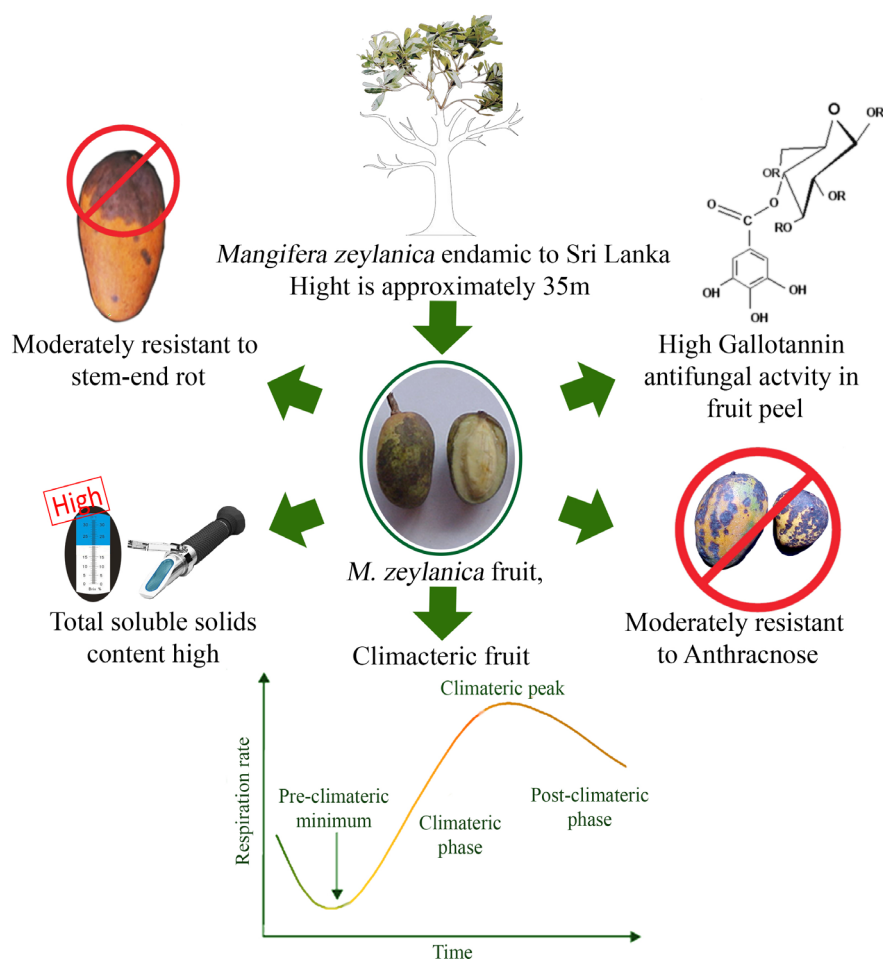


Response of endemic *Mangifera zeylanica* (Blume) Hook. f. fruit to common postharvest pathogens of cultivated mango (*Mangifera indica* L.) fruit in Sri Lanka

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Highlights

- *Mangifera zeylanica* fruit shows a climacteric pattern of respiration.
- *M. zeylanica* fruit is moderately resistant to *Colletotrichum* spp. and *Lasiodiplodia* sp. causing anthracnose and stem-end rot, respectively.
- Peel of unripe *M. zeylanica* fruit contains antifungal gallotannins that are present in the fruit peel of *M. indica*, contributing to its constitutive defences.

RESEARCH ARTICLE

Response of endemic *Mangifera zeylanica* (Blume) Hook. f. fruit to common postharvest pathogens of cultivated mango (*Mangifera indica* L.) fruit in Sri Lanka

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Abstract: Two species of *Mangifera* are found in Sri Lanka, the cultivated *Mangifera indica* L. and the endemic *M. zeylanica* (Blume) Hook.f. [Et-amba (S)]. *Mangifera indica* is a climacteric fruit, susceptible to infection by many fungal pathogens, causing postharvest diseases at ripe stage. Among them, *Colletotrichum* spp. and *Lasiodiplodia theobromae* that cause anthracnose and stem-end rot disease respectively, are considered the most destructive postharvest pathogens in ripe mangoes. Harvested fruit of *M. zeylanica* also showed a climacteric pattern of respiration and the pulp was sweet to taste, with acceptable TSS value for mango fruit. The present study investigated the resistance or susceptibility of the fruit of endemic *M. zeylanica* to these two pathogens. Artificial inoculation of fruits with *C. gloeosporioides* and *L. theobromae* separately, produced anthracnose as well as stem-end rot symptoms respectively, showing that *M. zeylanica* fruits are susceptible to the pathogens. However, considering the pattern and the extent of disease development, *M. zeylanica* fruits can be considered moderately resistant to both pathogens. *Alternaria* sp., *Pestalotiopsis* sp., *Lasiodiplodia theobromae*, *Curvularia* sp. and *Neofusicoccum* sp. were frequently isolated from the pedicel and the stem-end region of healthy fruits of *M. zeylanica* at harvesting maturity. However, *Colletotrichum* species could not be isolated from either the pedicel or the stem-end region of *M. zeylanica*. TLC-*Cladosporium* bioassay of

