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THE APPLICATION OF CHEMICAL ELICITORS IMPROVES THE FLAVONOID AND SAPONIN PROFILES OF BLACK BEANS AFTER SOAKING

LA APLICACIÓN DE ELICITORES QUÍMICOS MEJORA EL PERFIL DE COMPUESTOS FLAVONOIDES Y SAPONINAS EN EL FRIJOL NEGRO DESPUÉS DEL REMOJO

E.K. Díaz-Sánchez, D. Guajardo-Flores, D. Serna-Guerrero, J.A Gutierrez-Uribe, D.A. Jacobo-Velázquez*
Tecnológico de Monterrey, Escuela de Ingeniería y Ciencias, Centro de Biotecnología FEMSA, Eugenio Garza Sada 2501 Sur, C.P. 64849, Monterrey, N.L., México

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Abstract

Black beans are an excellent source of bioactive molecules such as flavonoids and saponins that prevent chronic diseases. In the present study, the effects of chemical elicitors [L-proline (L-pro), giberellins (GAs) and sucrose (Suc)], on the flavonoid and saponin content of black beans were evaluated. Elicitors were added in soaking water (0.5% w/v), and the concentration of flavonoids and saponins were determined after soaking and at 18 h of germination. The combination of Suc and L-pro resulted on the highest accumulation of most compounds quantified after soaking. For that treatment, quercetin-3*O*-glucoside, malvidin-3*O*-glucoside, and soyasaponins Af, increased by 68.9%, 42.8%, and 50.7% respectively, as compared with the control. Results indicated that adding Suc in combination with L-pro in soaking water could be used as an effective method to improve the concentration of bioactive compounds in black beans, which could be used as raw material to produce processed foods or nutraceutical supplements.

Keywords: black beans, chemical elicitors, L-proline, sucrose, nutraceuticals.

Resumen

El frijol negro es una excelente fuente de moléculas bioactivas como flavonoides y saponinas que previenen enfermedades crónicas. En este estudio, se evaluó el efecto de elicitores químicos [L-prolina (L-pro), giberelinas (GAs) y sacarosa (Suc)], en el contenido de flavonoides y saponinas del frijol negro. Los elicitores se adicionaron al agua de remojo (0.5% w/v), y la concentración de flavonoides y saponinas se determinó después del remojo y a las 18 h de germinación. La combinación de Suc y L-pro resultó en la mayor acumulación de la mayoría de los compuestos cuantificados después del remojo. Para ese tratamiento, la quercetina-3*O*-glucósido, la malvidina-3*O*-glucosido, y la soyasaponina Af, incrementaron 68.9%, 42.8%, and 50.7% respectivamente, comparado con el control. Los resultados indican que la adición de Suc en combinación con L-pro en el agua de remojo puede ser empleda como un método efectivo para mejorar la concentración de compuestos bioactivos en el frijol negro, los cuales pueden ser posteriormente utilizados como materia prima para la producción de alimentos procesados o suplementos nutracéuticos.

Palabras clave: frijol negro, elicitor químico, L-prolina, sacarosa, nutracéuticos.

1 Introduction

Common beans (*Phaseolus vulgaris* L.) have a high content of protein, fiber, prebiotics, vitamin B, and various phenolic compounds and it is the most important legume for human intake worldwide (Cámara *et al*., 2013; Tovar-Benítez *et al*., 2016). The common bean cultivars showing black seed coat, could be used for the elaboration of nutraceutical supplements to improve human health since they are an excellent source of preventive and therapeutic compound, such as flavonols, anthocyanins and saponins (Guajardo-Flores *et al*., 2013; Chávez-Santoscoy *et al*., 2013, Chávez-Santoscoy *et al*., 2014). Therefore, it is desirable to design procedures to increase the natural levels of nutraceutical in black beans.

