

Full Length Research Paper

Bot canker pathogens could complicate the management of *Phytophthora* black pod of cocoa

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Received 4 June, 2014; Accepted 4 August, 2014

Black pod is a major hindrance to cocoa production in Nigeria. It is caused by three different *Phytophthora* species with *Phytophthora megakarya* as the most important species in Nigeria and West African sub-region. *Phytophthora* spp. may enhance infections by opportunistic pathogens such as members of the Botryosphaeriaceae that cause branch and trunk cankers in many woody plants across the world. Botryosphaeriaceae has not been reported in cocoa nor in any woody plants in Nigeria to our knowledge. In the cocoa belt of Nigeria, research and understanding on cocoa black pod and *Phytophthora* is limited partly because of delayed or no access to some culture media, including required antibiotics. The objectives of this study were to: (1) use locally available materials to develop media for *Phytophthora* isolation from infected cocoa trees and pods samples and (2) to determine if members of Botryosphaeriaceae are associated with cankers of cocoa trees infected with black pod in Ondo State. The two formulated media, clarified tomato juice agar and cocoa pod agar supported the growth of *Phytophthora* spp. and were used for isolation from five cocoa producing local government areas, spanning all three senatorial districts of Ondo State. Based on morphological characteristics, four different species of Botryosphaeriaceae were identified from infected cocoa trees/pods but also from citrus and kola trees, which are similar to cocoa and usually planted in the same orchard with cocoa in Nigeria. These findings of new pathogens in cocoa and other hosts in Ondo State indicated the need for new strategies in the management of cocoa diseases in the State and across cocoa producing areas of Nigeria.

Key words: Cocoa, Canker, Black pod, pathogens, Botryosphaeriaceae, Phytophthora.

INTRODUCTION

Cocoa (*Theobroma cacao*) believed to have originated in the amazons and Orinoco basins of South America is now produced in many countries and about 70% of the

world total production is from West Africa. Nigeria is the 5th largest cocoa producer in the world with 160 thousand tons which amounted to 4.6% of the world

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production in 2007 (ICCO, 2010). Cocoa is the agricultural commodity that provides the highest foreign exchange earnings in Nigeria but also supports some companies in the country. Cocoa production is concentrated in the rainforest across the 'cocoa belt' region, which include six states of Ondo, Osun, Ogun, Delta, Edo, Cross-river, and Akwa-Ibom. Ondo State is responsible for over 50% of the total national production (Adejumo, 2005; Oyekale et al., 2009).

Black pod (pod rot) is a major hindrance to cocoa production in Nigeria. It is caused mainly by three different species of *Phytophthora*: *P. palmivora*, *P. megakarya*, and *P. capsici* but the most important species in Nigeria and the West African subregion is *P. megakarya* (Opoku et al., 2007). Diseases caused by *Phytophthora* are very common throughout the world (Adesemoye et al., 2011), especially in wet tropical regions of the world where it cause significant crop losses such as in cocoa and palms (Opoku et al., 2007; Adejumo, 2005; Drenth and Guest, 2004; Evans et al., 2003). Except for some species that are transmitted aerially, the spread of *Phytophthora* is mainly through infected soil, water and plants and plant materials. *Phytophthora* spp. can be isolated using a selective medium consisting of V8 juice, corn meal, or potato dextrose agar as base medium and five different antibiotics, pimaricin, ampicillin, rifampicin, pentachloronitrobenzene (PCNB), and hymexazole (Kannwischer and Mitchell, 1978).

Phytophthora spp. in many pathosystems have been reported to show certain level of interaction with other pathogens. For instance, co-infecting with *Fusarium* (Adesemoye et al., 2011) and it could pave the way for other pathogens such as members of the Botryosphaeriaceae (Adesemoye et al., 2014; McDonald and Eskalen, 2011). Botryosphaeriaceae are ascomycetes and rely on pruning wounds, earlier infections by other pathogens, insect infestation, and other stresses on the host for them to become opportunistic pathogens, causing branch and stem canker, gummosis, shoot blights, stem-end rot, fruit rot, and/or dieback of many woody plants (Abdollahzadeh et al., 2010; Adesemoye et al., 2013; Philips, 2002; Slippers et al., 2005).

