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Germplasm Characterization of Zolfino Landraces (*Phaseolus vulgaris* L.) by Flavonoid Content

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The flavonoid composition of three phenotypes of "Zolfino" landraces, a typical bean grown in Tuscany, has been elucidated by means of HPLC-DAD and HPLC-MS analysis. Flavonols, isoflavones, and anthocyanins have been separated and determined in the different samples chosen on the basis of their seed coat color. A flavonol that has not been previously found in *Phaseolus vulgaris* L. seeds has been characterized. The quantitative data show the presence of flavonols (ranging from 709 to 118 mg/kg of fresh weight), isoflavones (ranging from 14 to 2 mg/kg of fresh weight), and anthocyanins, in black beans only. These results show that this genotype could be very interesting from a nutritional point of view.

KEYWORDS: Polyphenols; anthocyanins; HPLC-DAD; HPLC-MS; beans; isoflavones

INTRODUCTION

Over 60 years ago veterinary scientists reported the presence in plants of compounds that induced estrus in immature animals or interfered with their normal reproductive processes (*1*−*3*). To this day, >300 plants have been found to possess compounds with this activity (*4*, *5*). These compounds have been defined as phytoestrogens and are most abundant in the plant family Leguminosae. Legumes include peas, beans, lentils, peanuts, and other podded plants that are used as food. Legumes have been cultivated for thousands of years and have played an important role in the traditional diets of many regions throughout the world. Beans have been recognized for their high protein content and more recently have been noted for their soluble-fiber content, but in general there has been relatively little research and discussion about the nutritional attributes of legumes. The exception to this is the soybean, which has been investigated intensively over the past 5−10 years. This is because several constituents of medical interest have been isolated from soybeans, including isoflavones, phytoestrols, protease inhibitors, inositol hexaphosphate, and saponins (*6*). Recently, many other legumes have also been reported to contain these same compounds as well. A number of studies have been undertaken in a search for links between soy phytoestrogens, sex hormone metabolism, biological activity, intracellular enzymes, protein synthesis, growth-factor action, malignant-cell proliferation, and angiogenesis on the molecular as well as on tissue and organism levels (*7*, *8*−*20*).

Phaseolus vulgaris L. cv. Zolfino, a typical Tuscan legume, comes from the hilly and mountainous region of Pratomagno, between the provinces of Arezzo and Florence. It is a small, round, pale yellow bean, with a white hilum and very fine hulls, and it is easy to cook. In the literature there are some reports about the polyphenol content of beans (*21*−*23*). The flavonols that are present in *P. vulgaris* L. include glycosides of quercetin and kaempferol (*21*, *22*). In some cases flavonoids are identified and quantitatively determined after hydrolysis (*24*, *25*); however, the qualitative and quantitative flavonoid composition of Zolfino beans has never been reported. The cultivation of Zolfino landraces is limited to a very small area, and the gene pool of this cultivar is susceptible to genetic drift. Therefore, it is important to maintain in cultivation the different possible genotypes found as segregant types in the populations. Some of these genotypes have different seed coat color, and normally they are eliminated through farmer selection.

The aim of this study was to characterize the flavonoid classes of *P. vulgaris* L. cv. Zolfino differing in seed coat color so as to find characteristics of these genotypes that would lead to improved cultivation and consequently reduce the germplasm drift of this landrace.

MATERIALS AND METHODS

Plant Material. Seeds of three different segregant phenotypes (four samples) belonging to populations of *P. vulgaris* L. cv. Zolfino with different seed coat pigmentations were collected in the Pratomagno

