*J Biosci* (2020)45:113
DOI: 10.1007/s12038-020-00086-7



# *Curvularia eragrostidis*, a new threat to large cardamom (*Amomum subulatum* Roxb.) causing leaf blight in Sikkim

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*MS received 8 April 2020; accepted 18 August 2020*

Large cardamom (*Amomum subulatum* Roxb.) is now affected by several diseases caused by both viruses and fungi. At present, leaf blight is considered a major threat to cardamom cultivation in Sikkim. During the past two decades, cultivation of the crop in this region has dropped by almost 60%. Hence, to quantify the severity of leaf blight damage and identification of the causal organism for the disease, a survey was conducted from May to August 2017 in different large cardamom growing regions of Sikkim. During this survey, a typical symptom of leaf blight was observed on cardamom leaves in many locations. The leaves with blights were collected, surface sterilized, and inoculated on potato dextrose agar (PDA). The pathogen was isolated as pure culture, and on the basis of morphological and microscopic characteristics, the fungus was identified species of *Curvularia*. Molecular characterization of the fungal isolate with ITS-rDNA partial gene amplification using universal primers (ITS4 and ITS5), showed 100% similarity with *Curvularia eragrostidis* (family: Pleosporaceae). The fungal isolate and nucleotide sequence was deposited in National Fungal Culture Collection of India (NFCCI), Pune and NCBI with accession numbers NFCCI 4541 and MN710527, respectively. This is the first report on the occurrence of *C. eragrostidis* pathogen causing leaf blight of large cardamom grown in Sikkim.

**Keywords.** *Curvularia eragrostidis*; large cardamom; leaf blight; Sikkim

## 1. Introduction

Large cardamom (*Amomum subulatum* Roxb.; family Zingiberaceae; order Scitaminae) is a principal cash crop cultivated in the north-eastern state of Sikkim and Darjeeling district of West Bengal. The crop has played a vital role in the economy of Sikkim and other large cardamom growing regions in the country for its exporting and foreign exchange earning potentiality. The crop grows in all the four districts of Sikkim ranging with different altitude from 800–3000 m amsl. Sikkim contributes maximum, i.e. 85%, of large cardamom production in India. It is also cultivated in parts of Uttarakhand and some other north-eastern states like Arunachal Pradesh, Manipur, Nagaland, Mizoram and Assam (Partap *et al.* 2014). Large cardamom cultivation is the source of income and livelihood of almost 88% population of Sikkim, and income from this particular crop is significantly higher than other livelihood options in this state. However, for the past 15–16 years, cultivation of the crop in the region has unfortunately dropped by almost 60% (Sharma *et al.* 2000). Experts are of opinion that there are multiple factors responsible for this drastic decrease in the production in the region, like climate change, alteration in cultivation practices, inadequate pollination system, incidence of pests and diseases (viral and fungal), etc. (Srivastava and Verma 1989; Saju *et al.* 2013; Gopi *et al.* 2018; Gurung and Bag 2018).


Published online: 14 September 2020

Large cardamom is a shade-loving crop that grows healthy under canopy in diffused filtered light. But nowadays, the open field plantation has become popular in the region where several foliar diseases are very frequent and several new foliar diseases have also been observed. Sometimes, the incident is so severe that the leaves are severely damaged by the pathogen and the entire plantation is affected by the infestation. Surprisingly, two most popular varieties, i.e. *Varlangey* and *Swaney*, are worse affected when grown in the open field conditions. For the present study, survey was conducted in leaf blight affected 30 villages of Sikkim. Interestingly a typical leaf blight of large cardamom was noticed in plantations of three villages, namely Andheri, Pakyong and Tumin of East Sikkim. In the present study, isolation, characterization, and identification of the causal organism of the newly noticed leaf blight of large cardamom were undertaken.

