JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Relationship between Cyanogenic Compounds in Kernels, Leaves, and Roots of Sweet and Bitter Kernelled Almonds

F. DICENTA,*,† P. MARTÍNEZ-GÓMEZ,†,‡ N. GRANÉ,§ M. L. MARTÍN,§ A. LEÓN,|| J. A. CÁNOVAS,† AND V. BERENGUER§

Departamento de Mejora y Patología Vegetal, CEBAS-CSIC, P.O. Box 4195, 30080 Murcia, Spain, Department of Pomology, University of California, Davis, California 95616, Departamento de Química Analítica, Facultad de Ciencias, Universidad de Alicante, P.O. Box 99, 03080 Alicante, Spain, and Departamento de Nutrición y Fisiología Vegetal, CEBAS-CSIC, P.O. Box 4195, 30080 Murcia, Spain

The relationship between the levels of cyanogenic compounds (amygdalin and prunasin) in kernels, leaves, and roots of 5 sweet-, 5 slightly bitter-, and 5 bitter-kernelled almond trees was determined. Variability was observed among the genotypes for these compounds. Prunasin was found only in the vegetative part (roots and leaves) for all genotypes tested. Amygdalin was detected only in the kernels, mainly in bitter genotypes. In general, bitter-kernelled genotypes had higher levels of prunasin in their roots than nonbitter ones, but the correlation between cyanogenic compounds in the different parts of plants was not high. While prunasin seems to be present in most almond roots (with a variable concentration) only bitter-kernelled genotypes are able to transform it into amygdalin in the kernel. Breeding for prunasin-based resistance to the buprestid beetle *Capnodis tenebrionis* L. is discussed.

KEYWORDS: Almond; *Prunus dulcis* Miller; bitterness; cyanogenic compounds; amygdalin; prunasin; *Capnodis tenebrionis* L.; mandelonitrile glucoside

INTRODUCTION

The biochemistry of cyanogenic glucosides is relatively well-known (*1*−*3*). In almond, Frehner et al. (*4*) observed the accumulation of amygdalin (which is associated with bitterness) in developing almond fruits, probably from prunasin.

Resistance to the buprestid beetle *Capnodis tenebrionis* L. (capnode) has been associated with the presence of prunasin in the roots of *Prunus* species (*5*−*7*). The relationship between prunasin and amygdalin in the different parts of the same plant remain unclear, however. A clear relationship for these compounds between kernels and roots would facilitate the breeding of resistant rootstocks with high concentrations of cyanogenic compounds, since the inheritance of the bitterness of the kernel is known (*8*−*10*). If this relationship would not exist, sweet-kernelled almonds with a high prunasin content in their roots might be obtained for cultivation on their own roots.

The objective of this work was to determine the relationship of the cyanogenic compounds (amygdalin and prunasin) among kernels, leaves, and roots of sweet-, slightly bitter-, and bitter-kernelled almond genotypes to design strategies for breeding almonds with a high concentration of cyanogenic compounds in their roots, thus probably conferring resistance to capnode.

* To whom correspondence should be addressed. Tel.: +34 968 396200. Fax: +34 968 396213. E-mail: fdicenta@cebas.csic.es.
† Departamento de Mejora y Patología Vegetal, CEBAS-CSIC.
‡ University of California.
§ University de Alicante.
|| Departamento de Nutrición y Fisiología Vegetal, CEBAS-CSIC.

MATERIAL AND METHODS

Plant Material. Fifteen seedlings of a cross between the cultivars Garrigues and Tuono almond were studied. The kernel flavor was determined by tasting the kernels by two different evaluators over 2 years. Five were characterized as sweet, 5 slightly bitter, and 5 bitter. Ten year old own-rooted trees were studied.

Roots (about 5 mm in diameter) and young leaves were sampled during the spring from each of the 15 genotypes. Mature fruits were collected, and the hull and shell were removed. Kernels, roots, and leaves were frozen, lyophilized in a Cryodos Telstar lyophilizer for 48 h at −85 °C and 10^{-2} mbar, ground, homogenized, and analyzed. At least two independent analyses per sample were performed.

Total Cyanide. The total cyanide determination involved two steps: release and measurement.

