Ornamental Horticulture
ISSN 2447-536X | HTTPS://ORNAMENTALHORTICULTURE.EMNUVENS.COM.BR/RBHO
SBFPO Sociedade Brasileira de Floricultura e Plantas Ornamentais

SCIENTIFIC ARTICLE

Plant hormones accumulation and its relationship with symplastic peroxidases expression during carnation-*Fusarium oxysporum* interaction

Leidy Johana Vanegas-Cano [1*], Sixta Tulia Martínez-Peralta [1], Ericsson Coy-Barrera [2], Harold Duban Ardila-Barrantes [1]

[1] Universidad Nacional de Colombia, Facultad de Ciencias, Departamento de Química, Bogotá, Colombia.
[2] Universidad Militar Nueva Granada, Facultad de Ciencias Básicas y Aplicadas, Departamento de Química, Cajicá, Colombia.

Abstract

The vascular wilting caused by *Fusarium oxysporum* f. sp. *dianthi* (*Fod*) is the most relevant disease for carnation cultivation. Understanding the biochemical mechanisms involved in resistance to *Fod* will allow the development of new disease control strategies. In this research, the levels of some phytohormones such as salicylic acid (SA), methyl salicylate (MeSA), and methyl jasmonate (MeJA) were evaluated in symplast of carnation roots infected with this pathogen. The accumulation of these hormones was then correlated with the expression levels of symplastic peroxidases, enzymes involved in the plant resistance against pathogen during interaction. Our results suggested that pathogen infection causes a differential accumulation of SA, MeSA, and MeJA in a resistant cultivar (*i.e. 'Golem'*), being earlier and higher than that observed in a susceptible one (*i.e. 'Solex'*). Simultaneously, an increase of guaiacol peroxidase enzymatic activity (GPX) and transcriptional levels of a gene coding for a symplastic peroxidase were presented as part of the resistance response. The positive statistical correlation between the accumulation of SA and MeJA and the expression of peroxidases (GPX activity and mRNA levels) indicates the possible cellular relationship of these phenomena during the activation of the resistance to *Fod*. Our findings suggested some hormonal signaling mechanisms acting at the roots during the regulation of the biochemical response associated with resistance against *Fod*.
Keywords: guaiacol peroxidase, methyl jasmonate, methyl salicylate, salicylic acid.

Resumo

Acúmulo de hormônios vegetais e sua relação com a expressão de peroxidases simplásticas durante a interação cravo-*Fusarium oxysporum*

A murcha vascular causada por *Fusarium oxysporum* f. sp. *Dianthi* (*Fod*) é a doença mais relevante para o cultivo de cravo. Compreender os mecanismos bioquímicos envolvidos na resistência ao *Fod* permitirá o desenvolvimento de novas estratégias de controle de doenças. Nesta pesquisa, os níveis de alguns fitohormônios como ácido salicílico (SA), salicilato de metil (MeSA) e jasmonato de metil (MeJA) foram avaliados em simplastos de raízes de cravo infectadas com este patógeno. O acúmulo desses hormônios foi então correlacionado com os níveis de expressão das peroxidases simplásticas, enzimas envolvidas na resistência da planta ao patógeno durante a interação. Nossos resultados sugeriram que a infecção do patógeno causa um acúmulo diferencial de SA, MeSA e MeJA em um cultivar resistente ('Golem'), sendo mais precoce e superior do que o observado em um suscetível ('Solex'). Simultaneamente, um aumento da atividade enzimática da peroxidase de guaiacol (GPX) e os níveis de transcrição de um gene que codifica para uma peroxidase simplástica foram apresentados como parte da resposta de resistência. A correlação estatística positiva entre o acúmulo de SA e MeJA e a expressão de peroxidases (atividade GPX e níveis de mRNA) indica a possível relação celular desses fenômenos durante a ativação da resistência ao *Fod*. Nossos resultados sugeriram alguns mecanismos de sinalização hormonal atuando nas raízes durante a regulação da resposta bioquímica associada à resistência contra *Fod*.
Palavras-chave: guaiacol peroxidase, jasmonato de metil, salicilato de metil, ácido salicílico.

*Corresponding author: ljvanegasc@unal.edu.co

https://doi.org/10.1590/2447-536X.v28i1.2412
Received: June 30, 2021 | Accepted: Oct 11, 2021 | Available online: Nov 8, 2021

Area Editor: Ana Maria Mapeli

Introduction

Carnation is a herbaceous plant that belongs to the family Caryophyllaceae and the *Dianthus* genus (Hernandez, 1983). *Dianthus caryophyllus* L. is native from Mediterranean and the origin of multiple varieties obtained mainly by cross-breeding. Carnation is one of the most important products of the global flower trade. However, a problem of great importance for the carnation crops worldwide is the prevalence of fungal diseases caused by fungal pathogens (Soto-Sedano et al., 2009).

