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Morphogenetic diversity of *Colletotrichum* species infecting *Sorghum bicolor* in the lake basin regions of Kenya

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Abstract

C. sublineolum is among the most destructive sorghum pathogens causing anthracnose in Kenya. Both preventive and breeding for resistance approaches have been developed. However, its continuous evolution into new strains is still a threat to sorghum production especially in tropics and sub-tropics. The pathogen is confirmed to be genetically and morphologically diverse but such information is lacking in Kenya and there is no known number of strains identified to cause the disease in Kenya. Systematic random and biased sampling was conducted in two districts within Lake Basin region where most sorghum is grown to collect anthracnose infected stem, leaf and panicle tissues only. Ten farms separated by four kilometers each were surveyed per district. Ten isolates were obtained per tissue per site, giving a total of sixty isolates for the whole study. Identification of the isolates was done using previous descriptions of the fungus and genetic diversity assessment was performed using simple sequence repeat (SSR) marker. A total of 15 morphologically diverse isolates were isolated from leaf, stem and panicle tissues. Leaf isolates were more diverse followed those infecting the panicle tissues. The stem was frequently infected by isolates resembling *C. gloeosporioides*. Genetic diversity assessment indicated the presence of two major sub-species with one sub-species showing low stability through evolution into two sub-groups. Only one universal primer amplified the bands thus there is need to involve several other primers to confirm these findings. This study also recommends Koch's postulate on *C. gloeosporioides* to ascertain its host range.

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Introduction

Anthracnose is one of the most destructive fungal diseases of sorghum due to its rapid spread on susceptible cultivars (Erpelding, 2010a; Erpelding, 2010b; Erpelding, 2011). The control of this pathogen is hindered by evolutionary mechanisms leading to the formation of several other subspecies (Mathur *et al*., 2003). Once formed, they overcome resistance and cause substantial yield loss especially under severe epidemics (Thakur and Mathur, 2000) through defoliation and tissue death.

Currently, only one *Colletotrichum* species, that is *C. sublineolum* has been confirmed to cause foliar, stem, panicle and grain anthracnose in sorghum and (Crouch *et al*., 2006). However, in the recent past, *C. gloeosporioides* was found to have pathogenic association with the local landraces of sorghum (Crouch and Inguagiato, 2009), increasing the threat to sorghum production.

Colletotrichum sublineolum was first reported in Togo, West Africa in 1902 (Thakur and Mathur, 2000) where it caused severe losses in yield and quality of grain. In Kenya, it was first reported in 1924 (Pande *et al*., 1998) and is mostly found in the Lake Basin Regions of Kenya where over 70% of the sorghum in the country is produced (Mukuru, 1993). In these regions of Kenya, 30 farms were earlier reported to have severe anthracnose (Ngugi *et al*., 2002).

Colletotrichum fungus exists in both sexual and asexual states and while in sexual state, it belongs to members of ascomycete, genus *Glomerella*. However, the sexual states (teleomorphs) are rare or absent and have not been reported from the field except in *C. falcatum*. Like *Glomerella* genus, graminicolous *Colletotrichum* produces erumpent acervuli with heavily melanized sterile hairs (setae), a characteristic distinguishing it from morphologically similar genus like *Gloeosporium* (Crouch and Beirn, 2009). *Colletotrichum* fungus infecting graminicolous plants exhibit falcate-shaped asexual conidia, a trait that is shared by other *Colletotrichum* infecting dicotyledonous and other non-graminicolous hosts' e.g. *C. capsici* in chilli (Shenoy *et al*., 2007).

For proper management and control of any disease causing organism including sorghum anthracnose, both genetic and morphological diversity of the pathogenic organism in question is of great importance especially in developing breeding objectives. None of these studies have been conducted in Kenya and there is urgent need for its proper management. Therefore, this study aimed at assessing the morphogenetic diversity of *Colletotrichum* species infecting *Sorghum bicolor* in the Lake Basin regions of Kenya.

Materials and methods

Morphological diversity assessment

Anthracnose infected leaf, stem and panicle tissues were obtained from Siaya and Kisumu district within the Lake Basin regions during the long rains. Biased sampling was used and most samples were collected from susceptible genotypes to maximize diversity of anthracnose pathotypes. Symptoms as observed during collection were recorded and samples brought into the laboratory for fungal isolation and morphological assessment. Isolation and culture purification was performed according to (Marley *et al*., 2001) and total of 60 isolates were obtained and pure cultures were raised from single spore of each isolate.

