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MORPHOLOGICAL AND MOLECULAR VARIABILITY OF *COLLETOTRICHUM TRUNCATUM* (SCHW.) ANDRUS AND MOORE ISOLATES PATHOGENIC TO SOYBEAN IN INDIA

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ABSTRACT
Among the eleven isolates collected from six different states exhibited morphological variability with respect to colony colour varied from cottony white to dark gray, spore ranging between 20.10-23.00 x 3.2-4.50 to 21.10-24.20 x 3.8-4.10 μm. The colony colour was dark gray in Bidar isolate of Karnataka, Ujjain isolate of Madhya Pradesh where as Bagalkot isolate of Karnataka and Kota isolate of Rajastan produced white colony colour. The rest of the other isolate produced distinct colony colour. There was no much difference between spore sizes of the different isolates collected. The molecular variability by using RAPD technique revealed genetic similarity coefficient of five isolates ranged from 37 to 66 per cent. The Maximum similarity of 66 per cent was observed between isolates Bagalkot (Bg) and Latur (La) followed by 58% between isolates Bagalkot (Bg), Ujjain (Uj). The similarity coefficient of 56 per cent was observed between isolates of Dharwad (Dh) and Ujjain (Uj) also in Umiam (Um) and Ujjain (Uj). Whereas, the least similarity of 37 per cent was observed between Dharwad (Dh) and Latur (La) isolates. The cluster analysis revealed sub cluster groups depending on same geographical locations with distinct variability.

INTRODUCTION

Soybean anthracnose has become one of the major threat in all soybean growing areas in recent years. In Karnataka, the disease has been noticed in severe form for the last two years owing to favourable weather conditions for growth and development of the pathogen at pod formation and pod development stage. The yield loss of 30 per cent has been recorded (Mahmood and Sinclair, 1992) due to *Colletotrichum truncatum*. The severity of disease and loss caused varies from location to location within a geographical area. In this context, Roy (1996) reported that the falcate spore isolates of *Colletotrichum* isolated from different parts of soybean resulted in two colony types *C. dematium f sp. truncatum* and *C. capsici*. *Colletotrichum capsici* was weakly pathogenic and *C. truncatum* was extremely virulent on soybean seedlings which caused considerable pre and post emergence seedling death. The morphology of both conidia and setae of *Colletotrichum truncatum* (Schw.) Andrus and Moore isolates from soybean were compared and found to be distinct. The Conidial shape was more useful than size of isolate to determine variability (Jagtap and Sontakke, 2009). The molecular variability in five isolates of *C. truncatum* collected from distinct areas in Brazil, isolated from soybean stems (4 isolates) and crotalaria (1 isolate). RAPD amplification, with five different primers, also showed polymorphic profiles among Brazilian *C. truncatum* isolates. Dendrogram analysis resulted in a similarity degree ranging between 0.331 and 0.882 among isolates and identified three RAPD groups (Juliane *et al.,* 2010). Sajjesh *et al.* (2014) reported use of ITS 1 and ITS 4 fungal primers for detection of seedborne infections of soybean in northern Karnataka. Though the available relevant information on taxonomy, biology, ecology, epidemiology, life cycle, host pathogen interaction, resistant sources and eco-friendly management aspects, has been reviewed on *Colletotrichum truncatum* Schw. Andrus and W D Moore by Sharma *et al.*, 2011. There was no limited information on variability of pathogenic isolates infecting soybean in major soybean growing areas. Hence, the present study aimed in identification of variability among different isolates of *Colletotrichum truncatum* based on morphological and molecular traits in the subcontinent.

MATERIALS AND METHODS

Morphological studies of *Colletotrichum truncatum*

The morphology of *Colletotrichum truncatum* on the infected seeds was studied by using infected seeds of the soybean cultivar JS 335. The seeds were incubated on wet blotters and the seeds which showed *C. truncatum* with acervuli and mycelium were selected for the study. The sections of seeds were taken and were mounted in lactophenol on glass slide for observations. Similarly, the morphology of the fungus grown on potato dextrose agar medium was also studied. The conidia produced on seeds as well as from the culture were measured after staining them with cotton blue. Conidia were thoroughly mixed so that a uniform spread was obtained and

a clean cover slip was placed over it. Fifty conidia were measured using ocular and stage micrometer. The microphotographs were taken to illustrate the morphology of the conidia.

