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J. Mycopathol, Res, 54(2) : 245-250, 2016;
ISSN 0971-3719

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J. Mycopathol. Res. ***54(2)*** *: 245-250, 2016;* (ISSN 0971-3719)

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Received : 30.10.2015 RMS Accepted : 16.03.2016 Published : 25.07.2016

Anthracnose is widely distributed at present in all major cowpea growing regions of the state leading to significant yield losses. Eight isolates of *Colletotrichum* spp., incitant of cowpea anthracnose were evaluated for their morphological, cultural variability and molecular characterization using ITS- rDNA sequence analyses. The isolates were tentatively identified as,*C.gloeosporioides* Penz. based on morphological and cultural characters such as nature of growth, colour, branching pattern, septation in hyphae, occurrence of sectors, vegetative and reproductive structures and the characters of acervuli and setae. The ITS- rDNA region of the isolates was amplified which confirmed the precise amplicon size of 540 bp. BLAST similarity search results also indicated that ITS- rDNA sequences of isolates in this study shared 99 - 100% sequence similarity with the existing sequences of *C. gloeosporioides* available in NCBI database. Hence, the experiment evidently distinguished the association of *C.gloeosporioides* with anthracnose of cowpea in Kerala based on morphological and cultural characters, ITS-rDNA sequence analyses and BLAST search in Genbank, facilitating effective disease management approaches.

Key words: Cowpea, anthracnose, characterization, ITS – rDNA, phylogeny

INTRODUCTION

Cowpea [*Vigna unguiculata* subsp. s*esquipedalis* (L.) Verdcourt] is a widely grown vegetable crop in the wetland fallows in Kerala. Due to favourable agro climatic conditions, the crop has gained much importance in Kerala and has come to occupy a prime position among the vegetable crops raised in the state. An array of fungal diseases affects the crop at the various stages of its growth, of which anthracnose caused by *Colletotrichum* spp. pose major concern. Enyiukwu and Awurum (2013) reported that cowpea anthracnose resulted in 50 % grain yield reduction in the affected crop. The disease is usually manifested as circular to irregular brown necrotic spots on leaves, spindle shaped lesions with light grey centre and reddish brown margin on the stem and vines and irregular deep seated reddish brown spots on the pods.

The principal *Colletotrichum* species that affect grain legumes are *C. lindemuthianum*, which has a worldwide distribution affecting common bean, cowpea, soybean and pea; *C. capsici* which is pan

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tropical on cowpea, chickpea, soybean, winged bean and peanut; *C. truncatum*, whose host range includes soybean, cowpea, lima bean, pigeonpea, peanut and lentil; *C. destructivum* and its teleomorph *Glomerella glycines*, affecting soybean and lentil in USA and Asia; *C. gloeosporioides* and its teleomorph *G. cingulata*, reported from pigeonpea, soybean and peanut in Asia and America. The association of several species of *Colletotrichum* with anthracnose of cowpea points out the complexity of the disease and foretell the chances of development of more severe and virulent strains through interspecific hybridization, mutation etc. which can lead to severe epiphytotics.

To understand the present plant disease situations and to predict the possible future development it is essential to learn as much as possible about the morphological and cultural variability in fungi that are pathogenic to plants (Chandran and Kumar, 2012). However, precise identification of species cannot be done due to variations in the phenotypic characters among species under various environmental conditions. The use of molecular marker techniques has improved the accuracy and speed of identification and classification of phytopathogenic fungi (Cai *et al,* 2009). The rDNA genes have been employed to analyze major evolutionary events because it is highly conserved, whereas the rDNA internal transcribed spacer (ITS 1 and ITS 2) is more variable so that it has been used for the investigation of the species level relationship and has been used in classifying fungal species due to its systematic and taxonomic usefulness.

Sequence analysis from ITS region has made progress towards a better understanding of the taxonomy of *Colletotrichum* spp. (Cunnington *et al*, 2004). Molecular analysis based on sequences of rDNA internal transcribed spacers (ITS 1 and ITS 2) of *Colletotrichum* spp. paralleled the morphological and cultural groupings too (Morlwakll *et al*, 2002 and Photita *et al,* 2005)

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Hence, keeping in view the importance of the crop and the severity of the disease, present study was taken up to investigate the morphological and cultural variability as well as molecular characterization through partial sequencing of Internal Transcribed Spacer (ITS) region of rDNA among the isolates of *Colletotrichum* spp. causing anthracnose of cowpea for the successful management of the disease.