(i) Release of Hydrogen Cyanide by Enzymatic Hydrolysis. Samples (0.2 g) were processed with 0.1 g of β-glucosidase (Sigma G 0395) in 4 mL of acetate buffer (pH 5.5) in a cylindrical glass vessel (3 cm diameter × 5 cm high) for 24 h at 35 °C. The hydrogen cyanide released was collected by microdiffusion in 1 mL of 0.2 M NaOH, located in a very small glass collector in the interior of the vessel, as described by Williams (*11*).

(ii) Measurement of Hydrogen Cyanide. Measurement of cyanide was carried out gravimetrically or spectrophotometrically, depending on the amount of cyanide. Gravimetric titration was performed with a standard solution of $AgNO_3$ and dimethyl-aminobencilidenrhodanine indicator, following the recommended procedure for samples containing more than 20 mg cyanide/100 g sample (*12*). Spectrophotometric determination at 580 nm, after derivatization with barbituric acid in pyridine (*13*), was used for samples with lower cyanide levels.

10.1021/jf0113070 CCC: $22.00
Published on Web 02/19/2002

Table 1. ANOVA (*p* Value) of the Amygdalin, Prunasin, and Total Cyanide Contents in Kernels, Leaves, and Roots for the 15 Genotypes and the 3 Flavor Groups (Bitter, Slightly Bitter, and Sweet Kernelled Genotypes) Assayed

		among genotypes	among groups
kernels	amygdalin	0.0001	0.0001
	total cyanide	0.0001	0.0001
leaves	prunasin	0.0001	0.0019
	total cyanide	0.0001	0.0014
roots	prunasin	0.0001	0.0173
	total cyanide	0.0001	0.0160

Amygdalin and Prunasin. Chromatographic determination of amygdalin and prunasin levels was carried out for 0.2 g samples (kernel, root, and leaf) and extracted with 10 mL of methanol for 12 h at room temperature. Extractions of roots and leaves were made in the presence of 0.1 g of polyvinylpolypirrolidone (Sigma) or active carbon (Norit CNR 115) to ensure the freedom from pigments, which can interfere with the chromatography. Determination was performed isocratically in a 446 Waters high-performance liquid chromatography (HPLC) system, following a procedure similar to that described by Kajiwara et al. (*14*), under the following conditions: Waters Symmetry column 250 mm × 4.6 mm, flow rate 1.5 mL/min, acetonitrile:water 20:80 as eluent, 20 μL of sample, and detection under UV at 218 nm.

To compare and correlate the contents of amygdalin and prunasin obtained by HPLC with those of cyanide obtained by microdiffusion, the HPLC values were transformed into cyanide values. Results (mg CN^-/100 g of dried sample) were tested using analysis of variance (ANOVA) to determine if the levels of cyanogenic compounds and cyanide in kernels, roots, and leaves were related to the genotype and the flavor of the kernel (sweet, bitter, or slightly bitter). Duncan's multiple range test was used to identify significant differences. Pearson's correlation coefficient was also calculated to test the relationships between the cyanogenic compounds in kernels, roots, and leaves.

RESULTS

Technique. The amygdalin peak appeared at 3.5 min, and that of prunasin appeared at 6.0 min under our chromatography conditions. Both were generally free from copurifying contaminants, as shown by runs made with diode array detection. The baseline was also sufficiently flat and clear so that peaks could be integrated automatically in most cases. Occasional disagreement with data obtained from the hydrolysis and diffusion methods forced us to repeat both determinations until results coincided. Reproducibility of the totals of amygdalin and prunasin from chromatography and those of cyanide from diffusion expressed as a coefficient of variation were estimated to be 5 and 10%, respectively, at a level of 50 mg CN^-/100 g sample. A significant source of variability in these figures could be the sampling itself. At higher levels, both determinations are similar in precision, but below 15 mg/100 g, chromatographic data appear to be more precise.

ANOVA. The ANOVA concludes significant differences among the 15 genotypes for the level of prunasin, amygdalin, and total cyanide in kernels, leaves, and roots (**Table 1**). ANOVA of groups (sweet, slightly bitter, and bitter genotypes) (**Table 1**) detected differences for amygdalin content and for total cyanide in the kernels. Significant differences were detected for prunasin and total cyanide for leaves. Differences were also observed, to a lesser extent, for prunasin and total cyanide in roots.