Studies on the variability of *C. truncatum* isolates by standard blotter method

During the year 2011-12 eleven soybean samples infected with anthracnose were collected from major soybean growing areas of India to find out the variability in *Colletotrichum truncatum*.To study the variability of *C. truncatum*, ten seed sample were subjected to standard blotter technique. For this purpose, ten seeds were placed on moistened blotter paper with the help of forceps in a petridish and 10 such petridishes were maintained for each seed sample from each location. The petridishes were incubated in a room temperature of 26 ± 2ºC for seven days. On eighth day, the petridishes were observed under stereo- binocular microscope to find out the presence of fruiting bodies (acervuli) on seed coat. Further, the standard tissue isolation procedure was followed to isolate the pathogen. The infected pieces of seeds were surface sterilized with 2 per cent sodium hypochlorite solution for two minutes and washed separately in sterile distilled water to remove traces of sodium hypochlorite if any and then transferred to sterilized petriplates containing PDA. Such petriplates were incubated at room temperature of 26ºC ± 2ºC and observed periodically for the growth of the fungal colonies which developed from the infected bits. Further, the colony colour, spore size, shape have been recorded (Annon.,1976).

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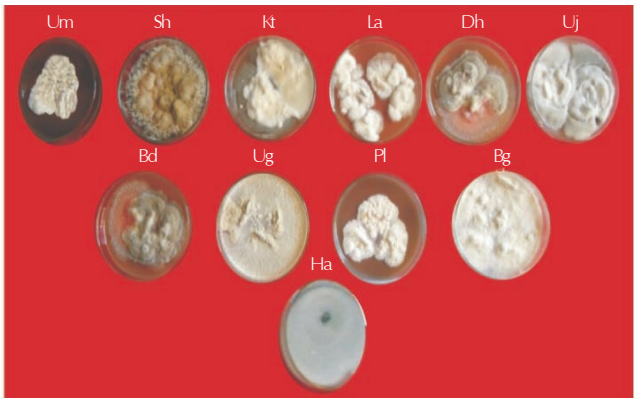
Study of molecular variability of *C. truncatum* isolates

Five geographically distinct isolates of *C. truncatum* representating from different regions were used in this study. Isolates *viz.*, Bg, Dh, La, Um and Uj representating two isolates from Karnataka, one from Maharastra, one from Meghalaya and one from Madhya Pradesh were selected. The pathogen was isolated by standard tissue isolation method. The cultures were maintained in slants at 5ºC under refrigerator. The preserved cultures are transferred to petriplates containing PDA and are incubated to get enough and fresh mycelial growth. Such mycelium was used for genomic DNA extraction. The total genomic DNA of *C. truncatum* isolates were extracted from young vegetative mycelium using the procedure given by Murray and Thompson (1980) with slight modifications. Fungal mat of 0.5g grounded to fine powder in liquid nitrogen and transferred to sterile eppendorf tube. To this, one mL of extraction buffer (1M Tris-5.0 mL; 5 M Nacl-14.0 mL; 0.5 M EDTA- 2.0 mL; 0.1% Mercaptoethanol-50.0 l; 2% CTAB-1.0 g; 1% PVP-0.5g) was added and incubated for 1h in water bath at 65ºC. Then the tubes were centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatant was transferred into other tubes. To the supernatant, equal mL of chloroform and isoamyl alcohol (24:1) and RNAse (1 mL/100 ml) was added and incubated at room temperature for 10-20 minutes. The tubes were centrifuged at 10,000 rpm for 10 minutes, separated the supernatant and added 0.6 vol of ice cold isopropanol + 0.1 vol of sodium acetate and incubated at -20ºC for overnight. Next day, the tubes were centrifuged at 13,000 rpm for 20 min at 4ºC, the supernatant was discarded and the pellet washed with 70 per cent ethanol and centrifuged at 13,000 rpm for 20 min at 4ºC. Again the supernatant discarded, the pellet air dried and dissolved in 100mL of sterile distilled water or TE. The DNA samples were stored at -20ºC for further studies.

