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Differential regulation of jasmonic acid pathways in resistant (Calcutta 4) and susceptible (Williams) banana genotypes during the interaction with *Pseudocercospora fijiensis*

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Abstract
One of the main biosecurity problems facing banana crops is black Sigatoka disease, caused by the fungus *Pseudocercospora fijiensis*. Disease control is achieved mainly by chemical sprays and generates more than 50% of the costs of production, with a trend to increase due to the rapid resistance that the fungus acquires to the main fungicide molecules. Thus, it is very important to acquire information on the molecular mechanisms of the plant–pathogen interactions in this pathosystem as a way to help design future control strategies. Hormonal plant responses of banana genotypes susceptible and resistant to *P. fijiensis* were identified and analysed in this work by transcriptomic (RNA-Seq and RT-qPCR) and metabolomic studies (ultraperformance liquid chromatography-mass spectroscopy). Differentially expressed genes related to signal transduction and biosynthesis pathways of jasmonic acid (JA) and ethylene (ET) were identified in the resistant variety Calcutta 4 during the early stage of interaction with *P. fijiensis*. Metabolomic analysis corroborated the induction of metabolites related to JA and ET pathways during the first 72 hr post-inoculation. Observed results are evidence that signalling via JA/ET could be key in the activation of defence response signals in the resistant variety Calcutta 4.

KEYWORDS
black Sigatoka, cultivar Calcutta 4, ethylene, jasmonic acid, *Pseudocercospora fijiensis*

1 | INTRODUCTION

Banana and plantain (*Musa* spp.) constitute one of the most important crops worldwide (after rice, wheat, and maize), ranking first among all fruits produced (Food and Agriculture Organization, 2020). The importance of banana and plantain production is not only because they represent a source of income for several developing countries but they also serve as staple food for more than 400 million people, particularly in Africa (International Institute of Tropical Agriculture, 2015). Among the whole group of banana

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plants, varieties of the Cavendish subgroup (*Musa acuminata* 'Williams', 'Grande Naine', 'Dwarf Cavendish', and 'Valery') are the most widely grown group of edible bananas for export due to their acceptance in the international market (Ploetz *et al.*, 2007). However, banana production is strongly threatened by a diverse range of pathogens such as viruses, bacteria, fungi, and insect pests (Dita *et al.*, 2013). Among them, the ascomycete fungus *Pseudocercospora fijiensis* (teleomorph *Mycosphaerella fijiensis*), which causes black Sigatoka disease, is the most serious agronomical problem in *Musa* plantations from an economic point of view (Arzanlou *et al.*, 2007).

Several banana genotypes resistant to *P. fijiensis* have been identified, such as *M. acuminata* subsp. *burmannicoides* 'Calcutta 4' (Miller *et al.*, 2010); however, due to its low fruit quality, this variety is not accepted in the markets. Resistant genotypes have been used as model plants to study defence mechanisms and as a source of resistance for breeding programmes. Although several efforts have been made to develop resistant banana plants, it has not yet been possible to release cultivars acceptable for the export markets, mainly because of disadvantages in the quality of the fruit and in maintaining the final progeny (Rowe and Rosales, 2000).

P. fijiensis colonizes plant tissues in a hemibiotrophic lifestyle with a long period of biotrophy before inducing necrosis in susceptible genotypes (Bévéraggi *et al.*, 1993). In resistant banana Calcutta 4, a rapid and early induction of defence-related genes and corresponding enzymes (peroxidase, phenylalanine ammonia lyase, β-1,3-glucanase), pathogenesis-related (PR) proteins PR4 and PR10, and disease resistance response 1, together with H_2O_2 production, have been observed associated with a hypersensitive-like reaction in response to infection by *P. fijiensis* (Torres *et al.*, 2012; Rodríguez *et al.*, 2016). In sharp contrast, these physiological plant responses have not been observed in infected tissues of the susceptible cultivars Grande Naine or Williams. Similarly, infected stomata varied from 0.95% in resistant Calcutta 4 to 11% in susceptible Grande Naine.

Phytohormones mediate several plant responses, including defence mechanisms against biotic or abiotic stress factors (Berens *et al.*, 2017). The most analysed phytohormones in defence processes are salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Kumar and Klessig, 2000; Glazebrook, 2005). Despite recent interest in the use of resistance inducers for disease control, few studies have been carried out in bananas and there is almost no knowledge of signalling pathways involved in the interaction between *P. fijiensis* and resistant cultivar Calcutta 4.

The objective of the present study was to study the behaviour of selected phytohormones in the susceptible banana cultivar Williams and the resistant banana Calcutta 4 (Ortiz and Vuylsteke, 1994; Miller *et al.*, 2010) during black Sigatoka disease development. SA, JA, and the conjugate jasmonic acid-isoleucine (JA-Ile) were detected, quantified, and correlated with the visual appearance of disease symptoms and histological determination of H_2O_2 induction. Furthermore, the expression of defence-related genes was studied via RNA-Seq and quantitative PCR techniques.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Plants of banana cultivars Calcutta 4 (diploid, AA genome group) and Williams (triploid, AAA genome group) were obtained from the in vitro culture facilities of Plant Biotechnology Unit of Universidad Católica de Oriente, Rionegro-Colombia. Two-month-old plants were kept under greenhouse conditions at 29 °C and relative humidity above 95% with standard fertilization and irrigation practices and a 12 hr:12 hr light/dark photoperiod.

