Short communication

Etiology of fungi causing postharvest crown rot of Robusta variety banana in Kerala

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Abstract

Crown rot of dehanded banana is the most important and prevalent postharvest disease of banana leading to heavy economic loss. Survey was conducted during March to May, 2018 in five banana growing districts of Kerala viz., Thiruvananthapuram, Alappuzha, Pathanamthitta, Palakkad and Wayanad to identify the major pathogen associated with crown rot of banana (var. Robusta) in Kerala. All the samples collected from the different locations of Kerala showed incidence of crown rot, indicating 100 per cent prevalence of crown rot in the state. Moreover, varying symptoms were noticed in the diseased fruit samples collected. This denoted the association of different pathogens in crown rot. Pathogens were isolated from the diseased samples collected from different locations surveyed. A total of 32 fungal isolates were obtained from the crown rot affected banana samples. The isolates were initially identified based on morphological characters including cultural as well as microscopic characters. Based on the observations, out of the 32 isolates obtained, there were ten *Lasiodiplodia* spp, eight *Colletotrichum* spp, five *Fusarium* spp, three *Aspergillus* spp, two each of *Cunninghamella* and *Verticillium* and one each of *Penicillium* and *Rhizopus*. Pathogenicity tests showed all the isolates to be pathogenic. Different pathogens produced different symptoms when inoculated separately on banana fruits. The pathogenicity test and subsequent virulence rating followed by molecular identification revealed *Lasiodiplodia theobromae* (GenBank accession number: MN046365) as the major as well as the virulent pathogen associated with the postharvest crown rot disease of Robusta variety banana in Kerala.

Key words: Banana, *Colletotrichum,* Crown rot, Kerala, *Lasiodiplodia theobromae*, Postharvest rot.

Banana is one of the tropical fruits that is exported in large quantities from India. Banana is an integral fruit component of most farming systems in Kerala. It is also an important commercial fruit crop of the country. The two primary postharvest rots of banana (*Musa* spp.) fruits are crown rot and anthracnose. Crown rot of dehanded banana is a major threat causing heavy losses during storage and marketing. Even though the infected fruits are almost safe for human consumption, the infection reduces fruit quality, shelf life and marketability.

The infection occurs before or during harvest, but the symptoms appear after overseas transportation. Postharvest loss of perishables in developing countries range from 5 to 50 per cent or more of the harvest. In India nearly 20 to 50 per cent of perishables are lost due to postharvest diseases, and for banana it accounts for 20 to 80 per cent (Yadav et al., 2013). Crown rot caused by a multitude of fungi has been reported in banana markets in Kerala. The management practice adopted is fungicide dip with carbendazim solution. The detectable residue of the fungicide on banana fruits has been reported by the Pesticide Residue Laboratory, at College of Agriculture, Vellayani. Resistance to fungicides

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such as carbendazim poses a serious threat. Moreover, since carbendazim is suspected to be an endocrine disruptor, the currently followed management practice is unsafe. For developing a safer alternate strategy for the management of the disease, it is essential to study the etiology of the causal agent. Robusta variety is most susceptible to this disease. Hence this study aimed to study the major pathogens associated with postharvest crown rot disease of Robusta variety in Kerala.

Survey and Symptomatology

The diseased fruits of Robusta variety banana were collected from major banana growing districts of Kerala. The districts selected included Thiruvananathapuram, Alappuzha, Pathanamthitta, Palakkad and Wayanad. Three locations were selected at random from each district for the survey *viz*., Pallichal, Kazhakkuttam and Varkala in Thiruvananthapuram; Edathuva, Cherthala and Kayamkulam in Alappuzha; Konni, Adoor and Pandalam in Pathanamthitta; Mannarkkad, Palakkayam and Olavakkode in Palakkad and Meenangadi, Karyambadi and Vaduvanchal in Wayanad. Surveys were conducted in the local markets of these 15 locations. Banana fruits with typical crown rot symptoms were collected. Diseased fruit specimens were brought to the lab and symptoms were recorded after careful observations.

As part of the survey conducted during the time period of March to May 2018, crown rot affected diseased samples of banana variety Robusta (AAA) were collected from Pallichal, Kazhakkuttam and Varkala in Thiruvananthapuram, Edathuva, Cherthala and Kayamkulam in Alappuzha, Konni, Adoor and Pandalam in Pathanamthitta, Mannarkkad, Palakkayam and Olavakkode in Palakkad and Meenangadi, Karyambadi and Vaduvanchal in Wayanad.

