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Original Research Paper

Etiology of mango fruit blackening caused by sooty blotch fungal complex in Eastern India

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ABSTRACT

New kind of superficial blackening caused by sooty blotch fungi was first noticed on mango (*Mangifera indica* L.) fruits in Odisha, Eastern India during late summers of 2015 and 2016. It emerged as a new threat to quality mango production since it affects the market value of the fruits drastically and its export potential. Hundred mango fruits from orchards in two locations were arbitrarily sampled and colonies of each mycelial type were counted on each mango fruits which revealed ramose type of mycelial colonies was more predominant. Representative colonies were isolated; cultures were purified and proved for its pathogenicity. Genetic identity was assigned through ITS-rDNA sequence analysis which revealed the association of four fungal genera such as *Pseudocercospora* sp., *Pallidocercospora* sp., *Zasmidium* sp. and *Passalora* sp. with sooty blotch of mango. Scanning electron microscopy studies confirmed the damage of waxy layer of fruits which led to shriveling of infected fruits in storage which highlights the significance of producing mangoes free from sooty blotch disease. Further investigations are required, particularly related to host-pathogen-weather interaction and spatiotemporal distribution across the major mango growing regions of India. This study established the association of sooty blotch fungal complex on mango for the first time in India.

Keywords: Eastern India, etiology, mango, sooty blotch disease

INTRODUCTION

Sooty blotch is a menace in number of fruit crops particularly in humid regions worldwide which is caused by group of epiphytic fungi (Gleason et al., 2011). The term "sooty blotch" designated to the fungi causing pigmented mycelial mat with or without sclerotium-like structures on surface of fruits or plant parts. These ectophytic fungi colonize the host's surface and lodge themselves in the waxy cuticle without damaging the host's epidermal cells (Gleason et al., 2019). Since, the mycelial growth, fruiting and survival structures were darkly coloured, signs of sooty blotch appear as black smudgy growth on colonized fruits. Subsequently, the affected fruits lost their visual appeal and therefore, the saleability of fruits was greatly reduced even though no adverse impact on pulp quality. Sooty blotch fly speck is a major production constraint in apple as well and led to drastic reduction in market value to the maximum extent of 90 per cent which in turn reported to cause severe economic loss for growers (Johnson et al., 1997; Willamson & Sutton, 2000).

SBFS fungi produce a varied range of colony morphology/ mycelial types on fruit skin. At present, the recognized mycelial types of SBFS include punctate, ramose, fuliginous, speck, discrete, compact speck, ridged honeycomb, flyspeck and fleck (Gleason et al., 2011). In apple, initially sooty blotch sign was thought of caused by *Gloeodes pomigena* (Colby, 1920) and fly speck by *Schizothyrium pomi* (Von Arx, 1959). But during late nineties, report emerged as three fungi, namely *Leptodonthium elatius*, *Peltaster fructicola* and *Geastrumia polystigmatis* causes sooty blotch (Johnson et al., 1997; Williamson & Sutton, 2000) and one fungal species *Schizothyrium pomi* causes flyspeck (Batzer et al., 2008). With the advent of molecular tools in combination with mycological methods, now worldwide the sooty blotch fly speck (SBFS) complex has extended to more than hundred named and putative fungal species as per a very recent review (Gleason et al., 2019).

SBFS on mango was previously reported in Europe (Perez-Martinez et al., 2009), China (Hongsanan et al., 2017), Japan (Ajitomi et al., 2017), and the United States of America (Gleason et al., 2011). Recently, two previously unknown *Peltaster*-like species were identified as casual fungi of SBFS on mango in Malaysia (Tham et al., 2023). Even though sooty blotch disease was known to occur on mango in India in recent years, it has been neglected with the presumption of sooty mould. Hence, objective of this present study was attempted to identify the key fungal genera involved in sooty blotch of mango based on sign, mycelial types, fungal morphology, pathogenicity and molecular characteristics.