2.2 | Fungal inoculation and disease development

One monoascosporic isolate of *P. fijiensis* from the microbial culture collection of the Plant Biotechnology Unit at Corporación para Investigaciones Biológicas (CIB) (Medellín, Colombia) was grown on potato dextrose agar (PDA, Becton Dickinson) and incubated at 25 °C. Banana plants were inoculated with conidia of *P. fijiensis* as reported by Álvarez *et al.* (2013). Inoculation experiments were repeated three times. Six leaf disk samples (c.10 cm diameter) per plant from two different plants were collected at 12, 18, 24, 48, 72, 144, 360, and 720 hr post-inoculation (hpi). Banana plants of both genotypes (i.e., Calcutta 4 and Williams) without fungal inoculation were used as controls. Black Sigatoka disease symptoms were described in six stages according to the Fouré scale (Fouré, 1987). Samples collected at each time point were cut, frozen in liquid nitrogen and stored at −80 °C until further use.

2.3 | Hydrogen peroxide accumulation

Presence and accumulation of hydrogen peroxide (H_2O_2) in banana leaves of Calcutta 4 and Williams at 12, 18, 24, 48, 72, 144, 360, and 720 hpi were determined by the method reported by Hao *et al.* (2011). Noninoculated banana leaves of the same cultivars and at the same time points were used as controls. Briefly, inoculated leaves were infiltrated with 1 mg/ml 3,3′-diaminobenzidine (DAB, Sigma Aldrich) dissolved in aqueous hydrochloric acid (pH 3.8), under low vacuum for 2 min. Leaves were incubated at room temperature for 2 hr to allow absorption of DAB and reaction with H_2O_2. Afterwards, leaves were cleared in ethanol:acetic acid (3:1 vol/vol) solution for 24 hr at 8 °C, with two changes of the solution. Finally, leaves were treated with gelatin solution (1%) and analysed in a Nikon Eclipse Ni microscope. Experiments were repeated three times using six leaf disk samples (c.10 cm diameter) per plant from two different plants.

2.4 | Gene expression analysis in inoculated plants

2.4.1 | RNA isolation and cDNA synthesis

Total RNA was extracted from Calcutta 4 and Williams banana leaves using the Small-Scale RNA Isolation kit (Invitrogen) according to the manufacturer's instructions. DNA in samples was eliminated using DNase I (Thermo Scientific). RNA quality was verified with the RIN algorithm (RNA Integrity Number) using the 2100 Bioanalyzer Instrument (Agilent). RNA concentration was measured using a ND-1000 spectrophotometer (NanoDrop Technologies). RNA (250 ng) was used to synthesize the first strand of cDNA by TransPlex Whole Transcriptome Amplification kit (Sigma) as reported by Rodríguez *et al*. (2016). Quality of cDNA was evaluated using a 2100 Bioanalyzer instrument (Agilent).

2.4.2 | RNA sequencing and data analysis

Total RNA from susceptible banana Williams and resistant Calcutta 4 was treated with RNAstable LD (Biomatrica) and sequenced at the High-Throughput Sequencing Facility (HTSF) of University of North Carolina (UNC). Thirty-six libraries were sequenced corresponding to three time points after inoculation (24, 72, and 144 hpi) using HTSF Illumina HiSeq 2000 technology. Three replicates per time point of each genotype with their respective controls were used for the study.

RNA sequence data were processed with the software Trimmomatic v. 0.27 (Bolger *et al*., 2014) to filter and clean reads. The software Bowtie v. 2 (Langmead and Salzberg, 2013) was used to align the sequenced reads with reference sequences from *M. acuminata* 'DH-Pahang' transcripts available in the banana genome hub (https://banana-genome-hub.southgreen.fr/pahang_v2, information released January 2016). Gene expression analysis was conducted using the computer package Bioconductor DESeq2 v. 1.12.4 in R (Anders and Huber, 2010). The point of cut-off where a gene was considered down- or up-regulated was 0.1 reads per kilobase per million mapped reads (RPKM). Rate of false discoveries (FDR) threshold was set to 0.1 and this value of dispersion of genes was calculated by adjustment of the curve in accordance with the mode “fit-only” implemented in the software DESeq.

RNA sequences obtained were sent to the sequence read archive database (SRA) available at the National Center for Biotechnology Information (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?, NCBI), submission number SUB4635266. Cluster analysis and visualization of RNA-Seq data were performed by MeV v. 4.9 software (http://mev.tm4.org), the distance metric for hierarchical clustering was the Pearson correlation with average linkage clustering.

2.4.3 | Validation of expression of genes in *Musa* after inoculation with *P. fijiensis*

cDNAs from Calcutta 4 and Williams plants inoculated with *P. fijiensis* were used for quantitative reverse transcription PCR (RT-qPCR), following the protocol described above (refer to section RNA isolation and cDNA synthesis), using 250 ng cDNA per reaction. The Maxima SYBR Green qPCR Master Mix (2×) Kit (Thermo Scientific) was used for amplification and detection of samples in real-time PCR following the manufacturer's instructions, in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). PCR conditions were: denaturation at 95 °C for 30 s, annealing at 58–62 °C (Table S1) for 20 s, and elongation at 72 °C for 30 s, for 35 cycles. All genes were analysed with three technical replicates. The relative expression software tool (REST) v. 2009 (Gene Quantification) (Pfaffl *et al*., 2002) was used to identify differentially expressed genes in group means with statistical significance. Pathogenesis-related protein 4 (*PR4*) was used as a reference gene involved in the plant defence of resistant genotype Calcutta 4. The 26S rRNA ribosomal gene was used as a housekeeping gene according to Rodríguez *et al*. (2016).

2.5 | Analysis of metabolites

2.5.1 | Preparation of samples

Lyophilized leaves (25 mg) were placed in a 2 ml glass vial and extracted using 1 ml of methanol:ethyl acetate:water solution (1:1:1 vol/vol/vol) at −20 °C, with 4 µl of internal standard. Samples were vortexed for 1 min, followed by sonication for 30 min at 8 °C and centrifugation at 5,000 × g for 10 min at 8 °C. The supernatant (600 µl) was collected for subsequent analyses and stored at −20 °C until use.

