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Peach Fruit: Metabolic Comparative Analysis of Two Varieties with Different Resistances to Insect Attacks by NMR Spectroscopy

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ABSTRACT: The metabolite profile of aqueous extracts of two peach varieties, Percoca Romagnola 7 and Flaminia, with different susceptibilities to *Ceratitis capitata* attack was investigated by means of 1D and 2D high-field NMR spectroscopy. Water-soluble metabolites belonging to different classes such as organic acids (citric, fumaric, malic, quinic, shikimic, and succinic acids), sugars (fucose, fructose, fructose-6-phosphate, glucose, glucose-6-phosphate, rhamnose, sucrose, and xylose), amino acids (alanine, asparagine, isoleucine, threonine, and valine) and other metabolites such as *myo*-inositol, choline, trigonelline, catechin, chlorogenic and neochlorogenic acids, orthophosphate, and α-L-glycerophosphorylcholine were identified. The metabolite profile together with a suitable statistical analysis was used to make a comparison between the two varieties. The levels of glucose, xylose, *myo*-inositol, choline, isoleucine, and valine were found to be higher in Flaminia than in Percoca Romagnola 7 samples, whereas the levels of fumaric acid, alanine, quinic acid, sucrose, fucose, and chlorogenic and neochlorogenic acid were found to be higher in Percoca Romagnola 7 than in Flaminia samples.

KEYWORDS: *peach fruit, NMR, metabolic profile, Ceratitis capitata*

INTRODUCTION

Among fruit-producing rosaceous crops, peach is the second most important fruit crop in Europe after apple and the third worldwide.[1] Fruit fly attack results in a peach disease, causing economically important losses. Mediterranean fly or medfly (*Ceratitis capitata*) is a species of fly diffused in tropical Africa, Australia, the Mediterranean area, and in some regions of North, Central, and South America. If uncontrolled, the damage can spread to up to 100% of the crop.[2] Estimates of the economical losses vary, but the severity of the damage is relevant. Costs to producers would range from $32 million to $370 million, depending on whether eradication is successful.[3,4] For instance, the state of Florida would incur an expected cost of $4.8 million each year if eradication were successful. In the Mediterranean Basin countries, if no control measures are applied against medfly, the annual fruits loss is estimated to be about U.S. $365 million. Under the current control programs, the direct damage (yield loss and control costs) and indirect damage (environmental impact and loss of export markets) amounts to U.S. $192 million per year. Add to this the cost of constructing and maintaining fruit treatment and any eradication facilities, and the full scale of the detriment resulting from the Mediterranean fruit fly in this area is difficult to gauge.[5]

Medfly is an extremely polyphagous insect with a host range of >250 species.[2] Larvae develop inside a very wide range of unrelated fruits and vegetables such as citrus, figs, apricots, nectarines, peaches, mandarins, kaki, strawberry, kiwifruit, and, to a lesser extent, apples and pears. Adult *C. capitata* lay their eggs under the skins of fruit, particularly in ripe ones and on damaged skin, where they hatch within 3 days and the larvae develop and feed inside the fruit. Moreover, the stinging marks due to ovipositing females cause damages that reduce fruit quality in the market. While the adults have a limited ability to disperse, at maximum 20 km from the host, the global fruit trade can transport infected fruit over thousands of miles. When an attack is detected, the damage is contained by destroying the fallen and infected host fruits. The absence of natural predators of this species makes the employment of chemicals paramount for the containment of population size, and the development of an effective integrated pest management protocol was accepted as a plant protection strategy for sustainable farming in Europe. As a consequence, numerous approaches have been developed to limit the chance of attack. Biological control has been tried with limited success,[6] and male annihilation coupled with sterile insect release have been used against some populations. Chemical sprays are not completely effective in protecting fruit because egg laying requires only a few minutes and chemical residues do not kill adults within this time frame. Proteinaceous liquid attractants in

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insecticide sprays are a recommended method of controlling populations in the vicinity of crops. The bait-insecticide sprays are applied to broadleaf plants that serve as refuge for flies and serve to encourage them to feed on the spray residue and can provide good rates of kill. To be effective, bait-insecticide sprays must be used in combination with good sanitation practices. These practices include destruction of unmarketable fruit on every harvest date and destruction of crop residues immediately after economic harvest has been completed.

Most of the aforementioned techniques are affected by considerable toxicity and environmental impact, and to reduce these collateral effects, a new strategy is needed. One of the most promising approaches is the realization of varieties naturally resistant to insect attacks. It is important to note that up to now, selective breedings (such as Fairtime, Redhaven, and Maria Bianca varieties) have been realized to improve peach taste and flavor[7,8] and are often linked to a decrease of the natural defenses of the plant. On the other hand, through the cross-breeding of varieties resistant to attacks with ones possessing good agronomic qualities, it might be possible to achieve a balance between those properties. To reach this target, it is important to determine the molecules and molecular mechanisms involved in defense against insect attacks.

In this study, the metabolic profiling of peaches from two varieties, Flaminia (FP) and Percoca Romagnola 7 (PR7P), is reported, to our knowledge, for the first time. FP is a Fayette−Fairtime cross-breeding made, selected, and tested by Istituto Sperimentale per la Frutticultura di Roma (Rome, Italy) and released in 1983 (selection IF 7310713). FP represents about 5% of the commercialized late maturation varieties.