Variations were noticed in symptoms on the diseased fruit samples collected from different locations. Rotting symptoms restricted to the crown region was noticed in some specimens whereas, in some other locations, along with the rot at crown region, irregular black spots were also observed throughout the fruit surface. Later on, the spots coalesced to form lesions which enlarged in size causing complete rotting of the fruits. Some specimens also exhibited crown end rot which extended to the entire fruit up to the distal end resulting in the rotting of the entire fruit. Some rotten fruits were also covered with fluffy mycelial growth of the fungus (Plate 1). Lassois et al. (2010) and Jagana et al. (2017) also reported similar symptoms in their study.

All the samples collected from different locations showed the incidence of crown rot. This indicated 100 per cent prevalence of crown rot in all the surveyed places and was in consistence with the study by Alemu (2014).

Isolation of pathogens

After the symptomatology studies, the diseased fruits were subjected to pathogen isolation. The diseased fruits were thoroughly washed in tap-water, followed by washing with sterile water and allowed to dry. Inside the laminar air flow chamber, small bits (6 sq. mm area) of peel were cut out from the rotting fruit (i.e. from the margin where the diseased and healthy tissues met). The bits were placed in 1 per cent sodium hypochlorite solution for 30 seconds for surface sterilization, and subsequently transferred to sterile water and rinsed to remove residue of the chemical. Washing was repeated three times. The bits were then allowed to dry over a sterile blotting paper. These bits were then transferred to Petri plates with solidified Potato Dextrose Agar (PDA) medium. The plates were wrapped using cling film or parafilm and kept for incubation at room temperature (28±2 ^{0}C).

When mycelial growth initiation from the bits commenced, pure culturing was done. To pure culture the fungi, hyphal tip method was used. Even within the same plate, mycelia showing difference in culture characters were separately pure cultured. In hyphal tip method, the tip of the mycelial growth