2.5.2 | Analytical equipment

To perform the analysis by ultra-performance liquid chromatography (UPLC), an Ultimate 3000 series RSLC device (Dionex) was used with an Acclaim RSLC 120 C18 column (150 × 2.1 mm, 2.2 µm, Dionex) and with a flow rate of 300 µl/min, in a binary solvent system of water (solvent A) and acetonitrile (solvent B) (grade LC-MS, Merck), both containing 0.1% (vol/vol) formic acid (eluent additive for LC-MS, Sigma Aldrich). This system was coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Samples were loaded onto the column and washed with a gradient as follows: linear increment from 0% solvent B to 100% solvent B in 15 min; 100% solvent B constant for 5 min; equilibrium time at 0% solvent B for 5 min. Ionization was achieved using electrospray ionization (ESI) with 4 kV for aerosol voltage and 35 V for capillary transfer voltage at a capillary temperature of 275 °C. Samples were measured in positive and negative ionization mode, in a mass range of m/z 100 to 1,000 using a resolution power of 30,000 m/m in an Orbitrap mass analyser.

2.5.3 | Data analysis

Data were evaluated and interpreted using the XCALIBUR program (Thermo Fisher Scientific). XCMS software was used for

analysis and comparison of samples over time; the centWave algorithm was used as a feature detection method using 2.5 parts per million (ppm) as maximal tolerated *m/z* deviation in consecutive scans, and a range of chromatographic peak widths of 5–20 s. Peak integration was found through descent on Mexican hat-filtered data, considering a minimum difference in *m/z* for peaks with overlapping retention time of 0.01. The Welch's *t* test (odd parametric) was used as a statistical test method with a significance *p* value threshold <0.05. Putatively annotated compounds were identified using the METLIN database according to *m/z* and retention time with a *m/z* absolute error less than 0.015 and a tolerance for database search less than 2 ppm (Tautenhahn *et al.*, 2012).

2.6 | Quantitative analysis of endogenous JA, JA-Ile, and SA

Leaves were collected from plants of Williams and Calcutta 4 at 12, 18, 24, 48, 72, 144, 360, and 720 hpi with *P. fijiensis*. Each leaf was cut and immediately frozen at −80 °C. A total of six samples per time point were harvested from each cultivar. Leaves were ground in liquid nitrogen and 250 mg was placed into a 2 ml Eppendorf tube. To each tube, 1 ml of a mixture of methanol and internal standards marked (D6JA, D4SA, 13C6JA-Ile) was added. Agitation in a shaker (20 min) was followed by centrifugation for 20 min at 16,000 × g. The aqueous phase was transferred to a new tube and the remainder was rinsed out with 800 µl of methanol without internal standards repeating shaking and centrifugation steps as described above. A volume of 500 µl of each sample was subjected to HPLC-MS to identify hormones SA, JA, and JA-Ile. For chromatography, an Agilent 1200HPLC system (Agilent Technologies) equipped with a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 µm, Agilent) was used. For mass spectrometry in the negative ionization mode, an API 5000 tandem mass spectrometer (AB Sciex) equipped with a Turbospray ion source was employed in MRM modus. Concentration of hydroxylated (OH) JA was calculated using 9,10-*D*2-9,10-dihydrojasmonic acid applying a response factor of 1.0 and 12-OH-JA-Ile was quantified using jasmonic acid-[$^{13}C_6$]-isoleucine applying a response factor of 1.0. Multifactorial analysis of variance was used to compare the response of the variables time, genotypes, and treatment. R v. 2.15.1 software was used for statistical analysis.

3 | RESULTS

3.1 | Qualitative black leaf streak disease symptoms

Differential progress of black leaf streak disease was observed in both *Musa* genotypes after *P. fijiensis* infection. In Williams, symptoms appeared 360 hpi and disease development reached stage 5 according to the scale of Fouré at 720 hpi (Fouré, 1987). In clear contrast, Calcutta 4 exhibited a hypersensitive-like response at 144 hpi with *P. fijiensis* (Figure S1).

3.2 | H_2O_2 accumulation in *Musa* genotypes

Hydrogen peroxide (H_2O_2) was not detected in the noninoculated controls (Figure 1a,d). In contrast, H_2O_2 was detected in the primary infected and mesophyll cells of leaves of Calcutta 4 at 72 hpi and 144 hpi with *P. fijiensis* (Figure 1b,c); in Williams,

