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The Role of Stomatal Traits and Epicuticular Wax in Resistance to *Mycosphaerella fijiensis* in Banana and Plantain (*Musa* spp.)

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Keywords: Banana - Black sigatoka - Epicuticular wax - *Musa* - *Mycosphaerella fijiensis* - Plantain - Stomata.

Summary

*Plantain (*Musa *spp., AAB group) cultivation is threatened by the black sigatoka disease caused by* Mycosphaerella fijiensis *Morelet, an airborne fungal pathogen. Twenty synthetic* Musa *hybrids of various ploidy, exhibiting a range of resistant and susceptible responses to the black sigatoka disease, were used to investigate the role of stomatal density, stomatal length and the thickness of epicuticular wax in resistance to* Mycosphaerella fijiensis. *The female parents of these hybrids were black sigatokasusceptible plantains, while the male parent was a wild, non-edible resistant banana. Stomatal length was negatively correlated with the initial development (incubation time) of black sigatoka in young host plant leaves in diploid but not in polyploid hybrids. Stomatal density on the abaxial surface of young leaves was negatively correlated with incubation time only in polyploids. Incubation time was positively correlated with the accumulation of epicuticular wax in both diploid and polyploid hybrids. Although the black sigatoka-resistant male parent lacks epicuticular wax, derived hybrids possessed epicuticular wax of various thickness which enhances black sigatoka resistance.*

Résumé

*La culture du plantain (*Musa *spp. groupe AAB) est menacée par la maladie de la cercosporiose noire causée par un pathogène cryptogame transmis par l'air,* Mycosphaerella fijiensis. *Morelet. Vingt hybrides synthétiques de* Musa *ayant divers degrés de ploïdie et une game variée de résistance et de sensibilité, ont été utilisés pour étudier le rôle de la densité stomatale, de la longueur des stomates, ainsi que de l'épaisseur de la cire épicuticulaire dans la résistance à* Mycosphaerella fijensis. *Les parents femelles de ces hybrides étaient des plantains sensibles à la cercosporiose noire, tandis que le parent mâle était une banane sauvage non comestible et résistante. La longueur des stomates était négativement corrélée au développement initial (période d'incubation) de la cercosporiose noire sur les jeunes feuilles des plantes hôtes diploïdes, mais non chez les hybrides polyploïdes. La densité stomatale sur la surface abaxiale des jeunes feuilles était négativement corrélée à la période d'incubation, mais uniquement chez les polyploïdes. La période d'incubation était positivement corrélée à l'accumulation de la cire épicuticulaire à la fois chez les hybrides diploïdes et polyploïdes. Bien que le parent mâle résistant à la cercosporiose noire soit dépourvu de cire épicuticulaire, les hybrides dérivés sont dotés de cire épicuticulaire de diverses épaisseurs ainsi que d'une plus grande résistance à la cercosporiose noire.*

Introduction

Plaintain and banana (*Musa* spp.) are important staple food crops in the humid and mid-altitude agro-ecological zones of sub-Saharan Africa (26). Banana leaf spot or black sigatoka, caused by *Mycosphaerella fijiensis* Morelet, is one of the most destructive diseases of banana and plantain. The extensive leaf necrosis caused by black sigatoka can reduce fruit yield by 30% to 50% (11, 18). Chemical control strategies exist but are environmentally unsound and unsustainable for the resource-poor smallholders that grow the crop in Africa.

Black sigatoka is an airborne fungal disease, which attacks the leaves of the plant. Ascospores are considered the main source of primary infection (18), while conidia play only a minor role (5, 18). Ascospores and conidia germinate on the leaf, especially on the exposed lower surface of the unfurling heart leaf (20). Spore germination is followed by epiphyllic growth of the germtubes, which enter the leaf through the stomata, and mycelial proliferation throughout the internal leaf tissues (10). During early stages of infection the leaf shows streaks, particularly on the lower surface of the leaf. Following continued fungal penetration and invasion, the streaks develop into lesions, then into large areas of black necrosis, followed by leaf death (3).

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An effective control strategy for black sigatoka is to provide high yielding resistant cultivars to farmers. Five distinct levels of host response to black sigatoka have been defined for breeding purposes; susceptible, less susceptible, partially resistant (14), highly resistant and extremely resistant (9). Triploid plantain cultivars and most triploid banana cultivars are susceptible to black sigatoka (8). Sources of resistance are, however, available in diploid bananas such as Calcutta 4, which appears to be extremely resistant to this leaf spot disease (14). Hybrids between plantains and Calcutta 4 show phenotypes ranging from highly resistant tot susceptible.