Morphological studies were conducted according to the protocol recommended by (Cai *et al*., 2009) where a five day old culture was incubated at 25^{o}C under 20% constant white fluorescent light inside Gallenkamp incubator/shaker. After the seventh day, colony characters such as conidia shape, presence of zonation or concentric rings, colour of mycelium (upper surface), colour of substrate (under surface), texture (fluffy or fibrous) and growth of aerial mycelium (luxuriant or scanty) were recorded. For growth rates of each isolate, pure cultures were assessed by measuring the diameter of the culture on daily basis for 7 days. The

morphologies were assessed under light microscope at eyepiece lense magnification of X10 and objective lense magnification of X40 (mg = X400) (Mathur *et al.*, 1989).

Genetic diversity assessment

Total mycelial DNA extraction and quantification was done (C.R.F, 2011) and out of fifteen morphologically diverse isolates, only ten produced adequate quantity of DNA for further studies (Figure 1).

Out of the four universal primers, (UBC 811, UBC 814, UBC 821and ERIC-a2) only ERIC-a2 (5' AAGTAAGTGACTGGGGTGAGCG 3') was successful. Amplification was carried out in 12.5 µl reaction mix [0.2 mm each of dNTP, 1x PCR buffer (Gitschier buffer), 0.5 units Taq DNA polymerase (Fermentas), 0.2 mm of primer, 1.5 mm MgCl2,0.16 mg⁄ml BSA, 10% DMSO, 20 ng DNA]. DNA amplification was performed in a PTC 200 thermal cycler (MJ Research) according to the following thermal profile: initial denaturation at 95°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 65°C for 8 min and by a final extension step at65°C for 15 min. Amplification products were separated in 1.5% agarose gel and detected by staining with ethidium bromide. The gels were photographed under UV light. Each PCR reaction was run in duplicate and only well defined and reproducible bands were scored (Bardas *et al.*, 2008).

The agarose gel electrophoresis bands were scored as present (1) and absent (0) and only polymorphic bands were considered for analysis. NTSYS-pc software version 2.1 was used for analysis of the diversity and similarity of the ten isolates whose DNA was amplified.

Results

Out of the 20 leaf isolates, 17 were *C. sublineolum* while 3 resembled *C. gloeosporioides*. All leaf tissues from Sega were infected by *C. sublineolum* isolates. The common characteristics observed in all the two species were scanty mycelial growth, fibrous mycelia and presence of setae, a further confirmation apart from conidia shapes that the isolates obtained from the sorghum tissues were *C. sublineolum*. The ascospores are normally enclosed within the ascus (asci) and are eight in number. When released, the ascospores germinate and form penetration peg which eventually penetrates plant tissues and cause infection (Fig. 2).

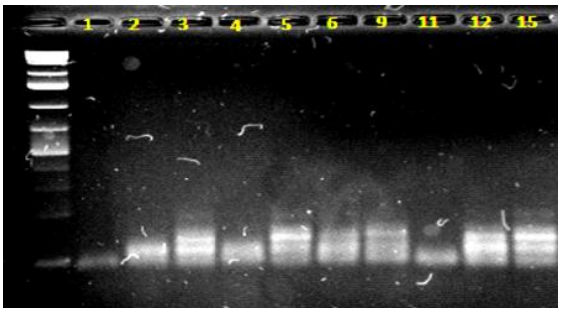

Fig. 1. Quantified DNA of ten *Colletotrichum* species isolated from leaf, stem and panicle tissues. Figures represent the isolate numbers as isolated.

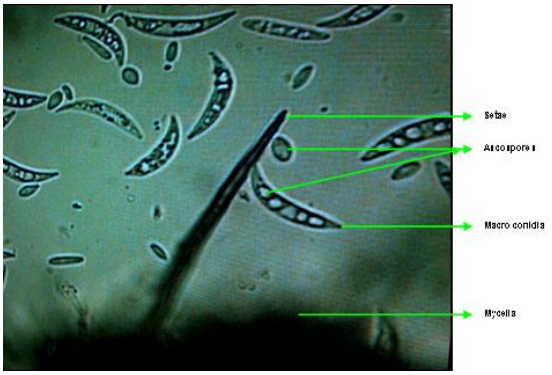

Fig. 2. Common features of *C. sublineolum* isolate infecting Sorghum bicolor under light microscope (Magnification = X400).

The *C. sublineolum* strains from leaf tissues had falcate shaped conidia with only isolate 1 having variable shapes from hokey stick to falcate shapes. On the other hand, *C. gloeosporioides* had either ovoid or fusiform shaped spores. The mycelia colour of all the *C. sublineolum* were grey but with varying colour intensities. Some isolates had concentric rings while others did not have rings. All colonies that formed concentric rings on half strength PDA had masses of pinkish conidia suspended in fluid matrix while those without rings had masses of

whitish to brownish conidia suspended on clear fluid matrix. At the centers of these rings, different colours ranging from black, grey, dirty white, cream and brown were observed (Fig. 3).