Kernels. Only the cianoglucoside amygdalin was detected in kernels by HPLC but with variable values (between 0 and 411 mg CN^-/100 g) depending on the genotype (**Table 2**). The highest amygdalin contents were observed in the bitter (between 205 and 411 mg CN^{-1}/100 g), with less in the slightly bitter (from 2 to 33 mg CN^{-1}/100 g), and the least in the sweet-kernelled (from 0 to 10 mg CN^{-1}/100 g) individuals. These values are reflected clearly in the means for the bitter (265 mg CN^{-1}/100 g), slightly bitter (13 mg CN^{-1}/100 g), and sweet (2 mg CN^{-1}/100 g) individuals. In the means of groups, Duncan's test differentiated only between the bitter kernels and the rest (**Table 2**). The correlation between amygdalin contents and total cyanide (obtained by using two different techniques) showed a correlation coefficient of 0.99 (**Table 3**).

Leaves. The only cyanogenic compound detected by HPLC in leaves was prunasin, with a smaller variability (from 6 to 88 mg CN^{-1}/100 g) and with a lower concentration (36 mg CN^{-1}/100 g on average) than for amygdalin in kernels (**Table 2**). The mean prunasin values in leaves were higher for the bitter individuals (56 mg CN^{-1}/100 g), intermediate for the slightly

Table 2. Cyanide Contents (mg/100 g) in Kernels, Leaves, and Roots of 15 Genotypes and 3 Flavor Groups by HPLC and Microdiffusion[a]

		kernels		leaves		roots	
flavor	genotype	HPLC[b]	microdiffusion	HPLC[c]	microdiffusion	HPLC[c]	microdiffusion
bitter	67	411.5 a	384.0 a	41.5 c	40.5 c	202.0 a	192.0 b
	60	287.5 b	281.0 b	88.0 a	96.5 a	106.5 f	109.0 e
	62	216.5 c	259.5 c	42.5 c	42.0 c	124.5 e	144.0 d
	56	209.0 d	222.0 e		33.0 d	127.0 e	137.5 d
	64	205.0 e	233.5 d	53.5 b	56.5 b	187.0 b	208.5 a
	group means	265.9 a	276.0 a	56.4 a	53.7 a	149.4 a	158.2 a
slightly bitter	79	33.0 f	16.0 f	28.0 d	15.5 g	58.5 hi	56.5 h
	63	16.0 g	14.5 f	54.0 b	55.0 b	64.5 ghi	78.0 fg
	88	9.0 h	6.0 g	33.0 d	21.5 f	165.5 d	178.0 c
	65	5.0 i	5.0 gh	6.5 f	0.0 i	62.5 ghi	69.0 g
	61	2.0 ij	2.0 gh	46.5 c	39.0 c	69.0 gh	82.0 f
	group means	13.0 b	8.7 b	33.6 b	26.2 b	84.0 b	92.7 b
sweet	66	10.5 h	3.0 gh	43.0 c	39.0 c	177.5 bc	181.5 c
	68	2.0 ij	1.0 gh	7.5 f	2.0 hi	52.5 ij	56.5 h
	57	0.0 j	0.0 h	15.5 e	5.5 h	41.5 j	43.5 i
	59	0.0 j	0.0 h	29.5 d	27.0 e	74.0 g	74.5 fg
	70	0.0 j	0.0 h	27.5 d	21.0 f	166.5 cd	171.0 c
	group means	2.5 b	0.8 b	24.6 b	18.9 b	102.4 b	105.4 b
	pooled means	93.8	95.2	36.9	32.9	111.9	118.8

[a] Values with different letters are statistically different according to Duncan's multiple range test ($\alpha = 0.05$). [b] Cyanide calculated from amygdalin content obtained by HPLC. [c] Cyanide calculated from prunasin content obtained by HPLC.

