First report of *Colletotrichum boninense* and *Pestalotiopsis microspora* infecting avocado fruits in Kenya.

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ABSTRACT

Avocado production is affected by anthracnose disease which leads to enormous economic loss to avocado farmers in Kenya. The disease affect the avocado fruits while in the field and after harvest during transport and storage. The causal agent of this disease have not been clear but presumed to be *Colletotrichum spp* mainly *C. gloeosporioides* as reported in regions where avocado is grown. The symptoms associated with the fungal infection are small blackish spots 'pepper spots' during early infection and black spots with raised margin which coalesce as invasion progresses. Due to fruit damages associated with the disease and new species of fungi being associated with the disease, this study was carried out to identify the causal agent(s) of the disease in Kenya. Fungal isolates were collected from diseased avocado fruits freshly picked from avocado trees in Murang'a County, Kenya. A total of forty six isolates sampled were morphologically identified as *Colletotrichum spp* based on their cultural characteristics mainly cream, greyish, whitish colour and cottony /velvety mycelia on the upper side of the culture. On the lower side, cultures were cream- grey having zonation which was concentric. Their spores were cylindrical, straight with round ends and with no septa. Whereas, thirty four isolates of the sample were identified as *Pestalotiopsis spp* based on their cultural and morphological characteristics; grey and white mycelium with black aecervuli on the upper side and greyish colour on the lower side. The spores are septate with 3-4 septa and 2 or 3 appendages at one end. Further molecular studies using ITS1 and ITS4 primers confirmed *Pestalotiopsis microspora*, *Colletotrichum boninense* and *Colletotrichum gloeosporioides* as the pathogens which causes anthracnose in avocado. The study showed a possible co-infection which need to be investigated further.

Key words*: anthracnose, avocado, Colletotrichum boninense, identification and Pestalotiopsis microspora,*

Introduction

Avocado (*Persea americana* Mill.) believed to have originated in Mexico, is currently cultivated all over the world including tropical areas of Africa ((Schaffer, *et al*., 2013). In Kenya, avocado is grown among other fruits by both small and large scale farmers for economic reasons. Avocado production in 2017 was 225,808 Metric tons accounting for Ksh 5.83 billion which was 5% of the total value of the fruits sub sector (HCD, 2018). The avocado fruit has nutrients such as minerals, vitamins and oil which make it popular for consumption by human (Schaffer et al., 2013).

Avocado production in Kenya however, is faced by constraints such as diseases, pests and poor agronomic practices. Among the diseases of great concern is anthracnose which mainly affect the fruits after harvest (Wasilwa, *et al*., 2004). The disease causes severe losses of up to 60% -100% avocado fruits while in the field and after harvest (Humble and Reneby, 2014; Siddiqui and Ali, 2014b; Pernezny and Marlatt, 2000). Management of anthracnose disease of avocado has been through the use of fungicides (Mattiuz et al., 2015; Obianom and Sivakumar, 2018; Arrebola,

2015; Smith, *et al*., 2011;). In Kenya, however use of fungicides to control the disease is limited due to lack of registered fungicide for use (PCPB, 2016). This has resulted to farmer using cultural practices such as pruning and field sanitation to reduce the severity of the disease.
Effective disease management requires the understanding of the interaction of the susceptible host, the virulence causal agent and the prevailing favourable environmental conditions for the disease to thrive ((Agrios, 2005). In view of this, identification of the causal agent of anthracnose disease of avocado in Kenya is paramount ((Kimaru, *et al*., 2018a)

Materials and Methods

Fungal isolation and culturing

Diseased avocado fruits having symptoms of anthracnose were collected from study area and brought to the laboratory for fungal isolation. The avocado fruits samples were cleaned using tap water and surface sterilized using 0.5% sodium hypochlorite for 30 seconds. Sections of the diseased area were cut aseptically from the margins of the lesion and placed on hardened potato dextrose agar (PDA) in petri dishes for fungal growth at room temperature (22 – 25^{o}C). The emerging fungi were sub-cultured to obtain pure cultures using single spore technique. To avoid bacterial contamination 0.5g/l of Streptomycin was added to PDA at molten state of about 50^{o}C (Choi *et al*., 1999). The pure cultures of the pathogens were preserved in the slant universal bottle and stored in the fridge at 4^{o}C for later use

Inoculation, Mycelial growth and sporulation of the isolates.

