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Morphological and Molecular Characterization of *Colletotrichum* Species Causing Anthracnose in Soursop (*Annona muricata*)

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ABSTRACT

Anthracnose is the principal postharvest disease of ripe soursop (*Annona muricata*) in Sri Lanka, causing reduced shelf life and marketing potential under warm, humid conditions. The disease was earlier believed to be caused by *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*, which are presently considered as species complexes. Identification of *Colletotrichum* species within complexes was facilitated by the use of multi-gene sequence comparison. For the present study, *Colletotrichum* was isolated from anthracnose lesions on ripe soursop, collected from the Central and North Western province of Sri Lanka. Morphometric analysis, using eleven characters ranging from colony, conidial to appressorial morphology, divided the twenty isolates into two main clusters. The conidial size was the most contributing factor for cluster separation. Ten isolates representing the two clusters were subjected to DNA sequence analysis, using Internal transcribed spacer of the ribosomal DNA (ITS), β-tubulin 2 (TUB2) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as gene regions. For resulting sequences, the species affiliations and identities were determined through similarity-based searches of the NCBI GenBank Database. Considering 95% similarity, *Colletotrichum siamense* and *Colletotrichum fructicola* were identified as pathogens associated with soursop anthracnose. Both species are belonging to the *C. gloeosporioides* complex. ITS region contributed in placing the taxa within *C. gloeosporioides* while TUB2 and GAPDH determined their identity to species level. This is the first report of *C. fructicola* and *C. siamense* from soursop anthracnose.

KEYWORDS: Anthracnose, *Colletotrichum*, Molecular data, Morphology, Soursop

INTRODUCTION

Soursop (*Annona muricata* L.), belonging to the family Annonaceae, originated in the lowlands of Central America. The fruit can be successfully grown in all three agro-ecological zones, Dry, Intermediate and Wet zone, except in the higher elevations (Anon, 2007). White edible pulp of soursop contains vitamins B_1, B_2, and C, high percentages of moisture, non-reducing sugars, and low percentages of fat and protein (Badrie and Schauss, 2009). Soursop can be eaten fresh or processed into juice, pulp, ice cream, cakes and candy (Love and Paull, 2011). When considering the medicinal values of soursop, in the recent past it was highlighted in the local media as a potential cancer fighting fruit (Jayawardhana, 2013). Soursop is also used as an antispasmodic, emetic, and sudorific in herbal medicine. The juice of the fruit is taken orally for hematuria, liver complaints, and urethritis while an extract of the stem bark has shown to reduce stress levels (Badrie and Schauss, 2009).

Soursop is affected by many insect pests and diseases. Out of these diseases, anthracnose (*C. gloeosporioides*) is the principal postharvest disease of soursop in warm humid environment (Badrie and Schauss, 2009). The spread of the disease is favored by windy conditions. Due to anthracnose (*C. gloeosporioides*), fruit production and availability may be severely limited through infection of flowers and fruit, which rot or shrivel. *Colletotrichum* has been ranked the eighth most important plant pathogen genus in the world due to its scientific and economic importance (Dean *et al.*, 2012). Symptoms of anthracnose are manifested mainly in two ways. In one, water soaked, sunken, dark brown to black necrotic spots are produced and the other produced pepper spot specks (Figure 1). The center of an older spot develops gelatinous pink or orange spore masses (Nelson, 2008). There are no direct evidences about any harmfulness of anthracnose to humans and animals, but the infection may result in unpleasant taste and texture in the pulp with ripening.

Colletotrichum represent the anamorphic (asexual) reproductive state, commonly associated with infection, while *Glomerella cingulata* represents the telomorphic (sexual) reproductive state, usually limited to dead host tissues (Sutton, 1992). The classification of the genus *Colletotrichum* to species level, based on the morphological features is now considered as unreliable and non-informative. Due to that reason the classification of genus

Colletotrichum is re attempted using molecular data.

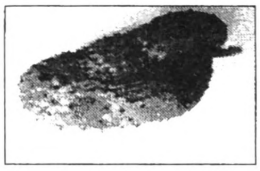

Figure 1. Soursop fruit showing anthracnose symptoms

Recent studies on *Colletortichum* species through epitypification and subsequent use of multi-gene phylogeny have revealed that some of the species, that were previously thought to be single species, are in fact species complexes (Cannon *et al.*, 2008). There are three main species complexes that have been proposed *viz. C. boninense* species complex with 18 species (Damm *et al.*, 2012), *C. acutatum* complex with 31 species (Damm *et al.*, 2012b) and *C. gloeosporioides* complex with more than 22 species (Weir *et al.*, 2012).

