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New Report of *Cercospora canescens* Isolates from Coastal Regions of Odisha, India causing Cercospora Leaf Spot (CLS) Disease in Mung Bean (*Vigna radiata* L.)

Sushree Suparna Mahapatra[1], Surjya Kanta Beura[1],
Dhaneswar Swain[2], Kundan R. Jadhao[2], Gyana Ranjan Rout[2]

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ABSTRACT

Background: Cercospora leaf spot (CLS) is one of the major diseases causing a major loss in production of mung bean of up to 60%. Although it is cultivated all round the world, its major contribution is attributed to the Asian continent especially the countries of Indian subcontinents which accounts for about 90% of the world's mungbean production. Due to its high nutritional content, mung bean is a major contributor to the Nation's economy. The present study was to isolate and characterize the new isolates of *Cercospora canescens* collected from coastal belts of Odisha, India.

Methods: Surveys were conducted in the mung bean growing fields of Bhubaneswar (20.26°N, 85.8°E) and Berhampur (19.36°N, 84.77°E), coastal regions of Odisha during 2018-20. New *Cercospora canescens* isolates were collected and characterised through morphological, biochemical and molecular basis.

Result: New *Cercospora* isolates were characterized on the basis of morphological, biochemical and molecular analysis to understand the gravity of the disease. Pathogenicity tests were conducted by artificial inoculation to identify the resistant and susceptible mung bean genotypes. On the basis of molecular analysis (internal transcribed spacer (ITS) region), there was a difference between the two isolates and forming different clades. These isolates were sequenced having the NCBI Acc. No. MZ475049 and MZ475050. Both isolates are different from each other because of transitional substitution of thymine and cytosine. The findings will be a complementary contribution for conservation and better management strategies of CLS disease in mung bean.

Key words: *Cercospora canescens*, Fungal isolates, Legume, Mung bean, Phylogenetic analysis.

INTRODUCTION

Mung bean or green gram (*Vigna radiata* L.) is an important pulse crop belonging to the family Fabaceae. Although it is cultivated all round the world, its major contribution is attributed to the Asian continent especially the countries of Indian subcontinents which accounts for about 90% of the world's mung bean production. India, being the largest producer of mung beans in the world, contributes around 54% of the global mung beanproduction (Singh and Singh, 2011) and has provided the Indian farmers a highly profitable source of income (Pal *et al*., 2021). But infestation of the crop with various diseases and pests poses serious threat to its cultivation and production (Raje and Rao, 2002). Cercospora leaf spot (CLS) caused by the fungus *Cercospora canescens* Ellis andMartin is one of the designated diseases causing a sizable loss in production upto 60% (Bharti *et al*., 2017). CLS disease is marked by typical characteristic symptoms initially on leaves producing irregular to angular lesions with reddish brown margin and yellow halo around the spots with greyish centre. The disease progresses better at 20-26°C and at a relative humidity of 90-100%, which is required for the optimum growth and sporulation of the pathogen (Kumar *et al*., 2011). The fungus *Cercospora canescens* can be identified and characterized based on the morphological structure, molecular characterisation of the genome, nature and intensity of the disease symptom caused on the host plant (Chand *et al*., 2000). Numerous *Cercospora* species till date have been identified based on the studies of the partial sequences of the fungi employing the ITS region because of its higher divergence and faster evolution than other coding regions of the rDNA genes (Crous *et al*., 2013). The analysis of phylogeny is majorly based on the partial sequence of the ITS region of the nuclear ribosomal DNA

[1]Department of Plant Pathology, College of Agriculture, Odisha University of Agriculture and Technology, Bhubaneswar-751 003, Odisha, India.
[2]Department of Agricultural Biotechnology, Odisha University of Agriculture and Technology, Bhubaneswar-751 003, Odisha, India.

Corresponding Author: Gyana Ranjan Rout, Department of Agricultural Biotechnology, Odisha University of Agriculture and Technology, Bhubaneswar-751 003, Odisha, India.
Email: grrout@rediffmail.com

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which is the most commonly sequenced regions for taxonomy and systematic affiliation of the fungi (Feliner and Rossello, 2007). It is regarded as the barcode for identification and characterization of fungi (Schoch *et al*., 2012). The present study is to identify and characterize the isolates of *Cercospora canescens* collected from the coastal regions of Odisha, India for CLS disease in mung bean.

