Occurrence of two strains of *Colletotrichum siamense* on *Jatropha curcas* leaves

Nkechi Gloria Ogbuji and Anthony Eromonsele Ataga

Department of Plant Science and Biotechnology, University of Port Harcourt, Port Harcourt, Rivers State, Nigeria.
Corresponding author's email: nkechi.ogbuji@uniport.edu.ng

Abstract

Jatropha curcas L., a plant of the great economic importance, is susceptible to attack by various microorganisms and the correct identification of these microbes is necessary for the development of sustainable disease prevention. This study aimed at identifying the fungi associated with the leaves of *J. curcas* collected from Aluu in Rivers State, Nigeria. Fungi were isolated using microbiological conventional culturing method and identified using molecular method. Genomic Fungal DNA was obtained using Zymo Fungal/Bacterial DNA Miniprep Kit. The internal transcribed spacer (ITS) region of isolates was amplified using universal ITS primers. BLAST search was carried out on National Centre for Biotechnology Information (NCBI) database and the species identity of the fungal isolates associated with *J. curcas* revealed that the organisms were two different strains of *Colletotrichum siamense*. Phylogenetic tree revealed the relationship among the two strains, other strains/isolates of *C. siamense* and other species of *Colletotrichum* deposited in the GenBank. The sequences of the isolates were deposited in GenBank under the accession numbers: OQ941591 for strain RCBBR_AEANK17 and OQ941592 for strain RCBBR_AEANK18. The use of molecular method for the identification of fungi gives accurate result and identifies organisms even to the strain level. This is because identification is based on the genetic constitution of organisms

Keywords: *Colletotrichum siamense*, DNA extraction, Fungi, *Jatropha curcas*, Phylogeny.

Introduction

Jatropha curcas L., a perennial plant of the family Euphorbiacae, is of great interest to Scientists because of its potential as a source of biodiesel; a renewable source of energy that is safer than fossil fuel (Achten *et al*., 2008; Yahuza *et al*., 2020). It is native to Central and South America (Yahuza *et al*., 2020). It is a perennial, monoecious small tree or shrub that grows up to 6 m high. Shrubs begin to mature at 4 to 5 months and reach full productivity at about three years. The female flowers out-numbers the male ones. It is a tropical plant that survives low or high rainfall regimes. It is cultivated as a hedge crop or as a commercial crop (Oliver, 1960). It grows at altitude between 0 to 500 m. The mean annual rainfall for its growth is about 300 to1000 mm or more. The plant grows well both in the tropics and subtropics. It can adapt to harsh conditions, the mean annual temperature for its growth is between 20 to 28 ºC, but it can grow at temperature of 15 to 40 °C (Kumar and Sharma, 2008). Well drained soils and good aeration favor its growth but it can also grow on poor dry soils or on soils with low nutrient content (Openshaw, 2000; Orwa *et al*., 2009). Root formation is reduced when plants are cultivated on heavy soils. *J. curcas* is tolerant to drought and can also withstand slight frost (Orwa *et al*., 2009). Several biologically active compounds have been isolated from *J. curcas*, which make it a potential medicinal plant. The plant is used by locals as a traditional medicine to cure and manage various diseases in Asia, Latin America and Africa, and is also used as an ornamental plant (Félix-Silva *et al*., 2014). It is also used as lubricants and in the production of soaps and cosmetics. Extracts from this plant have been reported to exhibit antiviral activity against HIV (human immunodeficiency virus) (Dahake *et al*., 2013).

