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Severe modifications in source-sink ratio influence the susceptibility of bananas to crown rot and its phenolics content

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Abstract

Banana susceptibility to crown rot is influenced by many biotic and abiotic preharvest factors, which include source-sink (So-Si) ratio modifications through trimming of leaves and fruit. Banana plant's resistance to biotic stress has been previously correlated to its phenolic content; it is hypothesized that the crown's phenolic content may influence the fruit's susceptibility. The aim of this work was to investigate the influence of severe So-Si ratio modifications, via the removal of leaves and fruit, and the involvement of phenolics in the fruit's susceptibility to crown rot. Fruit susceptibility was evaluated 13 days postinoculation (13 dpi) with *Colletotrichum musae*. Banana crowns obtained on the day of harvest before inoculation (dhbi) and 13 dpi were analysed for changes in phenolics using GC-MS, HPLC, and LC-MS devices. Severe So-Si ratio modifications had a significant effect ($p < .001$) on susceptibility, fruits of low So-Si ratio being most susceptible. It also significantly influenced ($p < .001$) some tree and fruit characteristics. The less susceptible (S−) crowns had higher amounts of phenolics compared to the more susceptible (S+) ones. Catecholamines were identified as the major phenolics in banana crown, notably dopamine compared to norepinephrine and normetanephrine. Hydroxycinnamic acids (ferulic acid and its derivatives) were significantly accumulated ($p < .001$) the dhbi in S− crowns compared to S+ crowns, but decreased 13 dpi. Phenolics have a possible role in the biochemical defence of banana crown and could be used by producers as a chemical criterion for estimation of the level of banana's susceptibility to crown rot.

KEYWORDS

chromatographic methods (GC-MS, HPLC, LC-MS), crown rot, fruit susceptibility, *Musa* spp., phenolic, source-sink ratio modifications

1 | INTRODUCTION

Crown rot is a postharvest disease of bananas affecting the tissue uniting fruit peduncles. Caused by a broad, unspecific parasitic complex, it is the most important postharvest disease affecting the quality of exported bananas. Within this complex, *Colletotrichum musae* is the most commonly described pathogenic species (Finlay and Brown, 1993; Krauss and Johanson, 2000; Lassois *et al*., 2010a; Nuratika *et al*., 2018). The disease develops during shipping, ripening, commercialization, and storage and has a negative impact on the market value of the bananas (Slabaugh and Grove, 1982). Crown rot control relies mainly on the application of postharvest fungicides (Slabaugh and Grove, 1982; de Lapeyre de Bellaire and Nolin, 1994). Nevertheless, seasonal and spatial variations in the performance of chemical control have been observed. It has been suggested that these spatiotemporal fluctuations may represent variations in

the quality potential of the fruit, which is influenced by preharvest factors such as agronomic practices and agroecological conditions (Chillet and de Lapeyre de Bellaire, 1996; Lassois *et al*., 2010a).

Fruit susceptibility to crown rot disease is an important component of this quality potential (Lassois *et al*., 2010a) and a specific methodology has been designed for its evaluation (de Lapeyre de Bellaire *et al*., 2008). More recently it has been shown that source-sink (So-Si) ratio modifications, through trimming of leaves and fruit, significantly influenced the susceptibility to crown rot disease of bananas (Lassois *et al*., 2010b). Therefore, using such So-Si ratio modifications is an ideal model to obtain fruits with contrasting susceptibility levels in order to understand underlying mechanisms of this plant–pathogen interaction. In previous work using cDNA-AFLP, Lassois *et al*. (2011) identified some genes involved in the susceptibility of bananas to crown rot disease in fruits exhibiting such contrasting disease responses. The results highlighted that metabolic pathways are potentially involved in this plant–pathogen interaction and particularly suggested that phenolics, notably dopamine, could be involved in the quantitative response of bananas to crown rot.

Dopamine and products that derive from its oxidation are the main phenolic compounds in bananas and these compounds have a fungitoxic activity against *C. musae* (Muirhead and Deverall, 1984), and antioxidant properties (Kanazawa and Sakakibara, 2000; Someya *et al*., 2002). Besides the high concentration of phenols, phenylalanine ammonia-lyase and oxidative enzymes like peroxidase and polyphenol oxidase (PPO) were reported in many resistant banana tissues such as in the interaction between *Musa* spp. and *Fusarium oxysporum* f. sp. *cubense* (cause of Panama disease) (Kavino *et al*., 2009). Amongst the many diverse secondary metabolites involved in banana's resistance to different pathogens, preformed and induced phenolic compounds have been widely reported in the literature as potential participants in the banana tree defence mechanisms (Ewané *et al*., 2012a).

Phenolics were shown to be involved in the preformed defence mechanism of the banana tree against black leaf streak disease, via specialized cells of the mesophyll that store phenolics in partially resistant cultivar tissues (Beveraggi *et al*., 1995; El Hadrami, 1997). Phenolics were also involved in induced defence mechanisms. For example, after a wound or an inoculation with *C. musae*, several phytoalexins (phenylphenalenones, irenolone, and emenolone types) were induced in the tissues of green bananas (Luis *et al*., 1993; Kamo *et al*., 1998, 2001). Higher contents of several phenolic compounds have been observed in roots tissues of banana cultivars resistant to phytophagous nematodes and the role of these phenolics in defence mechanisms has been reported (Valette *et al*., 1998; Collingborn *et al*., 2000; de Ascensao and Dubery, 2003; Wuyts *et al*., 2007). Despite convergent information on plant defence mechanisms involving phenolics in bananas, the fruit susceptibility to crown rot disease has not yet been linked to fluctuations in phenolic contents of the tissues of the banana crown.

The objective of this work was to understand better the role of phenolics in the susceptibility of bananas to crown rot disease. For this, we have first characterized the effect of a very broad range of So-Si ratio modifications on variations of susceptibility to crown rot disease. The objective was to select the most extreme situation for both susceptible (S+) and less susceptible (S−) fruits, exploring a broader range of So-Si ratios than former studies (Lassois *et al*., 2010a, 2010b, 2011). In a second step, preformed (preinoculation) and induced (postinoculation) phenolics were analysed in the crowns of both extreme situations (S+ and S−) using HPLC and LC-MS in order to identify phenolic compounds associated with a lower susceptibility to crown rot disease. Catecholamines, which might also play a specific role in plant defence mechanisms in bananas, were also studied through GC-MS analysis (Szopa *et al*., 2001).

2 | MATERIALS AND METHODS

2.1 | Plant material

Banana fruits were harvested from banana plants (*Musa acuminata* [AAA group, Cavendish subgroup] 'Grande Naine'), grown on a commercial banana farm belonging to the PHP (Plantations du Haut Penja) banana company, in the Littoral Region of Cameroon (Bouba, 100 m). The average temperature of the locality is 27°C and the mean annual rainfall is 2,500 mm. The banana plants were selected at flowering (horizontal finger stage) and their bunches covered with a plastic sleeve. Each bunch was tied with a belt, coloured to indicate the time for harvest, which was carried out at a constant physiological age when the sum of the daily mean temperature accumulated by the fruit at the 14°C threshold, between flowering and harvest, reached 900 degree days (dd) (Jullien *et al*., 2008). Therefore, temperatures were recorded on the experimental plot via an electronic probe (Tinytag Plus; Gemini Data Loggers) with regular data capture (every 15 min) and the calculation of an average daily temperature from all the daily data.

2.2 | Experimental design for source-sink ratio modifications

Various So-Si ratio modifications were performed at the flowering stage (fingers on horizontal position) by removing sources (leaves, L) and sinks (hands of the bunch, H, a hand being a cluster of fruits). The five following So-Si ratios were performed at that stage:

12L/1H: 12 leaves and 1 hand remaining, source-sink ratio = 8
12L/2H: 12 leaves and 2 hands remaining, source-sink ratio = 4
12L/8H: 12 leaves and 8 hands remaining, source-sink ratio = 1 (reference)
2L/8H: 2 leaves and 8 hands remaining, source-sink ratio = 0.17
1L/8H: 1 leaf and 8 hands remaining, source-sink ratio = 0.08

The values of the So-Si ratio were calculated as described by Lassois *et al*. (2010b), taking 12 leaves/8 hands as the reference for a banana tree where no leaves or hands were removed.