MATERIALS AND METHODS

Survey, collection and isolation of pathogen associated with anthracnose of cowpea

Potential cowpea growing areas of Thiruvananthapuram district of Kerala were periodically surveyed during 2012 – 2013 and samples showing typical anthracnose symptoms were collected. The infected tissues of the leaves and twigs were cut into small bits of one to two mm size and surface sterilized in 0.1 % mercuric chloride ($HgCl_2$) solution for one min and washed thrice in sterile distilled water before transferring them into sterile Petridishes containing solidified potato dextrose agar (PDA) under aseptic conditions. The plates were incubated at room temperature and the fungal growth that appeared on plates were purified by single spore isolation technique and transferred to PDA slants.

Morphological and cultural characterization of Colletotrichum spp

The nature of growth, colour, branching pattern and septation in hyphae of the different isolates of *Colletotrichum* spp. were recorded after seven days of incubation on PDA. The occurrence of sectors, vegetative and reproductive structures and the characters of acervuli and setae were described. Colony diameter was recorded daily for a week and the growth rate was calculated as the seven day average of mean daily growth (cm/day). The size and shape of conidia were measured using an image analyzer (Motic images plus 2.0 software). Color of the conidial mass and zonation were also recorded from the colonies grown on PDA plates at room temperature. The shape of appressoria was studied using a slide culture method modified from Hawksworth (1974).

Molecular characterization through DNA extraction and PCR amplification of ribosomal DNA internal transcribed spacer (ITS) regions of Colletotrichum

About 100 mg mycelia of each isolate of *Colletotrichum* spp. were harvested and powdered by liquid nitrogen and transferred to a microtube containing 600 µl of 10% CTAB buffer

and incubated at 65^{0}C for 1 h. An equal volume of phenol and chloroform (1:1) was added to the mixture and centrifuged at 12000 rpm for 10 min at 4^{0}C. To the supernatant 350 µl of ethanol was added and incubated at 20^{0}C overnight to precipitate DNA. The contents were centrifuged at 12000 rpm for 5 min and the DNA pellets were washed twice with 70% ethanol, dried and dissolved in 50 µl TE buffer (Than *et al*, 2008).PCR amplification reaction was carried out in a 20 µl reaction volume which contained 1X PCR buffer (contains 1.5 mM $MgCL_2$), 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 10 ng DNA, 0.4 µl DNA polymerase enzyme (Thermo scientific), 0.1 mg/ml BSA and 5 pM of forward and reverse primers. The primers used were ITS-1 (TCCGTAGGTGAACCTTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) (White *et al,*1990). The amplification reaction was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) with an initial denaturation of 30 sec at 98^{0}C, followed by 40 cycles of 5 sec at 98^{0}C, 10 sec at 60^{0}C and 15 sec at 72^{0}C, with a final extension for 60 sec at 72^{0}C. The PCR products were checked in 1.2 % agarose gels prepared in 0.5 X TBE buffer containing 0.5 µg/ml ethidium bromide. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

Sequence alignment and submission of sequences in NCBI

DNA sequencing reaction was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufacturer protocol. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al,* 2010). The identity of ITS-rDNA conserved region of the pathogen isolates associated with anthracnose of cowpea was established by performing a similarity search using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) database and the sequences were matched with existing available database for species confirmation. Based on the sequence matching results, the rDNA sequences were bankitted in the NCBI database and accession numbers were obtained.

Phylogenetic analysis

The data set based on the ITS- rDNA region of the pathogen isolates associated with anthracnose of cowpea and other *Colletotrichum* reference sequences were retrieved from NCBI Genbank database (USA) and compared. Multiple sequence alignment was done using ClustalW2 and phylogenetic analysis through Phylogeny.fr software (Dereeper *et al,*2008). A phylogeny tree was constructed using neighbour-joining (NJ) method. All traits had equal weight and gaps were treated as 'missing' values. Transitions and transversions were included in the analysis. The branch support of the trees resulting from the neighbour- joining (NJ) analysis was assessed by bootstrapping with 1000 replicates using the heuristic search option and indicated at the nodes as percentage.

