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Defence responses triggered during the plant-pathogen interaction between strawberry (*Fragaria* x *ananassa*) and *Colletotrichum acutatum*

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ABSTRACT

Strawberry (*Fragaria* x *ananassa* Duch.) production is an important economic activity in Argentina. Anthracnose, caused by *Colletotrichum* spp., is the most destructive fungal disease for strawberry and causes a huge economic loss every year. The gene-regulatory networks and metabolic pathways involved in the interaction between *Fragaria ananassa* and *Colletotrichum acutatum* are poorly understood. Sixteen strawberry cultivars were characterized in a local strain, M11, of *C. acutatum*, and the results showed that the Camarosa and Pajaro cultivars were the most tolerant and susceptible, respectively. Metabolic, biochemical and molecular analyses were used to study pathogen and strawberry interaction. The results showed that (1) after being infected by the pathogen, the Camarosa cultivar showed early induction of stomatal closure, callose and lignin accumulation; (2) the metabolic profile of the Camarosa cv. infected by *C. acutatum* showed high levels of carbohydrate accumulation, while the Pajaro cv. was characterized by accumulation of amino acids and their derivatives; (3) the expression of defence-related genes was induced earlier in the Camarosa cv. The above results demonstrated that an early and complex network of defence responses is triggered in the tolerant cultivar (Camarosa cv.), when infected by *C. acutatum*. Altogether, these results led us to expand the boundaries of knowledge of the metabolic pathways and gene-regulatory networks involved in the interaction between strawberry and *Colletotrichum acutatum*.

Introduction

Strawberry (*Fragaria* x *ananassa* Duch.) production is an important economic activity in Argentina, ranking thirty-fourth of all countries, with 14,750 t produced in 2019, and fifth in South America (FAO, 2023). Tucumán, a small region located in the northwest of Argentina, is the third most important strawberry producer in the country, producing fresh fruit throughout the year (Rodriguez et al., 2021). However, the mild temperatures under which the crop grows favour the appearance of fungal diseases, the main biotic stress affecting the crop (Garrido et al., 2016). Anthracnose is the most destructive fungal disease in strawberries, infecting practically all the plant's organs, mainly wounded and young tissues. It is caused by several *Colletotrichum* spp. clustered into five species complexes (Howard et al., 1992; Freeman and Katan, 1997; Ji et al., 2022), which greatly benefit from warm temperatures and high humidity. In Argentina, three species of *Colletotrichum* are the aetiological agents of strawberry anthracnose, *C. gloeosporioides, C. fragariae*, and *C. acutatum* (Peres et al., 2005). *C. acutatum* is the most prevalent,

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representing between 80 and 90 % of the total (Ramallo et al., 2000; Mónaco et al., 2000; Mena et al., 1974; Salazar et al., 2018).

Under a pathogen attack, the plant outcome could be a compatible interaction (successful infection) or an incompatible interaction (successful plant defence). However, in between those outcomes, there is a continuum gradient of susceptibility/resistance to a specific pathogen (Ponzio et al., 2016; Glazebrook, 2005). To prevent pathogen infection, plants have evolved an efficient immune system (Jones and Dangl, 2006), characterized by a set of defence responses such as changes in ion fluxes across the plasma membrane, production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), antimicrobial secondary metabolites, differential expression of defence-related genes, callose and lignin deposition, and stomatal closure (Boller and Felix, 2009; Dodds and Rathjen, 2010; Pieterse et al., 2012; Zipfel, 2008). Moreover, phytohormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), also accumulate as part of plants' systemic defence responses against pathogens, pests and abiotic stresses (Glazebrook, 2005; Bari and Jones, 2009). In recent years, technologies such as RNA-seq profiling allowed us to gain insight into the underlying molecular mechanisms associated with anthracnose resistance in strawberries. Wang et al. (2017) performed a transcriptomic study of the interaction between a resistant and a susceptible strawberry cultivar and *C. gloeosporioides* and identified numerous genes associated with defence mechanisms, plant-pathogen interaction, flavonoid biosynthesis, cell wall organization and response to biotic stimuli. Similarly, Zhang et al. (2018) provided evidence of metabolic changes occurring during *C. fructicola* infection in strawberry plants, and detected a large number of host/pathogen-responsive genes. In particular, the up-regulation of fungal genes encoding candidate effector proteins led to insights into the role of SA signalling during infection (Zhang et al., 2018). Additionally, a comparative transcriptome analysis of resistant and susceptible strawberry cultivars infected with *C. gloeosporioides* identified several up-regulated genes functionally associated with plant defence responses (Chandra et al., 2021). Amongst them, three genes belong to the genomic region *FaRCg1* and presented high expression levels. A previous study identified such genes to confer resistance to anthracnose by using two large multi-parental populations (Anciro et al., 2018). Moreover, Mehmood et al. (2021) identified a rapid induction of terpenoid metabolism in the wild strawberry *F. nilgerrensis* in response to *C. gloeosporioides*.

All the studies mentioned above provide an important catalogue of genes associated with the interaction between strawberry and *Colletotrichum*. However, the information available about the metabolic, cytological, and molecular networks associated with anthracnose disease resistance needs further exploration. To better understand such complex regulatory mechanisms underlying the defence responses to *Colletotrichum acutatum* infection in strawberry plants, it is necessary to perform a multi-disciplinary and integrative approach and evaluate cultivars with different degrees of disease resistance. Therefore, this study aims to characterize the *F. ananassa* and *C. acutatum* interaction by unravelling the metabolic, cytological and molecular defence responses underlying fungal infection. For this purpose, the responses of sixteen commercial cultivars of strawberry widely used in Argentina were analysed after infection by a local strain, M11, of *C. acutatum,* and the studies were further performed in the two most contrasting strawberry cultivars, namely "Pajaro" (susceptible) and "Camarosa" (tolerant).

