Original Research Article

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF MAJOR POSTHARVEST DISEASES OF MANGO

ABSTRACT

Mango (*Mangifera indica*), which is rich in nutritive value is affected by many biological factors including diseases. Postharvest diseases viz., anthracnose caused by *Colletotrichum gloeosporioides* and stem end rot caused by *Lasiodiplodia theobromae* are of economic importance due to loss in quality and quantity after harvesting. In this study, anthracnose and stem end rot infected mango fruits collected from farmer's field and fruit markets in Tamil Nadu were used to isolate the *Colletotrichum gloeosporioides* and *Lasiodiplodia theobroma* using tissue segment method and purified. The isolates were morphologically identified. Molecular characterization was carried through polymerase chain reaction using universal primers ITS1 and ITS4. Sequencing were done to identify the species and the sequence were deposited in Genbank. The morphological and molecular characterization will be useful in identifying the disease.

Key words: Mango, Postharvest diseases, Colletotrichum gloeosporioides, Lasiodiplodia theobromae.

1. INTRODUCTION

The king of all fruits, the mango, *Mangifera indica* L., is a member of the order sapindales and the family Anacardiaceae. It is often cultivated in Southeast Asian tropical and subtropical areas. Mangoes are said to have originated in India, Burma (Myanmar) and even the Malay area. It began to move to other continents in the sixteenth century [2]. Cultivation area available for the production of mango across India during 2022 was over 2.3 million hectares with a production of around 21 million metric tonnes. (www.statista.com).Among the diseases, antracnose caused by *Colletotrichum gloeosporioides* (Penz and Sacc.) and stem end rot caused by *Lasiodiplodia theobromae* (Pat.) Griffon & Maublanc, are of postharvest significance [8]. According to estimates, between 25 and 45 per cent of rotten, broken-textured, and microbiologically contaminated mangoes are wasted after harvest [1].

Identification of pathogens associated with mango anthracnose and stem end rot will be helpful for management practices. In this study the pathogens associated with anthracnose and stem end rot was isolated and they were morphologically characterized and molecularly confirmed.

2. MATERIALS AND METHODS

2.1 Isolation of *Colletotrichum gloeosporioides* and *Laisiodiplodia theobromae* infecting mango

Five different varieties of mango fruits infected with anthracnose and stem end rot was collected from farmer's field and fruit market of major mango growing areas of Tamil Nadu. The pathogens were isolated from the infected fruits using tissue segment method [18]. The fruits were surface sterilized with 70% ethanol and the infected portion was cut into sections. The sections were surface sterilized with 1% sodium hypochlorite and thrice washed with sterile distilled water. The sections were blot dried in sterile tissue paper to remove the excess water. The sterilized sections were placed on potato dextrose agar (PDA) medium and incubated at room temperature. The pure culture of the pathogens were done using hyphal tip method and was stored at 4°C for further studies.

2.2 Cultural and Morphological characterization of postharvest pathogens

The isolated pathogens were confirmed by cultural and morphological characters [17]. The pathogen on potato dextrose agar (PDA) medium produces conidial spores. The mycelium and conidia for both *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* were observed under phase contrast microscope. Mycelial character, colony morphology, growth pattern, zonation, conidial character were recorded for all the five isolates of *C. gloeosporioides* and *L. theobromae*.

2.3 Pathogenicity test

The mango var. sendhura was used for pathogenicity test. The pathogenicity test for anthracnose and stem end rot was performed on symptom free mango fruits. The fruits were surface sterilized with 70% ethanol and washed three times with sterile distilled water. The fruits were pin pricked and agar disc from 10 days old colonies were placed on pin pricked area [6]. The inoculated fruits were incubated at 25°C and observed periodically for symptom development. The pathogen was reisolated from the symptoms and were confirmed.