peel extract of the unripe *M. zeylanica* fruit resulted in a large inhibition zone at R_f 0.00 which corresponded with antifungal gallotannins contributing to the constitutive defences of *M. indica* fruit against invading pathogens.

Keywords: *Mangifera zeylanica*; endemic; postharvest; anthracnose; stem-end rot; mango fruit.

INTRODUCTION

The genus *Mangifera* (Family Anacardiaceae) consists of 69 species of Asian origin (Nakasone and Paull 1998) and all species do not bear edible fruit. The mango, *Mangifera indica* L., is the best known and most widely cultivated genus in the world. *Mangifera zeylanica* (Bl.) Hook f. is among the few species in the genus that bear edible fruit. *Mangifera zeylanica* is an endemic species to Sri Lanka and locally known as Et-amba (S) (Dassanayake and Fosberg, 1983). Although fruits of Et-amba are edible and the whole plant has medicinal properties, it is not cultivated due to the exceptionally smaller size (Fig. 1) (3 - 4 cm long) of the fruit (Dassanayake and Fosberg, 1983) and proportionately larger seed which occupies most of the fruit volume (Medagoda and Jayawardena, 1997).



Figure 1: (A) Twig of *M. zeylanica* with (a) lanceolate leaves (5 - 8 cm in length) arranged spirally, and (B) mature fruit of *M. zeylanica*, (b) fully mature fruit (4 cm in length), (c) longitudinal section of the fruit showing the seed and flesh, (d) seed of the fruit.



Weeraratne *et al.* (2005) carried out a study to establish the evolutionary relationship among eleven selected local *M. indica* cultivars and *M. zeylanica* using Random Amplified Polymorphic DNA (RAPD) markers. The dendrogram obtained showed four cultivated varieties of *M. indica* and the endemic *M. zeylanica*, that are all regular fruit bearers, grouped together. Based on the 2012 National Red List of Sri Lanka (MOE, 2012), *M. zeylanica* is categorized in the National conservation status 'Least Concerned' and as 'Vulnerable' according to Global Conservation status (Ariyaratne *et al.*, 2017).

Bark extracts of *M. zeylanica* show strong anti-cancer properties (Ediriweera *et al.*, 2016 a, b; 2017) and leaf extracts possess antimicrobial properties against certain pathogenic (human) bacteria and fungi (Dhanasiri *et al.*, 2014). Ethanol and 70 °C water extracts of leaves are effective against many microorganisms including Methiciline-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Pseudomonas aeruginosa* and non-albicans *Candida* (Dhanasiri *et al.*, 2014). Further, it has been shown that endophytic fungi colonizing leaves of *M. zeylanica* secrete potent antimicrobial substances against selected (human) pathogenic bacteria as well (Senevirathna *et al.*, 2015). However, there are no reports on diseases and the fungal pathogens causing diseases to *M. zeylanica* fruit. There is a common belief that certain endemic species or 'wild varieties' are more resistant to disease than cultivated varieties, yet no studies have been done so far to establish its validity, especially with *M. zeylanica*. The present study investigated the response of *M. zeylanica* fruits to major fungal pathogens causing two most common postharvest fruit diseases, anthracnose and stem-end rot (SER), in mango (*M. indica*). Mango anthracnose was earlier known to be caused by *Colletotrichum gloeosporioides* Penz and Sacc. (Dodd *et al.*, 1991; Arauz, 2000). More recent reports indicated that in Sri Lanka the mango anthracnose is caused by several species including *Colletotrichum asianum*, *C. siamense*, *C. fruticola* and *C. tropicale*, all belonging to the *Colletotrichum gloeosporioides* species complex (Komala Withanage *et al.*, 2014). The stem-end rot of mango is caused mainly by *Lasiodiplodia theobromae* (Pat.) Griff et Maubl. in tropical Asia (Johnson *et al.*, 1993) and *Dothiorella* spp., *Phomopsis mangiferae*, *Pestalotiopsis* sp. and *Cytosphaera mangiferae* are also known as causal agents (Johnson *et al.*, 1992). *Lasiodiplodia theobromae*, *Dothiorella* spp., *C. gloeosporioides*, *Phomopsis mangiferae* and *Pestalotiopsis mangiferae* have been identified as SER pathogens of mango, in Sri Lanka, while a study using ITS sequence data has reported two new Ascomycota species from the local cultivar 'Karuthacolomban', namely: *Xylaria* sp. and *Nodulisporium* sp. (Karunanayake *et al.*, 2014; Ekanayake *et al.*, 2019).