Materials and methods

Plant material and growth conditions

Sixteen strawberry varieties (*Fragaria* × *ananassa*) widely cultivated in Argentina were used in this study. Plants were obtained from the Strawberry Active Germplasm Bank (National University of Tucumán, Argentina) and in vitro propagated. Plantlets were then transferred to a soil-based substrate and maintained under nursery-controlled conditions (28 °C, 70 % relative humidity (RH), 16-h light cycle, and 350 μmol m^{-2} s^{-1}). Fourteen- to sixteen-week-old healthy plants were used for all the experiments.

Fungal isolates, propagation and inoculation

The local isolate M11 of *Colletotrichum acutatum* (Salazar et al., 2007, 2022) was used in this study. Disk stubs of fungal isolated colonies grown in potato dextrose agar (PDA) were transferred into Petri dishes containing fresh PDA and incubated in a growth chamber at 28 ± 2 °C under continuous white fluorescent light (200 μmol m^{-2} s^{-1}) for 10 days (Smith, 1990). Spore suspensions were prepared from fresh cultures grown on PDA plates, which were gently scraped and suspended in sterile water. The conidial suspensions were then filtered through sterile gauze, the concentration was adjusted to 1.5×10^6 conidia ml^{-1} using a Newbauer chamber, and 0.02 % Tween 20 was added (Salazar et al., 2007). The strawberry plants were spray-inoculated with *C. acutatum* suspension until run-off and then placed in infection chambers under controlled conditions (28 °C, 100 % RH, darkness) for two days. After this period, plants were moved to growth chambers (28 °C, 70 % RH, 16 h photoperiod, and 200 μmol m^{-2} s^{-1}) for 15 days. Control plants were sprayed with sterile water mixed with 0.02 % Tween 20 and incubated under similar conditions. The sixteen strawberry cultivars were infected with *C. acutatum,* and two independent experiments were performed, using 10 plants of each cultivar ($n = 10$).

Disease severity rating (DSR)

Strawberry cultivars' susceptibility was measured by assessing the DSR, according to a scale described by Delp and Milholland (1980). DSR was evaluated in petioles of treated plants using the following scale: (1) healthy petiole without lesions; (2) petiole with lesions <3 mm; (3) petiole with lesions from 3 to 10 mm; (4) petiole with lesions from 10 to 20 mm and girdling of petiole; (5) entirely necrotic petiole and a dead plant. After quantifying anthracnose symptoms, we found the two most contrasting cultivars were Pajaro and Camarosa, which were used for subsequent molecular analyses.

Cytological analysis

Stomatal closure analysis

For each experimental condition, Camarosa and Pajaro cv., infected and non-infected, six biological replicates were collected at 2, 5 and 9 days post-infection (dpi). The inner, younger, fully expanded leaves were immediately fixed in F.A.A. (Acetic acid, Formalin, Water and Alcohol 1:2:7:10). For stomatal closure analysis, the leaves were later diaphanized by a technique according to Dizeo de Strittmatter, 1973 and subsequently stained with crystal violet (D'Ambrogio de Argüeso, 1986). The preparations were mounted in glycerine water and observed in an optic microscope (Axiostar Plus, Carl Zeiss, Göttingen, Germany). Five random photographs were taken of each condition. Subsequently, stomatal closure measurements were taken from twenty-five stomata from each evaluated condition/time using ImageJ software (https://imagej.nih.gov/ij/).

Callose and lignin depositions

Callose depositions in leaves of control and infected plants were evaluated at 2, 5 and 9 dpi by a histochemical staining technique. The inner, younger, fully expanded leaves were decolorized using a solution mixture of 3:1 ethanol 96 %:lactic acid (previously diluted 1:2 in ethanol 96 %) until reaching translucent tissue. The samples were re-hydrated in 50 % ethanol for 2 h and then incubated in 67 mM K_2HPO_4 (pH 12) containing 0.01 % aniline blue (Sigma, USA) according to Caro et al. (2022). Samples were mounted on slides with 30 % glycerol and examined under a fluorescence microscope with a UV filter (excitation, 365/10 nm; emission, 460/50 nm). Photographs were taken of each

sample, and callose deposits were quantified using ImageJ software.

Lignin depositions in leaves of control and infected plants were evaluated at 2, 5 and 9 dpi through the qualitative phloroglucinol method (D'Ambrogio de Argüeso, 1986). Harvested leaves were clarified with bleach, washed with distilled water, treated with phloroglucinol (1 %) in an alcoholic solution and then subjected to a direct flame. Subsequently, hydrochloric acid (25 %) was added. The lignified cell walls were analysed for intensity of the red-violet colouration in an optic microscope (Axiostar Plus, Carl Zeiss, Göttingen, Germany).

Metabolic analysis

For each condition, six biological replicates were collected at 5 and 9 dpi. Metabolite extraction was performed via the extraction of lipophilic and polar compounds according to Roessner-Tunali et al. (2003). Samples were derivatised and injected (1 µl) into the GC-TOF-MS system (LECO Corporation, St. Joseph, MI, USA). Chromatography was performed on a 30 m SPB-50 column with 0.25 mm inner diameter and 0.25 µm film thickness (Supelco, Bellefonte, CA, USA). The temperatures of injection, interface, and ion source were set to 230 °C, 250 °C, and 200 °C, respectively. The carrier gas was He at a constant flow rate of 1 ml min^{-1}. The chromatograms and spectra were evaluated using the ChromaTOF (LECO Corporation, St. Joseph, MI, USA) and TagFinder (Luedemann et al., 2008). Ion spectra were compared to the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/). Sample variability was calculated, and an alpha value > 0.05 was considered as outlier and excluded from the analysis. Statistical analyses were performed using MetaboAnalyst 5.0 platform (Xia et al., 2009). Metabolites were first normalized to fresh weight and the internal control ribitol and standardized using the mean-centred and divided by the standard deviation of each variable; then, hierarchical clustering was performed by Euclidean distance. The fold-change was calculated against the Pajaro control 5 dpi using the normalized metabolites data.