MATERIALS AND METHODS

Sample collection and isolation of fungal isolates

Surveys were conducted in the mung bean growing fields of Bhubaneswar (20.26°N, 85.81°E) and Berhampur (19.36°N, 84.77°E), coastal regions of Odisha to identify plants infected with *Cercospora canescens* by visualising designated symptoms of CLS during 2018-2020. Samples collected from Bhubaneswar and Berhampur were denoted as BBS1 and BAM1 respectively. The infected samples were surface sterilized with 0.8% (v/v) sodium hypochlorite for 2 min. Lesions with dense and ample spores were selected after evaluation under microscope. The collected spore was spread over petri platescontaining 2% water-agar and incubated at 25±1°C for 6 hrs. The germinated spores were identified under a microscope (100×). The pure culture of the isolated fungus was maintained on PDA at 25±1°C and sub cultured as and when required. The cultures were visualised for structure, colour, texture, dimensions of the fungal colonies at different time intervals.

Pathogenicity test

The fungal mass of the pure culture was multiplied by mass production on sterilised parboiled sorghum grain for spore production and multiplication (Chand *et al*., 2013). The pure cultured fungal culture was transferred to the sorghum grains aseptically incubated at 25±1°C. Ten mung bean genotypes (5 highly resistant and 5 highly susceptible) along with Kopergaon as the susceptible check for CLS were artificially inoculated with the spore suspension (10^{-4} spore ml^{-1}) prepared by soaking the colonised sorghum grains in distilled water suspension was uniformly sprayed at 50% flowering stage or 35 days old mung bean plants during evening hours. Light irrigation was given immediately after inoculation to ensure proper humidity and further additional irrigations were applied as and when needed to maintain the moisture level.

DNA Isolation

The isolated fungal pure culture was sub-cultured in potato dextrose broth (PDB) mediumand incubated for 10-12 days at 25±1°C after shaking.The mycelia mats were harvested from the broth by filtration using Whatman No.1 filter paper and the fungal mats were well dried using sterile blotting paper. The freshly harvested mycelium (1 g) was ground to fine powder using liquid nitrogen in pre-chilled mortar and pestle. The fungal DNA was isolated using cetrimide tetradecyl trimethyl ammonium bromide (CTAB) method of DNA extraction and purification (Murray and Thompson, 1980).

PCR amplification

ITS 1 and ITS 4 primers were used for the amplification of the ITS region of the ribosomal nucleic region including the 5.8 rDNA of the fungus. The PCR was carried in 25 µl reaction volume using two primers, ITS1- (5-TCCGTAGGTGAACCTGCG G-3) and ITS4 (5- TCCTCCGCTTATTGATATGC-3) 1µll each, 1µll dNTP, 2.5 µllPCR buffer, 1µll of DNA, 0.5µll PFE polymerase. The PCR parameters were programmed in a Thermocycler (BIORAD, USA) as follows: initial denaturation at 95°C for 3min, primer annealing at 60°C for 40 secs, chain extension and final extension at 72°C for 1 min and 10 min respectively. The PCR amplification products were checked and characterized by gel electrophoresis in a 1×TAE agarose gel at 70V for 1 hr and the amplicons were visualized under UV with Ethidium bromide as staining agent. 100 bp ladder was used as reference standard molecular weight marker.

Elution of DNA fragment and Sequencing

The PCR amplified DNA product was purified using PROMEGA GEL kit and PCR purification kit. The desired DNA fragments obtained through electrophoresis were excised from the gel using a sterilized scalpel by visualising under UV light. The agarose slice containing DNA was transferred to microcentrifuge and incubated after adding membrane binding solution at 70°C to melt the gel. The DNA was recovered from the gel slice and purified through minicolumn assembly. Further, eluted fragment was kept in -20°C for sequencing. Sequencing was performed by a commercial service provider using Sanger sequencing method in 96 capillary array-based ABI 3730×l DNA analyzer. The sequence assembly and alignment were performed using Clustal × tool. The sequence of the fungal isolates was deposited in NCBI gene bank.

