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Does *Pestalotiopsis royenae* cause leaf streak of large cardamom?

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Leaf streak caused by *Pestalotiopsis royenae* (Guba) Steyaert has been reported as a new disease of large cardamom (*Amomum subulatum*). It is characterized by rectangular spots running parallel to the veins. *P. royenae* was isolated onto potato dextrose agar from the infected portion of the plant. In the pathogenicity test, disease symptoms were not present on inoculated plant and on detached leaf *in vitro* after 20 days. During 2014–2016, a survey in various large cardamom plantations of Sikkim revealed the presence of tea mosquito bugs on the infected parts of *A. subulatum*. The spots did not show any growth. Different sized spots have been noticed on the infected leaf produced by different instars. At the initial stage, these symptoms appear on the young and tender leaves of large cardamom. Tea mosquito bugs were collected from the infested leaves and allowed to feed under controlled conditions, which produced similar type of leaf streak symptoms in large cardamom and also in other non-host crops like maize and turmeric. The results reveal that the cause of leaf streak is due to feeding injury of tea mosquito bugs and rule out *P. royenae* as a pathogen causing leaf streak disease symptoms. *Pestalotiopsis* sp. was also isolated as endophyte from large cardamom.

Keywords: *Helopeltis theivora*, large cardamom, leaf streak, *Pestalotiopsis royenae.*

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LARGE cardamom (*Amomum subulatum* Roxb.), a member of Zingiberaceae under the order Scitaminae is the main cash crop cultivated in the sub-Himalayan state of Sikkim, Darjeeling district of West Bengal[1], Arunachal Pradesh, Nagaland, Mizoram, Manipur, Meghalaya, Assam and parts of Uttarakhand. Large cardamom is also cultivated in Nepal and Bhutan. Diseases like chirkey[2], leaf blight[3,4], leaf streak[5], capsule rot[6], wilt[7] and foorkey[8] have been reported in large cardamom, of which leaf streak caused by *Pestalotiopsis royenae* (Guba) Steyaert has been reported as a new disease of large cardamom from Sikkim[5]. Leaf streak caused by *P. royenae* often results in severe foliar damage and crop loss[9,10]. The disease is characterized by rectangular spots running parallel to the veins and elongated, rectangular, translucent streaks appearing on young leaves along the veins (Figure 1). The streaks turn reddish-brown within 3–4 days with a central straw-coloured necrotic area surrounded by a prominent dark-brown margin[5,11]. Srivastava[12] reported that the variety Golsey with crinkled leaf pattern was more susceptible than variety Sawney having smooth leaf pattern. During 2014–2016, a survey in various large cardamom plantations of Sikkim revealed the presence of tea mosquito bugs (*Helopeltis theivora*) on the infected parts of *A. subulatum* (Figure 2 *a* and *b*). It has been reported that the leaf streak disease of large cardamom is caused by *P. royenae*; however, the presence of tea mosquito bug and various reports on the endophytic nature of *Pestalotiopsis* spp. prompted us to examine the actual cause of leaf streak symptoms in large cardamom.

A study was initiated at ICAR Sikkim Centre (now National Organic Farming Research Institute), Gangtok, during 2014–2016 on leaf streak disease of large cardamom. A survey was conducted in different large cardamom plantations of Sikkim (Figure 3) to assess the incidence of the disease during May to July. Diseased leaves of large cardamom showing typical leaf streak symptoms were collected from the study area for further analysis. Disease incidence was calculated using the formula: per cent disease incidence = (no. of plants infected/total no. of plants assessed) × 100. The pathogen was isolated onto potato dextrose agar (PDA) from the diseased portion of the plant. The infected leaf samples were

Figure 1. Leaf streak symptoms in large cardamom caused by *Pestalotiopsis royenae*.

washed in sterilized distilled water. The diseased and healthy tissues were cut into small pieces and surface disinfected using 0.5% sodium hypochlorite for 3 min followed by three washings in sterile distilled water. The cut pieces were aseptically transferred to PDA plates and incubated at 28° ± 1°C. Fungal growth was periodically observed and cut pieces were transferred to PDA slant tubes for pure culture. Pure culture of the fungus was obtained by single spore isolation method (Figure 4). Microscopic examination of the pure culture fungus was carried out for identification. One-year-old plants of large cardamom were used for pathogenicity test. A conidial suspension of the fungus (1×10^6 conidia ml^{-1}) from PDA culture was sprayed on leaves of one-year-old plants. A total of three replications were maintained and each replication contained five plants. Plants kept as control were also sprayed with the same volume of sterilized distilled water and all inoculated plants were covered with polyethylene bags during incubation to maintain high moisture. Similarly, young leaves were collected and surface-disinfected with a solution of 0.5% sodium hypochlorite for 3 min. The leaves were subsequently rinsed in distilled water and dissected into 10 cm segments. The segments were placed abaxial on the surface of the water agar and inoculated with conidial suspension of the fungus (1×10^6 conidia ml^{-1}). The petri dishes were sealed using parafilm and kept in BOD at 28° ± 2°C with more than 85% relative humidity (RH). The pathogenicity test was carried out twice to prove Koch's postulates. This test was also performed with the help of mycelial plug (5 mm) instead of conidial suspension.

