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Review Article

Biology and Management Strategies of Cowpea Anthracnose Disease Caused by *Colletotrichum* Species

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ABSTRACT

Anthracnose remains an important biotic factor constraining the efficient production of cowpeas in tropical and sub-tropical regions of the world especially in Nigeria. Correct and accurate identification of its causal organism is essential for tailoring appropriate control or management techniques for this impediment. So far, many *Colletotrichum* organisms have been reported as responsible for causing this disease in the crop. It appears that majority of these reports were predicated on morphological and colony characteristics of the pathogens from culture studies and/or in some cases, on uncritical assumption of host-specificity of the pathogens. This makes for the identity of the causal organism of anthracnose in the crop to be generally unclear, confusing and a subject of much scientific debate. In the *Colletotrichum* patho-system, it is known that different species could cause anthracnose of the same host. Since isolates, show overlapping ranges of morphological, colony and phenotypic characteristics, molecular diagnostic approaches such as sequence analysis of the internal transcribed spacer (ITS) region (1.8S and 5.8S; 5.8S and 28S genes) and sequence analysis of β tubulin genes which offer comparative variability for resolving phylogenetic relationships of *Colletotrichum* species; as well as sequence analysis of introns from two genes (glutamine synthase and glyceraldehydes-3-phosphate) and MAT1-2 mating sequences which have allowed differentiation of isolates from species complexes could play vital roles in discriminating the causal organism(s). Though cultural strategies, tolerant and resisitant varieties against the disease and chemical interventions are used in managing the disease; resistance have been reported to be only temporary due to variability of *Colletotrichum* pathogens. Chemical interventions are disadvantaged in being human and eco-health disruptive; in addition to the fact that *Colletotrichum spp.* have shown resistance to carbendazim, thiophenate-methyl and benomyl. This review peers into the economic importance of anthracnose of cowpeas, its causal agent(s), management strategies for the disease and elaborated the response of the anthracnose organisms to phyto-chemicals from tropical plants for Integrated Disease Management (IDM) programs.

Key words: Anthracnose, *Colletotrichum spp*. Cowpea, Molecular identification, Characterization, IDM.

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) (*Fabaceae*) is reported as the most important grain legume in Nigeria. In Africa it is considered the second most important pulse crop and ranked amongst the top-five most important legumes in the world (Gibbon and Pain, 1991; Awurum and Enyiukwu, 2013). The crop is thought to have originated in the Transvaal region of South Africa or somewhere around West Africa (Aveling, 2007; Davies et al., 2012). It is used as food in the semi-arid and arid tropics of Asia, Africa and America (Tshovhote et al., 2003; Timko et al., 2007) where its protein-rich grains supplement cereals and scarce animal-derived protein for 110 million of its consumers (SADAFF, 2009). The leaves, pods and seeds are consumed; being prepared into several food forms in the continents. In addition, the crop plays roles in erosion control, and soil fertility restoration in various farming systems of the tropics; through symbiosis with nodular *Bradyrhizobium spp*. In these farming systems, the crop thrash is also used as fodder for feeding livestock (Enyiukwu and Awurum, 2013a).

Reports indicated that 2.0 million MT of its grains are produced annually in Nigeria on about 14 million ha of farmland (Singh et al., 2002). Between 2015 and 2020, production of cowpea grains in the country is projected to rise to 3.364 - 4.097 million MT (Abate et al., 2011). Expected demand however, in the same period is estimated at 5.273 – 6,906 million MT, creating a surplus demand of 1.909-2.809 million MT in the economy. Nigeria reported the authors shall require 0.90 – 1.33 trillion USD to cushion the surplus demand by importation of the grain from elsewhere if nothing is done to improve local productivity. Besides socio-economic constraints,

this deficiency in cowpea grain supply is reasoned to emanate from fungal disease pressures especially anthracnose.

Anthracnose characterized by sunken, black lesion is one of the major fungal diseases of cowpea which constrain its economic production (Enyiukwu and Awurum, 2013b). In affected cowpea plant, up to 50% yield reduction occurs. Its causal agent has been a subject of much scientific debate. It has been variously advanced and reported as a form of *Colletotrichum lindemuthianum*, *C. gloeosporoides, C. dematium* and recently as *C. destructivum* O'Gara (Emechebe and florin, 1997; Emechebe and Lagoke, 2002; Masangwa et al., 2013). Till date, the accurate identity of its causal agent is still unclear and shrouded in confusion (Hyde et al., 2009). Correct and accurate identification of the causal pathogen is essential for articulating proper management strategies for a given disease problem; in addition, it is required for good quarantine and regulatory purposes. This in turn will delay build up of resistance to a control agent (Gueber et al., 2003; Cannon et al., 2012; Enyiukwu et al., 2014a; 2014b).

Therefore, this communication presents a review of the biology of the cowpea anthracnose fungus; emphasizing needs for its molecular re-examination, proper identification and characterization; and the management strategies against the disease.

The genus *Colletotrichum*

Colletotrichum is made up of 750 species prior to the crucial pioneering work of Von Arx (1957) who reduced them to 11 (Cannon et al., 2000). Since then, there has been upward review of this number due to molecular phylogenic studies and re-examination of various strains in the world (Sato et al., 2012) with several workers reporting the genus to consist of 39 species, 1 variety and 7 *formae speciales* ((Sutton, 1992; Hyde et al., 2009). Recent reviews however recognized 66 species as constituting the genus which are listed in current literature (Shivaprakash, 2011; Cannon et al., 2012). The genus is ranked the fourth most studied pyhto-pathogenic fungi, being surpassed by *Fusarium, Phytophtora* and *Rhizoctonia* (Hyde et al., 2009) and the eight most important group of plant pathogens on a world scale based on economic-scientific perceptions (Cannon et al., 2012). *Colletotichum spp.* are worldwide in distribution, causing important diseases in humans, ornamentals and economic crops (Than et al., 2008; Hyde et al., 2009; Cannon et al., 2012). Generally, six members of the genus have so far been penciled down as human pathogens (Liu, 2012), while five have progressively been reported as aetiological agents of subcutaneous hyalohyphomycoses and keratomycoses (Yegneswaran et al., 2005). According to Cannon et al. (2012) subcutaneous infections, keratitis and corneal ulcers in humans have resulted from attacks from unusual phytonotic species of the genera such as *Colletotrichum dematium*, *C. truncatum*, *C. gloeosporioides, C. coccodes, C. crassipes* and *C. graminicola* (Cano et al., 2004; Mendiratta et al., 2005; Liu, 2012). For example, in India, report of unusual mycotic keratitis induced by *Colletotrichum graminicola* had been documented (Yegneswaran et al., 2010). The organism was implicated in corneal ulcer in a 44 year old man that refused to heal for more than 3 weeks. While Shivaprashkash et al. (2011) reported five cases of *C. truncatum* incited endophthalmitis in addition to keratitis in some parts of Asia, in Japan, a new implication of *C. taiwanense* in human cornea disease have been noted (Sato et al., 2012). Reports in recent times of disseminated mycotic infection caused by *C. acutatum* in some animals like sea turtle had also been documented (Manire e al., 2002).