Botryosphaeriaceae symptom expression could include dieback, bark cracking and discoloration, or death in some situations (Elliott and Edmonds, 2008; Smith et al., 1996) and cankers may exude reddish sap that dries to a whitish-beige powder. Their sporulation occurs on dead tissues and ascospores/conidia are discharged usually during wet periods. Botryosphaeriaceae possess both anamorphic (asexual stage) and teleomorphic (sexual stage) morphological characteristics. Teleomorphs of Botryosphaeriaceae are rarely seen in nature and their morphology differ little among species but there is a wide range of differences in anamorphs morphology and are used as the basis for species identification but may be combined with phylogenetic data (Abdollahzadeh et al.,

2010; Burgess et al., 2006; Luque et al., 2005). For instance, species in the genus *Diplodia*, *Dothiorella*, and *Lasiodiplodia* are separated from those in *Fusicoccum* by thick-walled conidia with a much smaller length to width ratio and are dark and septate when mature (Burgess et al., 2006; Philips et al., 2008). Phylogenetically, *Lasiodiplodia citricola* is closely related to *L. parva* but conidia of *L. citricola* are longer and wider than those of *L. parva* (Abdollahzadeh et al., 2010).

Many species of Botryosphaeriaceae have been identified in many woody plants in different parts of the world (Adesemoye et al., 2014; Brown-Rytlewski and McManus, 2000) but not in tree crops such as cocoa, citrus, or kolanut in Ondo State, Nigeria. It is common in Ondo State and many parts of Nigeria to find citrus trees planted within cocoa orchards, which make it easy for common pathogens to move between the two plants. Citrus is host to many *Phytophthora* spp. diseases such as gummosis, canker, and foot rot (Bawage et al., 2013; Drenth and Guest, 2004). Kola tree is an important woody cash crop, which is also commonly planted in cocoa orchards in Ondo State (Figure 1) and across the Nigerian cocoa belt. Though very little is known about the pathology, kola is related to cocoa and may share some common pathogens with cocoa as well as citrus. Kola tree in the genus *Cola* (Family Sterculiaceae) is native to the tropical rainforest of West Africa and is also cultivated in the American tropics (Burdock et al., 2009; www.nhm.ac.uk/seeds). The trees produce fruits, which are caffeine-containing nuts (Burdock et al., 2009). Among kola species, the most common in Nigeria are *Cola nitida* (Obi Gbanja), *Cola acuminata* (Obi Abata), *Garcina cola* (Orogbo), and *Buchholzia coriacea* ('wonderful kola'). Kola is an important economic cash crop in Nigeria, which in 2011 was reportedly worth about \$30,000,000 (Akinbode, 2011). It is valued for its perceived medicinal attributes (Adebayo and Oladele, 2012; Burdock et al., 2009; Ihesie, 2013) and kola is eaten across Nigeria and used during traditional events.

The scarcity and high cost of required antibiotics and other materials for preparing growth media continue to pose a problem in many developing countries, including Nigeria (Adesemoye and Adedire, 2005) and negatively affect research on *Phytophthora* and many other pathogens. The first objective of this study was to use locally available materials to develop two media for *Phytophthora* isolation from infected cocoa trees and pods samples. The second objective was to determine if members of Botryosphaeriaceae are associated with branch and trunk cankers of cocoa trees infected with black pod in Ondo State of Nigeria and identify the species involved based on morphology.

