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BASIC AREA - Article

Genotypic differences in cyanogenic glycosides levels of compatible *Prunus persica P. persica* and incompatible *P. persica P. mume* combinations

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ABSTRACT: Graft incompatibility is a phenomenon associated with complex physiological, biochemical, and genetic interactions between scion and rootstock. The main objective of this work was to assess the role of cyanogenic glycosides (CGs), amygdalin and prunasin, in the graft incompatibility of *Prunus* and possible biochemical effects in compounds of the phenylpropanoid pathway. Graft compatibility, amygdalin and prunasin content, phenylalanine ammonia-lyase activity, total phenolic compounds content and antioxidant activity, were studied in different graft combinations (Chimarrita/Capdeboscq; Chimarrita/Tsukuba 1; Chimarrita/Umezeiro; Maciel/Capdeboscq; Maciel/'Tsukuba 1; Maciel/Umezeiro) and ungrafted genotypes. The results indicate that there was graft incompatibility of Chimarrita and Maciel cultivars grafted into Umezeiro rootstock. Combinations identified as incompatible showed higher prunasin concentration and phenylalanine ammonia-lyase (PAL) activity in rootstock and greater concentration of total phenolics compounds and antioxidant activity in scion and rootstock. The results indicate that large differences in CGs concentration, especially prunasin, can be the graft incompatibility cause between *Prunus persica* and *P. mume*. The prunasin concentration may be considered a promising marker to predict graft compatibility between *P. persica* and *P. mume*.

Key words: graft incompatibility, amygdalin, prunasin, phenylalanine ammonia-lyase, oxidative stress.

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INTRODUCTION

The need for increasing peach adaptation to marginal areas is growing, especially due to adaptation to environmental changes, such as deficit of chilling hours and water. One central tool to improve adaptation is grafting, main among different species of *Prunus*. Like peach-almond hybrids (*Prunus amygdalus* × *P. persica*) largely used as rootstocks for peach trees in the Mediterranean countries (Mestre et al. 2015). On the other hand, interspecific graft might result in grafting incompatibility, as happens between peach/Japanese apricot (Telles et al. 2009), peach/Myrobalan plum (Moreno et al. 1993) and peach/nectarine (Zarrouk et al. 2006).

In general, this phenomenon is associated with disorders in anatomical, genetic, physiological, or biochemical interactions between scion and rootstock. Given the complexity of such interactions, early diagnosis is extremely difficult, and in most cases, no external symptoms are observed for several years (Pina and Errea 2008; Zarrouk et al. 2006, Pina et al. 2012; Pereira et al. 2014a).

Graft incompatibility has been classified into two types: translocated and localized (Errea 1998; Zarrouk et al. 2006). In the translocated incompatibility, degeneration of the sieve tubes and phloem companion cells is observed in the graft union region, causing translocation problems associated with leaf senescence and development of the radicular system. This graft-incompatibility is typically observed in peach grafted on Myrobalan plums (Moreno et al. 1993) where visual symptoms (symptoms of yellowing and reddening in leaves and wood, premature defoliation, early growth cessation, and acute leaf curl) can be usually observed during the first two growing seasons. The localized incompatibility, in turn, displays malformation in graft union due to physiological and morphological alterations resulting in impaired and even fractured union after several years of grafting (Errea 1998). The alterations include functionality disturbances in newly formed vascular cambium, lack of tissue differentiation, imperfect lignification and lack of vascular continuity, which might cause rupture of the graft union (Errea 1998, Zarrouk et al. 2006; Zarrouk et al. 2010; Pina et al. 2012).

Although the events involved in the grafting process are known, the mechanisms by which the incompatibility is expressed remain unclear (Pina and Errea 2008; Calatayud et al. 2013; Pereira et al. 2014a). Some authors attribute the incompatibility between pear and quince trees (Gur et al. 1968; Moore 1986; Nocito et al. 2010) and between peach and almond trees (Gur and Blum 1973; Pereira et al. 2014a) to the cyanide release in the graft interface. This process, known as cyanogenesis, occurs by the hydrolysis of cyanogenic glycosides (CGs). These compounds, mainly amygdalin and prunasin, are present in approximately 2500 species of plants, notably in the Rosaceae family (Vetter 2000). In general, such plants use their cyanogenic ability as a natural defense mechanism against herbivores and pathogens (Zagrobelny and Moller 2011). Cyanogenic-dependent incompatibility occurs through the hydrolysis of CGs by β-glucosidase, thereby which results in the release of cyanide (Conn 1980). Thus, grafting between genotypes with high differences in CG concentrations might result in incompatibility problems (Gur et al. 1973; Moore 1986, Pereira et al. 2015). Other substances related to graft incompatibility studies are phenolic compounds and enzymes involved in CG synthesis. Alterations in the profile of these compounds have been shown to be associated with the quantitative and qualitative differences exhibited among compatible and incompatible genotypes in the main stages of graft formation (Errea 1998; Usenik et al. 2006; Pina and Errea 2008; Zarrouk et al. 2010; Hudina et al. 2014; Irisarri et al. 2015; Irisarri et al. 2016).

Nocito et al. (2010) previously indicated the possibility of this relation of cause and consequence in their study about graft incompatibility between pear (*Pyrus communis* L.) and quince (*Cydonia oblonga* Mill.). The incompatibility possibly caused by the higher cyanogenic potential of quince compared to pear led to oxidative stress by the accumulation of reactive oxygen species (ROS), inducing the defense metabolism based on the increase in enzyme activity of the phenylpropanoid pathway. This way, *Prunus* grafts formed by a genotipe with high concentration of CGs and another with low catabolism capacity of these compounds, may be incompatible.

