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ORIGINAL RESEARCH ARTICLE

Characterization of *Colletotrichum* species causing new pre-harvest anthracnose symptoms on mango in Eastern India

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Abstract

Anthracnose caused by *Colletotrichum* species is an important disease of mango (*Mangifera indica* L.) affecting leaf, flowers and fruits in mango orchards worldwide. The fungus *Colletotrichum* sp. usually invade the fruits during its developmental stage but remains quiescent until fruit ripening culminating in devastating anthracnose disease, especially during post-harvest stage. In contrary, new kind of pre-harvest anthracnose symptoms were observed on green unripe mangoes with varying level of incidence at field level in the state of Odisha, situated in Eastern India. This study attempted to characterize the *Colletotrichum* species affecting mango cultivars and causing new kind of symptoms under field condition on maturing green mangoes in comparison to post-harvest phase using morphological methods and molecular tools as well as by pathogenicity tests on intact green as well as on detached mature fruits. Eight *Colletotrichum* isolates from pre-harvest phase and two from post-harvest phase isolated from different mango varieties were studied for their diversity through morphological examination as well as sequence analysis of internal transcribed spacer (ITS), chitin synthase (CHS-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin (TUB2) and ApMat genomic regions. Multigene phylogeny of all ten isolates revealed the identification of *C. siamense*. Pathogenicity assay of all 10 *Colletotrichum* isolates on green intact fruits in field as well as on detached ripening fruits in laboratory resulted in similar anthracnose symptoms on two selected test varieties Arka Anmol and Mallika. Results confirmed the association of *C. siamense* with both pre as well as post-harvest anthracnose symptoms of mango. Accurate pathogen identification provides a reliable basis for devising disease management schedules against anthracnose occurring at different phenological stages of the mangoes.

Keywords Mango · Pre-harvest anthracnose · Multigene phylogeny · Eastern India

Introduction

Mango is one of the most sought-after seasonal fruits growing in tropical and subtropical parts of the world. India is the home of thousands of varieties of mangoes which come in various sizes, shapes, and colors with a wide variety of flavors and tastes, with a definite eco-geographical need for optimum growth and yield. India is the world's largest producer of mango with a production of 24 million tons of mango in 2020 (FAO-STAT 2021). However, mango production is constrained by diseases, pests, and poor post-harvest handling of fruit. Anthracnose disease on mango fruits caused by *Colletotrichum* species is a serious problem encountered in many tropical and sub-tropical countries which not only reduces the fruit quality drastically, but also adversely affects shelf life and marketability of mature mango fruits in ripening stage. Typically, anthracnose disease result in fruit spots which start as oval to round, black, sunken spots on fruit skin when mangoes start ripening. *Colletotrichum* species also causes leaf spot and blossom blight in mango plants at different growth stages. In tropical conditions, fruit spoilage due to anthracnose disease caused by *Colletotrichum* species at pre and/ or post-harvest stages

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ranges from slight loss in quality, to total spoilage of the fruits, resulting in reduced sales while impacting domestic and export marketing (FAOSTAT 2021; Hindorf 2000).

In our experimental farm located in the state of Odisha, Eastern India, in past few years we observed a new kind of preharvest anthracnose symptoms (without tear stains or pepper spots) on few varieties of mango. The symptoms generally start as shiny, dark brown to black circular, sub-circular to irregular necrotic patches which enlarge to 1.0–2.5 inches diameter covering the major portion of the fruits, even though the symptoms varied with varieties. The infected skin became dry, flaky, with or without yellow halo, which sometimes manifested as big cracks in fruits as the disease advanced. The symptoms of preharvest of anthracnose were noticed in other varieties of mango during 2017–2018. According to our investigation, the incidence of preharvest anthracnose reached as high as 45% in highly susceptible varieties seriously affecting the fruit quality. It is known that once the spores of *Colletotrichum* land on peel of unripe fruits under field condition, spores germinate by producing germ tubes which will then form melanized appressoria that penetrate the cuticle directly. At this stage, this pathogen is noted for its ability to maintain itself in an extended dormant state until fruit ripening (Giblin et al. 2010) except on young fruits (Estrada et al. 2000). Once the fruits start ripening the pathogen embarks on necrotrophic infection and decay symptoms develop on the peel resulting in anthracnose disease symptoms (Dodd 1991; Akem 2006). In preliminary investigations, we observed that even though the fruiting bodies produced on the diseased green fruits indicated the association of *Colletotrichum* species, the peculiar difference in symptomatology from the post-harvest anthracnose necessitated the systematic investigation of the pathogen population with pre as well as post-harvest phases of mango.

Due to the economic relevance of mango in India, it was decided to undertake comprehensive work to determine the species composition of the *Colletotrichum* population involved in causing anthracnose in the pre-harvest phase of mango, under coastal Eastern India conditions. As the taxonomic intricacy among *Colletotrichum* species is well known further morpho-taxonomic identification alone potentially can be misleading. Although internal transcribed spacer (ITS) gene sequence data in many *Colletotrichum* species complexes show modest phylogenetic resolution (Willingham et al. 2012), the polyphasic method is advised for their correct identification. This study presents the symptomatology, pre-harvest anthracnose incidence on different mango varieties, taxonomy, and phylogenetic relationships of *Colletotrichum* isolates associated with pre-harvest anthracnose along with post-harvest anthracnose of mango in the state of Odisha, Eastern India through morphological, molecular, and pathogenicity analyses.

Materials and methods

Sampling and isolation of causal agents

Immature green mangoes showing dry anthracnose symptom were collected from eight different cultivars of mango viz., Arka Anmol (Alphonso x Janardhan Pasand), Arka Neelkiran (Alphonso x Neelum), Manjeera (Rumani x Neelum), Mahmood Bahar, Sai Sugandh (Totapuri x Kesar), Sindhu (Ratna x Alphonso), Totapuri from our experimental farm of ICAR-IIHR- Central Horticultural Experiment Station and Mallika farmer's field (Fig. 1). Ripened fruits of the above varieties showing post-harvest fruit rot symptoms were collected from the local market and var. Mallika and Amrapali used for comparison in this study (Fig. 2). Isolation of the pathogen was done as per Sharma et al. (2017). Small pieces (0.5 cm^2) of infected tissue sections partially consisting of healthy portions from the affected fruits were dissected and surface-sterilized with 1.0% sodium hypochlorite for 30–60 s and subsequently rinsed with sterile distilled water thrice. These tissues were blot dried on a sterile paper towel and placed aseptically on Potato Dextrose Agar medium (PDA, HiMedia™ Laboratories Pvt Ltd) amended with streptomycin sulphate (0.1 mg mL^{-1}) and incubated at 25–27 °C for 5 days. Pure culture of the fungi was obtained by single hyphal tip method and all isolates were subjected to morphological characterization and phylogenetic study. The eight *Colletotrichum* isolates resulted from preharvest anthracnose symptoms and two *Colletotrichum* isolates from ripe anthracnose symptoms were cultured at 25 °C for 7 days, PDA media. Subsequently, the cultures were subjected to morphological characterization (colony morphology, conidial measurements), and growth rate as well as assessed for color of the conidial masses (Than et al. 2008). Conidia were mounted in 100% lactic acid and digital images were recorded with an Olympus BX53 microscope (Olympus Co., Tokyo, Japan). The length and width of 30 conidia per isolate were measured with the Q capture Pro image analyzer. Each morphological feature was compared with *C. siamense-type* strains (Weir et al. 2012). After seven days, the colony's diameter was measured to calculate its growth rate (mm/day).

