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Characterization and quantification of saponins and flavonoids in sprouts, seed coats and cotyledons of germinated black beans

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ABSTRACT

Saponins, flavonols and isoflavones were quantified in sprouts, cotyledons and seed coats of black beans (*Phaseolus vulgaris* L.) subjected to germination over five days. Sprouts had a higher concentration of saponins compared to cotyledons or seed coats ($p < 0.05$). The saponins concentration in hilum increased 2.3-fold after soaking. After the first day of germination, the saponin concentration in sprouts and cotyledons increased 1.9 and 2.1-fold, respectively. Additional germination days decreased the amount of the most abundant soyasaponins in black bean sprouts. Flavonols and isoflavones were associated with seed coats and less than one third of the initial amount remained after the soaking process. The concentrations of flavonols were also reduced during germination process. Aglycones were detected only after soaking and their concentration remained unchanged during germination. Genistein was detected only after three days of germination. In general, one-day germinated black beans could be recommended for increasing the concentration of saponins and non-glycosylated flavonols in sprouts and seed coats, respectively.

1. Introduction

Saponins are present in trace amounts in common beans, approximately sevenfold less compared to soybeans (Rupasinghe et al., 2003). They can be divided into groups A, B or E based on the structures of aglycones (Rupasinghe et al., 2003; Shiraiwa, Harada, & Okubo, 1991a; Shiraiwa, Kudo, Shimoyamada, Harada, & Okubo, 1991b). Group A saponins have glycosyl groups attached to the C-3 and C-22 positions of the aglycone whereas those belonging to groups B and E are glycosylated only in the C-3 position. Group E saponins contain a ketone in C-22 instead of the hydroxyl group found in counterparts belonging to group B. Group B saponins also exist in the plant as conjugates of 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) at C-22 (Kudou et al., 1992). The DDMP conjugates are thermally labile and can easily be degraded due to its relatively weak covalent bond (Berhow, Kong, Vermillion, & Duval, 2006; Decroos et al., 2005).

Along with isoflavones, saponins change during germination due to synthesis and accumulation (Paucar-Menacho, Berhow, Mandarino, Chang, & de Mejia, 2010a; Paucar-Menacho, Berhow, Mandarino, de Mejia, & Chang, 2010b). The relevant change in composition enhances the overall nutritional value of the soybeans and the contents of health-promoting phytochemicals (Bau, Villaume, & Mejean, 2000). These changes have also been reported in black beans (*Phaseolus vulgaris* L.) but only for phenolic compounds (Diaz-Batalla, Widholm, Fahey, Castano-Tostado, & Paredes-Lopez, 2006).

To the best of our knowledge, however, no previous studies on the fate of saponins in the different anatomical parts of common beans subjected to germination. The aim of this research was to identify and quantify saponins in the sprouts, seed coats and cotyledons of black bean seeds (*P. vulgaris* L.) using HPLC–MS-TOF and HPLC–UV–ELSD techniques. Furthermore, we tested the effect of soaking, drying and sprouting (1–5 days) on the concentration of these compounds along with other bioactive phytochemicals such as flavonols and isoflavones.

2. Materials and methods

2.1. Plant materials

The black bean variety used for the experiments was "San Luis" collected from a local distributor. The seeds were first cleaned using sieves to remove dockage and splits and then with a damp cloth to remove surface dust. Malting tests were carried out with individual lots of seeds.

Each lot was soaked in distilled water (1:3, w/v) over 24 h with aeration at 500 ml/min provided by an aquatic pump. Then, the soaking water was discarded and the resulting wet seeds were

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placed on germination trays in a dark chamber set at 20 °C and 92% relative humidity for 5 days. Samples were taken daily for the determination of germination percentage and sprout length and then immediately dehydrated in an oven set at 60 °C for 4 h. The percentage of solid loss was calculated from the difference in weight between raw and germinated dried seeds. The germinated seeds were physically separated into sprouts, seed coats and cotyledons. Each anatomical part was weighed, ground into a powder (Coffee and Spice Grinder Krups GX4100, DF, Mexico) and stored in a freezer at −80 °C until analysis.

2.2. Extraction of bioactive constituents of black bean

After homogenization, 2.5 g of each sample were accurately weighed and extracted with 25 ml of 80% aqueous methanol (DEQ Monterrey, Mexico). Extraction was carried out in a Vortemp (Vortemp 1550, Labnet International, Inc. Edison, NJ) for 30 min at 2.87 g and 25 °C using eight 5 mm glass beads (KIMAX, Vineland, NJ). Then, the resulting extract was filtered using a No. 1 Whatman filter paper and the solids were washed with 80% methanol to adjust to a final volume of 25 ml. The extract was evaporated under vacuum at 60 °C (Speedvac concentrator, Savant SC210A, Thermo electron Co., Milford, MA) to eliminate the methanol and then freeze-dried (Virtis freezemobile Sentry 2.0, Gardiner, NY).