Phylogenetic analysis

Phylogeny study was performed by establishing the relationship between the isolated fungal strains with genus *Cercospora* and *Cercospora* related species based on ITS nucleotide sequences using neighbour joining method (Saitou and Nei, 1987) in MEGA 7.0 (Kumar *et al*., 2016).

RESULTS AND DISCUSSION

Morphological characterization

The Cercospora isolates were grown in PDA medium in petri plates for 30 days to evaluate the growth rate. The growth of the fungal colonies was very slow but the radial growth took place between 15 to 21 days of re-culture. The average radial growth of the BBS1 isolate was recorded as 4.2-5.5 cm diameter whereas, BAM1 about 2.8-4.3 cm diameter. The BBS1 fungal colony was marked by white fluffy cottony appearance with a radish brown outer border. Whereas, the BAM1 had a less fluffy growth with pale cream colour and was not marked with red or brown border but had a feathery growth at the margin (Fig 1a,b). Both the isolates had a very slow growth on the artificial medium but did not produce spore on the PDA medium. But the isolates sporulated on

sorghum grains and the spores were characterized with the conidia and branched conidiophore, pale olivaceous, straight to slightly bent and multi-septate. *Cercospora canescens* with slight stroma often with dense fascicle bearing conidiophores which were generally branched, straight, geniculate, bearing few to many septa, pale to medium dark brownin colouration (Videira *et al*., 2017).

Pathogenicity test

Leaf spots formation initiated after 10 days after inoculation. The findings showed that the inoculated plants showed

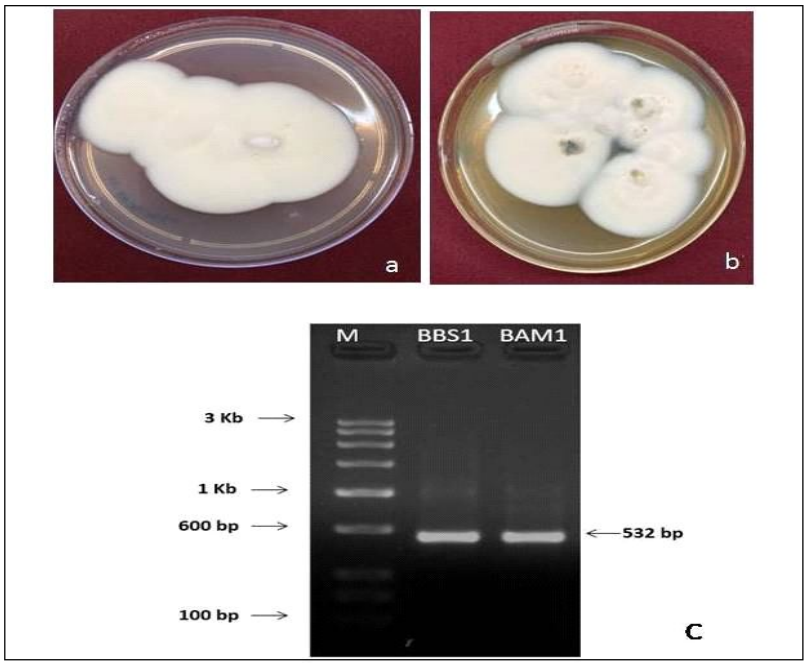


Fig 1: Fifteen-days-old culture of *Cercopora canescens* BBSI isolate (a), BAMI isolate (b) (c) Gel profile of internal transcribes spacer (ITS) region of BBSI and BAMI isolates of *Cercospora canescens*. M represents the reference molecular marker.