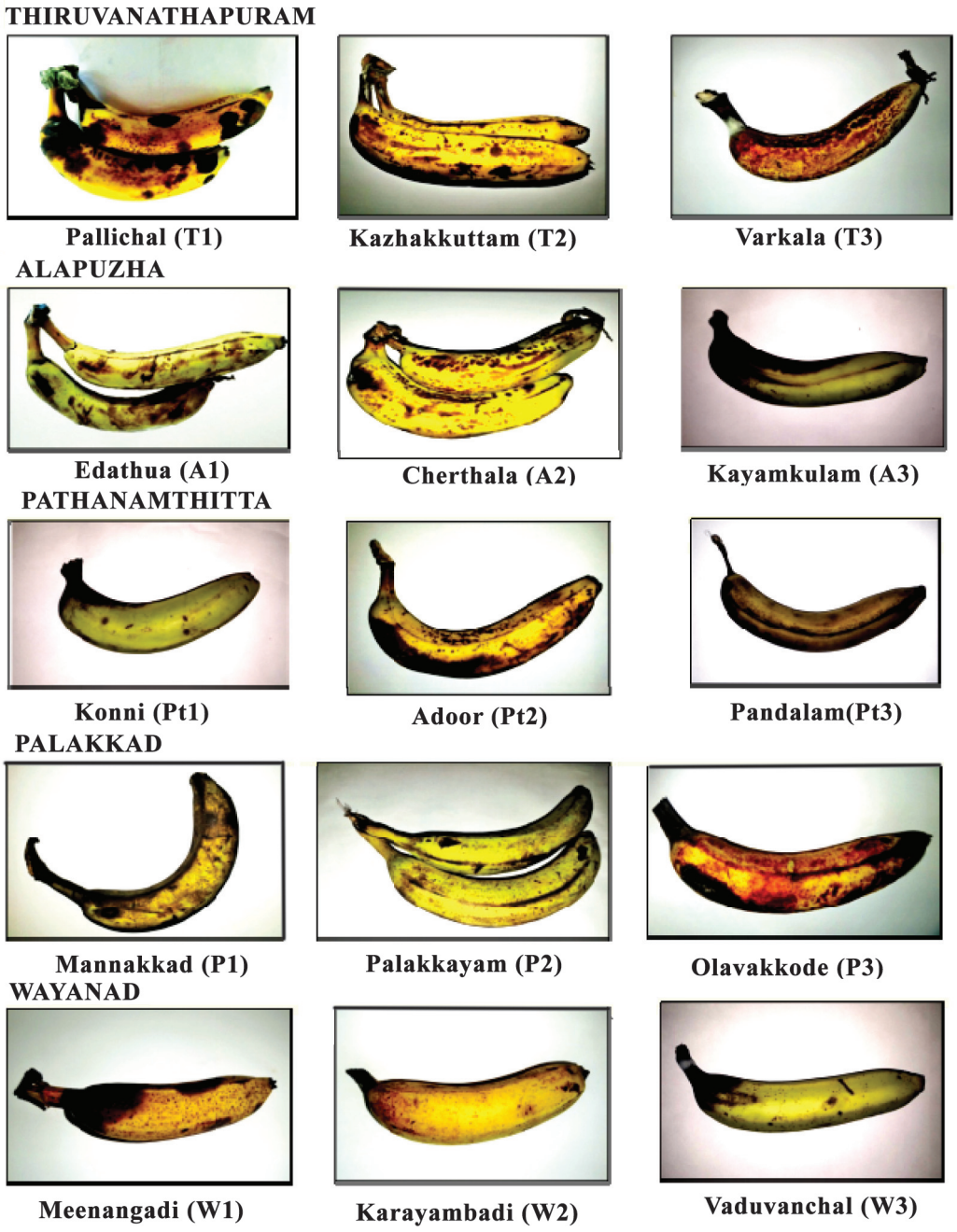


Plate 1. Fruits with crown rot symptoms collected from different locations in Kerala

in the media was cut out using a sterile inoculation needle or a cork borer and was inoculated into sterile Petri plates with solidified PDA medium. The Petri plates were separately labelled and kept for incubation at room temperature. When pure cultures were obtained, the fungi were transferred into separate PDA slants and maintained for further studies.

Thirty two isolates of different pathogens were isolated from the samples collected from the 15 locations in Kerala. These isolates were purified by

hyphal tip method. These pathogens were given designations/codes based on the location from where the diseased samples were collected (i.e., T for Thiruvananthapuram, A for Alappuzha, P_t for Pathanamthitta, P for Palakkad and W for Wayanad; 1, 2, 3 for the three different locations within the districts; and A, B, C etc. for different pathogens from the location). For example, P1A coded for the first pathogen (A) isolated from the sample collected from Mannarkkad (1) location of Palakkad (P) district.

Morphological studies of the 32 isolates were carried out (Table 1-5). Morphological characters selected for identifying the pathogens included cultural as well as microscopic characters. Cultural characters included colour of mycelia, nature of growth, etc whereas microscopic characters included mycelial septation, type, shape and colour of spore. Morphological studies revealed that out of the 32 isolates, there were ten *Lasiodiplodia* spp, eight *Colletotrichum* spp, five *Fusarium* spp, three *Aspergillus* spp, two each of *Cunninghamella* and *Verticillium* and one each of *Penicillium* and *Rhizopus*.

Pathogenicity tests and virulence rating

The pure cultures of the isolated fungi were

Table 1. Morphological characters of different isolates causing postharvest crown rot in banana (var. Robusta) in Thiruvananthapuram district

Isolate	Culture characters	Nature of mycelia	Spore characters
T1A (*Lasiodiplodia* sp.)	Regular margin, initially white and turns grey	Septate, melanised	Immature spore - hyaline, mature spore - dark, septate with longitudinal striations
T2A (*Lasiodiplodia* sp.)	Regular margin, initially white and turns grey	Septate, melanised	Immature spore - hyaline, mature spore - dark, septate with longitudinal striations
T2B (*Rhizopus* sp.)	Regular margin, fluffy, raised and off-white	Aseptate, hyaline	Sporangia with black spores
T2C(*Lasiodiplodia* sp.)	Regular margin, initially white and turns grey	Septate, melanised	Immature spore - hyaline, mature spore - dark, septate with longitudinal striations
T3A (*Fusarium* sp.)	Irregular margin, concentric zonations, purplish centre with white margin	Septate, purple	Multi-septate and fusiform macroconidia
T3B (*Colletotrichum* sp.)	Irregular margin, concentric zonations, orangish white	Septate, hyaline	Hyaline and cylindrical

Table 2. Morphological characters of different isolates causing postharvest crown rot in banana (var. Robusta) in Alappuzha district

Isolate	Culture characters	Nature of mycelia	Spore characters
A1B (*Penicillium* sp.)	Regular margin, concentric zonations, green centre with white margin	Septate, hyaline	Green and round conidia
A1C (*Colletotrichum* sp.)	Irregular margin, concentric zonation, orangish white	Septate, hyaline	Hyaline and cylindrical
A1D (*Aspergillus* sp.)	Irregular margin, concentric zonation, brown centre with white margin	Septate, hyaline	Brown and round conidia
A2A (*Lasiodiplodia* sp.)	Regular margin, initially white and turns grey	Septate, melanised	Immature spore - hyaline, mature spore - dark, septate with longitudinal striations
A2B (*Colletotrichum* sp.)	Irregular margin, concentric zonation, off-white mycelia	Septate, hyaline	Hyaline and cylindrical
A3A (*Colletotrichum* sp.)	Irregular margin, concentric zonations, orangish white	Septate, hyaline	Hyaline and cylindrical
A3B (*Lasiodiplodia* sp.)	Regular margin, initially white and turns grey	Septate, melanised	Immature spore - hyaline, mature spore - dark, septate with longitudinal striations