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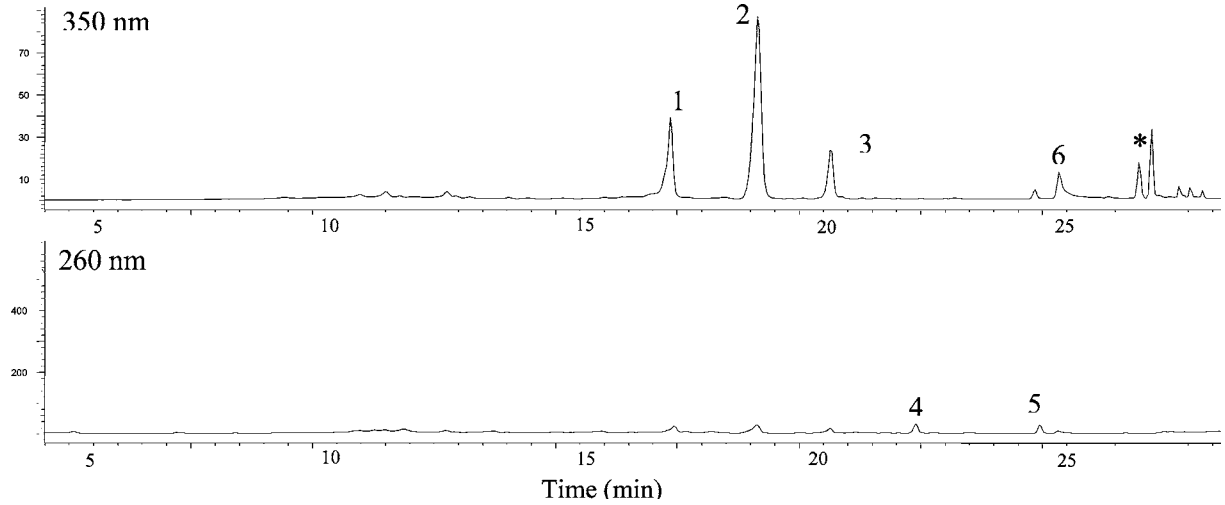

Figure 1. Chromatographic profile acquired by HPLC-DAD (350 and 260 nm) of the hydroalcoholic extract from *P. vulgaris* cv. Zolfino C at the relative maxima of absorbance of flavonols and isoflavonoid derivatives, respectively. Polyphenolic compounds: (1) kaempferol 3-*O*-xylosylglucoside; (2) kaempferol 3-*O*-glucoside; (3) kaempferol 3-acetylglucoside; (4) daidzein; (5) genistein; (6) kaempferol; (∗) uncharacterized.

area (Florence and Arezzo). The samples were as follows: Zolfino A, yellow seed coat, Querceto (Arezzo); Zolfino B, tobacco seed coat; Zolfino C, black seed coat; and Zolfino D, yellow seed coat (B−D were all from Leccio, Florence).

Standards. Authentic standards of daidzein, genistein, kaempferol 3-*O*-glucoside, rutin, delphinidin, and malvin were purchased from Extrasynthèse S.A. (Lyon, France).

Solvents. All solvents used were of HPLC grade purity (BDH Laboratory Supplies, Poole, U.K.).

Extraction and Purification of Flavonoids. A 1 g sample of ground dry seeds was extracted with 3 × 30 mL of 70% ethanol, adjusted to pH 2.0 with formic acid; each step involved an extraction for 3 h at room temperature. The extracts were combined and defatted with 3 × 30 mL of petroleum ether. The defatted extracts were evaporated to dryness under vacuum at room temperature and finally redissolved in EtOH/H_2O (70:30) adjusted to pH 2.0 with formic acid, to a final volume of 5 mL. The seed coats of 13 g of beans were removed and extracted with 3 × 15 mL of 70% ethanol adjusted to pH 1.8 with formic acid to avoid degradation of the anthocyanins. The extracts were combined and defatted with 3 × 10 mL of petroleum ether. The defatted extracts were evaporated to dryness under vacuum at room temperature and finally redissolved in H_2O/CH_3CN/MeOH/HCOOH (45:22.5:22.5:10), solution B used for anthocyanins in HPLC-DAD analyses, to a final volume of 3 mL.

