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Profile analysis and correlation across phenolic compounds, isoflavones and antioxidant capacity during germination of soybeans (Glycine max L.)

Fabiola A. Guzmán-Ortiz, Eduardo San Martín-Martínez, María E. Valverde, Yolanda Rodríguez-Aza, José De J Berrios and Rosalva Mora-Escobedo

Abstract

The objective of this research was to evaluate the effect of germination on the content of phenolic compounds, isoflavones and antioxidant activity of soybean extracts obtained from ungerminated soybeans and those germinated for 2 and 6 days. After 6 days of germination, the content of most phenolics, including isoflavones, significantly increased compared with that of samples from ungerminated soybeans and soybeans germinated for 2 days. The free phenolic compounds, benzoic and vanillic acids, decreased during the germination process. The antioxidant activity of the soybeans increased with germination time, and a high correlation was observed among total phenolic contents (TPCs), Trolox equivalent antioxidant activity (TEAC) and ferric reducing antioxidant power (FRAP). Isoflavones also showed high correlations with TEAC and FRAP assays, and only glycitin showed relatively low correlations. The process of germination allowed for increases in bioactive phenolic compounds and isoflavone aglycon. These effects enhanced the functional properties of the soybean.

Introduction

Soybean (Glycine max L.) is a widely consumed legume due to its high nutritional and technofunctional properties. Studies have shown that East Asian populations consuming soybean products regularly have lower risks of certain chronic diseases, most notably heart disease and cancer (McCue & Shetty, 2004). Interest in soy consumption has increased due to its notable health benefits. However, because of the presence of anti-nutritional compounds in soy, such as trypsin inhibitors, phytic acid and saponins, the consumption of soy has been relatively limited. Germination is one suitable process that can increase the use of soybean. Germination improves soybean nutritional quality, increases the level of bioactive compounds and decreases anti-nutritional factors (Guzmán-Ortiz, Robles-Ramírez, Sánchez-Pardo, Berrios, & Mora-Escobedo, 2014; López-Amorós, Hernández, & Estrella, 2006; Robles-Ramírez, Mora-Escobedo, Ramón-Gallegos, & Torres-Torres, 2012). Germination is a complex process in which the seed must undergo a physically quick recovery from maturation drying. This stage is followed by continuous and intense metabolic changes (including cellular events) to support embryonic development and seedling growth (Nonogaki, Bassel, & Bewley, 2010).

Germination involves three main phases: (a) seed hydration, when heavy water absorption by various tissues occurs to form the seed and increase respiratory activity (Simon, 1984); (b) germination, when water absorption decreases, stored reserves are mobilized, and new compounds are synthesized to provide nutrients necessary for the growth of a new seedling (some seed reserve materials are degraded during germination and used for respiration and
synthesis of new cell constituents of the developing embryo; this causes significant biochemical changes, which leads to the next phase; and (c) growth, where the radicle emerges (Guzmán-Ortiz et al., 2014). During growth, the seedlings may be damaged by microbial and environmental stresses or lack of nutrients. This makes it necessary to develop a defense mechanism through the synthesis of secondary metabolites, such as phenolic compounds, through different metabolic pathways (Dueñas, Hernández, Estrella, & Fernández, 2009; Kim et al., 2006; Michalak, 2006). Depending on their content and chemical structure, the contributions of a metabolic pathway to antioxidant activity (AA) are primarily achieved through two mechanisms: acting as hydrogen donors (or electron termination reactions) to break the free radical generation cycle or acting as metal chelators.

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses that counteract the harmful effects of free radicals and other oxidants. Free radicals are one of the causes of a large number of diseases, including cancers, cardiovascular diseases and atherosclerosis. Protection against free radicals can be enhanced by the intake of dietary antioxidant nutrients (Alam, Bristi, & Rafiquzzaman, 2013) as phenolic compounds. Factors affecting the antioxidant activities of phenolic compounds, in addition to structural properties, include the number and positions of hydroxyl groups and the presence of linked sugars. These factors ultimately determine the properties of phenolic compounds, such as solubility and the tendency to donate electrons or hydrogen atoms (Khokhar & Apenten, 2003; Rice-Evans, Miller, & Paganga, 1997). Some compounds, such as syringic, chlorogenic, gallic and ferulic acids and isoflavones, have been found in soybeans. These features all contribute to the functionality and health benefits of phenolic compounds.

