Colletotrichum siamense causing anthracnose-twister disease of onion (*Allium cepa*) in Sri Lanka

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Abstract

Symptomatic samples of anthracnose-twister disease of cultivated onion (*Allium cepa*) were collected from heavily infected fields at Matale district, Sri Lanka during a severe outbreak that occurred through late July to August in 2020. The pathogen was identified as *Colletotrichum siamense* based on morphology and combined gene sequence analysis of nuclear ribosomal internal transcribed spacers 1 and 2 with 5.8S region (ITS), glyceraldehyde-3-phosphate dehydrogenase gene (GPDH) and beta-tubulin (TUB) regions. *C. siamense* is a species with global distribution and a wide host range. This is the first report of *C. siamense* infecting onion in Sri Lanka.

Keywords Anthracnose · Molecular diagnostics · Onion diseases · Tropical pathogens

Onion (*Allium cepa*), an important crop grown for its flavorful bulbs and leaves, is consumed globally. Large scale cultivation of onion in Sri Lanka accelerated in the early 1980's. Overall, the extent of cultivation has increased since then with some annual fluctuations. However, in the year 2020 local cultivation did not meet even a quarter of the annual requirement of onion (DCS, 2020). One major factor contributing towards the reduction of onion yield in the country was the impact of plant diseases that occur both in the nurseries and field. Fungal pathogens are the causative agents of many severe diseases that reduce yield of onion worldwide. Among them, species of *Alternaria*, *Colletotrichum*, *Fusarium*, *Sclerotium*, and *Rhizoctonia* are widely encountered (Conn et al. 2012; Mishra et al. 2014).

Among the major onion producing regions in Sri Lanka, Matale district produces an estimated 50% of the total

production of big onion (DCS, 2020). Through late July to August in 2020, a severe outbreak of anthracnose-twister disease was reported from several localities of onion cultivations of Matale district (Central Province), in Sri Lanka leading to a significant yield loss. These affected onion fields in Matale district were reported to abandon because of this disease, causing a short-term scarcity of onion supply in the local market. The leaves and bulbs of onion plants showed typical anthracnose-twister disease symptoms (Alberto et al. 2019), such as yellow to dark spots of leaves and bulbs, irregular leaf yellowing, curling, twisting, chlorosis, abnormal elongation of the neck, formation of slender bulbs and ultimately the death of plants. Concentric rings of acervuli and orange conidial masses were abundantly observed on bulbs with anthracnose symptoms and twisted leaves of diseased plants.

Multiple samples of infected onion plants with disease symptoms were collected from heavily infected fields in Bulanawewa, Galewela (7.84 N, 80.58E) and Sigiriya area (7.95 N, 80.75E) in Matale District. The single spore isolation method (Choi et al. 1999) was used to obtain the pathogen from diseased plant tissue onto Potato Dextrose Agar (PDA) plates. The inoculated plates were incubated at 25 °C under dark conditions. Characteristics of conidia, conidiophores, and appressoria were assessed under a Carl Zeiss compound light microscope, using an Axiocam digital camera. (Carl Zeiss Microscopy, Thornwood, NY, USA). In total, 10 isolates were obtained from the infected

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samples and all the isolates were morphologically identical. Two representative pure cultures of the pathogen were deposited at University of Sri Jayewardenepura culture collection (USJCC) under the accessions USJCC-0056 and USJCC- 0058.

DNA was extracted from two isolates grown on PDA at 25 °C for 7 d using a modified protocol of Arnold and Lutzoni, (2007). The ribosomal internal transcribed spacer (ITS) region was amplified using the primer pairs,

ITS-1/ITS-4 (White et al. 1990) and Sanger sequenced by Macrogen, Korea. To confirm the identity of the pathogen in *Colletotrichum gloeosporioides* complex, the glyceraldehyde-3-phosphate dehydrogenase (GPDH) and beta-tubulin (TUB2) gene regions were amplified using the primer pairs, GDF1/GDR1 (Templeton et al. 1992) and T1/ Bt-2b (O'Donnell and Cigelnik 1997) respectively, following the methods described by Damm et al. (2009). Samples were Sanger sequenced using the same

Table 1 Isolates of *Colletotrichum* species used in the phylogenetic analyses with details of host and location, and GenBank accession numbers of the sequences