PR7P is a clone identified within a Percoca population widespread in Romagna (Italy) by Istituto di Coltivazioni Arboree of the University of Bologna in 1962−1963. Even if this variety is mainly commercialized in a local market, it is relevant in the selective breeding to increase the resistance to insect attacks.

FP used for fresh market and characterized by good sensorial properties is easily attacked by *C. capitata*, whereas PR7P, used also for the canning industry, with its greater firmness and aroma, is known to be able to resist such attack.

All of the identified metabolites and their relative amounts permit a comprehensive view of the investigated varieties, allowing the metabolite profile to be mapped as completely as possible. The comparison between the metabolite profiles of the two varieties can suggest which molecules are related to the resistance to *C. capitata* attacks. In this regard, NMR spectroscopy plays an important role[9−11] as it yields a comprehensive metabolite profile and also provides direct structural information regarding individual metabolites in the sample.

MATERIALS AND METHODS

Sampling. The two peach varieties, PR7P (10 fruits) and FP (10 fruits) were hand harvested in an experimental field located in Lazio region, Italy. Fruits were harvested at commercial ripening; that is, FP fruits in the second week of September and PR7P in the third one. Fruits of the same variety were collected from different 5-year-old plants in the same orchard. In this orchard the agronomic conditions were identical for both cultivars.

In this stage of ripening, due to the high sugar content, fruits are severely subject to medfly attack. In the case of FP, in the absence of any treatment, the level of damage ranges between 90 and 100%, whereas PR7P is resistant to the attack.

FP fruits are characterized by a medium-large size (180−190 g), a circumference of about 23 cm, and an ovate, symmetrical, tip midlight hollow, shallow suture shape. PR7P fruits are characterized by a large size (200−220 g), a height of about 68 mm, and a width of about 78 mm.

Sample Preparation. Fresh-cut pulp (1 g) was frozen in liquid N_2, finely powdered, and submitted to an extraction according to the modified Bligh−Dyer methodology[12,13] with methanol/chloroform/water at 2:2:1 volumetric ratio. Sample was kept at 4 °C for 1 h and then centrifuged for 20 min at 11000g at 4 °C. The upper hydroalcoholic phase was carefully separated and dried under an N_2 flow. The dried phase was stored at −80 °C until the NMR analysis. To evaluate the reproducibility of sample preparation, three portions of one of the fruits were used to perform three extractions. Samples were prepared according to the method reported above and analyzed by NMR, and the intensity of selected NMR signals was measured. The obtained values showed a very good repeatability (standard deviation < 2.5%) for all signals.

NMR Spectra. The dry residue of the hydroalcoholic phase was dissolved in 0.75 mL of D_2O phosphate buffer (150 mM, pD 7.0, measured by a glass BNC electrode (Sigma-Aldrich)) containing 4,4-dimethyl-4-silapentane sodium sulfonate (DSS) as an internal standard. The NMR spectra of peach aqueous extracts were recorded at 27 °C on a Bruker AVANCE600 spectrometer operating at the proton frequency of 600.13 MHz and equipped with a Bruker multinuclear *z*-gradient inverse probehead. ^{1}H spectra were referenced to DSS signal (δ = 0.00 ppm), whereas ^{13}C spectra were referenced to the CH-1 resonance of α-D-glucose (δ = 93.10 ppm).

The ^{1}H spectra of the aqueous extracts were acquired by co-adding 512 transients with a recycle delay of 3 s and using a 90° pulse of 10.8 μs and 32K data points. To minimize the variability of the signals intensity due to water suppression, a simple solvent presaturation pulse sequence was used, where a soft pulse, corresponding to an excitation window of about 6 Hz, centered at the offset frequency was applied during the relaxation delay.[14] Moreover, the offset calibration was performed in each spectrum to minimize residual HDO signal.

2D NMR experiments,[14] namely, ^{1}H−^{1}H COSY (cosygpqfpr), ^{1}H−^{1}H TOCSY (mlevphpr), ^{1}H−^{1}H NOESY (noesyphpr), ^{1}H−^{13}C HSQC (hsqcetgp), and ^{1}H−^{13}C HMBC (hmbcgplpndqf), were performed using the same experimental conditions previously reported.[11] The mixing time for the ^{1}H−^{1}H TOCSY was 80 ms, and the mixing time for ^{1}H−^{1}H NOESY was 400 ms. The ^{1}H−^{13}C HSQC experiments were performed using a coupling constant $^1J_{C-H}$ of 150 Hz, and the ^{1}H−^{13}C HMBC experiments were performed using a delay for the evolution of long-range couplings of 80 ms.

The ^{31}P{^{1}H} NMR experiments were performed at 242.94 MHz by co-adding 1000 transients with a recycle delay of 7 s, a 20 kHz spectral width, 8K data points, a GARP pulse sequence for proton decoupling, and a 90° ^{31}P pulse of 15 μs. Chemical shifts for the ^{31}P spectrum were given in parts per million with respect to an external standard of aqueous 85% solution of H_3PO_4.

