearance. Sulaiman and Patil (1966) [29] also reported the variation in the colony colour of *R. bataticola* isolates *i.e.* dark black, dark grey and partial grey appearance. Similar results also observed by Aghakhani and Dubey (2009) [2] in colony colour of *R. bataticola* isolates, white, dull white, creamy, grey and black. Sharma *et al.* (2012) [26] found variation in colony colour like light black to black and light grey to grey. Present results confirm the observations made by Gupta *et al.* (2012) [12] they reported that colony colour of *R. bataticola* varied from dark black to greyish cottony.

Sclerotial size

On the basis of microscopic observations of the sclerotia size varied from 71.5-102.4 x 62.4-85.7 µm (table 4). Where MP9 found to have largest sclerotial size (102.4 x 73.6 µm) followed by MP12 (95.0 x 80.1 µm), MP8 (90.5 x 83.3 µm), MP3 (90.3 x 83.1µm), MP11 (88.0 x 85.7 µm) MP1 (87.3 x 83.2 µm), MP15 (86.2 x 72.7 µm), MP14 (85.8 x 75.3 µm), MP6 (85.6 x 76.2 µm), MP2 (82.2 x 70.1 µm), MP5 (81.05 x 70.5 µm), MP13 (80.3 x 70.3 µm), MP10 (78.3 x 66.0 µm) and MP7 (77.5 x 66.2 µm). The smallest sclerotial size was observed in MP4 (71.5 x 62.4 µm). Gupta *et al.* (2012) [12] also experienced the variation pattern in the sclerotial size among the *R. bataticola* isolates. Sclerotia size ranged from 103.3 to 117.2 x 90.1- 106.5 µm to 72.7-87.5 x 57.1-73.5 µm. They also reported significant variation of sclerotia size within the isolates. On the basis of microscopic observations Varma and Pathe (2013) [31] found the size of sclerotia varied from 72.2-117.2 x 57.1-106.5 µm. Different workers had given wide range of dimensions of sclerotia size in herbaceous plants (50-150µm), roots of woody plants (80-100 µm) and in culture (50-200µm), 120µm or small, 120-200 µm or medium and 200µm or large, 1930), 60-165 x 57 -114 µm and 36-99 x 36-81 µm; 150-200 µm by Philip *et al.* (1969) [24]; biggest sclerotia 101.51 µm while smallest sclerotia 66.88 µm reported by Byadgi and Hegde (1985) [8]. Jharia and Khare (1985) [16] found sclerotia in the root isolate (84x93 µm), soil isolate (90 x75 µm) and seed isolate (85x70 µm). Monga and Sheo (1995) [20] and Mandal *et al.* (1998) [18] recorded the sclerotia size 58.83 to 126.63 µm and 66.14 to 128.25 µm respectively.

Table 4: Variability in morphology of sclerotia produced by different isolates of *M. phaseolina*

S. No.	Isolate	Size of sclerotia (µm)**	Shape	No. of sclerotia per microscopic field
1	MP1	87.3x83.2	Oval	141.95
2	MP2	82.2x70.1	Round	140.09
3	MP3	90.3x83.1	Oval	128.14
4	MP4	71.5x62.4	Round	171.94
5	MP5	81.5x70.5	Round	75.33
6	MP6	85.6x76.2	Oval	100.15
7	MP7	77.5x66.2	Oval	117.36
8	MP8	90.5x83.3	Round	87.32
9	MP9	102.4x73.6	Round	112.73
10	MP10	78.3x66.0	Round	119.70
11	MP11	88.0x85.7	Oval	155.23
12	MP12	95.0x80.1	Round	144.96

13	MP13	80.3x70.3	Oval	134.85
14	MP14	85.8x75.3	Round	150.49
15	MP15	86.2x72.7	Oval	169.64
S.Em ±				16.350
CD (5%)				47.223

** Average of 10 sclerotia

Sclerotial shape

On the basis of microscopic observations of sclerotial shape the isolates were classified in two groups that is round and oval. Round shape sclerotia recorded in isolates MP2, MP4, MP5, MP8, MP9, MP10, MP12 and MP14 whereas, oval shape sclerotia were found in MP1, MP3, MP6, MP7, MP11, MP13 and MP15 isolates (table 4). Present results are in agreement with the findings of Gupta *et al.* (2012) [12] who found that shape of sclerotia among the *R. bataticola* varied from round to oval among the isolates. Isolates collected from Chhattisgarh showed round and oval sclerotia. Sharma *et al.* (2012) [26] also observed the difference in *R. bataticola* sclerotial shape as oblong, ellipsoid, irregular and round.