Colletotrichum is well represented in the warm moist environment of the humid an sub-humid tropics where they occur as saprobes, endophyites or pathogens on leaves, stems, flowers and sometimes fruits of both field and perennial susceptible crops (Waller et al., 2000). Many economically important diseases including wilt, rot and anthracnose have been ascribed to attacks from members of the genus on a diverse array of crops including cereals such as maize, sorghum, as well as banana, sugar cane, strawberry, cowpeas and the *Proteacea* (Xie et al., 2010; Lubbe et al., 2012). It has been reported that *Colletotrichum* species may cause similar diseases on different crops; or distinct diseases of separate organs of the same crop (Waller et al., 2000). Perfect et al. (2011) reported that members of the genus use diverse strategies for invading host tissues ranging from intracellular hemibiotrophy to intramural necrotrophy (i.e. they form germ tubes, appressoria, intracellular hyphae and secondary hyphae). Infection occurs via the agency of appressoria that develop from germinating spore on the plant surface followed by turgor-driven penetration of the curticle or epidermal cells by infective hyphae. The fungus establishes within the host by host-induced virulence effectors. Infected tissues may remain asymptompless for 1-3 days or extended period of time as in post-harvest species. This biotrophic phase is then followed by necrotrophic phase resulting in death of plant cells and emergence of lesions (Cannon et al., 2012). Different forms of the fungus and in some cases other pathogens can co-inhabit lesions (Waller et al., 2000). It is reported for example that in the patho-system of anthracnose in chillis, different *Colletotrichum* species were found associated with the disease in the same host (Than et al., 2008).

Classical and molecular identification of *Colletotrichum* species

Morpho-taxonomic criteria such as conidial shape and size, appresoria morphology and size, setae morphology, temperature response of the organisms on Potato Dextrose Agar (PDA) are currently in use for identification of *Colletotrichum* species. For example *C. gloeosporioides* and *C. acutatum* associated with post-harvest anthracnose of apple were significantly differentiated on the bases of several morphological and

ecologicalparameters by Grahovac et al. (2012). According to this source, morphological features of most isolates of the genus however, may vary considerably with environmental conditions, making isolates to have overlapping ranges of conidial and colony characteristics. Therefore, Waller et al. (2000) submitted that the phenotypic plasticity of members of the genus *Colletorichum,* makes species differentiation based solely on morphological criteria to be difficult. So also does host range and host specificity (Freeman et al., 1998). These authors inferred that numerous cases of several species or biotypes being associated with a single host as well as a single pathogenic species on multiple hosts have been reported. This further compounds the problem. Several species of the genera according to many workers were assumed to be specific to the plant they infected (Hyde et al., 2009; Cannon et al., 2012). Freeman et al. (2000) asserted though, that some level of host specificity existed within a species of the genus in certain cases, due to the fact that no cross-infections occurred in closely planted almond, avocado and strawberry trees in the same geographic regions. In support to this, Waller et al. (2000) remarked that molecular tools like mitochondrial DNA (mtDNA), ribosomal DNA restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) have demonstrated that some level of host-specificity exists in the genus. Nevertheless, this crude assumption resulted in large numbers of taxa in the genus. Cannon et al. (2012) noted up to 750 species described in the genus in the late 19th century. This explosion of taxonomic activity was largely driven by this uncritical assumption of strong host-specificity in *Colletotrichum species*. Leading in effect to new taxon being erected each time a new disease was reported, even in the absence of unique morphological diagnostic characters and all too often with little or no comparison with previously described taxa. Unfortunately, therefore, many of the taxa were described based solely on morphological data without distinctive features apart from the identity of their plant partners. For example, confusion trails the assumption of host-specificity in legumes where any strain of *Colletotrichum* species with straight conidia attacking the crops has been regarded as *C. lindemuthianum* (Cannon et al., 2000; 2012).

Another important factor is the growth characteristics of members of the genus on culture media. *Colletotrichum* species grows slowly on PDA (Enyiukwu, 2011); and invariably take longer time to produce conidia, appressoria, and acervulus in routine culture media. Several species of the genera in culture studies are reported to produce secondary conidia directly from germinating primary spores, which are generally smaller and more varied in shape. This has been reported to contribute strongly to making morphological identification of species within the genus to be difficult or delayed (Cannon et al., 2000; Liu, 2012) Though morphology of conidia and appressoria have been successfully applied as a reliable means of discriminating certain *Colletotrichum* species such as those pathogenic to strawberry; in numerous other cases identification predicated on these criteria have been insufficient due to overlapping ranges in fungal morphology and variation in phenotypic and colony characteristics. On these bases therefore, the current taxonomic status of most species of *Colletotrichum* is unclear (Than et al., 2008). Hence, a recent review by Hyde et al. (2009) termed the genus a *Catalogue of confusion*.

This taxonomic confusion has prompted the need for other means of discriminating and differentiating members of the genus. Therefore. great potential is believed to exist for augmenting morphological classification system of members within the genus with molecular data (Cannon et al., 2000). In this regard, Sato et al. (2012) in a recent study in Japan for instance, discriminated 3 rare species of *Colletotrichum* attacking bamboo and crysanthenum based on morphological evidences and supportive molecular data. Molecular tools appropriate for the accurate identification of inter and intra-specific diversity within the genera *Colletotrichum*, or its broad species like *C. acutatum* or species complex is highly desirable (Gueber et al., 2003). Generally these molecular tools may involve low-cost analyses of electrophoretic band patterns (RAPD, RFLP etc) or relatively expensive internal transcribed spacer (ITS) sequence of the test fungal DNA.