The ^{1}H−^{31}P HMBC spectra were obtained using a recycle delay of 2 s, a 90° ^{1}H pulse of 11 μs, a 90° ^{31}P pulse of 15 μs, 6 and 10 kHz spectral widths in proton (F2) and phosphorus (F1) dimensions, respectively, 1K data points in F2, 512 increments in F1, and a linear prediction up to 1K points in F1. Data were processed using unshifted sinusoidal window functions in both dimensions. The delay for the evolution of J_{P-H} long-range couplings was 80 ms.

Pulsed field gradient spin echo (PGSE) experiments[15] were performed with a pulsed field gradient unit producing a magnetic field gradient in the *z*-direction with a strength of 55.4 G cm^{-1}. The stimulated echo pulse sequence using bipolar gradients with a longitudinal eddy current delay was used. The strength of the sine-shaped gradient pulse with a duration of 1.4 ms was logarithmically incremented in 32 steps, from 2 to 95% of the maximum gradient strength, with a diffusion time of 120 ms and a longitudinal eddy current delay of 25 ms. After Fourier transformation and a baseline correction, the diffusion dimension was processed using the DOSY[16] subroutine of the Bruker TOPSPIN 1.3 software package.

Table 1. Summary of the Metabolites Identified in the 600 MHz ^{1}H Spectrum of a Peach Aqueous Extract[a]

compound	assignment	^{1}H (ppm)	multiplicity (*J*, Hz)	^{13}C (ppm)
organic acids				
citric (CA)	α,γ-CH	2.76*		44.77
	α′,γ′-CH	2.88		44.77
fumaric (FA)	CH	6.51*	s	
malic (MA)	α-CH	4.41		69.33
	β,β′ CH_2	2.69*, 2.83		40.86
quinic (QA)	CH_2-1,1′	1.88*, 2.09		41.48
	CH-2	4.02		67.88
	CH-3	3.55		76.16
	CH-4	4.15		71.36
	CH_2-5,5′	2.05, 2.00		38.21
	C-6			78.06
shikimic (SHA)	CH_2-7	2.76, 2.22		
	CH-6	4.02		
	CH-5	3.75		
	CH-4	4.43		
	CH-3	6.69		
succinic (SA)	α,β-CH_2	2.41*	s	34.80
carbohydrates				
α-glucose (αGLC)	CH-1	5.22*		93.10
	CH-2	3.53		72.49
	CH-3	3.70		73.84
	CH-4	3.42		70.67
	CH-5	3.83		72.52
β-glucose (βGLC)	CH-1	4.63		96.97
	CH-2	3.24*		75.17
	CH-3	3.47		76.84
	CH-4	3.40		70.70
	CH_2-6,6′	3.71, 3.89		61.80
sucrose (SUCR)	GLC CH-1	5.41*		93.22
	CH-2	3.55		72.11
	CH-3	3.75		73.54
	CH-4	3.46		70.26
	CH-5	3.83		73.38
	CH_2-6	3.81		61.18
	FRU CH_2-1′	3.67		62.44
	C2′			104.85
	CH-3′	4.21		77.45
	CH-4′	4.04		75.04
	CH-5′	3.89		82.44
	CH_2-6	3.81		63.38
myo-inositol (MI)	CH-1	4.05		73.18
	CH-2,5	3.52		72.48
	CH-3,6	3.61		73.28
	CH-4	3.27*		75.28
α-D-fructofuranose (αFRUfu)	CH-3	4.10		83.00
	CH-5	4.05		82.33
β-D-fructofuranose (βFRUfu)	CH-3	4.10		76.49
	CH-4	4.10		75.56
	CH_2-6,6′	3.81, 3.65		63.87
β-D-fructopyranose (βFRUpy)	CH_2-1,1′	3.56, 3.70		64.99
	CH-3	3.79		68.70
	CH-4	3.88		70.72
	CH-5	3.99		70.25
	CH_2-6,6′	3.70, 4.02*		64.39
α-xylose (αXYL)	CH-1	5.19*	d (3.8)	93.24
	CH-2	3.52		
	CH-3	3.60		
	CH-4	3.64		
	CH_2-5,5′	3.68		
β-xylose (βXYL)	CH-1	4.57*	d (7.9)	97.50

