Content of the cyanogenic glucoside amygdalin in almond seeds related to the bitterness genotype

Contenido del glucósido cianogénico amigdalina en semillas de almendra con relación al genotipo con sabor amargo

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ABSTRACT

Almond kernels can be sweet, slightly bitter or bitter. Bitterness in almond (*Prunus dulcis* Mill.) and other *Prunus* species is related to the content of the cyanogenic diglucoside amygdalin. When an almond containing amygdalin is chopped, glucose, benzaldehyde (bitter flavor) and hydrogen cyanide (which is toxic) are released. This two-year-study with 29 different almond cultivars for bitterness was carried out in order to relate the concentration of amygdalin in the kernel with the phenotype (sweet, slightly bitter or bitter) and the genotype (homozygous: sweet or bitter or heterozygous: sweet or slightly bitter) with an easy analytical test. Results showed that there was a clear difference in the amount of amygdalin between bitter and non-bitter cultivars. However, the content of amygdalin did not differentiate the other genotypes, since similar amounts of amygdalin can be found in the two different genotypes with the same phenotype.

Key words: kernel taste, benzaldehyde, hydrogen cyanide, *Prunus*.

RESUMEN

Las semillas de almendras pueden ser dulces, ligeramente amargas y amargas. El amargor en almendro (*Prunus dulcis* Mill.) y en otras especies *de Prunus* se relaciona con el contenido de la amígdalina diglucósido cianogénico. Cuando una almendra que contiene amigdalina se tritura, produce glucosa, benzaldehído (sabor amargo) y ácido cianihídrico (que es tóxico). El estudio es realizado durante dos años, con 29 variedades de almendra diferentes para la amargura o amargor, se ha realizado con el fin de relacionar la concentración de la amígdalina en el núcleo con el fenotipo (dulce, ligeramente amargo y amargo) y el genotipo (homocigota: dulce o amargo o heterocigótico: dulce o amarga un poco) por un ensayo de análisis fácil. Los resultados mostraron que existía una clara diferencia en la cantidad de amigdalina entre amargo / cultivares no amargo. Sin embargo, el contenido de amigdalina no diferenciaba entre los otros genotipos, ya que cantidades similares de amigdalina se puede encontrar en los dos genotipos diferentes con el mismo fenotipo.

Palabras clave: sabor amargo, benzaldehído, ácido cianhídrico, *Prunus*.

Introduction

Prunus kernels accumulate cyanogenic diglucoside amygdalin, which is related to bitterness (McCarty *et al.,* 1952; Conn, 1980; Frehner *et al.,* 1990; Møller and Seigler, 1991; Swain *et al.,* 1992; Poulton and Li, 1994; Dicenta *et al.,* 2002; Franks *et al.,* 2008; Sánchez-Pérez *et al.,* 2008). Upon disruption of tissue containing amygdalin this will be degraded by b-glucosidases with release of glucose, benzaldehyde and hydrogen cyanide (Morant *et al.,* 2008). This two-component system, each of them chemically inert, will provide the plant with a chemical defense against herbivores, insects and pathogens (Conn, 1969; Nahrstedt, 1985; Jones, 1998; Morant *et al.,* 2003; Zagrobelny *et al.,* 2004; Nielsen *et al.,* 2006; Zagrobelny *et al.,* 2007a; Zagrobelny *et al.,* 2007b), as a source of energy reserves.

In the almond (*Prunus dulcis* Mill.), bitterness is controlled by a single gene, *Sk* (*Sweet kernel* gene), which is dominant over the bitter gene (Heppner, 1923; Heppner, 1926; Grasselly and L'amandier, 1972; Kester *et al.,* 1977; Gharbi, 1981; Vargas and Romero, 1988; Dicenta and García, 1993; Dicenta *et al.,* 2007). The *Sk* gene has been localized in linkage group five in different genetic linkage maps, but its function is still unknown. As a result of the development of genetic linkage maps, some molecular markers have been found close to the *Sk* locus (Joobeur *et al.,* 1998; Bliss *et al.,* 2002; Sánchez-Pérez *et al.,* 2007). However, a marker allele associated with the bitterness allele remains to be identified.

Recently, amygdalin and, its precursor, prunasin (cyanogenic monoglucoside) content, and the enzymes involved in the amygdalin pathway, have been studied in four

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different almond genotypes (Sánchez-Pérez *et al.,* 2008). Distinct cellular localizations of the enzymes involved in the degradation pathway, possibly involving a seed coat prunasin hydrolase, have also been suggested to be related to bitterness in the almond (Sánchez-Pérez *et al.,* 2008). Furthermore, an enzyme involved in the biosynthetic pathway has also been related to bitterness in the almond (Franks *et al.,* 2008).

