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Morphological Characterization and effect of Extracts of some Medicinal Plants on the Control of *Colletotricum* isolates Associated with Papaya Anthracnose Disease

Arumugam Vengadaramana[1] and Selvaratnam Laxmija[2]

[1]*Department of Botany, Faculty of Science, University of Jaffna, Sri Lanka*
[2]*Department of Botany, Faculty of Science, University of Jaffna, Sri Lanka*

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ABSTRACT

Anthracnose disease caused by *Colletotrichum* species is a common postharvest disease of papaya fruit in Sri Lanka. The present study was conducted to determine the morphological and pathogenic variations, control of pathogen by the application of extracts of some medicinal plants and commercial fungicides and influence of culture media on the growth of *Colletotrichum* isolates associated with papaya anthracnose disease in Jaffna. Four *Colletotrichum* isolates (ISO1, ISO2, ISO3 and ISO4) were isolated from anthracnose diseased papaya (*Carica papaya* L.) fruits. Length, width and length:width ratio of the spores differed significantly among 4 isolates under light microscope. Among the different culture media tested, potato and beetroot dextrose agar media supported the growth of *colletotrichum isolates* significantly. When the PDA medium was supplemented with different plant sources, amount of mycelium in cowpea medium of 4 isolates was high compared to other media. ISO2 had dense mycelium in coconut oil seed cake medium. When compared to the control, cinnamon extract showed 11, 18, 9 and 14% of inhibition on the growth of isolates ISO1, ISO2, ISO3 and ISO4 isolates respectively. Clove extract completely inhibited the growth of isolates ISO1 and ISO2 and showed more than 50 % of inhibition of growth of Isolates ISO3 and ISO4. Balloon vine extract showed significant inhibition in the growth of all four *Colletotrichum* isolates.

***Keywords*:** Anthracnose, Papaya, Medicinal plants, Colletotrichum.

Introduction

Papaya (*Carica papaya* L.) is considered as the largest contribution of tropical fruit production in developing countries. Papaya is a dominant fruit crop, belonging to the family *Caricaceae*. *Carica* is the largest of the four genera with 48 species, among which *Carica papaya* L. is the most important and cultivated all over the world.[1] Papaya fruits are very susceptible to diseases caused by many microorganisms especially fungi, as its high in moisture and nutrients.[2] Anthracnose of papaya is commonly caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (teleomorph *Glomerella cingulata* (Stoneman) Spauld & Schrenk) and is a major postharvest disease throughout papaya production areas in the tropics and subtropics.[3] Anthracnose results in pre and post-harvest losses. In general, pathogens associated with complex papaya anthracnose symptoms are classified as *Colletotrichum gloeosporioides*. However, studies have shown that this species is not always the only one involved in the disease.[4,5] There are several species of the *Colletotrichum* genus that cause numerous diseases in various hosts, whose symptoms are not always visible until the fruit begins the maturation process.[6] Symptoms of anthracnose infection of papaya fruit are typically seen as distinct circular sunken lesions on mature to ripe fruit. *Colletotrichum* species cause anthracnose disease in a number of hosts, including legumes, tree crops, fruits, and vegetables, and are among the most economically important plant pathogens worldwide. In some cases, several *Colletotrichum* species or biotypes can form an infection complex in a single host.[7] Therefore, it is essential to identify the prevailing species since the epidemiology of fungal species and the control methods vary.[8] Postharvest diseases of fresh fruits are traditionally being controlled by synthetic chemical fungicides.[9] Synthetic fungicides are currently used as primary means for the control of plant disease. However, the alternative control methods are needed because of the negative public perceptions about the use of synthetic chemicals, resistance to fungicide among fungal pathogens, and high development cost of new chemicals.[10] The uses of plant-derived products as disease control agents have been studied, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance.[11] Crude extracts of some well-known medicinal plants are used to control some of the plants pathogens.[12] Medicinal plants are used as excellent antimicrobial agents because it possess a variety of chemical constituents in nature recently much attention has been conducted towards extracts and biologically active compounds isolated from popular plant species. In general, studies of this nature are becoming popular for *Colletotrichum* spp. since the methods based on morphological and cultural tests have not always been satisfactory.[7] Molecular methods based on DNA sequences are indispensable especially for cryptic species.[13] Objective of this study was to identify different isolates of *Colletotrichum* sp. associated with papaya anthracnose disease by growth, morphological and pathogenic variations and control the mycelial growth of *Colletotrichum* sp. associated with papaya anthracnose disease by using extracts of selected medicinal plants and compare with commercial fungicides available in Sri Lanka.

