Vol. 19(5), pp. 231-239, May, 2020
DOI: 10.5897/AJB2020.17116
Article Number: D0662D063674
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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African Journal of Biotechnology

Full Length Research Paper

Morphological and molecular characterisation of *Colletotrichum gloeosporioides* (Penz) isolates obtained from *Dioscorea rotundata* (Poir)

Joseph Kwowura Kwodaga*, Elias Nortaa Kunedeb Sowley and Benjamin Kongyeli Badii

Department of Agronomy, Faculty of Agriculture, University for Development Studies, Nyankpala Campus, Tamale, Ghana.

Received 13 March, 2020; Accepted 23 April, 2020

Anthracnose disease is a major constraint to yam production in tropical West Africa and anywhere the crop is cultivated. This study determined the cultural characteristics and growth rates of mycelia and also characterised 6 isolates of *Colletotrichum gloeosporioides,* the causal agent of the yam anthracnose disease, obtained from *Dioscorea rotundata* leaves, vines and setts in the Tolon District of Ghana. The cultural characteristics and mycelial growth rates of the isolates were determined on Potato Dextrose Agar (PDA). The *C. gloeosporioides* isolates were characterised using polymerase chain reaction technique with the universal primer pairs ITS1/ITS4 and NS1/NS2, *C. gloeosporioides* species specific primer pairs CgInt/ITS4 and CgLac-f/CgLac-r, and *C. acutatum* species specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1. Based on the PCR, six isolates of *C. gloeosporioides* with distinct cultural characteristics were obtained. There were no significant differences ($P \leq 0.05$) in mycelial growth rates among the isolates. The *C. gloeosporioides* isolates produced characteristic band sizes on ITS1/ITS4, NS1/NS2, CgInt/ITS4 and CgLac-f/CgLac-r. None of the isolates produced a band on CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1. The proper identification of *C. gloeosporioides,* the pathogen responsible for the *D. rotundata* anthracnose is important for the proper management of the disease.

Key words: Anthracnose disease, cultural characteristics, DNA band, morphological characteristics, Mycelial growth rate, polymerase chain reaction (PCR).

INTRODUCTION

Yam, a staple crop is mainly cultivated in the tropical and subtropical regions for its tubers (Agrios, 2005; Achar et al., 2013). Unfortunately, these regions also provide favourable conditions that support the growth and survival of the yam anthracnose disease pathogen, *Colletotrichum gloeosporioides* (Penz); a major threat to yam production worldwide (Agrios, 2005; Chaube and Pundhir, 2009; Lebot, 2009; Reddy, 2015). In West African yam growing countries, the most important commercial yam species such as *D. alata* and *D. rotundata* are susceptible to the anthracnose disease (Ayodele et al., 2000; Lebot, 2009). The majority of

*Corresponding author. E-mail: kwodagaj@yahoo.com. Tel: +233 244 425262.

cultivated yams in Ghana are varieties of *D. rotundata* and *D. alata* (Demuyakor et al., 2013), with the former being the most preferred (Otoo et al., 2015). During cultivation, the disease affects the yam leaves and vines, and severe infection results in yam plant defoliation and vine dieback (Ayodele et al., 2000). *C. gloeosporioides* has also been reported to cause an orange-brown yam tuber rot known as "dead skin" (Green and Simons, 1994; Reddy, 2015).

Yams are mainly cultivated from yam setts, which if not obtained from certified sources could be infected with *C. gloeosporioides* which is capable of initiating anthracnose disease on yam crops (Aighewi et al., 2003; Asiedu and Sartie, 2010; Ayoola, 2012; Osei-Adu et al., 2016). *C. gloeosporioides* is not host specific and as such the presence of other susceptible crops in and around yam fields could also be a source of inoculum for the establishment of the disease on yam crops (Lebot, 2009). The spores of *C. gloeosporoides* require moisture, optimum temperature (20 - 30°C) and high relative humidity to germinate and establish on yam crops, with mature spores mainly disseminated by rain splash (Sharma and Kulshrestha, 2015).

