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Relationship between hydroxycinnamic acids and the resistance of apple cultivars to rosy apple aphid

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ABSTRACT

The phenolic profiles of apple cultivars from the SERIDA Asturian cider apple breeding program, including parents and progenies, were determined by ultrahigh-performance liquid chromatography-diode array detector-electrospray ionization-quadrupole time of flight/mass spectrometer in order to study the relationship between phenols and the resistance of apple tree cultivars to rosy apple aphid (RAA). A pattern recognition technique named partial least square discriminant analysis (PLS-DA) was used to classify apple cultivars based on resistance to RAA, resistant and susceptible, reaching scores with accuracy higher than 97% and 91% respectively. Hydroxycinnamic acids, particularly 4-caffeoylquinic acid (4-CQA) and 4-*p*-coumaroylquinic acid (4-pCoQA), were identified as the major player in RAA resistance by the PLS-DA model. Indeed, the isomerisation 5-CQA → 4-CQA is favoured in resistant cultivars, whereas the isomerisation 5-pCoQA → 4-pCoQA is favoured in susceptible cultivars. As a result, resistant cultivars accumulate higher amounts of 4-CQA than susceptible ones, and the opposite occurs for 4-pCoQA. Also, minor isomerisations of 5-CQA to 1-CQA or 3-CQA show opposite behaviour for resistant and susceptible cultivars. Cultivar resistance to RAA is concluded to be related with the phenylpropanoid pathway, the isomerisation reactions being the key metabolic reaction for a cultivar to be resistant or susceptible to RAA.

1. Introduction

Rosy apple aphid (RAA), *Dysaphis plantaginea* Pass. (Hemiptera: Aphididae), is one of the major insect pests of apple, *Malus domestica* (Borkh.), in Europe and North America [1–3]. RAA causes leaf-rolling and shoot distortion, and when the infestation is high, this aphid reduces the commercial value of the yield because fruits remain smaller and deformed [4]. RAA control relies primarily on pesticide sprays, because naturally occurring predators are not enough [5,6]. Moreover, resistance to aphicides has been already reported [7]. Thus, new strategies for the sustainable control of RAA are urgently needed [8].

The introgression of resistance genes in plants is a sustainable strategy to control pest and to reduce at the same time the collateral effects of pesticides [9]. Therefore, many breeding programs have been developed worldwide to improve the resistance to different pests and diseases [10–12]. Most breeding programs focused primarily on apple scab, caused by *Venturia inaequalis* (Cke.) Wint., that is considered the major concern in most apple-producing regions [13]. Indeed, numerous scab-resistant cultivars have been developed by various organizations. The cultivar named 'Florina' released by the Institut National de la Recherche Agronomique (INRA) in Angers (France) [14] is resistant to apple scab and RAA, and only slightly susceptible to fire blight, *Erwinia amylovora* (Burrill) Winslow et al. [15]. Other cultivars, such as 'GoldRush' and 'Galarina', are also resistant to RAA, whereas "Liberty', 'Priscilla', 'Redfree' and 'William's Pride' show low susceptibility [3]. The use of cultivars resistant to scab and resistant or only slightly susceptible to RAA would reduce pesticide use and increase opportunities for sustainable apple production. In this sense, in the Regional Service for Agri-Food Research and Development (SERIDA) of Asturias (NW Spain), a cider apple breeding program was started in 1999 which aims was the implementation of new cultivars of cider apple of high interest in terms of fruit quality, resistance to scab and RAA, low

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susceptibility to fire blight and regular bearing [12].

Phenolic compounds have been associated with plant resistance mechanisms and plant responses to biotic-stress. For example, an increased activity of the enzymes involved in the phenolic biosynthesis has been associated to fungal infection [16]; the accumulation of hydroxycinnamic acids, gallic acid, quercetins and catechin has been referred in the green walnut husk tissue after infection with bacteria *Xanthomonas arboricola pv. juglandis* [17,18]; the accumulation of benzoic acid has been observed in apple fruit after inoculation with *Nectria galligena* which causes latent infections in apple [19]. Different classes of compounds have been correlated with infection of *Venturia inaequalis*: a rapid and localised accumulation of phenylpropanoids has been reported as the base for scab resistance [20–24], higher levels of flavan-3-o1s were found in apple leaf tissues of scab-resistant cultivars [21,25], the phloridzin/flavanol ratio was observed to be higher in susceptible varieties [26], finally, an increasing content of 3-hydroxyphlorizin has been correlated with reduced susceptibility to scab and fire blight [27]. Likewise, higher levels of two *p*-coumaric acid derivatives were found in cultivars with the polygenic resistance character [26]. To our knowledge, no correlation between phenolic composition in apple tissues and resistance to RAA has been reported to date.

