

## Molecular Identification of Fungal Species Associated With Mango Anthracnose in Southwest Nigeria

Onyeani C. Awa<sup>1</sup> & O. Oguntade<sup>2</sup>

### Abstract

*Colletotrichum* species have been implicated as causal agents of fruit anthracnose in mango in Nigeria but due to the difficulties in distinguishing fungal isolates by their morphological characteristics using the traditional taxonomic systems, a quick and an unambiguous molecular test based on the amplification of fragments of the internal transcribed spacer (ITS1) region to distinguish *Colletotrichum* isolates from other pathogenic fungi was conducted in the speciation of 7 fungal species frequently found associated with anthracnose fruit rot in mango in Southwest Nigeria. The analysis of the nucleotide sequences of the ribosomal DNA (rDNA) fragment showed sufficient variability to clearly classify the 7 fungal species isolated from symptomatic mango fruits into *Colletotrichum gloeosporioides*, *Fusarium verticillioides* and *Lasiodiplodia theobromae*. Chromatogram produced from their rDNA data confirmed the identities of the fungal isolates. *Colletotrichum gloeosporioides* was identified to be the fungus responsible for anthracnose in mango in Southwest Nigeria.

**Keywords:** Chromatogram, *Colletotrichum gloeosporioides*, Fungal Species, Internal Transcribe Spacer, Ribosomal DNA.

### 1. Introduction

Mango (*Mangifera indica* L.) a member of the *Anacardiaceae* family, is one of the tropical crops grown abundantly throughout the southern humid Nigeria. The crop is a host to a large number of pathogens such as bacteria, fungi and viruses (Diedhiou *et al.*, 2007; Kumar *et al.*, 2007). Fungal pathogens are frequently encountered on rotting mango and are the major agents of fruit rot after harvest (Diedhiou *et al.*, 2007). Studies from several parts of the world where mango is grown have shown that anthracnose is the most devastating fungal disease which does not only reduce mango fruit yield but also render marketable fruits worthless. In Nigeria, mango production and exportation is greatly limited due to post harvest rot of fruits associated with anthracnose and over 30% of harvestable fruits are lost annually because of fruit abortions and abscission caused by Anthracnose (Onyeani *et al.* 2012).

While researchers from Sri Lanka, Australia, Panama, Phillipines, Thailand, Brazil and India implicated *Colletotrichum acutatum* J.H.Simmonds (Jayasinghe and Fernando, 2009; Fitzell and Prakash as cited in Akem, 2006) and recently *Colletotrichum karstii* and *Colletotrichum asianum* Prihastuti, L. Cai & K.D. Hyde 2009 (Index Fungorum) in Brazil and Sri Lanka respectively (Lima *et al.* 2013; Krishnapillai and Wijeratnam, 2014) as being responsible for anthracnose in mango. Majority of researchers from other countries have implicated *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. 1884 (Index Fungorum) as the fungal pathogen responsible for anthracnose in mango (Wharton and Dieguez-Uribeneondo, 2004; Than *et al.*, 2008; Kim *et al.*, 2008; Sangeetha and Rawal, 2009; Jayasinghe and Fernando, 2009).

<sup>1</sup> Department of Crop production, College of Agricultural Sciences, Olabisi Onabanjo University, Ayetoro Campus, Ogun State, Nigeria. [awa.onyeani@yahoo.com](mailto:awa.onyeani@yahoo.com), Cell Phone: +234803300529

<sup>2</sup> Germplasm Health Unit, International Institute of Tropical Agriculture, Ibadan, Nigeria

In Senegal, Diedhiou *et al.* (2007) reported the main role played by *C. gloeosporioides* in causing anthracnose of mango in the Niayes area of Senegal. Okigbo and Osuinde (2003) also reported the role of *C. gloeosporioides* in fungal leaf spots of mango in Nigeria though they did not report about mango fruit rotting which is the main economic effect of mango anthracnose.

