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Host–pathogen interactions of *Musa* spp. and *Mycosphaerella musicola* with epidemiological variables and leaf anatomy within the pathosystem of Yellow Sigatoka disease

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Abstract

Yellow Sigatoka, which is caused by *Mycosphaerella musicola/Pseudocercospora musae*, is the primary biotic problem for the Brazilian banana crop, with the causal agent presenting high pathogenic variability among the pathogen isolates. Few details concerning the host-pathogen interactions that occur during the infection process are available for this disease. Studies have been carried out under greenhouse conditions with artificial inoculation with 2 *M. musicola* isolates (1- Lavras, Minas Gerais state and 2- Cruz das Almas, Bahia state) of a group of banana varieties, including 'Grand Naine', 'Prata Anã', 'Calipso', 'Preciosa', 'Japira' and 'BRS Platina'. In each plant, two leaves were inoculated; F1- Leaf number one and F2 - Leaf number two. After the dynamics of host-pathogen interactions, the different bananas genotypes were evaluated and the fungus-associated changes in the leaf anatomy within the tissues and stomata were determined. Inoculations were carried out with a suspension of 4.10^4 conidia/mL that was sprayed onto micropropagated plants of the different genotypes. The lower limb surface of the number one leaves (F1) and number two leaves (F2) was sprayed until runoff. The data were collected weekly over ten weeks, although shorter incubation and latent periods were observed in the susceptible varieties (Grand Naine and Prata Anã). The Grand Naine genotype showed the greatest disease severity, confirming its susceptibility to yellow Sigatoka. The leaf inoculation of *M. musicola* altered the anatomy. In most cases, contamination increases the thickness of the epidermis, hypodermis and parenchyma. However, the thickness of leaf tissues was reduced in Prata Anã. The BRS Platina hybrid showed the greatest variation in these parameters. In general, infection with *Mycosphaerella* significantly decreased the stomatal density. Exceptions to this behavior occurred in the epidermis of the leaves of Grand Naine and BRS Platina. The resistant genotypes, Preciosa, Japira and BRS Platina, are promising for commercial plantations in regions where the yellow Sigatoka inoculum pressure is high. The isolate from Cruz das Almas, Bahia State, was more pathogenic (AUDSPC=7.9100) than was the one from Lavras, Minas Gerais State (AUDSPC=3.2737), in which the Grand Naine susceptible genotype was inoculated. Yellow Sigatoka infection changed the leaf anatomy by altering the leaf thickness and the number and size of the stomata.

Keywords: Disease; Banana; Progress curve disease; Plant anatomy; Brazil.
Abbreviations: AUDSPC_area under disease severity progress curve, PD_polar diameter, ED_equatorial diameter, SD_stomatal density, BOD_biochemical oxygen demand, IP_incubation period, LP_latency period, CRBD_completely randomized block design.

Introduction

Bananas (*Musa* spp.) are characterized as important sources of income, in addition to being a staple food for small producers and poor populations in many developing countries. In addition, the fruit is present in the daily diet of all social classes in Brazil. Brazil is one of the major producers and consumers of banana (*Musa* spp.). In 2011 alone, Brazil produced 7,349,379 t of bananas in an area of approximately 528,879 ha (IBGE, 2011). Despite the production of approximately 8% of the global banana supply, Brazil accounts for only 1% of world banana exports (Lichtemberg and Lichtemberg, 2011) because domestic production is almost entirely directed to the domestic market due to the large population and high per capita national consumption. Yellow Sigatoka, which is caused by *Mycosphaerella musicola/Pseudocercospora musae*, continues to be a great problem for the banana crop in Brazil, with the causal agent presenting high pathogenic variability (Cordeiro and Matos, 2005). This pathogen is responsible for reducing the production, productivity and profitability of this crop. In Brazil, yellow Sigatoka was first discovered in the Amazon region in 1944 and is now present in the entire country, although with greater effects in regions where rains are more frequent and where temperatures remain around the optimal level of 25 °C (Cordeiro and Matos, 2005). Yellow Sigatoka is a polycyclic disease. Reproductive structures are produced and disseminated continuously and may result in several disease cycles annually (Zapater et al., 2008). Thus, an increase in the pathogen population in a susceptible host may cause exponential growth for a short duration, provided the environment is favorable (Vale et al., 2004).

The disease progress curve describes the proportion of disease over time and is the best way to represent an epidemic (Madden et al., 2007). Other important parameters to quantify the disease include incubation and latency periods, which are the periods of time between inoculation and the appearance of symptoms and between inoculation and sporulation, respectively (Parlevliet, 1979). The incubation period may indicate the degree of resistance for the plant genotype. The best strategy to control the disease, both economically and environmentally, is the use of resistant cultivars (Pimentel et al., 2010). Yellow Sigatoka causes necrotic leaf lesions, which drastically reduce the leaf area and consequently the fruit quality (Chillet et al., 2009). The first line of defense against plant pathogens is the plant surface, to which microorganisms must adhere to penetrate and cause infection (Agrios, 2004). Some characteristics, such as the quantity and quality of the wax and cuticle that cover the epidermis; the size, location and shape of the stomata and lenticels; and the presence of thick cell walls, may avoid infection. Moreover, the physical strength and chemical stability of lignin play a secondary but important role not only in cellular protection against insects because of the indigestibility of lignin by insects but also often in ceasing pathogen growth (Taiz and Zeiger, 2004). Considering the existence of possible physical barriers to the fungus, the leaf anatomy (mainly leaf tissue thickness and density and size of stomata) was studied in different banana genotypes that were infected with *Mycosphaerella* sp. differential mechanisms were discovered and evaluated (Craenen et al., 1997; Valerio et al., 2002; Hernandez et al., 2006). To evaluate the possible relationship between some changes in the anatomic variables of the foliar epidermis in banana plants that were inoculated with *Mycosphaerella* sp., Rodríguez et al. (2009), the leaf lamina tissues were studied in the genotypes 'Titiaro' (AA), 'Grand Naine' (AAA), and 'FHIA-18' (AAAB). The authors observed the highest adaxial and abaxial cuticle thickness and low stomatal density on the abaxial epidermis on 'FHIA-18' as well as the presence of anatomic characteristics in the epidermis of 'FHIA-18' that could represent more effective physical barriers against the penetration and colonization of the fungi and could be associated with fungi resistance. 'Titiaro' has the highest stomatal density, which may be associated to its susceptibility to Sigatoka disease. Because yellow Sigatoka is the main limitation of banana plantations in Brazil, one goal of this study was to determine the dynamics of the host-pathogen interactions in banana genotypes that were artificially inoculated with monocyclic isolates of *M. musicola* from Lavras, Minas Gerais State, and Cruz das Almas, Bahia State, both in Brazil, in the Southeast and Northeast Regions, respectively. Another goal was to identify and assess the fungus-caused anatomical changes in the leaf tissues and stomata of the above artificially inoculated and infected banana plants.

