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Original article

Coumaroyl-isocitric and caffeoyl-isocitric acids as markers of pineapple fruitlet core rot disease

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Summary

Introduction – **Fruitlet core rot is the major postharvest disease affecting 'Queen' pineapple in La Réunion island. The symptoms are black spots located in the pineapple fruitlets.** *Materials and methods* – ***Fusarium ananatum*, the main causal agent of fruitlet core rot was inoculated in 'MD-2' and 'Queen' (Victoria), a tolerant and a susceptible cultivar, respectively. A metabolomics approach to healthy and inoculated fruitlets allowed to determine which putative metabolites are involved in fruitlet core rot disease. The evolution of fruitlet core rot was then followed-up with a focus on the phenolic compounds.** *Results and discussion* – **Based on the metabolomics results, the phenolic compounds seemed to be determining markers of black spots. Coumaroylisocitrate and caffeoylisocitrate dramatically increased in the infected fruitlets in both cultivars post-inoculation. The 'MD-2'-infected fruitlets reached higher hydroxycinnamic acid levels in a shorter time than those of the 'Queen'-infected fruitlets. In healthy fruits of 'MD-2', coumaroyl-isocitric acid and hydroxybenzoic acids were naturally produced as the fruit mature.** *Conclusion* – **These phenolic compounds play a major role in the pineapple disease resistance.**

Keywords
Indian Ocean, pineapple, *Ananas comosus*, *Fusarium ananatum*, phenolic compounds, metabolomics, postharvest management

Significance of this study

What is already known on this subject?

- Fruitlet core rot is the main postharvest disease of pineapple. The internal symptoms are black spots where polyphenol oxidase and laccase exhibit high activity in cv. Smooth Cayenne.

What are the new findings?

- Coumaroyl-isocitric acid and hydroxybenzoic acids accumulate respectively 5 and 4 times more in the tolerant cultivar than in the susceptible cultivar during natural ripening.
- Coumaroyl-isocitric and caffeoyl-isocitric acids are highly synthetized in both tolerant and susceptible pineapple cultivars in response to *Fusarium ananatum* inoculation.

What is the expected impact on horticulture?

- To enhance levels in phenolic compounds in the susceptible cv. Queen in order to contain fruitlet core rot disease.

Introduction

The 'Queen' pineapple cultivar (*Ananas comosus* var. *comosus*) is an emblematic product of the French Reunion island, located in the Indian Ocean. Well-known as a sweet and tasty fresh fruit, it is susceptible to disease and pests that affect crop yield and fruit quality.

The major postharvest disease affecting the 'Queen' pineapple is fruitlet core rot (FCR) disease caused predominantly by *Fusarium ananatum*, as observed in South Africa (Fournier *et al.*, 2015; Jacobs *et al.*, 2010). The fungus *Talaromyces stollii*, previously described as *Penicillium funiculosum*, can cause the same symptoms (Edmonstone-Sammons, 1955; Lim and Rohrbach, 1980). The fungal pathogen penetrates the plant at flowering stage through the stylar canals and nectary ducts, and remains latent until the fruit ripens (Rohrbach and Pfeiffer, 1976). Fruitlet core rot is often not detected until the fruit is cut open (Oxenham, 1953). Internal symptoms consist of a browning of the center of the fruitlet beginning immediately below the floral cavity and, in severe cases, extending to the core of 'Queen' pineapple cultivar (Tryon, 1898).

On the other hand, some cultivars exhibit no symptoms of FCR, like 'MD-2'. This pineapple is a hybrid produced by the Pineapple Research Institute of Hawaii, now the world's pre-eminent fresh fruit cultivar (Bartholomew *et al.*, 2012). Its success is partly due to its tolerance to biotic and abiotic stress. In the case of chilling injury, Raimbault *et al.* (2011) explained the absence of browning symptoms in the flesh of the fruit by inactivity of polyphenol oxidase (PPO) genes. 'Flhoran 53', a cultivar rarely affected by FCR, has been created by the CIRAD pineapple breeding program in the 1990s (Horry *et al.*, 2007).

Improved knowledge of the pathogen and the plant interaction in tolerant and susceptible cultivars will allow the

development of novel approaches to enhance the resistance in the susceptible pineapple. Plant defense mechanisms have been intensively studied but information concerning fruitlet core rot is scarce. Avallone *et al.* (2003) reported a higher activity of PPO in infected fruitlets of the 'Smooth Cayenne'. This suggests the role of secondary metabolites in response to the fungus infection.

