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Morphological Character Analysis and Signal Cryptic Speciation in *Lasiodiplodia theobromae* on Cashew (*Anacardium occidentale* L.)

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Abstract

Lasiodiplodia theobromae (Pat) Griffon & Maubl. is a pathogen causing inflorescence dieback disease of cashew in Nigeria and also a common pathogen with a wide host range in the tropics and sub-tropics. The character variations in this pathogen necessitate better understanding of it towards development of management strategies. Isolates identified as *L. theobromae* were cultured from inflorescence dieback disease of cashew across growing ecologies of Nigeria and studied base on morphological characters. Variability in mycelial texture and colour, conidia and septa sizes and pycnidia production were recorded in this study. The Principal Component Analysis (PCA) and WARD clustering analysis identified four well-supported traits within the isolate group. Isolates within each cluster was: 2, 2, 4 and 1 respectively and isolate CDA1416 (Obollo-Afor) and CDA2924 (Idi-Ayunre) in cluster III were the most similar. Members within clusters I and II united at the semi-partial R-Square distance of 0.0294 and 0.0278 respectively. Isolate CDA2308 (Oro) was distinguished among others and signal a potential cryptic specie, differences in these isolates were supported by conidial morphology and textural variations. This understanding will form the bases for development of diseases management strategy against the pathogen.

Keywords: cashew, clustering, dieback, *Lasiodiplodia theobromae*, morphotypes, ecologies, variations

1. Introduction

1.1 Pathogen of Disease on Cashew

The analysis of morphological characters of *Lasiodiplodia theobromae* (Pat) Griffon & Maubl. was carried out as part of general study on character variations in this pathogen. *Lasiodiplodia theobromae* is an important plant pathogen in the tropics and subtropics.

1.2 Status of Disease in Nigeria

This pathogen has been reported to cause numerous diseases, including dieback, fruit rots, leaf spot among others (Punithalingam, 1980). Inflorescence dieback of cashew was reported by Adeniyi et al. (2011) and is a major limiting factor affecting cashew nut production in Nigeria, causing annual crop loss of 40-45% (Olunloyo, 1983). This disease is a major focus of recent cashew pathology research in Nigeria. In-view of the wide spread of this pathogen, the large number of hosts and its character variability (Adeniyi et al., 2016), it is possible that *L. theobromae* on cashew composed of a number of traits considering diversity in their morphological characters expression. The purpose of this study was to determine variability within a collection of isolates previously identified as *L. theobromae* through analysis of their morphological quantitative parameters as part of measures to develop management strategies against the pathogen.

2. Methods

A collection of isolates previously identified as *L. theobromae*, pathogen of inflorescence dieback disease of cashew in Nigeria (Adeniyi et al., 2011) was studied (Table 1), to investigate its mycelia and morphological characters according to the methods of Adeniyi et al. (2016). Such characters include mycelia growth rate, colour pigmentation, pycnidial production and conidial dimension of the isolates.

2.1 Analyses Selected Parameters

The data of the quantitative parameters were subjected to analysis of variance, using PROC GLM in SAS. Means of the measured parameters were separated using the honest significant difference method of (Tukey, 1949). Variability among the isolates for the colony colour and texture at the obverse and reverse were nominally coded. Mean of the two quantitative traits were generated for each isolate. The quantitative means across the three replication and the nominal codes for each isolate for the four parameters gave a 9 × 6 multivariate data matrix. The data was standardized (mean = 0; standard deviation = 1) to harmonize the various units of measurements among the parameters following the approach of Adewale et al. (2013). The data matrix was then subjected to Gower genetic distance (Gower, 1971) analysis. The resulting product was further subjected to Principal Component Analysis (PCA) and WARD clustering analysis. Mean and standard deviation was estimated for the members of each of the four clusters identified at the 0.1 inflection point on the dendrogram to be able to understand variability within each cluster.

3. Results

The sources of the nine isolates of *L. theobromae* from inflorescence dieback disease of cashew were cultured from major cashew growing ecologies of Nigeria were presented in Table 1.

3.1 Sampling Location

The randomly selected farmers' fields in major cashew growing ecologies of Nigeria were established over a decade, the agronomic observation perepectives, characteristics and assessement of the cashew plots showed closed and interlocking cashew canopies and irregular spacing of the plantations. The farmers' fields also comprised of cashew stands of varied sources and accessions mixed on same plot and the plots lack proper and expected farm management practises.

