



Isoflavonoid composition and biological activity of extracts from soybean seedlings treated by different elicitors

DIEGO DURANGO¹, JENNIFER MURILLO¹, FERNANDO ECHEVERRI²,
GUSTAVO ESCOBAR² and WINSTON QUIÑONES²

¹Universidad Nacional de Colombia, Sede Medellín, Facultad de Ciencias, Escuela de Química, Grupo de Química de los Productos Naturales y los Alimentos, Calle 59ª, 63-020, Autopista Norte, P. O. Box 3840, Medellín, Antioquia, Colombia

²Química Orgánica de Productos Naturales, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia, Calle 70, 52-21, P. O. Box 1226, Medellín, Antioquia, Colombia

Manuscript received on October 9, 2017; accepted for publication on December 6, 2017

ABSTRACT

Time-course and dose-response experiments were carried out to establish the ability for synthesizing isoflavonoids of soybean seedlings (cv. Soyica P34) treated with salicylic (SA) and isonicotinic acids (INA). Then, 25 structurally-related compounds were evaluated for their isoflavonoid-eliciting activity. Next, the antimicrobial and antioxidant activities of EtOAc-soluble fraction from soybean seedlings treated with some synthetic elicitors were determined. Results showed that the concentration of isoflavonoids in soybean seedlings was significantly increased by the application of SA and INA. The major isoflavonoids detected were the malonyl-glycosidic isoflavones, followed by genistin and daidzin. The isoflavone aglycones (genistein, daidzein, and formononetin), coumestrol and glyceollins were found in lower concentrations. Maximum accumulation of glyceollins was detected after 48 and 144 h in soybean seedlings treated with 1.6 mM INA and SA, respectively. EtOAc-extracts from soybean seedlings treated with two structurally-related compounds to INA displayed a significant antimicrobial and antioxidant activity. Therefore, INA, SA and structurally-related compounds can be used to increase the amounts of natural antioxidant or antimicrobial compounds in soybean, either to protect the plant directly against pathogens or as a natural source for subsequent isolation of isoflavonoids or bioactive extracts, which have potential application in functional foods or pharmaceutical and personal care products.

Key words: elicitors, isoflavonoids, salicylic acid, soybean.

INTRODUCTION

Plants produce a wide array of fungitoxic secondary metabolites, which can be divided into constitutive or inducible (VanEtten et al. 1994, 2001).

Correspondence to: Diego Durango
E-mail: dldurango@unal.edu.co

* Contribution to the centenary of the Brazilian Academy of Sciences.

Constitutive metabolites, called phytoanticipins, exist in healthy plants in their biologically active forms or as inactive precursors, which are quickly activated in response to infection or cell injury (Piasecka et al. 2015). Constitutive metabolites represent one of the first chemical barriers to deter potential pathogens; however, in many cases, those metabolites are not enough to protect plants

against the infection. On the other hand, inducible secondary metabolites (named phytoalexins) are synthesized from remote precursors in response to pathogen attack (Hammerschmidt 1999). In general, these compounds are absent or present in low levels in healthy plants but induced when plant tissues are exposed to fungal hyphae (Arruda et al. 2016). They can also be induced by abiotic stressors such as natural organic (salicylic acid, SA; methyl jasmonate; chitosan), inorganic (potassium phosphonate, heavy metals), and synthetic compounds (2,6-dichloroisonicotinic acid, DCIA; benzo [1,2,3]-thiadiazole-7-carbothioic acid S-methyl ester, BTH). The accumulation of phytoalexins has been reported to be related to the resistance of the plant to pathogens.

Soybean (*Glycine max* L.) produces several constitutive and inducible secondary metabolites that belong to the isoflavonoid group. Glycoside isoflavones (6-*O*''-malonyl-genistin, 6-*O*''-malonyl-daidzin, genistin, daidzin) are often present in relatively high levels in healthy plants (Graham et al. 1990). In contrast, the aglycones genistein, daidzein, formononetin released by hydrolysis from the preformed conjugates or biosynthesized from isoflavonoid pathway, are present in very low amounts in soybean seedlings (Graham et al. 1990). Additional to these compounds, some other isoflavonoids are synthesized and accumulated in soybean tissues in response to biotic and abiotic stress. The phytoalexins coumestrol and glyceollins, derived from daidzein, are increased in soybean plants after exposure to microorganisms (Boué et al. 2000) (Figure 1). Interestingly, isoflavonoids, besides being related to the defense mechanisms of soybean, have important beneficial properties to health. Genistein and daidzein (and their β -glucoside, 6-*O*''-malonyl- β -glucoside, or 6-*O*''-acetyl- β -glucoside derivatives) have been associated with numerous biological activities and health benefits (i.e., prevention of neoplastic and circulatory diseases, osteoporosis, among others) (McCue and Shetty 2004, Wang et al. 2013). These

compounds, along with coumestrol and glyceollins have showed antioxidant, antimicrobial, estrogenic and antiestrogenic, and cancer preventive properties (Burow et al. 2001).

On the other hand, it has been determined that the use of elicitors enhance the concentration of bioactive compounds in plants (Boué et al. 2008). Salicylic acid (SA) and some structurally related compounds (like acetyl salicylic acid, methyl salicylate, trifluoroethyl salicylate) can act as powerful elicitors for secondary metabolism induction, including phytoalexins, in plants and plant cell cultures (Amaresh and Bhatt 1998, Qian et al. 2006). Similarly, exogenous application of isonicotinic acid (INA) and derivatives [i.e. 2,6-dichloroisonicotinic acid (DCIA), trifluoroethyl 2,6-dichloroisonicotinate, isonicotinamide, and 2-(2,6-dichloro-pyridine-4-carbonyloxy)-ethyl jasmonate] has shown to activate defense responses in different plants and a suspension culture of *Taxus chinensis* (Kauss et al. 1992, Dann et al. 1998, Qian et al. 2005). Due to the importance of soybean isoflavonoids for plant defense and human health, in the present paper, we evaluate the effect of SA, INA and some structurally related compounds on isoflavonoid induction in soybean seedlings (cv. Soyica P34). In addition, the antimicrobial and antioxidant activity of the EtOAc-soluble fraction from soybean seedlings treated with some synthetic elicitors was studied.

MATERIALS AND METHODS

PLANT MATERIAL

Certificated soybean (*G. max* L. cv. Soyica P34) seeds were obtained from Semillas del Pacífico (Cartago, Colombia). Seeds were surface-sterilized for 2 min in NaOCl (2.0 %), washed with tap water, and sown in moist vermiculite (2 cm) in plastic trays at room temperature. Six-day-old seedlings were harvested and washed with distilled water