In a previous study, Robles-Ramírez et al. (2012) reported the effects on cervical cancer cell lines of protein hydrolysates from soy germinated for 2 and 6 days at 27°C with and without phytochemicals. They found that soy protein hydrolysates with phytochemicals extracted from beans germinated for 2 days inhibited the growth of HeLa cells and C-33 cervical cancer cells. Moreover, the soy protein hydrolysates extracted from beans germinated for 6 days, but free of phytochemicals, showed a cytotoxic effect on cancer cells. González-Espinoza, Ramón-Gallegos, Torres-Torres, and Mora-Escobedo (2011) compared the effects of protein hydrolysates with and without phytochemicals in adipocyte-like cells after 3T3-L1 cell line differentiation. They reported that adipogenesis and triglyceride accumulation were strongly inhibited by the hydrolysates from soybeans germinated for 2 days at 28°C with ethanol-soluble phytochemicals compared with ungerminated soybeans. In both studies, the effects of germination on proteins and their peptides were studied. However, changes in phytochemicals and phenolic compounds have been reported to improve chronic degenerative diseases (Chen, Sam, & Chang, 2015). In this study, we evaluated the changes in phytochemicals, phenolic compounds and antioxidant capacity of a soybean variety grown in Mexico. We examined the effects of germination for 2 and 6 days at 27 ± 1°C on the profiles of phenolics and isoflavones and on AA.

### Materials and methods

#### Materials

Soybean (*Glycine max* L.) seeds were purchased from a local market in Mexico City. The reagents used in the analyses were of analytical or HPLC grade. Sodium carbonate, Folin–Ciocalteu phenol reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tripryridyl-s-triazine (TPTZ), hydrochloric acid, ferric chloride, acetate buffer, ferrous sulfate, ethanol and methanol were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The standards of isoflavones were daidzin, daidzein, genistin, genistein and glycitein. The phenolic standards were quercetin, kaempferol, gallic, benzoic, protocatechuic, syringic, p-coumaric, trans-cinnamic, chologenic, vanillic and ferulic acids. All standards were obtained from Sigma-Aldrich Co.

#### Germination

Germination was performed according to the method described by González-Espinoza et al. (2011) with some modifications. Soybean seeds were disinfested with sodium hypochlorite (0.05%) for 10 min and thoroughly rinsed with water to remove residual disinfectant. Subsequently, the seeds were placed in plastic trays and wetted to saturation. The trays were placed in a germination chamber (Ambi-Hi-Lo Chamber, Lab-line Instruments Inc., Melrose Park, IL, USA) at 27 ± 1°C in the dark (González-Espinoza et al., 2011; Robles-Ramírez et al., 2012). Seeds were disinfested again after 24 h with sodium hypochlorite (0.05%). Thereafter, the seeds were wetted by spraying with water every 12 h for 2 and 6 days of germination.

#### Sample preparation

After germination, the seeds were dried on dryer trays (901 Tsw brand S.A. of C.V.) for 24 h, at 40°C, ground in a mill 2HP Engine 16 in (Raymond, USA) and stored under refrigeration (4.4°C).

#### Extraction of phenolic compounds

Phenolic compounds were extracted according to Dueñas et al. (2009), León-López, Dávila-Ortiz, Jiménez-Martínez, and Hernández-Sánchez (2013) and Khoddami, Truong, Liu, Thomas, and Selle (2015) using an acidified methanol solution (methanol:chlorhydric acid, 99:1.0 v/v). A sample of 0.5 g was stirred into 10 mL of acidified methanol at room temperature for 16 h in complete darkness. The mixture was centrifuged at 10,300g for 10 min at 4°C. The supernatant was filtered through a 0.45 μm syringe filter (Whatman, Inc., Maidstone, UK) and stored at 5°C for later analysis of the phenolic content and profile by HPLC.