The present study was carried out to investigate the resistance or susceptibility of *M. zeylanica* to anthracnose and stem-end rot in ripe fruits, and also to assess the level of preformed antifungal activity in fruit peel of *M. zeylanica*, in comparison with three local mango (*M. indica* L.) cultivars showing variable susceptibility to the two postharvest fungal diseases considered in the study. 'Karuthacolomban' is more resistant to anthracnose

disease but highly susceptible to SER whereas the cultivar 'Willard' is highly susceptible to anthracnose while more resistant to SER. The cultivar 'Rata' ('Vellacolomban') is moderately susceptible to both diseases (Karunanayake *et al.*, 2014).

MATERIALS AND METHODS

Fruits

Three cultivars of *M. indica*, 'Karuthacolomban' (KC), 'Willard' and 'Rata' were used along with *M. zeylanica* for inoculation studies. The fruits of *M. indica* were bought from a wholesale market in 'Kiribath kumbura' (7° 27" N and 80° 57'E) (Kandy District, Central Province/ CP) which receives fruits regularly from orchards in Dambulla, (7° 52' 27.18" N and 80° 39' 4.06" E) (Matale District, CP). Unripe fruits at harvest maturity, selected for the study, had been harvested on the same day. Mature, unripe fruits of *M. zeylanica* were hand-picked (by employing climbers) from trees of 'Raththota' area (7° 30' 48" N, 80° 39' 42" E) (Matale District, CP). All fruits selected for studies were healthy and blemish free. Fruits were transported to the Plant Pathology laboratory at the University of Peradeniya within 8 h of harvest and taken for studies within 24 h. All fruits were washed in tap water and allowed to dry under ambient conditions, 28 ± 2 °C, in the laboratory.

Naturally occurring fungi in fruit of *M. zeylanica* - isolation studies

Small square segments (0.5 x 0.5 cm²) were cut from the peel at the stem-end of the healthy fruit and pieces (0.5 cm long) of the fruit pedicel were also cut from fruits of *M. zeylanica* to isolate fungi present, if any, in the outer cell layers of peel and fruit pedicel. The segments were surface sterilized in 1% NaOCl (Chlorox, USA) for 3 min, washed in sterile distilled water (SDW), cut in half (pedicel longitudinally) and transferred on to PDA in Petri dishes. The plates were incubated at 28 ± 2 °C for 10 days for initial isolations. Ten-day old colonies from tissue segments on PDA were examined for colony morphology and under the light microscope (Olympus CX31 Microscope with Digital Camera DP20) for conidial morphology and identified to genus level with the aid of CMI descriptions. Identified fungi were sub-cultured on fresh PDA and maintained as pure cultures for 4 to 6 weeks to observe structures such as pycnidia.

Symptoms of diseases which developed as natural infections on harvested *M. zeylanica* fruits were also recorded. The causal agents of these diseases were isolated from symptomatic fruit peel tissues as described above.

For isolation of endophytic fungi, tissue segments were aseptically cut from the inner layers of the peel and from the outer pulp, just below the peel. Visually healthy, unripe fruits were used for isolation studies. These segments were subjected to triple sterilization, by first placing in 70 % ethanol for 30 sec, then in 1% NaOCl for 2 min and again in 70% ethanol for 15 sec. (Petrini, 1986). The segments were removed using sterile forceps, cut into halves and transferred aseptically on to PDA in Petri dishes. The plates were incubated at room temperature (28 ± 2 °C).

Fruit inoculations

Anthraco nose pathogen

A suspension of conidia of *C. gloeosporioides* was prepared by pouring SDW into Petri plates and scraping the mycelium of a two-week-old pure culture, using a sterile glass rod, then filtering through glass wool (Karunanayake *et al.*, 2015). The concentration was adjusted to 10^5 conidia/ml. Three drops (20 μ l) were placed along the long axis of each fruit and the inoculated fruits were maintained in moist chambers to provide necessary humidity for conidia germination (100% RH and 28 ± 2 °C). When symptoms appeared, disease severity was assessed by measuring the diameter of the lesion along 2 axes right angles to each other and calculating the average diseased area. Measurements were taken daily for 7 days after inoculation.

