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Research Paper

First report of Phyllosticta capitalensis as one of the associated fungus of blight disease of large cardamom

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ABSTRACT
Large cardamom, an important spice and cash crop of Himalayan region of Indian-subcontinent is under severe crisis mainly due to the advent of new fungal diseases in the last two decades. The crop dies or the production decreases considerably within 4-5 years of plantation contrary to 20-25 years in earlier times. Number of fungal disease has been reported affecting the large cardamom at various levels. The present study revealed a new associated fungus which has been repeatedly isolated along with the already reported pathogenic fungus, Colletotrichum gloeosporioides. The identity of new associated fungus causing blight disease in large cardamom is established by morphological and molecular characterization as Phyllosticta capitalensis, asexual state of Guignardia mangiferae. Though, the P. capitalensis is an endophyte and is considered as weak pathogen in most of the host, further systematic testing of pathogenicity in large cardamom is needed to know the actual virulence level.

KEYWORDS: *Large cardamom, blight disease, Colletotrichum, P. capitalensi, ITS, BLASTn*

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I. INTRODUCTION

Large cardamom (*Amomum subulatum* Roxb.) belonging to the family Zingiberaceae is an important cash crop of the Sikkim Himalaya. It is believed to be originated in this region and has high concentration of cultivated varieties. Now, this spice crop is also cultivated in other hilly states of India including Uttarakhand and North Eastern states, mainly imported from Sikkim. Apart from hilly Indian states, countries like Nepal and Bhutan is also involved in large scale cultivation. The seeds encapsulated in capsules are aromatic and are widely used as flavoring agent in food, confectioneries and ayurvedic preparations. The dried large cardamom capsules are largely exported to other countries and considered as one of the foreign revenue earner horticulture commodity. However, due to the advent of fungal diseases in addition to the pre-existing two viral disease, 'Chirkey' and 'Furkey' infecting large cardamom crop, the production and quality decreased considerably during the last two decades. The infected plant produces fewer capsules which never mature. The infected seeds are whitish in color instead of black and are less aromatic. The farmers involved in the cultivation of large cardamom are directly affected due to the infestation of fungal disease as the plants starts dying during the peak year of fruiting. The production decreases drastically following years and whole field dies which appears as burnt-up. Baring few places, every cardamom field is infested with fungal disease. The first fungal disease appeared in 1999, as per farmers' representation, somewhere in Thoday-Tangta of Kalimpong sub-division of Darjeeling district near Indo-Bhutan border (Saju, 2010). The 'Varlangey' cultivars brought from this region was found to be infected with fungal disease. It is suspected that the native pathogen have developed virulence when it infected more susceptible cultivars and might have undergone genetic changes in new host due to highly favorable climatic conditions.

It was first reported that the leaf blight of large cardamom is caused by *Colletotrichum* state of *Glomerella cingulata* (Stoneman) Spauld. & SchrenK) from kitchen garden of Muzaffarpur, Bihar (Prasad et al., 1984). Later, it was described as anthracnose caused by *G. cingulata*, perfect state of *C. gloeosporioides* from Sikkim, the traditional area of crop origin and cultivation (Srivastava, 1989). The disease appears generally with the advent of pre-monsoon showers followed by clear sunny days during March-May and progress rapidly during the rainy season (Saju, 2013). First, water soaked lesions appear on leaves which enlarge rapidly, coalesce and cover major or entire lamina giving blighted appearance (Pun et al., 2006). In most cases, the

lesion on the pseudostem becomes necrotic and as a result the entire leaves dry out giving burnt-up appearance (Saju, 2013). Other fungal diseases of lesser prevalence such as collar rot(*Fusarium oxysporum*), leaf streak (*Pestalotiopsis versicolor* and *P. royenae*) and leaf rust (*Phakospora elettariae*) had also been recorded.

Further investigation was carried out to find out any other associated fungus causing blight disease in large cardamom. It has been found that a new fungus is repeatedly isolated along with the *Collectotrichum gloesporioides* from the blight region of the infected cardamom plant. Microscopic observation also revealed the presence plenty of semi-circular spores different from *Collectotrichum* spores on the infected leaves. The details of investigations and establishment of organism identity carried out through morphological and molecular characterization are described in this paper.