Fungal genomic DNA extraction and PCR amplification

All 10 fungal isolates were cultured in potato dextrose broth (PDB, HiMedia™ Laboratories Pvt Ltd) and incubated at 28 ± 2 °C for seven days under a static condition. Then the mycelia were harvested, dried on sterile filter paper on laminar hood, and homogenized

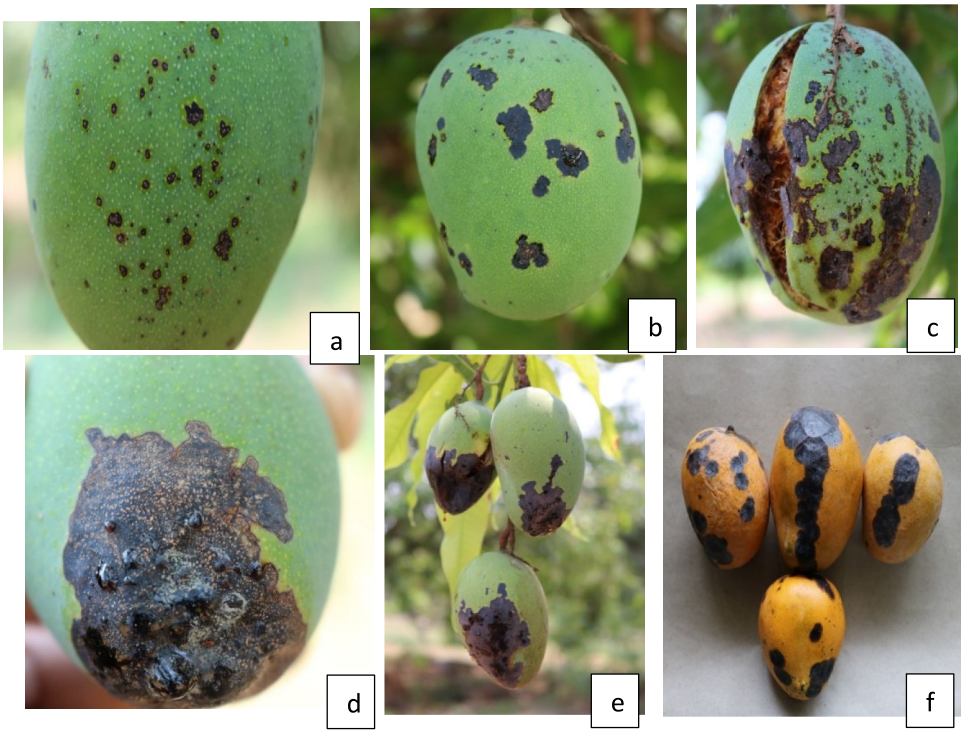

Fig. 1 Different stages of preharvest dry anthracnose in the highly susceptible var. Mahmood Bahar from minute lesion to big patch (**a**, **b**, **c**) and big irregular black lesion on Arka Anmol (**d**) and Prabha Shankar (**e**) at natural field condition, post-harvest anthracnose for comparison (**f**)

into a fine powder using liquid nitrogen. One hundred milligrams of powdered mycelia were subjected to DNA extraction using the using the Fungal DNA Purification Kit (HiPurATM; HiMedia, Maharashtra, India) according to the manufacturer's instructions. The Apn2/Mat1-2-1 intergenic spacer (ApMat), beta-tubulin (TUB2) gene, chitin synthase (CHS-1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and inter transcribed spacer (ITS) region were amplified using the primers as given in Table 1. The isolated genomic DNA was subjected to PCR using 25 µl 2X PCR Master Mix (Emerald Amp® MAX, DSS Takara Bio Inc.), 1 µl of each primer (10mM), 2 µl of DNA template, and the final volume were adjusted to 50 µl using sterile distilled water (nuclease-free). The PCR amplification cycle followed is shown in Table 1. PCR products were separated by gel electrophoresis in an

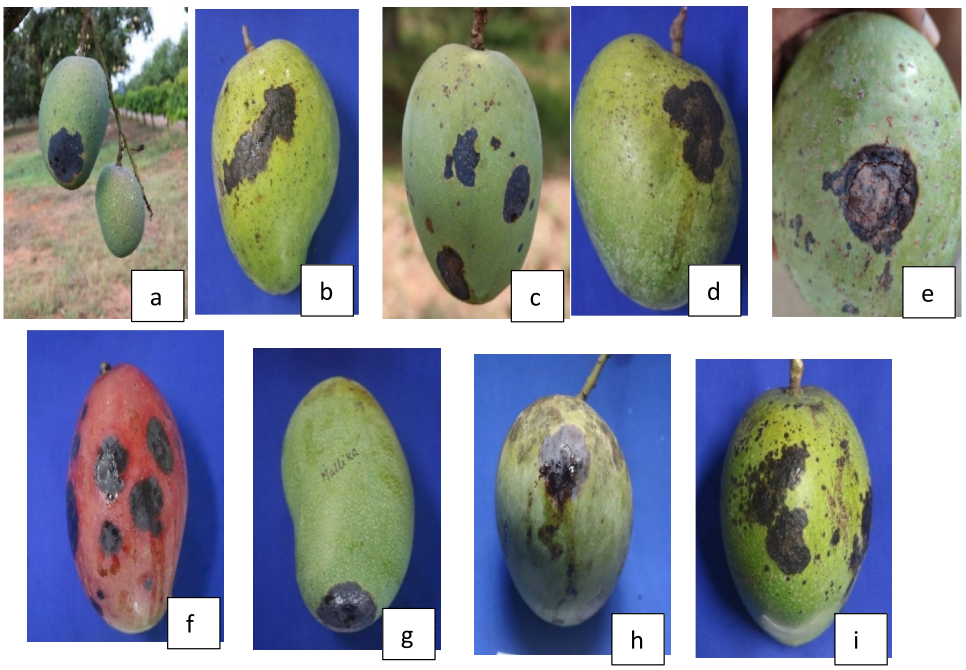

Fig. 2 Symptoms of pre-harvest anthracnose on var. Arka Aruna (**a**), Swarna Jahangir (**b**), Pusa Surya (**c**), Au-Rumani (**d**), Banganpalli (**e**), Lat Sundari (**f**), Mallika (**g**), Neelum (**h**), Rajapuri (**i**)

