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Histological and Molecular Characterization of the *Musa* spp. x *Pseudocercospora musae* Pathosystem

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Abstract: Yellow Sigatoka, caused by the fungus *Pseudocercospora musae*, represents one of the most challenging diseases in bananas, which is aggravated due to the genetic variability of this pathogen. The main objective of this study was to characterize the infection process of *P. musae* in two banana cultivars with different levels of resistance and to quantify the expression of resistance genes. Inoculated and non-inoculated leaf samples of the two cultivars, Yangambi km 5—resistant and Grande Naine—susceptible, were investigated by light microscopy, histochemistry, scanning electron microscopy, and RT-qPCR. The cultivar Grande Naine showed early signs of *P. musae* infection, including the production of lipophilic substances and phenolic compounds, while the cultivar Yangambi km 5 was not affected. In the Grande Naine cultivar, the protein kinase gene was upregulated, while in the Yangambi km 5, it was repressed. The WAK2 gene was initially upregulated in both cultivars; however, in Grande Naine, it was subsequently repressed, while in Yangambi km 5, it remained downregulated. These findings have the potential to contribute to more effective management strategies in the control of yellow Sigatoka and the development of banana varieties resistant to yellow Sigatoka.

Keywords: anatomy; gene expression; *Musa* spp.; pathogenesis; phenolic compounds; RT-qPCR; scanning electron microscopy (SEM)

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1. Introduction

Banana is a globally popular fruit, especially in tropical and subtropical regions. Brazil is the fourth largest producer of banana globally, trailing only behind India, China, and Indonesia [1]. World production of bananas in 2022 was of approximately 115.7 million tons, with a harvested area of around 5.7 million hectares [1]. Despite the remarkable numbers, the productivity of bananas in Brazil remains relatively low, averaging only 15 kg per hectare in 2022 [2]. This situation highlights the urgent need to improve agricultural practices, incorporating innovative technologies to optimize banana production in the country. Additionally, it is of paramount importance to address the challenge of diseases that often affect this crop, as these phytosanitary problems play a significant role in limiting productivity.

One of the most devastating diseases in bananas is yellow Sigatoka, caused by the fungus *Pseudocercospora musae* (Zimm.) Deighton [morfose sexual: *Mycosphaerella musicola*

R. Leach ex JL Mulder] [3,4]. In Brazil, yellow Sigatoka is present in all states, contributing to massive production losses in banana cultivation along with other fungal diseases caused by *Pseudocercospora fijensis* (black Sigatoka) and *Fusarium oxysporum* f. sp. cubense (Foc), which causes Fusarium wilt [5,6]. The occurrence of yellow Sigatoka is strongly linked to abiotic factors such as high temperatures, rainfall, and humidity, which create a conducive microclimate for the pathogen's infection and spread [7–9], causing lesions that are associated with profound modifications in leaf structure [10]. Given this challenging condition, plants need to develop effective defense strategies.

One of the first lines of defense of plants against pathogens occurs on the surface of organs, where microorganisms attempt to adhere and penetrate to cause infections [6,11]. In this context, characteristics such as the size, location, and density of stomata, along with the thickness of cell walls, play a crucial role as possible physical barriers in interactions between plants and pathogens [12,13]. Additionally, the production of phenolic compounds in cell walls is also described as a resistance mechanism, acting together with physical barriers against pathogen attacks [14,15]. Histological studies play a fundamental role in characterizing the infectious process, including characterization of anatomical modifications and analysis of metabolites produced after infection [16], as well as observation of pathogen interaction with the plant surface [16–18]. Investigation of these processes, therefore, allows for understanding plant responses to pathogens, contributing to a better understanding of the infectious process.

The structural and physiological modifications that occur during pathogen colonization are closely related to changes in gene expression [19]. In banana plants, some genes play a crucial role in inducing resistance, including pathogenesis-related genes (PR) [13,20,21].

Despite the importance of these genes, studies investigating gene expression patterns in the pathosystem involving *P. musae* and different banana cultivars have not yet been widely explored. Additionally, cultivars of great economic importance, such as Grande Naine, are highly susceptible to the disease [7], while other cultivars show some degree of resistance, such as the Yangambi km 5 cultivar. This contrasting scenario can be explained by the high genetic variability of *P. musae* resulting from the fungus's capacity for sexual reproduction, as well as the use of banana cultivars's susceptibility [22,23].

Given this scenario, comprehensive studies on the interaction between *Musa* spp. x *P. musae* are of utmost importance for banana-breeding programs, which aim to develop cultivars resistant to this disease, and to decide on the best strategy for economically and environmentally controlling and managing the disease [24]. In this study, we comprehensively describe the structural, histochemical, and molecular aspects of the infection process of *P. musae*, the causal agent of yellow Sigatoka. Additionally, we characterize alterations in resistant and susceptible cultivars at the molecular level and investigate candidate genes for resistance to this pathogen. The dynamics of resistance-associated genes in the Yangambi km 45 cultivar are also explored.

2. Materials and Methods

2.1. Plant Material, Experimental Assay, and Experimental Conditions

Two banana cultivars contrasting in yellow Sigatoka (YS) resistance, Grande Naine (susceptible) and Yangambi km 5 (resistant), were used. Seedlings of the cultivars were obtained from in vitro culture. The plants were acclimatized in styrofoam trays in a greenhouse and, at three months of age, transferred to polyethylene bags containing a mixture of soil, substrate, and coconut fiber (2:2:1), where they remained until the end of the evaluations with irrigation by spraying twice a day to maintain the ideal humidity for fungal development.