## 2. Material and methods

### 2.1 *Survey and sampling*

Survey and sampling was carried out in the large cardamom growing areas in Sikkim during the month of May to August 2017. As mentioned above leaves of large cardamom (varieties *Varlangey* and *Swaney*) with blight appearance were collected in triplicate each from three villages, namely Andheri, Pakyong and Tumin of East Sikkim for further experimental activities.

### 2.2 *Isolation of endophytic fungi*

The endophytic fungi were isolated from the lesions of leaves of large cardamom (varieties *Varlangey* and *Swaney*) showing the symptoms of blight, by culturing on potato dextrose agar (PDA, Hi-media, India) medium and incubating at 25°C following the protocol as described by Pandey and Palni (1998). Initially, leaves collected with lesions were cut into small pieces, surface sterilized with 0.1% mercuric chloride for 1 min followed by thorough washing with sterile distilled water thrice to remove any traces of mercuric chloride. Then sterilized leaf pieces were inoculated on PDA plates, sealed with Parafilm-M (Tarson, India) and kept in dark at 25°C for 5 days. Repeated subcultures were performed to get a pure culture and preserved at 4°C for further uses.

### 2.3 *Morphological characterization*

Preliminary experiments and microscopic observations confirmed that isolates isolated from both the large cardamom (varieties *Varlangey* and *Swaney*) were same and showed similar responses. Hence for the present study and further experiments, fungus isolated from the variety *Varlangey* was used. It is known that fungi exhibit variable morphological characters under the influence of different nutritional composition of the medium (Kim *et al.* 2005; Saha *et al.* 2008; Cagigal and Sanchez 2017). Therefore, five different medium Potato Dextrose agar (PDA), Sabouraud Dextrose Agar, Czapek Dox Agar, V8 Juice Agar and Potato Carrot Agar were used for the morphological characteristics, like colony size, radial growth of colony on different media, sporulation, pigmentation, etc. In the entire experiment, three replications were taken into consideration for each treatment. The fungal morphology was studied macroscopically. Samples were taken from the fungal isolate grown for 72 h on PDA at 25°C, mounted on microscopic glass slides, stained with lacto phenol cotton blue dye. To determine the vegetative and reproductive structures, fungal isolate was grown for 72 h on PDA. A small portion of the mycelium was taken from the freshly grown isolate and mounted on microscopic glass slides, stained with lacto phenol cotton blue and observed under compound microscope (Labophot-2, Nikon, Japan). Microscopic structures i.e., hyphae, conidia, conidiophores and arrangement of spores were characterized, measured and photographed. For identification and confirmation, isolate was sent to the Agharkar Research Institute Fungus Culture Collection (ARIFCC), Pune, India.

### 2.4 *Physiological characterization*

Wide ranges of temperatures (5–50°C) and pH (3–13) tolerance were tested by incubating the fungal isolate in PDA medium. Colony diameter with minimum of 5 mm on PDA plates was considered for growth at different temperature and pH (Kim *et al.* 2005; Saha *et al.* 2008).

### 2.5 *Molecular characterization*

Molecular identification of the isolated fungal pathogen was carried out on the basis of internal transcribed spacer (ITS) rDNA amplification and sequence analysis. Extraction of genomic DNA was done following CTAB method (Lee *et al.* 1988). Fungal ITS (internal transcribed spacer) region gene was amplified using ITS primers ITS5 [5′-GGAAGTAAAAGTCGTAACAAGG-3′] and ITS4

