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Full Length Research Paper

Genetic diversity of *Colletotrichum gloeosporioides* in Nigeria using amplified fragment length polymorphism (AFLP) markers

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***Colletotrichum gloeosporioides* is the causal agent of yam anthracnose disease in Nigeria. Differential cultivars and amplified fragment length polymorphic DNA markers were used to assess the extent of genetic diversity among 39 isolates of the pathogen. Fourteen (14) pathotypes of the pathogen were identified based on inoculation of a differential set of *Dioscorea alata* genotypes of which pathotype P11 was the most virulent attacking all differential *D. alata* host genotypes, while pathotype P8 was the least virulent. 52% of the isolates were avirulent, 23% were slightly virulent and 25% were virulent. Amplified fragment length polymorphism (AFLP) analysis confirmed genetic variation among the *C. gloeosporioides* isolates and was effective in establishing genetic relationships between them. However, the grouping of the isolates based on AFLP analysis was not directly related to virulence groups or geographical origin of the isolates. The genetic variation in *C. gloeosporioides* is important in choosing strategies to develop durable resistance**

Key words: Anthracnose disease, pathotypes, genetic diversity, amplified fragment length polymorphism (AFLP) markers, yam.

INTRODUCTION

Anthracnose disease is a major constraint to the production of *Dioscorea alata* (Abang et al., 2002; Egesi et al., 2009; Aduramigba et al., 2010), causing about 90% loss in yield. The disease is caused by the pathogen *Colletotrichum gloeosporioides* Penz. (Nwankiti and Ene, 1984; Abang et al., 2002; Aduramigba et al., 2010). *Colletotrichum* is one of the most important plant pathogenic fungi worldwide (Holliday, 1980). Species differentiation in the genus has been based primarily on conidia size and shape, and presence or absence of setae (Sutton, 1992). *Colletotrichum* species are ubiquitous and have been isolated from a variety of environments. They are best known, however, as the causal agent of the disease symptoms commonly known as anthracnose. *C. gloeosporioides* has been reported as a pathogen on a wide range of host species throughout the world (Bailey and Jeger, 1992; Cannon et al., 2008).

Anthracnose affects the leaves, petioles, stems and veins of the plant, causing leaf spots, leaf blotches, petiole blights, premature abscission, dieback and eventual death of the entire plant. The disease usually has a dramatic effect on infected plants, converting a field of initially healthy yam plants from 'green' to 'black' within a few weeks (Green and Simons, 1994).

Phytopathogenic fungi are usually classified as species and races based on morphology, host specialization, cultivar specificity and mode of parasitism (O'Nell et al., 1998). The application of methods in molecular analysis has helped to clarify the genetic relationships of fungal taxa which are not clearly distinguished by their

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morphology. Restriction fragment length polymorphism (RFLP), PCR-based random amplified polymorphic DNA (RAPD) and rDNA sequence analyses have been reported to give a significant improvement in the identification and classification of phytopathogenic fungi (O'Nell et al., 1998). However, limitations in using these techniques include difficulty in standardization, time and effort required, the relatively small numbers of polymorphisms generated, small and variable selective sampling of the genome and reproducibility.

RAPD markers which are based on the amplification of discrete DNA fragments in the genome by the use of oligonucleotide primers with random sequences have been largely used to identify physiological races of different *Colletotrichum* species (Guthrice et al., 1992).

Amplified fragment length polymorphism (AFLP) is a PCR-based fingerprinting technique that has been used in plants, bacteria, nematodes and fungi (O'Nell et al., 1998). AFLP markers assay the presence/absence of restriction enzyme sites in combination with sequence polymorphisms adjacent to these sites. Production of AFLP is based on selective amplification of restriction enzyme digested DNA fragments. Multiple bands are generated in each amplified reaction that contains DNA markers of random origin (Zabeau and Vos, 1993; Vos et al., 1995). AFLP have significant advantage over other procedures because variability can be assessed at a large number of independent loci and data are obtained more quickly and are reproducible (Zabeau and Vos, 1993). It represents a practical advance in fungal DNA finger-printing because of the greater resolution and the collection of more information than is possible by RADP and RFLP techniques. AFLP analysis is useful in identifying genetic diversity and analysis of population structure within complex genera of fungi (O'Nell et al., 1998). This study was conducted to determine the variation among isolates of *C. gloeosporioides* in terms of virulence and DNA polymorphism using AFLP molecular markers.

