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sin Fronteras**

## Effect of phosphites on the content of total phenols and chlorogenic acid in peach-*Monilinia fructicola* pathosystem

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## Efecto de fosfitos en el contenido de fenoles totales y ácido clorogénico en el patosistema durazno-*Monilinia fructicola*

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## Efeito de fosfitos no conteúdo de fenóis totais e ácido clorogênico no patosismo de pêsego-*Monilinia fructicola*

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## Abstract

*Monilinia fructicola* is a fungal pathogen responsible for fruit brown rot in many species of the *Prunus* genus. The use of abiotic elicitors as phosphites is being studied for the induction of fruit resistance as an alternative to the use of synthetic fungicides. The aim of this work was to evaluate the effect of phosphites on the content of total phenols, chlorogenic acid and changes in susceptibility to *M. fructicola* in peach fruits. Peach fruits of the cultivars Flordaking and Elegant Lady were treated with potassium- and calcium-phosphites, and harvested at three development stages: green fruit (GF), pit hardening (PH) and harvest time (HT). After harvesting, half of them were inoculated with a conidia suspension of *M. fructicola* under laboratory conditions. Total phenols and chlorogenic acid content were assayed on the skin of the fruits. Results showed different behaviors according to the cultivar. In Flordaking, the phosphite treatment allowed an increase in the content of total phenols in the stages of GF and PH, but not for chlorogenic acid. In the presence of inoculum, chlorogenic acid decreased, but not the total phenols. The Elegant Lady did not show differences in the content of total phenols and chlorogenic acid after the phosphite treatments. Both metabolites exhibited a seasonal behavior.

**Keywords:** brown rot, *Prunus persica*, resistance inductors

## Resumen

*Monilinia fructicola* es un patógeno fúngico responsable de la podredumbre morena de la fruta en muchas especies del género *Prunus*. Se está estudiando el uso de inductores abióticos como fosfitos para la inducción de resistencia en los frutos como alternativa al uso de fungicidas sintéticos. El objetivo de este trabajo fue evaluar el efecto de los fosfitos sobre el contenido de fenoles totales, ácido clorogénico y los cambios de susceptibilidad a *M. fructicola* en los frutos de durazno. Frutos de los cultivares Flordaking y Elegant Lady fueron tratados con fosfitos de potasio y calcio, y cosechados en tres estados de desarrollo: fruto verde (GF), endurecimiento de carozo (PH) y cosecha comercial (HT). Después de la cosecha, la mitad de los frutos tratados fueron inoculados con una suspensión de conidios de *M. fructicola* en condiciones de laboratorio. Las determinaciones de fenoles totales y ácido clorogénico se analizaron en la piel de las frutas. Los resultados fueron diferentes según el cultivar. En Flordaking, el tratamiento con fosfito condujo a un aumento en el contenido de fenoles totales en las etapas de GF y PH, no así para el ácido clorogénico. En presencia del inóculo, el ácido clorogénico disminuyó, pero no los fenoles totales. Elegant Lady no mostró diferencias en el contenido de fenoles totales y ácido clorogénico después de los tratamientos con fosfito. Ambos metabolitos exhibieron un comportamiento estacional.

**Palabras clave:** podredumbre morena, *Prunus persica*, inductores de resistencia

## Resumo

*Monilinia fructicola* é um patógeno fúngico responsável pela podridão-parda dos frutos em muitas espécies do gênero *Prunus*. O uso de indutores abióticos, como os fosfitos, para a indução de resistência nos frutos, está sendo estudado como alternativa ao uso de fungicidas sintéticos. O objetivo deste trabalho foi avaliar o efeito dos fosfitos no conteúdo de fenóis totais, ácido clorogénico e alterações na suscetibilidade a *M. fructicola* em frutos de pêssigo. Frutos das cultivares Flordaking e Elegant Lady, tratados com fosfitos de potássio e cálcio, foram avaliados e colhidos em três estágios de desenvolvimento: fruta verde (GF), endurecimento do carozo (PH) e colheita comercial (HT). Do total de frutos tratados e colhidos, metade deles foram inoculados com uma suspensão de conídios de *M. fructicola* em condições de laboratório. As determinações de fenóis totais e ácido clorídrico foram analisadas na casca dos frutos. Os resultados mostraram comportamentos diferentes de acordo com a cultivar. Nos frutos de 'Flordaking', o tratamento com fosfito expressou um aumento no conteúdo de