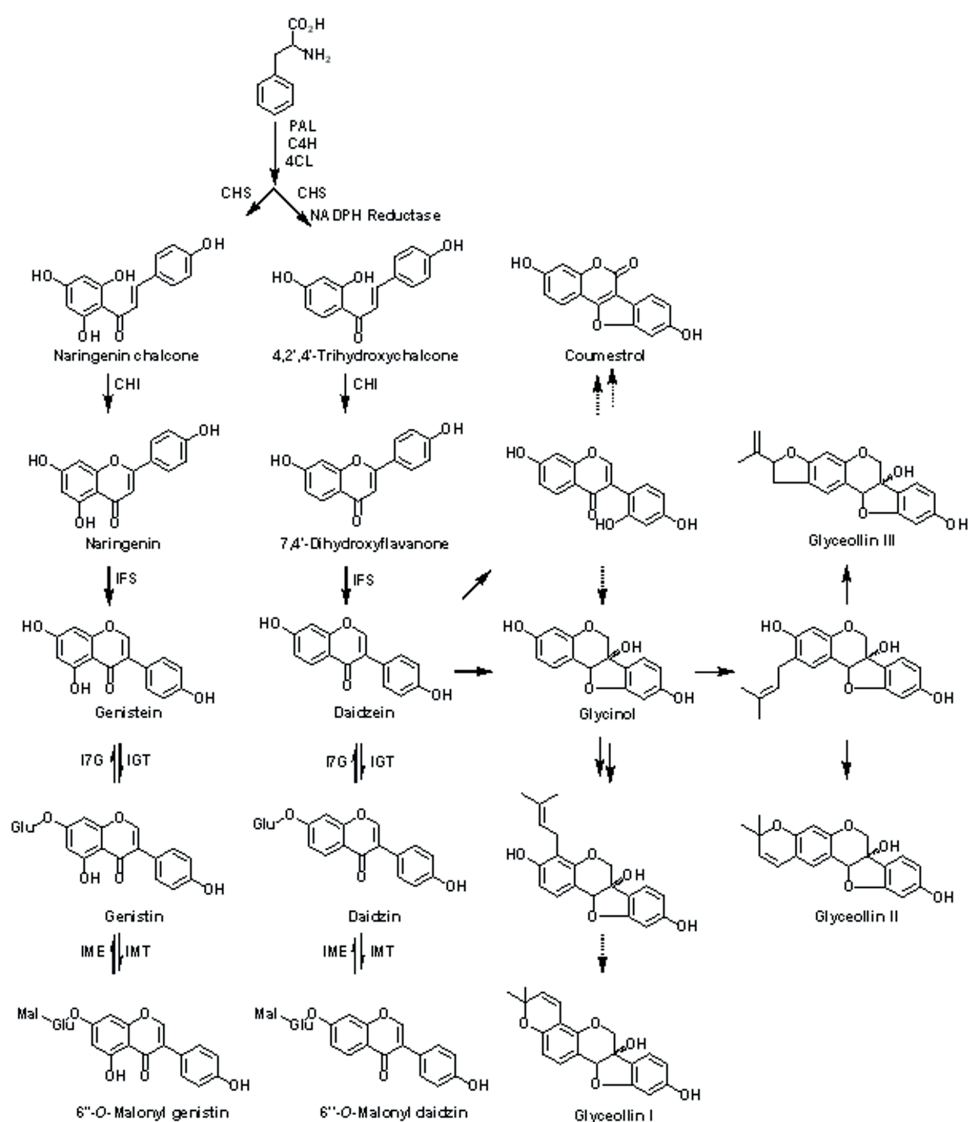


Figure 1 - Biosynthetic pathway of isoflavonoids in soybean (adapted from Graham and Graham 1991). Phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), isoflavone synthase (IFS), isoflavone-7-O-glucosidase (I7G), isoflavone-7-O-glucosyltransferase (IGT), isoflavone malonyltransferase (IME), isoflavone-7-O-malonyltransferase (IMT). Some enzymes have not been fully characterized in soybean.

to remove the vermiculite. Then, teguments were carefully removed from the seedlings.

CHEMICALS

Isoflavonoids genistein, daidzein, and formononetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Coumestrol was from Fluka Chemical Corp. (Milwaukee, WI, USA). 2-aminonicotinic acid (1), 2-hydroxynicotinic

acid (5), and 2-mercaptonicotinic acid (2) were from Alfa-Aesar Co. (Ward Hill, MA, UK). Salicylic acid (SA), isonicotinic acid (INA), nicotinic acid (4), citrazinic acid (3), isonipecotic acid (11), 4-pyridinethioamide (7), pyrrol-2-carboxylic acid (9), 2-methoxy isonicotinic acid (10), 2-hydroxyquinolein-4-carboxylic acid (8), 1,2,4-triazole-3-carboxylic acid (6), benzo (1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl

ester (BTH), 2,6-dichloropyridine-4-carboxylic acid (DCIA), thiosalicylic acid (**12**), 2-iodobenzoic acid (**13**), benzoic acid (**14**), acetylsalicylic acid (**15**), 2,3,5-triphenyltetrazolium chloride (TTC), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dihydroquinazolinones (**16-23**) were prepared from 2-aminobenzamide and benzaldehyde derivatives by conventional organic reactions (Dar et al. 2012). HPLC grade methanol and ethyl acetate (EtOAc) were obtained from Merck KGaA (Darmstadt, GER). H₂O treated with a Millipore system was used during HPLC analysis.

INDUCTION EXPERIMENTS

Dose-response profiles

Uprouted seedlings (7 g) were arranged vertically in sterile plastic trays and roots immersed for 4 hours in solutions of SA and INA to various concentrations (0.2, 0.4, 0.8, 1.6, 4.0 and 8.0 mM). Before preparing all solutions, SA and INA were dissolved in ethanol (0.2%). Then, SA and INA solutions were discarded and the roots were covered with cellucotton soaked with distilled water. The trays were closed with plastic film and seedlings incubated for 96 h at 25 °C in the darkness. Control experiments were performed using ethanol (0.2%) instead of elicitors and the same storage conditions. Experiments were done at least twice.

Time-course experiments

Soybean seedlings (7 g) were placed vertically in sterile plastic trays under aseptic conditions and the roots immersed for 4 hours in solutions of SA or INA (1.6 mM). Solutions were prepared in ethanol (0.2%). Then, elicitor solutions were discarded and the roots were covered with cellucotton soaked with distilled water. Polystyrene trays were covered

with stretch film and plant materials were stored at 25 °C during different times (24, 48, 72, 96, 120, and 144 h) in the darkness. Similarly, control experiments using seedlings treated with ethanol (0.2%) instead SA or INA solution and stored during 24 to 144 h were carried out. Experiments were done at least two times.

Inducer effect of structurally related compounds to SA and INA

Soybean seedlings (7 g) were placed vertically in sterile plastic trays and the roots immersed for 4 h in solutions 1.6 mM of nicotinic acid (**4**), citrazinic acid (**3**), 2-aminonicotinic acid (**1**), 2-hydroxynicotinic acid (**5**), 2-mercaptonicotinic acid (**2**), isonipecotic acid (**11**), 4-pyridinethioamide (**7**), pyrrol-2-carboxylic acid (**9**), 2-methoxyisonicotinic acid (**10**), 2-hydroxyquinolein-4-carboxylic acid (**8**), and 1,2,4-triazole-3-carboxylic acid (**6**). Treatments with benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), and 2,6-dichloropyridine-4-carboxylic acid (DCIA) served as positive controls. All solutions were prepared dissolving the elicitors in 0.2% ethanol. Subsequently, elicitor solutions were discarded and the roots were covered with cellucotton soaked with distilled water. The trays were closed with stretch film and seedlings incubated for 96 h at 25 °C in the darkness.

SAMPLE PREPARATION

Soybean seedlings were ground in a mortar with 20 mL ethanol (95%). Then, the solutions were centrifuged for 6 min (3400 rpm) and filtered through Whatman No. 1 filter paper. The filtrate was concentrated at 40 °C under vacuum (Rotavapor Buchi R-210 with vacuum controller V-850) and the remaining aqueous phase was partitioned three times with ethyl acetate (EtOAc, 3 x 20 mL). The organic phases were combined and brought to dryness under reduced pressure. Extracts were redissolved in methanol (HPLC-grade MeOH, 5.0

mL), filtered through a syringe sterile filter with a 0.45 mm pore size (Sartorius Biotech GmbH, Goettingen, Germany). The resulting solution (a 0.5 mL aliquot) was used without further purification for HPLC analysis. The samples were kept in amber glass vials and stored at -20 °C until HPLC analysis.

HPLC-DAD ANALYSIS

The analysis of isoflavonoids were performed by high performance liquid chromatography (HPLC) on a Gilson chromatograph equipped with a Gilson model 170 diode array detector (DAD), using a Phenomenex Security Guard cartridge C18 (4.0 x 3.0 mm) followed by a Phenomenex Luna 5 μ C18 (2) reverse-phase column (150 mm x 4.6 mm i.d., 5 μ m) (Torrance, USA). The compounds were eluted at a flow rate of 0.7 mL/min with the solvents A = methanol, and B = 0.5% acetic acid in water, as follows: from 10% A to 70 % A in 40 min, then 70% A to 90% A in 20 min, and subsequently by holding for 8 min to reequilibrate the column, for the next injection. Injection volume was 20 μ L. Isoflavonoids were monitored at the wavelengths of 248, 254, 270, 286 and 310 nm, although diode-array detection was used over a wavelength range of 200-600 nm to collect spectral data.