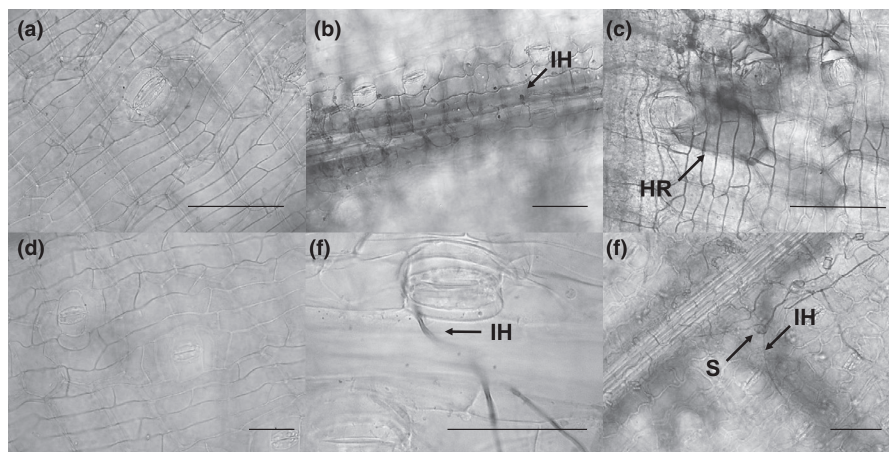

FIGURE 1 Histochemical observation of hydrogen peroxide (H_2O_2) accumulation in banana leaves of cv. Calcutta 4 (resistant) and cv. Williams (susceptible) inoculated with *Pseudocercospora fijiensis* using 3,3′-diaminobenzidine (DAB). (a) Control plant Calcutta 4 without inoculation at 144 hr post-inoculation (hpi); (b) DAB-positive browning of the primary infected and mesophyll cells in Calcutta 4 at 72 hpi (intracellular hypha, IH); (c) DAB-positive H_2O_2 accumulation in the cell wall appositions in Calcutta 4 at 144 hpi (hypersensitive response, HR); (d) Williams plants without inoculation at 360 hpi; (e) successful invasion of fungal hyphae in Williams with no visible plant cellular responses at 144 hpi; (f) formation of a stomatopodium (s) and some accumulation of H_2O_2 at 360 hpi in Williams. Scale bar: 50 µm

deposition of H_2O_2 was detected later, after 360 hpi, when the necrotrophic phase of the infection was in progress (Figure 1f).

3.3 | Differential gene expression of the whole *Musa* transcriptome

Overall, 34 differentially expressed genes related to signal transduction regulated by plant hormones such as JA and ET were identified. Thirteen of those genes were related to signal transduction mediated by ET. Most genes in this group were up-regulated in Calcutta 4 at 144 hpi including those for ET response factors (ET insensitive protein 3 (*EIN3*), *ERF1*, *ERF4*, *ERF9*, *ERF10*, *ERF11*, *ERF17*); *ERF4* had two isoforms with similar profiles of expression in Calcutta 4, but one of them was down-regulated in Williams at 72 hpi. In addition, three genes for ET response factors (*ERF9*, *ERF5*, *ERF105*) were identified as overexpressed in Calcutta 4 at 24 hpi (Figure 2), and *ERF1B* was up-regulated in Calcutta 4 at 72 hpi. Six genes were related to the biosynthesis and signal transduction pathway of JA, four were up-regulated in Calcutta 4 at 144 hpi (*COI*, *OPRI*, *LOX*, and *MYC2*), *MYB* was up-regulated at 24 hpi and *OPR2* was up-regulated at 72 hpi in Calcutta 4. With a different profile, the gene for dehydration-responsive element-binding protein 1E (*DREB1E*) was up-regulated in Calcutta 4 at 24 and 144 hpi, suggesting a possible important role in the activation of defence responses in Calcutta 4.

Eleven genes related to the JA signalling pathway were found to be overexpressed in Calcutta 4 after inoculation with *P. fijiensis*. Lipoxygenase (*LOX*), 12-oxophytodienoate reductase (*OPRI*), allene oxide synthase (*AOS*), allene oxide cyclase (*AOC*) and OPDA reductase (*OPR2*) genes are involved in JA biosynthesis, whereas *COI*, *COI1*, *MYC2*, *MYC4*, *MYB*, and *TIFY* genes are involved in JA signal transduction pathways (Figure 3). Genes related to JA biosynthesis were significantly, highly up-regulated early after inoculation with *P. fijiensis* (at 12–24 hpi) in the resistant genotype Calcutta 4, in clear contrast to the low induction in the susceptible genotype Williams, with the exception of the *JAR1* gene that was up-regulated at 72 hpi in Williams. Nevertheless, this expression of *JAR1* in Williams was lower than the earlier peak expression level observed in Calcutta 4 at 24 hpi (Figure 3).

Overall, the expression of all genes studied followed a response similar to that of the pathogenesis-related gene *PR4* during the early stages (at 12–24 hpi) of the Calcutta 4–*P. fijiensis* interaction.

3.4 | Analysis of the phytohormone-related metabolic pathways by UHPLC-ESI-Orbitrap-MS

In resistant banana Calcutta 4, metabolites belonging to SA biosynthesis were up-regulated only at 12 hpi, while metabolites of JA biosynthesis were up-regulated at 72 and 144 hpi, and down-regulated at 360 hpi. In addition, an important precursor of ET biosynthesis, 1-aminocyclopropane-1-carboxylic acid, was up-regulated at 144 hpi (Table S2).