Thus, the main objective of this work was to assess the role of cyanogenic glycosides, amygdalin and prunasin, in the graft incompatibility of *Prunus persica*/*Prunus mume* and possible biochemical effects in compounds of the phenylpropanoid pathway.

MATERIAL AND METHODS

Plant material

The study was performed in Chimarrita (*Prunus persica* L. Batsch) and Maciel (*P. persica* L. Batsch) peach

cultivars grafted into three rootstocks: the peaches, 'Capdeboscq' (*P. persica* L. Batsch) and 'Tsukuba 1' (*P. persica* L. Batsch), and the apricot 'Umezeiro' (*Prunus mume* Sieb. et Zucc.), as well as in the ungrafted genotypes. The cultivars and rootstocks materials were collected from the *Prunus* collection, maintained at the Federal University of Pelotas (Pelotas, Brazil). The buds of two cultivars were T-budding in situ on the three rootstocks (obtained by seeds) on November 2007.

The orchard was established in October 2008, with density of 1,333 plants·ha^{-1} (5 m × 1.5 m) and Y-shaped tree form. Samples were collected in the winter (2009) and summer (2010). The research was conducted in an Udult soil at Federal University of Pelotas, Brazil (lat 31°48'12.25"S, long 52°30'41.09"W).

Evaluation of graft compatibility

Translocated incompatibility was evaluated by estimating the chlorophyll content using a Soil Plant Analysis Development meter (Chlorophyll Meter SPAD-502 Konica-Minolta, Osaka, Japan) according to Zarrouk et al. (2006) and Calatayud et al. (2013). The measurements were made on upper side of 50 leaves (on the upper and lower sides), on Feb. 2010, approximately 150 days after full bloom. Localized graft incompatibility was evaluated by anatomical analysis of the graft interface (Herrero 1951; Zarrouk et al. 2006) as follows: class A = perfect union; class B = satisfactory union; class C = discontinuous union in the bark; class D = unions with vascular discontinuity; and class E = union rupture in the orchard or nursery conditions. Combinations A, B and C were considered compatible, whereas D and E were incompatible. The evaluation was made on plants sampled on Aug. 2011, three years and four months after grafting. Three graft unions per plot were cut 5 cm above and below of the union region. Each graft union (100-200 mm in diameter) was cut transversally into two slices to evaluation.

Determination of cyanogenic glycosides (CGs) concentration

Bark and cambium (5-10 g) fresh samples were excised from the scion (5 cm above the union) and rootstock (5 cm below the union) of 20-month (June = second dormancy period trees) and 28-month-old trees (February = second-growing vegetative station). Samples were immediately frozen in liquid nitrogen, stored at – 80 ºC and subsequently lyophilized. The extraction of CGs was performed using 500 mg of lyophilized samples, with 8 mL of pure methanol and 2 g of activated carbon. This mixture was agitated for 16 h at room temperature. Then, centrifugation was performed at 2,500 *g* for 20 min, and the supernatant was vacuum filtered using a 0.1-µm nylon filter. Twenty microliters of the total volume filtered were submitted to chromatographic analysis. The concentrations of the CGs amygdalin and prunasin were determined using high-performance liquid chromatography (HPLC). HPLC-specific prunasin and amygdalin standards (Sigma-Aldrich, Sigma-Aldrich Corporation, United States), activated carbon, and methanol were used for the analyses. Water filtered using a 0.1 µm nylon filter (Milli-Q water purification system; Millipore) was used for the experiments. An Inertsil ODS-3 (150 × 4.6 mm, 4 µm) analytical column and its equivalent pre-column were used for analyses at 25 °C. The mobile phase used was water–methanol (60:40, v/v) with a flow rate of 1.3 mL·min^{-1}, and UV detection at a wavelength of 254 nm. The results were expressed in milligrams per gram (mg·g^{-1}) of the CG of dry weigth.

Determination of phenylalanine ammonia-lyase activity

The phenylalanine ammonia-lyase (PAL) activity was determined according to the methods described by Hyodo et al. (1978) and modified by Campos et al. (2003). For the extraction, 500 mg of fresh bark tissue was collected, macerated using a micro-triturator (at ± 4 °C) with 4 mL of 50 mM sodium borate buffer (pH 8.5) containing 25 g·L^{-1} of polyvinylpyrrolidone and 4 mL·L^{-1} of mercaptoethanol. After maceration, another 4 mL of borate buffer was added and centrifuged at 4,000 g for 30 min (0 °C). Subsequently, 0.06 mL of phenylalanine and 4 mL of 50 mM sodium borate buffer (pH 8.5) were added to a 0.25 mL aliquot, and this mixture was incubated for 60 min (40 °C). The readings were performed at 290 nm by using a UV spectrophotometer (Model UV-1601 PC; Shimadzu Corporation, Japan). The enzymatic activity was expressed according to the amount of enzyme (µmol) used for producing 1 mm of

trans-cinnamic acid (Sigma-Aldrich, Sigma-Aldrich Corporation, United States) per minute per gram of fresh tissue (μmol·min^{-1}·g^{-1}). The analyses were performed in four biological replicates and two analytical replicates.

Determination of total phenolic content

Determination of total phenolic content in the bark tissues from scions and rootstocks in the grafted combinations and ungrafted genotypes was analyzed according to Swain and Hillis (1959), modified by Kosuge (1969). Three solvents were used: water, water-methanol (50:50, v/v), and methanol. The concentration of phenolic compounds was calculated using a standard curve of gallic acid (Sigma-Aldrich, Sigma-Aldrich Corporation, United States). The results were expressed in grams of gallic acid per 100 g of product (g·100 g^{-1}). The analyses were performed in four biological replicates and two analytical replicates.