CONFIRMATION OF IDENTITY OF ISOFLAVONOIDS BY LC-DAD-MS

Isoflavonoids identity was confirmed by liquid chromatography with mass spectrometry detection (LC-MSD) and diode array detector (DAD) on an HP 1100 series HPLC apparatus (Agilent Technologies, Waldbronn, Germany) interfaced to an HP series 1100 mass selective detector equipped with an API-ES chamber, using negative and positive ion modes, and the same chromatographic conditions as described above. MSD conditions were programmed as follows: capillary voltage, 3 kV; nebulizing pressure, 60 psi; drying gas temperature, 350 °C; drying gas flow, 12 L/min.

Their spectral characteristics are as follows: *daidzin*, t_R , 22.5 min; UV λ_{max} (MeOH-AcOH at chromatographic conditions, nm): 230.5sh, 248.6, 260.3, 304.2; EM-IE m/z (rel. int.): negative mode data, quasimolecular ion at 415 (5)(M-1)⁻, 253 (100)(M-1-Glu)⁻(aglycone fragment daidzein, base peak); positive mode data: 255 (100)(M+1-Glu)⁺(aglycone fragment daidzein, base peak); *genistin*, t_R , 27.5 min; λ_{max} (MeOH-AcOH at chromatographic conditions, nm): 259.2, 328sh; EI-MS m/z (rel. int.): negative mode data, ion quasimolecular at 431(5)(M-1)⁻, 269 (100)(M-1-Glu)⁻(aglycone fragment genistein, base peak); *malonyl-daidzin*, t_R , 30.4 min; UV λ_{max} (MeOH-AcOH at chromatographic conditions, nm): 230.5sh, 249.7, 304.0, 325.0sh. EI-MS m/z (rel. int.): negative mode data, 253 (100)(M-1-Glu-Malonyl)⁻(aglycone fragment daidzein, base peak); positive mode data: 503 (M+1)⁺; *malonyl-genistin*, t_R , 32.7 min; UV λ_{max} (MeOH-AcOH at chromatographic conditions, nm): 259.2, 325.0sh. EI-MS m/z (rel. int.): negative mode data, 269 (100)(M-1-Glu-Malonyl)⁻ (aglycone fragment genistein, base peak); positive mode data: 519 (M+1)⁺; *daidzein*, t_R , 34.9 min; UV λ_{max} (MeOH-AcOH at chromatographic conditions, nm): 234.1sh, 244.1, 306.0sh. EIMS m/z (rel. int.): negative mode data, quasimolecular ion at 253 (100)(M-1)⁻, positive mode data, quasimolecular ion at 255 (100)(M+1)⁺; *genistein*, t_R , 38.6 min; UV λ_{max} (MeOH-AcOH at chromatographic conditions, nm): 260.3, 335sh. EI-MS m/z (rel. int.): negative mode data, quasimolecular ion at 269 (100)(M-1)⁻; positive mode data, quasimolecular ion at 271 (100)(M+1)⁺; *coumestrol*, t_R , 41.4 min; UV λ_{max} (MeOH-AcOH at chromatographic conditions, nm): 242.5, 349.2. EI-MS m/z (rel. int.): negative mode data, quasimolecular ion at 267(100)(M-1)⁻; positive mode data, quasimolecular ion at 269 (100)(M+1)⁺; *formononetin*, t_R , 42.9 min; UV λ_{max} (MeOH-AcOH at chromatographic conditions, nm): 234.6, 282.7. EI-MS m/z (rel. int.): negative

mode data, quasimolecular ion at 267 (100)(M-1)⁻; glyceollins (I, II, and III), t_R , 44.2, 49.5, 50.6 min; UV λ_{max} (MeOH-AcOH at chromatographic conditions, nm): 236.3, 282.7, 315.6sh. EI-MS m/z (rel. int.): negative ion data, quasimolecular ion at 337 (100)(M-1)⁻; positive ion data, quasimolecular ion at 339 (100)(M+1)⁺.

QUANTIFICATION OF ISOFLAVONOIDS

Quantification of isoflavonoids was performed using standard calibration curves (peak areas vs. compound concentration for different concentrations). Five working solutions were prepared for each standard in methanol containing genistein, daidzein, coumestrol, formononetin, and phaseollin in 1, 10, 25, 50, and 100 mg/L concentrations. Assuming the presence of glycosyl, malonyl or acetyl moieties did not affect absorption properties (Doerge et al. 2000), the standard curves of genistein and daidzein were used for quantifying the malonyl conjugates and glucosides, and adjusted based on differences in molecular weight. Therefore, for isoflavonoids without a pure standard, genistin and malonyl-genistin, concentrations were estimated from the calibration curve for genistein. Similarly, daidzin and malonyl-daidzin were quantified from the calibration curve for daidzein. The quantity of glyceollins was determined as the total peak of three isomers and using the calibration curve for phaseollin (a structurally related pterocarpin). All calibration curves presented high linearity (correlation coefficient $r^2 > 0.96$). The regression equations were: genistein, $Y = 2.0 \times 10^{-7}X - 0.2445$ ($r^2 = 0.9970$); daidzein, $Y = 5.0 \times 10^{-7}X - 3.9883$ ($r^2 = 0.9928$); coumestrol, $Y = 5.0 \times 10^{-6}X + 7.6532$ ($r^2 = 0.9651$); formononetin, $Y = 8.0 \times 10^{-7}X - 1.7193$ ($r^2 = 0.9985$), and phaseollin, $Y = 1.0 \times 10^{-6} - 1.5738$ ($r^2 = 0.9970$). Data for each peak were collected using the wavelength that provides a maximum response. The results were expressed as μg phytoalexin/g

fresh material and presented as mean values \pm standard deviation.

BIOLOGICAL ACTIVITY

Antimicrobial activity

Antibacterial assay. Determination of MIC and MBC values. Extracts proceeding from soybean seedlings treated with water (control) and 1.60 mM of (4) and (5) (treatments) and incubated for 96 h at 25 °C in the darkness (according to *Inducer Effect of Structurally Related Compounds to SA and INA*) were evaluated for their antibacterial activity. Each extract was tested on selected Gram-positive (*Staphylococcus aureus*, *Enterobacter faecalis*, and *Bacillus cereus*) and Gram-negative bacteria (*Escherichia coli*) with the microdilution method with 2,3,5-triphenyl tetrazolium chloride (TTC) in 96-well microtiter plate (Basri et al. 2012). The stock solutions were made by dissolving the soybean extracts in ethanol/distilled water (50/50 v/v) to a concentration of 10000 $\mu\text{g}/\text{mL}$. Then, the extracts (40 μL) were serially diluted 50% with Mueller-Hinton broth (Becton Dickinson, Sparks, MD). Subsequently, 140 μL Mueller-Hinton broth and 50 μL bacterial suspension (1.0×10^8 UFC/mL) were added to the microtiter plate to obtain the final concentration of extracts ranging from 0.98-2000 mg/mL. As positive control, ampicillin sulbactam (Pfizer) solutions (0.85-1000 mg/mL) were used. The extracts in broth and the bacterial suspensions were used as negative control to ensure medium sterility and the adequacy of the broth for bacterial growth, respectively. Each extract was assayed in triplicate. The micro plates were covered with a cling film and incubated for 24 h at 37 °C. Following incubation, the plate was added with 20 μL TTC (5 $\mu\text{g}/\text{mL}$). The plate was then incubated again for another 30 min in the dark. The MIC value was taken as the lowest concentration of extract that showed no color changes of indicator after addition (Basri et al. 2012). Otherwise, the

minimum bactericidal concentration (MBC) value was determined by subculture of the wells, which showed no color changes on the sterile agar plate. The least concentration, which showed no visible growth on agar plate, was considered as MBC value.

Antifungal assay. The antifungal activity of untreated and elicitor-treated soybean extracts was evaluated against plant pathogenic fungi (*Colletotrichum lindemuthianum* and *Fusarium oxysporum*) by using poisoned food technique. Extracts were mixed with 15 mL of cooled (45 °C) molten PDA medium and allowed to solidify at room temperature for thirty minutes (concentration approx. 200 µg/mL). A mycelial disc 6 mm diameter, cut out from periphery of 3-day old cultures, was aseptically inoculated onto the agar plates containing the soybean extract. PDA plates with thymol were used as positive control. Fungal growth inhibition was determined after three days, until the negative control plates were completely full. All tests were run in triplicate. Results are reported as an average with its respective standard deviation.