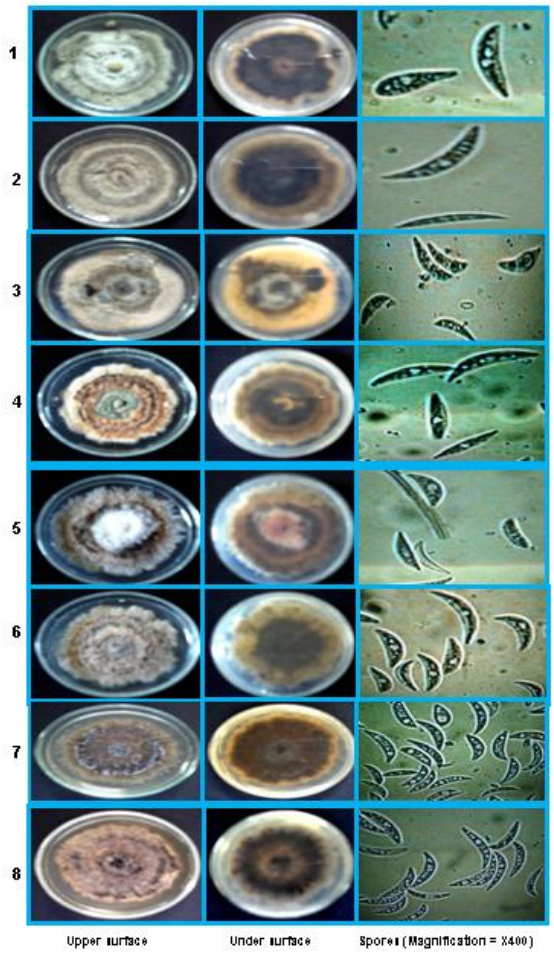

Fig. 3. Diversity of *C. sublineolum* isolates infecting leaf tissues in Siaya (1-6) and Kisumu (7-8) sites. These isolates were cultured in half strength PDA for 14 days.

Even from the same colony, there were many variations in sizes of conidia among the leaf isolates. In addition, the morphology of *C. sublineolum* spores from Siaya and Kisumu districts differed (Figure 2, 1-6 plates for Siaya and 7-8 plates for Kisumu isolates).

Morphological diversity of panicle/ear isolates of Colletotrichum species

Eighteen isolates from panicle tissues were *C. sublineolum* with two resembling *C. gloeosporioides*. However, unlike the leaf isolates, there were a lot of variations in terms of spore shapes in most isolates e.g. isolate 9. Also, few *C. sublineolum* isolates had falcate conidia only while majority had a mixture of falcate, hokey stick, oval and deformed falcate (variable) conidia. Presence of white conidia was also a frequent observation for the panicle isolates of *C. sublineolum* e.g. isolate 15. Moreover, isolate 15 of *C. sublineolum* from Sega site exhibited an unusual mycelial morphology and presence of white falcate conidia. Not all the *C. sublineolum* pathotypes formed clear ring and this observation is similar to those of the leaf tissues. The *C. gloeosporioides* obtained from the panicles were morphologically similar to those from the leaf tissues. There were three main morphological similarities among the *C. sublineolum* isolates and these include: grey mycelia, pinkish spore masses and formation of concentric ring on culture media. However, all colonies from Kisumu had brownish mycelia with whitish spore masses suspended in colourless fluid matrix (isolates 7, 8 and 12) and in common, all Kisumu isolates had scanty growth and fibrous mycelia texture (Figure 3).

Morphological diversity of stem isolates of Colletotrichum species

Unlike the leaf and panicle isolates, majority of *Colletotrichum* species isolated from the stem tissues looked like *C. gloeosporioides* which constituted 70% and the remaining 30% were *C. sublineolum*. However, the *C. sublineolum* from the stem tissues (pathotypes 2, 8 and 11 from figures 3 and 4) did not differ morphologically from those isolated from the leaf and panicle tissues. Same observation was made for isolate 13 and 14 (*C. gloeosporioides*) (Figure 4). Most importantly, *C. gloeosporioides* was frequently isolated from the stem than the leaf and panicle tissues.

Genetic diversity of Colletotrichum species

Genetic analysis showed the existence of two major sub-species of *Colletotrichum* in the lake basin regions of Kenya. The first group was genetically similar and consisted of isolates 3, 5, 6, 9, 12 and 15. The second group was diverse and evolved into two strains with the first group having isolate 1 while the

second group had 2, 4 and 11. These findings are similar to those in other countries (Chala *et al*., 2010; Crouch *et al*., 2008; Meirelles *et al*., 2009) (Fig. 5).