fenóis totais nos estágios de GF e PH, porém não para o ácido clorogênico. Na presença do inóculo, o ácido clorogênico diminuiu, mas não o conteúdo de fenóis totais. Para 'Elegant Lady' não se obtiveram diferenças no conteúdo de fenóis totais e ácido clorogênico após tratamentos com fosfito. Ambos os metabólitos exibiram comportamento sazonal.

**Palavras-chave:** podridão-parda, *Prunus persica*, indutores de resistência

## 1. Introduction

*Monilinia fructicola* is a fungal pathogen responsible for fruit brown rot, blossom and twig blight in many species of *Prunus*<sup>(1)</sup>. This disease is considered as a major limiting factor in stone fruit production worldwide<sup>(1)(2)(3)</sup>. Susceptibility of freestone peach (*Prunus persica* (L.) Batsch) to infection by this pathogen has been shown to change during fruit growth<sup>(4)</sup>. Thus, peach and nectarine (*Prunus persica* var. nectarina Maxim.) fruits are prone to *M. fructicola* infection during the early stage of rapid expansion of the pericarp (stage I), and then again at harvest maturity (stage III). Developmentally intermediate stage II fruit (pit hardening) is less susceptible to infection<sup>(1)(3)(5)(6)</sup>.

Fruit of Brazilian clingstone peach Bolinha possesses a high level of brown rot resistance due to differences in the architecture of epidermal tissue and a thicker cuticle with high phenolic content<sup>(2)</sup>. The accumulation of phenolic compounds is one of the typical defense responses of plants against the pathogens attack<sup>(7)</sup>. Chlorogenic and caffeic acids are major phenolic acids in the epidermis and sub-epidermal cell layers of peach fruit<sup>(2)</sup>.

Currently, the main strategy to control diseases in fruit plants is through pre-harvest fungicide use<sup>(8)(9)</sup>. However, synthetic fungicides offer risks to human health and to the environment. In addition, certain fungicides are no longer efficient due to the emergence of resistant strains of *Monilinia* spp.<sup>(10)(11)</sup>. Thus, there is a growing need to develop alternative approaches for disease control<sup>(9)(12)</sup>, like the use of abiotic elicitors to induce the fruit resistance<sup>(8)(9)</sup>, phosphites among them<sup>(13)</sup>. Phosphites are not-phytotoxic compounds that have a high fungicidal activity<sup>(14)(15)</sup>. Their mode of action may be direct or indirect, through the activation of plant defense mechanisms by phytoalexin production<sup>(14)(16)(17)(18)</sup>.

Changes in the susceptibility of peach —*Monilinia fructicola*— pathosystem could be associated with chemical inhibitors, including phenolic compounds, and the preventive treatments with phosphites could activate plant defense mechanisms.

The aim of this work was to evaluate the effect of potassium and calcium phosphites on the content of total phenols, chlorogenic acid and changes of fruit susceptibility to *Monilinia fructicola*.

## 2. Materials and methods

### 2.1 Plant material and fungal inoculum

Peach (*Prunus persica* L. Batsch) fruits, Flordaking and Elegant Lady, from the EEA INTA San Pedro (33°44'12.7"S, 59°47'58.2"W) experimental station, were used to evaluate the effect of potassium and calcium phosphites (FFK and FFCA, respectively) in three developmental stages: I) green fruit (GF), II) pit hardening (PH), and III) harvest time (HT).

A fungal inoculum of *Monilinia fructicola* provided by the Laboratory of Phytopathology from EEA INTA San Pedro (Buenos Aires, Argentina) was cultivated on Potato Glucose Agar medium, and incubated at 25 °C, during 15 days until spore formation. Then, a suspension of  $1 \times 10^6$  conidia mL<sup>-1</sup> was prepared for fruit inoculation.

