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BIOTECHNOLOGY

Identification of Donors and Molecular Characterization of *Corynespora cassiicola* Causing Fungal Leaf Spot of Mungbean and Urdbean

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ABSTRACT

In the wake of changing climatic scenario, dimnishing natural resources and growing food demands, the availability of germplasm capable to withstand biotic and abiotic pressures is crucial to ensure sustainability in agriculture system. Among the biotic stresses, fungal leaf spot disease cause significant yield reduction in mungbean (*Vigna radiata* L.) and urdbean (*Vigna mungo* L.), which are predominently grown in India and many parts of South and Southeast Asia. The present investigation was designed to identify resistant donors against the fungal leaf spot in mungbean (200) and urdbean (100) accessions by phenotyping under natural field conditions during *kharif* 2019. Though none of the 200 mungbean lines were found resistant to fungal leaf spot, 8 lines displayed moderate resistance. Likewise, out of 100urd bean accessions three germplasm lines unveiled resistance and 6 moderate resistance against the disease leaving others suceptible to highly sucepetible. The DNA of pathogen causing fungal leaf spot were isolated (*Jhansi isolate*) from the fungal mycelium and infected plants, respectively for molecular characterization and it was amplified using published primers for ITS and β-tubulin region for sequencing. A phylogenetic analysis of ITS sequence of the Jhansi isolates along with the reference sequences from NCBI, GenBank revealed maximum identity (98.53 per cent) of isolate with *Corynespora cassiicola* of cowpea reported from Ghana. Further β-tubulin sequence also showed 88.55 per cent similarity with *C. cassiicola* isolate from China. This was further confirmed by leaf sensitivity test against crude fungal toxin cassicoin secreted by *C. cassiicola*.

HIGHLIGHTS

- Molecular characterization of *Corynespora cassiicola* infecting mungbean and urdbean in India.
- Identified few mungbean and urdbean germplasm lines resistant against leaf spot induced by *Corynespora*.

Keywords: Germplasm, Screening**,** Resistant, Susceptible, Phenotyped and *Corynespora cassiicola*

Mungbean (*Vigna radiata* L., greengram) and urdbean (*Vigna mungo* L., black-gram) are important short duration grain legumes with wide adaptability, low input requirement and have the ability to improve soil fertility by fixing atmospheric nitrogen. (Karamany 2006; Bhanu *et al.* 2017). These crops are well adapted in large number of cropping systems and constitute a major source of protein in many developing countries worldwide. India

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produced 2.64 and 2.38 million tonnes of mungbean and urdbean, respectively in the year 2020-21 (3rd Advance Estimates, DAC & FW, 2020-21).

Although Uttar Pradesh is one of the major states contributing to the total production of mungbean and urdbean in India, their production is stagnant over the last few years due to frequent invasion of insect pests and fungal, bacterial and viral diseases besides drought, water logging, salinity, vagaries of climate etc. Although disease management practices, including physical, chemical, and biological methods have been researched and described in the literature, few of these are available or have been used by growers (Pandey *et al.* 2018). Over the last few years, resistance breeding has emerged as one of the most effective ways to manage biotic stresses in an economical, eco-friendly and sustainable manner. However, resistance breeding requires continuous efforts of enriching the reservoir of required genes to effectively tackle the specific diseases (Vasudevan *et al.* 2014). Further, higher temperatures and erratic weather pattern are likely to change the geographical pathogen distribution. This in turn might decrease the effectiveness of existing resistance genes in crop varieties (Garrett *et al.* 2006; Milus *et al.* 2009; Vasudevan *et al.* 2014) by promoting more aggressive races of pathogens and thus fostering chances of breakdown of resistance. Therefore, identification of resistant sources focused on developing varietal lines resistant to pests and diseases is a continuous process (Akhtar et al. 2011) and could be region specific for adaptation to different agro-ecosystems (Sharma and Ortiz 2002).

Leaf spot is an important fungal disease of mungbean and urdbean leading to significant damage to the production. The disease occurs on other legumes, including cowpea and soybean. Warm temperature, frequent rain, and high humidity favour the development of the disease. Numerous attempts have been made to screen mungbean and urdbean germplasm lines against leaf spot diseases in natural field/artificial conditions in few specific locations (Akhtar *et al.* 2014; Pandey *et al.* 2018; Reddi *et al.* 2018 and Bhaskar 2017). However, not much work has been reported related to evaluation of resistant sources against fungal diseases of mungbean and urdbean from Bundelkhand region,which has a leading position in terms of area and production of pulses in India. The experiment reported here highlights efforts to screen mungbean and urdbean germplasm lines under natural field conditions for identification of resistant lines against fungal foliar diseases for their possible use as resistant donors to develop varieties capable to withstand outbreak of fungal leafspot in the drought prone Bundelkhand region.