There have been several reports of diseases affecting *J. curcas*. These diseases include root rot caused by *Rhizoctonia bataticola* (Kumar *et al*., 2011), collar rot and root rot caused by *Lasiodiplodia theobromae* (Latha *et al*., 2009), black rot caused by *Botryosphaeria diplodia* (Rao *et al*., 2011), inflorescence blight caused by *Alternaria alternata* (Espinoza-Verduzco *et al*., 2012), and anthracnose caused by *Colletotrichum gloeosporioides* (Kwon *et al*., 2012). *Rhizopus nigricans* and *Aspergillus flavus* have been reported to affect germination and seedling health of *J. curcas* (Anjorin *et al*., 2011). Some phytopathogenic fungi have been isolated from stored *J. curcas* seeds (Dharmaputra *et al*., 2009). Field fungi (*Fusarium* spp., *Colletotrichum* sp. and *Cladosporium* spp.) were present at the early period of storage but their population decreased as the storage period prolonged. These fungi were later replaced by post-harvest fungi (*Penicillium* spp. and *Aspergillus* spp.). Viability, vigor and lipid content of seeds decreased with increase in storage time (Dharmaputra *et al*., 2009).

The method of isolation of pathogenic

microorganisms directly or indirectly determines the success of prevention/control strategies of plant diseases. Many plant pathologists made use of only conventional microbiological methods in the identification of microorganisms. This involves the use of various growth media to isolate microbes, and the use of biochemical tests and identification manuals to characterize microbes. These techniques have been shown to have a lot of limitation as many microbes cannot be grown on any known media and so cannot be isolated and studied. Also, the use of these techniques can lead to misinterpretation and misidentification of microorganisms since microbes might look alike morphologically. The advent of molecular method for the identification of microorganisms provides detailed and precise information, in view of the fact that identification is based on the genetic constitution of organisms (Khan and Javaid, 2021; Khan *et al.,* 2021). The aim of this study was to employ both microbiological (microbial culturing) and molecular methods in the characterization of fungi associated with *J. curcas* leaves.

Materials and Methods

Sample collection and fungi isolation

Diseased leaves of cultivated *J. curcas* showing anthracnose symptom were collected from household compounds in Aluu, Rivers State, Nigeria. Fungi were isolated from diseased *J. curca*s leaves using potato dextrose agar (PDA) as growth medium. The leaves were surface sterilized by rinsing with tap water and soaking in 70% ethanol for 5 min. Leaves were then rinsed with sterilized distilled water for three times, cut into small pieces and then plated on sterilized Petri dishes containing autoclaved PDA. Incubation of culture plates lasted for 7 days at room temperature. Observed fungal colonies in Petri dishes were separately cultured on PDA medium to obtain pure cultures which were used for the DNA extraction.

DNA extraction, quantification and gel electrophoresis

DNA was extracted and subjected to quantity-purity and quality checks using Nanodrop 2000c Spectrophotometer and gel electrophoresis respectively.

Polymerase chain reaction and sequencing of ITS region

Amplification of the ITS regions 1 and 2 of fungal isolates was carried out using the primer pair, ITS4 (5´-TCCTCCGCTTATTGATATGS-3´) and ITS5 (5´-GGAAGTAAAAGTCGTAACAAGG-3´). This primer set is known to amplify the ITS region I between the 18S and 5.8S rDNAs and the ITS region II. The PCR cocktail (25 µL, total volume) contained DMSO (1.0 µL), Taq polymerase (0.1 µL), 3 µL of genomic DNA (10 ng μL^{-1}), 10X PCR buffer (2.5 µL), 2.5 mM DNTPs (1.0 µL), 1.0 µL of each primer (concentration of 5 µM), 25 mM $MgCl_2$ (1.0 µL) and Nuclease-free water (13.4 µL). Amplifications were done in a thermocycler and consisted of thirty-six cycles. Each cycle included denaturation at 94 °C (30 s), annealing at 54 °C (30 s), and elongation at 72 °C (45 s). An extension step at 72 °C (7 min) was performed after the 36^{th} cycle. The final reaction products were held at 10 °C in the thermocycler till when removed. Reaction products were separated on agarrose gel, and amplicons were purified before sequencing on ABI 3500 Genetic Analyzer.