In the present work, the relationship between the detailed phenolic profile and the susceptibility to RAA was studied by chemometrics in an experimental population derived from a controlled cross of 'Meana' and 'Florina' created and maintained by the above mentioned breeding program of SERIDA. This population was chosen because of the characteristics of the parents; 'Meana' is one of the apple varieties accepted for the production of Asturian cider under Protected Designation of Origin (PDO) with high phenol content and 'Florina' is a cultivar resistant to scab and highly tolerant to fire blight. The pattern recognition model achieved was applied to predict the RAA resistance of other apple varieties of the PDO and preselected genotypes of the breeding program to verify its universality and robustness.

2. Materials and methods

2.1. Chemicals, solvents and standards

Water, methanol, acetonitrile, 2-propanol and formic acid were of Optima® LC/MS grade (Fisher Scientific, Fair Lawn, NJ, USA). Glacial acetic acid provided by Merck (Darmstadt, Germany) was of Suprapur® quality. Sodium fluoride (Fluka Chemie, Buchs, Switzerland) and ascorbic and formic acid (Panreac, Barcelona, Spain) were of ACS grade.

The phenolic standards (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, ferulic acid, quercetin-3-*O*-galactoside, isorhamnetin, isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-rutinoside were supplied by Extrasynthèse (Genay, France); procyanidin C1, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-arabinofuranoside, by Chromadex (Santa Ana, CA, USA); caffeic acid, 5´-*O*-caffeoylquinic acid (5-CQA), *p*-coumaric acid, phloretin, phloretin-2′-*O*-glucoside and quercetin, by Sigma-Aldrich Chemie (Steinheim, Germany); phloretin-2′-*O*-xylosylglucoside, by Polyphenols Biotech (Bordeaux, France); and quercetin-3-*O*-rutinoside, by PhytoLab (Vestenbergsgreuth, Germany).

2.2. Apple samples

For this study two different set of individuals were used. The first, the *MxF* sample set, included a population of 155 individuals from a cross of 'Meana' x 'Florina' and the two parents. The second, the *Other Cultivars* sample set, included 100 apple cultivars divided as follow: 14 PDO and local cultivars, 10 PDO parents, 5 cultivars used as parents in the SERIDA breeding program and 71 individuals from various crosses of this breeding program. For the *MxF* population, during 2012 and 2013 a total of 214 apple juices were sampled (59 collected in 2012 and 2013, 3 collected only in 2012 and 93 collected only in 2013). For each accession of the *Other Cultivars* panel a batch of apple juice were collected during 2012, 2013 and 2014. Cider apples were harvested at the optimum stage of maturity at Regional Service for Agri-Food Research and Development (SERIDA) in Villaviciosa (Asturias, Spain).

2.3. Sample preparation for phenols analysis

Three apple batches of each cultivar were processed as follows. Each apple batch was crushed and pressed, and 5 mL of sodium fluoride (1 g/L) was added to 65 mL of the juice. Then, it was centrifuged (8000 rpm, 10 °C, 10 min), and the supernatant was kept at − 20 °C until analysis. Afterwards, an aliquot of 0.5 mL of apple juice was diluted with 1.5 mL of methanol–water–acetic acid (30:69:1, v/v/v) with 2 g/L of ascorbic acid (w/v), vortexed and filtered through a 0.45 µm PTFE filter (Waters, Milford, CA, USA) prior to injection into the ultrahigh-performance liquid chromatography-diode array detector-electrospray ionization-quadrupole time of flight/mass spectrometer (UHPLC-DAD-ESI-QToF/MS) system.

2.4. UHPLC-DAD-QToF/MS analysis

Apple juice samples were analyzed by UHPLC-DAD-ESI-QToF/MS using a previous validated method [28] using an ACQUITY UPLC™ system from Waters (Milford, MA, USA), equipped with a binary solvent delivery pump, an autosampler, a column compartment and a DAD detector. A reverse-phase column (Kinetex™ C18 1.7 µm, 2.1 mm × 100 mm) and a filter (Krudkatcher ™ ULTRA HPLC in-line filter, 0.5 µm depth filter × 0.004 in ID) from Phenomenex (Torrance, CA, USA) were used at 40 °C. Mobile phases consisted of 0.1% (v/v) acetic acid in water (A) and 0.1% (v/v) acetic acid in methanol (B). Separation was carried out in 18 min under the following conditions: 0–0.87 min, 0% B isocratic; 0.87–2.14 min, 0–15% B gradient; 2.14–5.04 min, 15% B isocratic; 5.04–7.63 min, 15–20% B gradient; 7.63–9.00 min, 20–23% B gradient; 9.00–14.00 min, 23–35% B gradient; 14.00–16.00 min, 35–51% B gradient; 16.00–18.00 min, 51–100% B isocratic; and re-equilibration of the system with 100% A (v/v) for 4 min prior to the next injection. The flow rate was 0.35 mL/min, the injection volume, 5 µL; and the autosampler temperature, 4 °C. UV–visible spectra of the chromatographic peaks were recorded from 210 to 500 nm (20 Hz, 1.2-nm resolution). Flavan-3-ols and dihydrochalcones were monitored and quantified at 280 nm; hydroxycinnamic acids at 320 nm; and flavonols at 370 nm.