Vascular wilting caused by *Fusarium oxysporum* f. sp. *dianthi* (*Fod*) is the most important disease for carnation crops (Hegde et al., 2017). The development of new strategies for environmentally-friendly control of fungal diseases is necessary for this crop. Alternatives such as the cultivation of resistant cultivars, biological control, and resistance inducers (RIs) application have attracted the attention of the scientific community (Alexandersson et al., 2016; Pérez Mora et al., 2021). Indeed, understanding the biochemical phenomena involved in the plant pathogen resistance is a current challenge for the phytopathologist (Kou et al., 2021; Miyaji et al., 2021; Nunes da Silva et al., 2021). As part of the plant defense response against pathogenic infection, there are several biochemical phenomena such as production of reactive oxygen species (ROS), accumulation of pathogenesis-related (PRs) proteins, and reinforcement of the cell wall through the lignin production and the callose deposition (Heller and Tudzynski, 2011; Alexandersson et al., 2016; Li et al., 2019). Specifically, the lignin accumulation and the regulation of highly oxidizing species are determining mechanisms of resistance to vascular pathogens. In this sense, peroxidase enzymes play a determining role since their participation in this type of phenomenon has been widely reported and discussed (Hakeem et al., 2014; Prakasha and Umesha, 2016). For instance, an increase of the activity levels of guaiacol peroxidase (GPX) has been reported as an important response against *Fusarium* pathogens (Cuervo et al., 2009; Anthony et al., 2017), including the causal pathogen of carnation vascular wilting, i.e., *Fod* (Cuervo et al., 2009; Ardila et al., 2011 Ardila et al., 2014). However, the regulatory mechanisms related to the expression of this enzyme and the signaling molecules involved in these biochemical responses are not previously described.

Phytohormones are important components of the regulating processes involved in a wide range of biotic and abiotic stresses. Among such components, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are molecules that regulate the response of plants against various types of pathogens (Zehra et al., 2017b; Gulyani et al., 2018). In general, the plant response to biotic stress depends on the plant species (Qi et al., 2016; Boba et al., 2017). However, SA is usually related to resistance to biotrophic pathogens which require living host cells to take their nutrients. In contrast, necrotrophic pathogens derive their nutrients from dead host cells, and the related plant resistance is more sensitive to JA/ET-mediated responses (Hakeem et al., 2014; Gulyani et al., 2018). Similarly, methyl jasmonate (MeJA) can activate the defense mechanisms of the host plant against a wide range of pathogens (Di et al., 2016; Thatcher et al., 2016). In the case of plant-pathogen interactions involving hemibiotrophic pathogens, an initial biotrophic phase occurs and, subsequently, a necrotrophic phase is activated. The hormones involved in the defense response activation against certain hemibiotrophic pathogens belonging to the genus *Fusarium* depend on the parasitized plant species (Di et al., 2016; Qi et al., 2016). Therefore, the particular accumulation of SA and JA as a response to Fusarium presence is specifically determined by the plant-pathogen interaction. In this regard, the SA, JA, and ET pathways were found to interact positively in order to activate the basal resistance against *Fusarium oxysporum* (Qi et al., 2016).

For carnation (*Dianthus caryophyllus* L.), despite the negative impact of the hemibiotrophic pathogen *Fod*, the signaling process involved in pathogen resistance still remain unknown. Therefore, the study's aims were oriented to evaluate i) the accumulation of SA, JA, and MeJA, ii) the expression levels of peroxidase enzymes from the symplast of the carnation roots and, finally, iii) the statistical correlation of these biochemical variables. The main plant defense mechanisms are expected to be activated at the cytoplasmatic level and this strategy allows to detect the earliest signaling resistance phenomena. The simultaneous evaluation of the biochemical process occurred during this interaction, both in resistant and susceptible cultivars, is an important step in the search for those processes that can ultimately be decisive for the disease resistance.

Materials and Methods

Plant material

Carnation cuttings used in our study were donated by the floriculture company Florval S.A.S. QFC headquarters. This company is located in the central part of Colombia, specifically in Gachancipá town (4°59′27″N 73°52′23″W), Cundinamarca Department. According to the Köppen–Geiger climate classification system, this town is classified as subtropical highland (Cfb), typically found in mountainous locations in some tropical countries (altitude = 2568 masl). Winters are cold and summers cool with a small annual temperature oscillation (14.5 ± 5.0 °C). The precipitations are abundant and well distributed although with a winter maximum (annual precipitation = 1493 mm).

Three-week-old carnation cuttings (*Dianthus caryophyllus* L.) of two cultivars with contrasting levels of resistance to vascular wilting named '*Golem'* (R, Resistant) and '*Solex*' (S, Susceptible) were used in this study. A *Fod* isolate obtained from carnation plants with typical wilting symptoms was used in the present study. This fungal isolate was plated on Potato Dextrose Agar (PDA) medium plates at 20 ° C in the dark until surface saturation. Genus and race were confirmed applying conventional PCR, using specific primers for both genus and race (Chiocchetti et al., 1999; Abd-elsalam et al., 2003). The inoculum was prepared in Czapek-Dox broth previously sterilized by stirring for 7 days at 25 ° C. The content of the medium was diluted with

sterile water to afford a 1.0×10^6 conidia * mL^{-1} suspension (Ardila et al., 2014).