Data analysis and phylogeny

PCR artifacts and sequencing noise were removed from sequences using MEGA X software (Kumar *et al.,* 2018). Edited sequences were identified using BLAST on NCBI database. Sequence were aligned and a phylogenetic tree was constructed to show evolutionary relationships.

Results

Fungal isolates from *J. curcas*

Two organisms were isolated from *Jatropha curcas* and they both formed white colonies with dry surface (Fig. 2).

DNA concentration and Purity

The concentration and purity of fungal genomic DNA showed that extracted DNA met the quality check and was suitable for downstream processing such as sequencing and PCR. The DNA concentration of samples 1 and 2 were 187.3 and 180.8 respectively while the DNA purity were 1.9 and 1.8, respectively

PCR Products

PCR products obtained after amplification showed clear bands on gel when viewed under ultraviolent light (Figure 3). Sample 1 had 565 base pairs while sample 2 had 567 bp.

Molecular characterization of fungi associated with *J. curcas*

Only amplified DNA samples were sequenced. The sequence of each organism was subjected to BLAST on NCBI database. A BLAST search using the ITS sequences obtained showed that the sequence alignments of the two isolates were above 500 base pairs This is of high confidence limit for the identification of microorganisms.

The species identity of the two fungal isolates revealed that the organisms are two different strains of *Colletotrichum siamense* Prihastuti, L. Cai and K. D. Hyde. Sample 1 was most closely related to *Colletotrichum siamense* isolate1 (MH368104.1) with a similarity percentage of 99 while the best BLAST hit for sample 2 was *Colletotrichum*

siamense isolate SII1 (MH005091.1) with the similarity percentage of 90. The ITS sequences were submitted on GenBank and accession numbers were assigned to each sequence. *Colletotrichum siamense* strain RCBBR_AEANK17 was given the accession number OQ941591 while *Colletotrichum siamense* strain RCBBR_AEANK18 was given the accession number OQ941592

Phylogenetic analysis

There was genetic diversity between the strains and three other species of *Colletotrichum*. *C. siamense* isolate 1, *C. siamense* isolate SII1, *C. gloeosporioides* strain ALE-96, *C. gloeosporioides* isolate HZ20, *C. endophyticum* isolate AVS28, and *C. fructicola* strain C1-2 were closely related to the fungal strains of *C. siamense* from this study (Fig. 4).

Discussion

The two fungal isolates were identified to be two strains of *Colletotrichum siamense* using ITS primers for PCR amplification. For many years, the ITS region of nrDNA (nuclear DNA) has been in use for fungal identification and diversity studies, and has been designated as the generally accepted marker for fungi identification. ITS is the preferent marker for both identification of pure fungal cultures and mixed samples (Bellemain *et al*., 2010). It exhibits the highest variation among other DNA markers for fungi identification (Bruns *et al*., 2003; Mitchell and Zuccaro, 2006). ITS was selected as the official barcode for fungi by a group of mycologists because it is easy to use, has a widespread use and an appropriate large barcode gap (Schoch *et al*., 2012). The ITS region was found to be among the DNA markers with highest ability to provide accurate identification for several fungi (Schoch *et al*., 2012). Other researchers have also opined that ITS is the appropriate region for fungi identification (Dentinger *et al*., 2011; Kelly *et al*., 2011). *Colletotrichum* consists of plant pathogenic fungal species responsible for anthracnose and post-harvest rots on various vegetables, fruits and ornamentals, especially in the tropics and sub-tropics (Hyde *et al*., 2009; Damn *et al*., 2012a). Despite this, *Colletotrichum* species are considered as fungal pathogens of great economic value (Dean *et al*., 2012). *Colletotrichum* species can survive as endophytes in plant tissues without showing any symptom or causing any disease (Liu *et al*., 2013). They can also survive as saprophytes in infected plants, seeds, crop residues and soil. Disease initiation and development marked by a necrotic phase (lesion development) and variable virulence may be preceded by endophytic and epiphytic life phases and dormant infection stages (Rojas *et al*., 2010; Cannon *et al*., 2012).