MATERIALS AND METHODS

Thirty-eight (38) isolates of *C. gloeosporioides* obtained from infected yam leaves and one reference isolate (Cg 33) from IITA were studied. The isolates were purified by single spore isolation. A dilute spore suspension of each isolate was prepared by picking conidia from sporulating acervuli and placing them in sterile vials containing 1 ml of sterile distilled water. These suspensions were shaken thoroughly and streaked onto thin plates of tap water agar using a sterile wire loop. Single-spore isolations for all the isolates were done on tap water agar using two plates per isolate. Streaked plates were labelled and incubated at 28°C for 12 to 24 h. After incubation, a Swann Morton No. 11 blade fitted onto a holder was used to transfer four single germinated spores of each *C. gloeosporioides* isolate to Petri dishes containing 1/4 potato dextrose agar. Single germinating conidia were identified with the aid of a binocular dissecting microscope used at high power (x40). Plates were sealed, labelled and incubated at 28°C for 3 to 5 days. The isolates were maintained on potato dextrose agar with periodic sub-culturing. The isolates were characterized based on morphological and cultural criteria (Baxter et al., 1983; Hawskworth et al., 1995) as well as virulence characteristics on both local and improved yam genotypes.

Determination of *C. gloeosporioides* pathotypes

The reactions of four differential *D. alata* genotypes (TDa 289, TDa 85/00250, TDa 94-126 and TDa 94-72) were used to differentiate isolates of *C. gloeosporioides* into pathotypes. These genotypes were selected based on their differential reactions to anthracnose on the field (Aduramigba-Modupe et al., 2008). This was carried out using a rapid bioassay method developed by Green et al. (2000) for screening yam for the response to anthracnose disease. Spore suspensions of the isolates were prepared by washing the surface of 7 to 10-day old pure cultures of *C. gloeosporioides* isolated from infected yam leaves with sterile distilled water. The suspension was then passed through four layers of muslin cloth to remove fungal mycelia and other debris and then adjusted to 1×10^6 spores ml^{-1} using a haemocytometer. The leaves were inoculated with spore suspensions of individual isolates of *C. gloeosporioides* using an artist's paint brush. Leaves used as control were inoculated with sterile distilled water (SDW). Two sterile filter papers were placed inside a 9 cm glass Petri dish and moistened with SDW. An inoculated whole leaf was placed in each dish, covered and sealed with parafilm. The experiment was set up in a randomized complete design with three replications. Incubation was done at 28°C under 12 h/day fluorescent light and lasted for 4 days. Leaves were rated for disease reaction using the scale of Sweetmore et al. (1994).

DNA extraction from mycelia

DNA extraction was done according to Reeder and Broda (1987) with some modifications. Approximately, 0.3 g of washed mycelia was suspended in 200 ml of 2x CTAB buffer (50 mM Tris, pH 8.0; 10 mM EDTA; 2% hexadecyltri-methylammonium bromide; 0.1% 2-mercaptoethanol), followed by 100 ml of 20% solution of sodium dodecyl sulphate (SDS) in double distilled water and was incubated at 65°C for 10 min.

DNA was isolated by two extractions of chloroform isoamyl alcohol (24:1) and precipitated with absolute ethanol at -20°C. DNA was washed with 70% ethanol, dried and re-suspended in 100 µl of sterile distilled water. DNA samples were subjected to electrophoresis to check their integrity in 1% agarose gels along with undigested Lambda DNA standards using 3 µl of DNA sample loaded unto agarose gel 0.5x TBE (45 mM Tris- acetate, 1M EDTA, pH 8) as running buffer.