### 2.2 Experimental treatments

Phosphites were applied in the field, biweekly, from a month after full flowering till harvest, in both cultivars. Three treatments were assayed: a) 0.3%-FFK (300 mL per hL, CS 12.9% assimilable phosphorus; 16.3% soluble potassium), b) 0.3%-FFCA (300 mL per hL, CS P2O5 29.7%, Ca 12.7%), and c) control (water), in a block design with 3 replications. The day after applying the treatments, 20 fruits per



treatment of each replication were harvested and taken to the laboratory. The developmental stage of the fruits was determined based on their size by using a caliber, and on the evaluation of the pit and embryo formation, according to the ease of opening it with a knife. Ten of the harvested fruits per treatment of each replication were inoculated on the suture with 30  $\mu\text{L}$  of a conidial suspension of *M. fructicola* isolate grown on Potato Glucose Agar; while the remaining 10 fruits were not inoculated. The conidial concentration was measured with a hemocytometer, and the suspension was adjusted to the desired concentration ( $1 \times 10^6$  conidia  $\text{mL}^{-1}$ ) with sterile water. Inoculated and non-inoculated fruits were kept for 6 hours at room temperature, until they were taken to the laboratory to extract phenols.

### 2.3 Phenolic compounds determination

After treatment, samples were transferred to the Molecular Biology laboratory from Faculty of Agricultural Sciences of the National University of Rosario (Santa Fe, Argentina), where the extraction and determination of phenols were carried out based on the methodology described by Coseteng and Lee<sup>(19)</sup>.

Extracts were obtained from the skin of fruits, inoculated with *M. fructicola* under controlled laboratory conditions and without inoculation, for both cultivars.

A 50-g sample of fruit skins was homogenized in a blender with 100 mL of 80% ethanol and boiled for 5 minutes under a hood. The extract was filtered through paper and the residue was mixed with an additional 100 mL of the same ethanol solution and boiled for 10 minutes to re-extract phenolics. The extracts were combined, and the solution was allowed to cool before it was made up to a final volume of 250 mL.

The alcohol extract was diluted to obtain an absorbance reading within the range of the standards (10–100  $\text{mg mL}^{-1}$ ). One mL of the diluted extract was added to 10-mL distilled water. Two mL of Folin–Ciocalteu phenol reagent was added, then the sample was mixed, and after 5 minutes, 2-mL saturated  $\text{Na}_2\text{CO}_3$  solution was added and shaken. Absorbance at 640 nm was measured in a UV-Vis Spectrophotometer (Perkin Elmer, Ohio, USA) after

1 hour. The amount of total phenolics was calculated from a standard curve of catechol prepared at the same time.

Chlorogenic acid was determined by a modification of the procedure described by Mapson and others<sup>(20)</sup>. It was necessary to dilute the alcohol extract to obtain an absorbance reading  $<1.00$ . Two mL of 5% sodium molybdate solution in 50% ethanol was added to 10 mL of the diluted alcohol extract. After mixing, a 10 mL aliquot of the above solution was mixed with 2 mL of 50% ethanol, and the absorbance at 370 nm was read in a UV-Vis Spectrophotometer (Perkin Elmer, Ohio, USA). The concentration of chlorogenic acid was determined from a standard curve (10–50  $\mu\text{g}$  chlorogenic acid  $\text{mL}^{-1}$ ) prepared at the same time. The results were expressed as  $\mu\text{g}$  chlorogenic acid per g fresh fruit.

### 2.4 Data Analysis

Experiments were carried out in triplicate. The data were subjected to analysis of variance, and the significance of the differences between means was estimated by the Tukey's test ( $p \leq 0.01$  or  $p \leq 0.05$ ).

The entire data set that supports the results of this study is published in the article itself.

