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Blossom Blight Resistance in Peach: Phenotyping and Antioxidants Content in Petals

Maximiliano Dini[1,2*]
https://orcid.org/0000-0003-1118-7803

Rodrigo Cezar Franzon[3]
https://orcid.org/0000-0002-0942-9714

Maria do Carmo Bassols Raseira[3]
https://orcid.org/0000-0002-0648-5526

Bernardo Ueno[3]
https://orcid.org/0000-0002-0355-6907

Priscila Monalisa Marchi[1,4]
https://orcid.org/0000-0001-7505-1142

Marcia Vizzotto[3]
https://orcid.org/0000-0002-8071-4980

[1]Universidade Federal de Pelotas, Faculdade de Agronomia Eliseu Maciel, Programa de Pós-Graduação em Agronomia, Pelotas, Rio Grande do Sul, Brasil; [2]Instituto Nacional de Investigación Agropecuaria (INIA), Sistema Vegetal Intensivo, Estación Experimental INIA Las Brujas, Rincón del Colorado, Canelones, Uruguay; [3]Empresa Brasileira de Pesquisa Agropecuária, Embrapa Clima Temperado, Pelotas, Rio Grande do Sul, Brasil; [4]Faculdade Santo Ângelo (FASA), Agronomia, Santo Ângelo, Rio Grande do Sul, Brasil.

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*Correspondence: mdini@inia.org.uy; Tel.: +598-96256299 (M.D.)

HIGHLIGHTS

- Detailed protocol for phenotyping blossom blight susceptibility in peach.
- Could the petals content in phenolic compounds, anthocyanins or antioxidants be a new approach to estimate disease susceptibility?
- Suggestion of a possible new and practical approach to estimate peach flower reaction to *Monilinia fructicola*, enabling an indirect form of phenotyping for this trait.

Abstract: Brown rot and blossom blight caused by fungi of the genus *Monilinia* are the most important peach diseases. The increased concern with the environment and the health of workers and consumers, as well as the emergence of fungus isolates resistant to the main fungicide molecules favor control strategies such as genetic resistance. The objective of this study was to adjust a phenotyping protocol for evaluation of resistance/susceptibility to blossom blight in peach, as well as to quantify the antioxidant compounds present in the petals of these flowers and their correlation with the disease incidence and severity. The experiment was arranged in a randomized complete block split-split plot design, the plot being four concentrations of *Monilinia fructicola* conidia; the subplot two phenological flower stage; and the sub-subplot four peach genotypes. The quantification of antioxidant compounds and their correlation with susceptibility to blossom blight was performed in the four genotypes analyzed. Phenotyping was more efficient when concentrations between 400 and 4,000 conidia mL^{-1} were used, regardless of phenological flower stage. The phenolic compounds, anthocyanins and antioxidant activity are positively correlated among them, and negatively correlated with the blossom blight incidence and severity. In order to estimate the blossom blight susceptibility, it is recommended to use flowers at the pink or bloom stage, inoculum equivalent to 20-200

conidia per flower, and perform the evaluation at 96 hours after inoculation. This study suggests that more intense pink flowers have a higher content of antioxidant compounds and less blossom blight susceptibility.

Keywords: *Prunus persica* (L.) Batsch; *Monilinia fructicola* (Winter) Honey; phenolic compounds; anthocyanins; antioxidant activity.

INTRODUCTION

Brown rot is considered one of the most important diseases of the peach culture and it is mainly caused by either of three species of the genus *Monilinia* - *M. laxa* (Aderh. & Ruhl.) Honey, *M. fructigena* Honey, and *M. fructicola* (Winter) Honey – the latter being responsible for the disease in Brazil and in most of the world [1–6]. In the Americas, the fungus *M. fructicola* has the ability to cause damage during the entire peach cycle, being the blossom and fruit ripening the most susceptible stages [5,7]. The main disease symptoms are blossom blight, twig cankers and fruit rot [5,6,8] which, under conditions of high humidity and mild temperatures such as in Brazil, may be visible 48 hours after infection [6]. Symptoms begin from the first blossoms, causing necrosis and flowers death. Normally, the flowers remain attached to the twig which can be infected by the fungus, resulting in cankers and blight of one year old twigs [5,8]. The blossom blight is considered a primary infection, and has a great epidemiological importance, since it is an inoculum source for secondary fruit infections, directly by the production of conidia, or in the form of quiescent infections in developing fruits, manifesting only at the fruit ripening [5,6,8]. The brown rot may cause up of 60% in fruit losses, under hot and humid weather conditions; reduction in yield due to flower damage; loss of plant vigor by the death of buds and branches, from sprouting to harvesting; besides the high costs for its control (cultural and chemical) [3,6,8].