Determination of antioxidant activity

The antioxidant activity of phenolic extracts was determined according to Brand-Williams et al. (1995). Briefly, 0.5 g of bark tissue was extracted using 20 mL of methanol (Sigma-Aldrich, Sigma-Aldrich Corporation, United States), 2.8 mL of DPPH (2,2-diphenyl-1-picryl-hydrazyl) and 0.17 mL of methanol. The reaction was produced during 3 h at room temperature, protected from light. The optical density was measured in a spectrophotometer (Model UV-1601 PC; Shimadzu Corporation, Japan) at 515 nm. The antioxidant activity was expressed as mM of trolox equivalent antioxidant capacity (TEAC) in DW (mM TEAC·g^{-1}). The analyses were performed in four biological replicates and three analytical replicates.

Statistical analysis

Data were evaluated by analysis of variance and Tukey's multiple range test by the statistical program WinStat 2.11 (Machado and Conceição 2005).

RESULTS AND DISCUSSION

In this study, we evaluated combinations that presented or not symptoms of grafting incompatibility and related the occurrence or not of this phenomenon to the levels of cyanogenic glycosides, PAL activity and concentration of phenolic compounds from cultivar and rootstock of each combination.

According to SPAD readings, no translocated incompatibility was detected (Table 1). However, the anatomical analyses indicated severe vascular discontinuity in peach/*P. mume* combinations (Chimarrita/Umezeiro and Maciel/Umezeiro). These grafts were diagnosed with localized graft incompatibility Class D (Figure 1c,f). On the other hand, in peach/peach grafts (Chimarrita/Capdeboscq, Maciel/Capdeboscq, Chimarrita/Tsukuba 1 and Maciel/Tsukuba 1) good continuous vascular union were observed, and were classified as compatible grafts Class A (Table 1 and Figures 1a,b,d,e). Previous studies also identified similar compatibility problems between peach and *P. mume* (Comioto et al. 2012, Telles et al. 2009, Pereira et al. 2013; Pereira et al. 2014b). Pereira et al. (2015), also suggested that other graft incompatibility symptoms between peach and Japanese apricot are higher susceptibility of plants to *Xanthomonas arboricola* pv. Pruni, lower stem diameter above and below of union, besides of lower leaf area and fruiting. These results →

Table 1. SPAD values and internal examination of the graft unions between the peach cultivars Chimarrita and Maciel grafted on the Capdeboscq, Tsukuba 1 and Umezeiro rootstocks.

Combination	SPAD values (Translocated incompatibility)	Incompatibility category** (Localized incompatibility)
Chimarrita/Capdeboscq	37.05 ± 1.04 ^{ns} *	A
Chimarrita/Tsukuba 1	37.05 ± 0.36	A
Chimarrita/Umezeiro	37.59 ± 0.41	D
Maciel/Capdeboscq	36.28 ± 0.40	A
Maciel/Tsukuba 1	36.67 ± 0.28	A
Maciel/Umezeiro	37.64 ± 1.07	D

*Mean separation within lines by Tukey's multiple range tests at $p < 0.05$. ^{ns} Means not significantly different at the $p < 0.05$ level of significance (Tukey's multiple test). Means ± SE, n = 4; **Categories A, B, C, D, and E: classification of the rating of 'localized' graft incompatibility according to Mosse and Herrero (1951).

indicate that, although not observed in this study, translocated graft incompatibility is also present between peach and Japanese apricot.

No significant differences were observed between amygdalin levels in the scion and rootstock combinations involving Chimarrita (Figure 2a). However, among →