The quality and quantity of DNA was analyzed by running 2 ml of each sample mixed with 2 ml of 10x loading dye in 1% agarose gel. The DNA from all isolates produced clear sharp bands in one per cent agarose gel indicating the good quality of DNA. The DNA has been quantified by comparing with the



Bg-Bagalkot, Bd-Bidar, Dh-Dharwad, Ha-Haveri, Ug-Ugarkhurd, La-Latur, Um-Umiam, Pl-Palampur, Sh-Sehore, Ujjain, Kt-Kota

Plate 1: Morphological variability of *Colletotrichum truncatum* isolates

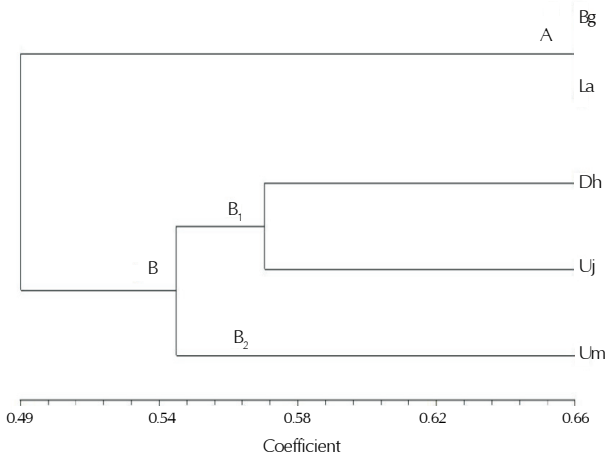


Figure 1: Dendrogram showing genetic similarity between isolates of *C. truncatum* based on RAPD profiles

1 kb size marker (Genei, Bangalore) and by spectrophotometer (Nanodrop ND1000). Commercial kit of OPA and OPB of decamer DNA primers were obtained from M/S Bangalore Genei Pvt. Ltd. Bengaluru. The four individual dNPT's such as dATP,dGTP and dTTP were obtained from M/S Bangalore Genei Pvt. Ltd. Bengaluru. Taq DNA polymerase and 10 x Taq buffer were obtained from M/S Bangalore Genei Pvt. Ltd. Bengaluru.

Corbett research gradient PCR supplied by JH BIO innovation Pvt. Ltd. R. T. Nagar, Bengaluru was used for cyclic amplification of DNA. The PCR amplification of RAPD analysis was performed according to Williams *et al.* (1990) with certain modifications. The optimum condition for DNA amplification used were as follows.

Sl. No.	Step	Temperature (°C)	Duration (min)	Number of cycles
1	Initial denaturation	94	4	1
2	Denaturation	94	1	35
3	Annealing	36	1	
4	Extension	72	2	
5	Final extension	72	10	1
6	Total temperature	4	Forever	-

After the completion of the PCR, the products were stored at 4°C until the gel electrophoresis was done. Totally ten random primers with the sequences were used in the study.

PCR amplifications were carried out in 0.2 mL eppendorf tubes with 20 mL reaction mixture which consisted of 2.0 mL of 10x Taq buffer, 2.0 mL of 25 mM MgCl2, 2.0 mL of primer (1 picomolar/mL), 1.5 mL of dNTP, 0.3 mL of Taq polymerase enzyme and 12.2 mL of sterile PCR water (Genei, Bangalore) and 2 mL of DNA sample. Amplification was carried out by 4 minutes of initial denaturation at 94°C followed by 40 cycles of denaturation of 94°C for 1 minutes; annealing at 37°C for 1 minutes; extension at 72°C for 2 minutes with final extension at 72°C for 10 minutes. Amplified PCR products were subjected to 1.0 per cent agarose gel electrophoresis with 1 x TAE as running buffer. The banding patterns were visualized under UV trans-illuminator with ethidium bromide (10mg/mL) staining. The DNA banding profiles were documented in the gel documentation system and compared with 1kb DNA ladder.Separation of amplified products by agarose gel electrophoresis. One gram of agarose was weighed and added to a 250mL conical flask containing 100 mL of 1x TAE buffer. The agarose was melted by heating the solution on a oven and the solution was stirred to ensure even mixing and complete dissolution of agarose.The solution was cooled to about 50°C. Two to three drops of ethidium bromide (0.5 μg/mL) was added. The solution was mixed and poured into the gel casting platform after inserting the comb in the trough. While pouring sufficient care was taken for not allowing the air bubbles to trap in the gel. The gel was allowed to solidify and the comb was removed after placing the solidified gel into the electrophoretic apparatus, containing sufficient buffer (1x TAE) so as to cover the wells completely. The amplified products (20μL) to be analysed were carefully loaded into the sample wells, after adding bromophenol blue with the help of micropipette. Electrophoresis was carried out at 60 volts, until the tracking dry migrated to the end of the gel. Ethidium bromide stained DNA bands were viewed under UV-transilluminator and photographed for documentation as per protocol given by Williams *et al.* (1990) with certain modifications.