Table 3. Morphological characters of different isolates causing postharvest crown rot in banana (var. Robusta) in Pathanamthitta district

Isolate	Culture characters	Nature of mycelia	Spore characters
Pt1B (*Cunninghamella* sp.)	Raised and fluffy, yellowish white	Aseptate, hyaline	Vesicles on branched sporangiophores bear globose sporangioles
Pt1C (*Colletotrichum* sp.)	Irregular margin, concentric zonations, orangish white	Septate, hyaline	Hyaline and cylindrical
Pt1D (*Aspergillus* sp.)	Irregular margin, concentric zonations, green centre with white margin	Septate, hyaline	Green and round conidia
Pt2A (*Aspergillus* sp.)	Irregular margin, concentric zonations, dark brown centre with white margin	Septate, hyaline	Dark brown and round conidia
Pt2B (*Verticillium* sp.)	Irregular margin, off-white mycelia	Septate, hyaline	Ellipsoidal, verticillate phialides bear conidia
Pt3A (*Colletotrichum* sp.)	Irregular margin, concentric zonations, orangish white	Septate, hyaline	Hyaline and cylindrical

Table 4. Morphological characters of different isolates causing postharvest crown rot in banana (var. Robusta) in Palakkad district

Isolate	Culture characters	Nature of mycelia	Spore characters
P1B (*Lasiodiplodia* sp.)	Regular margin, initially white and turns grey	Septate, melanised	Immature spore - hyaline, mature spore - dark, septate with longitudinal striations
P2A (*Fusarium* sp.)	Irregular margin, yellowish white	Septate, orange	Multi-septate fusiform macroconidia
P3A (*Colletotrichum* sp.)	Irregular margin, concentric zonations, orangish whitish	Septate, hyaline	Hyaline and cylindrical

Table 5. Morphological characters of different isolates causing postharvest crown rot in banana (var. Robusta) in Wayanad district

Isolate	Culture characters	Nature of mycelia	Spore characters
W1B (*Verticillium* sp.)	Irregular margin, off-white mycelia	Septate, hyaline	Ellipsoidal, verticillate phialides bear conidia
W1C (*Lasiodiplodia* sp.)	Regular margin, initially white and turns grey	Septate, melanised	Immature spore - hyaline, mature spore - dark, septate with longitudinal striations
W2A (*Colletotrichum* sp.)	Irregular margin, concentric zonations, orangish white	Septate, hyaline	Hyaline and cylindrical
W2B (*Cunninghamella* sp.)	Irregular margin, off-white mycelia	Aseptate, hyaline	Vesicles on branched sporangiophores bear globose sporangioles
W2C (*Lasiodiplodia* sp.)	Regular margin, initially white and turns grey	Septate, melanised	Immature spore - hyaline, mature spore - dark, septate with longitudinal striations
W3A (*Fusarium* sp.)	Irregular margin, yellowish white	Septate, orange	Multi-septate fusiform macroconidia

subjected to pathogenicity tests. For pathogenicity test, first emerged healthy mature banana hands were collected from the farmer's field / market. Hands with four fingers (four replications) were cut out and fingers were separated. The fruits were washed in tap water followed by subsequent washing in sterile water and thereafter allowed to dry and de-latex. Inside the laminar air flow chamber, the banana hands were wiped with 50 per cent ethyl alcohol using sterile cotton pads. Five-day old fungal cultures, isolated from the diseased fruits, were used for the inoculation. Each isolate was inoculated singly on separate fruits (four replications). For inoculating the fungal cultures, small incision was made in the crown region of the banana fruit using cork-borer. Fungal bits needed for inoculation were taken from the 5-day old culture using cork-borer. The culture bit was then placed onto the incision made in the crown region of the fruit. Moistened cotton lining was placed over