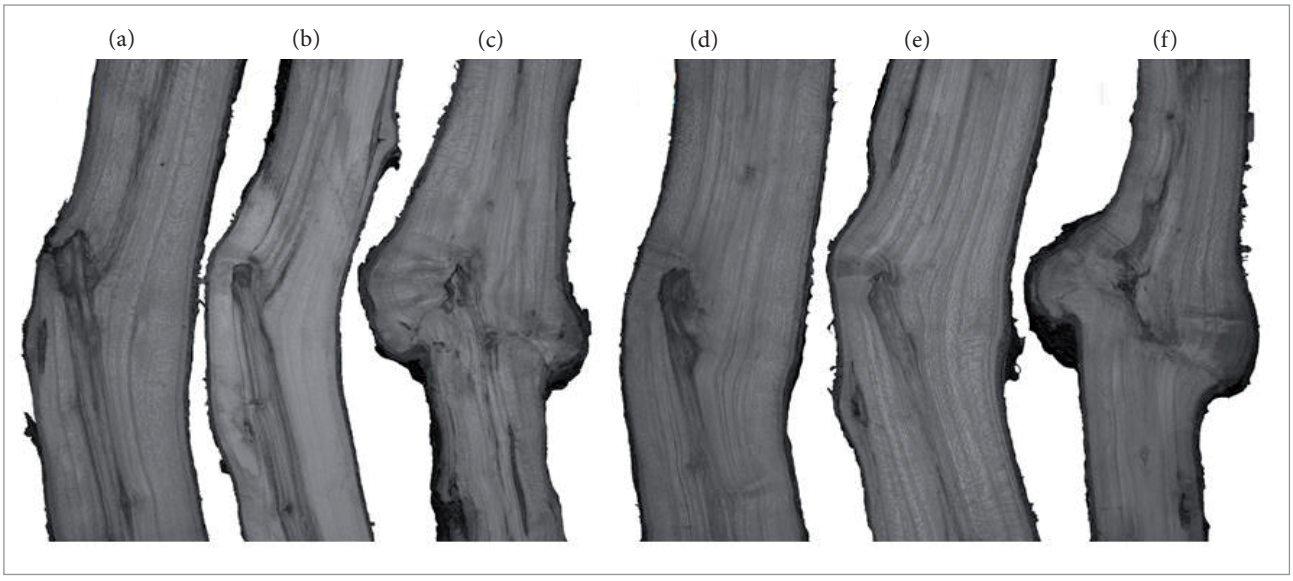

Figure 1. Longitudinal section union region of (a) Chimarrita/Capdeboscq; (b) Chimarrita/Tsukuba 1; (c) Chimarrita/Umezeiro; (d) Maciel/Capdeboscq; (e) Maciel/Tsukuba 1 and (f) Maciel/Umezeiro combinations.

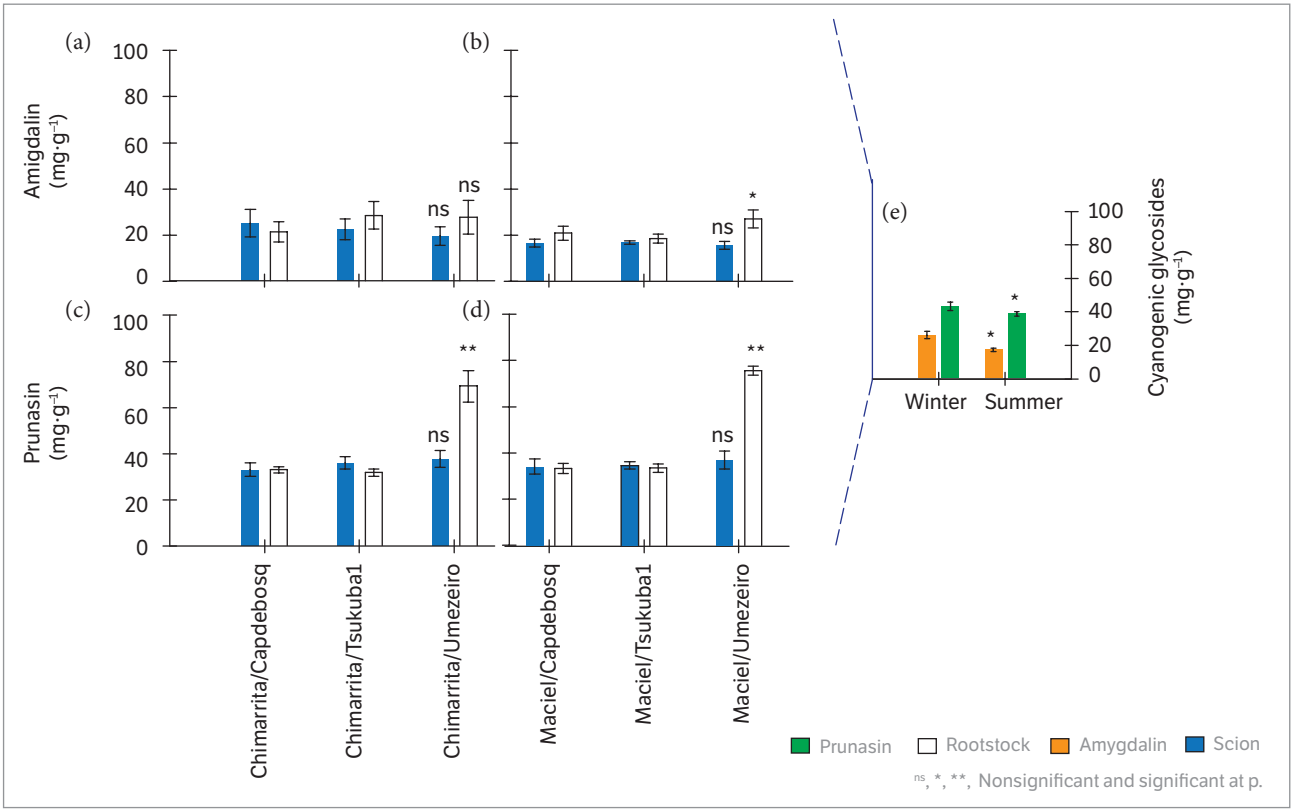

Figure 2. (a, b) Amygdalin, (c, d) prunasin concentration (mg·g-1) in the scion and rootstock of the combinations and (e) Amygdalin and prunasin concentration in the winter and summer.

combinations including Maciel the highest amygdalin content was observed in the rootstock of the Maciel/Umezeiro combination (Figure 2b).

The prunasin concentration showed no significant differences in the scions from six studied combinations (Figures 2c,d). However, when grafted with Chimarrita, the rootstock Umezeiro presented 2.1 and 2.2-fold higher levels of prunasin than those detected in Capdeboscq and Tsukuba 1, respectively (Figure 2c). When combined with Maciel, Umezeiro also showed 2.3 and 2.2-fold higher levels of prunasin as compared to Capdeboscq and Tsukuba 1, respectively (Figure 2d).

No significant differences were found between amygdalin concentrations of scion and rootstock in the investigated combinations (Figures 2a,b). However, differences in prunasin levels were noted between partners from the incompatible combinations. In combinations with symptoms of graft incompatibility (Chimarrita/Umezeiro and Maciel/Umezeiro), Umezeiro rootstock showed prunasin concentration 1.9-fold and 2.2-fold higher than Chimarrita and Maciel cultivars, respectively (Figures 2c,d).