MATERIALS AND METHODS

Seed collection and raising of plants for germplasm screening

During *kharif*-2019, 200 mungbean and 100 urdbean accessions were collected from ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi and ICAR-Indian Institute of Pulses Research (ICAR-IIPR), Kanpur. The seeds were sown in experimental field of RLBCAU, Jhansi (25.51° N and 78.56° E at an altitude of 227 m above mean sea level) in two replications of 4 m row length with a uniform spacing of 45 cm between the rows and 10 cm between the plants. Timely watering and fertilizer application as per standard agronomic package were done for proper growth of plants, except plant protection methods. Along with these, known mungbean varieties viz., Samrat and urdbean variety, IPU 2-43, were used as resistant checks. Bundelkhand local was used as susceptible checks for both, mungbean and urdbean crop.

Disease incidence

The disease incidence in the field was recorded starting from the first appearance of the disease till maturity of crop at a regular interval of 15 days. Ten plants were randomly selected in each row and tagged for regular recording of the observations. Scoring was done based on the disease symptoms using standard rating scale (Manandhar *et al.* 2016) for fungal leaf spot symptoms, regular recording of occurrence and spread of disease. The entire data were pooled together and the Percent Disease Index was computed from the above scales depending on the disease by using the following formula (Wheeler 1969) and the score was given.

Symptomatology and collection of the samples

To study the symptoms, regular recording of occurrence and spread of disease was done from

15 DAS onwards at a regular interval of 15 days till harvesting. Diseased samples were collected from the plants showing severe leaf spot symptoms under field condition and were carefully placed in polythene bags, properly tagged, and brought to the laboratory.

Morphological characterization

Isolation, purification and identification of the fungal pathogen

Infected plant parts such as leaves showing typical symptoms of leaf spot were used for isolation of pathogens. The leaves were washed with tap water to remove dust. Then the infected portion of the leaves was cut into small pieces/ bits (2-3 mm) and washed in sterile distilled water for one minute, and then surface sterilized with 1% Sodium hypochlorite solution for 30 seconds, and rinsed thoroughly thrice with sterile distilled water. The infected pieces were air-dried for few minutes to remove water present on the sample. The diseased bits were then transferred to the Petri plates containing Potato Dextrose Agar (PDA) media under aseptic conditions and incubated at 25±1°C for 4 to 5 days.

The fungal culture was purified by single spore isolation method (Riker and Riker 1936) and was maintained in the laboratory under refrigerated conditions for further studies. The fungi were identified according to cultural characteristics described by Gilman (1957), Barnett and Hunter (1972) and Nelson *et al.* (1982).

Pathogenicity test

Pathogenicity test was conducted by inoculating one-month-old plants raised in earthen pots. The fungal suspension was made by adding mycelial discs into sterile distilled water and the suspension was sprayed on plants using hand atomizer. Later, inoculated plants were covered with plastic bags for 12 hours photoperiod to create high humidity for two days. The plants were watered regularly and observed for the appearance of disease symptoms. The pathogen was re-isolated and compared with the original culture.

Molecular characterization

DNA isolation and quantification: The total genomic DNA was extracted from the mycelial mat of pure fungal culture, obtained from the susceptible genotypes of mungbean (PM 2, SML-134 and ML-5) and urdbean (STY-2115, U-135, T-9) grown on potato dextrose broth, using of Cetyl Trimethyl Ammonium Bromide (CTAB) (Murray and Thompson 1980). The quantification of DNA was done by observing it at 260 nm and 280 nm wavelength using a UV- spectrophotometer. DNA quantification was done to check its quantity, purity, and intactness.

Primer Synthesis and PCR amplification

Primer synthesis: The universal primers viz. ITS 1 and ITS 4 for ITS region and Tub aF and Tub aR for β-tubulin region (White *et al.* 1990; Glass and Donaldson, 1995; Chethana *et al.* 2015) published already were synthesised (Table 1) and used for Polymerase Chain Reaction (PCR) amplification of the DNA.

PCR amplification: The extracted DNA was subjected to amplification following standard PCR conditions. The standard components of the PCR mix using Dream taq PCR master mix were used for amplification of DNA. After completion of the PCR amplification, the products were stored at 4°C until gel electrophoresis is done.

Agarose gel electrophoresis (Horizontal gel electrophoresis): One per cent gel was prepared by melting 1g of agarose in 100 ml of 1x TAE (Tris buffer) and added 2-3 µl Ethidium bromide.

Table 1: Details of primer sequences used for amplifying the DNA

Primers		Sequence information	Annealing temperature ºC	Expected amplicon size
ITS	ITS 1 (Forward)	CGGATCTCTTGGTTCTGGGA	54ºC	550 bp
	ITS 4 (Reverse)	GACGCTCGAACATGCC		
β-tubulin	Tub aF (Forward)	GGTAACCAAATCGGTGCTGCTTTC	55ºC	450 bp
	Tub aR (Reverse)	ACCCTCAGTGTAGTGACCCTTGGC		

A sufficient amount of 1x TAE buffer was added in the electrophoresis tank to cover gel up to 10 mm depth. Each well of the gel was loaded with 8 µl PCR products along with 4 µl loading dye (HIMEDIA) along with 50 bp DNA ladder (HIMEDIA). Electrophoresis was done for about 40-45 minutes under 50 volts. Agarose gel with migrated DNA fragments were visualized under gel documentation (BioRad Laboratories). Further, the amplified products were submitted for sequencing at M/S Eurofins Scientific India Pvt Ltd., Bengaluru, India.