In vitro antioxidant and antiradical activity

Ferric reducing antioxidant power (FRAP) assay. Reducing power was determined using the method prescribed by (Berker et al. 2007) with slight modification. An amount of 125 µL extracted samples were mixed with 125 µL distilled water, 250 µL with sodium phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 250 µL potassium ferricyanide [$K_3Fe(CN)_6$] (1%), and the mixture was incubated at 50 °C for 20 min. Next, 250 µL of trichloroacetic acid (10%) were added to the reaction mixture, which was then centrifuged at 3000 rpm for 10 min. Then, the upper layer of the solution (500 µL) was mixed with distilled water (400 µL) and ferric chloride ($FeCl_3 \cdot 6H_2O$, 100 µL, 0.1%), and the absorbance was measured at 700 nm. Methanolic

solutions of known Trolox concentration ranging from 0.1 to 1.0 mM were used for the preparation of the calibration curve (the regression equation was $Y = 1.2007X(mM \text{ trolox/mL}) + 0.0734$). The results were expressed as mg Trolox equivalent/g soybean extract and presented as mean values \pm standard deviation.

DPPH radical scavenging activity. The 2,2-diphenyl-1-picryl-hydrazyl (DPPH[•]) radical scavenging activity of soybean extracts was estimated as described by (Hatano et al. 1989). Briefly, 0.02 mg/mL solution of DPPH radical reagent in methanol was prepared and 1980 µL of this solution was added 20 µL of ethanolic extracts. After 30 min of reaction at room temperature in the darkness, the absorbance of the solution was measured at 517 nm. Blank sample was prepared using 20 µL of methanol instead of the extract. The free radical scavenging activity of each extracts was determined by comparing its absorbance with that of a blank solution. The ability to scavenge the DPPH radical was calculated using the following equation: DPPH scavenging activity (%) = $(A_b - A_s) / A_b \times 100$ where A_b is the absorbance of the control and A_s is the absorbance of the sample. The DPPH radical scavenging activity values were determined as milligrams of Trolox equivalents by computing with standard calibration curve constructed for different concentrations of Trolox ($Y = 23.980 X(mg \text{ Trolox/mL}) - 23.654$). Results were expressed in mg Trolox Equivalent/g soybean extract and presented as mean values \pm standard deviation.

STATISTICAL ANALYSIS

Results were analyzed by a one-way ANOVA and mean values were compared with the Fisher's least significant differences (LSD) at the 0.05 probability level.

RESULTS AND DISCUSSION

DOSE-RESPONSE EXPERIMENTS

Plants exposed to high concentrations of SA, INA or derivatives may exhibit symptoms of phytotoxicity (Durango et al. 2013, War et al. 2011, Kedare and Singh 2011). Therefore, dose-response experiments were initially carried out in order to choose a safe concentration. The effect of elicitor concentration on the levels of isoflavonoid compounds in soybean seedlings was evaluated in the range 0.2 to 8.0 mM and after 96 h post-induction. A representative HPLC profile showing the response of soybean seedlings induced by INA is shown in Figure 2.

The accumulation of isoflavonoids in response to the treatment with SA and INA is presented in Figure 3. Overall, the application of both elicitors resulted in significant increase of isoflavonoid concentrations, compared to the water-treated seedlings. According to dose-response curves, maximum amount of each isoflavonoid was reached using different concentrations of SA and INA. Thus, maximum accumulation of malonyl-daidzin (174.56 $\mu\text{g/g}$ f.w. almost 10-fold the amount

found in control seedlings), malonyl-genistin, and glyceollins (98.62 $\mu\text{g/g}$ f.w., representing an increase of 433% compared to control) was seen at 1.6 mM SA. The maximum concentration of daidzin (129.44 $\mu\text{g/g}$ f.w.), coumestrol (49.19 $\mu\text{g/g}$ f.w.), and genistin (53.44 $\mu\text{g/g}$ f.w.) was found when soybean seedlings were treated with SA at 8.0, 0.8, and 0.4 mM, respectively. Meanwhile, INA caused the same isoflavonoid-eliciting effect between 0.2 and 0.8 mM, being daidzin the major component in soybean seedlings (about 42.49 $\mu\text{g/g}$ f.w.). The application of INA at 1.6 mM resulted in a slight reduction in the amount of daidzin and genistin accompanied by a dramatic increase in the level of malonyl-daidzin (132.11 $\mu\text{g/g}$ f.w., more than six times the amount found in the control seedlings) and malonyl-genistin.

Treatments with INA and SA at 4.0 and 8.0 mM afford a decrease in the biosynthesis of some isoflavonoids. It has been reported that the phytotoxicity of SA may have led to low phenolic contents (Rajjou et al. 2006). Due to SA being a physiological inhibitor of some enzymes, it seems possible to think that high concentration of elicitor may inhibit enzymes involved in the biosynthesis of

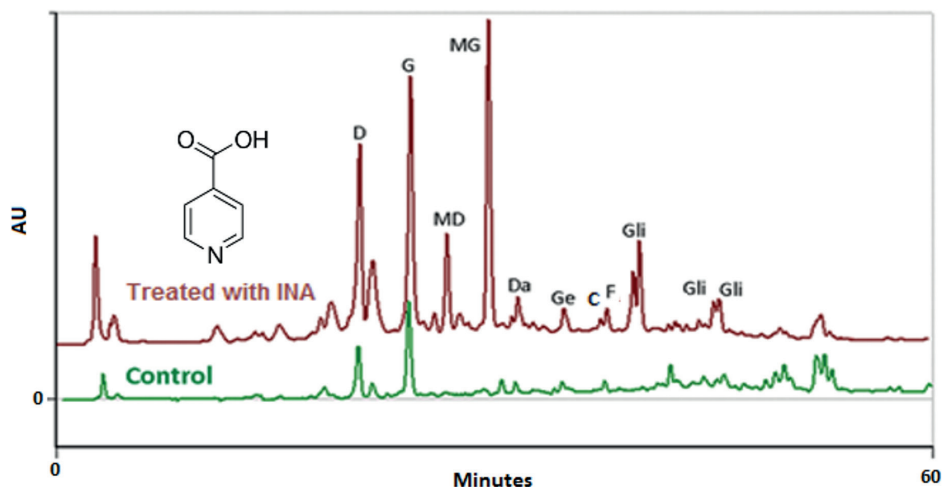


Figure 2 - HPLC profile of soybean (cv. Soyica P34) seedlings after treatment with INA. Daidzin (D), genistin (G), malonyldaidzin (MD), malonylgenistin (MG), daidzein (Da), genistein (Ge), coumestrol (C), formononetin (F), glyceollins I, II, III (Gli). Tissues were harvested at 48 h for HPLC. Analysis wavelength: 270 nm.