2.4 Molecular confirmation of *Colletotrichum gloeosporiodes* and *Lasiodiplodia theobromae*

2.4.1 Genomic DNA extraction

DNA extraction was performed using modified CTAB method [16]. The mycelial mat was taken from the pure isolates of both the pathogens. The mycelial mat was grinded with CTAB buffer (10 per cent CTAB,1M Tris base, 5M Nacl, 0.5M EDTA) using pestle and mortar and transferred to an eppendorf tube. The mixture was incubated at 65°C for 30 min. The phenol, chloroform and isoamyl alcohol was added to the mixture at a ratio of 25:24:1 and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a new eppendorf tube. The equal volume of ice cold isopropanol was added to the supernatant. The tube was incubated for overnight at -20°C. After incubation, it was centrifuged for 10 min at 13,000 rpm and discard the supernatant. 70% ethanol was added and centrifuged for 10 min at 10,000 rpm. Then the pellet was air dryed and resuspended in 30µl of double sterile distilled water [7]. The DNA concentration was quantified using Nano drop spectrophotometer.

2.4.2 PCR amplification of genomic DNA using universal primers

The DNA was subjected to amplification by polymerase chain reaction using universal primers of the forward ITS1 primer (5'-TCCGTAGGTGAACCTGCGC-3') and reverse ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3'). PCR amplification reaction mixture of 10 µl was prepared using 2µl of 2X master mix, 1 µl of forward primer, 1 µl of reverse primer, 2 µl of nuclease free water and 1 µl of DNA template. The amplification conditions were an initial denaturation of 94°C for 4 min, followed by 35 cycles of denaturation of 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension of 72°C for 5 min [14]. The amplified product was confirmed through agarose gel electrophoresis. One gram agarose dissolved in 100 ml of 1X TAE buffer amended with 2 µl of EtBr were casted and used for gel electrophoresis. The gel was documented in gel documentation unit. PCR amplified product was sent for sequencing and the sequence was deposited in Genbank database.

3. RESULTS

3.1 Isolation of postharvest pathogens

The postharvest pathogens isolated from five different varieties of mango revealed that the fruits infected with anthracnose and stem end rot with typical symptoms [Fig 1, 2] produced pathogens of *C. gloeosporioides* and *L. theobromae* respectively. The isolates of *C. gloeosporioides* and *L. theobromae* from different varieties of mango were listed in Table 1.

Table 1. Isolates of *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* of mango varieties.

Pathogen	Isolate	Variety	Location	District
Colletotrichum gleosporoides	Scg	Sendhura	Hosur	Krishnagiri
	Bcg	Banglora	Shoolagiri	Krishnagiri

	Hcg	Himampasand	Fruit market	Coimbatore
	Acg	Alphonso	Fruit market	Coimbatore
	Ncg	Neelam	Farmer’s field	Krishnagiri
Lasiodiplodia theobromae	BLt	Banglora	Hosur	Krishnagiri
	Slt	Sendhura	Shoolagiri	Krishnagiri
	Hlt	Himampasand	Fruit market	Coimbatore
	Alt	Alphonso	Fruit market	Coimbatore
	Nlt	Neelam	Farmer’s field	Krishnagiri

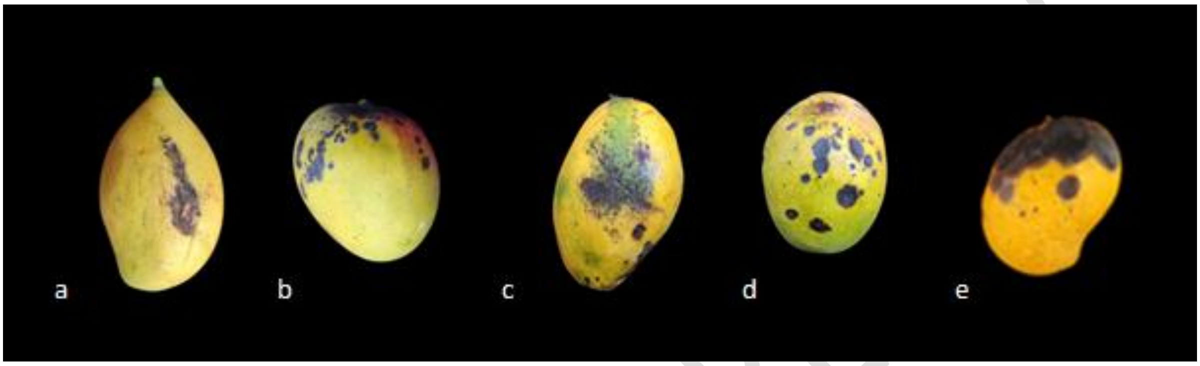


Fig 1. Symptoms of anthracnose on mango varieties (a) Banglora (b) Sendhura, (c) Himampasand, (d) Alphonso, (e) Neelam.