[5′-TCCTCCGCTTATTGATATGC-3′] (White *et al.* 1990; Hou *et al.* 2012; Manamgoda *et al.* 2015; Peterson *et al.* 2016). PCR was conducted in 25 µl reaction volume. Reaction tube contained approximately 2 µl DNA template, GoTaq® Green Master Mix, 2X (11 µl; GoTaq® DNA Polymerase 2X contains reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$), 1 µl each of 10 µM forward and reverse primers, Nuclease-Free Water to 10 µl (in this reaction except DNA template, all ingredients used were procured from Promega, USA). PCR reaction was performed in Master cycler gradient (Eppendorf, Germany) with 95°C for 5 min for initial denaturation followed by 30 cycle of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The PCR product was verified by staining with ethidium bromide on 1% agarose gel electrophoresis (Bio-Rad, USA). Amplified fragments were sequenced using ABI 3100 automated DNA sequencer (SeqGen, Inc. 1725 Del Amo BlvdTorrance, CA 9001, USA) with ABI-Big-Dye® Terminatory 3.1 cycle sequencing kit at MACS-Agharkar Research Institute, Pune.

### 2.6 *Evolutionary analysis by maximum likelihood method*

Raw sequence obtained was manually edited for inconsistency. The retrieved sequences were identified and compared using NCBI BLASTn tool (Altschul *et al*. 1990). The evolutionary history was inferred by using the Maximum Likelihood method and Jukes-Cantor model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio (NJ) algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Eight nucleotide sequences were involved for this analysis. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 555 positions in the final dataset. The analysis of Phylogenetic evolutionary was carried out in MEGA X (Kumar *et al.* 2018).

### 2.7 *Pathogenicity confirmation*

Pathogenicity test was determined by Koch's postulates (1876). For this experiment, fresh and healthy plants of large cardamom (Variety: *Varlengey*) were used as test plants. Conidial suspension of the test fungus was prepared by flooding the lawn of 5 days old pure fresh culture of the test fungi and $1\times10^4$ $ml^{-1}$ conidial count was maintained in the inoculums. The conidial suspension was then sprayed on the fresh leaves of large cardamom plants grown in the nursery poly house at normal temperature (25–28°C), at 6th Mile, Gangtok (25.85° N, 93.77° E and 1200 m amsl). The experiment was carried out with three replications for all the treatments. Control plants were kept in isolation to avoid mechanical contamination. Initial symptoms were noticed after 18–20 days of inoculation. Hence, after 20 days of inoculation, infected leaves with spots were taken for the further study. Procedures followed for pathogen isolation, analysis, etc., were the same as mentioned in the previous section.

### 2.8 *Statistical analysis*

Least significant difference (LSD) was carried out using the method of Snedecor and Cochran (1968).

## 3. Results

In the present study, the disease was detected in two varieties (*Varlangey, Swaney*) of the six varieties cultivated in the region. Till now the disease has been identified in three villages of East district out of total 50 villages of all four districts of Sikkim, studied. This is a typical leaf blight disease initially started with the symptoms of 7–12 dark brown spots per leaf. Leaf spots are usually scattered over the leaf surface, circular or irregular with variable shape, light brown center and reddish brown margins (figure 1C, D). At early stage, these spots were very tiny with 1.0–2.5 mm diameter, yellow central core with gradual discoloration towards the leaf surfaces. In due course of time, these spots increased in size and spread to the entire leaves and turned to dark brown (figure 1A, B) causing severe foliar damage to the plants. Eventually, these spots turned necrotic blight appearance and finally the infected leaves dried out.

Radial mycelial growth of the isolate was significantly ($P<0.05$) affected by the culture media. Results revealed that out of five different media tested for the fungal isolate, three, i.e., Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar and V8 Juice Agar were found to be significantly different ($P<0.05$) and gave satisfactory performance. In Potato Carrot Agar and Czapek Dox Agar medium, colony growth was