Five banana trees at the same flowering stage were selected for each treatment. This selection was repeated three times for three

successive weeks. At harvest, for each harvested banana tree, two clusters consisting of four banana fruits were cut off from the second hand (except for the treatment 12L/1H). The first cluster was used for identification of preformed phenolic compounds on the day of harvest before inoculation (dhbi); the banana crown was immediately frozen in liquid nitrogen and freeze-dried after removal of the green parts for further biochemical analyses. The second cluster was used for artificial inoculations and evaluation of susceptibility to crown rot at 13 days postinoculation (13 dpi). At this time, crowns were also frozen in liquid nitrogen, and freeze-dried after removal of the green and necrotic parts (Figure 1) for identification of induced phenolic compounds.

The grade (i.e., fruit diameter) and length of the same medial fruit harvested on the second hand as well as the number of leaves remaining at harvest (NLH) were also assessed for each sampled banana plant.

2.3 | Evaluation of susceptibility to crown rot disease

The susceptibility of bananas to crown rot was evaluated through the artificial inoculation of banana clusters with a single spore isolate of *C. musae* (de Lapeyre de Bellaire *et al.*, 2008). Bunches were harvested on the day of experimentation and their second hands were transported to the laboratory. The medial part of the hand was cut into two clusters of four banana fingers. The cuttings were squared, with regular and clear-cut sections in order to obtain similar crowns between the clusters. Once latex ran out, crown tissues were dried with absorbent paper and sterilized by dipping them into 50% alcohol. Fruits were then laid out at room temperature for 2 hr to allow the crowns to dry. A droplet of 50 µl of a *C. musae* suspension (10^4 conidia/ml) was then deposited at the top of the crown. A sterilized square filter paper of 1 cm^2 was placed on the droplet in order to keep the inoculum in place. Three hours after inoculation, the clusters were packed in perforated plastic bags in commercial boxes and stored under stable conditions at 13°C for 13 days (Ewané *et al.*, 2012b). At the end of this storage period, an evaluation of the internal progression of the rot within the crown was carried out. The clusters were divided into two parts and the transverse cutting of the crown allowed a visualization of the spread of rot into the crown. The internal necrotic surface (INS) expressed in mm^2 was calculated by assuming a rectangular shape to the internal necrosis. Its value was taken as a measure of the fruit's susceptibility to crown rot.

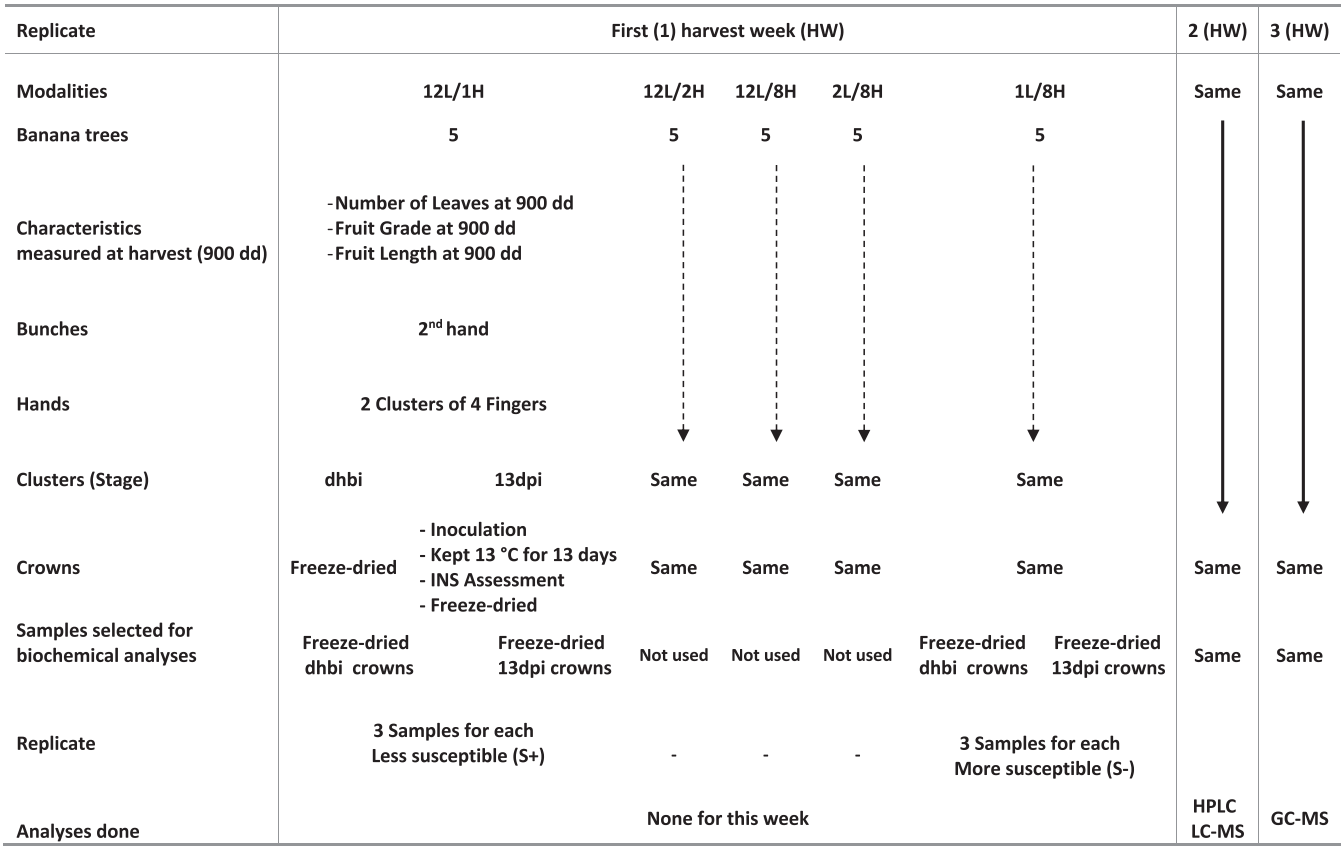


FIGURE 1 Experimental design for the study of the effect of source-sink ratio modifications on the susceptibility of bananas to crown rot disease. L, leaves; H, hand or cluster of fruits; dhbi, day of harvest before inoculation; dpi, days postinoculation; INS, internal necrotic surface

2.4 | Analysis of phenolic compounds of banana crowns

Two different extraction methods were needed for these analyses, because banana crown tissues might contain free soluble (or lightly bound to cell wall) phenolic compounds (catecholamine and soluble phenolics) and compounds bound to the cell walls (bound phenolics). These methods are described below. The free soluble phenolic fraction was analysed by HPLC + LC-MS. However, a more specific analysis was required for catecholamine as it coelutes with ascorbic acid (added as a protective in the solvent), as previously reported by Wuyts *et al.* (2007). Therefore, catecholamines were analysed by GC-MS. The bound phenolics were analysed by HPLC + LC-MS (Figure 2).