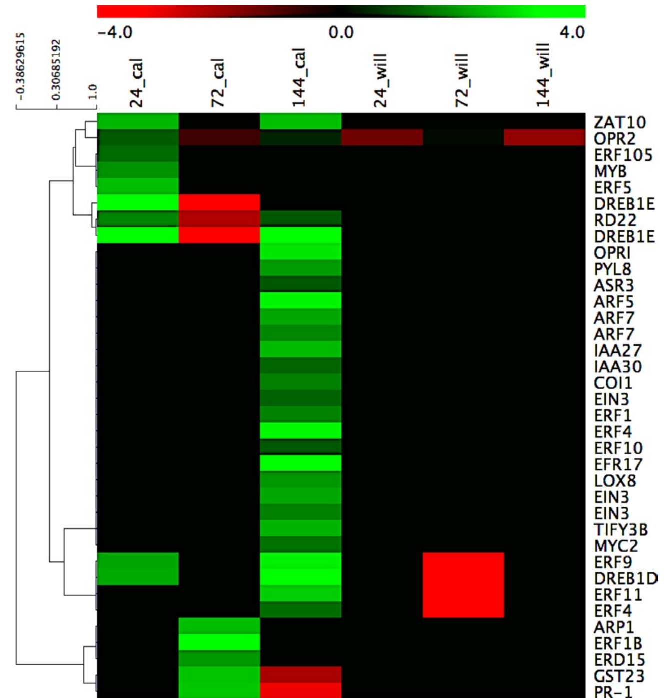

FIGURE 2 Hierarchical cluster of differentially expressed genes related to jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) in banana cv. Calcutta 4 (Cal, resistant) and cv. Williams (Will, susceptible) after inoculation with *Pseudocercospora fijiensis*. The figure was drawn by means of MeV v. 4.9 software; the distance metric for hierarchical clustering was the Pearson correlation with average linkage clustering. Sample names are shown in the top of the figure, gene names are shown on the right side of the figure. Log_2 fold change expression values representation ranges from red (−4.0 lowest value, down-regulated) to green 4.0 (highest value, up-regulated). Dehydration-responsive element-binding protein 1E, DREB1E; 12-oxophytodienoate reductase 1, OPRI; abscisic acid receptor PYL8, PYL8; abscisic stress-ripening protein 3, ASR3; auxin response factor 5, ARF5; auxin response factor 7, ARF7; auxin response factor 7, ARF7; auxin-repressed protein, ARP1; auxin-responsive protein IAA27, IAA27; auxin-responsive protein IAA30, IAA30; coronatine-insensitive protein, COI1; dehydration-responsive element-binding protein 1D, DREB1D; dehydration-responsive protein RD22, RD22; dehydration-responsive element-binding protein 1E, DREB1E; ET-insensitive 3, EIN3; ET-responsive transcription factor 1, ERF1; ET-responsive transcription factor 11, ERF11; ET-responsive transcription factor 1B, ERF1B; ET-responsive transcription factor 4, ERF4; ET-responsive transcription factor 4, ERF4; ET-responsive transcription factor 5, ERF5; ET-responsive transcription factor 9, ERF9; ET-responsive transcription factor ERF010, ERF10; ET-responsive transcription factor ERF01, EFR17; lipoxygenase 8, LOX8; MYB family transcription factor, MYB; protein EARLY RESPONSIVE TO DEHYDRATION 15, ERD15; protein ET INSENSITIVE 3, EIN3; protein ET INSENSITIVE 3, EIN3; protein TIFY 3B, TIFY3B; ET-responsive transcription factor ERF105, ERF105; transcription factor MYC2, MYC2; zinc finger protein ZAT10, ZAT10; glutathione transferase 23, GST23; 12-oxophytodienoic acid reductase, OPR2; pathogenesis-related protein 1, PR-1 [Colour figure can be viewed at wileyonlinelibrary.com]

In Williams, differential expression of metabolites involved in SA biosynthesis was found at 72 and 360 hpi. Metabolites involved in ET and JA biosynthesis were detected up to 360 hpi (Table S3).