Colletotrichum is a genus in the phylum Ascomycota. *Colletotrichum* is the asexual reproductive phase of the fungus (anamorph) while *Glomerella is the* sexual reproductive phase (telemorph) of the genus. Species of this genus have conidia that are thin-walled and transparent. The appressoria are brown in color with margins. Conidiomata are acervular, sub-cuticular or epidermal, and may have setae (Sutton, 1980). Eleven species complexes of *Colletotrichum* are recorded. The two most common species complexes are *C. acutatum* and *C. gloeosporioides* species complexes comprising of 36 and 38 species, respectively (Marin-Felix *et al*., 2017). A plant can be prone to infection by only one species of *Colletotrichum* (Doyle *et al*., 2013). Many members of *C. gloeosporioides* and *C. acutatum* spp. complexes have been recorded to be associated with anthracnose on citrus (Huang *et al*., 2013; Guanaccia *et al*., 2017). About seven species of *Colletotrichum* belonging to the *C. gloeosporioides* spp. complexes have been reported as the cause of anthracnose on camellia (Liu *et al*., 2013). *C. gloeosporioides* is a well known plant pathogen that causes huge crop losses worldwide (Ajay, 2014). It has been recorded to infect various commercially important crops in various countries. It causes canker and anthracnose in many plant species including those growing in the wild. Species of *C. gloeosporioides* and *C. boninense* complexes were reported to be associated with olive anthracnose (Schena *et al*., 2013). C. *gloeosporioides* was reported on rubber trees to be responsible for Colletotrichum leaf disease (Wastie *et al*., 1970; Radziah and Omar, 1998). New species of *Colletotrichum* have been revealed on rubber tree, some of which are, *C. laticiphilum*, *C. acutatum*, *C. citri*, *C. nymphaeae*, and *C. simmondsii* in the *C. acutatum* species complex (Damn *et al*., 2012a; Hunupolagama *et al*., 2017) and *C. annellatum* in the *C. boninense* complex (Damn *et al*., 2012b). Two new species of *C. acutatum* species complex, *C. australisinense* and *C. bannaense* are also associated with anthracnose on rubber trees (Liu *et al*., 2018).

C. truncatum was reported on *J. curcas* in India and Burkina Faso where it caused anthracnose and stem canker respectively (Ellison *et al*., 2014; Farr and Rossman, 2020). In Mexico, *C. capsici* was reported on *J. curcas* seedlings where it caused death of apical buds and canker of the stem (Torres-Calzada *et al*., 2011). Symptoms were necrotic lesions with or without yellow halo on centre or edges of leaves. *C. gloeosporiodes* was recorded to be responsible for anthracnose disease on *Jatropha curcas* (Kwon *et al*., 2012) and *Ledebouriella seseloides* (Lee and Kwak, 2013) in Korea, on yam in Nigeria (Abang *et al*., 2002), and on *Arabidopsis* sp. (Gao *et al*., 2021). In Taiwan, *C. gloeosporiodes* and *C. truncatum* were reported to cause anthracnose on vegetable soyabeans (Chen *et al*., 2006). *C. truncatum* was reported as the cause of anthracnose of pepper (Mahmodi *et al*., 2013) and chili (Auyong *et al*., 2011). *C. siamense* was reported to be

responsible for *Amorphophallus konjac* anthracnose in China (Wu *et al*., 2019).

Colletotrichum siamense have been isolated from eight plants: jackfruit, coffee, Japanese plum, fig tree, mint, black pepper, rosemary and cocoa (James *et al*., 2014). *C. siamense* has been recorded in Africa, China, Vietnam, USA and Thailand on several host plants (Prihastuti *et al*., 2009; Weir *et al*., 2012). The fungus has also been recorded in Australia in the States of New South Wales and Queensland (Weir *et al*., 2012), and the Northern Territory (James *et al*., 2014). *C. siamense* is a species within *C. gloeosporioides* species complex (Prihastuti *et al*., 2009) but there is very little information about its epidemiology and ecology.