MATERIALS AND METHODS

Experimental site

The present study was carried out in the mango orchards during consecutive mango fruiting seasons of 2015-2019. Two distant mango orchards with minimum of 3000 tree from each was taken for study in both the location. The first orchard is located in experimental farm of IIHR-Central Horticultural Experiment Station (20°142 17 N, 85°462 E, 25.5 m above MSL) in Bhubaneswar, coastal plains Khorda district of Odisha (Location 1) and second one at farmer's orchard (19^{o} 102 to 19^{o} 122 N, 83^{o} 012 to 83^{o} 012 E, 800 to 823 m above MSL) located in Rayagada, a southern district of Odisha state (Location -II). Location -I have 3500 mango tress and location II has 3000 mango trees. 'Location -I' have been characterized by hot and humid tropical climate with annual rainfall is 1628 mm. Most of the precipitation falls in the month of July averaging 401. Location II has been classified as tropical climate but the summers have a good amount of rainfall with annual rainfall (1527 mm). Most of the precipitation falls in the month of July, averaging 357 mm and more than 200 mm rain fall was received from the month June-September (Data accessed on 27-7-2021-https://en.climate-data.org/asia/india/odisha/rayagada-24333/).

Disease incidence, severity

Disease incidence was recorded from random 20 trees selected per location and the severity of disease was recorded in random 100 fruits as follows

Disease incidence (%) = Number of diseased fruits observed/total number of fruits observed x100.

Disease severity (percent disease index) = [Sum of individual ratings/total number units observed × maximum disease grade] ×100

Disease rating scale includes 0-5 scale where, 0 indicates healthy, no disease, 1 indicates 1-10 per cent fruit surface covered with sooty blotch, 2 indicates <11-25 per cent fruit surface covered with sooty blotch, 3 indicates <26-50 per cent fruit surface covered with sooty blotch, 4 indicates <51-75 per cent fruit surface covered with sooty blotch, 5 indicates > 75 per cent fruit surface covered with sooty blotch.

Symptomatology and mycelial types

Colony morphology of blotch infected fruits were examined for the difference in mycelial characters using ZEISS SteREO Discovery. V20 Motorized Stereo microscope and recorded.

Isolation

Fruits of mango var. Amrapali, Mallika and Totapuri displaying sooty blotch infection during survey were collected from both the orchards and brought to the laboratory in a sealed aseptic cover. Isolation was performed with thorough rinsing of infected fruits in running tap water for 10 mins followed by gentle swabbing of fruit surface having blotch colonies with sterile cotton swabs dipped in sterile water and surface sterilization step was skipped sooty blotch being the epiphyte and fruits were allowed to air dry. Then single knot-like fungal structures of mycelial growth (for fuliginous type) from each blotch colonies picked with a sterile scalpel and transferred to half-strength potato dextrose agar (PDA) and acidified water agar (15 g) of agar-agar in 1000 mL water with a post-autoclave addition of 40 drops of 50% lactic acid per liter (AWA) as mentioned by Ivanovic et al. (2010). All the plates were incubated at 28±2°C for 2 weeks and observed periodically for fungal growth and the representative fungal isolates exhibiting similar colony morphology were chosen and taken for further studies. The purified cultures were stored under -4°C for further use. During isolation, contaminating fast emerging microorganisms, mainly bacteria and fungi from genera *Curvularia, Penicillium, Alternaria, Cladosporium* etc. were eliminated based on its morphology. The fungal isolates which produced sooty blotch symptoms on fruits upon artificial inoculation were subjected to identification. The respective fungal isolates were grown on PDA by placing 8 mm

diameter mycelial plug of respective cultures from month-old colonies and incubated at 28± 2°C in an alternate cycle of dark and light for 30 days as they were very slow growing in nature. After one month of growth on PDA, the colony texture, colour, growth of each fungal colony was documented and observed under microscope (BX 53 Olympus make) for micromorphological characters.