To enhance yam production, there is the need to manage the anthracnose disease on the crop. The proper identification of the causative agent of the disease is crucial for appropriate disease management. Identification of *C. gloeosporioides* based on cultural, mycelial growth rate and morphological characteristics can be confused with other species within the genus, more especially *C. acutatum* (Chowdappa and Kumar, 2012; Reddy, 2015). According to Serra et al. (2011), different species of *Colletotrichum* are capable of infecting a single host. The foliage infection of *C. acutatum* and *C. gloeosporioides* are difficult to differentiate in terms of their symptoms and cultural morphology (Shi et al., 2008). This makes it difficult to differentiate between *C. acutatum* and *C. gloeosporioides;* hence the need to use molecular techniques for the proper identification of *Colletotrichum* isolates (Serra et al., 2011). The polymerase chain reaction (PCR) technique has been documented as one means of properly identifying *C. gloeosporioides* isolates (Shi et al., 2008; Serra et al., 2011; Raj et al., 2013; Chagas et al., 2017). The proper identification of the *D. rotundata* anthracnose disease pathogen is essential for selecting appropriate strategies in managing the disease to enhance the crop's productivity. This study sought to determine the cultural characteristics, mycelial growth rates and molecular characteristics of *C. gloeosporioides* isolates obtained from infected *D. rotundata* leaves, vines and setts in the Tolon district of Ghana.

MATERIALS AND METHODS

Study site

The study was conducted in the Spanish Laboratory at the Nyankpala Campus of the University for Development Studies, during the 2016 and 2017 main cropping seasons. The site which is located in the Tolon district of Ghana (latitudes 9° 15' and 10° 02' North and Longitudes 0° 53' and 1° 25' West), were the *D. rotundata* leaves, vines and setts with anthracnose symptoms were obtained for the study. The mean annual rainfall ranges from 950 to 1,200 mm and humidity between April and October can be as high as 95% in the night, falling to 70% in the day. The soil is generally of the sandy loam type and the vegetative cover is basically Guinea Savanna interspersed with short drought resistant trees and grassland.

Sampling of anthracnose infected *D. rotundata* plant parts

Ten anthracnose infected *D. rotundata* leaves were randomly obtained from each of 48 yam farms in the Tolon district. Infected *D. rotundata* vines (a total of 5) were obtained by a complete survey of each of the 48 farms for die-back symptoms. One hundred and fifty *D. rotundata* planting setts (5 per farmer) were randomly obtained from 30 yam farmers. These setts were carefully examined and those with "dead skin" symptoms selected for the study. A total of 21 symptomatic "dead skin" setts were obtained for the study. The samples were then conveyed in well labelled envelops to the laboratory.

Preparation of culture media

The medium was prepared as directed by the manufacturer's (Sigma-Aldrich Co., Spain) recommendation of 39 g of Potato Dextrose Agar (PDA) per litre of distilled water. Two hundred and fifty (250) mg of amoxicillin was added to suppress bacterial growth. The mixture was heated to completely dissolve the solutes and then autoclaved at 1.03 kg cm^{-2} pressure at 121°C for 15 min. About 20 ml each of the molten media were poured into Petri dishes (9 cm diameter) and allowed to solidify before use.

Isolation of *C. gloeosporioides*

Fragments (1 cm) of symptomatic tissues consisting of diseased and healthy parts were obtained from the anthracnose infected *D. rotundata* leaves, and vines affected by die-back. Similarly, 0.5 cm^3 fragments were obtained from portions of the yam setts with the "dead skin" symptoms. These tissues were each washed with tap water and surface sterilised in 70% alcohol for 3 min. The tissues were then rinsed with three changes of sterilised distilled water. Each tissue was inoculated on the PDA and incubated at ambient temperature (28±2°C) for 7 days. The mycelia that grew were sub-cultured onto fresh PDA, and further sub-cultured until pure cultures of *C. gloeosporioides* were obtained.

Identification of *C. gloeosporioides*

The cultural and microscopic view of the morphological characteristics of 7 days old pure culture of the isolates under a compound microscope (Leica DME, Leica Microsystems, Shanghai, China) were compared to those documented by Barnett and Hunter (2006).

Determination of mycelial growth rate

The method of Than et al. (2008) was employed with some modifications. For each *C. gloeosporioides* isolate, the mycelial growth on PDA plate was measured daily until the fifth day. The

Table 1. Primer pairs and their annealing temperatures.

