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ORIGINAL ARTICLE

Identification of *Lasiodiplodia* species inciting stem rot of dragon fruit in India through polyphasic approach

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Abstract

Lasiodiplodia species commonly thrive as endophytes, saprobes, and plant pathogens in tropical and subtropical regions. Association of *Lasiodiplodia* species causing stem rot in dragon fruit in the coastal belt of Odisha, eastern India, has been illustrated here. The stem rot disease was characterized by yellowing of the stem, followed by softening of the stem tissues with fungal fructifications of the pathogen in the affected tissues. On the basis of macro- and micromorphological characteristics, the four fungal isolates recovered from diseased stems were identified initially as *Lasiodiplodia* species. By comparing DNA sequences within the NCBI GenBank database as well as performing a multigene phylogenetic analysis involving the internal transcribed spacer region (ITS-rDNA), β-tubulin (*β-tub*), and elongation factor-alpha (*EF1-α*) genes, the identity of *Lasiodiplodia* isolates was determined. The isolate CHES-21-DFCA was identified as *Lasiodiplodia iraniensis* (syn: *L. iranensis*) and the remaining three isolates, namely CHES-22-DFCA-1, CHES-22-DFCA-2, and CHES-22-DFCA-3, as *L. theobromae*. Although pathogenicity studies confirmed both *L. iraniensis* and *L. theobromae* were responsible for stem rot in dragon fruit, *L. iraniensis* was more virulent than *L. theobromae*. This study established the association of *Lasiodiplodia* species with stem rot in dragon fruit using a polyphasic approach. Further investigations are required, particularly related to on host–pathogen–weather interaction and spatiotemporal distribution across the major dragon fruit–growing areas of the country to formulate prospective disease management strategies. This is the first report on these two species of *Lasiodiplodia* inflicting stem rot in *Hylocereus* species in India.

Keywords Dragon fruit · Stem rot · *Lasiodiplodia* species · Multigene phylogeny · India

Introduction

Dragon fruit (*Selenicereus* spp., formerly *Hylocereus*) is a perennial cactus belonging to family Cactaceae. It has drawn attention in many countries during recent years as an edible fruit. This fruit is native to Central America, Mexico, and South America (Barthlott and Hunt 1993). The former genus *Hylocereus* comprised 14 species and among them, *H. undatus*, *H. polyrhizus*, *H. costaricencis*, and *H. megalanthus* were the most cultivated species in dragon fruit–growing countries (Tel-Zur et al. 2011). This fruit is rich in vitamin C, phosphorus, calcium, salt, potassium, and vitamin A, and contains up to 16.6% of total solids (Tel Zur 2015). Vietnam is one among the leading producers of dragon fruit and is the foremost exporter in the world (Mercado-Silva 2018). *Hylocereus* species were introduced to India in the late 1990s (Karunakaran et al. 2019). Dragon fruit is known by numerous vernacular names such as pitaya, pitahaya, strawberry pear, and buahnaga. This tropical fruit crop starts bearing fruits in the year following planting and attains maximum yield potential in 5 years (Hamidah and Zainuddin 2007). It has great potential as a new, water-efficient, and very adaptable crop for India. Cultivation of dragon fruit is witnessing a momentum in many states of India, including the east Indian states such as Odisha. The ever-increasing demand for healthy and nutritious fruit is favoring the expansion of dragon fruit cultivation. However, major constraints for dragon fruit cultivation are diseases and insect pests. Numerous plant pathogens were reported to cause diseases

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in dragon fruit (Balendres and Bengoa 2019). Although dragon fruit is cultivated and thrives well in diverse tropical regions, under newly introduced environmental conditions, diseases may be a bottleneck for its successful production if not managed well in time. Dragon fruit diseases so far reported from India include anthracnose from Andaman and Nicobar Islands (Abirami et al. 2019), viral disease from Telangana state (Parameswari et al. 2021), and stem canker caused by *Neoscytalidium dimidiatum* from Pune, Satara, and Solapur districts of Maharashtra (Salunkhe et al. 2022).

In 2018, in an experimental farm at Bhubaneswar, in the coastal belt of the Odisha state, eastern India, red and white pulp varieties of dragon fruit exhibited stem rot symptoms characterized by yellowing of the stem, followed by softening of stem tissues. In most cases, rotting typically initiated along the stem, from growing tips and stem margins, but it was also observed in the middle of the stem involving part of, or the entire section of, the stem. As rotting progressed, fungal fructifications appeared on the cankers. Finally, the rotten portion detached from stem leaving behind only the central core. On both red and white pulp varieties, the incident of rotting was more prominent during summer. Previous studies in different countries indicated that *Lasiodiplodia* and other species in the Botryosphaeriaceae family were responsible for stem rot of dragon fruit (Mohd et al. 2013; Briste et al. 2019, 2022; Serrato-Diaz and Goenaga 2021; de Mello et al. 2022). A similar stem canker of cactus pear (*Opuntia ficus-indica*), another member of the family Cactaceae, was caused by *Neofusicoccum batangarum*, a fungus that like *Lasiodiplodia* species belongs to the family Botryosphaeriaceae (Aloi et al. 2020) and toxins produced by this fungus are hypothesized to be responsible for the symptoms (Masi et al 2020). As morphological features between *Lasiodiplodia* species and other Botryosphaeriaceae overlap in some cases, molecular methods have been used to identify these fungi and, in a broader context, to elucidate the phylogeny of the Botryosphaeriaceae (Slippers et al. 2005, 2017). Botryosphaeriaceae is the largest family within the order Botryosphaeriales, which encompasses at least 24 major genera including *Diplodia*, *Lasiodiplodia*, *Botryosphaeria*, *Dothiorella*, and *Neofusicoccum* (Burgess et al. 2018; Phillips et al. 2019; Zhang et al. 2021). Among them, *Lasiodiplodia* spp. are cosmopolitan and known to be associated with approximately 500 hosts, mostly woody plants, and different fruit trees in subtropical and tropical zones where they cause numerous diseases like cankers, die-back, fruit rot, and root rots in tree species such as mango, citrus, eucalyptus, neem, avocado, apple, pear, and peach (Punithalingam 1980; Alves et al. 2008; Rodríguez-Galvez et al. 2015). *Lasiodiplodia* species have different lifestyles, traversing from saprophytic to endophytic and to pathogenic roles (Slippers and Wingfield 2007; Abdollahzadeh et al. 2010; Liu et al. 2012; Chen 2015; Dissanayake 2015).

