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Molecular Variability of *Colletotrichum* spp. Associated with Anthracnose of Soybean

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ABSTRACT

Background: Anthracnose is a major foliar disease of soybean associated with *Colletotrichum* species. In India, *C. truncatum* is the most widely associated species, whereas, other species are also being reported to cause anthracnose disease in soybean.

Methods: Twenty-eight soybean infected samples were collected from different location of India, in order to obtain the pure cultures and were subjected to pathogenicity test. Microscopic observations were made for morphological characterization of all the isolates. Molecular detection was carried out using ITS primers and the sequences were deposited in the NCBI GenBank. Phylogenetic analysis was made using the MegaX bioinformatics tool.

Result: The pure cultures were subjected to pathogenicity test, which had reproduced similar symptoms found under field conditions. Microscopic observations on spore morphology were made which revealed that truncate conidia were associated in twenty-seven isolates and cylindrical conidia in one isolate. Molecular detection made showed sequence similarity to *Colletotrichum truncatum* and the isolate with cylindrical spore was sharing homology with *C. plurivorum*. Phylogeny analysis clustered the two species into two groups. Hence, the study shows the association of two distinct species in causing anthracnose of soybean.

Key words: *C. plurivorum*, *C. truncatum*, Detection, *Glycine max*, Phylogeny.

INTRODUCTION

Soybean (*Glycine max* L.) is one such protein crop that has high economic importance across the globe due to its usage as food, feed and as by-products in industrial sector. It is very well known for its high protein and oil content accounting to nearly 40 and 20 per cent respectively and hence known as “Golden Nugget”. This crop is a source of vegetable oil, proteins for human and animal feeds and is grown throughout the tropical, sub-tropical and temperate regions. The crop coverage is about 125 million hectare in the world with a production and productivity of 358.85 million tons and 2870 kg/ha respectively. Major soybean producing countries include USA, Brazil, Argentina, China and India. In India, it is cultivated over an area of 11.30 m ha, with production of 10.93 mt and productivity of 960 kg/ha, wherein the Madhya Pradesh state is a massive producer and thus obtaining the title as “Soya State”. Followed by Madhya Pradesh is the Maharashtra, Rajasthan, Karnataka and Telangana. Due to its multifaceted uses it has been an increase in production, but still face biotic and abiotic constraints during the production practices. Anthracnose in particular, imposes a major threat as it infects the plants and seeds, which would lead to a yield loss upto 100 per cent (Yang and Hartman, 2016).

Nakata and Takimoto (1934) disclosed for the first time about anthracnose leaf spot of soybean from Korea during 1917. *Colletotrichum* species can infect soybean at all stages (Sharma *et al*., 2011) and is associated with wide number of species, of which *C. truncatum* is the most commonly associated species (Sharma *et al*., 2011; Dias *et al*., 2018).

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Symptoms manifested after infection on the crop includes chlorotic dull brown lesions, which coalesce and turn to dark brown and necrotic, thus leading to premature drying of foliage. Cankerous lesions can also be observed on the infected petiole and stem region. Lesions are also recorded on infected pods where they appear concentric, wherein severely affected pods bear chaffy and shrivelled seeds (Yang *et al*., 2015; Uddhav, 2017). *C. truncatum* is the widely associated species in all soybean growing countries. In India, soybean anthracnose associated with *C. truncatum* was first reported by Verma and Upadhyay (1973). The other species associated with soybean anthracnose includes, *C. gloeosporioides*, *C. incanum* and *C. dematium, C. chlorophyti*, *C. brevisporum, C. musicola*, *C. plurivorum* and *C. sojae*. So, this study aims to identify the association of *Colletotrichum* species with soybean anthracnose in India. As, there is limited information on variability of *C. trunactum* and diversity of species infecting soybean.