On the other hand, most of the cultivated almond cultivars are heterozygous for this trait, mainly sweet, although some of them are slightly bitter. When two heterozygous cultivars are crossed in breeding programs, 25% of the offspring will be bitter and therefore eliminated from the breeding process, since the industry is mainly interested in sweet-kernelled cultivars. This disadvantage reduces the efficiency of breeding programs, and it is especially frequent when a self-compatible progenitor is used since most of the self-compatible cultivars are heterozygous for bitterness (Grasselly and Crossa-Raynaud, 1980; Dicenta, 1991). This is indeed happening in the American Breeding Program, since the bitter *P. webbi* has been used as a self-compatible progenitor. Consequently, 50% of the offspring will be bitter when the other progenitor used in the cross is heterozygous, such as Carmel or Livingstone (Ledbetter and Pyntea, 2000; Gradziel *et al.,* 2001). Fortunately, a study of these descendants has enabled the breeders to classify the cultivars used as progenitors as homozygous or heterozygous (Grasselly and Crossa-Raynaud, 1980; Dicenta, 1991; Vargas *et al.,* 2001), which is a very arduous (3-4 years), and therefore expensive, task.

The main objective of this research is to study the variability of the amygdalin content in a representative set of almond cultivars, its relation with the phenotype (sweet, bitter or slightly bitter) and the genotype (sweet homozygous, bitter homozygous or heterozygous), in order to identify the bitterness genotype of an almond cultivar with an easy analytical test.

Material and methods

Plant material

The content of amygdalin was analyzed for two years in 23 cultivars and in six descendants of the cross Garrigues × Tuono, provided by the Almond Breeding Program of the Centre of Edafology and Applied Biology of the Segura-Consejo Superior de Investigaciones Científicas (CEBAS-CSIC) CEBAS-CSIC, Murcia (Spain).

The phenotype and genotype of some of these cultivars were previously determined by different authors (Grasselly and Crossa-Raynaud, 1980; Dicenta, 1991; Vargas *et al.,* 2001):

- *Sk/Sk* (sweet): Del Cid, Ferragnès, Peraleja, Primorskii, Ramillete, Titan.
- *Sk/sk* (sweet): Marcona, Desmayo Largueta, Atocha.
- *Sk/sk* (slightly bitter): Garrigues, Genco, Tuono.
- *sk/sk* (bitter): S3060, S3062, S3076, S3108, S3112, S3126.
- *Sk/Sk or Sk/sk* (genotype unknown, but sweet): Achaak, Bonita, Carretas, CEBAS, Colorada, Ferraduel, La Mona, Pajarera, Planeta, Rumbeta, and Tioga.

Fifty mature almond fruits were collected per cultivar and the hull and shell removed. Kernels were frozen, lyophilized in a Cryodos Telstar lyophilizer for 48h at -85°C at 1 Pa, ground, homogenized, and analyzed. Two independent analyses per sample were performed.

Amygdalin content

Chromatographic determination of amygdalin levels was carried out for 0.2 g of kernel samples and extracted with 10 mL of methanol for 12 h at room temperature. Kernel extractions were done with 0.1 g of polyvinyl-polypirrolidone (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 waters high-performance liquid chromatography, Controler model 600 (HPLC) system, following a procedure similar already described (Kajiwara *et al.,* 1983), under the following conditions: Waters Symmetry column 250 x 4.6 mm, flow rate 1.5 mL min^{-1}, acetonitrile:water 20:80 as eluent, 20 µL of sample, and detection under UV at 218 nm.

Results, expressed as mg amygdalin/100 g of dry weight (DW), were tested using analysis of variance and Tukey´s test to determine differences between years and cultivars.

Results and discussion

Analysis of variance of the amygdalin content showed significant differences between cultivars and between years as well as the year x cultivar interaction (Tab.1).

For the majority of the cultivars (Ramillete, Bonita, Marcona etc.), the content of amygdalin was similar in both years (Tab. 2). However, in the first year the content of some cultivars was slightly lower (Achaak, Colorada, Carretas,

Ferraduel, S3060, S3076, etc.), or slightly higher (Desmayo Largueta, Garrigues, S3126, S3062, and S3112).

In general, there was a high variability of the amygdalin content between the studied cultivars. The average content of the bitter cultivars was much higher than the non-bitter ones (Tab. 2).

Tukey's test with all the cultivars showed clear differences between bitter and non-bitter (sweet and slightly bitter) cultivars, but did not between or within sweet and slightly bitter ones. Within the bitter cultivars, four different groups were identified.