Stem-end rot pathogen

The fruit pedicel was removed and the peel at the stem end of each fruit was slightly damaged by scraping with a sterile scalpel (Nisansala *et al.*, 2015). A mycelial plug of *L. theobromae* was placed with the mycelium touching the freshly cut surface of the stalk-end. The inoculated fruits were incubated in moist chambers at 100% RH and 28 ± 2 °C. The mycelia plug was removed after 24 h and the fruits were returned to the moist chambers. When the symptoms appeared, the severity of the stem-end rot was measured daily by tracing the affected area onto a transparent graph papers with 1 mm² squares and recording the number of squares occupied by the disease area. Measurements were taken for 7 days after inoculation.

Data analysis

Each experiment consisted of 8 replicate fruits from each cultivar and the trial was carried out twice. Data of the two trials were pooled and the variance was analyzed as a completely randomized design at the 5% probability level with the software package SAS ver.6.12.

Antifungal activity of the peel of M. zeylanica

Thin (1 - 3 mm) peels were obtained from mature unripe fruits of *M. zeylanica* and extracted into dichloromethane and methanol (1:1 v/v) using vacuum infiltration (Karunanayake *et al.*, 2011). Aliquot (100 μ l) of crude peel extract equivalent to 0.5 g fresh peel tissue was spotted on TLC, developed with chloroform:methanol:ethyl acetate (90:5:5 v/v/v) and subjected to a thin layer chromatography bioassay with *Cladosporium cladosporioides* as the assay organism. Inhibition zones corresponding to antifungal activity were observed after 72 h incubation in moist chambers (Karunanayake *et al.*, 2011).

Rate of respiration

Twenty-four freshly harvested fruits of uniform maturity were taken for the study. The rate of respiration and variation in total soluble solids (TSS) were measured daily using three fruits for up to 8 days after harvest. Each fruit which was to be used for the study on a given day was weighed and then placed inside an air-tight glass vessel, and the vessel was sealed for one hour prior to the measurement

of CO₂. The composition of air in the vessel was assessed using CO₂ measurements obtained from a gas analyzer (280 Combo gas analyzer). The rate of respiration was calculated using the highest CO₂ reading using the equation below (Kays, 1991).

Rate of respiration =

$$\%CO_2 \times \frac{\text{Volume of vessel} - \text{volume of fruit (mL)}}{\text{Time of incubation (h)} \times \text{fresh weight of fruit (g)}}$$

The weight of the fruit was used as the volume, assuming that the fruit density to be equal to that of water. Three fruits were used daily, and the average value was calculated.

Total soluble solids content in pulp

The middle one-third of fruit mesocarp was obtained separately from each of the three fruits on days 3 to 7 after harvest. The mesocarp tissues obtained for each day were combined and blended (ULTRATURRAX omnimixer ®, T25 basic) for 1 min. A portion of this pulp mixture (approximately 5 g) was then centrifuged at 3000 g (Sigma 3K30 Laboratory centrifuge) for 10 min. A drop (20 μ l) of the juice extracted from the pulp by this process was placed on the glass prism of a hand-held refractometer (Leica, Model 10430) and the °Brix reading at room temperature (28 ± 2 °C) was obtained (Samane *et al.*, 2012).

RESULTS AND DISCUSSION

Naturally occurring fungi in fruit peel of *M. zeylanica*.