Metabolomics offers the possibility to monitor changes in secondary metabolism during resistance and disease development. Biochemical approaches have also been proposed to describe mechanisms governing host-pathogen interactions (Dixon and Paiva, 1995; Knepper and Day, 2010; Wojtaszek, 1997).

This study had three specific objectives: (i) to target a family of marker metabolites induced by fruitlet core rot disease; (ii) to monitor their accumulation following an inoculation with the fungal pathogen *Fusarium ananatum* in the tolerant and the susceptible cultivars; and (iii) to compare their eventual accumulation during natural ripening in both cultivars.

Materials and methods

Plant material

This study was conducted in an experimental field of three pineapple cultivars more or less susceptible to fruitlet core rot: the tolerant 'MD-2', intermediate 'Flhoran 53' and susceptible 'Queen' (Barral *et al.*, 2017; Bartholomew *et al.*, 2012; Horry *et al.*, 2007). The pineapples were grown at the CIRAD Research Station in La Réunion island (21°10'S, 55°30'E) according to standard agricultural practices (Fournier, 2011). Fruits were inoculated in November 2014 and January 2015 at a mature green stage corresponding to 80% of their expected harvest date, using the SIMPIÑA pineapple crop model (Dorey *et al.*, 2015). Fruit maturity was determined visually based on shell color. In La Réunion, the outer color of 'Queen' pineapples is closely related to the fruit ripeness stage (Fournier *et al.*, 2015; Soler, 1992). The code G (green shell) defined a fruit at 80% of the expected harvest. C0 corresponded to the basal section with a yellow hue, C2 to a half basal yellow coloration and C4 to a totally yellow fruit (Darnaudery *et al.*, 2016).

Inoculum and inoculations

The *Fusarium ananatum* Clp001 strain used for the inoculations was taken from a collection of fungal pathogens. The fungus was isolated from naturally infected 'Queen' pineapple on Réunion Island and purified in selective media. The *Fusarium*-specific primer elongation factor tef-1α and tef-2α made it possible to identify Clp001 as a *Fusarium ananatum*. The single-spore isolate was grown on potato dextrose agar (PDA) for two weeks at 25 °C. A fungal suspension was made in sterile water and adjusted to 10^3 conidia mL^{-1}. Fruits were immediately inoculated after this step. Twenty-five µL of the conidial suspension were injected into the parenchyma adjacent to the remnant floral cavity. Four fruitlets were inoculated per fruit in order to form the modality, "infected fruitlet". The uninfected side of the fruit corresponded to the "healthy fruitlet". Parallel to fungal inoculation, 25 µL sterile water were also inoculated as a control into separate fruits, referred to as "H_2O fruitlet". Pathogenicity tests were conducted to ensure that the black spots observed were due to the Clp001 strain.

Metabolomics

The "infected fruitlet" and "healthy fruitlet" of the tolerant 'MD-2' and susceptible 'Queen' cultivars were sampled 13 days after inoculation. Since the signs of infection were invisible from the outside, we used a Posca marker to mark the injection site. The frozen dissected fruitlets were ground in