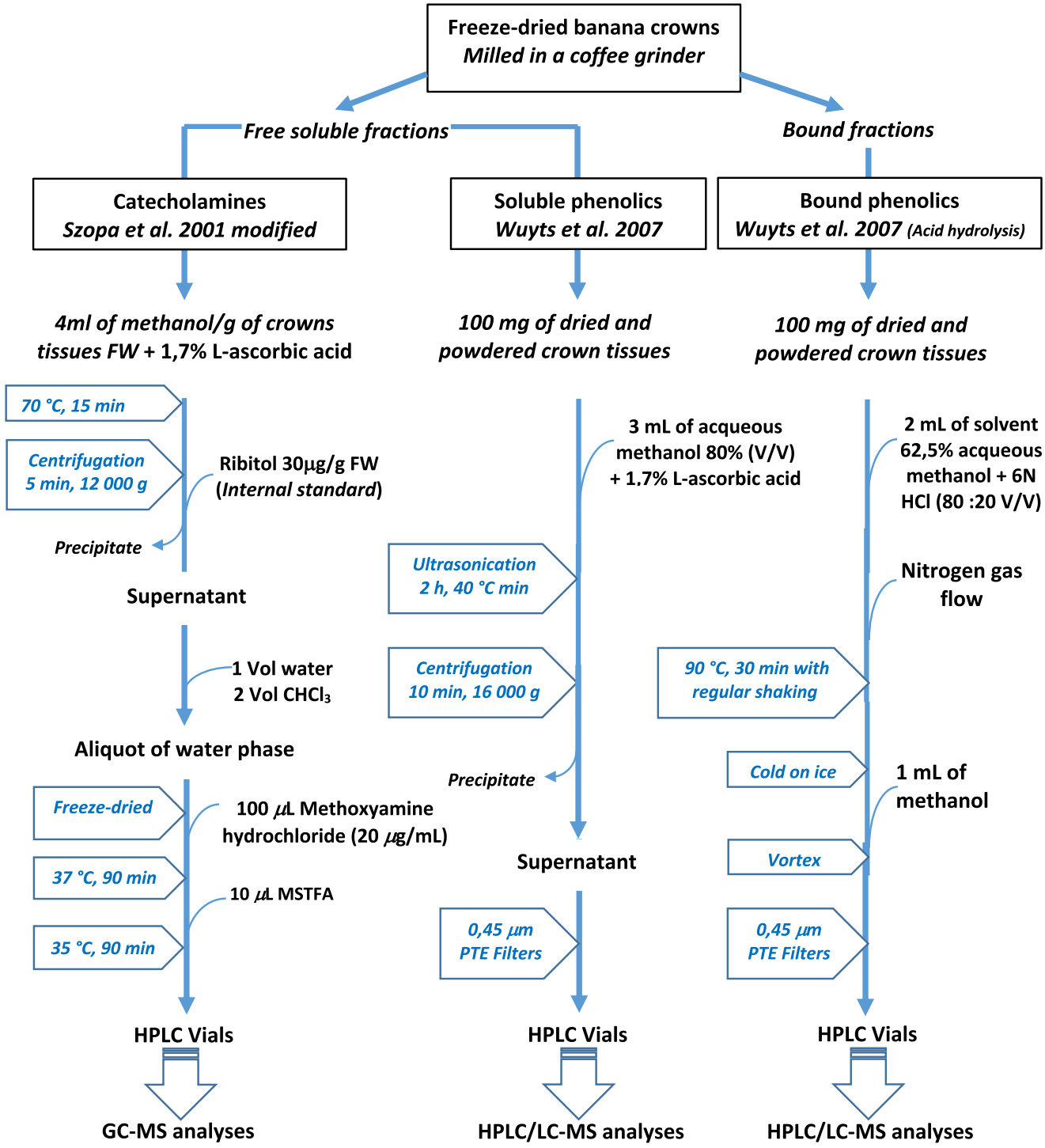


FIGURE 2 Methods of extraction and analysis of catecholamines and phenolics (soluble and bound) from freeze-dried banana crowns

2.4.1 | Sample preparation

Crowns for analysis were selected from two So-Si ratio treatments that led to high and low susceptibility: S+ (high susceptibility, treatment 1L/8H, So-Si = 0.08) and S− (low susceptibility, treatment 12L/1H, So-Si = 8; Figure 1). The crowns were collected at two stages: the day of harvest before inoculation (S+/dhbi and S−/dhbi) and at 13 dpi (S+/13 dpi and S−/13 dpi). Only crowns of the same field replicate were compared. For GC-MS analysis, three S+ and three S− banana crowns harvested on bunches from the third replicate were selected and for HPLC and LC-MS analyses, three S+ and three S− crowns harvested on bunches from the second replicate were selected. Prior to the analyses, the freeze-dried crowns were milled in a coffee grinder.

2.4.2 | Methanolic extraction of phenolic compounds

Extraction of free soluble fraction

For the soluble phenolics, 100 mg of dried and powdered crown tissues were placed in a Pyrex tube with a Teflon-coated cover and extracted with 3 ml aqueous methanol 80% (vol/vol) containing 1.7% L-ascorbic acid (wt/vol). The mixture was ultrasonicated for 2 hr at 40°C and centrifuged at 16,000× **g** for 10 min. The supernatant was passed through 0.45 μm filters into HPLC vials.

Catecholamine extractions were carried out similarly, but, according to the method reported by Szopa *et al.* (2001), modified by the addition of L-ascorbic acid (1.7% wt/vol) to the extraction solvent as an antioxidant (Figure 2).

Extraction of bound fraction

Bound phenolic compounds were extracted by acid hydrolysis following the method used by Wuyts *et al.* (2007). Dried and milled crown tissue (100 mg) and 2 ml of a solvent containing 62.5% aqueous methanol and 6 M HCl (4:1 vol/vol) were suspended in a Pyrex tube with a Teflon-coated cover, placed under nitrogen gas flow to prevent oxidation and incubated 30 min at 90°C with regular shaking. Samples were then cooled on ice and 1 ml methanol was added. The tubes were vortexed and the extract passed through 0.45 μm filters into HPLC vials.

2.4.3 | Chromatographic analysis of banana crown methanolic extracts

GC-MS analysis

GC-MS analyses were performed on a HP-6890 GC system coupled with a 5973N mass detector and equipped with a HP-5MS fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness). Injection temperature was 230°C, the interface set to 250°C and the ion source adjusted to 180°C. The temperature programme started at 70°C for 5 min, followed by a 5°C/min temperature ramp up to 300 and 310°C was maintained for 1 min. The system was then equilibrated at 70°C for 6 min before the next injection. The flow rate of helium, the carrier gas, was 1 ml/min. Mass spectra were recorded at 2 scans/s with an *m/z* 50–600 scanning range. Two microlitres of sample was used for analysis. The chromatograms and mass spectra were evaluated using the MSD ChemStation program (Hewlett Packard). Dopamine, epinephrine, norepinephrine, and normetanephrine (Sigma) were used as standards. The extracts were spiked with dopamine, epinephrine, norepinephrine, and normetanephrine, and estimated recoveries were 78%, 65%, 83%, and 80% respectively. Each catecholamine was quantified by internal standardization with ribitol and their respective calibration curve, using the following ions: ribitol (*m/z* 307; 319), dopamine (*m/z* 174; 338; 426), epinephrine (*m/z* 174; 294; 355), norepinephrine (*m/z* 174; 355; 514), normetanephrine (*m/z* 174; 297; 456).

HPLC analysis

HPLC analyses were performed on an Agilent HP 1100 system using a C18 Inertsil ODS2 (250 × 2.1 mm, 5 μm) reverse phase analytical column with a diode array detector. Phenolic compounds were monitored at five wavelengths: 254, 280, 330, 366, and 450 nm and the peak spectra were recorded. Fluorescence detection was also carried out with an excitation wavelength of 305 nm and emission at 410 nm. Methanolic extracts were separated with a mobile phase consisting of acetonitrile and Milli-Q water acidified at pH 3.0 with phosphoric acid and a flow rate of 0.3 ml/min at 35°C. A gradient of acetonitrile was programmed (0% for 5 min, increase to 50% in 70 min, 50% to 100% in 3 min, and 100% for 7 min). The column was then re-equilibrated with 0% acetonitrile for 10 min. The volume of extracts injected into the column was 20 μl. Retention time (t_R) and UV absorption spectra of the peaks of the extracts were compared with the ones obtained with the phenolic standards from Sigma-Aldrich, (ascorbic, gallic, chlorogenic, caffeic, coumaric, ferulic, and sinapic acids; catechin, rutin, and naringin) and Fluka (hesperidin, phloridzin, quercetin, cinnamic acid, naringenin, and kaempferol), previously prepared under the same conditions as crown samples to monitor degradation and avoid underestimation, and analysed under the same HPLC conditions in order to confirm their identification. The identified compounds were quantified by external calibration with the corresponding standard. The unidentified compounds were quantified in dopamine equivalents and the hydroxycinnamic acids in ferulic acid equivalents.