Isolations made from healthy stem-end area showed the presence of several Botryosphaeriaceae species; *Lasiodiplodia theobromae* (Syn. *Botryodiplodia theobromae*) (Fig. 2 a, b), and *Neofusicoccum* sp. (figure not provided). Isolations from healthy fruit pedicel showed the presence of *Alternaria* sp., *Neofusicoccum* sp., *Curvularia* sp. (figures not provided) and *Pestalotiopsis* sp. (Fig. 2 c, d), sp. Endophytes were not isolated from the internal fruit tissue of *M. zeylanica*. *Lasiodiplodia theobromae*, *Dothiorella* spp., *C. gloeosporioides*, *Phomopsis mangiferae* and *Pestalotiopsis mangiferae* have been identified as the most frequently observed SER pathogens of mango, in Sri Lanka. (Karunanayake *et al.*, 2014; Ekanayake *et al.*, 2019). Stem-end rot (SER) symptoms developed from natural infections in *M. zeylanica* fruits during ripening (Fig. 3). Typical soft, pale brown rots originated at the stem-end and extended towards the stylar-end. It is noteworthy that in *M. zeylanica*, the SER did not develop covering a larger portion of the fruit within 4 to 5 days and the pulp or mesocarp tissue was not completely softened and macerated as seen in the cultivar '*Karuthacolomban*'. Isolations of the diseased tissue on to PDA showed the growth of a greyish white colony which darkened with time to become a dark grey-black following incubation at room temperature (28 ± 2 °C). Hard raised structures, which were identified as pycnidia developed on the surface of the colony after 28 days incubation at room temperature. Pycnidia development enhanced when the cultures were exposed to near UV light for a period of 12 h daily. When the pycnidia were crushed and observed under the light microscope, dark brown, two-

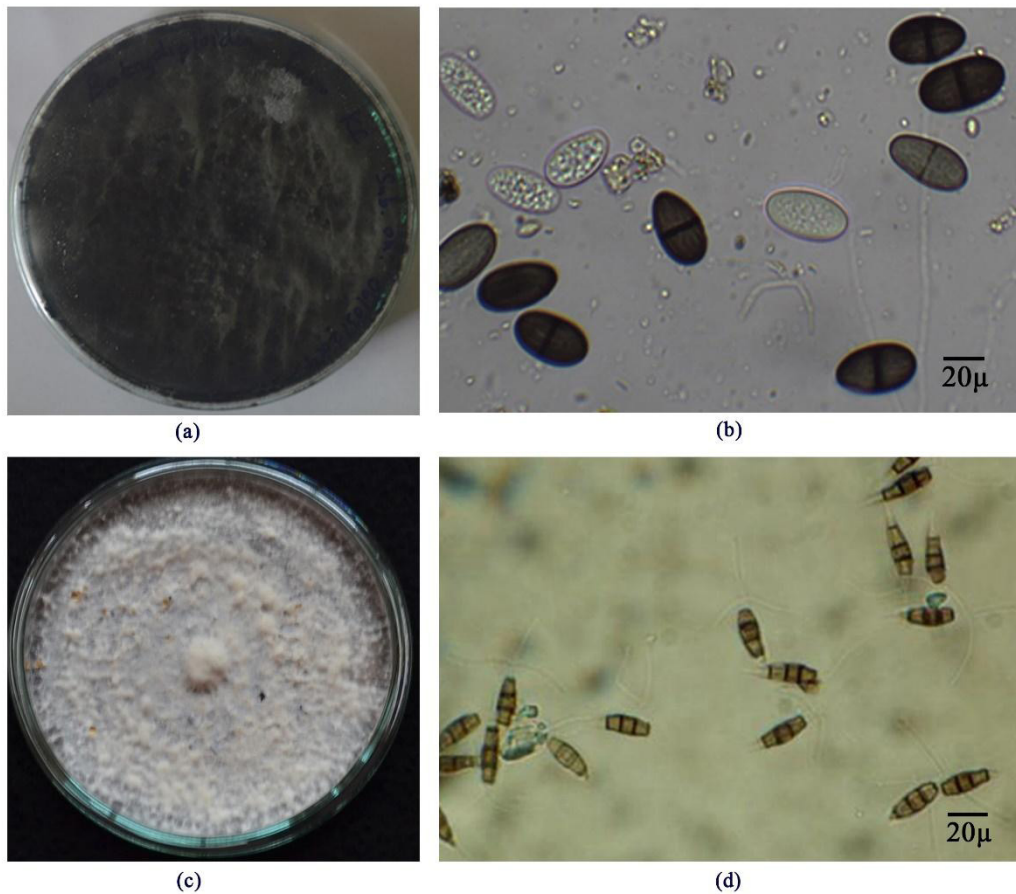


Figure 2: Two fungi isolated from the fruit of *M. zeylanica*, (a) A colony of *L. theobromae* grown on PDA, (b) Mature conidia of *L. theobromae*, (c) *Pestalotiopsis* sp. colony on PDA and, (d) Conidia of *Pestalotiopsis* sp.