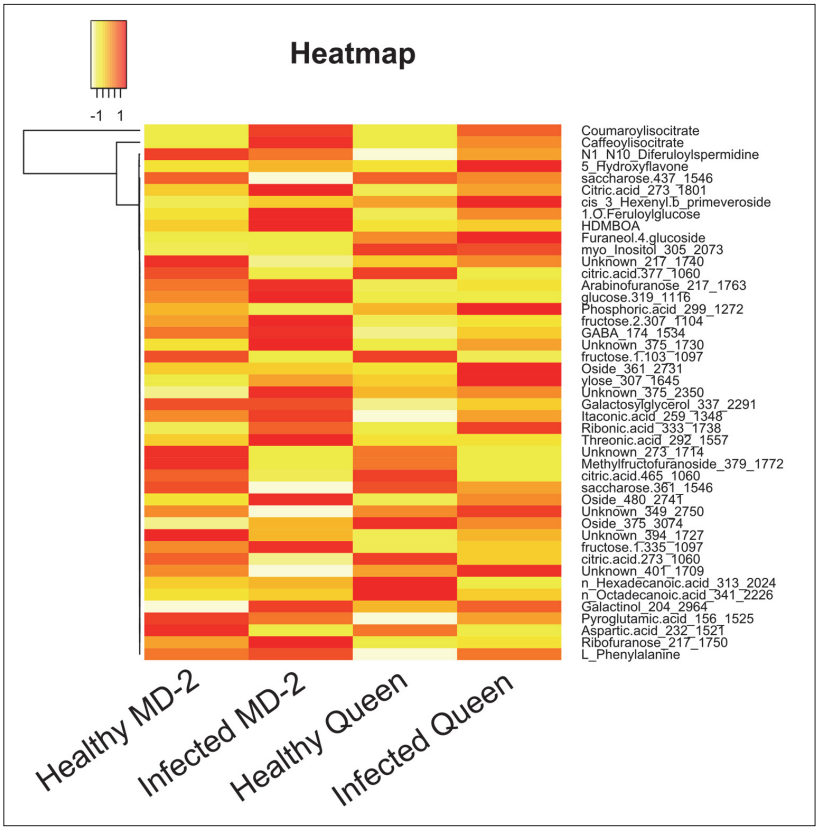

Figure 1. Hierarchical clustering analysis of metabolites in samples obtained from healthy and infected fruitlets of 'MD2' and 'Queen' cultivars. Red highlighting indicates high levels of metabolite and yellow highlighting indicates low levels of metabolite.

a Grindomix blender (Retsch, Haan, Germany). An aliquot of the powder was lyophilized for 72 h at -52 °C. Twenty mg of lyophilized powder were diluted in 600 µL methanol, 150 µL chloroform and 400 µL water. The aqueous fraction was used for GC-EI-MS and UPLC-HRMS analyses. For GC-EI-MS, metabolites were putatively identified based on their spectral similarity and their retention index relative to compounds of the Golm Metabolome Database (GMD). For UPLC-HRMS, putative structures were inferred from MS and MS/MS spectra matched against public databases and previous studies (Ma *et al.*, 2007; Steingass *et al.*, 2015).

Evolution of free phenolic compounds

Free phenolic kinetics were monitored in the "healthy fruitlet", "Infected fruitlet" and "H_2O fruitlet" of the tolerant 'MD-2', intermediate 'Flhoran 53' and susceptible 'Queen' cultivars. Analyses were carried out at 0, 2, 4, 6, 8, 10, 13, 21 and 28 days after inoculation (DAI) for 'MD-2'; 0, 2, 4, 6, 8, 10, 14 and 25 for 'Flhoran 53' cultivars and 0, 2, 4, 6, 8, 10, 13 and 22 DAI for 'Queen'. For each cultivar, three fruits were randomly handpicked at each sampling date. The content of phenolic compounds in "infected fruitlet" and "H_2O fruitlet" are presented according to the number of days after inoculation. Preliminary analyses revealed that the kinetics of phenolic compounds in 'healthy fruitlet' are more coherent according to the degree of ripening than to the number of days after inoculation. Indeed, during the experiment, the fruits matured in a heterogeneous way.

Fruitlet sampling was similar to metabolomics but the extraction differed. Five hundred mg of lyophilized powder were added to 10 mL of an ethanol-water solution (80:20, vol:vol) and 100 µL of methyl 4-hydroxybenzoate (1 g L^{-1}) was additionally added as an internal standard. The solution was sonicated for 10 min at 35 °C and then filtered (Transsonic, TI-H15 MF2, Fisher, Bioblock Scientific). Ultrasound-assisted extraction was repeated after the extract was recovered on the filter. The total solution was concentrated under low pressure and the residual was precisely adjusted to 10 mL in methanol. Ultrasound-assisted extraction was chosen after a preliminary test. The optimal condition for the extraction was determined based on a comparison of different solvents, times, frequencies and temperatures in both stirring and ultrasound extraction. Samples were filtered with a 0.45-µm filter (Millipore) and injected into a high-pressure liquid chromatography (HPLC) column.