Reference solutions containing phenolic standards were used for identification. However, quantification of phenolic compounds in apple juice samples was carried out selecting only one standard of each phenolic family: flavan-3-ols were quantified as (−)-epicatechin; hydroxycinnamic acids as 5-CQA or *p*-coumaric acid; dihydrochalcones as phloretin-2′-*O*-glucoside; and flavonols as quercetin-3-*O*-galactoside. Solutions of these standards at concentration between 0.01 and 100 µg/mL in methanol–water–acetic acid (30:69:1, v/v/v) were used to build the corresponding calibration curves. The identification of phenolic compounds was confirmed by means of an UHPLC-DAD-ESI-QToF-MS^E strategy using a SYNAPT™ G2 HDMS mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI source operating in both positive and negative modes, as reported in a previous work [29].

2.5. Phenotypic analysis of the resistance level to RAA

The response to RAA was evaluated after infestation with aphids in a greenhouse in Villaviciosa, Asturias (Spain). The number of replicates per individual plant genotype varied from three to eight depending on the cultivar/crossing. The same numbers of plants of 'Florina' and 'Golden Delicious' were used as resistant and susceptible controls, respectively. Plants were grafted on M.7 rootstocks and kept outdoors in 4-liter pots. Plants were fertilized with 8 g of Osmocote plus. In mid-June, when new shoots were about 20 cm, plants were introduced into

the greenhouse and randomly distributed in the greenhouse. Plants were infested when new shoots were about 30–50 cm, and aphid movements from one plant to another were prevented by putting the pots in dishes filled with water. Secondary shoots were periodically pruned to keep the aphids on the principal stem. There were no overlapping branches.

Aphids for infestation were field-collected from different apple cultivars to capture some of the natural variability. Individuals from each cultivar were reared separately on susceptible apple plants. Thus, several distinct populations of RAA were maintained in the laboratory. Four apterous adults from at least two different populations were carefully placed with a small paintbrush on the youngest leaf of each evaluated plant. When necessary, reinfestations were performed during the first week of the experiment to make sure that the initial number of aphids was four per plant.

To assess damage on plants, observations were made once a week from the day after the infestation to the end of the experiment, 21 days later. Shoot damage was coded from 0 to 5 based on Rat-Morris (1993): 0 = no damage; 1 = leaf slightly curled at the edge; 2 = leaf curled longitudinally; 3 = typical RAA leaf rolling; 4 = 2–5 typically-rolled leaves; and 5 = more than 5 typically-rolled leaves. Plants exhibiting shoot damage classes of 0, 1 or 2 were considered resistant and classes 3–5, susceptible.

2.6. Data analysis

Datasets were made up of the concentrations of individual phenolic compounds (variables in columns) measured by UHPLC-DAD in the apple juices (samples in rows). Two datasets were studied: *i*) the *MxF* data matrix, containing the data of 220 (including parents) or 114 (in the subset) apple juice samples of the progeny of 'Meana' and 'Florina', was used to build a classification model of apple cultivars according to RAA resistance; and *ii*) the *Other Cultivars* data matrix, containing the data of 225 or 89 (in the subset) apple juice samples of the *Other Cultivars* sample set, was used to validate the classification model achieved with *MxF* data matrix. Firstly, datasets were analyzed by univariate procedures (ANOVA, Fisher index and Box–Whisker plots), and afterwards, by unsupervised and supervised multivariate techniques, such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), respectively [30]. Data analysis was performed by means of the statistical software packages SPSS Statistics 17.0 (IBM Corporation, Armonk, NY, 1993–2007), Statistica 6.1 (StatSoft Inc., Tulsa, OK, 1984–2004) and The Unscrambler 9.1 (Camo Process AS, Oslo, Norway, 1986–2004). The multivariate techniques (PCA and PLS-DA) were applied to the autoscaled (or standardized) *MxF* data matrix of original variables. PLS-DA was carried out following these steps: (*i*) the data set was divided into a training-test set and an external data set; (*ii*) the training-test set was subsequently divided into a training set and a test set several times to perform cross-validation; (*iii*) the training-test set was used for the optimization of the number of PLS components by threefold cross-validation (3-fold CV); (*iv*) a mathematical model was built using all of the samples of the training-test set and the optimized parameters; (*v*) this model was validated using an independent test set of samples (external data set), that is, performing an external validation; and (*vi*) a final mathematical model was built using all of the samples of *MxF* data matrix and the optimized parameters, and was used to predict the RAA resistance of the samples in the *Other Cultivars* data matrix. In PLS-DA, Root Mean Square Error in Prediction (RMSEP) is plotted against the number of PLS components in order to find the optimal number of the latter. Sometimes there are several almost equivalent local minima on the curve; the first one should be preferred to avoid overfitting (according to the principle of parsimony). The model with the smallest number of features should be accepted from among equivalent models on the training set. Once PLS components are estimated by cross-validation, the predictions in the training-test set are represented in a box and whisker plot in order to define the boundary. Binary classification models can lead to artifacts if they are not used and validated properly [31]. The reliability of the classification models achieved was studied in terms of recognition ability (percentage of the samples in the training set correctly classified during the modelling step), prediction ability in the cross-validation (percentage of the samples in the test set correctly classified by using the model developed in the training step), and the prediction ability in the external validation (percentage of the samples of the external set correctly classified by using the optimized model) [30].