RNA isolation and qPCR analysis

For each condition, three biological replicates were collected at 2, 5 and 9 dpi; each biological replicate consisted of a pool of the inner, younger, fully expanded leaf from two randomly selected plants. The sampled leaves were immediately frozen in liquid nitrogen upon collection and stored at −80 °C until processing. High-quality total RNA was isolated from 100 mg of frozen tissue using the RNAqueous Total RNA Isolation Kit (Invitrogen, USA) following the manufacturer's instructions. The genomic DNA was eliminated after treatment with DNase I (Invitrogen, USA) for 20 min at room temperature. The RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The purity and integrity of total RNA were determined by the 260/280 nm ratio. For each sample, 500 ng DNase-treated RNA was reverse transcribed using the Superscript III first strand synthesis system (Invitrogen, USA) and random hexamer primers following the manufacturer's instructions. qPCR was carried out using iTaq Universal SYBR Green Supermix (Bio-Rad, USA) in an ABI 7500 thermocycler (Applied Biosystems, USA). Amplification efficiencies and Ct values were determined using LinRegPCR (Ruijter et al., 2009). Gene relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The expression of the selected genes was evaluated relative to the reference genes *Actin* and *Elongation Factor −1α* (*EF-1α*) (Table S1).

The genes evaluated in the current study included the allene oxide synthase (*FaAOS*), involved in the JA biosynthetic pathway (Wasternack, 2007); acetyl-CoA synthetase (*FaACS*), involved in endogenous ET biosynthesis (Van de Poel and Van Der Straeten, 2014); the SA-responsive gene (*FaPR1)* (Spoel et al., 2009); phenylalanine ammonia-lyase1 (*FaPAL1*), a gene marker of the phenylpropanoid pathway (Dixon et al., 2002); the transcription factor *FaMYB30,* a positive regulator of the pathogen-induced hypersensitive response (HR) (Vailleau et al., 2002); the respiratory burst oxidase protein D (*FaRBOHD*), associated with reactive oxygen species (ROS) production (Torres et al., 2005); and a calcium-dependent protein kinase (*FaCDPK*) (Ranf et al., 2011).

Results

Phytopathological characterization

After inoculating sixteen of the most commonly used strawberry cultivars in northwest Argentina with the virulent strain M11 of *C. acutatum*, we identified two of the most contrasting varieties (Fig. 1A). The most susceptible varieties were Pajaro, Milsei and Seascape, which showed the first anthracnose symptoms as early as 2 - 3 dpi, including small lesions on leaves, petioles and crown. These symptoms were more severe at 9 dpi (Fig. 1B) and increased until the plant was dead at 20 dpi. The most tolerant varieties were Camarosa and Sweet Charlie, which did not present anthracnose symptoms (*DSR* ≅ 1); instead, healthy plants were observed at 9 dpi (Fig. 1B). Based on the above results, for further study, we selected Camarosa and Pajaro as the tolerant and susceptible varieties, respectively, for anthracnose disease, and for further characterization of the plant-pathogen interaction between *F. ananassa* and *C. acutatum*.

Stomata aperture and callose and lignin depositions during Colletotrichum acutatum infection

Early induction of stomatal closure was observed in infected Camarosa plants at 5 and 9 dpi compared to the control plants (Fig. 2). On the contrary, no differences were observed between the control and infected plants of the Pajaro cv. at the same time-point (Fig. 2). Additionally, the tolerant cultivar Camarosa presented a significant increase in callose deposits at 5 dpi, and the accumulation progressed by 9 dpi (Fig. 3A). On the contrary, the susceptible cultivar Pajaro showed only minor callose deposits at 9 dpi (Fig. 3A).

In comparison to control plants, higher depositions of lignin in the xylem cell walls were observed in infected Camarosa plants in comparison to control plants, mainly at 5 dpi. At this specific time, the lignin depositions in infected Camarosa plants were distributed more uniformly in the periphery of the xylem vessels, especially in the smaller diameter elements, compared to infected Pajaro plants (Fig. 3B). No differences were detected between infected and control plants of the Pajaro cultivar at any time-point evaluated (Fig. 3B).

Metabolic analysis during Colletotrichum acutatum infection

To identify metabolites and metabolic pathways associated with defence responses to anthracnose, we performed metabolic profiling for primary metabolism using the GC–MS method. In this analysis, 47 metabolites were identified from control and infected plants at 5 and 9 dpi, respectively (Fig. 4). Hierarchical clustering analysis (HCA) allowed us to classify the metabolites into two main clusters. Most of the amino acids and their derivatives were accumulated in the susceptible cultivar Pajaro, under the infected condition. Conversely, we found an accumulation of sugars in Camarosa plants in both control and infected plants, but infected plants presented higher levels (Fig. 4). Additionally, we performed a detailed analysis of the metabolite changes and evaluated and compared them to the control plants of the susceptible variety Pajaro at 5 dpi (Fig. 5). We observed a significant accumulation of several amino acids, such as tryptophan, tyrosine, phenylalanine, isoleucine, lysine, valine, proline, threonine, ß-alanine and cysteine, and some amino acid derivatives such as GABA and putrescine. On the contrary, most such compounds were decreased or remained unaltered when comparing control and infected plants of Camarosa. Carbohydrate metabolites such as sucrose, fructose, rhamnose, xylose, fucose, trehalose, glucose and glucose-6-phosphate were detected at higher basal levels in Camarosa control plants, and levels markedly increased after