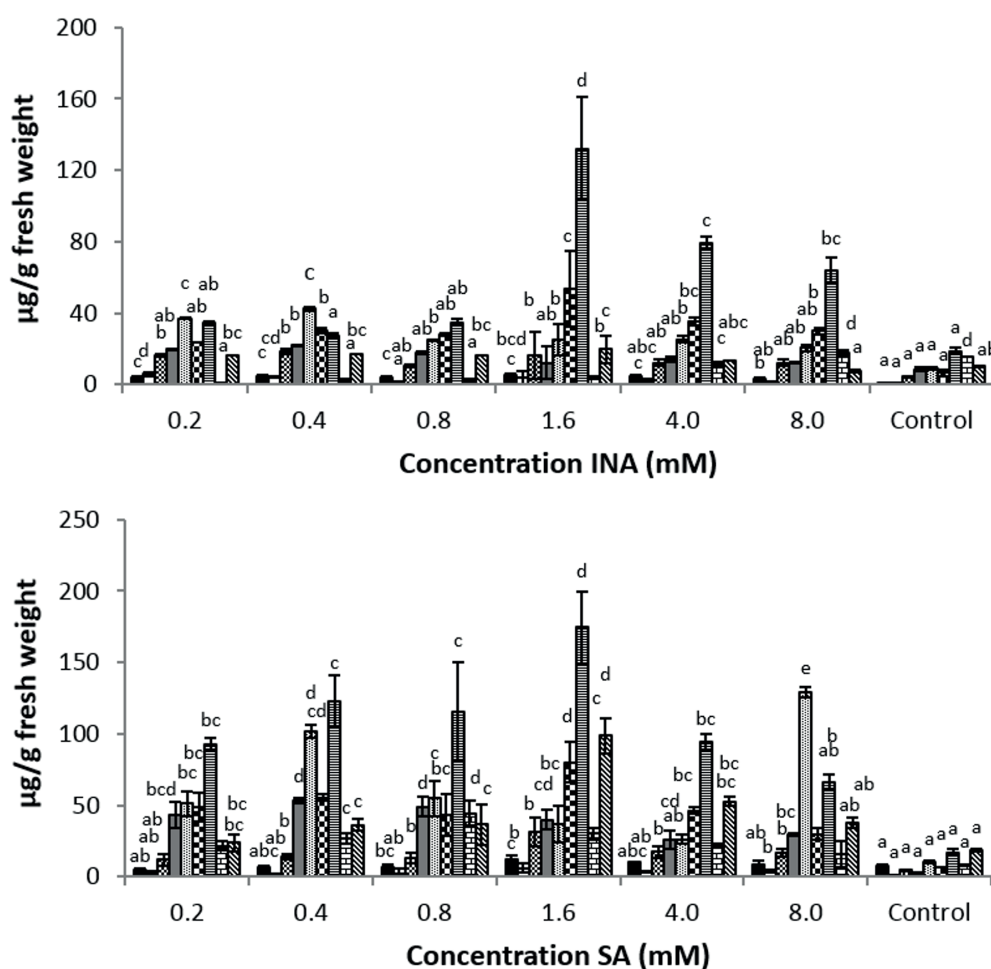


Figure 3 - Accumulation of isoflavonoids on soybean (cv. Soyica P34) seedlings treated with INA (top) and SA (down) at different concentrations. ■, Formononetin; □, Genistein; ▨, Daidzein; ▩, Genistin; ▤, Daidzin; ▥, Malonyl-genistin; ▦, Malonyl-daidzin; ▧, Coumestrol; ▨, Glyceollins. Bars represent the mean concentration of isoflavonoids \pm standard deviation ($n = 3$). For each isoflavonoid, bars with different letters are significantly different ($p = 0.05$). 96 h post-induction.

isoflavonoids. Daidzein and its glucosyl conjugates (daidzin and malonyl-daidzin) were present in the soybean seedlings treated with each elicitor and with water at levels higher than those of genistein and its glucosyl conjugate (genistin and malonyl-genistin), in agreement with (Graham and Graham 1991, Romani et al. 2003).

TIME-COURSE EXPERIMENTS

In order to study the isoflavonoid accumulation in the time-course, roots of soybean seedlings were immersed in 1.6 mM SA or INA. After

staying for 4 h in the solution, it was discarded and seedlings were stored during 144 h. Every 24 h, the isoflavonoid contents were quantitatively measured by HPLC-DAD. Isoflavonoid contents were dependent on post-induction time and were significantly increased in response to SA and INA as compared to control. The isoflavonoid accumulation in the time-course is shown in Figure 4. Analysis indicates that the isoflavonoid content varied significantly ($p = 0.05$) between treatments (time intervals), except for genistein and formononetin. Isoflavonoid content in INA-

treated seedlings was significantly increased as compared to water-treated seedlings, even at 24 h post-induction. Under this condition, the content of daidzin, malonyl-genistin, glyceollins and genistin increased respectively 468, 590, 173, and 4026% with respect to control. Malonyl-daidzin was the major isoflavonoid detected in the analysis, with a maximum amount of almost 157.15 $\mu\text{g/g}$ f.w. after 72 h. In general, it was found that malonyl-daidzin showed a behavior with several transient maximum at 24, 72 and 144 h. The maximum levels of coumestrol and glyceollins were achieved at 48 h after induction, being respectively 20.53

and 98.56 $\mu\text{g/g}$ f.w. Then, the concentration of both declined rapidly.

Data shows that the application of SA in soybean seedlings results in the higher accumulation of malonyl-daidzin, which reached a maximum amount of about 174.56 $\mu\text{g/g}$ f.w. after 96 h post-induction. Then, the content of malonyl-daidzin was gradually declined. Maximum accumulation of the malonyl-genistin (near 80.00 $\mu\text{g/g}$ f.w.) and glyceollins (111.04 $\mu\text{g/g}$ f.w.) was attained at 96 and 144 h respectively. Glucosyl conjugates of daidzein (108.39 $\mu\text{g/g}$ f.w.) and genistein (61.60 $\mu\text{g/g}$ f.w.), along with coumestrol (44.60 $\mu\text{g/g}$

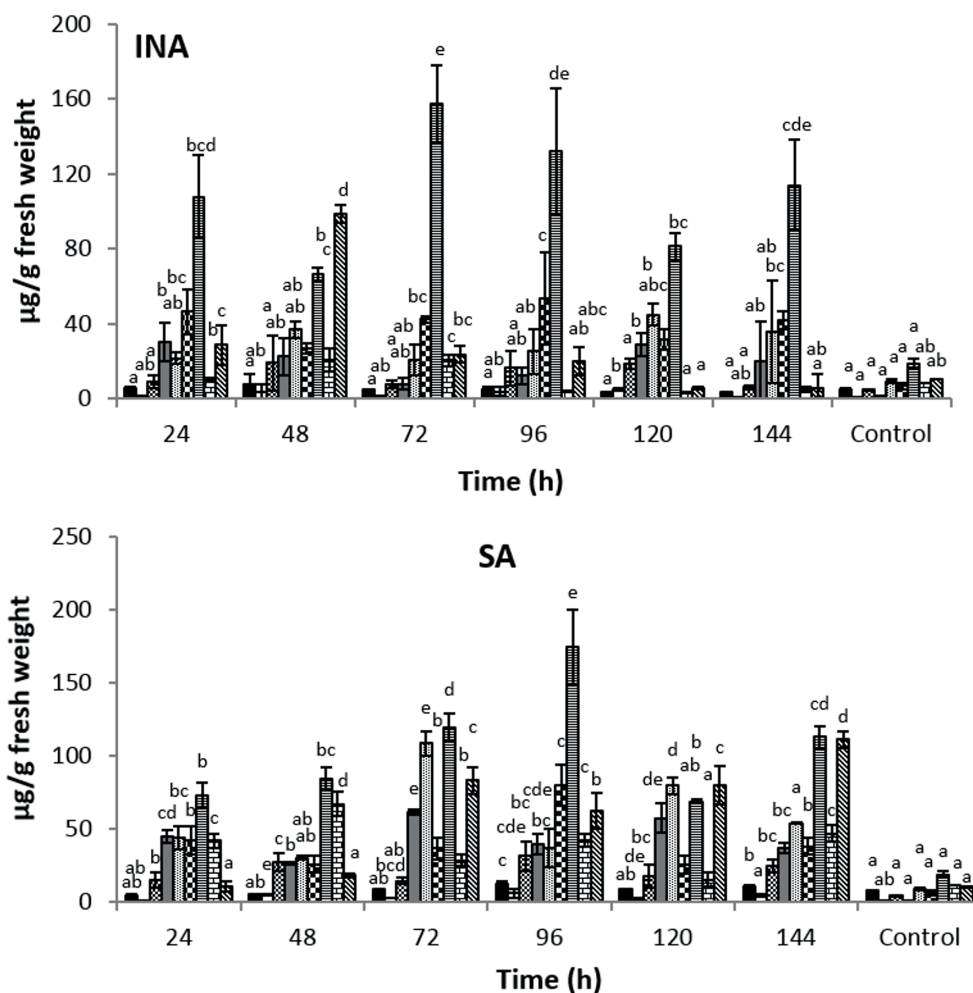


Figure 4 - Time-course accumulation of isoflavonoids in soybean seedlings treated with INA (top) and SA (down). ■, Formononetin; □, Genistein; ▨, Daidzein; ▩, Genistin; ▤, Daidzin; ▥, Malonyl-genistin; ▦, Malonyl-daidzin; ▧, Coumestrol; ▨, Glyceollins. Bars represent the mean concentration of isoflavonoids \pm standard deviation ($n = 3$). For each isoflavonoid, the bars headed by the same letter do not differ at $p = 0.05$.

f.w.), reached the maximum content at 72 h. The decrease in the concentration of malonyl-daidzin and malonyl-genistin coincided with the increase in the level of daidzin, coumestrol and glyceollins after 120 h.