*Corresponding author. E mail: avengad19@yahoo.com
Tel: 0094212229645

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Materials and Methods

Isolation of fungi

Papaya fruits with symptoms of anthracnose were collected in May, 2017 from 15 locations belonging to 15 divisional secretariat (DS) divisions of Jaffna District (Figure 1). Fungi were isolated from anthracnose lesions of papaya fruits. The infected fruits which showed typical symptoms were surface sterilized in 1 % Clorox solution for three minutes and washed repeatedly thrice in sterile distilled water then air-dried on a clean bench. Then surface sterilized peels of fruit were cut into small bits measuring about 1-2 cm and transferred to sterile petri plates containing Potato Dextrose Agar (PDA) medium with streptomycin 100 µg/mL under aseptic conditions then incubated at room temperature (give the temp range). The fungal colonies that appeared on the papaya fruit peel after incubation period were inoculate on to fresh sterile PDA medium to obtain pure cultures and for identification.

Growth characteristics of *Colletotrichum* isolates in different media

Five millimeter diameter discs of each fungus obtained from pure cultures were transferred at the center of sterile Petri dishes (in triplicates) containing five different growth media e.g. (i) Commercial Potato Dextrose Agar (CPDA) [CPDA 39 g; Distilled H_2O 1L] (ii) Potato Dextrose Agar (PDA) [Potato (peeled) 200 g; Dextrose 20 g; Agar 15 g; Distilled water 1L] (iii) Yam Dextrose Agar (YDA) [Yam (peeled) 200 g; Dextrose 20 g; Agar 15 g; Distilled water 1L] (iv) Cassava Dextrose Agar (CDA) [Cassava 200 g (peeled); Dextrose 20 g; Agar 15 g; Distilled water 1 L] (v) Beet root Dextrose Agar (BDA) [Beet root (peeled) 200 g; Dextrose 20 g; Agar 15 g; Distilled water 1L) and (vi) Sweet potato Dextrose Agar (SDA) [Sweet potato (peeled) 200 g; Dextrose 20 g; Agar 15 g; Distilled water 1L). The Petri dishes of three replicates in each medium were then incubated for 6 days at 32 ± 3 ^{o}C at room temperature and macroscopic features (e.g. Colour of the upper surface and reverse side, texture of colonies) of colony characters and rate of colony growth (cm/day) on each medium were recorded.[14]

Growth characteristics of Colletotrichum isolates in Agar medium substituted with different natural plant sources

Five millimeter diameter discs of each fungus obtained from pure cultures were transferred at the center of sterile Agar (15 g/L) with six natural plant sources such as 39 g/L (instead of CPDA) of Cowpea, Sesame oil seed cake, Soy bean, Horse gram, Coconut oil seed cake and Corn separately. Plates of three replicates in each medium were incubated for 6 days at room temperature (32 ± 3 ^{o}C). Then macroscopic features (e.g. colour of the upper surface and reverse side, texture of colonies) of colony characters and rate of colony growth (cm/day) on each medium were recorded.[14]

Variation of spore morphology of Colletotrichum isolates

Spore shapes and dimensions observed by light microscope under high power (MC-20, Microse, Ostia) were measured. Thirty spores from each fungal isolate were selected randomly and used to measure width and length by a calibrated ocular micrometre.[14]