Figure 3: Fruit of *M. zeylanica* with characteristic symptoms of stem-end rot, from natural infections.

celled conidia were seen. Based on colony characteristics and the morphology of conidia, the causal organism was confirmed as *L. theobromae* [Fig 2 (a), (b)]. Based on literature and phylogenetic studies the genus *Lasiodiplodia* is reported to be the dominant genus associated with mango diseases worldwide (Trakunyingcharoen *et al.*, 2014). Further, *L. theobromae* is reported as the most dominant of the species from the most number of mango growing countries in association with mango diseases world-wide (Trakunyingcharoen *et al.*, 2014) and is also the most common causal agent for SER in tropical Asia (Johnson *et al.*, 1993).

Neofusicoccum sp. and *Pestalotiopsis* sp. are reported to be present in stem-end rots (Johnson *et al.*, 1992) and *Alternaria* sp. is a known mango fruit pathogen (Droby *et al.*, 1986) although not associated with postharvest disease in Sri Lanka. Seven taxa of Botryosphaeriaceae are reported to be associated with SER in Brazil, *Neofusicoccum* sp., *Botryosphaeria dothidea* being among the reported. *Pseudofusicoccum stromaticum* and *B. dothidea* were the most frequently isolated while *Neofusicoccum parvum* and *Neoscytalidium dimidiatum* were the most virulent (Marques *et al.*, 2013). However, it is interesting to note that *Colletotrichum* spp., known to form quiescent infections

in mango (Arauz, 2000) and cause anthracnose in ripe fruit, were not isolated from *M. zeylanica*. It could be that *Colletotrichum* spp. are not naturally found in this locality or that other microbes present as microflora on the fruit's surface out-compete *Colletotrichum* sp. for nutritional and space requirements. Anthracnose development due to natural infections was also not observed in harvested fruits of *M. zeylanica*, however, fruits were susceptible to the disease and expressed characteristic symptoms when inoculated.

Inoculation studies

Anthracnose pathogen

Mangifera zeylanica fruits developed anthracnose disease following artificial inoculation. In fruits of cultivar 'Willard', measurable anthracnose lesions appeared five days after inoculation and after six days in fruits of the cultivars 'Rata' and *M. zeylanica*. Anthracnose symptoms appeared in 'Karuthacolomban' fruits only eight days after of inoculation (Fig. 4). There was no significant difference among the time taken for development of anthracnose lesions in fruits among *M. zeylanica*, 'Karuthacolomban' and 'Rata'. However, all three cultivars developed significantly ($P < 0.05$) smaller lesions of anthracnose upon inoculation and hence are significantly more resistant to anthracnose when compared with 'Willard'.

Stem-end rot pathogen

Disease development commenced two days after inoculation in all the considered cultivars. The lesion areas of 'Rata', 'Willard' and *M. zeylanica* were not significantly different from each other. Stem-end rot area was significantly higher ($P < 0.05$) in cultivar 'Karuthacolomban' compared to the other three cultivars used in the study from disease initiation onwards (Fig 5). *Mangifera zeylanica* is significantly

resistant to SER when compared with 'Karuthacolomban' ($P < 0.05$).

It is significantly less susceptible to anthracnose when compared with the highly susceptible 'Willard' and significantly less susceptible to SER when compared with highly susceptible 'Karuthacolomban'. Therefore, its resistance seems comparable with cultivars such as 'Rata', and 'Dilpasan' (Karunanayake *et al.*, 2014) which show moderate resistance to both diseases. The study confirms that *M. zeylanica* is moderately resistant to both SER and anthracnose diseases.

Antifungal activity in the fruit peel of *M. zeylanica*

The crude peel extract of *M. zeylanica*, when spotted on TLC and subjected to the *Cladosporium* bioassay, show the presence of antifungal activity (Fig. 6). A clear large inhibition zone was present in the peel extract of *M. zeylanica* at R_f 0.00. This zone of inhibition was identical to that in peel extracts of *M. indica* species, at R_f 0.00, which constitutes gallotannins (Adikaram *et al.*, 2010; Karunanayake *et al.*, 2011). Additional inhibition zones were also seen at R_f 0.72 - 0.75 and 0.8 - 0.92 which are not so prominent.