The increasing concern over environmental protection and consumers and workers health [9,10] together with the emergence of fungal isolates resistant to the main fungicide molecules used [11–15], emphasized control strategies such as genetic resistance, seeking to reduce the use of pesticides. That is the most effective way to control the disease, reducing production costs and environmental impact. However, despite all efforts, the selection of resistant genotypes is still limited due to the scarcity or lack of knowledge of good resistance or immunity sources [16]. In addition, there are few studies in the literature focused on flower resistance, and these indicate that there is no correlation between flower and fruit resistance to *M. fructicola* [16,17–21]. Different methodologies were tested in some studies to adjust a phenotyping protocol for blossom blight susceptibility [17,18,20,22]. Although all these studies have flaws, therefore, a phenotype protocol for this character must be adjusted in order to correctly differentiate genotypes with varying levels of genetic resistance as a tool for peach breeding programs.

The plant resistance to pathogens may be due to structural and/or biochemical mechanisms, either constitutive or induced. The structural mechanisms are physical barriers against pathogen penetration and/or colonization, whereas the biochemical mechanisms include substances capable of inhibiting (preforming) or producing (post formed) adverse conditions for its survival in host tissues, in response to its presence [23–25].

“Phenolic compounds found in the flesh and epidermis of peach fruits were found to be negatively associated with brown rot [26], particularly with sporulation, being the epidermis more effective than the flesh. Anthocyanin content has been reported in peach skin and the incidence of brown rot (-0.55), severity of the lesion (-0.48), and sporulation (-0.49) [27]. Scariotto [26] reported a wide range of anthocyanin content in the flesh and skin of numerous peach and nectarine genotypes, ranging from 0 to 18.0 and 0 to 525.1 g de Cyanidin 3-glucoside per $100g^{-1}$ of dry weight in the flesh and skin, respectively. Chaparro and coauthors [28] observed four times more cyanidin-3-glycoside (anthocyanin) in a red flower genotype than in a pink flower genotype when studying the distribution of anthocyanins in different tissues of the peach tree. The expression of genes responsible for reactive oxygen species (ROS) and hydrogen peroxide production in peach blossom petals was studied in response to host (*M. fructicola*) and non-host (*Penicillium digitatum*) fungus pathogens [29]. In that study, exogenous antioxidant application in blossoms significantly reduced blossom blight, and hydrogen peroxide accumulation was higher in response to *M. fructicola*. Similarly, applications of exogenous methyl jasmonate and salicylic acid to apricot fruits inoculated or not with *M. laxa* significantly reduced brown rot and lesion diameter [30]. Furthermore, in that same study, the lignin content, total soluble phenol content, total antioxidant capacity, phenylalanine ammonia-lyase activity, and superoxide dismutase activity in treatments with exogenous methyl jasmonate and salicylic acid were higher in inoculated and non-inoculated fruits.

Thus, the aim of this work was to adjust a protocol to perform the phenotyping of the susceptibility to blossom blight in the peach, as well as to estimate the content of phenolic compounds, anthocyanins, and

antioxidant activity in the petals of the flowers, testing their correlation with the incidence and severity to blossom blight.

MATERIAL AND METHODS

The study was conducted in the laboratories of Fruit Breeding, Phytopathology and in the Food Science and Technology of Embrapa Clima Temperado, in Pelotas, RS, Brazil, in the year 2017. Plants from the Embrapa Clima Temperado peach breeding program's active germplasm bank were used (Latitude 31º41'S, Longitude 52º26'W, altitude 57m), and the four genotypes were located in the same orchard and subjected to the same chemical and cultural management. This orchard was installed in 2002 and the trees were grafted onto the 'Capdeboscq' rootstock and spaced 2 m within rows and 5 m between rows; each genotype has three trees. The climatic conditions in the 40 days preceding the study (July 01 to August 10, 2017) were favorable for disease development, with average temperatures of 15.1°C and maximums of 28.6°C, average relative humidity of 82.5% and maximums of 97.0%, and 20 episodes of rain totaling 93.4 mm [31].

Phenotyping for blossom blight resistance

The experiment was arranged in a randomized complete block split-split plot design, with the four conidia concentrations of *M. fructicola* (CC) (0, 400, 4.000 e 40.000 conidia mL^{-1}) as the plot; two phenological flower stages (PFS) (balloon and open flower stages), the subplot; and the four peach genotypes with showy flowers (Gen) ('Bolinha', 'Eragil', 'Ônix' and Conserva 1526), the sub-subplot.

For testing the reaction to blossom blight, it was used the technique of detached flowers, cited by Fabiane [17], as the most efficient technique for this purpose. Twigs containing flower buds at half-inch green and pink stages, stages 3 and 4 according to Chapman and Catlin [32] were collected from the four genotypes, on the same date (August 11, 2017). The branches were prepared by removing the open or damaged flowers and then kept in buckets with water, inside a cold room (4±1°C), in order to standardize flowering [18], and also to reduce the contamination with pathogens [8,33]. After 48 hours in the cold room, the branches were left for another 24 hours at room temperature (20±5°C) in the laboratory. Later, the flowers with no damage and pathogen-associated symptoms were then chosen at the balloon and open stages, stages 4 and 5, respectively [32].