The study used the isolate 41MG of *P. musae* from the culture collection of the Phytopathology Laboratory at Embrapa Mandioca e Fruticultura, preserved by the Castellani method [25]. The isolate was cultivated in a V8 medium (150 mL L^{-1}) consisting of Campbell's V8 Vegetable Juice, 18 g/L of agar, and 2 g/L of calcium carbonate in a final volume of 500 mL, and maintained in a Biochemical Oxygen Demand (B.O.D.) incubator for 20 days

at 25 °C with a 12 h photoperiod. Subsequently, the isolate was subcultured to promote better mycelial growth and maintained in a B.O.D. incubator at 25 °C under constant light for 11 days [4]. The spore suspension obtained was properly filtered, and the concentration adjusted to 4×10^4 using a Neubauer chamber.

Forty-five six-month-old plants of each cultivar, Grande Naine (YS-susceptible) and Yangambi km 5 (YS-resistant), were inoculated. For each plant, leaves 1 and 2 were selected, as they were the youngest and most recently expanded leaves, and the solution of isolate 41MG (4×10^4) was sprayed onto the abaxial surface until runoff according to [4]. The experimental design used was completely randomized with 45 plants for each cultivar, consisting of two treatments: inoculated cultivar and non-inoculated with the pathogen.

2.2. Light Microscopy, Histological Analysis, Leaf Clarification and Stomatal Index and Density

For anatomical, histochemical, and leaf clarification characterization, leaf samples from inoculated and non-inoculated cultivars were collected. Parts of the middle third of each leaf were obtained at 0, 24, 48, 72, and 120 h after inoculation (h.a.i.), which were immediately fixed in FAA (formaldehyde, acetic acid, and 50% ethanol; 1:1:18 in volume) [26], subjected to vacuum for approximately 12 h, and then stored in 70% ethyl alcohol. Subsequently, samples from each leaf were dehydrated in a series of tertiary butyl and embedded in histological paraffin [26]). For light microscopy, serial sections of variable thickness (12–16 µm) were made with a rotary microtome (Leica RM2245, Leica Biosystems, USA), stained with safranin and Astra blue, and mounted in synthetic resin [27].

For leaf clarification, samples of 2 cm × 2 cm were collected from the same leaf for all time points evaluated, ensuring that the leaf area analyzed remained consistent throughout the experiment. The samples were placed in 5% sodium hydroxide for 24 h, washed in distilled water for 30 s (3×), and then submerged in sodium hypochlorite containing 2.5% active chlorine for 4 h. After this process, the samples were stained with 1% aqueous safranin and mounted on slides with glycerinated gelatin. An image analysis to determine stomatal density and stomatal index was performed using the Anati Quant 2® v. 2.0 software. The experimental design was completely randomized with three replicates. Means were compared using Tukey's test at a 5% probability level, using Sisvar® v. 5.8 software [28].

Epidermal and stomata cell counts in order to obtain the stomatas index and stomatal density, were calculated using the Quanti 2® v. 2.0 [29], whereas:

$$\text{Stomatal index (\%)} = C2 \times 100/(C1 + C2) \text{ and Stomatal densitity (mm}^2\text{)} = C2/\text{total area}) \quad (1)$$

where C1 = epidermal cells and C2 = stomata.

Sampling from the same leaf and leaf area at different time points aimed to eliminate variations due to heterogeneity between leaves, allowing for a more accurate comparison across the time points analyzed.

2.3. Detection of Phenolic Compounds

For histochemical tests, ferric chloride and potassium dichromate were used to detect general phenolic compounds [30]. For the tests, slides of the Grande Naine and Yangambi km 5 cultivars at 120 h.a.i. were selected. The slides were mounted with glycerinated gelatin. The choice of the 120 h.a.i. time point was based on preliminary studies, indicating this as the critical period during which significant changes occur in the interaction between the pathogen *P. musae* and the banana cultivars. This period allows for the observation of relevant biochemical and histochemical responses, providing a better understanding of the plant's defense mechanisms against infection.

All optical microscopy analyses were conducted at the Plant Anatomy Laboratory (LAV) of the Federal University of Recôncavo da Bahia (UFRB), using an Olympus BX51 photomicroscope (Olympus system Microscopes, Japan) equipped with an Olympus A330 digital camera (Olympus Corporation, Japan). Serial transverse sections were performed on a rotary microtome (Leica RM2245) with varying thicknesses (12–16 µm), stained with

safranin and Astra blue (GERLARCH, Cheshire, UK, 1969), and the slides were mounted with synthetic resin.

2.4. Scanning Electron Microscopy (SEM)

For scanning electron microscopy (SEM), the samples were dehydrated in a series of ethanol and dried to the critical point (Leica EM CPD 030, Leica Microsystems, Germany) using liquid CO_2. Leaf samples were mounted on metal holders and coated by sputter coating using a Denton Vacuum Desk IV (Moorestown, NJ, USA) and SEM (Jeol, Tokyo, Japan, JSM-6390LV). The analyses were conducted at the Electron Microscopy Service of the Gonçalves Moniz Electron Microscopy Center (CPqGM), FIOCRUZ (Salvador, Bahia, Brazil).

2.5. Reverse Transcription, cDNA, and Real-Time PCR (qPCR)

Genes used in this study were identified from previous analysis of RNA-seq of the *Musa* spp. x *M. musicola* pathosystem. RNA was extracted from leaf samples of both cultivars, originating from inoculated and control plants using the Gambino et al. [31] protocol. After obtaining total RNA, cDNA synthesis was performed, which were used as templates in real-time PCR evaluations.

2.6. Primer Design

Eighteen pairs of specific primers were designed using Primer Express® Version 3.0 software (Applied Biosystems, Foster City, CA, USA). The sequences were obtained from previous RNA-Seq sequencing data (Helixxa—Illumina HighSeq 2000—TruSeq) that generated 30 G of data from 8 libraries of the *Musa* spp. x *M. musicola* interaction [32]. The sequences were successfully tested and primers are listed in Table 1.

Table 1. Primers used for gene expression evaluation in contrasting banana genotypes for resistance to yellow Sigatoka.