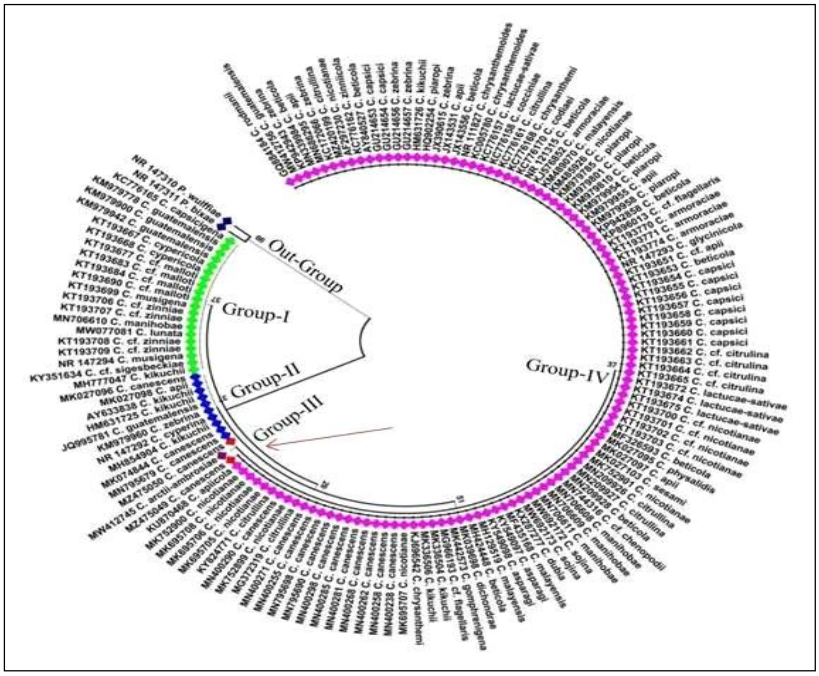
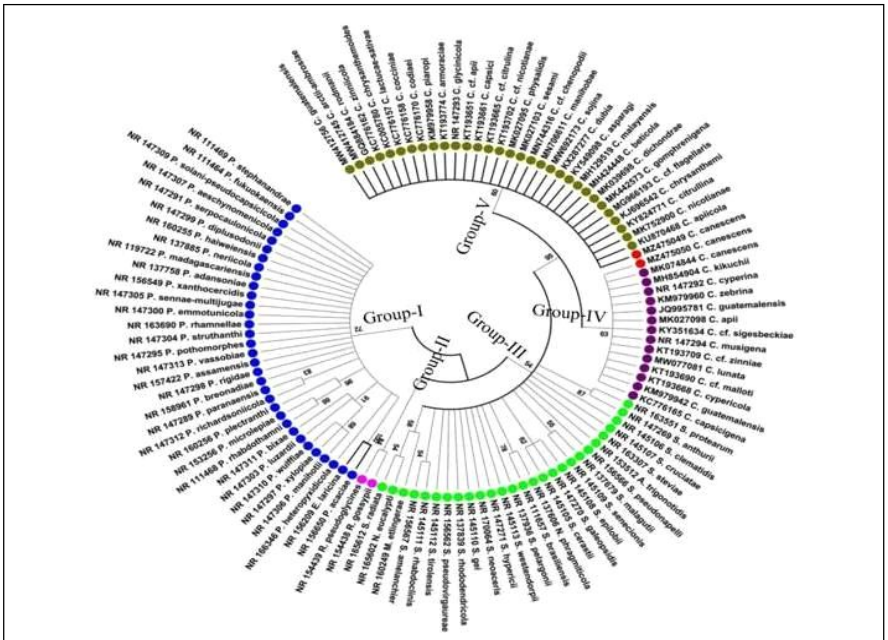


Fig 2A: Phylogenetic tree of germs *Cercospora* based on ITS nucleotide sequences constructed using neighbor joining method in MEGA 7.0. The *Cercospora canescens* isolate of the present study marked in red color in the phylogenetic tree. Black bold font on tree node indicates bootstrap values in per cent.

typical lesions on the leaves which were irregularly circular with dark reddish-brown outer margin with palecentre. The smaller spots further coalesced together to form irregular lesions of yellow chlorotic halo around the brown margin and on maturity formed a greyish centre with spores. The susceptible plants showed higher incidence of the disease with a greater number of spots with greyish centres on the leaves which further led to the infection of the pods.