MATERIALS AND METHODS

During 2019, twenty-eight fields were sampled for anthracnose-infected soybean, which includes, Karnataka, Maharashtra, Madhya Pradesh, Uttarakhand, Nagaland, Jharkhand and Rajasthan. The samples were washed; dried and the pathogen was isolated using the standard tissue isolation method (Rangaswamy and Mahadevan, 1972) on sterilized Potato Carrot Agar (PCA) medium, subsequently incubated at 28°C. Sub-culturing was carried out to purify the cultures and was maintained on Potato Dextrose Agar (PDA) slants for further use. Phenotypic characterization was carried out with respect to twenty-eight isolates collected from different locations (Table 1) were cultured on Potato Carrot Agar (PCA) medium for sporulation. The mycelium and spores of all the isolates was examined under the microscope for morphological characters.

To prove the pathogenicity, spore suspension (1×10^6 spores per ml) of all isolates was prepared using sterile distilled water. Forty days old seedlings of soybean, which is susceptible to anthracnose, *i.e.,* JS35, was used, upon which the spore suspension was sprayed. The seedlings were covered with polythene bags for 48 hours to provide favorable condition for infection to commence. The plants were regularly observed for symptom development.

The genomic DNA of all the 28 isolates was carried as per Murray and Thompson (1980) with slight modification. Mycelium from 4 to 5 days old pure broth culture was harvested using sterile Whattman filter paper and transferred to pre-chilled pestle and mortar. The mycelium was homogenized to fine powder using liquid nitrogen and about 100 mg of the fine powder was transferred to pre-sterilized micro-centrifuge tubes containing freshly pre heated CTAB buffer. 10 microlitre of beta-mercaptoethanol was added to each tube and mixed well by inversion. The tubes were incubated in hot water bath at 65°C for 45 minutes with intermittent mixing of the tubes. After incubation, equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed well. The tubes were centrifuged at 10000 RPM for 10 minutes. The upper aqueous phase was collected into fresh tubes to which equal volume of chloroform: iso-amyl alcohol was added and mixed. Later, the tubes were centrifuged at 10000 RPM for 10 minutes in order to obtain the three phases in the tube, from which, the upper aqueous phase was collected and transferred into fresh micro-centrifuge tubes. Equal volume of pre-chilled iso-propanol followed by mixing and incubation at -20°C overnight. The tubes were centrifuged at 10000 RPM for 20 minutes and decanted to retain the pellets. The pellets were washed with 70% ethanol, air-dried and dissolved in TE buffer and stored at -20°C for further use.

Molecular detection was carried out using primers complementary to 5.8S RNA gene with the flanking ITS, namely ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al.*, 1990). The PCR cocktail was prepared, which consists of 2 mM $MgCl_2$, 200 µM of each dNTP's and 2.5 U of Taq polymerase (Takara, Japan) in a 0.2 ml micro-centrifuge tube and to each tube, 50 ng of DNA was dispensed to make a final volume of 20 µl. The tubes were vortexed very gently. PCR was performed in a thermocycler with conditions as: Initial denaturation of 94°C for 5 minutes, 32 cycles each of denaturation at 94°C for 50 seconds, 52°C for 45 seconds and extension of 72°C for 1 minute, followed by one cycle of final extension of 72°C for 5 minutes. A water control (no template control) was maintained along with the twenty-eight samples.

Nucleotide sequences of the isolates were subjected to BLAST analysis for homology search in the NCBI platform and all the twenty-eight sequences were deposited in GenBank of NCBI and accession numbers assigned were obtained (Table 1). Phylogenetic analysis for the 28 isolates was executed using MEGAX tool. Further, a maximum likelihood tree was developed to assess the relationship within these isolates.

RESULTS AND DISCUSSION

Symptoms noticed in anthracnose infected soybean plants under field situation was, light to brown colored lesions of which, some appeared necrotic which gave rise to blighted condition in severely affected plants. During the microscopic study made, the conidia of SeMP22 were aseptate and cylindrical in shape, however truncate conidia were observed for the other 27 isolates. In common among all the isolates was the cup shaped acervuli (Plate 1). On PCA medium, vegetative mycelium initially appeared white which subsequently turned to greyish colour (Plate 2). The mycelium appeared hyaline, septate and branched. During pathogenicity test, symptoms produced were noticed initially at 6 days after inoculation, which were similar to those found in field conditions (Plate 3). The infected pods were malformed and chaffy. The pathogen was re-isolated and observed for morphological characters, which were similar to Plate 1. Several evidences revealed the symptoms of soybean anthracnose as appearance of necrotic lesions on leaf that leads to premature drying, formation of concentric rings on pods with acervuli as sign of infection and also the emergence of necrotic spots on petiole and stem (Nagaraj *et al.*, 2014; Yang *et al.*, 2015; Uddhav, 2017, Yang and Hartman, 2016). These instances give the evidence that formation of such symptoms being associated with *Colletotrichum* spp.