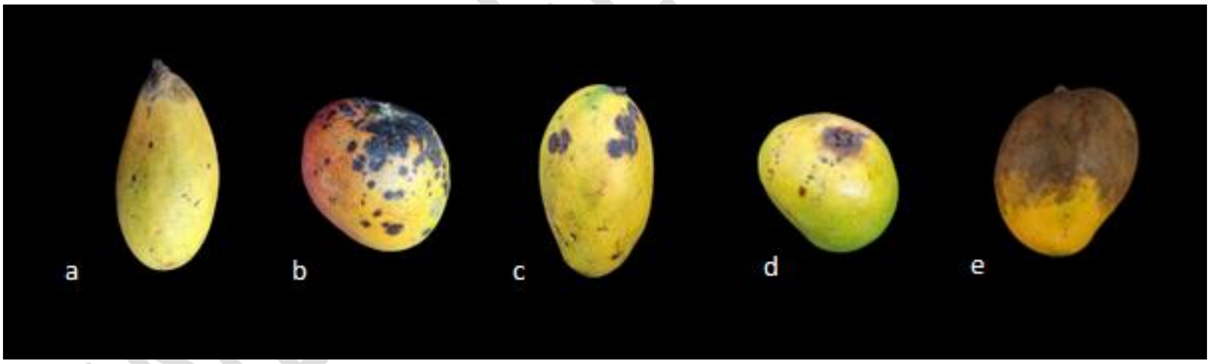


Fig 2. Symptoms of stem end rot on mango varieties (a) Banglora (b) Sendhura, (c) Himampasand, (d) Alphonso, (e) Neelam.

3.2 Cultural and Morphological characterization of postharvest pathogens

The cultural and morphological characters of *C. gloeosporioides* and *L. theobromae* were listed in Table 2. The isolates of *C. gloeosporioides* and *L. theobromae* isolated from different varieties of mango revealed that the mycelial growth pattern showed variation among the isolates. The mycelium of *C. gloeosporioides* were hyaline, septate and branched. The colour of the mycelium ranges from white fluffy to dull grey colour. The pathogen produced cylindrical shaped conidia with two oil globules [Fig 3 and 4] [3, 13]. The mycelium of *L. theobromae* were brown coloured, septate and branched. It produced pycnidia in cultural plates which consists of conidial spores. The conidia were bicelled with straiations and brown coloured [Fig 5 and 6] [11]. Based on this, the isolates were identified as *C. gloeosporioides* and *L. theobromae.*

Table 2. Cultural and Morphological characters of isolates of *C. gloeosporioides* and *L. theobromae* in mango varieties.

Pathogen	Variety	Isolate	Colony character	Mycelium	Conidia
Colletotrichum gloeosporioides	Sendhura	Scg	Dull white	Hyaline	Cylindrical
	Banglora	Bcg	Pinkish brown	Hyaline	Cylindrical
	Himampasand	Hcg	Brownish white	Hyaline	Cylindrical
	Alphonso	Acg	Whitish	Hyaline	Cylindrical
	Neelam	Ncg	Dull white	Hyaline	Cylindrical
Lasiodiplodia theobromae	Sendhura	Slt	Grey	Brown coloured	Bicelled
	Banglora	Blt	Greyish	Brown coloured	Bicelled
	Himampasand	Hlt	Greyish black	Brown coloured	Bicelled
	Alphonso	Alt	greyish	Brown coloured	Bicelled
	Neelam	Nlt	Greyish white	Brown coloured	Bicelled