Invariably, it has been very difficult to separate *C. gloeosporioides* and *C. acutatum* responsible for causing anthracnose on mango by traditional taxonomical methods, probably because both species are morphologically very similar. This could be the reason why there are conflicting reports about the etiology of mango anthracnose in Nigeria. Nigeria Plant Quarantine Service (NPQS) in the Department of Nigeria Agricultural Quarantine Service in 2001 listed *C. gloeosporioides* as the causal agent of mango anthracnose in Nigeria whereas Center for Agricultural Bioscience International (CABI) in 2007 listed *C. acutatum* and the perfect stage of *C. gloeosporioides* (*Glomerella cingulata*) as the species that are infesting mango in Nigeria. Suffice is it to say that, traditional approach to fungal identification sometimes causes wrong classification of fungal species resulting sometimes to practical problems in fungal disease management.

In the last few decades, molecular approaches employing the Polymerase Chain Reaction (PCR) has been found to provide sensitive means of characterizing and classifying plant pathogenic fungal species (Etebu and Osborn, 2012). In this study, molecular approach was adopted in a view to using region of DNA sequence best suitable for accurate identification of the fungal pathogens associated with anthracnose in mango fruits. This work will no doubt enhance the management of mango fruit anthracnose in Nigeria.

## **2. Materials and Methods**

### **2.1. Tissue Preparation**

Pure cultures of 7 fungal species isolated from symptomatic mango fruits were each grown in Potato Dextrose Broth and filter to get 50mg-100mg mycelia.

### **2.2. DNA Extraction Protocol**

100mg of different fungal mycelia was mixed separately with 1ml of DNA Extraction Buffer (DEB) containing proteinase K (0.05mg/ml) and macerate in a sterile mortar following Byrd *et al.* (1990) method slightly modified. The resultant extract was dispensed into 1.5ml eppendorf tube. 50 $\mu$ l of 20% Sodium Dodecyl Sulphate (SDS) was added into the tube and incubated in a water bath at 65°C for 30min. The tubes were allowed to cool to room temperature before adding 100 $\mu$ l of 7.5M Potassium Acetate and centrifuging at 13000rpm for 10min. The supernatants were transferred into new freshly autoclaved tubes added with 2/3 volumes of cold Isopropanol and Isopropyl alcohol. The tubes were gently inverted 3-5 times before incubating at -20°C for 1 hour. Thereafter, the tubes were centrifuge at 13000rpm for 10min and the supernatant discarded. 500 $\mu$ l of 70% ethanol was added into each tube and centrifuged for 5min at 13000rpm before carefully discarding the supernatant with the DNA pellet intact. Traces of ethanol were removed before drying the DNA pellets at 37°C for 10-15 min. The DNA pellets were resuspended in 50 $\mu$ l of Tris-EDTA (TE) buffer.

### **2.3. PCR Amplification of 5.8S rRNA gene and Internal transcribed spacer region**

ITS universal primer set which flank ITS1, 5.8S (ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3' and ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3') described by White *et al.* (1990) were used to amplify regions of the RNA polymerase I transcription units (5.8S and ITS) gene in all the 7 isolates in this study with amplicons size of 550bp. The PCR conditions included a cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles (each cycle comprised of 30s) of denaturation at 94°C. Primers annealing was done at 55°C for 30s, followed by 1.5min extension at 72°C and a final extension for 7min at 72°C. The ITS gene segment amplified from the isolates were sequenced in both direction from purified products.

#### **2.3.1. Purification of PCR products**

Two volumes of 95% ethanol (stored at -20°C) were added to each tube and incubated at -80°C for 20min. The tubes were thereafter spinning at 13,000rpm for 10min and ETOH removed from the tubes. 500 $\mu$ l of 70% ethanol (stored at -20°C) was added to each tube and spin at 13,000rpm for 5min. The pellet was air-dried for few minutes and the samples re-suspended in 20 $\mu$ l sterile distilled water.