Sequence analyses of the intergenic transcribed spacer (ITS 1, ITS 2) of ribosomal DNA (rDNA) have proven valuable for delineating species of *Colletotrichum* (Freeman et al., 2000; Lubbe et al., 2004). For example, Afanodor-Kafuri et al. (2003) discriminated species of *Colletotrichum* attacking tamarillo, pisiflora and mango in Columbia on the bases of complete ITS1-5.8S and ITS-2 region molecular-guided identification of the fungi to their respective species as *C. acutatum* on tamarillo, *C. gloeosporioides* associated with mango and *Colletotrichum sp*. on pissiflora. Simiarly, *Colletotrichum gloeosporioides, C. acutatum, C. coccodes,* and *C. dematium* were reported implicated in the initiation and development of tomato anthracnose in literature. However, Swetlana et al. (2010) confirmed from recent molecular genomic species-specific Calnt2/ITS4 studies that only *C. acutatum* was responsible for the disease in the crop.

Conversely, *C. acutatum sensu lato* (s.l) represents a species that encompasses a wide range of morphological and genetic diversity. Characterization of *Colletotrichum acutatum s. l.* has been enhanced by the use of molecular markers such as sequence analysis of two gene introns (mtDNA and intro RFLPs) and mating compatibility. These have identified genetically distinct and perhaps biologically discreet groups amongst the morphologically similar isolates of *C. acutatum s.l.* (Gueber et al., 2003).

Randomly amplified polymorphic DNA (RAPD), reported Zakaria et al. (2009), differentiated *Colletotrichum* species attacking banana, apple and guava. In the investigation, isolates from banana and guava clustered together in an unweighed pair group method analysis (UPGMA). The banding patterns of *C. musae* isolates in the trial were highly similar but showed intra-specific variations. The results reported these investigators ascribed *Colletotichum musae* as responsible for the attacks on banana and guava and *C. gloeosporioides* on apple . Conventional identification of a fungus has been done majorly by its spores

characteristics. *Colletotrichum acutatum* has been identified predominantly traditionally by its ellipsoidal or fusiform conidia, often pointed at both ends. However, based on molecular criteria, isolates with more or less atypical conidia, with one or both ends rounded have been identified and ascribed as *C. acutatum* also (Gueber et al., 2003). This otherwise would not be possible on the premises of morphological descriptive identification. Hence, this thus sustains the submission that discriminating *C. gloeosporioides* and *C. acutatum* solely on morphological criteria is difficult (Waller et al., 2000).

Limitation of molecular identification

Though molecular techniques especially those involving the sequencing of ITS and D1-D2 regions of rDNA of *Colletotrichum spp* had helped tremendously in rapid identification of the fungi; however many sequences deposited in GenBank are either wrongly labeled due to multiple names of the same species or misidentification of the fungus on the basis of morphological character. Hyde et al. (2009) is of the opinion that much of the confusion especially with regard to *Colletotrichum destructivum* is based on the fact that the fungus has not been epitypified and that types are not available for sequence data comparison. This limitation therefore informs the necessity for the epitypification of all the *Colletotrichum* species and especially *C. destructivum;* and this invariably may help to resolve the many taxonomic problems besetting the genus and aid in accurate species identification (Liu, 2012). For instance it will enable us to compare species named as *destuctivum* against the epitype and to determine whether the strains from cowpea in the infection studies are correctly named (Hyde et al., 2009). Accurate species identification will invariably translate to formulation of good management techniques against the disease (Than et al 2008)

Anthracnose of legumes: A highlight

Anthracnoses incited by *Colletotrichum spp.* occur commonly in a wide variety of legumes where they cause significant yield losses (Masangwa et al., 2013). Soybean for example is susceptible to anthracnose at all stages of growth. The disease characterized by crowded black acervuli borne on well developed stomata, was first reported in Korea in 1917 (Akem, 1994). Symptoms usually appear in the early reproductive stages on stems, pods and petioles as irregular brown lesions, which later turn black from presence of acervuli that produce minute black spines (setae) visible to the naked eye. This disease caused by *Colletotrichum truncatum* and *C. glycines* according to this author is attended by necrosis of lamina veins, premature defoliation, pod blanking and shriveled seeds; resulting in 16-26% yield reduction or total crop failure in severe instances.

Bean (*Phaseolus vulgaris* L.) anthracnose is initiated by the fungus *C. lindemuthianum* (Sacc. and Magn.) Bri and Cav. (Amadioha, 2003; Masangwa et al., 2013). This pathogen, noted Mohammed (2013), has been severally reported as associated also with anthracnose of mung bean (*Vigna radiata* L.), cowpea (*V. unguiculata* L.) and broad bean (*Vicia fibia*). The disease is worldwide in distribution and the pathogen has many races. For instance more than 25 races of the pathogen have been isolated in Brazil. The fungus survives for 2 years in infected thrash or seeds and up to 5 years at controlled temperature of 4^{o}C as mycelia or spores. All levels of its conidia germination, incubation and sporulation require high level of humidity (92%) or free water on the crop with ambient temperature remaining at about 28^{o}C. On the pod infected seeds may be shriveled and discoloured and have dark brown to black cankers which are more conspicuous on white testa seeds. Anthracnose is a major fungal disease of bean and has high importance especially in the tropics where it causes serious crop losses. *Colletotrichum lindemuthianum* is hemibiotrophic pathogen. It produces cell wall degrading enzymes and low molecular weight phyto-toxins which participate in killing host cells in advance of the invading hyphae and thereby contribute to the necrotrophic growth of the fungus. Yieid losses of 40-80% have been attributed to attacks of the disease in susceptible bean lines in Tanzania, 90% in Sudan; with 92% and 95% documented in Malawi and Columbia respectively. However losses could reach 100% in very susceptible cultivars under favourable conditions of cool, wet weather (Enyiukwu, 2011; Mohammed, 2013).

Anthracnose of pigeon pea (*Cajanus cajan* L.) is caused by *Colletotrichum cajani* Rangel. According to Thakur et al. (2011) the disease is destructive and of high importance in India affecting leaves pods and seeds of the crop especially during the rains. Symptoms include spots on pods and leaves, blackening and shriveling of veins of infected leaves, defoliation and seed discolouration. Generally affected pea plants, show up to 86% pods infection and 36% unmarketable seeds.

