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Short Communication

First report of *Lasiodiplodia theobromae* causing leaf spot on *Flacourtia montana,* a wild edible fruit tree of Western Ghats, India

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ABSTRACT

***Flacourtia montana* J. Graham wild edible fruit tree, endemic to the Western Ghats, India was found infected with leaf spot disease. Based on morphological characteristics, molecular analyses (ITS and LSU) and pathogenicity, the pathogen was identified as *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Botryosphaeriaceae).This is the first report of *L. theobromae* causing leaf spots on *F. montana* from Western Ghats, India.**

Keywords: *Flacourtia montana*, *Lasiodiplodia theobromae,* leaf spot, Western Ghats

Flacourtia montana J. Graham (Salicaceae), is a wild edible fruit tree, endemic to Western Ghats, India, commonly known as Mountain Sweet Thorn and distributed in evergreen and semi-evergreen forests at an altitude upto 1000 m asl. It is a potential fruit-bearing tree as the fruits are rich in reducing and non-reducing sugars, proteins, essential micro and macronutrients (Mundaragi *et al*., 2015, 2017). The fruits were good source of natural antioxidants, so can be used as a functional food and for pharmaceutical applications (Chand and Azeez, 2021). The commercially viable wine prepared from the fruit contains major phenolic acids and had free radical scavenging property (Mundaragi *et al*., 2019). Traditional practitioners residing in the Kattunaikka tribe in Wayanad Wildlife Sanctuary, Kerala, use the bark decoction for liver disorders (Ratheesh *et al.,* 2011). The leaves possess hepatoprotective, anti-inflammatory and antioxidant activities (Joshy *et al*., 2016).

Lasiodiplodia theobromae (syn. *Botryodiplodia theobromae*), is a ubiquitous pathogen associated with the dieback of woody trees and horticultural crops in tropical and subtropical regions. The pathogen could cause severe damage to various tissues (twigs, bark, vascular tissue and fruits) of affected plants and lead to economic loss (Pavlic *et al*., 2007). The study aimed to identify and characterize the causative agent associated with the leaf spot of *F. montana* based on morphological, molecular and pathogenicity studies.

Infected leaves of *F. montana* showing typical symptoms of leaf spot diseases were collected from Vazhachal forest areas (N 10° 17.028' E 076° 37.726'; ± 272m asl), Vazhachal Forest Division, Thrissur Dist., Kerala during February-March 2021 (Fig.1a). The initial symptoms of this disease appear as very small rust brown zone that gradually increases from 5 to10 mm in diameter, changing from circular to elliptical lesions on the leaves (Fig. 1b & 1c). Gradually lesions enlarge and coalesce; causing diseased leaves to become blighted (Fig. 1d).

Infected leaves were cut into small pieces; surface sterilized with mercuric chloride for 1 min, washed in sterile distilled water, placed on potato dextrose agar (PDA) plates and incubated at 27°C for 7 days. After incubation, morphologically distinct colonies were selected, purified and used for further studies (John *et al*., 2021). Colony morphology including colour, shape and growth rate was determined after 7 days of incubation on PDA at 25°C in darkness. Slide cultures were prepared by agar cubes and incubated at 25±2°C for 3-5 days, until adequate growth and conidiogenesis had occurred. After incubation, the slide culture and fungal morphological structures were observed

under an Olympus SZX2 stereomicroscope and Leica DM2000 LED compound microscope with a DS-5Mc camera.

Pathogenicity test was evaluated on healthy *F. montana* leaves using the universal protocol (Koch's postulates). It was performed by inoculating actively pathogens to healthy leaves and the fresh leaves without inoculation sprayed with distilled water served as control. The inoculated leaves were maintained at 27°C for 7 to 12 days in a plastic box with wet sterile filter paper for the observation of disease symptoms (John *et al.*, 2021).