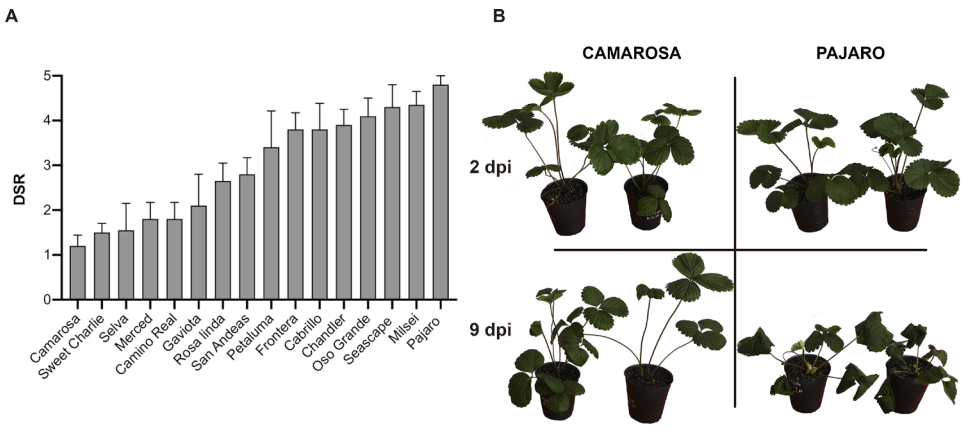

Fig. 1. Phenotypic analysis. (A) Disease Severity Rating (DSR) evaluated in petioles of treated plants at 9 dpi using the following scale according to Delp and Milholland (1980): (1) healthy petiole without lesions; (2) petiole with lesions <3 mm; (3) petiole with lesions from 3 to 10 mm; (4) petiole with lesions from 10 to 20 mm and girdling of petiole; (5) entirely necrotic petiole and a dead plant. (B) Anthracnose symptoms in Camarosa and Pajaro plants at 2 and 9 days post-infection (dpi).

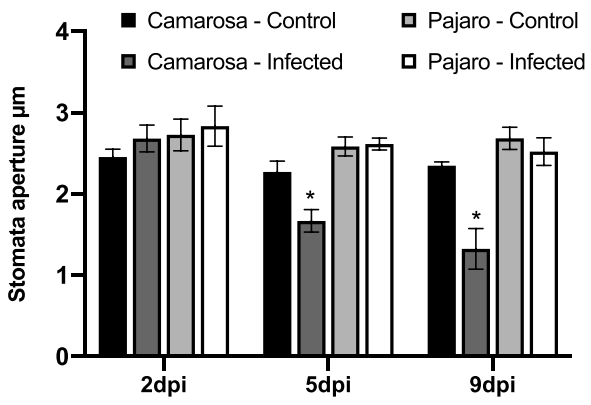

Fig. 2. Stomata closure analysis. Data represent the stomata aperture averages ±SE from twenty-five independent stomata in each condition at 2, 5 and 9 days post-infection (dpi). Asterisks indicate significant difference between control and infected plants (*$p < 0.05$; Student's *t*-test).

C. acutatum infection. Additionally, citrate and 2-oxoglutarate metabolites were up-regulated in Camarosa control and infected plants as well as in infected Pajaro plants.

Expression profile of defence-related genes during Colletotrichum acutatum infection

Upon *C. acutatum* infection, *FaAOS* expression was significantly up-regulated in infected Camarosa plants at 2, 5, and 9 dpi, respectively. However, overexpression levels were lower in infected Pajaro plants (Fig. 6A). Likewise, *FaACS* exhibited higher expression levels in infected Camarosa plants at 2, 5, and 9 dpi, with a slight increase detected in infected Pajaro plants at 9 dpi (Fig. 6B). In contrast, *FaPR1* expression showed significant up-regulation in infected Pajaroplants at 2, 5, and 9 dpi, respectively, while it was significantly down-regulated in infected Camarosa plants (Fig. 6C). Furthermore, *FaPAL1* expression was significantly increased in infected Camarosa plants at early stages post-infection, specifically at 2 and 5 dpi. No significant differences were observed in the Pajaro cultivar at any analysed time-point (Fig. 6D). *FaMYB30* exhibited significant up-regulation in infected Camarosa plants at 2 and 5 dpi, with no differences detected in the Pajaro cultivar at any analysed time-point (Fig. 6E). Additionally, *FaRBOHD* expression was significantly up-regulated in infected Camarosa plants at 2, 5, and 9 dpi. In contrast, its expression was significantly increased in infected Pajaro plants only at 9 dpi (Fig. 6F). Finally, *FaCDPK* demonstrated high expression levels in infected Camarosa plants at 2 and 5 dpi, followed by a sharp drop at 9 dpi. In infected Pajaro plants, its expression level increased at 5 and 9 dpi (Fig. 6G).

Discussion

Characterization of the strawberry-Colletotrichum acutatum interactions

Anthracnose is one of the most devastating fungal diseases in strawberries, causing important economic losses. In this study, we first characterized the phytopathological interactions amongst sixteen strawberrys cultivars that are widely grown in Tucumán, a small region located at the northwest of Argentina, against the virulent strain M11 of the hemibiotrophic fungus *C. acutatum* (Salazar et al., 2007). The cultivars showed differential responses to the fungus (Fig. 1A). For instance, Camarosa presented an incompatible interaction and was classified as the most tolerant cultivar (Fig. 1B). It exhibited small lesions, less than 3 mm, in a limited number of the analysed plants. On the other hand, Pajaro presented a compatible interaction and was classified as the most susceptible cultivar. It presented infection symptoms in leaves, petioles and crown at 9 dpi (Fig. 1B). The other fourteen cultivars were classified in between.

Cytological profile induced during Colletotrichum acutatum infection in the tolerant and susceptible strawberry cultivars

The spread of an invading pathogen involves the participation of physical and chemical barriers at the early stages. At the cellular level, stomata closure is one of the first host defence layers to prevent the invasion of a pathogen. Different bacteria, oomycetes, and fungi could exploit stomatal openings as major invasion routes (Melotto et al., 2008; Arnaud and Hwang, 2015; Ye et al., 2020). In our previous study, we observed that the virulent strain M11 of *C. acutatum* infects strawberry cells either by penetrating the epidermal cells, by appressorium formation, or by accessing the stomata (Salazar et al., 2007). In this study, stomata closure occurred earlier in tolerant Camarosa plants than in susceptible Pajaro ones (Fig. 2). Thus, the defence response is likely to function as an early and efficient physical barrier against fungus infection.