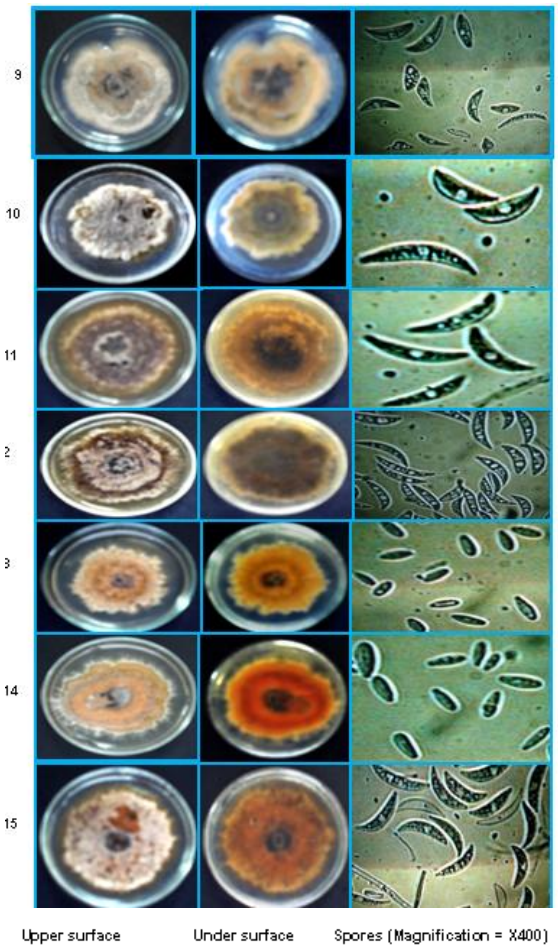

Fig. 4. Diversity of *C. sublineolum* and *C. gloeosporioides* isolates infecting panicle in Siaya (9, 10, 11, 14, and 15) and Kisumu (12, 13). Isolates cultured in half strength PDA for 14 days.

Discussion

Existence of different *Colletotrichum* morphologies indicates the presence of several sub species of *C. sublineolum* infecting sorghum in the lake basin regions of Kenya. By use of genetic data, it is clear that there are only three sub-species of *C. sublineolum* affecting sorghum production in the lake basin. The high variation in spore shape may be due to attempt by the pathogen to overcome panicle resistance to infection (Zanette et al., 2009). Moreover, the presence of many strains of *C. sublineolum* concurs with the pathogen – host evolution and differentiation theory which states that as the number of host species increases through breeding for resistant varieties of the same host, the pathogens of the same species feeding on these different hosts also change in their genetic makeup after a given period of time. This evolutionary mechanism has been confirmed on *Colletotrichum sublineolum* (Chala *et al*., 2010), European corn borer (*Ostrinia nubilalis*) (Martel *et al*., 2003) and leaf beetles (*Neochlamisus bebbianae*) (Scott *et al*., 2008).

A pathogen may also undergo evolutionary changes and this may alter the genetic make-up in order to infect a different host plant. For example, the unstable nature of *C. gloeosporioides* known to cause anthracnose of fruits and vegetables may have led to the formation of ex isolates of *C. gloeosporioides* (Du *et al*., 2005) and this may have conferred the pathogenic association of this pathogen with the local landraces of sorghum as found by Crouch and Inguagiato (2009).

High frequency of variable morphologies in terms of spore shapes (strain 9) among the *C. sublineolum* may be an attempt by the newly formed strains to overcome panicle resistance. Further, the presence of unique colony of *C. sublineolum* exhibiting pinkish mycelia colour and presence of white falcate conidia which lacks conidiogenous cells may confirm the presence of newly evolving strains like strain 15. Panaccione *et al*., (1989) also observed the production of oval conidia lacking conidiogenous cells and at present, even the falcate conidia found in this study lacks the cells, confirming that several evolutionary changes are taking place in *C. sublineolum*.

For *C. gloeosporioides* like isolates, the changes attempt to expand the host range and this may be due to continuous changes in the environmental factors coupled with host(s) factors especially the genotype. High frequency of stem infection by the *C. gloeosporioides* than *C. sublineolum* strains may show some degree of specificity or preference for certain parts of the host plant and this may be due

to host resistant genes which regulate the site of infection (Thakur and Mathur, 2000). Morphological differences in conidia shape for *C. sublineolum* obtained from the two districts show that certain environmental and sorghum genotype factors play significant roles in genetic and physiological changes within the pathogenic strains (Chala *et al*., 2010).

Conclusions and recommendations

There are 13 morphologically diverse species of *C. sublineolum* in Kisumu and Siaya districts of Kenya. However, these morphologies are further divided into three groups based on their genetic differences, indicating the existence of three sub-species affecting sorghum production in the region. However, it is not clear whether the suspected C. gloeosporioides were true to type and this study recommends proper study on the host range of this pathogenic fungi.

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