### 2.3.2. DNA sequencing

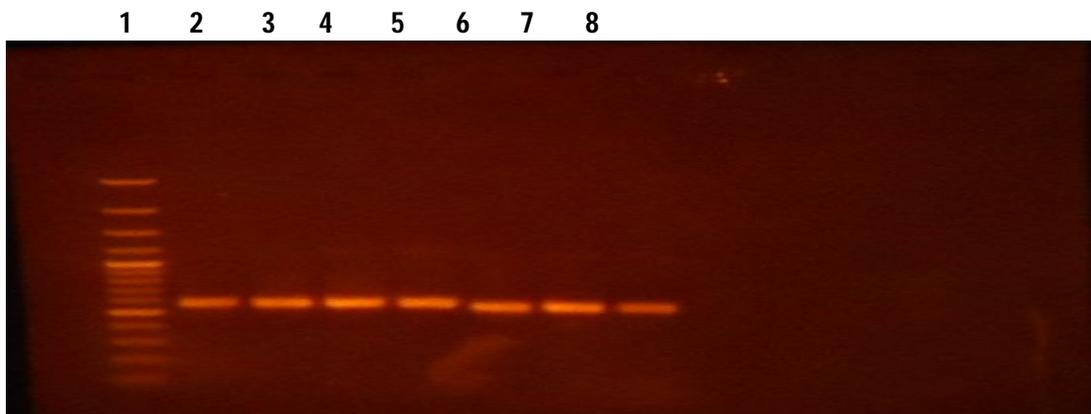
Purified post PCR DNA samples were sequenced on both strands using electrophoresis on an ABI automated DNA capillary sequencer. Sequences generated were analysed using sequencing analysis software BioEdit 7.2.5 (Hall, 1999). Isolates identities were confirmed using NCBI/BLAST ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov))

### 2.4. Pathogenicity Test

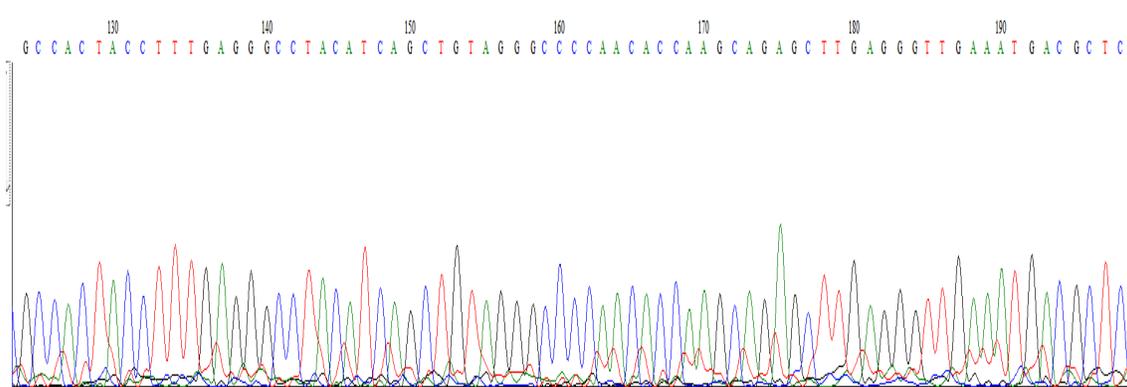
Twelve healthy freshly harvested green matured mango fruits were surface sterilized by swabbing with 70% ethanol and later with 1% NaOCl solution. The fruits were inoculated with spore suspension of *C. gloeosporioides*, *L. theobromae* and *F. verticillioides* prepared following the procedure of Sivakumar *et al.* (1997). Isolation and re-isolation of pathogens from fruits that showed symptoms of anthracnose after 5 days of incubation was carried out following Koch's postulate for proof of pathogenicity as described by Schumann and D'Arcy (2006)

### 3. Results

The DNA extracted from all the isolates were successfully amplified with fungal species universal primer ITS1 and ITS4 primer. Amplicon size of PCR product was 550bp long (Figure 1). The analysis of the nucleotide sequences of ITS1 region of the isolates and the chromatogram (Figure 2) produced from their rDNA data showed that 4 out of the 7 isolates were *C. gloeosporioides*, 2 were identified as *Fusarium verticillioides* while 1 isolate was identified as *Lasiodiplodia theobromae*. Pathogenicity tests on the identified fungal pathogens conducted showed that only *C. gloeosporioides* isolates reproduced anthracnose symptom typical of the original symptom observed on the symptomatic mango fruits from which they were originally isolated.



**Figure 1:** Agarose gel electrophoresis of genes amplified by PCR using ITS1 and ITS4 from DNA extracted from fungal Isolates.



**Figure 2:** Chromatogram of rDNA of fungal isolates from mango. Red is Thymine; Blue – Cytosine; Green – Adenine and Black – Guanine.