In ungrafted genotypes, higher amygdalin and prunasin concentration were verified in the Umezeiro rootstock, while no significant differences were observed between peach cultivars Chimarrita, Maciel and peach rootstocks Capdeboscq and Tsukuba 1 (Table 2). These results indicate that the greatest cyanogenic potential presented by Umezeiro rootstocks in the grafts is a natural characteristic of this genotype. The Umezeiro rootstock presents important characteristics, as resistance to nematodes *Meloidogyne javanica* and *M. incognita* and control of tree vigor (Mayer et al. 2003, Mayer and Pereira, 2006; Pereira et al. 2007).

There were isolated and significant effects of season (winter and summer) on concentration of CGs, being amygdalin and prunasin concentrations higher in the winter than in summer (Figure 2e). These results are in agreement with a study of Gur et al. (1968), who also found higher concentrations of CGs in the pear/quince combinations during the winter.

PAL activity were not statistically significant between scions from six combinations (Figures 3a,b). However, among the rootstocks, Umezeiro showed higher activity when compared to Capdeboscq and Tsukuba 1. When grafted with Chimarrita, Umezeiro showed 33% and 48% higher PAL activity than 'Capdeboscq' and 'Tsukuba 1', respectively (Figure 3a). Grafted with Maciel, the PAL activity of the Umezeiro rootstock was 41% and 38% higher than that observated in Capdeboscq and Tsukuba 1, respectively (Figure 3b). Between the ungrafted genotypes, Umezeiro also showed higher PAL activity than the Capdeboscq and Tsukuba 1 rootstocks and the Chimarrita and Maciel cultivars (Table 2).

The PAL activity showed a similar profile to the prunasin concentration in the combinations studied. This probably occurred because the amino acid L-phenylalanine is the progenitor of many CGs, including prunasin (Ganjewala et al. 2010), and is also the precursor for biosynthesis of many phenolic compounds, where the PAL is the first enzyme in this pathway (Wang et al. 2012; Irisarri et al. 2016). Hypothesis strengthened by the high correlation ($r = 0.79$; $p < 0.01$) between these compounds in the rootstock (Table 3). Probably, there was correlation only in the rootstock, because the higher prunasin concentration and PAL activity were observed only in the Umezeiro rootstock. On the other hand, the absence of correlation in the scion is possibly because the cultivars showed low and similar concentrations of the prunasin and activity of PAL.

The total phenolic compounds concentration was significantly high in Chimarrita and Maciel cultivars →

Table 2. Amygdalin (mg·g^{-1}) and prunasin (mg·g^{-1}) concentration, phenylalanine ammonia-lyase (PAL) activity (μmol·min^{-1}·g^{-1}), total phenolics (g·100 g^{-1}) concentration and antioxidant activity (mM TAEC·g^{-1}) in ungrafted genotypes, Maciel, Chimarrita, Capdeboscq, Tsukuba 1 and Umezeiro.

Genotype	Amigdalin	Prunasin	PAL	Total phenolics	Antioxidant activity
Chimarrita	12.35 ± 1.77 b*	21.51 ± 1.18 b	0.84 ± 0.03 b	0.65 ± 0.05 b	502.28 ± 0.19 c
Maciel	10.31 ± 0.96 b	20.25 ± 0.31 b	0.87 ± 0.03 b	0.66 ± 0.07 b	553.35 ± 2.34 a
Capdeboscq	13.09 ± 0.91 b	22.29 ± 3.73 b	0.87 ± 0.02 b	0.78 ± 0.08 b	530.78 ± 3.95 b
Tsukuba 1	12.48 ± 1.42 b	17.74 ± 2.86 b	0.85 ± 0.01 b	0.74 ± 0.05 b	535.19 ± 2.54 b
Umezeiro	21.92 ± 1.05 a	39.90 ± 1.16 a	1.17 ± 0.02 a	0.91 ± 0.06 a	568.65 ± 5.84 a

*Mean separation within lines by Tukey's multiple range tests at $p < 0.05$. Means ± SE, n = 6.