Table 3. Pearson's Correlation Coefficient for the Relationships between the Amygdalin, Prunasin, and Total Cyanide Contents in Kernels, Leaves, and Roots of the 15 Almond Genotypes Studied

		kernels	leaves		roots	
		total cyanide	prunasin	total cyanide	prunasin	total cyanide
kernels	amygdalin	0.99	0.55	0.58	0.52	0.49
	total cyanide		0.56	0.59	0.52	0.50
leaves	prunasin			0.98	0.33	0.36
	total cyanide				0.33	0.36
roots	prunasin					0.98

bitter ones (33 mg CN^{-1}/100 g), and lower for the sweet (24 mg CN^{-1}/100 g). As in the case of amygdalin in the kernel, Duncan's test differentiated the bitter individuals from the rest (**Table 2**). Despite this, some bitter genotypes showed lower prunasin values than the slightly bitter ones and some of the sweet genotypes (**Table 2**). The correlation between the values of prunasin and the values of total cyanide obtained using the two techniques exhibited a correlation coefficient of 0.98 (**Table 3**).

Roots. The only cyanogenic compound in the roots, prunasin, showed wide variability (from 41 to 202 mg CN^{-1}/100 g) and a mean of 112 (**Table 2**). The prunasin contents were greater in the bitter (mean of 149 mg CN^{-1}/100 g), intermediate in the sweet (102 mg CN^{-1}/100 g), and lower in the slightly bitter genotypes (84 mg CN^{-1}/100 g). As in the leaves, Duncan's test differentiated the bitter individuals from the rest, although some bitter genotypes showed lower values than the slightly bitter and sweet genotypes (**Table 2**). The values of prunasin and total cyanide obtained by means of the two techniques had a correlation coefficient of 0.98 (**Table 3**).

Relationships between Cyanogenic Compounds in Kernels, Leaves, and Roots. Results showed a relationship between the presence of the cyanogenic compounds in the kernels, leaves, and roots (**Table 3**). The correlation coefficients for total cyanide were 0.59 (kernel−leaves), 0.50 (kernel−roots), and 0.36 (root−leaves).

DISCUSSION

Variability and Distribution of Cyanogenic Compounds. There was considerable variability in the concentrations of cyanogenic compounds in the genotypes studied. A specific distribution of each compound (amygdalin and prunasin) was observed in the kernels, leaves, and roots. Prunasin (a monoglucoside) was only found in the vegetative parts, while amygdalin (a diglucoside) was localized mainly in bitter kernels. Prunasin thus appears to be the form of cyanogenic glycoside transported in the plant while amygdalin is utilized for storage, as previously suggested by Frehner et al. (*4*). Usai and D'hallewin (*6*), studying three sweet- and one bitter-kernelled almond cultivars, found both amygdalin (mainly in the bitter cultivar Sassari 11) and prunasin in the kernels. Prunasin has never been detected by us in mature kernels, but it was detected in kernels during fruit development (own nonpublished data). Other nonpublished work of our group showed that when the extraction was carried out with methanol:water (80:20), like in the work of Usai and D'hallewin (*6*), some amygdaline is hydrolyzed to prunasine. Prunasin was never detected in mature kernels when methanol was used.

As in other species (*15*), cyanogenic compounds in almond could have a function in protecting the plant (roots and aerial parts) and the seed against plant eaters (insects, mammals, or birds). This would suggest a reproductive advantage for bitter-kernelled over sweet-kernelled genotypes. In fact, most of wild almonds are bitter-kernelled (*16*).

Relationship between Kernel Flavor and Prunasin in Vegetative Parts. The concentrations of cyanogenic compounds (amygdalin) in the kernel are in agreement with the sweet or bitter flavor of the kernel. Differences in cyanogenic levels between bitter and nonbitter kernels were also observed by Usai and D'hallewin (*6*) and Dicenta et al. (*17*).

For slightly bitter individuals, this relationship is less clear since while they generally possessed more amygdalin than sweet genotypes, certain sweet genotypes had higher amygdalin levels than some slightly bitter genotypes.

Other compounds may also be responsible for the slightly bitter flavor. Even among bitter kernels, we found a wide variability (genotype 67 had double the amygdalin content of genotype 64), which we were not able to distinguish when tasting the bitter kernels. In general, the bitter genotypes had more prunasin in the vegetative parts (root and leaf) than the nonbitter ones. However, individual analysis does not show a close relationship between the amygdalin content in the kernel (bitter trees) and the prunasin content in the vegetative part, since some bitter genotypes had less prunasin than some sweet ones.