Table 1 List of primers along with the PCR conditions used in the present study

Gene	Primer Name	Primer Sequence (5'-3')	PCR conditions	Reference
Apn2/Mat1-2-1 intergenic spacer (ApMat)	AM-F	TCATTCTACGTATGTGCCCG	94 °C: 3 m 94 °C: 45 s 62 °C: 45 s × 30 72 °C: 1 m 72 °C: 7 m	Silva et al. (2012)
	AM-R	CCAGAAATACACCGAACTTGC		
beta-tubulin (TUB2) gene	Bt2b	GGTAACCAAATCGGTGCTGCT TTC	94 °C: 4 m 94 °C: 15 s 65 °C: 40 s × 35 72 °C: 1 m 72 °C: 5 m	Glass and Donaldson (1995)
	Bt2a	ACCCTCAGTGTAGTGACCCTT GGC		
Chitin synthase (CHS-1)	CHS-79 F	TGGGGCAAGGATGCTTGGAAG AAG	94 °C: 3 m 94 °C: 45 s 62 °C: 45 s × 30 72 °C: 1 m 72 °C: 7 m	Carbone and Kohn (1999)
	CHS-345R	TGGAAGAACCATCTGTGAGAG TTG		
glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GDF	GCCGTCAACGACCCCTTC ATTGA	94 °C: 4 m 94 °C: 15 s 60 °C: 30 s × 35 72 °C: 40 s 72 °C: 5 m	Templeton et al. (1992)
	GDR	GGGTGGAGTCGTACTTGAGCA TGT		
Ribosomal RNA (rRNA) gene	ITS1	TCCGTAGGTGAACCTGCGG	94 °C: 4 m 94 °C: 15 s 52 °C: 40 s × 35 72 °C: 1 m 72 °C: 5 m	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC		

agarose gel (1.2%) stained with ethidium bromide (EtBr), viewed and photographed in the Gel Documentation system (Vilber, Marne-la-Vallée, France). The target amplicon was eluted from gel using Gel Extraction Kit (QIAquick from Qiagen India Pvt Ltd, New Delhi, India) following the manufacturer's instructions. Subsequently sequencing for amplified regions of respective genes were performed with 10 picomoles of each primer in both directions for the identification of isolates by Sanger sequencing method (Medauxin sequencing services). The resultant sequences were edited, and assembled with the Bio Edit software V.7.0.9.0) (Hall 1999) and the obtained sequences were subjected to nBLAST analysis. The forward and reverse sequences were assembled and final consensus sequences thus obtained were submitted in NCBI database and the (http://www.ncbi.nlm.nih.gov) accession numbers were obtained. Table 2 provides the gene sequences of list of type strains that belong to the *Colletotrichum* species complex used for phylogenetic analysis.

Single locus and multi-locus phylogenetic analysis

For each of the five loci, respective sequences of reference strains of *Colletotrichum* species were retrieved from the NCBI GenBank database via Basic Local Alignment Search Tool (BLAST) (Altschul 1990) and included in this study for phylogenetic analysis (Table 2). First phylogenetic trees were constructed for individual gene sequences. Subsequently, the concatenated gene sequences for all 5 genes viz., ApMat, TUB2, CHS-1, GAPDH, and ITS, sequenced of present 10 isolates as well as other selected references sequences given in Table 2 were generated using software Sequence Matrix version 1.8 (Vaidya et al. 2011). The concatenated sequences of the present 10 isolates as well as other reference sequences were aligned using software MEGA X (Kumar et al. 2018). All the regions that were unambiguously aligned were removed from the analysis and gaps were regarded as missing data. To get the maximum parsimonious tree, analysis was performed through the software PAUP 4.0 (Phylogenetic Analysis using Parsimony v. 4.0b10) (Swofford 2002). The bootstrap replication was kept at 1000 and all the tree statistics viz., tree length, CI (consistency index), RI (retention index), RC (rescaled consistency index), and HI (homoplasy index) were calculated. The phylogenetic tree was constructed using the neighbour joining method and was rooted through *Colletotrichum boninense* CBS123755.