Pathogenicity

Pathogenicity studies were performed under field condition as suggested by Batzer et al. (2014) with slight modifications during the months of May and June, favourable conditions were simulated under field condition by covering the whole productive mango tree with 50% shade net and soil surface were laid with paddy straw and it was sprayed with water on alternative days to maintain the favorable condition for the development of sooty blotch fungi inside the structure. The Koch's postulates were modified according to the need of epiphytic pathogens like sooty blotch (Díaz Arias et al., 2010). Surface-disinfested fruits were swabbed with one-month old fungal suspension (~5 × 10^5 infection units such as mycelial fragments and/ or conidia per ml adjusted with hematocytometer were added with 0.5% mango juice and 0.1% Tween 20) (n=6). For control, sterile distilled water added with 0.5% mango juice and 0.1% Tween 20 were used with no fungal suspension. Inoculated fruits were covered with polythene covers with holes for air circulation. After complete symptom expression the infested fruits were brought to lab and confirmed for mycelial morphology with original fungal isolate.

Molecular identification by ITS-rDNA sequence analysis and phylogeny

Representative fungal colonies (ISO 60, ISO 115, ISO 117, ISO 118, ISO 135) based on mycelial morphology were subjected to ITS-rRNA sequence analysis to assign the genetic identity. Genomic DNA was isolated from mycelial disc as per the protocol mentioned by Doyle & Doyle (1990). Further PCR amplifications were performed for 18s rDNA using universal fungal primer pairs ITS1/ITS4 (White et al., 1990). Each PCR reaction consists of total reaction volume of 25 µl and the reaction was performed in an Eppendorf Thermal Cycler (made in Germany) with reaction cycles of initial denaturation for 2 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and a final extension of 10 min at 72°C. Amplified PCR products were subsequently sent to sequencing (Eurofins Scientific India Pvt Ltd, Bengaluru) and the resulting forward and reverse sequences were aligned using Clustal-W (Thompson et al., 1994). Contigs were subjected to blast search at NCBI GenBank database against available nucleotide sequences (https://blast.ncbi.nlm.nih.gov) and the putative genus identity of each fungal isolate was assigned (Lim et al., 2019). All the sequences were submitted to GenBank and accession numbers were derived. Further, combined phylogenetic tree was constructed using Mega 7 software (Kumar et al., 2016) to understand the genetic relationship among the fungal genus infecting mango sooty blotch along with other strains of NCBI BLAST search.

Scanning electron microscopy

The blotch infected mango fruit peels were subjected to scanning electron microscopy (SEM) studies to assess the damage of sooty blotch infection in fruit surface under TM 3030 Plus tabletop microscope (Hitachi high Tech corporation).

RESULTS AND DISCUSSION

Incidence and severity of SBFS on mango

In this study, sooty blotch disease incidence on three different mango varieties with varying level of disease severity has been recorded in two different orchards for two successive years (Table 1 & Fig. 1). Results

Table 1 : Incidence and severity of sooty blotch disease on late maturing mango varieties

Year	2016				2017			
Variety	Location I		Location II		Location I		Location II	
	Incidence	Severity	Incidence	Severity	Incidence	Severity	Incidence	Severity
Amrapali	14	3.4	78	56.9	17	5.4	87	51.9
Mallika	10	2.1	32	11.6	7	2.1	22	9.6
Totapuri	7	1.1	13	2.6	5	1.6	16	3.3

Fig. 1 : Sooty blotch infected mango fruit

revealed that in both the years mango variety Amrapali recorded highest sooty blotch incidence (14% and 17% disease incidence in location I during 2016) and (17% and 87% in disease incidence in location II during 2017) in both the two locations surveyed. However, sooty blotch, incidence as well as severity was less in Totapuri compared to other two varieties (Table 1). This varying level of disease incidence and severity in different mango varieties might be due to difference in harvesting time and coincidence of mango maturity with rain. In general, 80% of fruits of Totapuri mango variety were harvested before onset of monsoon, hence it escaped from sooty blotch fungal colonization.