ID	Primer	Description	Sequence (5′-3′)	Amplicon (pb)
GSMUA_AchrUn_randomT23380_001	MUSA_3	protein kinase domain containing protein, expressed	F: CAGTGGTGGATTTGTTGTGC R: TACGGGAATTCCATTGGGTA	250
GSMUA_Achr1T09200_001	MUSA_5 (WAK2)	Putative Wall-associated receptor kinase 2	F: ACGTCATTGGCATCGTCATA R: GTCGGCAACGAAGGTAAAAG	230
TUB *	MUSA_17	Beta Tubulin	F: TGTTGCATCCTGGTACTGCT R: GGCTTTCTTGCACTGGTACAC	112
25S *	MUSA_18	25S rRNA	F: ACATTGTCAGGTGGGGAGTT R: CCTTTTGTTCCACACGAGATT	106

* Reference genes.

For validation of cDNAs and validation of primers, a conventional PCR was performed for all cDNA samples. For this purpose, a reaction mix for a final volume of 20 µL was used, containing autoclaved Milli-Q water; 1 U of Taq DNA Polymerase enzyme (Fermentas), 5 U/µL in appropriate buffer; 0.2 µM of each primer (forward and reverse); 2.0 mM of $MgCl_2$; 0.2 mM of dNTPs; and 1 µL of cDNA at a dilution of 1:200 (*v*/*v*).

For the negative control, 1 µL of autoclaved ultrapure water was pipetted instead of cDNA, and for the positive control, banana DNA was used. The amplification products were verified on 1% agarose gel in 1× TAE buffer stained with ethidium bromide.

2.7. RT-qPCR, Differential Gene Expression, and Data Analysis

Individual cDNA from each time point under inoculated and non-inoculated conditions was used as a template in quantitative gene expression analysis. For each gene (Table 1), three biological replicates were conducted for each analyzed time point (24, 48, and 72 h after inoculation), one sample from all non-inoculated time points of the Yangambi Km 45 cultivar, and the negative control (autoclaved DEPC-treated water). RT-qPCR analyses were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

The amplification efficiency of each primer pair was determined by standard curve, with five serial dilutions of the cDNAs used, and the resulting coefficient of determination (R^2). The expression levels of target genes were determined by quantification, using the expression: $QR = 2^{-\Delta\Delta CT}$ [33].

3. Results

3.1. Anatomical Characterization, Stomatal Density, and Stomatal Index

The leaf anatomy of the Yangambi km 5 and Grande Naine cultivars is similar, presenting the following characteristics: uniseriate epidermis, paracytic stomata, dorsiventral mesophyll, with palisade parenchyma on the adaxial surface and spongy parenchyma on the abaxial portion, including intercellular spaces known as aerenchyma, and collateral vascular bundles surrounded by a parenchymatous sheath that extends to the hypodermis on both surfaces (Figure 1A,E).

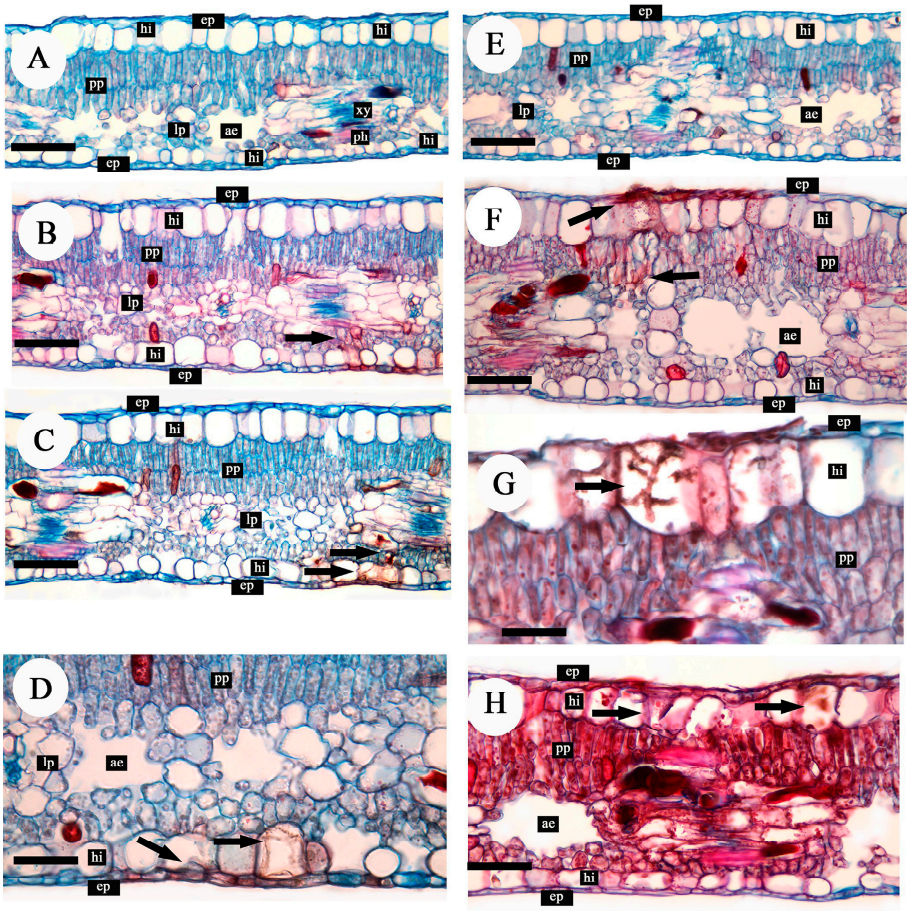

Figure 1. Cross sections of the leaf of the Grande Naine (**A**–**D**,**F**–**H**) and Yangambi km 5 (**E**) banana cultivars: (**B**–**D**) plants inoculated with the pathogen at 72 h.a.i and (**F**–**H**) plants inoculated with the pathogen at 120 h.a.i. (**A**) = Grande Naine cultivar, not inoculated; (**E**) Yangambi km 5 cultivar, not inoculated. Abbreviations: ae, aerenchyma; ep, epidermis; hi, hypodermis; pp, palisade parenchyma; lp, lacunose parenchyma; ph, phloem; xy, xylem. The arrows indicate suberized tissue. The arrows indicate suberized tissue. Scale bar = 50 µm.