These studies have initiated research on the re-evaluation of *Colletotrichum* species infecting different food crops such as, tropical, sub-tropical and temperate fruits, chilies, *etc.* However, even globally, only few studies on characterization of *Colletotrichum* species infecting soursop have been done. One such study was done by Wier *et al.*, (2012) in Panama, using ITS, GAPDH, CAL, alpha(1)-antichymotrypsin (ACT) and chorionic somatomammotropin hormone 1 (CSH-1) gene regions. According to their studies, *C. tropicale* was the species causing anthracnose in soursop. Another study by Álvarez *et al.*, (2006), conducted in Colombia using ITS region, found out that *C. gloeosporioides* and *C. acutatum* are associated with anthracnose in soursop. ITS region has been useful only for the identification of *Colletotrichum* isolates into the broad complex level but not for the species level (Cai *et al.*, 2009). Furthermore, there were no previous attempts to characterize the causative agent of soursop anthracnose at species level in Sri Lanka. Therefore, this study was undertaken to accurately identify *Colletotrichum* species causing anthracnose in soursop in Sri Lanka. The results of this study would help growers, handlers and exporters to take effective measures to control soursop anthracnose and increase availability of harvested fruit. Also it will be important in Bio-security and quarantine.

MATERIALS AND METHODS

The study was carried out at the Plant Pathology Laboratory in the Department of Botany, University of Peradeniya from December, 2015 to May, 2016.

Isolation of Colletotrichum

Ripe soursop fruits, showing anthracnose symptoms, were collected from fruit markets and orchards in Peradeniya, Eriyagama, Galagedara, Kandy (Central Province) and Kurunagala (North Western Province) of Sri Lanka. *Colletortichum* was isolated from anthracnose lesions on 10 soursop fruits. Small (5×5 mm^2) segments were cut from infected areas and surface sterilized by dipping in 1% sodium hypochlorite (Clorox, USA) for 1-3 min. The segments were rinsed twice in sterilized distilled water (SDW) and finally dried on sterilized tissue paper. The tissue segments were aseptically transferred onto Potato Dextrose Agar (PDA) plates (four pieces per plate), supplemented with 50 µg/mL tetracycline to suppress bacterial growth. The plates were incubated at room temperature (28-30 °C) for 5-7 days. The isolates, labeled with acronyms SB01-SB20, were sub-cultured by transferring discs (6 mm diameter) of mycelium onto fresh PDA plates and allowed to grow at 28 °C for seven days.

Preparation of Monoconidial Isolates

A suspension of conidia was prepared by suspending mycelia scraped from seven day old cultures and filtering through sterile glass wool. A loopful of conidia suspension, from each isolate was streaked on thin tap water agar plates and the plates were incubated at 28 °C for 18 h. A small piece of agar with a single germinated conidium, located by a light microscope (Olympus CX22, made in Philippines), was transferred onto fresh PDA. The plates were incubated for seven days.

Morphological Studies

The morphological characteristics were studied using seven day old isolates in triplicate. To study colony characteristics, the colony color (white or blackish ash), texture (cottony or wooly), elevation of colonies (elevated or flat), presence or absence of concentric rings, pigmentation underneath, visible pink conidia masses and sectoring were recorded and the images were digitized.

Drops (20 µL) of a conidial suspension (1×10^7 conidia/mL) of each isolate were placed on clean microscopic slides. After placing a cover slip, the slides were observed under a light microscope (ZEiZZ Lab.A1 with AxioCamERc 5s camera and ZEN lite 2012 software) and the shapes of conidia were recorded and photographed while the length and width of 50 randomly selected conidia per each isolate were measured using ZEN lite 2012 software and averaged.

To produce appressoria, two drops (20 µL) of a conidia suspension (1×10^5 conidia/mL) were placed on either side of three replicate glass slides for each isolate. The slides were incubated in moist chambers at 28 °C for 12-15 h. The shape of appressoria was recorded and photographed.

Morphometric Analysis

All the morphological characters were coded into a data matrix and a morphometric analysis was carried out using the PAST version 2.17 (Ryan *et al.*, 2001). First, the Euclidean similarity measure option was selected to generate a range-normalized Manhattan distance matrix. A hierarchical cluster analysis (CA) was performed by selecting the 'paired group' (UPGMA) option to depict the phenetic relationships among all isolates as a dendrogram. An ordination analysis was carried out using the Euclidean measure option (transformation exponent C=2) to generate a distance matrix for use in a Principal Coordinates Analysis (PCoA). Similarity Percentage Analysis (SIMPER) and Principal Component Analysis (PCA) were performed to obtain more accurate dissimilarity levels of the groups and to identify the morphological characters that contributed the most for the separation of clusters, and thereby to find the best morphological characters to differentiate between the *Colletotrichum* species.