#### Determination of total phenolic content (TPC)

The total phenolic content (TPC) was assessed according to Singleton, Orthofer, & Lamuela-Raventós (1999) and Jiménez Martinez et al. (2012) using Folin–Ciocalteu reagent. The sample extracts were placed in the dark for 2 h before analysis. Absorbances were measured at 765 nm using a UV/Vis
spectrophotometer (BOECO S-22, Hamburg, Germany). The TPC was calculated based on a standard curve plotted with gallic acid as a standard. The final results were expressed as milligram of gallic acid equivalents (GAE) per 100 gram dry basis (db).

**Identification and quantification of phenolic compounds using HPLC**

**Chromatographic conditions**

To identify free phenolic compounds, the crude extracts of phenolic compounds were studied using the HPLC method described by Harbaum, Hubbermann, Zhu, and Schwarz (2008) with some modifications. An HPLC-diode array detector (Agilent Technologies 1200, Santa Clara, CA, U.S.A.) was equipped with an Atlantis C18 column (5 μm, 4.6 mm × 150 mm) for chromatographic separations. The UV–vis absorption spectra were monitored by the DAD from 200 to 600 nm, and the phenolic compounds were detected at 280 nm. The mobile phases comprised 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 100% acetonitrile (solvent B). The injection volume was 5 μL, and the mobile phase gradient was as follows: at start, A:B = 90:10; from 0 to 0.1 min, A:B = 90:10; from 0.1 to 30 min, A:B = 60:40; from 30 to 60 min and A:B = 55:45 at end. The total HPLC run time was 60 min, and the flow rate was 1 mL/min. Benzoic, catechin, chlorogenic, ferulic, gallic, p-cinamic, p-cumaric, protocatechuic syringic and vanillic acids and quercetin were used as quantification standards. The stock standard solutions were prepared with ethanol at 1 mg/mL. Calibration curves were generated by serial dilutions. The retention times of the phenolic compounds in the samples were compared with those of the standards, and the contents of individual phenolics were evaluated using the calibration curves of the different standards.

**Determination of isoflavones by HPLC**

**Extraction of isoflavones**

Isoflavones in germinated soybeans were extracted according to the method reported by Carrão-Panizzi, Favoni, and Kikuchi (2002). Briefly, 100 mg of each sample was placed in tubes with 4.0 mL of 70% aqueous ethanol containing 0.1% acetic acid, stirred at room temperature for 1 h and centrifuged at 10,304 g for 10 min at 4°C. The supernatant was filtered through a 0.45 μm syringe filter (Whatman, Inc.) and stored at 5°C for profile analysis by HPLC later.

**Chromatographic conditions**

Analyses were carried out by HPLC (Agilent Technologies 1200) according to the method of Carrão-Panizzi et al. (2002) with slight modifications. The column used in this experiment was a YMC-Pack ODS-AM C18 (5 μm, 250 mm × 4.6 mm ID). Isoflavones were detected at the wavelength of 260 nm. The binary mobile phases were composed of water with 0.1% acetic acid (solvent A) and acetonitrile with 0.1% acetic acid (solvent B). The injection volume was 5 μL, and the gradient of mobile phases was as follows: 0–20 min, A:B = 80:20; 20–25 min, A:B = 0:100; 25–40 min, A:B = 80:20. The total HPLC run time was 40 min, and the flow rate was 1 mL/min. The control standards used were daidzin, daidzein, genistin, genistein and glycitein. All standards were dissolved in methanol to 1 mg/mL. Calibration curves were prepared by serial dilutions. The retention times of isoflavones in samples were compared with those of the standards, and the contents of individual isoflavones were determined from the calibration curves.