Results

Monocyclic parameters of the M. musicola isolate from Cruz das Almas, Bahia State, Northeast Region of Brazil

The areas under disease severity progression curve (AUDSPC) varied significantly with the leaf position on the banana plants (Tables 1 and 2). As shown in Table 1, both the 'Grand Naine' and 'Prata Anã' cultivars had proportionately higher disease and severity as evidenced by a greater AUDSPC than that of the other two genotypes. The AUDSPC that were related to the leaf position were obtained from the inoculations of the second leaves-F2 (3.2975) (Table 2). Shorter average incubation periods, denoting greater isolate aggressiveness, were observed in the Grand Naine (21.87 days) and the Prata Anã (26.25 days) cultivars (Table 1) due to their susceptibility to yellow Sigatoka. These two genotypes are different in terms of IP values and showed the highest susceptibilities to yellow Sigatoka (Table 1).

During the latency period, the inoculated leaf position × genotype interaction (Table 3) was significant. When leaf number one was inoculated, Grand Naine showed the shortest period of 38.5 days. However, when leaf number two was inoculated, Prata Anã and Grand Naine behaved similarly and showed the shortest latency periods of 31.50 and 34.25 days, respectively. A comparison of the latency periods between the two inoculated leaf positions within the genotypes was statistically significant only for Prata Anã, which showed the shortest latency period for leaf number 2 (Table 3).

Monocyclic parameters of the M. musicola isolate from Lavras, Minas Gerais State, in the Southeast Region of Brazil

For the isolate from Lavras, Minas Gerais, there was no significant difference for the incubation period variable (Table 1) , but for variable AUDSPC significant effect (Tables 1 and 2). When the plants were inoculated with *M. musicola*, Grand Naine produced the greatest AUDSPC value of 3.2737. The other cultivars showed significantly smaller AUDSPC values (Table 1). A significantly larger value of 1.1954 for the AUDSPC occurred with inoculations to leaf number two (F2) (Table 2). As the experiments were performed at different times, the favorable weather during Experiment 1 (average temperature of 21.9°C, 4.77 mm of precipitation and 88.39% relative humidity) involving the isolate from Cruz das Almas might have promoted disease progression (Table 4). For Experiment 2, the conditions were warmer, drier and less humid (average temperature of 24.15°C, 1.22 mm of precipitation and 79.37% relative humidity) (Table 4). The interaction between the genotype and inoculated leaf position significantly affected the duration of the incubation period, which is related to the aggressiveness of the isolate. The shortest incubation periods were observed for leaf number two - F2 for the Grand Naine (22.75 days), Prata Anã (28.00 days) and Calipso (29.75 days) cultivars as shown in Table 3. For leaf number one, Calipso had the shortest incubation period (29.75 days). The latency period was also significantly affected by the genotype x inoculated leaf position interaction (Table 3). The shortest latency period of 29.75 days was observed for Grand Naine, which is susceptible to yellow Sigatoka, when the inoculation was performed on leaf number two - F2. The differences between the two leaf positions within the genotypes were statistically significant for the Grand Naine and Prata Anã cultivars (Table 3), and leaf number two - F2 displayed the shorter latency period.

Leaf anatomy of the banana genotypes that were inoculated with M. musicola

Table 5 shows the thickness of the epidermis, hypodermis and the palisade and spongy parenchymal layers in healthy leaves and in leaves that were infected by *M. musicola*.

In 50% of the genotypes, thickness changes occurred in the epidermis of the leaves. In these genotypes, the thickest epidermis and therefore the greatest changes were observed in leaf number two – F2 (Fig 1).

Table 1. Area under the disease severity progress curve (AUDSPC) and incubation period (IP) of *Mycosphaerella musicola* isolates from different locations infecting different banana genotypes.

Genotypes	IsCA		IsL
	AUDSPC	IP	AUDSPC
Japira	1.0025 C	34.62 B	0.2162 B
BRS Platina	1.0025 C	37.87 A	0.4725 B
Calipso	1.6237 C	32.37 C	0.2162 B
Preciosa	1.6237 C	32.37 C	0.4887 B
Prata Anã	4.1500 B	26.25 D	1.1787 B
Grand Naine	7.9100 A	21.87 E	3.2737 A

Means within the columns followed by the same letter do not differ by Scott-Knott's test at the 5% probability level ($p \leq 0.05$). Legend: IsCA - Cruz das Almas' isolate; IsL - Lavras' isolate.