Plastic boxes (50 × 35 × 10 cm) with phenolic foam (Green-up®) previously washed in flowing water during 30 minutes were used, and in each cell of the foam (2.5 × 2.5 × 3.8 cm), one flower with a small portion of the twig was fixed.

The inoculation was done by spraying with a fine drop sprinkler, on August 14, 2017. Using water sensitive cards, the volume of suspension sprayed was adjusted to ensure proper flower coverage. The four conidia per mL concentrations were chosen based on the average number of conidia that would reach each flower. The conidia suspension volume used per box was 0.8 mL, and each box contained four blocks (replicates) of ten flowers from each of the four genotypes, being a total of 160 flowers per box. As a result, 400 conidia per mL was expected to correspond to about 2 conidia per flower, 4000 conidia per mL to about 20 conidia per flower, and 40,000 conidia per mL to about 200 conidia per flower.

The fungus isolates, inoculum preparation, inoculation form and incubation conditions were described by Dini and coauthors [22]. In summary, the fungus was isolated from mummified fruits infected with *M. fructicola* and collected from Embrapa Clima Temperado peach orchards. The isolates were incubated in Petri dishes with Potato Dextrose Agar (PDA) culture medium for seven to ten days in a 252°C growth chamber with 12 hours of light. The fungus` isolate was purified of contaminants using successive replicates under the same conditions. Conidia were removed from Petri dishes containing seven-day cultures of *M. fructicola* using a brush and 10 mL of distilled water. After filtering the suspension, the concentration of conidia was determined using an optical microscope and a Neubauer chamber, which was then adjusted to the concentrations used in this study.

The incidence and severity of blossom blight were evaluated at 72, 96 and 120 hours after inoculation (hai), and the flowers with necrotic spots in the petals were considered as infected. The severity was evaluated according to the scale (Figure 1), modified in relation to that proposed by Dini and coauthors [22], using the ImageJ program and photographs of flowers evaluated 72, 96 and 120 hai with *M. fructicola* under the same conditions of the experiment. The original scale had only five classes [22], and the authors mentioned the difficulty in identifying genotypes with greater or lesser susceptibility. Thus, the evaluation scale was modified to nine classes, being the first class (0) referring to the absence of symptoms of blossom blight, the following four (1 to 4) referring to necrotic spots between 1% and 20% of flower surface, and the remaining four classes (5 to 8) referring to necrotic spots > 20% of the flower surface (Figure 1).

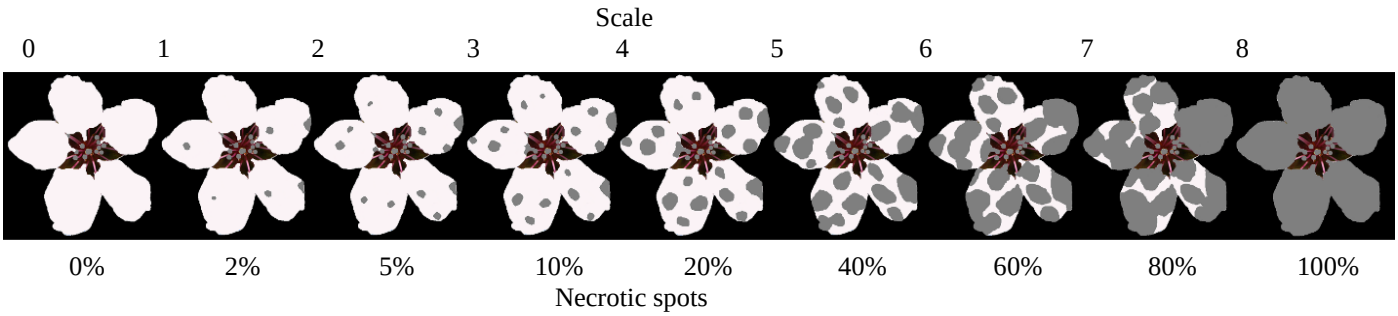

Figure 1. Scale used for the evaluation of infection and severity of blossom blight on artificially inoculated peach flowers with spray.

Infections in the anthers and/or pistil were not evaluated, because these organs are more sensitive to fungi [5,6] and present growth of other genera such as *Cladosporium*, *Penicillium*, *Alternaria* and *Botrytis* [4,6]. Furthermore, the flowers were not previously disinfected, having influence of the natural inoculum from the field.

The blossom blight incidence and severity data were transformed using arcsin $\sqrt{x}$ and $\sqrt{x}$, respectively, for the residuals to fit to the normal distribution, according to the Shapiro-Wilk test. They were then subjected to analysis of variance (ANOVA) using a split-split plot design. The means were compared with Tukey test ($p \leq 0.05$).