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Table 1. continued

compound	assignment	^{1}H (ppm)	multiplicity (*J*, Hz)	^{13}C (ppm)
	CH-2	3.21		
	CH-3	3.43		
	CH-4	3.61		
	CH_2-5, 5′	3.31, 3.92		66.22
α-fucose (*α*FUC)	CH-1	5.19	d (4.3)	
	CH_3	1.19	d (6.7)	
β-fucose (*β*FUC)	CH-1	4.54	d (8.0)	
	CH_3	1.23*	d (6.6)	
α-rhamnose (*α*RHA)	CH-1	5.10	d (1.2)	
	CH_3	1.26	d (6.2)	
β-rhamnose (*β*RHA)	CH-1	4.86	d (1.2)	
	CH_3	1.28	d (5.9)	
fructose-6P (FRU6P)	CH_2-6,6′	3.95		(^{31}P) 1.13
α-glucose-6P (*α*GLC6P)	CH_2-6,6′	4.13, 4.07		(^{31}P) 1.39
β-glucose-6P (*β*GLC6P)	CH_2-6,6′	4.04, 4.02		(^{31}P) 1.39
amino acids				
alanine (ALA)	*α*-CH	3.78		
	β-CH_3	1.49*		
	COOH			177.0
asparagine (ASN)	*α*-CH	4.01		52.27
	β,*β*′-CH_2	2.86*, 2.94		35.48
isoleucine (ILE)	*α*-CH	3.68		
	β-CH	1.97		36.80
	γ-CH_2	1.26 1.47		
	γ′-CH_3	1.00*	d (7)	
	δ-CH_3	0.92	t (7)	11.96
phenylalanine (PHE)	CH-3,5	7.42	m	
	CH-4	7.36	m	
	CH-2,6	7.32	m	
threonine (THR)	*α*-CH	3.59	d (6.6)	
	β-CH	4.25		66.91
	γ-CH_3	1.32*		20.53
valine (VAL)	*α*-CH	3.62		
	β-CH	2.28		
	γ-CH_3	0.99	d (7)	
	γ′-CH_3	1.04*	d (7)	19.05
miscellaneous metabolites				
choline (CHN)	$N(CH_3)_3^+$	3.22*		54.98
chlorogenic acid (CGA)	CH_2-2′	2.06, 2.21		38.92
	CH-3′	5.32		72.07
	CH-4′	3.88		
	CH-5′	4.24		
	CH-8	6.41*	d (16)	115.74
	CH-7	7.67	d (16)	
	CH-2	7.22	d (2)	116.22
	CH-5	6.96	d (8.2)	117.31
	CH-6	7.14	dd (8.2, 2)	123.72
neochlorogenic acid (nCGA)	CH_2-2′	1.92, 2.09		
	CH-3′	4.07		
	CH-4′	3.62		
	CH-5′	5.38		74.06
	CH_2-6′	2.09 2.23		
	CH-8	6.43*	d (16)	115.95
	CH-7	7.66	d (16)	
	CH-2	7.22	d (2)	
	CH-5	6.96	dd (8.2,2)	
	CH-6	7.15	d (8)	
trigonelline (TRIG)	CH-1	9.12		
	CH-3,5	8.83		
	CH-4	8.08		
	CH_3	4.43	s	

Table 1. continued

compound	assignment	^{1}H (ppm)	multiplicity (*J*, Hz)	^{13}C (ppm)
catechin (CTH)	CH-8	6.12*	d (2)	
	CH-6	6.03	d (2)	
	CH-2′	6.96	d (1.8)	
	CH-3′	6.94	d (8.3)	
	CH-6′	6.87	dd (8.3;1.8)	
adenosine triphosphate (ATP)	CH-2	8.51	s	
	CH-8	8.26	s	
	Rib CH-1′	6.14	d	
	Rib CH-3′	4.56		
L-α-glycerophosphorylcholine (αGPL)	CH_2-1′	4.32		(^{31}P) 0.58
	CH_2-1	3.94, 3.87		
	CH_2-3	3.67		
uridine (URI)	CH-5; CH-1′	5.97*		
U1		0.95*	d (6.7)	
U2		1.22*	d (6.5)	
U3		1.27*	d (6.8)	
U4		5.56*	d (8.3)	
U5		5.57*	d (8.3)	
U6		5.91*	s	

[a]Abbreviations are given in parentheses. Signals selected for the statistical analysis are denoted by an asterisk.

The intensities of 29 selected ^{1}H resonances due to water-soluble metabolites (see Table 1) were measured with respect to the intensity of DSS signal used as internal standard and normalized to 100.

Statistical Analysis. The statistical analysis of NMR data was performed using the Statistica package for Windows (version 5.1). Before the principal component analysis (PCA) and the analysis of the variance (ANOVA) were performed, the intensity of the 29 selected variables were mean-centered and each variable was divided by its standard deviation (autoscaling). PCA results are shown reporting the scores of the principal components and also as a plot of the variable loadings. In the ANOVA the variables with the highest index of variability were selected according to their *p* level and *F* values. The *p* level represents the decreasing index of reliability of a result and gives the probability of error involved in accepting a result as valid. A *p* level of 0.05 (5% probability of error) was chosen as a borderline acceptable error level. The *F* value is the ratio between groups' variability to within-group variability: the larger is this ratio, the higher is the discrimination power of the corresponding variable.

RESULTS AND DISCUSSION

Metabolite Profiling. To have a complete view of the peach fruit, a detailed study of the metabolite profile of the aqueous extracts was performed.

The assignment of the ^{1}H spectrum of a peach aqueous extract (see Figure 1), obtained by 1D and 2D NMR experiments and, when necessary, by adding standard compounds, is reported in Table 1 and will be discussed for each class of compounds. For abbreviations, see also Table 1.

Organic Acids. In the ^{1}H spectrum of aqueous peach fruit extracts citric acid (CA), fumaric acid (FA), malic acid (MA), quinic acid (QA), succinic acid (SA), and shikimic acid (SHA) were identified by means of their diagnostic peaks.

Sugars and Phosphorus-Containing Compounds. Different sugars are present in peach. Fructose, glucose, and sucrose, the main carbohydrates in peach fruit previously detected by liquid chromatography,[17,18] were identified in the ^{1}H spectrum by means of their diagnostic signals (see Table 1 and Figure 1). α-Xylose and β-xylose were also identified by means of their diagnostic anomeric ^{1}H doublets at 5.19 and 4.57 ppm, respectively (see Table 1), and the assignment was confirmed by 2D experiments. Fucose and rhamnose were also identified by adding the corresponding standard compounds (see Table 1). *myo*-Inositol was identified in the ^{1}H spectrum owing to its characteristic spin system in the TOCSY map.