Genomic DNA was extracted from the pure cultured fungal plate isolate (KFRIMCC315) using the NucleoSpin ® Plant II Kit (Macherey-Nagel). The internal transcribed spacer (ITS1–5.8S-ITS2) region of rDNA was amplified using the universal primers ITS-1F & ITS-4R and larger subunit (LSU) using the universal primers LROR & LR7 to verify the identity of the fungus (White *et al.,* 1990). The PCR product was electrophoresed in a 1.2% agarose gel with a 2-log DNA ladder marker. The PCR amplified product was purified by gel ExoSAP-IT (GE Healthcare) and subjected to direct sequencing using BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufacturer protocol. Obtained Sequences (ITS & LSU) were compared with those from the Genbank database using the BLAST software on the NCBI website. The multiple sequence alignment was performed by using the Clustal W program for the KFRIMCC315 sequence along with 3 sequences of other isolates of the *Lasiodiplodia theobromae* and *Diplodia alatafructa* and *Aspergillus candidus* as outer group (Thompson *et al.,* 1994). Phylogenetic tree was constructed by MEGA6 software using the maximum likelihood method with a bootstrap of 1000 replicates (Tamura *et al.*, 2013).

Cultures of the isolates grew and spread as velvety, effuse white on PDA after 7 days (Fig.1e). The isolated fungal pathogen was observed with white-grey fluffy mycelia (Fig. 1f) and become brownish black with age (Fig. 1g). Blister like fruiting bodies was produced on PDA after 20-25 days (Fig.1h). The hyaline and cylindrical pycnidial paraphyses were observed. Conidia were ellipsoidal with a broadly rounded apex and thick-walled, contents granular. Initially, the conidia were hyaline and aseptate (Fig.1i), and then become dark brown with 1-septa (Fig.1j&1k). The average size of the conidia is 24-27×10-12µm (n=20). From these morphological characteristics, we concluded that the isolated fungal species belonged to the genus *Lasiodiplodia*. A reference specimen (KFRIMCC315) was deposited in the culture collection of the Plant Pathology Department of Kerala Forest Research Institute (KFRI), Peechi, Thrissur, Kerala.

After the molecular study, the obtained sequences of the present pathogenic fungus (ITS-365 bp, and LSU-661bp) were deposited in the Genbank as MZ707764 and MZ707765 respectively. The sequence was analyzed through BLAST homology search the against NCBI database (https://blast.ncbi.nlm.nih.gov/Blast) and showed 100% similarity with the ITS and LSU sequences of *L. theobromae* isolates originated from different crops (Table 1). In the phylogenetic tree, the representative isolate KFRIMCC315 (Dot marked) was placed within the same clade comprising reference strains of *L. theobromae* (Fig. 2 a&b). Therefore, the pathogenic fungus was correctly and authentically identified as *L. theobromae* based on both morphological and molecular characteristics.

For the pathogenicity test, control leaves remained symptomless and healthy (Fig. 3a), while inoculated leaves shows brown, necrotic, margins hairy with yellow hallow circular spots after 6-7 days of inoculation (Fig. 3b & 3c). The initial lesions were observed after three days. The symptoms on the inoculated plants were similar to those observed in the infected plant in the field. Fungi re-isolated from lesions developing on the inoculated leaves were found to be morphologically and microscopically identical to the original isolates used for inoculation studies thus fulfilling Koch's postulates. The experiment was performed in triplicate. The results revealed that *L. theobromae* was found as causal agent of leaf spot of *F. montana.*

Based on morphology, molecular analyses and pathogenicity, the pathogen was identified as *Lasiodiplodia theobromae* from leaves of *F. montana.* The Literature survey indicates that there was no record of *L. theobromae* on *F. montana* from all over the world (Farr and Rossman, 2021).