The cell wall is another initial physical barrier, together with stomatal closure, that helps cope against pathogen invasion. Cell wall reinforcement is a typical physiological response to plant immune activation (Luna et al., 2011; Underwood, 2012; Wan et al., 2021). In the present study, we found a significant callose and lignin accumulation upon *C. acutatum* infection in the tolerant Camarosa cultivar between 5 and 9 dpi, whereas only scarce callose deposits were detected in infected plants of the cultivar Pajaro, just at the later time analysed (Fig. 3). Thus, it is likely that callose and lignin depositions serve as other early defence responses against *C. acutatum,* making the cell wall more resistant to fungal mechanical pressure and restricting the pathogen to the infection site, as reported also by other authors (Yang et al., 2018; Lee et al., 2019). Altogether, these results suggest that early cytological defence

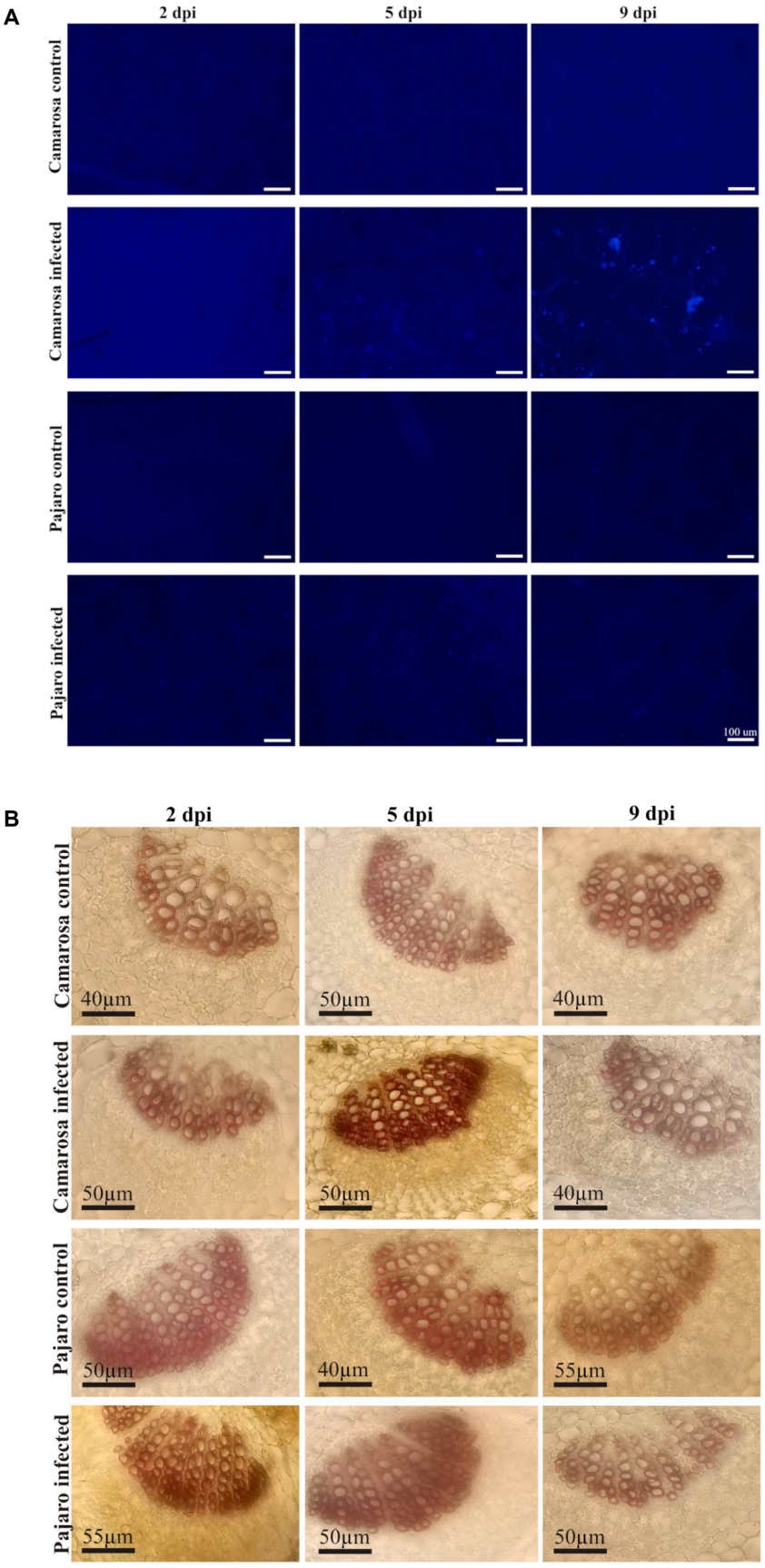

Fig. 3. Callose and lignin deposition. (A) Callose depositions in leaves of control and infected plants were evaluated at 2, 5 and 9 days post-infection (dpi), stained with aniline blue and analysed by fluorescence microscopy. (B) Accumulation of lignin in the main vascular bundle in leaves of control and infected plants was evaluated at 2, 5 and 9 days post-infection (dpi). The level of lignin deposit on cell walls was evaluated by estimating the intensity of the red-violet colour.