The presence of glucose-6-phosphate (αGLC6P and βGLC6P), fructose-6-phosphate (FRU6P), α-L-glycerophosphorulcholine (αGPC), and orthophosphate was evidenced by the ^{31}P{^{1}H} NMR spectrum reported as a projection in the F1 dimension of the ^{1}H−^{31}P HMBC map (see Figure 2). Furthermore, the long-range contacts observed in the map allowed the assignment of methylene protons in position 6 of GLC6P and FRU6P and the methylene protons of αGPC, namely, CH_2-1, CH_2-3, and CH_2-1′ (see Table 1 and Figure 2). It is worth noting that both α and β anomers of GLC6P were detected. The assignment was further confirmed by adding the corresponding standard compounds. The ^{31}P{^{1}H} NMR spectrum also shows two unassigned resonances at 0.64 and 1.63 ppm. The ^{31}P resonance at 0.64 ppm suggests the presence of a compound with a phosphodiester moiety similar to that present in αGPC.

Free Amino Acids. The ^{1}H spectrum of peach extracts allowed the free amino acid composition to be obtained. Six amino acids, namely, alanine (ALA), threonine (THR), aspartate (ASP), valine (VAL), isoleucine (ILE), and phenylalanine (PHE), were identified, as reported in Table 1.

Phenolics and Other Compounds. In the ^{1}H NMR spectrum, chlorogenic (CGA) and neochlorogenic (nCGA) acids were identified on the basis of their diagnostic spin systems (see Table 1). CGA (see the sketch in Figure 3) shows characteristic aromatic signals at 7.14 ppm (CH-6), 6.96 ppm (CH-5), and 7.22 ppm (CH-2) and double-bond protons at 7.67 ppm (CH-7) and 6.41 ppm (CH-8). The assignment of the quinic acid moiety protons at 2.06 and 2.21 ppm (CH_2-2′), 5.32 ppm (CH-3′), 3.88 ppm (CH-4′), and 4.24 ppm (CH-5′) was obtained by ^{1}H−^{1}H TOCSY.

nCGA (see the sketch in Figure 3) shows characteristic aromatic signals at 7.15 ppm (CH-6), 6.96 ppm (CH-5), and 7.22 ppm (CH-2) and double-bond protons at 7.66 ppm (CH-7) and 6.43 ppm (CH-8). Again, the assignment of the quinic acid moiety protons at 1.92 and 2.09 ppm (CH_2-2′), 4.07 ppm

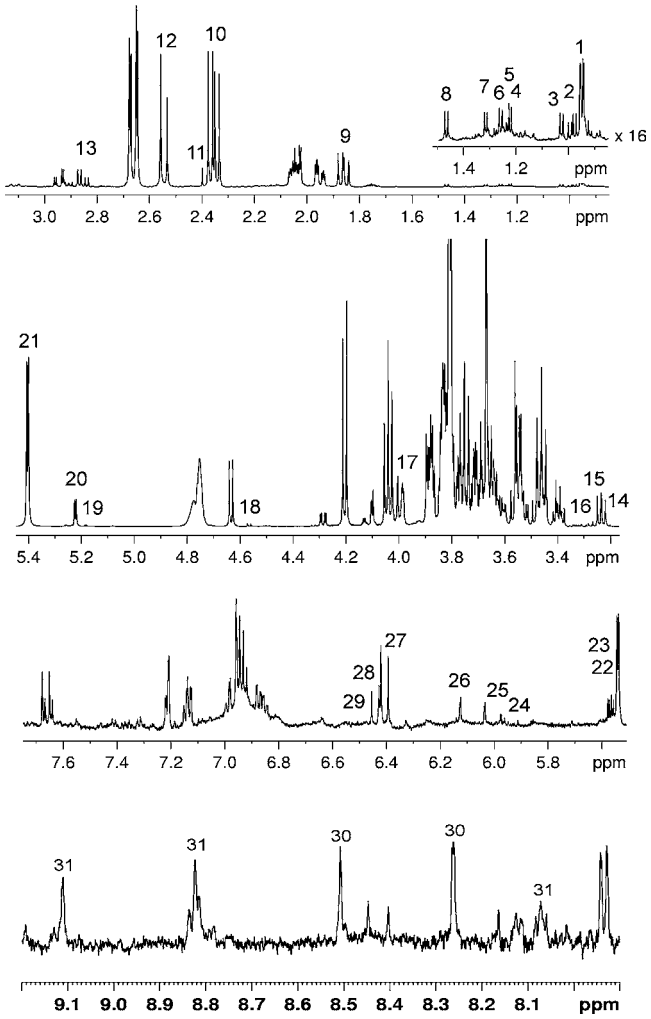

Figure 1. 600.13 MHz ^{1}H NMR spectrum of a peach extract in aqueous solution at 27 °C. The assignments of some peaks are also reported: 1, U1; 2, ILE; 3, VAL; 4, U2; 5, FUC; 6, U3; 7, THR; 8, ALA; 9, QA; 10, MA; 11, SA; 12, CA; 13, ASN; 14, CHN; 15, *β*GLC; 16, MI; 17, *β*FRUpy; 18, *β*XYL; 19, *α*XYL; 20, *α*GLC; 21, SUCR; 22, U4; 23, U5; 24, U6; 25, URI; 26, CTH; 27, CGA; 28, nCGA; 29, FA; 30, ATP; 31, TRIG.