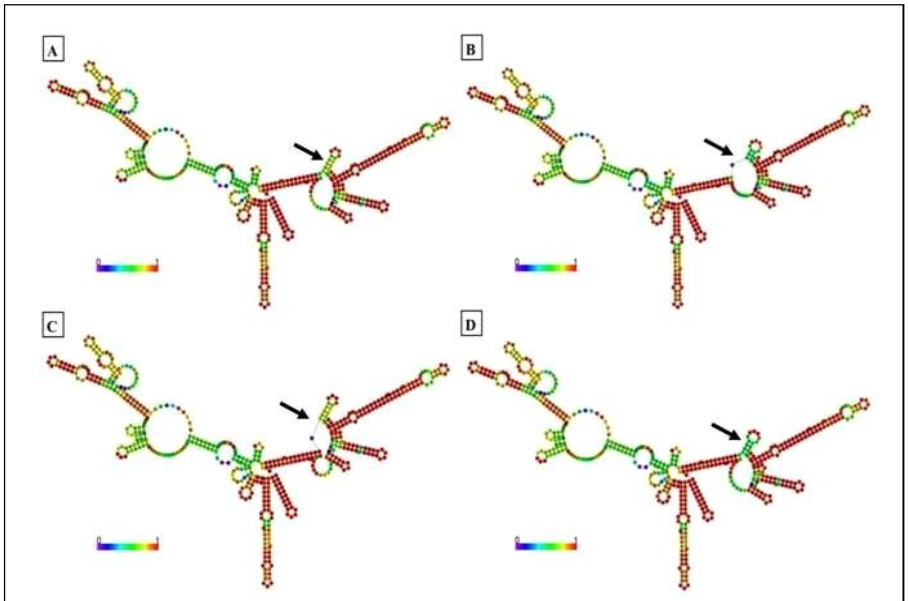
Molecular characterization

Molecular analysis showed that the amplicons separated on agarose gel through electrophoresis showed a similar



The *Cercospora canescens* of the present study is marked in red color in the phylogenetic tree. Black bold font on tree node indicates bootstrap values in pre cent.

Fig 2B: Neighbour-Joining tree representing the phylogenetic relationship of *Cercospora* germs and its closely related genera derived from ITS region in MEGA 7.0.



A: Rrepresents group-I species, B: Represents group-II species, C: Represent group III species, D: Represents group-IV species. Black arrow indicates the variation in helices structures of these species.

Fig 3: Secondary structure model of *Cercospora* genus derived from ITS region with variable sites that distinguish related species in four different groups in pgylogenetic analysis.

banding pattern under UV trans-illumination (Fig 1c). The sequence of the fungal isolates BBS1 and BAM1 were deposited in NCBI gene bank and assigned the accession numbers *viz*. MZ475049 and MZ475050 respectively. The evolutionary history was inferred using the phylogenetic comparison was based on the ITS sequences of the two isolates of *Cercospora* in the present study and the sequences of other related species available in the NCBI GenBank database to infer the genetic relationships among the isolates and establish the level of genetic diversity (Fig 2A). The phylogenetic analyses were performed based on alignments and comparisons of the sequences using ClustalX integrated into MEGA 7 software. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Videira *et al*., 2017). The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) to assess the clade stability are shown next to the branches (Felsenstein, 1985). The dendrogram based on the neighbour joining method in MEGA 7.0 establishing the relationships between different species of *Cercospora* depicts that the species can be delineated into four broad groups namely Group-I, II, III and IV of related species. Among the 4 groups, most of the *Cercospora canescens* isolates are constituted under the group II and IV.The isolate BBS1 (Acc. No. MZ475049) falls under group IV and BAM1 (Acc.No. MZ475050) under group II (Fig 2B). Based on the phylogenetic tree representing the relationships among different *Cercospora* species and other closely related genera, it is implied that genus Cercospora shares common ancestors with other genera like *Septoria, Pseudocercospora, Exutisphaerella, Neodeightoniella, Ramularia*etc (Fig 3). The genera *Cercospora* and *Septoria* were found to share a and more immediate ancestor than between *Cercospora* and *Pseudocercospora* (To-Anun *et al*., 2011). These genera are clustered into 5 different groups based on their relatedness and *Cercospora* species clearly fall under group IV and V (Fig 3). It is evident from the secondary structure model of *Cercospora* genus derived from ITS region with variable sites and parsimony informative

Table 1: Comparison of the parsimony informative sites of genus*Cercospora species.*Red colour indicate the new isolates *i.e*. BBS1 (MZ475049) and BAM1 (MZ475050).