Determination of total concentrations of phenolic compounds, anthocyanins and antioxidant activity in petals of the peach blossom

The experiment was in a completely randomized design, where each of the four peach genotypes ('Bolinha', 'Eragil', 'Ônix' e Conserva 1526) was considered as a treatment. The flower petals were collected in the same date as for the previous experiment. Three replicates of approximately 1g of petals (fresh weight) from each genotype were used and for each of the quantifications.

In each sample, 14 mL methanol was added for phenolic compound extraction and antioxidant activity determination, and 13 mL of ethanol acidified with hydrochloric acid (85:15, 95% ethanol: 1.5N HCl) was added for anthocyanin extraction.

Total phenolic compounds were quantified based on the method adapted by Swain and Hillis [34]. The absorbance was measured at 725 nm wavelength with a spectrophotometer. Chlorogenic acid was used as the standard for the calibration curve. The concentration of total phenolic compounds was calculated and expressed in mg Chlorogenic acid equivalent per 100 g of tissue (mg CAE 100 g^{-1}).

The total anthocyanins content was quantified by the method adapted from Fuleki and Francis [35]. The reading was performed in a spectrophotometer at an absorbance of 535 nm. Cyanidin 3-glucoside was used as the standard for the calibration curve and the results were expressed as mg Cyanidin 3-glucoside equivalent per 100g of tissue (mg C3GE 100g^{-1}).

The total antioxidant activity was estimated by the technique adapted by Brand-Williams et al. [36] using the stable radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH). The absorbance was measured at a 515 nm wavelength in a spectrophotometer. Trolox was used as standard for the calibration curve and the results were expressed in µg Trolox equivalent per g of tissue (µg TE g^{-1}).

The quantified values for phenolic compounds, anthocyanins and antioxidant activity were submitted to ANOVA and the means were compared using Tukey's test ($p \leq 0.05$). These values were also submitted to the Spearman's correlation analysis, with the values referring to the incidence and severity of *M. fructicola* obtained from the previous experiment.

RESULTS

Phenotyping for blossom blight resistance

There was no significant interaction between the three factors studied (CC, PFS, and Gen) for the incidence and severity of brown rot in any of the three evaluations (72, 96, or 120 hai). Regarding the double interactions, it should be noted that the CC × Gen interaction was highly significant ($p < 0.001$) for the two parameters (incidence and severity) and in all three evaluation periods (hai). The main factor CC as well as Gen was also highly significant ($p < 0.001$).

Regarding to the averages for each genotype within each CC (Table 1), it was observed that in the evaluation performed at 72 hai, even when no inoculum was used (0 conidia mL^{-1}), high values were obtained for the average incidence of blossom blight (54.45 to 72.38%), and no significant differences between the genotypes were found.

Table 1. Incidence and severity according to conidial concentration of *Monilinia fructicola* (CC) and genotype, evaluated at 72, 96 and 120 hours after inoculation (hai).

Parameters	hai	Genotype	CC (conidia mL^{-1})			
			0	400	4,000	40,000
Incidence (%)	72	'Bolinha'	54.45 aA [1]	68.75 aA	72.23 aA	92.50 aB
		'Eragil'	60.63 aA	64.46 aA	77.81 aA	98.44 aB
		'Ônix'	72.38 aA	95.14 bB	100 bB	100 aB
		Conserva 1526	72.10 aA	93.16 bB	97.23 bB	100 aB
	96	'Bolinha'	69.45 aA	80.00 aA	83.48 aA	97.50 aB
		'Eragil'	70.00 aA	81.88 aAB	91.56 abBC	100 aC
		'Ônix'	76.19 aA	96.53 bB	100 bB	100 aB
		Conserva 1526	79.09 aA	96.11 bB	100 bB	100 aB
	120	'Bolinha'	74.18 aA	80.00 aA	84.73 aA	100 aB
		'Eragil'	79.69 aA	85.63 aA	91.43 aA	100 aB
		'Ônix'	80.48 aA	97.36 bB	100 bB	100 aB
		Conserva 1526	82.90 aA	97.5 bB	100 bB	100 aB
Severity (0 to 8) [2]	72	'Bolinha'	0.86 aA	0.93 aA	1.25 aA	1.89 aB
		'Eragil'	1.00 aA	1.13 aA	1.36 aA	3.03 bB
		'Ônix'	1.23 abA	1.83 bB	2.25 bBC	2.99 bC
		Conserva 1526	1.53 bA	2.08 bAB	2.40 bBC	2.84 bC
	96	'Bolinha'	1.46 aA	1.53 aA	1.74 aA	3.28 aB
		'Eragil'	1.56 aA	1.78 abA	2.08 aA	4.01 abB
		'Ônix'	1.91 aA	2.34 bcAB	3.05 bB	4.30 bcC
		Conserva 1526	1.84 aA	2.81 cB	3.90 cC	4.98 cD
	120	'Bolinha'	1.91 aA	1.88 aA	2.05 aA	3.93 aB
		'Eragil'	2.38 abA	2.65 bA	2.70 bA	4.63 abB
		'Ônix'	2.51 bA	2.83 bA	3.93 cB	4.89 bC
		Conserva 1526	2.75 bA	3.50 cB	4.39 cC	5.19 bC

[1] Averages followed by the same lowercase letter in the column and followed by the same capital letter in the row do not differ by the Tukey test ($p < 0.05$). [2] Scale of 0 to 8 used is shown in Figure 1.