Botryosphaeriaceae in general survives in a latent condition as endophytes for extended time, but under the influence of stress factors, may cause disease (Slippers and Wingfield 2007). The external stimuli in the form of high temperature or drought stress trigger these fungi to transition into potential pathogens, which ultimately cause the disease (Aloi et al. 2021). The accurate identification of the causative agent of a disease is critical for developing appropriate disease management strategies. This investigation was carried out with the objective to study the symptomatology of stem rot of dragon fruit observed at Bhubaneswar as well as identify and characterize the causal agent of the disease.

Material and methods

Sampling and isolation

Dragon fruit stems with rotting symptoms were collected from the experimental orchard located at Bhubaneswar, Odisha, eastern India (20°14′ N, 85°46′ E). They were carried to a research laboratory for pathogen isolation and subsequent identification of the causative agent. Briefly, three 5×5 mm^2 pieces of symptomatic tissue along with bordering uninfected healthy portion were cut from progressing edge of the lesions. Further, the tissues were surface sterilized with 1% sodium hypochlorite for 1 min and washed consecutively three times with sterilized distilled water. The tissue bits were allowed to air-dry on sterilized blotting paper and transferred aseptically to Petri dishes on a fresh potato dextrose agar (PDA) medium amended with streptomycin sulfate (100 ppm) to avert bacterial contamination. The dishes were incubated at 28 ± 2 °C under room condition with continuous illumination and observed periodically for fungal growth. The emerging hyphal tips from the infected tissues were aseptically transferred to new PDA dishes and incubated at 28 ± 2 °C with a 12 h photoperiod. Pure cultures of the isolates were coded as CHES-21-DFCA, CHES-22-DFCA-1, CHES-22-DFCA-2, and CHES-22-DFCA-3, and the initial identification was carried out based on macro- and micro-morphological features. Pure cultures of the isolates were maintained at 4 °C on PDA slants for further study.

Pathogenicity

The pathogenicity of fungal isolates was evaluated on mature and healthy, uniformly sized dragon fruit stems, with a total of 10 stem segments allocated per isolate. In brief, healthy stems were washed under flowing tap water, followed by disinfection with 70% ethanol. They were rinsed twice with sterile distilled water. After air-drying in a laminar flow chamber, the stems were wounded with a sterilized needle at regular intervals (2.5–5 cm) depending on the stem size.

An 8 mm diameter mycelial plug, punched from the margin of a 7-day-old colony in a PDA dish, was placed on each wound, with the mycelial surface facing down. To prevent desiccation, the mycelial plug was covered with wet sterile cotton. The stems were placed in plastic containers lined with two layers of sterilized wet blotting papers and incubated at 28 ± 2 °C and more than 80% relative humidity with a 12 h light and dark cycle at ambient room conditions. The inoculated stems were observed daily up to 15 days post inoculation. The experiment was carried out twice. Tissues from the margins of the lesions were picked up and placed into PDA plates and incubated for a week to recover the inoculated fungi. The isolates recovered from artificially induced lesions were identified and compared with the original isolates, fulfilling Koch's postulates. To know the ability of pathogenic fungi in causing rot at different temperatures, tests were also conducted. These involved inoculating the highly virulent isolate CHES-21-DFCA and incubating the stem segments at different temperatures ranging from 10 to 40 °C. The severity of symptom was rated at several time intervals after inoculation (2, 4, 6, 8, 10, 12, 14, and 16 days post inoculation).

Phenotypic characterization

The isolates were cultured on PDA medium to examine the colony characteristics. Three 8 mm discs were cut out from 7-day-old colonies of each isolate, and these mycelial discs were transferred to PDA dishes. The dishes were incubated at 28 ± 2 °C for 1 week. The mean radial growth (mm per day) and colony color of each isolate were determined. Sterilized toothpicks were kept near to the fungal discs on water agar (2%) and incubated at 28 ± 2 °C for 2–3 weeks for sporulation. Observations on conidial characters were recorded under an Olympus Bx53 microscope equipped with a digital camera. Conidia were mounted in lactic acid (100%). The dimensions of conidia and pycnidia were also recorded. Preliminary identification of isolates was done based on colony color and morphology and color, as well as shape, size, septation, and striations of conidia, according to the criteria described by Phillips et al. (2013).

Molecular characterization

All four isolates characterized in the current study were grown in potato dextrose broth (PDB) at 28 ± 2 ºC for 12 days. The mat of mycelium was separated from the broth by filtering through sterilized filter paper (Whatman No.1) and washed with sterile distilled water. A fungal DNA purification kit (HiPurATM; HiMedia, Maharashtra, India) was used to extract the total genomic DNA of the isolates according to the manufacturer's instructions. The respective primer pairs used for amplification of ITS region of ribosomal DNA (ITS-rDNA), partial elongation factor 1-alpha (*EF-1α*), and β-tubulin (*β-tub*) genes were ITS1/ITS4 (5′-TCCGTAGGTGAACCTGCGG-3′/5′-TCCTCCGCTTATTGATATGC-3′), EF1-688F/EF1-1251R (5′-CGGTCACTTGATCTACAAGTGC-3′/5′-CCTCGAACTCACCAGTACCG-3′), and Bt2a/Bt2b (5′-GGTAACCAAATCGGTGCTGCTTTC-3′/5′-ACCCTCAGTGTAGTGACCCTTGGC-3′), according to White et al. (1990), Alves et al. (2008), and Glass and Donaldson (1995), respectively. PCR reaction mixtures consisted of green dye-added 25 µl of 2 × PCR MAX Master Mix (Takara Bio Inc.), 1 µl of each primer (10 mM), and 2 µl of DNA template. The volume was brought to 50 µl using sterile nuclease-free water. The PCR amplification cycles were as follows: an initial denaturation at 94 ºC for 4 min; 35 cycles of denaturation at 94 ºC for 15 s; annealing at 52 ºC (ITS1/4), 55 ºC (EF1-688F/1251R), and 65 ºC (Bt2a/Bt2b) for 40 s; elongation at 72 ºC for 1 min, followed by a final elongation step at 72 ºC for 5 min. The PCR products were separated by gel electrophoresis in agarose gel (1.2%) stained with ethidium bromide (EtBr) viewed under ultraviolet light and photographed in a gel documentation system (Vilber, Marne-la-Vallée, France). The target amplicon was eluted from gel using a gel extraction kit (QIAquick; Qiagen India, New Delhi, India) following the manufacturer's instructions and sequenced by Sanger sequencing method (Eurofins India Pvt Ltd, Karnataka, India). The resultant sequences were edited and assembled with the BioEdit software, V.7.0.9.0 (Hall 1999). Nucleotide sequences were assembled and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis

For each of the three loci, sequences of reference strain and *Lasiodiplodia* species homologous to the isolates of the present study were retrieved from the NCBI GenBank database via Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) and aligned with the isolates of this study for phylogenetic analysis (Table 1). The concatenated sequences of three genes (*ITS*, *EF1-α*, and *β-tub*) of the present isolates (both pathogenic and nonpathogenic isolates) were included as well as additional selected reference sequences of *Lasiodiplodia* species were constructed using the Sequence Matrix software, version 1.7.8 (Vaidya et al. 2011). The maximum parsimonious tree was constructed through Phylogenetic Analysis Using Parsimony (PAUP), v. 4.0b10, with 1000 random stepwise addition and TBR (tree bisection and reconnection) as branch swapping algorithms. To understand the robustness of the tree, the tree length (TL), retention index (RI), homoplasy index (HI), consistency index (CI), and rescaled consistency index (RC) and homoplasy index were calculated along with bootstrap

Table 1 Details of species of *Lasiodiplodia* used in this study with their NCBI GenBank accession numbers

S. no	Species	Isolate	GenBank accession numbers			Host	Country
			EF1-α	*β-tub*	ITS		
1.	*L. iraniensis*	CHES-21-DFCA[a]	ON461991	MZ447869	MZ314206	Dragon fruit	India
2.	*L. theobromae*	CHES-22-DFCA-1[a]	OP832011	OP832008	OP935661	Dragon fruit	India
3.	*L. theobromae*	CHES-22-DFCA-2[a]#	OP832012	OP832009	OP935662	Dragon fruit	India
4.	*L. theobromae*	CHES-22-DFCA-3[a]	OP832013	OP832010	OP935663	Dragon fruit	India
5.	*L. pseudotheobromae*	UACH259	MH286522	MH279919	MH277926	*Citrus latifolia*	Mexico
6.	*L. pseudotheobromae*	UACH261	MH286516	MH279926	MH277919	*Citrus latifolia*	Mexico
7.	*L. pseudotheobromae*	UACH265	MH286513	MH279930	MH277916	*Citrus latifolia*	Mexico
8.	*L. pseudotheobromae*	UACH274	MH286520	MH279922	MH277923	*Citrus latifolia*	Mexico
9.	*L. pseudotheobromae*	UACH278	MH286524	MH279918	MH277927	*Citrus latifolia*	Mexico
10.	*L. theobromae*	UACH263	MH286528	MH279908	MH277691	*Citrus latifolia*	Mexico
11.	*L. theobromae*	UACH264	MH286534	MH279909	MH277692	*Citrus latifolia*	Mexico
12.	*L. theobromae*	UACH280	MH286527	MH279910	MH277695	*Citrus latifolia*	Mexico
13.	*L. theobromae*	UACH285	MH286531	MH279913	MH277697	*Citrus latifolia*	Mexico
14.	*L. theobromae*	UACH288	MH286529	MH279911	MH277699	*Citrus latifolia*	Mexico
15.	*L. citricola*	XGWY42	MT856964	MT856967	MT849762	Mulberry	China
16.	*L. citricola*	UACH262	MH286541	MH279934	MH277948	*Citrus latifolia*	Mexico
17.	*L. mahajangana*	IRNKB208	MN633995	MN633436	MN634041	walnut	Iran
18.	*L. mahajangana*	BPPCA158	MK562446	MK573993	MK542017	Mangifera indica	Malaysia
19.	*L. viticola*	UCD2553AR	HQ288269	HQ288306	HQ288227	Grapevine	Arkansas and Missouri, United states
20.	*L. viticola*	UCD2604MO	HQ288270	HQ288307	HQ288228	Grapevine	Arkansas and Missouri United states
21.	*L. iraniensis*	IRAN1502C	GU945335	KP872416	GU945347	*Juglans* sp.	Iran
22.	*L. iraniensis*	IRAN1520C	GU945336	KP872415	GU945348	*Salvadora persica*	Iran
23.	*L. iraniensis*	UACH275	MH286542	MH279933	MH271621	*Citrus latifolia*	Mexico
24.	*L. iraniensis*	CP/VPC-3	MT162471	MT212401	MT103323	*Pinus elliottii* var. elliottii x *Pinus caribaea* var. hondurensis	Mexico
25.	*L. iranensis* (previously identified as *L. jatrophicola*	CMM3610	KF226690	KF254927	KF234544	*Jatropha curcas*	Brazil
26.	*L. mediterranea*	BL101	KJ638330	KU720483	KJ638311	Grapevine	Italy
27.	*L. mediterranea*	BL1	KJ638331	KU720482	KJ638312	Holm oak	Italy
28.	*L. missouriana*	UCD2193MO	HQ288267	HQ288304	HQ288225	Grapevine	Arkansas and Missouri, USA
29.	*L. missouriana*	UCD2199MO	HQ288268	HQ288305	HQ288226	Grapevine	Arkansas and Missouri, USA
30.	*L. plurivora*	STE-U5803	EF445395	KP872421	EF445362	prunus species	South Africa
31.	*L. plurivora*	STE-U4583	EF445396	KP872422	MT649616	Woody hosts surrounding vineyards	South Africa
32.	*L. hormozganensis*	CBS:168.28	MT592138	MT59262	MT587427	*Cocos nucifera*	Indonesia
33.	*L. hormozganensis*	GBLZ16BO-019	MN539211	MN539187	MN540683	*Litchi chinensis* Sonn	China
34.	*L. subglobosa*	UACH270	MH286539	MH279917	MH271619	*Citrus latifolia*	Mexico
35.	*L. subglobosa*	UACH282	MH286540	MH279917	MH271620	*Citrus latifolia*	Mexico
36.	*L. parva*	*Lth*-soj3	MZ643247	MZ643243	MZ613157	*Styphnolobium japonicum*	China
37.	*L. parva*	*Lth*-soj2	MZ643246	MZ643244	MZ613155	*Styphnolobium japonicum*	China
38.	*L. euphorbicola*	CMM3609	KF226689	KF254926	KF234543	*Jatropha curcas*	Brazil
39.	*L. euphorbicola*	IBL329	KT247492	KT247494	KT247490	Tropical fruit trees	Northeast Brazil