Molecular Studies

DNA Extraction

Ten isolates (SB01, SB02, SB05, SB08, SB09, SB12, SB15, SB16, SB18 and SB20) were randomly chosen for molecular studies. DNA was extracted using the protocol described by Živković *et al.*, (2010). Aerial mycelium (0.5 g) scraped from seven day old cultures, using a sterile inoculation loop, was placed in a sterile micro centrifuge tube (1.5 mL), containing 300 µL of extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 25 mM EDTA, and 2% sodium dodecyl sulfate, pH 8.5) and crushed finely. Uncapped tubes were then placed in a boiling water bath for 5 min and allowed to cool at room temperature (25 °C). Two hundred micro liters of phenol that was equilibrated with extraction buffer (vol/vol), together with an equal volume of chloroform was added. The tubes were vortexed for about 2-3 min, and centrifuged at 12 000 rpm for 5 min. The resulting supernatant was then transferred into a new 1.5 mL micro centrifuge tube containing 200 µL of chloroform and vortexed for 30 sec followed by the centrifugation at 12 000 rpm for 15 min. The supernatant was pipetted out into a new 1.5 mL tube and 200 µL of ice cold isopropanol was added. Tubes were inverted several times for the DNA to precipitate and centrifuged at 12 000 rpm for 15 min. The pellet was retained and washed with 400 µL of ice cold ethanol and centrifuged at 12 000 rpm for 5 min. The pellet was air-dried for 10 min, and re-suspended in 50 µL in low-TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.5) to dissolve DNA. Finally 3 µL of Ribonuclease A was added and incubated for 1 h at 37 °C and stored at -20 °C.

PCR Amplification

Three gene regions, β-tubulin 2 (TUB2), [(BT2a 5'-GGTAACCAAATCGGTGCTTTC-3'), (BT2b 5'-ACCCTCAGTGTAGTGACCC TTGC3')], GAPDH [(GD92F1 5'-GCCG TCAACGACCCCTTCATTGA-3'), (GDR1 5'-GGGTGGAGTCGTACTTGAGCATGT-3')] and ITS [(ITS-1F 5'-CTTGGTCATTTAGA GGAAGTAA-3'), [(ITS-4 5'-TCCTCCGCTT ATTGATATGC-3')] were amplified (Weir *et al.*, 2012 and reference there in).

All PCR amplifications were carried out in a total 40 µL volume containing 1X PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate (dNTPs), 0.2 µM each forward and reverse primer, 1 U of Taq DNA polymerase (Promega) and 3 µL of unquantified DNA extract, using a Thermal Cycler (Applied Bio systems Veriti). The PCR program consisted of an initial denaturation at 95 °C for 4 min, 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55, 60, 52 and 54 °C for TUB2, GAPDH, ITS and GS respectively for 30 sec, extension at 72 °C for 45 sec and a final extension at 72 °C for 7 min (Weir *et al.*, 2012). The PCR products were separated by electrophoresis in 1% agarose gel, stained with ethidium bromide, and visualized with a UV trans illuminator. The PCR products were sequenced for both directions (Applied Bio systems, 3500 genetic analyzer) at the Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Sri Lanka.

RESULTS AND DISCUSSION

Morphological Characteristics

The morphological analysis initially divided the twenty isolates into two major clusters, cluster A and cluster B, at a dissimilarity of approximately 25%, which is very low, and each of these clusters were further divided into cluster C, D, E and F (Figure 2.).

The Principal Coordinates Analysis (PCoA) also supported clustering of two phenetic groups resulted in the Cluster analysis. PCoA analysis resulted in two separate groups

with non-overlapping distribution of coordinates (Convex hulls).