2.3. Identification and quantification of saponins, flavonols and isoflavones

Saponins and flavonoids were quantified using an HPLC–DAD–ELSD (Agilent Technologies, Santa Clara, CA) system. Separation was performed in a Zorbax SB-Aq 4.6 mm ID × 150 mm, 3.5 μm reverse column (Agilent Technologies, Santa Clara, CA) with a flow of 0.5 ml/min. Elution was conducted with (A) HPLC-grade water adjusted to pH 2 with trifluoroacetic acid (Sigma, St. Louis, MO) and (B) HPLC-grade acetonitrile. Separation was achieved with 20% B for the first 6 min, increasing the B concentration to 50% at 12 min and to 100% at 30 min. This last solvent concentration was maintained for the next 10 min. Chromatograms were acquired at 220, 280 and 320 nm and integrated by the HP-Agilent Software (Chemstation for LC Copyright Agilent Technologies, 1990–2003). Peak identification of flavonoids was based on retention time and UV spectra. Aglycones were compared to authentic standards of kaempferol, quercetin, myricetin and genistein. To confirm the detection of the DDMP-conjugated saponins their absorption maximum at 295 nm was obtained.

The identification of saponins in black bean extracts was confirmed by HPLC–MS-TOF (Model G1969A Agilent 1100 Santa Clara, CA). Chromatographic conditions used were the same as those described for the HPLC–DAD–ELSD analysis. Mass spectra were collected using electrospray source in positive mode (ESI+) under the following conditions: *m/z* range, 100–1500; nitrogen gas; gas temperature, 350 °C; drying gas flow rate, 13 l/min; nebulizer pressure, 50 psig; capillary voltage, 4000 V; and fragment voltage, 70 V. The identification of saponins based in the mass spectra was easier in view of the fact that they always presented the $[M+Na]^+$ and/or $[M+K]^+$ ions (Lee, Chen, Hwang, & Hsu, 1999). Extracted ion chromatograms were obtained considering accurate mass obtained for the saponins or their adducts with Na or K with an error range of 0.01 units using the Analyst QS 1.1 software (Applied Biosystems, Carlsbad, CA). The mass spectrum of each ion was obtained to confirm the presence of the different ions forms of the saponins and/or corresponding fragments previously reported.

All saponins were quantified using the evaporate light scattering detector (ELSD) (Decroos et al., 2005; Rupasinghe et al., 2003) and calculated by using a standard curve obtained from soyasaponin I purified in our laboratory. Flavonoids were quantified using standard curves from authentic standards of aglycones. For glycosylated flavonols the concentrations were reported as equivalents of aglycones.

2.4. Statistical analysis

All analyses were done in triplicate and results were expressed as mean ± standard deviations. Statistical analyses were conducted by one-way ANOVA, and differences among means were compared with Tukey's tests with a level of significance of $p < 0.05$ using the JMP® Version 5 software (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Physical changes during germination

The solids loss after soaking averaged 10.7% and did not increase significantly during germination (Table 1). These losses were attributed to the degradation and oxidation of starch and sugars during the respiration process (Rupasinghe et al., 2003) and the leaching of soluble compounds into the soaking water. According to (Berrios, Swanson, & Cheong, 1999), the solid losses from soaked black beans (*P. vulgaris* L.) were in the range of 6.5–11.5% and differed as a consequence of changes in storage conditions such as time, temperature, pressure and relative humidity.

Almost 90% of the seeds had clear evidences of sprouting after 3-days of germination (Table 1). The sprout weight of the 3-day germinated black bean was 3.9% of the total seed (Table 1) and the length increased from 0.5 to 2.1 cm during the first four days of germination. Interestingly, the sprouts doubled their length from the day 4–5 of germination. Similar results have also been reported by Berrios et al. (1999).

3.2. Identification of saponins

Saponins of groups A, B, E, and DDMP-conjugated forms were found (Table 2). Some of these molecules had previously been reported in black beans, such as phaseoside I belonging to group A

Table 1
Change of physical properties of black bean during the germination process.

		Number of days germinated				
	Soaking	1	2	3	4	5
Solid loss (%)	10.7 ± 0.4a	11.8 ± 1.2a	7.7 ± 3.3a	10.8 ± 0.2a	11.0 ± 1.8a	9.3 ± 1.7a
Germination percentage (%)	n.d.	48.2 ± 2.6c	73.5 ± 4.9b	89.4 ± 0.5a	93.7 ± 0.9a	95.0 ± 1.0a
Sprout length (cm)	0.5 ± 0.0e	0.5 ± 0.0e	1.5 ± 0.1d	2.1 ± 0.5c	3.3 ± 0.6b	4.2 ± 0.3a
Sprout (%)	1.3 ± 0.1c	1.4 ± 0.0c	1.4 ± 0.2c	3.9 ± 0.2b	4.7 ± 0.3a	3.7 ± 0.1b
Cotyledon (%)	89.9 ± 0.1a	89.4 ± 0.6a	89.4 ± 0.6a	89.0 ± 0.2a	85.9 ± 0.3a	85.7 ± 1.1a
Seed coat (%)	8.9 ± 0.2b	9.1 ± 0.1b	9.2 ± 0.1b	7.1 ± 0.2c	9.4 ± 0.3b	10.5 ± 0.2a

n.d.: not determined.
Mean values in each row sharing the same letter are not significantly different ($p < 0.05$).