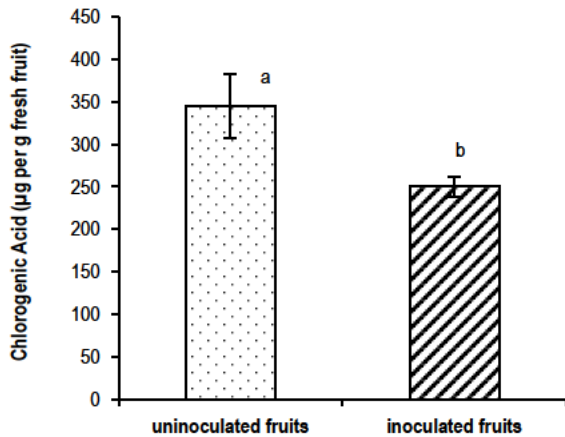
## 3. Results and discussion

The two cultivars studied showed different behavior regarding the amount of total phenols determined. The Flordaking showed differences ( $p \leq 0.05$ ) between treatments for the content of total phenols expressed as  $\mu\text{g}$  per g fresh fruit. General means were: FFca =  $374.8 \pm 47.04$  a, FFK =  $362 \pm 16.85$  a, and Control =  $306.55 \pm 58.65$  b. This effect was observed in GF and PH stages, but not in HT. On the other hand, the treatments did not express differences in the content of chlorogenic acid. The total phenol content did not vary between inoculated and non-inoculated fruits, although chlorogenic acid did, which showed a fall in the presence of the pathogen (Figure 1). This pattern was observed in the different stages of development for all treatments. The accumulation of phenolic compounds is one of the typical defense responses of plants against the attack by pathogens<sup>(7)</sup>. This behavior could be due to



the possibility that the pathogen developed attack pathways that counteract the defense of the fruit.

Figure 1. Content ( $\mu\text{g}$  per g fresh fruit) of chlorogenic acid in the skin of inoculated and non-inoculated fruits, Flordaking



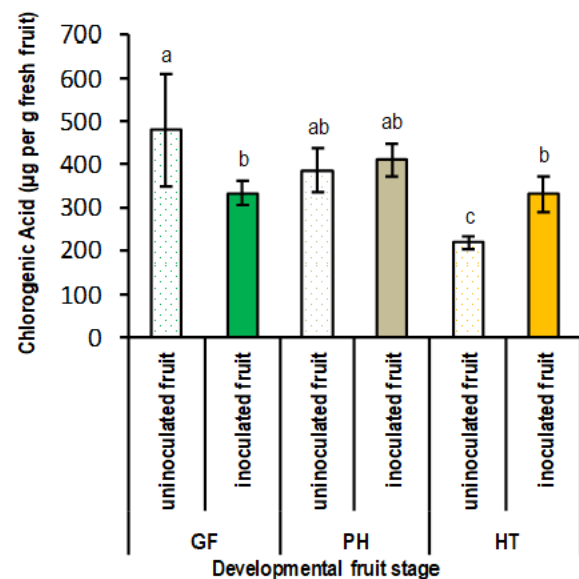
\*Values represent mean  $\pm$  S.E of the fruits treated with water, potassium and calcium phosphites, inoculated and not inoculated. Letters denote significant differences ( $p \leq 0.05$ )

The contents of total phenols and chlorogenic acid were not affected by the phosphite treatments in Elegant Lady. Total phenols in the skin of inoculated fruits ( $413 \mu\text{g}$  per g fresh fruit) decreased more than half compared to non-inoculated fruits ( $867 \mu\text{g}$  per g fresh fruit) in PH and HT status ( $p \leq 0.01$ ). In GF stage, the content of chlorogenic acid was higher in the absence of the pathogen ( $p \leq 0.01$ ); however, this effect was reversed ( $p \leq 0.01$ ) in the HT stage, unlike Flordaking (Figure 2). There are known reports in which changes in phenolic metabolism have been found after plant-pathogen interaction<sup>(7)</sup>.

Both metabolites showed significant seasonal behavior ( $p \leq 0.01$ ) for the two cultivars studied, with a peak in PH and a drop at HT (Figure 3), matching the time of lower and higher susceptibility of the fruit to the disease, respectively. Lee and Bostock<sup>(1)</sup> found higher levels of total phenols in the skin of fruits in

stage II of growth and significantly lower in harvest maturity (end of stage III). In addition, they identified chlorogenic acid as the main phenolic compound in the skin of peaches, which declined during the transition from stage II to stage III of the fruit, in agreement with the results presented in this work.