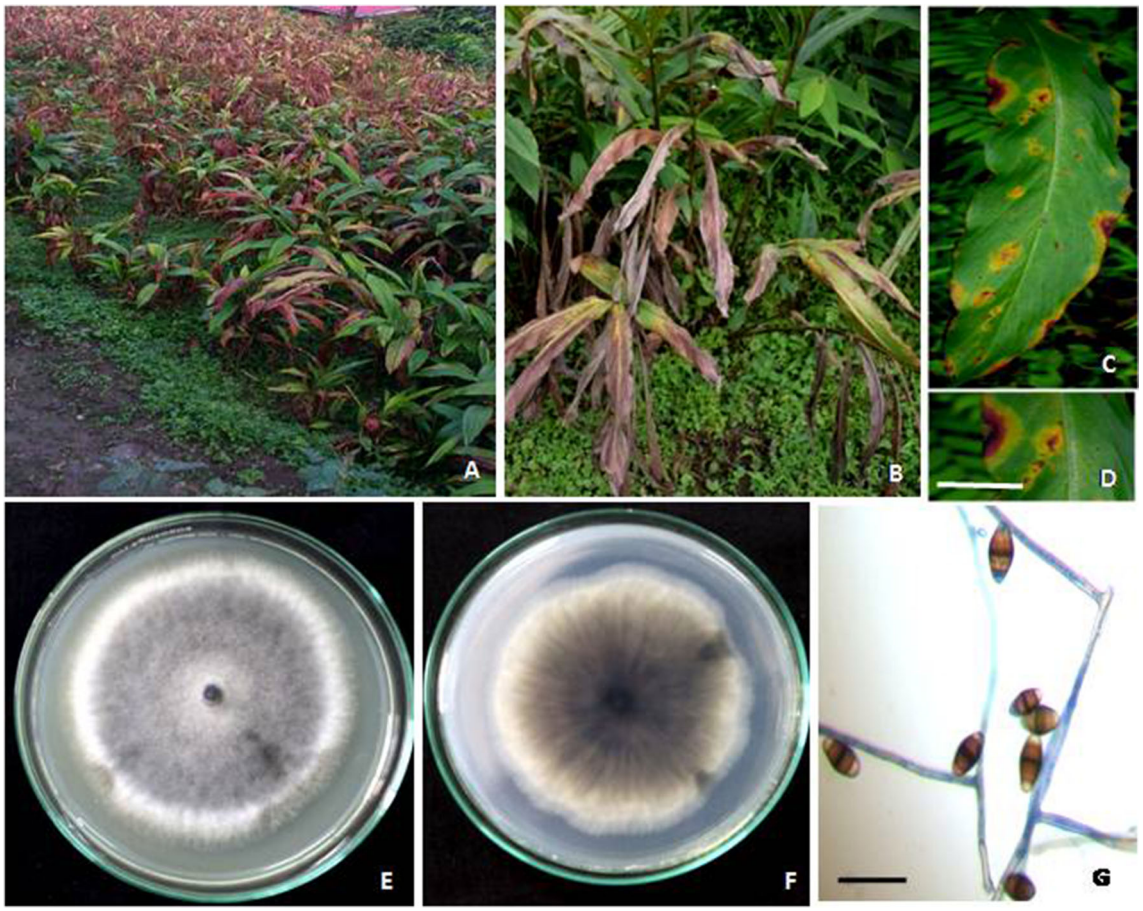


**Figure 1.** Leaf blight of large cardamom (*Amomum subulatum* Roxb.) var. *Varlangey* in Sikkim caused by *Curvularia eragrostidis.* (**A**) Leaf blight effected large cardamom field. (**B**) Close view of infected plant. (**C**–**D**) Leaf blight symptoms in large cardamom caused by *C. eragrostidis* ((**C**), bar = 10 cm; (**D**), bar = 30 cm). (**E**–**F**) *In vitro* growth of *C. eragrostidis* ((**E**), front view; (**F**), view from lower side). (**G**) Mycelium and conidiospores of *C. eragrostidis* (Bar = 100 μm).

significantly low ($P>0.05$) and with 60.5 ± 0.41 mm and 51.5 ± 0.41 mm average colony diameter in comparison to 64.5 ± 0.41 mm, 63.5 ± 1.24 mm and 64.5 ± 0.41 mm when grown on (PDA), Sabouraud Dextrose Agar (SDA) and V8 Juice Agar respectively. As PDA is the most common and widely used medium for fungal experiment, PDA was used for further study.