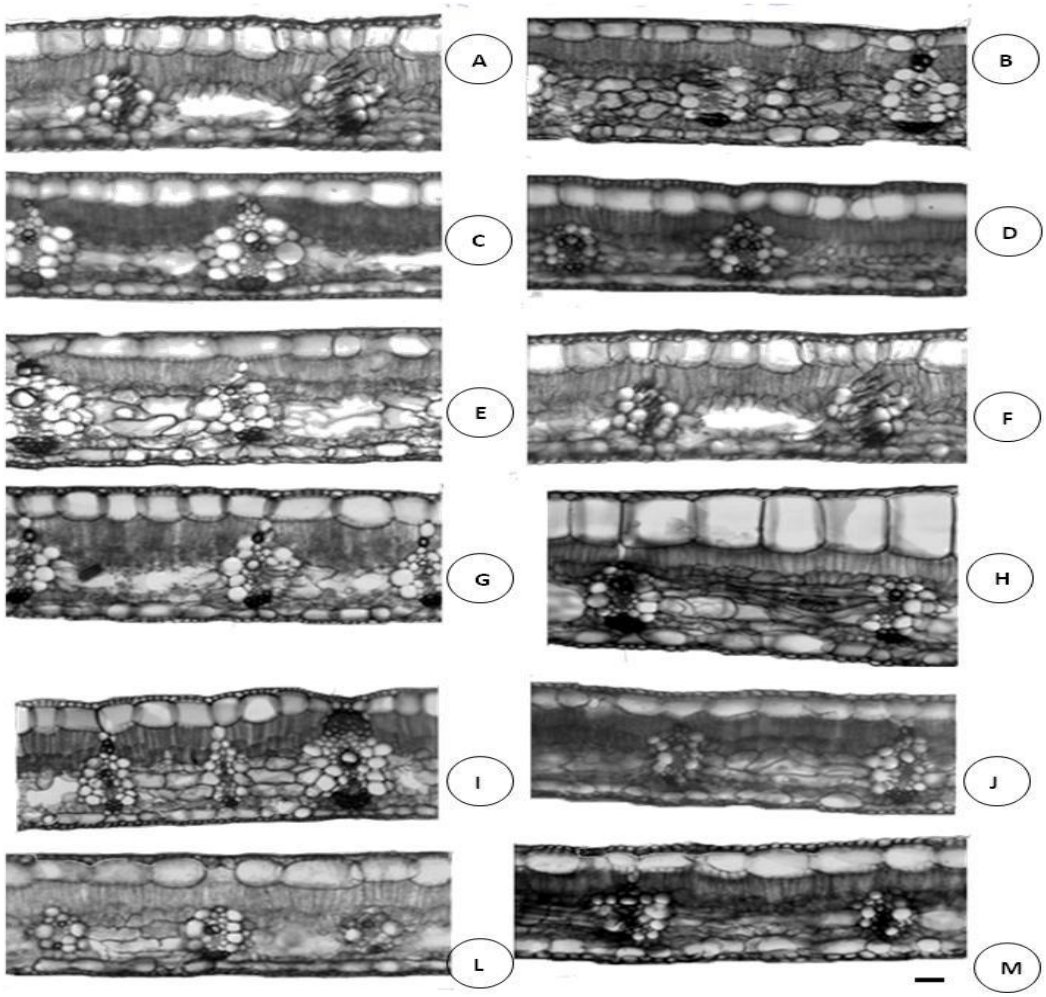

Fig 1. Cross sections of healthy leaves (left) and F2-type leaves that were infected (right) with *Mycosphaerella musicola* Leach in the Calipso (A-B), Grand Naine (C-D), Japira (E-F), BRS Platina (G-H), Prata Anã (I-J) and Preciosa (L-M) cultivars of banana.

The hybrid BRS Platina cultivar showed the greatest variation in the thickness of the tested leaves (Table 5). With the exception of the Calipso genotype, the changes occurred in the abaxial surface of the epidermis, and only the F2 leaves showed epidermal sheets with an abaxial surface that was thicker than that in the leaves of the control plants. The leaves at other positions showed varied behaviors (Table 5).

The fungal inoculations also altered the hypodermis and the thickness of the infected leaf tissue (Table 5). The changes in the parenchyma affected all but the BRS Platina genotype and differed among the other genotypes and between the leaf positions. The Japira cultivar, which is resistant to yellow Sigatoka (Azevedo et al., 2010), has the healthiest leaves and the thickest palisade among all of the cultivars. Fungal infections also caused changes in the spongy parenchyma of the leaves of the dwarf cultivar Silver, which is considered susceptible (Cordeiro et al., 2006). These results indicate that inoculation with *M. musicola* changes the leaf anatomy. In most cases, the contamination increases the thickness of the epidermis and hypodermis parenchyma. However, in some genotypes, the Prata Anã thickness decreased in these tissues. In addition to Calipso, the number two - F2 leaves of all of the genotypes were the most anatomically altered (Table 5).

Table 6 shows that in all of the evaluated genotypes, a greater stomatal density was observed on the abaxial surface of the epidermis, the main place of fungal infection. As occurred in

Table 2. Area under the disease severity progress curve (AUDSPC) for different types of leaves from six banana genotypes (Japira, BRS Platina, Calipso, Preciosa, Prata Anã, Grand Naine) that were inoculated with different *Mycosphaerella musicola* isolates.

Leaves	IsCA	IsL
Leaf 1 (F1)	2.4733 B	0.7533 B
Leaf 2 (F2)	3.2975 A	1.1954 A

Means within the columns followed by the same letter do not differ by Scott-Knott's test at the 5% probability level ($p \leq 0.05$). Legend: IsCA - Cruz das Almas' isolate; IsL - Lavras' isolate.