Pathogenic variation

Pathogenicity of the isolated fungi were performed under laboratory conditions. Fully matured (percentage maturity 25%) green unripe papaya fruits were collected from the field (Thirunelvelly, Jaffna), washed thoroughly under running tap water. The fruits were surface sterilized with 1 % Clorox solution for 3 minutes and rinsed with sterilized distilled water three times. Surface disinfected fruits were air dried under a laminar air flow cabinet and subjected to pathogenicity trials. Concentration of the collected spore suspension of each isolate was adjusted by Hemocytometer to 10^5 conidia/mL (grown for six days on PDA) separately.[15] Two wounds were made on each papaya fruit using cork-borer (5 mm diameter) and filled with 20 µL spore suspension. The fruits inoculated with sterile distilled water served as the control. Inoculated fruits and control were covered with plastic tray at the laboratory of Department of Botany and incubated at room temperature (32 ± 3 ^{o}C). The papaya fruits were arranged according to a completely randomized design (CRD) with three replicates. Fungi were re-isolated from the artificially inoculated fruits showing typical anthracnose symptoms and the cultures obtained were confirmed for morphology and colony characters.

Antifungal activity of plant extracts and fungicides in vitro

Preparation of plant extracts

Twenty grams of dried clove (*Syzygium aromaticum*) flower buds, dried cinnamon (*Cinnamomum zeylanicum*) bark, fresh holy basil (*Ocimum tenuiflorum*) leaves and fresh balloon vine (*Cardiospermum halicacabum*) leaves were washed with sterile distilled water and soaked in 150 mL of absolute ethanol (99.98 %) for 5 successive days separately at room temperature. The supernatant was filtered through Whatman filter paper. After the extraction with absolute ethanol, the filtrates were then evaporated under reduced pressure at 50◦C using a rotary evaporator to yield the crude extract. Crude extracts were mixed with 5 mL sterile distilled water

In vitro assay

In vitro experiment was conducted by mixing 2 mL of clove, cinnamon, holy basil and balloon vine ethanol extracts with 10 mL PDA medium supplemented with streptomycin (100 µgmL^{-1}) and pouring it into sterile petri dishes separately. From each isolate of *Colletotrichum* spp., a myclelial plug having diameter of 5 mm was placed separately on PDA medium supplemented with clove, cinnamon, holy basil and balloon vine extracts and incubated for six days at room temperature (32 ± 3 ^{o}C). Each fungal isolate was replicated three times. Controls were maintained for each isolate by placing a mycelia plug on PDA medium without any plant extracts. Control and tests plates were supplemented with streptomycin (100 µgmL^{-1}). Radial growth of the colony was measured for each isolate grown on PDA with and without the plant extracts (control). Relative inhibition of colony growth (%) was calculated for each isolate by using the growth data values measured after six days on control plates and plates amended with plant extracts.[16]

$$\text{Percentage (\%) of inhibition} = \frac{\text{Diameter of control colony} - \text{Diameter of treated colony}}{\text{Diameter of control colony}} \times 100$$

Sensitivity to Fungicide in vitro

Homai (Thiophanate-methyl 50% + Thiram 30% WP), Topsin (Thiophanate-methyl 50%) and Green mancozeb (Mancozeb 80% (W/W) WP) fungicides recommended by the Department of Agriculture, Sri Lanka were used for *in vitro* assays. The recommended dosage of Homai (1.8 g/L), Topsin (1.2 g/L) and Green mancozeb (1.2 g/L) were dissolved in sterile distilled water, added to molten PDA medium supplemented with streptomycin (100 µgmL^{-1}) then mixed thoroughly by gentle shaking. From each isolate of *Colletotrichum*, a myclelial plug having diameter of 5 mm was placed separately on PDA medium supplemented with Homai, Topsinn or Green mancozeb separately and incubated for six days at room temperature (32 ± 3 ^{o}C). Each fungal isolate was replicated three times. Controls were maintained for each isolate by placing a mycelial plug on PDA medium supplemented with the antibiotic but not containing the fungicide. Radial growth of the colony was measured for each isolate grown on PDA with and without the fungicide (control). Relative inhibition of colony growth (%) was calculated for each isolate by using the growth data values measured after six days on control plates and plates amended with fungicides.[16]

Statistical analysis

Data were statistically analyzed by Analysis of Variance (ANOVA) using a SAS statistical package (version 9.1.3) and mean separation was done by Least Significance Difference (LSD).