RESULTS AND DISCUSSION

Eight isolates (C1 to C8) of *Colletotrichum* spp. were obtained from infected samples collected from major cowpea growing areas of Thiruvananthapuram district (Table 1). The colonies appeared sparse, dense or fluffy with whitish aerial mycelium becoming grey. Colony reverse was light-dark greyish to black. Light orange colored slimy spore mass were produced either outward from the centre of the colony or near the inoculation point. Black acervuli and abundant setae were observed. While, sporulation was sparse and acervuli and setae were lacking in C4 and C6. The mycelia appeared hyaline, septate and branched. Conidia were hyaline, straight, cylindrical with both apices rounded or with one apex rounded and the other end pointed. Size of conidia varied from 8.6 - 11.3 x 3.5 - 4.3 µm. Appressoria formed were irregular, clavate or ovoid and light to dark brown in color (Table 2).The nature of growth, colour, branching pattern, septation in hyphae and the characters of acervuli and setae of different iso-

Table 1 : *Colletotrichum* isolates obtained and their respective locations

Isolate code	State	Location
C1	Kerala	Vellayani
C2	Kerala	Pappanchani
C3	Kerala	Kalliyoor
C4	Kerala	Balaramapuram
C5	Kerala	Pothencode
C6	Kerala	Azhur
C7	Kerala	Kattakada
C8	Kerala	Kulathoor

Table 2 : Morphological and cultural characters of *Colletotrichum* isolates inciting anthracnose of cowpea

Isolate No.	No. of days taken to cover 9 cm Petridish	Average growth rate (cm/day)	Colony texture	Colony colour		Acervulate (Yes/No)	Setae	Perithecia	Conidia			Shape of appressoria
				Upper	Lower				Shape	Average length (µm)	Average breadth (µm)	
C1	6	1.38	Sparse	Initially white turning to grey	Dark greyish to black	Yes	Present	Scattered and globose	Cylindrical with round apices	9.8	4.3	Irregular
C2	7	1.21	Sparse	Initially white turning to grey	Dark greyish to black	Yes	Present	Not formed	Cylindrical with round apices	10.3	3.8	Irregular
C3	6	1.38	Sparse	Initially white turning to grey	Dark greyish to black	Yes	Present	Scattered and globose	Cylindrical with round apices	9.0	4.2	Irregular
C4	9	1.04	Dense	Off-white	Grey	No	Absent	Not formed	Straight obtuse apex	8.6	3.6	Clavate/ Irregular
C5	6	1.39	Sparse	Initially white turning to grey	Dark greyish to black	Yes	Present	Scattered and globose	Cylindrical with round apices	11.3	3.7	Irregular
C6	10	0.80	Dense	Off-white	Grey	No	Absent	Not formed	Cylindrical with a pointed end	8.8	3.7	Clavate
C7	7	1.35	Dense	White turning to grey White	Dark grey	Yes	Present	Scattered and globose	Cylindrical	9.5	3.5	Irregular /ovoid
C8	7	1.30	Dense	turning to grey	Dark grey	Yes	Present	Not formed	Cylindrical	9.6	3.8	Irregular/ ovoid

lates of *Colletotrichum* spp. obtained in this study were in accordance with the characters of *C. gloeosporioides* (Chowdappa *et al,* 2012). The conidial measurements of isolates examined in this study also fit within the measurements of spore size of *C. gloeosporioides* reported previously (Chowdappa *et al,* 2012). Thus, based on the morphological and cultural characters as well as in comparison with the standard keys the isolates of the pathogen associated with anthracnose of cowpea were tentatively identified as, *C. gloeosporioides* Penz. *C. gloeosporioides*, is a species complex comprising morphologically indistinguishable but genetically isolated species and has been reported on broad range of hosts (Cai *et al,*2009). The identification of *C. gloeosporioides* is mainly based on conidial morphology, which is extremely variable. Wide variations in cultural and morphological characters, pathogenicity and host range have been reported among isolates of *C. gloeosporioides*.The identity of the species was further confirmed through ITS- rDNA sequence analyses. The internal transcribed spacer region of rDNA is the most used target sequence in the

Table 3 : *Colletotrichum* isolates and their identity as revealed by molecular characterization using ITS- rDNA sequence analysis

Isolate code	Species identification	GenBank Accession No.	Matching organism in NCBI GenBank with accession number	Identity (%)
C1	*C. gloeosporioides*	KJ584648	*C. gloeosporioides* FJ172225.1	99
C2	*C. gloeosporioides*	KJ584649	*C. gloeosporioides* FJ172225.1	99
C3	*C. gloeosporioides*	KJ584650	*C. gloeosporioides* FJ172225.1	99
C4	*C. gloeosporioides*	KJ584651	*C. gloeosporioides* KF053199.1	100
C5	*C. gloeosporioides*	KJ584652	*C. gloeosporioides* FJ172225.1	100
C6	*C. gloeosporioides*	KJ584653	*C. gloeosporioides* JX258732.1	99
C7	*C. gloeosporioides*	KJ584654	*C. gloeosporioides* JX161648.1	100
C8	*C. gloeosporioides*	KJ584655	*C. gloeosporioides* JX161648.1	100