***In vivo* assay and maintenance of plant material**

The seedling inoculation was carried out according to the previously-reported procedure (Pérez Mora et al., 2021). The main experiment consisted by 4 treatments, i.e., "*T1*" R Non-inoculated, "*T2*" R inoculated, "*T3*" S Non-inoculated and, "*T4*" S inoculated, under a completely randomized experimental design. 150 rooted seedlings for each treatment were maintained under the same conditions, i.e., sprinkling water irrigation, average temperature 20 °C, photosynthetically active radiation 5 µmol m^{-2} s^{-1}, and relative humidity 65.8%. Root sampling was carried out at different post-inoculation times (i.e., 0, 1, 2, 7 and, 14 *dpi*, *days post-inoculation*) (Ardila et al., 2014). The vascular wilting incidence was also calculated as the number of symptomatic plants from the total inoculated plants at 2 months after inoculation, expressing this relationship as a percentage (Equation 1).

$$\% \text{ Incidence} = (\text{Plants with vascular wilt symptoms/Inoculated plants}) * 100$$

Quantification of salicylic acid, methyl salicylate, and methyl jasmonate in *Fod*-inoculated and non-inoculated carnation root symplast

To obtain the root symplast from those plants comprising treatments *T1-T4*, the apoplast from the plant tissue was firstly removed by following a previously reported protocol (Martínez et al., 2017). The symplastic hydromethanolic extracts were then obtained by mixing for 15 min the respective macerated, symplast-enriched roots (0.2 g) and 80% methanol (1 mL) (Ardila et al., 2013). The homogenate was centrifuged at 12 000 g during 15 min. The resulting supernatant was concentrated using vacuum to afford the raw extract and, subsequently, the extract was redissolved in a acetonitrile/methanol/water (1:1:12, v/v/v) mixture (Floerl et al., 2012). This solution was filtered through a PTFE filter (0.22 µm), and the filtered sample (10 µL) was injected into a HPLC system coupled to a diode array detector (DAD) Shimadzu® Prominence. The separation was carried out on an RP-18 column (Synergi® (Phenomenex), 250 mm x 4.60 mm, 5 µm). The mobile phase consisted of a mixture of a phase A (HCOOH 0.1%) and a phase B (Acetonitrile 100%), using the following gradient elution: 0-3 min A 100%, 18-21 min B 7%, 40-45 min B 30%, 59-63 min B 100% and finally 66-70 min B 0%. All three hormones (SA, JA, and MeJA) were detected at 270 nm and their identification was achieved by comparing their retention time with certified standard standards (Sigma-Aldrich). The content of these compounds in the test carnation roots was calculated from a standard curve previously constructed from known concentrations of each certified standard and expressed as µg·mg^{-1} fresh plant material.

Preparation of symplast fraction for guaiacol peroxidase (1.11.1.7) activity evaluation from *Fod*-inoculated and non-inoculated carnation roots

Symplast-enriched carnation roots (0.2 g) from *T1-T4* plants were macerated with liquid nitrogen. Then, cold acetone (1 mL, -20 °C) was added to remove interferent phenolic-type compounds. After stirring for 30 s, the macerated material was centrifuged at 10,000 g for 10 minutes. This procedure was carried out twice with the complete removal of the solvent after each centrifugation. The extraction of the symplast-enriched fraction was carried out for 1 h with the buffer Na_2HPO_4-NaH_2PO_4 pH 6.5 100 mM at 4 °C. After centrifuging at 12,000 *g* for 30 minutes, the resulting symplastic fractions were stored at -20 °C for further analysis of GPX enzymatic activity (Cuervo et al., 2009).

Determination of symplastic guaiacol peroxidase activity in *Fod*-inoculated and non-inoculated carnation roots

The quantification of the guaiacol peroxidase (GPX, E.C. 1.11.1.7) activity was carried out using the spectrophotometric method to measure the oxidation of guaiacol to tetraguaiacol (Ardila et al., 2014). The results were expressed as µmol of tetraguaiacol produced min^{-1}·mg^{-1} protein. The total protein content was determined according to the linearized Bradford method (Zor and Selinger, 1996).

