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Molecular and morphological characterization of *Colletotrichum lagenarium* (Pass.) Ellis and Halsted causing Anthracnose disease in Bottle gourd [*Lagenaria siceraria* (Mol.) Standl.]

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The pathogen was identified by using species primers such as Co-M-3(37)R_1/Co-M-337F_1, used as the primers which are specific to *Colletotrichum lagenarium,* DNA fragment of the expected length (480 bp) was obtained. Among eight solid media tested, potato dextrose agar (86.66 mm) supported the highest growth of *Colletotrichum lagenarium* and maximum sporulation was observed on potato dextrose agar and the sporulation was moderate on rest of the media except Sabouraud's agar in which fair sporulation was observed. The conidia were hyaline, single celled cylindrical in shape and measured 3.2-8.6 × 3.2-4.8 μm in size.

Key words: Characterization, molecula, morphological, *Colletotrichum lagenarium*, Bottle gourd

INTRODUCTION

Bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] is one of the important cucurbitaceous vegetable crops, which belongs to the family Cucurbitaceae. It is a warm season crop, monoecious climber and propagated through seeds. In India, it is cultivated in an area of 149 thousand hectare with production of 2458 thousand metric tonnes (Anon, 2017). In Karnataka, bottle gourd is cultivated in an area of 0.65 thousand hectare with production of 8.36 thousand metric tonnes and (Anon, 2017). The fruits harvested at mature stage are used for making a wide range of articles of common use, including bowls, bottles, containers and musical instruments. As per nutrient data base of USDA 100g of bottle gourd consists water (96%), carbohydrate (2.9%), protein (0.2%), fat (0.5%), mineral matter (0.5%), 20 mg calcium, 10 mg phosphorous, 11mg of vitamin C per 100g fresh weight and traces of vitamin A and vitamin B with a calorific value of 1.2 cal.

The symptoms of *Colletotrichum lagenarium* on leaves, stem, fruits, sometimes on the entire seedling and rarely on stem are found. The symptoms appear as small, raised, water soaked areas on the leaves located along the vines near the crown of the plant. The centre of the spot contains saucer shaped fruiting body acervuli. This disease has been first described during 1867 on gourd fruits in Italy. The bottle gourd anthracnose has been first reported from USA, and is described as *Colletotrichum lagenarium* (Pass.) Ell. and Halst. In India it is first reported by Mundkur on long melon (*Cucumis melo* var. *utilissimus Roxb.*) and bottle gourd (*Lagenaria siceraria* Mol. Standl) to be very serious near Ferozepur in Punjab.

The pathogen requires several specific compound for their growth. A wide range of media can supports the radial growth and sporulation. Various media compositions also influence the different colony morphology of the pathogen. Keeping all these points in view, the present research work was undertaken on morphological and molecular characterization of *Colletotrichum lagenarium* causing anthracnose disease in bottle gourd.

MATERIAL AND METHODS

Isolation of the pathogen

Colletotrichum sp. was isolated from the infected fruit of bottle gourd which was showing water soaked sunken lesions like symptoms. The

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samples were then cut into small pieces and surface sterilized with 0.1 per cent sodium hypochlorite solution for 60 seconds and then washed thoroughly for three times in sterile distilled water under aseptic condition. The samples were then placed aseptically in sterile Petri plates containing potato dextrose agar medium amended with streptomycin sulphat, incubated at 25±2° C for eight days. Further, purification and subculturing of this pathogen was done by following single spore isolation technique. The cultures of *Colleotrichum* spp were maintained at 5-8^{0}C in the refrigerator and subcultured periodically at an interval of one month during the course of investigation.

Proving pathogenicity

The bottle gourd seedlings were raised by sowing healthy seeds in plastic pots, filled with sterilized soil. When plants were 25 days old, they were inoculated with the isolated pathogen. Pin prick method was followed for proving pathogenecity, before inoculation leaves were washed with sterilised water and air dried. The sharp sterilised tooth picks were used for inoculation. The sharp tip of the tooth pick was dipped in one week old cultures separately and then sharp tip of the tooth pick was pressed gently on growing young leaves without creating major injury. The leaves were covered with polythene bags to maintain sufficient humidity and to ensure successful penetration of the pathogen in to the host tissue. Control plants were maintained by atomizing sterile distilled water in the similar manner. Observations were made at regular intervals to know the symptom development on the inoculated leaves up to seven days. The fungus was reisolated and identified from the leaves showing characteristic disease symptoms to confirm the pathogenicity.