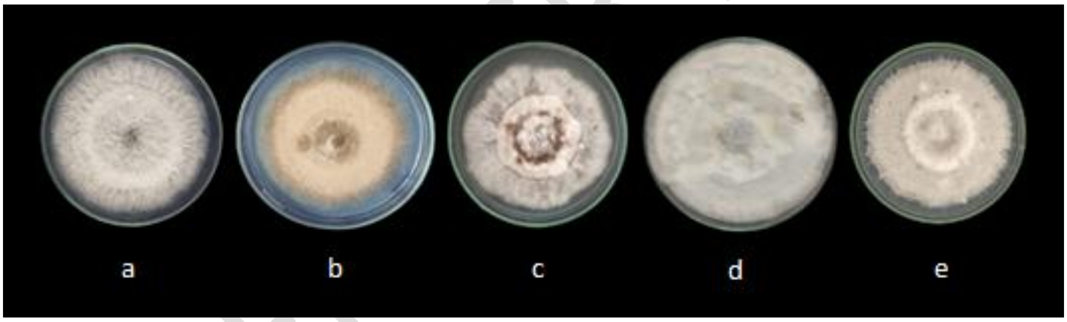


Fig 3. Colony character of *C. gloeosporioides* (a) Scg, (b) Bcg, (c) Hcg, (d) Acg, (e) Ncg

a b

Fig 4. Mycelium and conidial character of *C. gloeosporioides* (a) Hyaline, branched mycelium, (b) Cylindrical conidia.

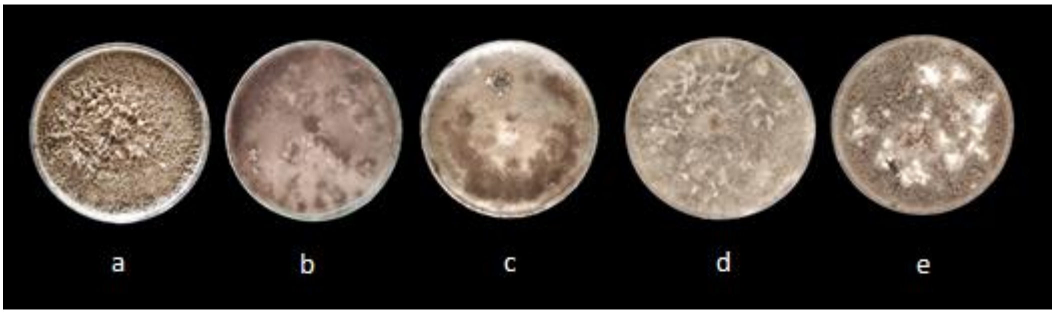
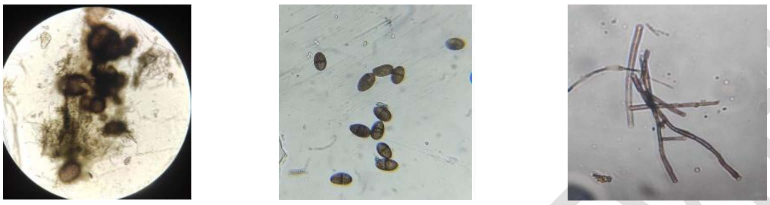


Fig 5. Colony characters of *L. theobromae* isolates (a) Slt, (b) Blt, (c) Hlt, (d) Alt, (e) Nlt



a b c

Fig 6. Mycelial and conidial character of *L. theobromae* (a) Pycnidia, (b) Bicelled conidia with striations, (c) Brown coloured septate mycelium.

3.3 Pathogenicity test

Artifically inoculated fruits recorded typical symptoms as that of naturally infected fruits. The *C. gloeosporoiodes* inoculated fruits produced dark black coloured sunken spots while *L. theobromae* inoculated fruits produced dull brown coloured rotting symptom [Fig 7] [4]. The isolate Acg produced black coloured sunken spots within 5 days whereas isolate Slt produced rotting symptom within 4 days. The pathogen reisolated from the artificially inoculated fruits were morphologically similar to the pathogen isolated.