Figure 2. Content ( $\mu\text{g}$  per g fresh fruit) of chlorogenic acid in the skin of inoculated and non-inoculated fruits, in different stages of developments for the cultivar Elegant Lady: green fruit (GF), pit hardening (PH) and harvest time (HT)

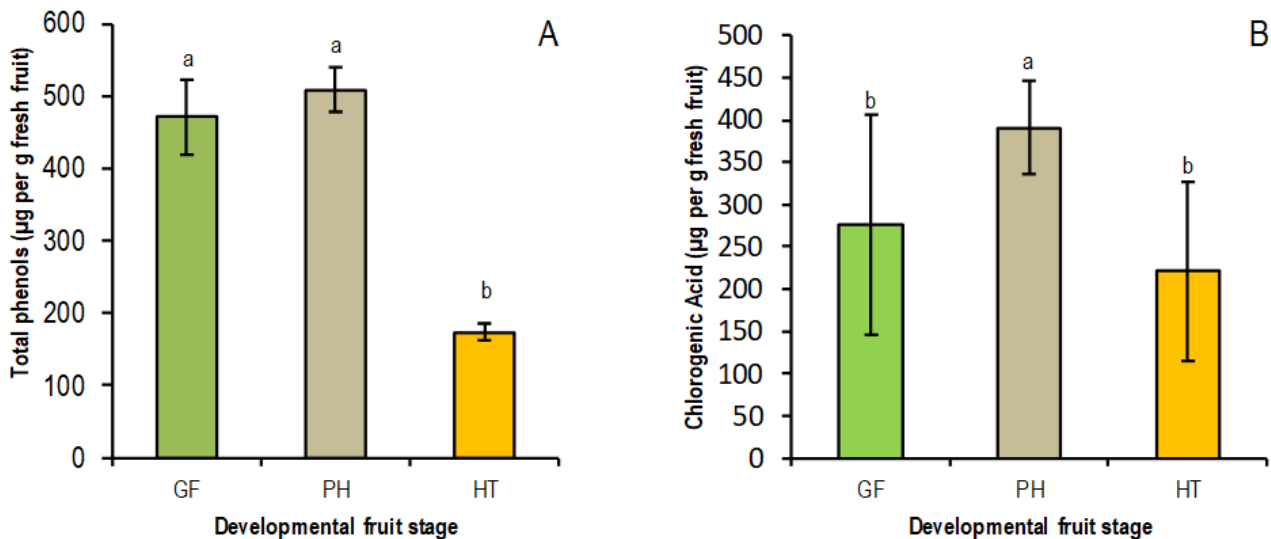


\*Values represent mean  $\pm$  S.E. Letters denote significant differences ( $p \leq 0.01$ )

Phosphites can stimulate rapid production of phytoalexins, which are important mechanisms of plant defense<sup>(14)(21)(22)</sup>. In this work, the preventive application of phosphites did not show a clear relationship regarding the content of secondary metabolites.



Figure 3. Evolution in the content of total phenols (A) and chlorogenic acid (B) ( $\mu\text{g}$  per g fresh fruit) in the fruit skin of cultivar Flordaking during its development stages: green fruit (GF), pit hardening (PH) and harvest time (HT)



\*Values represent mean  $\pm$  S.E. Letters denote significant differences ( $p \leq 0.01$ )

#### 4. Conclusions

The content of total phenols and chlorogenic acid presents a seasonal behavior, which accompanies the curve of peach fruit susceptibility to infection by *Monilinia fructicola*. However, up to now, it is not possible to confirm that this differential susceptibility is exclusively associated with the content of these chemical inhibitors.

Phosphites showed random responses in both metabolites and cultivars. We did not observe activation of defense mechanisms against preventive applications. Further investigations are necessary to define the effect of phosphites at the enzymatic level in the peach-*Monilinia fructicola* pathosystem.

#### Author contribution statement

AL conducted the experiments, collected the data, performed the formal analysis and wrote the original draft. HP and MM provided assistance and supervision in the experiments, writing and language editing.

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