Polymerase chain reaction (PCR) amplification

The modified method of Vos et al. (1995) was employed in the AFLP analysis. Three primers: EAA/MO, EAC/MA and EAA/MG were used for amplification. Amplification products were separated by electrophoresis in 2% agarose gels.

Data analysis

Bands from lanes of the gel plates were scored visually for their presence or absence. A band was considered polymorphic if it was

Table 1. Pathotype designation of *C. gloeosporioides* in the *D. alata* differential set differential host *D. alata*.

Pathotype	TDa 289	TDa 85/00520	TDa 94-126	TDa 94-72
P1	+	-	+	-
P2	-	-	+	+
P3	+	-	+	+
P4	-	+	+	-
P5	-	+	+	+
P6	-	+	-	-
P7	+	+	-	-
P8	-	-	-	-
P9	-	-	+	-
P10	+	-	-	+
P11	+	+	+	+
P12	+	+	-	+
P13	-	-	-	+

+: Susceptible host reaction; -: resistant host reaction.

present in one or more of the isolates used in this study. Positions of unequivocally scorable AFLP bands were transformed into a binary character matrix for the presence ("1") or the absence ("O") of a band at a particular position. Pair-wise distance matrices were compiled using the Jaccard coefficient of similarity (Jaccard, 1908) present in NTSYS-PC 2 software packages (Rohlf, 1993). Dendrograms were created by unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis (Sneath and Sokal, 1973).

RESULTS

Determination of *C. gloeosporioides* pathotypes

Classification of the isolates was done according to the disease reactions of four differential *D. alata* cultivars. Thirteen (13) possible pathotypes were identified from the 39 isolates used in this study (Table 1). The four *D. alata* cultivars varied in their reactions to the isolates. P11 was the most aggressive and accounted for about 8% of the isolates, while P8 was not virulent on any genotype in the differential set, and accounted for about 18% of the isolates. P2 and P5 were the most common and they accounted for 13 and 16%, respectively. Different virulence patterns were found for some of the field isolates (Table 1).

Molecular variation

Fifty eight (58) polymorphic bands were observed. The similarity matrices were generated by each of the three primer pairs and were combined to determine the genetic relationships among *C. gloeosporioide*s isolates. AFLP analysis was effective in establishing genetic relationships between different isolates of *C. gloeosporioides.* The dendrogram generated by complete cluster analysis showed 4 main clusters at 55% similarity level (Figure 1). All the isolates were distinct at 100% similarity level. The 4 main clusters could not be related to pathotype, host origin or geographical location where the isolates were obtained (Table 2). The isolates within the clusters were as follows:

Cluster I: It contained 5 isolates, 53, 48, 40, 27 and 10 in two subgroups, 'a' and 'b'. Subgroup 'a' consists of 53, 48 and 40 from Ebonyi, Cross river and Akwa-Ibom States in the humid forest area of Nigeria, while subgroup 'b' consists of 27 and 10 from Benue State in the guinea savannah.
Cluster II: This comprises two isolates, 26 from Ebonyi State and 34 from Cross river State, Nigeria.
Cluster III: Twenty-two (22) isolates, divided into three subgroups, 'a', 'b' and 'c'. Subgroup 'a' has isolates 39, 31, 33, 30 and 22 from Ebonyi State. Subgroup 'b' consists of isolates 38, M33, 45, 42, 41, 43 and 35. Subgroup 'c', has isolates 4, 7, 9, 25, 27, 36, 49, 52, 55 and reference isolate F37 from cassava.
Cluster 1V: There were 2 subgroups 'a' and 'b'. 'a' has 2 isolates, 29 and 24 and 'b' has 8 isolates 1, 2, 15, 16, 17, 18, 20 and 8.

The most closely related isolates were 7 and 49, as well as 52 and 56 at 62 and 98% similarity coefficient, respectively.