**Determination of antioxidant activity (AA) by ABTS assay**

Antioxidant activity (AA) can be measured by several methods. ABTS⁺ is one of the most commonly used methods due to its ability to evaluate hydrophilic and lipophilic antioxidants based on interactions with hydrogen or electron donor species (Alam et al., 2013). The total phenolic extracts were used for antioxidant tests. Antioxidant activities of the samples were determined using ABTS radical according to the methodology described by Pastrana-Bonilla, Akoh, Sellappan, and Krewer (2003) with slight modifications. ABTS was dissolved in water to 7 mM and allowed to react with a 2.45 mM potassium persulfate solution for 16 h in the dark. This reaction formed ABTS radical cations. The ABTS⁺ solution was diluted in absolute ethanol until reaching an absorbance of 0.70 (±0.02) at 734 nm. Diluted ABTS⁺ (990 μL of ABTS and 10 μL of each extract) were added to cuvettes. Then, Trolox was added. The absorbance was measured at 734 nm each minute for 5 min. Percent inhibition was calculated using the equation below and plotted as a function of content of antioxidants and Trolox for the standard reference data.

\[
\text{Inhibition} (%) = \left( \frac{\text{Abs sample } t_0 - \text{Abs sample } t_5}{\text{Abs sample } t_0 - \text{Abs solvent } t_0} \right) \times 100
\]

where ‘Abs sample’ is the absorbance of the extract at \( t_0 \) (initial) or at \( t_5 \) (5 min), and ‘Abs solvent’ is the absorbance of the extraction solvent (acidified methanol) at \( t_0 \) (initial) or at \( t_5 \) (5 min).

A calibration curve of 0–25 μmol of Trolox was used to calculate AA. The total antioxidant activity (TAA) unit was defined as the Trolox content with equivalent antioxidant activity (TEAC) expressed as micromols of Trolox per gram of sample dry basis (db).

**Ferric reducing antioxidant power (FRAP) assay**

Ferric reducing antioxidant power (FRAP) is another assay used to determine the ability for a sample to reduce a ferric complex (Benzie & Strain, 1996). It is one of the most rapid tests and is very useful for routine analyses (Jagtap, Panaskar, & Bapat, 2010). The FRAP assay was carried out according to the method described by Benzie and Strain (1996) with slight modifications. FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM hydrochloric acid with 2.5 mL of 20 mM FeCl₃ solution and 25 mL of 0.3 M acetate buffer (pH 3.6). The assay solution consisted of a mixture of 20 μL of the extract, 30 μL of water and 300 μL of the FRAP reagent. The absorbance was measured after 30 min at 593 nm. Ferrous sulfate (FeSO₄·7H₂O) at 20–100 μM was used to plot a standard curve. The results were expressed as micromols of Fe(II) per 100 gram of db.

**Statistical analysis**

The results were analyzed using Statgraphics Plus 5.1 software (Warrenton, VA, U.S.A.). Data were expressed as the means and standard deviations (SDs) of triplicate
determinations. Analyses of variance (ANOVA) and least significant difference (LSD) tests were used to determine the significant differences between means. Pearson correlation test was evaluated to utilize the correlation between the total phenolic content, individual free phenolics, isoflavones and AA. A probability (p) value of less than 0.05 was considered significant.

Results and discussion

Determination of total phenolic content (TPC)

The ungerminated sample showed a concentration of phenolic compounds of 548.0 ± 21.0 mg GAE/100 g db (milligrams equivalents of gallic acid in 100 grams of db sample), which showed no significant difference (p > 0.05) from the sample germinated for 2 days. This last sample showed a concentration of 581.4 ± 15.0 mg GAE/100 g db; however, both samples showed significant differences (p < 0.05) compared with soybeans germinated for 6 days, which showed the highest concentration, 1159.5 ± 74.5 mg GAE/100 g db, increasing more than 100% compared with the sample without germination. Chen et al. (2015) reported values of 950 and 910 mg/100 g on yellow and green soybean sprouts, respectively, germinated at 21°C by 6 days, while Wang et al. (2015) reported lower concentrations, approximately 35 mg/100 g, in Chinese soybean cultivar ZH13 germinated at 30°C.