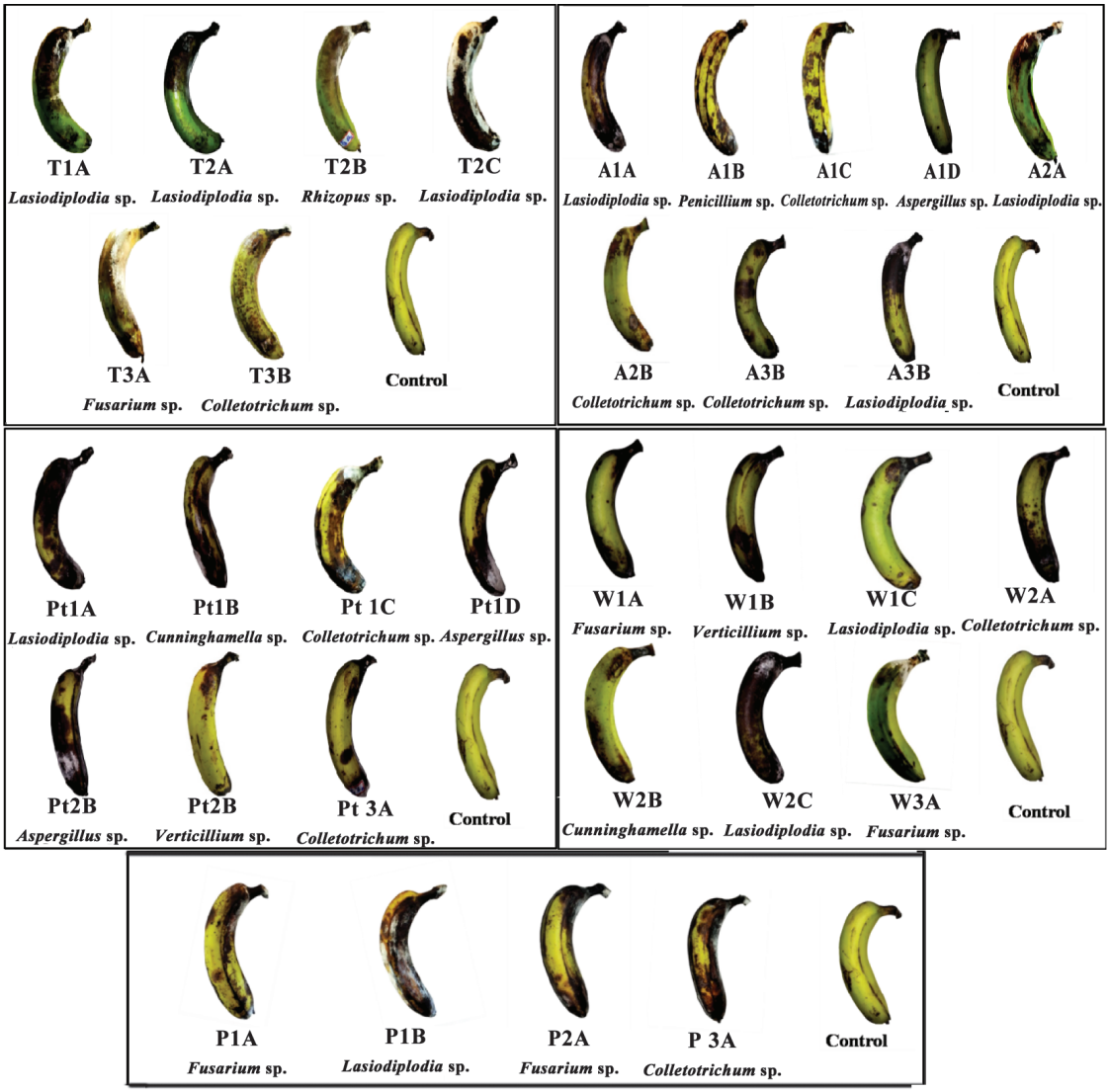
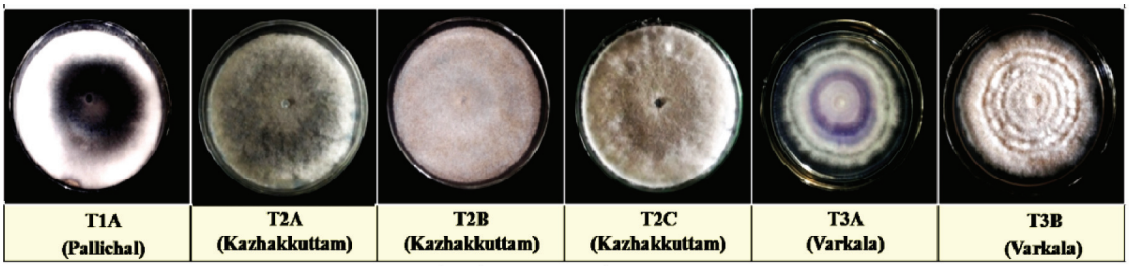


Plate 2. Symptom at five days after artificial inoculation of isolates from different locations in Thiruvananthapuram, Alappuzha, Pathanamthitta, Wayanad and Palakkad

the inoculated region to ensure proper moisture. The inoculated fruits were kept in polythene covers, labeled, tied and tagged; and pin pricks were given to polythene cover to ensure aeration. The inoculated fruits were kept in humid chamber for incubation (30 ± 2°C). Control fruits were also kept without inoculating any fungi. Four replications were maintained for each treatment including the control. The fruits were observed for symptom development on fifth day after pathogen inoculation. The fruits showing the disease symptoms were subjected to re-isolation of the pathogens in order to prove Koch's postulates. The identity of isolated pathogen was tested with the initial inoculated pathogen. Those fungi which confirmed the identity with inoculated fungi on re-isolation were confirmed to be pathogenic.