MATERIALS AND METHODS

Sample collection

Cocoa pods showing symptoms of black pod and infected branches



Figure 1. Kola nut tree (in front) and cocoa trees (at the background) planted in close proximity and both were infected with canker but kola tree had severe mixed symptoms as shown. There was invasion by many pests on the kola tree and possibly many other opportunistic infections.

were collected from different cocoa plantation in five local government areas (LGAs) of Ondo State, Nigeria: (i) Akoko southeast, (ii) Ose, (iii) Idanre, (iv) Ondo west, and (v) Odigbo. These five LGAs spread across all the three Senatorial districts of the State that is (i) and (ii), Northern district; (iii); (iv), Central district; and (v), Southern district. Production is concentrated in the Central district. Samples showing *Botryosphaeriaceae*-like branch and/or trunk canker symptoms were aseptically collected from two other plants - citrus (sweet orange), and kolanut from two of the locations where cocoa black pod samples were collected in Oke-origbo, Akoko southeast and Ose-oba, Ose LGAs. All samples were labeled appropriately and transported to the laboratory on ice for organisms to remain viable and isolations were made from samples.

Formulation of media for isolation of *Phytophthora*

Culture media were formulated by modifying the methods of Adesemoye and Adedire (2005). Fresh and ripe tomato fruits were obtained from Ibaka market, Akungba-Akoko, Ondo State. In preparing clarified tomato juice agar (CTJA), 200 g of fresh tomato

fruits without any visible infections, was weighed with a digital weighing balance (Mettler-Toledo International Inc, UK). The fruits were washed with tap water and blended. The blended chime was then sieved into a clean conical flask to remove the chaff and a volume of 100 ml of the sieved tomato chime was poured into an Erlenmeyer flask. Then, 18 g of agar-agar powder was added and sterile water was added to make a total volume of 1 L. The formulation of the cocoa pod agar (CPA) followed similar steps as CTJA. Two mature cocoa pods looking free of infection were obtained from the cocoa orchard at Oke-origbo sample location in Akungba-Akoko, Ondo State. Pods were washed, broken open, and 200 g of the pod was mashed in a mortar and the same formulation rate as CTJA was used. Each medium was homogenized, the CTJA and CPA were sterilized in the autoclave, allowed to cool to about 45°C and poured into sterile Petri dishes.

Pathogen isolation from samples

Infected cocoa pods were washed with tap water and disinfected by dropping in ethanol for 3 s, in 10% sodium hypochlorite for 10 s and again in ethanol for 2 s. Due to the nature of the infection, there may be high population of saprophytes if pods are not well disinfected but the pathogen cannot be eliminated by this disinfection as they grow well into infected plant tissues. The pods were rinsed three times in sterile water to eliminate any trace of disinfectant. Aseptically, superficial tissues were removed, cuts of 2-4 mm size were made in three locations per pod using a sterile small knife and placed on the newly developed media. The inoculated plates were then incubated at room temperature (25°C) in the laboratory for 72 h. Isolates were purified by transferring hypha tips to new sterile agar plates (Adesemoye et al., 2014). Macroscopic observation of the culture was done. Microscopic examination of the sporangia was carried out by preparing slide mounts from the incubated culture plates.

Branch and trunk canker samples were wiped with serviette to remove dirt and surface-sterilized by dipping in 95% ethanol for 3 s and then passed through Bunsen flame. Samples were placed on a tray that had already been swabbed with 75% ethanol, with the aid of a small knife, each time dipping the knife into ethanol and passing it through Bunsen flame, small cut pieces of about 2-4 mm were made at the margin of healthy and infected tissues. Six pieces of each sample were placed aseptically onto a solidified potato dextrose agar with tetracycline (PDA-tet) and incubated at room temperature for 3-5 days in the dark at 25°C. Pure cultures were obtained by transferring hypha tips from the resulting colony onto fresh PDA-tet plates and incubated similarly.

For sporulation, the isolates were grown on oatmeal agar prepared as reported by Adesemoye et al. (2014) and incubated for about 3-4 weeks with light to encourage pycnidia formation. After sporulation, spores were observed under the compound microscope. Morphological examination of the conidia (spores) produced by each isolate were done, noting their colour, shape, and presence or absence of septations.