Number of sclerotia per microscopic field

Significant variation in number of sclerotia per microscopic field of *M. phaseolina* was also observed in all the fifteen isolates. Highest number of sclerotia per microscopic field was noted in MP4 (171.94) followed by MP15 (169.64), MP11 (155.23), MP14 (150.49), MP12 (144.96), MP1 (141.95), MP2 (140.09), MP13 (134.85), MP3 (128.14), MP10 (119.70), MP7 (117.36), MP9 (112.73), MP6 (100.15) and MP8 (87.32). Least sclerotia per microscopic field were formed in MP5 (75.33). Varma and Pathe (2013) [31] grouped the *R.bataticola* isolates according to number of sclerotia per microscopic field into three categories i.e. sparse, medium and abundant. Gupta *et al*., 2012 [12] reported the variation in number of sclerotia from 9.7 to 22.2.

Pathogenic variability

***In vitro* blotter paper technique**

The data presented in (table 5 and Fig. 4) revealed that the isolates were variable in their aggressiveness. The disease reaction of all the fifteen isolates on cultivar CG Soya-1, JS 97-52, JS 93-05, JS 335 and RSC 10-46 varied between 5-9 grades. Isolates showed varying degree of disease rating after inoculation on the particular variety. All the isolates of *M. phaseolina* categorized into aggressive and highly aggressive on the basis of reaction shown on varieties. It is evident from the table 5 that individual disease response of all the *M. phaseolina* isolates on soybean variety CG Soya-1. Aggressiveness pattern of different *M. phaseolina* isolates behaved differently. Based on their aggressiveness the isolates were classified in two group MP1, MP2, MP5, MP7, MP8, MP9, MP10, MP12 and MP13 produced clear but small lesion on roots with new roots free from infection. Second group comprised of MP3, MP4, MP6, MP11, MP14 and MP15 with many lesions on roots, completely discoloured and damaged. While, control plants without inoculation scored grade 1 with no infection on roots. Individual disease response of all the *M. phaseolina* isolates was observed on soybean variety JS 97-52. Aggressiveness pattern of different *M. phaseolina* isolates behaved differently. Based on their aggressiveness the isolates were classified in two groups MP2, MP3, MP5, MP7, MP8, MP9, MP11 and MP12 and MP13 produced clear but small lesion on roots with new roots free from infection. Second group comprised of MP1, MP4, MP6, MP10, MP14 and MP15 with many lesions on roots, completely discoloured and destroyed. While, control plants without inoculation scored grade 1 with no infection on roots. Individual disease response of all the *M. phaseolina* isolates was observed on soybean variety JS 93-05. Aggressiveness pattern of different *M. phaseolina* isolates behaved differently. Based on their aggressiveness the isolates were classified in two group MP1, MP2, MP3, MP4, MP5, MP6, MP7, MP8, MP9, MP10, MP11, MP12 and MP13 produced clear but small lesion on roots with new roots free from infection. Second group comprised of MP14 and MP15 with many lesions on roots, completely discoloured and destroyed. While, control plants without inoculation scored grade 1 with no infection on roots. Individual disease response of all the *M. phaseolina* isolates was observed on soybean variety JS 335. Aggressiveness pattern of different *M. phaseolina* isolates behaved differently. Based on their aggressiveness the isolates were classified in two groups MP3, MP5, MP6, MP8, MP9, MP11 and MP13 produced clear but small lesion on roots with new roots free from infection. Second group comprised of MP1, MP2, MP4, MP7, MP10, MP12, MP14 and MP15 with many lesion on roots, completely discoloured and destroyed. While, control plants without inoculation scored grade 1 with no infection on roots. Individual disease response of all the *M. phaseolina* isolates was observed on soybean variety RSC 10-46. Aggressiveness pattern of different *M. phaseolina* isolates behaved differently. Based on their aggressiveness the isolates were classified in two groups MP1, MP3, MP4, MP5, MP7, MP8, MP9, MP10, MP11, MP13 and MP15 produced clear but small lesion on roots with new roots free from infection. Second group comprised of MP2, MP6, MP12 and MP14 with many lesions on roots, completely discoloured and destroyed.