Evaluation of transcriptional levels of a gene coding for a symplastic peroxidase in *Fod*-inoculated and non-inoculated carnation roots using RT-qPCR

Total RNA was isolated from carnation roots (inoculated and non-inoculated) at 0, 24, 48, 168, and 336 h after inoculation, using TRIZOL reagent (Invitrogen, Madison, WI, USA) and subjected to RT-PCR (Ardila et al., 2014; Monroy-Mena et al., 2019), therefore the determination were done on cDNA. Three independent biological replicates were performed for each experiment. The expression levels were determined using the relative approximation (Pfaffl, 2001; Monroy-Mena et al., 2019). For the design of primers, a symplastic isoform of peroxidase reported in carnation (*Dca26220.1*) (http://carnation.kazusa.or.jp/) was selected. Absence of peptide signals for extracellular localization was also evaluated, according to the protocol suggested for apoplastic protein studies (Martínez et al., 2017). Designed primers from the sequence of accession number *Dca26220.1* are described in Table 1.

Table 1. Primers for real-time PCR quantification of mRNA levels for the gene encoding GPX.

Protein name	Primers	Sequence 5´-3´	Fragment size
Guaiacol peroxidase	GPX forward GPX reverse	CGCCAACACGACTAATACGA TGACAGCGAAACTCTTGACAG	161 pb
Histone	HIS forward HIS reverse	CACAGGTACCGTCCTGGAAC GTGCCAACACAGCATGACTC	160 pb

Statistical analysis

All results including phytohormone quantification (SA, MeSA, JA), GPX enzymatic activity, and transcriptional levels by RTqPCR were expressed as means ± standard deviation (SD). The normal distribution of data was checked by the Shapiro-Wilks test. Once it was verified, the data were examined by one-way ANOVA, followed by a Fisher's test ($p < 0.05$) (Ardila et al., 2014; Cuervo et al., 2009). Differences between cultivars were determined using Statgraphics® Software version 5.1. The significance of the Pearson's correlation coefficient was calculated with a ($p < 0.05$), according to Azzimonti (2003).

Results

Assessment of vascular wilting incidence for susceptible and resistant carnation cultivars

The differential resistance response of the carnation cultivars was evaluated as the vascular wilting incidence at 60 days post-inoculation (Figure 1). The *Fod*-inoculated susceptible cultivar treatment "*T4"* presented wilting symptoms in most of the plants, involving stem inclination and chlorosis at lower and upper leaves for 80% of the T4 plants. For the non-inoculated susceptible cultivar treatment "*T3"*, 8% of the cuttings presented a weak chlorosis at some lower leaves. On the other hand, the *Fod*-inoculated resistant cultivar treatment *"T2"* showed a slight wilting for 15% of the cuttings, while only 5% of the cuttings from the non-inoculated resistant cultivar treatment *"T1"* presented a weak chlorosis. The results for the non-inoculated treatments *"T1 and T3"* were not associated with the typical wilting symptoms caused by *Fod*, but were related to an adaptive outcome due to the environmental conditions of this field test. An important condition to point out is related to the testing time employed in this study in order to observe the biochemical responses, since such an evaluation was carried out up to a maximum of 14 days after inoculation, during which time the plants were healthy and vigorous.

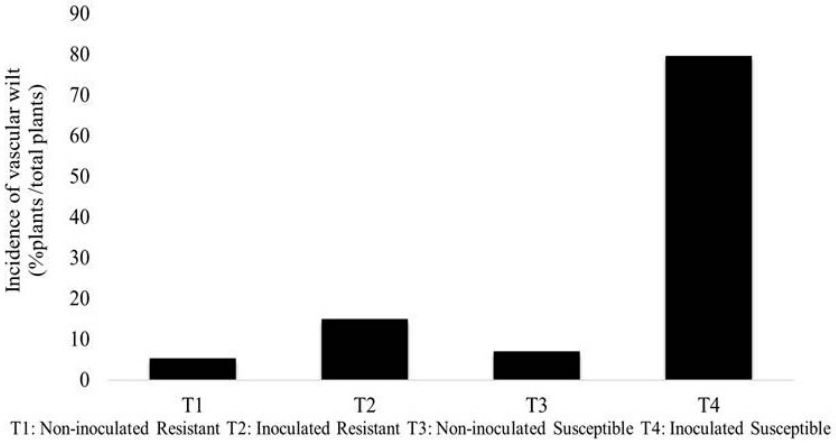

Figure 1. Incidence of vascular wilt caused by *Fod* during the *in vivo* assay. Each bar corresponds to a total of 113 plants of each test treatment.

Quantification of salicylic acid, methyl salicylate, and methyl jasmonate in root symplast from non-inoculated and *Fod*-inoculated carnation cultivars

SA accumulation in root symplast for the *Fod*-inoculated resistant treatment *"T2"* occurs at 1 and 14 dpi. The *Fod* presence seemed to increase the SA content in root symplast up to 0.18 µg SA·mg^{-1}, which corresponds to a *ca.* 2-fold content to that presented in the non-inoculated treatment *"T1"*. In contrast, concerning the *Fod*-inoculated susceptible cultivar treatment *"T4"*, there were no significant differences regarding the non-inoculated one, except for 1 dpi, when a significant SA content decrease was presented (Figure 2).