Table 2 Accession numbers of gene sequences used in the present study

S. No.	Species	Isolate	Host	Country	ApMat	Btub	CH0S	GADPH	ITS
1.	*C. siamense*	GM473	*Mangifera indica*	India	JQ894553	JQ894592	JQ894607	JQ894622	JQ894673
2.	*C. siamense*	NK24, MU1	*Mangifera indica*		JQ894582	JQ894602	JQ894617	KU642501	KU642480
3.	*C. siamense*	GM172	*Mangifera indica*		JQ894562	JQ894591	JQ894606	JQ894621	JQ894662
4.	*C. siamense*	GM390	*Mangifera indica*		JQ894570	JQ894597	JQ894611	JQ894627	JQ894670
5.	*C. aenigma*	ICMP_18608	*Persea americana*	Israel	KM360143	JX010390	JX009774	JX010044	JX010244
6.	*C. aenigma*	ICMP 18686	*Pyrus pyrifolia*	Japan	MG717319	JX010389	MN525845	JX009913	JX010243
7.	*C. alienum*	ICMP 12071, LF322	*Malus domestica*	New Zealand, China	KJ954545	KJ955279	JX009882	JX010028	KJ955131
8.	*C. fructicola*	LF132, UMC006	*Camellia sinensis*	China	KJ954501	KJ955234	MW091991	KJ954786	KJ955085
9.	*C. fructicola*	LF133, B03_43034	*Camellia sinensis*	China	KJ954502	KJ955235	MW091983	KJ954787	KJ955086
10.	*C. musae*	CBS:116870	*Musa* sp.	USA	KC888926	JX010413	JX009896	JX010050	JX010146
11.	*C. musae*	ICMP17817, LC0872	*Musa* sp.	Kenya	JQ899270	HQ596280	JX009896	JX010050	JX010142
12.	*C. aeschynomenes*	T19_1812, 3-1-1, COL02	*Shorea siamensis*	Thailand	MK224880	JX010392	JX009799	MK792457	OP27897
13.	*C. aeschynomenes*	ICMP_17673, 578F15F_AM, 98_359A	*Aeschynomene virginica*	USA	KM360145	MK225115	MN964926	JX009930	JX010176
14.	*C. artocarpicola*	1-Mandarin, UMC001	*Citrus reticulata*	Australia	MG572170	MG572148	MW091986	MG572126	MG572137
15.	*C. artocarpicola*	2-Orange, UMC002	*Citrus sinensis*	Australia	MG572171	MG572149	MW091987	MG572127	MG572138
16.	*C. australianum*	BRIP-63698, AUS19	*Capsicum annuum*	Australia	KU923730	KU923696	MW092001	MN442116	KU923680
17.	*C. australianum*	BRIP_63699, AUS20	*Capsicum annuum*	Australia	KU923731	KU923697	MW092002	MN442117	KU923681
18.	*C. queenslandicum*	ICMP:1778, LM104	*Carica papaya*	Australia	KC888928	JX010414	JX009899	JX009934	JX010276
19.	*C. queenslandicum*	ICMP18705, CMM323, C956.1	*Coffea* sp. *Anacardium occidental*	Fiji, Brazil	MF110639	JX010412	JX009890	JX010036	JX010185
20.	*C. asianum*	GM595, MTCC 11680	*Mangifera indica*	India	JQ894554	JQ894601	JQ894616	JQ894623	JQ894679
21.	*C. asianum*	ICMP 18580, CBS 130418	*Coffea arabica*	Thailand	FR718814	JX010406	JX009867	JX010053	FJ972612
22.	*C. tropicale*	CBS:124949	*Theobroma cacao*	Panama	KC790728	JX010407	JX009870	JX010007	MH863435
23.	*C. tropicale*	GC3	Grape cv. Black Queen	Taiwan	MT648529	MT648526	MW684717	MT648519	MT555315
24.	*C. syzygiicola*	WC27	*Syzygium samarangense*	Taiwan	ON745310	ON745312	ON745311	ON646460	ON629752
25.	*C. syzygiicola*	DNCL021	*Syzygium samarangense*	Thailand	-	KF254880	-	KF242156	KF242094
26.	*C. gloeosporioides*	LF318, UMC013	*Camellia sinensis*	China	KJ954541	KJ955275	MW091998	KJ954828	KJ955127
27.	*C. gloeosporioides*	LF534, UMC011	*Camellia sinensis*	China	KJ954569	KJ955305	MW091996	KJ954859	KJ955158
28.	*C. alatae*	ICMP:17919	*Dioscorea alata*	India	KC888932	JX010383	JX009837	JX009990	JX010190
29.	*C. alatae*	ICMP 18122	*Dioscorea alata*	Nigeria		JX010449	JX009846	JX010011	JX010191
30.	*C. aotearoa*	ICMP:18532	*Vitex lucens*	New Zealand	-	JX010421	JX009764	JX009906	JX010220
31.	*C. aotearoa*	ICMP:18537	*Coprosma* sp.	New Zealand	KC888930	JX010420	JX009853	JX010005	JX010205
32.	*C. camelliae*	ICMP:18542, LF899	*Camellia sasanqua*	USA	KJ954627	JX010429	JX009857	JX009994	JX010223
33.	*C. camelliae*	LF207, CGMCC 3.14925	*Camellia sinensis*	China	KJ954511	KJ955244	MZ799255	KJ954796	KJ955095
34.	*C. clidemiae*	ICMP:18658	*Clidemia hirta*	USA	KC888929	JX010438	JX009877	JX009989	JX010265

Table 2 (continued)

S. No.	Species	Isolate	Host	Country	ApMat	Btub	CH0S	GADPH	ITS
35.	*C. clidemiae*	ICMP:18706	*Clidemia hirta*	USA	-	JX010439	JX009777	JX009909	JX010274
36.	*C. jiangxiense*	LF488, CGMCC 3.17361	*Camellia sinensis*	China	KJ954561	OK236389	MZ799257	KJ954850	KJ955149
37.	*C. jiangxiense*	LF687, KUMCC 21–0466	*Camellia sinensis*	China	KJ954607	KJ955348	OM744410	KJ954902	KJ955201
38.	*C. henanense*	LF238, CGMCC 3.17354	*Camellia sinensis*	China	KJ954524	KJ955257	MZ799256	KJ954810	KJ955109
39.	*C. henanense*	LF24 CMF043	*Cirsium japonicum*	China	KM610174	KM610184	MN685852	KM610178	KM610182
40.	*C. siamense*[a]	CHES2020-CS-AA	*Mangifera indica*	India	OP381818	OP174948	OP174932	OP174940	MZ491825
41.	*C. siamense*[a]	CHES2020-CS-AN	*Mangifera indica*	India	OP174954	OP174946	OP174930	OP174938	MZ491826
42.	*C. siamense*[a]	CHES2020-CS-MA	*Mangifera indica*	India	OP174958	OP174951	OP174935	OP174943	MZ491829
43.	*C. siamense*[a]	CHES2020-CS-MB	*Mangifera indica*	India	OP174959	OP174952	OP174936	OP174944	MZ491828
44.	*C. siamense*[a]	CHES2020-CS-SS	*Mangifera indica*	India	OQ732753	OP174953	OP174937	OP174945	MZ491830
45.	*C. siamense*[a]	CHES2020-CS-SIN	*Mangifera indica*	India	OP174957	OP174950	OP174934	OP174942	ON945555
46.	*C. siamense*[a]	CHES2020-CS-TP	*Mangifera indica*	India	OP174955	OP174947	OP174931	OP174939	MZ491827
47.	*C. siamense*[a]	CHES2020-CS-MM	*Mangifera indica*	India	OP174956	OP174949	OP174933	OP174941	MZ491824
48.	*C. siamense*[a]	MBCO	*Mangifera indica*	India	MW116160	MW116164	MW116162	MW093446	MW093446
49.	*C. siamense*[a]	RP6	*Mangifera indica*	India	MW116161	MW116165	MW116163	MW093514	MW093514

[a]Isolates used in the study

Pathogenicity evaluation on intact green mangoes under field condition

Field experimentation was commenced in March 2018 at CHES farm on 15-year-old Arka Anmol and Mallika trees. Fruits of cricket ball-sized stage (6–10 cm) on were selected. One representative isolates CHES2020-CS-AA from pre-harvest anthracnose symptoms of Arka Anmol and RP6 from post-harvest anthracnose symptoms collected from the local market taken for pathogenicity study. No chemical treatment was applied to the fruit during the entire season. Ten trees were selected for study and 10 fruit per tree were selected randomly, ensuring that mangoes were free of any apparent marks or disease and were tagged. On each tree, a control fruit was tagged, giving a total of 10 control fruit. Fruits were pinpricked on the shoulder area and an 8 mm diameter mycelial plug of each isolate was placed on the wounds covered with moist sterile absorbent cotton and sealed with parafilm to ensure that the plug should not fall as the fruits were hanging on the tree. Each fungal isolate was inoculated onto randomly chosen five fruits on all 10 trees. Control fruits were inoculated with a sterile PDA plug. Fruit was enclosed for 48 h in a polypropylene bag and plastic bag with both ends tied. After 48 h the bottom end was untied however the bags were not removed till the experimentation. Fruits were assessed for visible lesions, and the lesion was measured after a week of inoculation and at weekly intervals up to 6 weeks of inoculation. Fungi were re-isolated from the lesions and reidentified to verify the Koch's postulates. The experiment was repeated twice.