Analytical Techniques and Equipment. *HPLC-DAD Analysis.* Analyses for flavonols, isoflavones, and anthocyanins were carried out using an HP 1100L liquid chromatograph equipped with a DAD and managed by an HP 9000 workstation (Agilent Technologies, Palo Alto, CA). Flavonols and isoflavones were separated by using a 150 × 3.9 mm i.d., 4 μm Nova-Pak C18 column (Waters) operating at 26 °C, according to the method of Romani et al. (*26*). Anthocyanins were separated by using a 250 × 4.6 mm, 4 μm, Synergy MAX-RP80A column (Phenomenex, Torrence, CA) operating at 26 °C (*27*). In brief, the eluent was composed of (A) H_2O/HCOOH (95:5), (B) H_2O/CH_3-CN/MeOH/HCOOH (50:22.5:22.5:5)), and (C) MeOH. A three-step linear solvent gradient system was used, starting from 0 to 70% of solution B for a 70-min period, at a flow rate of 1 mL/min. The percentage of solution B reached 9% from 0 to 11 min, then 55% from 21 to 60 min, and finally 70% from 64 to 70 min. The contribution of solution C to the eluent was 2% throughout the entire run. UV−vis spectra were recorded in the 190−600 nm range, and the chromatograms were acquired at 260, 280, 305, 330, 350, and 520 nm.

HPLC-MS Analysis. HPLC-MS analyses were performed using an HP 1100L liquid chromatograph linked to an HP 1100 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies). Spectra were recorded in negative ion mode, fragmentor 80, for flavonols and isoflavones and in positive ion mode, fragmentor 120, for anthocyanins, and the same chromatographic conditions as previously described were applied. The mass spectrometer operating conditions were as follows: gas temperature, 350 °C; nitrogen flow rate, 10.0 L/min; nebulizer pressure, 40 psi; quadrupole temperature, 40 °C; and capillary voltage, 3500 V.

The identity of polyphenols was ascertained using data from HPLC-DAD and HPLC-MS analyses, by comparison and combination of their retention times and UV−vis and mass spectra with those of authentic standards. In particular. the anthocyanins were identified by comparing data to those of *Vitis vinifera* L. grape extracts (*28*).

Identification and Quantification of Individual Flavonoids. Identification of individual flavonoids was carried out using their retention times and both spectroscopic and mass spectrometric data. Quantification of individual polyphenolic compounds was directly performed by HPLC-DAD using a five-point regression curve ($r^2 \geq 0.998$) in the range of 0−30 μg on the basis of authentic standards. In particular, genistein derivatives were determined at 260 nm using genistein as reference compound, whereas daidzein derivatives were determined at 305 nm using daidzein as reference compound. Flavonols such as kaempferol and quercetin derivatives were determined at 350 nm using kaempferol 3-*O*-glucoside and quercetin 3-*O*-rutinoside (rutin) as reference compounds, respectively. Delphinidin and petunidin derivatives were determined at 520 nm using delphinidin as reference compound, whereas malvidin derivatives were calculated at the same wavelength but with malvin as reference compound. In all cases, actual concentrations of the derivatives were calculated after corrections for differences in molecular weight had been applied. From each site three samples were collected, so as to express the analytical results as an average with its standard deviation.

RESULTS AND DISCUSSION

This study focused on the comparison of the different flavonoid classes in four samples of *P. vulgaris* L. cv. Zolfino based on their seed coat color. The extraction procedure for flavonoids from *Phaseolus* seeds was one which ensures that all of the polyphenol classes were obtained. The flavonol, isoflavone, and anthocyanin classes were identified using data from HPLC-DAD and HPLC-MS analysis by comparison and combination of their retention times and mass spectrometry and UV spectra. For example, the chromatographic profile of Zolfino A, recorded at 260 and 350 nm, is presented in **Figure 1**. The figure reveals the qualitative composition of the seeds. Among flavonols and isoflavones we identified kaempferol 3-*O*-xylosylglucoside, kaempferol 3-*O*-glucoside, kaempferol acetylglucoside, kaempferol, quercetin 3-*O*-glucoside, daidzein, and genistein. The mass spectrum of kaempferol acetylglucoside