Results and Discussion

Four *Colletotrichum* isolates (ISO1, ISO2, ISO3 and ISO4) were isolated from anthracnose infected papaya fruits. Identification of *Colletotrichum* isolates was based on morphological characters such as colony characters and size and shape of conidia (Figure 2 and 3)

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and descriptions of Sutton and Waterson[17], Lim et al.[18] and Afanador-Kafuri *et al*..[19]

Growth characteristics of pathogen in PDA medium

Four morphologically different *Colletotrichum* isolates (*i.e.* ISO1, ISO2, ISO3 and ISO4) could be categorized based on the shape of the spores (Table 1). ISO2 isolate produced straight, cylindrical larger and hayline spores, which fit well with the description of *C. gloeosporioides* by Sutton.[20] They also produced greyish-white colonies with dark gray to black reverse colony colour which is a common macroscopic feature of *C. gloeosporioides* colonies.[21] ISO1 isolates produced fusiform hayline spores, acute at both ends. Both upper and reverse surfaces of the colonies grown on PDA were white to orange in colour, with slight shades of pink and light mouse grey aerial mycelium (Table 1). On the reverse side, the center was orange. The spore shape and the colony characters observed for the ISO1 is similar to descriptions of *C.* acutatum.[22] *Colletotrichum acutatum* is one of the most frequently reported species causing the disease commonly known as anthracnose on numerous host plants worldwide.[23] ISO3 isolate of *Colletotrichum* produced very small-sized hyaline spores which are cylindrical, straight to spindle shaped (Table 1). However, it was not possible to place them at a species level based on the available information of colony and spore morphology. Isolate ISO4 produced falcate shaped, hyaline and relatively large spores (Table 1). Both upper and reverse surfaces of the colonies grown on PDA were white in colour, which fit well with the description of *C. capsici*.[24]

Variation of spore morphology of Colletotrichum isolates

All four isolates produced straight, cylindrical relatively larger and hayline spores with length to width ratio of about 4:1. Generally, conidial shape of most isolates looked very similar (Figure 2). Results summarized in Table 1 demonstrated considerable variation in conidial dimensions, particularly the width. Variability of conidial length within the isolates was greatest in *Colletorichum* isolate. There was significant difference among 04 in conidial length. Conidia of isolates ISO1, ISO3 and ISO4 were in the range of 12.89- 33.13 X 3.68- 6.58 µm. Isolate ISO3 was very distinct because of its size (4.68 X 3.44 µm). Such conidia were 7 times smaller than that of the biggest isolate ISO4.

Growth characteristics of pathogen in different media

The growth characters of *Colletotrichum* isolates were studied on five different solid media. The colony growth rate and colony colour (upper and riverside) were considered as growth characters. All the four *Colletotrichum* isolates produced very low dense colony in YDA medium and upper and lower surface were pale in colour (Figure 4). Hence YDA medium is not suitable for growth of *Colletotrichum* isolates. Colonies in CPDA, PDA F, CDA. SPDA and BDA showed difference in surface and reverse colouration of the four isolates. Growth rates showed a significant difference among four *Colletotrichum* isolates ($P < 0.0001$) (Figure 4) in different media at 6 days after incubation at room temperature ($32 \pm 3^{o}C$). There was no significant difference in ISO4 on SPDA, BDA, CDA and YDA (Figure 4). ISO3 did not show significant difference among media PDA, SPDA, CDA and YDA. Type of culture media and their chemical compositions have significantly affected the mycelial growth rate and conidial production of *Phoma exigua*[25]