Pathogenicity evaluation on detached (ripening) mango fruits under laboratory condition

Physiologically matured, unblemished fruits, de-sapped mango fruits were collected from the experimental orchard and ensured that these mangoes were not sprayed with any fungicides during maturation. The fruits were washed under running tap water for 2 mins followed by surface sterilization with 70% ethanol and rinsed with sterile distilled water to inoculation. The same representative isolates CHES2020-CS-AA and RP6 were taken for a pathogenicity study on detached ripening fruits to determine the pathogenicity. The samples were inoculated using the wound inoculation method (Lin et al. 2002) which included a 3 mm depth pin-prick using a sterile needle on the mid portion of

fruit including the control fruits. Then the fruits were inoculated at the wounded portion with a 5 mm mycelial disc taken from the actively growing region of seven-day-old culture. Fruits inoculated with sterile agar plug served as the negative control. The inoculated set of fruits were placed in plastic containers lined with paper towels wetted with sterile distilled water to maintain a high humidity necessary for infection and arrangements were made to avoid direct contact of fruits with water and were removed out of containers after 48 h of inoculation and maintained at room temperature (28 ± 2 °C) and lesion development was monitored periodically. The experiments were arranged in a completely randomized design with three replications containing four fruits for each fungal isolate. The experiment was repeated twice. Disease assessments were done at 7 DAI depending on the presence of visible anthracnose lesions and by measuring the lesion size. Fungi were re-isolated from the lesions and reidentified to verify the Koch's postulates.

Results

Symptomatology and incidence of pre-harvest anthracnose on different mango varieties

New kind of anthracnose symptoms were observed on green intact mango fruits on the trees in contrast to usual post-harvest anthracnose wherein anthracnose spots develop on fruits during its ripening phase. Even though disease incidence varies with varieties, these dry anthracnose symptoms were observed in several varieties grown in our germplasm block, which includes many commercial varieties. In a few susceptible varieties, symptoms appear in all stages of fruit development starting from the pea stage, lemon stage, cricket ball stage, and mature fruits with varied levels of incidence and severity. Pre-harvest dry anthracnose caused 1 mm to several centimeter wide black, big elliptical, round to irregular patches on the skin leading to the typical spreading lesion and eventual cracking of infected fruits. Lesions on fruits produced orange, conspicuous, spore masses in a concentric ring pattern with gum-like exudates in dry conditions. The lesions were mostly confined to the rind only with 1–3 mm brownish lesion touching the resin canal however not extending to a pulp. However, when fruits were harvested and kept in room condition, the lesions exhibited during the field stage started extending to pulp to cause a maximum of up to 1.0–3.0 cm deep lesions but not further. The preharvest dry anthracnose spots are raised, flaky, and dry in contrast to sunken, wet with slightly raised edges of lesions on ripe fruits. Nonetheless, even superficial disease development resulted in serious aesthetic damage and rejection of fruit along the marketing chain. Different stages of pre-harvest dry anthracnose in the highly susceptible var. Arka Anmol and Mahmood Bahar occurred at natural field conditions has been elaborated in Table 3 and depicted in Fig. 1. The incidence and susceptibility levels of various mango cultivars grown in our experimental farm are listed in Table 4 and depicted in Fig. 2. During our study, the incidence of pre-harvest anthracnose symptoms was observed only on a few non-commercial varieties during 2013 with the incidence of up to 45% in highly susceptible cultivars, but slowly it was observed on noted commercial varieties such as Deshahari, Langra and Totapuri (Banglora) up to 5–10% incidence (Table 4).

Fungal isolation and morphological characterization

A total of 8 fungal isolates were isolated from symptomatic green fruits belonging to different varieties showing pre-harvest anthracnose (7 isolates from our experimental orchard, one from a farmer's field) and two isolates from ripe fruits showing post-harvest anthracnose symptoms under market condition (var. Amrapali and Kesar) were taken for study. The uniform colony emerged out of infected anthracnose spots from pre-harvest dry anthracnose is depicted in Fig. 3a, b. The isolates showed little difference according to the colony characteristics like colony color texture, and pattern of sporulation. Colonies produced from isolates varied in color from off-white to dull pink with dense, cottony mycelium, and salmon orange conidial masses around the inoculation point, black acervuli were observed to be randomly distributed on culture plates. On average, the growth rates were varying from 9.61 to 13.05 mm per day. Length and width of conidia were recorded and the details are presented in Table 5. Conidia were all hyaline, cylindrical, one-celled, guttulate, and fusiform with obtuse ends with an average length of 14.74–18.44 µm and width of 4.53–5.91 µm (Fig. 3c). Zhang et al. (2020) studied the morphological characteristics of *C. siamense* infecting straw berry and reported the average length and width of conidia as 13.9 to 19.1 µm and 4.7 to 6.5 µm respectively wherein the conidial morphology is almost in agreement with our present study.

Single and multigene-based phylogenetic analysis

All the 5 genes viz., ITS, GAPDH, CHS-1 TUB2 and ApMat of present 10 isolates were successfully amplified and sequenced. All 5 regions when used individually to construct the phylogenetic tree resulted in topographically different trees. Moreover, individual genes did not result in a robust phylogenetic tree with reliable species delimitation. Different isolates of the same *Colletotrichum* species clustered in different clades. The BLAST analysis of individual genes indicated that all the present isolates belong to the *C. gloeosporoides* species complex. ITS sequences of these isolates

Table 3 Incidence of preharvest dry anthracnose at different stages of fruit development in the highly susceptible var. Arka Anmol under natural field condition in the state of Odisha located in Eastern India

Critical Stages	Infection % in highly susceptible cultivar Arka Anmol
Pea stage	Common on 1–5% of fruits
Lemon size / Fruits at 30% of final size.	Rarely infected
Cricket ball size/ fruits at 50% of final size.	Common on 1–5% of fruits
Fruit at standard cultivar size, shoulders fully developed flesh creamy green colour	Common on 25% of fruits
Fruit fully developed, flesh pale yellow colour	Infection common on 25% of fruits

showed more than 99% identity with the available sequences of *C. aenigma, C. siamense*, *C. gloeosporoides, C. fructicola*, and *C. queenslandicum*; CHS-1 sequences showed 98–100% similarity with the sequences of *C. fructicola* and *C. siamense*; GAPDH, TUB2 and ApMat sequences showed 99–100% identity with the sequences of *C. siamense*. The single gene phylogenetic trees are shown in Figs. S1–S5.