3. Results

3.1. Phenolic contents

Forty individual phenolic compounds were quantified in the apple juice of all cultivars studied [28]; statistics of the concentration values for each phenolic family are gathered in the supplementary material (Tables S1 and S2 for cultivars in *MxF* dataset, and Tables S3–S5 for cultivars in *Other Cultivars* dataset in supplementary information; see details for composition of datasets in the Experimental section).

In the *MxF* dataset, the concentration of phenolic compounds ranged from 465 to 3445 g/L apple juice. Flavan-3-ols represented 27–70% of total phenolics, followed by hydroxycinnamic acids (16–55%), dihydrochalcones (10–42%), and flavonols (0.4–2%). The distribution of phenolic families was in accordance with the results obtained in previous studies [32]. 'Florina' presented the lowest phenolic content (290–350 mg/L of juice) in the *MxF* dataset, while 'Meana' showed a content of phenols similar to the descendants with the highest concentrations (1131–1475 mg/L of juice). The concentration distribution of the phenolic families was also different among the parents cultivars. In 'Meana' the most abundant classes of compounds were flavan- 3-ols (60–64% of total phenols), dihydrochalcones (22–26%) and hydroxycinnamic acids (14%); being flavonols (0.3–0.4%) the less represented family. 'Florina' presented lower percentages of dihydrochalcones (9–11%) and flavan-3-ols (41–52%), and higher percentages of hydroxycinnamic acids (37–47%) and flavonols (2.5–2.7%). The percentage of flavonols in 'Florina' was eight times the one found in 'Meana'. The phenolic profiles of these genitors agreed with the type of apple cultivar they are known to belong to: 'Meana' is a bitter sharp cider apple cultivar included in the Asturian cider PDO, and 'Florina' is a dessert apple cultivar with low phenolic content and a bicolour fruit, pigmentation that could be related to its high flavonol content. In the other hand, distribution of phenolic families for descendants and genitors of *Local Cultivar* data set was mainly within ranges for 'Meana' × 'Florina' progeny.

3.2. Resistance to RAA

The susceptibility to RAA was determined in those individuals in the 157 cultivars of *MxF* and in 89 cultivars within of the *Other Cultivars* dataset. For 27 *MxF* individuals was impossible to determine the resistance to RAA because not enough growth of the plants or problems in the process of infestation. 55 of the *MxF* individuals were classified as resistant and 75 as susceptible (for the 114 cultivars of *MxF* subset, 50 were resistant and 64 susceptible). In the subset of *Other Cultivars* dataset 9 were resistant and 80 susceptible. For other individuals in dataset, either the susceptibility was not determined or the separated concentrations of 4- CQA and 5-CQA were not measured.

3.3. Pattern recognition to determine apple cultivar resistance to RAA

Data analysis of the phenolic profiles of *MxF* data matrix was performed in order to investigate the relationship between phenolic composition and the resistance of apple tree cultivars to RAA, as well as to develop a chemical assay to determine the resistance/susceptibility of an apple cultivar to RAA according to phenolic composition. The univariate analysis (ANOVA, Fisher test, box, and whiskers plots) of the concentrations of phenols in the apple juices disclosed that none of the phenolic compounds was able to discriminate between resistant and susceptible