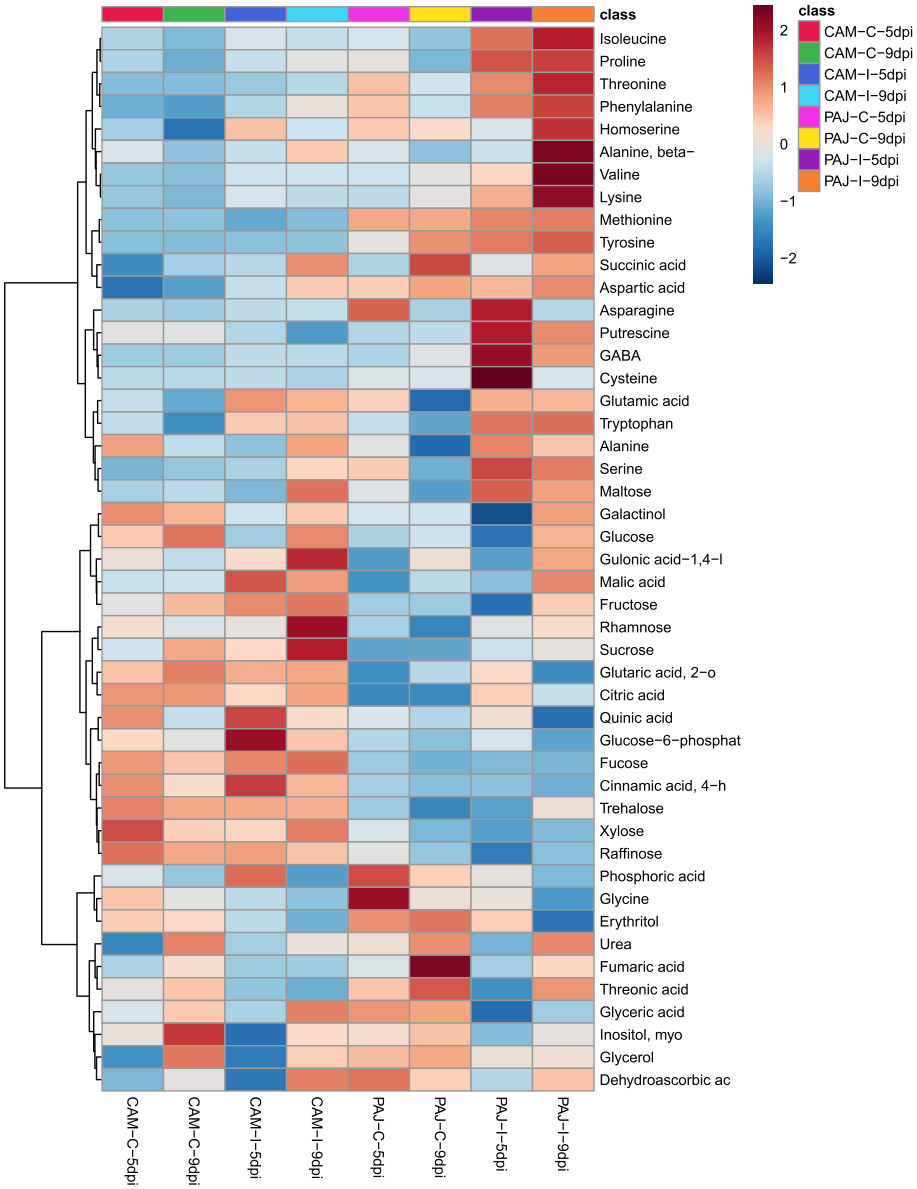

Fig. 4. Hierarchical clustering heatmap. Hierarchical clustering was performed by Euclidean distance using MetaboAnalyst 5.0 (Xia et al., 2009). CAM-C, CAM-I, PAJ-C and PAJ-I correspond to Camarosa control, Camarosa infected, Pajaro control and Pajaro infected, respectively.

responses are time-modulated and play an important role in preventing the spread of the hemibiotrophic fungus *C. acutatum* in the tolerant strawberry variety Camarosa.

Metabolic profile induced during Colletotrichum acutatum infection in the tolerant and susceptible strawberry cultivars

Primary metabolism has a very well-described role as an energy provider for physiological processes like growth, development and cell reproduction, and also as signalling molecules that trigger and regulate a plant's defence responses upon an invading pathogen (Zaynab et al., 2019; Rojas et al., 2014). For instance, the metabolite analysis performed during the infection of the hemibiotrophic bacteria *P. syringae* revealed that the levels of several branched-chain amino acids (such as valine, leucine, and isoleucine) and aromatic amino acids (such as phenylalanine, tyrosine, tryptophan, and lysine) significantly increased upon infection, whereas the aspartate levels decreased (Návarová et al., 2012; Zeier, 2013). Similarly, here we found that such amino acids accumulated in the susceptible cultivar Pajaro upon *C. acutatum* infection (Fig. 5).

Putrescine is the central product of the polyamine (PA) biosynthetic pathway and the most abundant PA in nature. PAs play important roles in diverse plant growth and developmental processes as well as in biotic and abiotic stress responses (Chen et al., 2019; González-Hernández et al., 2022). However, the mechanism by which PAs act during plant-microbe interaction is sometimes contradictory. Different researchers suggest that PA and PA oxidation exert positive and negative influences on plant resistance to (hemi)biotrophic and necrotrophic pathogens, respectively (Zeier, 2013). For instance, higher PA levels – achieved either by the overexpression of arginine decarboxylase (ADC) in transgenic tobacco, or by the exogenous application of putrescine – led to the increase of *S. sclerotiorum* necrosis and disease severity. On the contrary, the inhibition of PA led to the reduction of disease severity. In another study, enhanced PA levels induced resistance to the biotrophic bacterium *P. viridiflava* in tobacco, and this effect was reduced when PA oxidation was inhibited (Marina et al., 2008). Here, we discovered a notable accumulation of putrescine in infected Pajaro plants, which likely contributed to the heightened disease severity observed in this