* *Corresponding author. E-mail*: djacobov@itesm.mx
Tel. +52-818-358-20-00, ext. 4820, Fax +52-818-328-4136.
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During water imbibition of dried seeds, the metabolic activity of the plant cell is restarted showing biosynthesis of secondary metabolites as a response to oxidative stress (Taiz and Zeiger 1998). Therefore, the application of chemical elicitors in soaking water to enhance the metabolic activity of the growing seedling would be a simple and effective method to improve their nutraceutical content. Chemical elicitors such as L-proline (L-pro), sucrose (Suc) and gibberellins (GAs) have shown to improve the phenylpropanoid metabolism in plants (Shetty *et al*., 2003; Solfanelli *et al*., 2006; Baque *et al*., 2012; Liang *et al*., 2013). However, scientific information regarding the effect of chemicals elicitors on the biosynthesis of saponins in black beans is scarce. Therefore, the objective of this study was to determine the effects of adding L-pro, GAs and Suc, alone or in combination in soaking water on the accumulation of flavonoids and saponins in black bean seed coats and sprouts, immediately after soaking and at 18 h of germination.

2 Materials and methods

2.1 Chemicals

HPLC grade water, methanol and acetonitrile were obtained from BDH (Poole, UK). Formaldehyde was obtained from DEQ (Monterrey, NL, México). Trifluoroacetic acid (TFA), L-pro, Suc, kaempferol, quercetin, myricetin, malvidin-3*O*-glucoside and soyasaponin I were obtained from Sigma Aldrich (St. Louis, MO). GAs were obtained from IMAISA (Monterrey, NL, México).

2.2 Biological material and chemical elicitor treatments

The black bean variety "San Luis" used for the experiments was purchased from a local distributor. The effects of chemical elicitors (L-pro, Suc and GAs) on the phytochemical content were tested by their addition (0.5% w/v) to soaking water either alone or in combination. Flavonoid and saponins content was determined immediately after soaking and at 18 h of germination.

For germination, black beans were soaked during 24 h in distilled water (1:3 w/v) with aeration at 500 mL/min provided by an aquatic pump. After soaking, water was drained and the beans were placed on germination trays with moistened Kimtowels sheets (Kimberly-Clark, Irving, TX). The germination trays with the black beans were placed in a biosafety cabinet Labconco Co. (Kansas City, MO) at 25 ºC during 18 h. Soaked and germinated seeds were immediately dehydrated in an oven set at 60 ºC for 4 h and then, seeds were physically separated into seed coat, sprout and cotyledon. Only the seed coat and sprout were grounded into a homogeneous powder (Coffee and Spice Grinder Krups GX4100, D.F., México) and stored at -20ºC until phytochemical analyses.

2.3 Extraction of phytochemicals

For the extraction of flavonoids and saponins, powdered seed coats (1 g) or sprouts (0.5 g) were extracted with 10 mL or 5 mL of 80% aqueous methanol, respectively. Extraction was carried out in a vortemp 1550 (Labnet International Inc., Edison, NJ) for 30 min at 9000 rpm and 25 ºC using eight 5 mm glass beads (KIMAX, Vineland, NJ). After centrifugation (12,000xg for 10 min). Extracts were passed through nylon syringe filters (0.45 μm, Agilent Technologies, Santa Clara, CA) before injection to the chromatographic system.

2.4 Detection and quantification of flavonoids and saponins from black beans seed coats and sprout extracts by HPLC-DAD-ELSD

Flavonoids and saponins were quantified using an HPLC-DAD-ELSD (Agilent Technologies, Santa Clara, CA) system. Separation was performed in a Zorbax-SB-Aq 4.6 x 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 and (B) HPLC-grade acetonitrile. Separation was achieved with 20% B for the first 6 min, increasing the B concentration to 50% at the 12 min and at 100% at 30 min. This last solvent concentration was maintained for the next 10 min. Flavonols and anthocyanins were quantified using the chromatograms acquired at 365 and 520 nm, respectively. Peak identification of flavonoids was based on retention time and UV spectra, in a range of 190 nm to 600 nm, as compared with a previous report (Guajardo-Flores *et al*., 2012). Flavonols were quantified as equivalents of the authentic standards of aglycones (kaempferol, quercetin and myricetin). Anthocyanins were quantified using standard curve of malvidin-3*O*-glucoside. To confirm the detection of

the DDMP-conjugated saponins, their UV maximum absorption at 280 nm was obtained. All saponins were quantified using the evaporative light scattering detector (ELSD) set at a temperature of 50 ºC and 1 mV offset, and a standard curve of soyasaponin I.