It was not possible to differentiate the genotypes regarding the incidence of the disease when a CC of 40,000 conidia mL^{-1} was used, observing extremely high averages, between 92.5 and 100% of blossom blight incidence, indicating that the CC used was very high.

When the concentrations of 400 and 4,000 conidia mL^{-1} were used, it was possible to significantly differentiate two groups between the tested genotypes, a group of lower susceptibility ('Bolinha' and 'Eragil'), and another group with higher susceptibility to blossom blight ('Onix' and Conserva 1526), in the three evaluations (72, 96 and 120 hai) (Table 1).

Analyzing the behavior of the severity of blossom blight, the results were different from the incidence. In most cases, there were significant differences between genotypes regarding the severity at the four inoculum concentrations, at the three evaluation times. Significant differences were not detected only when no inoculum (0 conidia mL^{-1}) was used, in the evaluation performed at 96 hai (Table 1). This shows that the scale used was efficient to quantify the severity of blossom blight and differentiate genotypes.

When the concentrations of 400 and 4,000 conidia mL^{-1} were used, it was possible to significantly differentiate up to three levels among the tested genotypes in the evaluations performed at 96 and 120 hai. With 4,000 conidia mL^{-1} and, in the evaluation at 96 hai, 'Bolinha' and 'Eragil' presented less severity of blossom blight, followed by 'Onyx 'and finally Conserva 1526. In the evaluation at 120 hai, 'Bolinha' presented lower severity, followed by 'Eragil', and finally 'Onix' and Conserva 1526, the latter two being significantly different from 'Bolinha' but not from 'Eragil'.

When the CC x PFS interaction was analyzed, it was observed that it was significant in only two cases; the first was for incidence when evaluated at 72 hai, and the second for severity when evaluated at 120 hai.

In the first case, for the concentrations of 0, 400 and 4000 conidia mL^{-1} there were observed significant differences between the two phenological flower stages, with the balloon presenting a lower incidence than the open flower (Table 2). When the concentration of 40,000 conidia mL^{-1} was used and the evaluation

performed 96 and 120 hai, there was no significant difference. However, the difference observed may be due to the larger surface of the open flower, since they received a higher proportion of conidia per flower when compared to the balloons.

Table 2. Incidence and severity according to conidial concentration of *Monilinia fructicola* (CC) and phenological flower stage (PFS), evaluated at 72, 96 and 120 hours after inoculation (hai).

Parameters	hai	PFS [1]	CC (conidia mL^{-1})			
			0	400	4,000	40,000
Incidence (%)	72	Pink (4)	53.94 aA [2]	70.13 aB	81.27 aC	96.09 aC
		Bloom (5)	75.84 bA	90.63 bB	92.36 bB	99.38 aC
	96	Pink (4)	71.53	86.63	93.91	98.75
		Bloom (5)	75.84	90.63	93.61	100
	120	Pink (4)	77.02	87.74	93.21	100
		Bloom (5)	81.6	92.5	94.86	100
Severity (0 to 8) [3]	72	Pink (4)	0.86	1.14	1.48	2.44
		Bloom (5)	1.44	1.84	2.15	2.93
	96	Pink (4)	1.59	2.03	2.60	4.11
		Bloom (5)	1.79	2.19	2.78	4.18
	120	Pink (4)	2.22 aA	2.84 aB	3.30 aB	4.75 aC
		Bloom (5)	2.56 aA	2.58 aA	3.23 aB	4.56 aC

[1] Phenological flower stage: pink (4) and bloom (5) stages of the phenological classification of Chapman and Catlin [32]. [2] Averages followed by the same lowercase letter in the column and followed by the same capital letter in the row do not differ by the Tukey test ($p < 0.05$). [3] Scale of 0 to 8 used is shown in Figure 1.

Comparing the CCs tested in each PFS, it is observed that the treatments in which inoculum was used were always different from the treatment without the use of inoculum, and that when 40,000 conidia mL^{-1} was used, the average incidence of blossom blight was very high (between 96.09 and 100%).

The severity of the disease presented significant differences when evaluated at 120 hai, due to the CC factor (Table 2). There were no significant differences between the two PFS within each CC level. Comparing the severities between the CCs tested in flowers at balloon and bloom stages, it was observed that in both PFS, the severity for 40,000 conidia mL^{-1} was higher than all other CCs. Regarding the flower stage, there were no differences between the uninoculated flowers and those with 400 mL^{-1} conidia.