Table 1 (continued)

S. no	Species	Isolate	GenBank accession numbers			Host	Country
			EF1-α	*β-tub*	ITS		
40.	*L. venezuelensis*	WAC12540	DQ103569	KP872427	DQ103548	*Acacia mangium*	Venezuela
41.	*L. venezuelensis*	CBS129759	JX545121	JX545141	JX545101	*Acacia mangium* and *Pinus caribaea* var hondurensis	Venezuelan region of Guayana
42.	*L. jatrophicola*	CMW36236	KU886997	KU887498	KU887120	*Adansonia* sp.	South Africa
43.	*L. jatrophicola*	DSYWK05	KP297435	KP297437	KP297436	*Caryota mitis*	China
44.	*L. thalandica*	BJFU DZP160123-13	KY676798	KY676795	KY676789	Unknown	China
45.	*L. thalandica*	BJFU DZP160119-9	KY676797	KY676794	KY676788	Unknown	
46.	*L. hyalina*	CGMCC3.17975	KX499917	KX499992	KX499879	*Acacia confusa* Merr	China
47.	*L. hyalina*	BJFU DZP160121-9	KY751302	KY751299	KY767661	Unknown	China
48.	*Diplodia mutila*	CBS230.30	MZ073946	MZ073930	MW810264	*Phoenix dactylifera*	USA

[a]Isolates of present study

analysis involving 1000 bootstrap replications. The phylogenetic tree was rooted through *Diplodia mutila* CBS230.30.

Results

Symptomatology

The dragon fruit stem rot was detected in the experimental orchard at Bhubaneswar throughout the year with different degrees of severity spanning from partial to complete rotting of the stem. Observations were made in three randomly selected distinct plots, each comprising 100 plants aged between 3 and 5 years for each season. Each plot included both red and white pulp dragon fruit types. During the summer months of 2018, a few dragon fruit plants showed symptoms of stem rot, which did not result in considerable crop loss. However, by mid-May 2019, the stem rot incidence was observed in a high proportion, affecting 26% of red-fleshed plants and 34% of white-fleshed plants. Although the disease appeared throughout the year, the manifestation of symptoms peaked during April–June, which correspond to summer months. Usually, symptoms initiated along the margin or tip of the stem, although instances were also observed in the middle of the stem without any physical injury or wounds. The typical symptom of stem rot was characterized by yellowing and softening of the stem, followed by rotting, involving partial or complete length of the stem (Fig. 1A–H). Subsequently, circular to irregular cankerous lesions were observed in the middle or at the margin of the lesions. When the rot became old, the cankerous growth containing fungal fructifications involving host tissues was also seen (Fig. 2A–E). The cankerous grayish lesions contained black-headed pycnidia arranged in a circular to irregular manner, which expanded overtime, covering the entire lesion In advanced stages of the disease, the host epidermis became dried and shredded, leaving characteristic circular holes if rotting occurred on the stem margin. In some cases, rotten portion detached from the stem leaving behind only the central core (Fig. 2F). When the rot was confined on the margin of stems, the infected host tissues along with fungal structures separated in a semi-circular shape. The stem rot disease occurred in the middle of stem without any physical damage or wound. The symptoms later led to a general decline in the vigor of the plant. Eventually, the severely infected plants become less productive in subsequent seasons.

Isolation and pathogenicity

Isolation was carried out from the infected symptomatic stem and four representative isolates (CHES-21-DFCA, CHES-21-DFCA-1, CHES-22-DFCA-2, and CHES-22-DFCA-3) were further selected for pathogenicity evaluation and characterization. In pathogenicity assays, two isolates viz., CHES-21-DFCA and CHES-22-DFCA-2, produced disease symptoms comparable to those observed in the field. Other two isolates did not show any symptoms. Upon artificial inoculation, CHES-21-DFCA induced a yellow discoloration around the inoculation site, which appeared 3 days post inoculation. Then, yellowing enlarged rapidly, leading to stem softening and rotting within 10 days (Fig. 3). Conversely, the isolate CHES-22-DFCA-2 showed very mild symptoms, with rotting confined to the site of inoculation and not progressing further. No rotting symptoms were observed in stems inoculated with isolates CHES-21-DFCA-1 and CHES-22-DFCA-3 even after 15-day incubation. The isolates recovered from artificially inoculated symptomatic stems were identical to CHES-21-DFCA and CHES-22-DFCA-2, fulfilling Koch's postulates. Notably, the symptoms produced by the isolate CHES-21-DFCA

Fig. 1 Symptoms and signs of stem rot disease of dragon fruit (*Selenicereus* spp.,) observed in the field. Stem rot initiation and progression. **A–D** Along the stem margin. **E** and **F** On the growing tip. **G** At middle of the stem **H** At the cut portion of stem

were much more severe than those incited by the isolate CHES-22-DFCA-2. Similar kind of extensive rotting symptoms were observed under field conditions more often than milder rotting patterns associated with the isolate CHES-22-DFCA-2. The ability of the isolate CHES-21-DFCA to induce stem rot was tested at temperatures ranging from 10 to 40 °C. The pathogen was able to induce rotting within a week at temperatures ranging from 15 to 40 °C. Maximum severity of rotting was recorded at 35 and 40 °C (Fig. 4A–F). Although rotting expanded slowly at temperature between 15 and 30 °C, all stem portions were rotten completely at the site of inoculation within 2 weeks. In contrast, at 10 °C, it took at least 35 days for the rot to reach a diameter of 8 mm.