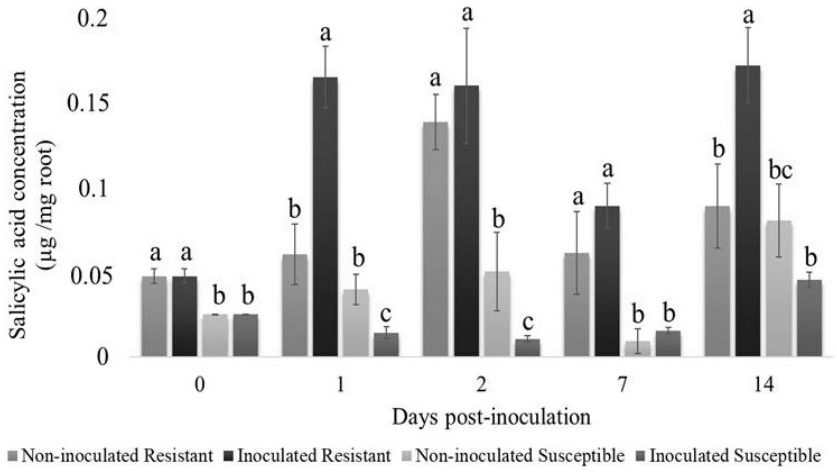

Figure 2. Salicylic acid (SA) content in carnation root symplast, during interaction with *Fod* in Resistant and Susceptible cultivars. The vertical bars correspond to the standard deviation of each mean (n = 3). Different letters indicate statistically significant differences ($p < 0.05$).

Quantification results of MeSA accumulation in the root symplast of plants infected with *Fod* are presented in Figure 3. In resistant cultivar, the inoculation caused an increase at 2 dpi of this MeSA, while a late and less significant increase at 7 dpi in the susceptible cultivar was found.

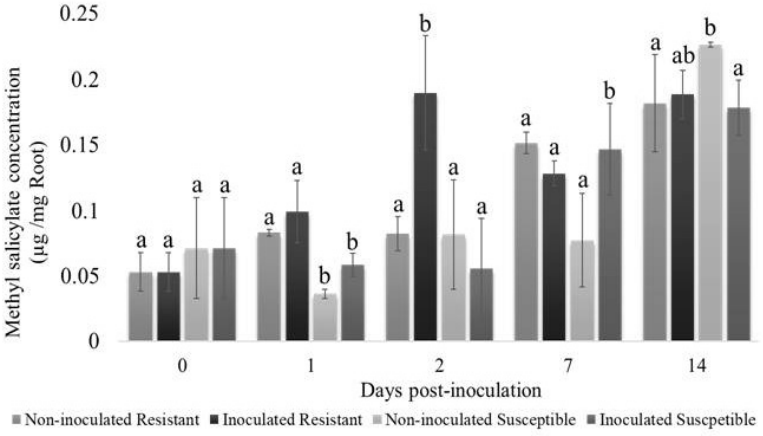

Figure 3. Concentration of methyl salicylate (MeSA) in carnation root symplast, during the interaction with *Fod* in Resistant and Susceptible cultivars. The vertical bars correspond to the standard deviation of each mean (n = 3). Different letters indicate statistically significant differences ($p < 0.05$).

The MeJA accumulation in root symplast caused by the pathogen inoculation occurred in both cultivars at 1 and 14 dpi (Figure 4). However, the levels in the resistant cultivar (4.8 µg·mg^{-1} plant material and 2.9 µg mg^{-1} plant material) were *ca.* 3-fold higher than its respective non-inoculated treatment (2.4 and 0.4 µg mg^{-1} plant material). On the other hand, the MeJA accumulation at 14 dpi in the inoculated susceptible cultivar was found to be 2-fold higher to that of the content found in the non-inoculated treatment. These results suggest that inoculation with *Fod* in the resistant cultivar *Golem* generates a greater and earlier content increase of this phytohormone than those found in the susceptible cultivar.