Pathogenic variation

Characteristic symptoms of anthracnose were displayed by all the isolates of *Colletotrichum* on papaya fruits after wound inoculation of the pathogen (Figure 5). Lim and his co-workers[18] suggested that wound-inoculation with conidial suspension was the most efficient among the inoculation methods. All *Colletotrichum* isolates were pathogenic on papaya fruits. Infections stimulate ripening of fruits, and lesions enlarge with ripening. Diameter of the anthracnose lesions varied among the four *Colletotrichum* isolates significantly ($p< 0.0001$). The highest diameter of lesion of *Colletotrichum* isolate was observable due to ISO4 (Table 2). Isolate ISO3 gave the lowest diameter. According to definition by Taylor et al.[26] and Taylor and Ford,[27] the variations of quantitative measurements on disease development provide an idea of the diverse nature of the pathogen population in terms of their aggressiveness *i.e*. natural variation in virulence. The level of aggressiveness of isolates of a given pathogen is also an important consideration in resistance breeding programmes and disease control management. Host genotypes with partial resistance would result in lower level of infection which eventually will decrease the inoculum amount in the field to limit the potential of epidemics.[21]

Antifungal activity of plant extracts in vitro

The antimicrobial activity of plant extracts have been explained by many researchers due to the presence of phytochemicals in them.[29] Antifungal activity of four botanical extracts namely, Clove (*Syzygium aromaticum*), Cinnamon (*Cinnamomum zeylanicum*), Holy basil (*Ocimum tenuiflorum*) and Balloon vine (*Cardiospermum halicacabum*) were assayed and the effect of plant extracts on the growth of *Colletotrichum* isolates were observed. The data revealed that significant reduction in mycelia growth of *Colletotrichum* isolates against four medicinal plants (Table 3). Cinnmon has shown low 9.3 % inhibition for ISO3 (Table 2). The antifungal effects of the studied plant ethanol extracts of clove, cinnamon, holy basil and cardiospermum on the tested fungi strains were compared with the control. The results showed that all the plant extracts exhibited different degrees of antifungal activity against *Colletotrichum* isolates (Table 3).

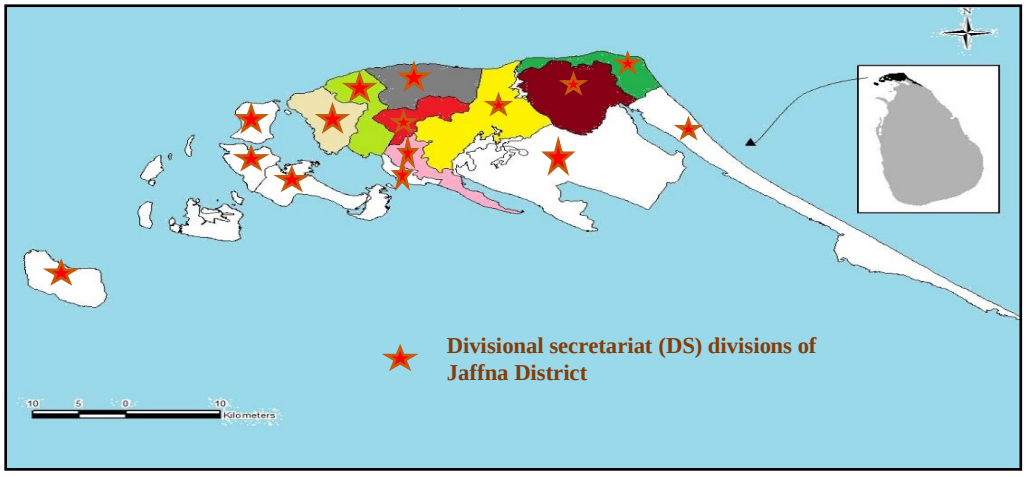

Figure 1: Divisional secretariat divisions of Jaffna District from where the anthracnose infected papaya fruit samples were collected