Quantification of phenolic acids was performed using commercial standards of *trans*-*p*-coumaric acid, chlorogenic acid, *trans*-sinapic acid and gallic acid purchased from Sigma-Aldrich (France). HPLC analysis was performed using a Dionex Ultimate 300 apparatus (Dionex Co., Sunnyvale, CA, USA) equipped with a diode array detector. The column used was a reverse phase, Waters Symmetry Shield C18 250 × 4.6 mm, 5 µm ID. Peak areas with maximum absorption at 280 nm were converted to µg g^{-1} dry weight of gallic acid equivalent (GAE) using a standard curve prepared with different concentrations of gallic acid. Peak areas at 320 nm of *trans*-*p*-coumaric acid, chlorogenic acid, *trans*-sinapic acid and their derivatives were also converted from their corresponding standards.

Statistical analysis

Statistical analyses were conducted in R statistical software (R Development Core Team, 2015). Analyses of variance (ANOVA) on phenolic acids level between cultivars for the first ten days after inoculation were carried out. Comparisons of means in terms of phenolic acids among the cultivars for each sampling date were made by Tukey's multiple comparison tests.

Results and discussion

Metabolomics

A hierarchical clustering was used to arrange the metabolites on the basis of their relative levels across samples (Figure 1). Metabolites mainly clustered according to inoculation status. The effect of inoculation was also evident within each cultivar. Among the perturbed metabolites, phenolic compounds could be candidates for markers of fruitlet core rot disease. In the infected fruitlets, 28 metabolites are found at higher levels in the 'MD-2' cultivar compared to the 'Queen' cultivar. The dendrogram distinguished two compounds as markers of the disease: *p*-coumaroyl-isocitric acid and caffeoyl-isocitric acid.

Phenolic compounds evolution in healthy fruitlet during ripening

Our results only show an accumulation of *p*-coumaroyl-isocitric acid and hydroxybenzoic acids in healthy fruitlets of 'MD-2' during ripening (Figure 2). Levels of those metabolites started to increase at the C1 maturity stage, when the shell color changed from green to yellow. The level in hydroxybenzoic acids reached 2,231 µg g^{-1} dry weight (DW) at C2 in 'MD-2'. The *p*-coumaroyl-isocitric acid level continued to increase until the C4 stage to reach 345 µg g^{-1} DW. In 'Queen', the highest level of hydroxybenzoic acids was 573 µg g^{-1} DW monitored at C3 maturity. For all of the cultivars, the caffeoyl-isocitric acid level remained close to zero. The 'MD-2' tolerant cultivar accumulated four times more hydroxybenzoic acids and five times more *p*-coumaroyl-isocitric acid in healthy fruitlets than the susceptible 'Queen' cultivar during ripening.

Most of the studies describe a decrease in phenolic acid content during fruit ripening (Burda *et al.*, 1990; Castrejón *et al.*, 2008; Gruz *et al.*, 2011; Lima *et al.*, 2005), whereas our results showed an increase in coumaroyl-isocitric acid and hydroxybenzoic acids. In agreement with our results, McKeehen *et al.* (1999) showed an increase in phenolic compounds on wheat grain during maturation. Because bibliographic data concerning the evolution of phenolic acids during pineapple maturation and ripening are scarce, it is not possible to compare the increase we observed with previous reports at this time.

The accumulation of phenolic compounds was related to maturity in the tolerant variety. In fact, early accumulation in the tolerant cultivar is consistent with the first symptoms in the susceptible cultivar. These compounds are known for their antifungal properties (Aziz *et al.*, 1998; Maher *et al.*, 1994; Zabka and Pavela, 2013). The tolerant cultivar is already armed if the pathogen tries to penetrate the fruitlet. The response to the attack of the fungus is therefore immediate.

Accumulation in hydroxycinnamoyl isocitrates after *Fusarium ananatum*

Our results show a high increase of *p*-coumaroyl and caffeoyl isocitrates following inoculation with *Fusarium ananatum* fungus in infected fruitlets (Figures 3A–B). 'MD-2' accumulated higher levels of coumaroyl-isocitric and caffeoyl-isocitric acids in the infected fruitlets in a shorter time compared to 'Flhoran 53' and 'Queen'. For example,