LC-MS analysis

LC-MS analyses were carried out with an Agilent HP 1100 system coupled to an HCT mass spectrometer (Bruker Daltonics) to corroborate the identifications made by HPLC. The extracts were diluted with methanol in order to obtain a total concentration of 10 μg/ml. The column and elution gradient used were the same as described above for the HPLC-UV analysis. After the column, the flow was split and 0.05 ml/min was sent to the source for electrospray ionization (ESI). Column effluent was monitored in negative and positive ion modes. The ESI conditions were: N_2 pressure = 35 psi, N_2 flow

rate = 8.5 L/min, and 365°C. The ionic trap was set to scan from 150 to 1,000 m/z (scan rate of 26,000 m/z/s). The trap was emptied either after 200 ms or as soon as a total ionic current of 100,000 was reached. The t_R and mass spectra of the peaks of the extracts were compared with those of standards previously analysed under the same LC-MS conditions in order to confirm their identification.

2.5 | Statistical analysis

The effects of severe So-Si ratio modifications on banana susceptibility to crown rot were analysed by the subjection of INS values calculated for the three clusters and each fruit grade and length values to a partially nested mixed three-way analysis of variance (ANOVA; Minitab v. 15.1). Each tree was taken as an experimental unit, and treatment, week, and bunch as factors. Multiple comparisons of the different means were performed by applying Tukey's test at a 5% probability level. Data of crown phenolics quantified by HPLC analyses were subjected to a two-way ANOVA (Minitab v. 15.1), with treatment and stage as factors.

3 | RESULTS

3.1 | Effects of severe So-Si ratio modification on susceptibility to crown rot and on some fruit and tree characteristics

The modifications of the So-Si ratio at flowering had a highly significant effect ($p < .001$) on susceptibility of bananas to crown rot disease (Table 1). The level of fruit susceptibility decreased with increasing So-Si ratio (Figure 3). The highest INS average value of 524.0 mm^2 was obtained with the lower So-Si ratio (1L/8H, So-Si ratio = 0.08) and the lowest value of 246.2 mm^2 was obtained with the higher So-Si ratio (12L/1H, So-Si ratio = 8; Table 2). Samples of these extreme treatments (12L/1H and 1L/8H) with differential susceptibilities to crown rot were then selected for chromatographic analyses.

The severe So-Si ratio modifications, which occurred during flowering, had a very significant effect ($p < .001$) on grade and length of fruits distinguishing three statistically different groups amongst treatments (Table 3). Grade and fruit length increased with increasing So-Si ratio.

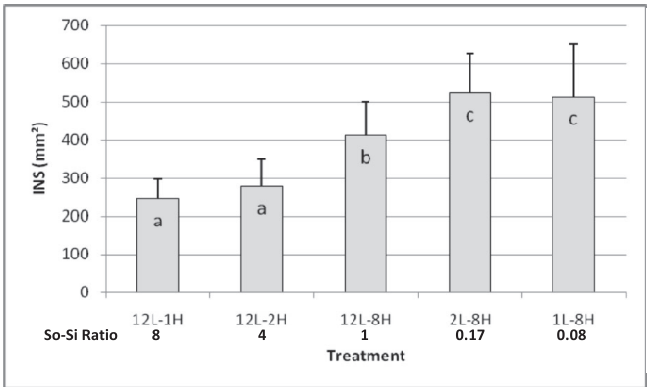


FIGURE 3 Effect of source-sink ratio modifications on the susceptibility of bananas to crown rot disease. The area of the internal necrotic surfaces (INS) were evaluated at 13 days postinoculation (13 dpi). Each bar represents the average of 15 replicates and *SD* is shown. L, leaves; H, hand

TABLE 2 Susceptibility level of banana crown samples used for chromatographic analysis

	INS for GC-MS (mm^2)		INS for HPLC & LC-MS (mm^2)	
Sample	S−	S+	S−	S+
1	294	513	252	646
2	176	494	264	620
3	252	560	231	576

Note: Samples for GC-MS analyses, and for HPLC and LC-MS analyses were collected in two different replicate experiments.

Abbreviations: INS, internal necrotic surface; S−, low susceptibility; S+, high susceptibility.

3.2 | Phenolic constituents of banana with differential susceptibility to crown rot disease

3.2.1 | Analysis of standard products

The HPLC method enabled a good separation of the 20 phenolic standards (see SolC t_R in Table 4, which corresponds to the t_R of the pure compounds), except for catecholamines that eluted in the same peak and could not be separated. Our LC-MS conditions allowed the

TABLE 1 Analysis of variance of the effect of modification of source-sink ratio on susceptibility of banana to crown rot disease

		INS[a]		Grade[b]		Length[c]	
Source	*df*	*F*	*p*	*F*	*p*	*F*	*p*
Treatment	4	24.25	<.001	121.66	.001	121.20	<.001
Week	2	8.88	.001	1.99	.151	1.29	.288
Treatment × week	8	2.36	.035	0.33	.948	2.38	.034
Bunch (treatment)	20	1.77	.062	1.10	.383	1.60	.104

[a]Internal necrotic surface (mm^2).

[b]Fruit grade (diameter in mm).

[c]Length of fruit (cm).

TABLE 3 Mean values and standard deviations of some tree and fruit characteristics: fruit grade (diameter), fruit length, and number of leaves at harvest (NLH), for various source-sink ratio modifications

Treatment	Source-sink ratio	Grade (mm)	Length (cm)	NLH
12L/1H	8.00	39.0 ± 1.5 a	33.1 ± 1.4 a	6.9
12L/2H	4.00	38.3 ± 1.5 a	32.3 ± 1.4 a	6.7
12L/8H	1.00	34.8 ± 1.2 b	29.5 ± 1.1 b	6.2
2L/8H	0.17	30.1 ± 1.3 c	25.3 ± 0.7 c	2.0
1L/8H	0.08	29.5 ± 1.8 c	24.7 ± 1.4 c	1.0

Note: Means are the result obtained after 3 weeks here represented with standard deviation in the table. The different letters represent groups showing statistically significant differences.
Abbreviations: H, hands or clusters of fruit; L, leaves.

detection and confirmed the identification of only 16 of these standards not submitted to acid hydrolysis; the *m/z* of the base peak of the spectra, which correspond to the $[M-H]^-$ ion are presented in Table 4 (SolC $[M-H]^-$). The t_R of standards identified with HPLC was no different from that obtained with LC-MS. Under the conditions used for extraction of the bound fraction of phenolic compounds (acid hydrolytic conditions, AHC), some of the standards gave one chromatographic peak at the same t_R, as found with extraction conditions used for the soluble phenolics (Table 4). It was assumed that the hydrolytic conditions did not affect these molecules (dopamine, epinephrine, norepinephrine, normetanephrine, quercetin, naringenin, and kaempferol). However, such extraction conditions modified the chemical composition of other compounds like gallic acid, catechin, caffeic acid, coumaric acid, ferulic acid, sinapic acid, naringin, and cinnamic acid, for which two peaks were observed after they had been subjected to hydrolytic conditions. One peak corresponded to the unmodified standard (almost the same t_R) and the other to an acid extraction by-product (Table 4). For chlorogenic acid, in addition to the unmodified molecule, two supplementary peaks were detected.

The GC-MS method allowed a good separation, identification, and quantification of the four catecholamines under study (dopamine, epinephrine, norepinephrine, and normetanephrine; Figure 4).