Primer pair	Sequence (5´→3´)	Annealing temperature (°C)
ITS1/ITS4	Forward: TCC GTA GGT GAA CCT GCG G Reverse: TCC TCC GCT TAT TGA TAT GC	56
NS1/NS2	Forward: GTA GTC ATA TGC TTG TCT C Reverse: GGC TGC TGG CAC CAG ACT TG	53
CgInt/ITS4	Forward: GGC CTC CCG CCT CCG GGC GG Reverse: TCC TCC GCT TAT TGA TAT GC	65
Ca-f1/Ca-r1	Forward: TGA ACA TAC CTA ACC GTT GC Reverse: AGG GTC CGC CAC TAC CTT TA	55
CaGlu-f1/CaGlu-r1	Forward: CGT TCA CGA CAA ACA CCT TG Reverse: ATC GAG TCG TGA TCG AAT CC	55
CgLac-f/CgLac-r	Forward: GAA GAT CTC GGC ACC ATC AT Reverse: AAC AAC AGG GAC CAG GTC AG	56

mycelial growth rate was calculated as 5 days average of the mean daily growth. Each isolate was replicated four times.

DNA extraction using the CTAB protocol

Extraction of DNA from the fungal mycelia was done according to the CTAB protocol (Lodhi et al., 1994). The mycelia were grinded to a fine paste in 400 µl of extraction buffer in microfuge tubes using a pestle and then incubated in a re-circulating water bath at 65°C for 15 min, followed by centrifugation at 12000 rpm for 5 min. Four hundred (400) µl of supernatant was transferred into new Eppendorf tubes and 250 µl of Chloroform: Iso Amyl Alcohol (24:1) was added to each tube, mixed with the solution by inversion and centrifuged at 13,000 rpm for 1 min. The upper aqueous phase was transferred into a clean microfuge tube and 50 µl of 7.5 M Ammonium Acetate, followed by the addition of 400 µl of ice-cold ethanol to each tube to precipitate the DNA. This was then mixed by slow inverted movements that caused the DNA to precipitate at the bottom of the tubes. The tube containing the DNA was then centrifuged at 13,000 rpm for 5 min after which the propanol was decanted. This was washed twice with 0.5 ml of 70% ethanol and centrifuged at 15,000 rpm for 5 min. The DNA was then dried and 50 µl of TE buffer was added to dissolve it. It was then stored at -20°C until required.

PCR amplification of *C. gloeosporioides* strains

The reaction volume was 20 µl containing 2 µL of genomic DNA, 2X Master Mix with standard buffer (New England Biolab, UK) and 1 µL of each primer. PCR amplification was started at an initial denaturation step at 94ºC for 5 min followed by 35 cycles of denaturation at 93ºC for 1 min; for each primer pair, DNA annealing was done at a specific temperature (Table 1) for 1 min with extension at 72ºC for 2 min and a final extension at 72ºC for 5 min. The formation and size of the PCR products were checked by electrophoresis in 1.0% agarose gel and stained with ethidium bromide.

Pathogenicity test

This was determined by a modified detached leaf method described by Shivanna and Mallikarjunaswamy (2009) with some modifications. Fully expanded apparently healthy *D. rotundata* leaves were detached from their plants and washed with tap water to remove any dust particles on them. The leaves were then surface sterilized with 70% alcohol for 3 min and rinsed in three changes of sterile distilled water and left to air-dry in a microflow laminar flow workstation. A leaf was then placed on moistened blotter discs (filter paper) in a Petri dish, wounded by gentle pricking with a sterilized needle and inoculated with 1 ml spore suspension (2 x 10^6 spore/ml) of *C. gloeosporioides* and then incubated under light-dark cycle of 12/12 h at 23±2°C for 7 days. The leaves that served as control were prick inoculated with sterile distilled water. Fungal pathogens were re-isolated on PDA plates and isolates compared with the inoculants culture based on colony and morphological characteristics.

Data analysis

The mycelial growth rate data were subjected to one-way Analysis of Variance (ANOVA) with GenStat (12th edition) statistical software. Treatment means were separated with the least significant difference of Tukey's multiple-range test at 5% significance level.