Stomatal density and stomatal index showed significant variations between cultivars and evaluated periods. On the abaxial surface of the Grande Naine cultivar, a higher stomatal density was observed at 24 h after inoculation (h.a.i.) (105.31 stomata/mm^2) and at 48 h.a.i. (96.36 stomata/mm^2), compared to the period of 72 h.a.i. (57.12 stomata/mm^2), which recorded the lowest value for this variable. In contrast, the Yangambi Km 5 cultivar exhibited a higher stomatal density at the periods of 48 h.a.i. (36.73 stomata/mm^2) and

72 h.a.i. (35.29 stomata/mm^2), while the lowest densities were observed around 120 h.a.i. (27.91 stomata/mm^2) and 24 h.a.i. (31.35 stomata/mm^2) Table 2 and Figure 2.

Table 2. Stomatal density of the abaxial surface (S.D.) and stomatal index of the abaxial surface (S.I.) in paradermal sections of two banana cultivars, Yangambi Km 45 (Caipira) and Grande Naine, inoculated with *Pseudocercospora musae*.

Time	Caipira	Grande Naine	Caipira	Grande Naine
	I.D. (%)	I.D. (%)	D.E. est.mm^{-2}	D.E. est.mm^{-2}
24 h.a.i.	19.70 bA	61.37 aA	31.35 bB	105.31 aA
48 h.a.i.	20.78 bA	45.38 aB	36.73 bA	96.36 aA
72 h.a.i.	27.43 aA	19.36 bD	35.29 bA	57.12 aB
120 h.a.i.	16.76 bA	31.34 aC	27.91 bB	64.17 aB

Means followed by the same lowercase letter in the row and uppercase letter in the column do not differ statistically according to Tukey's test at 5% probability. I.D (%) = stomatal index; D.E. = stomatal density.

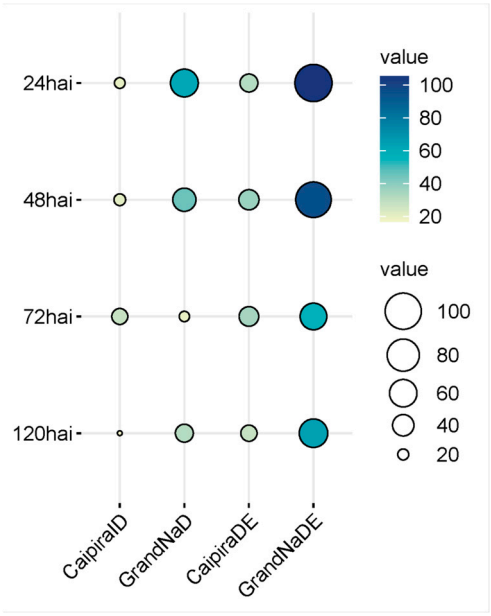

Figure 2. Ballon plot depicting the stomatal density (SD) of the abaxial surface, stomatal index (SI) of the abaxial surface in paradermal sections of two banana cultivars, Yangambi Km 45 (Caipira) and Grande Naine, inoculated with *Pseudocercospora musae*.

Regarding the stomatal index, the Grande Naine cultivar showed the highest stomatal index 24 h after inoculation (h.a.i.) (61.37%) and the lowest index at 72 h.a.i. (19.36%). The Yangambi Km 5 cultivar did not show significant differences in stomatal indices over time.

3.2. Pre-Penetration Process of P. musae in Resistant and Susceptible Banana Cultivars

In all analyses conducted by scanning electron microscopy (SEM) (Figure 3), hyphae were visible inside the Grande Naine cultivar starting from 24 h.a.i. with growth on stomatal cells beginning at 48 h.a.i. on both leaf surfaces (Figure 3F–H); signs of infection were observed from 72 h.a.i. (Figure 2). In the Yangambi Km 5 cultivar, hyphae were visible from 24 h.a.i. but did not grow on stomatal cells (Figure 3B–D). Although hyphae were observed at 24 h.a.i. in the Grande Naine cultivar, this did not indicate the onset of the infection process, as even when these structures were observed in the stomata, as seen in cross sections using light microscopy, signs of infection were observed from 72 h.a.i.