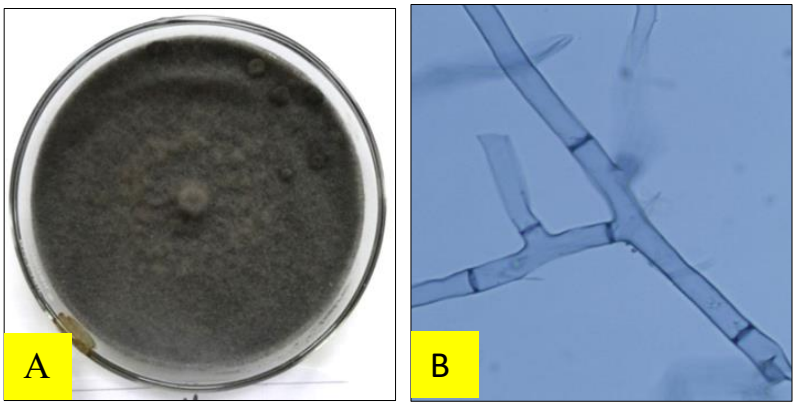

Fig 3: Identification of *M. phaseolina (*A) Black colony colour of *M. phaseolina* (B) Branching pattern and septation

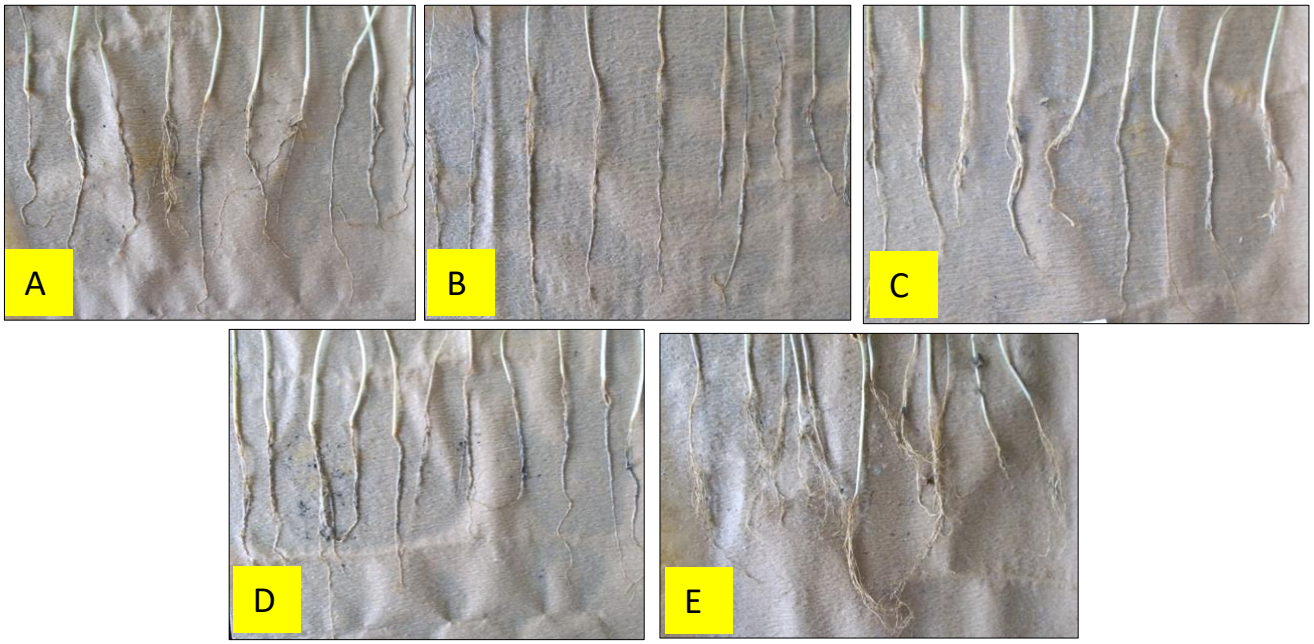

Fig 4: Extent of root damage in *in vitro* aggressiveness test of *M. phaseolina* isolate MP14 through blotter paper on soybean varities, (A.) CG Soya-1, (B) JS 97-52, (C) JS 93-05, (D) JS 335 and (E) RSC-10-46