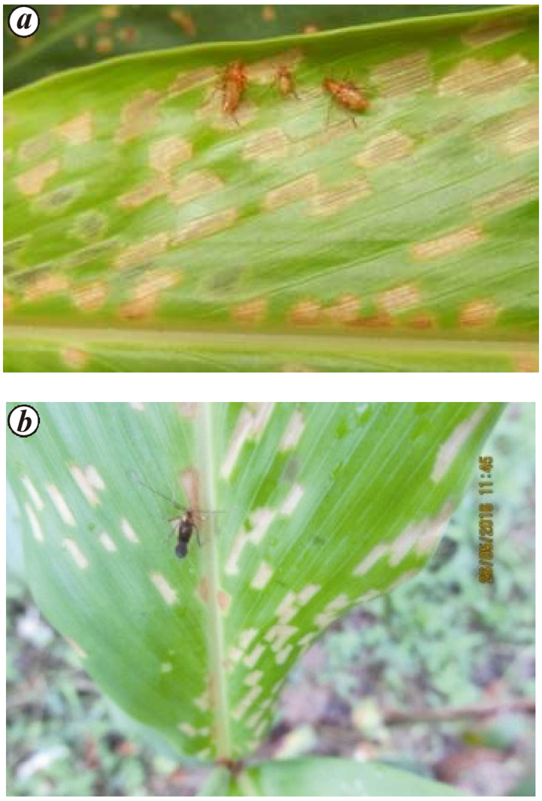

Figure 2. ***a***, Nymphs of tea mosquito bug; ***b***, Adult of tea mosquito bug.

Figure 3. Map of surveyed large cardamom areas in Sikkim. (1) ICAR Tadong, (2) Assam Lingzey, (3) Pangthang, (4) Raigaon (Pakyong), (5) Thekabung, (6) Darap, (7) Kecheoperli, (8) Sakyong, (9) Soreng, (10) Hee-Gyathang, (11) Gnon, (12) Naga, (13) Kabi, (14) Jaubari, (15) Borong, (16) Linguee Payong, (17) Tinkitam and (18) Ravangla.

First and second instar nymphs of tea mosquito bug were collected from the infested large cardamom leaves to study the role of this insect on leaf streak symptoms. A mass culture of *Helopeltis theivora* was maintained in the laboratory at 26° ± 4°C and 70–90% RH on detached large cardamom shoots inside rearing cages (Figure 5). Shoots were renewed every alternate day. Shoots containing eggs were separated from the rearing cage and kept in separate glass cages (26 cm × 18 cm × 15 cm) providing tender flushing shoots of large cardamom for hatching. Five nymphs of each first, second, third and fourth instars

Figure 4. Culture of *P. royenae* with acervuli.

were released to separate cages providing young shoots of large cardamom for feeding. The feeding spots of each instar of nymph were observed on the third, fifth and seventh day after release. In case of adult, five pairs of male and female were released to the cages providing young shoots for feeding. Similarly, the feeding spots of adults were observed on the third, fifth and seventh day after release. A total of four replications for each stage were maintained. Nylon mesh net was used to confine these nymphs on tender flushing shoots of large cardamom in the glass house. Further, nymphs and adults of tea mosquito bug were released on maize and turmeric to confirm similar type of streaks after feeding injury by the insect.

Figure 5. Insect cage with large cardamom leaf.

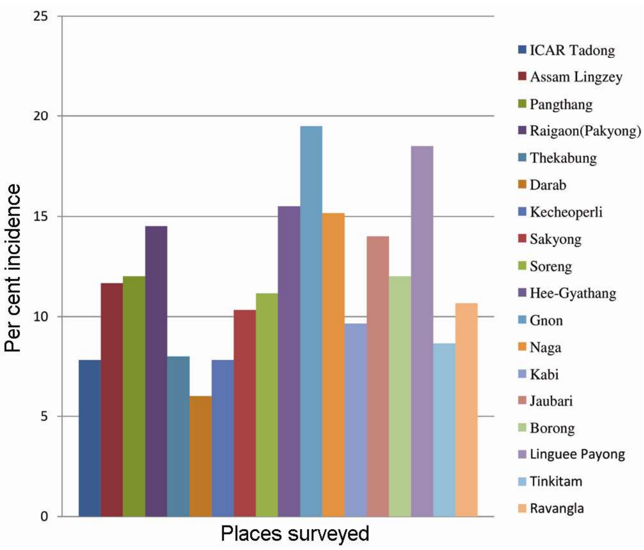

Figure 6. Leaf streak incidence in different large cardamom areas of Sikkim.