Sections of mycelial plugs were cut aseptically from pure cultures preserved in slant and placed on PDA in 9cm-diameter petri dishes and incubated for 10 days. Mycelia plugs from 10 day –old pure cultures of *Colletotrichum spp* and *Pestalotiopsis spp* were aseptically cut using five millimetre -diameter cork borer and placed individually at the centre of hardened PDA in 9cm-diameter petri dishes. The cultures were incubated at room temperatures in the range of 22-25^{o}C and mycelial characteristics studied and recorded. On eleventh day, the cultures were flooded with distilled water and scrapped to bring the spores into suspension. The suspension was filtered through double layer cheese cloth to remove mycelia. The spore suspension was serially diluted to 10^{-6} for ease of studying spores characteristics.

Conidial morphology and size.

A drop of spore suspension (10^{-6}) was placed on a microscope slide, covered with a cover slip and observed under microscope. To enhance visibility, the spores were stained with lactophenol (cotton blue). The shape and sizes of the spores from different isolates was noted.

Determination of genetic diversity of fungal isolates.

DNA extraction

Pure fungal cultures derived from a single spore from the original fungal isolates were used. An improved fungal extraction protocol as described by Liu *et al*., 2012 and Kimaru *et al*., 2018a was used.

Polymerase chain reactions

The extracted DNA from fungal isolates was used as templates in polymerase chain reaction. The primers used were in two sets; ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5-'TCCTCCGCTTATTGATATGC-3'), CgInt; (5'-GGGGAAGCCTCTCGCGG-3') and ITS4

(TCCTCCGCTTATTGATATGC). Primers CgInt is specific to *Colletotrichum gloeosporioides* while (ITS1 and ITS4) are universal primers.

DNA cleaning and Sequencing

The amplified products of the target fragments obtained above were cleaned using the Qiagen PCR cleaning kit according to the manufacturer instructions. The cleaned fragments were submitted for Sanger sequencing at Inqaba Africa Genomic platform in South Africa together with the primers used for amplification (ITS1 and 4 and ITS4 and CgInt). The sequences obtained were used to identify the isolates.

RESULTS

Fungal isolates

Avocado fruits showing symptoms of anthracnose disease (Plate 1) collected from the study area from which the fungal isolates were obtained. The isolates were identified based on cultural morphological characteristic on PDA and spore characteristics as observed under microscope (Domsch *et al.,* 1980; Rabha *et al.,* 2016).

Plate 1: Diseased avocado fruits showing symptoms of anthracnose disease.

A total of forty six isolates sampled had mycelia colour ranging from being whitish, greyish or cream. Further, the mycelial texture was either cottony or velvety on the top side and grey- cream with circular zonation on the reverse side Plate (i) Their spores were cylindrical, straight with rounded end (Plate ii), typical of *C. gloeosporioides* and *C. boninense* (Silva and Michereff, 2014). The remaining thirty four isolates had greyish white mycelium with black aecurvuli on the upper side and grey- black on the lower side (Plate iii). Their spores had 3-4 septa and 2 or 3 appendages at one end, characteristics of *Pestalotiopsis microspora* (Hyde *et al*., 2014) (Plate iv).