Each amplified band was considered as RAPD marker and recorded for all samples. Data was entered using a matrix in which all observed bands or characters were listed. The RAPD pattern of each isolate was evaluated, assigning character state '1' to all the bands that could be reproducible and detected in the gel and 'O' for the absence of band. The data matrix thus generated was used to calculate Jaccard similarity coefficients for each pair wise comparison. The similarity coefficients were subjected to Unweighted Pair-Group Method on Arithmetic Average (UPGMA) cluster analysis to group the isolates based on their overall similarities. Statistical Package for Social Science (SPSS) was used for the cluster analysis and subsequent dendrogram preparation and per cent polymorphism was calculated by using the formula.

$$\text{Per cent polymorphism} = \frac{\text{No. of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Sequences of random primers used in RAPD analysis for different isolates of *Colletotrichum truncatum* are as follows

Sl. No.	Primer	Sequence
1	OPA-01	5- CAGGCCCTTC-3
2	OPA-05	5-AGGGGTCTTG-3
3	OPA-07	5-GAAACGGGTG-3
4	OPA-10	5-GTGATCGCAG-3
5	OPA-11	5-CAATCGCCGT-3
6	OPA-16	5-AGCCAGCGAA-3
7	OPA-17	5-GACCGCTTGT-3
8	OPB-11	5-GTAGACCCGT-3
9	OPB-13	5-TTCCCCCGCT-3
10	OPB-18	5-CCACAGCAGT-3

RESULTS AND DISCUSSION

Morphological characters of isolates of *Colletotrichum truncatum*

The results on morphological characters recorded on different isolates of *Colletotrichum truncatum* are presented in Table 1, Plate 1. Irrespective of the isolates that are collected, the colony colour of *C. truncatum* varied from cottony white to dark gray. The spore shape was only truncate type with a size ranging from 20.10-23.00 x 3.2-4.50 to 21.10-24.20 x 3.8-4.10 μm. Among the different isolates collected, the colony colour was dark gray in Bidar isolate of Karnataka, Ujjain isolate of Madhya Pradesh while, the Bagalkot isolate of Karnataka and Kota isolate of Rajastan produced white colony colour. The rest of the other isolate produced distinct type of colony colour. Among the five isolates of Karnataka, the colony colour varied from light gray to dark gray while the two isolate of Madhya Pradesh produced cottony gray to dark gray. The Maharastra isolate produced cottony white while Meghalaya produced creamy white. The colony colour of Ugarkhurd was distinct from others producing creamy yellow. There was no much difference between spore sizes of the different isolates

Table 1: Morphological characters of isolates of *Colletotrichum truncatum*

Sl. No.	Isolates	Place of collection	Morphological traits Colony Colour	Spore size (μm)(25 spores) Length	Width	Mean Length	Width
1.	Bg	Bagalkot	White	20.0 - 23.4	3.5 - 4.40	21.7	3.95
2.	Bd	Bidar	Dark gray	20.10 - 23.0	3.5 - 4.00	21.55	3.75
3.	Dh	Dharwad	Light gray	20.0 - 23.10	3.8 - 4.10	21.55	3.95
4.	Ha	Haveri	Gray	20.0 - 24.00	3.5 - 4.40	22.00	3.95
5.	Ug	Ugarkhurd	Creamy yellow	20.10 - 24.0	3.5 - 4.40	22.05	3.95
6.	La	Latur	Cottony white	20.10 - 24.0	3.5 - 4.40	22.05	3.95
7.	Um	Umiam	Creamy white	20.10 - 23.0	3.5 - 4.00	21.55	3.75
8.	Pl	Palampur	Creamy	20.5- 24.30	3.2 - 4.50	22.4	3.85
9.	Sh	Sehore	Cottony gray	20.10 - 24.5	3.3 – 4.50	22.3	3.9
10.	Uj	Ujjain	Dark gray	21.10 -24.20	3.6 - 4.20	22.65	3.9
11.	Kt	Kota	White	21.10-24.20	3.6 - 4.20	22.65	3.9

Note: Bg- Bagalkot, Bd-Bidar, Dh-Dharwad, Ha-Haveri, Ug- Ugarkhurd, La-Latur, Um-Umiam, Pl-Palampur, Sh-Sehore, Uj-Ujjain, Kt-Kota