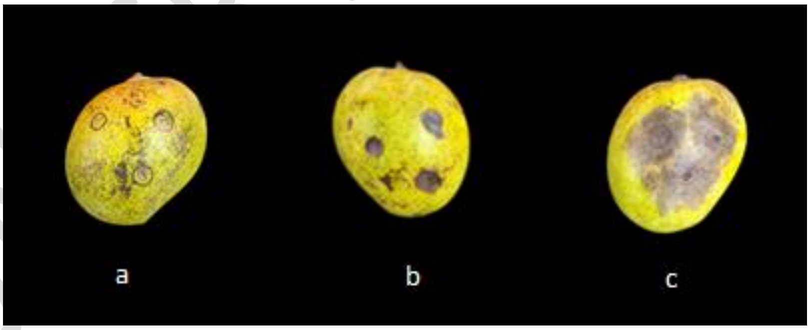


Fig 7. Pathogenicity test a) Control, b) *C. gloeosporioides* inoculated fruit exhibiting black coloured sunken spots, c) *L. theobromae* inoculated fruits exhibiting rotting symptom.

3.4 Molecular characterization of *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*

Five isolates each of *C. gloeosporiodes* and *L. theobrome* were subjected to amplification by polymerase chain reaction using universal primers ITS1 and ITS4. The PCR product were identified by using gel electrophoresis which yielded an amplicon size of 560 bp [fig 8, 9] [14]. The products were further partially sequenced and the sequence was submitted in Genbank and accession number were obtained. Genbank accession number for *C. gleosporioides* was ACG OR145043 and Accession number for *L. theobromae* was SLT OR145046. The *Colletotrichum* spp was identified as *C. gloeosporioides* through blasting the sequence in NCBI software which recorded maximum identity of 100 per cent as shown in Fig10. The *Lasiodiplodia* spp was identified as *L. theobromae* through blasting the sequence in NCBI software which recorded maximum identity of 88 per cent as shown in Fig 11.

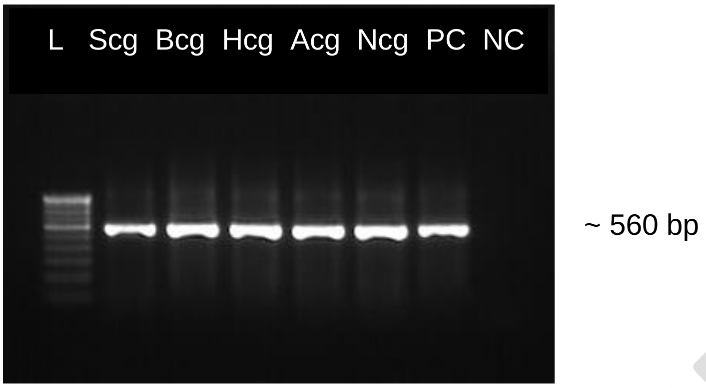


Fig 8. PCR amplification for isolates of *C. gloeosporioides*. *PC- positive control, NC – negative control, L-100bp ladder

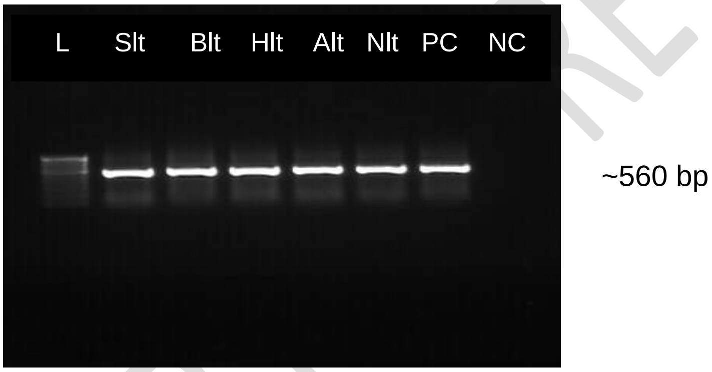


Fig 9. PCR amplification for isolates of *L. theobromae*. *PC- positive control, NC – negative control, L-100bp ladder.