3.2.2 | Analysis of free soluble fraction compounds

For the extracts of soluble phenolics (ESP), HPLC profiles were very similar for both treatments (S+ and S−) and both stages (dhbi and 13 dpi). Only one major compound was eluted, with approximately the same amount for all treatments, after 2.5 min (peak 1). It was identified as catecholamine by comparison of its t_R, UV spectrum and major *m/z* (152) observed in ESI-MS (negative mode) with those obtained from the pure catecholamine standard. However, in the chromatographic profile of the ESP, this catecholamine peak coeluted with ascorbic acid (Figure 5). Due to poor resolution, catecholamine was not quantified with this method.

The catecholamine presence in the methanolic extracts of crowns was clearly confirmed through GC-MS analysis on the basis of the t_R and mass spectra of their standards (Figure 4). For both stages studied (dhbi and 13 dpi), dopamine content was not significantly ($p > .05$) different in the S− (12L/1H) and S+ (1L/8H) crown samples (Table 5). Norepinephrine and normetanephrine contents significantly ($p < .05$) differed between the S− and S+ banana crowns at harvest stage (dhbi); their average amount was six and two times higher, respectively, in the S+ banana crowns than in the S− crowns (Table 5). Their concentration decreased strongly in banana crowns analysed 13 dpi. They were detected in very low amounts at unquantifiable concentrations. Epinephrine was not detected in banana crowns. Octopamine, another derivative of tyrosine, and other polar metabolites such as phenolic acids, carbohydrates (polysaccharides), free amino acids, fatty acids, phytosterols and derivatives were also identified in the crown extracts (not shown).

3.2.3 | Analysis of the bound fraction of phenolic compounds

HPLC chromatograms obtained from the extracts of the bound phenolics (EBP) of the S− and S+ treatments at both stages (dhbi and 13 dpi) are shown in Figure 6. These chromatographic profiles differ quantitatively, but not qualitatively. Ten major peaks, all common to both treatments and both stages, were detected (peaks 1–10 in Figure 6).

Peak 1 was the major compound detected and was identified as catecholamine, as for the ESP, by its t_R, UV spectrum, major *m/z* (152) observed in ESI-MS, and GC-MS analysis. The UV spectra from compounds of peaks 2–10 are shown in Figure 7. Compounds 2–4 had maxima in their spectrum at 280 nm and compounds 5–10 close to 310–330 nm. Peaks 2–4 were quantified in dopamine equivalents (λ_{max} 280 nm) while peaks 5–10 in ferulic acid equivalents (λ_{max} 330 nm). Vanillin and rutin were detected in traces, as well as traces of many other phenolic and derivatives (not shown).

The UV/visible spectra of peaks 2 (t_R 16 min) and 3 (t_R 27 min) were closest to the those of cinnamic acid amongst the 20 phenolic standards but did not correspond exactly to any of them (Figure 7) and had very different retention times from cinnamic acid (51 and 69 min under hydrolytic conditions). In addition, the spectrum of peak 4 was closest to that of gallic acid. However, this peak had a retention time (29 min) very different from gallic acid (12 and 26 min). The base peak obtained for the ESI-MS spectra (negative mode) of peaks 2, 3, and 4 were respectively ions of *m/z* 531, 545, and 143 (Table 6). Peak 2 is not a distinct peak but a large one, which seems to be constituted by more than one product. The compounds in peaks 2, 3, and 4 could not be assigned to any of the standards used here (Figure 7, Table 6).

Peaks 5 and 9 had the same t_R, UV spectra, and base peaks in ESI-MS (*m/z* 163 and *m/z* 177, attributed to $[M-H]^-$) to those of coumaric acid standard and its acid extraction by-product (coumaric acid methyl ester), respectively. Coumaric acid methyl ester probably resulted from the methylation of the acid by the methanol used for the extraction in the presence of HCl. Therefore, peaks 5 and 9

TABLE 4 HPLC retention time (t_R) and LC-MS spectral information of phenolic standards separated on the Inertsil ODS 2 column under soluble conditions (SolC) and after acid hydrolytic conditions (AHC)

				HPLC analysis		LC-MS analysis	
No.	Standard	Formula	Molar mass	SolC t_R (min)	AHC t_R (min)	SolC $[M-H]^-$	AHC $[M-H]^-$
1	Dopamine[a]	$C_8H_{11}NO_2$	153.08	2.5	2.5	151.8	151.9
2	Norepinephrine[a]	$C_8H_{11}NO_2$	153.08	2.5	2.5	ND	ND
3	Epinephrine[a]	$C_9H_{13}NO_3$	183.09	2.5	2.5	ND	ND
4	Normetanephrine[a]	$C_9H_{13}NO_3$	183.09	2.5	2.5	ND	ND
5	Ascorbic acid	$C_6H_8O_6$	176.03	4.1–5.7	ND	174.7	ND
6	Gallic acid	$C_7H_6O_5$	170.02	11.5	11.8, 25.8	168.9	168.9, 182.7
7	Catechin	$C_{15}H_{14}O_6$	290.08	27.0	27.0, 45.8	288.9	288.9, 427.0
8	Chlorogenic acid	$C_{16}H_{18}O_9$	354.10	28.1	28.6, 35.7, 41.6	352.9	ND, 366.9, ND
9	Caffeic acid	$C_9H_8O_4$	180.04	28.5	28.8, 43.5	178.9	179, 192.7
10	Coumaric acid	$C_9H_8O_3$	164.05	34.2	33.5, 51.1	162.9	ND, 176.7
11	Ferulic acid	$C_{10}H_{10}O_4$	194.06	36.7	36.7, 52.9	192.9	192.5, 377.9
12	Sinapic acid	$C_{11}H_{12}O_5$	224.07	37.2	37.2, 52.4	222.8	222.6, 236.6
13	Rutin	$C_{27}H_{30}O_{16}$	610.15	38.7	51.2	609.1	ND
14	Naringin	$C_{27}H_{32}O_{14}$	580.18	41.8	42.3, 55.1	579.2	270.9, 433.0
15	Hesperidin	$C_{28}H_{34}O_{15}$	610.19	42.8	57.8	609.1	ND
16	Phloridzin	$C_{21}H_{24}O_{10}$	436.14	44.5	58.5	435.0	ND
17	Quercetin	$C_{15}H_{10}O_7$	302.04	50.7	50.7	300.9	ND
18	Cinnamic acid	$C_9H_8O_2$	148.05	51.2	51.4, 69.1	ND	ND, ND
19	Naringenin	$C_{15}H_{12}O_5$	272.07	55.3	55.3	271.0	ND
20	Kaempferol	$C_{15}H_{10}O_6$	286.05	57.5	57.3	284.9	285.0

Abbreviation: ND, not detected.

[a]Catecholamine.

were identified as coumaric acid and its methyl ester, respectively (Figure 7, Table 6).

Similarly, peaks 6 and 10 had the same t_R (36.9 and 52.9 min), UV spectra, and base peaks in ESI-MS (*m/z* 193 and *m/z* 378, attributed to $[M-H]^-$) as the ferulic acid standard and its acid extraction by-product, respectively. Therefore, peaks 6 and 10 were identified by HPLC, UV, and LC-MS as ferulic acid and its acid extraction by-product, respectively (Figure 7, Table 6).

Peaks 7 (t_R 41.0 min) and 8 (t_R 41.7 min) had the same UV spectrum as chlorogenic acid. However, the retention times of these two peaks, as well as the results of the LC-MS analysis, could not corroborate the nature of these products (Figure 7, Table 6).

Overall, most phenolic compounds had higher contents in S− crowns than in S+ crowns, and their content was also higher in crowns at harvest stage (dhbi) than at 13 dpi (Table 7).