2.5 *Statistical analysis*

Statistical analyses were performed using 3 repetitions. Data represents the mean value of samples and their standard error. Analyses of variance (ANOVA) were conducted using JMP software version 11.0 (SAS Institute Inc. Cary, NC), and mean separation was performed using the Tukey's HSD test ($p < 0.05$).

3 Results

3.1 *Effects of chemical elicitors and germination time on the flavonoid content in black bean seed coats*

Flavonoids were only detected in methanol extracts of black bean seed coats (Tables 1 and 2). Quercetin and myricetin as aglycones and their corresponding 3*O*-glucosides were the major flavonols quantified. Likewise, 3*O*-glucoside forms of delphinidin, petunidin and malvidin anthocyanins were also identified in black bean seed coats (Table 2). Similar flavonoid profiles in black bean seed coats have been previously reported by other authors (Oomah *et al*., 2010; Guajardo-Flores *et al*., 2012; Mojica *et al*., 2015).

Seed coats of black beans treated with Suc in combination with L-pro (Suc+L-pro) showed the highest content of flavonoids after soaking (Tables 1 and 2).

Table 1. Effect of gibberellins (GAs), sucrose (Suc) and L-proline (L-pro) in the accumulation of flavonols in black bean seed coat.

Germination time (h)	Treatment [i]			Flavonols (mg/100g dry seed coat)			
	GAs	Suc	L-Pro	Myricetin-3*O*-glucoside	Quercetin-3*O*-glucoside	Myricetin	Quercetin
0	0	0	0	17.0 ± 0.3bc(yz)	86.5 ± 1.4bcd(yz)	12.0 ± 0.3cde(xy)	35.3 ± 0.9b(y)
	0	1	0	19.9 ± 1.4b(y)	103.5 ± 7.4b(y)	15.0 ± 1.2ab(vw)	47.7 ± 3.9a(x)
	1	0	0	18.5 ± 1.35b(y)	88.4 ± 5.0bc(yz)	12.5 ± 0.7bcde(wxy)	32.4 ± 2.0bcd(yz)
	0	0	1	17.7 ± 0.6bc(y)	98.0 ± 2.2bc(y)	9.8 ± 0.2ef(yz)	35.2 ± 1.5b(y)
	1	1	0	19.3 ± 0.1b(y)	95.1 ± 2.0bc(y)	13.0 ± 0.2bcd(wx)	36.7 ± 1.1b(y)
	0	1	1	27.5 ± 0.5a(x)	146.1 ± 2.2a(x)	16.3 ± 0.2a(v)	52.4 ± 0.6a(x)
	1	0	1	12.9 ± 0.7d(z)	68.9 ± 2.9de(z)	7.8 ± 0.4ffg(z)	24.7 ± 1.3def(z)
	1	1	1	24.9 ± 1.2a(x)	130.2 ± 5.2a(x)	11.8 ± 0.4de(xy)	35.0 ± 1.2b(y)
18	0	0	0	18.0± 0.1bc(w)	83.9 ± 0.6cd(x)	15.0 ± 0.1ab(x)	36.2 ± 0.4b(x)
	0	1	0	14.1 ± 1.6cd(x)	63.7 ± 7.9e(y)	14.8 ± 1.3abc(x)	34.0 ± 3.5bc(x)
	1	0	0	8.4 ± 0.3ef(y)	39.4 ± 1.7fg(y)	5.8 ± 0.2g(z)	13.8 ± 0.4g(z)
	0	0	1	12.8 ± 0.2d(x)	58.1 ± 1.5ef(y)	13.1 ± 0.2bcd(x)	33.3 ± 0.5bc(x)
	1	1	0	8.3 ± 0.3f(yz)	41.7 ± 2.1fg(z)	7.8 ± 0.3fg(yz)	18.3 ± 1.0efg(z)
	0	1	1	7.7 ± 0.2f(yz)	35.7 ± 0.7g(z)	7.1 ± 0.2fg(yz)	17.0 ± 0.6fg(z)
	1	0	1	12.4 ± 0.4cde(x)	60.8 ± 1.3e(z)	8.9 ± 0.2f(y)	26.0 ± 0.6cde(y)
	1	1	1	5.4 ± 0.0f(z)	27.1 ± 0.3g(z)	5.5 ± 0.0g(z)	13.4 ± 0.0g(z)