In general, highest levels of isoflavonoids were present in seedlings treated with SA. Although INA induced a rapid accumulation of glyceollins during the first 48 h, then the concentration of these was rapidly decreased. Aglycones were present in all treatments at low concentrations, while glucosides (daidzin and genistin) and malonyl-glucosides were the major components. These results are in agreement with those reported by (Zhang et al. 2006). On the other hand, the levels of isoflavonoids reached in cultivar Soyica P34 were notoriously lower than those that have been reported for other cultivars (Boué et al. 2000, Zhang et al. 2006). For SA, the reduction in the amount of malonyl-glucosyl conjugates of daidzin coincided with the increase in the level of daidzin, coumestrol and glyceollins. The above is in agreement with the fact that daidzin is biosynthetically formed from malonyl-daidzin, and then daidzin is transformed in coumestrol and glyceollins (Graham et al. 1990, Graham and Graham 1991).

INDUCER EFFECT OF STRUCTURALLY RELATED COMPOUNDS TO INA AND SA

Nowadays, there is an increasing interest in finding new elicitors to improve the yield of specific metabolites obtained from plants and cell cultures, and to control important agronomic diseases. Considering that the largest increases for isoflavonoids in soybean seedlings were caused by treatment with SA and INA, the eliciting effect of 25-structurally related compounds was evaluated (Figure 5).

According to the time-course and dose-response experiments, soybean seedlings were immersed for 4 h in solutions containing the compounds at 1.6 mM and subsequently allowed

to incubate for 96 h. In the analysis were included benzoic acid derivatives, nicotinic and isonicotinic acid derivatives, dihydro-quinazolinones, and two recognized elicitors DCIA and BTH (Bokshi et al. 2006). Soybean isoflavonoids content after treatments is presented in Figure 6. Soybean isoflavonoids were grouped in five classes: malonyl-glycosides (malonyl-genistin, malonyl-daidzin), aglycones (daidzein, genistein, and formononetin), glycosides (daidzin, genistin), coumestrol and glyceollins.

There was a significant increase in isoflavonoid production under all treatments compared to that of the control. In Figure 6 (up), the isoflavonoid-eliciting effect of several nitrogen-containing cyclic compounds is shown. As can be seen, maximum concentration of malonyl-glycosides was obtained when compounds **4** ($228.4 \pm 20.4 \mu\text{g/g}$ f.w.), INA ($185.8 \pm 58.1 \mu\text{g/g}$ f.w.), **3** ($154.9 \pm 10.3 \mu\text{g/g}$ f.w.), and **10** ($144.7 \pm 10.3 \mu\text{g/g}$ f.w.) were used as elicitors. Meanwhile, highest accumulation of aglycones was found in soybean seedlings treated with **1** ($57.2 \pm 11.4 \mu\text{g/g}$ f.w.), **6** ($44.7 \pm 2.2 \mu\text{g/g}$ f.w.) and **4** ($40.6 \pm 1.4 \mu\text{g/g}$ f.w.). In addition, treatments with **7**, **2**, **6**, and **9** resulted in a strong increase in the content of malonyl-glucosyl conjugates, being respectively 7.2, 6.9, 6.7, and 6.4-fold over water-treated seedlings. As much as 2.2, 2.1, and 1.6-fold was increased the content of coumestrol over control seedlings, using compounds **2**, **5**, and **6** respectively. In addition, the highest amount of glyceollins was obtained ($93.55 \mu\text{g/g}$ f.w.) in the soybean seedlings treated with compound **5**. According to our results, the highest inducer activity of malonyl-glucosyl isoflavones was found for **4** (nicotinic acid). On the other hand, the 2-substituted nicotinic acids (**1**, **2**, and **5**) displayed a glyceollins-eliciting effect higher than the other nitrogen-containing cyclic compounds.

Similarly, Figure 6 (down) shows the effects of treatment with benzoic acid derivatives, dihydro-quinazolinones, and BTH on the accumulation of

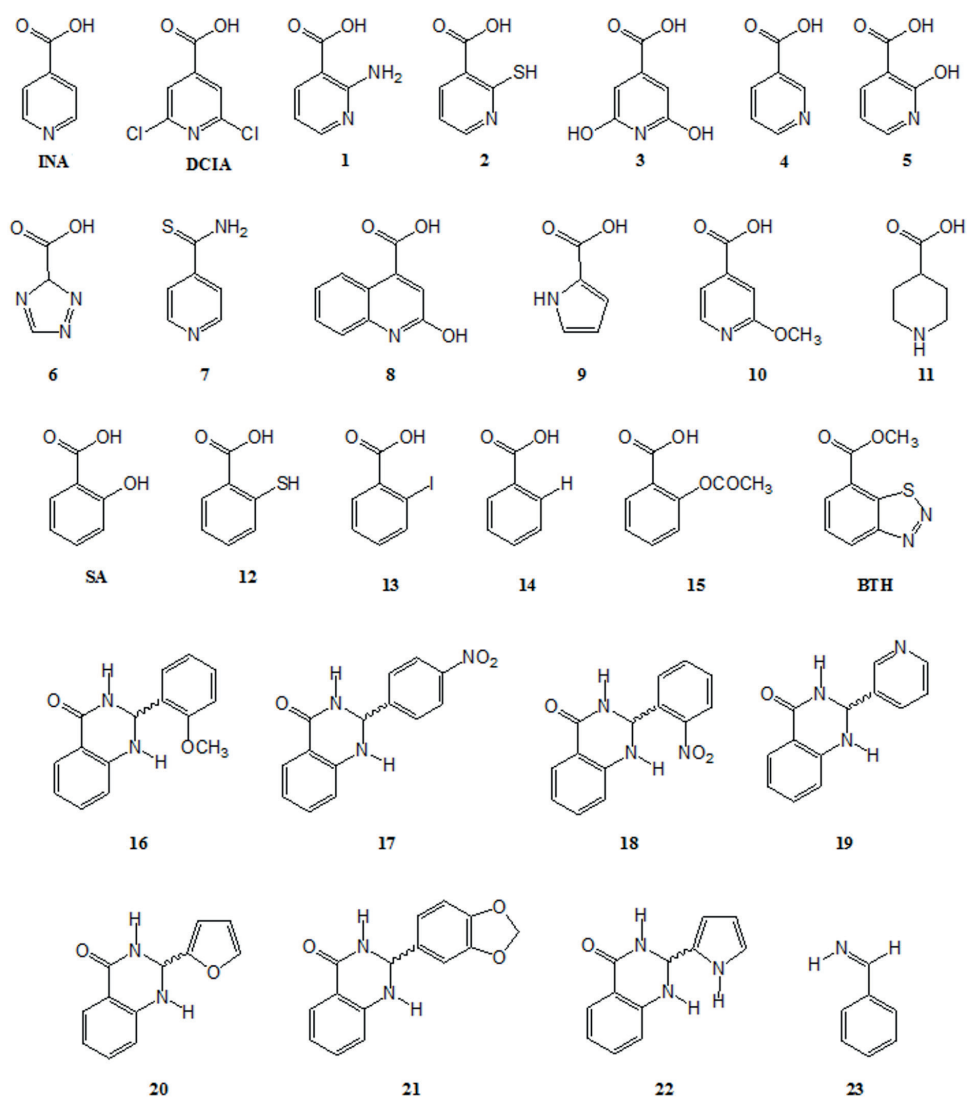


Figure 5 - Chemical structures of compounds evaluated as elicitors. Structurally related compounds to INA (DCIA and **1-11**) and SA (BTH, and **12-23**) were taken out.

isoflavonoids in soybean seedlings. In general, the application of SA and dihydro-quinazolinones (**22**, **21**, **16**, and **17**) produced a higher content of malonyl-glycosides and aglycones (especially for **16**) compared with water-treated seedlings. Soybean seedlings elicited with SA, **22** and **21**, show malonyl-glycosides concentrations of about 252.71, 153.81, and 132.82 $\mu\text{g/g}$ f.w. respectively. Aglycones reached respectively concentrations of 80.10, 35.04, and 31.19 for **16**, **2**, and SA. Furthermore, malonyl-glucosyl conjugates

exhibited maximum levels when SA and **15** were used as elicitors, being respectively 104.27 and 96.25 $\mu\text{g/g}$ f.w. As shown in Figure 6 (down), dihydro-quinazolinones **17** and **20** showed to be potent inducers of coumestrol reaching respectively amounts of 119.17 and 59.30 $\mu\text{g/g}$ f.w. On the other hand, glyceollins displayed the maximum production when soybean seedlings were treated with SA and **20**.