Table 2
Saponins divided by groups (A, B and E) found in black bean ordered by elution time and identified comparing ion mass with previous reports of these molecules in black bean and other bean seeds.

Group A			
Name[1]	*m/z*	Positive MS assignment[2]	References
Phaseoside I	1291	$[M+K]^+$	Kinjo et al. (1998) and Aparicio-Fernandez et al. (2005)
	1275	$[M+Na]^+$	
	1253	$[M+H]^+$	
	1091	$[M-glc+H]^+$	
	959	$[M-glc-arab+H_2O+H]^+$	
	599	$[M-glc-arab-glc-gal-H_2O+H]^+$	
	441	$[M-glc-arab-glc-gal-glu+H_2O+H]^+$	
	423	$[M-glc-arab-glc-gal-glu+H]^+$	
Soyasaponin Af (acetyl A2)	1275	$[M+H]^+$	Shiraiwa et al. (1991b)
	1107	$[M-Acetylgroups+H]^+$	
	599	$[M-arab-glc-gal-2H_2O+H]^+$	
	439	$[M-arab-glc-gal-glu+H]^+$	
	423	$[M-arab-glc-gal-glu-O+H]^+$	
Deacetylated soyasaponin Af (A2)	1129	$[M+Na]^+$	Shiraiwa et al. (1991b)
	1107	$[M+H]^+$	
	439	$[M-arab-glc-gal-glu+H]^+$	
	423	$[M-arab-glc-gal-glu-O+H]^+$	
Deacetylated soyasaponin Ae (A5)	1113	$[M+Na]^+$	Shiraiwa et al. (1991b)
	1091	$[M+H]^+$	
	599	$[M-arab-glc-gal+H_2O+H]^+$	
	423	$[M-arab-glc-gal-glu+H]^+$	
Deacetylated soyasaponin Ah (A3)	1083	$[M+Na]^+$	Shiraiwa et al. (1991b)
	1061	$[M+H]^+$	
	423	$[M-glc-arab-arab-glu+H_2O+H]^+$	
Group B and E			
Soyasaponin Ba and methyl ester	995	$[M+Na]^+$	Dong et al. (2007)
	811	$[M-glc+H]^+$	
	441	$[M-glc-gal-glu-ME+H]^+$	
Soyasaponin Ba (V)	997	$[M+K]^+$	Ayet et al. (1996), Kinjo et al. (1998), Lee et al. (1999) and Dong et al. (2007)
	981	$[M+Na]^+$	
	959	$[M+H]^+$	
	599	$[M-glc-gal-2H_2O+H]^+$	
	441	$[M-glc-gal-glu+H]^+$	
	423	$[M-glc-gal-glu-H_2O+H]^+$	
Soyasaponin Bb (I)	981	$[M+K]^+$	Ayet et al. (1996), Kinjo et al. (1998) and Lee et al. (1999)
	965	$[M+Na]^+$	
	797	$[M-rha+H_2O+H]^+$	
	599	$[M-rha-gal-H_2O+H]^+$	
	441	$[M-rha-gal-glu+H_2O+H]^+$	
	423	$[M-rha-gal-glu+H]^+$	
Soyasaponin Bb' (III)	819	$[M+Na]^+$	Decroos et al. (2005)
	797	$[M+H]^+$	
	441	$[M-gal-glu+H]^+$	
Soyasaponin Bd	979	$[M+Na]^+$	Yoshiki and Okubo (1995) and Lee et al. (1999)
	957	$[M+H]^+$	
	439	$[M-glc-gal-glu+H]^+$	
Soyasaponin αg	1107	$[M+Na]^+$	Lee et al. (1999) and Aparicio-Fernandez et al. (2005)
	1085	$[M+H]^+$	
	923	$[M-glc+H]^+$	
	743	$[M-glc-gal-H_20+H]^+$	
	725	$[M-glc-gal-2H_20+H]^+$	
	567	$[M-glc-gal-glu+H]^+$	
	423	$[M-glc-gal-glu-DDMP-H_20+H]^+$	
Soyasaponin βg	1107	$[M+K]^+$	Kudou et al. (1992) and Aparicio-Fernandez et al. (2005)
	1091	$[M+Na]^+$	
	1069	$[M+H]^+$	
	923	$[M-rha+H_2O+H]^+$	
	725	$[M-rha-gal-H_2O+H]^+$	
	423	$[M-rha-gal-glu-DDMP+H]^+$	
Soyasaponin γg	945	$[M+Na]^+$	Kudou et al. (1992) and Kudou et al. (1993)
	923	$[M+H]^+$	
	423	$[M-rha-gal-glu-DDMP+H]^+$	

[1] Nomenclature is according to Shiraiwa et al. (1991a, 1991b) and names in parenthesis are according to Kitagawa et al. (1982).
[2] rha, rhamnopyranosyl; gal, galactopyranosyl; ara, arabinopyranosyl; glu, glucuronopyranosyl; glc, glucopyranosyl; DDMP, 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one and ME, methyl ester.

saponins (Aparicio-Fernandez, Yousef, Loarca-Pina, de Mejia, & Lila, 2005), soyasaponin Ba and Bb from group B (Dong, He, & Liu, 2007; Lee et al., 1999), soyasaponin Bd from group E and soyasaponins αg from DDMP conjugates (Lee et al., 1999). Ions for soyasaponin βg were previously detected by Aparicio-Fernandez et al. (2005) in black beans but probably incorrectly attributed to proanthocyanidin oligomers. This is due to the UV maximum absorption at 280 nm that is given by the DDMP substituent of this sort of

saponin. Other saponins found in the current study have been reported in different legume seeds (Table 2).