II. MATERIALS AND METHOD

Isolation of pathogenic fungi

The infected leaves of large cardamom showing the symptoms of blight disease were collected from the germplasm maintained at Biotechnology Research and Extension Centre at Sajong, Rumtek, East Sikkim. The samples were brought to the molecular biology laboratory at Vigyna Bhawan, Deorali, Gangtok, East Sikkim. The infected regions of leaves were washed, dried with tissue paper and cut into 1cm.sq. pieces. They were dipped in 0.1% Mercuric Chloride for 30 second and then rinsed with sterile distilled water thrice. Each surface sterilized pieces were cultured on Potato Dextrose Agar (PDA) at 28^{o}C. Based on the type of fungal colony characteristics, the mycelium were picked with the help of forceps tip and sub-cultured in fresh media for pure isolation. Each isolate were maintained with subsequent sub-culture for further investigation.

Microscopic observation

Mycellia and the spores were observed under compound microscope (Leica DM 300, Germany) after staining with lactophenol-cotton blue.

Isolation of fungal DNA

The DNA was isolated from the 4-6 days old mycelia depending on the growth of each isolate. The protocol followed by T.R. Prabha was adopted to isolate the fungal DNA. The quality and quantity of isolated DNA was checked with Biospectrophotometer (Denovix, USA) at 260/280nm. The DNA having optical density (OD) value of around 1.8 is taken for PCR. The quality of DNA was also checked in gel electrophoresis using 1.2% agarose gel prepared in 1X TAE.

PCR and primers

The ITS-1(5'-TCCGTAGGTGAACCTGCGG-3') & ITS-4(5'-CCTCCGCTTATTGATATGC-3') were used as forward and reverse primer respectively. The PCR conditions for amplification of target region were set as; initial denaturation at 95^{o}C for 5 min. followed by 38 cycles of denaturation at 95^{o}C for 30 Sec., primer annealing at 55^{o}C for 1 min. and extension at 72^{o}C for 1 min. Final extension at 72^{o}C for 6 min and infinite hold at 4^{o}C. The PCR product was visualized in 1.6% gel aligned with 100 bp DNA ladder.

Sequencing and BLASTn

The gene sequencing was done by Biokart India Pvt. Ltd, Bangalore, India duly adopting Sanger dideoxy sequencing method. The sequences generated by reverse and forward primer were used to generate consensus sequence using Bioedit software. BLASTn search tool of NCBI was used to compare the consensus sequence with the nucleotide sequence from GenBank.

III. RESULTS AND DISCUSSION

Culture of blight infected leaves of large cardamom on PDA repeatedly resulted in the development of two distinct culture colonies, one totally black compact and another fluffy off-white. Sometimes, a single sample produces two distinct regions one developing on each side. Microscopic observation revealed that off-white isolate was C. *gloesporioides* whereas black region shows different types of semi-circular spores. Microscopic observation of blight infected leaves also shows similar type of spores. In order to establish the identity of the organism, the gene sequences of the black isolate and fluffy off-white isolate were subjected to BLASTn run in NCBI GenBank. BLASTn run result of black isolate revealed 100% similarity with *Phyllosticta capitalensis*. The query cover is 100% and e value 0.0. It also shows 100% gene identity with *Guignardia mangiferae* which is the sexual state of *P. capitalensis*. Whereas, the BLASTn result of off-white isolate shows 100% gene identity with C. *gloesporioides*. The partial gene sequence of both the organism i.e. C. *gloesporioides* and *P. capitalensis* were submitted to NCBI GenBank and published under accession no. **MZ148583** and **MZ076514** respectively. Morphological comparison of culture colony, mycelia, conidia/spore of the new fungus also shows similarity with the *P. capitalensis*. Thus, the identity of a new associated fungus

of Large Cardamom blight is morphologically and molecularly identified as *Phyllosticta capitalensis*, asexual state of *Guignardia mangiferae.* Though, the *P. capitalensis* is an endophyte and is considered as weak pathogen in most of the host, further systematic testing of pathogenicity in large cardamom is needed to know the actual virulence level.

IV. CONCLUSION

Large cardamom blight is caused by number of fungi either as primary infection or as opportunist fungus which has its own significance in causing disease in the long run. *P. capitalensis* is found to be one of the associated fungus of large cardamom blight disease. The actual level of pathogenicity needs further investigation.

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