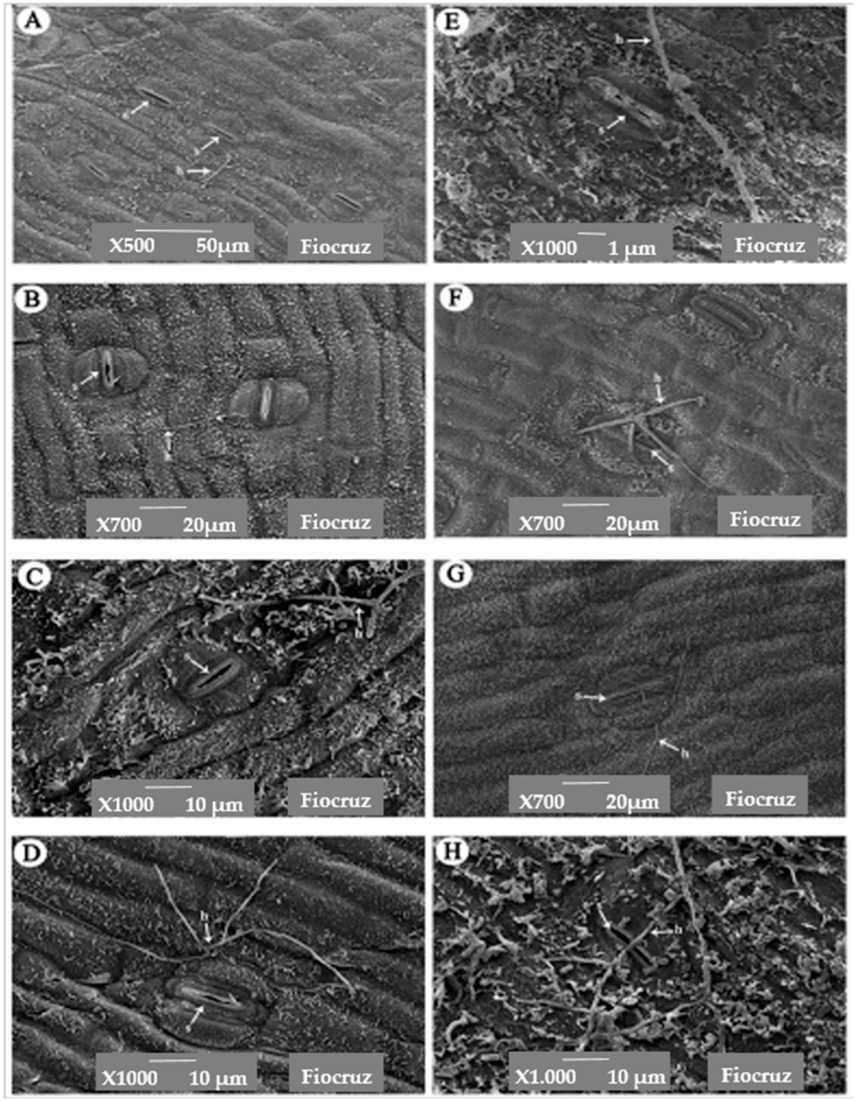

Figure 3. Scanning electron microscopy (SEM) for observation of the interaction of *P. musae* from the abaxial surface of leaves of banana cultivars Yangambi Km 5 (**A**–**D**) and Grande Naine (**E**–**H**). (**A**–**D**) Cultivar Yangambi Km 45 at 24, 48, 72, and 120 h.a.i., respectively. (**E**–**H**) Cultivar Grande Naine at 24, 48, 72, and 120 h.a.i., respectively. Abbreviations: h.a.i., hours after inoculation; h, hypha; s, stomata.

3.3. Colonization of P. musae in Resistant and Susceptible Banana Cultivars

The leaves of the Grande Naine cultivar exhibited differences in their anatomy after infection by the fungus *P. musae*, primarily due to the production of chemical compounds, as indicated by positive histochemical tests (Figure 4). The production of phenolic compounds was initially observed along with signs of infection after 72 h of inoculation, with more significant alterations noted after 120 h.a.i. (Figure 4). At 72 h.a.i., the presence of chemical compounds on the cell walls of various tissues was observed, but there were no significant changes in cell anatomy. However, at 120 h.a.i., there was a greater deposition of infection-related compounds, along with some ruptures in the epidermal and subepidermal cells.

The Grande Naine cultivar exhibited positive results for both the potassium dichromate and ferric chloride tests (Figure 4B–D) indicating that the content present in the palisade and spongy parenchyma cells resulting from the infectious process consists of phenolic compounds. The amount of phenolic compounds gradually increased throughout the interaction becoming more evident at 120 h after inoculation (h.a.i). In contrast, the Yangambi Km 5 cultivar tested negative for the presence of phenolic compounds.

No signs of chemical compounds were observed in the Yangambi Km 5 cultivar (Figure 4), also indicated by negative histochemical tests for this substance (Figure 4). At 72 h.a.i., the presence of chemical compounds was observed on the cell walls of various tissues, but there were no significant changes in cell anatomy. However, at 120 h.a.i., there was a greater deposition of infection-related compounds along with some ruptures in the epidermal and subepidermal cells. Furthermore, the Grande Naine cultivar tested positive for phenolic compounds, while the Yangambi Km 5 cultivar tested negative for these compounds.

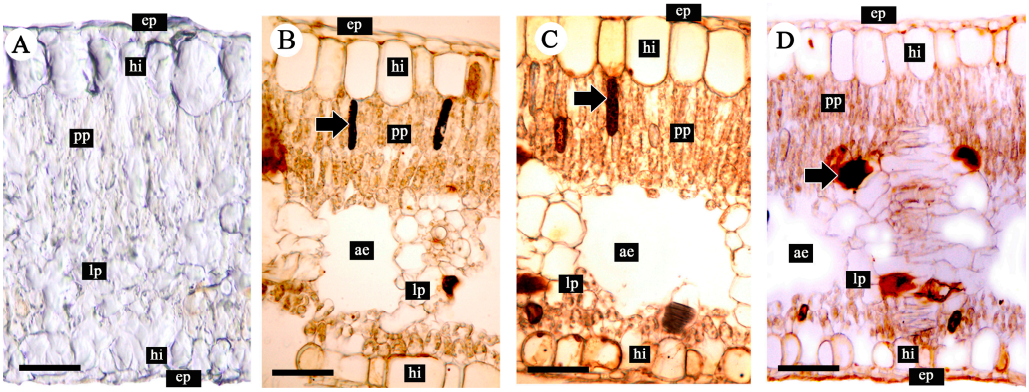

Figure 4. Cross sections of leaves from the Grande Naine banana variety inoculated with *P. musae* 120 h.a.i. (**A**) = Control. (**B**–**C**) Histochemical test with ferric chloride. (**D**) Histochemical test with potassium dichromate. Arrows indicate production of phenolic compounds. Scale bar: 40 µm.

3.4. Gene Expression Analysis

3.4.1. Validation of Genes by RT-qPCR

From the 18 primers designed after large-scale analyses of the data obtained from RNA-seq (Table 1), two genes (MUSA_3 and MUSA_5) related to plant defense response (Table 1) were selected. These genes were chosen due to their high expression (log2fold change) and the production of a single amplification product (Table 3). Additionally, reference genes were selected for their stability in *Musa* spp. [34]. From the generation of the standard curve for each primer, it was possible to calculate the amplification efficiency (E) of the primers, which ranged from 97.0 to 110.0% (Table 3).