Temperature was found to be another important physical factor influencing growth of fungal isolates. In the present study, the mycelial growth of the isolate followed linear trends of response with the changes in temperature up to 35°C and then decreased sharply as temperature increased thereafter. Colony growth was least at 45°C and at 10°C with colony diameter about 20.5 mm. The optimum temperature for colony growth was found to be between 25–35°C with colony diameter 64.5 ± 0.41 mm after 5 days of incubation. At 20°C, moderate growth with average 41 ± 0.83 mm colony diameter was recorded which was significantly low ($P>0.05$) in comparison to the growth at 25–35°C. No colony growth was observed at 5°C.

Medium pH also significantly affected radial mycelial growth of the isolate. Colony growth of the isolate was observed in a wide range of pH (5.0–9.0) of medium. Maximum colony growth was obtained at medium pH 6.0–8.0 with 64.5–63.5 mm colony diameter (table 1). Then a sharp decline of the growth occurred when medium pH increased to 9 or more. No colony growth was observed when medium pH was 3 and 13.

Colony morphology of the fungus obtained on PDA is presented in figure 1E, F. Colony diameter at 25°C was 64.5 mm, greenish with white margin from the top view, and dark green and white from invert plate view, with wavy margins. The isolated fungal culture was observed to be fast growing on PDA with scanty mycelia, and cottony with fast fungal growth. The mycelium was septate, hyaline, and 4–8 μm wide. The conidiospores were simple oval shaped, septate, pale

**Table 1.** Phenotypic and genotypic characters of the endophytic fungi

| Sl. no. | Character | Description |
|---|---|---|
| 1. | Colony morphology | Greenish with white margin and dark green and white from invert plate view along with wavy margins with 64.5 ± 0.41 mm colony diameter |
| 2. | Microscopic features | Mycelium septate, hyaline and 4–8 µm wide. Size of conidia: length 78.81 ± 1.65 µm; width in the middle 42.16 ± 1.51 µm |
| 3. | Physiological characterization (pH and temperature) | Temperature requirement between 10°C to 45°C, optimum 25°C and pH 5 to 11 optimum 7 |
| 4. | Accession number and nucleotide sequence number | NFCCI 4541 and MN710527, respectively |
| 5. | Phylogenetic relationship of the isolate (18rRNAs analysis) and list of top five hits upon BLASTn analysis | Maximum (100%) similarity with *Curvularia eragrostidis* (KU856617.1), *C. eragrostidis* (KU232927.1), *C. eragrostidis* (KU232931.1), *C. eragrostidis* (KU232929.1) and *C. eragrostidis* (KU232927.1) |

brown with hyaline base and fertile tip. The conidia were olive brown, 3-septate, more or less curved at the 3rd cell or nearly straight in the middle. Size of conidia is: length 78.81 ± 1.65 µm; width in the middle 42.16 ± 1.51 µm (table 1; figure 1G). The 3rd cell from the base was larger and darker than the basal and apical cells which were hyaline with smooth tip. On the basis of colony morphology and microscopic features, the isolate was identified as *Curvularia* sp. by National Fungal Culture Collection of India (NFCCI), Agharkar research institute, Pune.