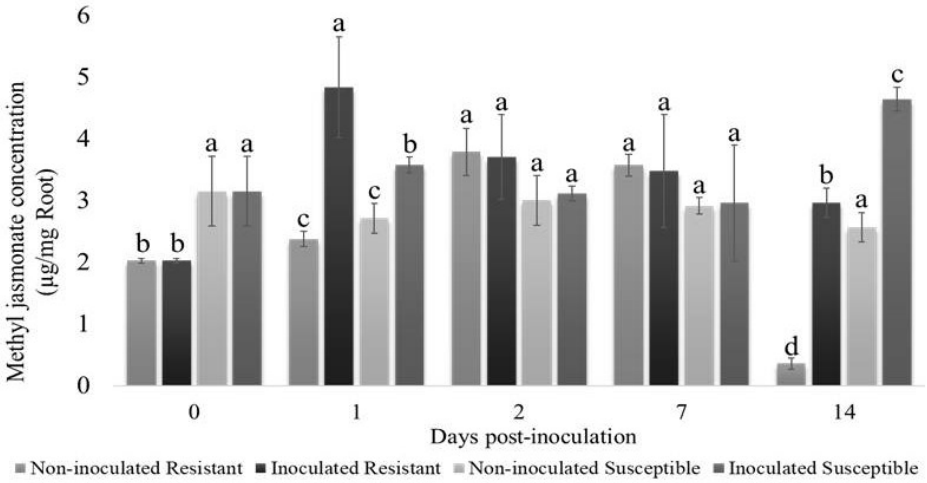

Figure 4. Concentration of methyl jasmonate (MeJA) in carnation root symplast, during the interaction with *Fod* in Resistant and Susceptible cultivars. The vertical bars correspond to the standard deviation of each mean (n = 3). Different letters indicate statistically significant differences ($p < 0.05$).

Determination of the enzymatic activity and transcriptional levels of the GPX enzyme in root symplast from non-inoculated and *Fod*-inoculated carnation cultivars

The symplastic GPX activity in carnation roots increased at 1 dpi in the inoculated resistant cultivar treatment *"T2"* (Figure 5). On the other hand, the inoculation with *Fod* in the susceptible cultivar did not generate any activity increase when compared to its control. In fact, inoculated susceptible *"T4"*, there are significantly lower values to that of its control at all test times. These results suggested that differential response between cultivars includes a significant increase of GPX activity at the root symplastic level for the resistant cultivar.

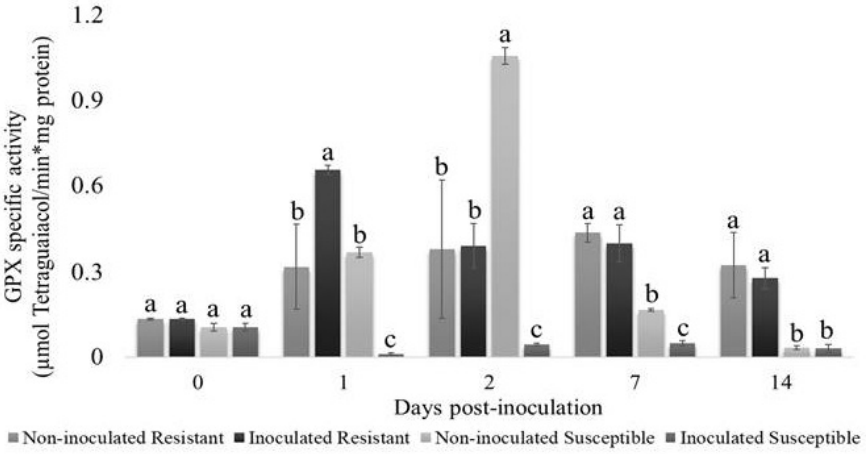

Figure 5. Activity levels of GPX in carnation root symplast during the interaction of Resistant and Susceptible cultivars with *Fod*. The vertical bars correspond to the standard deviation of each mean (n = 3). Different letters indicate statistically significant differences ($p < 0.05$).

The transcriptional analysis of the gene coding for a symplastic peroxidase by real-time PCR technique for both cultivars is shown in Table 2. For the resistant cultivar, the expression of this gene in the inoculated treatment *"T2"* exhibited a 3-fold increase over control. These differences are also presented for the other times (2, 7, and 14 dpi), but involving a lesser extent. For the susceptible cultivar, the inoculation with *Fod* caused an increase at 2 dpi (Table 2).

Table 2. Relative expression of the *Dca26220.1* gene coding for a symplastic peroxidase at the root level during the interaction of Resistant and Susceptible cultivars with *Fod*. The results are expressed as mean ± SD, obtained from 3 biological replicates and a technical duplicate. Different letters indicate statistically significant differences ($p < 0.05$).

Post-inoculation time (days)	Non-inoculated Resistant cultivar	Inoculated Resistant cultivar	Non-inoculated Susceptible cultivar	Inoculated Susceptible cultivar
0	1±0[b]	1±0[b]	1±0[a]	1±0[a]
1	13.0±1.59[c]	31.27±3.15[b]	0.11±0.04[a]	0.13±0.02[a]
2	9.27±2.43[c]	29.49±9.01[b]	0.008±0.001[b]	0.37±0.16[a]
7	0.25±0.079[c]	0.81±0.11[b]	0.11±0.009[a]	0.02±0.002[b]
14	1.30±0.41[c]	2.66±0.81[b]	0.0044±0.00026[a]	0.002±0.001[a]

A positive statistical correlation between SA and MeJA was observed. In addition, these phytohormones exhibited a significant correlation with GPX activity and mRNA levels of the gene *Dca26220.1* coding for a symplastic peroxidase. In the same way, the transcriptional levels for this gene were also correlated with the GPX enzymatic activity. In general, the susceptible cultivar did not present any significant positive or negative correlation between the evaluated parameters (Table 3).