Multi-locus phylogenetic analysis was conducted among 51 strains including 10 present isolates and *C. boninense* as out-group (*C. boninense* CBS123755) (Table 1). The concatenated sequences for all 5 genes included 3141 positions. The gene boundaries in the multigene sequence dataset included ApMat: 1-1061; BTUB: 1062-1854; CHS: 1855-2163; GADPH: 2164-2460; and ITS: 2461-3141. Of all the characters, 1157 characters were constant, 440 variable characters were parsimony uninformative, and 1106 characters were parsimony informative. This parsimony analysis resulted in the most parsimonious tree presented in Fig. 4 (TL: 2762, CI: 0.730, HI: 0.270, RI: 0.938, RC: 0.685). All the present isolates were phylogenetically similar and clustered with *C. siamense* isolates viz., GM473, MU1, GM172, and GM390 derived from Mango, and thus were identified as *C. siamense*. The phylogenetic tree depicted in Fig. 1 differentiated the species of *Colletotrichum* genus very clearly which were included in the analysis. According to the morphological characterizations and phylogenetic analyses, the causal agent of pre-harvest as well as post-harvest anthracnose infecting *Mangifera indica* from Eastern India was identified as *C. siamense*.

Pathogenicity evaluation on intact green mangoes under field condition

Both tested isolates could infect intact green mangoes inoculated under field conditions as well as detached ripe mangoes inoculated under laboratory conditions. Similarly, both the isolates successfully produced dark, prominent, black lesions on all three inoculated fruits which were on the tree (Fig. 5). The fruits began to show obvious symptoms after a week of inoculation under field conditions. On and near the inoculation point, a near-circular blackish necrotic lesion was observed on the skin (Fig. 5). The rind of wounded points was rotted severely, followed by spread to the surrounding area were also rapidly infected and became necrotic black brown. The lesion became sub-circular to irregular measuring 1–2-inch diameter and taking different shape during the phase of infection (Fig. 6). However, the fruits did not fall from the tree. The fruits inoculated with non-colonized sterile agar plugs showed no symptoms. Koch's postulates were verified by reisolating the same fungal isolates from the symptomatic fruits of mango and confirming their identification through morphological characteristics.

Table 4 Response of various mango cultivars to preharvest dry anthracnose under field condition in the state of Odisha located in Eastern India

Cultivars	Incidence (%)[a]
Alfazli, Arka Aruna, Arka Puneet, Au Rumani, Mallika, Neeleshan, Neeleshan Gujarat, Neelgoa, Sabri, Swarna Jehangir, Navneetham, Ambika, Arunika (H39), H949, H1084, H1739, Pusa Arunima, Latsundari, Bombay Green, Chausa, Janardan Pasand, Kesar, Niranjan, Zardalu, Rumani, Hamilton Sundari	0 (Nil incidence)
Alphanso, Arka Neelachal Kesri, Bombay green, Himsagar, Manjeera, Neeleswari, PKM-1, PKM-2 Rajapuri, Ratna, Sindhu, Sundar Langra, Swarna Rekha, Banganpalli, Gulabkhas, Neelum	< 5
Arka Neelkiran, Dashehari, Langra, Neelphanso, Prabha Shankar, Totapuri (Bangalora)	5–10
Sai Sugandh, Mahmood Bahar, Neeludin	10–25
Arka Anmol	> 25

[a]Sample size 250 fruits counted from 10 trees covering all four directions as well as from up, middle and lower level of tree canopy

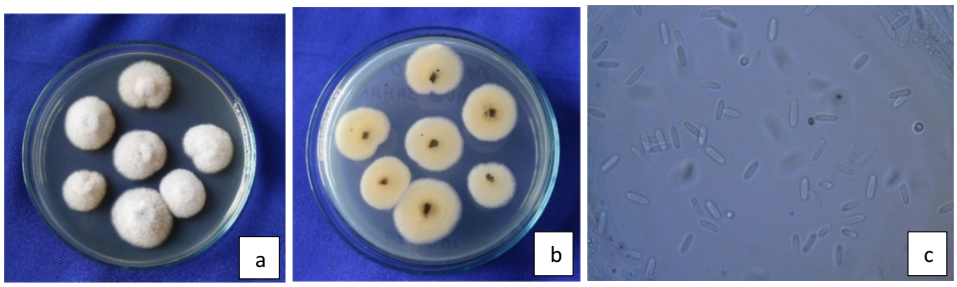

Fig. 3 *Colletotrichum* species isolated from infected fruit tissues from preharvest anthracnose symptoms of var. Arka Anmol (**a**, **b**) spores of *C. siamense* isolated from var. Arka Anmol (**c**)

Pathogenicity evaluation on detached ripening mango fruits under laboratory condition

Two representative isolates namely CHES2020-CS-AA and RP6 were inoculated on detached mature mangoes of Var. Arka Anmol and Mallika. The dark, sunken, and black lesions started appearing at the site of inoculation five days after inoculation, it progressed further and developed as big lesions of 2–5-inch diameter in var. Arka Anmol as well as Mallika. The fruits of Arka Anmol inoculated with isolates CHES2020-CS-AA and RP6 have been depicted in the picture (Fig. 7). The fruits of control groups inoculated with non-colonized sterile agar plugs showed no symptoms. The morphological characteristics of fungus obtained from the lesions on inoculated fruits of mango matched those of the inoculated fungal isolate, thusconfirming Koch's postulates.

Discussion

Anthracnose stands as a significant threat to the production of quality mango fruits in almost all mango-producing nations globally. Even though, on fruits, the post-harvest phase of anthracnose is much more prevalent, the pre-harvest anthracnose phase has not been reported as a common phenomenon. Nelson (2008), documented the occurrence of "tear stain" symptoms on fruits intact on the tree in Hawaii leading to the "alligator skin" effect and even causing fruits to develop wide, deep cracks in the epidermis that extend into the pulp. In Australia, the occurrence of tear stains on green mango referred to as pepper spots, caused by *C. gloeosporioides* has been described (Giblin et al. 2010). Apart from this, there were no reports available in the Web of Science about the occurrence of anthracnose on green

Table 5 Morphological characterization and growth rate of *C. siamense* isolates from pre as well as post-harvest anthracnose infecting mango