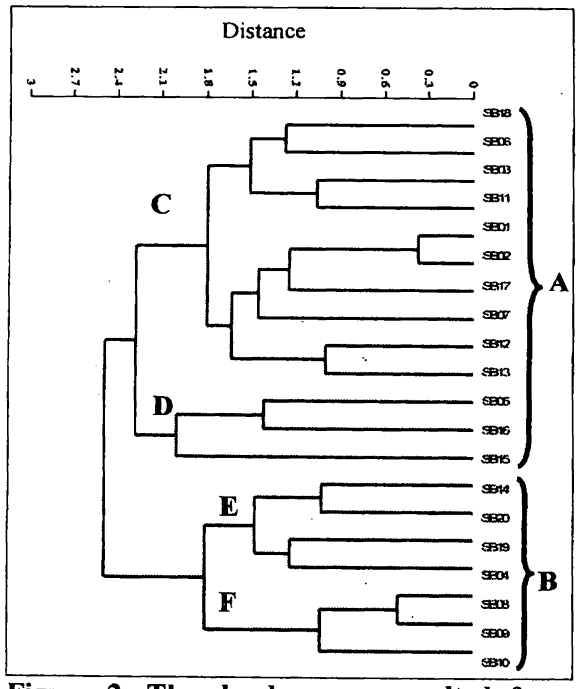

Figure 2. The dendrogram resulted from cluster analysis, showing the two clusters of *Colletotrichum* isolates A and B

According to the SIMPER analysis, conidia length is the most contributed quantitative character with a contribution of 1.172, while production of conidia is the mostly contributed qualitative character with a contribution of 0.8244 for the clustering of A and B. Appressoria shape did not support for the separation of *Colletotrichum* into clusters while elevation of the colony, pigmentation underneath and colony colour showed a significant contribution for the separation (Table 1).

Table 1. SIMPER analysis summarizing the contribution of each of quantitative and qualitative characters for the cluster analysis

Taxon	Contribution
Conidial Length	1.172
Conidial Width	0.8663
Production of conidial masses	0.8244
Elevation of the colony	0.737
Pigmentation underneath	0.5942
Colony colour	0.5942
Concentric rings	0.5498
Conidial shape	0.5062
Colony Texture	0.4439
Sectoring	0.08042
Appressoria shape	0

However, classification of the genus *Colletotrichum* based on the morphological features, is now considered unreliable and non-informative as morphology can vary with environmental factors and incubation conditions such as culture media temperature and light regime (Cannon *et al.*, 2000). Further, these characters can change or disappear with continuous sub-culturing (Cai *et al.*, 2009). Also Appressorial morphology does not appear to be a useful character for identification of *Colletotrichum* spp. This was also confirmed by previous studies (Prihastuti *et al.*, 2009).

Identification of Colletotrichum Isolates Based on Molecular Data

Ten isolates, SB01, SB02, SB05, SB08, SB09, SB12, SB15, SB16, SB18 and SB20, used for molecular studies represented the main clusters separated based on morphological variations. All the sequences obtained for different gene regions, TUB2 [~700 bp], GAPDH [~300 bp], which are protein coding genes for the identification of *Colletotrichum* isolates to the species level, as been recommended by Weir *et al.*, (2012), and ITS [~500 bp] were subjected to BLAST search with available nucleotide sequence in GenBank (htttp://www.ncbi.gov), and the first three identities that gave >95% similarity were mainly considered. Only the *Colletotrichum* isolates SB20 and SB12 were identified unambiguously as *C. fructicola* and *C. siamense*, respectively. Even in cluster analysis these two isolates were distinctly separated from each other, since SB12 was grouped in cluster A and SB20 was in cluster B. However the identification of other isolates were problematic, as it showed >95% similarity for both *C. fructicola* and *C. siamense*. Although the two gene regions that were used along with the ITS, have been considered to be the most effective gene regions with maximum resolving ability for the identification of *Colletotrichum* spp. (Cai *et al.*, 2009, Weir *et al.*, 2012), in certain instances both TUB2 and GAPDH regions are insufficient in verifying the identity of *C. fruticola* (Weir *et al.*, 2012). According to their studies this taxon can be distinguished using Glutamine synthetase (GS) and Manganese-superoxide dismutase (SOD2) gene regions. Moreover *C. Fruticola* and *C. siamense* are considered to be very closely associated within the same host (Phoulivong *et al.*, 2010). This may be the reason for having very low dissimilarity (25%) for the cluster analysis. Hence further studies should be conducted to differentiate these isolates among *C. fruticola* and *C. siamense*.

CONCLUSIONS

Based on DNA sequence analysis of this study, *C. fructicola* and *C. siamense* were among the causal organisms of soursop anthracnose in Sri Lanka. This is the first record

of the association of *C. fructicola* and *C. siamense* with soursop anthracnose. Also low morphological dissimilarity between the two isolates may indicate that the two species are closely related. Since accurate identification of causal agents is crucial for meaningful disease management, the findings of the present study would be helpful in the selection of new disease management strategies for soursop anthracnose.

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