Anthracnose of cowpea

Members of the Genus *Colletotrichum* have been reported to cause two major diseases in cowpea. These are anthracnose and brown blotch. These diseases are very destructive due to susceptibility of many cowpea lines to them (Adegbite and Amusa, 2008). Fungal metabolites have been implicated in the development of these diseases while leaf topography and specific physical and in some cases, chemical signals are reported to trigger appressoria formation from these organisms on leave surfaces (Podila et al., 1993; Amusa et al., 2001; Mohammed, 2013).

Brown blotch is locally important in the savannas of Africa (Aveling, 2007). The pathogen attacks all above ground parts of susceptible cowpea. The disease is symptomized by pre- and post-emergence damping-off, girding of stem or branches, flower abortion, pod mumification and lesions on pods and leaves (Emechebe and Florini, 1997). This highly important disease of the crop is cosmopolitan in distribution affecting countries like India, Thailand, Zambia, Cote d'Ivoire and Nigeria (Aveling, 2007). In a study in Southwest Nigeria, 100% of all cowpea lines evaluated had the disease (Ajibade and Amusa, 2001). Ayodele and Kumar (2011) reported that since the 1970s up to 56% of the fields surveyed in Brazil had the disease. In an impact study also in Western Nigeria, brown blotch reduced stand establishment of cowpea from 88% in healthy seeds to 24% in infected ones. In the savannas of Northern Nigeria, yield loss of 46% has been attributed to the disease peaking at 75% in very wet years (Enyiukwu, 2011).

The conidia of the causal agent of brown blotch are bluntly tapered, curved, unicellular and hyaline; and are borne singly on conidiophores. The disease was initially thought to be caused by two species of the genera *Colletotrichum* namely *C. capsici* and *C. truncatum* (Emechebe and Florini, 1997). However, modern molecular investigations have suggested that both are the same organisms (Emechebe and Lagoke, 2002; Adegbite and Amusa, 2008). Even *C. truncatum,* reported Cannon et al. (2012) In effect, is actually a member of the clade *Colletotrichum destructivum* O'Gara

Anthracnose disease characterized by black lesions, usually sunken, is another major disease of cowpea (Emechebe and Florini, 1997; Awurum et al., 2005). The disease is pan-tropical in distribution being widely recorded in West and East Africa, Asia and Brazil where conditions are wet and humid for the main part of the growing season. It has wide host range affecting alfalfa, Lucerne, pigeon pea, soybean, hyacinth bean, asparagus bean, common bean, mung bean, broad bean and cowpeas etc. (Infonet-Biovision, 2014; Cropgenebank, 2014). It has also been reported to attack *Brassica campestris* and *Arabidopsis thaliana* (O'Connell et al., 2002). In Nigeria, the disease is one of the major fungal diseases of cowpea crop (Aveling, 2007). The fungus overwinters in the previous crop debris, and can also be seed-borne as dormant mycelia within the seed coat or as spores between the cotyledons; from where it initiates infection of hypocotyls and young leaves in the field (Hill and Waller, 1990).

The infection process in susceptible seedlings involves hemibiotrophy. Spores germinate on the host within 6-9 h (Cropgenebank, 2014) to form melanized appressoria and penetrate the seedlings at the anticlinal walls of the epidermal cells by the appressoria and infection peg. Infection vesicles then form in the penetrated host cells within 48 h and secondary hyphae from such multi-lobed infection vesicle at 60 h which then penetrate the host cell wall to initiate necrotrophic phase of the infection. Acervuli with a single melanize seta form at the plant surface after 96 h (Latunde-Dada et al., 1996; Shen et al., 2001). The fungus attacks all above ground parts of the cowpea plants. Affected plants generally develop lesions which are tan to brown, sunken and lenticular; which enlarge rapidly and coalesce to girdle stems, peduncles and petioles. Generally, lesions appear at the under-leaf surfaces before the upper sides.

Lesions from infected cultivars sporulate profusely. Spores of the pathogens are water-borne and easily transmitted in wet weather by air currents, insects, man, machinery and, rain splash to cause secondary infection on petioles, leaves and stems of the plants. Cowpea anthracnose is favoured by warm wet weather. Temperature range of 25-30^{o}C (27^{o}C optimum) with relative humidity of 92% or free moisture on the plant surface have been found to encourage spore germination of the pathogen and initiation of the disease in the plant (Mohammed 2013; Infonet-Biovision, 2014)

Seeds are possible sources of long distance distribution of the fungus into pathogen-free locations (Awurum and Ucheagwu, 2013). Reports from Pakistan for instance, suggested that the anthracnose fungus was introduced into the country from infected seeds from Nigeria (Qureshi et al., 1985). In a survey, up to 88% infection was recorded on seed samples from seed-lots in India; and seed germination decreases with increased infection of such seed-lots resulting in rots and seedling mortality (Prassana, 1985). The disease affects yield, seed quality and marketability Grain yield losses of 35,925.00 MT have been estimated due to anthracnose in Rwanda (Cropgenebank, 2014). In the humid rainforest agro-ecology of Nigeria however, 50% grain yield loss has been reported from attacks of the disease in susceptible cultivars (Aveling, 2007). Thus, translating to losses of about1.0 million MT of grains per annum; in the country as a whole (Singh et al., 2002). *Colletotrichum spp.* as seed-borne organisms may produce mycotoxins in affected grains. Mycotoxins are possible weapons of biological warfare. They have been implicated in some forms of cancers, abortions, allergies, kwashiorkor, immune dysfunction and deaths in humans especially in sub-Saharan Africa (Enyiukwu, 2011; Viljoens, 2013; Enyiukwu et al., 2014b).

Causal agent of anthracnose in cowpeas

Anthracnose is pan-tropical in distribution (COPR, 1981; Aveling, 2007); and that a single or multiple species of *Colletotrichum* could inflict the disease is well documented (Freeman et al., 2000). Many *Colletotrichum* species in like manner have been reported associated with anthracnose of cowpea in several regions of cowpea producing nations in Africa (Table 1).