Healthy leaves were collected to study the endophytes of large cardamom. The plant parts were brought to the laboratory in sterilized bags. Endophytes were isolated using a modified method described by Arnold *et al.*[13].

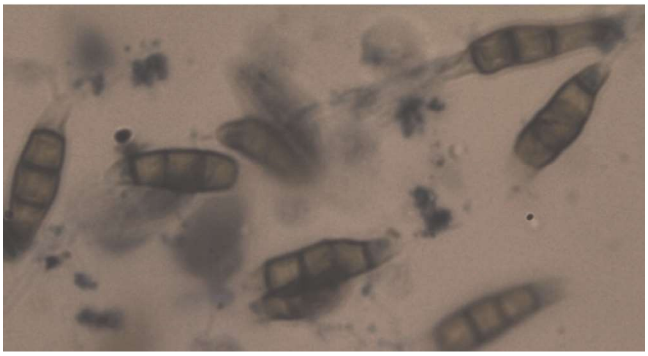

Figure 7. Spores of *P. royenae*.

Figure 8. Feeding symptoms on the leaf after release of insect.

Figure 9. Symptoms on the large cardamom plant after artificial release of insect.

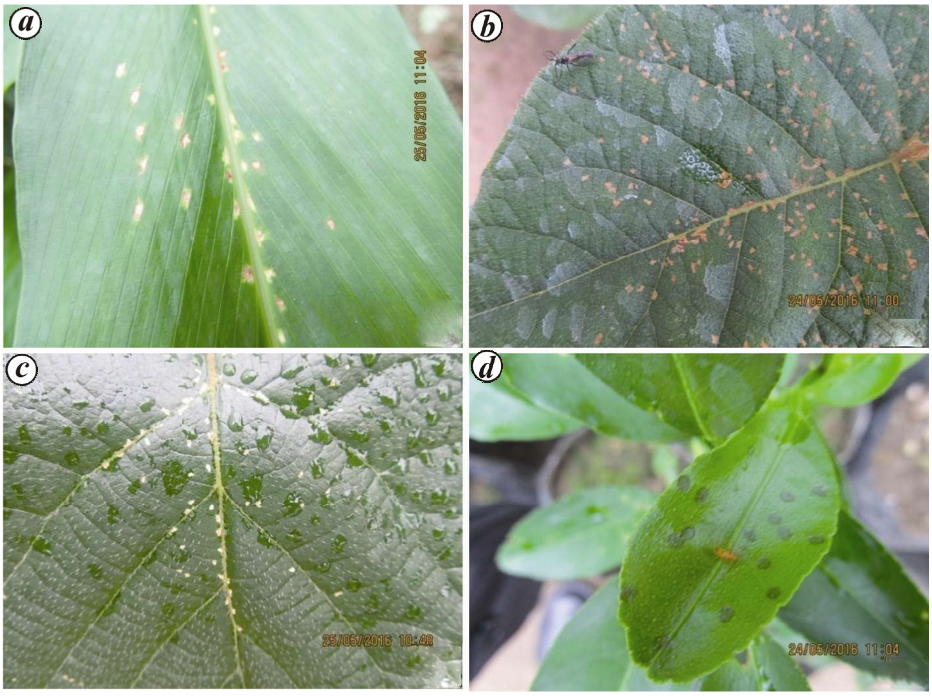

Figure 10. Symptom of tea mosquito bug feeding on (***a***) leaf of large cardamom; (***b***, ***c***) kiwi fruit leaf, and (***d***) Sikkim mandarin.

The leaves were washed in sterile water, surface-disinfected by soaking in 70% ethanol for 30 sec and 0.1% mercuric chloride ($HgCl_2$) solution for 2 min and subsequently rinsed in sterile demineralized water. Small pieces of leaf tissue were placed on aqueous agar (distilled water and 1.5% agar-agar) supplemented with antibiotic streptomycin (3 mg/100 ml) in petri plates and incubated. The tips of the fungal hyphae were removed from the aqueous agar and placed on PDA. After several days of incubation, the fungal culture was assessed by colony morphology. The fungi were subcultured as described above, and the final pure cultures were transferred to PDA slant tubes. As controls, uncut, surface-disinfected and non-disinfected leaf pieces were also placed on the same agar to check for contaminated fungi.