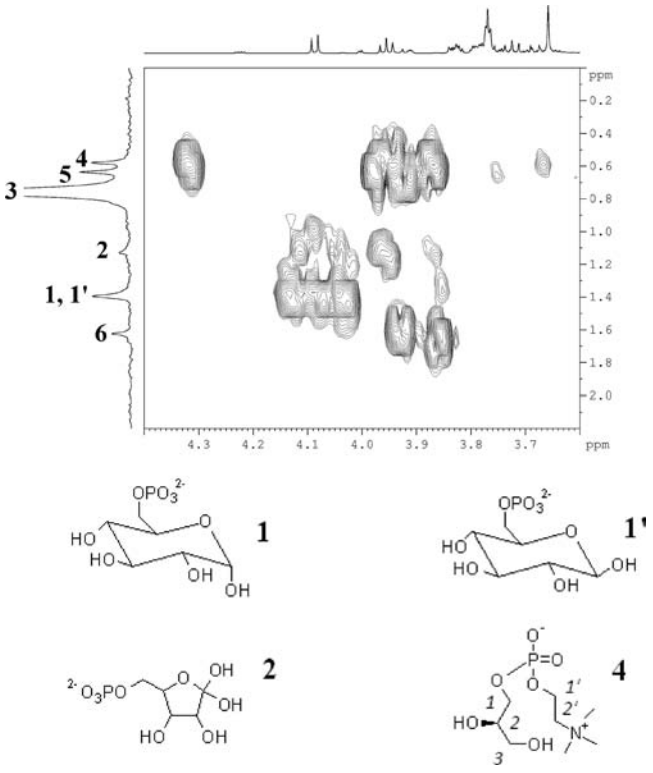

Figure 2. ^{1}H−^{31}P HMBC map of an aqueous extract of peach fruit. The ^{1}H and ^{31}P{^{1}H} NMR spectra are reported as projections in the F2 and F1 dimensions, respectively. **1**, **1′**, *α*-glucose-6-phosphate and *β*-glucose-6-phosphate; **2**, fructose-6-phosphate; **3**, orthophosphate; **4** *α*-L-glycerophosphorylcholine; **5**, **6**, unassigned compounds.

(CH-3′), 3.62 ppm (CH-4′), 5.38 ppm (CH-5′), and 2.09 and 2.23 ppm (CH_2-6′) was obtained by ^{1}H−^{1}H TOCSY.

CGA and nCGA acids have been quantified and identified in three peach varieties by Villarino et al.[19] using HPLC-DAD and HPLC systems coupled to a hybrid quadrupole time-of-flight mass spectrometer. It has been suggested[20] that the high concentration of CGA and/or nCGA in immature fruits might contribute to their reduced susceptibility or increased resistance to brown rot infection by interfering with fungal melanin production. Both metabolites are known for their inhibitory effect on herbivores as well as pathogens, and it has been observed that the presence of CGA could be related to host plant resistance to insect attacks.[18] Isomers of chlorogenic acid have been previously identified and quantified in prune (*Prunus domestica* L.) by HPLC. It has been also found that all isomers showed almost the same antioxidative activity.[21]

In our case, CGA and nCGA were found to be more abundant in PR7P, which is known to resist *C. capitata* attack, than in the FP variety (see Figure 3). This result is in agreement with the data reported in the literature, which suggest that the phenylpropanoid pathway plays a role in the resistance against insect attacks.[20]

The ^{1}H spectrum shows also the presence of catechin identified by the diagnostic chemical shift of the doublet signals at 6.03 ppm (CH-6) and 6.12 ppm (CH-8) (see Figure 1). Moreover, the assignment of the aromatic protons at 6.96 ppm (CH-2′), 6.94 ppm (CH-3′), and 6.87 ppm (CH-6′) was obtained by ^{1}H−^{1}H TOCSY.

Trigonelline, a product from the metabolism of vitamin B_3, was also identified by means of the diagnostic spin system (see Figure 1 and Table 1). The characteristic peaks of trigonelline were observed at 9.12 ppm (CH-1), 8.83 ppm (CH-3,5), 8.08 ppm (CH-4), and 4.43 ppm (CH_3).

The presence of ATP was revealed by the observation of singlets at 8.51 ppm (CH-2) and 8.26 ppm (CH-8) of the adenine moiety and two signals of the ribose moiety at 6.14 ppm (CH-1′) and 4.56 ppm (CH-3′) (see Figure 1 and Table 1). The addition of the corresponding standard compound confirmed this assignment.

Finally, choline was identified by means of the characteristic methyl signal at 3.22 ppm.