Table 1. *Colletotichum* species reported as causing anthracnose in cowpeas in Africa

Region	Pathogen	Source
Southwest Nigeria	*Colletotrichum lindemuthianum*	COPR, 1981
Southwest Nigeria	*Colletotrichum lindemuthianum*	Amusa et al., 1994
Southeast Nigeria	*Colletotrichum lindemuthianum*	Amadioha, 2003
Southeast Nigeria	*Colletotrichum lindemuthianum*	Amadioha and Obi, 1998; 1999
Euro-Asia	*Colletotrichum lindemuthianum, Colletotrichum gloeosporioides*	Emechebe and Florini, 1997
Southwest Nigeria	*Colletotrichum destructivum*	Latunde-Dada et al., 1996; 1997
	Colletotrichum destructivum	Allen et al., 1998
Southeast Nigeria	*Colletotrichum destructivum*	Awurum et al., 2005, Awurum and Enyiukwu, 2013
Southeast Nigeria	*Colletotrichum destructivum*	Enyiukwu and Awurum, 2013
Southwest Nigeria	*Colletotrichum destructivum*	Ogu and Owoye, 2013
Southaest Nigeria	*Colletotrichum destructivum*	
South Africa	*Colletotrichum lindemuthianum var truncatum, Colletotrichum dematium, Colletotrichum frageriae.*	Aveling, 2007
South Africa	*C. dematium*	Pakeia 2006
Uganda	*C. gloeosporioides';*	Moses, 2006
South Africa	*Colletotrichum lindemuthianum, Colletotrichum dematium*	Masangwa et al., 2013

Cowpea anthracnose is considered by Masangwa et al (2013) to be initiated by both *C. dematium* (Fr) Grove var. *truncatum* and *C. lindemuthianum,* the causal agent of the disease in many bean (*Phaseolus vulgaris* L.) growing regions of the world. Subsequent articles had advanced several forms of *Colletotrichum* such as *C. dematium, C. frageriae, C. gloesporioides* as responsible for the disease in Table 1. The actual identity of the fungus responsible for anthracnose in cowpea has however, been shrouded in confusion and is a subject of much scientific debate (Adegbite and Amusa, 2008). The fungus was variously thought to be a form of *Colletotrichum lindemuthianum, C. dematium* or *C. frageriae* in several early literature (COPR, 1981; Aveling, 2007); and a recent review in the advances in cowpea research considered the fungus more appropriate to be regarded as a form of *C. gloeosporioides* (Emechebe and Florni, 1997). However, some molecular evidences from investigations by Latunde-Dada et al. (1996; 1997) in Nigeria and adopted in the review by Allen et al. (1998) now regarded the pathogen to be closely related to and strongly considered to be a form of *C. destructivum* O'Gara; which ideally attacks lucerne, tobacco and alfalfa rather than *C. lindemuthianum*. Shen et al. (2001) confirms that the rDNA of the fungus tallied with that from alfalfa and tobacco. These seemed to give the strongest impetus ascribing the identity of the causal pathogen of the disease to *C. destructivum* O'Gara (Emechebe and Lagoke, 2002). However, some authorities did not consider *C. destructivum* as an acceptable species. According to Hyde et al. (2009) such authorities thought of it as a synonym of *Glomerella cingulata* L. No clear separation or distinction in other words was observed by investigators from ITS1 and ITS2 based dendogram between *Colletotrichum destructivum and Colletotrichum linicola;* while *Colletotrichum truncatum* (Syn. C. *dematium f. truncatum*) was seen as most similar (96%) to the fungus (Shen et al., 2001). From nucleotide sequence investigation of the D2 and ITS regions of rDNA Latunde-Dada and Lucas (2007) showed however that *C. destructivum, C. truncatum* and *C. linicola* had very high similarities ranging between 97-99%. These molecular studies in addition to a combination of investigations on phylogenic relationship, morphology, infection process, intra-cellular infection structures propelled these authors therefore to propose that *Colletotrichum destructivum* O'Gara is a species aggregate; including *Colletotrichum truncatum and C. linicola*. The questions that beg to be answered in regard to the causal agent of anthracnose in cowpeas are:

1. Is cowpea anthracnose actually caused by several species of *Colletotrichum*?
2. Or that the seemingly new species of *Colletorichum* involved in the patho-system of anthracnose of cowpeas are variants, mutants, races or biotypes of a single species?

Colletotrichum capsici and *C. truncatum* were asserted cause of brown blotch a closely related disease of cowpea anthracnose with >90% and <10% incidence of recovery of these pathogens from infected tissues of cowpea respectively. However, mixed infections on the same infected plant part were recorded (Emechebe and Florini, 1997). Recent reviews by some authorities reported that *C. capsici* and *C. truncatum* is the same organism, with *C. truncatum* adopted as the name of the organism (Hyde et al., 2009; Cannon et al., 2014) in contrast to *C. capsici* preferred by Emechebe and Lagoke (2002). *Colletotrichum truncatum* is actually depicted

as a member of the clade: *Colletotrichum destructivum* (Cannon et al., 2014). Latunde Dada et al. (1996; 1997) inferred from some investigations in Nigeria that the anthracnose of cowpea is induced by *Colletotrichum destructivum* O'Gara. Masangwa et al., (2013) on the other hand reported the disease as caused by *C. lindemuthianum* var. *truncatum*. A catalogue of confusion, a plethora of uncritical taxonomy; these no doubt are. One therefore, is impelled to pose some very serious questions all the more here:

1. Is brown blotch which is without controversy a closely related disease to anthracnose of cowpeas actually a new disease or variant of the well known disease anthracnose itself: Or is brown blotch probably or possibly arising from inducement from a mutant, variant, race or biotype of *C. lindemuthianum*?
2. Is the causal agent of anthracnose actually host-specific which was the presumed building block of the numerous taxa of *Colletotrichum* species? Or that cross-infections did occur in the instances leading to the cowpea anthracnose diseases?

Urgent and well articulated works to answer these questions and unravel the correct and accurate identity of the *Colletotrichum* entity responsible for inciting anthracnose in cowpeas are suggested and pressingly warranted.