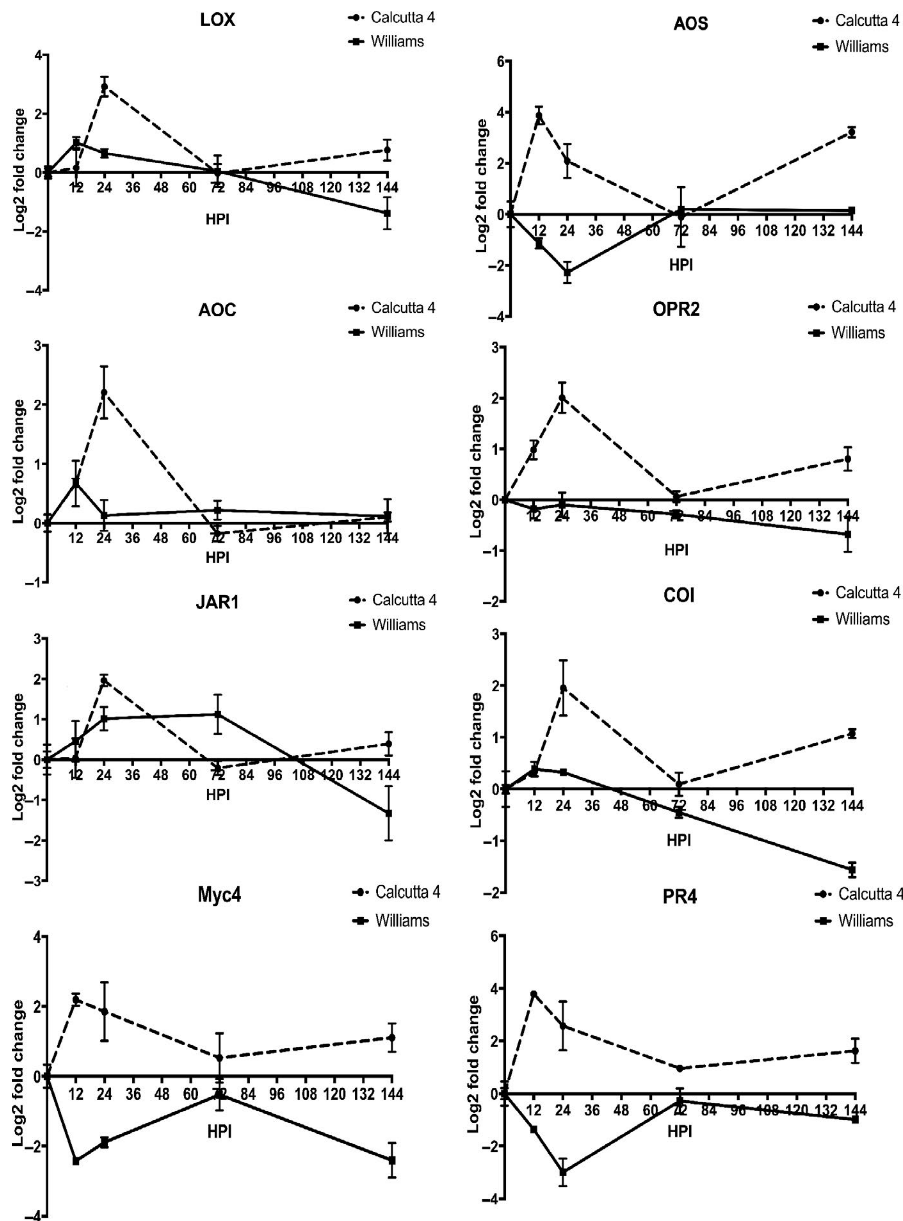

FIGURE 3 Expression of genes of banana cultivars Calcutta 4 (resistant) and Williams (susceptible) with time after inoculation (hr post-inoculation, hpi) with *Pseudocercospora fijiensis*, determined by quantitative reverse transcription PCR. Pathogenesis-related protein 4 (*PR4*) was used as a reference for defence expressed in the resistant cv. Calcutta 4 and 26S rRNA was used as a housekeeping gene according to Rodríguez *et al*. (2016). Lipoxygenase, LOX; allene oxide synthase, AOS; allene oxide cyclase, AOC; 12-oxophytodienoate reductase 2, OPR2; coronatine-insensitive protein, COI1; jasmonate-amido synthetase, JAR1; transcription factor MYC4, Myc4

3.5 | Quantitative analysis of endogenous JA, JA-Ile, and SA by HPLC-MS

The level of JA in banana Calcutta 4 was significantly increased at 72, 144, and 360 hpi, while in Williams no changes were observed between the control and treatments. The maximum induction of JA in Calcutta 4 was reached at 144 hpi with a concentration of 170.5 ng/g. JA-Ile was overexpressed in Calcutta 4 at 12 and 144 hpi, and the highest value was measured at 360 hpi. No significant variation was observed for JA-Ile in Williams. Induction of SA in Calcutta 4 was observed at 360 hpi. In Williams, SA was overexpressed at 12 and 360 hpi (Figure 4), but levels, though significant, were low at most time points.

4 | DISCUSSION

Plant hormones such as JA, ET, and SA play a central role in systemic defence responses through signalling activation in different plant tissues (Adie *et al*., 2007; López *et al*., 2008). In general, it is believed that signalling associated with JA and ET triggers resistance against necrotrophic pathogens, whereas SA activates resistance responses against biotrophic and hemibiotrophic pathogens (Glazebrook, 2005). The balance of plant hormones has an important influence in the outcome of plant–pathogen interactions, as well as in the establishment of successful systemic immunity. Analysis of molecular and biochemical responses to *P. fijiensis* in the banana variety Calcutta 4, which is

Jasmonic acid

JA ng/g (fold change)

45
35
25
15
5
−5
−15
−25
−35

12 18 24 48 72 144 360

Hours post inoculation

Calcutta
Williams

(±)-Jasmonic acid-isoleucine

JA-Ile ng/g (fold change)

45
35
25
15
5
−5
−15
−25
−35

12 18 24 48 72 144 360

Hours post inoculation

Calcutta
Williams

Salicylic acid

SA ng/g (fold change)

45
35
25
15
5
−5
−15
−25
−35

12 18 24 48 72 144 360

Hours post inoculation

Calcutta
Williams

FIGURE 4 Results of analysis of endogenous jasmonic acid (JA), JA-isoleucine, and salicylic acid (SA) in leaves of banana cultivars Calcutta 4 (resistant) and Williams (susceptible) with time after inoculation with *Pseudocercospora fijiensis*, determined by HPLC-MS. Fold change values were calculated with respect to the uninoculated control for each time point. Average fold change was calculated using five replicates. Errors bars correspond to the standard error of the mean calculated using values from five replicates

resistant to black Sigatoka disease, could contribute to unravelling defence mechanisms that may be useful in banana breeding and disease management. Previous work has shown that external application of SA and methyl jasmonate (MeJA) significantly reduced disease index and lesion diameter in banana fruits caused by the fungal pathogen *Colletotrichum musae*. In addition, it was found that SA and MeJA enhanced the expression of the defence-related genes *MaWRKYs*, *MaPR1-1*, *MaPR2*, *MaPR10c*, *MaCHI3*, *MaCHI4*, and *MaCHIL1* (Tang *et al*., 2013).

Biosynthesis of JA starts from α-linolenic acid, which is processed by LOX, AOS, and AOC to produce *cis*-(+)-12-oxo-phytodienoic acid (OPDA). JA biosynthesis takes place in peroxisomes and 12-oxophytodienoate reductase (OPR2) is one of the key enzymes to produce JA, JA-Ile, or MeJA (Wasternack and Hause, 2013). In our work, *LOX*, *AOS*, *AOC*, and *OPR2* genes were up-regulated in resistant banana Calcutta 4 during the first 24 hpi. Up-regulation of *OPR2* with simultaneous activation of *PAL*, *POX*, phytoalexins, pathogenesis-related (PR) proteins and detoxification processes have been correlated with increase in defence in pea (*Pisum sativum*) against the pathogenic fungus *Mycosphaerella pinodes* (Fondevilla *et al*., 2011). Similarly, Rodríguez *et al*. (2016) reported overexpression of *PAL*, *POX*, *PR-4*, *PR10*, and disease resistance response 1 genes, at 72 hpi of resistant banana cv.Calcutta 4 with *P. fijiensis*.