In general, the kernel taste of cultivars is related to amygdalin content, although with some variability. In sweet cultivars such as Primorskii, Ramillete, Bonita and CEBAS, amygdalin was not detected, but in others such as Ferraduel or Pajarera it was until 27 mg/100 g DW. In slightly bitter cultivars such as Garrigues or Tuono, amygdalin content

TABLE 1. Amygdalin content media values (X) obtained for each cultivar, in two different years and the Tukey test including all the cultivars and subtracting the bitter ones.

						Tukey test						
			Means (mg/100 g muestra)				All			Non-bitter		
Flavor	Genotype	Cultivar	Year 1	Year 2	X	SE	Year 1	Year 2	X	Year 1	Year 2	X
Sweet	*SkSk*	Primorskii	nd	nd	nd		e	e	e	m	j	j
		Ramillete	nd	nd	nd		e	e	e	m	j	j
		Titan	0.43	0.62	0.53	0.021	e	e	e	m	ij	ij
		Del Cid	-	2.15	2.15	0.007	-	e	e	-	hi	hi
		Peraleja	1.76	2.56	2.16	0.021	e	e	e	l	h	hi
		Ferragnès	5.16	5.5	5.33	0.007	e	e	e	h	fg	f
	Sksk	Marcona	1.87	1.87	1.87	0.014	e	e	e	kl	hi	hij
		Desmayo	8.11	6.86	7.49	0.064	e	e	e	f	ef	e
		Atocha	7.28	8.02	7.65	0.007	e	e	e	g	e	e
	Sk--	Bonita	nd	nd	nd		e	e	e	m	j	j
		CEBAS	nd	nd	nd		e	e	e	m	j	j
		Tioga	0.34	0.52	0.43	0.007	e	e	e	m	ij	ij
		Colorada	2.41	2.72	2.57	0.021	e	e	e	jk	h	gh
		Carretas	2.49	2.65	2.57	0.014	e	e	e	j	h	gh
		Planeta	3.71	4.65	4.18	0.007	e	e	e	i	g	fg
		La Mona	4.63	6.1	5.37	0.007	e	e	e	h	fg	f
		Rumbeta	-	5.39	5.39	0.014	-	e	e	-	fg	f
		Achaak	10.22	11.25	10.74	0.028	e	e	e	e	d	d
		Ferraduel	21.96	23.36	22.66	0.021	e	e	e	c	b	b
		Pajarera	-	27.26	27.26	0.028	-	e	e	-	a	a
S.Bitter	*Sksk*	Genco	17.34	18.74	18.04	0.042	e	e	e	d	c	c
		Garrigues	23.81	22.93	23.37	0.014	e	e	e	b	b	b
		Tuono	25.05	25.81	25.43	0.014	e	e	e	a	a	a
Bitter	*sksk*	S3126	2.439	2.36	2.4	0.141	d	d	d	-	-	-
		S3108	3.799	3.81	3.805	0.004	c	c	c	-	-	-
		S3062	3.87	3.784	3.827	0.001	c	c	c	-	-	-
		S3060	4.915	5.036	4.976	0.003	b	b	b	-	-	-
		S3112	5.206	5.011	5.109	0.006	b	b	b	-	-	-
		S3076	5.894	6.028	5.961	0.043	a	a	a	-	-	-

TABLE 2. ANOVA of amygdalin content among varieties and years (1997, 1998), for the studied cultivars.

SV	Sum of squares	DF	Mean square	F ratio	Significance F
Main effects	571790283	29	19716906	18779.41	< 0.001
Year	9362	1	9362	8.92	0.004
Variety	571786321	28	20420990	19447.97	< 0.001
Year x variety	107439	22	4883	4.65	< 0.001
Explained	571897721	51	11213681	10680.00	< 0.001
Residual	112342	107	1050		
Total	572010063	158	3620317		

was until 25 mg/100 g DW. In bitter cultivars, the detected levels were between 2400 mg/100 g DW (S3126) and almost 6000 mg/100 g DW (S3076).

The number of plant species with cyanogenic glucosides is more than 2650 (Seigler and Brinker, 1993; Bak *et al.,* 2006). Studies on passion fruit seeds (*Passiflorae*) obtained 3.1 mg/100 g DW (Chassagne *et al.,* 1996), in lime 7000 mg/100 g DW of linamarin, and in bamboo 500 mg/100 g DW of taxiphyllin (Shahidi and Wanasundara, 1997). Furthermore, in linen seeds, linamarin (300 mg/100 g DW) and linustatin (6000 mg/100 g DW) were detected.