Table 3. Efficiency of primers used in the analysis of differential gene expression in the *Musa* spp. x *P. musae* pathosystem.

ID	Gene	Description	Sequence (5′-3′)	Amplicon (pb)	Efficiency	Ta *
GSMUA_AchrUn_randomT23380_001	Musa_3	protein kinase domain containing protein, expressed	F: CAGTGGTGGATTTGTTGTGC R: TACGGGAATTCCATTGGGTA	250	0.98	60
GSMUA_Achr1T09200_001	Musa_5	Putative wall-associated receptor kinase 2 (WAK2)	F: ACGTCATTGGCATCGTCATA R: GTCGGCAACGAAGGTAAAAG	230	1.10	60
25S	25S	25S rRNA	F: ACATTGTCAGGTGGGGAGTT R: CCTTTTGTTCCACACGAGATT	106	0.99	60
TUB	Tubulin	Beta Tubulin	F: TGTTGCATCCTGGTACTGCT R: GGCTTTCTTGCACTGGTACAC	112	0.97	60

* Ta = primer-specific annealing temperature.

3.4.2. Gene Expression of Protein Kinase (MUSA_3) and Protein Kinase 2 (MUSA_5)

The expression of the MUSA 3 gene was higher in the compatible interaction with the susceptible cultivar (Grande Naine) for all evaluated time points (Figure 5A). For the resistant cultivar (Yangambi Km 5), the detected expression was lower than the control, with a decrease in expression over the course of the interaction.

For the MUSA 5 gene, which encodes the receptor protein of protein kinase 2, the expression was lower than that of the control at all analyzed time points for the Yangambi Km 5 cultivar (resistant). The susceptible cultivar exhibited higher differential expression

compared to basal expression at 24 and 48 h.a.i. For both cultivars, there was higher expression at 24 and 48 h.a.i. (Figure 5B).

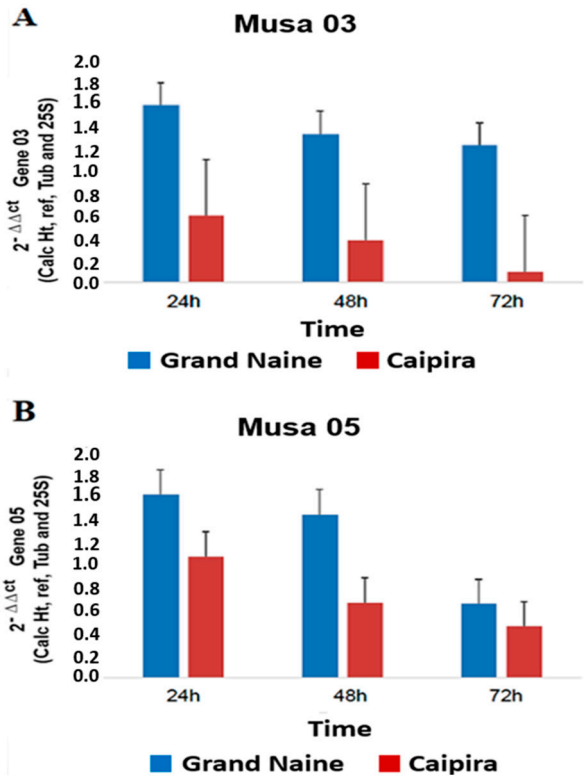

Figure 5. Relative expression based on RT-qPCR data of two candidate resistance genes calculated at three inoculation time-periods (24, 48, and 72 h.a.i) in Grande Naine and Yangambi km 5 plants in the *Musa* spp. x *P. musae* pathosystem. (**A**) Profile of relative expression analysis of protein kinase gene (Musa_3) for Grande Naine and Yangambi km 5 cultivars. (**B**) Profile of relative expression analysis of protein kinase 2 genes (Musa_5) for Grande Naine and Yangambi km 5 cultivars.

4. Discussion

4.1. Histological Studies

The results of this study are in line with recent research conducted by Madail et al. [10], reinforcing the consistency of the anatomical characteristics of the cultivars Yangambi km 5 and Grande Naine with those described in the existing literature. The observed features, such as the uniseriate epidermis, paracytic stomata, dorsiventral mesophyll, and the differentiated mesophyll structure composed of palisade parenchyma on the adaxial surface and lacunous parenchyma on the abaxial surface, along with intercellular spaces known as aerenchyma, correspond well to the descriptions of previous studies, including those of Skutch [35].

This congruence between different studies highlights the robustness and reliability of these anatomical features as possible diagnostic markers for these banana cultivars. By corroborating the anatomical details presented by previous research, our study supports the existing body of knowledge and provides indicative results that these traits may be stable and reproducible under different environmental conditions and study methodologies.

In addition, the agreement of our results with those of Madail et al. [10] suggests that the anatomical characteristics of these banana cultivars are consistent, which may be crucial for several practical applications. For example, these anatomical markers can aid in the accurate identification and classification of banana varieties, which is essential for breeding programs, genetic studies, and agricultural practices. The consistency in leaf anatomy also suggests a potential resilience of these cultivars to environmental stresses, as stable anatomical characteristics generally correlate with adaptability and robustness in plants.

In conclusion, the results of this study not only corroborate the findings of previous research but also enhance our understanding of the leaf anatomy of the Yangambi km 5 and Grande Naine cultivars. This improved understanding could have significant implications for the fields of botany, agriculture, and horticulture, providing a reliable basis for future studies and practical applications related to these important banana varieties.