Species	Isolate code/Culture collection	Host	Country	GenBank accessions		
				ITS	GPDH	TUB2
<i>C. aenigma</i>	ICMP 18608 ^T	<i>Persea americana</i>	Israel	JX010244	JX010044	JX010389
<i>C. aeshynomenes</i>	ICMP 17673, ATCC 201874 ^T	<i>Aeschynomene virginica</i>	USA	JX010176	JX009930	JX010392
<i>C. alatae</i>	CBS 304.67, ICMP 17919 ^T	<i>Dioscorea alata</i>	India	JX010190	JX009990	JX010383
<i>C. alienum</i>	ICMP 12071 ^T	<i>Malus domestica</i>	New Zealand	JX010251	JX010028	JX010411
<i>C. aotearoa</i>	ICMP 18537	<i>Coprosma</i> sp.	New Zealand	JX010205	JX010005	JX010420
<i>C. asianum</i>	ICMP 18580, CBS 130418 ^T	<i>Coffea arabica</i>	Thailand	FJ972612	JX010053	JX010406
<i>C. boninense</i>	CBS 123755, MAFF 305972 ^T	<i>Crinum asiaticum</i> var. <i>sinicum</i>	Japan	JQ005153	JQ005240	JQ005588
<i>C. camelliae</i>	CGMCC 3.14925 T, LC1364	<i>Camellia sinensis</i>	China	KJ955081	KJ954782	KJ955230
<i>C. clidemiae</i>	ICMP 18658 T	<i>Clidemia hirta</i>	USA, Hawaii	JX010265	JX009989	JX010438
<i>C. cordylinicola</i>	MFLUCC 090551, ICMP18579 ^T	<i>Cordyline fruticosa</i>	Thailand	JX010226	JX009975	JX010440
<i>C. fruticola</i>	ICMP 18581, CBS 130416 ^T	<i>Coffea arabica</i>	Thailand	JX010165	JX010033	JX010405
<i>C. gloeosporioides</i>	IMI 356878, ICMP 17821, CBS 112999 ^T	<i>Citrus sinensis</i>	Italy	JX010152	JX010056	JX010445
<i>C. horii</i>	ICMP 10492 T, MTCC 10841	<i>Diospyros kaki</i>	Japan	GQ329690	GQ329681	JX010450
<i>C. kahawae</i> subsp. <i>kahawae</i>	IMI 319418 T, ICMP 17816	<i>Coffea arabica</i>	Kenya	JX010231	JX010012	JX010444
<i>C. musae</i>	CBS 116870 T, ICMP 19119, MTCC 11349	<i>Musa</i> sp.	USA	JX010146	JX010050	HQ596280
<i>C. nupharicola</i>	CBS 470.96, ICMP 18187 ^T	<i>Nuphar lutea</i> subsp. <i>polysepala</i>	USA	JX010187	JX009972	JX010398
<i>C. psidii</i>	CBS 145.29, ICMP 19120 ^T	<i>Psidium</i> sp.	Italy	JX010219	JX009967	JX010443
<i>C. queenslandicum</i>	ICMP 1778 ^T	<i>Carica papaya</i>	Australia	JX010276	JX009934	JX010414
<i>C. salsolae</i>	ICMP 19051 ^T	<i>Salsola tragus</i>	Hungary	JX010242	JX009916	JX010403
<i>C. siamense</i>	ICMP 18578, CBS 130417 ^T	<i>Coffea arabica</i>	Thailand	JX010171	JX009924	JX010404
	USJCC 0056 (C6)	<i>Allium cepa</i>	Sri Lanka	MW440478	MZ803133	MZ803137
	USJCC 0058 (C10)	<i>Allium cepa</i>	Sri Lanka	MW444844	MZ803135	MZ803138
<i>C. syzygicola</i>	DNCL021 MFLUCC 100624 ^T	<i>Syzygium samarangense</i>	Thailand	KF242094	KF242156	KF254880
<i>C. theobromicola</i>	MTCC 11350, CBS 124945 ^T , ICMP 18649	<i>Theobroma cacao</i>	Panama	JX010294	JX010006	JX010447
<i>C. ti</i>	ICMP 4832 ^T	<i>Cordyline</i> sp.	New Zealand	JX010269	JX009952	JX010442
<i>C. tropicale</i>	CBS 124949, ICMP 18653, MTCC 11371 ^T	<i>Theobroma cacao</i>	Panama	JX010264	JX010007	JX010407
<i>C. xanthorrhoeae</i>	BRIP 45094, ICMP 17903, CBS 127831 ^T	<i>Xanthorrhoea preissii</i>	Australia	JX010261	JX009927	JX010448

ATCC American Type Culture Collection, BRIP Queensland Plant Pathology Herbarium, Australia, CBS-Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, CGMCC-China, General Microbiological Culture Collection, Beijing, China, ICMP-International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand, IMI Culture collection of CABI Europe UK Centre, Egham, UK, LC Working collection of Lei Cai, housed at CAS, China, MFLUCC Mae Fah Luang University Culture Collection, Chiang Rai, Thailand, MTCC The Microbial Type Culture Collection and Gene Bank, Department of Biotechnology (DBT) and the Council of Scientific and Industrial Research (CSIR), Government of India, USJCC University of Sri Jayewardenepura culture collection, Department of Botany, University of Sri Jayewardenepura, Nugegoda, Sri Lanka. All the cultures in the table are ex-type cultures (T) except the isolates in the study (bold). Isolate codes and accession numbers in bold represents the isolates used in this study