DNA sequencing and Phylogenetic analysis: The amplified products of fungal ITS and β-tubulin regions were sequenced by Sanger method at M/S Eurofins Scientific India Pvt Ltd., Bengaluru, India. The obtained sequences were deposited in the GenBank, National Centre for Biotechnology Information (NCBI) database and Accession numbers were obtained. The sequences thus obtained were also subjected to nBLAST to find out homology with available nucleotide sequences from the fungal DNA database available at NCBI. The reliable reference sequences retrieved from GenBank and present study sequences for fungal DNA were aligned using the ClustalW program available in MEGA X software (https://www.megasoftware.net/) with default parameters. The phylogenetic tree was constructed by MEGA X software using the Maximum Likelihood method adopting 1000 bootstrap replications (Kumar *et al*. 2018).

Extraction of fungal crude toxin using acetone for leaf sensitivity test

The extraction of toxin using acetone was done as per the method adopted by Kumar *et al*. (2017) with the slight modifications. The fungal pathogen was grown on PDA medium by placing 5 mm of mycelial plug obtained from the margin of the original culture. From a 20 days old culture, mycelial agar plugs of 7 mm were excised with the help of cork-borer. About 5 mycelial plugs were kept inside the test tube and 10 ml of acetone was added into the same tube and kept under dark conditions in order to extract the fungal toxin. After 4 hrs, the extract was centrifuged at 10000 rpm for 10 minutes. The supernatant was transferred into a tube using a micropipette. The treatments for pathogenicity evaluation of cassiicolin crude toxin extracted from fungal culture are described in Table 2.

Study on plant growth parameters of mungbean and urdbean genotypes

Data on plant growth parameters was recorded by randomly selecting five plants in each of the genotypes of both mungbean and urdbean at maturity. The mean values of five competitive plants of identified resistant and moderately resistant were averaged and expressed as the mean of the character along with the exhibited range. The characters recorded in the study were plant-height, pod length, number of clusters per plant, number of pods per cluster and seed per pod. Plant height was measured in centimetres (cm) from the base of the plant to the tip at maturity. Number of pods and number of clusters were counted at the maturity stage of the selected plants. Pod length was measured from the base to the tip of the pod in cm. Five pods per plant were randomly selected, threshed separately and the average number of seeds per pod was also calculated.

Table 2: Pathogenicity evaluation of cassiicolin crude toxin extracted from fungal culture

Treatments	
Culture crude suspension dilutions	1000 µl spore suspension (100 %)
	750 µl spore suspension + 250 µl distilled water (75%)
	500 µl spore suspension + 500 µl distilled water (50 %)
	250 µl spore suspension + 750 µl distilled water (25%)
Extracted Toxin	Crude suspension
10% acetone (9ml acetone + 1ml water)	Control to confirm only acetone reaction
Water	Negative control

RESULTS AND DISCUSSION

Symptomatology

The symptoms due to fungal leaf spot disease were recorded 35 days after sowing when the sympotoms first appeared on the older leaves. Initially small chlorotic spots with necrotic centres appear on the leaves and later, the necrotic spots become light brown and surrounded by a chlorotic halo (Fig. 1 and 2). Finally the infected leaves become dry and drop. The symptoms were quite distinct from the *Cerospora* leaf spot disease (caused by *Cercospora canescens*) and later after morphological and molecular characterization, the causal organism for fungal leaf spot was identified as *Corynespora cassiicola.*

Screening of mungbean germplasm

200 germplasm lines of mungbean were evaluated for occurrence of fungal leaf spot disease under natural environmental conditions prevailing in the region. Scoring for the corynespora leaf spot (LS) disease was done using already available scale (Manandhar *et al.* 2016). During the present study no germplasm line was found to be resistant to corynespora LS disease. However, eight germplasm lines viz. IC 314322, IC 73401, IC 76499, DGG 5, NM 1, VMS-6, ML 1464 and ML 2037 exhibited moderate resistance (MR) against corynespora leaf spot disease having the disease severity of 33.3%. Of the remaining germplasm lines, 79 were recorded as moderately susceptible (MS) with the disease severity range between 41.7 to 58.3 per cent. Further, in addition to these 79 lines, the check varieties viz. SML 668 and Samrat also showed moderately susceptible reaction to corynesporaLS disease with the disease severity of 58.5 % and 41.7 %, respectively. Further, among the identified susceptible lines, few entries viz. IC 314347, IC 348964, IC 314649, Sona Yellow, IPM 312 43K, KM 2241, IPM 2-2-3, CO 8, NH 805, MH 2-15, Pusa Bold