Table 1. Baseline information on isolate sources

Isolate number	Specie	Host	Locality
*CDA0715a	*L. theobromae*	*A. occidentale* L.	Otukpa, NC, Nigeria
CDA0715b	*L. theobromae*	*A. occidentale* L.	Odoba, NC, Nigeria
CDA1416	*L. theobromae*	*A. occidentale* L.	Obbollo-Afor, SE, Nigeria
CDA2205	*L. theobromae*	*A. occidentale* L.	Ochaja, NC, Nigeria
CDA2214	*L. theobromae*	*A. occidentale* L.	Ejule, NC, Nigeria
CDA2305	*L. theobromae*	*A. occidentale* L.	Ganmo, NC, Nigeria
CDA2308	*L. theobromae*	*A. occidentale* L.	Oro, NC, Nigeria
CDA2921	*L. theobromae*	*A. occidentale* L.	Ogbomoso, SW, Nigeria
CDA2924	*L. theobromae*	*A. occidentale* L.	Idi-Ayunre, SW, Nigeria

Note. *CDA: Culture of Dele Adeniyi. NC: North Central. SE: South Eastern. SW: South Western.

3.2 Description of the Pathogen and Isolates

The paraphyses of *L. theobromae* showed dark flask-shaped. The hollow structure of mature pycnidia was elongated to form a neck having circular pore through which conidia were liberated. The immature conidia observed in this study were globose and light brown but becoming dark-brown with longitudinal striation and single septation when matured.

The nine isolates varied significantly ($P < 0.001$) for Pycndia with a range of 0 to 260 and a mean of 68.22. The coefficient of variation for the parameter was very low (4.75) compare to the 39.83% obtained from colony growth rate (Table 2). Grand mean for colony growth rate among the nine isolates in this experiment was 13.42 (Table 2). Although all isolates in this study has a similar rate of mycelial growth but they can be separated on the bases of their conidial morphology and mycelial texture.

Table 2. ANOVA and mean performances of isolates *Lasiodiplodia theobromae* for pycnidial production and colony growth rate

Sources of variation	DF	MS_Pycnidial production	MS_Colony Growth Rate
Isolates	8	24263.83***	4.73
Error	16	10.50	28.57
Isolates		Mean_Pycnidia	Mean_Colony Growth Rate
**CDA0715a		171.00[b]	14.17[a]
CDA0715b		11.00[f]	13.33[a]
CDA1416		49.00[d]	13.33[a]
CDA2205		34.00[e]	12.02[a]
CDA2214		0.00[g]	14.10[a]
CDA2305		260.00[a]	14.17[a]
CDA2308		0.00[g]	11.95[a]
CDA2921		12.00[f]	14.17[a]
CDA2924		77.00[c]	13.57[a]
Grand Mean		68.22	13.42
CV (%)		4.75	39.82

Note. DF: Degree of freedom, Mean Square; ***: Significance at 0.001; **CDA: Culture of Dele Adeniyi. Means followed by the same super script letter in each column are not statistically different (P = 0.05). CDA0715a: Benue (Otukpa), CDA0715b: Benue (Odoba), CDA1416: Enugu (Obbollo-Afor), CDA2205: Kogi (Ochaja), CDA2214: Kogi (Ejule), CDA2305: Kwara (Ganmo), CDA2308: Kwara (Oro), CDA2921: Oyo (Ogbomso), CDA2924: Oyo (Idi-Ayunre).

3.3 Character Variation of Isolates

Conidia of all isolates are septated with single septa but the septa size varies. While CDA2214 and CDA2308 produce no conidia, similarities were recorded in the septa size of CDA0715a, CDA0715b, CDA1416 and CDA2205 but all the isolates however differ in their conidia sizes (Table 3).

Table 3. Conidial dimension of *Lasiodiplodia theobromae* isolates

Isolate number	Conidia size (µm)	Septa size (µm)
*CDA0715a	27.3 – 46.8 × 19.5 23.4	11.7 – 15.6 × 3.9 - 11.7
CDA0715b	27.3 – 35.1 × 15.6 – 19.5	11.7 – 15.6 × 3.9 – 11.7
CDA1416	31.2 – 42.9 × 15.6 – 19.5	11.7 – 15.6 × 3.9 – 11.7
CDA2205	35.1 – 50.7 × 19.5 – 23.4	11.7 – 19.5 × 3.9 – 11.7
CDA2214	**Nil	Nil
CDA2305	27.3 – 39 × 19.5 – 23.4	11.7 – 19.5 × 3.9 – 7.8
CDA2308	Nil	Nil
CDA2921	31.2 – 42.9 × 11.7 – 23.4	11.7 – 15.6 × 3.9 – 7.8
CDA2924	27.3 – 42.9 × 15.6 – 23.4	11.7 – 15.6 × 3.9 – 11.7