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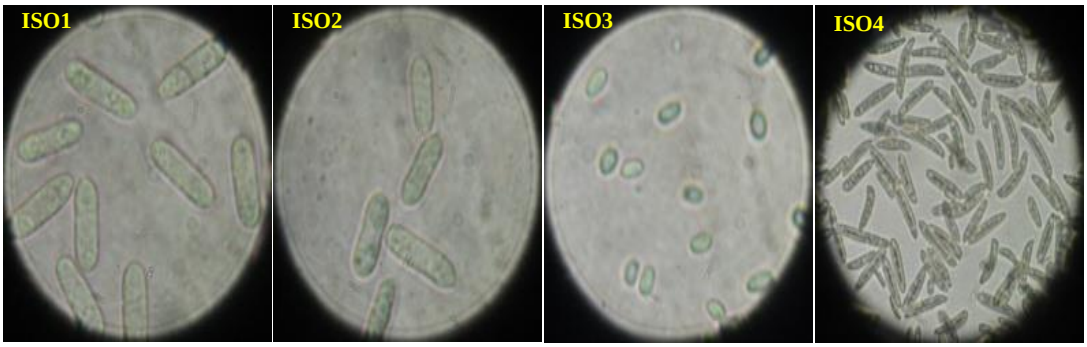

Figure 2: Shapes of spores of *Colletotrichum* isolates on PDA at 6 days after incubation at room temperature (32 ± 3 °C)

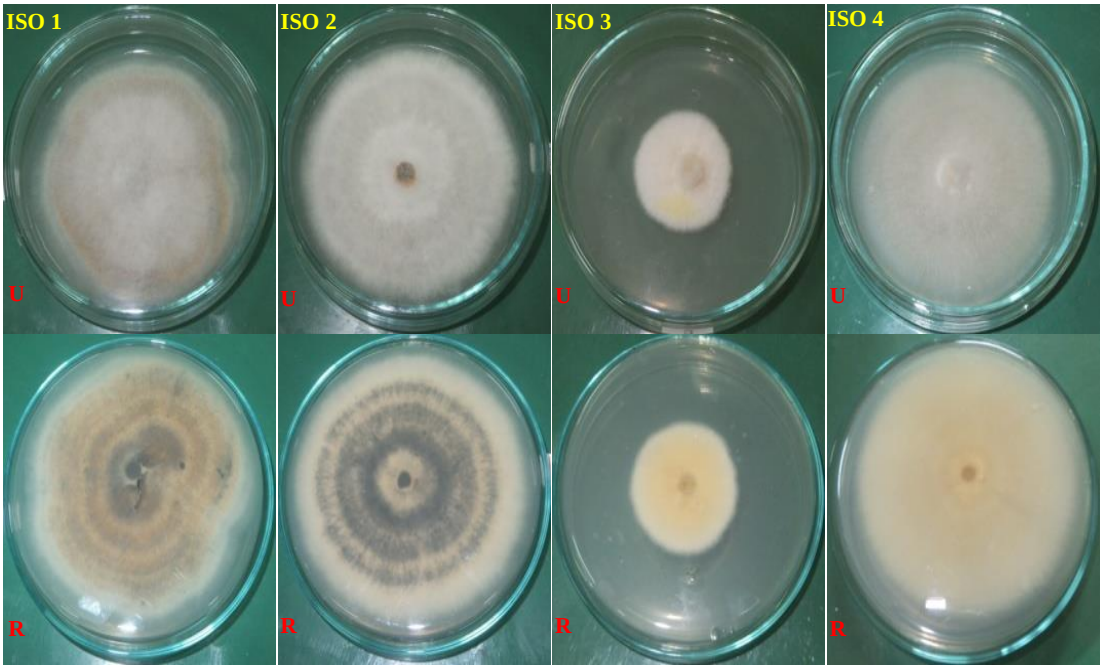

Figure 3: Colony appearance (U: Upper side; R: Reverse side) of *Colletotrichum* isolates on PDA at 6 days after incubation at room temperature (32 ± 3 °C)