cultivars to RAA by itself. Hence, it was necessary to move on to multivariate data analysis in order to achieve the discrimination required. The presence of outliers in the *MxF* and *Other Cultivars* datasets were analyzed by PCA, and no outliers or extreme samples were detected. First, the multivariate data analysis was performed on the autoscaled datasets containing 39 variables, i.e. 39 phenolic chromatographic peaks (the peaks of 4-CQA and 5-CQA were not resolved for some samples, so they were quantified together). When PCA was carried out on *MxF* dataset (220 × 39 matrix), bidimensional plots of the sample scores in the spaces defined by the four first principal components (accounting for 57% of total system variability: PC1 for 26%, PC2 for 13%, PC3 for 10%, PC4 for 8%) did not show any clustering of the samples according to the year of harvest (2012 and 2013); samples being distributed in a compact cluster. Regarding the *Other Cultivars* dataset (225 × 39 matrix), bidimensional plots of the scores in the spaces defined by the four first principal components (accounting for 57% of total system variability: PC1 for 28%, PC2 for 14%, PC3 for 8%, and PC4 for 7%) showed that 2012 and 2013 samples were distributed in a compact cluster, but 2014 samples formed a partially overlapped cluster, due to PC2, which contained information related to the variability of the harvest year. It is well-known that environmental and seasonal aspects affects agricultural samples, therefore in the modelling it is important to have chemical data of several harvests to obtain general classification models that include the seasonal variability as well. On the other hand, the PCA score plots did not show any clusters related to the resistance/susceptibility of apple cultivars to RAA. This indicates that the direction of maximum variability in the data set did not correspond to the direction of maximum discrimination between resistant and susceptible cultivars. This suggests the presence of other sources of variability. Indeed, the year of harvest was confirmed to be one of them as said above.

In order to extract the useful information contained in the apple juice phenolic profiles related to the resistance of apple tree cultivars to RAA, binary PLS-DA classification models were developed (Table 1). First, a PLS-DA analysis was performed on the *MxF* dataset (179 × 39 matrix), affording a PLS-DA model (three PLS components selected; the boundary at 0.583; class codes: resistant = 0, susceptible = 1) with recognition and prediction abilities in the cross-validation of 94% and 88% respectively for the resistant apple cultivars, and 87% and 82% respectively for the susceptible cultivars. The predictions in the external validation were 96% and 82% for resistant and susceptible cultivars, respectively. The facts that in the cross-validation the recognition ability was higher but close to the prediction ability, and the prediction ability in the external validation close to them disclosed that the model achieved was feasible and not random, as well as well represented by the samples in the data set. The regression coefficients of the PLS models indicate the importance of the variables on the model: the larger the regression coefficient, the higher the influence of the variable on the PLS model [33]. The variables that presented the highest regression coefficients in the PLS-DA model were the concentrations of 4-*p*-coumaroylquinic acid (4-pCoQA), 1- CQA and the sum of 4-CQA and 5-CQA; presenting opposite sign that of 4-pCoQA respect to those of the caffeoylquinic acids. In fact, the box & whisker plots of these variables showed that the susceptible apple cultivars contained higher levels of 4-pCoQA than the resistant ones; and that the resistant cultivars presented higher contents of the caffeoylquinic acids (1-CQA and 4-CQA + 5-CQA) than the susceptible ones. If these variables were removed from the dataset, no PLS-DA model was achieved to classify samples according to their resistance to RAA; which confirmed that the variables containing information related to apple tree susceptibility to RAA were 4-pCoQA, 1-CQA and 4-CQA + 5-CQA. Indeed, a better PLS-DA model was achieved using only the two most important variables, i.e. 4-pCoQA and 1-CQA (Table 1). These findings suggested that 4-CQA and 5-CQA separately might help to understand the relationship between these phenolics and the apple cultivars resistance to RAA. Thus, a PLS-DA analysis was carried out on a *MxF* data subset (114 × 40 matrix), containing only the samples for which 4-CQA and 5-CQA were quantified separately. The classification results of the PLS-DA model achieved were very similar to those afforded by the previous model using 39 phenolic variables (Table 1). The most influent variables on the model were 4-CQA, 4-pCoQA and 1-CQA; again caffeoylquinic acids presenting opposite sign to that of *p*-coumaroylquinic acid. The best PLS-DA model was achieved using the two most influent variables, 4-CQA and 4-pCoQA (Table 1), affording recognition and prediction

Table 1
PLS-DA models for the determination of apple tree cultivar resistance to RAA (best model in bold).[a].