The microscopic observations made for spores were similar to that described earlier (Plate 1). Such similar results of morphological characterization under microscope were discussed by various researchers (Roy, 1996; Photita *et al.*, 2005). Association of *C. plurivorum* was noticed whose morphology was also described by de Silva *et al.* (2019) as cylindrical conidia without septa and hence this was confirmatory with our present findings.

In the molecular detection, all the isolates amplified at 550 bp, which was measured using the 1Kb DNA marker (Plate 4). On contrary there was no amplification foreseen

Table 1: Anthracnose infected disease samples collected across different soybean growing regions and their sequence information deposited in NCBI database.

State	Isolate name	Location	GenBank accession ID assigned*	Similarity hit: Host	Per cent similarity hit
Karnataka	HBKA 1	Hebbal	MT804659	*Colletotrichum truncatum* strain BRIP 28371: *Capsicum annuum*	100
Karnataka	MBKA 2	Mudhol	MT806021	*Colletotrichum truncatum* strain CTM5: legume	99.82
Karnataka	ABKA 3	Anigol	MT806088	*Colletotrichum truncatum* isolate BU-Ch-C002: *Capsicum annum*	99.68
Karnataka	KBKA 4	K K Koppa	MT804660	*Colletotrichum truncatum* isolate AD41123: *Glycine max*	100
Karnataka	BBKA 5	Belavadi	MT806022	*Colletotrichum truncatum* isolate AD41123: *Glycine max*	100
Karnataka	NBKA 6	Nayanagar	MT806089	*Colletotrichum truncatum* strain BRIP 62368: *Glycine max*	100
Karnataka	UBKA 7	Ugarkhurd	MT804661	*Colletotrichum truncatum* strain SCL3: *Citrus limon*	100
Karnataka	MuBKA 8	Munavalli	MT806023	*Colletotrichum truncatum* isolate AD41123: *Glycine max*	100
Karnataka	BiBKA 9	Bidar	MT806090	*Colletotrichum truncatum* isolate AD41123: *Glycine max*	100
Karnataka	ADKA 10	Adargunchi	MT804662	*Colletotrichum truncatum* strain BRIP 62368: *Glycine max*	100
Karnataka	MDKA 11	MARS, Dharwad	MT806024	*Colletotrichum truncatum* strain CTM5: legume	100
Karnataka	YDKA 12	Yarikoppa	MT806091	*Colletotrichum truncatum* isolate AD41123: *Glycine max*	100
Karnataka	KDKA 13	Kalaghatgi	MT804663	*Colletotrichum truncatum* strain BRIP 28371:*Capsicum annum*	99.42
Karnataka	AHKA 14	Aladakatti	MT806025	*Colletotrichum truncatum* strain BRIP 28371: *Capsicum annum*	100
Karnataka	NHKA 15	Nandihalli	MT806092	*Colletotrichum truncatum* strain BRIP 28371:*Capsicum annum*	100
Karnataka	SHKA 16	Shivapur	MT804664	*Colletotrichum truncatum* strain BRIP 28371: *Capsicum annum*	99.82
Karnataka	NeHKA 17	Nelogal	MT806026	*Colletotrichum truncatum* isolate AD41123: *Glycine max*	100
Karnataka	ShHKA 18	Shiggaon	MT806093	*Colletotrichum truncatum* strain BRIP 28371: *Capsicum annum*	99.64
Madhya Pradesh	JMP 19	Jabalpur	MT804665	*Colletotrichum truncatum* strain BRIP 28371: *Capsicum annum*	100
Madhya Pradesh	MMP 20	Mandsuar	MT806027	*Colletotrichum truncatum* isolate TG611: *Capsicum frutescens*	99.82
Madhya Pradesh	SMP 21	Sagar	MT806094	*Colletotrichum truncatum* isolate C-17:bean	98.51
Madhya Pradesh	SeMP 22	Sehore	MW308523	*Colletotrichum plurivorum* strain BRIP 28669: *Carica papaya*	99.82
Maharashtra	LMH 23	Loni	MT804666	*Colletotrichum truncatum* strain BRIP 28371: *Capsicum annum*	100
Maharashtra	AMH 24	Amaravati	MT806028	*Colletotrichum truncatum* strain BRIP 28371: *Capsicum annum*	100
Rajasthan	KRJ 25	Kota	MT806095	*Colletotrichum truncatum* strain BRIP 28371: *Capsicum annum*	100
Jharkhand	RJH 26	Ranchi	MT804667	*Colletotrichum truncatum* strain CTM5: legume	99.82
Nagaland	NNL 27	Nagaland	MT806029	*Colletotrichum truncatum* isolate AD41123: *Glycine max*	100
Uttarakhand	PUK 28	Pantnagar	MT806096	*Colletotrichum truncatum* strain BRIP 28371: *Capsicum annum*	100