Table 5: Aggressiveness of different isolates of *M. phaseolina* against different varieties of soybean under *in vitro* condition by blotter paper method

S. No.	Isolate / Variety	MP1	MP2	MP3	MP4	MP5	MP6	MP7	MP8	MP9	MP10	MP11	MP12	MP13	MP14	MP15	Control	Mean
1	CG Soya-1	7.0	6.3	7.6	7.6	5.0	7.6	5.6	6.3	5.6	5.6	7.6	7.0	7.0	8.3	7.6	1.0	6.82
2	JS 97-52	7.6	7.0	5.6	8.3	5.6	8.3	6.3	7.0	6.3	7.6	5.6	5.6	5.6	8.3	7.6	1.0	6.87
3	JS 93-05	5.6	7.0	7.0	7.0	6.3	5.6	5.0	5.6	5.0	5.6	6.3	7.0	7.0	9.0	8.3	1.0	6.51
4	JS 335	8.3	7.6	7.0	7.6	5.6	5.0	7.6	7.0	7.0	7.6	6.3	8.3	6.3	9.0	8.3	1.0	7.27
5	RSC 10-46	7.0	7.6	5.6	7.0	6.3	8.3	6.3	6.3	5.6	7.0	5.6	7.6	5.0	9.0	7.0	1.0	6.78
	Mean	7.13	7.13	6.60	7.53	5.80	7.00	6.20	6.47	5.93	6.73	6.33	7.13	6.20	8.73	7.80	1.0	7.13

Average performance of all the isolates on soybean variety revealed that MP14 found to be highly aggressive showing disease reaction (8.73) and less aggressive, isolate MP5 disease grade (5.80), table 5. A positive correlation between the mycelium growth and aggressiveness of MP14 and MP5 is reported during present investigation. Gupta *et al.* (2012) [12] also used blotter paper to screen chick pea against pathogen *R. bataticola* on the basis of disease reaction shown on plant. Further they also reported that fast growing isolates are highly virulent compare to slow growing isolates. According to Gupta *et al.* (2006) [13] the varying degree of virulence among forty chickpea isolates were observed, through blotter paper technique. The disease reaction of the isolates varied from 6.6 – 9.0 on a scale of 1-9. Pawar *et al.* (2014) [23] reported the diversity among different isolates of *Rhizoctonia bataticola* collected from various locations of Maharashtra while studying the pathogenicity. Nagma *et al.* (2015) [21] also follow blotter paper technique for screening of chickpea germplasm against dry root rot.

Cut stem inoculation technique

The data presented in table 6 and Fig.5 revealed that the isolates were variable in their aggressiveness. The disease reaction of all the fifteen isolates on cultivar CG Soya-1, JS 335 and JS 97-52 in the form of linear stem necrosis (4cm to 15 cm) from the cut stem portion of soybean plant (Fig. 5). All the isolates of *M. phaseolina* categorized into less aggressive (4.0-6.9 cm), aggressive (7.0-9.9 cm) and highly aggressive (10.0- 14.0cm) on the basis of reaction shown on varieties.

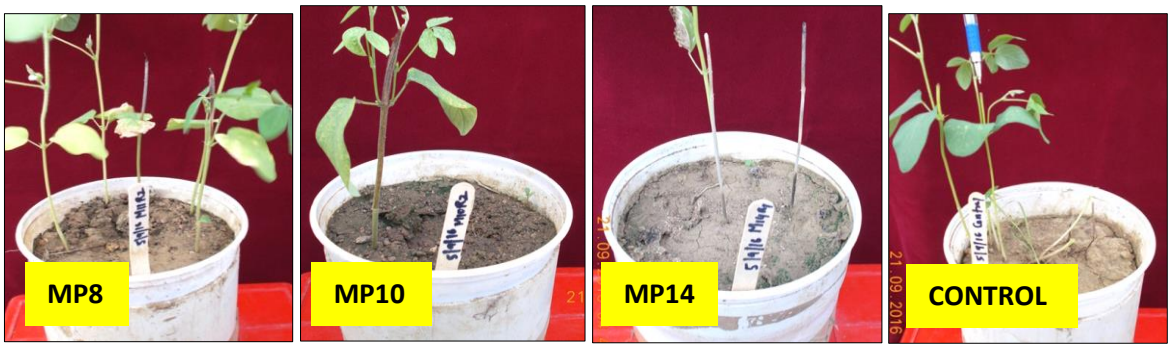

Fig 5: Aaggressiveness of *M. phaseolina* isolates causing linear stem necrosis through cut stem inoculation technique on soybean variety JS 97 - 52