Table 2: RAPD banding profile of different primers for different isolates of *Colletotrichum truncatum*

Sl. No.	Primer	Total No. of bands	No. of Polymorphic bands	Polymorphism (%)
1	OPA-01	8	5	75.0
2	OPA-05	8	8	100.0
3	OPA-07	7	3	71.4
4	OPA-10	5	2	40.0
5	OPA-11	7	3	71.4
6	OPA-16	10	10	100.0
7	OPA-17	9	8	88.9
8	OPB-11	9	7	77.8
9	OPB-13	7	7	100.0
10	OPB-18	5	4	80.0

Table 3: Similarity coefficient values for RAPD studies for *Colletotrichum truncatum* isolates

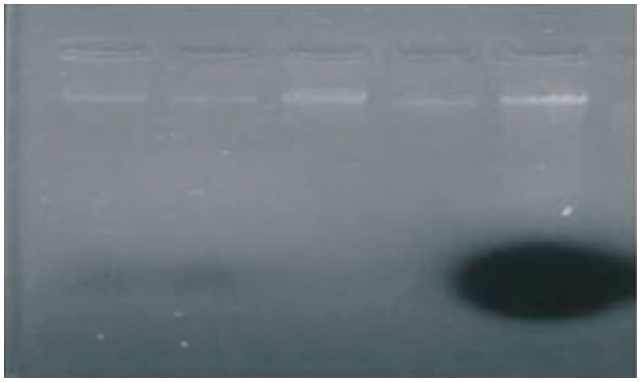
	Bg	Dh	La	Um	Uj
Bg	1				
Dh	0.44	1			
La	0.66	0.37	1		
Um	0.5	0.51	0.54	1	
Uj	0.58	0.56	0.51	0.56	1

collected. The maximum length of the spore was ranged between 20.10-24.5 μm in Sehore isolate followed by 20.5-24.30 μm in Palampur isolate, while the spore length was minimum in Bidar isolate (20.10-23.00 μm). The maximum spore width was observed 3.3- 4.50 μm in Sehore isolate. The minimum spore width of 3.5- 4.00 μm in Umiam isolate of Meghalaya and Bidar isolate of Karnataka. There was no significant difference with respect to spore size. The reports of Jagtap and Sontakke, 2009 while studying the morphology of both conidia and setae of *C.truncatum* isolates from soybean were compared and found to be distinct.Conidial shape was more useful than size in isolates determination. To understand still more existence of variation among the isolates *C. truncatum* PCR based RAPD technique was used in the present investigation.

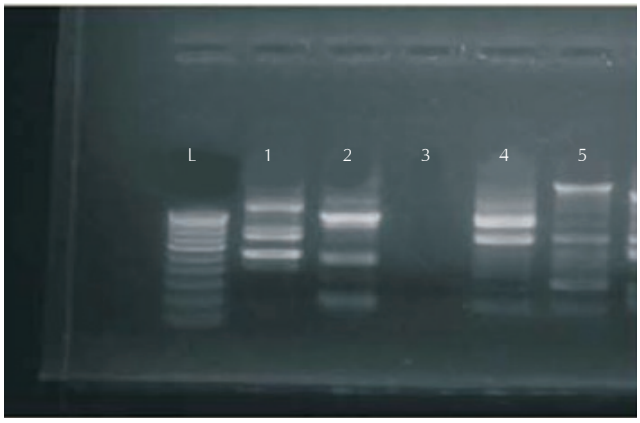
Molecular variability of isolates of *Colletotrichum truncatum*.

The suitability of Random Amplified Polymorphic DNA (RAPD) was used to detect the variations among the isolates of *C. truncatum* OPA and OPB series primers were used to determine genetic distance between the isolates and to construct a dendrogram. Banding profile of different primers for five different isolates of *C. truncatum* representating different geographical area is given in Table 2, Plate 2. Out of the 10 primers used for amplification, OPA-05, OPA-16 and OPB-13 showed cent per cent polymorphism. Information on banding pattern for all the primers was used to determine genetic distance between and to construct a dendrogram.