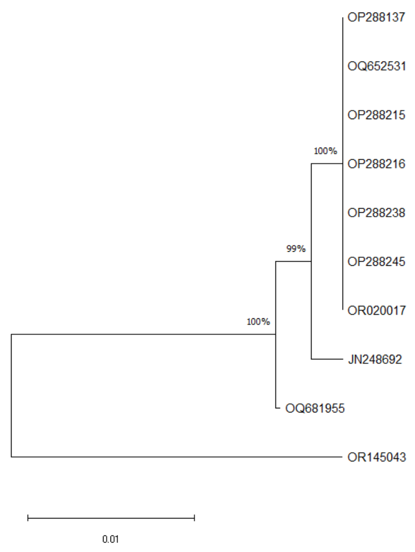


Fig 10. Phylogenetic tree of *Colletotrichum gloeosporioides*.

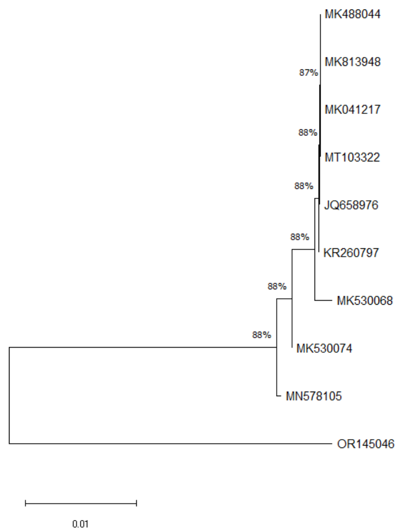


Fig 11. Phylogenetic tree of *Lasiodiplodia theobromae*.

4.DISCUSSION

Among five isolates of *Colletotrichum gloeosporioides*, each isolate varied in morphological characters such as colony morphology from dull grey to white fluffy mycelium and mycelial growth rate which are on par with the results of Udhayakumar *et al.*, 2019. Only isolate Bcg, Hcg and Ncg produced zonations [3]. The *C. gloeosporioides* isolates produced cylindrical shaped conidia with two oil globules. The isolates of *L. theobromae* also recorded difference in colony morphology, pycnidia production and sporulation. It exhibited colour variation from grey to dark greyish black colour which are on par with the results of Dheivam *et* al, 2020 and Kanyakumari *et al.*, 2022. The *Lasiodiplodia theobromae* isolates produced immature hyaline conidia which doesn't have septum and in later stages dark bovoid mature conidia with middle septum and longitudinal striations were observed. These results are in line with Munirah *et al*., 2017.

While proving pathogenicity, isolate Acg produced dark brown to black lesions initially and later these spots coalesce and formed large blighted areas. The spots were sunken and depressed. It recorded high disease incidence when compared to other isolates. Hence, it is taken as virulent isolate of *C. gloeosporioides* [3]. Isolate Slt produced dark brown to black rot symptoms and the rot was dark streaked and water conducting tissues which helps to differentiate stem end rot from anthracnose. The cultural and morphological characteristics were used for initial confirmation of pathogens. However, many *Colletotrichum* spp are associated with anthracnose disease. The fruit rotting of mango was not only associated with *L. theobromae* but also with *L. pseudotheobromae*. The rotting symptom produced by both *L. theobromae* and *L. pseudotheobromae* were similar and the colony morphology of both the pathogens were dull grey to dark black [10]. Hence, species identification was difficult with morphological characterization.

In the present study, molecular characterization was done to identify the species associated with *Colletotrichum* and *Lasiodiplodia* through Internal Transcription Factor (ITS). The observed amplicon size ranged from 500-600 bp which were on par with the results of Munirah *et al*, 2017. The difficulty in differentiating between *C. gleoeosporioides and C. acutatum* become necessary for sequencing the PCR product. The PCR product were sequenced and the genome sequence was obtained.

5. CONCLUSION

The pathogen associated with major postharvest diseases of mango were identified as *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*. These pathogens were phenotypically identified by morphological and cultural characters. Morphologically identified isolates were amplified by polymerase chain reaction and the PCR product were partially sequenced and accession number were obtained for virulent isolate.