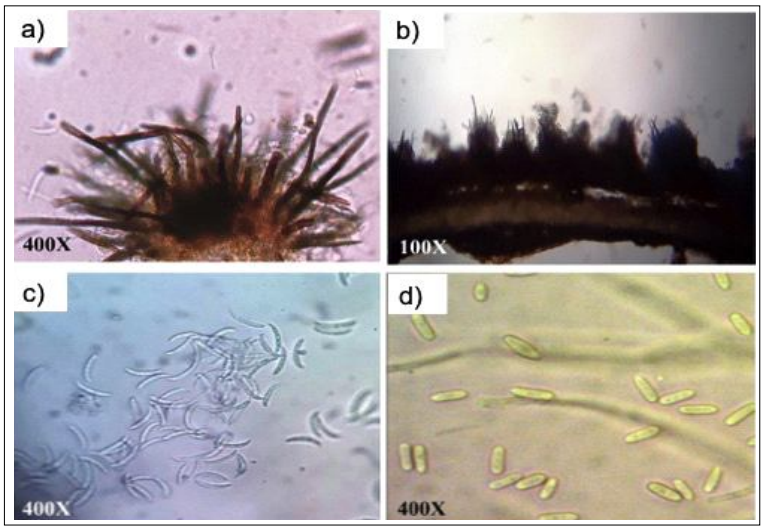

Plate 1: Microscopic observation of the anthracnose infected soybean samples; acervuli in 400X (a) and 100X (b) magnification, conidia of *Colletotrichum truncatum* (c) and conidia of *C. plurivorum.*

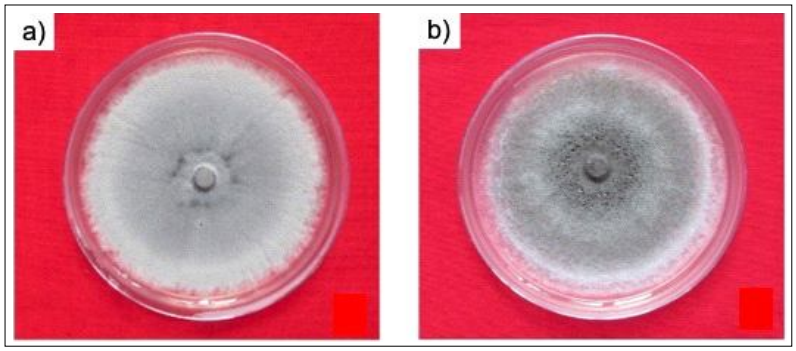

Plate 2: Vegetative mycelia of *C. truncatum* and *C. plurivorum* on potato carrot agar medium.