			3-fold cross-validation				External validation	
Dataset	**Model**	**Resistance to RAA**	**N**	**prior prob**	**% R**	**% P**	**N**	**% P - EV**
MxF (179 × 39)	3 PLS comp.;	resistant	48	*0.39*	93.8	87.5	23	95.7
	boundary = 0.5831	susceptible	74	*0.61*	86.5	82.4	34	82.4
MxF (179 × 2)	1 PLS comp.;	resistant	48	*0.39*	95.8	95.8	23	87.0
	boundary = 0.6203	susceptible	74	*0.61*	89.2	86.5	34	91.2
	2 variables: 4-pCoQA & 1-CQA							
MxF (114 × 40)	2 PLS comp.; boundary = 0.5368	resistant	38	*0.44*	92.1	84.2	12	100
		susceptible	48	*0.56*	85.4	81.3	16	81.3
***MxF* (114 × 2)**	**1 PLS comp.;**	**resistant**	**38**	***0.44***	**97.4**	**97.4**	**12**	**100**
	boundary = 0.5473	**susceptible**	**48**	***0.56***	**91.7**	**91.7**	**16**	**100**
	2 variables: 4-CQA & 4-pCoQA							
prediction of		**resistant**					**9**	**88.9**
Other cultivars		**susceptible**					**80**	**98.8**
MxF (114 × 3)	1 PLS comp.;	resistant	38	*0.44*	100	100	12	100
	boundary = 0.5559	susceptible	48	*0.56*	91.7	91.7	16	93.8
	3 variables:							
	4-CQA/5-CQA,							
	4-pCoQA/5-pCoQA,							
	1-CQA/3-CQA							
MxF (114 × 2)	1 PLS comp.;	resistant	38	*0.44*	97.4	97.4	12	100
	boundary = 0.5559	susceptible	48	*0.56*	91.7	91.7	16	100
	2 variables:							
	4-CQA/5-CQA,							
	4-pCoQA/5-pCoQA							

[a] Abbreviations: PLS-DA, partial least square discriminant analysis; prior prob, prior probability; PLS comp, number of PLS components selected; % R, percentage of recognition ability; % P, percentage of prediction ability in cross-validation; % P-EV, percentage of prediction ability in the external validation; class codification: 0, resistant; 1, susceptible.

Table 2
Statistics of the concentrations of hydroxycinnamic acids (mg of 5-CQA equivalents/L of juice) in the apple juice of descendants of `Meana´x`Florina´ crossing according to their resistance to RAA: resistant (n = 50) and susceptible (n = 64).[a].

Resistance to RAA	1-CQA	3-CQA	4-CQA	5-CQA	4-pCoQA	5-pCoQA	ΣCQA	ΣCoQA
resistant								
mean	3.5	1.6	119	335	2.0	1.9	458	3.8
SD	2.0	0.7	60	105	1.2	1.0	156	2.0
Min	0.4	0.4	21	184	0.5	0.6	230	1.0
Max	7.8	3.3	313	632	6.5	4.6	908	9.5
median	2.9	1.4	99	305	1.5	1.6	418	3.0
%	0.74	0.35	24.98	73.93	49.18	50.82		
susceptible								
mean	1.2	2.4	20	340	21	2.3	364	23
SD	1.1	1.4	31	112	18	1.8	122	19
Min	0.3	0.2	4	195	1	0.5	201	2
Max	8.7	7.4	218	710	96	9.2	737	106
median	0.9	2.0	12	319	16	1.6	343	17
%	0.31	0.67	5.26	93.76	86.21	13.79		

[a] Abbreviations: SD, standard deviation; Min, minimum value; Max, maximum value; %, mean percentage of each CQA or CoQA isomers related to the sum of all quantified CQAs (ΣCQA) or CoQAs (ΣCoQA) respectively.

abilities in the cross-validation of 97% for the resistant apple cultivars and 92% for the susceptible cultivars, and 100% of hits in the external validation for both classes. This PLS-DA model was used to predict the resistance to RAA of the apple cultivars contained in *Other Cultivars* dataset (Table 1). Percentages of correct predictions for resistant and susceptible cultivars were of 89% and 99% respectively; showing a good performance when applying the PLS-DA model to other cultivars and crossings different from those used to build the model.

Tables 2 and 3 gather information related to hydroxycinnamic acid concentrations in the apple juice of resistant and susceptible cultivars and their ratios, in order to understand their relationship with apple cultivar resistance to RAA taking into account the phenolic metabolic route in plants (Fig. 1). Box & whisker plots of the ratios 4-CQA/5-CQA, 4-pCoQA/5-pCoQA and 1-CQA/3-CQA showed that none of these ratios were completely discriminant between resistant and susceptible cultivars, but it was possible to establish borders between both classes as half of the distance between the whiskers of both classes (when 100% of the samples of both classes were not overlapping, except for outliers and extreme samples) for 4-CQA/5-CQA, and as half of the distance between the two samples that cross their numerical values when coming back sample to sample from the overlapping whiskers of each class (when at least of 75% of the samples of both classes were not overlapping) for the 4-pCoQA/5-pCoQA and 1-CQA/3-CQA ratios (Fig. S1 in supplementary information): For resistant cultivars, 4-CQA/5-CQA > 0.074, 4-pCoQA/5-pCoQA < 1.975, and 1-CQA/3-CQA > 0.757; for susceptible cultivars: 4-CQA/5-CQA < 0.074; 4-pCoQA/5-pCoQA > 1.975; and 1-CQA/3-CQA < 0.757. The PLS-DA models achieved using these ratios as variables achieved the same classification results than using 4-CQA and 4-pCoQA concentrations; the only difference was one resistant sample that was wrongly classified as susceptible in cross-validation by the latter model.