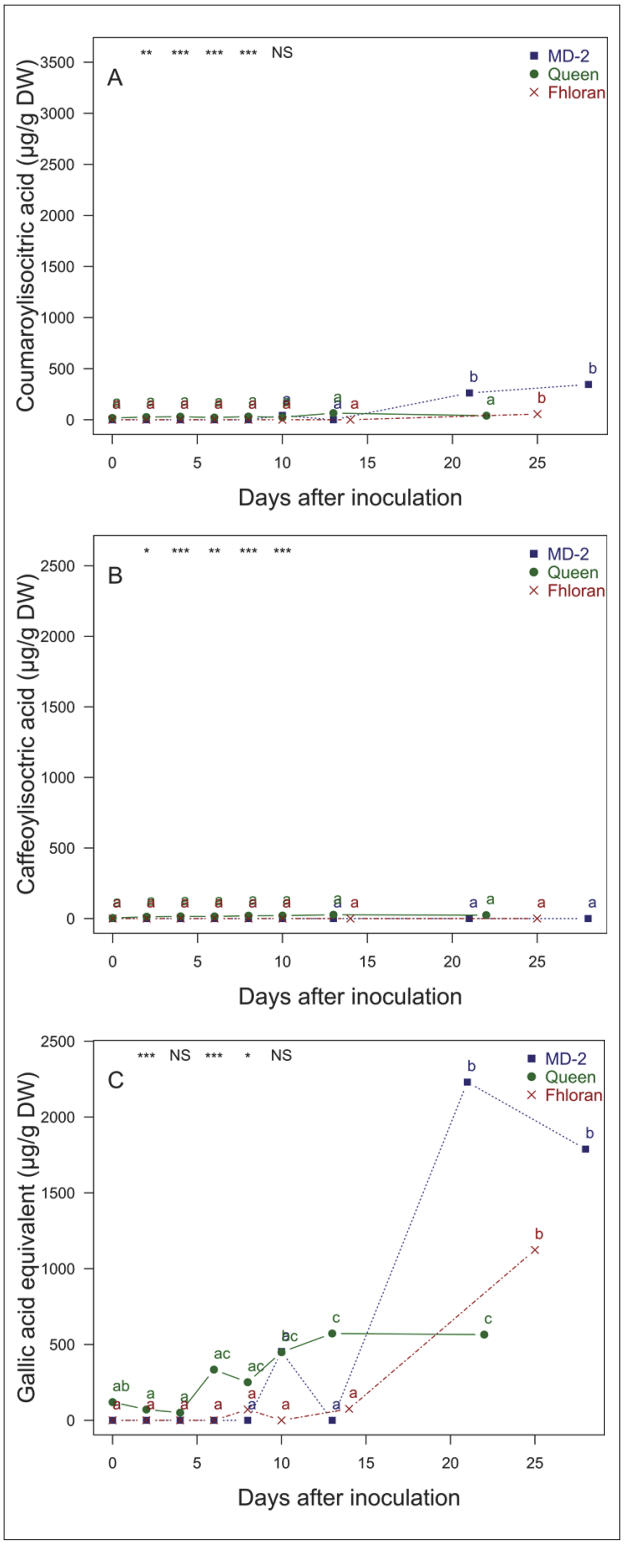

Figure 2. Evolution of *p*-coumaroylisocitrate (A), caffeoyl-isocitrate (B) and hydroxybenzoic acids (C) in healthy fruitlet during fruit ripening starting at green maturity stage (G) to fruit maturity (C4) for the 'MD-2' tolerant cultivar (■), 'Flhoran 53' intermediate cultivar (×) and the 'Queen' susceptible cultivar (•). Data points are mean values of 3 fruits. Differences of phenolic acids level between cultivars were either significant at $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***), or non-significant (NS) for each sampling date. Different letters indicate that data are significantly different at $P<0.05$ between DAI for each cultivar (according to Tukey's multiple comparison test). DW: Dry weight.