L. theobromae is a pervasive pathogen belonging to the family Botryosphaeriaceae associated with approximately 500 hosts including perennial fruit and

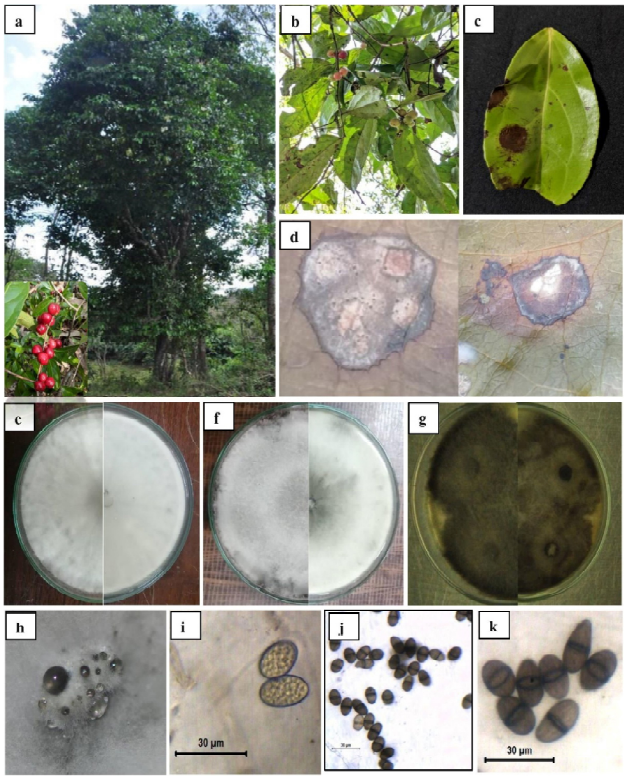

Fig. 1 (a) : *Flacourtia montana* habit (inset) Close up of ripened fruits (b) Infected leaves (c) Upper surface infected leaf (d) Enlarged view of infected portions (e) Upper and lower view of 7-day-old colony on PDA (f) Upper and lower view of 30-day-old colony on PDA (g) Upper and lower view of more than one month -old colony (h) Fruiting body (i) Immature and hyaline single celled conidia (j) & (k) Mature, brown and septate (2 celled) conidia. Scale bar = 20µm and 30µm

nut trees, vegetable crops, and ornamental plants (Punithalingam, 1980) and occurs also as an endophyte (Rubini *et al*., 2005; Mohali *et al.,* 2005). It causes damage to several crops and trees in India including root rot and collar rot disease of physic nut in India (Latha *et al*., 2009); peduncle blight of tuberose in India (Durgadevi *et al*., 2019); leaf spot of *Parthenium* (Kumar *et al.,* 2000); dieback of cocoa (Kannan *et al.,* 2019); immature nut rots in cashew (Prathibha *et al*., 2017); tip blight disease of *Dracaena fragrans* (Banerjee *et al.,* 2017); top dying disease of *Rauvolfia serpentina* (Dadwal *et al*., 2011).

The accurate identification of Botryosphaeriaceae species is necessary to determine the global distribution of these pathogen contribute to develop effective disease management strategies, because these species differ considerably in their interactions with different hosts and environmental conditions (Britton and Hendrix, 1986) Denman *et al*., 2003.

In the current study, based on morphological characteristics and molecular analyses and pathogenicity, the pathogen isolated from *F. montana* was identified as *L. theobromae.* This is the first report of *L. theobromae* causing leaf spots on *F. montana* from India. Since, the plant is ecologically and economically very important, the leaf spot caused by *L. theobromae* is of great concern. Therefore, the early detection and appropriate remedy against this pathogen is necessary to protect the plants from this disease.

Table 1 : Isolates of *Lasiodiplodia theobromae, Aspergillus candidus, Diplodia alatafructa* retrieved from Genbank.

Species name	Isolate	GenBank accession number	
		ITS	LSU
*L. theobromae**	KFRIMCC315	MZ707764	MZ707765
L. theobromae	MRR-153	MT075447.1	N/A
L. theobromae	MRR-130	MT075443.1	N/A
L. theobromae	MRR-126	MT075440.1	N/A
L. theobromae	L3	N/A	MN181372.1
L. theobromae	N/A	N/A	KC442316.1
Aspergillus candidus	ATCC1002(ITS) TUMS1390(LSU)	NR_077149.1	JQ846017.1
Diplodia alatafructa	CBS 124931	NR_111416.1	N/A