RESULTS

The two formulated media, clarified tomato juice agar (CTJA) and cocoa pod agar (CPA) both supported the growth of *Phytophthora* spp. The hyphae on the CTJA and CPA appeared whitish throughout the period of 72 h of incubation and had covered the entire length of the 100 mm Petri plate on the 4th day of incubation. With these two media, *P. megakarya* was isolated from all the five local government areas of the state where samples

Table 1. Species of Botryosphaeriaceae identified and their conidial morphology.

Isolate ID	Location of sampling	Conidial morphology	Host	Identification
OKR01	Oke-Origbo	Conidia smooth, aseptate, unicellular, cylindrical with broadly rounded ends, some with a large central guttule, smooth, with glassy wall that remains hyaline even after the conidia have been discharged from the pycnidium.	Kolanut	<i>Botryosphaeria stevensii</i>
OKR02	Oke-Origbo	Conidia oblong to subcylindrical, septate, occasionally slightly constricted at septum, moderately thick-walled, externally smooth, internally finely verruculose, ends rounded often with a truncate base.	Kolanut cocoa	& <i>Dothiorella viticola</i>
OKR03	Oke-Origbo	Dark brown unicellular Paraphyses, aseptate, thick-walled.	Cocoa	<i>Lasidiplodia theobromae</i>
OSE01	Ose-Oba	Conidial pigmented, verruculose, ovoid, 1-septate	Citrus	<i>Lasidiplodia citricola</i>
OSE02	Ose-Oba	Dark brown unicellular Paraphyses, aseptate, thick-walled.	Cocoa	<i>Lasidiplodia theobromae</i>
OSE03	Ose-Oba	Dark brown unicellular Paraphyses, aseptate, thick-walled.	Cocoa	<i>Lasidiplodia theobromae</i>



Figure 2. (A) Conidia of *Botryosphaeria stevensii* OKR01 isolated from kola tree. (B) Front. (C) Reverse view of *Botryosphaeria stevensii* on oatmeal agar at 3 weeks. Mycelium was dense and aerial. Pycnidia were visible.

were collected: Akoko southeast, Ose, Idanre, Ondo west, and Odigbo LGAs.

Characterization of isolates from branch and trunk canker samples revealed four species of *Botryosphaeriaceae* from cocoa as well as two other hosts (citrus and kola tree) obtained from two sampling locations (Akungba-Akoko and Ose) in Ondo State (Table 1). No sexual structures were observed and observations

were based on anamorph structures produced by the isolates on oatmeal agar at 3 weeks. The species identified included *Lasidiplodia theobromae* and *Dothiorella viticola* isolated from cocoa, *Botryosphaeria stevensii* and *Dothiorella viticola* isolated from kolanut tree, and *Lasiodiplodia citricola* isolated from citrus, details of which are presented in Figures 2 to 5.

B. stevensii isolated from kola tree has similarities to



Figure 3. (A) Conidia of *Dothiorella viticola* OKR 2. (B) Front. (C) Reverse view of *Dothiorella viticola* on oatmeal agar at 3 weeks. Possesses aerial mycelium, colonies cottony, dark olive to grayish, darkening from the centre of the colony. Individual pycnidia were visible, spherical to globose, black, immersed, partially erumpent and thick-walled at maturity.



Figure 4. (A) Young conidia of *Lasiodiplodia theobromae* OKR3. (B) Paraphyses, young and matured conidia of *Lasiodiplodia theobromae* OSE2. (C) Front view of *Lasiodiplodia theobromae* on oatmeal agar at 3 weeks. Hyphae greyish and fluffy with abundant aerial mycelium; the reverse of the colony ranges from fuscous to dark at 4 weeks. Pycnidia were looking pronounced and raised.