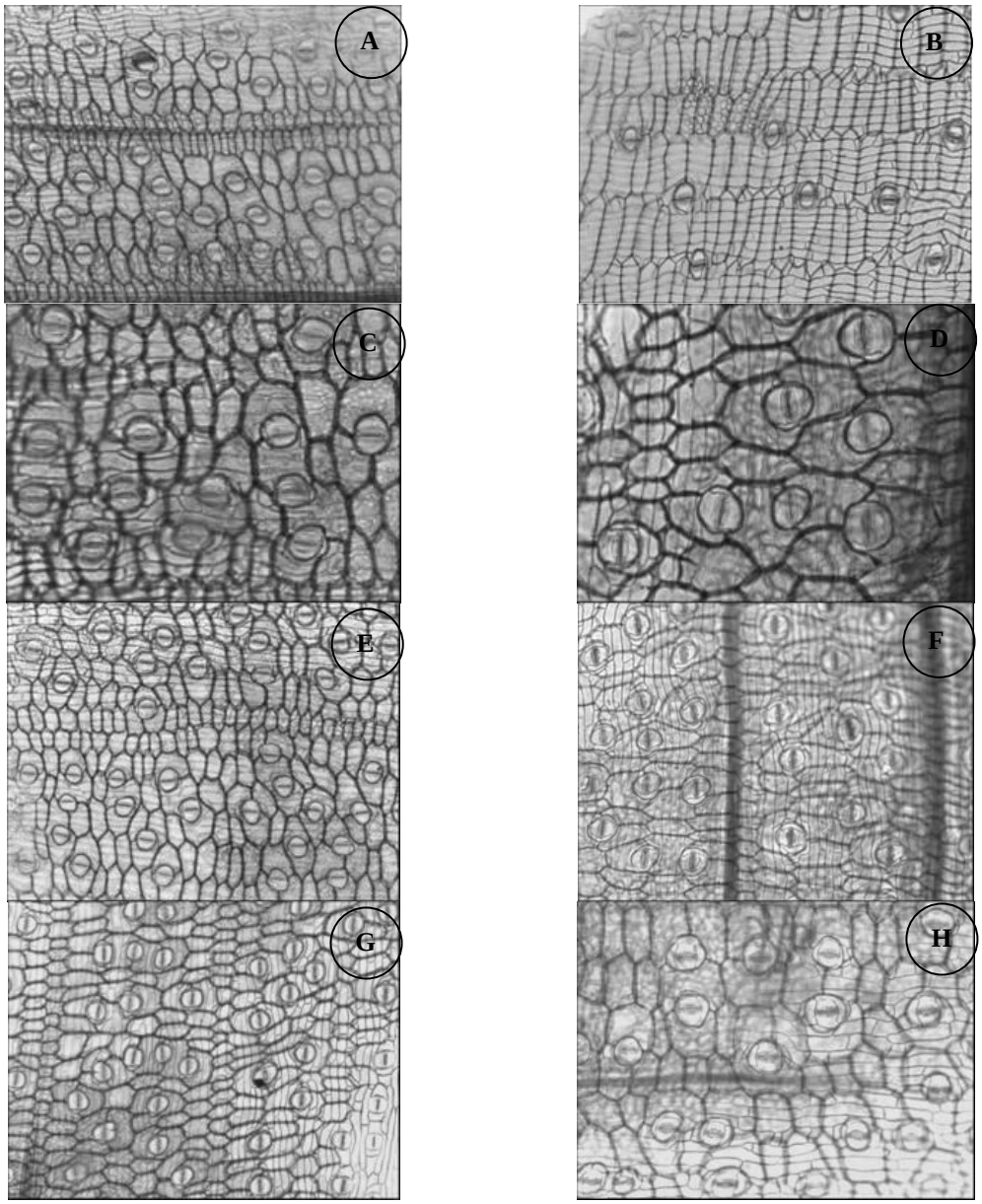

Fig 2. Paradermal sections of healthy leaves (left) and leaves that were infected (right) with *Mycosphaerella musicola* Leach in the Calipso (A-B), Prata Anã (C-D), Japira (E-F) and Preciosa (G-H) cultivars of banana.

the anatomical structure of the infected leaves, fungal infection also changed the stomata structure. On both sides of the leaves, changes occurred in the polar diameters (PD) of the stomata of infected leaves with a tendency to increase in size. The disease also changed the equatorial diameter (ED), mainly in the adaxial epidermis stomata (Table 6). The ratio of the polar over equatorial diameters was also altered on the abaxial surface of the epidermis. On this surface, there was an increase in the ratio between the diameters. On the adaxial surface, there were also changes in the relationship between the PD and ED in half of genotypes (Table 6). These modifications occurred in different types of leaves' lamina, depending on the genotype. In general, infection by *M. musicola* significantly decreased the stomatal density (Fig 2). Exceptions to this behavior occurred in the epidermis of the leaves of Grand Naine and BRS Platina. In the abaxial epidermis of Grand Naine, which is susceptible to yellow Sigatoka, no differences were observed between the infected and healthy leaves. However, on the adaxial surface of the hybrid BRS Platina, there was an increase in the stomatal density (SD) of F2 leaves.

Table 3. Incubation (IP) and latency (LP) periods of *Mycosphaerella musicola* in relation to the different banana genotypes, fungal isolates and types of inoculated leaves.

Genotypes	Lavras				Cruz das Almas	
	IP		LP		LP	
	Leaf 1	Leaf 2	Leaf 1	Leaf 2	Leaf 1	Leaf 2
BRS Platina	35.00 Cb	36.75Aa	56.00 Aa	57.75 Aa	56.00 Aa	56.00 Ab
Preciosa	35.00 Ca	35.00 Ba	49.00 Bb	49.00 Bb	51.75 Ba	50.75 Ca
Japira	32.00 Db	33.20 Ca	45.50 Cb	47.25 Cb	56.00 Aa	52.50 Ba
Calipso	29.75 Ea	29.75 Da	42.00 Eb	42.00 Da	48.25 Da	42.00 Da
Prata Anã	42.00 Aa	28.00 Eb	49.00 Bb	37.25 Ea	50.75 Ca	31.50 Fb
Grand Naine	36.75 Ba	22.75 Fb	42.75 Da	29.75 Fb	38.50 Eb	34.25 Ea

Means within the columns and rows followed, respectively, by the same upper- and lowercase letters do not differ by Scott-Knott's test at the 5% probability level ($p \leq 0.05$). IP=Incubation Period; LP= Latency Period.

Discussion

Monocyclic parameters of the M. musicola isolate from Cruz das Almas, Bahia State, in the Northeast Region of Brazil

The AUDSPC is inversely proportional to the degree of resistance; therefore, genotypes with very low AUDSPC values are resistant, while those with high values are susceptible (Table 1). At 24°C, Grand Naine also showed greater AUDSPC values compared to those of other cultivars, such as Caipira, Prata Zulu and Pacovan, which did not differ in this characteristic, which is consistent with findings from Rocha (2008). The predominant susceptibility of younger leaves to *M. musicola* was previously reported by Romero (1995) and Stover (1980). One must consider, however, that the mentioned related research works were performed with *Mycosphaerella fijiensis.* The largest (AUDSPC) that were related to the leaf position were obtained in inoculations of leaves F0 and F1, at 1.83 and 1.49, respectively (Rocha, 2008). The fungus infects young leaves, including leaves zero (unopened leaf), one, two, three and, exceptionally, leaf four (Stover, 1980). The initial symptom of yellow Sigatoka is slight discoloring in the form of spots between leaf venation; these spots become long necrotic lesions with yellowing edges. In severe attacks, these lesions may coalesce and affect as much as greater than 50 % of the leaf area, causing the premature death of the leaf (Rocha Júnior et al., 2010).

However, the appearance of stripes of greenish to brown color as seen by the naked eye on the second leaf depends on the genotype and environmental conditions, which explains the results of the variable AUDSPC in this work (Wardlaw, 1961). Generally, the older leaves present a greater number of lesions during more developed stages. It is possible that the higher level of disease development in leaf number F2 with a greater stomata density caused this result because the spore concentration in both of the inoculated leaves was the same. The incubation periods results are similar to those published by several authors (Meredith, 1970; Gauhl, et al., 1993), who claim that the small differences between the results can be explained by local climatic conditions in each experiment (Marin et al., 2003). Banana genotypes that are susceptible to *M. musicola* and *M. fijiensis* have a lower incubation period and greater number of sporulated spots on the leaves compared to those of the resistant genotypes. With the increased level of resistance, a correspondent increase in the transition time between disease stages also occurs. In some resistant genotypes, the progress of the symptoms is interrupted during the early stages (Fouré et al., 1990). It is noteworthy that these genotypes, in fact, are tolerant of yellow Sigatoka, which explains the appearance of symptoms that are significantly smaller and of a longer lesion developmental time. In Costa Rica under favorable weather