Present studies also recorded that comparatively more disease incidence and severity in location II compared to location I in both the year of study. In location I, the mango varieties which were harvested during peak summer days escaped from blotch infection and only those variety where maturity period got extended and coincided with rain by June end received the mild infection. Similarly, in Location II, the harvesting of said two varieties Amrapali and Mallika extended up to July to mid-august, where blotch severity has been recorded high. As a result of analyzing 25 years of meteorological records. Kirby (1954) found that severity of sooty blotches was positively related to rainfall levels in orchards and the amount of sooty blotch present on fruit in unsprayed orchards was directly proportional to the amount of rainfall received in July and, to a lesser extent, in August and September months.

Symptomatology and mycelial types

In present study, various mycelial types on fruit surface we could observe with differences in type of mycelial growth and arrangement of fruiting bodies on mango (Fig. 2). The estimated mean number of sooty blotch colonies per mango is in the range of 80-150. 2-3 mycelial types per mango was observed during our sampling. However, ramose mycelial type was recorded predominantly. The appearance of the blotch colonies was closely associated with the weather parameters. The ramose mycelial type increased with increasing rainfall and temperature which is prevalent in the coastal plain region.

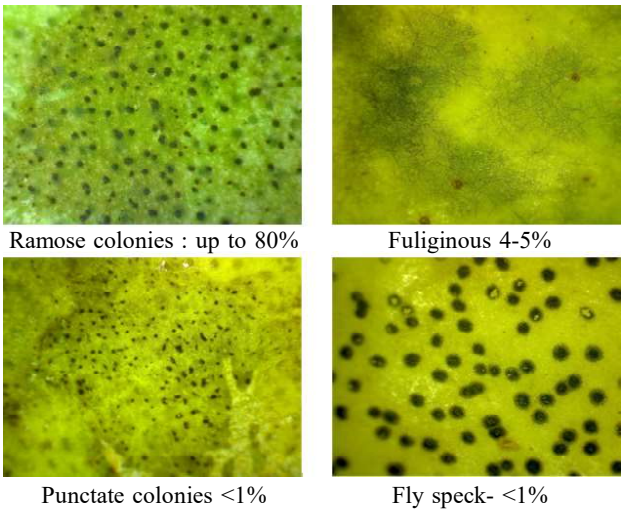

Fig. 2 : Mycelial types of sooty blotch and fly speck fungi on mango

Isolation and pathogenicity

Lot of difficulty had been encountered to isolate the sooty blotch fungi because of its very slow growing nature and no surface sterilization with disinfectants possible as it will kill the epiphytic fungi. Very small pieces (1-2 mm approximately) of blotch infected tissues were excised in aseptic condition and plated on water agar and subjected to incubation. Mostly the blotch fungi produced rubbery, heaped thallus on PDA where in part of thallus was submerged inside the

media. The colour varied from olive greenish gray to dark brown or black. Sooty blotch fungi grow extremely slow and, in some case, it took maximum 30 days to grow into 20 mm colony on PDA. No spores/reproductive structures associated with blotch colonies neither on cultures on different media obtained in isolations nor on sooth blotch infested fruit surface. In similar kind of work, Gleason et al. (2011) reported that many sooty blotches and fly speck species sporulate rarely or not at all, either on fruit or in culture, making morphological description of species difficult.

Pathogenicity was performed with twenty-eight representative isolates isolated from different mycelial types *viz.*, ramose, fuliginous and punctate. Mango being a climacteric fruit the pathogenicity tests were performed under simulated field conditions. Results showed that only five fungal isolates ISO115, ISO117, 1SO118, ISO135 and ISO211 could reproduce similar sooty blotch colony morphology upon artificial inoculation on mango and given the representative images in Fig. 3a-f).