Usai and D'hallewin (*6*) and Mulas (*7*) also observed this lack of correlation, although the interpretation of their results was misleading. They compared the amygdalin contents of the kernels of selected cultivars, with prunasin contents in the shoots or roots of the seedlings of these cultivars obtained by open pollination. Although pollinizer does not affect almond kernel bitterness (*18*), the seedlings resulting from open pollination of bitter-kernelled almonds could be sweet (Ss) or bitter (ss), depending on the pollinizer (*8*−*10*). Genetically heterozygous sweet almonds (Ss), could give sweet- (SS or Ss) or bitter (ss)-kernelled seedlings, as could occur with the cultivar Texas (Ss) (*19*), used by Usai and D'hallewin (*6*).

Mulas (*7*) obtained a correlation coefficient of 0.97 between the prunasin concentration in shoots and roots of seedlings of several *Prunus* species (sweet and bitter almond seedlings included) but did not study the kernels. This high correlation coefficient is very different from that found in the current work between leaves and roots, although they are different organs (shoots instead of leaves) and the plants were of a very different age (18 months instead of 10 years).

Genetic Control of Prunasin and Amygdalin. Our results indicate that prunasin and amygdalin production occur by different processes. Prunasin is present in the vegetative part of most almond genotypes, although only some genotypes are able to store it in the kernels. These individuals, the bitter ones, would be those that traditionally have been considered homozygous recessive (ss), so the bitter flavor would be related to the possibility of transforming the prunasin into amygdalin in the kernel. Frehner et al. (*4*) observed the accumulation of amygdalin in developing almond fruits, probably from prunasin, but noted that the UDPG:prunasin glucosyltransferase presumably involved was still undescribed. So, the prunasin concentration in the vegetative part seems to be a polygenic trait while the amygdalin presence in the kernel is monogenic, as was previously demonstrated (*10*). If kernel bitterness was regulated by this enzyme, due to the recessive genotype (ss) of bitter individuals, regulatory genes would be involved in bitterness control.

Breeding for Resistance to Capnode. If the resistance to capnode is related to the prunasin concentration in the roots as

suggested by Malagón and Garrido (*5*), Usai and D'hallewin (*6*), and Mulas (*7*), our results offer promise for breeders as they could select individuals with higher prunasin concentrations in roots, independent of the sweetness or bitterness of kernel. This could be particularly useful for sweet almonds with high prunasin contents in their roots, which could then be vegetatively propagated and grown on their own roots.

Similarly, if all bitter-kernelled almonds had high levels of cyanogenic compounds in the roots, it would facilitate the breeding of highly cyanogenic rootstocks, by obtaining bitter-kernelled almonds. In this aspect, it is interesting to note that Atocha, DesmayoLarguez, and Garrigues, the main Spanish cultivars used as sources of seedling rootstocks (*20*), could generate bitter-kernelled rootstocks, since they are heterozygous for bitterness. Finally, breeding for highly cyanogenic rootstocks may also increase scion productivity (*21*) and resistance to nematodes (*22*).

ACKNOWLEDGMENT

The authors thank Mr. Mariano Gambín for technical assistance in the experiments presented in this paper.