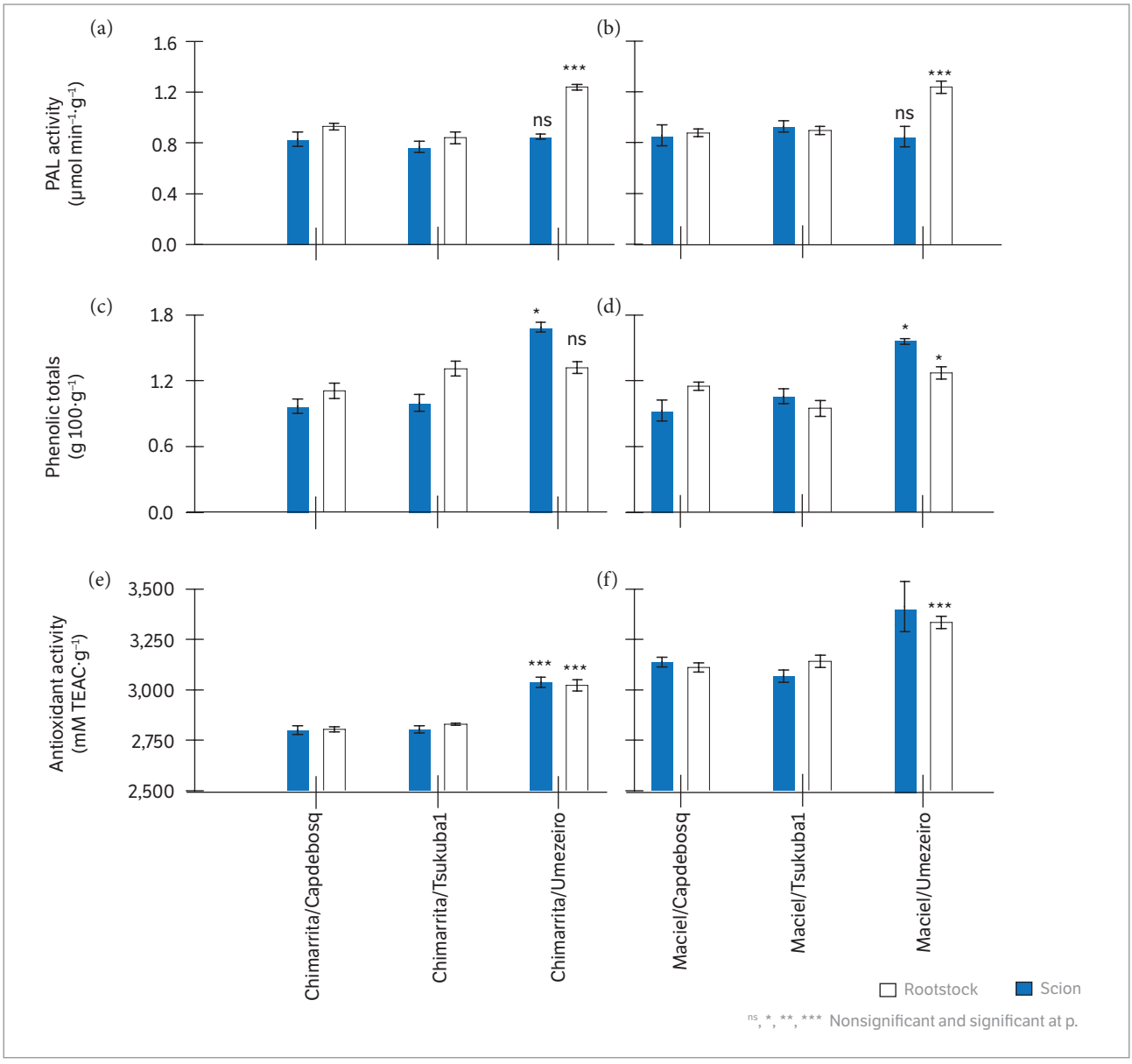

Figure 3. Phenylalanine ammonia-lyase (PAL) activity (µmol min-1·g-1), concentration of phenolic compounds (g 100·g-1) and antioxidant activity (mM TEAC·g-1) in the scion and rootstock of the combinations.

grafted on Umezeiro (Figure 3c,d). In Chimarrita grafted on Umezeiro, the phenolic content was 43% and 41% higher than that in the same cultivar grafted on Capdeboscq and Tsukuba 1, respectively (Figure 3c). In Maciel grafted on Umezeiro, the levels were 40% and 32% higher than that in Maciel grafted on Capdeboscq and Tsukuba 1, respectively (Figure 3d). No difference was observed in the concentration of total phenolic compounds of the rootstocks combined with Chimarrita (Figure 3d), while between rootstocks grafted with Maciel, Capdeboscq showed the lowest concentration (Figure 3e). In Chimarrita/Umezeiro and Maciel/Umezeiro the concentrations of phenols were highest in the scion that in the rootstock, while in the other grafts, the concentrations of phenols between grafted partners was superior in the rootstocks (Figure 3e,f). Among the ungrafted genotypes, it was observed that Umezeiro naturally presented higher total phenolics concentrations compared to the other genotypes (Table 2).

The antioxidant activity was also higher in the cultivars Chimarrita and Maciel when grafted on Umezeiro, and also in the rootstock Umezeiro grafted with both cultivars (Figures 3e,f). In the evaluation of the ungrafted genotypes, →

Table 3. Pearson's correlation coefficients between, amigdalin (Ami), prunasin (Prun), phenylalanine ammonia-lyase (PAL), phenolic compounds (Phe) and antioxidant activity (AntOx) parameters, in the scion and rootstock.

Parameters		Scion					Rootstock				
		Ami	Pru	PAL	Phe	AntOx	Ami	Prun	PAL	Phe	AntOx
Scion	Ami		0.44*	-0.10^{ns}	- 0.09^{ns}	- 0.41*	0.29^{ns}	- 0.23^{ns}	- 0.35^{ns}	0.02^{ns}	- 0.46*
	Pru	0.44*		0.11^{ns}	- 0.11^{ns}	- 0.13^{ns}	0.48*	- 0.03^{ns}	- 0.13^{ns}	0.01^{ns}	- 0.12^{ns}
	PAL	- 0.09^{ns}	0.11^{ns}		0.19^{ns}	0.12^{ns}	0.07^{ns}	0.06^{ns}	0.03^{ns}	- 0.22^{ns}	0.18^{ns}
	Phe	- 0.09^{ns}	- 0.11^{ns}	0.19^{ns}		0.47*	0.40^{ns}	0.84**	0.78**	0.57**	0.42*
	AntOx	- 0.41*	- 0.13^{ns}	0.12^{ns}	0.47*		0.02^{ns}	0.64**	0.53**	0.25^{ns}	0.89**
Rootstock	Ami	0.29^{ns}	0.48*	0.07^{ns}	0.40^{ns}	0.02^{ns}		0.29^{ns}	0.21^{ns}	0.41^{ns}	- 0.01^{ns}
	Pru	- 0.23^{ns}	- 0.03^{ns}	0.06^{ns}	0.84**	0.64**	0.29^{ns}		0.79**	0.47*	0.58**
	PAL	- 0.35^{ns}	-0.13^{ns}	0.03^{ns}	0.78**	0.53**	0.21^{ns}	0.79**		0.41^{ns}	0.48^{ns}
	Phe	0.02^{ns}	0.01^{ns}	-0.22^{ns}	0.57**	0.25^{ns}	0.41*	0.47*	0.41*		0.14^{ns}
	AntOx	- 0.46*	- 0.12^{ns}	0.18^{ns}	0.42*	0.89**	- 0.01^{ns}	0.58**	0.48*	0.14^{ns}	

^{ns} Nonsignificant; * significant at $p < 0.05$ and ** significant at $p < 0.01$.