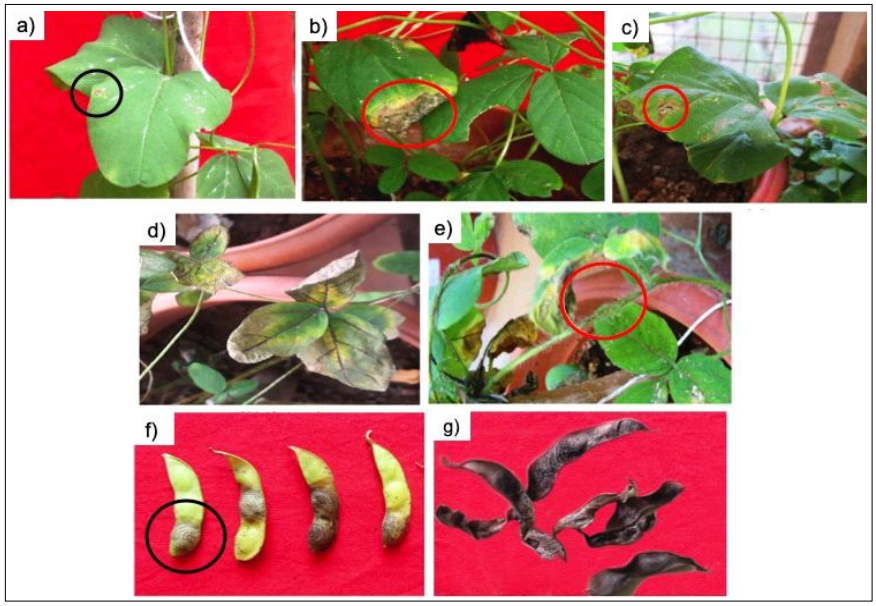

Plate 3: Pathogenicity test for anthracnose disease in glasshouse condition. Symptoms include initial brown colored lesion (a), necrosis on leaf with dot like acervuli (b), shot hole symptom on leaf (c), veinal necrosis (d), cankerous spot on stem (e), concentric rings on pods comprising of acervuli (f), shriveled and chaffy pods (g).

in the no template control (NTC). Among the 28, 18 isolates were obtained from different locations of north Karnataka, where soybean was extensively grown. During the homology search, they showed similarity of more than 98 per cent to *C. truncatum* in the NCBI database, of which some reported from different host other than soybean (Table 1). Isolates namely JMP19, MMP20, SMP21 and SeMP22 (Table 1) were collected from parts of Madhya Pradesh showed similarity to *Colletotrichum* species. However, the isolate, SeMP22 was 99.82 per cent similar to *C. plurivorum* reported on papaya, while the other isolates showed more than 99 per cent homologous with *C. truncatum.* Isolates collected from Maharashtra, namely, LMH23 and AMH24 showed 100 per cent homology to *C. truncatum* reported on *Capsicum annuum* (Table 1). The other isolates namely, KRJ25 from Rajasthan, RJH26 from Jharkhand, NNL27 from Nagaland and PUK28 from Uttarakhand were more than 99 per cent homology to *C. truncatum*.

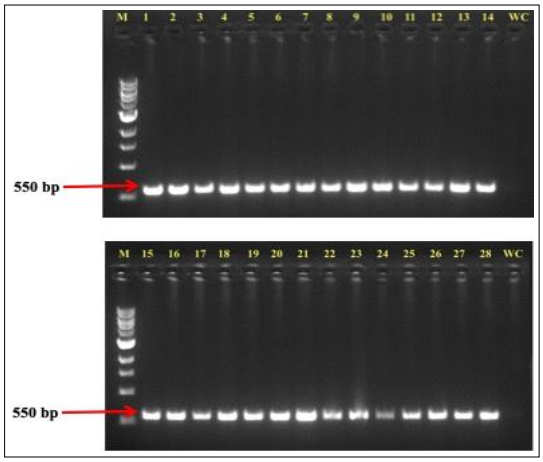

Plate 4: Gel electrophoresis for PCR product of ITS4/5 regions. Lane M is the 1kb DNA marker, WC is the water control (no template control), 1-28 = HBKA 1, MBKA 2, ABKA 3, KBKA 4, BBKA 5, NBKA 6, UBKA 7, MuBKA 8, BiBKA 9, ADKA 10, MDKA 11, YDKA 12, KDKA 13, AHKA 14, NHKA 15, SHKA 16, NeHKA 17, ShHKA 18, JMP 19, MMP 20, SMP 21, SeMP 22, LMH 23, AMH 24, KRJ 25, RJH 26, NNL 27, PUK 28 respectively.