Table 3. Pearson correlations between the test biochemical parameters in the carnation-*Fod* interaction. N variable defined by the data matrix. *Significant values ($p < 0.05$).

Parameter	Resistant cultivar				Susceptible cultivar			
	SA	MeJA	GPX	*gpx*	SA	MeJA	GPX	*gpx*
SA	1				1			
MeJA	0.5408*	1			-0.0436	1		
GPX	0.5060*	0.5719*	1		0.2473	-0.1856	1	
gpx	0.5735*	0.5657*	0.50*	1	-0.2802	-0.0483	-0.2111	1

Discussion

Understanding the signaling mechanisms involved in the resistance against plant pathogens is necessary for the development of new control strategies. In our study, two cultivars of carnation with different resistance levels to vascular wilting caused by *Fod* were inoculated with the pathogen, and endogenous levels of hormones such as SA, MeSA, and MeJA were evaluated at the root symplastic level. For the most vascular wilting pathogens, including *Fod*, the first contact point between both organisms is the root and, therefore, knowing the early responses in this plant part is essential for understanding the resistance mechanisms.

According to our results, *Fod* inoculation generated a significant increase in the phytohormones at the symplastic level of carnation roots for the resistant cultivar '*Golem*'. In particular, the accumulation of MeSA in this cultivar agrees with the reported information about other plant-pathogen models (Boba et al., 2017). This hormone accumulation was found to be at 2 dpi, one day after the SA accumulation (Figures 2 and 3). This sequential accumulation is likely presented by the activation of salicylic acid carboxyl methyltransferase (SAMT), which has been reported to play an important role in the activation of resistance responses in the plant, such as Systemic Acquired Resistance. It has been revealed that high SA levels in tobacco leaves lead to an increase of MeSA levels (Boba et al., 2017; Li et al., 2018), and this accumulation has been related to the hypersensitive response (HR) (Seskar et al., 1998; Anthony et al., 2017). According to our data and a possible hemibiotrophic *Fod* lifestyle in carnation, it is likely that the accumulation of MeSA and SA regulates the defense against *Fod* during days 1 and 2 of interaction when the biotrophic phase of the pathogen might be predominating. It has been previously demonstrated a role for this signaling pathway in the response to pathogens of the genus *Fusarium* in other plants, such as chickpea, flax, and arabidopsis (Boba et al., 2017; Bhar et al., 2018).

Jasmonic acid and ethylene signaling pathways are reported to be important in plant-pathogen interactions, acting antagonistically to the SA pathway (Hakeem et al., 2014; Di et al., 2016). In our study, there was no detectable accumulation of jasmonic acid using HPLC-DAD (results not shown), being probable since this metabolite is rapidly converted to its methyl ester (Hickman et al., 2017). However, accumulation of MeJA and SA occurred simultaneously at early times (1 dpi) in symplastic roots of the resistant cultivar ($p < 0.05$) (Figures 2 and 4, Table 3). These results agreed with pathosystems involving other specialized forms of *Fusarium oxysporum*, whose accumulation of these signaling hormones may be accumulated simultaneously (Makandar et al., 2010). In this context, the role of jasmonic derivatives varies

depending on the plant model. For instance, JA signaling attenuates the SA signaling pathway in *Arabidopsis thaliana* (Makandar et al., 2010) and, therefore, the susceptibility in the host plant. In another study, tomato seeds (*Lycopersicum esculentum* L.) treated with MeJA showed an increase in resistance against *Fusarium oxysporum* f. sp. *lycopercisi*, by increasing signaling molecules such as SA and the phenylalanine ammonium lyase enzyme (Zehra et al., 2017b). These results would suggest that resistance against *Fusarium oxysporum* pathogen is based on a multicomponent response involving both signaling pathways. Undoubtedly, the resistance against vascular pathogens is complex and should be the subject of complementary studies using other biochemical or molecular markers.

The phytohormone accumulation in carnation root symplast was statistically correlated with the expression of peroxidases using guaiacol as substrate. This is a protein associated with resistance against *Fod* (Ardila et al., 2014; Prakasha and Umesha, 2016). The peroxidase-like GPX activity at the symplastic level can be related to the detoxification of the cytoplasmic hydrogen peroxide (H_2O_2) produced due to infection with *Fod*. In carnation have shown an H_2O_2 accumulation in resistant cultivars at 48 hpi (Cuervo et al., 2009; Ardila et al., 2014). Thus, the activity of peroxidases may be necessary to regulate the H_2O_2 released by vacuoles, peroxisomes, and mitochondria (Heller and Tudzynski, 2011; Dalvi et al., 2017). H_2O_2 is a reactive oxygen species (ROS), which increase its intracellular levels as part of the signaling mechanisms activated during infection (Gulyani et al., 2018). The regulation of antioxidant species at the cytosolic level is essential to avoid cell death that can be favorable for necrotrophic and hemibiotrophic pathogens (Heller and Tudzynski, 2011). Additionally, H2O2 release by cells leads to the formation of substances with antifungal activity, activation of defense-related genes, and the regulation of ROS, which have been previously reported in this plant-pathogen interaction (Heller and Tudzynski, 2011 Ardila et al., 2014).