Fig. 3 : Pure culture of one month old sooty blotch fungal isolates ISO118 (a), ISO60 (b), ISO135 (c), pathogenicity evaluation of ISO118 (d), ISO60 (e), ISO135 (f) on fruits

Genetic identity and phylogeny

Those fungi which could prove for pathogenicity under field condition were identified by ITS-rDNA analysis and attempted to assign putative genus status. The partial ITS sequences of five fungi described in this study were deposited in NCBI GenBank and received accession numbers. As all these five fungi causing sooty blotch on mango were sterile, Latin binomial could not be assigned hence we given only putative genus status based on a mega blast search in GenBank nucleotide database relying on only ITS sequence data. The nearest hits of ITS sequence of ISO115 (MZ701953) shown 100% identity with *Pseudocercospora cruenta*. (MK430966), 1SO118 (MZ701955) has shown 100% per cent similarity with *Pallidocercospora thailandica* (KX672151) and ISO135 (MZ701956) shared 100% identity with *Pseudocercospora* sp. (MG712852) hence given the putative status such as ISO115-*Pseudocercospora* sp., 1SO118-*Pallidocercospora* and ISO135-*Pseudocercospora* sp. Further, MW889007 has shown 100% similarity with *Zasmidium queenslandicum* (MH874742) and ISO117 (MZ701954) has shown 98.72 per cent identity with *Passalora intermedia* (FJ790251), hence, ISO60 and ISO117, assigned the putative status of *Zasmidium* sp. and *Passalora* sp., respectively (Table 2).

Earlier reports revealed that in apple, *Pseudocercospora* sp. LLS1 and *Pseudocercospora* sp. LLS2 (family: Mycosphaerellaceae) and *Passalora* sp. Isolate FG3 (family Micropeltidaceae) has been reported to cause SBFS and exhibiting fuliginous mycelial type (Batzer et al., 2005; Gleason et al., 2011). From China and USA, one species of *Zasmidium* belongs to Mycospahaerellaceae and several other new taxa belongs to Dissoconiaceae such as species of *Pseudoveronaea, Ramichloridium* and *Uwebraunia* and members of an undescribed genus

Table 2 : Putative fungal genus associated with mango sooty blotch complex

Isolate number	Designated putative species based on ITS phylogeny	Mycelial type on host	GenBank accession
Location I			
ISO115	*Pseudocercospora* sp.	Ramose	MZ701953
1SO118	*Pallidocercospora* sp.	Ramose	MZ701955
Location II			
ISO 60	*Zasmidium* sp.	Fuliginous	MW889007
ISO117	*Passalora* sp.	Fuliginous	MZ701954
ISO135	*Pseudocercospora* sp.	Ramose	MZ701956