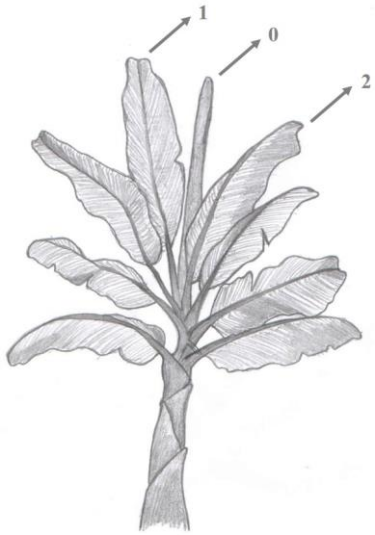

Fig 3. Numeric position of the leaves on the banana plants. Legend: 0 - F0; 1 - F1 and 2 - F2. (Photo courtesy of: Jonathan Faria Bonette).

conditions and susceptible hosts, the incubation period can vary from 13-14 days, while under unfavorable weather conditions, this duration may extend for up to 35 days for *M. fijiensis* (Marin et al., 2003). Comparing the progress of black and yellow Sigatoka in bananas and plantains in various ecological zones of Cameroon, Mouliom-Pefoura and Mourichon, (1990) observed incubation periods for *M. musicola* that were lower compared to those of *M. fijiensis* (15 to 18 days and 22 to 25 days, respectively). This fact, according to the authors, may be attributed to the dominance of yellow Sigatoka in regions of high altitude. The first visible symptoms appear between 11 and 106 days after germination, and this variation depends on the infected genotype (Meredith, 1970). The susceptible cultivars Grand Naine and Pacovan had the lowest incubation periods (20.83 and 20.66, respectively), while the resistant genotypes, such as Caipira (26.33) and Prata Zulu (26.16), showed the greatest incubation periods, agree with the results of Rocha (2008). Likewise, the incubation period and latency period also vary according to the climatic conditions, host susceptibility and infection intensity (Marin et al., 2003). In Costa Rica, this variable in Grand Naine lasted 25 to 70 days during the wet and dry seasons, respectively, for *M. fijiensis.* Thus, the shorter are the incubation and latency periods, the

Table 4. Mean values of the maximum (T. max), minimum (T. min) and average (T. average) temperatures, relative humidity (RH) and precipitation (P). Cruz das Almas, Bahia State, Brazil.

Month in 2010	T. max (°C)	T. min (°C)	T. average (°C)	RH (%)	P (mm)
June	26.35	19.86	22.69	87.32	3.19
July	25.29	19.4	21.77	91.35	8.53
August	25.60	18.11	21.34	86.50	2.60
Sept.	26.82	19.02	22.34	82.53	2.37
October	30.11	21.43	24.60	82.09	1.12
Nov.	31.61	21.49	25.51	73.51	0.16

Source, Meteorological Station of Embrapa Cassava and Fruits (2010).

Table 5. Thicknesses in µm of the adaxial (E.Ad.) and abaxial epidermis (E.Ab.), adaxial (H.Ad.) and abaxial hypodermis (H.Ab.) and the palisade (PP) and spongy parenchyma (SP) of healthy banana leaves and leaves that were inoculated with *Mycosphaerella musicola*.

Treatment	E.Ad.	E.Ab.	H.Ad.	H.Ab.	PP	SP
Calipso C	7.92 b	8.67 a	41.42 b	26.50 a	76.50 a	114.25 b
Calipso F0	8.92 b	8.00 a	52.00 a	30.67 a	67.92 b	99.75 b
Calipso F1	8.61 b	8.48 a	50.17 a	21.23 b	74.26 a	91.88 b
Calipso F2	10.50 a	9.33 a	51.92 a	27.58 a	62.25 b	119.92 a
CV (%)	13.80	15.11	12.25	16.93	15.91	13.62
Grand Naine C	9.12 a	6.79 b	46.64 c	16.46 d	67.83 a	71.84 c
Grand Naine F0	9.50 a	6.33 b	39.83 c	20.67 c	45.58 b	49.25 d
Grand Naine F1	7.92 a	7.67 a	60.42 b	25.50 b	65.33 a	109.83 b
Grand Naine F2	8.20 a	8.26 a	71.44 a	29.20 a	70.13 a	159.64 a
CV (%)	19.46	20.18	17.08	19.24	13.57	14.64
Platina C	8.42 d	8.08 b	45.83 b	23.25 b	75.50 a	97.75 b
Platina F0	10.55 b	7.54 b	54.94 b	26.63 b	72.04 a	94.70 b
Platina F1	9.75 c	8.92 b	47.83 b	29.08 a	65.33 a	86.50 b
Platina F2	12.08 a	10.42 a	68.33 a	30.58 a	71.42 a	108.83 a
CV (%)	14.74	19.37	26.84	19.56	15.25	14.25
Japira C	7.92 b	6.75 b	49.92 b	24.25 a	82.17 a	107.33 a
Japira F0	7.17 b	7.25 b	32.17 c	18.08 b	45.42 d	63.42 b
Japira F1	9.83 b	8.92 a	42.25 b	29.00 a	57.50 c	72.25 b
Japira F2	10.17 a	9.75 a	53.92 a	27.67 a	73.33 b	110.08 a
CV (%)	11.13	16.48	14.60	22.04	12.79	12.81
Prata Anã C	9.17 a	6.92b	51.50 a	24.75 a	75.58 a	114.67 a
Prata Anã F0	10.08 a	7.42b	40.83 b	18.33 b	72.58 a	58.66 c
Prata Anã F1	10.30 a	9.12 a	45.84 b	25.12 a	54.54 b	85.47 b
Prata Anã F2	10.92 a	9.08 a	43.00 b	21.92 a	76.83 a	76.42 b
CV (%)	16.23	17.75	18.50	24.94	16.55	16.12
Preciosa C	9.08 a	8.08 b	41.00 c	18.33 b	63.50 b	72.08 b
Preciosa F0	9.75 a	9.67 a	58.75 a	30.25 a	77.50 a	138.58 a
Preciosa F1	10.25 a	8.08 b	42.50 c	22.17 b	62.42 b	80.92 b
Preciosa F2	10.00 a	11.08 a	50.50 b	28.25 a	66.58 b	81.00 b
CV (%)	15.23	22.72	19.61	21.78	10.56	15.43

Values within the columns followed by the same letter are not significantly different by Scott-Knott's test at the 5% probability level. C = Control Treatment, F0 = Leaf number 0, F1 = Leaf number 1, and F2 = Leaf number 2.

more aggressive is the disease. Cordeiro and Matos (2005), working with susceptible genotypes (Prata Anã and Nanicão) that were inoculated with *M. musicola*, observed latency periods ranging, on average, from 26 to 42.5 days, similar to those found in this work, and recorded the increase in this variable from 48 to 59.5 days in the same cultivars when the plants were previously submitted to acquired resistance.