Previous studies have shown that in Calcutta 4, peroxidase activity is enhanced during the early stages of interaction with *P. fijiensis* (Torres *et al*., 2012; Rodríguez *et al*., 2016). These results are consistent with the histochemical responses found in the present work in leaves of Calcutta 4 inoculated with *P. fijiensis*, where H_2O_2 accumulation was found at early stages of infection. This reaction is usually related to hypersensitive-like reactions in resistant plant genotypes, and has been reported widely for Calcutta 4 when inoculated with *P. fijiensis* (Torres *et al*., 2012). Furthermore, we have found that expression of the *GPX* gene decreased in resistant Calcutta 4 and increased in susceptible Williams during the interaction with *P. fijiensis*. This gene is responsible for detoxification of H_2O_2 in the cell and plays an important role in controlling the oxidative burst and accumulation of reactive oxygen species (Nanda *et al*., 2010).

In our research, the gene coding for JA ZIM-domain protein (JAZ) was down-regulated in Calcutta 4, and genes coding for COI1 and transcription factor MYC2 proteins were up-regulated. MYC2 is important for transcriptional activation of JA pathways (Shoji and Hashimoto, 2011). In addition, JAZ and JAI3 have been reported as MYC2 repressors (Chini *et al*., 2007). Our results suggest that a similar mechanism to that reported by Chini *et al*. (2007) may exist in resistant banana Calcutta 4, where COI1 induction would degrade JAZ by ubiquitin-mediated proteolysis. In the absence of its repressor, MYC2 would be up-regulated, which eventually activates expression of defence-related genes such as PR protein genes and transcription factors from the WRKY family. In addition, overexpression of the ET-responsive transcription factor gene (*ERF*), could be a factor in the activation of ET synthesis. According to Berrocal-Lobo and Molina (2004), overexpression of ERF1 in *Arabidopsis thaliana* enhanced plant resistance to fungal pathogens such as *Botrytis cinerea* and *Plectosphaerella cucumerina*. ERFs identified in the present work have been reported as repressing or activating plant defence responses (Huang *et al*., 2016). ERF11 and ERF4 contain an EAR motif that functions in negative regulation of ET-responsive genes via the GCC box in *A. thaliana* and *Nicotiana attenuata* against necrotrophic pathogens (Yang *et al*., 2005; Lu *et al*., 2011; Huang *et al*., 2016). However, another study of hemibiotrophic interactions by Zheng *et al*. (2019) suggests that transcriptional activation of *BT4* by ERF11 is a key step in SA/ET-regulated plant resistance against *Pseudomonas syringae* pv. *tomato*. In our work, *ERF4* and *ERF11* were overexpressed in cv. Calcutta 4 at 144 hpi, whereas in cv. Williams they were down-regulated at 72 hpi, suggesting that both genes may be implicated in the activation of cv. Calcutta defences against *P. fijiensis*.

Consistent with gene expression patterns, levels of metabolites related to JA biosynthesis changed after infection of banana by *P. fijiensis*. Metabolites 12-oxo-*cis*-10,15-phytodienoate, 12,13(*S*)-epoxylinolenate and 3-oxo-2-(*cis*-2′-pentenyl)-cyclopentane-1-octanoate were up-regulated in Calcutta 4 at 72 hpi (Figure 5). Similarly, OPC6-*trans*-2-enoyl-CoA and 1-aminocyclopropane-1-carboxylic acid, metabolites related to JA biosynthesis, were up-regulated in Calcutta 4 at 144 hpi, suggesting activation of JA biochemical pathways after infection by *P. fijiensis*. Meanwhile, 12,13(*S*)-epoxylinolenate and linoleate were down-regulated in susceptible variety Williams at 144 hpi. Ncho *et al*. (2016) showed that when MeJA was applied to leaves of banana cv. Grand Naine, which is susceptible to black Sigatoka disease, it stimulated production of defence-related phenolic compounds better than SA; moreover, the highest accumulation was found at 72 hr after treatment with MeJA. These results are similar to our findings, where Calcutta 4 modulates genes for biosynthesis of JA at 72 hpi, suggesting that JA plays a role in, or is a component of, the complex system of banana defence responses.

Plants have different layers of defence responses to pathogens that may intercommunicate. When pathogens penetrate plant tissues and colonize the apoplast, molecular patterns from microorganisms and from plant damage (i.e., PAMPs, MAMPs, or DAMPs) may be recognized by pattern recognition receptors (PRR), which, upon activation, induce intracellular signalling leading to pattern-triggered immunity (PTI). Pathogens may suppress PTI activation by a number of effector proteins, which may also be recognized in the apoplast or inside the host cell by plant defence-related proteins named R proteins. When an effector protein is recognized by a R protein, signalling leading to the hypersensitive response, which is a form of programmed cell death, is activated. This form of defence response is named effector-triggered immunity (ETI). Effector proteins from well-adapted pathogens may also suppress ETI, causing disease, but additional R proteins from the plant may recognize these effector proteins and induce plant cell death, in a never-ending coevolution for recognition and evasion of recognition in plant–pathogen interactions. In both PTI and ETI, JA and ET are involved as key players in the induction of processes that contribute to the resistant phenotype observed in a number of pathosystems, including banana