S. No	Stage of anthracnose	Mango variety	Isolate	Growth rate[a] (mm/day)	Conidia[b]	
					Length (µm)	Width (µm)
1	Pre-harvest	Arka Anmol	CHES2020-CS-AA	(9.50–15.50) 12.50 ± 0.52	(12.50–19.50) 15.01 ± 0.63	(4.10–5.9) 4.67 ± 0.18
2		Arka Neelkiran	CHES2020-CS-AN	(11.00–14.00) 12.83 ± 0.31	(13.60–19.50) 15.26 ± 0.56	(4.15–7.20) 5.78 ± 0.24
3		Manjeera	CHES2020-CS-MA	(12.50–14.50) 13.05 ± 0.24	(15.10–20.90) 18.44 ± 0.76	(4.13–6.90) 5.91 ± 0.29
4		Mahamood Bahar	CHES2020-CS-MB	(9.00-12.50) 10.82 ± 0.34	(12.50–19.40) 15.18 ± 1.02	(4.50-6.00) 5.20 ± 0.21
5		Sai Sugandh	CHES2020-CS-SS	(11.30–13.00) 12.26 ± 0.20	(14.50–22.90) 17.24 ± 1.22	(4.10–5.20) 4.53 ± 0.13
6		Sindu	CHES2020-CS-SIN	(8.00-12.50) 10.51 ± 0.49	(13.00-22.70) 16.73 ± 1.22	(4.15–6.20) 5.62 ± 0.20
7		Totapuri	CHES2020-CS-TP	(1.90–15.20) 12.61 ± 1.24	(14.50–18.50) 15.92 ± 0.40	(0.60–6.90) 5.25 ± 0.57
8		Mallika	MBCO	(8.00-11.50) 9.61 ± 0.33	(12.00-18.50) 14.85 ± 0.70	(5.10-7.00) 5.68 ± 0.17
9	Post-harvest	Mallika	CHES2020-CS-MM	(9.60–12.50) 10.48 ± 0.26	(13.50–17.20) 14.74 ± 0.43	(5.00-6.60) 5.67 ± 0.20
10		Amrapali	RP6	(9.00–15.00) 11.52 ± 0.59	(14.00-18.70) 15.21 ± 0.41	(4.50–7.10) 5.33 ± 0.31

[a]The colony diameter data were used to calculate the mycelial growth rate (mm/day) and colony diameter (mm) was measured in two perpendicular directions

[b]The length and width of 30 conidia per isolate were measured after a week of incubation at 30 °C

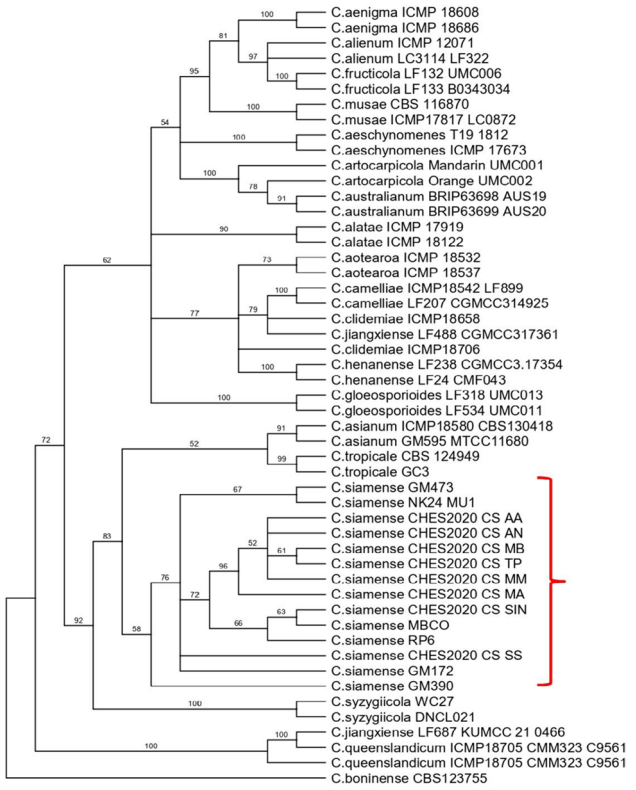

Fig. 4 Phylogenetic tree generated from maximum parsimony analysis based on alignment of ApMat, TUB2, GADPH, CHS-1 and ITS sequences. The tree is rooted with *C. boninense* CBS123755

maturing mangoes before harvest. Instead of pepper spots or tear stain symptoms as described by earlier workers. In our study, on single green fruit, we observed either one single large circular anthracnose lesions of several centimeters in size with concentrically arranged salmon orange-colored fruiting bodies of the fungi or otherwise many isolated smaller individual lesions with or without fungal fruiting bodies. Further, these anthracnose lesions were dry, hard, flaky confined to the rind, and not able to reach the fruit pulp until the fruit started ripening. As per the general perception of scientists, on green mango fruits, conidia of *Colletorichum* often remain latent and imperceptible until ripening, however, this fact has to be re-visited in days to come. This above scenario necessitated us to conduct a detailed investigation on the etiology of pre-harvest anthracnose occurring on green fruits of different mango varieties in comparison with a known post-harvest phase of anthracnose.

Across the world, the involvement of several *Colletotrichum* species causing anthracnose on ripened mango fruits has been documented by several researchers. *Colletotrichum* isolates causing anthracnose disease generally consist of pathogenically and genetically different populations of *C. gloeosporioides* which were studied by many researchers across the world (Hodson et al. 1993; Alahakoon et al. 1994; Hayden et al. 1994). Johnston et al. (2010) reported the cryptic nature of the *Colletotrichum* species complex in which the species are morphologically similar but genetically different. In India, studies based on either morphology and or ITS gene-sequence data reported *C. gloeosporioides* as a causal agent of mango anthracnose (Kumar et al. 2017; Sangeetha and Rawal 2009; Gupta et al. 2010; Lakshmi et al. 2011). Chowdappa and Mohan Kumar (2012) from India reported that as *C. gloeosporioides* isolates infecting mango varied in their level of virulence during pathogenicity

Fig. 5 Pathogenicity evaluation of *Colletotrichum* isolates under field condition on variety Arka Anmol

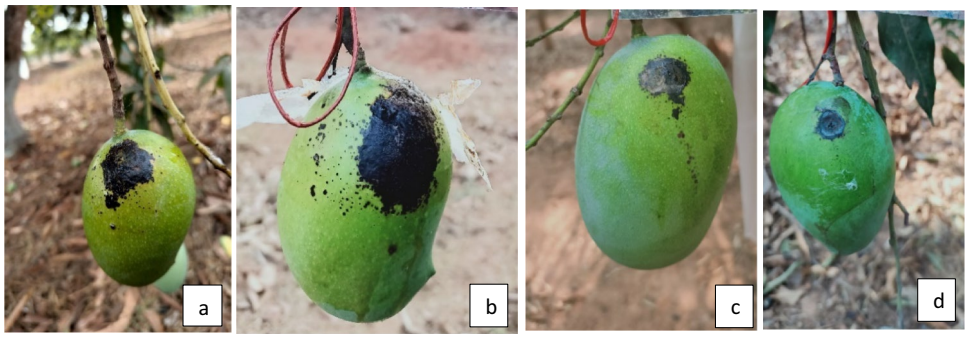

Fig. 6 Field inoculation of CHES2020-CS-AA and RP6 isolates on mango varieties Arka Anmol (**a**, **b**) and on var. Mallika (**c**, **d**)

testing, hence authors opined that the disease may be caused by more than one species. Worldwide most records post-2008 related to mango anthracnose pathogen were based on morphological identification or similarity with the non-type sequences submitted to GenBank (Cai et al. 2011). The necessity to re-examine the occurrence of *C. gloeosporioides sensu lato* on tropical fruits was emphasized by Phoulivong et al. (2010). Hence, several studies which re-examined the *Colletotrichum* species associated with mango anthracnose and other crops during the past decade were based on multigene phylogeny given the resolution of *C. gloeosporioides sensu lato* into several cryptic species (Weir et al. 2012; Jayawardena et al. 2016).