Conclusion

Fungal organisms on plants can lead to destructive effects even when these organisms are existing as endophytes because under favourable conditions, endophytic organisms become pathogenic. Close monitoring of plants and accurate identification of organisms on plants is vital for the development of control strategies. To the best of our knowledge, this is the first report of *Colletotrichum siamense* on *J. curcas* in Nigeria at the time of writing this report. The use of molecular method for the identification of fungi associated with plants is recommended as accurate identification to the species and strain level is assured.

Author's contributions

Both the authors conceived and designed the topic. NGO did the laboratory analysis. Author AEA did the bioinformatics analysis. Author NGO wrote the first draft of the manuscript. Both authors read and approved the final version of the manuscript.

Conflict of interests

The authors declare that there is no conflict of interest between them.

Fig. 1: *Jatropha curcas* plant

Fig. 2: Pure cultures of the fungal isolates from *Jatropha curcas* leaves, grown on potato dextrose agar at room temperature.

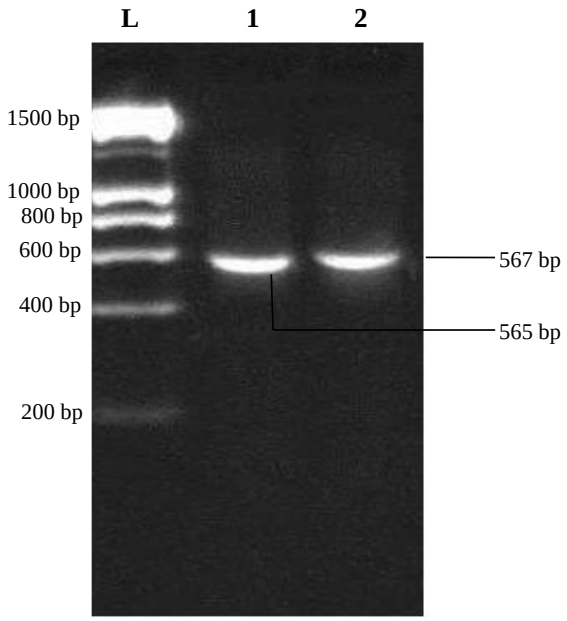

Fig. 3: PCR products generated from amplification of ITS genes. L- 1Kb DNA Ladder from Bioline. **1** and **2** represent the PCR product for each DNA sample.

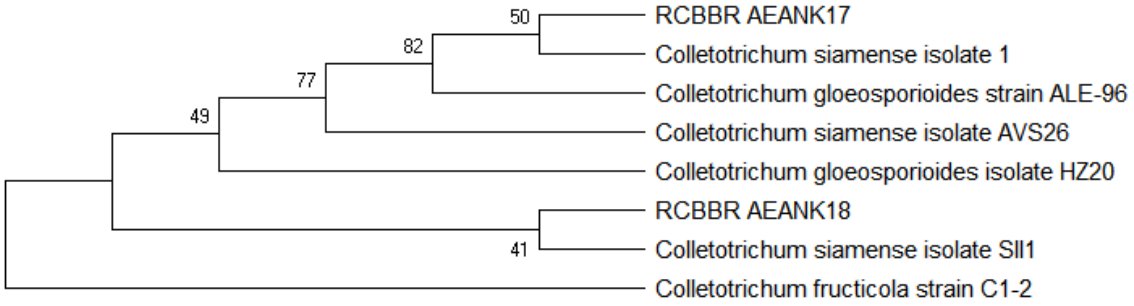

Fig. 4: Phylogenetic tree showing the relationship between two strains of *Colletotrichum siamense* isolated from *Jatropha curcas* and other *Colletotrichum* species on GenBank.

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