Based on ITS sequences and phylogenetic analysis top five hits upon BLASTn analysis are *Curvularia eragrostidis* (KU856617.1), *C. eragrostidis* (KU232927.1), *C. eragrostidis* (KU232931.1), *C. eragrostidis* (KU232929.1) and *C. eragrostidis* (KU232927.1) with maximum (100%) similarity with the isolate. The tree has shown the highest log likelihood (− 769.39). The percentage of replicate trees in which the associated taxa clustered together is shown next to the branches. The submission number of the gene sequence alignment at NCBI is MN710527, was identified as *Curvularia eragrostidis* (figure 2). The ITS rDNA sequence data of the fungal species showed 100% similarity with sequences of *Curvularia eragrostidis* available in GenBank. Total score and query coverage showed 100% similarities between the DNA sequences of ITS gene of the isolate and *C. eragrostidis* from NCBI database. The fungal isolate and nucleotide sequence has been deposited in National Fungal Culture Collection of India (NFCCI) and Gene Bank (Benson *et al.* 2012) with accession numbers NFCCI 4541 and MN710527, respectively (figure 2). To the best of our knowledge, it is the first report on *Curvularia eragrostidis* of family pleosporaceae causing leaf blight, and is likely to be a new foliar threat to large cardamom in the region.

## 4. Discussion

For the last two decades, large cardamom cultivation in the region is passing through a very bad phase. The regions of undulating mountains that once offered the perfect climatic conditions and fertile soil for this king of spice crop, have turned into a breeding ground for pathogens and large cardamom production has gone down drastically mainly due to various types of viral and fungal diseases. The cultivation has dropped by half within the last decade in Sikkim. Reports say in Sikkim during 2004 the area under large cardamom cultivation was 23,000 hectares and that has now reduced to just 12,500 hectares, which is 45% less to that of 2004 (Sharma *et al.* 2000; Anonymous 2010). Foliar damages are considered as one of the major limitations in many crops, which include leaf blight, leaf spot, leaf streak and many more, resulting reduction of crop growth as well as production. Reports say reduction of large cardamom plantation in the region is due to different kind of diseases, like chirkey, foorkey, leaf blight, leaf spot, anthracnose, wilt, collar rot, capsule rot and leaf streak, etc. (Sharma *et al.* 2000; Anonymous 2010; Saju *et al.* 2011, 2013; Gurung and Bag 2018). Therefore, new plantations with healthy looking planting materials are also being infected soon as they may contain germ spores of the fungus. Several national agencies are working to control the problem, but with limited or negligible success. Only a few

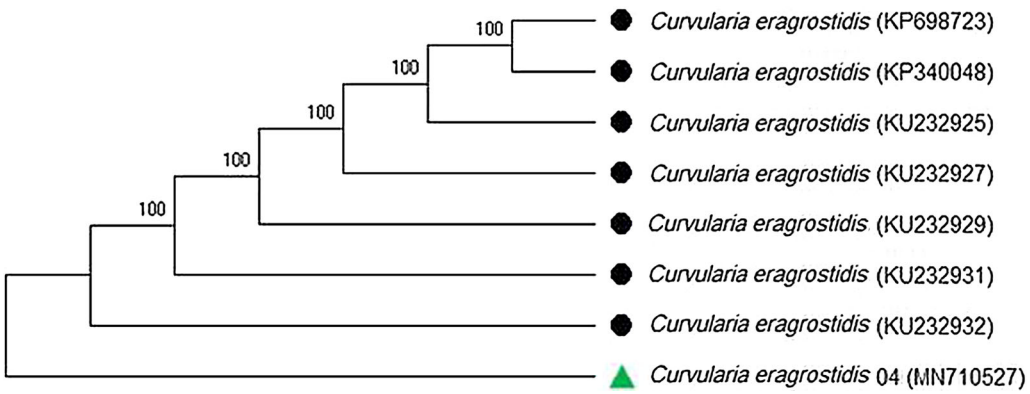


**Figure 2.** Phylogenetic tree constructed with neighbor-joining (NJ) methods with ITS nucleotide sequence of fungal species isolated from large cardamom using MEGA X. Bootstrap values = 1000.

survey and scientific reports are available mainly describing the severity of the problem but there is no proper identification of causal organism, recommendation towards controlling the problem and the disease persists with the same intensity or even more.