Fig. 1: Symptom of fungal leaf spot on mungbean leaves

Fig. 2: Symptom of fungal leaf spot on urdbean leaves

2, PLM 771, IC 76466, IC 285532, IC 76377, PLM 187 and IC 76418 were having the disease severity of > 90 per cent. It was also recorded that a germplasm line viz. IC 305291 was even having the disease severity upto 100%. The disease severity of these lines was even more than the susceptible check (Bundelkhand local) where it was recorded 83.3 per cent only. Therefore, all the screened mungbean germplasm lines were grouped from moderately resistant lines to susceptible lines based on the disease severity. The list of the mungbean entries showing moderately resistant and moderately susceptible reaction to corynespora leaf spot disease is presented in table 3 and rest were susceptible to highly susceptible.

Limited information is available in literature on *C. cassiicola* infecting *Vigna* pulses, therefore the results were compared with the closely related fungus i.e. *Cercospora canescens*, belonging to the same phylum, causing fungal diseases on mungbean and urdbean crops with almost similar symptoms (Iqbal *et al.* 2004; Singh and Gurha 2007; Akhtar *et al.* 2014). Further, the varied response of the germplasm accessions might be due to more inoculum load of *C. cassiicola* that prevailed in *kharif* 2019. The absence of resistant lines may attributed to limited number of germpalsm lines, mostly from north Indian states, included in the present study for screening. The absence of resistant source in the test germplasm therefore highlights clearly the need to have enough genetic material in hand for development of new lines, resistant to disease or bearing specific characteristics. Further, the effectiveness of the genetic resistance also varies with the seasonal changes (Davidson and Kimber, 2007).Therefore, the importance of genetic diversity and biodiversity cannot be emphasized enough in the context of resistence breeding against biotic and abiotic stresses.

Screening of urdbean germplasm

The corynespora LS disease severity in 100 urdbean germplasm lines was recorded from 16.67 to 100 per cent. Based on mean disease severity three germplasm lines viz. IC-530501, IC-570221 and IC-570263 were found resistant to *C. cassiicola* during *kharif*, 2019 experiment. The disease incidence of resistant germplasm was recorded in the range of 15.2 to 16.67 per cent as per the scale. Six germplasm lines viz. IC-565276, TPU-99-232, IC 565247, PLU79, PLU81 and IC 41718 were found to be moderately resistant with the disease severity range of 25.0 to 33.33 per cent. Out of 100 germplasm lines that were screened, 39 were found moderately susceptible with disease severity ranging from 41.67 to 58.33 per cent. Remaining 52 germplasm lines were found to be susceptible with disease severity range of 66.67 to 100 per cent. Similar to mungbean lines, the urdbean lines are also grouped from resistant to susceptible lines based on the disease severity and are the entries showing resistant, moderately resistant and moderately susceptible reaction to corynespora leaf spot disease is presented in table 3and rest were found to be suspetible to highly suspetible.

67 urdbean genotypes were screened for *C. cassiicola* under field condition (Naik *et al.* 2014) and the accessions TU-94-2, P 703, LBG 709, IPU-02-33, IPU-94-2, AKU-2, AKU-99-04, VBN 6 and P 512 showed resistant reaction. However, none of these genotypes were available in the set of the genotypes screened in this study. Similar work on screening of urdbean genotypes against fungal leaf spot was undertaken by several workers (Gunasri *et al.* 2018; Chandra *et al.* 2019) where Gunasri *et al.* (2018) observed out of sixteen genotypes evaluated, only one genotype LBG 645 was moderately resistant to *C. cassiicola* and *C. canascens*. These studies indicated that these crops are being infected by *C. cassiicola* apart from *C. canascens.* As the disease is comparatively less known in northern and central India, the results obtained on varying reactions against *C. cassiicola* need to be further confirmed in future studies.

Morphological and Molecular characterization

Morphological characterization

Initially colony colour was greyish at the centre and whitish towards periphery, brown in the lower surface. Later, the colony colour turned grey in colour. The texture of the fungal colony was fluffy giving the velvety look. The colour of the mycelium varied from white to grey with respect to age of the culture (Fig. 3). The conidia of the fungi were also observed under compound microscope (LEICA DM 2500 LED) at 10X and 40X magnification (Fig. 4). Conidia were hyaline, smooth, septate (5-6), straight or curved and medium to large in size. The size of the conidia ranged from 18-12 µm in length and 6-7

Table 3: Disease reaction of mungbean and urdbean entries against corynespora leaf spot disease during *Kharif* 2019