Table 1: Groups of *Colletotrichum* isolates from Jaffna District based on spore shapes and spore dimensions, isolates grown on PDA 6 days after incubation at room temperature (32 ± 3°C)

Isolates	Shape of spores	Conidial Dimensions		
		Mean Length (µm)	Mean Width (µm)	Mean Length / Width
ISO1	Cylindrical, straight one or both ends are rounded, hyaline, unicellular - *C. acutatum*[22]	12.89[c]	03.50[b]	03.68[c]
ISO2	Straight, cylindrical, hyaline, one or both ends are pointed, unicellular[20] - *C. gloeosporioides*	15.10[b]	03.50[b]	04.31[b]
ISO3	Very small in size, cylindrical, straight to spindle shaped, hyaline, unicellular - Unknown species	04.68[d]	03.44[b]	01.37[d]
ISO4	Falcate, both ends are pointed, hyaline, unicellular [24] - *C. capsici*	33.13[a]	05.19[a]	06.58[a]

Means in columns followed by same letter are not significantly different by LSD at 5 % level

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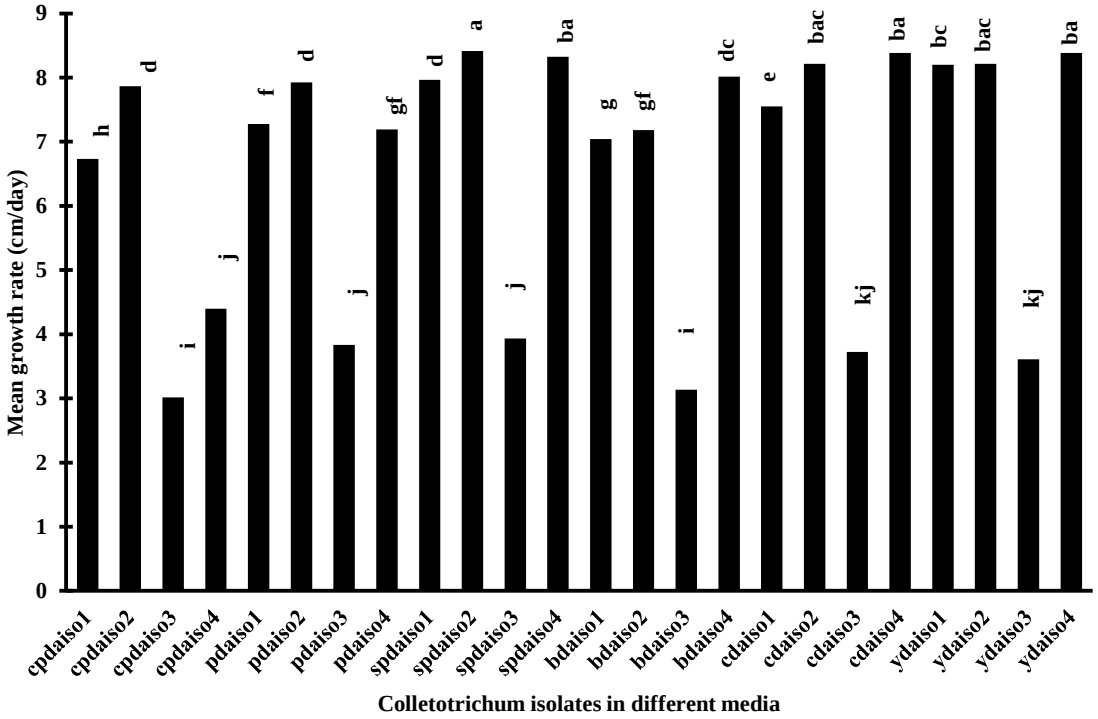

Figure 4: Colony growth rate of *Colletotrichum* isolates on different media at 6 days after incubation at room temperature (32 ± 3°C)
Means followed by same letter are not significantly different by LSD at 5% level

Figure 5: Pathogenic behavior of *Colletotrichum* isolates on papaya fruits; C: control; isolates ISO1, ISO2, ISO3 and ISO4.