*From this study

Fig. 2a : Phylogenetic tree based on ITS sequences

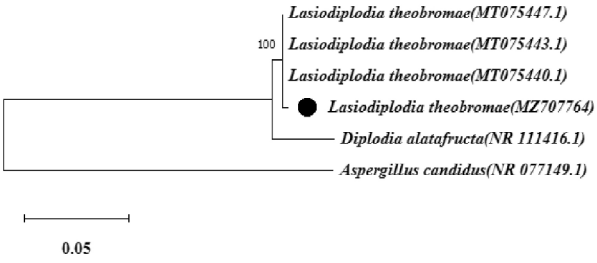

Fig. 2b : Phylogenetic tree based on LSU sequences

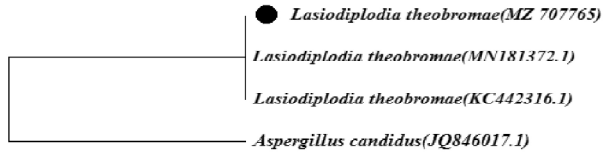

Fig. 2a, b : Phylogenetic tree representing the genetic relatedness of *Lasiodiplodia theobromae* with other isolates of *Lasiodiplodia theobromae*. *Aspergillus candidus, Diplodia alatafructa* is used as out group retrieved from Genbank, inferred by the maximum likelihood method using the ITS (2a) and LSU (2b) sequences. The robustness was evaluated with 1,000 bootstrap replicates. Our isolates are shown in bold dot mark.

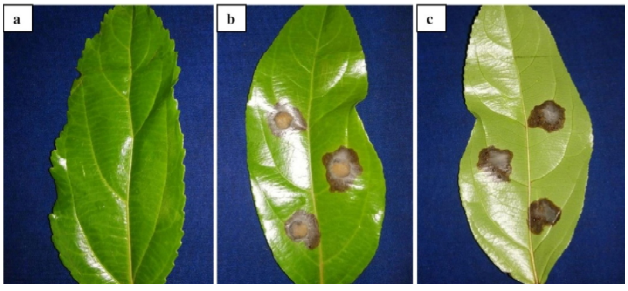

Fig. 3 (a) : Uninoculated leaves; (b) & (c) upper and lower view of leaves inoculated with fungal mycelium disc.

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REFERENCES

Banerjee,A., Panja,B. and Saha, J. 2017. Tip blight disease of *Dracaena fragrans* caused by *Lasiodiplodia theobromae* (Botryosphaeriaceae), from India. *Austrian J. Mycol.*, **26**: 63-67

Britton, K.O. and Hendrix, F.F. 1986. Population dynamics of *Botryosphaeria* spp. In peach gummosis cankers. *Plant. Dis*., **70**:134-136.

Chand, A.R.J. and Azeez, K. 2021. Underutilised fruits of Agasthyamala Biosphere Reserve: a potential source of natural antioxidants. *Vegetos*., **34**:447–452.

Dadwal, V.S., Bhartiy, S. and Verma, R.K. 2011. A New Top Dying Disease of *Rauvolfia serpentina*, Caused by *Lasiodiplodia theobromae*. *J. Mycol. Plant Pathol*., **41**(2): 246-248

Denman, S., Crous, P.W., Groenewald, J.Z., Slippers, B., Wingfield, B.D. and Winfield, M.J. 2003. Circumscription of *Botryosphaeria* species associated with Proteaceae based on morphology and DNA sequence data. *Mycology*., **94**: 294-307.

Durgadevi, D. and Sankaralingam, A. 2012. First report of peduncle blight of tuberose caused by *Lasiodiplodia theobromae* in India. *New Dis. Rep*., **26**:5.

Farr, D. F. and Rossman, A.Y. 2021.Fungal Databases, U.S. National Fungus Collections, ARS, USDA. https://nt.ars-grin.gov/fungaldatabases/ (Retrieved August 15, 2021).