Reaction	No. of entries	Range of PDI (%)	Disease rating scale	Entries
Mungbean entries				
MR	8	31.2-33.3	2	IC 314322, IC 73401, IC 76499, NM 1, DGG 5, VMS-6, ML-2037, ML- 1464
MS	79	41.7-58.3	3	IC 314568, IC 121301, IC 417873, IC 488524, IC 19420, IC 15567, IC 373199, IC 76451, IC 103821, COGG 912, ML 2056, PDM 281, ML 1256, PRATEEKSHA NEPAL, PUSA 9531, PAIRY MUNG, MASH 338, RMG-353, SONA GREEN, IPM 5-2-8, IC 76322, IC 305241, IC 8422, IC 305291, IC 121220, IC 52046, LBG 623, IPM409-4, IC 9127-1, IC 76338, IC 76474, IC 314512, IC 52046, IC 314697, IC 39400, IC 121203, PUSA 672, IPM02-3, ML 1299, BDYR 2, HUM 1, MGG295, BM 63, ML 515, IPM 2K 14 9, IPM 99 125, OMG 1030 (PMR), KM11-584, KOPERGAON, OMG-1045(PMR), DAAV-2, IC 11443-1, IC 76414, IC 76361, PL M188, IC 764-76, PLM 1032, PLM 772, PLM 646, PLM 653, IC 76448, IC 314854, PM 6, IC 296672, EC 496839, IC 76389, IC 305284, GANGA 8, BANGLADESH LONG POD, TARM-15, PDM 178, BRAZIL, IPM 2-19, VAMBAN 2, IC 8924, IC 76444
Urdbean entries				
R	03	15.2- 16.67	1	IC 530501, IC-570221, IC 570263
MR	06	25.00-33.33	2	IC 565276, TPU-99-232, IC 565247, PLU79, PLU81, IC 41718
MS	39	41.67-58.33	3	NG-2119, UH-8038, NPU-180, UH-85-15, STY-2187, IPU-99-213, IPU-99-123, IC-16511, STY-2834, IPU-99-221, IPU-99-222, IPU-99-6, JU-78-27, PLU-65, MASH-1, IPU-917, IC-21003, IPU-98-36, PGRU-95014, K-614830, IC-604265, IC-530658, IC616487, PLU-53A, IC 605329, PGRU-99028, IC 584696, IC 616493, IC 545207, IC 616494, IC 570274, IC 616495, IC 530452, IC 545200, IC600266, PLU54, IC 530611, PDU-8, IPU-99-40

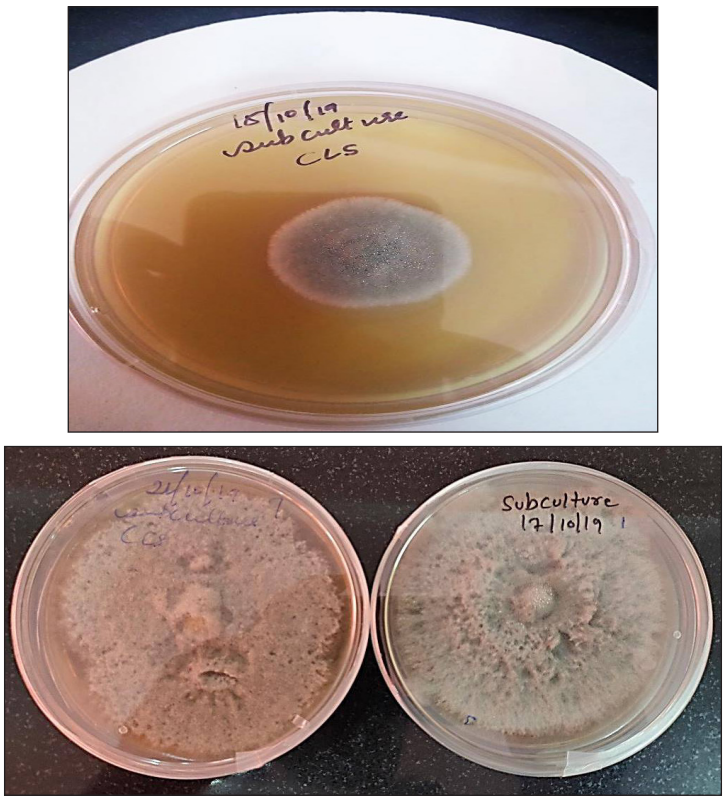

Fig. 3: The growth and texture of the fungal colony of *Corynespora cassiicola*

μm in width. The hypha was unbranched, septate with light brown colour. The fruiting bodies of the fungus were amphigenously scattered i.e. were grown on both sides of leaves. The conidiophores were unbranched, and medium dark in colour bearing conidia in chains or singly.