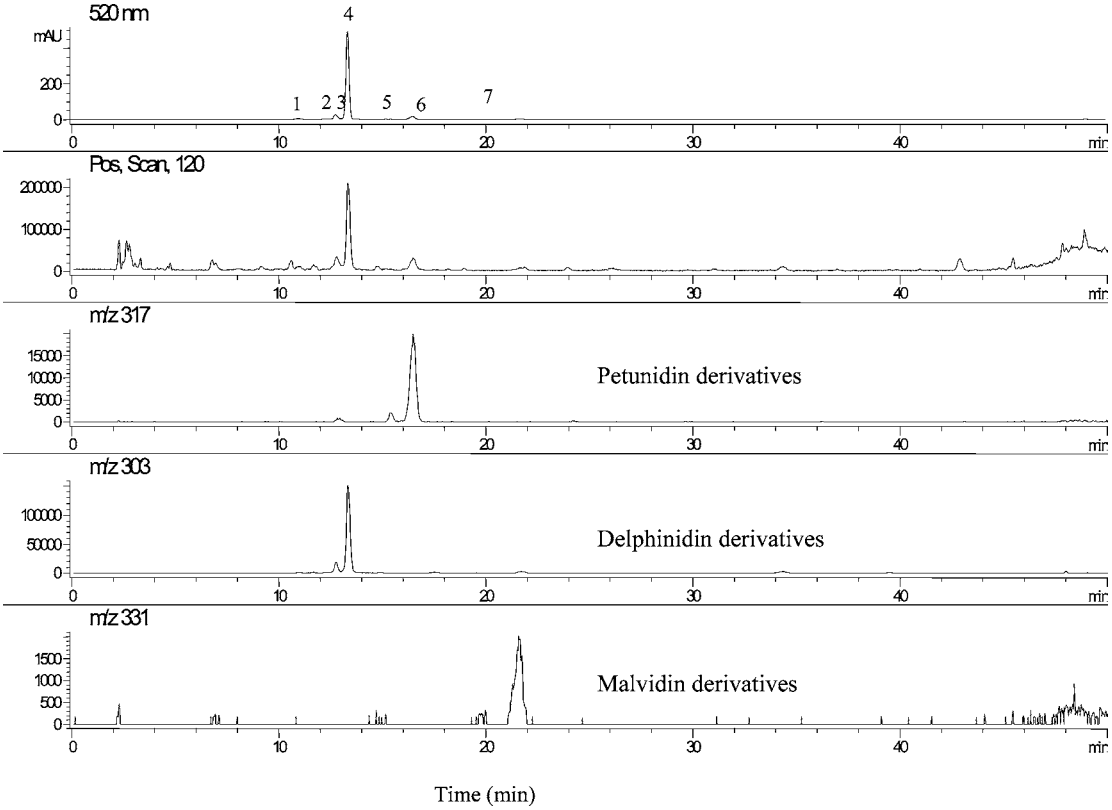

Figure 2. Chromatographic profile acquired by HPLC-DAD (520 nm) of the aqueous alcoholic extract from seed coats of *P. vulgaris* cv. Zolfino C at the relative maxima of absorbance of anthocyanins. Identified anthocyanins: (1) delphinidin 3,5-*O*-diglucoside; (2) delphinidin feruloylglucoside; (3) petunidin 3,5-*O*-diglucoside; (4) delphinidin 3-*O*-glucoside; (5) petunidin 3-*O*-rhamnoside; (6) petunidin 3-*O*-glucoside; (7) malvidin 3-*O*-glucoside.

Table 1. Contents of Flavonoids, Isoflavones, and Anthocyanins in Zolfino Landraces A–D[a]

	zolfino A	zolfino B	zolfino C	zolfino D
flavonols				
quercetin 3-*O*-glucoside	nd	tr	tr	nd
kaempferol 3-*O*-xylosylglucoside	109 ± 1.6	nd	nd	186 ± 2.2
kaempferol 3-*O*-glucoside	476 ± 5.2	545 ± 6.5	66 ± 0.73	307 ± 3.7
kaempferol 3-acetylglucoside	75 ± 1.2	164 ± 1.9	34 ± 0.47	68 ± 0.81
kaempferol	tr	nd	18 ± 0.23	nd
total	660	709	118	561
isoflavones				
daidzein	4 ± 0.06	tr	8 ± 0.09	2 ± 0.02
genistein	2 ± 0.03	2 ± 0.03	6 ± 0.09	2 ± 0.03
total	6	2	14	4
anthocyanins				
delphinidin 3,5-*O*-diglucoside	nd	nd	tr	nd
delphinidin feruloylglucoside	nd	nd	11	nd
delphinidin 3-*O*-glucoside	nd	nd	145 ± 1.5	nd
petunidin 3,5-*O*-diglucoside	nd	nd	tr	nd
petunidin 3-*O*-rhamnoside	nd	nd	tr	nd
petunidin 3-*O*-glucoside	nd	nd	8 ± 0.09	nd
malvidin 3-*O*-glucoside	nd	nd	6 ± 0.09	nd
total	nd	nd	170	nd
total flavonoids	666	711	302	565