A maximum likelihood tree was constructed (Fig 1) for the twenty-eight sequences using the MEGAX bioinformatics tool. The analysis showed formation of two main clusters, in which *Colletotrichum plurivorum* (SeMP22) obtained from Madhya Pradesh (Sehore) solely belonged to one cluster and all the other 27 *C. truncatum* isolates were grouped together to form another cluster.

To date, this is the first report of *C. plurivorum* infecting soybean in India. *C. plurivorum* was initially known as *C. sichuanensis*, which infected *Capsicum annuum* in Sichuan Province of China, later, it was regarded as *C. plurivorum* as a distinct species with a wide host range (Damm *et al*., 2019). Recently, it has been reported to cause anthracnose in papaya (Sun *et al*., 2019), okra (Batista *et al*., 2020) and in chilli (Saktivel *et al*., 2018).

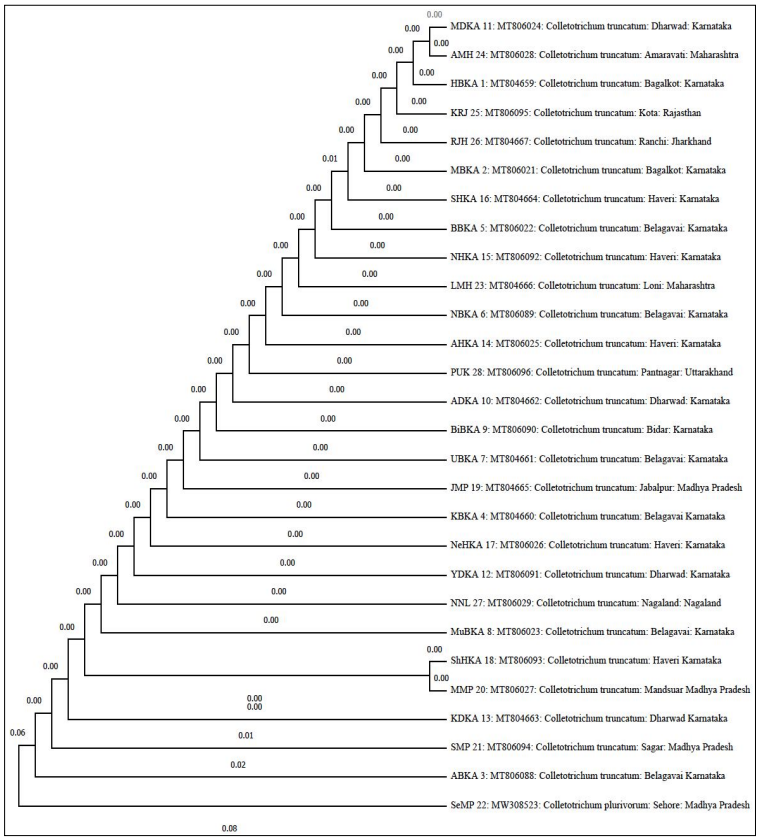

Fig 1: Maximum likelihood tree constructed for twenty-eight isolates.

CONCLUSION

Soybean [*Glycine max* (L.) Merill] is an internationally recognized oil seed crop, which gets infected by several fungal diseases, out of which *Colletotrichum truncatum* causing anthracnose or pod blight is an economically important disease. In our study, we hereby report the infection of soybean with *Colletotrichum plurivorum*, which has not been reported earlier in India. Soybean anthracnose is associated with wide number of pathogenic and endophytic *Colletorichum* sps. In the present investigation we report the association of *C. truncatum* and *C. plurivorum* in soybean.

Conflict of interest: None.

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