The principal component analysis of polymorphic bands

The binary data generated from the 39 isolates were

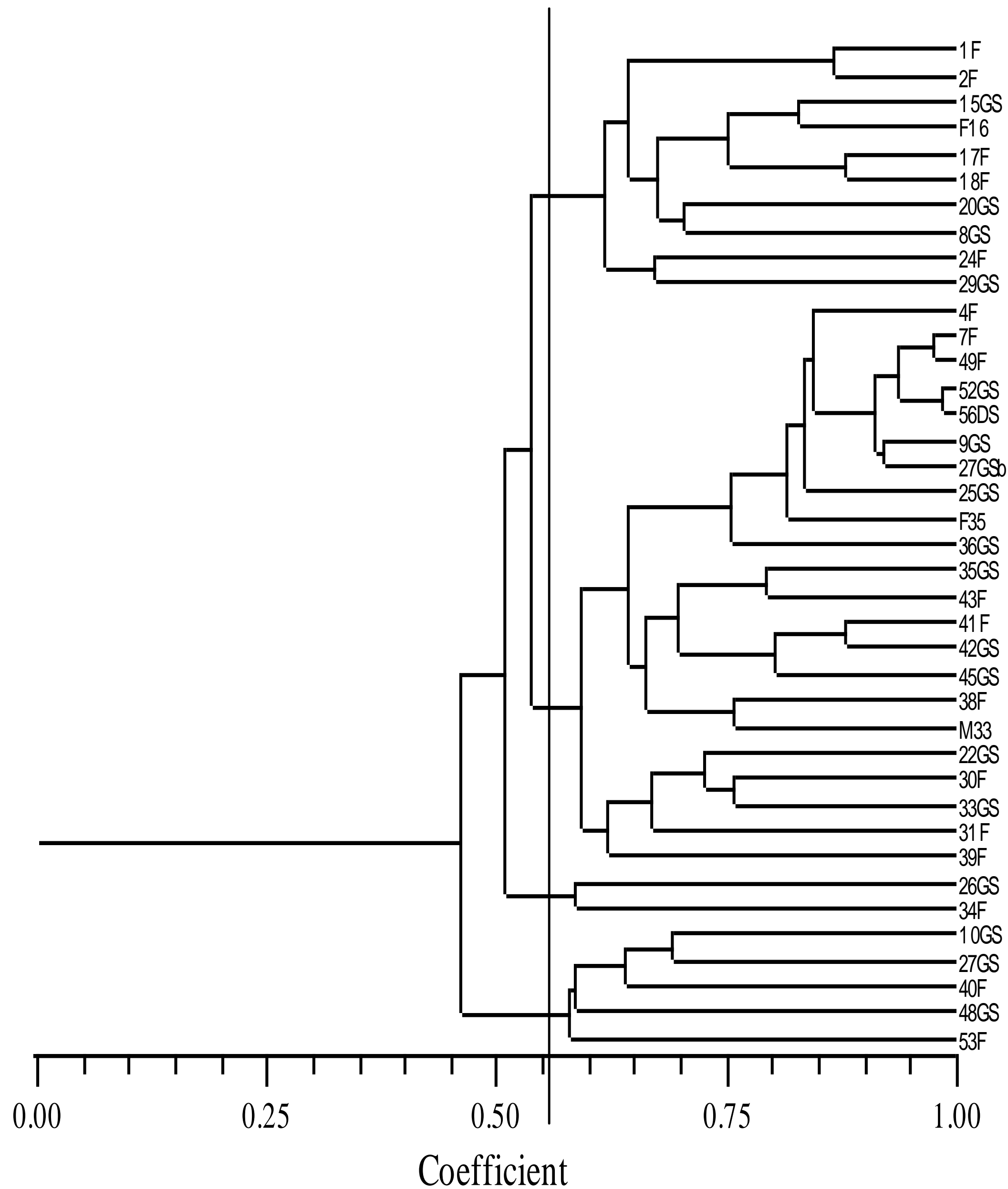

Figure 1. Dendrogram showing the genetic similarity of 39 *C. gloeosporioides* isolates based on UPGMA using NTSYS v.2.02j.