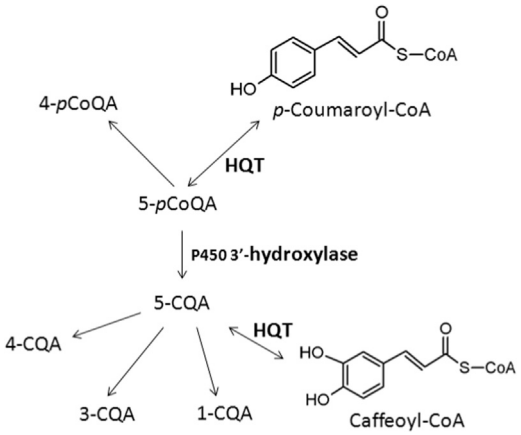

Fig. 1. Scheme of the final steps of hydroxycinnamic acid biosynthesis.

Using the first two ratios and their corresponding border values, the

Table 3
Statistics of hydroxycinnamic acids concentration ratios in the apple juice of descendants of `Meana´x`Florina´ crossing according to their resistance to RAA: resistant (n = 50) and susceptible (n = 64).[a].

Resistance to RAA	1-CQA/3-CQA	4-CQA/5-CQA	4-pCoQA/5-pCoQA	5-CQA/5-pCoQA	4-CQA/4-pCoQA	ΣCQA/ΣCoQA
resistant						
Mean	**2.3**	**0.4**	**1.1**	**202**	**73**	**138**
SD	1.2	0.1	0.4	77	33	52
Min	0.3	0.1	0.4	102	11	40
Max	7.3	0.6	2.1	480	147	294
Median	2.0	0.3	0.9	181	70	131
susceptible						
Mean	**0.7**	**0.06**	**10**	**210**	**5**	**31**
SD	1.2	0.09	7	106	17	36
Min	0.2	0.02	1	69	0.3	4
Max	8.9	0.53	29	556	110	178
Median	0.4	0.04	9	192	0.7	18
border	0.757	0.074	1.975		8.741	

[a] Abbreviations: See in Table 2.

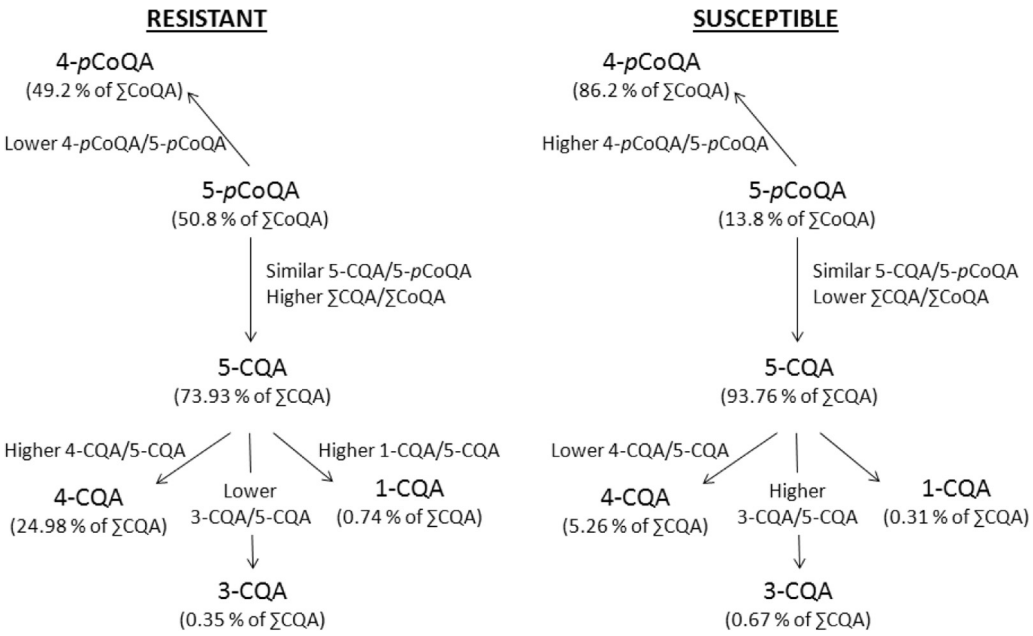

Fig. 2. Scheme of the key metabolic pathways involved in the apple tree resistance to RAA, indicating the percentage (%) of each CQA and CoQA isomers regarding the amount of total CQAs and total CoQAs respectively, and the relative results of different hydroxycinnamic acid ratios comparing resistant and susceptible cultivars.