Table 2: Mean diameter of anthracnose lesions of anthracnose symptoms by four different *Colletotrichum* isolates when inoculated on papaya fruits at room temperature (32 ± 3°C)

Isolate	Lesion diameter (cm)
ISO1	2.11[b]
ISO2	1.18[c]
ISO3	0.86[d]
ISO	3.25[a]

Means followed by same letter are not significantly different by LSD at 5% level

Clove extracts completely inhibited (100%) the growth of ISO1 and ISO2 at room temperature (32 ± 3°C) which may be due to the presence of antimicrobial compounds such as eugenol, acetyl eugenol, iso-eugenol and *β*-caryophyllene[30,31,32] in clove. Other plants extracts did not completely control the fugal growth. In this study only water soluble antimicrobial compounds were extracted but Cowman[33] revealed that most of the antimicrobial active compounds that have been identified are soluble in polar solvents such as methanol, ethanol etc. than in water.

Sensitivity to Fungicide

In vitro screening revealed that the growth of all four isolates of *Colletotrichum* (Table 4) was completely inhibited (100%) by Homai (Thiophanate-methyl 50% WP + Thiram 30%). Further, ISO1, ISO2 and ISO3 were completely inhibited by Topsin (Thiophanate-methyl 50%) but not ISO4 (Table 4). Twenty-two percent inhibition was observed in the growth of ISO4 in PDA medium by Topsin. Green mancozeb (Mancozeb 80% (W/W) WP) was not completely inhibitory to the growth of ISO1 and ISO4 but completely inhibited the growth of ISO2 and ISO3 (Table 4). Green mancozeb inhibited the growth of ISO1 and ISO4 at 6 days after incubation at room temperature (32 ± 3°C) at percentages of 36 and 46% respectively.

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Table 3: Fungicidal activity of various medicinal plants extracts against the growth of *Colletotrichum* isolates 6 days after incubation at room temperature (32 ± 3°C)

Colletotrichum Isolates	Inhibition % of fungal growth			
	Cinnamon	Clove	Holy basil	Cardiospermum
ISO1	11.5[c]	100[a]	7.5[a]	26.0[b]
ISO2	18.2[a]	100[a]	5.0[b]	28.7[a]
ISO3	09.3[d]	53[c]	0.0[c]	19.7[d]
ISO4	15.0[b]	59[b]	0.0[c]	20.7[c]

Means followed by same letter are not significantly different by LSD at 5% level

Table 4: Fungicidal activity of different commercial fungicides against the growth of *Colletotrichum* isolates 6 days after incubation at room temperature (32 ± 3°C)

Colletotrichum Isolates	Inhibition % of fungal growth		
	Homai	Topsin	Green mangozep
ISO1	100[a]	100[a]	36[c]
ISO2	100[a]	100[a]	100[a]
ISO3	100[a]	100[a]	100[a]
ISO4	100[a]	22[b]	46[b]

Means followed by same letter are not significantly different by LSD at 5% level

Conclusion

The present study revealed the different *Colletotrichum* isolates associated with papaya anthracnose disease in Jaffna district of Sri Lanka. Antifungal activity was confirmed by all of the selected plant species. Clove was the most effective inhibitor for the mycelial growth of four tested *Colletotrichum* isolates associated with papaya anthracnose disease. Media tested i.e., Potato, Sweet potato, Cassava and Yam dextrose agar supported the maximum growth of all the four *Colletotrichum* isolates. The findings of the present investigation could be an important step towards the possibility of using natural plant products as bio-pesticides in the control of plant diseases caused by *Colletotrichum* isolates.

Conflict of interest

The authors declare no conflict of interest.

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Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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