Analyzing the interaction PFS x Gen it is observed that the incidence was significant when evaluated at 120 hai. Comparing the two PFS, it was only significant in the Eragil cultivar, presenting a higher incidence in open flower PFS (93.13%) compared to the floral bud (85.24%) (Table 3).

Table 3. Incidence and severity according to suspension genotype and phenological flower stage (PFS), evaluated at 72, 96 and 120 hours after inoculation (hai).

Parameters	hai	PFS [1]	Genotype			
			'Bolinha'	'Eragil'	'Ônix'	Conserva 1526
Incidence (%)	72	Pink (4)	64.38	63.17	87.51	86.38
		Bloom (5)	79.59	87.5	96.25	94.86
	96	Pink (4)	84.38	83.59	90.11	92.74
		Bloom (5)	80.84	88.13	96.25	94.86
	120	Pink (4)	85.42 aA [2]	85.24 aA	92.67 aB	94.64 aB
		Bloom (5)	84.03 aA	93.13 bB	96.25 aB	95.56 aB
Severity (0 to 8) [3]	72	Pink (4)	1.11 aA	1.23 aA	1.74 aB	1.84 aB
		Bloom (5)	1.35 aA	2.03 bB	2.41 bBC	2.58 bC
	96	Pink (4)	2.16 aA	2.22 aA	2.71 aB	3.24 aC
		Bloom (5)	1.84 aA	2.49 aB	3.09 aC	3.53 aC
	120	Pink (4)	2.71 bA	3.06 aB	3.43 aBC	3.91 aC
		Bloom (5)	2.17 aA	3.11 aB	3.64 aC	4.01 aC

[1] Phenological flower stage: pink (4) and bloom (5) stages of the phenological classification of Chapman and Catlin [32]. [2] Averages followed by the same lowercase letter in the column and followed by the same capital letter in the row do not differ by the Tukey test ($p < 0.05$). [3] Scale of 0 to 8 used is shown in Figure 1.

In the severity parameter, the three evaluations showed significant differences (Table 1). Mainly due to the effect of the genotype not to the PFS (Table 3). In the most cases the cultivar Bolinha presented the lowest severities and the selection Conserva 1526 the highest.

Phenolic compounds, anthocyanins and antioxidant activity, and their correlation with blossom blight

The four tested genotypes presented variability regarding the total content of phenolic compounds, anthocyanins and antioxidant activity in its flower petals. The phenolic compounds content was higher in the cultivar Bolinha, with an average of 1356.0 mg CAE 100 g^{-1}, without presenting significant differences with 'Eragil' (1299.5 mg CAE 100 g^{-1}). 'Ônix' e Conserva 1526 presented the lowest mean values, with 1171.1 and 1155.9 mg CAE 100 g^{-1} respectively. These last two genotypes also did not present significant differences with 'Eragil' (Figure 2A).