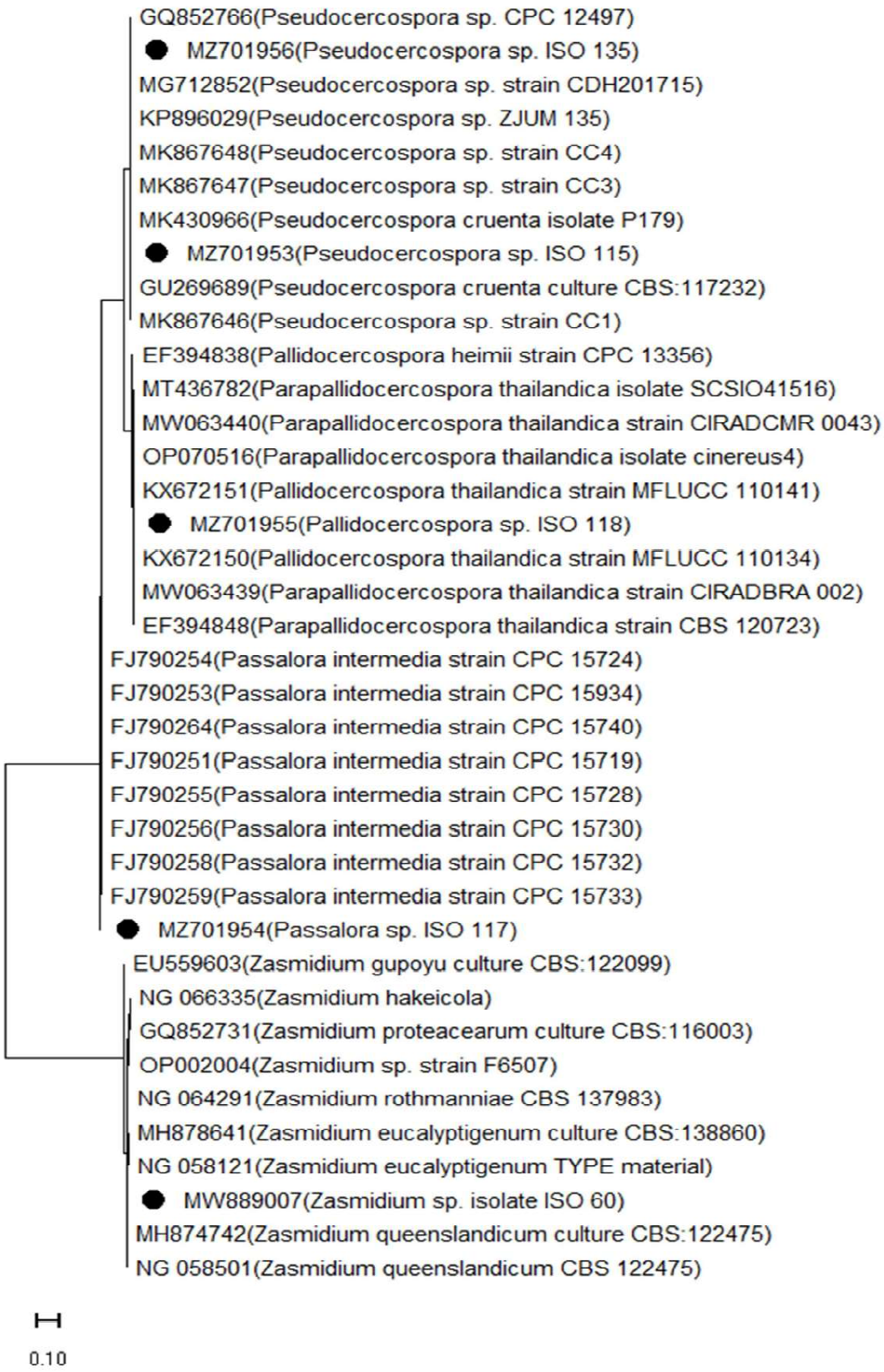

Fig. 4 : Phylogenetic relationship of fungi associated with sooty blotch complex inferred by the neighbour-joining method using ITS sequences (with 1000 bootstrap replications)

were identified to be associated with cause SBFS blemishes in apple and winter squash (Li et al., 2012). From Serbia and Montenegro, four SBFS genera namely *Pseudocercosporella*, *Pseudocercospora*, *Peltaster*, *Schizothyrium* and five putative species were identified from the signs of SBFS in apple (Ivanović et al., 2010). Diaz Arias et al. (2010) explained that several putative species reside in Dothideomycetes involved in causing SBFS could not be placed to the order level such as sterile mycelia sp. FG6, *Ramularia* sp. CS2 and *Sybren* sp. CS1. Phylogenetic relationship of fungi associated with sooty blotch complex of mango documented in these studies such as *Pseudocercospora* sp, (ISO115, ISO135), *Pallidocercospora* sp. (ISO 118), *Zasmidium* sp (ISO 60) and *Passalora* sp. (ISO117) were studied through neighbour-joining method along with isolates of closest matches from NCBI and represented in Fig. 4. In present study also, all five described genera belong to Dothideomycetes. In Malaysia, two previously undescribed *Peltaster*-like species were the predominant causes of SBFS on mango plantation belongs to Dothideomycetes, however, these two *Peltaster*-like species did not group with the previously described *Peltaster* spp. Mango isolate L111H grouped strongly with undescribed '*Pseudopeltaster*' isolates (Tham et al., 2023).