On the other hand, pathogen infection generated changes in the mRNA levels for the gen coding for the symplastic peroxidase (*Dca26220.1*). According to our study, transcriptional increase in the resistant cultivar at 1 dpi is statistically correlated with the GPX enzymatic activity ($p < 0.05$). This means a possible regulation at the transcriptional level for this enzyme at early times of the interaction and indicates that the isoenzyme, encoded by the sequence *Dca26220.1*, can contribute significantly to the GPX activity. However, taking into account the mRNA increase on the other days, there were no significant changes in enzyme activity at later times (Table 2). It is possibly due to the existence of post-transcriptional regulatory mechanisms that are affecting the stability, location, and/or activity of the active enzyme (Yin et al., 2019). On the other hand, when comparing the mRNA levels with the activity of GPX in the susceptible cultivar (Table 2), the results are not consistent. Post-transcriptional regulatory mechanisms would be likely presented in this cultivar to regulate the final levels of the active enzymes. Considering the high number of genes that code for peroxidases in different plants (Mika et al., 2008; Wu et al., 2019), other enzymes may be also affecting the final activity, since expression of only one of the sequences was evaluated herein.

According to the Pearson's correlations, a positive relationship between the SA and MeJA levels with the peroxidase expression is evidenced (Table 3). The expression of this type of enzyme is probably regulated by the levels of these phytohormones in the resistant cultivars of carnation during the response against *Fod*. Taking into account that plants have an antioxidant system to prevent damage caused by the ROS accumulation, it is suggested that the accumulation of MeJA and SA causes ROS homeostasis in the carnation-*Fod* model by regulating the activity of enzymes with GPX activity (Ardila et al., 2014; Wu et al., 2019). This proposal is also supported by the fact that the mRNA increase of the gene *Dca26220.1* is statistically correlated with the SA and MeJA accumulation in the resistant cultivar at the root symplastic level. These phytohormones may play a central role in the transcriptional activation of different genes associated with stress response (Chowdhury et al., 2017; Hickman et al., 2017). The activation of similar mechanisms has been documented in other plant models infected with Fusarium species, where the hormones accumulation participated within the plant defense events against infection such a kind of phytopathogens (Zehra et al., 2017a; 2017b).

Results obtained in our research suggested that the resistance activation in carnation against *Fod* involves the simultaneous accumulation of those phytohormones related to salicylic acid and jasmonic acid signaling pathways. Likewise, this role would be associated with the activation of mechanisms such as ROS homeostasis at the symplastic level, by the action of peroxidase-type enzymes, such as that encoded by the sequence *Dca26220.1*. Functional studies with carnation phytohormone-targeted knockouts will allow in the future to corroborate whether these biochemical processes are effectively affected. The use of such functional assays has granted important knowledge advances on these signaling routes in different plant-pathogen models (Makandar et al., 2010; Thatcher et al., 2016). Hence, understanding the signaling routes involved in the *Fod*-related resistance of this model will promote the future development of new control strategies of this pathogen that affects the floriculture sector worldwide.

Conclusions

In conclusion, we observed some relevant insights into the signaling process involved in carnation pathogen resistance. Hence, the following concluding remarks were disclosed: i) in general, we determined the accumulation of SA, MeSA, MeJA hormones in both cultivars. The resistant cultivar presented an early increase in the SA, MeSA levels, indicating their plausible role in the activation of defense mechanisms associated with the biotrophic phase of *Fod* , ii) the interaction with the pathogen *Fod* at the root level generated changes in the enzymatic activity

and transcriptional levels of the guaiacol peroxidase enzyme and, iii) the Pearson's correlations revealed a positive correlation among the analyzed hormones and the expression levels of peroxidase enzyme. Thus, our findings indicated that these signaling components comprised some related biochemical and molecular factors during the early carnation response against *Fod*. These considerations provided useful insights into this pathogen-plant interaction to incorporate additional alternatives for disease management (e.g., fortifying the plant defense) and minimize the loss of the respective commercial commodities.

Author contribution

LVC: Experimental activities, data curation, writing original draft. **HDAB**: supervised the research, designed the experiment, writing revision. **STMP**: Supervised the biochemical analysis experiments, writing revision. **ECB**: Supervised the chromatographic analyses, writing revision.

Acknowledgments

Authors thank Florval SAS company for supply of plant material and the DIEB of the Universidad Nacional de Colombia for financing the project with code No 41753 and Minciencias with the projects 110165842786 and 110180864094.

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