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Association of Arthrinium Species as a New Fungal Disease of Large Cardamom

Kul Bahadur Subba[1], Arundhati Bag[2], Bharat C. Basistha[3]

[1]PhD scholar, Sikkim Manipal University, 5th Mile, Tadong, E. Sikkim, Gangtok, India & Scientific Officer, Department of Science and Technology, Sikkim, India
[2]Head, Medical Biotechnology, Sikkim Manipal University, 5th Mile, Tadong, E. Sikkim, India
[3]Principal Director, Department of Science and Technology, Government of Sikkim, Vigyan Bhawan, Gangto, E. Sikkim, India

Abstract

Amomum subulatum Roxb., commonly known as Large Cardamom or Black Cardamom, is an important cash crop of Himalayan region of Indian sub-continent. Due to various factors including the infestation of crop by fungal and viral disease, the production and productivity has decreased considerably which largely affected the farmers depending on this crop. Number of fungal disease has been reported to be associated with the crop. The microscopic exploration of infected region of leaves, their isolation and further molecular characterization revealed the association of *Arthrinium* species, a new un-reported fungus. In order to know the virulence level of this associated fungus, further investigation is needed. The detail description of the finding of is presented in this research paper.

Keywords: Large Cardamom, Fungal Disease, Gene Sequencing, Molecular Characterization

Introduction

Amomum subulatum Roxb., belonging to the family Zingiberaceae is a rhizomatous spice crop grown in the humid Himalayan region of Indian sub-continent. It originated in trans-Himalayan region of Eastern Nepal and present day Indian state of Sikkim in ancient times. Lepchas, the aboriginal tribes of Sikkim are believed to be the first to collect wild capsule mainly for food and medicine. Roxburgh collected the plant from Eastern Nepal and described in his book “Plants of the Coast of Coromandel”. It is a shade loving plant and prefers to grow near the perennial streams in humid areas of hilly regions. There are number of cultivar evolved naturally over a period of time. It is commonly known as Large Cardamom or Black Cardamom due to its large capsule which contains numerous aromatic seeds. The dried capsules are mainly used in food preparations, medicine and confectioneries industries. It is an important cash crop of Sikkim, an Indian Himalayan state. The crop is also grown in Nepal and Bhutan.

Due to various biotic and abiotic factors, number of fungal disease has emerged in addition to pre-existing two viral diseases. Some of the fungal disease has developed high virulence and greatly affected the production and productivity. Among the number of fungal disease reported, *Colletotrichum gloeosporioides* is considered as main causal organism causing blight disease in Large Cardamom. The disease first appeared, as per farmers’ representation, in Thoday-Tangta under Kalimpong sub-division of Indian state of West Bengal (Saju, 2010). 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). It was, later, 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 fungal disease causing collar rot (*Fusarium oxysporum*), leaf streak (*Pestalotiopsis versicolor* and *P. royenae*) and leaf rust (*Phakospora elettariae*) of lesser prevalence has also been reported.

New pathogenic fungus *Epicoccum nigrum* was found to be associated with Large Cardamomthrough morphological and molecular characterization (Gurung, 2020). Some other pathogenic fungus recorded were *Pestalotiopsis maculans, Verticillium lecani, Curvularia eragrostidis, Colletotrichum gloeosporioides* and *Phoma carva* (Gurung, 2020). It has been observed that among the six varieties of *A. subulatum* grown in Sikkim, *Varlangey*, *Sawney*, *Ramla* and *Ramsey* were found to be blight infected (Gurung, 2020).

The microscopic observation of infected leaves revealed the presence of numerous medium sizes roundish spores unlike the spores of reported *C. gloeosporioides* on many occasions. On further culture, microscopic observation and molecular characterization, it is identified as species belonging to genus *Arthrinium*. The detail of report of new fungal species associated with large cardamom is described in this paper.

The genus *Arthrinium* belonging to Apiosporaceae in Xylariales under class Sardariomycetes has number of species which are well-known plant pathogens, endophytes or saprobes. Among the pathogenic *Arthrinium*, the *A. phaeospermum* is a widely distributed pathogenic fungus infecting wide range of hosts. The host not only includes plants but also humans and animals, (Shujiang, 2020). This pathogenic fungus infects the plant species like cowpea, garden pea, French bean, sugarcane, bamboo etc.

Material and methods

Microscopic observation

The infected leaves samples were collected from different locations and brought to the laboratory for microscopic observation and isolation of fungus. The infected leaves were scrapped with surgical blade and placed on the lactophenol cotton blue solution. It was observed under the compound microscope (Leica DM 1000). The size of the spore was measured with the scale mounted on microscope.

Isolation of fungus

The infected leaves were lightly scrapped using surgical blade and washed with sterile distilled water. The spores were collected on the sterile distilled water. The floating spores were pipetted with sterile Pasteur pipette and placed over the petridish containing potato dextrose agar (PDA). The cultures were allowed to grow at 28^{o}C. After 6 days, they were sub-cultured on fresh PDA.