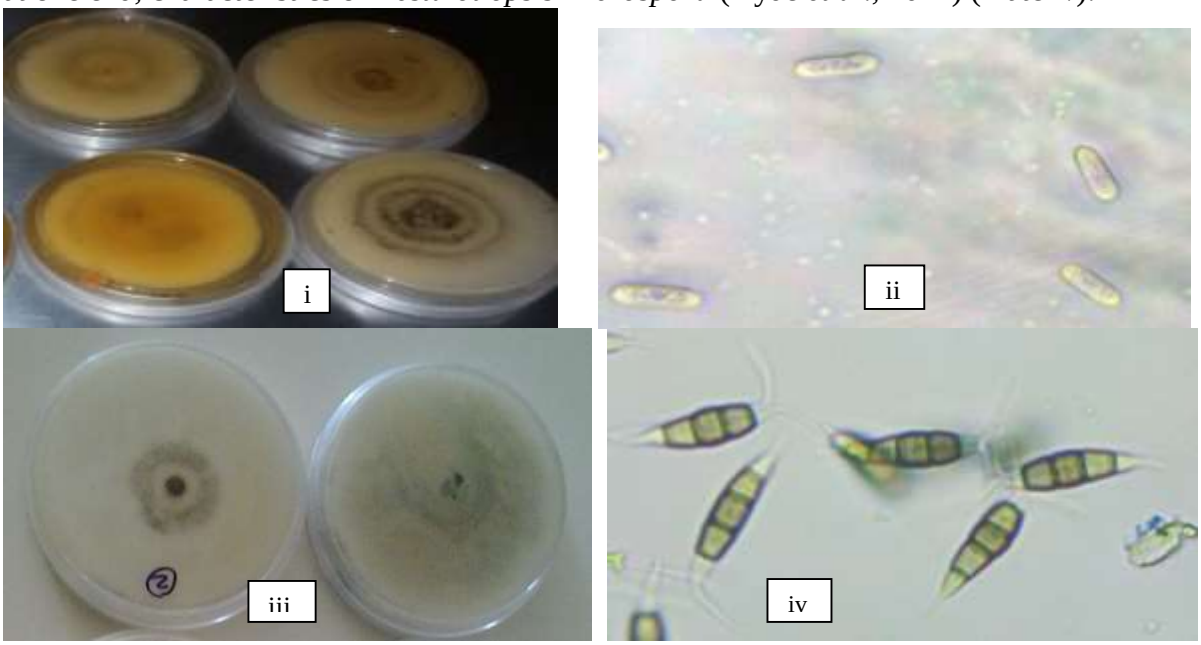

Plate 2: Mycelial of *C. gloeosporioides* (i), *C. gloeosporioides* spores (ii)(x400), Mycelial of *Pestalotiopsis microspora* (iii), and *Pestalotiopsis microspora* spores (iv) (x400) (Kimaru *et al*., 2018a).

The Mycelial growth of the fungal isolates

The fungal isolates grew steadily on the PDA medium covering the petri dish in 12 days after inoculation. The mycelial colour varied from greyish white, cream and greyish pink on the top side of the culture (Table 1). Likewise, the lower side of the cultures had cream- grey, light grey and dark grey (Table 1). The mycelial texture showed some variation where, cottony was observed in 58 isolates as compared to velvety observed in 22 isolates (Table 1). Based on these cultural characteristics alone, it is not possible to identify the causal agents because of shared cultural characteristics in terms of colour of the cultures. However, *Colletotrichum* spp showed concentric zonation while *Pestalotipsis microspora* had non-concentric zonation. Further, *C. gloeosporioides* and *C. boninense* had similar conidial shape which was straight and cylindrical while *Pestalotiopsis microspora* the spores were septate and with appendages.

Table 1: Cultural characteristics of the *C. gloeosporioides, C. boninense and P. microspora* on PDA

		Upper side		**Lower side**		
Pathogen	**No of Isolates**	**Colony colour**	**Texture**	**Colour**	**Zonation**	**Conidial shape**
C. gloeosporioides	24	Greyish white	Cottony	Cream-grey	Concentric	Cylindrical and straight
	12	Cream	Velvety	Greyish orange		
C. boninense	10	Greyish pink	Velvety	Light Grey		
P. microspora	34	Greyish white	cottony	Dark Grey	Non - concentric	Septate with 2-3 appendages

Phylogetic studies.

The phylogenetic identification of the fungal isolates was based on sequence similarities with sequences in the Genbank. The isolates *C. gloeosporioides, C. boninense* and *Pestalotiopsis microspora* identification was inferred from 12, 1 and 10 sequences, respectively (Table 2). The phylogenetic analysis of the isolates and reference sequences from the GenBank resulted into three clades; Clade1 (*Colletotrichum gloeosporioides*), Clade 2 (*Colletotrichum boninense*) and Clade 3 (*Pestalotiopsis microspora*) (Kimaru *et al*., 2018a).