The average incidence of leaf streak varied from 6% to 19.5%. The incidence was more severe in shaded areas than open areas and was recorded highest in Gnon north Sikkim (19.5%; Figure 6). The incidence was more during rainy season. The test fungus was identified and deposited in Indian Type Culture Collection, IARI, New Delhi (ID No. 10468.17). Mycelium septate, white and produced many acervuli. Conidia five-celled, narrow and fusiform shaped. Conidia were 21.63–28.84 × 5.78–6.75 μm with two hyaline, cylindrical to conical apical cells typical of *Pestalotiopsis* spp. with 2–4 apical appendages, three intermediate cells of conidia versicolorous, upper two cells fuliginous, usually opaque and lowest cell olivaceous-brown (Figure 7). The length of apical and basal appendages was 11.9–16.6 and 6.93–10.56 μm respectively. In pathogenicity test, disease symptoms were not observed on the inoculated leaves and the control even after 20 days. Species of *Pestalotiopsis* have been reported as a pathogen in different crops[14–18]. *Pestalotiopsis* spp. have also been reported as endophytes in diverse plant species[19–21]. In general, *Pestalotiopsis* spp. initiate symptoms as small flecks; later they increase in size. In case of grey leaf spot on coconut, initially minute, yellow lesions appear on the leaflets. Gradually, the spots enlarge, become brownish and turn greyish-white at the centre and develop dark brown margins surrounded by yellow halos. Many spots coalesce into irregular necrotic patches[22]. The symptoms of *Pestalotiopsis microspora* Speg. on Japanese spicebush (*Lindera obtusiloba*) start as grey or dark brown lesions surrounded by yellowish halos; later they enlarge by coalescing and become more irregular causing leaf blight[23,24]. Zhang *et al.*[25] reported that the spots on hidcote (*H. patulum*) are small, round and pale brown initially and later expand to 5–12 mm in diameter, round to irregular-shaped with pale brown centres and dark brown margins.

However, in large cardamom crop, the spots are rectangular from the beginning and do not show any gradual increase in size. In some leaves, spots coalesce among themselves as a result of secondary infection due to some saprophytes growing on the dead tissue of the infected areas. These symptoms appear on the younger leaves of large cardamom whereas in other crops, the symptoms caused by *Pestalotiopsis* spp. were first noticed on older leaves. For example, *Pestalotiopsis palmarum* (Cooke) Steyaert infects palms; the symptoms are usually observed on older leaves[26]. Insects prefer tender parts and more importantly, tea mosquito bug feeds only on tender leaves. The symptoms on tender leaves have also been noticed in various other crops like chilli and seedlings of mandarin and kiwi. It has also been found that the tea mosquito bug is polyphagous type, and feeds on other hosts.

The symptoms of leaf streak were initiated from the second day of release of tea mosquito bug on the young leaves (Figure 8). The feeding was not noticed on older leaves, which indicates the preference of young leaves for food. The young leaves in the field also showed similar symptoms (first or second) and the incidence was severe in shaded areas. The size of the streak varied from 1 to 12 × 1 to 3 mm. The streaks were initially white in colour and later turned brown (Figure 9). This has also been observed on other hosts like kiwi, red cherry pepper and duranta. The feeding pattern on large cardamom was similar to that of other hosts (Figure 10 *a*). In other hosts, the spots are oval, round or triangular in shape (Figure 10 *b–d*), whereas in large cardamom the spots are rectangular in shape. This may be due to parallel venation pattern of large cardamom leaves, which is characteristic of monocot plants. Similar type of spots was also noticed on non-host crops like turmeric (Figure 11) and maize (Figure 12) upon artificial release of tea mosquito bug.