The soyasaponin Af, soyasaponin αg and soyasaponin Ba (V) were the most abundant saponins in black bean tissues (Tables 3 and 4). The soyasaponin Af ion mass spectra (Fig. 1) presented characteristic ions at 1297 *m/z* ($[M+Na]^+$), 1275 *m/z* ($[M+H]^+$), 599 *m/z* [(M−arab−glc−gal−$2H_2O$+H)], 439 *m/z* [(M−arab−glc−gal−glu+H)], and specially at 1107 *m/z* which may come from the loss of the tetra-*O*-acetyl group of the C-22 position (Shiraiwa et al., 1991b). Soyasaponin Ba exhibited the presence of major ions at 997 *m/z* ($[M+K]^+$), 981 *m/z* ($[M+Na]^+$) and 959 *m/z* ($[M+H]^+$) previously reported in black beans (Ayet et al., 1996; Dong et al., 2007; Kinjo et al., 1998). The ions at 599 *m/z* [(M−glc−gal−$2H_2O$+H)] and 441 *m/z* [(M−glc−gal−glu+H)] show similarity to the molecules of soyasaponins Af and Ba. These were oleanolic acid derivatives conjugated in the first instance with glucuronic acid. Soyasaponin αg showed major ions at 1107 *m/z* ($[M+Na]^+$) and 1085 *m/z* ($[M+H]^+$) and fragment ions at 923 *m/z* [(M−glc−H_2O+H)], 743 *m/z* [(M−glc−gal−H_2O+H)], 725 *m/z* [(M−glc−gal−$2H_2O$+H)] and 567 *m/z* [(M−glc−gal−glu+H)] (Lee et al., 1999).

3.3. Saponin profiles of hilum, sprouts, seed coats and cotyledons of black beans

Hilum contained the highest amount of saponins compared to seed coats or cotyledons (Table 3) as previously reported for soybeans (Yoshiki, Kudou, & Okubo, 1998). Rupasinghe et al. (2003) demonstrated that the soybean hilum contributed more soyasaponins from group A than those from group B. Similar results were obtained in this study considering that the mass concentration ratio of soyasaponins from group A in hilum:cotyledon:seed coat was 40:1:2.7 and only 8.7:1:1.13 for group B (Table 3). The main constituent in these anatomical parts was soyasaponin Af. Group B soyasaponins were in lower amounts compared to group A and most of these molecules were detected as DDMP-conjugated molecules. For most saponins associated to hilum, the drying treatment did not have any significant effect on the concentration (Table 3), indicating that the conditions used were not deleterious for the acetylated and DDMP-conjugated forms which were previously reported to be sensitive to temperature (Jyothi, Kanya, & Rao, 2007; Kurosawa, Takahara, & Shiraiwa, 2002). In contrast, the total amount of saponins in cotyledons decreased from 27.62 to

Table 3
Concentrations of soyasaponins in bean hilum, cotyledon and seed coats in non-germinated black bean (mg/100 g of sample).