resistance of the apple cultivars in the *Other Cultivars* dataset for which the ratios could be calculated (n = 89) was predicted. For 21 apple cultivars (23.6%), the ratios could not establish their resistance to RAA (ratios provided opposite predictions). For the other cultivars, 89% were correctly predicted as resistant, and 98% as susceptible. The predictions of the PLS-DA model (using 4-CQA and 4-pCoQA) for the samples in the *Other Cultivars* dataset agreed with those obtained by the ratios, and all the 21 uncertain samples were classified as susceptible. The 4-CQA/4-pCoQA ratio was able to attain an even better discrimination (excluding outlier samples) (Fig. S1 in supplementary information); 89% of resistant cultivars (4-CQA/4-pCoQA > 8.741) and 99% of susceptible cultivars (4-CQA/4-pCoQA < 8.741) were correctly predicted.

4. Discussion

From the results achieved in the present study, it can be proposed that hydroxycinnamic acids are related to the resistance of apple tree cultivars to RAA. This observation agrees with previous knowledge on the multiple roles of these important secondary metabolites in plants. For instance, high levels of hydroxycinnamic acids showed to give increased protection from harmful UV light in transgenic tomato plants [34], enhanced microbial resistance [35], or act as pest resistance factors in ornamental plants [36].

The hydroxycinnamic acids and shikimate esters are synthesized via the phenylpropanoid pathway. Enzymes involved in the early stages of this pathway have been known for several years [37,38], however those involved in the last steps are still not so clear. It is currently thought that the primary route for 5-CQA formation in higher plants is via *p*-coumaroyl-CoA and quinic acid [35] by the combined activities of two acyl transferases (HCT, hydroxycinnamoyl transferase with preference for shikimate; and HQT, hydroxycinnamoyl transferase with preference for quinate) and a P450 3´-hydroxylase. 3´-hydroxylation is not catalyzed on the free *p*-coumaric acid, but on its conjugates with shikimic or quinic acids [39–41]. *In vitro* studies on HCT and HQT from Robusta coffee showed that 5-caffeoylshikimic acid (5-CSA) and 5-CQA were the major enzymatic products and that the subsequent 3- or 4-isomerisation of 5-CQA occurred non-enzymatically in solution [42]. It is currently unclear what level of hydroxycinnamic acid isomerisation occurs in vivo [42]. Taking into account these bibliographic data and the results presented above, a scheme of the final steps of hydroxycinnamic acid biosynthesis is presented in Fig. 1.

The ratio 5-CQA/5-pCoQA was similar for both classes of cultivars (Table 3 and Fig. 2), resistant and susceptible to RAA, indicating a similar 3´-hydroxylase activity in both. However, ratio ΣCQA/ΣCoQA was significantly different between the two classes of cultivars due to different isomerisation activities. Thus, whereas in resistant cultivars the isomerisation 5-pCoQA → 4-pCoQA decreased and the isomerisation 5-CQA → 4-CQA increased; in susceptible cultivars, the opposite occurred. As a consequence, resistant descendants accumulated more CQAs than susceptible ones, whereas the susceptible cultivars accumulated higher amounts of CoQAs than the resistant ones. Moreover, although 1-CQA and 3-CQA isomers were present in lower amounts, a clear different isomerisation behaviour was observed between both classes of cultivars respect to RAA. In this sense, resistant cultivars favoured the isomerisation 5-CQA → 1-CQA, and susceptible cultivars, the isomerisation 5-CQA → 3-CQA; therefore the ratio 1-CQA/3-CQA helped to differentiate between both classes of cultivars (Fig. 2).

Therefore, these results support an interaction between hydroxycinnamic acids and RAA resistance. The RAA resistance locus is already known to be at the bottom of the LG8 [43,44]. Moreover, four candidate genes putatively involved in the RAA resistance response have been identified [45]. Further studies are needed to test the implication of hydroxycinnamic acids in the response shown by RAA-resistant cultivars and the involvement of those candidate genes in metabolic pathways correlated to the compounds that have been identified as associated with RAA resistance by PLS-DA.

5. Conclusions

The present study confirms that the phenolic profile of apple cultivars is related to the resistance of apple tree to RAA. The PLS-DA classification models described allowed the prediction of the resistance to RAA in apple cultivars according to their phenolic composition and in particular their content of phenolic acids. Indeed, certain hydroxycinnamic acid ratios are characteristic of most resistant cultivars: 4-CQA/5-CQA higher than 0.074, 4-pCoQA/5-pCoQA lower than 1.975, 1-CQA/3-CQA higher than 0.757, and 4-CQA/4-pCoQA higher than 8.741. These observations can be explained by the metabolic routes of phenolic compounds.

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Declarations of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2018.05.040.

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