Source of inoculum

Observations made during study period in both the orchard locations revealed that mango twigs themselves act as a source of inoculum wherein plenty of blotch colonies on the upper side of the mangoes close to the twigs. In addition, existence of other reservoir hosts like jackfruit, sapota, star goose berry, Acacia, simaruba, fig, piasala and *Calotrophis* were also observed wherein these blotch fungi colonized the young twigs of these plants which were present inside the orchard as well as on borders. For sooty blotch and flyspeck of apple, more than 50 reservoir hosts have been documented including vines, woody and herbaceous plants trees and shrubs that were present near or on borders of apple orchards (Williamson & Sutton, 2000).

Evidence of damage of mango fruit skin by sooty blotch fungi by scanning electron microscopy

In scanning electron microscope (SEM) studies, it was observed that epicuticular wax crystals present on mango skin looked dissolved around the hyphae of sooty blotch fungi (Fig. 5), while, healthy mangoes, wax crystals remained intact. The earlier worker put forth few possible advantages of embedding of SBFS fungi on fruit cuticle rather than simply settling above the fruit apart from enhanced access to nutrients, stronger attachment to the hydrophobic fruit surface (Xu et al., 2016), otherwise it will be washed off during rain. Fungal penetration of cuticle of fruit cell wall was recently confirmed through recent studies with SEM in ectophytic fungi *Peltaster fructicola* and *Ramichloridium luteum* (Xu et al., 2016; Wang et al., 2017).

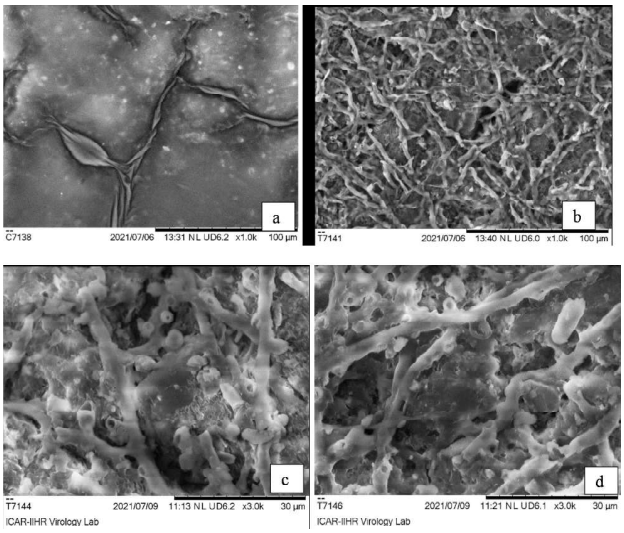

Fig. 5 : Scanning electron micrographs of healthy mango skin cv. Amrapali (a), sooty blotch infected mango skin at the magnification of 1000 (b) and 3000 (c, d). Bar presents 10 µm colonized by sooty blotch fungi. Epicuticular wax crystals appear dissolved by hyphae

CONCLUSION

In this study, the association of sooty blotch fungal complex on mango was documented for the first time in mango growing regions of Odisha, India through systematic preliminary studies. Technology-based sooty blotch disease management, such as disease-warning systems is need of the hour to enhance the effectiveness and cost-efficiency of sooty blotch management. More research on sooty blotch disease of mango involving additional mango-producing states of India is need of the hour for better understanding on the biology of this disease under Indian condition and efficient management of this emerging problem at field level.

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