The pathogen was reisolated from the artificially inoculated plants and compared with the original culture to confirm the Koch's postulates.

Molecular characterization of the pathogens

DNA of *Colletotrichum* species was extracted from fungal mycelia by following modified CTAB method and used for Polymerase chain reaction by using a set of species- specific oligonucleotide primers. Genomic DNA of the isolate was diluted to 2 ng of 50 ng/µl DNA template, 10 µl of 2X PCR master mix, 1 µl primer and 7 µl of nuclease free water.

Specific primers for identification of *Colletotrichum* sp:

Organism	Primers	Primer sequence
Colletotrichum lagenarium	Co-M-3(37)R_1	5^1 TCCGTAGGTGAACCTGCGG 3^1
	Co-M-337F_1	5^1 TCCTCCGCTTATTGATATGC 3^1
Colletotrichum gleosporoides	MKCgF	5^1 ACGCAAAGGAGGCTCCGGA 3^1
	MkCgR	5^1 ATGGATGCTAGACCTTTGCTGAT 3^1
Colletotrichum capsici	CcapF	5^1 GTAGGCGTCCCCCTAAAAGG 3^1
	CcapR	5^1 CCCAATGCGAGACGAAAT 3^1

PCR amplification was performed in a thermal cycler with the temperature profiles of 95^{0}C for 5 minutes of initial denaturation, followed by 30 cycles of denaturation at 95^{0}C for 30 sec annealing cycle and extension at 72^{0}C for 30 sec with final extension at 72^{0}C for 10 min. After completion of the polymerase chain reaction, the products were subjected for gel electrophoresis to confirm the amplicon.

Seperation of amplified products by agarose gel electrophoresis

1.5 per cent agarose gel was prepared and allowed to cool to 60^{0}C. After cooling, 2 µl of ethidium bromide (10 mg) was added. The solution was mixed and poured into the gel casting platform after inserting the comb. While pouring, sufficient care was taken for not allowing the air bubbles to trap in the gel. The gel was allowed to solidify. Solidified gel was placed into the electrophoresis apparatus containing sufficient buffer (1X TBE) so as to cover the wells completely. Then 15 µl of amplified products were carefully loaded into the sample wells after adding orange dye. Electrophoresis was carried out at 60V until the tracking dye migrated to the end of the gel. Ethidium bromide stained DNA bands were viewed under transilluminator and photographed for documentation.

Morphological characterization of the isolated pathogen

The growth characters of the pathogen was studied on semi-synthetic solid media *viz* potato dextrose agar, oat meal agar, malt extract agar, corn meal agar and synthetic solid media Sabouraud's agar, Richards's agar, Czapek's dox agar and glucose aspergine agar. Fifteen ml of each of the sterilized medium was poured to each Petri plate separately.

Such Petri plates were aseptically inoculated with 5 mm disc cut-outs from periphery of the seven

day old culture and incubated at 25±2 °C for a period of seven days. Each treatment was replicated thrice following completely randomized design. Observations like colony size, mycelia colour, margin of the colony and topography of the colony, colony diameter were recorded .The data on radial growth was analyzed statistically.

RESULTS AND DISCUSSION

Isolation of the pathogen and proving pathogenecity

Colletotrichum lagenarium was isolated from the infected fruit having sunken necrotic lesions.

Pathogenicity test was proved for *Colletotrichum* sp. on bottle gourd plants. Pathogen was artificially inoculated on the leaves by pin prick method. After eight days of inoculation, the typical symptoms of anthracnose disease on the leaves appeared as small sunken necrotic lesions, initially on the lower leaves. Such spots coalesced and developed into irregular patches which dried up eventually. In the early stages, these spots had greyish white centre. The coalesced spots later gave rise to blighted appearance .