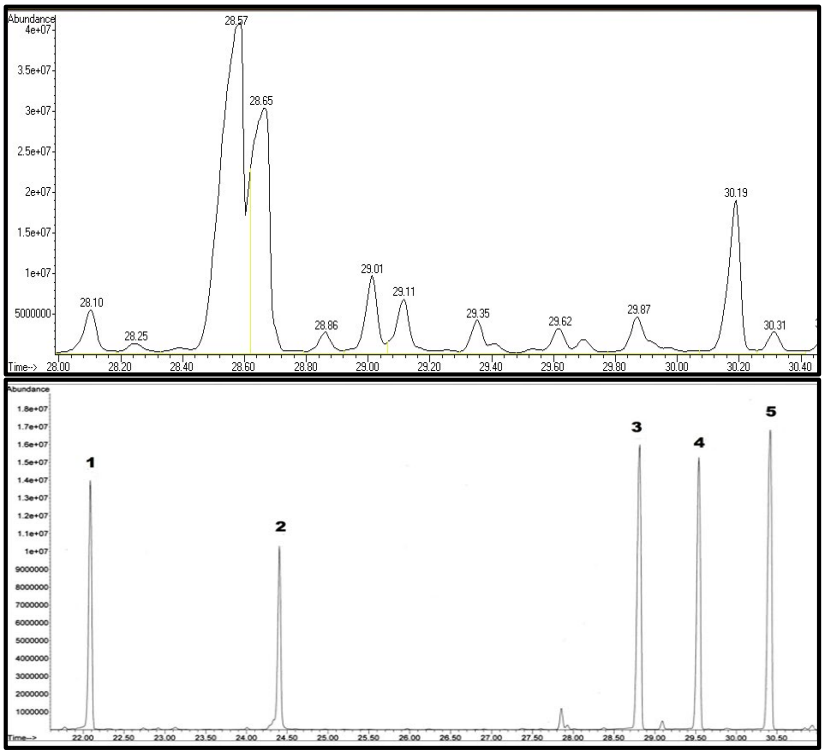
The product present in peak 2 was the second most important compound in the EBP after peak 1 (catecholamines). Its amount was significantly higher ($p = .014$) in the S− crowns than the S+ crowns on the day of harvest (dhbi): 153.8 and 15.6 µg dopamine equivalents per g FW respectively. Its level significantly decreased ($p = .044$) at 13 dpi to 36.4 and 4.3 µg dopamine equivalents per g FW for S− and S+ crowns, respectively (Table 7).

For samples from dhbi, the amount of phenolic compounds accumulated in the S− crown samples as compared to the S+ ones were significantly higher ($p < .05$) in peak 3 and peak 5 (coumaric acid), and more significantly higher ($p < .001$) in peak 6 (ferulic acid), peak 10 (ferulic acid extraction by-product), peak 7, and peak 8. For peak 4 and peak 9 (methyl ester of coumaric acid), no significant difference was noticed between S− and the S+ crown samples (Table 7).

At 13 dpi, the amount of phenolic compound was significantly lower ($p < .05$) than at the dhbi stage for peak 6 (ferulic acid), peak 10 (ferulic acid extraction by-product), peak 7, and peak 8. This decrease was more important for S+ crown samples. For peak 3, the decrease was most pronounced in the S− crown samples (Table 7). For peak 5 (coumaric acid), peak 4, and peak 9 (methyl ester of coumaric acid) there was no significant difference ($p > .05$) between either stage (dhbi and 13 dpi).

4 | DISCUSSION

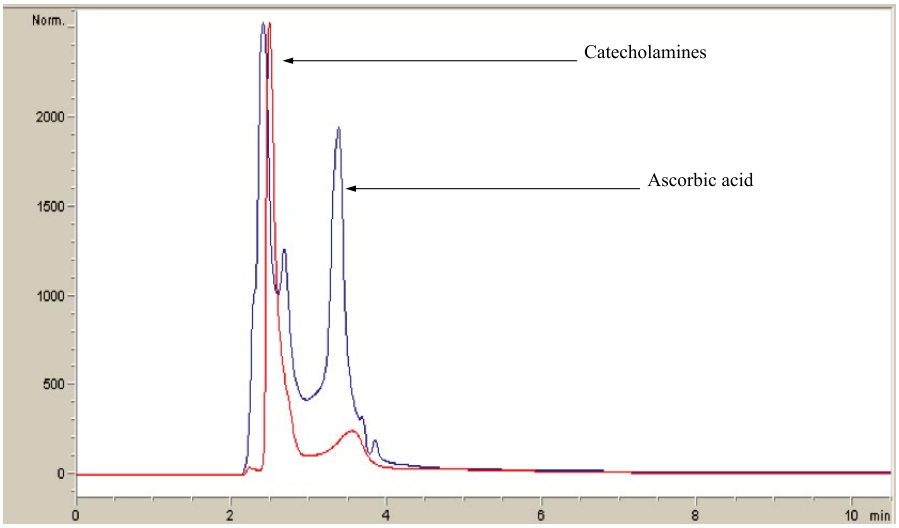
The severe modification of So-Si ratio was found to influence the susceptibility of fruits to crown rot disease. Banana susceptibility to



Peak N°	1	2	3	4	5
R_T (min)	22.09	24.40	28.78	29.52	30.39
m/z ( )	307; 319	174; 294; 355	174; 338; 426	174; 355; 514	174; 297; 456

FIGURE 4 GC-MS profiles of banana crown extract between 28 and 30.5 min retention time (a) and catecholamines standards from Sigma from 22 to 30.5 min (b). 1, Ribitol (internal standard); 2, epinephrine; 3, dopamine; 4, normetanephrine; 5, norepinephrine

FIGURE 5 HPLC chromatogram at 280 nm of soluble methanolic fraction of banana crown between 0 and 10 min retention time. Chromatograms of phenolic compounds present in the susceptible banana crown sample (S+, blue) are superimposed on those from the less susceptible banana crown sample (S−, red). Identities of the known peaks are indicated



this postharvest disease increased when the So-Si ratio was severely decreased. Our results are in accordance with those of Lassois *et al.* (2010b) that revealed a similar effect of So-Si ratio modifications. However, in our study we explored a broader range of So-Si ratio modifications, from 12L/1H (So-Si = 8) to 1L/8H (So-Si = 0.08), than former studies (12L/2H, So-Si = 4 to 5L/8H, So-Si = 0.4). In such extreme conditions, the differences between susceptible (S+) and less susceptible (S−) fruits were more pronounced and sufficient to allow a differential study of phenolics. As expected, the removal of some hands at flowering reduced fruit susceptibility (Lassois *et al.*, 2010b). In the same way, severe defoliations led to an increase of fruit susceptibility. In a previous study, we showed that black leaf streak disease (BLSD) increased banana susceptibility to crown rot disease (Ewané *et al*, 2013). BLSD is a foliar disease that might cause severe defoliation levels (comparable to treatments 2L/8H and 1L/8H; So-Si ratio 0.17 and 0.08, respectively) in commercial banana

farms and would then lead to an important increase of fruit susceptibility to crown rot. Our results in the present study support the fact that, besides already reported impacts of BLSD on yield and fruit conservation (greenlife), this foliar disease might also affect other fruit quality traits.

Lassois *et al.* (2010b, 2011) hypothesized that modifications in So-Si ratio lead to a change in partitioning of assimilates between various sinks, which would influence the formation of secondary metabolites involved in a plant–pathogen interaction. Our results support such a hypothesis because there were significantly higher amounts of phenolic compounds in less susceptible fruits (S−). This is the first study dedicated to the evaluation of phenolic compounds in banana crowns and to their possible involvement as a plant defence mechanism to crown rot disease.

Among the 10 phenolic compounds identified in the EBP from crown tissues, only five (corresponding to peaks 1, 5, 6, 9, and 10) could be identified: catecholamines (peak 1), coumaric acid (peaks 5 and 9), and ferulic acid (peaks 6 and 10). Coumaric acid and ferulic acid might play a significant role in lower crown rot susceptibility because their amounts were significantly higher in S− fruits at harvest stage and their content decreased significantly in S− fruits 13 days after infection. Indeed, such compounds and their conjugates have been previously identified in banana tissues, recently in banana peel (Manthey and Jaitrong, 2016), and have been related to defence mechanisms (Valette *et al.*, 1998; Kanazawa and Sakakibara, 2000; de Ascensao and Dubery, 2003; Wuyts *et al.*, 2007). Ferulic acid and its esters can be toxic for root nematodes (Wuyts *et al.*, 2006) or bound to cell walls for their protection against enzymatic attacks (Wuyts *et al.*, 2007). However, here, the amount of ferulic acid was more important in the banana crown samples than in the root samples. Coumaric acid and its methyl ester possess antifungal properties and increase after infection, being associated with phytoalexin-like behaviour in many plants (Daayf *et al.*, 1997; Li *et al.*, 2018; Yuan *et al.*, 2019).