Microscopic observation

The spores/conidia and mycelium were observed under compound microscope.

Isolation of fungal DNA and Polymerase chain reaction

Isolation of fungal DNA

The fungal DNA was isolated from the 4-5 days old mycelia after sub-culture. 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 PCR amplification was carried out using primer; ITS-1(5'-TCCGTAGGTGAACCTGCGG-3') & ITS-4(5'-CCTCCGCTTATTGATATGC-3') as forward and reverse primer respectively. The PCR conditions for amplification of target region were set as; initial denaturation at 95ºC for 5 min. followed by 38 cycles of denaturation at 95ºC for 30 Sec., primer annealing at 55ºC for 1 min. and extension at 72ºC for 1 min. Final extension at 72ºC for 6 min and infinite hold at 4ºC. The PCR product was visualized in 1.6% gel aligned with 100 bp DNA ladder.

Sequencing and BLASTn

The gene sequencing was carried out by Biokart India Pvt. Ltd, Bangalore, India by 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.

Results and Discussion

The microscopic observation of infected region of leaves of large cardamom revealed the presence of numerous roundish spores in number of samples. These spores are unlike the spores of C. gloeosporioides, the main causal organism of blight disease in large cardamom. The cultures of these spores have produces large size mycelium and also produced numerous similar spores in the cultures. Some of the old cultures of about 20-25 days old produced large conidia of about 25 µm by 20 µm with equatorial slit, the characteristic features of Arthrinium species.

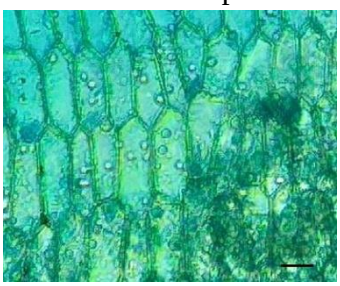

Figure 1: Microscopic observation of spores on infected leaf surface(Bar=20µm)

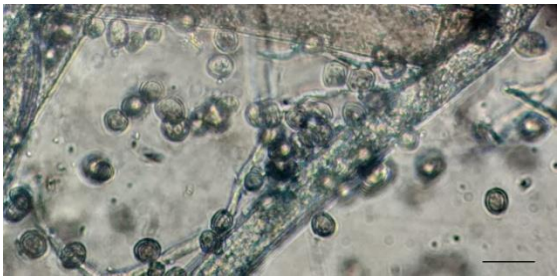

Figure 2: Microscopic observation of spores formed in cultures (Bar=20µm)

Figure 3: Microscopic observation of conidia formed in cultures (Bar=20µm)

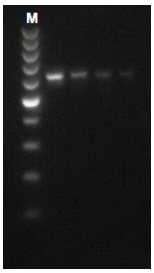

Figure 4: PCR product of *Arthrinium* sp. (M=Marker)

Table 1: **Microscopic Observation of Different Types of Spores Present on the Infected Leaf Surface**

Sample ID	Roundish spore (4-8 µm)	Ovoid to fusiform spore/conidia (15-25µm length & 6-10µm width)	Round spore (3-5 µm)	Cylindrical round end spore (12µm length and 6µm width)	Oval shaped conidia tapering at end, septate(20µm by 3.5µm)
GPC-01	+	-	+	-	-
GPC-02	+	-	-	-	-
GPC-03	+	+	+	-	+
GPC-04	+	+	-	-	+
CFC-E1	+	+	-	-	-
CFC-E2	-	-	+	-	-
CFC-E3	+	-	+	-	-
CFC-E4	+	-	-	+	-
CFC-W1	+	-	-	-	-
CFC-W2	-	+	-	-	-
CFC-W3	+	-	-	-	-
CFC-W4	+	-	+	-	-
CFC-N1	+	-	-	+	+
CFC-N2	-	-	+	-	-

GPC: Germplasm collection; CFC-E; Cardamom field collection, East; CFC-W: Cardamom field collection, West, CFC-N: Cardamom field collection, North,: Presence(+): Absence (-)

Further molecular characterization was carried out through sequence comparison with the existing gene sequence in GenBank. The BLASTn results showed the 94.12% similarity with *Arthrinium* sp. CGPY-6 isolate with the accession number KR709044.1. The result also indicated 100% query cover

having 0.0 E value with maximum and total score value of 854. The partial gene sequence was submitted in NCBI GenBank as *Arthrinium* sp. Isolate LCF1OW internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence with accession no. MW091027.1. The study indicated the association of *Arthrinium* species as new pathogen of Large Cardamom.

The presence of large number of roundish spore on the infected leaves which is unlike the spore of *C. gloeosporiodes*, the main causal organism of blight disease in large cardamom and further molecular characterization indicated the association of *Arthrinium* species as new pathogenic fungus. Further study is needed to understand the level of virulence of the reported fungus.

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