Chung, Seo, Ahn, and Kim (2011) determined the TPC in raw soybean seeds and reported a content of 366 mg/100 g db. These values were lower than those found in our research. However, Cevallos-Casals and Cisneros-Zevallos (2010) reported similar TPC values; they found seeds germinated at 7 days with the highest content of phenols; additionally, Troszyńska et al. (2011) reported the highest concentration of polyphenols in lentils after 7 days of germination. Conversely, Randhir, Lin, and Shetty (2004) reported that the TPC in mung bean samples decreased with germination.

The observed variations in content of a few individual phenolic compounds could be due to the specific biosynthesis pathways for these compounds. The primary metabolism pathways generate a large number of simple molecules, such as shikimic acid, acetates and amino acids, which constitute the starting materials for the biosynthetic pathways of many secondary metabolites (García, 2004; Prakash, Upadhyay, Singh, & Brahma, 2007). The changes produced in the concentration of phenolic compounds are associated with the action of endogenous esterases. The first key enzyme in phenylpropanoid metabolism is phenylalanine ammonium lyase, which catalyzes the l-phenylalanine produced by the shikimic acid pathway to remove ammonia residues and produce transcinnamic acid. Transcinnamic acid can be converted into an intermediate through the phenylpropanoid metabolic pathway. The intermediates include coumaric acid, asaftetide, syringic acid and so on, which may further be converted into coumarin, chlorogenic acid and COA ester. In this study, we found on the second day, low concentration of one of the intermediates such as p-coumaric acid, which explain the low concentration of chlorogenic acid (Rena & Sun, 2014; Kim, Kim, & Park 2004). The late increase of the phenylalanine ammonium lyase activity during the germination process may be another cause of the decrease in the concentration of some free phenolics such as chlorogenic acid in the first days of germination.

Identification and quantification of free phenolic compounds

Phenolic profiles of ungerminated soybeans and soybeans germinated for 2 and 6 days are presented in Table 1. The increase in the phenolic content in the seed samples was not noticeable after 2 days of germination, but it was higher after the sixth day.

| Phenolic compound | 0 Days | 2 Days | 6 Days |
|-------------------|--------|--------|--------|
| Benzoic acid      | 127.12 ± 3.48 | 111.77 ± 0.44 | 75.14 ± 0.10 |
| Catechin          | 38.38 ± 1.16  | 42.60 ± 0.05  | 159.95 ± 0.62 |
| Chlorogenic acid  | 7.80 ± 0.45   | 5.55 ± 0.38   | 142.27 ± 1.31 |
| Ferulic acid      | 48.48 ± 1.92  | 47.56 ± 1.77  | 48.60 ± 2.09 |
| Gallic acid       | 134.22 ± 2.14 | 125.09 ± 1.10 | 420.10 ± 3.97 |
| p-Cumaric acid    | 20.95 ± 0.9   | 22.03 ± 1.2   | 52.79 ± 0.52 |
| Protocatechuic acid | 39.06 ± 0.14 | 36.28 ± 1.12  | 173.21 ± 2.71 |
| Quercetin         | 523.82 ± 0.87 | 419.81 ± 2.31 | 955.29 ± 1.49 |
| Syringic acid     | 44.18 ± 0.13  | 40.01 ± 0.64  | 323.46 ± 2.48 |
| Vanillic acid     | 4.32 ± 0.16   | 6.36 ± 0.43   | ND |

ND: not detected.
ND: no detectado.

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Correa et al. (2010) and Prakash et al. (2007) reported lower contents of gallic acid, p-cumaric acid, ferulic acid and quercitin in a few varieties of soybean (G. max L). The

Table 1. Phenolic compounds in germinated soybean (Glycine max L.)