In the late 1990s rot disease was first noticed in the region and people described it as some mysterious disease. Then after more than two decades in 2010 a group of scientists from the Indian Cardamom Research Institute reported that *Colletotrichum gloeosporioides* is responsible for causing the leaf blight (Saju *et al.* 2011). Another group of scientists tried to control the disease using a chemical pesticide, like Copper Oxychloride with limited success. So far other pathogens like *Fusarium* sp., *Rhizoctonia* sp. are also reported for other fungal diseases of large cardamom (Anonymous 2014). During the present study attempts were made for scientific evaluation and identification of causal organisms for blight of large cardamom in the region. Interestingly, out of the four districts of Sikkim, samples collected from three villages of East district was distinct with symptoms. The pathogen was identified as *C. eragrostidis*, which is first report of the pathogen causing blight disease to large cardamom in three villages of East district of Sikkim. On the basis of the morphological behavior of the isolate and species characteristics the pathogen has been identified as *C. eragrostidis.* Further in addition to morphological parameters and characterization using two partial ITS rDNA sequence evaluated in the NCBI database also confirmed the isolate as *C. eragrostidis* with 100% sequence similarity, a newly identified pathogen causing blight disease to large cardamom grown in Sikkim.

The genus *Curvularia* is having of more than 40 species which are distinguished by differences of conidia structure, number of septa and the colony morphology (Zhang *et al.* 2004; Chung and Tsukiboshi 2005). Most of species of *Curvularia* are pathogenic and give rise to substantial loss losses in agricultural produced all over the world. Generally, the genus caused leaf spot disease which is necrotic for several plant families (Dasgupta *et al.* 2005). For example the pathogen *Curvularia* sp. causes leaf spot disease in rice, wheat, grass, maize, tea, and sorghum, etc. (Dasgupta *et al.* 2005; Fajolu *et al.* 2012; Dey *et al.* 2016; Garcia-Aroca *et al.* 2018; Seephueak *et al.* 2019). It is also reported that the *Curvularia* sp. causes leaf spot disease is one of the major draw backs in rice cultivation as well, as it inhibits the germination of the rice seeds also. The disease is characterized by the symptoms of long and wide grayish white spot with brown color surrounding around it with irregular shape (Bawa *et al.* 2018) with the brown border surrounded by a yellow halo (Fajolu 2012; Sarkar *et al.* 2018; Seephueak *et al.* 2019). Surprisingly, in the present study, phenotypic characteristics are similar as reported above and were recorded with distinct conidiospores causing blight of large cardamom and gradually infected leaves damaged severely and dried up.

In conclusion, viral diseases namely *chirke* and *foorkey* of large cardamom are also reported which are causing severe damage to the crop. There are other fungal diseases (i.e., wilt, leaf rot, leaf spot, anthracnose, rust, leaf blight and leaf streak) found in large cardamom that have affected the productivity sharply (Sharma and Rai 2012; Saju *et al.* 2013; Gopi *et al.* 2018). So far, other pathogens like *Fusarium* sp., *Colletotrichum* sp. and *Rhizoctonia* sp. have reported for fungal diseases of large cardamom (Anonymous 2014). With the limitations of the study indicated that this disease with distinct symptoms caused by *C. eragrostidis* is a new threat for the crop but till now restricted within the East district of Sikkim. But the

presence of the disease indicated that the *C. eragrostidis* is a new minor fungal pathogen associated with leaf blight of large cardamom growing in the region. So further studies are needed to determine the distribution and severity of the disease in the region. Hence, the present study will help to develop strategy to prevent crop losses due to *C. eragrostidis* infection.

## Acknowledgements

Financial support received from G.B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi-Katarmal, Almora, under the research grant 'Integrated Eco-development Research Programme (IERP)' is gratefully acknowledged. Agharkar Research Institute Fungus Culture Collection (ARIFCC), Pune, India, is also acknowledged for identification of the pathogen. The authors are thankful to the reviewers for valuable comments to improve the manuscript.

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