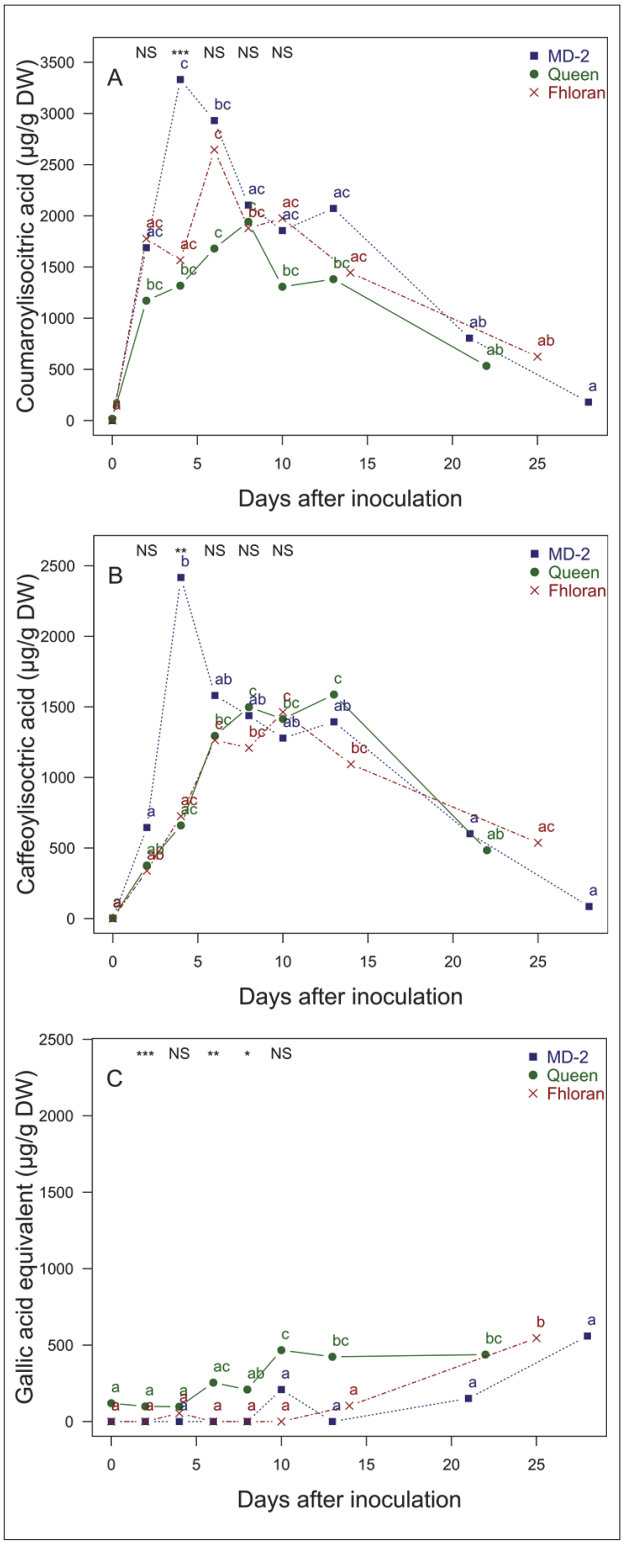

Figure 3. Evolution of *p*-coumaroylisocitrate (A), caffeoyl-isocitrate (B) and hydroxybenzoic acids (C) after inoculation with *Fusarium ananatum* in infected fruitlets of the 'MD-2' tolerant cultivar (■), 'Flhoran 53' intermediate cultivar (×) and the 'Queen' susceptible cultivar (•). Data points are mean values of 3 infected fruitlets. Differences of phenolic acids level between cultivars were either significant at $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***), or non-significant (NS) for each sampling date. Different letters indicate that data are significantly different at $P<0.05$ between DAI for each cultivar (according to Tukey's multiple comparison test). DW: Dry weight.