Plant bugs of genus *Helopeltis* are serious pests of many cultivated crops worldwide, particularly major cash crops like tea, cocoa, cinchona, cashew and pepper[27]. There are more than 40 known species spread across Western Africa, Sri Lanka, Bangladesh, India, Papua New Guinea and Northern Australia. *H. theivora* has been reported to cause 80% infestation resulting in crop loss to the tune of 10–50% in tea from North East India[28]. Kalita *et al.*[29] reported more than 13 hosts from Sikkim. It was found that the incidence of the symptoms coincided with the emergence of new leaves on the plants. The symptoms on the plants first started on the emerging leaves as a small streak of light brown colour; later they became dark brown with straw colour in the centre. The incidence of the disease symptoms varied from 15% to 30%. Close examination of the diseased plants showed the presence of nymphs and adults of tea mosquito bug. Kalita *et al.*[29] also reported that symptoms of tea mosquito bug resembled the leaf streak disease. In the inoculated leaves under laboratory conditions, the inoculated nymphs produced the symptoms as rectangular lesions from the second day onwards. After six days the rectangular streaks became brown and after 10 days they produced straw colour. Immediately after infestation, the streaks were light green and transparent but not brown. Gradually the streaks changed to brown colour. The size of the spots varied with the age of the nymphs. The biggest spot was observed in case of adults. The nature and physical appearance of the spots produced by different instar nymphs and adults were similar to those caused by *P. royenae* in large cardamom.

Pestalotiopsis sp. has also been isolated as endophyte from large cardamom. It has been frequently isolated from the blighted leaves of large cardamom and was reported as the pathogen causing blight in large cardamom leaves. There is a possibility that *Pestalotiopsis* sp. is a natural inhabitant of large cardamom as an endophyte or saprophyte. When the insect feeds on large cardamom leaves, the endophytic association might have converted into saprophytic association or the feeding spots might have been colonized by saprophytic *Pestalotiopsis* sp. Some endophytic *Pestalotiopsis* spp. act either as parasites or saprophytes due to the different stages of host plants and (or) environmental conditions[30]. *Pestalotiopsis microspora* (syn. *P. royenae*) has been reported as endophyte in *Taxus wallachiana*[20], plants of Podocarpaceae, Theaceae and Taxaceae[21], and mangroves[31]. The results revealed that the cause of leaf streak was due to feeding injury of tea mosquito bug and ruled out *P. royenae* as the pathogen causing leaf streak disease symptoms.

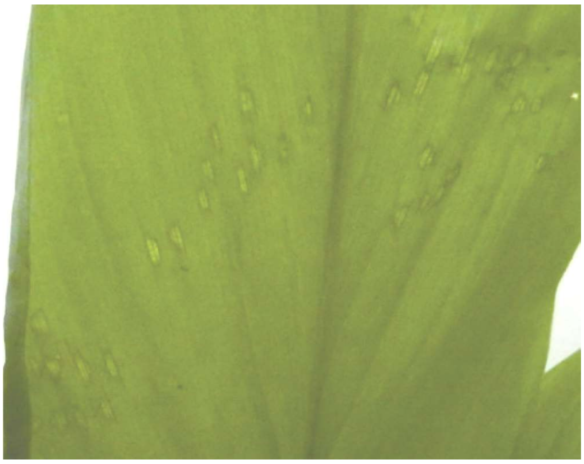

Figure 11. Leaf streak-like symptoms after insect release on turmeric.

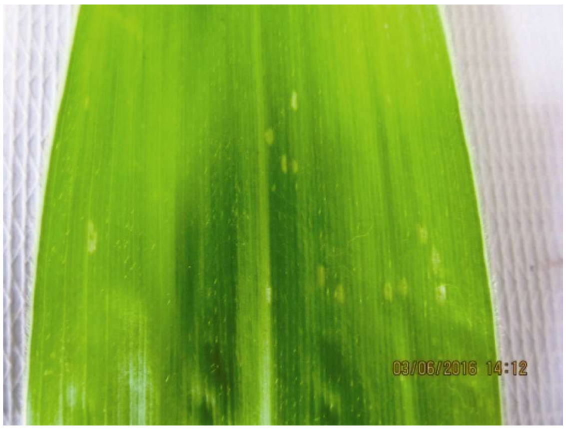

Figure 12. Leaf streak-like symptoms after insect release on maize.

Leaf streak is one of the major problems in large cardamom and has been long considered as a disease caused by the pathogen *P. royenae*. The study has demonstrated that leaf streak is not a disease caused by *P. royenae* as previously reported, but purely feeding spots caused by *H. theivora*. This is an important finding in large cardamom and will be useful for farmers, scientists and extension functionaries to understand the real cause of the problem and take up timely and proper control measures. Excess and unwanted spraying of chemicals on account of wrong diagnosis will also affect other beneficial insects and antagonistic microbes present in large cardamom ecosystem. However, detailed study is essential in future on other aspects like impact of tea mosquito bug on growth and yield characters of large cardamom, weather parameters which help in the build-up of insect population, alternate hosts and management options for tea mosquito bug, etc. for sustainable production in large cardamom. As there is a decline in the yield of large cardamom in recent times due to various biotic and abiotic factors, the present study will help address this problem.

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