Monocyclic parameters of the M. musicola isolate from Lavras, Minas Gerais State, in the Southeast Region of Brazil

The AUDSPC is inversely proportional to the degree of resistance; therefore, genotypes having very low AUDSPC values are resistant, while those with high AUDSPC values are susceptible (Irfaq, 2009). The results for AUDSPC are different from those found by the same authors (26.27) with black Sigatoka, who reported that younger leaves are more susceptible than are older leaves. However, these authors agree with the results found in Experiment 1 for the isolate from Cruz das Almas, Bahia, which also demonstrated the superiority of cv. Grand Naine for the variable AUDSPC. This result is explained by the appearance of lesions depending on the genotype (Fouré et al., 1990) and environmental conditions in which the plants are cultivated (Marin et al., 2003). The data that were obtained for incubation periods agree with other authors who claim that the increased level of resistance also increases the transition time between the disease stages. In some resistant genotypes, the progress of the symptoms is interrupted during the early stages (Fouré et al., 1990). Banana genotypes that are susceptible to *M. musicola* and *M. fijiensis* have lower incubation periods, a greater number of spots on the leaves and greater sporulation than those of resistant genotypes (Mouliom-Pefoura and Mourichon, 1990). The first symptoms of Sigatoka are visible between 11 and 106 days after germination and vary according to the banana genotypes (Meredith, 1970). The susceptible genotypes Nanicão and

Table 6. Polar diameter (PD) and equatorial diameter (ED) in µm, polar and equatorial diameter ratio (PD/ED) and stomatal density (SD) of the adaxial and abaxial epidermis of healthy banana leaves and leaves that were inoculated with *Mycosphaerella musicola*.

Treatment	Abaxial				Adaxial			
	PD	ED	PD/ED	SD	PD	ED	PD/ED	SD
Calipso C	30.65 b	17.61 b	1.75 b	111.05 a	30.28 c	17.13 d	1.77 a	111.05 a
Calipso F0	34.66 a	17.52 b	1.99 a	87.61 b	33.94 b	20.84 b	1.69 a	48.53 b
Calipso F1	30.84 b	17.20 b	1.80 b	101.88 a	35.55 b	18.87 c	1.89 a	36.68 c
Calipso F2	35.56 a	20.98 a	1.72 b	60.11 c	41.58 a	23.20 a	1.80 a	24.45 c
CV (%)	4.07	10.28	9.42	15.05	7.47	7.73	9.86	19.98
Grand Naine C	28.64 c	17.12 a	1.68 b	121.24 a	31.71 b	19.81 a	1.62 b	34.64 b
Grand Naine F0	27.79 d	17.69 a	1.55 b	115.12 a	28.73 c	14.04 b	2.06 a	12.23 c
Grand Naine F1	31.53 b	16.47 a	1.93 a	110.03 a	34.06 a	21.80 a	1.60 b	39.73 a
Grand Naine F2	33.80 a	17.43 a	1.99 a	114.10 a	34.35 a	21.05 a	1.64 b	42.79 a
CV (%)	2.73	10.79	12.03	14.89	6.58	12.33	12.04	22.00
Platina C	32.72 b	16.78 b	1.96 b	137.54 a	33.45 b	18.63 a	1.81 b	29.55 b
Platina F0	32.76 b	19.40 a	1.70 c	80.48 c	29.73 c	16.78 b	1.80 b	16.30 c
Platina F1	34.27 a	17.52 b	1.97 b	99.84 b	36.36 a	20.17 a	1.82 b	29.54 b
Platina F2	34.29 a	15.91 c	2.17 a	93.73 b	37.90 a	17.65 b	2.20 a	37.69 a
CV (%)	3.11	7.69	8.28	15.36	9.64	13.92	14.28	32.86
Prata Anã C	28.08 b	15.65 a	1.80 b	133.52 a	27.51 b	16.64 b	1.66 a	50.94 a
Prata Anã F0	26.33 c	15.00 a	1.77 b	76.41 c	25.78 b	15.83 b	1.64 a	12.23 c
Prata Anã F1	31.81 a	15.03 a	2.12 a	103.92 b	31.82 a	19.89 a	1.61 a	13.25 c
Prata Anã F2	31.80 a	16.14 a	1.99 a	122.26 a	33.63 a	20.67 a	1.64 a	28.53 b
CV (%)	4.27	9.34	9.49	27.02	7.84	9.85	11.55	36.67
Japira C	31.00 b	16.00 b	1.98 a	115.12 a	29.54b	19.41 b	1.54 b	34.64 a
Japira F0	34.17 a	19.08 a	1.92 a	88.63 b	38.08a	19.67 b	1.92 a	12.23 c
Japira F1	32.25 b	18.50 a	1.75 b	87.61 b	31.33b	22.75 a	1.43 b	12.23 c
Japira F2	33.67 a	17.67 a	1.94 a	87.61 b	37.58a	19.50 b	1.93 a	19.36 b
CV (%)	4.84	8.23	8.60	13.27	7.38	12.29	13.58	33.33
Preciosa C	31.36 c	17.31 b	1.81 b	115.12 a	34.73 b	18.25 a	1.91 a	25.47 a
Preciosa F0	30.72 c	16.34 b	1.89 b	90.67 b	36.41 b	18.20 a	2.01 a	24.45 a
Preciosa F1	34.18 b	19.43 a	1.78 b	72.33 c	39.68 a	19.89 a	2.02 a	21.40 a
Preciosa F2	40.18 a	19.46 a	2.07 a	62.14 c	40.19 a	18.59 a	2.21 a	16.30 b
CV (%)	3.83	8.64	8.79	14.64	6.63	10.96	13.34	31.29

Values within the columns followed by the same letter are not significantly different by Scott-Knott test's at the 5% probability level. C = Control Treatment, F0 = Leaf number 0, F1 = Leaf number 1, and F2 = Leaf number 2.

Prata Anã highlighted *M. musicola* latency periods ranging on average from 26 to 42.5 days (Cordeiro and Matos, 2005). The incubation period varies depending on the climatic conditions, host susceptibility and intensity of infection (Marin et al., 2003). In Costa Rica, Grand Naine obtained latency periods ranging between 25 to 70 days during the wet and dry seasons, respectively, for *M. fijiensis* and *M. musicola* (Marin et al., 2003). From the observed results, it can be concluded that the shorter are the incubation period and the latency period, the more aggressive is the disease.Considering the climatic diversity in Brazil and the ability to place different degrees of virulence of this pathogen in banana-producing regions, it is necessary to study the behavior of different commercial banana cultivars that are launched by the National Banana Breeding Program to assist in developing management measures to reduce the impacts of yellow Sigatoka in these regions.