Umezeiro and Maciel showed the highest antioxidant activities, followed by Tsukuba 1, Capdeboscq, and Chimarrita (Table 2).

In overall, biochemical and anatomical results were correlated, as the same combinations identified as incompatible showed higher prunasin and PAL activity in rootstock and greater total phenolic content and antioxidant activity in scion and rootstock.

Probably, the graft incompatibility was caused by differences in prunasin levels between peach cultivars (Chimarrita and Maciel) and *P. mume* rootstock (Umezeiro). Fact also suggested by Telles et al. (2009), when they verified graft incompatibility between peach and Japanese apricot. It was also noted that the high levels of amygdalin and prunasin represented the natural characteristic of Umezeiro, in relation to other genotypes. In contrast, the excellent graft compatibility observed between peach cultivars (Chimarrita and Maciel) and peach seedlings rootstocks (Capdeboscq and Tsukuba 1), is likely related to the similar prunasin levels.

The incompatibility caused by grafting genotypes with distinct cyanogenic potentials has been previously shown in pear and quince trees (Gur et al. 1968, Moore 1986, Nocito et al. 2010) and between peach and almond trees (Gur and Blum 1973). Furthermore, the incompatibility identified in this study was very similar to that reported by Gur and Blum (1973), between peach (*Prunus persica*) and almond (*Prunus dulcis*) trees. Even though in both cases, the cultivar and the rootstock were cyanogenic species, one of the components showed high potential for cyanide production. In this study, the genotype with greater concentration of CGs had an average potential twice as large as the lowest. Probably, the grafting process initializes the incompatibility, since the enzyme β-glucosidase responsible for the hydrolysis of prunasin is compartmentalized (Conn 1980). Thus, during the grafting, occur the ruptures of a significant number of the cells from scion and rootstock, allowing the enzyme action on the CGs and consequently the release of cyanide in the graft union. As the Umezeiro apricot showed high concentration of prunasin, possibly the levels of cyanide released are also high, reaching levels that are toxic to peach cultivars (Chimarrita and Maciel).

As the apricot 'Umezeiro' showed high prunasin concentration, possibly the levels of released cyanide are also high, reaching levels that are toxic for peaches cultivars (Chimarrita and Maciel).

In general, cyanogenic species are capable of metabolizing cyanide, although this ability is related to the production potential of each genotype (Zagrobelny and Moller, 2011). Thus, considering the lower production potential showed by genotypes of peach, compared to apricot, it is possible that the ability of these cultivars in catabolizing cyanide is also lower. This situation indicates that the scion is the most affected component in these incompatible grafts. This hypothesis is correlated with the swelling observed in the scion of the peach/*P. mume* combinations (Figure 1). The results indicate a close relationship between prunasin content and the graft incompatibility phenomenon and suggest that the prunasin →

content can be utilized as a promising biochemical marker of graft incompatibility between peach and *P. mume*. However, further studies are needed with other *Prunus* species to verify whether prunasin could be a reliable marker of compatibility for the entire genus.

Moreover, Umezeiro showed a higher PAL activity than that Capdeboscq and Tsukuba 1 rootstocks, as well as Chimarrita and Maciel cultivars, suggesting that PAL may also play an important role in the incompatibility process (Figure 3a,b). This result is in agreement with that found by Pereira et al. (2014b), which studied the same combinations (Chimarrita/Capdeboscq, Chimarrita/Tsukuba 1 and Chimarrita/Umezeiro) and observed increased PAL activity in the Umezeiro rootstock. In the same study, the authors also found that *PAL1* and *PAL2* gene expression were highly influenced by different combinations, as well as, the Chimarrita/Umezeiro combination showed the higher expression of both PAL genes in both graft partners compared to Chimarrita/Capdeboscq and Chimarrita/Tsukuba 1. These results are in agreement with those of Pina and Errea (2008) and Irisarri et al. (2016), who showed that PAL gene expression was higher in incompatible combinations when studying compatible and incompatible callus unions of *Prunus*. These studies also showed no induction of PAL gene expression in ungrafted genotypes, suggesting that the gene is expressed because of the incompatibility phenomenon. However, in this study, the elevated activity seems to be a natural characteristic of the rootstock Umezeiro in incompatible combinations, and increased PAL activity was also observed in Umezeiro compared to the other genotypes in ungrafted situations (Table 2).

PAL activity may also be associated with the high level of total phenolics in Umezeiro. The main source of phenolics synthesized in plants is the phenylpropanoid pathway, in which PAL is considered as key enzyme (Wang et al. 2012). PAL catalyzes the deamination of phenylalanine for the production of *trans*-cinnamic acid, which is subsequently converted to *p*-coumaric acid by an oxidation reaction catalyzed by cinnamate 4-hydroxylase (C4H). Subsequently, C4H undergoes thioesterification that is activated by 4-coumarate CoA-ligase, producing *p*-coumaroyl-CoA, which is directed toward the production of flavonoids, anthocyanins, and lignins (Xu et al. 2010).