[i]1: 0.5% w/v of the elicitor in soaking water. 0: No use of GAs, Suc or L-Pro
Different letters indicate statistical difference by the Tukey's HSD test ($p < 0.05$). Means within time results with different letters (a-g) are different at $\alpha = 0.05$. Means within treatments results with different letters (v–z) are different at $\alpha = 0.05$.

Table 2. Effect of gibberellins (GAs), sucrose (Suc) and L-proline (L-pro) in the accumulation of anthocyanins in black bean seed coat.

Germination time (h)	Treatment [i, ii]			Anthocyanins mg/100g dry seed coat		
	GAs	Suc	L-Pro	Delphinidin-3*O*-glucoside	Petunidin-3*O*-glucoside	Malvidin-3*O*-glucoside
0	0	0	0	3.3 ± 0.1cd(xy)	3.2 ± 0.0de(y)	1.4 ± 0.0ef(y)
	0	1	0	3.5 ± 0.2bc(x)	3.6 ± 0.2abcd(xy)	1.8 ± 0.1abcd(wx)
	1	0	0	3.4 ± 0.2bc(x)	3.3 ± 0.2cde(y)	1.6 ± 0.1cde(xy)
	0	0	1	2.6 ± 0.1bc(x)	2.4 ± 0.1bcd(y)	1.1 ± 0.0cde(xy)
	1	1	0	3.7 ± 0.1de(yz)	3.5 ± 0.0fg(z)	1.6 ± 0.0g(z)
	0	1	1	4.7 ± 0.1a(w)	4.3 ± 0.1a(x)	2.0 ± 0.0a(w)
	1	0	1	2.2 ± 0.1efg(z)	2.1 ± 0.1fgh(z)	1.0 ± 0.0gh(z)
	1	1	1	3.4 ± 0.1bc(x)	3.5 ± 0.2bcd(y)	1.6 ± 0.1de(xy)
18	0	0	0	4.0 ± 0.0b(w)	4.1 ± 0.0ab(x)	1.9 ± 0.0abc(wx)
	0	1	0	3.7 ± 0.3bc(w)	4.0 ± 0.3abc(x)	2.0 ± 0.2ab(w)
	1	0	0	1.8 ± 0.0gh(z)	1.8 ± 0.1gh(z)	0.9 ± 0.0gh(yz)
	0	0	1	3.7 ± 0.1fgh(yz)	3.8 ± 0.1gh(z)	1.6 ± 0.0gh(yz)
	1	1	0	1.9 ± 0.0 bc(w)	1.5 ± 0.1abcd(x)	0.7 ± 0.0bcde(x)
	0	1	1	2.2 ± 0.1efg(xy)	2.1 ± 0.1fgh(z)	0.9 ± 0.0gh(yz)
	1	0	1	2.5 ± 0.1ef(x)	2.7 ± 0.0ef(y)	1.1 ± 0.0fg(y)
	1	1	1	1.4 ± 0.0h(z)	1.5 ± 0.0h(z)	0.7 ± 0.0h(z)

[i]GAs: gibberellins. Suc: sucrose. L-pro: L-proline.
[ii]1: 0.5% w/v of the elicitor in soaking water. 0: No use of GAs, Suc or L-Pro
Different letters indicate statistical difference by the Tukey's HSD test ($p < 0.05$). Means within time results with different letters (a-h) are different at $\alpha = 0.05$. Means within treatments results with different letters (w–z) are different at $\alpha = 0.05$.

For flavonols, the Suc+L-pro treatment showed 61.7%, 68.9%, 35.8% and 48.4%, higher levels of myricetin-3*O*-glucoside, myricetin, and quercetin, respectively, as compared with the control (Table 1). Regarding anthocyanins, the 3*O*-glucosides of delphinidin, petunidin and malvidin in the Suc+L-pro treatment increased by 42.4%, 34.3% and 42.8%, respectively, as compared with the control (Table 2).