Virulence rating of the pathogens was carried out using the disease score chart (Fig.1) developed by Nath et al. in 2015 and PDI (Per cent Disease Index) was calculated as per the formula described by



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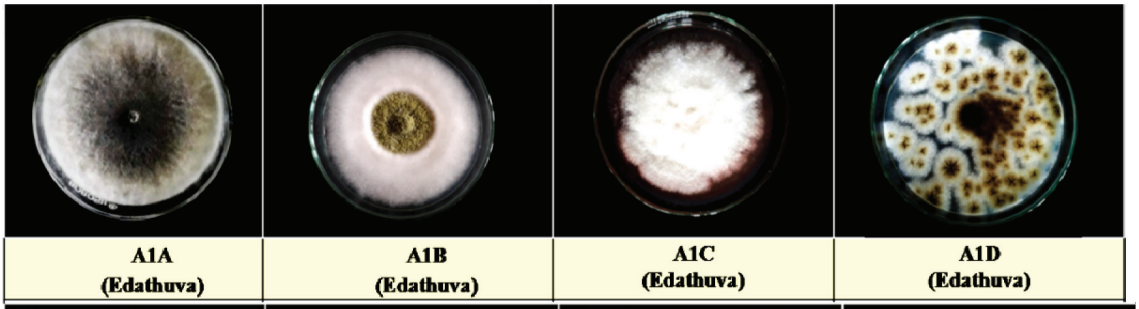
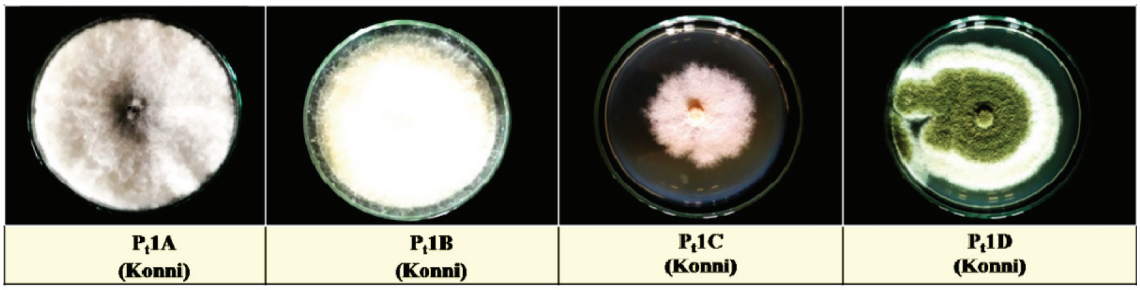
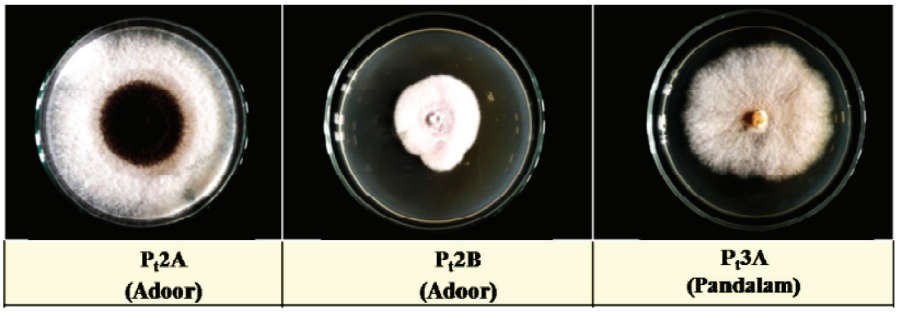