*Not*e. *CDA: Culture of Dele Adeniyi. CDA0715a: Benue (Otukpa), CDA0715b: Benue (Odoba), CDA1416: Enugu (Obbollo-Afor), CDA2205: Kogi (Ochaja), CDA2214: Kogi (Ejule), CDA2305: Kwara (Ganmo), CDA2308: Kwara (Oro), CDA2921: Oyo (Ogbomso), CDA2924: Oyo (Idi-Ayunre). **Nil: No sporulation.

With the four morphological traits employed in the study, the first three principal component axes captured 91.4% of the total variation (Table 4). Eigenvalues and variance proportion consistently decreased from PC1 to PC4. The proportional contribution of each character to the total variance (eigenvector) varied in dimension and quantity in the four PC axes. Most positive contribution in each axis was much higher than 0.3 and each character differently made highest contribution to the variance in each axis (Table 4).

Table 4. Eigenvalues, variance proportion of four PC-axes and eigenvectors of four morphological characters

	Principal Component Axes			
	PC1	PC2	PC3	PC4
Eigenvalues	1.940	1.001	0.713	0.346
%Variance per PC-axis	0.485	0.250	0.178	0.086
%Cumulative variance across PC-axes	0.485	0.735	0.914	1.000
Morphological Traits	Eigenvectors			
Pycnidia	0.498	0.156	0.814	-0.255
Colony Growth Rate	0.632	-0.132	-0.125	0.753
Colony Texture and Colour-Obverse	0.146	0.951	-0.271	-0.001
Colony Texture and Colour-Reverse	-0.576	0.230	0.498	0.606

Four clusters significantly evolved in the dendogram at the inflection point of 0.1 (Figure 1). Membership of isolates within each cluster was: 2, 2, 4 and 1 respectively. CDA1416 and CDA2924 (both in cluster III) were the most similar isolates with a point of union at 0.0026 semi-partial R-Square distance. The duo members in clusters I and II united at the semi-partial R-Square distance of 0.0294 and 0.0278 respectively. Isolate CDA2308 was distinguished and tied with the remaining eight isolates at 0.3677 semi-partial R-Square distance (Figure 1).