Phenotypic characterization

The colonies of all four fungal isolates had a grayish-black cottony growth texture, accompanied by an olivaceous green-to-black color on the reverse/bottom side. The diameter of the colony grew to 90 mm after 4–5 days of incubation at 28 ± 2 °C and a 12:12 h (light:dark) photoperiod. The isolates were identified as *Lasiodiplodia* species based on morphological and conidial characteristics.

Lasiodiplodia iraniensis

The colony of the *L. iraniensis* isolate CHES-21-DFCA was initially grayish white with fluffy aerial mycelia on the PDA medium and became olivaceous gray at the surface whereas the reverse showed greenish gray after 2 weeks of incubation at 28 ± 2 °C. Blister-like, thick-walled, globose, dark brown pycnidia were produced on the sterilized toothpicks within 3 weeks of incubation. These pycnidia measured 230–535 µm in diameter. The isolates lacked conidiophores whereas paraphyses were cylindrical and hyaline. Initially aseptate, they became septate when matured and very rarely branched. Conidia, which were initially hyaline, aseptate, and ovoid with blunt/rounded ends, became dark brown, thick-walled, with one middle septum and longitudinal striations when matured. The conidia measured 17.5–24 × 11–14.5 µm size ($n = 50$) (Table 2). The teleomorph form remained unknown.

Lasiodiplodia theobromae

The colonies of the *L. theobromae* isolates CHES-21-DFCA-1, CHES-22-DFCA-2, and CHES-22-DFCA-3

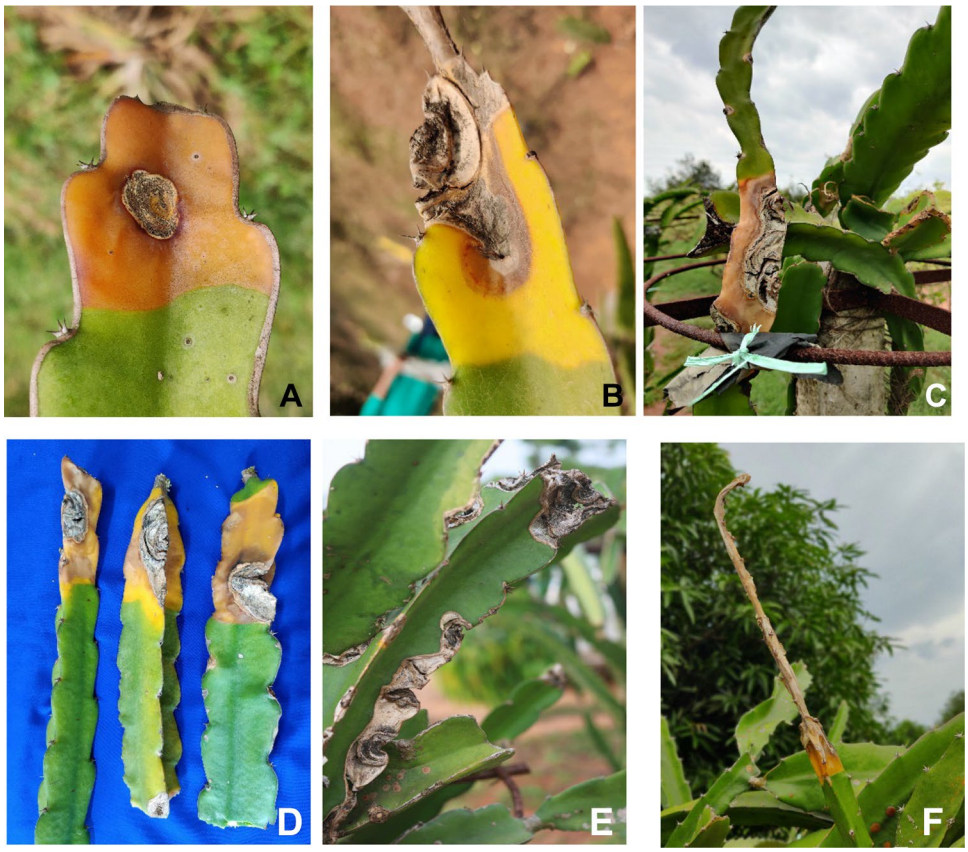

Fig. 2 **A–E** Development of cankerous growth consist of fruiting body of causative fungi within the rotten stem portion. **F** Rotten portion detached leaving behind the central core of stem

Fig. 3 Pathogenicity assay showing the stem rot symptom on dragon fruit stem upon artificial inoculation of test pathogen *L. iraniensis* 10 days after inoculation Arrow mark in yellow indicates the site of pathogen inoculation, arrow mark in red indicates control which received no inoculation

were initially white with fluffy aerial mycelia on the PDA medium. They became smoky gray after 15 days when incubated at 28 ± 2 °C. All three isolates later produced abundant black pigmentation, which was visible from the reserve side of the PDA media. The morphological features of all three *L. theobromae* isolates were similar in nature. Dark gray to black color knot-like pycnidia were produced on water agar overlaid with sterilized darbha grass (*Desmostachya bipinnata*) within 2 weeks. The pycnidia were solitary, globose, uniloculate, and thick-walled. They were semi- or fully immersed, measuring 250–550 µm. Conidiophores were cylindrical, hyaline, rarely septate, and branched. Fungal paraphyses were hyaline and aseptate. Young conidia were hyaline and aseptate, and mature conidia were dark brown, striated, with one middle septation. Conidial measurements ($n = 50$) of all three isolates are given in Table 2. The teleomorph form remained unknown.

Molecular characterization

For molecular characterization, the nucleotide sequences of *ITS*, *EF1-α*, and *β-tub* genes were generated and used to identify the *Lasiodiplodia* isolates at the species level. The amplified PCR products of *ITS* (~ 550 bp), *EF1-α* (~ 500 bp), and *β-tub* (~ 450 bp) genes were sequenced using the custom sanger sequencing services and were subjected to NCBI Blastn analysis. Both the forward and reverse sequences were compiled and submitted to NCBI GenBank and accession numbers were obtained (Table 1).