Table 2. Variability in colour of mycelium, pathotype and molecular group of *C. gloeosporioides* isolates from different agroecological zones.

Isolate number	Agroecological zone	Colour	Pathotype	Molecular group
30	Forest	Grey	P12	3
38	Forest	Grey	P13	3
40	Forest	Grey	P13	1
43	Forest	Grey	P2	3
4	Forest	Grey	P3	3

Table 2. continues

17	Forest	Grey	P8	4
31	Forest	Orange	P12	3
`41	Forest	Orange	P8	3
18	Forest	Orange	P9	4
1	Forest	Salmon	P1	4
39	Forest	Salmon	P1	3
34	Forest	Salmon	P11	2
2	Forest	Salmon	P2	4
49	Forest	Salmon	P2	3
7	Forest	Salmon	P4	3
24	Forest	Salmon	P5	4
16	Forest	Salmon	P7	4
53	Forest	Salmon	P9	1
8	Guinea savannah	Grey	P5	4
27a	Guinea savannah	Grey	P11	1
27b	Guinea savannah	Grey	P11	3
45	Guinea savannah	Grey	P2	3
52	Guinea savannah	Grey	P2	3
42	Guinea savannah	Orange	P11	3
26	Guinea savannah	Orange	P6	2
35	Guinea savannah	Salmon	P1	3
22	Guinea savannah	Salmon	P10	3
33	Guinea savannah	Salmon	P10	3
20	Guinea savannah	Salmon	P2	4
29	Guinea savannah	Salmon	P2	4
10	Guinea savannah	Salmon	P3	1
25	Guinea savannah	Salmon	P5	3
36	Guinea savannah	Salmon	P5	3
15	Guinea savannah	Salmon	P6	4
48	Guinea savannah	Salmon	P5	1

subjected to principal component analysis using SAS. It made use of the matrix data to produce six Eigen vectors. The first six principal components were associated with 20.24, 13.76 and 7.55, 6.92, 6.07 and 4.71%, respectively, of the variance of the genetic distance and contributed about 59.25% of the total variation found among the 39 isolates (Table 2). The plot of the first principal component scores generated a scatter graph of the isolates (Figure 2).

DISCUSSION

The genetic studies of *C. gloeosporioides* isolates in this study contributed to our understanding of the genetic structure of *C. gloeosporioides* population in yam growing zones of Nigeria and will help in selecting strains of the pathogen for screening yam germplasm for resistance to anthracnose disease in Nigeria.

Breeding and effective deployment of durable plant resistance require an understanding of genetic diversity among *C. gloeosporioides* from yam-based cropping systems, and the geographical distribution of the pathotypes. A differential host series is based on vertical resistance of lines, such as host genotypes that are differentially resistant to some physiological races but not others, and which do not show a significant genotype x environment (G x E) interaction with respect to disease response. The use of molecular tools has provided pathologists with numerous markers for analysing populations of *C. gloeosporioides* from different host plants. The results of this study indicated that the populations of *C. gloeosporioides* are highly heterogenous for both virulence and DNA pattern.

Four cultivars of *D. alata* selected on the basis of their performances in field screening, varied in their reaction (resistant to susceptible) to inoculations with *C. gloeosporioides* isolates in the laboratory. The disease