| Phenolic compound | 0 Days | 2 Days | 6 Days |
|-------------------|--------|--------|--------|
| Benzoic acid      | 127.12 ± 3.48 | 111.77 ± 0.44 | 75.14 ± 0.10 |
| Catechin          | 38.38 ± 1.16  | 42.60 ± 0.05  | 159.95 ± 0.62 |
| Chlorogenic acid  | 7.80 ± 0.45   | 5.55 ± 0.38   | 142.27 ± 1.31 |
| Ferulic acid      | 48.48 ± 1.92  | 47.56 ± 1.77  | 48.60 ± 2.09 |
| Gallic acid       | 134.22 ± 2.14 | 125.09 ± 1.10 | 420.10 ± 3.97 |
| p-Cumaric acid    | 20.95 ± 0.9   | 22.03 ± 1.2   | 52.79 ± 0.52 |
| Protocatechuic acid | 39.06 ± 0.14 | 36.28 ± 1.12  | 173.21 ± 2.71 |
| Quercetin         | 523.82 ± 0.87 | 419.81 ± 2.31 | 955.29 ± 1.49 |
| Syringic acid     | 44.18 ± 0.13  | 40.01 ± 0.64  | 323.46 ± 2.48 |
| Vanillic acid     | 4.32 ± 0.16   | 6.36 ± 0.43   | ND |

ND: not detected.
ND: no detectado.
reported differences in the phenolic compound contents, among different studies, could be due to several factors, including the chosen analytical method and extraction, as well as plant genetics, environmental factors, farming systems, irrigation, location and temperature where seeds were cultivated (Kim, Padilla, & Griffiths, 2004; Lee et al., 2008).

Identification and quantification of isoflavones

The two aglycones (daidzein and genistein) and three glucosides (daidzin, genistin and glycitin) were separated and identified using HPLC. The contents of the compounds were determined at 280 nm and are presented in Table 2. The total isoflavone content was 610.3 µmol/100 g db for raw soybean seeds and comprised daidzin (48.06%), genistin (26.09%), glycitin (13.25%), daidzein (9.51%) and genistein (3.11%). The germination process affected the isoflavone content. In soybean seeds germinated for 2 days, the aglycones forms, daidzein and genistein, decreased by 0.44% and 7.31%, respectively, whereas the glycosylated forms increased. Daidzin had the highest increase at 47.60%, followed by genistin and glycitin with 26.73% and 15.34% increase, respectively. In soybean germinated for 6 days, the total isoflavones increased to 314.59% mainly due to a considerable increase in aglycone forms. The contents of the glycosylated forms also increased over 2 days of germination: by 47.69% for daidzin, 74.17% for genistin and 7.6% for glycitin. However, these increases were smaller than those determined in the soybean samples germinated for 2 days.

The observed increase in aglycones with the germination process is associated with the hydrolysis of glycosylated metabolites, due to the action of glucosidase enzymes activated during the germination process (Ribeiro et al., 2006). However, there could also be a tendency for the hydrolysis of glycosidic bonds in disaccharide and polysaccharide molecules and thereby, increasing the availability of free sugars. Martín-Cabrejas et al. (2008) and Khetarpaul and Chauhan (1990) have reported increased soluble carbohydrate contents during germination and can be readily linked to aglycones through OH group binding to yield glycosylated forms. This is reflected in increases in the contents of these compounds (Paucar-Menacho, Berhow, Mandarino, Chang, & De Mejia, 2010; Zhu, Hettiarachchy, Horax, & Chen, 2005).

The germination process promotes the rapid consumption of carbohydrates and leaching of water-soluble compounds to increase the content of isoflavones (Kim et al., 2013). Changes in isoflavone content have been reported to be influenced by β-glucosidase enzyme activity, germination stage, physiological conditions of the seed (Ribeiro et al., 2006) and isoflavone biosynthesis through phenylpropanoid (Yu et al., 2000) and malonate pathways (Hahlbrock & Scheel, 1989). Therefore, the variability observed in reported values of different forms of isoflavones by different researchers may be associated with these different factors and the type of seed under study.

The concentrations were similar to those reported by Romani et al. (2003), who observed a higher content of daidzin when compared with other isoflavones. Huang, Cai, and Xu (2014) reported that 3 days of germination was the optimal time for the production of germinated soybean with the highest total isoflavone content. Tepačević et al. (2010) reported in a study with different soybean cultivars that the contents of daidzein, genistin and daidzin were lower than those indicated in Table 2 in the present study. They reported contents of 7.51–17.85 µmol/100 g for daidzin, 3.25–40.11 µmol/100 g for genistein and 54.32–163.91 µmol/100 g for daidzin. A content range of 40.72–79.68 µmol/100 g was reported for glycitin which was similar to those determined in the present study. Paucar-Menacho et al. (2010) found a similar trend in the content of isoflavones during germination; however, their reported values were lower than ours. Chen et al. (2015) also reported lower values. In this research, glycitein was not determined because Paucar-Menacho et al. (2010) and Chen et al. (2015) reported it as not detectable during soy germination.