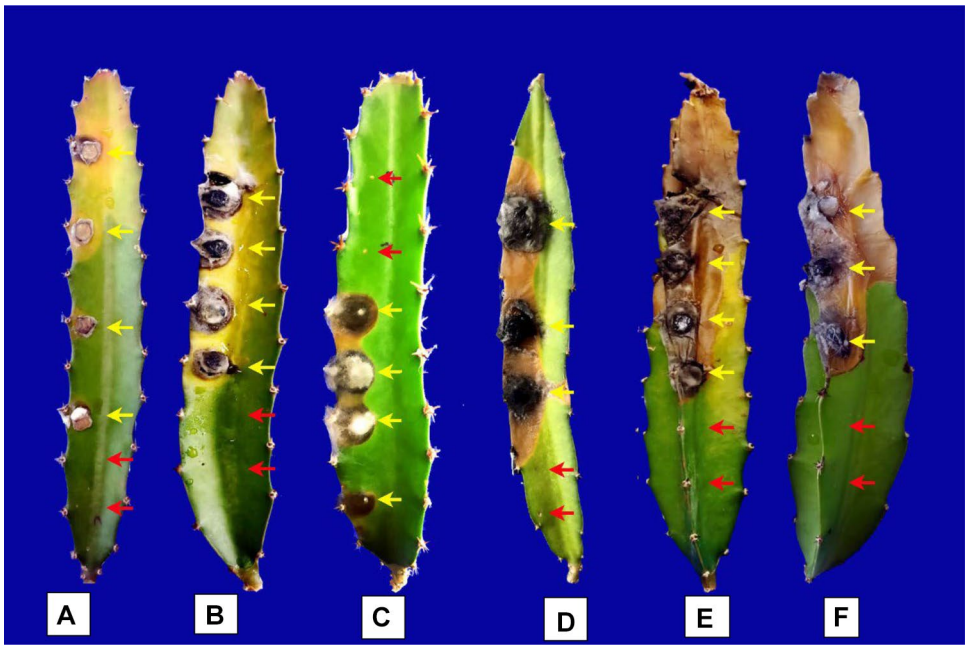

Fig. 4 **A–F** Stem rot caused by *L. iraniensis* at 15 °C (**A**), 20 °C (**B**), 25 °C (**C**), 30 °C (**D**), 35 °C (**E**), 40 (**F**) °C temperature on dragon fruit stem 10 days after inoculation. Arrow mark in yellow indicates the site of pathogen inoculation, red arrow mark indicates control which received no inoculation

Table 2 Morphological characteristics of *Lasiodiplodia irnaiensis* and *L. throbromae*

Isolate	Pathogen	Conidia	Septation	Paraphyses	Source of data
CHES-21-DFCA	*L. iraniensis*	17.5–24 × 11–14.5	1	septate	This study
CHES-21-DFCA-1	*L. theobromae*	24.5–28.0 × 16.5–18 µm	1	septate	This study
CHES-22-DFCA-2	*L. theobromae*	22.0–26.0 × 14.0–15.6	1	septate	This study
CHES-22-DFCA-3	*L. theobromae*	23–26.8 × 12.4–15.0	1	septate	This study

The BLAST searches of the isolate CHES-21-DFCA in the NCBI GenBank database showed that the *ITS* sequence of this isolate exhibited 98.55–98.76% similarity with *L. pseudotheobromae* (MN341226, MN887206) and 98.55% similarity with *L. theobromae* (MK166047, MH865367) and *L. iranensis* (MK282705). The *EF1-α* gene showed 100% similarity with *L. hyalina* with 91% query cover (KX499917, KY751302), 98.19%–99.42% similarity with *L. iranensis* isolates with 100% query cover (MF580812, MW725045, OL455942), 95%–96% similarity with *L. theorbromae* isolates (OL455945, MK570085, MF580814), and more than 97% similarity with *L. thailandica* (KY751303) and *L. jatrophicola* (KT325583, KT325582, KU507447). Notably, a stretch of 8 nucleotides (AGCGCT GC) found in the *EF1-α* gene sequences of all the *L. thailandica* isolates was missing in the examined isolates (*L. iranensis*, *L. hyalina*, and *L. jatrophicola*) (Table 1). The *β-tub* gene sequence of this isolate exhibited more than 99% similarity with *L. pseudotheobromae* (MN867365, MN243787), *L. theobromae* (KR260823, KR260821), *L. iranensis* (MK294103, MK294101), *L. jatrophicola* (MH251965, MH251964), *L. lignicola* (KT852958), and *L. hormozganensis* (OL405589, OL405587) isolates.

The *ITS* sequences of isolates CHES-22-DFCA-1, CHES-22-DFCA-2, and CHES-22-DFCA-3 displayed 99%–100% similarity with different *L. theobromae* isolates (MN831964, MN831965, MK584592, MT103322) and > 99% similarity with *L. hormozganensis* (JX464085, JX464085). Likewise, the *EF1-α* gene of these three isolates, viz., CHES-22-DFCA-1, CHES-22-DFCA-2, and CHES-22-DFCA-3, was 100% similar to *L. theobromae* isolates HB2 and ML1001 (MF580814, JN542563) and 98.85% similar to *L. mahajangana* isolates (OL455923, OL455922). The *β-tub* gene of these three isolates was also 100% identical to *L. theobromae* isolates (MN172230, MK587448, MW118596). Hence, multigene phylogenetic analysis was carried out by combining all three gene sequences, in order to establish the identity of all four isolates to the species level.

Multigene phylogenetic analysis

Owing to conflicts observed in single-gene phylogenies in case of all the four isolates (data not shown), the three genes were concatenated. The combined dataset comprised 623 characters of *ITS* (1–623), 792 characters of *EF1-α* (624–1416), and 680 characters of *β-tub* (1420–2100) genes.