[a] Data are expressed in mg/kg of fresh weight of seed flour. Moisture content = 13–14%. Average value ± SD. nd = not detected. tr = traces, <0.2 mg/kg of fresh weight (*35*).

with peaks at *m*/*z* 489 and 285 corresponded to the quasi-molecular ion and to the aglycon, respectively. To our knowledge this is the first report on the identification and quantification of this compound in *P. vulgaris* L. seeds. On the basis of the literature data (*22*, *23*) the linkage of the acetylglucose should be at the C3 position of kaempferol.

The identification of anthocyanins, found only in the black seeds (Zolfino C), was established by comparison with anthocyanins of previously studied *Vitis vinifera* L. (*28*) analyzed under the same analytical conditions. **Figure 2** shows the HPLC-DAD, the TIC profile in positive mode at 120 eV and the extracted ion chromatograms for petunidin (*m*/*z* 317), delphini-

din (*m*/*z* 303), and malvidin (*m*/*z* 331), obtained from the aqueous alcoholic (ethanol/water, 70:30, v/v, pH 2) extract of Zolfino C seed coats. In this extract we found delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and malvidin 3-*O*-glucoside, as reported in a previous work about the characterization of black bean anthocyanins (*29*). We also found petunidin 3-*O*-rhamnoside, delphinidin feruloylglucoside, and traces of delphinidin 3,5-*O*-diglucoside and petunidin 3,5-*O*-diglucoside, which were characterized through their MS fragmentation spectra.

The fragmentation pattern of delphinidin 3-*O*-glucoside shows signals at *m*/*z* 465 and 303, corresponding to the quasi-molecular ion $[M + H]^+$ and to the fragment after the loss of a glucose moiety $[M - 162 + H]^+$. The mass spectrum of delphinidin feruloylglucoside, which has not been previously reported from dry beans (*29*), has peaks at *m*/*z* 641, 465, and 303, corresponding to the quasi-molecular ion, to the fragment after the loss of a feruloyl moiety $[M - 176 + H]^+$, and to the aglycon, respectively. The mass spectrum of petunidin 3-*O*-rhamnoside shows peaks at *m*/*z* 463 and 317, corresponding to the quasi-molecular ion and the fragment after the loss of the rhamnose moiety $[M - 14 6 + H]^+$, respectively.

In **Table 1**, the quantitative data for the four phenotypes of *P. vulgaris* L. cv. Zolfino differing in seed coat color are reported. Flavonols are always the most represented compounds, ranging from 118 to 709 mg/kg. According to Beninger et al. (*23*), kaempferol 3-*O*-glucoside is the main flavonol found in all samples, ranging from 66 to 545 mg/kg. The amount of kaempferol 3-acetylglucoside is quite high in these phenotypes and ranges from 34 to 164 mg/kg. Isoflavones (daidzein and genistein) were also found in the 2−14 mg/kg range in all samples analyzed.

From a quantitative point of view, the isoflavone content is comparable with the content of other *P. vulgaris* beans as reported by Mazur et al. (*30*), whereas it is lower than that of soy food (*31*). The flavonol content was in most cases higher than that reported in yellow and green beans (*21*). It should be noted that the flavonol level, in contrast to the isoflavone level (*32*), is not much affected by heat treatment (*25*). This is very important because flavonols are antioxidant and chelating molecules with beneficial health effects (*33*). It should be kept in mind that kaempferol 3-*O*-glucoside, the most representative flavonol, is reported as an anticancer molecule (*34*). The variability in flavonoid content within the different phenotypes could be very interesting for future breeding programs to improve the use of common bean as a food that can be chosen by the consumer for the presence of these classes of nutraceutical compounds with antioxidant activity and chemoprotective properties (*34*).

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