Germination time (18 h) decreased the concentration of flavonoids in seed coats treated with elicitors during soaking. Interestingly, for the control a different behavior was observed after germination, where increases of 25.0%, 21.2%, 28.1%, 35.7% for myricetin, delphinidin 3*O*-glucoside, petunidin 3*O*-glucoside, and malvidin 3*O*-glucoside, respectively, were observed as compared with control samples after soaking (Tables 1 and 2).

3.2 Effects of chemical elicitors and germination time on the saponin content in black bean sprouts

The concentration of saponins was only determined in black bean sprouts since they were not detected in the seed coats. Saponins detected in sprouts included the soyasaponins Af, I, V, αg, βg, and γg. The saponin profiles of black bean sprouts reported herein agrees with previous reports where soyasaponin αg was regarded as the major saponin (Guajardo-Flores *et al*., 2012; Guajardo-Flores *et al*., 2014).

Interestingly, as observed for flavonoids (Tables 1 and 2), the Suc+L-pro treatment showed the highest levels of soyasaponins Af, I, and V after soaking, where increases of 50.7%, 6.8 % and 1.8%, respectively, were detected as compared with the control (Table 3). On the other hand, the GAs+Suc treatment showed 33.9% and 9.68% higher levels of soyasaponins αg and βg, respectively, whereas

Table 3. Effect of gibberellins, sucrose and L-proline in the accumulation of saponins in black bean sprouts.

Germination time (h)	Treatment [i, ii]			Soyasaponins mg/100g sprout		
	GAs	Suc	L-Pro	Soyasaponin Af	Soyasaponin I	Soyasaponin V
0	0	0	0	616.2 ± 2.8cde(z)	547.6 ± 0.5def(yz)	545.8 ± 0.3bcde(yz)
	0	1	0	636.9 ± 6.9cd(z)	547.4 ± 0.3def(z)	543.4 ± 0.3e(z)
	1	0	0	626.6 ± 3.6cde(z)	547.2 ± 0.2def(z)	545.3 ± 0.2bcde(yz)
	0	0	1	773.0 ± 31.7b(y)	550.0 ± 0.3cd(xy)	543.9 ± 0.2de(z)
	1	1	0	641.2 ± 6.8c(z)	549.0 ± 0.1cde(yz)	547.1 ± 0.2b(y)
	0	1	1	928.9 ± 23.3a(x)	585.3 ± 0.8a(v)	555.0 ± 1.4a(x)
	1	0	1	615.3 ± 1.6cde(z)	553.9 ± 0.4b(w)	546.9 ± 0.6b(y)
	1	1	1	605.1 ± 2.8cde(z)	552.3 ± 0.8bc(wx)	546.3 ± 0.8bcd(yz)
18	0	0	0	577.9 ± 2.9e(z)	545.0 ± 0.2f(z)	544.1 ± 0.3cde(yz)
	0	1	0	581.6 ± 4.3de(z)	546.1 ± 0.2ef(z)	544.9 ± 0.5bcde(xyz)
	1	0	0	614.2 ± 1.7cde(xy)	546.2 ± 0.1def(z)	543.9 ± 0.1de(z)
	0	0	1	578.0 ± 4.4e(z)	548.3 ± 1.7def(yz)	545.7 ± 0.6bcde(xyz)
	1	1	0	585.7 ± 6.2cde(yz)	546.6 ± 0.5def(z)	544.1 ± 0.0cde(yz)
	0	1	1	627.4 ± 5.6cde(x)	554.0 ± 0.9b(x)	545.9 ± 0.3bcde(xy)
	1	0	1	604.9 ± 13.7cde(xyz)	552.7 ± 0.9bc(xy)	546.7 ± 0.6bc(x)
	1	1	1	580.6 ± 3.1de(z)	549.2 ± 1.3cde(yz)	545.4 ± 0.4bcde(xyz)

[i]GAs: gibberellins. Suc: sucrose. L-pro: L-proline.
[ii]1: 0.5% w/v of the elicitor in soaking water. 0: No use of GAs, Suc or L-Pro
Different letters indicate statistical difference by the Tukey's HSD test ($p < 0.05$). Means within time results with different letters (a-f) are different at $\alpha = 0.05$. Means within treatments results with different letters (v–z) are different at $\alpha = 0.05$.

soyasaponin γg content was not affected by the elicitors applied (Table 3). Likewise, germination time did not show a positive effect on the accumulation of soyasaponins in black beans sprouts, since none of the treatment or the control showed increases after 18 h. Indeed, the treatments that showed the highest content of soyasaponins after soaking showed decreases in their concentration after germination time.