Compounds 2, 3, 4, 7, and 8 remain unknown. Compound 4 is likely to have an insignificant role in this pathosystem because its variation is poorly linked with fruit susceptibility. However, compounds 2, 3, 7, and 8 probably play an important role in banana crown defence and should be further extracted, purified, and completely characterized by MS and nuclear magnetic resonance techniques. In this regard, compound 2 would require particular attention because it is at very high levels in the crowns of less susceptible fruits (S−) compared with susceptible fruits (S+) and could be a potential marker for crown rot susceptibility.

There are good reasons to speculate that all phenolic compounds in banana crowns may create a toxic environment for fungal invasion and growth, notably in the S− crown samples. Indeed, the hydroxycinnamic acids and their derivatives (*p*-coumaric, ferulic) serve as precursors for a wide variety of chemically diverse phenylpropanoid derivatives with antimicrobial functions, such as the plant-specific phytoalexin hydroxycinnamic acid amides (HCAA) and flavonoids (Dixon *et al.*, 2002; Facchini *et al.*, 2002). Thus, the phenolic content of the banana crown could be part of the biochemical response mechanism to infection and may be involved in the variation of banana susceptibility. With LC-MS conditions used in the present study, it was not possible to identify all the phenolics involved in banana susceptibility to crown rot or test their fungicidal activity in vitro. Consequently, we cannot speculate on their particular antifungal properties.

The amount of most phenolic compounds decreased significantly from harvest (dhbi) to 13 dpi, especially in the S+ crown. Possible maturation effects could explain this decrease because previous authors have mentioned that the amount of phenolics decreases during fruit ripening (Kanazawa and Sakakibara, 2000). Given that all phenolic compounds were present in uninfected banana crowns (dhbi) and that their amount decreased after inoculation, they should be considered as constitutive compounds and not induced. However, the evaluation of the synthesis of induced compounds 13 days after inoculation is probably too late for the assessment of biochemical events occurring in banana crown tissues in the first hours and days after inoculation and the establishment of infections.

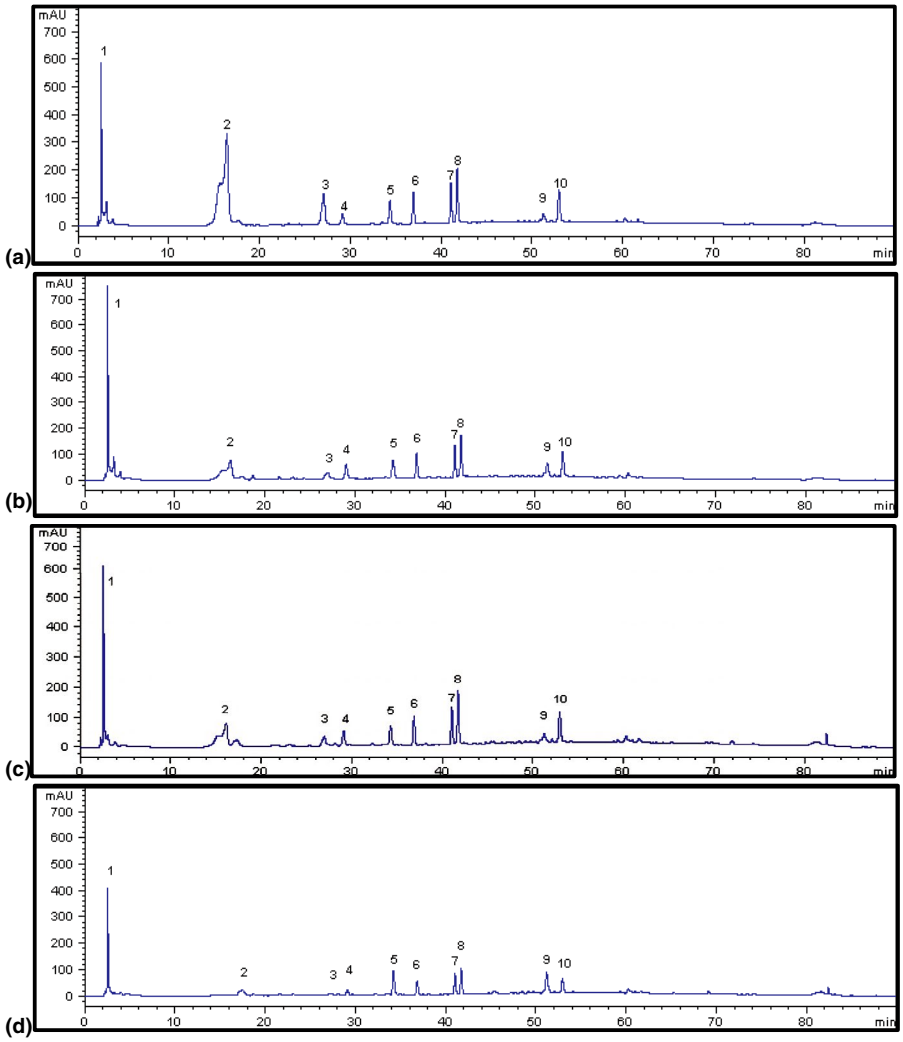
Catecholamines were found in the less susceptible (S−) as well as in the susceptible (S+) banana crowns. GC-MS enabled them to be identified as dopamine and its derivatives (norepinephrine and normetanephrine), while HPLC detected the presence of catecholamines in banana crowns without specific identification of the different compounds. The amounts of dopamine were similar in S− and S+ fruits, while norepinephrine and normetanephrine contents were higher in susceptible fruits. Our result seems contradictory to a previous study where the overexpression of the dopamine-β-monooxygenase gene was highlighted in bananas less susceptible (S−) to crown rot (Lassois *et al.*, 2011). However, another study has shown that the dopamine level did not differ between healthy and infected banana roots of resistant and susceptible cultivars (Wuyts *et al.*, 2007). This reveals the degree of complexity of the regulation mechanisms for

TABLE 5 GC-MS analysis of catecholamine contents for susceptible (S+) and less susceptible (S−) banana crowns at two sampling stages

Catecholamine	Treatment	S− (mg/g crown FW)	S+ (mg/g crown FW)
Dopamine	dhbi	21.23 ± 0.68 aA	19.76 ± 0.29 aA
	13 dpi	20.46 ± 0.28 aA	17.91 ± 2.00 aA
Norepinephrine	dhbi	1.03 ± 0.53 a	6.34 ± 1.35 b
	13 dpi	–	–
Normetanephrine	dhbi	1.73 ± 0.09 a	3.22 ± 0.58 b
	13 dpi	–	–

Note: Means are the result of three replicates ± *SD*. Values followed by the same letter, lower case for treatment (S+/S−) or upper case for sampling stages (dhbi/13 dpi), represent groups showing no statistically significant differences ($p < 0.05$).

Abbreviation: dhbi, day of harvest before inoculation; dpi, days postinoculation; FW, fresh weight.