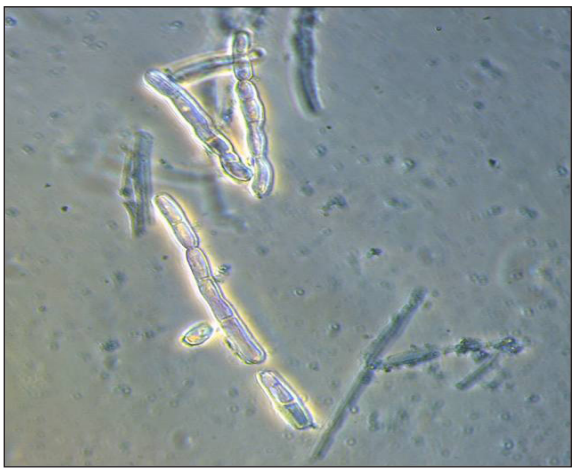

Fig. 4: 40X magnification of microscopic view of condia of *C. cassiicola*

The morphology of the isolated fungus resembled to that of *C. cassiicola,* which was supported by the morphological study conducted by Souza *et al.* (2009) of the fungus on the leaf surface of the coffee plant. The similar mycelia and conidial characteristics, as observed in the present study, were also reported in the C.M.I. description of pathogenic fungi and bacteria, in rubber, in chilli, and in tomato for *C. cassiicola* infection (Ellis and Holliday 1971; Qi *et al.* 2011; Suresh *et al.* 2017; Kamei *et al.* 2018). Accordingly, the pathogen causing fungal leaf spot of mungbean and urdbean at Jhansi was identified as *C. cassiicola* confirmed further by pathogenicity test and molecular characterization.

Pathogenicity test

The pathogenicity test showed jhansi isolate was capable of causing the disease in both the crops. Ferreira and Bentes (2017) have evaluated the pathogenicity of *C. cassiicola* isolates in tomato, eggplant and papaya and found that all isolates caused disease in their original host giving positive reaction. Their study supported the present investigation confirming the Koch's postulate.

Molecular characterization

The identity of fungal pathogen causing leaf spot disease in mungbean and urdbean was confirmed through molecular approach by subjecting the fungal DNA to PCR amplification using already designed universal primers for ITS and β-tubulin as described in material and methods. After gel electrophoresis the band size of 550 bp and 450 bp for ITS (Fig. 5) and β-tubulin (Fig. 6), respectively were observed under gel documentation unit for the selected susceptible genotypes of mungbean and urdbean. The resulting PCR product was sent for sequencing to M/s Eurofins Scientific India Pvt. Ltd and the nucleotide sequence for ITS and β-tubulin region was obtained. The similar findings were reported on molecular characterization studies using ITS for amplification of *C. cassiicola* infecting cotton (Conner *et al.* 2013), rubber plants (Prosper *et al.* 2018), *Acanthus ilicifolium* (Xie *et al.* 2020) where the amplicon size of 545- 550 bp were obtained.

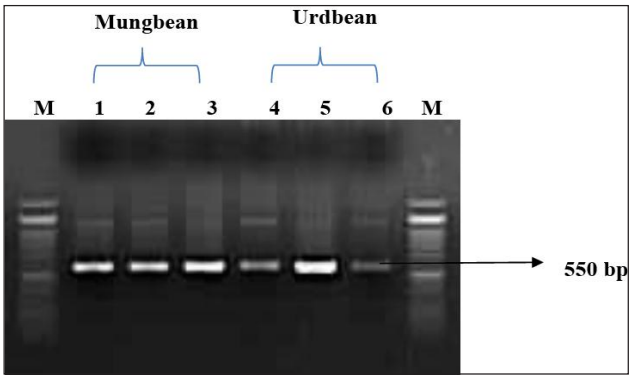

Fig. 5: PCR amplification of DNA of *C.cassiicola* using ITS specific primers. M: Marker; Lane 1: PM 2; Lane 2: SML-134; Lane 3: ML-5; Lane 4: STY-2115; Lane 5: U-135; Lane 6: T-9

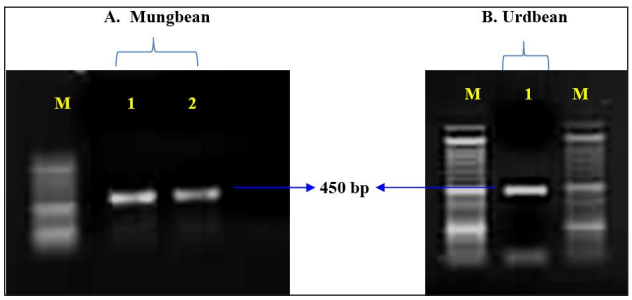

Fig. 6: PCR amplification of DNA of C.cassiicola using β-tubulin specific primers. **(A)** M: Marker; Lane 1: PM 2; Lane 2: SML-134 **(B)** Lane M: Marker; Lane 1: STY-2115; Lane M: Marker

DNA sequencing and Phylogenetic analysis

The BLAST homology analysis of the amplified ITS and β-tubulin region sequences was carried out.