molecular detection of fungi and is also the most employed marker used to infer lower level taxonomy in fungi (Bruns, 2001). The amplification of the ITS- rDNA region of eight isolates of *Colletotrichum* spp. using universal primers ITS 1 and ITS 4 yielded an amplicon of approximately 540 bp long which was in accordance with the findings of Chowdappa *et al,* (2012) confirming that all the isolates were of genus *Colletotrichum*. Adhipathi *et al,* (2013) had also attempted the amplification of 5.8S rDNA region for molecular based confirmation of *C. capsici*. Photita *et al.* (2005) noted that the correlation between morphological and molecular based clustering demonstrated the genetic relationships among the isolates and species of *Colletotrichum* and indicated that ITS rDNA sequence data were potentially useful in taxonomic species determination. Sequences of *Colletotrichum* isolates were deposited in GenBank and accession numbers were obtained. The BLAST similarity search confirmed the results obtained as the ITS - rDNA sequences from *C. gloeosporioides* in this study shared 99 - 100% sequence similarity with the existing sequences of *C. gloeosporioides* available in NCBI database (Table 3). Confirmation of the identity of *Colletotrichum* species using sequence data from ITS-rDNA region had been attempted by other workers as well (Forseille *et al*, 2011; Arzanlou and Torbati, 2013; Raj *et al*, 2013).Phylogenetic analysis grouped the *Colletotrichum* isolates into three clusters with *Pyricularia grisea* (FN555114.1) as an outgroup strain. All *Colletotrichum* isolates grouped in cluster I included *C. gloeosporioides* isolates in this study *viz.,* C1, C2, C3 and C5 along with three reference isolates from Genbank; KJ002768.1 (*C. lindemuthianum*), JX669450.1 (*C. gloeosporioides*) and FJ172225.1 (*C. gloeosporioides*) with 50 % bootstrap support. The top of cluster II had three *C. gloeosporioides* isolates; C4, C7 and C8 grouped

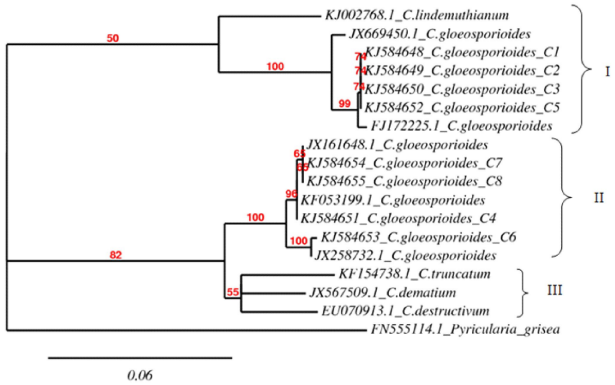

Fig. 1 : Phylogenetic tree generated from ITS- rDNA sequences of *Colletotrichum* spp. by Neighbour Joining (NJ) analysis (Scale bar = 0.06 substitutions per site)

with reference isolates; JX161648.1 and KF053199.1 with 95 % bootstrap support and the other branch of this cluster comprised of isolate C6 and the reference isolate JX258732.1 with 100 % bootstrap support. Cluster III included other principal *Colletotrichum* reference isolates; KF154738.1 (*C. truncatum*), JX567509.1 (*C. dematium*) and EU070913.1 (*C. destructivum*) infecting grain legumes, with 55 % bootstrap support (Figure 1).The phylogenetic tree constructed from ITS- rDNA sequences using neighbor-joining (NJ) method grouped the isolates of *C. gloeosporioides* into two clusters indicating the complexity of the species while, other principal *Colletotrichum* spp. infecting grain legumes were grouped into a third cluster. The results were in accordance with the findings of Adhipathi *et al,* (2014) whom attempted the amplification of ITS region and sequencing of*C. gloeosporioides* causing leaf spot disease in turmeric. Similar attempts had been performed by other workers as well (Photita *et al*, 2005; Forseille *et al*, 2011; Arzanlou and Torbati, 2013 and Raj *et al*, 2013). Hence, the present study concluded the association of *C. gloeosporioides* with anthracnose of cowpea in Kerala and these findings will enable in formulating effective management strategies for disease control thereby avoiding heavy crop losses.

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