Studies have highlighted the importance of leaf anatomy in the investigation of potential physical barriers against the yellow Sigatoka fungus, with a special focus on the quantity and size of the stomata [12,36–38]. Stomatal density, pointed out by Rodríguez et al. [37] as one of the most crucial variables, plays a significant role in determining resistance or susceptibility to yellow Sigatoka. These authors also underlined that anatomical characteristic such as the thickness of the cuticle and epidermis, the length of the stomata, and stomatal density are associated with a more effective physical barrier against the penetration of the fungus that causes Sigatoka. The stomatal index, which indicates the number of stomata among epidermal cells, is a vital structure for gas exchange and transpiration in plants.

Variations in the stomatal index may reflect the response of plants to fungal infection, affecting their metabolism and water balance [39]. The data of this study are in line with the referenced research, highlighting the importance of leaf anatomical characteristics, such as stomatal density, cuticle thickness, stomata length, and stomatal density, as determining factors in resistance or susceptibility to yellow Sigatoka.

In addition to physical barriers, the study revealed that a specific compound (maybe suberin—but not tested) appears to be a key factor in plants' resistance to infection. Healthy cells at the periphery of the lesion begin to produce this compound in large quantities, limiting the pathogen's advance and protecting healthy plant tissues. However, resistance in the cultivar Grande Naine seems to be delayed, and the production of this compound was not fast enough to prevent infection, given the high susceptibility of this cultivar to the fungus.

In contrast, the cultivar Yangambi Km 5 showed no signs of infection, suggesting that the faster or more effective production of this chemical compound, along with other resistance characteristics, may have been crucial for the resistance of Yangambi Km 5 to the fungus *P. musae*. These findings suggest that the combination of physical barriers and fast and effective chemical responses are determinants of plant resistance to the pathogen, offering valuable insights for management strategies and improvement of cultivars with greater resistance to yellow Sigatoka.

4.2. Scanning Electron Microscopy (SEM) and Phenolic Compounds

Scanning electron microscopy revealed that both cultivars analyzed presented hyphae of the fungus on the surface of the leaves as early as 24 h after inoculation, with the hyphae directed to the stomata. However, there were notable differences between cultivars in the development of infection. In the Grande Naine cultivar, hyphae started to grow in the stomatal cells from 48 h, while in the Yangambi Km 5 cultivar, the growth of the hyphae was more limited, and after 72 h, the hyphae were observed moving away from the stomatal cells.

These results corroborate previous studies indicating that Sigatoka fungus infection occurs predominantly through the stomata, with visible signs of infection appearing approximately 72 h after inoculation. The absence of symptoms in the Yangambi Km 5 cultivar suggests a significant difference in the response between resistant and susceptible cultivars, especially in the initial phase of the infectious process, known as the pre-penetration phase. This may be associated with defense mechanisms already present in the Yangambi Km 5 cultivar, such as the control of the stomata opening period, which can act as an additional barrier to pathogen penetration. These observations point to a complex interaction between the pathogenic fungus and the plant's defense mechanisms. The fact that the hyphae of the fungus in the cultivar Grande Naine continued to develop in the stomatal cells, while in the Yangambi Km 5, the growth was limited and the hyphae moved away, suggesting that

the cultivar Yangambi Km 5 has resistance mechanisms which inhibit progression of the infection. These mechanisms may include specific immunological responses or anatomical adaptations which hinder penetration of the fungus.

However, to fully understand the resistance mechanisms of Yangambi Km 5, further studies are needed. The relationship between the infectious process and penetration via stomata is complex and not yet fully understood. Future investigations should focus on elucidating the details of these resistance mechanisms, potentially revealing new strategies for the management and improvement of disease-resistant cultivars such as yellow Sigatoka.

The stomatal density on the abaxial surface of the Grande Naine cultivar was higher in the first 24 and 48 h after inoculation (105.31 and 96.36 stomata/mm^2, respectively) but decreased significantly to 57.12 stomata/mm^2 at 72 h.a.i. For the Yangambi Km 45 cultivar, the highest stomatal density occurred at 48 and 72 h after inoculation (36.73 and 35.29 stomata/mm^2, respectively), while the lowest densities were observed at 120 and 24 h after inoculation (27.91 and 31.35 stomata/mm^2, respectively).

However, the results of this study demonstrate that stomatal density may be related to pathogen penetration. The cultivar Yangambi Km 5, known for its resistance, showed a lower stomatal density in all times evaluated. Studies on leaf anatomy in relation to possible physical barriers against P. musae have mainly focused on the quantity and size of stomata [12,36,40]. According to Rodriguez et al. [37], stomatal density is one of the most important variables to determine resistance to yellow Sigatoka.

Our results reveal that the Grande Naine cultivar had a higher stomatal density compared to the Yangambi Km 5 cultivar. However, the stomatal density of Grande Naine was reduced over the study period. Araújo et al. [12] observed an increase in stomatal density for this cultivar in the same pathogenic system. These authors mentioned that the infection caused by *P. musae* significantly reduced the stomatal density for the banana genotypes evaluated, except for Grande Naine, which is susceptible and BRS Platina resistant to yellow Sigatoka. The difference in results between the studies can be explained by the methodology adopted. In this study, the measurements of stomatal density were taken from the same leaf area and the same leaves over time, which allowed for a more precise analysis of the evolution of stomatal density in relation to the progression of the infection. This approach helps to better understand how the stomatal density of Grande Naine may be related to its susceptibility to the pathogen, especially considering that the reduction in stomatal density observed over time may reflect a plant response to the stress caused by the infection.

These findings indicate a complex interaction between stomatal density and pathogen resistance. The fact that the Yangambi Km 5 cultivar, with lower stomatal density, demonstrates resistance to the fungus suggests that a reduced stomatal density may be associated with lower penetration of the pathogen. On the other hand, the Grande Naine cultivar, with initially higher stomatal density, experienced a reduction in this density over time, which may be related to the progression of the infection.