Table 4: *C. cassiicola* isolates and corresponding GenBank accessions used in the phylogenetic tree analysis based on partial nucleotide sequences of ITS region

Original Host	Geographic location	Name of the Isolate	Query cover (%)	Identity (%)	GenBank Accession number
Cowpea	Ghana	unid7	100	98.53	MN809263
Rubber tree	India	6M	99	97.55	JX087444
Coleus	USA	RWB321	100	98.37	FJ852592
Rubber tree	India	8G	100	98.19	JX087446
Rubber tree	Malaysia	CKT05D	100	98.36	EU364539
Sorghum	China	FXSB-1	100	98.19	MK139711
Clinacanthus	Malaysia	SAL1	100	98.19	KY806119
Cotton	India	CC7	100	98.19	MG976657
Guava	Malaysia	183PG/F	100	98.36	GU066725
Rambutan	Malaysia	118NL	100	98.36	GU066681
Tomato	USA	AS49	100	98.19	FJ852574
Golden trumpet	Brazil	—	99	98.35	MF962868
Rubber tree	China	CATAS001	100	98.18	EF198115
Cotton	India	CICR-NCS	100	98.01	MN945374
Strobilanthes	Malaysia	PBR5	100	98.01	KY828943
Unknown	India	BAB-5556	100	98.01	KU504327
Syzygium	Malaysia	81SS/F	100	98.01	GU066654
Soybean	Columbia	AGSV13	100	98.00	MN298749
Kadsura	China	HLH-1-1	100	98.00	MH255527
Avocado	USA	DMW1038	100	98.00	KU593529

Table 5: *C. cassiicola* isolates and corresponding GenBank accessions used in the phylogenetic tree analysis based on partial nucleotide sequences of β-tubulin (tub A) gene

Original Host	Geographic location	Name of the Isolate	Query cover (%)	Identity (%)	GenBank Accession number
Rubber tree	China	LC001	93	88.55	MN604075
Cowpea	China	JD001	93	88.55	MN604072
Papaya	China	FMG001	93	88.55	MN564946
Acanthus	China	ALY1-3	93	88.55	MN887509
Castor	China	RLT-3	93	88.55	MN512639
Avocado	China	YLBB1125	93	88.55	MN737729
Cucumber	China	HNLY1707	93	88.55	MH763700
Kadsura	China	HLH-1-1	93	88.55	MH263734
Kiwifruit	China	BS8	93	88.55	KJ954133
Straw berry	China	FCC16	93	88.55	KX755447
Golden Privet	China	ST3	93	88.55	KX429665
Unknown	USA	EH-1026	93	88.55	KU605287
Honey suckle	China	J1	93	88.55	KU564092
Sweet potato	China	LP138	93	88.55	KU167044
East India lotus	Japan	Nelumbo04	93	88.55	AB539201
Cucumber	Japan	KE1	93	88.55	AB539181
Perilla	Japan	PC95010	93	88.55	AB539172
Mop heads	China	XQ3-1	93	88.30	MH572688
Perilla	Brazil	491	93	88.31	HQ641092
Benghal dayflower	Brazil	631.1	93	88.57	HQ641082

The ITS and β-tubulin region sequences of Jhansi isolate were submitted to NCBI GenBank and the accession numbers MT731034 and MT 647836 were obtained respectively. The ITS nucleotide sequence was found to have 97.55 to 98.53 percent similarity with *C. cassiicola* infecting different crop species from different geographical locations (Table 4). The ITS sequences of study isolate shared maximum identity (98.53%) with *C. cassiicola* of cowpea (GenBank Accession No. MN809263), reported

from Ghana. Similarly, β-tubulin region nucleotide sequences showed maximum nucleotide sequence homology 88.30 to 88.55 per cent with *C. cassiicola* isolates (Table 5). The majority of *C. cassiicola* isolates reported from different parts of the world showed maximum of 88.55% nucleotide sequences identity for the β-tubulin region with Jhansi isolate.

A phylogenetic analysis was also performed of ITS and β-tubulin sequences along with the reference sequences obtained from NCBI, GenBank. This analysis showed that the Jhansi isolates (MT731034 and MT647836) are placed in the cluster of *C. cassiicola* and thus supported the findings of morphological identification of the pathogen as *C. cassiicola*. Further, the phylogenetic tree revealed that *C. cassiicola* causing leaf spot in urdbean and mungbean is closely related to *C. cassiicola* isolates of China and Ghana (GenBank Accession No. MN809263 and MN604072, respectively) which were isolated from the cowpea plant (Fig. 7 and 8).

As the morphology, pathogenicity and leaf sensitivity test by cassicolin toxin gave similar results in both mungbean and urdbean genotypes, it was confirmed that the pathogen infecting both