Our investigation led to the identification of *C. siamense* as a causative agent for both pre as well as post-harvest anthracnose phases of mango fruits based on morphological characteristics of the pathogen, pathogenicity study as well as multigene phylogenetic analysis. It ruled out which ruled out our curiosity about the involvement of additional new species of *Colletotrichum* which might have had the ability to cause the pre-harvest phase of anthracnose. However, the association of *C. siamense* on mango has been reported in mango-growing countries of the world including India (Sharma et al. 2013), China (Liu et al. 2017; Qin et al. 2017), Mexico (Tovar-Pedraza et al. 2020), Taiwan (Wu et al. 2020) and Thailand (Rattanakreetakul et al. 2023). In India, four known species of *Colletotrichum* namely *C. frgariae sensu stricto, C. fructicola, C. jasmine-sambac* and *C. melanocaulon* and five *Colletotrichum* lineages (with no species names) were found to be associated with mango tissues/ anthracnose (Sharma et al. 2013). Involvement of several other *Colletotrichum* species namely, *C. asianum, C. alienum, C. cliviicola, C. cordylinicola, C. endophytica, C. fructicola, C. fioriniae, C. gloeosporioides, C. grossum, C. gigasporum, C. karsti, C. liaoningense, C. plurivorum, C. musae, C. queenslandicum, C. scovillei, C. simmondsii, C. tropicale* and *C. theobromicola* has also been reported to be associated with mango anthracnose by number researchers around the world (Liu et al. 2013; Lima et al. 2013; Ismail et al. 2015; Pardo-De la Hoz et al. 2016; Qin et al. 2019; Manzano León et al. 2018; Li et al. 2019; Vitale et al. 2020; Tovar-Pedraza et al. 2020; Alvarez et al. 2020).

Apart from mango, *C. siamense* has been reported to infect many hosts growing in tropical and sub-tropical regions of the world (Prihastuti et al. 2009; Phoulivong et al. 2010, Weir et al. 2012, Liu et al. 2013), even though it was first described on *Coffea arabica*. (Prihastuti et al. 2009. In India, *C. siamense* has been reported to be associated with other host crops namely Chilli (Sharma and Shenoy 2013), Cliff Banana (Kumar et al. 2017), *Pongamia pinnata* (Dwarka et al. 2016), Cotton (Salunkhe et al. 2020) and elephant foot yam (Prasad et al. 2017).

Our pathogenicity study at the field and laboratory demonstrated that *C. siamense* isolated from the pre-harvest anthracnose phase of mango has the potential to induce both pre-harvest as well as post-harvest anthracnose

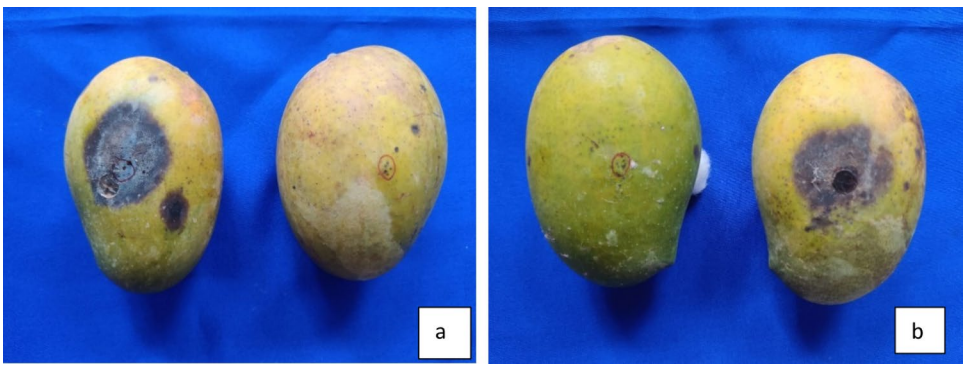

Fig. 7 Pathogenicity evaluation of isolates CHES2020-CS-AA (**a**) and RP6 (**b**) on detached fruits of var. Arka Anmol under laboratory condition

symptoms on green maturing as well as ripened mango respectively. Similarly, *C. siamense* isolated from the post-harvest anthracnose phase of mango has the potential to induce both post-harvest as well as pre-harvest anthracnose symptoms on ripened mango as well as on green maturing mangoes respectively. This result necessitated us to re-verify the claim of the latent phase of *Colletotrichum* on green maturing mangoes.

As scientific community believes that the conidia of *Colletotrichum* remain dormant in green mangoes till fruits enter to ripening phase, as green fruits have a constitutive defence barrier, known as dienes (Prusky et al. 1982), anti-fungal resorcinols in fruit peel and latex (Hassan et al. 2007) and elaborate constitutive defence system comprising anti-fungal gallotannins, resorcinols in latex, and the enzyme chitinases (Karunanayake et al. 2011). However, as evident from our current study, *C. siamense* causing pre-harvest anthracnose symptoms on the peel of mango must have happened by breaking the defense barrier as listed by earlier researchers which shows the unparallel adaptation of *C. siamense* on green mango fruits by breaking the concept of preformed defense barriers. Additional studies are warranted for a better understanding of biochemical and physiological mechanisms that led to the establishment of preharvest anthracnose on green mangoes to tackle the emerging pre-harvest phase of mango anthracnose.

Conclusion

The identity of *Colletotrichum* associated especially with pre-harvest anthracnose rot of mango was confirmed by morphology, multigene phylogeny as well as by pathogenicity study conducted on intact green mango fruits at field level as well as on detached ripening mango fruits. Supplementary studies are warranted for a better understanding of the physiological machineries leading to pre-harvest anthracnose on green fruits. Further, with the advances in genomics and transcriptomics, there would be more new vistas in the exploration of the molecular mechanism of anthracnose occurring on green mango fruits where fungi could break the constitutive defense barrier.

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Declarations

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

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

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