Peak N°	1	2	3	4	5	6	7	8	9	10
R_T (min)	2.5	16.0	27.0	29.1	34.3	36.9	41.0	41.7	51.2	52.9

FIGURE 6 HPLC chromatogram at 280 nm of the bound methanolic fraction of banana crown for the different treatments (susceptible/less susceptible [S+/S−]) and sampling stages (day of harvest before inoculation/13 days postinoculation [dhbi/13 dpi]). (a) S−/dhbi, (b) S+/dhbi, (c) S−/13 dpi, and (d) S+/13 dpi. Numbers over the peak indicate: 1, catecholamines; 5, coumaric acid; 6, ferulic acid; 9, coumaric acid methyl ester; 10, ferulic acid HCl extraction by-product; 2, 3, 4, 7, and 8 indicate unknown phenolics

dopamine and its derivatives. Nevertheless, dopamine and its derivatives seem to be involved in the banana crown defence mechanism. Indeed, dopamine is an intermediate in the biosynthesis of benzylisoquinoline alkaloids (Trenchard *et al.*, 2015) that can also form conjugates with phenolic acids like *p*-coumaryladrenaline, implicated in plant defence (Roepenack-Lahaye *et al.*, 2003).

To conclude, the influence of severe So-Si ratio modifications on banana susceptibility to crown rot has been demonstrated, as well as the differential accumulation of phenolics in crown tissues. This is the first study dedicated to the evaluation of phenolic compounds in banana crowns and their possible involvement as a plant defence mechanism against crown rot disease; it is also the first report of catecholamine content in banana crowns. The results suggest an effect of So-Si ratio modifications on banana fruit quality potential in terms of phenolic accumulation, and present a useful implication that phenolics may be involved in the biochemical basis of variation in banana susceptibility to crown rot. Thus, this study represents a starting point for a better understanding of the function(s) of these intriguing molecules in the banana crown's defence mechanisms.

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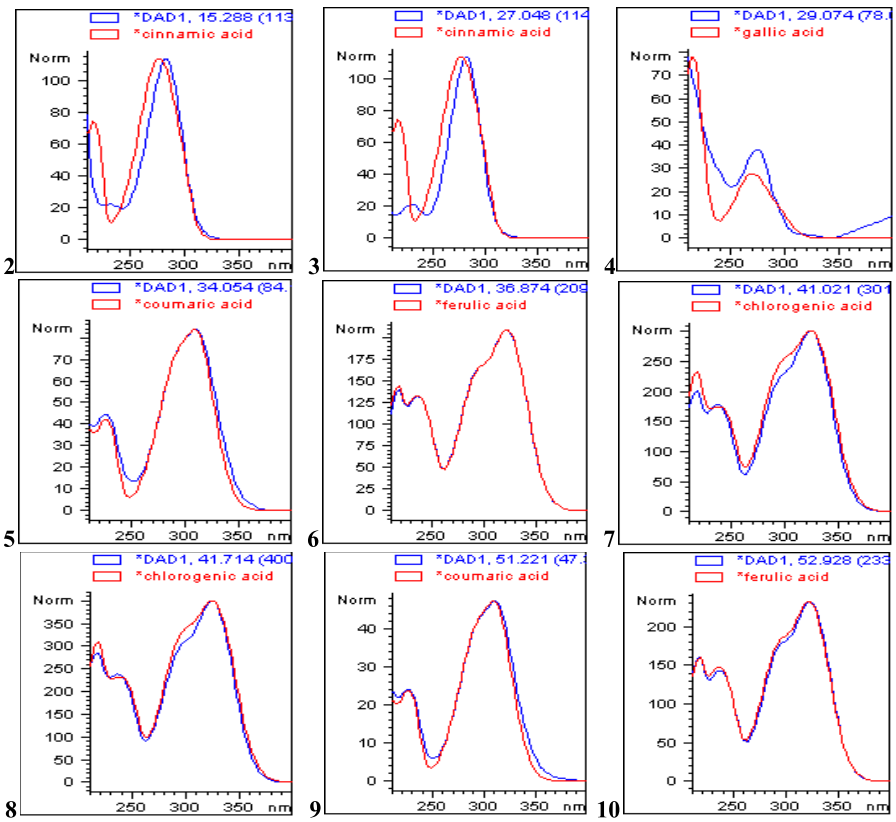

FIGURE 7 UV spectra of HPLC peaks 2–10. DAD1, diode array detector 1. Absorbance (A) = f(λ) obtained during identification of peaks. Spectra of phenolic compounds present in the banana crown sample (blue) are superimposed on those of the corresponding phenolic standard (red) measured under the same experimental conditions

TABLE 6 HPLC/LC-MS analysis data of phenolic compounds in banana crown extracts, according to their UV absorption spectra, retention time (t_R), and their negative ionization mass spectra

Peak	λ_{max} (nm)	t_R (min)	HPLC compound	$[M-H]^-$	LC-MS compound
1	280	2.5	Catecholamine	151.6	Dopamine $[M-H]^-$
2	280	16.0	Unknown	530.7	Unknown $[M-H]^-$
3	280	27.0	Unknown	544.8	Unknown $[M-H]^-$
4	280	29.1	Unknown	142.5	Unknown $[M-H]^-$
5	330	34.3	Coumaric acid	162.6	Coumaric acid $[M-H]^-$
6	330	36.9	Ferulic acid	192.6	Ferulic acid $[M-H]^-$
7	330	41.0	Chlorogenic acid	416.7	Unknown $[M-H+64]^-$
8	330	41.7	Chlorogenic acid	338.7	Unknown $[M-H-14]^-$
9	330	51.2	Coumaric acid ME	176.6	Coumaric acid by-product
10	330	52.9	Ferulic acid Eb	377.6	Ferulic acid Eb

Abbreviations: Eb, extraction by-product; ME, methyl ester.

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TABLE 7 Phenolic contents of the extracts of bound phenolics (EBP) for different susceptibility levels and sampling stages of banana crown

Peak	Compound	Stage	Less susceptible crown (S−)	Susceptible crown (S+)	*p*	
					Treatment	Stage
2	Unknown	dhbi	152.80 ± 0.66 ax	15.64 ± 0.03 bx	.014	.044
		13 dpi	36.42 ± 0.15 ay	4.30 ± 0.01 by		
3	Unknown	dhbi	86.00 ± 0.99 ax	3.63 ± 0.00 bx	.032	.051
		13 dpi	13.11 ± 0.11 ay	0.79 ± 0.00 bx		
4	Unknown	dhbi	5.60 ± 0.03 ax	5.89 ± 0.01 ax	.144	.092
		13 dpi	5.56 ± 0.03 ax	1.81 ± 0.01 ax		
5	Coumaric acid	dhbi	11.86 ± 0.05 ax	7.26 ± 0.02 bx	.008	.057
		13 dpi	8.73 ± 0.05 ax	5.56 ± 0.02 bx		
6	Ferulic acid	dhbi	23.15 ± 0.02 ax	12.23 ± 0.02 bx	<.001	<.001
		13 dpi	16.51 ± 0.02 ay	5.46 ± 0.01 by		
7	Unknown	dhbi	27.82 ± 0.08 ax	15.85 ± 0.01 bx	<.001	.008
		13 dpi	20.92 ± 0.05 ay	7.42 ± 0.02 by		
8	Unknown	dhbi	35.89 ± 0.11 ax	20.17 ± 0.02 bx	<.001	.008
		13 dpi	26.87 ± 0.07 ay	9.28 ± 0.02 by		
9	Coumaric acid ME	dhbi	7.43 ± 0.01 ax	7.31 ± 0.01 ax	.843	.358
		13 dpi	8.09 ± 0.01 ax	7.94 ± 0.02 ax		
10	Ferulic acid Eb	dhbi	38.32 ± 0.10 ax	20.15 ± 0.07 bx	<.001	.001
		13 dpi	28.74 ± 0.09 ay	9.38 ± 0.03 by		

Note: Peak 6 is ferulate and expressed in μg/g crown FW. Peaks 2, 3, and 4 are expressed in μg of dopamine equivalents/g FW while peaks 5, 7, 8, 9, and 10 are expressed in μg of ferulate equivalents/g FW. *p* is the probability obtained with analysis of variance. Means are the result of three replicates ± *SD*. The different letters a and b for treatment (S+/S−), x and y for sampling stages (dhbi/13 dpi) represent groups showing statistically significant differences.

Abbreviation: ME, methyl ester; Eb, extraction by-product; dhbi, day of harvest before inoculation; dpi, days postinoculation; FW, fresh weight.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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