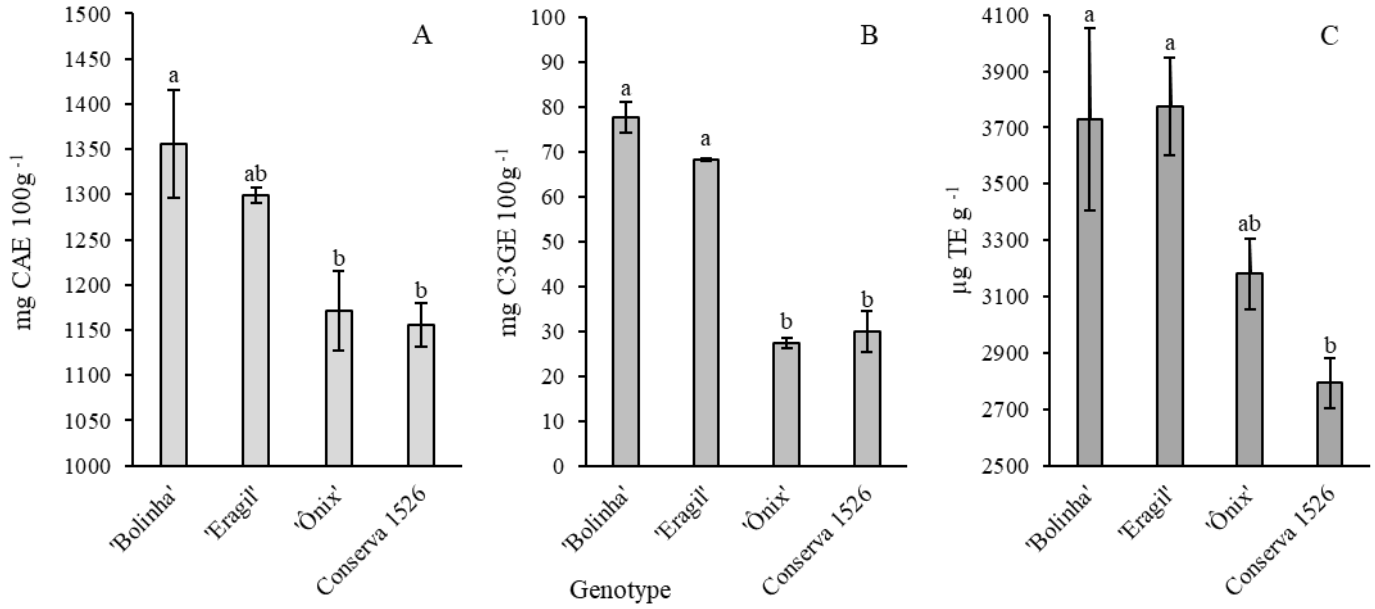

Figure 2. Means of total phenolic compounds (A) expressed as mg Chlorogenic acid equivalent per 100 g of tissue (mg CAE 100 g^{-1}), total anthocyanins (B) expressed as mg Cyanidin 3-glucoside equivalent per 100 g of tissue (mg C3GE 100g^{-1}), and total antioxidant activity (C) expressed as µg Trolox equivalent per g of tissue (µg TE g^{-1}) of the flower petals of four peach genotypes. Columns with the same letter do not differ by the Tukey test ($p < 0.05$); the bars represent the standard error of the mean.

For the anthocyanin content, the cultivars Bolinha and Eragil presented the highest averages, 77.7 e 68.3 mg C3GE 100g^{-1}, respectively (Figure 2B). The four genotypes have showy flowers but differ in the intensity of the pink color of their petals, being 'Bolinha' and 'Eragil' darker than 'Onyx' and Conserva 1526, which was confirmed by the difference in the anthocyanins content found.

The antioxidant activity estimates were higher in Eragil Bolinha and Onyx cultivars (3775.0, 3729.1 and 3180.3 µg TE g^{-1}, respectively), however, 'Onix' did not differ in relation to Conserva 1526 (Figure 2C).

When the correlations between the three compounds quantified in the petals were tested, high positive correlations were observed, 0.87 between phenolic compounds and anthocyanins, 0.83 between phenolic compounds and antioxidant activity, and 0.70 between anthocyanins and antioxidant activity (Table 4). In the present study, the PFS was not significant to quantify the blossom blight susceptibility. Thus, PFS was not considered when the correlations were tested. Spearman correlations and their significance (p-value) for the incidence and severity data, evaluated at 96 hai, are presented. Evaluations at 72 and 120 ha had similar values, however, the absolute values in the correlations were lower (data not shown).

Table 4. Spearman´s correlation between phenolic compounds (PC), anthocyanins (Ant), antioxidant activity (AA), incidence (Inc) and severity (Sev) of blossom blight.

		PC	Ant	AA	0 [1]		400		4,000		40,000	
					Inc [2]	Sev	Inc	Sev	Inc	Sev	Inc	Sev
PC		-	*** [4]	**	ns	ns	***	***	***	***	ns	*
Ant		0.87 [3]	-	**	*	*	***	***	***	***	ns	*
AA		0.83	0.70	-	ns	ns	**	**	**	***	ns	*
0	Inc	-0.49	-0.52	-0.41	-	ns	ns	*	*	*	ns	*
	Sev	-0.36	-0.52	-0.24	0.56	-	ns	ns	*	*	ns	*
400	In	-0.83	-0.74	-0.70	0.28	0.36		***	***	***	ns	*
	Sev	-0.85	-0.75	-0.65	0.59	0.39	0.81	-	***	***	ns	**
4,000	Inc	-0.82	-0.89	-0.68	0.61	0.64	0.77	0.83	-	***	ns	***
	Sev	-0.82	-0.83	-0.77	0.62	0.54	0.75	0.80	0.91	-	ns	***
40,0000	Inc	-0.36	-0.36	-0.20	0.34	-0.14	0.11	0.23	0.21	0.28	-	ns
	Sev	-0.69	-0.72	-0.65	0.6	0.52	0.57	0.67	0.78	0.85	0.42	-

[1] 0, 400, 4.000 and 40.000 = number of *Monilinia fructicola* conidia mL^{-1}. [2] Incidence and severity of blossom blight correspond to the evaluation 96 hours after inoculation for 'Bolinha', 'Eragil', 'Ônix' and Conserva 1526 peach genotypes. [3] In the lower diagonal the Spearman´s correlation value. [4] In the upper diagonal: ns, *, **, ***; nonsignificant and significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively.

All correlations between incidence and severity of blossom blight and antioxidant compounds in the petals were negative (Table 4). When the concentrations of 400 and 4,000 mL^{-1} conidia (more effective concentrations to test the susceptibility of this disease, according to the previous experiment) were used, the correlations ranged between -0.65 and -0.89, and were all significant.