#### 4. Discussions

Traditionally, isolates of *Colletotrichum acutatum* have so often been wrongfully identified as *Colletotrichum gloeosporioides* (Wharton and Dieguez-Uribeneondo, 2004) and many of *Colletotrichum* species are unable to be reliably distinguished due to complexes of the species (Weir, Johnson and Damm, 2012). In this study, a fragment was amplified by PCR from fungal isolates with universal primer specific for distinguishing fungal species. The nucleotide sequences of the ITS1 region of *Colletotrichum* species differed considerably from *C. acutatum* reference isolate, implying that all the *Colletotrichum* species isolated were *C. gloeosporioides* and not *C. acutatum*. This result agrees with Nigeria Plant Quarantine Service (2002) which listed *C. gloeosporioides* as the causal agent of mango fruit anthracnose in Nigeria although there were no representation of pathogen isolation and identification in their study. The identification of all the 4 fungal isolates that belonged to *Colletotrichum* genus to be *C. gloeosporioides* and the pathogenicity test that confirm the pathogenesis of *C. gloeosporioides* implies that, *C. gloeosporioides* was the pathogen responsible for anthracnose in mango in Nigeria and not *C. acutatum* as listed by CAB International (2007).

In addition, this result is consistent with reports by several workers implicating *C. gloeosporioides* as the causal agent of anthracnose in mango (Wharton and Dieguez-Uribeneondo, 2004; Than *et al.*, 2008; Kim *et al.*, 2008; Sangeetha and Rawal, 2009; Jayasinghe and Fernando, 2009). This study identified *F. verticillioides* and *L. theobromae* to be associated with anthracnose of mango, implying that other fungal species may act in synergy with *C. gloeosporioides* to cause fungal disease of mango. Several workers including Johnson (2008), Rawal (1998) and Sangchote (1991) had implicated other fungal species in earlier reports, to be responsible for postharvest diseases of mango associated with fruit rotting during ripening worldwide. Okereke *et al.* (2010) also reported the isolation of these fungal species from infected mangoes in their study.

#### 5. Conclusion

This identification method clearly in real time classified the 7 fungal species isolated from mango fruits. In addition, *C. gloeosporioides* was found to be the fungus responsible for anthracnose of mango in Southwest Nigeria.

#### Acknowledgement

We wish to express our sincere gratitude to Dr. O.S. Sosanya for his encouragement and guidance during this study.

#### References

- Akem, C.N. 2006. Mango Anthracnose Disease: Present Status and Future *Research Priorities*. *Plant Pathology Journal* 5(3): 266 – 273.
- CAB International 2007. Pests excluded from Phytosanitary measure assessments because they already occur in Nigeria. Retrieved December 13, 2008, from CABI Crop Protection Compendium software.
- Diedhiou, P.M., Mbaye, N. Drame, A. & Samb, P.I. 2007. Alteration of post harvest diseases of mango *Mangifera indica* through production practices and climatic factors. *African Journal of Biotechnology*, 6(9): 1087 -1094.
- Etebu, E. & Osborn, A. M. 2012. Molecular approach to plant fungal identification in agricultural soils. *Nigerian Journal Plant Protection*, 26: 18 -34.
- Hall, T.A. 1999. BioEdit: A User-friendly biological sequence alignment editor and analysis program for window 95/98/NT, *Nucl. Acids Symp. Ser.* 41: 95 – 98.
- Jayasinghe, C.K. & Fernando, T.H.P.S. 2009. First Report of *Colletotrichum acutatum* on *Mangifera indica* in Sri Lanka. *Cey. Jour. Sci. (Bio. Sci.)* 38(1): 31 – 34.
- Johnson, G.I. 2008. Status of mango post harvest disease management R and D: Options and solutions for the Australian mango industry. *Horticulture Australia final report for project MG08017*. *Horticulture Australia* p.130
- Kim, J.K., Park, S., Choi, W., Lee, Y. & Kim, H.T. 2008. Characterization of *Colletotrichum* Isolates Causing Anthracnose of Pepper in Korea. *Plant Pathol. Jour.* 24(1): 17 – 23.
- Krishnapillai N. & Wijeratnam, R.S.W. 2014. First Report of *Colletotrichum asianum* causing anthracnose on Willard mangoes in Sri Lanka. *Plant Disease Report*. [Online] Available: <http://dx.doi.org/10.5197/j.2044-0588.2014.029.001> (Accessed January, 20, 2014)
- Kumar, A.S., Reddy, N.P.E., Reddy, K.H. & Devi, M.C. (2007). Evaluation of Fungicidal Resistance among *Colletotrichum gloeosporioides* Isolates Causing Mango Anthracnose in Agri Export Zone of Andhra Pradesh, India. *Plant Patho. Bull.* 16: 157 – 160.