Tissue	Compound	Treatment		
		Raw	Dry	Soaked
Hilum	Phaseoside I	8.71 ± 1.22a	8.84 ± 1.47a	14.83 ± 1.88b
	Soyasaponin Af (acetyl A2)	244.62 ± 103.15b	368.27 ± 29.06b	569.08 ± 76.17a
	Deacetyl soyasaponin Af (A2)	7.15 ± 1.11b	7.85 ± 1.25b	12.01 ± 1.37a
	Deacetyl soyasaponin Ae (A5)	7.52 ± 1.16b	8.08 ± 1.63b	13.99 ± 2.36a
	Deacetyl soyasaponin Ah (A3)	6.31 ± 1.32b	7.94 ± 0.93b	13.78 ± 2.38a
	Total Group A	274.29	400.98	623.69
	Soyasaponin Ba (V)	21.72 ± 9.14b	24.18 ± 9.99b	67.53 ± 11.31a
	Soyasaponin Bb' (III)	6.54 ± 1.14a	7.23 ± 1.89a	11.38 ± 2.47a
	Soyasaponin αg	139.46 ± 71.52b	215.31 ± 42.60ab	313.22 ± 83.01a
	Soyasaponin βg	6.62 ± 0.98c	18.85 ± 1.81b	32.12 ± 0.92a
	Soyasaponin γg	7.32 ± 1.60b	8.44 ± 0.18b	15.68 ± 2.07a
	Total Group B	181.66	274.01	439.93
	Total soyasaponins	455.95	674.99	1063.62
Cotyledon	Soyasaponin Af (acetyl A2)	6.79 ± 0.56a	2.02 ± 0.50b	3.13 ± 2.62b
	Deacetyl soyasaponin Af (A2)	n.d.	n.d.	1.15 ± 0.14
	Deacetyl soyasaponin Ah (A3)	n.d.	n.d.	1.01 ± 0.14
	Total Group A	6.79	2.02	5.29
	Soyasaponin Ba (V) ME	4.44 ± 0.03	n.d.	n.d.
	Soyasaponin Ba (V)	n.d.	1.00 ± 0.22a	1.08 ± 0.20a
	Soyasaponin Bb (I)	5.48 ± 0.78a	1.25 ± 0.30b	1.64 ± 0.51b
	Soyasaponin Bb' (III)	3.71 ± 0.05a	0.90 ± 0.26b	0.84 ± 0.10b
	Soyasaponin αg	5.90 ± 0.97a	1.53 ± 0.29b	1.27 ± 0.21b
	Soyasaponin βg	5.74 ± 0.20a	1.13 ± 0.22b	2.52 ± 1.28b
	Total Group B	20.83	5.81	7.35
	Total soyasaponins	27.62	7.83	12.64
Seedcoat	Soyasaponin Af (acetyl A2)	15.67 ± 5.09a	7.52 ± 1.12b	2.40 ± 0.66b
	Deacetyl soyasaponin Af (A2)	3.13 ± 0.09a	3.18 ± 0.10a	n.d.
	Total Group A	18.80	10.70	2.40
	Soyasaponin Ba (V) ME	3.81 ± 0.32a	3.18 ± 0.17b	0.70 ± 0.04c
	Soyasaponin αg	11.03 ± 4.08a	4.37 ± 0.89b	1.26 ± 0.55c
	Soyasaponin βg	5.99 ± 1.26a	3.26 ± 0.28b	0.60 ± 0.03c
	Soyasaponin γg	2.65 ± 0.07a	n.d.	0.57 ± 0.09b
	Total Group B	23.48	10.81	3.13
	Total soyasaponins	42.28	21.51	5.53

ME, methylester; n.d., not determined.
The data represent the mean ± SD of at least three replicates.
Different letters by row indicate significant differences, $p < 0.05$.
Nomenclature is according to Shiraiwa et al. (1991a,1991b) and names in parenthesis are according to Kitagawa et al. (1982).

Table 4
Concentrations of soyasaponins in sprouts, cotyledon and seed coats of black beans (*Phaseolus vulgaris*) subjected to different germination times (mg/100 g of sample).