4 Discussion

In the present study, the effects of L-pro, GAs and Suc applied alone or in combination during soaking, on the accumulation of saponins and flavonoids in black bean seed coats were evaluated. Flavonoids were only detected in the seed coats, whereas saponins were detected in the sprouts. Saponins have antimicrobial properties, and thus it is reasonable that sprouts resulted in the accumulation of these compounds (Vincken *et al*., 2007; Yendo *et al*., 2010).

Interestingly, the treatments containing GAs (except for the Suc+L-pro+GAs treatment) did not show enhancement of flavonoids in black bean seed coats. It is well known that GAs accelerates germination of seeds (Rademacher, 2000; Hayashi *et al*., 2014), and during germination reactive oxygen species (ROS) are produced (Rhandir *et al*., 2004; El-Maarouf-Bouteau and Bailly, 2008). Likewise, flavonoids play a key role on neutralizing ROS produced during germination, because free radicals need to be maintained below toxic concentration for the plant (Mittler, 2002). Therefore, it is likely that although flavonoids were synthesized during germination their biosynthesis rate was like their utilization rate to neutralize ROS, and thus, no accumulation of flavonoids were detected in most GAs treatments. However, this behavior was not observed when analyzing the accumulation of certain saponins, since the GAs+Suc treatment showed the highest content of soyasaponins αg and βg. Saponins are not associated with the neutralization of free radicals, and their biosynthesis is elicited by jasmonic acid (Yendo *et al*., 2010), which is produced during germination (Creelman and Mullet, 1997).

Table 3. (Continued) Effect of gibberellins, sucrose and L-proline in the accumulation of saponins in black bean sprouts.

Germination time (h)	Treatment [i, ii]			Soyasaponins mg/100g sprout		
	GAs	Suc	L-Pro	Soyasaponin αg	Soyasaponin βg	Soyasaponin γg
0	0	0	0	1801.7 ± 57.1ab(yz)	681.9 ± 6.4abcd(yz)	574.6 ± 3.6a(x)
	0	1	0	2153.7 ± 109.7ab(xy)	727.4 ± 11.5ab(xy)	583.0 ± 3.5a(x)
	1	0	0	2171.5 ± 73.7ab(xy)	711.3 ± 9.3abc(xy)	575.5 ± 9.2a(x)
	0	0	1	2008.3 ± 171.7ab(xy)	690.0 ± 13.4abcd(xyz)	570.5 ± 9.0a(x)
	1	1	0	2412.7 ± 111.5a(x)	747.5 ± 23.7a(x)	583.0 ± 12.7a(x)
	0	1	1	1472.7 ± 34.2ab(z)	632.5 ± 3.4abcd(z)	567.0 ± 0.7a(x)
	1	0	1	2061.0 ± 49.3ab(xy)	689.9 ± 5.2abcd(xyz)	572.1 ± 1.0a(x)
	1	1	1	1885.7 ± 51.5ab(yz)	672.7 ± 11.9abcd(yz)	572.0 ± 2.0a(x)
18	0	0	0	1861.2 ± 81.4ab(x)	686.6 ± 10.8abcd(x)	572.9 ± 1.5a(yz)
	0	1	0	1923.4 ± 120.2ab(x)	699.9 ± 13.8abcd(x)	572.5 ± 2.5a(z)
	1	0	0	1782.3 ± 52.8ab(x)	667.1 ± 2.8bcd(x)	558.1 ± 3.9a(yz)
	0	0	1	1905.2 ± 207.16ab(x)	670.4 ± 24.2abcd(x)	574.1 ± 5.2a(yz)
	1	1	0	1973.4 ± 200.11ab(x)	682.9 ± 18.0abcd(x)	565.3 ± 4.8a(yz)
	0	1	1	1914.9 ± 53.0bab(x)	675.9 ± 9.1d(x)	571.7 ± 1.6a(yz)
	1	0	1	2132.6 ± 342.7ab(x)	700.0 ± 29.1abcd(x)	580.4 ± 8.5a(y)
	1	1	1	1618.4 ± 104.4b(x)	645.1 ± 10.9cd(x)	564.7 ± 2.5a(yz)