Comparison between FP and PR7P Varieties. The metabolite profiles of the two peach varieties with different degrees of protection against insect attacks were compared. As reported, the NMR profiles show the presence of the same metabolites, although in different concentrations. To evaluate the presence of latent variables correlating the single metabolites, PCA was applied to the intensity of 29 ^{1}H resonances (see Table 1 and Figure 4). The first two PCs account for 52.9% of the variability within the data, PC1

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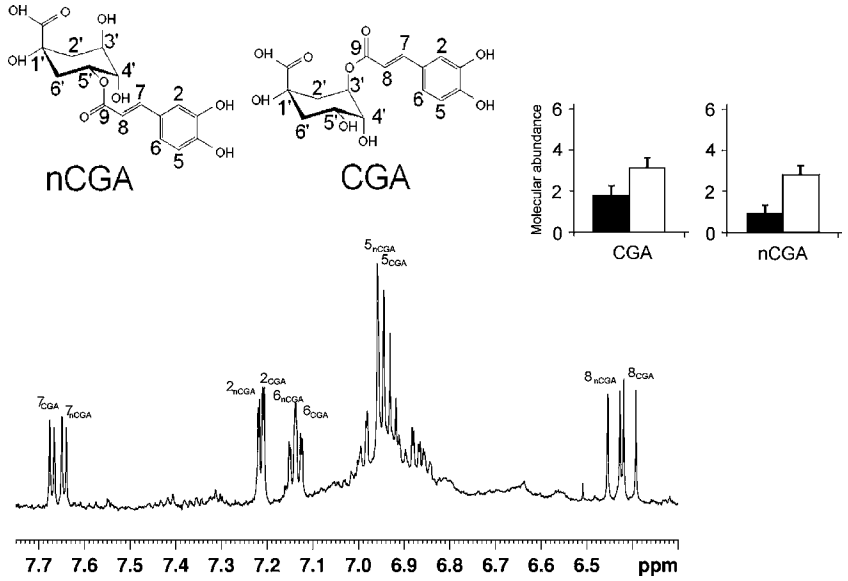

Figure 3. Aromatic spectral region of a peach extract in aqueous solution. Resonances of chlorogenic (CGA) and neochlogenic (nCGA) acids are labeled according to the numbering reported in the schemes. Histograms of CGA and nCGA in FP (black bars) and PR7P (white bars) resulting from the quantitative NMR spectroscopic analysis of the corresponding CH-8 resonances are also reported.

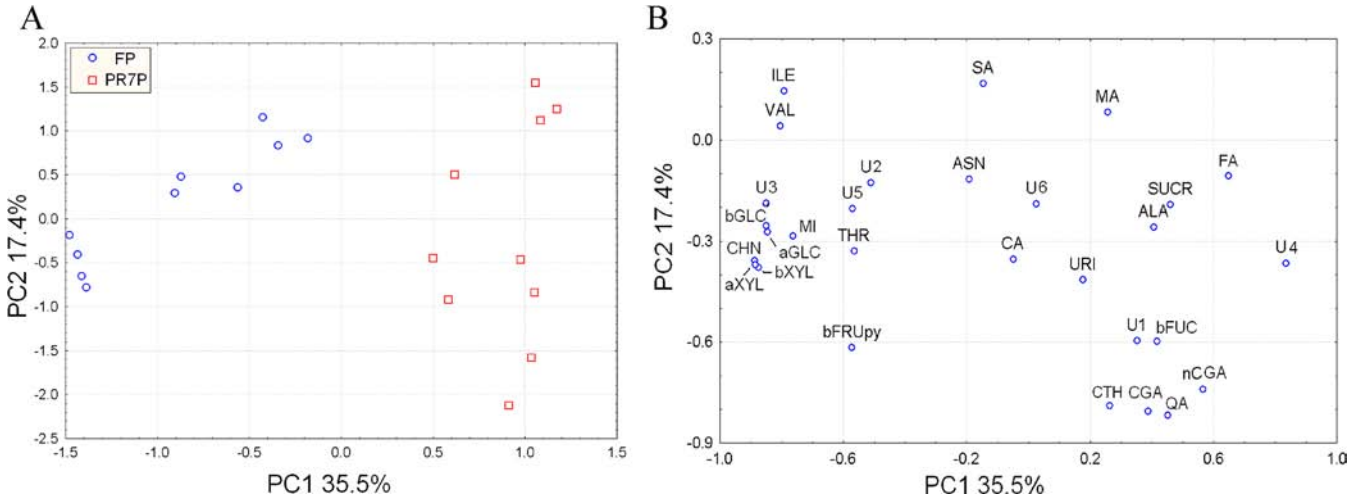

Figure 4. PCA applied to the intensity of 29 NMR resonances: (A) plot of sample scores; (B) plot of variable loadings; (○) FP variety; (□) PR7P variety.

providing for 35.5% and PC2 for 17.4%. The contribution of the variables (the metabolites) to the principal components is given by the variable loadings reported in Figure 4B.

Variables with loadings lying on the left side of the PC1 axis, namely, VAL, ILE, αGLC, βGLC, αXYL, βXYL, MI, U3, and CHN, are present in the highest concentration in FP samples, whereas variables with loadings lying on the right side of PC1 axis, such as ALA, CGA, nCGA, U4, QA, FA, SUCR, and βFUC, are more abundant in PR7P samples. The ANOVA (see Table 2) confirmed the statistical significance of these variables in the discrimination between FP and PR7P varieties.