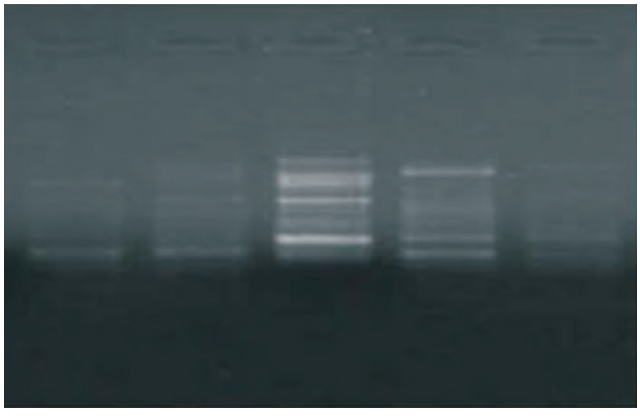
Similarity coefficient of five isolates of *C. truncatum* based on RAPD analysis is given in Table 3, Coefficient ranged from 37 to 66 per cent. Maximum similarity of 66 per cent was observed between isolates Bagalkot (Bg) and Latur (La) followed by 58 per cent in Bagalkot (Bg) and Ujjain (Uj) isolates. The similarity coefficient of 56 per cent was observed between isolates of Dharwad (Dh) and Ujjain (Uj) also in Umiam (Um) and Ujjain (Uj). The similarity coefficient of 51 per cent was observed between isolates of Latur (La) and Ujjain (Uj) also in Dharwad (Dh) and (Umiam) Um Whereas, the least similarity of 37 per cent was observed between Dharwad (Dh) and Latur (La) isolates. RAPD data distinguished the various isolates into major cluster A and B represented in Fig. 1. Majority of the isolates (3 isolates) were in cluster B under various sub-clusters. Remaining isolates fall under cluster A which has two isolates. In case of sub cluster B1, which was comprising a two isolates Dharwad (Dh) and Ujjain (Uj), differed from all other isolates. RAPD data distinguished the five isolates in to major cluster A and B. Out of five isolates two isolates were in cluster A. Remaining three isolates were under major cluster B, where sub cluster B1 has got a two isolates Dharwad (Dh) and Ujjain



DNA check



OPB-11



OPB-18

Amplified products of five isolates of C. truncatum in polyacilamide gel. RAPD profiles generated by the primer OPB - 11 and Primer OPB-18. Columns 1 to 5 represent isolates Bg (Bagalkot), Dh (Dharwad), La (Latur), Um (Umiam) and Uj (Ujjain).

(Uj), cluster B2 has only one isolate Umiam (Um) which differed from all other isolates in the major cluster B. It is clear from present investigation that there is a great molecular variation exists among the isolates of *C. truncatum* which could be used to distinguish variation among the isolates of *C. truncatum*. The study also brought out of pathogen showed molecular variation in over locations and also in a location depending on the genotype grown. These results are in agreement with Juliane *et al*. (2010), who studied the molecular variability in nine soybean isolates of *C. truncatum* based on RAPD analysis, a polymorphism was detected among the wild isolate using RAPD amplification, with five different primers, also showed polymorphic profiles among Brazilian *C. truncatum* isolates. Dendrogram analysis resulted in a similarity degree ranging among isolates and identified three RAPD groups. Maria *et al*. (1994) who studied the molecular variability in five soybean isolates of *C. truncatum* based on RAPD analysis, Purified DNA from each isolate was amplified by the random amplified polymorphic DNA (RAPD) technique with the aid of arbitrary oligonucleotide primers. Amplification products visualized in agarose gels showed specific patters (fingerprints) for each isolate. Genetic dissimilarities and cluster analyses showed that the five isolates could be separated into three distinct groups. These results confirm RAPD as a useful technique to study genetic variability among isolates of *C. truncatum*. The isolate such as Dharwad (Dh) from Karnataka and Ujjain (Uj) from Madhya Pradesh showed similarity with respect to morphology and molecular variability. The morphologically Dharwad (Dh) isolate produced light gray and Ujjain (Uj) isolate produced dark gray. The molecular variability of these two isolates formed a similarity cluster B1 with more than fifty per cent similarity coefficient. In case Bagalkot (Bg) and Latur (La) isolates showed similar morphological character with production of white and cottony white colony colour and the molecular variability of these two isolates formed a similarity cluster A with more than sixty per cent similarity coefficient.

Thus, the results brought out a new information that based on morphological and molecular variability, Bagalkot (Bg) isolate from Karnataka and Lature (La) isolate Maharastra similarly Dharwad (Dh) from Karnataka and Ujjain (Uj) isolate of Madhya Pradesh have evolved and coexisted over years. This information will help in further identification of pathotypes/ races prevalent in *C truncatum* in India and that help in breeding for durable resistance against anthracnose disease.

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