According to Than et al. (2008) some *Colletotrichum* species respond differently to various fungicide control measures. For instance *C. acutatum* is moderately susceptible to benzimidazole while *C. gloeosporioides* is highly susceptible to the fungicide. Correct and accurate identification of species will ultimately lead plant pathologists to formulate more effective and efficient anthracnose management strategies through selecting appropriate fungicides or longer lasting resistant varieties. On the other hand, it will aid governmental authorities for quarantine and regulatory purposes. This source however, explained that molecular techniques will invariably aid in accurately discriminating and naming taxonomically difficult genera such as *Fusarium, Macrosphaerella* and to a large extent *Colletotrichum*. The authors noted that molecular diagnostic approaches such as sequence analysis of the internal transcribed spacer (ITS) region (18S and 5.8S; 5.8S and 28S genes) and sequence analysis of β tubulin genes which offer comparative variability have proved useful in resolving phylogenetic relationships of *Colletorichum* species. Others are sequence analysis of introns from two genes (glutamine synthase and glyceraldehydes-3-phosphate) and MAT1-2 mating sequences which has allowed differentiation of isolates from species complexes involving *C. acutatum, C. graminicola and C. gloeosporoides* in chilli anthracnose (Dubber et al., 2003; Du et al., 2005). Sequence analysis of conserved protein coding genes such as β-tubulin and translation elongation factor 1-alpha, which contain highly variable introns, could be noted by Gueber et al. (2003) to be particularly helpful for the phylogenic examination of fungal species, including Colletotrichum species.

In a similar study for example in Thailand, *C. capsici, C. acutatum and C. gloeosporioides* implicated in anthracnose of chilli pepper were differentiated by a combined application of morphological characterization and molecular diagnostic gene sequencing based on rDNA-ITS region and β-tubuline gene studies (Than et al., 2008).

Control of cowpea anthracnose disease

Anthracnose is an important disease. Many factors including seed transmissibility of the disease, ability of the causal fungus to survive up to 22 months in the crop thrash coupled with occasional development of sclerotia by the pathogen, and lack of cost effective chemical control method are some of the constraints reported to limit effective control of the disease. In a bid to check the development and advancement of anthracnose of cowpea, several control strategies cutting across seed treatment, cultural measures, sanitation procedures, fungicide spraying and use of resistant cultivars have been employed (Melotto et al., 2000). Such cultural method involves the use of clean seeds, field sanitation whereby previous cowpea residues are deeply buried or properly destroyed by burning, cowpea rotation with non-host crops of the disease, mixed or intercropping and destruction of alternate weed hosts of the pathogens among others (Awurum et al., 2001; Than et al., 2008). These and many other cultural practices are mainly prophylactic. They may not effectively check the disease during epidemics and thus, a significant drawback (Enyiukwu et al., 2014).

Another notable control technique is the use of tolerant or resistant varieties of the crop where available. Numerous studies affirm that resistance to *Colletotrichum lindemuthianum* induced anthacnose for example in common bean is controlled by major genes acting singly, as duplicate or complementary factors, or as members of an allelic pair (Melotto et al., 2000). Cowpea cultivars such as IT93K-452-1, IT86D-719 and IT89K-288 developed by the IITA, Ibadan Nigeria have been reported from cropping system and varietal interaction trials in Southeast Nigeria to be tolerant to brown blotch a closely related disease of cowpeas (Awurum, 2014) while TVX 3236 according to Emechebe and Florini (2007) is out-rightly resistant to anthracnose of the crop. Nevertheless, many pathogenic *Colletotrichum* species are reported to be highly variable making resistance to the disease(s) to be only temporary. This thus constitutes a major disadvantage of use of resistant cultivars in managing the disease (Mohammed, 2013).

Biological control of the pathogen *C. destructivum* has been attempted *in vitro* with *Pseudomonas forescens* and *Bacillus subtilis. P. florescens* was more antagonistic and antagonists inhibited the fungus

effectively in the trial than *B. subtilis* (Akinbode and Ikotun, 2008). Dressing common bean seeds with culture isolates of *Trichoderma haziarum, T. viride* and *Gliocladium virens* for about 15 minutes before sowing restricted infection by *C. lindemuthianum* had increased the bean seed germination (Mohammed, 2013). An isolate of *T. haziarum* (T3) harvested from the rhizosphere of some crops impeded *C. dematium* by 89.44% in a trial better than the synthetic fungicide Vitavax-200. In like manner, On-farm biological control of anthracnose of cowpea has been demonstrated to be feasible also with *Trichoderma viride* (Adegbeti and Amusa, 2008). In a similar vein, Jagtap et al. (2012) demonstrated the efficacy of *T. viride, T. hamatum* and *P. flourescens* against *C. truncatum* implicated in causing anthracnose and pod blight in soybean. These treatments reduced the mean colony diameter of the fungus by 79.4%, 73.74% and 69.31% respectively in culture studies. Bio-agents have been presumed to produce toxic volatile metabolites (Mohammed, 2013). Competition for space and nutrients, antibiosis, direct parasitism; as well as rapid and effective colonization of the seeds, rhizosphere or phyllosphere to the disadvantage of the invading pathogens; have been suggested for their activity. However, formulating bio-control agents in such a way that low-input growers can easily use them has been retarding its adoption on a broad scale (Suprapta, 2012; Enyiukwu et al., 2014b).

Another important management strategy of the disease is by chemical fungicides. Chemical control of anthracnose is very effective and fast acting; and such interventions are administered through seed or foliar sprays. An assessment by Shovan et al. (2008) on radial elongation and dry weight of *C. dematium* with Tilt-250 EC (100, 200, 400ppm) showed that the fungicide completely impeded the fungus while Vitavax-200 restricted these attributes of the pathogen by 77.41% and 83.45% respectively. Chemicals such as benomyl, carbendazim, thiophanate--methyl, thiobendazole, Quadric, Fint and Cabrio are registered and used in commercial scales against cowpea anthracnose. For instance, Mohammed (2013) reported that seed-dressing bean seeds with carbendazim 2g/kg seeds, followed by 0.5kg/ha of foliar application of the fungicide in the field reduced the incidence and severity of anthracnose in bean (*Phaseolus vulgaris*).

Control of cowpea anthracnose using synthetic chemicals

Synthetic pesticides save labour in crop production. They enforce instant effects especially in therapeutic interventions. In high value crops such as avocados, mango and legumes they make possible production of crops with pleasant appearances and lend strong contribution to yield increases and cultivation of large hectares of monoculture farmlands by small numbers of people (Enyiukwu, 2011). For instance, benomyl and carbendazim reduced losses due to anthracnose from greater than 40% to less than 5% in cowpea (Emechebe and Florinin, 1997). In India, anthracnose of mung bean induced by *Colletotrichum truncatum* was effectively checkmated with Baristin (0.05%), Benlate (0.10%) and Topsin (015%) (Singh, 2001). Similar studies in Uganda by Edema and Adipala (1994) confirmed that the fungicide Mancozeb improved cowpea production by reducing brown rust (*Uromyces vignae*) impact by 67%.