RESULTS

Cultural characteristics and mycelial growth rate of the *C. gloeosporioides* isolates

Based on the cultural and morphological characteristics, the *C. gloeosporioides* isolates obtained from the anthracnose infected yam leaves, vines and planting setts were grouped into six (CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6) (Table 2). Each of the isolates had cylindrical conidia with both ends rounded. Setae were not recorded for the isolates CDr1, CDr2, CDr3, CDr4, and CDr6, except for CDr5. The mycelial growth rate of the *C. gloeosporioides* isolates ranged from 4.55±0.287 (CDr4) to 5.35±0.263 (CDr3) mm per day (Table 2). There were no significant differences ($P \leq 0.05$) in mycelial growth rate among the various isolates (Table 2).

Table 2. *Colletotrichum gloeosporioides* isolates obtained from *D. rotundata* crops, their cultural characteristics and mycelial growth rate.

Isolate	Yam part of isolation			Cultural characteristics	Mycelial growth rate per day (mm)
	Leaves	Vines	Setts		
CDr1	+	-	-	Mycelial growth with a pinkish centre and whitish towards the margins.	5.30 ± 0.208[a]
CDr2	+	+	+	Whitish mycelial growth with concentric rings and having abundant orange conidia masses at the centre.	5.20 ± 0.141[a]
CDr3	+	+	-	White cottony sparse mycelial growth.	5.35 ± 0.263[a]
CDr4	+	-	-	White cottony dense mycelial growth.	4.55 ± 0.287[a]
CDr5	+	-	+	Whitish cottony mycelial with dark conidia masses occurring in concentric masses.	5.05 ± 0.171[a]
CDr6	+	+	-	White mycelial growth with concentric rings.	4.95 ± 0.377[a]
F (pr)					**0.291**

Means ± standard errors in the same column followed by the same letter are not significantly different ($P \leq 0.05$) as determined by Tukey's multiple-range test. Key: (+) = isolated, (-) = not isolated.

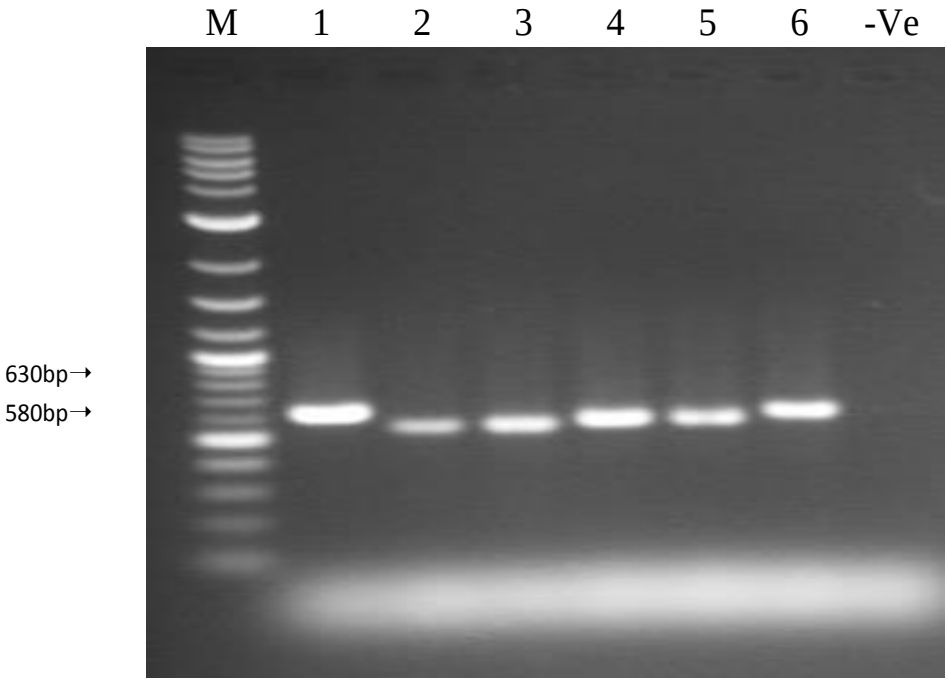

Figure 1. Amplified band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using the universal primer pair ITS1/ITS4. M = Molecular size marker (Quick-Load Purple 2-Log DNA Ladder, 0.1-10.0 kb); 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