Antioxidant activity (AA) by the ABTS and FRAP assays

Antioxidant activity (AA) was evaluated using ABTS and FRAP assays. Values are expressed as micromols of Trolox per 100 gram of sample db and micromols of Fe(II) per 100 gram of sample, respectively (Table 3). The AA for soybeans germinated for 2 days was not significantly different (p > 0.05) from that in ungerminated soybeans. However, the AA in soybeans germinated for 6 days was significantly higher (p < 0.05) by 188.03% and 293.60% as measured by the ABTS and FRAP assays, respectively, when compared with ungerminated soybeans. The AA in soybeans germinated for 6 days was also significantly greater (p < 0.05) than those germinated for 2 days, resulting in increases of 165.51% and 266.80% as measured by the ABTS and FRAP assays, respectively. Similar values were reported by Wang et al. (2015) for different soybean cultivars, that is the AA significantly increased as the germination process advanced. Fernández et al. (2008) carried out a kinetic study of the antioxidant compounds and antioxidant capacity during germination.

Table 2. Isoflavone concentrations of soybean (Glycine max L.) at different germination times

| Isoflavones (µmol/100 g db) | 0 Days | 2 Days | 6 Days |
|---------------------------|--------|--------|--------|
| Aglycones                 |        |        |        |
| Daidzein                  | 58.06 ± 0.41 | 57.80 ± 1.01 | 822.90 ± 3.29 |
| Genistein                 | 18.99 ± 1.20  | 17.62 ± 0.82  | 122.60 ± 2.01  |
| Glucosides                |         |        |        |
| Daidzin                   | 293.33 ± 0.32 | 348.13 ± 0.74 | 514.16 ± 0.58 |
| Genistin                  | 159.04 ± 1.78 | 195.49 ± 1.37 | 340.49 ± 0.43 |
| Glycitin                  | 80.88 ± 2.49  | 112.23 ± 1.09  | 119.81 ± 1.24  |
| Total                     | 610.3   | 731.26  | 1919.96 |

Table 3. Antioxidant activity in germinated soybean (Glycine max L.).

| Germination time (Days) | µmol Trolox/100 g sample | µmol Fe (µl) /100 g sample |
|------------------------|--------------------------|---------------------------|
| 0                      | 1699.3 ± 164.4ab         | 279.01 ± 8.18ab           |
| 2                      | 1930.4 ± 159.6ab         | 307.04 ± 15.47b           |
| 6                      | 3195.1 ± 320.60a         | 819.17 ± 78.47a           |

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| 6                      | 3195.1 ± 320.60a         | 819.17 ± 78.47a           |

Data were expressed as mean and standard deviation of triplicate determinations. Analysis of variance and least significant difference tests were used to determine the significance differences of their means. In same line, different superscript lowercase letters indicated statistical significance (p < 0.05). Los datos se expresaron como la media y la desviación estándar de determinaciones por triplicado. Se aplicó el Análisis de Varianza y la prueba de Diferencia Menos Significativa de sus medias. Diferentes letras en el mismo renglón indican diferencia estadísticamente significativa (p < 0.05).
7 days of germination of soybean Glycine max cv. Jutro and Glycine max cv. Merit. They found that germination promoted a higher content of phenolic compounds when compared with ungerminated seeds. Moreover, the antioxidant capacity (TEAC) increased during the germination process, resulting in a TEAC retention of 11–14% at the end of the germination period. Similar impacts of germination on phenolic contents and AA were reported by Cevallos-Casals and Cisneros-Zevallos (2010) in soybeans and other legumes.