Acc. no.	Species name	T	A	C	Acc. no.	Species name	T	A	C
KT193707	*C. cf. zinniae*	.	.	T	MK752899	*C. nicotianae*	C	.	.
KT193699	*C. musigena*	.	.	T	KU870468	*C. apiicola*	C	.	.
KT193690	*C. cf. malloti*	.	.	T	MG372319	*C. citrullina*	C	.	.
KT193667	*C. cypericola*	.	.	T	MZ475049	*C. canescens*	C	.	.
KM979942	*C. guatemalensis*	.	.	T	KJ696542	*C. chrysanthemi*	C	.	.
KC776165	*C. capsicigena*	.	.	T	MK336506	*C. kikuchii*	C	.	.
MH777047	*C. kikuchii*	.	.	T	MG966193	*C. cf. flagellaris*	C	.	.
KY351634	*C. cf. sigesbeckiae*	.	.	T	MK442573	*C. gomphrenigena*	C	.	.
MW077081	*C. lunata*	.	.	T	MK039698	*C. dichondrae*	C	.	.
MN706610	*C. manihobae*	.	.	T	MH424448	*C. beticola*	C	.	.
Group-I					MH129519	*C. malayensis*	C	.	.
MZ475050	*C. canescens*	T			KY549098	*C. asparagi*	C	.	.
MN795679	*C. canescens*	T	.	.	KX287277	*C. dubia*	C	.	.
NR 147292	*C. cyperina*	T	.	.	MW692173	*C. sojina*	C	.	.
KM979960	*C. zebrina*	T	.	.	MN706608	*C. manihobae*	C	.	.
JQ995781	*C. guatemalensis*	T	.	.	MN744316	*C. cf. chenopodii*	C	.	.
HM631725	*C. kikuchii*	T	.	.	MK027103	*C. sesami*	C	.	.
MK027098	*C. apii*	T	.	.	MK027097	*C. apii*	C	.	.
MH854904	*C. kikuchii*	T	.	.	MK027095	*C. physalidis*	C	.	.
Group-II					KT193700	*C. cf. nicotianae*	C	.	.
MW412745	*C. arctii-ambrosiae*	C	.	T	KT193672	*C. lactucae-sativae*	C	.	.
Group-III					KT193665	*C. cf. citrulina*	C	.	.
KT193770	*C. armoraciae*	C	.	.	KT193661	*C. capsici*	C	.	.
KM979958	*C. piaropi*	C	.	.	KT193651	*C. cf. apii*	C	.	.
KC776170	*C. codiaei*	C	.	.	NR 147293	*C. glycinicola*	C	.	.
KC776158	*C. cocciniae*	C	.	.	NR 111827	*C. chrysanthemoides*	C	.	.
KC776162	*C. zinniicola*	C	.	.	GU214657	*C. zebrina*	C	.	.
GQ884184	*C. rodmanii*	C	.	.	MW412756	*C. guatemalensis*	C	.	.
Group-IV					**Group-IV**				

sites of the genus (Table 1) that the related species can be distinguished into four different groups in phylogenetic analysis, which implies that species differ from one another based on transitional substitution of thiamine (T) and cytosine (C) (Fig 3). New *Cercospora* isolates, BBS1(MZ475049) falls under group IV and BAM1 (MZ475050) falls under group II.

CONCLUSION

The present phylogenetic study showed that clade *Cercospora* appears as a sister group to *Septoria* establishing a closer relationship. *Cercospora* and *Septoria* share similar morphological characteristics in the pattern of conidiophores and structure of conidia. But they differ from each other with respect to production of pycnidial conidiomata by *Septoria* only (To-Anun *et al*., 2011). Although the two isolates BBS1 and BAM1 showed similar banding pattern but they differ from each other with regard to the position of C and T which make them fall into two different groups. This report can be brought in accordance with the fact that single morphological species do not necessarily reflect a single phylogenetic unit (Taylor *et al*., 2000). *Cercospora* and related genera like *Septoria, Pseudocercospora, Exutisphaerella, Neodeightoniella, Ramularia* share a common teleomorph genus *Mycosphaerella.* The *Mycosphaerella* is considered to be a complex constituted of a large number of species of diverse group of plant pathogenic pathogens. It was previously thought to be monophyletic on the basis of the phylogenetic analysis of the ITS region (Goodwin *et al*., 2002). Later on, with advances in the molecular data, it concluded that the genus to be poly- and paraphyletic and closeto the taxa-bearing genera *Ramularia* anamorphs. This investigation will help to identify the occurrence and distribution of new *Cercospora canescens* isolates for CLS disease in mung bean.

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Conflict of interest: None.

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