In summary, the isoflavonoid-eliciting activity of a series of benzoic acid derivatives, nitrogen-

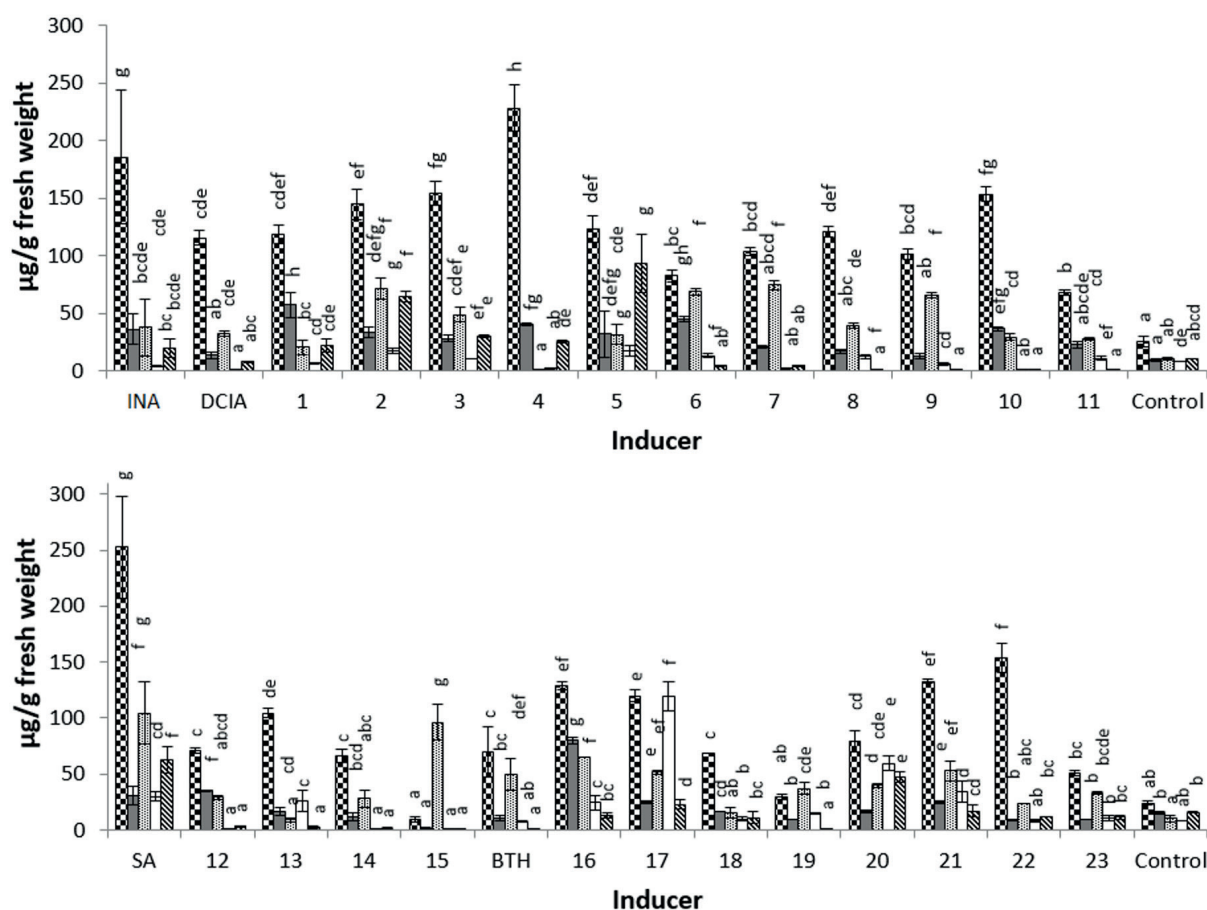


Figure 6 - Accumulation of isoflavonoids in soybean seedlings treated with structurally related compounds to INA (top) and SA (down). Malonyl-glycosides (malonyl-genistin, malonyl-daidzin); aglycones (daidzein, genistein, and formononetin); glycosides (daidzin, genistin); coumestrol; glyceollins. Bars represent the mean concentration of isoflavonoids \pm standard deviation ($n = 3$). For each isoflavonoid, the bars headed by the same letter do not differ at $p = 0.05$.

containing cyclic compounds, and dihydro-quinazolinones showed that highest concentration of malonyl-glycosides were induced by SA (252.71 $\mu\text{g/g}$ f.w.) and **4** (228.42 $\mu\text{g/g}$ f.w.). Meanwhile, maximum aglycone contents were observed in soybean seedlings treated with **16** (80.10 $\mu\text{g/g}$ f.w.) and **1** (57.19 $\mu\text{g/g}$ f.w.). Glucosyl conjugates reached their rated capacity of synthesis after treatments with SA (104.27 $\mu\text{g/g}$ f.w.), followed by **15** (96.25 $\mu\text{g/g}$ f.w.). Compounds **17** and **5** had the strongest impact on coumestrol (119.44 $\mu\text{g/g}$ f.w.) and glyceollins (93.55 $\mu\text{g/g}$ f.w.), respectively. BHT (Actigard® or Bion®, commercially available from Syngenta) and DCIA, two efficient

defense activators in a wide variety of plant species (Bektas and Eulgem 2014), induced low levels of isoflavonoids under the conditions used.

According to our analysis, dihydro-quinazolinones and 2-substituted nicotinic acids possess a strong isoflavonoid-eliciting effect being even higher than that showed by the BTH and DCIA. The upper glyceollin accumulation was found to be induced by **5**, followed by **2** and SA. The fact that soybean seedlings treated with dihydro-quinazolinones and 2-substituted nicotinic acids accumulate lower levels of malonyl-glucosyl conjugates and daidzin compared to SA but higher amounts of aglycones, coumestrol and glyceollins

indicate an efficient biosynthetic conversion of the precursors as a result of the induction. Interestingly, while 2-substituted nicotinic acids (i.e. **5** and **2**) induced highest levels of glyceollins, the dihydro-quinazolinones (i.e. **17** and **20**) elicited upper amounts of coumestrol. The above may suggest the possibility to modulate the response of soybean seedlings and the conversion of daidzein to glyceollins or coumestrol through the application selective of these compounds. The isoflavonoid elicitor effects of dihydro-quinazolinones in common bean was previously demonstrated by us (Durango et al. 2013).

BIOLOGICAL ACTIVITY OF ETHYL ACETATE-SOLUBLE FRACTION FROM SOYBEAN SEEDLINGS TREATED WITH ELICITORS

Several studies have shown that isoflavones genistein and daidzein, their glycosides, and coumestrol have antioxidant activity (Lee et al. 2005). Besides acting as phytoalexins, daidzein, genistein, coumestrol and glyceollins have been reported having *in vitro* antibacterial activity (Rivera-Vargas et al. 1993, Ulanowska et al. 2006, Fett and Osman 1982, Kim et al. 2010). Some authors have suggested that genistein displays a bacteriostatic effect inhibiting DNA, RNA and protein synthesis (Ulanowska et al. 2006)

while coumestrol is active against *S. aureus*, *B. megaterium* and *E. coli* inhibiting membrane-associated transport processes (Fett and Osman 1982). In addition, glyceollins have revealed remarkable antifungal effect against *Fusarium oxysporum*, *Phytophthora capsici*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea* with growth inhibitions ranging from 10.9-61.0% (Kim et al. 2010). Therefore, the induction of isoflavonoid compounds in soybean using elicitors may be a valuable alternative for accessing bioactive compounds or extracts, which may have possible utilization in functional foods, pharmaceutical supplement or personal care products.