However, these successes notwithstanding, in glorious and harmful drawbacks associated with synthetic pesticides used in crop production such as residues in crops and the environment, mammalian toxicity, effects on non-target species, extinction of edaphons, and poisonous influences on human organs and immune systems abound in literature (Enyiukwu and Awurum, 2013; Enyiukwu et al., 2014a). Placental transfers account for most of the exposures of developing fetuses to pesticide residues while lactation for neonates. In like manner, exposures in adults occur through inhalation, ingestion of contaminated foods and water; and by skin absorption (Gourounti et al., 2008).

According to Enyiukwu (2011), even low levels of synthetic pesticides have been found detrimental to the health of human fetuses, neonates and young children. Thus lending strong support to earlier reports from the British Medical Association (BMA) (1992), that long term intake or intermittent doses of pesticides could cause allergies, cancer, mutagenecity, neurotoxicity and anti-immune effects etc. Besides being implicated in human male sterility, pesticides could cause deaths (Taiga 2009; 2011). Two million people are believed to be exposed to agricultural pesticide poisoning every year while 10% of victims actually die from such exposures (Enyiukwu and Awurum, 2013). In the view of Gourounti et al. (2008) majority of these effects on human health may be due to the ability of synthetic pesticides especially organochlorines to impair or alter the levels of certain hormones, enzymes, growth factors and neuro-transmitters; and to induce key genes (cytochrome p450 1A1 gene) involved in the metabolism of steroids and xenobiotics.

Synthetic fungicides encourage build up of agricultural pest species. It has been reported that excessive and inappropriate application of broad-spectrum synthetic pesticides has led to 150 pathogens to exhibit resistance to fungicides (Enyiukwu and Awurum, 2013). Reports from India showed that strains of *Colletotrichum lindemuthianum* the incitant of bean (*Phaseolus vulgaris*) anthracnose are resistant to powerful and effective fungicides including carbendazim, benomyl and thiophanate–methyl (Emechebe and Florini, 1997). To prosecute the war against crop attacking fungi with synthetic chemicals costs the world 4.2 billion dollars annually (Enyiukwu, 2011). Huge expenditure you might say. Therefore a cheap user and ecologically friendly alternatives that can be integrated with the low-input farming systems of sub-Saharan Africa are without doubt welcome (Awurum and Enyiukwu, 2013; Enyiukwu and Awurum, 2013a). Phyto-pesticides are one of such viable alternative plant disease control strategies (Enyiukwu and Awurum, 2011; 2012).

Use of plant-derived pesticides in cowpea anthracnose control

Records show that plant-derived pesticides have been used since 1763 in plant protection (Enyiukwu, 2011). In the recent times, reports of the effectiveness of plant-derived pesticides for control of farmer-saved seeds, stored grains, root rots, anthracnoses and other field fungal diseases of cowpea, French bean and various legumes have been documented (Mogle, 2013; Awurum et al., 2014). In chillis reported, Than et al. (2008) *in vitro and in vivo* studies showed that crude extracts from rhizome, leaves and creeping branches of *Acorus calamus* (sweetflag), *Cymbopogon martii* (palmorosa) oil, *Ocimum sanctum* leaf, and *Azadirachta indica* (neem) oil restricted the growth of the *Colletotrichum spp* involved in anthracnose of the crop with *A. calamus* being the most fungitoxic. Aqueous extract of *Haplophyllum sieversii* retarded the growth of *Colletotrichum fragariae, C. gloesporioides and C. acutatum*. Bioassay guided fractionation of this specimen resulted in the isolation of bioactive alkaloids

Cowpea anthracnose is seed-borne and seed transmissible. Awurum *et al.* (2005) reported that extracts of *Uvaria chamae* were effective as seed-dressing phytochemical against the disease. This view was supported by both Awurum and Ucheagwu (2013) and Awurum and Enyiukwu (2013) who found extracts of some tropical spice plants (*Piper guineense, Xylopia aethiopica, Monodora myristica*) strongly effective as storage protectants and seed-dressing phytochemicals against anthracnose. Corroborative studies from Ogu and Owoye (2013) in Southwest Nigeria also demonstrated the efficacy of pesticides derived from *Cyathula prostata* and *Diodia scandens in vitro* against the disease. These extracts effectively checked not only the mycelial growth but also the spore germination of these pathogenic fungi. Also in Western Nigeria, glasshouse evaluations by Owolade and Osikanlu (1999) indicated that brown blotch (*C. capsici*) was inhibited by extracts from *Acalypha ciliata* and *Ocimum gratissimum*. In a similar study conducted in Southeast Nigeria, *Carica papaya* and *Piper guineense* seeds and roots-derived pesticides retarded the development and growth of *Colletotrichum destructivum* induced anthracnose in the same crop both in culture and glasshouse (Enyiukwu and Awurum, 2013).

Obi and Bariuso-Vargus (2013) demonstrated that *Azadirachta indica, Cymbopogon citratus, Ocimum gratissimum* and *Xylopia aethiopica* proved effective in inhibiting *Colletotrichum destructivum* both *in vitro* and *in vivo*; with *X. aethiopica* and *A. indica* being the most toxic to the fungus. On the other hand, leaf extract of *Agemone mexicana, Semecarpus anacardium, Cassia fistula* and *Tephrosia purpurea* in an in vivo investigation showed fungitoxic attributes against *C. destructivm* in cowpea seeds. However, observations revealed that *Nicotinia tabacum* checked the growth of this fungus in culture more than other assayed extract (Akinbode and Ikotun, 2008).