Figure 5. (A) Matured conidia of *Lasiodiplodia citricola* OSE1. (B) Front view of *Lasiodiplodia citricola* on oatmeal agar at 3 weeks. Presented abundant aerial mycelium reaching to the lid of the petri plate, becoming smoke-grey to iron grey at the surface and greenish grey at the reverse of the plate. Pycnidia were superficial, dark brown to black, covered with mycelium.

the characteristics described by Alves et al. (2004) and Philips (2002) with conidia that are smooth, unicellular, cylindrical with broadly rounded ends, a thick glassy wall that remains hyaline even after the conidia have been discharged from the pycnidium. It had previously been reported as the cause of branch canker in grapevine (Philips, 2002; Larignon and Dubos, 2000). *D. viticola* isolated from kola tree were similar to those described by Philips et al. (2005) and Luque et al. (2005), with brown and 1-septate conidia that darkens from early stages of development.

L. citricola isolated from citrus had similar characteristics to the description by Abdollahzadeh et al. (2010) with conidia initially hyaline, aseptate, ellipsoid to ovoid, both ends broadly rounded becoming pigmented, verruculose, ovoid, and 1-septate with longitudinal striations. On the plate, *L. theobromae* showed fluffy, irregular and cottony white appearance and later turned black similar to the description by Abdollahzadeh et al.



Figure 6. Detached cocoa pods left in the plantation that may act as sources of further infection for *Phytophthora* spp. and members of the Botryosphaeriaceae.

(2010) and the mature conidia had 2-celled dark brown conidia with striation (Burgess et al., 2006).

DISCUSSION

The developed media was helpful in the isolation of *P. megakarya* from all five sampling locations. The results indicate that *P. megakarya* is the common species of *Phytophthora* in all the three districts of Ondo State. The problem usually encountered in Nigeria from the unavailability of several antibiotics that are added to the conventional or popular medium used in *Phytophthora* studies may be circumvented. The clarified tomato juice agar (CTJA) and cocoa pod agar (CPA) did not require any antibiotic.

The pattern of growth of the pathogen on the formulated media was different from the popular medium. On the conventional medium, *Phytophthora* growth is usually slower appearing whitish with appressed mycelia growth pattern while on the newly formulated CTJA and CPA, the mycelia growth had fluffy aerial whitish appearance and fast growth on the two media, which might be due to the absence of antibiotics on the two formulated media but growth was slightly faster on CTJA than CPA. On both media, the aerial mycelia growth first became obvious after 72 h of incubation at 25°C. Further studies for the isolation of *Phytophthora* species with these media are recommended for necessary improvements and modifications and possible commercialization.

Cocoa is an important cash crop in Nigeria and West Africa on which about one million people, mostly small-holder farmers, directly derive their livelihood (Opoku et

al., 2007) and the livelihood of many more are indirectly dependent on it. The infestation of cocoa trees by members of Botryosphaeriaceae which cause dieback and their possible interaction with *Phytophthora* black pod disease constitute additional concerns to cocoa production in West Africa. New strategies for managing both diseases are urgently needed to improve cocoa production in Nigeria, where production has been decreasing. Canker disease will add more complexity to disease management in cocoa as the pathogen has now been isolated from cocoa but also from two other cash crops - citrus and kola trees in the region.

Botryosphaeriaceae are difficult to control once established. It has been shown that their spores could persist in the soil and leaf litter in the orchard and they are likely to invade plants particularly during establishment (seed and soil transmission), during stress, and shortly after pruning, as pruning wounds provide a major entry point for potential pathogens (Adesemoye et al., 2014; McDonald and Eskalen, 2011). Sanitation by total removal of infected parts and pruned branches as well as treatment of wounds on trees appear to be the best option to reduce disease. Though chemicals are used, none is labeled for the pathogen (Peterson and Helmer, 1992). Pods that are usually left within cocoa plantations (Figure 6) should be removed as this will help to reduce potential inoculum sources of *Phytophthora*, Botryosphaeriaceae, and other related pathogens.

Conflict of Interests

The authors have not declared any conflict of interests.

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