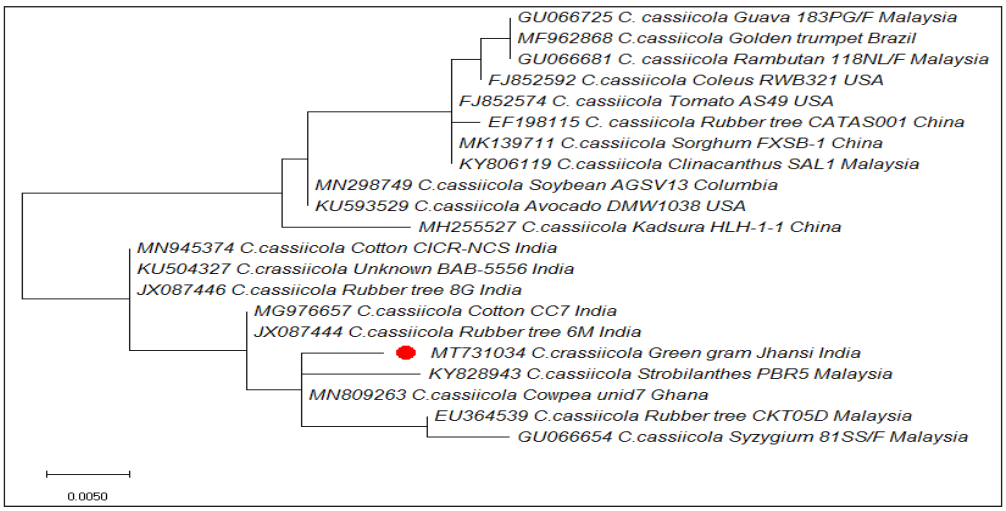

Fig. 7: Maximum likelihood phylogenetic tree generated from ITS region of *C. cassiicola* isolated from mungbean and reference sequences from NCBI at bootstrap values of 1000 replicates (MEGA X)

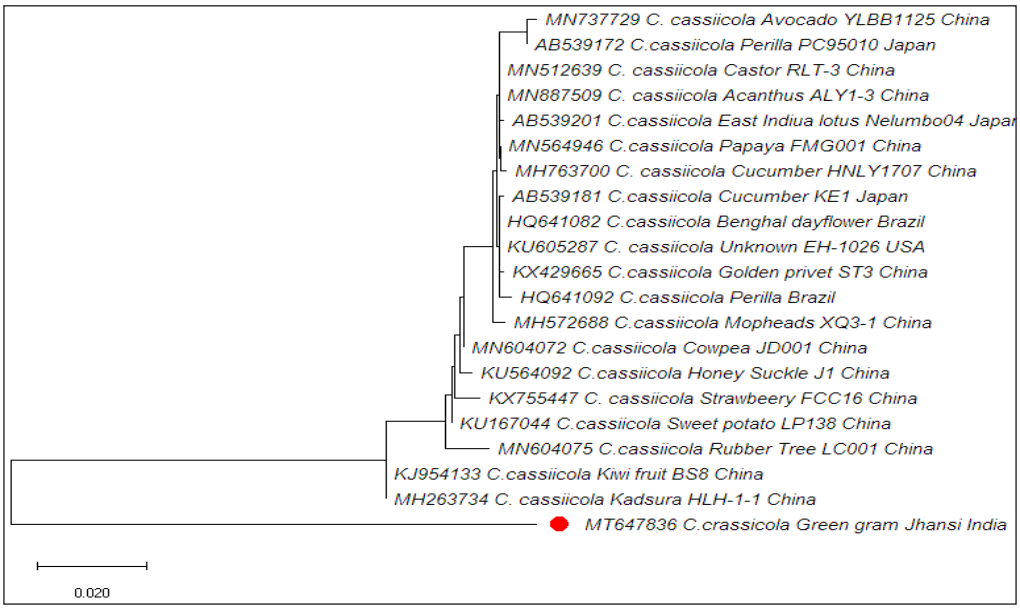

Fig. 8: Maximum likelihood phylogenetic tree generated from β-tubulin (tub A) region of *C. cassiicola* isolated from mungbean and reference sequences from NCBI at bootstrap values of 1000 replicates (MEGA X).

these crops is *C. cassiicola*. Further, there are several reports available for *C. cassiicola* infecting urdbean (Sontirat *et al*. 1991; Naik *et al.* 2014; Gunasri *et al.* 2018) and no reports are available for molecular characterization of *C. cassiicola* on mungbean, therefore, the sequencing and phylogenetic analysis was done only for the mungbean genotype for its confirmation at molecular level.

CONCLUSION

Of the accessions of mungbean (200) and urdbean (100) that were phenotyped under natural field conditions, only 8 lines of mungbean showed moderately resistant reaction while 3 and 6 germplasm lines of urdbean displayed resistant and moderately resistant reaction to the leaf spot disease, respectively.

The pathogen causing fungal leaf spot disease was isolated and characterized morphologically as well as at molecular level. The isolate shared maximum identity (98.53 per cent) with *C. cassiicola* of cowpea reported from Ghana. Further β-tubulin sequence also showed the similarity (88.55%) with *C. cassiicola* isolate from China. Based on the screening of the genotypes, the mungbean genotypes viz. IC 314322, IC 73401, IC 76499, NM 1, DGG 5, VMS-6, ML-2037, ML- 1464 and urdbean genotypes, viz. IC 530501, IC-570221, IC 570263, IC 565276, TPU-99-232, IC 565247, PLU79, PLU81, IC 41718 were identified as potential donors against *C. cassiicola* for their use in mungbean and urdbean programme.

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