Table 6: Aggressiveness of different isolates of *M. phaseolina* against different varieties of soybean under *in vitro* condition by cut stem inoculation method

Variety	CG Soya-1 Disease progress in (cm)			JS 335 Disease progress in (cm)			JS 97-52 Disease progress in (cm)		
Isolates	**7th day**	**9th day**	**14th day**	**7th day**	**9th day**	**14th day**	**7th day**	**9th day**	**14th day**
MP1	4.10	4.5	4.9	4.8	5.5	6.1	5.5	5.5	6.4
MP2	4.6	4.9	5.2	4.9	4.9	5.0	5.0	5.2	5.2
MP3	4.3	5.0	5.4	3.7	4.7	8.1	4.5	5.0	5.7
MP4	4.7	5.2	6.7	4.9	5.6	5.6	4.8	5.3	5.3
MP5	4.4	5.2	5.2	4.9	5.4	5.4	4.9	5.4	5.4
MP6	6.3	7.3	11.0	6.6	7.6	11.3	6.3	7.8	10.0
MP7	4.7	5.2	5.8	4.8	5.6	5.6	4.9	5.4	6.0
MP8	4.2	5.2	5.9	4.8	5.4	6.6	4.9	5.3	7.2
MP9	4.9	7.0	9.0	5.0	6.4	8.5	4.9	6.8	8.8
MP10	5.4	6.1	6.3	4.9	5.8	6.7	5.0	5.6	6.3
MP11	4.6	5.1	6.9	4.6	6.0	7.7	4.9	6.5	7.6
MP12	5.7	6.7	7.8	5.6	7.0	8.1	5.0	6.1	7.3
MP13	6.7	7.9	8.5	6.3	7.5	7.8	5.4	6.5	7.3
MP14	8.3	12.8	15	7.3	12.2	14.5	7.1	9.8	14.3
MP15	3.8	4.7	5.5	4.0	4.7	5.9	4.2	5.0	5.9

The information found in the (table 6) shows that disease response of all the *M. phaseolina* isolates on soybean variety CG Soya-1 revealed that isolates MP1, MP2, MP3, MP4, MP5, MP7, MP8, MP10, MP11 and MP15 had shown lesser disease incidence and kept in the category of less aggressive. Isolates MP9, MP12 and MP13 categorized into group two i.e. aggressive. Remaining two isolates MP6 and MP15 were placed in the third group highly aggressive.

Soybean variety JS 335 showed variable level of disease reaction after inoculation with all the fifteen isolates. Nine isolates MP1, MP2, MP3, MP4, MP5, MP7, MP8, MP10 and MP15 were categorized as less aggressive. Isolate MP3, MP9, MP11, MP12 and MP13 behaved aggressive. MP6 and MP14 found to be highly aggressive and caused higher disease incidence.

Disease reaction of all the isolates on the variety JS 97-52 classified into three groups. Disease reaction revealed that isolate MP1, MP2, MP3, MP4, MP5, MP7, MP10 and MP15 were categorized as less aggressive. Isolate MP8, MP9, MP11, MP12 and MP13 fall in the category of aggressive. Similar to earlier isolate MP6 and MP14 behaved highly aggressive.

Comparative study of all the isolates on three different variety revealed that isolate MP1, MP2, MP3, MP4, MP5, MP7, MP10 and MP15 showed less aggressive reaction on all the soybean varieties. Isolate MP9, MP12 and MP13 showed aggressive reaction and MP6 and MP14 showed highly aggressive reaction. The entire fact is in agreement with the findings of Twizeyimana *et al.* (2012) [30] who reported that the soybean genotypes had different levels of resistance to *M. phaseolina* infection and isolates had different level of aggressiveness on the basis of relative amount of necrosis caused by each isolates.

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