Fig. 1 Morphology of *Colletotrichum siamense* (a–c) Anthracnose-twister disease symptoms of onion plants d) Culture on PDA. e) Conidial masses on PDA. f,g) Conidia. h,i) Conidiophores. j) Conidia. k–m)

Appressoria produced on slide cultures of PDA. n–r) anthracnose-twister symptoms observed in pathogenicity testing. Scale bars: f=33 μ m; g–i=23 μ m; j, k=10 μ m; l, m=5 μ m

Fig. 2 Maximum Likelihood Phylogram generated using the combined analysis of ITS, GPDH and TUB2 for the *Colletotrichum gloeosporioides* species complex with representative isolates from each taxon. Isolates from this study are indicated in red. Ex-type isolates

are indicated in bold. The ML bootstrap values greater than 50% are displayed above the branches. The tree is rooted with *C. boninense* (CBS 123755)

primers and resulted DNA sequences were deposited in GenBank (Table 1). Individual and combined Maximum Likelihood (ML) gene trees based on the three gene regions (ITS, GPDH and TUB2) were estimated using the software RAxML 8.2.8 Black Box (Stamatakis et al. 2008) in the CIPRES Science Gateway platform (Miller et al. 2010). For the concatenated dataset, all free-modal parameters were

estimated by RAxML with an ML estimate of 25 per site rate categories. The concatenated dataset was partitioned by locus and the gaps were treated as missing data. The RAxML analysis utilised the GTRCAT model of nucleotide substitution with the additional options of modeling rate heterogeneity (G) and proportion invariable sites (I).

Pathogenicity tests were carried out using healthy onion plants grown in potting mixture under greenhouse conditions. Fungal isolates were inoculated in non-wounded plants using colonised agar plugs of 7.5 mm diameter with actively growing mycelia and by spraying of conidial suspension (10^5 – 10^7 conidia/ml). Each method of inoculation consisted of three replicates and a control per each isolate. Controls were maintained by using non-colonised agar plugs and autoclaved distilled water. Plants were incubated at 25 °C.

Morphological observations revealed that the fungal colonies grown on PDA were flat with entire margin, white surface with an abundant cottony aerial mycelium, and orange conidial masses visible in concentric rings. Conidia measured were $13.2 - 20.1 \times 3.7 - 5.9 \mu\text{m}$ ($\bar{x} = 15.2 \pm 1.5 \times 4.7 \pm 0.6$, $n = 30$), unicellular, hyaline, aseptate, smooth-walled, cylindrical with rounded apex. Appressoria measured were $7.2 - 12.3 \times 3.1 - 6.8 \mu\text{m}$ ($\bar{x} = 9.1 \pm 2.2 \times 5.6 \pm 1.4$, $n = 5$), unicellular, brown, thick-walled, ellipsoidal to irregular. Conidiophores were hyaline, cylindrical to subcylindrical (Fig. 1). Based on the morphological features, the isolates were tentatively identified as a species belong in *Colletotrichum gloeosporioides* complex (Weir et al. 2012).

The BLASTn search of newly generated ITS sequences, against ex-type material revealed that the isolates C6 (Culture collection: USJCC 0056) (GenBank: MW440478) and C10 (Culture collection: USJCC 0058) (GenBank: MW444844) are 99.8% identical to the ex-type of *Colletotrichum siamense* strain C1315.2 (culture collection(s): ICMP:18,578/ CBS130417, GenBank: JX010171). The standard BLASTn search of newly generated GPDH sequences of C6 (GenBank: MZ803133) and C10 (GenBank: MZ803135) revealed 99.8% identity with *C. siamense* isolates LC 2876 (GenBank: KP703334) and YJMB27 (GenBank: MK963091). Similarly, the BLASTn analysis of newly generated TUB2 sequences of C6 (GenBank: MZ803137) and C10 (GenBank: MZ803138), are 99.8% identical with *C. siamense* YJMB29.1 (GenBank: MK963154) and YJMB22.1 (GenBank: MK963153) isolates.

A ML phylogenetic tree generated based on the combined DNA sequence alignment of three gene regions (ITS, GPDH and TUB2) with ex-type species in *C. gloeosporioides* species complex revealed that the two new isolates (C6 and C10) obtained in this study are conspecific with ex-type isolate of *C. siamense* (strain C1315.2/ ICMP:18578/ CBS130417) (Fig. 2).

Pathogenicity experiments resulted in the appearance of symptoms similar to original specimens, after 5 d of incubation. The control plants remained symptomless. Koch's postulates were completed by re-isolating the pathogen from symptom-bearing plants. *C. siamense* had been previously found affecting onion plants in Southwest India causing

anthracnose (Chowdappa et al. 2015; Farr and Rossman 2021). To our knowledge, this is the first report of *C. siamense* affecting *A. cepa* in Sri Lanka. Further studies will be required to understand the epidemiology of this disease and foster disease management programs in Sri Lanka to avert potential outbreaks in future.

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Declarations

Conflict of interests The author(s) declare no conflict of interest.

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