The pathogen was reisolated from artificially inoculated leaves and the morphological characters of the reisolated organism were compared with the original cultures of the pathogen and it was confirmed that the causal agent of anthracnose was *Colletotrichum* sp.

Molecular characterization of the isolated pathogen

Species specific primers set Co-M-3(37)R_1/Co-M-337F_1, MKCgF/MkCgR, CcapF/CcapR were used to detect the pathogen as *Colletotrichum lagenarium*, *Colletotrichum gleosporoides* and *Colletotrichum capsci* respectively. When PCR primed with MKCgF/MkCgR and CcapF/CcapR did not amplified DNA, in PCR mixture primers Co-M-3(37)R_1/Co-M-337F_1, used as specific to *Colletotrichum lagenarium,* amplified DNA fragment of the expected length (480 bp) was obtained, which confirmed the template DNA is of *Colletotrichum lagenarium* (Fig. 1). This result is in agreement with Sharma (2018) in which the product with a size approximately 500 bp was obtained.

Morphological characterization of the isolated pathogen

The pathogen *Colletotrichum lagenarium* produced hyaline branched septate mycelium. The conidia were hyaline, single celled cylindrical in shape and

Table 1: Morphological characteristics of Colletotrichum lagenarium on different solid media

Media	Mean mycelial diameter (mm)	Colony character			Sporulation	Pigmentation
		Colony Colour	Colony margin	Topography		
Corn meal agar	46.00	creamy white	Regular	Sparse mycelium	++	-
Czapek's Dox Agar	74.03	white	Uniform	Sparse mycelium	+ + +	Sunrise
Glucose Aspergine agar	54.30	white	Regular	Aerial mycelium	+ +	Light brown
Malt Extract Agar	63.55	dull white	irregular	Aerial mycelium	++	Dark brown
Oat Meal Agar	83.00	bright white	Regular	Compact submerged mycelium	++	Light brown
Potato Dextrose Agar	86.66	dull white	Regular	Profuse aerial mycelium	+ + + +	Light brown
Richard's agar	77.34	light pinkish White	Wavy, irregular	Aerial mycelium	+++	-
Sabouraud's Agar	73.30	creamy white	Regular	Submerged myceliun	+	-
S.Em ±	0.62					
CD (0.01)	1.89					

+Fair, 0-10 conidia per microscopic field + + + Very Good, 20-30 conidia per microscopic field

+ + Good 10-20 conidia per microscopic field + + + + Excellent, more than 30 conidia per microscopic field

measured 3.2-8.6 × 3.2-4.8 *µ*m in size. The saucer shaped fruiting body acervulli was black coloured measuring 12.8-23.6 × 4.65-8.10 size. On the basis of the morphological characteristics, it can be concluded that species closely resembled to *Colletotrichum lagenarium.*

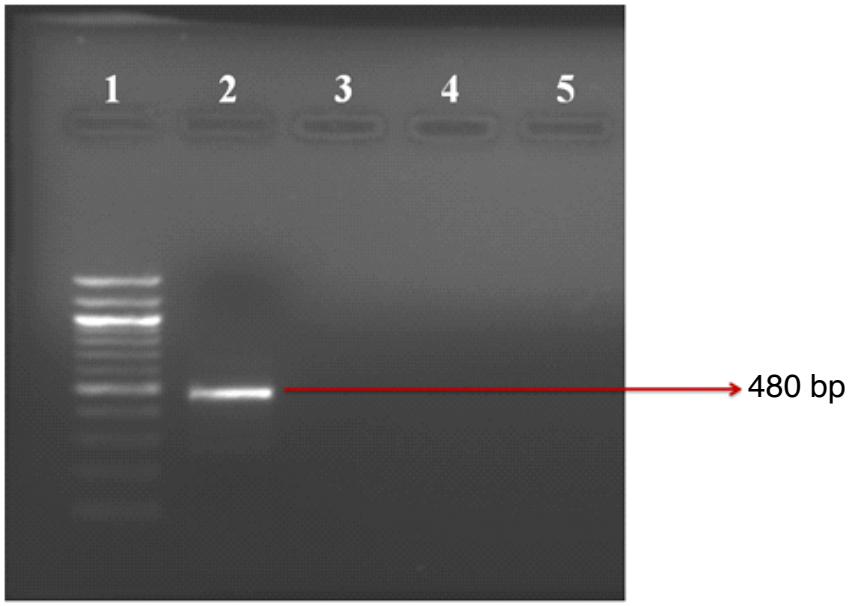
Effect of solid media on the growth of the pathogen