Polymerase chain reaction

The PCR analysis carried out for the detection of the ITS region of the *C. gloeosporioides* isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 produced characteristic band sizes of approximately 580 bp when run on the universal primer pair ITS1/ITS4 (Figure 1). A band size of 560 bp was amplified when the DNA of each of the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 were run with the 18S rRNA universal primer pair NS1/NS2 (Figure 2). The species-specific primer pair for *C. gloeosprioides,* CgInt/ITS4 produced a band size of 463 bp for each of the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 (Figure 3). Also, CgLac-f/CgLac-r primer pair which is specific to *C. gloeosporioides* produced band sizes which ranged from 200 bp to 1300 bp for the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 (Figure 4). The *C. acutatum* species specific primer pairs

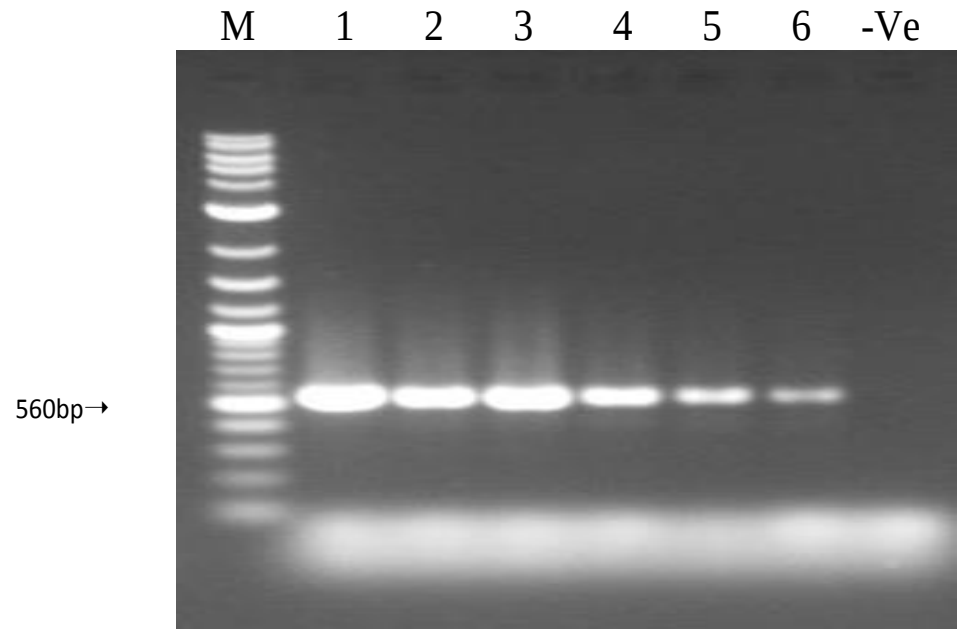

Figure 2. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using the universal primer pair NS1/NS2. M = Molecular size marker (Quick-Load Purple 2-Log DNA Ladder, 0.1-10.0 kb; 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