Table 2: *Colletotrichum gloeosporioides, C. boninense* and *Pestalotiopsis microspora* isolates with their sequence number (Genbank accession number)

Species	**Culture**	**Location**	**Genbank accession number**
Colletotrichum gloeosporioides	14a	Murang'a County	MG013524
	39a		MG013525
	4b		MG013527
	5b		MG013528
	6b		MG013529
	8b		MG013530
	10b		MG013531
	11b		MG013532

	12b	MG013533
	13b	MG013534
	9b	MG013535
	7b	MG013536
Colletotrichum boninense	26a	MG013526
Pestalotiopsis microspora	27a	MG013537
	30a	MG013538
	6a	MG013539
	28a	MG013540
	2a	MG013541
	31a	MG013542
	32a	MG013543
	4a	MG013544
	25a	MG013545
	18a	MG013546

DISCUSSION AND CONCLUSION

Cultural and morphological characteristics of *Colletotrichum gloeosporioides, Colletotrichum boninense* and *Pestalotiopsis microspora* isolates.

The fungal isolates showed varied cultural characteristics on PDA media in form of colour and texture. *C. gloeosporides* cultures had white, grey or cream colour and velvety / cottony mycelium on the upper side. While on the reverse side, it was greyish cream with concentric pattern of zonation. This is in agreement with observations made on *Colletotrichum gloeosporioides* isolates from avocado fruits. (Chowdappa, *et al*., 2012; Dean et al., 2012; Sharma and Kulshrestha, 2015a). The isolates produced spores that were cylindrical and straight a feature of *Colletotrichum gloeosporioides* which was also reported by Chowdappa *et al*. (2012). However, there were some variations observed in cultural characteristic of the fungus, *C. gloeosporioides*. The variations observed could be associated to their genetic variations among isolates and repeated sub –culturing during the study (Vidyalakshmi and Divya, 2013; Weir *et al*., 2012).
The *P. microspora* isolates had grey-white mycelium with arcevuli on the upper side and dark greyish lower side. These isolates produced spores having 3-4 septa and 2 or 3 appendages as reported by El-argawy, (2016).

Phylogenetic studies of *Colletotrichum gloeosporioides* and *Pestalotiopsis microspora*.

The phylogenetic study identified *C. gloeosporioides, C. boninense* and *P.microspora* as the causal agent of anthracnose of avocado. This was based on the sequences of the isolates, showing acceptable similarity index with published fungal sequences in the NCBI, Genbank. Among the three pathogen, *C. gloeosporioides* has been reported as the most common and wide spread pathogen in avocado, worldwide (Honger *et al*., 2016; Siddiqui and Ali, 2014; Twizeyimana *et al*., 2013). *C. boninense* identified in the study area, although not common, it is also associated with the disease ((Silva-Rojas and Ávila-Quezada, 2011).

The sequences of *P. microspora* used in this study, showed 100% identity with the published sequences in the NCBI genbank thereby confirming their identity ((Kimaru, *et al*., 2018a). *P. microspora* has not been widely associated with disease despite being prevalent in areas where the host is grown. It has been identified as a causal agent of scab disease in guava in Hawaii and more research on this fungus to establish its host range is inevitable (Keith *et al*., 2006). It has been regarded both as an endophyte and as a pathogen causing post -harvest diseases (Metz *et al*., 2000). In this study, the fungus was isolated from diseased avocado fruits showing symptoms associated with anthracnose disease of avocado.

Conclusively, this study identified *C. gloeosporioides, C. boninense and P. microspora* as the causal agents of anthracnose disease of avocado in Kenya.

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The fungi, *C. gloeosporioides*, *C. boninense* and *Pestalotiopsis microspora* were morphologically identified based on cultural and microscopical characteristics using published fungal key (Freeman *et al*., 1998; Nagamani *et al*., 2009).