REFERENCES

1. Alam SM, Rahman MA, Reza MH, Amin MN, Hussen MA. Postharvest loss assessment of mango at different stages of supply chain through traditional and improved handling practices. Adv. Plants Agric. Res. 2019;9:384-8.

2. Purseglove JW. Mangoes west of India. InSymposium on Mango and Mango Culture 24 1969 Jun 30 (pp. 172-176).

3. Udhayakumar R, Usharani S, Muthukumar A. Pathogenicity variation, morphological and cultural characteristic of Colletotrichum gloeosporioides isolates. Plant Arch. 2019;19(1):425-30.

4. Sarkhel S. Post-harvest Diseases of Mango and their Integrated Management.

5. Dheivam M, Manonmani K, Kalpana K, Senthilraja C, Theradimani M, Rajangam J. Studies on Morphological Variability of the Fungal Pathogen, Lasiodiplodia theobromae causing Dieback in Mango. Int. J. Curr. Microbiol. App. Sci. 2020;9(12):2446-54.

Available: https://doi.org/10.20546/ijcmas.2020.912.289

6. Goudarzi A, Samavi S, Amiri Mazraie M, Majidi Z. Fungal pathogens associated with pre-And postharvest fruit rots of mango in southern Iran. Journal of Phytopathology. 2021 Sep;169(9):545-55.

7. Carter-House D, Stajich JE, Unruh S, Kurbessoian T. Fungal CTAB DNA extraction. Protocols. io. 2020 Oct 26.

8. Iram S, Ahmad HM. Major post harvest diseases of Mango and their management. International Journal of Agronomy and Plant Production. 2013;4(12):3470-84.

9. Kanyakumari R, Somasekhara YM, Basavaraj S, Deeksha MG. Morpho-molecular identification of Lasiodiplodia theobromae causing root rot of mulberry in India. National Academy Science Letters. 2022 Oct;45(5):451-6.

10. Munirah MS, Azmi AR, Yong SY, Nur Ain Izzati MZ. Characterization of Lasiodiplodia theobromae and L. pseudotheobromae causing fruit rot on pre-harvest mango in Malaysia. Plant Pathol Quar. 2017;7(2):202-13.

11. Sathya K, Parthasarathy S, Thiribhuvanamala G, Prabakar K. Morphological and molecular variability of Lasiodiplodia theobromae causing stem end rot of mango in Tamil Nadu, India. Int J Pure App Biosci. 2017;5(6):1024-31.

Available: http://dx.doi.org/10.18782/2320-7051.5892

12. Kwodaga JK, Sowley EN, Badii BK. Morphological and molecular characterisation of Colletotrichum gloeosporioides (Penz) isolates obtained from Dioscorea rotundata (Poir). African Journal of Biotechnology. 2020 May 31;19(5):231-9.

13. Zakaria L, Juhari NZ, Vijaya SI, Anuar IS. Molecular characterization of Colletotrichum isolates associated with anthracnose of mango fruit. Sains Malaysiana. 2015 May 1;44(5):651-6.

14. Kaviyarasi M, Kamalakannan A, Rajendran L, Rajesh S, Kavino M, Lakshmi KR, Basha JS. Morphological and molecular characterization of Colletotrichum gloeosporioides causing mango anthracnose. International Journal of Plant & Soil Science. 2022 Jul 7;34(20):837-44.

15. Silva Neto JA, Ambrósio MM, Araújo MB, SILVA R, Pinto PS, Holanda IS. Morphological, molecular and pathogenic characterization of Colletotrichum gloeosporioides isolated from mango. Revista Caatinga. 2022 Aug 22;35:514-27.

16. Doyle JJ. Isolation of plant DNA from faesh tissue. Focus. 1990;12:13-5.

17. Freeman S, Katan T, Shabi E. Characterization of Colletotrichum species responsible for anthracnose diseases of various fruits. Plant disease. 1998 Jun;82(6):596-605.

Available: https://doi.org/10.1094/PDIS.1998.82.6.596

18. Poongothai M, Viswanathan R, Malathi P, Ramesh Sundar A. Sugarcane wilt: Pathogen recovery from different tissues and variation in cultural characters. Sugar Tech. 2014 Mar;16:50-66.