Leaf anatomy of banana genotypes that were inoculated with M. musicola

Cordeiro and Matos (2005) characterized the defense mechanism of plants against *M. musicola* through the formation of lesions in the form of barely perceptible streaks or spots, similar to a hypersensitivity reaction with the formation of necrotic local lesions with late visualization. In contrast, the phenotypic expression of a high-strength block is characterized by the development of symptoms during disease stages I and II by blocking the sexual and asexual sporulation of the pathogen. The thickening of the evaluated tissues may be related to a system of plant defense against pathogen development. There is a direct relationship between the thickness of the epidermis and resistance to *Mycosphaerella musicola* (Rodríguez et al., 2009). According to Rodríguez et al. (2009), the genotypes that were classified as susceptible had a thinner abaxial epidermis than that of the resistant genotypes. There is a relationship between the thickness of the palisade, where the development of the fungus occurs, and cultivar resistance to Sigatoka (Valerio et al., 2002). For these authors, a thicker the leaf palisade offers more resistance to the fungus causing yellow Sigatoka. The increased leaf thickness that was observed in this study appears to be a strategy that plants have developed to prevent inoculation and fungal development. According to our results, different genotypes have different attitudes towards yellow Sigatoka. The cultivars Prata Anã and Grand Naine are considered susceptible to this disease (Borges et al., 2011), whereas the Preciosa, Japira and BRS Platina genotypes are resistant (Azevedo et al., 2010; Borges et al., 2011; Pimentel et al., 2010). The Calipso cultivar is only resistant to black Sigatoka (Willadino et al., 2011), whereas other authors (Mattos et al., 2010) also consider Calipso resistant to yellow Sigatoka. Cordeiro and Matos (2005) noted in a study of banana genotypes that none of the studied genotypes was considered resistant to any of the tested isolates of the fungus *M. musicola.*The stomatal size and number are associated with plant responses to pathogens, specifically that the presence of small, scarce stomata acts as a barrier to fungal penetration (Valerio et al., 2002). According to Rodríguez et al. (2009), stomatal density (SD)

is one of the most important variables for determining the resistance or susceptibility to yellow Sigatoka. For these authors, SD is more relevant than are the polar or longitudinal diameters because the probability of disease increases as the number of natural leaf openings increases. In addition, the percentage of the pathogens that enter their host plants via the stomata is 70, suggesting a clear relationship between the stomatal density and the susceptibility of a cultivar to Sigatoka (Rodríguez et al., 2009). The results of the analyses involving the different host genotypes and the positions of the inoculated leaves indicate that *M. musicola* affects the epidermis and alters the stomatal anatomy and density and the relationship between the polar and equatorial stomatal diameters. Such changes may affect the photosynthetic activity of the plant and the degree of host resistance to the pathogen isolate.

Materials and Methods

Experimental station

The experiments were conducted in a greenhouse at Embrapa Cassava and Fruits in Cruz das Almas City in Bahia State, Brazil, from June to November 2010.

Genotypes

Resistant genotypes to yellow Sigatoka

1) BRS Platina (AAAB) – Prata Anã hybrid FHIA-2) Japira (AAAB) - Pacovan hybrid (Embrapa) and 3) Preciosa (AAAB)

Susceptible genotypes to yellow Sigatoka

4) Grand Naine (AAA) – Cavendish cultivar, 5) Prata Anã (AAB) – Santa Catarina cultivar and 6) Calipso (AAAA) – High Gate hybrid (Jamaica).

Mycosphaerella musicola isolates

Two *Mycosphaerella musicola* isolates were used for infection: the first was collected in Lavras City (IL = Isolated Lavras) (Latitude 21º 14' 43''S, Longitude 44º 59' 59''W and 919 m of Altitude) in Minas Gerais State in the Southeast Region of Brazil, and the second was isolated from plants that were cultivated in Cruz das Almas City (IL = Isolated Cruz das Almas) (Latitude: 12º 40' 12"S, Longitude: 39º 06' 07" W and 220 m of altitude) in the Northeast Region of Brazil.

Isolation and mycelial growth of Mycosphaerella musicola

Leaves with characteristic lesions of yellow Sigatoka were collected during stages IV and V, washed with a soft sponge and detergent in tap water and then dried on paper towels following the methodology described by Cordeiro and Matos (2005). The rectangular portions of the dried leaves (10 cm × 5 cm) were removed and surface-disinfected by immersion in 70% ethanol for one minute followed by transfer to a solution of 2.5% sodium hypochlorite for five minutes and then a triple-rinsed in sterile distilled water. In a vertical flow chamber, the dimensions of the dried leaf samples were reduced to 5.0 cm × 2.5 cm using forceps and scalpel and removing the borders between the injured edges and the healthy tissue (Rocha 2008). The rectangles were transferred to Petri dishes containing water-agar (2%) medium and kept in a biochemical oxygen demand incubator (BOD type) that was set to 26°C±1°C with a 12-hour photoperiod. After 48 hours, the Petri dishes were opened under a stereoscopic microscope (magnifying glass) to identify the sporodochia, and the conidia were selected and transferred using a thin, sterile needle stylus to test tubes containing malt agar culture medium. The cultures were incubated for 30 days in a BOD that was set to 26°C±1°C and 12 hours of light daily, enabling the growth and successive subculturing of the cultures (Rocha, 2008).

Induction of sporulation

The mycelia colonies of the isolates were macerated under aseptic conditions using a mortar and pestle and subsequently diluted in sterile distilled water; 700 µL of the suspension was then distributed over the surface of Petri dishes containing 20 mL of V8 medium. The dishes were sealed with plastic wrap and transferred to a BOD chamber that was set at 26°C±1°C with a 12-hour photoperiod as described by Rocha (2008).
After 10 days of incubation, 10 mL of distilled water was poured into the dish so that a soft brush that was smoothly passed over the colonies would induce conidia release. After 15 minutes, the resulting spore suspension was filtered through a sieve (0.42 mm) and a double layer of gauze, and the number of spores was determined using a Neubauer chamber. With appropriate dilutions, the conidia suspension was adjusted to 4.10^4 conidia/mL and was used to inoculate the plants using of a manual plastic atomizer.