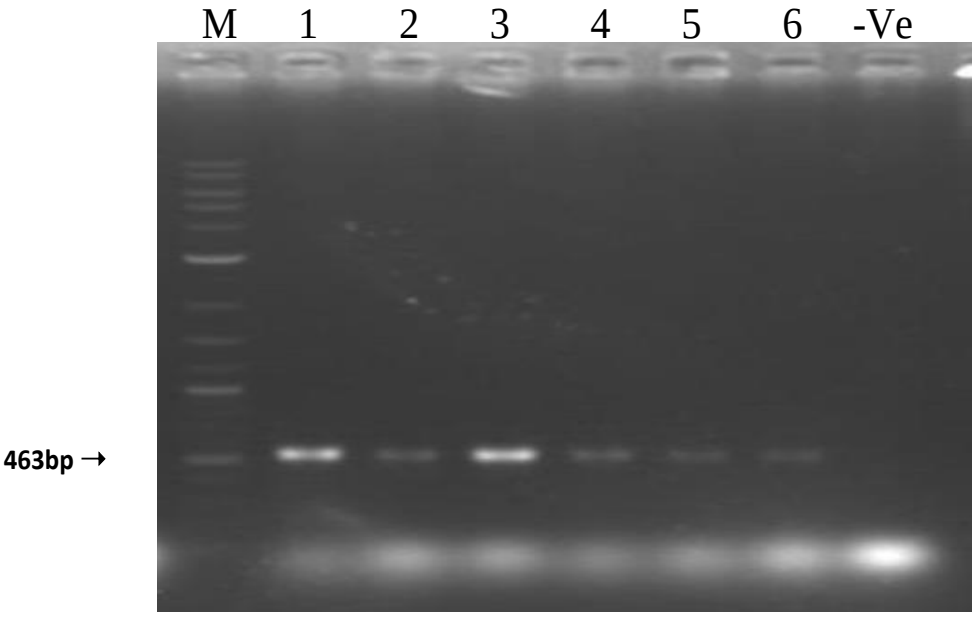

Figure 3. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using *C. gloeosporioides* species specific primer pair CgInt/ITS4. M = Molecular size marker (Quick-Load Purple 2-Log DNA Ladder, 0.1-10.0 kb; 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1 each did not produce bands for all the isolates of *C. gloeosporioides* (Figures 5 and 6). There was no PCR amplification for the negative control (nuclease-free PCR water) on the various primer

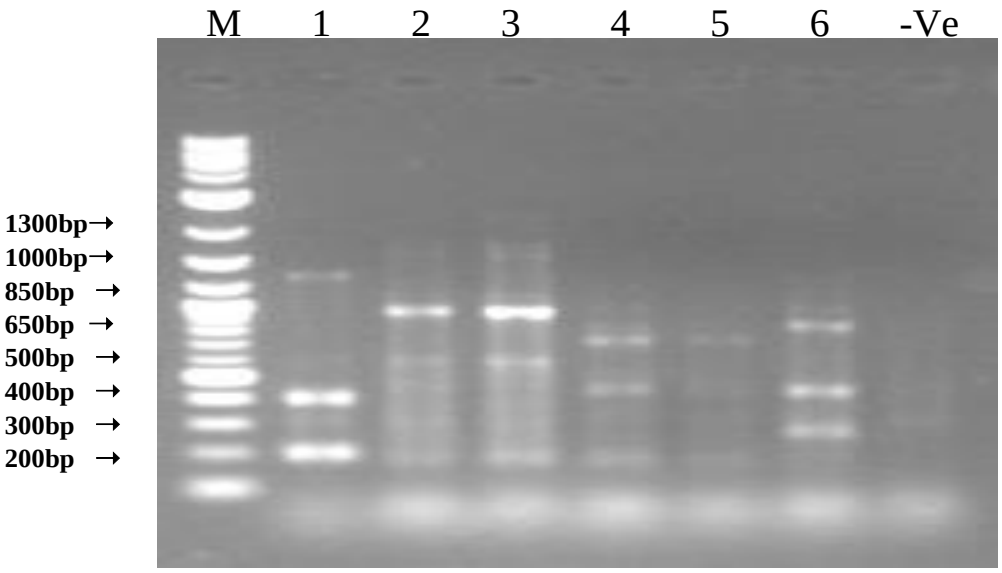

Figure 4. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using C. *gloeosporioides* species specific primer pair CgLac-f/CgLac-r. M = Molecular size marker, Quick-Load Purple 2-Log DNA Ladder (0.1-10.0 kb); 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