Previous reports suggest that stomatal density and the presence of epicuticular wax may play a role in host plant resistance to black sigatoka (14, 24). Vasquez *et al.* (24) found a negative correlation between the density of the stomata and the degree of resistance. Stomata play a keyrole in the infection process. As in most vascular plants, the stomatal apparatus in banana and plantain comprises two specialized epidermal cells (guard cells), joined at their ends but capable of separating in the middle to generate a pore through the epidermis into the intercellular space system (17). Stomata open due to increased turgor of the guard cells, facilitating infection by *Mycosphaerella fijiensis*. Stomatal opening and closure is stimulated by a great variety of external and internal factors. Elongation of the guard cells seems to be the main mechanism to pen the pore (7); however the overall length of the stoma changes very little with opening.

Stomatal density increases from the base to the middle of the lamina (20). The adaxial side of the leaf has fewer but larger stomata than the abaxial side. Stomatal size and ploidy are positively correlated, while stomatal density and ploidy are negatively correlated in banana and plantain (2, 16, 23). The appearance and ultrastructure of epicuticular waxes of plantain and banana vary between different genotypes (4) from non-existant to extensive waxiness. It has been suggested that epicuticular was may play a role in resistance to *Mycosphaerella fijiensis* (15). Wax on the leaf surface reduces the accumulation of moisture on all leaves and therefore may retard the establishment and germination of spores (1, 14, 24).

In this experiment the role of stomatal traits and epicuticular wax in disease development and host resistance to black sigatoka in 20 euploid (diploid, triploid and tetraploid) half- and full-sib hybrids was assessed.

Material and Methods

F_1 euploid hybrids were derived from interspecific interploidy crosses (27). Locally adapted French plantain (*Musa* spp., AAB group) cultivars Obino l'Ewai (OL) from Nigeria and Bobby Tannap (BT) from Cameroon were used as female parents (21) while the wild, non-edible diploid banana *M. acuminata* ssp. *burmannicoides* Calcutta 4 (C4) from Burma was used as the male parent due to its high level of resistance to black sigatoka. TMPx 1206-2 (an F_1 hybrid derived from the cross between the somaclonal variant French Reversion of the Nigerian False Horn plantain cultivar Agbagba and C4) was self-pollinated to produce an F_2 population.

The field layout was a completely randomized design with five plants per genotype. Plants were spaced 3 m between rows and 2 m within rows. The plot was surrounded by the black sigatoka-susceptible Cavendish banana cv. Valery. The experiment was located at the IITA High Rainfall Station in Onne (4°51'N, 7°3'E), southeastern Nigeria. Annual rainfall averages 2400 mm and temperatures average 27°C. Black sigatoka is ubiquitous in this area, thus negating the need for artificial inoculation.

Data were collected from 1993 to 1995 on the four parental genotypes, six euploid hybrids of BT x C4, nine euploid hybrids of OL x C4 and four F_2 progenies. The susceptible False Horn plantain cv. Agbagba was included in the trial as a reference susceptible clone. Each genotype of the segregating population was individually assessed for response to black sigatoka. Host response to black sigatoka for the selected hybrids was established from previous trials (14, 25, 28) by recording at flowering the number of the youngest leaf spotted (YLS), counting down from the first (top) open leaf (19, 22). Those hybrids which have a host response similar to the susceptible cv. Agbagba were rated as susceptible. The other hybrids, showing a significantly greater number of functional leaves than the cv. Agbagba, were rated according to their disease severity as less susceptible, partially resistant, and highly resistant.

Estimation of the incubation time for *M. fijiensis* on different host genotypes started four months after planting. All plants were observed every other day to score Brun's stage B of the leaf (9). At this stage the cigar leaf has emerged from the preceding leaf, and is still fully rolled but has not reached its full length. Similarly, the second streak stage on the Fouré scale, i.e., brown stripe on the inderside of the leaf (3), was scored for each leaf. The incubation time was calculated as the number of days elapsing between these two stages at the early phase of plant growth (IT_y, based on an average of third and fourth leaves) and throughout the growth cycle (IT).

Leaf samples of 3 cm^2 were excised from the centre of the third and the fourth leaf of each plant and stored in 95% ethanol for decoloring. The samples were then mounted in glycerine and observed using a light microscope (16). The density and length of stomata on adaxial and abaxial surfaces was measured at 400x magnification using an ocular micrometer. Data were recorded from a random selection of 10 0.3 mm^2 fields per leaf per plant. The width and persistence of the apertures of the stomata depends mainly on the light intensity and degree of water stress (7). Hence, leaf samples were taken at the same time each day to avoid possible differences due to opening or closing of the stomata. Leaf waxiness was evaluated according to a scale with three distinct categorles: 1 (none), 2 (moderate) and 3 (extensive).