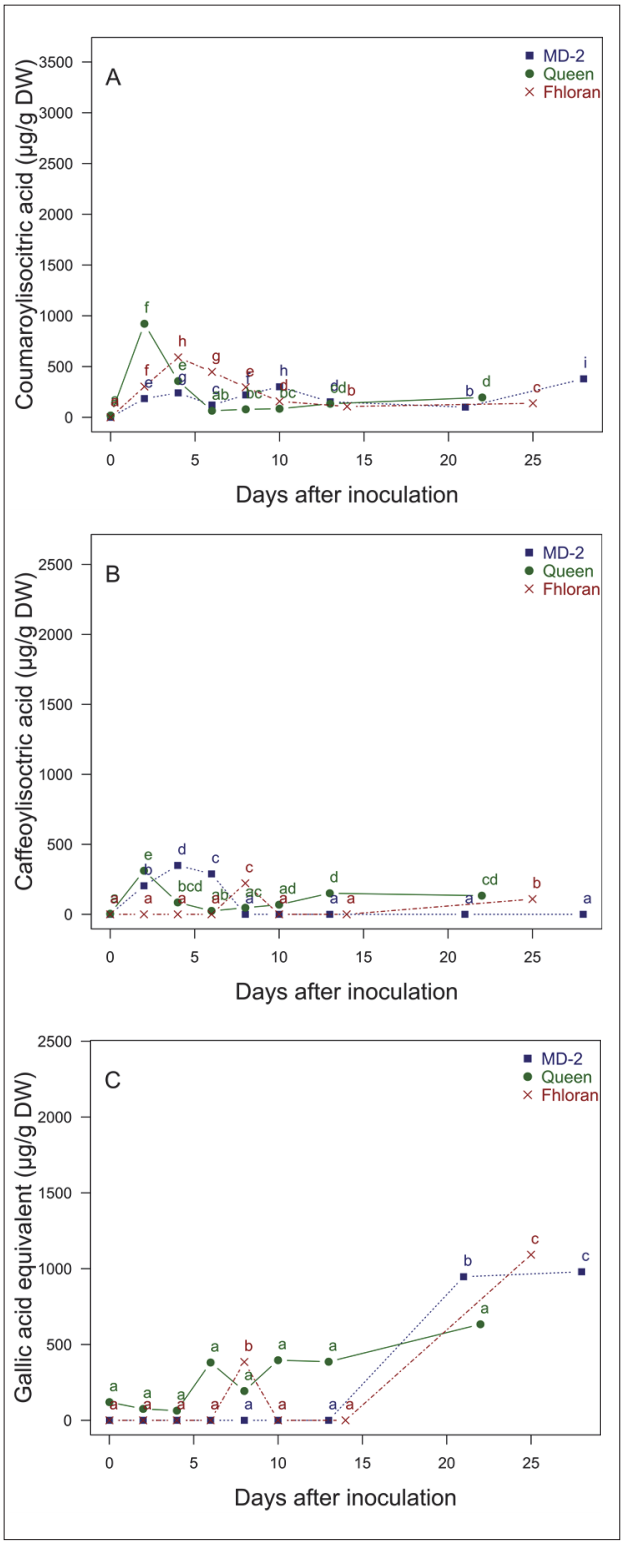

Figure 4. Evolution of *p*-coumaroylisocitrate (A), caffeoyl-isocitrate (B) and hydroxybenzoic acids (C) in H_2O inoculated fruitlet of the 'MD-2' tolerant cultivar (■), 'Flhoran 53' intermediate cultivar (×) and the 'Queen' susceptible cultivar (•). Data points are mean values of 2 infected fruitlets. Different letters indicate that data are significantly different at $P < 0.05$ between DAI for each cultivar (according to Tukey's multiple comparison test). DW: Dry weight.

p-coumaroyl-isocitric acid reached a concentration of 3,331 µg g^{-1} DW four days post-inoculation for 'MD-2'. In the infected fruitlet of 'Flhoran 53', the highest amount was 2,645 µg g^{-1} DW at 6 DAI, and 'Queen' reached 1,941 µg g^{-1} DW at 8 DAI. The caffeoyl-isocitric acid levels followed the same trend as coumaroyl-isocitric acid in the infected fruitlet, with a peak at 2,416 µg g^{-1} DW at 4 DAI in 'MD-2' and 1,587 µg g^{-1} DW at 13 DAI in 'Queen'.

The coumaroyl and caffeoyl isocitrates are depsides composed of an isocitric acid and a hydroxycinnamic acid linked by an ester bound (Masike *et al.*, 2017; Parveen *et al.*, 2008). This type of phenolic compound is known to be involved in plant stress response (Varbanova *et al.*, 2011). Petkovšek *et al.* (2009) described an accumulation of hydroxycinnamic acid depside in apple in response to infection by a fungus. In the general phenylpropanoid pathway, hydroxycinnamic acid depsides, such as coumaroyl-quinic and caffeoyl-quinic acids, are key molecules leading to lignin formation (Vanholme *et al.*, 2010). Lignin biosynthesis can be induced upon pathogen infection and act as a physical barrier from microbial degradation (Miedes *et al.*, 2014; Tronchet *et al.*, 2010).