LITERATURE CITED

(1) Conn, E. E. Cyanogenic compounds. *Annu. Rev. Plant Physiol.* **1980**, *31*, 433−451.
(2) Seigler, D. S. Cyanogenic glucosides and lipids: Structural types and distributions. In *Cyanide in Biology*; Vennesland, B., et al., Eds.; Academic Press: London, 1981; pp 133−143.
(3) Halkier, B. A.; Scheller, H. V.; Moller B. L. Cyanogenic glucosides: the biosynthetic pathway and the enzyme system involved. In *Cyanide Compounds in Biology*; Evered, D., Harnett, S., Eds.; John Wiley & Sons: New York, 1988; pp 49−66.
(4) Frehner, M.; Scalet, M.; Conn, E. E. Pattern of the cyanide-potential in developing fruits. *Plant Physiol.* **1990**, *94*, 28−341.
(5) Malagón, J.; Garrido, A. Relación entre el contenido de glicósidos cianogénicos y la resistencia a *Capnodis tenebrionis* L. en frutales de hueso. *Bol. San. Veg. Plagas* **1990**, *16*, 499−503.
(6) Usai, M.; D'hallewin, G. Cyanogenic glucosides contained in different organs of bitter and sweet almond. *Amélioration génétique de deux espèces de fruits secs méditerranéens*; Rapport EUR 14081 FR.; Comisión des Communautés européenes: 1990; pp 233−236.
(7) Mulas, M. Almond genetic resources and resistance to *Capnodis tenebrionis*. *Acta Hortic.* **1994**, *373*, 41−48.
(8) Heppner, J. The factor for bitterness in the sweet almond. *Genetics* **1923**, *8*, 390−392.
(9) Heppner, J. Further evidence on the factor for bitterness in the sweet almond. *Genetics* **1926**, *11*, 605−606.
(10) Dicenta, F.; García, J. E. Inheritance of the kernel flavour in almond. *Heredity* **1993**, *70*, 308−312.
(11) Williams, W. J. *Handbook of Anion Determination*; Butterworth: London, U.K., 1979.
(12) Clesceri, L. S.; Greenberg, A. E.; Eaton, A. D. *Standard Methods for the Examination of Water and Wastewater*, 20th ed.; The American Public Health Association, the American Water Works Association and the Water Environment Federation: Washington, DC, 1998; 1220 pp.
(13) Asmus, E.; Über die Verwendung der Barbitursäure für die photometrische Bestimmung von Cyanide und Rhodanid. *Z. Anal. Khim.* **1953**, *138*, 414.
(14) Kajiwara, N.; Tomiyama, C.; Ninomiya, T.; Hosogai, Y. Determination of amydalin in apricot kernel and processed apricot by high performance chromatography. *J. Food Hyg. Soc. Jpn.* **1983**, *24*, 42−46.
(15) Jones, D A. Cyanogenesis in animal-plant interactions. In *Cyanide Compounds in Biology*; Evered, D., Harnett, S., Eds.; John Wiley & Sons Ltd.: Chichester, U.K., 1988; pp 151−165.
(16) Grasselly, Ch. Les espéces sauvages d'amandiers. *Options Méditerranéennes* **1976**, *32*, 28−43.
(17) Dicenta, F.; Martínez-Gómez, P.; Grané-Teruel, N.; Martín-Carratalá, M. L.; Berenguer, V.; Mejía, D. M. Herencia del contenido de amigdalina y del sabor de la semilla en una descendencia de almendro. *Actas de Horticultura* **1999**, *25*, 35−40.
(18) Dicenta, F.; Martínez-Gómez, P.; Ortega, E.; Duval, H. Cultivar pollinizer does not affect almond flavour. *HortScience* **2000**, *35*, 1153−1154.
(19) Grasselly, Ch.; Crossa-Raynaud, P. L'amandier. *Maisonneuve et Larose*; Paris, France, 1980; 446 pp.
(20) Simard, M. H.; Olivier, G.; Vargas, F. J.; Romero, M. A.; Gomes Pereira, J.; Maia de Souza, R.; Isaakidis, A. Selection de semis de *Prunus amygdalus* comme porte-greffe de l'amandier. *Options Méditerranéennes* **1997**, *16*, 53−72.
(21) Spiegel-Roy, P.; Weinbaum, S. A. Increasing productivity in sweet almond using selected clones of bitter almond. *Euphytica* **1985**, *34*, 213−217.
(22) Kochba, J.; Spiegel-Roy, P. Alnem 1, Alnem 88, Alnem 201 almonds; nematode resistant rootstock seed source. *HortScience* **1976**, *11*, 270.

Received for review October 4, 2001. Revised manuscript received December 26, 2001. Accepted January 2, 2002. This work was financed by the projects "Influencia de los compuestos cianógenos en la resistencia de patrones de almendro (Prunus dulcis Miller) al gusano cabezudo (Capnodis tenebrionis L.)" (1F.D.97-0989-C03-01) and "Análisis de los compuestos cianógenos en almendro (Prunus dulcis Miller) en relación con la resistancia al gusano cabezudo (Capnodis tenebrionis L.)" (1F.D.97-0989-C03-03) and was cofinanced by both the Comisión Interministerial de Ciencia y Tecnología and FEDER resources.

JF0113070