Factorial analysis of variance with unequal number of observations was performed for stomata traits, following the additive liner model

$$Y_{ijkl} = \mu + L_i + G_j + LG_{ij} + e_{ijk} + S_{ijkl.}$$

where Y_{ijkl} is the phenotype of i^{th} leaf surface, measured in the l^{th} sample of the k^{th} plant in the j^{th} genotype, μ is the mean, L_i and G_j are the leaf surface and genotype effects, LG_{ij} is the effect of the interaction between leaf surface and genotype, and e_{ijk} and S_{ijkl} are the experimental and sampling errors, respectively. In addition the effect of genotype on incubation time was analyzed by a one-way ANOVA using the additive linear model

$$Y_{jkl} = \mu + G_j + e_{ijk} + S_{jkl.}$$

The coefficient of variation was calculated to determine the degree of precision with which the traits are compared and the reliability of the experiment. $LSD_{0.05}$ was used to analyze differences in stomatal length and density between the tested genotypes. Phenotypic correlations were calculated for segregating materials at the same ploidy level to determine associations between morphological descriptors and incubation time. Also, regression models ($IT = \alpha + \beta_i X_j$) were developed to dertermine the effect (β_i) of morphological traits (X_j) on the dependent variables IT_y and IT (6). All statistical analyses were done using MSTAT-C (13) software.

Results and Discussion

The two plantains (Bobby Tannap and Obino l'Ewai) and the banana parent (Calcutta 4) of the genotypes were first assessed to dertemine the extent of phenotypic polymorphism for stomatal traits and epicuticular wax on the leaves (Table 1). The black sigatoka-susceptible triploid cultivars Bobby Tannap and Obino l'Ewai had fewer but longer stomata than the extremely black sigatoka-resistant diploid banana Calcutta 4. This contrasts with the report of Vasquez *et al.* (24), who indicated that germplasm resistant to black siga-

Table 1
Black sigatoka (BS) reaction, stomatal density and length, waxiness of the leaves and disease incubation time in young leaves (ITy) and throughout the growth cycle (IT) of parents and their respective diploid, triploid and tetraploid offspring.

Clone	Genome/ cross	Ploidy	Black sigatoka reaction[1]	ITy (days)	IT (days)	Stomatal Density (mm^{-2}) adaxial	Stomatal Density (mm^{-2}) abaxial	Stomatal Lenght (µm) adaxial	Stomatal Lenght (µm) abaxial	Leaf Waxines
Obino l'Ewai (OL)	AAB	3x	S	25.3	21.4	6.65	54.75	36.40	31.00	3
9243-2	OL x C4	2x	S	26.8	21.7	29.22	81.39	30.63	24.70	1
1549-7	OL x C4	2x	LS	24.9	24.8	32.22	96.89	30.06	24.20	1
1448-1	OL x C4	2x	PR	26.6	28.4	33.36	96.31	29.26	24.38	2
9593-1	OL x C4	3x	S	24.2	24.0	12.04	50.01	38.80	32.75	2
5860-1	OL x C4	4x	S	22.8	24.4	11.94	43.58	39.70	33.74	2
597-4	OL x C4	4x	S	27.4	25.4	11.16	43.53	39.90	34.18	2
5706-1	OL x C4	4x	LS	26.5	22.2	9.52	47.25	38.76	33.44	2
548-9	OL x C4	4x	PR	23.2	24.7	11.56	51.04	40.93	34.79	2
6930-1	OL x C4	4x	HR	24.9	24.6	9.80	47.52	37.84	32.66	2
Calcutta 4 (C4)	AA	2x	ER	57.9	54.8	27.45	88.95	26.80	21.10	1
Bobby Tannap (BT)	AAB	3x	S	25.9	22.3	6.3	52.65	34.00	28.20	3
9007-4	BT x C4	2x	S	22.5	20.0	30.05	83.40	31.70	24.70	1
5233-2	BT x C4	2x	LS	22.5	22.3	23.79	65.13	34.17	28.27	1
1518-4	BT x C4	2x	PR	24.0	30.6	27.13	79.36	32.34	25.44	2
1187-8	BT x C4	4x	S	27.4	25.4	8.71	55.61	39.66	37.12	2
4479-1	BT x C4	4x	LS	25.9	25.6	10.31	38.37	40.64	34.78	2
582-4	BT x C4	4x	PR	26.4	30.6	12.04	48.30	42.26	35.60	3
1206-2	Fr. rev.xC4	2x	S	31.0	22.8	20.16	73.28	29.49	23.60	1
F_2 progenies										
8256-5	1206-2 S1	2x	S	27.0	20.5	22.40	69.69	31.60	26.45	1
8096-15	1206-2-S1	2x	LS	28.0	26.0	26.93	72.53	30.98	25.24	2
8096-12	1206-2-S1	2x	PR	29.5	23.3	30.41	86.19	27.53	22.33	1
8256-4	1206-2-S1	2x	HR	28.0	27.8	38.48	89.69	30.18	24.73	1
Agbagba	AAB	3x	S	23.2	22.6	10.78	52.74	36.12	30.98	2
Mean				27.1	26.0	19.41	64.26	35.26	29.59	
$LSD_{0.05}$				4.1	5.0	------- 3.19 -------		------- 0.66 -------		
Coefficient of variation (%)				17.0	15.3	------ 12.27 ------		------- 3.27 -------		