Masangwa et al. (2013) demonstrated from culture and greenhouse studies that aqueous extracts of *Carica papaya* and *Syzygium cordatum* were actively toxic against the anthracnose agent *C. lindemuthianum* with minimum inhibitory concentration (MIC) of 1.5mg/mL. So did *S. cordatum, Allium sativum* and *Chlorophytum sp.* to *C. dermatum* with MIC of 3.13, 6.25 and 12.5 mg/mL respectively. An evaluation of the fungitoxic effects of garlic, onion and neem *in vitro* in Bangladesh revealed that *C. dematium* the agent responsible for anthracnose of soybean was most sensitive to garlic (Shovan et al., 2008). Makherjee et al. (2011) found that the anthracnose pathogen *C. gloeosporoides* was sensitive to garlic (*A. sativum*). The phyto-pesticide retarded the fungus by 60.45%. *C. lindemuthianum* was sensitive to neem seed extract in an evaluation reported Mohammed (2013). It restricted the conidial germination and mycelia elongation of the fungus while *Lawsonia inermus* as seed treatment and field sprays improved seedling emergence and impeded development of anthacnose of the bean plant in the field. Toxicities of indigenous spices (*A. sativum, C. citratus, O. gratissimum, X. aethiopica* and *Afromonium meleguata*) were recently reported. According to Adesegun et al. (2011) *C. capsici* inciting anthracnose which could result in yield loss of 95% in pepper were sensitive to these spice extracts with *O. gratissimum* being the most toxic to the fungus. In a parallel study, Onyeani and Osunlaja (2012) demonstrated that *Annona squamosa, A. indica* and *Vernonia amygdalina* impeded the development and severity of anthracnose lesions incited by *C. gloeosporoides* on mango. The pathogen was most sensitive to *A. squamosa* giving mean disease index of 0.27 which was statistically superior to the disease index of 0.33 obtained from benomyl.

Field studies by Amadioha and Obi (1999) and Amadioha (2003), established that natural products derived from *Piper nigrum, Ocimum sanctum* and *Citrus lemon* significantly reduced the incidence and severity of anthracnose of cowpea. Eno (2011) working also in the field, noted that extracts from *Anacardium occidentalis* potently checked the development and spread of anthracnose induced by *C. lindemuthianum* against cowpea plants. In several instances these plant-derived preparations were found superior in fungitoxicity to benomyl against assayed fungal pathogens of anthracnose of the crop both in culture and in the field (Amadioha and Obi, 1998; Awurum and Ucheagwu, 2013).

Integrated disease management (IDM) approach

Because no one strategy of disease management is effective in all instances, a holistic approach whereby these control measures may be applied in sequences or in combinations to maximize their effects is advocated (Hill and Waller, 1990; Ogu and Owoye, 2013). Such holistic method -- integrated disease management (I D M) –

involves environment-sensitive approach to disease management, in which case synthetic chemical interventions are considered as last resorts to a given disease condition. In IDM, the disease control methods are carefully selected in such a way that the disadvantage of one intervention is offset by another in the sequence. This option has been adjudged the best approach to checking anthracnose disease in legumes (Mohammed, 2013). For instance, the soil could be incorporated with ash, leaves or twigs of some tropical plants such as *Chromolaena odoranta, Carica papaya,* etc. (Onwusiri, 2000; Ononuju and Kpadobi, 2008). These materials reportedly release toxic compounds that retard activities of *Meloidogyne* nematodes which gall cowpea roots predisposing them to fungal attacks.

Clean cowpea seeds obtained by dressing with isolates of bio-antagonists such as *Trichoderma viride* or phyto-extracts such as neem extracts or in some cases certified resistant cultivars where available; should then be cropped using good agronomic practices (GAPs). GAPs involving optimum plant density make for good air circulation in the farm and reduce humidity around the crop canopy (Mohammed, 2013). Furrow irrigation instead of sprinklers should be used also to disallow build up of humidity that could help to initiate or exacerbate the disease condition; while proper and timely weeding to destroy alternate weed-hosts of the disease should be emphasized (Awurum, 2001). In low-input agriculture, intercropping or rotating cowpea with non-host crops such as cereals or cassava should be adopted over sole or continuously cropped cowpea (Awurum, 2014). This could then be followed by protectant sprays of phyto-extract preparations such as neem or black pepper 1-2 times before flowering of the crop (Amadioha, 2003; Oparaeke, 2007: Enyiukwu and Awurum, 2013b). However, how best to manage the disease, is largely determined by the factors of farm economy, consumer demands (organically or conventionally produced foods) and environmental regulations on pesticides as stipulated by national regulatory agencies.

CONCLUSION

Cowpeas are important dietary components of African and other tropical cuisines. Anthracnose no doubt is one of the major constraints to its efficient production especially in Nigeria. Managing this yield and grain quality reducing disease involves a number of strategies. However the efficacy of control strategy(ies) is/are dependent on proper identification of the causal agent of the disease. In cowpeas, identification of the causal agent has been largely done by morphological characteristics and assumption of host-partner specificity of the fungus. These have spelt far-reaching problems to sustainable resistant cultivar development. In the light of the fact of reports of several species being involved in the patho-system of *Colletotrichum*, it is therefore strongly necessary that in addition to classical identification, we re-evaluate, and characterize the fungus causing cowpea anthracnose by molecular techniques. This will not only aid in good control techniques of the disease, help in stemming the tide of development of resistance to known synthetic antifungal agents, aiding plant breeders in developing resistant cultivars with sustained immunity against the disease; but also in quarantine and legislative controls.

Managing the disease for sustainable cowpea grain production in the overall, should be holistic; taking into account integrated approach since no one control strategy is grossly effective at all times and conditions. Besides eco-disruption, use of standard fungicides may not be economically feasible in many low-input farming systems of sub-Saharan Africa. Therefore cowpea health management for sustainable grain production in such farming systems should emphasize the use of plant extracts in IDM programmes in the stead of synthetic chemicals.

SUGGESTIONS

Considering the importance of cowpeas in Nigeria and Africa at large, advancements in cowpea researches in the coming years should focus on and take into account the following:

- Integration of phyto-extracts in managing anthracnose of cowpeas in sub-Saharan Africa (SSA). Phyto-extracts are eco-compatible; and present multiple bio-active agents against a pathogen such that development of resistance to them is most unlikely. Therefore incorporating them into plant health management of cowpeas in SSA will not only enhance sustainable cowpea grain production, aid in the delay of development of resistance to powerful fungicides, but in addition will reduce expenditure of scarce foreign exchange on importation of synthetic fungicides.
- Molecular re-examination of the cowpea anthracnose fungus is urgently warranted and should be thoroughly pursued. There is so much confusion about the taxonomy of this fungus; and until this confusion is adequately cleared, efficient and effective control of the disease especially with chemical agents will remain a mirage, while development of cultivars with appreciable sustainable resistance to the fungus will be elusive.

COMPETING INTERESTS

The authors declare no potential conflict of interests.

AUTHORS' CONTRIBUTIONS

D N Enyiukwu designed and drafted the manuscript. A N Awurum and C C Ononuju assisted with critical revision of the manuscript; while J A Nwaneri contributed in drafting the manuscript. All authors gave their final approval to the submitted manuscript.

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