PATHANAMTHITTA



PALAKKAD



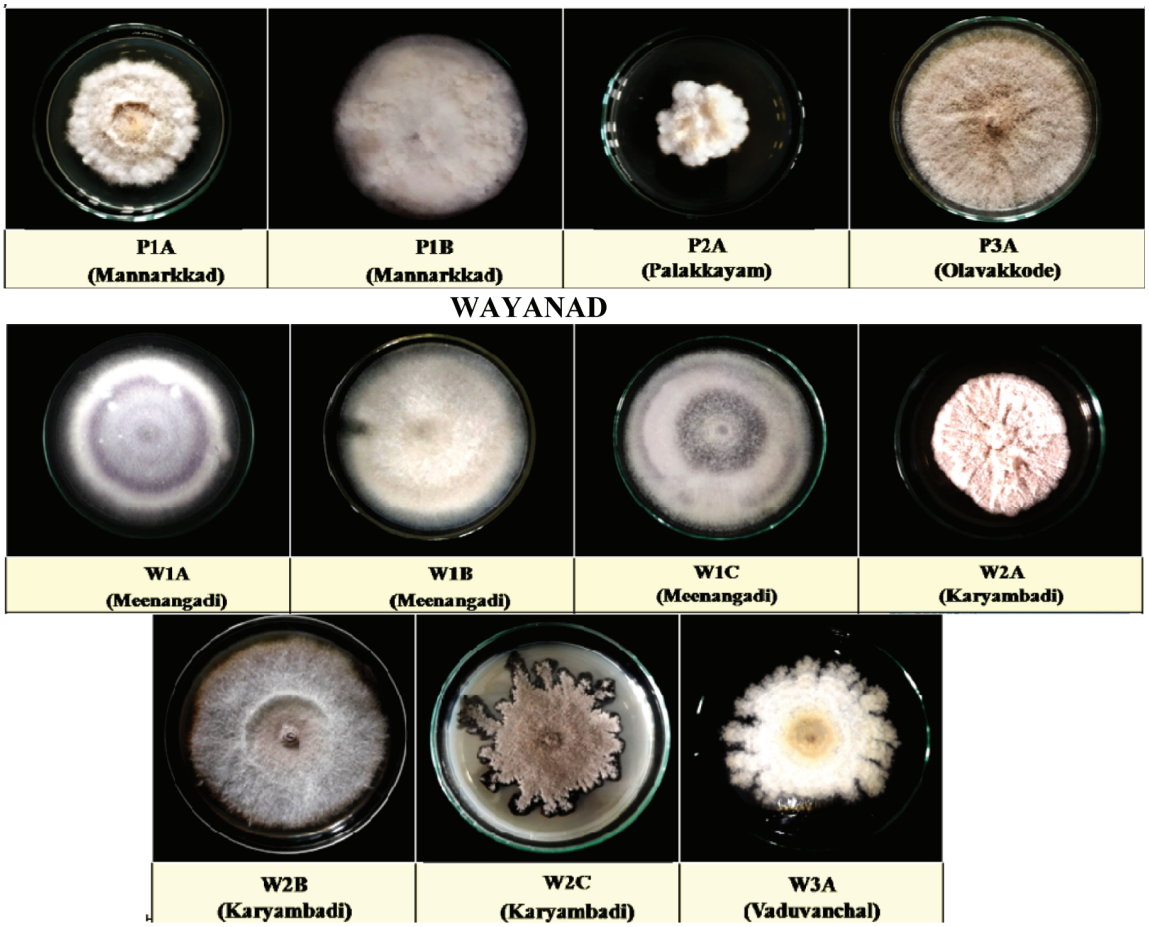


Plate 3. Isolates from diseased banana collected from different locations in Thiruvananthapuram, Alappuzha, Pathanamthitta, Palakkad, Wayanad

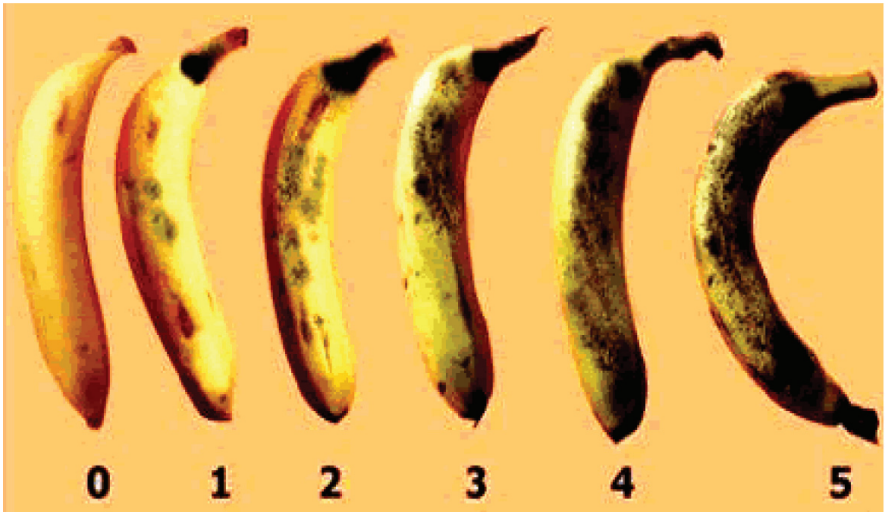


Figure 1. Assessment key for fruit rot of banana (Nath *et al*., 2015)