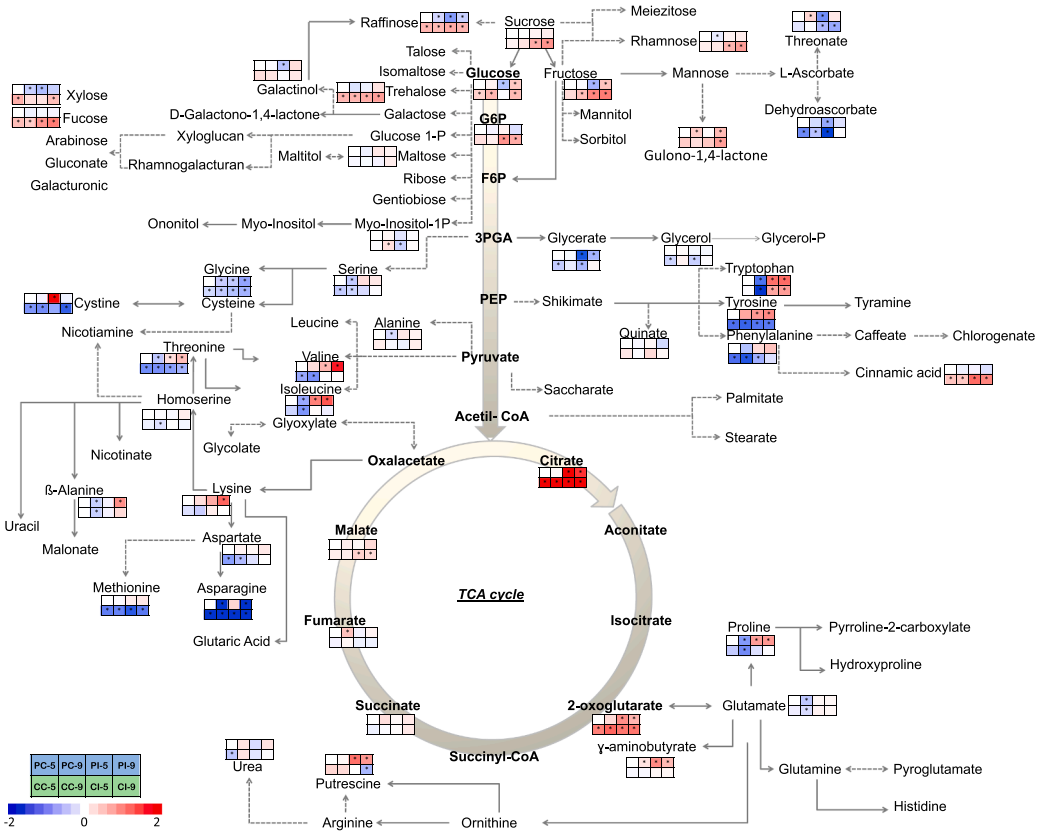

Fig. 5. Metabolic profile. Metabolite profile was analysed by GC-TOF-MS. Each graph represents the eight different conditions: PC-5, PC-9, PI-5 and PI-9 correspond to Pajaro Control −5, −9 dpi and Pajaro Infected −5, −9 dpi, respectively; CC-5, CC-9, CI-5 and CI-9 correspond to Camarosa Control −5, −9 dpi and Camarosa Infected −5, −9 dpi, respectively. Colour scale corresponds to the fold-change against Pajaro control 5 dpi as a control, blue: down-regulated, red: up-regulated. The asterisks indicate significant differences ($*p < 0.05$).

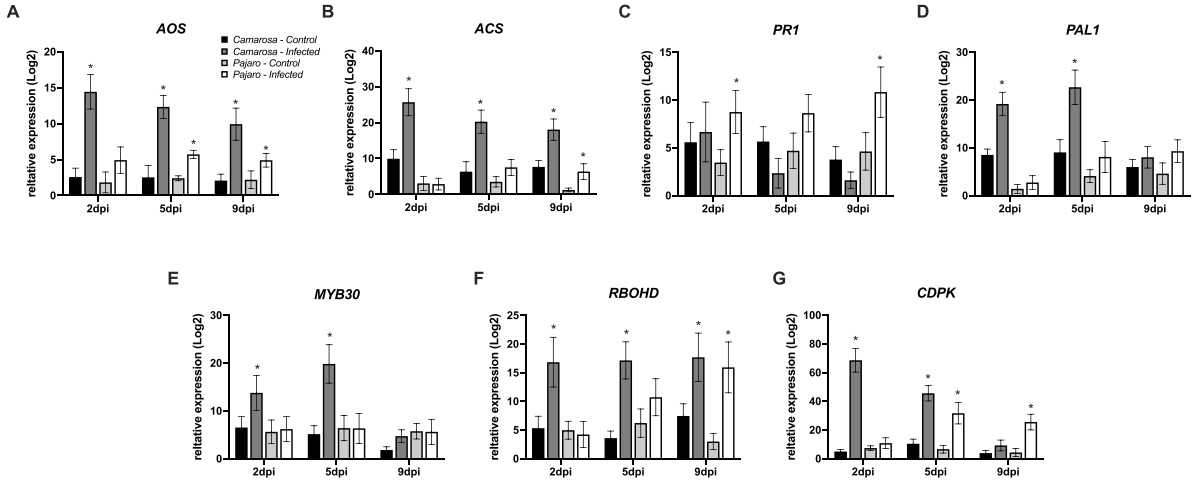

Fig. 6. Relative expression of defence related genes. The relative mRNA levels of *AOS, ACS, PR1, PAL1, MYB30, RBOHD* and *CDPK* were determined at 2, 5 and 9 days post-infection (dpi) in both Camarosa and Pajaro varieties under control and infected conditions. The expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The expression of the selected genes was evaluated relative to the reference genes *Actin* and *Elongation Factor −1α* (*EF-1α*). The asterisks indicate significant differences in relation to the respective control plants ($*p < 0.05$).

particular cultivar (Fig. 5). By contrast, the tolerant cultivar Camarosa showed a lower level of putrescine in infected plants.

Sugar metabolites constitute the primary substrate providing energy and structural material for defence responses in plants (Morkunas and Ratajczak, 2014). The increase in sugar demand during defence responses includes cell wall strengthening, biosynthesis of phytoalexins, stimulation of the synthesis of flavonoids, and the induction of defence-related proteins. Moreover, accumulated soluble sugars themselves may activate the expression of defence-related genes (Liu et al., 2022; Herbers et al., 2000; Sonnewald et al., 2012; Gebauer et al., 2017). Some sugars also influence the plant immune system as priming molecules inducing higher plant resistance to pathogens, known as "sweet immunity" and "sweet priming" (Morkunas and Ratajczak, 2014; Moghaddam and Van Den Ende, 2013; Moghaddam and Van Den Ende, 2012). In the present study, we found an accumulation of sugars in plants of Camarosa in comparison to Pajaro ones (Fig. 5). The increased levels were also found under the control condition, evidencing higher basal levels in this specific cultivar. Additionally, during the infection,

Camarosa plants exhibited a significant increase in sugar levels, which positively correlated with a heightened disease tolerance. Likewise, the pre-treatment of rice and Arabidopsis plants with exogenous sucrose, glucose, or fructose led to an increased resistance to the fungus *Magnaporthe oryzae* and the bacterial pathogen *Pseudomonas syringae,* respectively (Gómez-Ariza et al., 2007; Qian et al., 2015), giving solid evidence of the importance of sugars in plant–pathogen interactions.