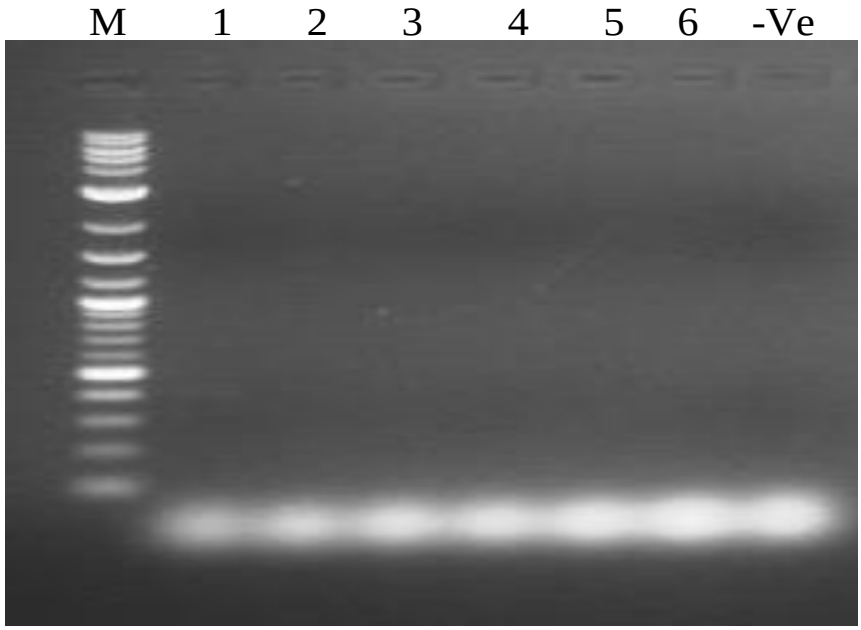

Figure 5. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using *C. acutatum* species specific primer pair CaGlu-f1/CaGlu-r1. M = Molecular size marker (Quick-Load Purple 2-Log DNA Ladder, 0.1-10.0 kb; 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

pairs ITS1/ITS4, NS1/NS2, CgInt/ITS4, CgLac-f/CgLac-r, CaGlu-f1/CaGlu-r1 and Ca-f1/ Ca-r1 (Figures 1, 2, 3, 4, 5 and 6).

Pathogenicity test

The yam leaves inoculated with the *C. gloeosporioides*

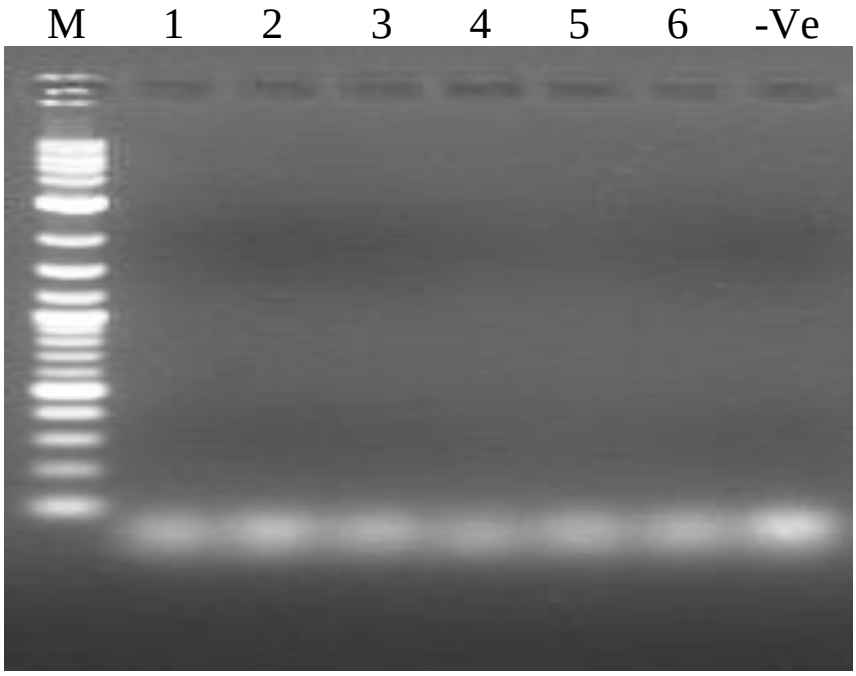

Figure 6. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using *C. acutatum* species specific primer pair Ca-f1/Ca-r1. M = Molecular size marker, Quick-Load Purple 2-Log DNA Ladder (0.1-10.0 kb); 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

conidia suspension developed symptoms of anthracnose disease. The re-isolation of the pathogen from these diseased leaves on PDA produced similar cultural and morphological characteristics as the mother culture. Anthracnose disease symptoms were not developed on the negative control leaves. These outcomes satisfied Koch's postulate.

DISCUSSION

The cylindrical shaped conidia with rounded ends recorded for each of the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 were similar to the observations made by Abera et al. (2015) who also worked on *C. gloeosporioides.* The mycelial growth rate per day for the various isolates which ranged from 4.55±0.287 to 5.35±0.263 mm were within the growth rate range of 3.6 to 11.2 mm recorded for *C. gloeosporioides* (Than et al., 2008; Abera et al., 2015). The insignificant differences ($P \leq 0.05$) in growth rate among the *C. gloeosporioides* isolates confirmed Than et al. (2008) report. Interestingly, Abera et al. (2015) recorded significant differences in mycelial growth rate among *C. gloeosporioides* isolates. These contradictory reports on significant differences in mycelial growth rate among *C. gloeosporioides* isolates attested that, it is not reliable for distinguishing among *Colletotrichum* isolates.