[1] BS reaction as measured by the youngest leaf spotted at flowering (14, 25, 28): S (susceptible), LS (less susceptible), PR (partially resistant), HR (highly resistant), ER (extremely resistant).

[2] 1 (none) 2 (moderate), 3 (extensive).

toka had low stomatal densities. Nevertheless, the different ploidy levels of the parental genotypes may confound the relationship between length of stomata and resistance. Hence, our investigation was carried out on segregating full-sibs of the same ploidy level which differed significantly in their resistance to black sigatoka.

Based on the coefficients of variation, the measurements of stomatal length were more precise than those of stomatal density (Table 1). This reflects the measurement procedures, which were more precise for length than for density. Stomatal length was determined with the aid of an eyepiece with micrometer scale whereas stomatal density was recorded by counting numbers of stomata in randomly selected 0.3 mm^2 eyefields. There were no significant differences ($P > 0.05$) in stomatal density and length between full-sib and half-sib F_1 hybrids of similar ploidy, which exhibited different levels of resistance.

Most correlations between stomatal traits and incubation time (IT) were not significant ($P > 0.05$) However, length of stomata in the adaxial leaf surface was negatively correlated with IT_y, but only in the diploid (2x) hybrids ($r = -0.757$; $P = 0.006$). IT_y was shortened one day by increasing 1 µm in stomatal length in the third and fourth leaf ($IT_y = 63.2 - 1.2$ stomatal length). Also, a decrease of stomata density on the adaxial leaf surface increased IT_y in polyploids (3x and 4x) ($IT_y = 36 - 0.98$ stomatal density; $R2 = 0.475$; $P = 0.036$). Similarly, a positive correlation between IT and length of stomata on the adaxial surface was observed in polyploid (3x and 4x) hybrids ($r = 0.712$; $P = 0.028$). On average an increase of 1 µm in stomatal length on the adaxial leaf surface resulted in a 1.4-days increase in the IT in the polyploid hybrids ($IT = -31.4 + 1.4$ stomatal length). However, the lack of a significant correlation ($P > 0.05$) between IT_y and IT in both diploid and polyploid hybrids suggested that stomatal traits such as density in polyploids and length in diploids are potential mechanisms of resistance to black sigatoka only in early stages of plant growth.

There was a strong association between stomatal traits and ploidy. Supporting the findings of Borges (2), Simmonds (16), and Vandenhout *et al.* (23), diploids on average have short stomata but high stomatal density, whereas triploids and tetraploids have long stomata at low density.

A significant phenotypic correlation between IT and epicuticular wax was observed for diploid ($r = 0.723$, $P = 0.011$) and polyploid ($r = 0.889$; $P = 0.001$) hybrids. This was surprising since Calcutta 4 lacks epicuticular wax on its leaves. However, this trait seems to enhance host plant resistance by lengthening the incubation time of the disease in the leaves of the black sigatoka resistant hybrids. The presence and/or increase of epicuticular wax (wx) on the leaf surface lengthened the incubation time by 5 days on average in the diploids ($IT = 18 + 5.3wx$) and 7 days in the polyploids ($IT = 11.5 + 6.5$ wx). Hence, accumulation of epicuticular wax on the leaves, which is due to a wx recessive gene (15), might play a role in resistance to black sigatoka. An increase in the frequency of the wx allele could be related to an enhanced host plant resistance to black sigatoka in the hybrids (15). This would explain why the coefficient of correlation was higher in the polyploids which may have more copies of the wx allele (i.e., dosage effect) than their diploid full-sibs.

Host plant resistance to *Mycosphaerella fijiensis* may be due to a delay in the development of the disease. Morphological traits such a stomatal density should not be regarded as influencing resistance to this disease after fungal infection. However, epicuticular wax and stomata trait may affect incubation time, especially in secondary disease cycles. In addition, it is likely that resistance to black sigatoka is also affected by biochemical factors which influence the development of the disease (12, 14).

Our research was based on observation of segregating materials with similar genetic background and ploidy levels which should be a more powerful tool for investigation of resistance mechanisms than those based on phenotypic observations of cultivated landraces. Indeed, putative trait associations may be observed in triploid crops such as banana and plantain due to spurious genetic linkages and vegetative propagation.

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