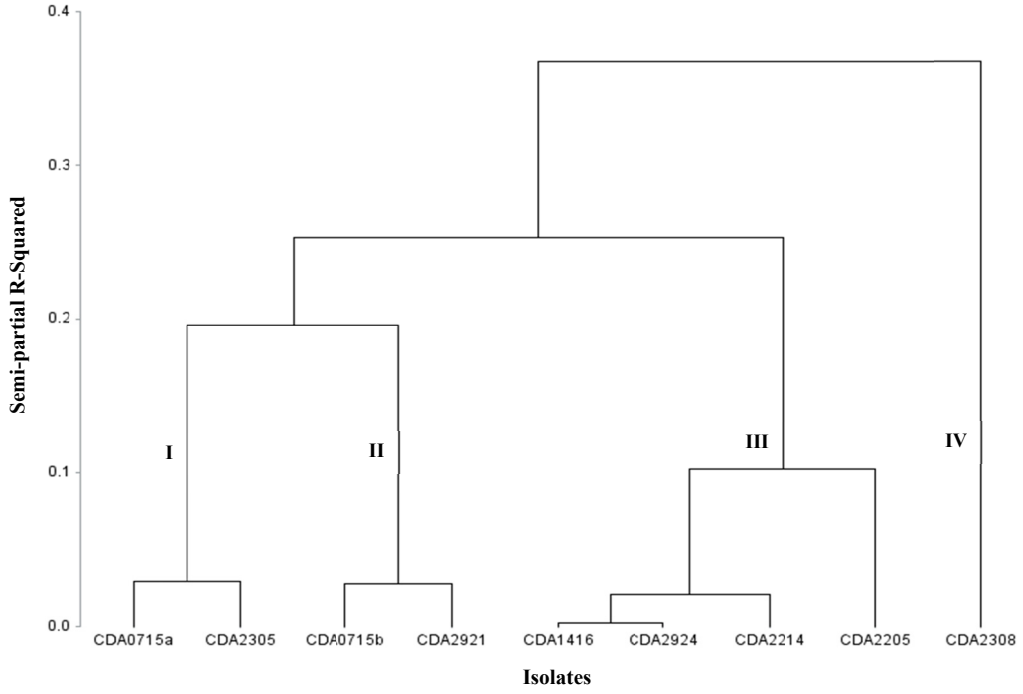

Figure 1. Dendogram showing the relationship among the nine isolates

Note. CDA0715a: Benue (Otukpa), CDA0715b: Benue (Odoba), CDA1416: Enugu (Obbollo-Afor), CDA2205: Kwara (Oro), CDA2214: Kogi (Ejule), CDA2305: Kwara (Ganmo), CDA2308: Kwara (Oro), CDA2921: Oyo (Ogbomoso), CDA2924: Oyo (Idi-Ayunre).

The colour and texture on the reserve of the colonies for members in clusters I, II and III was greyish blue (Table 5). The two isolates in cluster I had the highest (215.50) mean for Pycnidia and mean colony growth rate of 14.17. The four isolates in cluster III had mean Pycnidia and colony growth rate content of 40 and 13.25 respectively. From Table 3, mean Pycnidia and colony growth rate for the two isolates in cluster II was 11.50 and 13.75 respectively. The proportion of the colony colour and texture on the obverse side was halved between fluffy olivaceous grey and fluffy mouse grey (cluster I) and fluffy groh grey (cluster II). However, the colony colour and texture on the obverse side for cluster III was shared in the ratio of 1:3 for fluffy mouse grey and fluffy dark mouse grey (Table 5).

Table 5. Intra-cluster variability of the nine isolates based on two quantitative and two qualitative morphological characters

Morphological characters	Cluster						
	I(2)		II(2)		III(4)		IV(1)
	Mean	StDev	Mean	StDev	Mean	StDev	Mean
Pycnidia	215.50	62.93	11.50	0.71	40.00	32.07	0.00
Colony growth rate	14.17	0.00	13.75	0.59	13.25	0.89	11.95
CTCO	50% Fluffy mouse grey, 50% Fluffy olivaceous grey		50% Fluffy groh grey, 50% Fluffy olivaceous grey		25% Fluffy mouse grey, 75% Fluffy dark mouse grey		Fluffy mouse grey
CTCR	All Greyish blue		All Greyish blue		All Greyish blue		Sky grey

Note. StDev: Standard deviation; CTCO: Colony Texture and Colour-Obverse; CTCR: Colony Texture and Colour-Reverse. Cluster population is in parenthesis.

4. Discussion

The inflorescence dieback disease affect cashew at varied stages of flowering and different cashew accession are susceptible to this disease in Nigeria (Adeniyi et al., 2017) The paraphyses of *L. theobromae*, the dark flask-shaped and hollow structure of mature pycnidia in this study is in agreement with the description of (Ekundayo & Haskin, 1969a, 1969b) as the ostiole was elongated to form a neck having circular pore through which conidia were liberated. The natures of conidia of *L. theobromae* observed in this study are similar to the descriptions of Meredith (1961) for the structure and variation in conidia sizes of *L. theobromae*. Alves et al. (2008) also reported presence of paraphyses within the conidiomata and the conidia initially hyaline and aseptate but with time a formation of septum and dark brown and melanin deposition gave conidia a striated appearance. Adeniyi et al. (2016) reported significant variations observed in characters of *L. theobromae* isolates from dieback diseases of cashew and the similarity was observed in this study on conidia size (31.3 – 42.9 × 15.6 – 19.5 µm) of CDA1416 and conidia size of *L. gonubiensis* reported by Pavlic et al. (2004) except for the multi-septate conidia nature of *L. gonubiensis*. However, conidia sizes of isolates in this study were somewhat larger than those reported by Burgess et al. (2006) and Alves et al. (2008) for *L. theobromae.* The diversity and cryptic speciation in the isolates of *L. theobromae* can be further established using molecular characterization tools. Wider isolate collection areas/locations will substantiate the level of biodiversity of this pathogen on cashew agro-ecologies of Nigeria and effect of good agricultural practices could also be evaluated.

5. Conclusion

The differential characters in the isolates of *L. theobromae* were evident in the conidial morphology and textural variations. This understanding will form the bases for development integrated crop and diseases management strategy against the pathogen to enhance yield of cashew.

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