Mc Kinney in 1923.

$$\text{Per cent Disease Index} = \frac{\text{Sum of grades of each infected finger}}{\text{No. of fingers assessed x Maximum grade used}} \times 100$$

The pathogenicity and virulence of the 32 isolates obtained during the survey were assessed by artificial inoculation on healthy fruits. The lesion size and days taken for symptom development were observed to identify the most virulent isolate among the pathogens. Pathogenicity tests showed that all the 32 isolates were pathogenic (Plate 2). Different pathogens produced different symptoms when inoculated separately on banana fruits. In case of *Lasiodiplodia* sp., rotting started at crown region and later covered whole fruit with fluffy mycelia. Oozing of liquid was also observed in the diseased fruits as a result of severe rotting. Rotting in crown region along with spots on whole fruit was observed in fruits inoculated with *Colletotrichum* sp. and *Penicillium* sp. In case of *Aspergillus* sp., *Cunninghamella* sp. and *Verticillium* sp., rotting was observed in the crown region as well as in the distal end. Rotting restricted to the crown region was noticed in *Fusarium* sp. and *Rhizopus* sp.

The observations from pathogenicity test followed by morphological identification (Plate 3) revealed *Lasiodiplodia* sp. as the common as well as the major pathogen associated with postharvest crown rot disease of Robusta variety in Kerala. It was associated with crown rot in all the districts surveyed indicating its ubiquitous nature. Similar findings were made by Anthony et al. (2004) who reported that the main pathogen associated with the crown rot infected Embul variety of banana was *L. theobromae.*

It was observed that out of the seven isolates obtained from Thiruvananthapuram, T2C (PDI-100%) was found to be more virulent (Table 6). Similarly, out of the eight isolates from Alappuzha, seven isolates from Pathanamthitta, four isolates from Palakkad and seven isolates from Wayanad, i.e., A1A (PDI- 80 %), P_t1A (PDI-86.67%), P1B and P3A (PDI- 73.33%) and W2C (PDI- 93.33%) respectively were found to be more virulent in the respective districts (Table 6). Molecular studies of the virulent pathogen were carried out and it was identified as *Lasiodiplodia theobromae* (GenBank accession number: MN046365).

Table 6. Per cent disease index of crown rot caused by isolates collected from different locations in Kerala

Survey locations	Isolate	PDI (%) *
Thiruvananthapuram	T1 A	73.33 (58.93)
	T2 A	68.33 (55.82)
	T2 B	46.67 (43.09)
	T2 C	100.00 (89.36)
	T3 A	71.67 (57.91)
	T3 B	28.33 (32.02)
Alappuzha	A1 A	76.67 (61.15)
	A1 B	46.67 (43.09)
	A1 C	41.67 (40.20)
	A1 D	31.67 (34.18)
	A2 A	53.33 (46.91)
	A2 B	33.33 (35.25)
	A3 A	25.00 (29.93)
	A3 B	73.33 (58.93)
Pathanamthitta	Pt1 A	86.67 (68.66)
	Pt1 B	46.67 (50.79)
	Pt1 C	73.33 (58.93)
	Pt1 D	63.33 (55.73)
	Pt2 A	60.00 (68.66)
	Pt2 B	33.33 (43.09)
	Pt3 A	40.00 (52.74)
Palakkad	P1 A	48.33 (39.21)
	P1 B	73.33 (58.93)
	P2 A	53.33 (44.04)
	P3 A	73.33 (58.93)
Wayanad	W1 A	33.33 (35.25)
	W1 B	26.67 (30.95)
	W1 C	46.67 (43.09)
	W2 A	31.67 (34.18)
	W2 B	21.67 (27.60)
	W2C	93.33 (75.24)
	W3 A	33.33 (35.25)
CD (0.05)		2.305
SE (m) ±		4.606

PDI- Per cent Disease Index

Observation taken 5 days after inoculation of pathogen isolates

* Values in parentheses are angular transformed data

The result confirmed T2C isolate (*Lasiodiplodia theobromae* from Kazhakkuttam location of Thiruvananthapuram district) to be the most virulent pathogen with 100 per cent PDI. From this study, it

could also be interpreted that *Lasiodiplodia* sp. was the major pathogen and specifically, T2C isolate of *Lasiodiplodia theobromae* (GenBank accession number: MN046365) was the most virulent pathogen associated with postharvest crown rot disease of Robusta variety in Kerala.

Lasiodiplodia theobromae was isolated from the major banana growing districts of Kerala selected in the study which confirmed its uniform distribution and virulence. This called for an urgent need to formulate an effective and sustainable strategy for the management of postharvest crown rot of banana.

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