Once they reached their maximum levels, the two hydroxycinnamic acids significantly decreased until the end of the trial. Atanasova-Penichon *et al.* (2012) observed similar kinetics of phenolic acid level in maize after infection with *Fusarium graminearum*. Free phenolic acid content reached an absolute maximum few days after fungus inoculation and then progressively decreased until the last harvest time. During our experiment, a lightening of the flesh in the infected fruitlet occurred the first days after inoculation with *F. ananatum* for the three treated cultivars. The characteristic browning due to the oxidation of phenolic compounds started only 4 DAI. The 'MD-2' had the latest and most confined symptoms. High levels of polyphenol oxidase and laccase activity were measured in well-formed pineapple infected fruitlets (Avallone *et al.*, 2003). The PPO is able to oxidize hydroxycinnamic acid esters, which then react with other quinones, forming high-molecular-weight polymers with brown pigmentation (Mayer and Harel, 1979; Parveen *et al.*, 2008; Zhou *et al.*, 2003).

Hydroxybenzoic acids in infected fruitlet

The levels of hydroxybenzoic acids did not increase following inoculation but only six days after the inoculation for 'Queen', eight days for 'Flhoran 53' and 12 days for 'MD-2' (Figure 3C). In comparison with the healthy fruitlet (Figure 2C), the synthesis of hydroxybenzoic acids was repressed in the infected fruitlet. The phenylpropanoid pathway leading to lignin synthesis could be up-regulated at the expense of the hydroxybenzoic acids during a pathogenic attack. Some studies demonstrate the existence of a channeling pathway in the biosynthesis of phenolic compounds. For example, phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) enzymes create metabolic channeling for the early steps in monolignol biosynthesis (Rasmussen and Dixon, 1999; Winkel-Shirley, 1999). Lee *et al.* (2012) also suggest a metabolic channeling for two monolignol biosynthetic enzymes involved in lignin formation, caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase. The activation or suppression of genes encoding enzymes of the phenylpropanoid pathway should be investigated to support our hypothesis. Finally, Gauthier *et al.* (2016) demonstrated the ability of *F. graminearum* to bio-transform some simple phenolic acids into a diversity of metabolites. This alternative metabolic pathway could explain the de-

crease of hydroxybenzoic acids concentration in the infected fruitlet. The increase could be correlated with the evolution of maturation during the trial.

Phenolic compound evolution in H_2O-inoculated fruitlet

The fruitlet reacted to H_2O inoculation (Figure 4). The *p*-coumaroyl and caffeoyl isocitrates accumulated in all three cultivars from the second day after inoculation. Maximum values were obtained at 2 to 4 DAI at levels ten times lower than those found in the infected fruitlets. Thereafter, levels in hydroxycinnamic acids decreased to reach similar values as healthy fruitlets. Hydroxybenzoic acids did not appear to be affected by inoculation, like in the case of infected fruitlets. Moreover, the symptoms were not visible except for the occasional trace of the needle (data not shown).

This inoculation with sterile water confirms that the pattern of evolution of the phenolic compounds observed in the infected fruitlet is due to the presence of the pathogenic fungus *Fusarium ananatum*.

Conclusion

Metabolomics showed that content in primary and secondary metabolites varied considerably among the pineapple cultivars analysed. Hierarchical clustering made it possible to identify hydroxycinnamic acids depside as markers of fruitlet core rot disease. Based on these preliminary results, the kinetics focused on this family of compounds confirm the differences between cultivars. During the natural ripening, healthy fruitlet of the tolerant cultivar accumulates up to 5 times more coumaroyl-isocitric acid and 4 times more hydroxybenzoic acids than the susceptible cultivar. And after inoculation with *Fusarium ananatum*, coumaroylisocitrate and caffeoylisocitrate are abundantly synthesized in the infected fruitlet of each cultivar, with higher levels reached for the tolerant cultivar. The fruit response to the fungal attack was both higher and faster for 'MD-2'. Regarding hydroxybenzoic acids, levels in infected fruitlet after inoculation increased much less than in healthy fruitlet during ripening. These differences in phenolic content are caused by the regulation of the phenylpropanoid synthetic pathway. Phenolics pre-formed at the critical stage of disease appearance and a different regulation of the phenylpropanoid pathway after fungus infection could give an advantage to the tolerant pineapple. The results of this study provide evidence that the phenolic compounds played a dominant role in the pineapple-*Fusarium ananatum* pathosystem. Given the success of 'Queen' in La Réunion island, the goal will not be to replace it with a resistant cultivar but to find ways to increase the levels of phenolic compounds through elicitors and new agricultural practices.

Acknowledgments

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