John, C.J., Ge, M. and Noushad, N. 2021. Probiotic rhizospheric *Bacillus* sp. from *Zingiber officinale* Rosc. displays antifungal activity against soft rot pathogen *Pythium sp*. *Curr. Plant Biol*., **27**: 100217.

Joshy, C., Thahimon, P.A., Arunkumar, A., Betty, C. and Christudas, S. 2016 Hepatoprotective, anti-inflammatory and antioxidant activities of *Flacourtia montana* J Grah leaf extract in male Wistar rats. *Bulletin Faculty Pharmacy Cairo Univ*., **54**(2): 209-217.

Kannan, C., Karthik, M. and Priya, K. 2010. *Lasiodiplodia theobromae* causes a damaging dieback of cocoa in India. *Plant Pathol*., **59**(2): 410.

Kumar, P.S. and Singh, S.P. 2000. First report of *Lasiodiplodia theobromae* as a foliar pathogen

of *Parthenium hysterophorus*. *Plant Dis*., **84**(12): 1343.

Latha, P., Prakasam, V., Kamalakannan, A., Gopalakrishnan, C., Raguchander, T, Paramathma, M. and Samiyappan, R. 2009. First report of *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl causing root rot and collar rot disease of physic nut (*Jatropha curcas* L.) in India. *Australas. Plant Dis. Notes.,* **4**: 19-20.

Mohali, S., Burgess, T.I. and Wingfield, M.J. 2005.Diversity and host association of the tropical tree endophyte *Lasiodiplodia theobromae* revealed using simple sequence repeat markers. *For. Pathol*., **35**: 385-396.

Mundaragi, A. and Thangadurai, D. 2015. Proximate composition, nutritive value and antioxidant activity of a wild fruit, *Flacourtia montana* J. Graham. (*Salicaceae*). *Vegetos.,* **28**(4): 181-187

Mundaragi,A.C., Thangadurai, D., Sangeetha, J., Byahatti, S.D. and Anu Appaiah, K.A. 2017.Chemical analysis of volatile constituents of F. montana J. Grahamm (Salicaceae) : An edible and wild fruit of Western Ghats of India. *Plant Arch*., **17**(1): 506-508.

Mundaragi, A., Thangadurai, D., Anu Appaiah. K.A., Chethan, J. D., Sangeetha, J., 2019. Physicochemical characterization, antioxidant potential and sensory quality of wine from Wild Edible Fruits of *Flacourtia montana* J. Graham. *Braz Arch Biol Technol*. **62**.

Pavlic, D., Slippes, B., Cautinho, T.A., Gryzenhout, M. and Wingfield, M.J. 2007. The Botryosphaeriaceae occurring on the *Syzgium cordatum* in South Africa and their potential threat to Eucalyptus. *Plant Pathol*., **56**:624–636.

Prathibha, V.H., Hegde, V. Sharadraj, K.M. and Chowdappa, P. 2017. First report of *Lasiodiplodia theobromae* causing apple and immature nut rots on cashew in India. *J. Plant Pathol.*, **99**: 295.

Punithalingam, E. 1980. Plant diseases attributed to *Botryodiplodia theobromae* Pat. J. Cramer, Vaduz.

Ratheesh, N.M.K., Mithunlal, S., Sujanapal, P., Kumar, A. N., Sivadasan, M. and Ahmed, H.A. 2011. Ethnobotanically important trees and their uses by Kattunaikka tribe in Wayanad Wildlife Sanctuary, Kerala, India. *J. Med. Plants Res*., **5**(4): 604-612.

Rubini, M.R., Silva-Ribeiro, R.T., Pomella, A.W., Maki,C.S., Araujo, W. L., Dos Santos, D.R. and Azevedo, J.L. 2005. Diversity of endophytic fungal diversity13 fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis perniciosa*, causal agent of Witches' Broom disease. *Int. Res. J. Biol. Sci*., **1**: 24-33.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol*., **30**: 2725–2729.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*., **22**: 4673-4680

White, T. J., Bruns, T., Lee, S. and Taylor, J. W.1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications,* (Eds.), Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York. p. 315-322

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