[i]GAs: gibberellins. Suc: sucrose. L-pro: L-proline.
[ii]1: 0.5% w/v of the elicitor in soaking water. 0: No use of GAs, Suc or L-Pro
Different letters indicate statistical difference by the Tukey's HSD test ($p < 0.05$). Means within time results with different letters (a-f) are different at $\alpha = 0.05$. Means within treatments results with different letters (v–z) are different at $\alpha = 0.05$.

Thus, the application of GAs during soaking of black beans may be accelerating germination, resulting on higher levels of jasmononic acid, which elicits the accumulation of certain saponins.

The addition of Suc in combination with L-pro in soaking water resulted on a synergistic effect on the accumulation of most bioactive compounds evaluated. This could be explained in terms of availability of carbon sources needed for secondary metabolite production, as well as with the activation of metabolic pathways involved on the synthesis of primary metabolites required for phenolic biosynthesis. Metabolic pathways involved on the production of carbon skeletons required for phenolic biosynthesis include respiration, glycolysis, oxidative pentose phosphate pathway (OPPP), and shikimate pathway (Umbarger, 1973). The combination of Suc and L-pro as elicitors are contributing to a higher activation of such metabolic pathways which are part of the primary metabolism of the seeds. In the specific case of sucrose, it is the main sugar translocated in plants, and is the substrate for glycolysis and respiration, which produces substrates for both, the OPPP and shikimate pathway (Umbarger, 1973). On the other hand, proline is involved on overexpressing the OPPP (Shetty *et al*., 2003). The OPPP produces erythrose 4-phospate, which is required as substrate in the shikimic acid pathway, where aromatic amino acids (phenolic precursors) are produced. Thus, adding Suc in combination with L-pro to soaking water resulted on higher accumulation of phenolics by giving the fuel required to produce the carbon skeletons needed for their production, as well as by accelerating the biosynthesis rate of phenolic precursors (primary metabolites, i.e. aromatic amino acids). Other elicitors such as glutamic acid or folic acid have also shown differential effects to enhance the concentration of flavonols in kidney bean (Dueñas *et al*., 2015).

The concentration of most bioactive compounds decreased or remained constant after 18 h of germination of black beans treated with the elicitors. However, for the control slight increases were detected for certain flavonoids (myricetin, delphinidin 3*O*-glucoside, petunidin 3*O*-glucoside, and malvidin 3*O*-glucoside) after 18 h of germination as compared with soaked seeds (time 0 h). As earlier described for GAs, it is likely that the application of elicitors is generating higher oxidative stress during germination of seeds, which affects the rate of utilization of flavonoids to neutralize free radicals, showing lower levels of phenolics at 18 h of germination. On the other hand, for the control results suggest that the rate of flavonoid synthesis was higher than utilization rate and thus phenolic accumulation was observed. As in previous reports, saponins content did not increase after germination under chemical induced stress (Mendoza-Sánchez *et al*., 2016). But it is important to point out that elicitors affected positively during the soaking process, representing a significant advantage to produce functional ingredients from black beans.

Conclusions

Results presented herein demonstrated that the nutraceutical content of black beans could be enhanced if a combination of Suc and L-pro (0.5% w/v) are added to soaking water during water imbibition of the seeds. The method is simple and effective, and the seeds could be used as raw material to produce processed foods or nutraceutical supplements. Further investigations should be directed into increase our understanding on the physiological and molecular mechanisms that induces the accumulation of flavonoids and saponins in the seeds treated with the elicitors, as well as on the design of bioprocesses to extract and purify bioactive compounds produced by the stress.

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