Tissue	Compound	Number of days gemrinated				
		1	2	3	4	5
Sprout	Phaseoside I	29.05 ± 3.74a	n.d.	n.d.	n.d.	n.d.
	Soyasaponin Af (acetyl A2)	1127.96 ± 99.51a	403.44 ± 121.82b	125.87 ± 28.03c	82.33 ± 11.52c	60.14 ± 7.92d
	Deacetyl soyasaponin Af (A2)	22.95 ± 5.61a	22.22 ± 3.14a	16.01 ± 0.62a	n.d.	n.d.
	Deacetyl soyasaponin Ae (A5)	25.61 ± 5.16a	23.67 ± 5.68a	n.d.	15.21 ± 4.80a	20.21 ± 5.05a
	Deacetyl soyasaponin Ah (A3)	18.06 ± 6.30a	25.90 ± 5.37a	19.88 ± 1.06a	n.d.	n.d.
	Total Group A	1223.63	475.23	141.88	97.54	80.35
	Soyasaponin Ba (V)	111.58 ± 43.34a	70.47 ± 32.01ab	62.04 ± 18.52b	17.92 ± 2.66d	37.01 ± 8.77c
	Soyasaponin Bb (I)	n.d.	n.d.	25.28 ± 3.28a	15.89 ± 2.96a	18.81 ± 6.19a
	Soyasaponin Bb' (III)	19.39 ± 6.16a	18.17 ± 6.60a	n.d.	n.d.	n.d.
	Soyasaponin Bd	n.d.	n.d.	26.98 ± 5.78a	n.d.	16.51 ± 2.21b
	Soyasaponin αg	583.07 ± 117.94a	221.08 ± 145.39b	43.67 ± 10.10c	45.17 ± 9.35c	24.18 ± 3.48d
	Soyasaponin βg	56.91 ± 6.80a	34.33 ± 9.12b	21.12 ± 5.60c	21.97 ± 2.58c	16.95 ± 5.56c
	Soyasaponin γg	22.62 ± 3.09a	19.92 ± 6.56a	n.d.	n.d.	12.04 ± 1.23b
	Total Group B + E	793.57	363.97	179.09	100.95	125.5
	Total soyasaponins	2017.20	839.20	320.97	393.57	205.85
Cotyledon	Phaseoside I	nd	nd	nd	nd	5.52 ± 0.68
	Soyasaponin Af (acetyl A2)	5.52 ± 1.02c	11.60 ± 2.09b	11.86 ± 1.11b	18.67 ± 3.66a	10.68 ± 0.41b
	Total Group A	5.52	11.60	11.86	18.67	16.20
	Soyasaponin Ba (V)	3.66 ± 0.52c	7.51 ± 0.81ab	6.39 ± 0.54bc	10.19 ± 1.40a	5.16 ± 0.96bc
	Soyasaponin Bb (I)	4.00 ± 1.35c	7.59 ± 1.69ab	6.69 ± 0.56bc	9.67 ± 0.87a	5.06 ± 0.61bc
	Soyasaponin Bb' (III)	4.03 ± 0.55ab	5.44 ± 0.61a	5.63 ± 0.54a	n.d.	3.66 ± 0.15b
	Soyasaponin αg	5.35 ± 0.43d	8.89 ± 1.43ab	7.74 ± 0.48bc	10.64 ± 0.73a	6.49 ± 0.45 cd
	Soyasaponin βg	4.27 ± 0.18c	7.85 ± 1.59ab	6.78 ± 0.61b	8.70 ± 0.54a	4.73 ± 0.19c
	Total Group B + E	21.31	37.28	33.23	39.20	25.10
	Total soyasaponins	26.83	48.88	45.09	57.87	41.30
Seed coat	Soyasaponin Af (acetyl A2)	1.95 ± 0.19b	0.75 ± 0.08b	1.89 ± 0.72b	7.38 ± 1.73a	0.76 ± 0.10b
	Deacetyl soyasaponin Af (A2)	n.d.	n.d.	n.d.	0.33 ± 0.02a	0.30 ± 0.05a
	Deacetyl soyasaponin Ae (A5)	n.d.	n.d.	0.37 ± 0.02a	0.34 ± 0.02a	0.25 ± 0.01b
	Total Group A	1.95	0.75	2.26	8.05	1.31
	Soyasaponin Ba (V)	0.62 ± 0.13a	0.40 ± 0.01b	0.46 ± 0.04ab	0.43 ± 0.08ab	0.27 ± 0.04b
	Soyasaponin Bb (I)	n.d.	n.d.	n.d.	0.31 ± 0.02a	0.26 ± 0.04a
	Soyasaponin Bd	n.d.	n.d.	n.d.	n.d.	0.21 ± 0.01
	Soyasaponin αg	1.23 ± 0.07b	0.46 ± 0.27b	0.52 ± 0.08b	3.14 ± 0.66a	0.49 ± 0.19b
	Soyasaponin βg	n.d.	0.41 ± 0.04a	n.d.	0.62 ± 0.11a	0.38 ± 0.16a
	Soyasaponin γg	n.d.	n.d.	n.d.	0.38 ± 0.01	n.d.
	Total Group B + E	1.85	1.27	0.98	4.88	1.61
	Total soyasaponins	3.8	2.02	3.24	12.93	2.92

n.d., not determined.
The data represent the mean ± SD of at least three replicates.
Different letters by row indicate significant differences, $p < 0.05$.
Nomenclature is according to Shiraiwa et al. (1991a, 1991b) and names in parenthesis are according to Kitagawa et al. (1982).

7.83 mg/100 g probably due to a reduction in the extractability of these compounds in this complex matrix. The reduction was less drastic in seed coats because it only decreased from 42.28 to 21.51 mg/100 g. Particularly in cotyledons, the methyl ester form of soyasaponin Ba was most affected and appeared as a non-methylated form after drying. Soyasaponin αg was the most affected in seed coats with a loss of approximately 60%.

After soaking, the concentration of saponins associated to the hilum increased 2.3-fold (Table 3) probably due to the intrinsic enzymatic activity of the activated seed in preparation for germination and generation of the new plant (Kurosawa et al., 2002; Yoshiki et al., 1998). The soyasaponin Af concentration increased from 244.62 to 569.08 mg/100 g, representing more than the 53% of total saponins detected in this tissue after soaking.

The amount of total soyasaponins in cotyledons and seed coats was reduced after soaking (Table 3) in agreement with previous reports in bean seeds (Shi et al., 2004). Contrary to the effect of drying, soaking produced a higher loss of saponins in seed coats than the observed for cotyledons. Soyasaponin Af in cotyledons was reduced from 6.79 to 3.13 mg/100 g after soaking and in the deacetylated form was found in this tissue only after soaking. Another deacetylated saponin from group A (deacetyl soyasaponin Ah) at a concentration of 1.01 mg/100 g was found in cotyledons only after soaking, probably due to the migration or translocation of compounds from the developing sprout to this reserve tissue (Rupasinghe et al., 2003).