The amounts of phenolic compounds and isoflavones synthesized during germination influenced the AA (Figures 1(a, b) and 2(a,b)). Daidzein was observed to have a significant effect (p < 0.05) on AA. This effect was greater on soybean samples germinated for 6 days. Although the genistein content was not as high as observed for daidzein, it had an important contribution to the AA at the end of 6 days of germination. This may be due to the hydroxyl groups at positions 4', 5' and 7', which were exposed and enabled the stable free radical delocalization of electrons in the double bonds of the aromatic ring (Michalak, 2006).

The AA evaluated by ABTS and FRAP indicated that the extracts from soybean germinated for 6 days had the greatest capacity to donate hydrogen and transfer electrons to complete the oxidation process. During germination, seeds synthesize larger amounts of phenolic compounds with AA to protect the hypocotyl from oxidative reactions caused by environmental factors (Cevallos-Casals & Cisneros-Zevallos, 2010). This favorably increases the AA in germinated soybeans.

The use of ABTS and FRAP measurement methods provides a better AA analysis of the samples due to the distinct selectivity of each method. The ABTS assay is applicable to both hydrophilic and lipophilic antioxidants because the reagent used in the assay is soluble in aqueous and organic solvents (Fernández et al., 2008). The FRAP assay is specific to water-soluble antioxidants (i.e. hydrophilic antioxidants) (Tyug, Nagendra, & Ismail, 2010). Most phenolic compounds synthesized in soybeans exhibited good AA, except for ferulic acid and glycinin. Their contributions to TPC, TEAC and FRAP were low. Vanillic acid did not contribute to TEAC or FRAP. The FRAP and TEAC assays exhibited a high and positive correlation (r = 0.995), suggesting that the two assays were suitable for evaluating the antioxidant capacity in soybean extracts.

Correlations

Correlations among the antioxidant capacity assays, that is, TEAC, FRAP, individual free phenolic compounds and isoflavones were statistically compared as shown in Table 4. Strong and positive correlations (p ≤ 0.05) existed between TEAC and catechin (r = 0.994), chlorogenic acid (r = 0.987), gallic acid (r = 0.985), p-cumaric acid (r = 0.993), protocatechuic acid (r = 0.987), quercetin (r = 0.947), syringic acid (r = 0.988), daidzein (r = 0.990), genistein (r = 0.988), daidzin (r = 0.995) and genistin (r = 0.999). Ferulic acid and glycinin showed a low correlation. Benzoic and vanillic acids had a negative correlation because their contents decreased with germination. The correlations between FRAP and individual free phenolic compounds and isoflavones had the same trends as those of TEAC.

The same trend was observed with TEAC and FRAP (r = 0.995). Each of the free phenolics and isoflavones had high contributions in TPC and had strong reducing ability toward the Folin–Ciocalteu reagent. Phenolic compounds and isoflavones...
components were oxidized, and the metal ion was reduced (Agbor, Vinson, & Donnelly, 2014). This was reflected in the results of the TEAC and FRAP assays. Phenolic compounds synthesized in soybeans exhibited good AA, except for ferulic acid and glycitin, which had low contributions to TEAC and FRAP. Vanillic acid did not contribute to TEAC and FRAP. The FRAP and TEAC assays exhibited a high and positive correlation \( r = 0.995 \), suggesting that the two assays were suitable for evaluating antioxidant capacity in the soy extracts.

### Conclusions

Germination is a cost-effective process by which the bioactive compounds’ content and AA of soybean seeds can be increased. Biochemical changes in soybeans result in the generation of value-added bioactive compounds. AA, isoflavone content and total and free phenolic compounds increased due to the germination process. Six days of germination showed the greatest increases in bioactive compounds. The isoflavone content increased up to 214.59%. Among the free phenolic compounds, quercetin had the greatest increase. Other phenolic compounds also increased. AA was largely influenced by these compounds and was highly correlated with ABTS and FRAP. Germination is a suitable process to obtain a functional food with high AA and potentially important health benefits. These beneficial effects may have applications in chronic degenerative diseases, such as cancer and obesity.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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