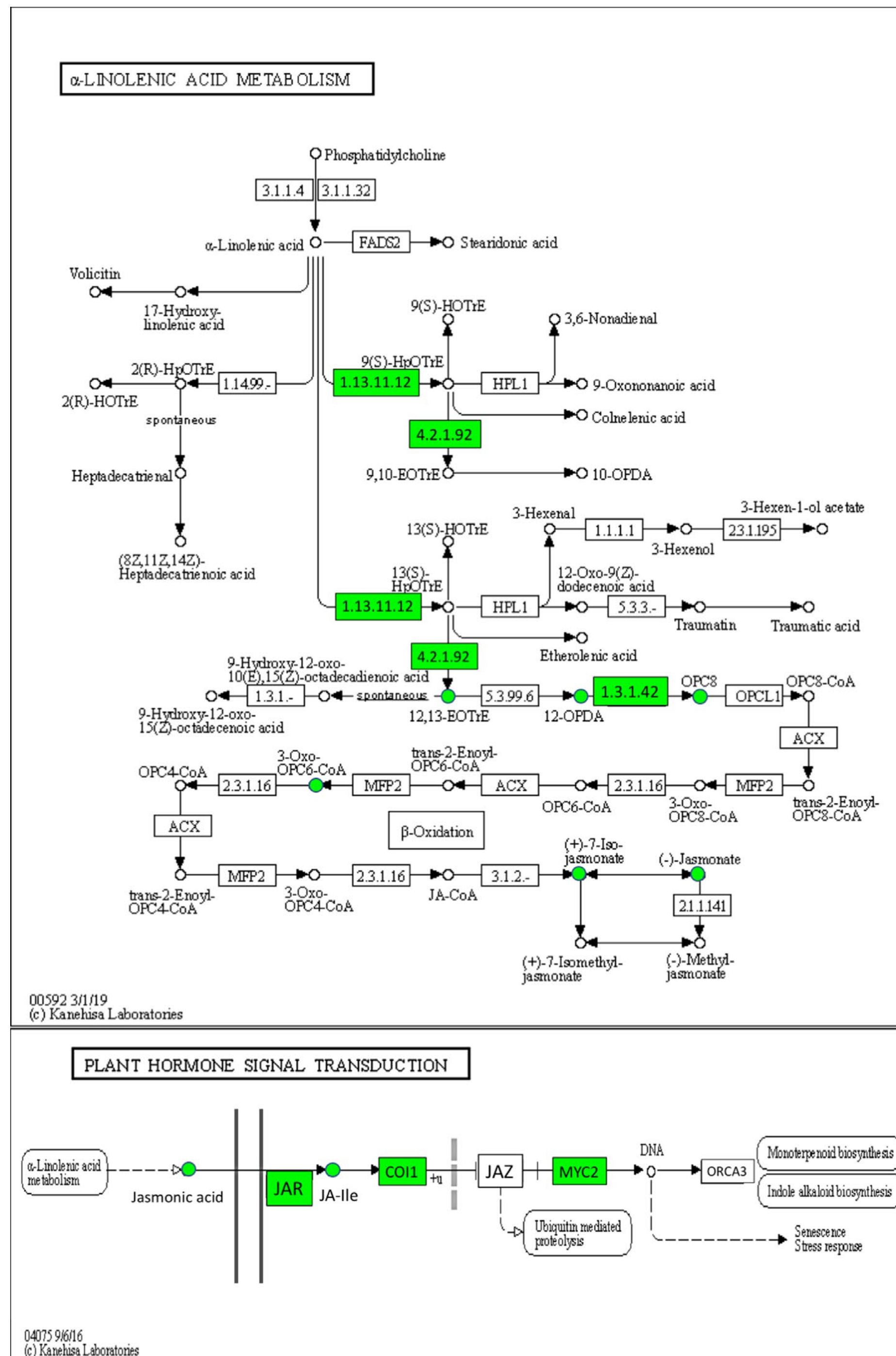

FIGURE 5 KEGG representation of up-regulated genes related to jasmonic acid (JA) biosynthesis (α-linolenic acid metabolism (ko00592)) and JA signal transduction pathways (ko04075) in banana cv. Calcutta 4 after inoculation with *Pseudocercospora fijiensis*. Rectangles are gene products, mostly proteins but including RNAs; circles are chemical compounds, DNA or other molecules. Genes or chemicals up-regulated at any time point were highlighted in green. Lipoxygenase (LOX) [EC:1.13.11.12]; allene oxide synthase (AOS) [EC:4.2.1.92]; 12,13(*S*)-epoxylinolenate (12,13-EOTrE) [C04672]; 12-oxophytodienoic acid reductase (OPR2) [EC:1.3.1.42]; 12-oxo-10,15(*Z*)-phytodienoic acid (12-OPDA) [C01226]; 3-oxo-2-(*cis*-2′-pentenyl)-cyclopentane-1-octanoate (OPR8) [C04780]; OPC6-*trans*-2-enoyl-CoA (3-oxo-OPC6-CoA) [C16334]; (+)-7-isojasmonic acid (JA-Ile) [C16317]; jasmonic acid (JA) [C08491]; jasmonic acid-amino synthetase (JAR1) [EC:6.3.2.52]; coronatine-insensitive protein 1 (COI1) [K13463]; transcription factor MYC2 (MYC2) [K13422] [Colour figure can be viewed at wileyonlinelibrary.com]

cv. Calcutta 4–*P. fijiensis* (Campos *et al.*, 2014). Early and strong induction of defence-related genes has been identified as a key factor in the resistant phenotype observed for Calcutta 4 when compared to susceptible Williams (Torres *et al.*, 2012; Rodríguez *et al.*, 2016). Results shown here indicate that early activation of JA and ET biosynthesis pathways is at least part of the response of the resistant phenotype Calcutta 4 to inoculation with *P. fijiensis*. In contrast, in the susceptible banana cv. Williams, activation of JA

and ET defence-related responses are weak, late, or not present, suggesting that in this variety, regulation of JA signalling occurs by a different mechanism to that in Calcutta 4 or that it is suppressed by pathogen effectors, as has been observed in other pathosystems (Campos *et al.*, 2014).

Results of the present study, together with previous research, open a myriad of possible applications for the integrated management of black leaf streak disease in banana crops, from the future development of improved banana cultivars with resistance to *P. fijiensis*, to novel and better molecules for induced resistance with greater effectiveness in commercial banana fields.

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DATA AVAILABILITY STATEMENT

Data available in article supplementary material and RNA sequences obtained were sent to the sequence read archive database (SRA) available at the National Center for Biotechnology Information (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?, NCBI), submission number SUB4635266.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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