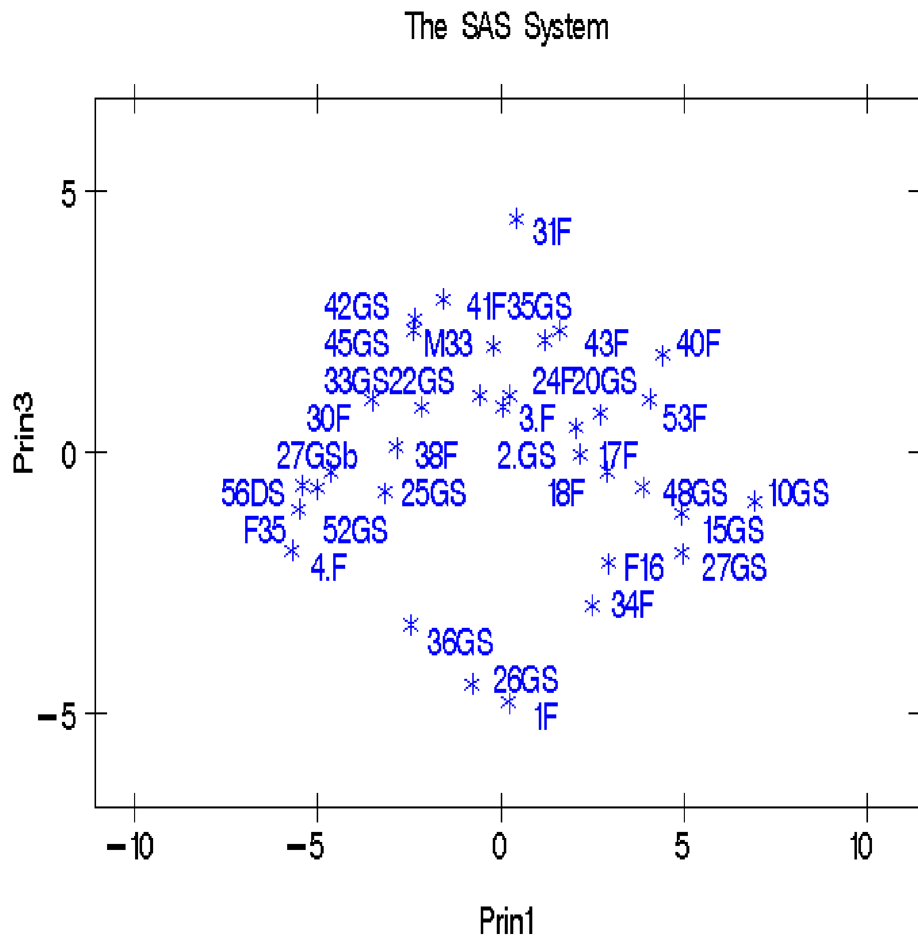

Figure 2. Scatter diagram of the first two principal components for 39 isolates of *C. gloeosporioides*.

severity of the host genotypes varied according to which isolate was used, suggesting the occurrence of vertical resistance within the cultivars (Van der Plank, 1984). This finding supports the work of Mignouna et al. (2001), who described the presence of a single dominant resistance gene in one cultivar of *D. alata* (TDa 95/00328).

Thirteen(13) pathotypes identified from the 39 isolates used in this study confirms the high variability observed by Kelemu et al. (1996) and Thottappilly et al. (1999). The extreme pathogenic variation among isolates of *C. gloeosporioides* should be considered when selecting isolates to use in an anthracnose resistance screening program. This implies that response may change depending on which isolate is used, and different combinations of isolates should be used when screening for resistance. Isolates within the group of the pathotype 11 would be appropriate for use in initial screening as they exhibited susceptibility reaction on all the host series.

Progress in identifying and monitoring variability in *C. gloeosporioides* is possible with the use of molecular markers that are isolate specific or pathotype specific. Molecular markers are used extensively to characterise plant pathogens (Guthrice et al., 1992; Michelmore et al., 1987; Thottappily et al., 1999). When combined with data on virulence, these markers often can elucidate the population genetic structure and evolutionary relationship of plant pathogens (McDonald and McDermott, 1993; Screenivasaprasad et al., 1992). This information can suggest novel strategies for the control of plant pathogens. Results reported here, however, showed there were no clear relationships between results of AFLP analysis, virulence tests and geographical origin.

In conclusions, high variability exists within the population of *C. gloeosporioides* isolates from infected leaf samples. The extreme pathogenic variation among isolates of *C. gloeosporioides* should be considered when selecting isolates to be used in an anthracnose resistance screening program. DNA-based molecular markers represent a potent tool for the identification of

distinct populations of *C. gloeosporioides* and thus, the development of *D. alata* genotypes with stable genetic resistance to anthracnose.

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