These could be due to resorcinols which are known to be antifungal compounds in unripe peel of *M. indica* fruits (Cojocar *et al.*, 1986; Prusky and Keen, 1993; Droby *et al.*, 1986; Hassan *et al.*, 2007; Karunanayake *et al.*, 2011) and are also reported in *M. zeylanica* (Ediriweera *et al.*, 2017) although not in connection with antifungal properties. The presence of Mangiferin has been reported from the bark of *M. zeylanica* (Herath *et al.*, 1970) and several other polyphenols, flavonoids and halogenated constituents with anti-cancer properties (Ediriweera *et al.*, 2016a; 2016b). The inhibition area produced by the dried peel extract, equivalent to 0.5 g was quite high,

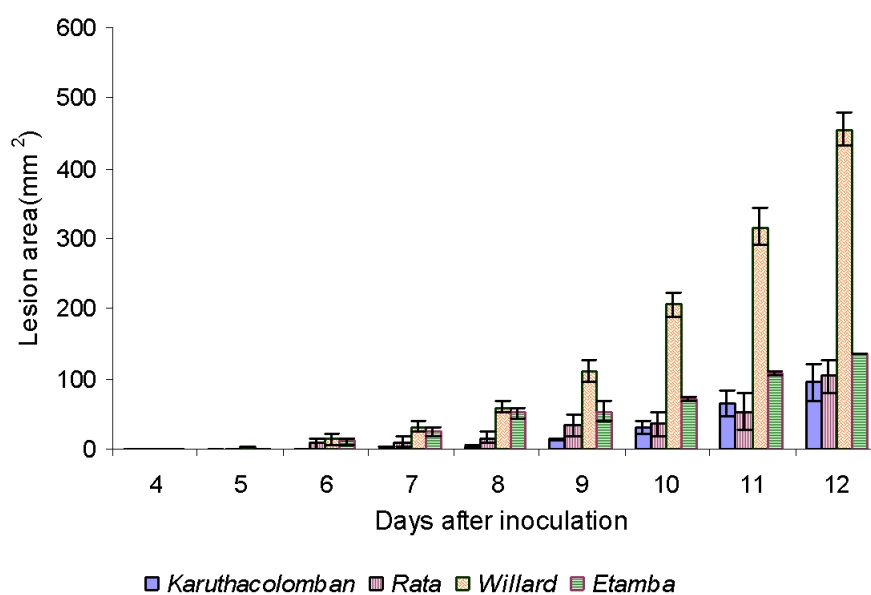


Figure 4: Anthracnose disease development after inoculation, in a few *M. indica* cultivars; 'Karuthacolomban', 'Rata', 'Willard' and *M. zeylanica* ('Et-amba').

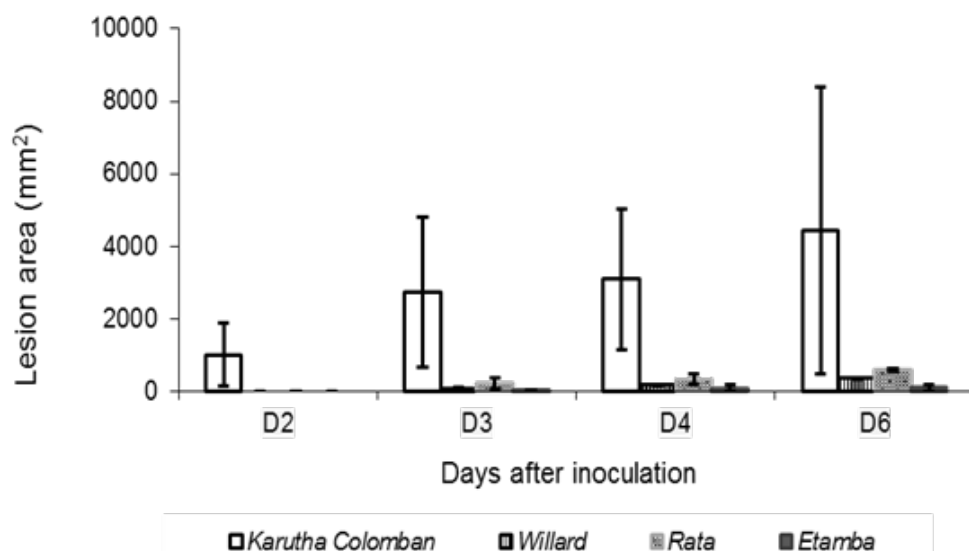


Figure 5: Stem-end rot development in selected *M. indica* cultivars and *M. zeylanica* ('*Et-amba*'), after inoculation with SER pathogen.