A multigene phylogenetic analysis was carried out using the *ITS*, *β-tub*, and *EF1-α* genes combined dataset of *Lasiodiplodia* species, including 43 isolates from GenBank corresponding to a wide range of known *Lasiodiplodia* species and one outgroup taxa (*Diplodia mutila*-CBS230.30) and 4 isolates (CHES-21-DFCA, CHES-22-DFCA-1, CHES-22-DFCA-2, and CHES-22-DFCA-3) from this study (Table 1). The maximum parsimonious tree is shown in Fig. 5. The resultant phylogenetic tree is given with bootstrap support values above the nodal branches. The isolate CHES-21-DFCA represented a distinct lineage from *L. hyalina*, *L. thailandica*, and *L. jatrophicola* and clustered along with *L. iranensis* isolates. Thus, it was identified as *L. iranensis*. The remaining three isolates were identified as *L. theobromae* as they clustered with *L. theobromae* reference strains. The maximum parsimony analysis constructed a single most parsimonious tree with TL = 1019, CI: 0.745, RI: 0.776, RC: 0.578, and HI: 0.255. There were 214 parsimony informative characteristics among the 1203 constant characters. The combined dataset analysis improved phylogenetic resolution. Thus, the pathogenic isolates CHES-21-DFCA and CHES-22-DFCA-2 were identified as *L. iraniensis* and *L. theobromae*, respectively.

Discussion

The symptoms of stem rot were observed in 2018 on the newly introduced crop in the state of Odisha, located in the eastern part of India. Because dragon fruit is a low-maintenance crop, stem rot has been found to have a significant impact on crop health. Over the 3-year investigation period, the disease increased significantly and rendered the plants less productive. Hence, this study was aimed to identify the causal agent of the stem rot disease of dragon fruits and to develop suitable management strategies. Investigations based on morphology, pathogenicity assessment, and multiple gene phylogenetic analysis revealed the association of Botryosphaeriaceae fungi in the stem rot disease of dragon fruit. To the best of our knowledge, this is the first report of *L. iraniensis* and *L. theobromae* as the causal agents of stem rot in dragon fruit plants in India. Among the two *Lasiodiplodia* species, *L. iraniensis* proved to be more virulent than *L. theobromae*. However, out of three *L. theobromae* isolates, only one isolate (CHES-21-DFCA-2) was able to cause rotting at the site of inoculation (only) whereas other two isolates (CHES-22-DFCA-1 and CHES-22-DFCA-3) were not able to cause any kind of rotting symptom on artificial inoculation. Hence, it can be inferred that in nature nonpathogenic strains of *L. theobromae* coexist as endophytes along with pathogenic strains. Similar observations were made by Sosa et al. (2016) in the cacao cushion galls disease caused by *L. theobromae* and *Fusarium decemcellulare*.

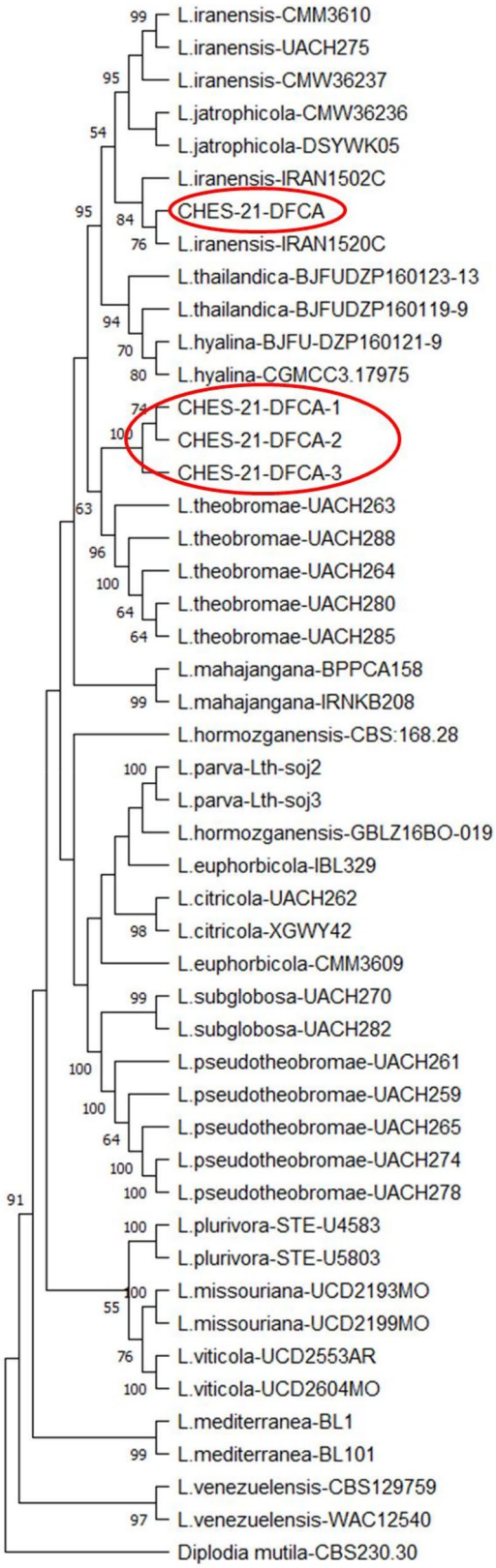

Fig. 5 Phylogenetic tree constructed from multigene (ITS, *EF1-α* and *β-tub*) dataset (Maximum parsimony bootstrap value (MPBS) displayed at the nodes

In the current study, *L. iraniensis* was found to cause a variety of rotting symptoms on the stems of dragon fruits including, marginal rotting, tip rot, partial, or complete stem rot. The symptoms were more severe in white-pulped varieties. In several dragon fruit–growing countries such as Israel, Taiwan, Malaysia, China, and the United States, canker disease affecting dragon fruit has been attributed to another member of the Botryosphaeriaceae family, *N. dimidiatum*. Moreover, canker disease caused by *N. dimidiatum* was devastating in Vietnam, which is a leading global exporter of dragon fruits. In Vietnam, this disease affected approximately more than 10,000 ha area and yield losses ranged from 30 to 70% in individual fields. Stem canker incited by *N. dimidiatum* has also been reported from India in commercially grown dragon fruit orchards of Pune, Solapur, and Satara districts of Maharashtra with a disease incidence of approximately 40% (Salunkhe et al. 2022). Additionally, stem rot and canker diseases of dragon fruit caused by *Diaporthe phaseolorum* and *L. theobromae*, respectively, have been reported from Bangladesh (Karim et al. 2019; Briste et al. 2022).