The observed variation in colour and pattern of growth among the pure cultures of CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 agreed with the findings of Than et al. (2008), Gautam (2014), Abera et al. (2015) and Appiah-Kubi et al. (2016) who also documented differences in cultural characteristics among isolates of *C. gloeosporioides*. The differences in cultural characteristics among the *C. gloeosporioides* isolates could be attributed to environmental rather than genetic factors. This is because according to Than et al. (2008), the use of morphological and phenotypic characteristics in distinguishing among C*olletotrichum* species can be deceptive, since different environmental conditions can cause variation among these traits. This makes it unreliable to depend solely on the cultural and morphological characteristics of *Colletotrichum* species for their identification. According to Cannon et al. (2000), the DNA traits of an organism are not directly influenced by environmental conditions and hence the most reliable method of distinguishing among *Colletotrichum* species will be the use of molecular techniques such as PCR.

When the DNA extracts of the C*. gloeosporioides* isolates were subjected to PCR run with the universal primer pairs ITS1/ITS4, the band size amplification of approximately 580 bp was yielded for all isolates. This agreed with the findings of Raj et al. (2013) who also

observed band amplifications of 580 bp when *C. gloeosporioides* isolates were subjected to PCR using the universal ITS1/ITS4 primer pair. Also, the various isolates were not separated when run on NS1/NS2 primer pair, because each of them produced a band size of 560 bp. This agreed with the findings of Shi et al. (2008) who also observed a 560 bp band size for *C. gloeosporioides* isolates after subjecting them to PCR using the universal primer pair NS1/NS2. The 463 bp detected with the C. gloeosporioides specific primer pair CgInt/ITS4 for isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 also confirmed all the isolates as the same strain of *C. gloeosporioides.* A similar observation was made by Shi et al. (2008). Also, Serra et al. (2011) reported that *Colletotrichum* isolates that produced band sizes on the primer pair CgInt/ITS4 was a confirmation of their identity as *C. gloeosporioides.*

The PCR analysis of the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 on the *C. gloeosporioides* species specific primer pair CgLac-f/CgLac-r produced band sizes for the various isolates; also revealing them as *C. gloeosporioides.* This agreed with the observation of band size production by *C. gloeosporioides* on the primer pair CgLac-f/CgLac-r documented by Shi et al. (2008) and Chagas et al. (2017).

The difficulty in distinguishing between *C. gloeosporioides* and *C. acutatum* using cultural and morphological characteristics necessitated the PCR run of all isolates on the *C. acutatum* species specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/ Ca-r1 to further clarify their identity as *C. gloeosporioides*. The lack of band production by the various *C. gloeosporioides* isolates when they were subjected to PCR on each of the *C. acutatum* species specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1 further confirmed their identification as *C. gloeosprioides*. A similar observation was made by Shi et al. (2008) who also recorded no DNA amplification for the PCR analysis of *C. gloeosporioides* on each of the *C. acutatum* species specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1. The pathogenicity test which showed that the *C. gloeosporioides* isolates produced anthracnose disease symptoms on the yam leaves confirmed the isolates as the causative agents of the disease.

The isolates of this study were identified as *C. gloeosprioides* based on their cultural and morphological characteristics, as well as mycelial growth rate. The isolates were confirmed as *C. gloeosprioides* as they produced characteristic bands on the universal primer pairs ITS1/ITS4 and NS1/NS2, and *C. gloeosprioides* species specific primer pairs CgInt/ITS4 and CgLac-f/CgLac-r. Also, the lack of bands amplification by CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 on the *C. acutatum* specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1 further confirmed all the isolates as *C. gloeosporioides*. The proper identification of *C. gloeosporioides* as the pathogen causing the *D. rotundata* anthracnose disease would aid in the development of appropriate strategies and tactics in the management of the disease in the Tolon district.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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