These results reinforce the idea that stomatal density is a critical variable in fungal resistance, but also shows the need for a more detailed analysis. The interaction between stomatal density and the plant's ability to resist infection is complex and can be influenced by other factors, such as the state of the stomatal cells and the plant's immune responses. Future studies should investigate these interactions to elucidate the exact mechanisms by which the stomatal density and other anatomical characteristics contribute to resistance to yellow Sigatoka, which may direct management and banana-breeding strategies.

Certainly, because penetration occurs via stomata, the lower stomatal density of the Yangambi Km 5 cultivar observed in this study may be a strategy that plants have developed to prevent penetration and, consequently, the development of the fungus.

Experiments carried out by Araújo et al. [12] demonstrated that there were changes in the leaf anatomy of banana plants when inoculated with *P. musae*, especially in the thickness of the leaves and in the number and size of the stomata. From these analyses, it

was possible to conclude that the anatomical changes can affect both the photosynthetic activity and the degree of resistance of the banana genotypes.

Differences in stomatal indices were observed. For the stomatal index of the cultivar Grande Naine, the times of 24 ha.i.a. (61.37%) and 72 ha.i.a. (19.36%) were considered, with the highest and lowest percentages, respectively. The stomatal indices of the cultivar Yangambi Km 5 were not significantly different. This result, according to Salisbury [41], is related to the fact that the stomatal index is relatively constant within a species. Therefore, it may be important in the characterization of cultivars, since this characteristic does not change after cell differentiation. The production of phenolic compounds was evaluated in this study, and there was greater deposition of compounds related to the infection at 120 ha.i. Phenolic compounds are known for their fungitoxic, antibacterial, and antiviral properties [42], which can inhibit spore germination, mycelial growth, and microbial enzyme activity. These compounds play an important role in plant resistance mechanisms, protecting them against attacks from microorganisms and animals. They are present in various parts of plants, such as vacuoles, cytoplasm, and cell walls [43].

However, it is important to highlight that the degree of involvement of structural and biochemical factors in the resistance response of plants can vary depending on the pathosystem, nutritional conditions, and environment. Although the presence of phenolic compounds was observed as a response to infection by pathogens in the Grande Naine cultivar, the production of these compounds was not sufficient to confer an effective resistance to the interaction between banana and *P. musae*. This suggests that the Grande Naine cultivar is susceptible and may need to employ other defense mechanisms to overcome infection by the pathogen.

The results indicate that, despite the production of phenolic compounds, the Grande Naine cultivar was not able to effectively resist the infection. This fact reinforces the idea that plant resistance is a complex and multifaceted phenomenon, which can involve a combination of structural and biochemical mechanisms. The Yangambi Km 5 cultivar, on the other hand, has other mechanisms of resistance which still need investigation. The resistance observed in this cultivar may be related to additional defense strategies beyond simple production of phenolic compounds.

Therefore, the results of this study highlight the need for further investigation into the resistance mechanisms of the Yangambi Km 5 cultivar. The variation in the effectiveness of structural and biochemical factors in plant response may be closely linked to the specific characteristics of the pathosystem and environmental conditions. A complete understanding of the resistance mechanisms of cultivars can provide valuable information for the development of more effective disease control and management strategies, such as yellow Sigatoka, and can contribute to the improvement of banana cultivars with greater resistance to pathogens.

4.3. Analysis of Gene Expression

In this study, we investigated the variations in gene expression during infection of bananas, using next-generation sequencing (RNA-seq) data to analyze the early stages of the interaction between banana and the fungus *P. musae*. The main objective was to understand the behavior of selected candidate genes in commercial banana cultivars with different levels of resistance.

The Yangambi km 5 cultivar is resistant to both black and yellow Sigatoka, as well as Fusarium wilt. In contrast, the Grande Naine cultivar is highly susceptible to yellow Sigatoka, which can lead to significant yield losses depending on environmental conditions. We observed that genes related to protein kinases, which play important roles in the processes of metabolic and cellular regulation, showed distinct expression patterns between the two cultivars during the interaction with *P. musae*. In studies carried out by Li et al. [44], the MUSA 3 gene was induced in the Grande Naine cultivar, while in the Yangambi km 5, there was repression. The MUSA 5 gene showed a similar pattern of induction and repression, but with variations in the time points evaluated.

The genes chosen for this analysis were selected based on validation from the genomic library cited in the work, ensuring the relevance and accuracy of the data obtained.

These genes are linked to the response to disturbances in the cell wall but also play roles related to abiotic stress and cell development [45,46]. Additionally, the wall-associated kinases (WAKs) genes, which are part of the wall-associated kinase family, play a crucial role in communication between the cell wall and the cytoplasm, which is crucial for systemic acquired resistance in plants [46,47]. Previous studies in different plants, such as Arabidopsis, rice, and wheat, have highlighted the importance of WAK genes in pathogen resistance. Receptor kinases, including Receptor-Like Kinases (RLKs), play an essential role in recognizing pathogen-associated molecular patterns, leading to the activation of defense genes [48–50].

In our research, the WAK2 gene (MUSA_5) emerged as a crucial factor in the response of banana cultivars to infection by the fungus *P. musae*. This finding suggests that the fungus may have triggered the activation of these genes in the Grande Naine cultivar, which, in turn, could have facilitated its penetration into the plant. In contrast, in the Yangambi km 5 cultivar, the activation process of the WAK2 gene conferred greater resistance to the infection.

These results have significant implications in the context of the search for banana varieties more resistant to yellow Sigatoka, a disease that can cause substantial production losses. However, it is important to note that gene expression is not an isolated process and may be influenced by a range of environmental factors and complexities inherent to the interaction between the plant and the pathogen.

Consequently, when planning and executing future research, as well as when employing advanced techniques such as genetic transformation or gene editing in banana cultivars to improve resistance to Yellow Sigatoka, it is essential to consider these multifaceted and dynamic aspects. An in-depth understanding of the molecular and physiological mechanisms underlying the response of plants to pathogens such as *P. musae* is crucial for the development of effective management strategies and for the cultivation of banana varieties with improved resistance to this disease [21].

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