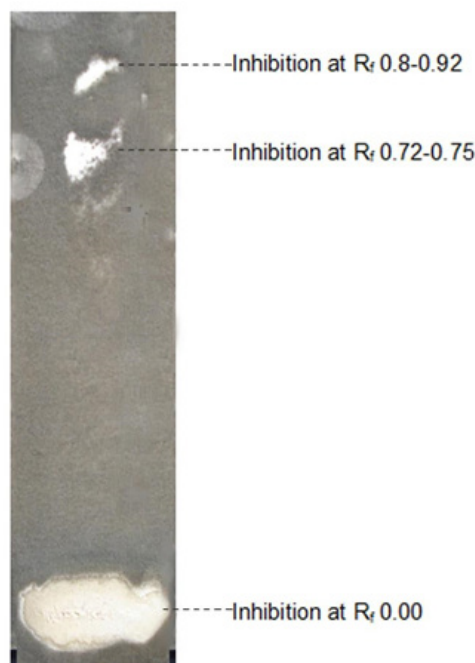


Figure 6: Thin layer chromatogram of the unripe fruit peel extract of *M. zeylanica* bio-assayed with *Cladosporium cladosporioides* sp.

424 mm², which is comparable with cultivars such as '*Gira*' and '*Karuthacolomban*' which are more resistant to anthracnose (Karunanayake *et al.*, 2014). Further, it is also noteworthy that the peel of '*M. zeylanica*' fruits also did not become yellow when ripe, as seen in anthracnose resistant '*Karuthacolomban*', '*Gira*' and moderately resistant '*Rata*'. Gallotannins are directly fungitoxic to *C. gloeosporioides* and *L. theobromae* (Adikaram *et al.*, 2010). The presence of Gallotannins at high concentrations should contribute to the relative resistance of '*M. zeylanica*' fruit to both anthracnose and SER diseases.

Rate of respiration

The rate of respiration of the *M. zeylanica* fruits was initially 0.1 CO₂ mL⁻¹ g⁻¹h⁻¹ then it rose to a maximum of 0.2 CO₂ mL⁻¹ g⁻¹h⁻¹ five days after harvest and again declined to 0.125 CO₂ mL⁻¹ g⁻¹h⁻¹. Therefore, *M. zeylanica* appears to

follow a climacteric pattern in respiration (Fig 7).

Total soluble solids content in pulp

The total soluble solids (TSS) content was initially 4.2 °Brix in the unripe fruit and gradually increased to 11.2 in the ripe fruit (Fig 7). °Brix value for sugar content is given as 4% for poor, 6% for average, 10% for good and 14% for excellent in mango fruit (Harrill, 1998) of *M. indica*. Therefore, it is apparent that *M. zeylanica* fruits also have comparable values and are in the range of 'good' in terms of TSS when compared with *M. indica* species.

CONCLUSION

The study confirms that the fruit of *M. zeylanica* is moderately resistant to both postharvest anthracnose and SER diseases that affect the cultivated mango fruits in Sri

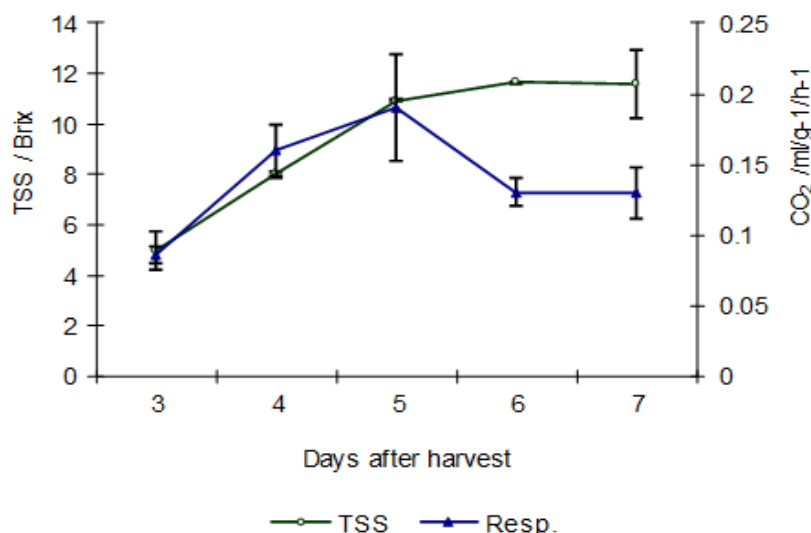


Figure 7: Variation in total soluble solids content (TSS) and rate of respiration (Resp.) from the day 3 after harvest, up to day 7 in fruits of *M. zeylanica*.

Lanka. Antifungal gallotannins which are found in the peel of *M. indica* are present in relatively high concentrations in the peel of *M. zeylanica* and may have contributed towards its resistance to postharvest fungal pathogens. *M. zeylanica* follows a climacteric pattern of respiration and the flesh is sweet to taste with acceptable TSS value.

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DECLARATION OF CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

AVAILABILITY OF RAW DATA

Only printable images are included. However, the raw data is available upon request.

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