Sucrose and hexoses were also reported to play an important role in the resistance to fungal pathogens through stimulation of phenylpropanoid metabolism (Morkunas and Ratajczak, 2014; Morkunas et al., 2011; Giberti et al., 2012). The phenylpropanoid pathway contributes to plant defences with a range of defences, including preformed or inducible physical and chemical barriers, and secondary metabolites that act as signal molecules involved in local and systemic signalling (Dixon et al., 2002; Ferri et al., 2011). For instance, in this study, high levels of cinnamic acid, a phenylpropanoid precursor, were detected in the tolerant cultivar Camarosa in both control and infected plants, suggesting the importance of this metabolic pathway for fungal resistance.

Molecular profile and role of phytohormones during Colletotrichum acutatum infection in the tolerant and susceptible strawberry cultivars

Phytohormones play major roles in the regulation of plant defence responses against pathogens, pests and abiotic stresses (Glazebrook, 2005; Bari and Jones, 2009). In general, the SA signalling pathway plays a significant role in plant immunity against biotrophic and hemibiotrophic pathogens by triggering systemic acquired resistance (SAR) (Zeier, 2013; Wildermuth et al., 2001; Grant and Lamb, 2006; Caro et al., 2020), whereas the JA and ET signalling pathways are usually associated with defence against necrotrophic pathogens and herbivorous insects (Bari and Jones, 2009). Despite that SA and JA/ET defence pathways often influence each other negatively, evidence of synergistic interactions have also been reported (Bari and Jones, 2009; Mur et al., 2006; Beckers and Spoel, 2006).

To further investigate the role of such phytohormones' signalling pathways during *C. acutatum* infection, we analysed the expression profile of a set of marker genes related to each one of them in the tolerant and susceptible strawberry cultivars (Fig. 6). *AOS* encodes an allene oxide synthase that functions as a key enzyme in the initial steps of the JA biosynthetic pathway (Laudert and Weiler, 1998), and *ACS* is involved in specific phases of ET production (Tarun and Theologis, 1998). The expression of both genes was rapidly induced in infected Camarosa plants at the three evaluated time-points. In contrast, infected Pajaro plants displayed a lower expression level, albeit only observed during later stages of infection. By contrast, *FaPR1*, a classical marker gene of the SA defence pathway (Graham et al., 2003; Van Loon et al., 2006), was up-regulated in infected Pajaro plants at the three evaluated times and down-regulated in infected Camarosa plants. Altogether, these results suggest that both phytohormone pathways are activated against the isolate M11 of the hemibiotrophic fungus *C. acutatum*. Yet, the JA/ET signalling pathways are likely to play an important role in the tolerance of Camarosa plants, which has been shown to be beneficial to strawberry plants to cope with *Colletotrichum* spp. at the necrotrophic stage (Bai et al., 2022), whereas the SA pathway is activated in the susceptible Pajaro plants, but rather inducing tolerance; it seems to influence the JA/ET pathways negatively, and thus, they are not enough to cope with the infection.

Phenylalanine ammonia-lyase genes such as *PAL1* play a crucial role in secondary phenylpropanoid metabolism and are associated with biotic and abiotic stresses in plants. Overexpression of *PAL1* has been observed in response to different pathogens and/or endogenous elicitors, and the enzyme is considered a chemical marker of induced resistance in many plant species (Kavil et al., 2021). In this study, *FaPAL1* expression was significantly increased in infected Camarosa plants at early stages, whereas no differences were detected in Pajaro (Fig. 6B). Similarly, we found accumulation of cinnamic acid metabolite, a phenylpropanoid precursor, suggesting the participation and importance of the phenylpropanoid pathway in the induced resistance against anthracnose in strawberry plants.

The transcription factor *MYB30* acts as a positive regulator of cell death, conditioning the hypersensitive response (HR) (Vailleau et al., 2002). We found that *FaMYB30* was up-regulated at the early stages of Camarosa plant infection (Fig. 6E). In another study, Kishi-Kaboshi et al. (2018) identified three MYB transcription factors in rice (*MYB30, MYB55* and *MYB110*) involved in MAMP perception and the induction of a large number of genes encoding enzymes related to the phenylpropanoids cinnamate/monolignol pathway that has an important role in plant immunity.

The perception of a pathogen-associated molecular pattern (PAMP) normally leads to the activation of a cascade of defence reactions, including ion fluxes, ROS production, activation of MAPK- and Ca^{2+}-dependent protein kinases (CPKs or CDPKs), transcriptional reprogramming, callose deposition and, ultimately, immunity (Boller and Felix, 2009; Zipfel, 2014). After pathogen perception, the production of reactive oxygen species (ROS) is induced by NADPH oxidases such as RBOHD. RBOHD is mainly controlled by Ca^{2+} via direct binding to EF-hand motifs and phosphorylation by Ca^{2+}-dependent protein kinases CDPK (Kadota et al., 2015). At the same time, CDPK also participates in the activation of innate immunity against pathogens (Romeis and Herde, 2014). In this work, we found up-regulation of *FaRBOHD* and *FaCDPK* genes in infected tolerant Camarosa plants at early infection stages, whereas in susceptible Pajaro plants such genes were expressed only at later times post-infection (Fig. 6F and G).

Conclusion

The current study provides valuable insights to better understand the intricate metabolic, cytological, and molecular mechanisms underlaying the defence responses triggered during the plant-pathogen interaction between *Fragaria ananassa* and *Colletotrichum acutatum*. Amongst sixteen cultivars of strawberry widely used in Argentina, we identified Camarosa and Pajaro as the most tolerant and susceptible cultivars, respectively, to the local strain M11 of *C. acutatum*. Camarosa triggers early stomata closure, cell wall fortification by callose and lignin depositions, induction of the JA/ET pathways, and the up-regulation of defence-related genes such as *FaPAL1, FaMYB30, FaRBOHD* and *FaCDPK*, helping to effectively cope with *C. acutatum* infection, whereas the susceptible strawberry cultivar Pajaro is unable to trigger such timely defence responses, allowing the fungal infection to advance. Altogether, these results led us to expand the boundaries of knowledge of *Fragaria ananassa-Colletotrichum acutatum* interaction.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in

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