3.4. Changes in saponins profile of sprouts, seed coats and cotyledons of black beans during germination

Seed germination had a marked effect on both composition and concentration of saponins present in seedlings (Table 4). The sprouts contained higher concentrations of groups A, B and E saponins compared to cotyledons or seed coats (Table 4) as previously reported in soybeans (Taniyama, Yoshikawa, & Kitagawa, 1988). Even if saponins were lost from the seed coat during the first germination day, the total amount in the seed increased due to the higher concentration detected in sprouts and cotyledons which represented 90.8% of the total seed weight (Table 1). After one day of germination, the total soyasaponin content of sprouts and cotyledons increased 1.9 and 2.1-fold, respectively. Different studies on soybean saponins have reported a similar increment of these

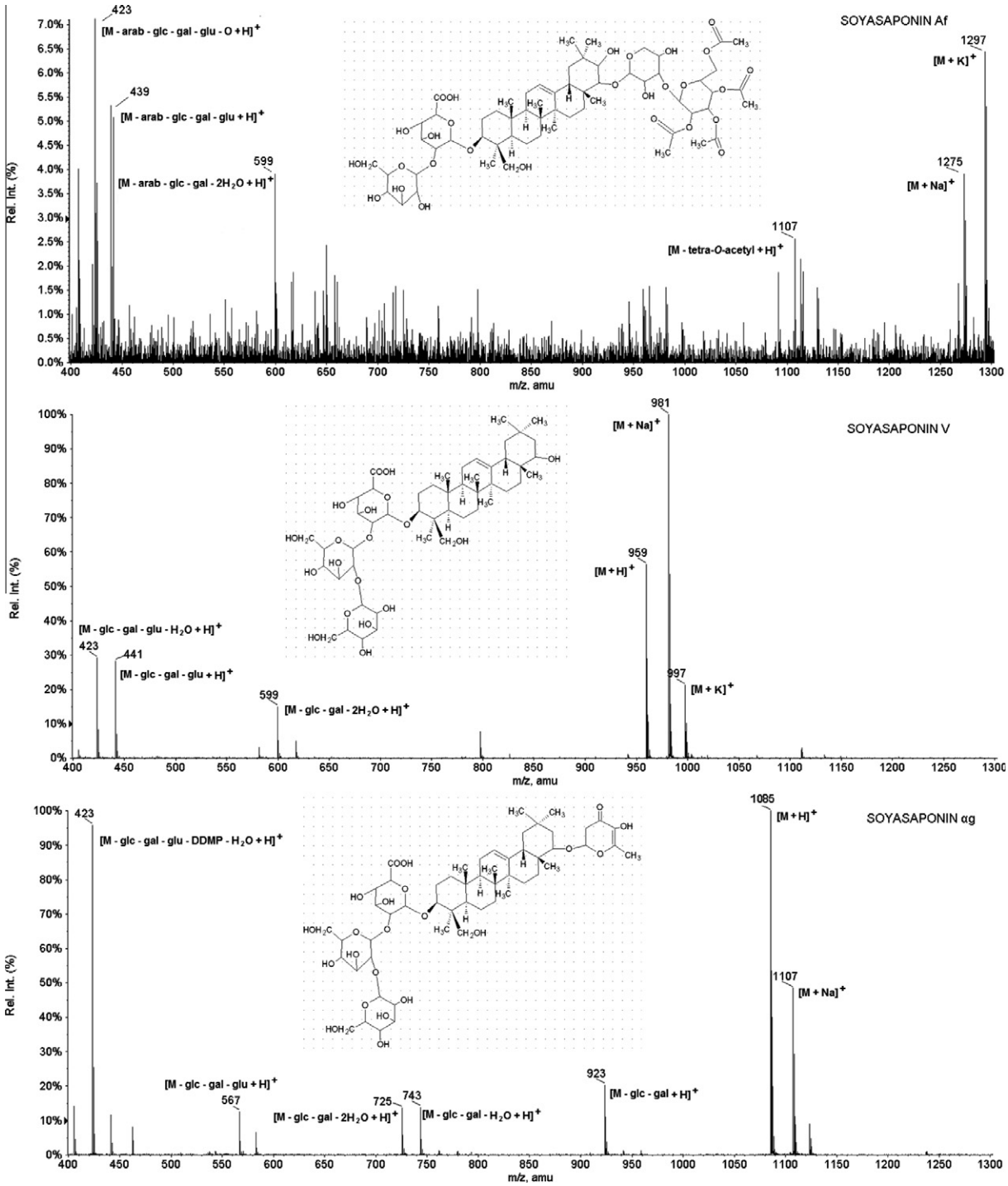

Fig. 1. Ion mass spectra and structures of soyasaponin Af (Top), soyasaponin Ba (V) (Middle) and soyasaponin αg (Bottom).

phytochemicals over the same time period (Jyothi et al., 2007; Paucar-Menacho et al., 2010a, 2010b; Rupasinghe et al., 2003; Shimoyamada & Okubo, 1991).

Among the saponins associated to sprouts, the group A moieties increased from 623.69 to 1223.63 mg/100 g and those belonging to groups B and E increased from 439.93 to 793.57 mg/100 g (Tables 3 and 4) likely due to synthesis and activation of different enzyme systems in the germinating seed that enhanced the production of these secondary metabolites and the weakening and modification of the seed structure that facilitated or enhanced the solvent extraction procedure (Kurosawa et al., 2002; Mwikya, Van Camp, Rodriguez, & Huyghebaert, 2001; Yoshiki et al., 1998). However, longer germination times significantly decreased the concentrations of soyasaponins Af, αg and βg.