The pulp of PR7P, the more resistant variety, presents greater amounts of ALA, QA, CGA, and nCGA than FP. These molecules are reported to be related to the defense against fungal and insect attacks in other members of the Rosaceae family, suggesting that the phenylpropanoid pathway is at least partially involved in the repulsion of *C. capitata*. In particular, this metabolic pathway leads to the synthesis of volatile 2-phenylacetaldehyde and 2-phenylethanol, the precursors (quinic and shikimic acids) of which are nonvolatile. Both volatile polyphenols, as well as other molecules developed from the same pathway such as cinnamic acid, its derivatives, and some volatile variants of phenylalanine, are known to possess antimicrobic and antimycotic activities.[22]

Several studies have been carried out on *C. capitata* to identify the volatile compounds that the insect finds more appealing,[23,24] and the strongest responses were given by medium chain length (from C5 to C8) alcohols, acids, and aldehydes, both linear and branched. The linear chains are synthesized starting from polyunsaturated fatty acids and the corresponding branched molecules from aliphatic amino acids such as ALA, VAL, ILE, and LEU. Moreover, whereas VAL- and ILE-derived volatiles are mainly involved in fruit aroma,[25] ALA is also the precursor of compounds involved in plant defense.[26] Of particular interest is the evidence that the PR7P variety, the more resistant and the less appealing variety,

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Table 2. Results of ANOVA Applied to 29 Selected Metabolites To Discriminate between FP and PR7P Peach Varieties: *F* Value, *p* Level, and FP/PR7P Mean Value Ratio

variable	*F* value	*p* level	FP/PR7P[a]
ALA	8.9	0.008	0.51
ASN	0.1	0.825	
CA	0.0	0.990	
CHN	21.1	0.00023	1.58
CGA	5.3	0.034	0.57
nCGA	11.4	0.0034	0.32
CTH	2.0	0.174	
FA	12.8	0.0022	0.47
βFRUpy	6.1	0.024	1.20
βFUC	8.5	0.0093	0.74
ILE	20.2	0.00028	2.61
αGLC	37.2	0.000009	1.54
βGLC	37.6	0.000009	1.54
MA	0.2	0.657	
MI	13.9	0.0015	1.21
QA	10.3	0.0049	0.72
SA	1.4	0.255	
SUCR	4.5	0.047	0.88
THR	2.2	0.156	
U1	8.0	0.011	0.81
U2	2.8	0.115	
U3	19.9	0.00030	1.34
U4	83.0	0.00000004	0.55
U5	3.0	0.099	
U6	0.1	0.823	
URI	0.9	0.347	
VAL	15.7	0.00092	1.79
αXYL	21.4	0.00021	1.63
βXYL	19.2	0.00036	1.54

[a]Reported only when significant at the statistical level.

possesses a lower amount of VAL and ILE along with significantly higher levels of ALA compared to FP.

On the basis of this literature data, it is also possible to identify the biological importance of the aforementioned principal components: PC1, with its emphasis on defense metabolites, describes the capacity to repel insect attacks, whereas PC2, due to its higher levels of flavor precursors and free carbohydrates, is an index of good sensorial properties.

This study demonstrated, on the basis of the analysis of nonvolatile precursors, that the resistance of PR7P toward medfly attacks could be ascribed to a different volatile metabolic profile compared to the vulnerable cultivar FP because the former showed the presence of molecules, ALA and QA especially, the metabolism of which leads to the biosynthesis of volatile compounds such as benzaldehyde and benzyl alcohol characterized by the ability to repel herbivore attacks. At the same time, the variety was poorer in VAL and ILE correlated with the formation of branched medium-length chain alcohols, acids, and aldehydes, which are volatiles known to attract the insect. The observation that ALA is also a possible precursor of volatile compounds has a great practical importance because it has been observed[27] that the quality of peach fruits was negatively correlated to the amount of quinic acid, one of the main intermediates of the phenylpropanoid pathway. The presence of another pathway related to resistance against insect attacks can lead to the breeding of resistant cultivars without compromising the necessary sensorial properties.

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ABBREVIATIONS USED

ATP, adenosine triphosphate; ALA, alanine; ASN, asparagine; CHN, choline; CA, citric acid; CGA, chlorogenic acid; COSY, correlated spectroscopy; CTH, catechin; DOSY, diffusion ordered spectroscopy; DSS, 4,4-dimethyl-4-silapentane sodium sulfonate; αFRUfu, α-D-fructofuranose; βFRUfu, β-D-fructofuranose; βFRUpy, β-D-fructopyranose; αFRU6P, α-fructose-6-phosphate; βFRU6P, β-fructose-6-phosphate; αFUC, α-fucose; βFUC, β-fucose; FA, fumaric acid; GARP, globally optimized alternating phase rectangular pulse; αGLC, α-glucose; βGLC, β-glucose; αGLC6P, α-glucose-6-phosphate; βGLC6P, β-glucose-6-phosphate; αGPC, α-L-glycerolphosphorylcholine; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum coherence; ILE, isoleucine; MA, malic acid; MI, *myo*-inositol; nCGA, neochlorogenic acid; NOESY, nuclear Overhauser and exchange spectroscopy; PGSE, pulsed field gradient spin echo; PHE, phenylalanine; QA, quinic acid; αRHA, α-rhamnose; βRHA, β-rhamnose; Rib, ribose; SHA, shikimic acid; SA, succinic acid; SUCR, sucrose; THR, threonine; TOCSY, total correlation spectroscopy; TRIG, trigonelline; URI, uridine; VAL, valine; αXYL, α-xylose; βXYL, β-xylose.

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