Detailed species-level morphological characterization of *Lasiodiplodia* isolates using morphology is nearly impossible as the size of the spores is extremely variable. In the past, when identification was based on morphology, many *Lasiodiplodia* species were identified as *L. theobromae* (Punithalingam 1976). Until 2000, species within the Botryosphaeriaceae family were identified exclusively by their morphological features (Denman et al. 2000). As conidial septation and pigmentation are strongly affected by cultural conditions, misidentification has become common in literature (Alves et al. 2006). Many new species of *Lasiodiplodia* have been described since 2004 based on DNA sequencing together with morphological features. As pointed out by Phillips et al. (2013), morphology of spores should only be used to distinguish between genera as it is not appropriate for identifying the species in *Lasiodiplodia.* To precisely identify the *Lasiodiplodia* species, multigene phylogenetic analysis is crucial, as suggested by Phillips et al. (2013).

Sequences of *ITS*, *EF-1α*, and *β-tub* regions are commonly used to differentiate the *Lasiodiplodia* species (Slippers et al. 2014; Bautista-Cruz et al. 2019). Alves et al. (2008) used morphological data together with *ITS* and *EF-1α* to characterize a group of *Lasiodiplodia* isolates that were earlier identified as *L. theobromae*. In Peru, sequence data of *ITS* and *EF-1α* were used to establish the association of five *Lasiodiplodia* species causing die-back of mango, which were earlier described as *L. theobromae* (Rodriguez-Galvez et al. 2017). *Lasiodiplodia theobromae*, *L. pseudotheobromae*, *L. subglobosa*, *L. brasiliense*, *L. iraniensis*, and *L. citricola* were reported to cause canker and die-back symptoms in Persian lime wherein the identity of organisms was confirmed by molecular tools and multigene phylogeny (Bautista-Cruz et al. 2019). In the current study, we used *ITS*, *β-tub*, and *EF-1α* sequence data to identify and investigate the phylogenetic relationships of *L. iraniensis* and *L. theobromae* with other closely related *Lasiodiplodia* species. *L. jatrophicola*, *L. hyalina*, and *L. thailandica* are phylogenetically close to but clearly distinct from *L. iraniensis.* However, a few researchers believe that *L. iraniensis* and *L. jatrophicola* have to be regarded as synonyms, as these two species cannot be divided into different species by using *ITS* and *EF1-α* sequence data (Rodriguez-Galvez et al. 2017; Cruywagen et al. 2017). However, in the present study, the combined gene such as *ITS*, *β-tub*, and *EF-1α* could clearly separate *L. iraniensis* from *L. jatrophicola*. *Lasiodiplodia iraniensis* was first reported from Iran on *Salvadora persica*, *Eucalyptus* sp., *Juglans* sp., mango, *Citrus* sp., and almond (Abdollahzadeh et al. 2010). Later, it was reported from various countries on economically important trees, such as mandarin (Al-Sadi et al. 2013), mango (Marques et al. 2013; Al-Sadi et al. 2013; Rodriguez-Galvez et al. 2017), *Anacardium occidentale* (Netto et al. 2017), and *Bougainvillea* (Li et al. 2015). Wither tip in citrus (*Citrus reticulata* cv. Kinnow) caused by *Colletotrichum siamense* and *L. iraniensis* has been reported from Pakistan, causing 40% yield loss (Fayyaz et al. 2018). Recently, *L. iraniensis* has been identified as the causal agent of rotting of Yam (*Dioscorea* spp.) in Florida (Jibrin et al. 2022). Several studies indicate that Botryosphaeriaceae fungi shows endophytic behavior within healthy tissues of plants. They shift into a pathogenic lifestyle, implying that these fungi become aggressive when plants are stressed (Schoeneweiss 1981; Blodgett and Stanosz 1995; Jami et al. 2013). This occurs in nonoptimal disturbed environments (Slippers and Wingfield 2007). Abiotic factors such as severe sunburn, drought, and freezing predispose plants, including citrus, to xylem dysfunction, which results in branch canker and die-back (Raimondo et al. 2010; Khanchouch et al. 2017; Aloi et al. 2021). In dogwoods (*Cornus florida*), plant stress has been demonstrated to be a crucial factor in triggering the pathogenic behavior of *L. theobromae,* and this was corroborated by the failure of artificial inoculations in pathogenicity trials (Mullen et al. 1991).

Although dragon fruit is a crassulacean acid metabolism (CAM) plant, in our study, the symptoms were more pronounced during summer months (March–May; data not shown). The fungi belonging to the Botryosphaeriaceae family grow well within the temperature range of 15–37 °C. In spite of having the ability to grow between 9 and 39 °C, the optimal temperature of 27–33 °C has been reported for the fungi (D'souza and Ramesh 2002). The extracellular enzymatic activity of the fungi also varies according to the temperature, which was confirmed by pathogenicity test on dragon fruit stems infected by *L. iraniensis.* Symptoms were more severe when stems were incubated at 30, 35, and

40 °C compared to those incubated at 15, 20, and 25 °C. On Chinese hackberry, canker disease outbreak caused by *L. pseudotheobromae* occurred between July and August (Liang et al. 2020; Zhang 2012).

Although dragon fruit adapt and thrives well in diverse tropical regions, infections by new pathogens can pose a challenge to its successful production. In Vietnam, an unproductive diseased dragon fruit orchard was rejuvenated into a healthy and high yielding productive one by implementing strict field sanitation and effective fungicide programs (Fullerton et al. 2018). However, management of stem rot/canker caused by *Lasiodiplodia* species is challenging because of limited information about this new pathogen on this new host plant and nonavailability of registered fungicides for this newly introduced crop. Currently, pruning and destruction of the infected stem is the best management strategy available. Hence, the stem rot disease should be monitored closely in various dragon fruit growing regions of the country to tackle this disease effectively.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

Ethical approval This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

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