Soyasaponins Bb and Bd were detected after the third day germination probably as a result of the loss of the DDMP group of βg and αg soyasaponins. The detection of soyasaponin Bd on germinated soybean seeds had previously been reported as a product of the degradation of DDMP-conjugated αg soyasaponin (Gu, Tao, Gu, & Prior, 2002). The deacetylated form of soyasaponin Ae was not affected by germination in accordance with previous reports in soybeans (Paucar-Menacho et al., 2010a, 2010b).

Table 5
Concentrations of flavonoids in non-germinated and germinated black bean seed coat (mg/100 g of sample).

	Myricetin 3-*O*-glucoside	Quercetin 4-*O*-galactoside	Kaempferol 3-*O*-glucoside	Quercetin	Genistein	Kempferol	Total
Non-germinated							
Raw	115.72 ± 29.87ab	643.18 ± 138.82ab	6.60 ± 1.41a	n.d.	n.d.	n.d.	765.50
Dry	154.71 ± 58.73a	1187.37 ± 370.90a	8.64 ± 2.68a	n.d.	n.d.	n.d.	1350.72
Soaking	24.83 ± 3.90b	214.90 ± 8.11b	2.20 ± 0.12b	0.97 ± 0.21	n.d.	0.16 ± 0.06	243.06
Number of days germinated							
1	18.15 ± 2.39a	141.55 ± 30.05a	1.36 ± 0.30a	1.28 ± 0.49a	n.d.	0.15 ± 0.06a	162.49
2	11.10 ± 1.81ab	82.02 ± 6.90b	0.67 ± 0.09b	1.51 ± 0.24a	n.d.	0.18 ± 0.04a	95.48
3	16.06 ± 4.46a	95.74 ± 18.27b	0.73 ± 0.13b	1.48 ± 0.48a	0.12 ± 0.03a	0.22 ± 0.01a	114.35
4	11.87 ± 1.81ab	80.88 ± 16.88b	0.58 ± 0.13bc	2.24 ± 0.24a	0.31 ± 0.12a	0.30 ± 0.07a	96.18
5	7.66 ± 2.99b	30.00 ± 5.07c	0.24 ± 0.03c	1.83 ± 0.27a	0.16 ± 0.06a	0.29 ± 0.07a	40.18

n.d.: not detected.
The data represent the mean ± SD of at least three replicates.
Different letters in each row in non-germinated or germinated samples indicate significant differences, $p < 0.05$.

For cotyledons, on the fourth day of germination, soyasaponin Af increased from 5.52 to 18.67 mg/100 g (Table 4), this was the only group A saponin initially found in this tissue. Likewise, groups B and E saponins associated with cotyledons increased from 21.31 to 39.2 mg/100 g after the fourth day of germination. The higher amount of soyasaponins in cotyledons observed after germination was probably a result of the translocation of stored phytochemicals from the sprouts (Rupasinghe et al., 2003). This mechanism could also explain the presence of phaseoside I in cotyledons after five days of germination. A similar change in the concentration of saponins in seed coats was noticed when the amounts present in these tissues obtained from the one and fourth day germinated seeds were compared. Particularly, group A saponins increased from 1.95 to 8.05 mg/100 g and group B and E saponins from 1.85 to 4.88 mg/100 g (Table 4).

3.5. Changes in flavonoid profile on seed coats of non-germinated and germinated black beans

The main flavonoids associated with black beans were detected only in the seed coat as previously reported (Aparicio-Fernandez et al., 2005). The total amounts of these flavonols in seed coats were 20-fold higher compared to a previous study that quantified these phytochemicals as aglycones (Diaz-Batalla et al., 2006). More importantly, these flavonols were quantified in the spent residue obtained after extraction with 80% methanol (Diaz-Batalla et al., 2006) and our results were obtained from the methanolic extract.

Flavonols concentrations were not affected by temperature during drying but soaking enhanced the leaching of these compounds into the water (Table 5) as previously reported by Ranilla, Genovese and Lajolo (2009). The aglycone forms of flavonols were detected after soaking due to the enzymatic activity of glycosidases (Chiarello et al., 2006). The loss of glycosylated flavonols and the presence of their aglycones during sprouting evidenced the effectiveness of germination (Chiarello et al., 2006; Yuan, Liu, Peng, Wang, & Liu, 2009). Genistein was detected after three days of germination in concentrations that ranged from 0.12 to 0.31 mg/100 g, in accordance with previous reports (Diaz-Batalla et al., 2006).

4. Conclusions

Acetylated and non-acetylated forms of group A soyasaponins, as well as groups B and E soyasaponins with their DDMP-conjugated forms were detected in significant amounts in black bean tissues. Soyasaponin Af and soyasaponin αg, a DDMP-conjugated, were the main molecules from groups A and B, respectively. Germination had a significant effect on the saponin and flavonoid profile in different black bean tissues. Soyasaponins were found in higher concentrations in sprouts compared to the other black bean tissues. Soaking leached out flavonols and saponins from seed coats but triplicated the amount of saponins in sprouts. Degradation or migration effects were observed after the third day of germination such as: degradation of DDMP groups from soyasaponins βg and αg to soyasaponins Bb and Bd, a noticeable increment in saponin concentration in cotyledons, and the detection of aglycone isoflavones in seed coats.

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