The growth performance of *Colletotrichum lagenarium* was studied on the eight different solid media as described in material and methods. The radial growth of the pathogen was measured and the results are presented in Table 1.

agar, having wavy irregular, irregular, uniform margin respectively. The mycelial growth was sparse in corn meal agar and Czapek's dox agar and it was profuse in potato dextrose agar. Wavy irregular margin was seen in Richard's agar and Sabouraud's agar having submerged mycelium and Czepek's dox agar with uniform colony margin. Oat meal agar produced submerged mycelium.

Maximum sporulation was observed on potato dextrose agar and the sporulation was moderate on rest of the media except Sabouraud's agar in which fair sporulation was observed. Dark brown pigmentation was found in potato dextrose agar

1- DNA ladder 100 bp.
2- Primer for *Colletotrichum lagenarium*
3- Primer for *Colleotrichum capsici*
4- Primer for *C. gleosporoides*
5- Water Control



Amplification of Colletotrichum lagenarium with primer Co-M-337F$_1$ and Co-M-3(37) R $_1$

Fig.1: Molecular identification of *Colletotrichum lagenarium*

Out of eight different solid media evaluated against the growth of *Colletotrichum lagenarium* potato dextrose agar (86.66 mm) recorded highest colony diameter which was significantly superior to other media, followed by oat meal agar (83.00mm) and Richards's agar (77.34 mm). Least growth was observed in corn meal agar (46.00 mm) and glucose aspergine agar (54.30).The results indicated that the pathogens grew well on both synthetic and non-synthetic media (Table1). The data in Table 1 depict that the growth on potato dextrose agar was dull white in colour. Colony margin was regular and with profuse aerial mycelium. All media produced white mycelia except Richard's agar showing light pinkish white coloured mycelia. Margin was regular in most of the media *viz.,*Czapek's dox agar, Richard's agar, malt extract and malt extract agar except Sunrise pigmentation was found in Czapek's dox agar. Corn meal agar, Richard's agar and Sabouraud's agar showed no pigmentation (Table 1).

These result is in agreement with the findings of Sharma (2018) who recorded the maximum growth of *Colletotricum lagenarium* on Potato dextrose agar medium (84.16 mm), followed by Czapek's dox Agar (87.50 mm), while least growth on V-8 juice agar medium (44.33 mm).

The pathogen requires several specific compound for their growth. A wide range of media can supports the radial growth and sporulation. Various media compositions also influence the different colony morphology of the pathogen. In plants,

carbohydrates are available in simple as well as in complex form and fungi convert the complex forms into simple water soluble sugars of low molecular weight before utilisation. In potato dextrose agar media the carbohydrate dextrose is available in simple form which can be easily utilised by the pathogen. Thus potato dextrose agar supported the maximum growth of the pathogens.

The pathogen was identified by using species primers such as when Co-M-3(37)R_1/Co-M-337F_1, used as the primers which are specific to *Colletotrichum lagenarium,* DNA fragment of the expected length (480 bp) was obtained. Among eight solid media tested, potato dextrose agar (86.66 mm) supported for highest growth of *Colletotrichum lagenarium*. The conidia were hyaline, single celled cylindrical in shape and measured 3.2-8.6 × 3.2-4.8 μm in size. Maximum sporulation was observed on potato dextrose agar and the sporulation was moderate on rest of the media except Sabouraud's agar in which fair sporulation was observed.

REFERENCES

Anonymous. 2017. *Horticultural statistics at a glance* p.286

Sharma,V, 2018. *Studies on anthracnose disease of cucumber*. M. Sc. Thesis, Chandra Shekar Krishi Vidyalaya. Palampur (India).