The present study indicates that the high level of total phenolics is a natural characteristic of Umezeiro genotype (Table 2). However, the high phenol concentrations in Chimarrita and Maciel grafted on the rootstock Umezeiro possibly represents the response of these cultivars to graft incompatibility (Figure 4). Pina and Errea (2008) observed similar results in incompatible combinations between Moniqui (*Prunus armeniaca*) and Marianna 2624 (*Prunus munsoniana* × *Prunus cerasifera*), with high levels of PAL transcription associated with the accumulation of soluble phenolic compounds in the incompatible combination. Pereira et al. (2013) studied the expression profile of three 4CL isoforms in the combinations Chimarrita/Capdeboscq, Chimarrita/Tsukuba 1 and Chimarrita/Umezeiro and observed greater transcription in the incompatible combination Chimarrita/Umezeiro. However, according that study, it remained unclear whether increased expression of 4CL isoforms is related to the cause or is a consequence of graft incompatibility. In general, several authors point these differences as a possible cause of incompatibility (Errea 1998, Zarrouk et al. 2010, Hudina et al. 2014). However, the results of the present study suggest that these differences between of phenolic concentration from cultivar and rootstock may be a defense reaction of plant against the stress. High antioxidant activity observed in cultivars and rootstocks from incompatible combinations also suggest this possibility. This might be related to the defense mechanism of the plant to combat oxidative stress by increasing the concentrations of secondary metabolic compounds with antioxidant actions (Figure 4).

Nocito et al. (2010) found evidence that the graft incompatibility between pear (*Pyrus communis*) and quince (*Cydonia oblonga*), occurs by a dysfunction of the respiratory pathway, causing respiration to follow an alternative pathway that includes the alternative oxidase enzyme (AOX), and that can be caused by cyanogenesis. This behavior has been related to different forms of stress and resulted in secondary oxidative damage due to accumulation of reactive oxygen species (ROS) (Pellinen et al. 2002, Wang et al. 2012; Irisarri et al. 2015). When the increase of ROS is relatively small, the antioxidant capacity of the cells is sufficient to maintain the balance between synthesis and degradation (Van Breusegem and Dat 2006). However, this balance is disturbed under conditions of severe stress such as those caused by graft incompatibility, leading to the accumulation of ROS and damage to proteins, lipids, DNA, and consequently to

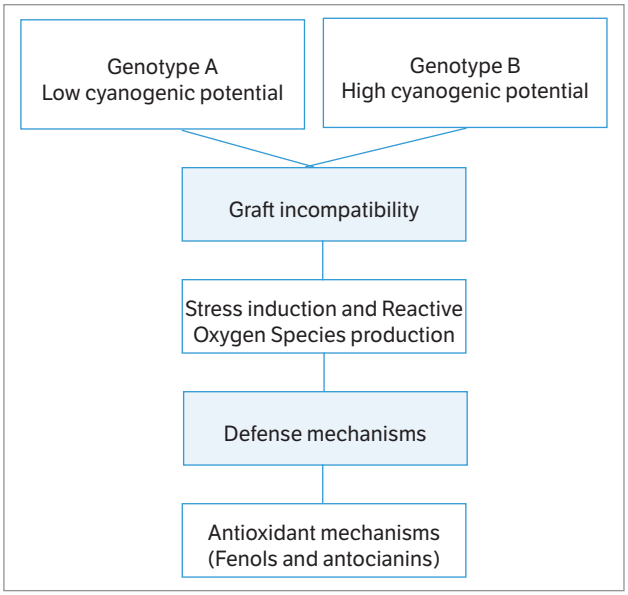

Figure 4. Scheme indicating the probable graft incompatibility mechanism manifested between *P. persica* (genotype A) and P. mume (genotype B) grafts.

membranes and other cell structures. This situation might lead to cell death (Van Breusegem and Dat 2006) and can be related with the vascular discontinuity (Figure 1) verified in the incompatible combinations.

The results of the Pearson's correlation study (Table 3) also support the hypothesis synthesized in the scheme of Figure 4, suggesting that the higher CG concentration, especially of prunasin, is the main cause of the incompatibility between peach and Japanese apricot. It is noted that there is a significant correlation between prunasin concentration in the rootstock and the PAL activity ($r = 0.79$; $p < 0.01$), phenols concentration ($r = 0.47$; $p < 0.05$) and antioxidant activity ($r = 0.58$; $p < 0.01$) in the rootstock (Table 3). As well, between prunasin concentration in the rootstock and the concentration of phenols ($r = 0.84$; $p < 0.01$) and antioxidant activity ($r = 0{,}64$; $p < 0.01$) in the cultivar of combinations.

Thus, is possible to believe that the greater activity of PAL, concentration of phenols and activity of antioxidant are plant responses to the stress generated by the incompatibility caused by the hydrolysis of prunasin and release of cyanide in the grafting region (Figure 4). Being, in the next studies, beyond of the CGs concentration, it would be important to determine directly the cyanide concentration released in the cultivar and rootstocks.

CONCLUSION

In the present investigation, it was possible to suggest biochemical methods to detect graft incompatibility in peach/Japanese apricot combinations. Prunasin concentration may be a good marker for assessing graft incompatibility between peach and Japanese apricot.

In addition, the results showed a higher level of phenolic compounds and antioxidant activity in peach/Japanese apricot grafts on response to incompatibility stress. Further studies are required to demonstrate details of the implication of CG in the graft incompatibility process.

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