Amygdalin has also been detected in other fruit tree kernels such as plum (4,100 mg/100 g), apricot (2,394 mg/100 g), cherry (2,306 mg/100 g) and apple (739 mg/100 g) (Lucas and Sotelo, 1983). In the case of apricot, different concentrations of amygdalin have been detected: 4,400 and 6,500 mg/100 g DW (Femenia *et al.,* 1995), or 5,500 and 7,000 mg/100 g DW (Stoewsand *et al.,* 1975; Briggs and Yuen, 1978; Mandenius *et al.,* 1983; Stosic *et al.,* 1987; Voldrich *et al.,* 1989; Gómez *et al.,* 1998; Negri *et al.,* 2008).

In bitter almond kernels, amygdalin levels were 5,808 mg/100 g DW (Corradi and Micheli, 1982; Shahidi and Wanasundara, 1997). Another analysis determined 926 mg of amygdalin/100 g DW for the bitter variety Sassari 11 and 106 mg/100 g DW in the sweet variety Arrubia, but in the sweet variety Texas, amygdalin was not detected (Usai and D'hallewin, 1992). Amygdalin content in sweet cultivars and slightly bitter ones from Sicily and Apulia was higher than the American or Russian ones, except for the American variety Perlees (Barbera *et al.,* 1987). In fact, the content detected by these authors was much higher than the content detected in this study for cultivars such as Genco (390 versus 18 mg/100 g) and Tuono (750 versus 25 mg/100 g). Recently, 9 µmol/100 mg fresh weight (FW) of amygdalin (4,082 mg amygdalin/100 g FW) was detected in the bitter genotype S3067 (Sánchez-Pérez *et al.,* 2008).

In general, the differences in amygdalin levels found in bitter cultivars of different *Prunus* species are within the range determined in this study (2,400-6,000 mg/100 g), with the exception of the results already mentioned (Barbera *et al.,* 1987; Shahidi and Wanasundara, 1997).

In order to detect some differences within the non-bitter group, we analyzed the data without the bitter cultivars, since their amygdalin content was much higher. Analysis of variance only with non-bitter cultivars showed significant differences between cultivars and between years, the same as when all the genotypes were included (Tab. 3).

The Tukey's test did not differentiate sweet and slightly bitter cultivars (Tab. 2). In general, sweet cultivars had less amygdalin than the slightly bitter ones, although some sweet cultivars (Ferraduel and Pajarera) had similar or higher amygdalin content than some slightly bitter ones.

Tukey's test neither distinguised between homozygous and heterozygous sweet cultivars. In general, sweet homozygous cultivars (*Sk/Sk*) contained lower levels of amygdalin than the sweet heterozygous ones, but Marcona (*Sk/sk*) had a lower content than Del Cid, Peraleja and Ferragnès (*Sk/Sk*).

Despite the lack of differences between *Sk/Sk* and *Sk/sk* sweet cultivars, we tried to classify the cultivars of an unknown genotype into these groups. So, Bonita, CEBAS and Tioga may be *Sk/Sk* and Achaak, Ferraduel and Pajarera *Sk/sk*. The rest of the sweet cultivars could not be classified based on the amygdalin content.

It has been shown that the heterozygous slightly bitter cultivars, like Garrigues, tend to generate more slightly bitter descendants than other heterozygous sweet cultivars (Dicenta and García, 1993). Therefore, the slightly bitter cultivars may pass to the offspring the capacity to produce the slightly bitter taste, apart from the level of amygdalin.

Conclusions

We can conclude that there is a high variability in amygdalin content among the studied cultivars, being this content characteristic for each variety and quite stable year by year.

TABLE 3. ANOVA of amygdalin content among varieties and years (1997, 1998), for the sweet and slightly bitter varieties.

SV	Sum of squares	DF	Mean square	F. ratio	Significance F
Main effects	9196.734	23	399.858	4761.249	< 0.001
Year	1.930	1	1.930	22.985	< 0.001
Variety	9176.640	22	417.120	4966.793	< 0.001
Year x variety	16.713	17	0.983	11.707	< 0.001
Explained	9213.448	40	230.336	2742.693	< 0.001
Residual	6.887	82	0.084		
Total	9220.334	122	75.577		

The bitter cultivars showed amygdalin contents much higher than the non-bitter ones; and slightly bitter ones had higher contents than sweet ones, but with some exceptions.

Finally, the amount of amygdalin is not a useful tool to identify *Sk/Sk* and *Sk/sk* cultivars. Therefore, further studies will be focused on the development of molecular markers for screening populations of fruit trees such as the almond with an easy PCR-based method.

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