- Lima, N.B., M. Marques, S. Michereff, M. Morais Jr., M.A. Barbosa, & M.P.S. Camara, 2013. First Report of Mango Anthracnose Caused by *Colletotrichum karstii* in Brazil. *Plant Disease*. [Online] Available: <http://dx.doi.org/10.1094/PDIS-01-13-0002-PDN> (Accessed April 4, 2013)
- Nigeria Plant Quarantine Service 2001. Check-List of Plant Pests in Nigeria. NPOS Publ. Ibadan Univ. Printrty, Ibadan, Nigeria.
- Okereke, V.C., Godwin-Egein, M.I. & Arinze, A.E. 2010. Assessment of Postharvest Rot of mango at Different Stages of market in Port Harcourt, Nigeria. *Int'l Jour. Curr. Res.*,11:006 – 010.
- Okigbo, R.N. & Osuinde, M.I. 2003. Fungal Leaf Spot Diseases of Mango (*Mangifera indica* L.) in Southeastern Nigeria and Biological Control with *Bacillus subtilis*. *Plant Protection Sciences*, 39(2): 70 – 77.
- Rawal, R.D. 1998. Management of Fungal Diseases. In: Tropical Fruits. In Arora, R.K. and V. Ramanatha Rao (eds). Tropical Fruits in Asia: Diversity, Maintenance, Conservation and Use. Proceedings of the IPGRI-ICAR-UTFANET Regional Training Course on the Use of Germplasm of Tropical Fruits in Asia held at India Institute of Horticultural Research, 18 – 31 May, 1997, Bangalore, India.
- Sangchote, S. 1991. *Botryodiplodia* stem end rot of mango and its control. *Acta Horticulturae* 291: 296 – 304.
- Sangeetha, C.G. & Rawal, R.D. 2009. Temperature Requirement of Different Isolates of *Colletotrichum gloeosporioides* Isolated from Mango. *American-Eurasian Journal of Scientific Resources* 4(1): 20 – 25.
- Schumann, G.L. & D'Arcy, C.J. 2006. Essential Plant Pathology. APS Press, St. Paul, Minnesota USA. Pp 8 – 10.
- Sivakumar, D., Wijeratnam, R.S.W., Wijesundera, R.L.C. & Abeysekera, M. 1997. Post-harvest Diseases of Rambutan (*Nephelium lappaceum*) in the Western Province. *Jour. Natn. Sci. Coun. Sri Lanka*, 25(4): 225 – 229.
- Than, P.P., Prihastuti, H., Phoulivong, S., Taylor, P.W.J. & Hyde, K.D. 2008. Chilli anthracnose disease caused by *Colletotrichum* species. *Jour. of Zhejiang Univ. Sc.* Vol. 9 No. 10, pp. 764 – 778. [Online] Available: <http://www.zju.edu.cn/jzus/2008/B0810/B081003.htm> (June 7, 2009)
- Wharton, P.S. & Dieguez-Uribeondo, J. 2004. The biology of *Colletotrichum actatum*. *Anales del Jardin Botanico de Madrid* 61(1): 3 – 22. [Onlin] Available: [www.rjb.csic.es](http://www.rjb.csic.es) (Accessed March 20, 2009)
- White, T.J., Bruns, T., Lee S. & Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, Y.J.) Aced. Press Inc., San Diego, California pp 315 - 322
- Weir B.S., Johnston P.R. & Damm U. 2012. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology*, 73: 115-180. [Online] Available: <http://dx.doi.org/10.3114/sim0011> (Accessed March 19, 2014)