DISCUSSION

Phenotyping for blossom blight resistance

This high incidence of blossom blight even without previous inoculation has already been reported [18,37] and is due to the high pressure of the inoculum naturally present in the peach orchards in southern Brazil at the flowering time [3-5].

Blossom blight varying between 0 and 100%, evaluated at 72 hai and without previous inoculation, was observed by Santos and coauthors [18]. These differences were attributed, besides the resistance/susceptibility, to the temporal (flowering date) and spatial (geographical position within the orchard) differences of the different genotypes. In the same study [18], cultivar Bolinha had a 30 to 50% incidence of blossom blight when no inoculum was used, which is a similar incidence to that obtained in the present study (between 30 to 70%) when the evaluation was at 72 hai.

This suggests that for a more detailed study about genetic resistance to this disease, would be necessary to keep the plants under controlled conditions, free from the presence of the natural inoculum, or to use a more efficient technique for twig disinfection before flowering and to cover them until its use. Thus, it might be possible to decrease the environmental effect and more accurately assess the genetic portion of the phenotypic expression [38,39].

According to some studies, the Bolinha cultivar has a high incidence of blossom blight (between 62 and 100%) when inoculated with *M. fructicola* [4,5,18], even though it has interesting resistance in the fruits. The mean incidence values in 'Bolinha' were similar in any of the three CC tested in the current study. (Table 2). It might not be among the most resistant genotypes if a larger number of genotypes were used, because the averages were so high. Previous studies identified genotypes with a much lower average incidence, despite the fact that the conditions and inoculation technique were not directly comparable. Outstanding genotypes with lower averages include 'Jubileu' and Conserva 930 [18], Conserva 1070 and Conserva 1055 [17], 'Magno', 'Leonense', and four other selections from the Embrapa peach breeding program [20].

This absence of significant effects due to the effect of the PFS (Tables 2 and 3), justifies the use of flowers in any of the two tested phenological stages and validates the previous works that used flowers at balloon stage [17,18,20] and those that used open flowers [17,21,22,37].

In summary, after analyzing all the possible combinations and the factors that present greater effects regarding the blossom blight susceptibility in the peach. It is suggested as a protocol for the phenotyping of this character, the use between 400 to 4,000 conidia mL^{-1} (20 to 200 conidia per flower - depending on whether the phenotyping is done in the absence of natural inoculum or not), with flowers in the balloon stage and/or open flowers, and make the evaluations preferably at 96 hai.

Phenolic compounds, anthocyanins and antioxidant activity, and their correlation with blossom blight

The total content of phenolic compounds has been linked to antioxidant activity in flowers of various species [40]. In our study, the content of phenolic compounds and anthocyanins, as well as antioxidant activity, were found to be positively correlated. In turn, the Bolinha and Eragil cultivars had the highest values for these three parameters, being that they present darker pink petals when compared to the Onix cultivar and the Conserva 1526 selection. This suggests that the darker the intensity of the pink color of the petals, the higher the content of phenolic compounds and antioxidant activity, since anthocyanin accumulation is responsible for the color of peach flowers [28,41,42].

Phenolic compounds including anthocyanins content present in the flesh and epidermis of peach fruits were negatively associated with the incidence and severity of brown rot [26,27]. In other stone fruit trees, such as apricots, the incidence of brown rot and the diameter of the lesion also were both negatively related to the content of phenolic compounds and antioxidant activity [30]. It was also reported a significant reduction in blossom blight with exogenous application of antioxidants during flowering [29]. There were no exogenous compound applications in our study, but blossom blight was lower in genotypes with higher levels of phenolic compounds, anthocyanins, and antioxidant activity.

These findings imply that blossom blight susceptibility may be indirectly selected. The susceptibility to blossom blight can be reduced by selecting flower petals with high levels of phenolic compounds, anthocyanins, and/or antioxidant activity. This is critical for peach breeding programs, particularly where blossom blight is a problem and genetic resistance is the best way to avoid the disease. Based on the intensity of the pink color of the petals, a much faster phenotyping may be suggested, but this hypothesis must be proven in studies with a larger number of genotypes and evaluation over several harvest seasons.

CONCLUSION

Phenotyping for blossom blight was more efficient when a concentration between 400 and 4,000 conidia mL^{-1} was used, regardless of the phenological state of the flower, whether balloon or open flower.

The scale used in this study, was efficient to quantify the severity of blossom blight.

For phenotyping the blossom blight susceptibility, it is recommended to use flowers at balloon and/or open stages, and a fungus suspension equivalent to 20-200 conidia of *M. fructicola* per flower, performing the evaluation at 96 hours after inoculation.

The total contents of phenolic compounds and anthocyanins, in addition to the antioxidant activity, are positively correlated with each other, and negatively correlated with the incidence and severity of flower blight.

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