Pathogenicity test

Initially, a test was performed with both of the *M. musicola* isolates to evaluate their pathogenicity.
For the inoculations, a suspension of 4.10^4 conidia/mL and micropropagated plants of the Prata Anã cultivar, which is susceptible to yellow Sigatoka, were used.
In addition, the suspension was sprayed onto the surfaces of the lower limb of leaves on leaf number one (F1) and leaf number two (F2) until runoff. The first symptoms were observed 21 days after the inoculations, verifying the pathogenicity of the isolates.

Inoculation of plantlets for epidemiological measurements

Micropropagated plants of three genotypes that are resistant to yellow Sigatoka, including Japira, BRS Platina and Preciosa, and three susceptible genotypes, including Calipso, Prata Anã and Grand Naine, were used for the inoculations. Two *Mycosphaerella musicola* isolates were used (IL and ICA); the IL was tested in first experiment, and the ICA was tested in a second experiment. Totally acclimatized banana plantlets that were derived from tissue culture and that were approximately 20 cm in length were transplanted to plastic bags (18 cm × 25 cm) containing a mixture of soil and bovine manure (2:1 v/v). After three months of vegetative growth, seedlings of approximately 50 cm in height were inoculated on the abaxial surfaces of leaves one and two with either distilled water (control plants) or a suspension of *M. musicola* containing 4.10^4 conidia/mL. The inoculations were performed using a manual plastic atomizer until runoff. Before inoculation, the leaves were marked with colored plastic tape [blue for leaf number one (F1) and orange for leaf number two (F2)] to ensure that the evaluations were always conducted on the same leaves. For the first five days after inoculation, the irrigation of the seedlings consisted of

10 minutes of nebulization 3 times a day (morning and early and late evening) and a daily manual wetting of the seedlings. After the initial five days, a manual irrigation and 10-minute nebulization (once in the afternoon) were conducted daily.
The following response variables were recorded: 1) the incubation (IP) and latency (LP) periods for each genotype and leaf position and 2) the progression of the disease as measured by severity using a Stove scale modified by Gauhl (Gauhl et al., 1993). The evaluations were performed for ten weeks after inoculation.

Area under the disease severity progress curve (AUDSPC)

The AUDSPC was calculated using the percentage of infection measured separately for the F1 and F2 leaves of each plant and the formula described below (Campbell & Madden, 1990).

$$\text{AUDSPC} = \sum_{i=1}^{n-1}\left(\frac{y_i + y_{i+1}}{2}\right)\left(t_{i+1} - t_i\right)$$

Where, AUDSPC = area under the disease severity progress curve, y_i= proportion of the disease in the i^{th} observation, t_i= time in days of the i^{th} observation, and n = total number of observations.
According to Madden et al. (2007), the disease progression curve, which is described as the proportion of disease over time, is the best way to represent an epidemic.

Incubation period (IP)

The IP is the number of days between inoculation and the appearance of symptoms in the inoculated leaves (Brun, 1963).

Latency period (LP)

The LP is the number of days between inoculation and the appearance of sporulation (Brun, 1963).

Statistical analysis

Grand Naine plantlets were used as controls because this genotype serves as an international standard for measuring plant susceptibility to Sigatoka leaf disease. Each experiment was organized in a double factorial (6 × 2) scheme that consisted of six genotypes and two types of inoculated leaves (Number one Leaf - F1 and Number two Leaf - F2) in each plant. The first experiment used a Lavras city, Minas Gerais State, isolate, and the second experiment used a Cruz das Almas city, Bahia State, isolate. The data were evaluated separately for each isolate. The experimental scheme was in completely randomized block design (CRBD) with four replicates, and the data were analyzed using the SISVAR statistical program (Ferreira, 2011). The data were evaluated used Scott-Knott's test at a given probability level 5% ($p<0.05$).

Leaf anatomy of the banana genotypes that were inoculated with M. musicola

Leaf samples measuring 2 cm × 2 cm were collected ten weeks after the plants were inoculated with the *M. musicola* isolate from Cruz das Almas, which was the more aggressive of the two tested isolates. The samples were collected within the transition regions between the lesions and the healthy tissue and then fixed in a solution of 70% (v/v) ethanol.

To study the anatomical tissue changes, cross sections were cut using a table microtome and cleared for 10 minutes in sodium hypochlorite solution 2.5% (v/v) before being rinsed in triple-distilled water. The sections were then stained with safrablau (1% safranin and 0.1% astra blue in a ratio of 7:3) for subsequent mounting in water and glycerin (1:1) on a slide beneath a coverslip (Kraus and Arduin, 1997). In the tissue sections, we evaluated the thickness of the adaxial and the abaxial epidermis and hypodermis and of the palisade and spongy layers. To evaluate the stomatal characteristics, paradermal sections were taken from the abaxial surface of the leaf samples and subsequently clarified in 2.5% sodium hypochlorite (v/v) for 10 minutes before rinsing in triple-distilled water. The rinsed sections were stained with 1% safranin and then mounted onto a slide using methodology described by Laboriau et al. (1961). The evaluations were performed using an Olympus CBB microscope and a clear camera. The studied stomatal variables were the density (SD), the polar diameter (PD), the equatorial diameter (ED) and the ratio of the polar to the equatorial diameter (PD/ED). For both the cross and paradermal sections, four leaves (two samples per sheet) totaling up eight fields were observed per treatment. The anatomy of leaf numbers zero (F0), one (F1) and two (F2) (Fig 3) from six banana genotypes that were artificially inoculated with a *M. musicola* isolate from Cruz das Almas, Bahia State, was evaluated. The leaves (F2) of uninoculated plants were used as controls, and only two sheets of the control leaves were examined because the symptoms were apparent from the visual observation of leaves from this position. The experiment consisted of a double factorial (6×4) scheme with six genotypes and four types of leaves, including F0, F1 and F2 from inoculated plants and F2 from control plants (without inoculation). The design was a completely randomized block design (CRBD) with eight treatment replicates, and the data were analyzed using the SISVAR statistical program (Ferreira, 2011) with Scott-Knott's test at a given probability level 5% ($p<0.05$).

Conclusions

Resistant banana cultivars, including Preciosa, Japira and BRS Platina, are promising genotypes for commercial plantations in regions with high inoculum pressure from yellow Sigatoka. The *M. musicola* isolate from Cruz das Almas City, Bahia State, is more pathogenic than is the isolate from Lavras City, Minas Gerais State. *M. musicola* changes the leaf tissues and stomata of infected banana plants, and these changes vary with the cultivar and the analyzed parameter. The anatomical modifications made by plants in response to infection may be related to different strategies of resistance to fungal genotypes.

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