In order to evaluate the effect of treatment with the elicitor in the biological activity of soybean, some extracts from seedlings elicited with water, **4**, and **5** were prepared and their antioxidant, antibacterial and antifungal activities were evaluated. The results are shown in Table I. EtOAc-soluble fractions from soybean seedlings were evaluated without any further refining. Two different methods were used to determine antioxidant properties: DPPH free radical scavenging and ferric reducing antioxidant power (FRAP); both are widely used to investigate the scavenging activities of several natural compounds and crude extracts of plants. In the DPPH assay,

TABLE I
Biological activity of EtOAc-soluble fraction from soybean seedlings treated with water, **4** and **5**.

Extract from soybean seedling treated with	DPPH scavenging activity		FRAP activity (mg TE/g Ext)	MIC ($\mu\text{g/mL}$) [†]				Growth inhibition (%) [§]	
	(%)	(mg TE/g Ext)		<i>E. coli</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>E. faecalis</i>	<i>C. lindemuthianum</i>	<i>F. oxysporum</i>
Water	7.89±0.16	9.3±0.0	33.6±3.7	n.d.	2000	1000	2000	8.8±1.0	28.0±4.4
4	22.22±2.52*	12.5±0.5*	44.2±4.3*	n.d.	2000	500	1000	17.8±2.5*	28.8±2.6
5	18.11±2.36*	13.6±1.1*	53.8±7.7*	n.d.	2000	500	1000	25.2±2.2*	42.0±5.0*
Ampicillin®**	--	--	--	15.6	15.6	125	15.6	--	--
Thymol**	--	--	--	--	--	--	--	26.4±3.2*	38.3±2.5*

Values are means \pm SD of triplicate assays. *Significantly different compared to water-treated seedlings ($p = 0.05$, Fisher's least significant differences). **Positive controls for antibacterial and antifungal assays, respectively. n.d. not detected (MIC > 2000 $\mu\text{g/mL}$). [†]MIC: Minimum Inhibitory Concentration. [§]Growth inhibition (%) = [1 - (radial growth treatment/radial growth control)] x100; all values were corrected considering the solvent effect (control). EtOAc-extracts were evaluated to 200 $\mu\text{g/mL}$.

the absorbance decreases at wavelength 517 nm as a result of a color change from violet to yellow as radical is scavenged by antioxidants through donation of hydrogen to yield the stable DPPH molecule (Kedare and Singh 2011). FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} . The results of the different EtOAc extracts showed that percentage scavenging of DPPH by the extract from soybean seedlings treated with **4** ($22.22 \pm 2.52\%$) was highest, while the water-treated seedlings revealed the lowest value ($7.89 \pm 0.16\%$). In the same way, FRAP assay revealed that extract from seedlings treated with **5** displayed the highest activity. Significant differences in the DPPH scavenging and FRAP assays between the soybean seedlings treated with **4** and **5** and those treated with water were found. The above is in agreement with the fact that application of **4** and **5** resulted in a higher content of isoflavonoids, particularly glycosides and aglycones.

The inhibitory effects of the ethyl acetate fraction of soybean seedlings treated with water, **4** and **5** against *E. coli*, *S. aureus*, *B. cereus*, *E. faecalis*, *C. lindemuthianum* and *F. oxysporum* are shown in Table I. Antibacterial and antifungal activity was evaluated using the microdilution method with TTC (2,3,5-triphenyl tetrazolium chloride) in 96-well microtiter plate and the poisoned food technique, respectively. The experimental data were obtained at the third day after inoculation for fungi and after 24 h for bacteria. The antimicrobial activity of the extracts was determined through the Minimal Inhibitory Concentration (MIC) and Minimal Bactericide Concentration (MBC). In general, all extracts exhibited a low inhibitory effect against bacteria under the conditions used, compared with Ampicillin®. MIC values corresponding to EtOAc-extracts from soybean seedlings induced by **4** and **5** were significantly lower for *B. cereus* (Gram +) and *E. faecalis* (Gram +); however, none of the tested extracts showed bactericidal activity. On the other hand, inhibitions of *C. lindemuthianum* and *F.*

oxysporum using the extract from seedlings elicited by **5** were significantly higher as compared to the water-treated seedlings. This extract displayed an antifungal activity like thymol. This can be attributed to the presence of new and more abundant isoflavonoid compounds, specifically aglycones and glyceollins, which have been recognized by their fungistatic properties (Fett and Osman 1982, Kim et al. 2010).

CONCLUSIONS

Soybean isoflavonoids has been linked to defense mechanisms against pathogens and many health benefits. The application of exogenous elicitor on plants has been suggested as a good alternative to increase the isoflavonoid contents and consequently, the resistance to plant pathogenic microorganisms and the biological activity of extracts (especially, antioxidant activity). In the present work, the application of salicylic and isonicotinic acids and structurally-related compounds resulted in a higher content of isoflavonoids compared to water-treated soybean seedlings (var. Soyica P34). The accumulation of isoflavonoids varied depending on the dose and post-induction time. Generally, maximum levels of isoflavonoids were reached for salicylic acid at 1.6 mM and between 96 and 144 h incubation. Malonyl-daidzin, coumestrol and glyceollins were the major compounds present in the extracts from soybean induced by these elicitors. In addition, some structurally related compounds to salicylic acid and isonicotinic acid showed a strong elicitor effect. Soybean seedlings treated with dihydro-quinazolinones and 2-substituted nicotinic acids displayed the highest amounts of glyceollins and coumestrol. These compounds could be considered good candidates for the design of new phytoprotectants and to understand the molecular events related to the production of phytoalexins. Finally, there was significant increase in the antioxidant and antimicrobial

activity in the extracts from soybean seedlings treated with nicotinic acid and 2-hydroxynicotinic acid with respect those treated with water. Thus, these elicitors may be used to increase the amount of natural antioxidant or antimicrobial compounds in soybean and its extracts, for subsequently, be isolated with a higher yield and used in functional foods or pharmaceutical and personal care products.

ACKNOWLEDGMENTS

This study was supported by Departamento Administrativo de Ciencia, Tecnología e Innovación Colciencias (No. 111874558342, contract: 057-2017).

REFERENCES

- AMARESH C AND BHATT RK. 1998. Biochemical and physiological response to salicylic acid in reaction to systemic acquired resistance. *Photosynthetica* 35: 255-258.
- ARRUDA RJ, PAZ ATS, BARA MTF, CORTES MVCB, FILIPPI MCC AND CONCEIÇÃO EC. 2016. An approach on phytoalexins: function, characterization and biosynthesis in plants of the family Poaceae. *Cienc Rural* 46: 1206-1216.
- BASRI DF, TAN LS, SHAFIEI Z AND ZIN NM. 2012. *In vitro* antibacterial activity of galls of *Quercus infectoria* Olivier against oral pathogens. *Evid Based Complement Alternat Med*, Article ID 632796.
- BEKTAS Y AND EULGEM T. 2014. Synthetic plant defense elicitors. *Front Plant Sci* 5: 804.
- BERKER K, GUCLU K, TOR I AND APAK R. 2007. Comparative evaluation of Fe(III) reducing power-based antioxidant capacity assays in the presence of phenanthroline, batho-phenanthroline, tripyridyltriazine (FRAP), and ferricyanide reagents. *Talanta* 72: 1157-1165.
- BOKSHI AI, MORRIS SC, MCCONCHIE RM AND DEVERALL BJ. 2006. Pre-harvest application of 2,6-dichloroisonicotinic acid, β -aminobutyric acid or benzothiadiazole to control post-harvest storage acquired resistance (SAR). *J Hort Sci Biotechnol* 81: 700-706.
- BOUÉ SM, CARTER CH, EHRLICH KC AND CLEVELAND TE. 2000. Induction of the soybean phytoalexins coumestrol and glyceollin by *Aspergillus*. *J Agric Food Chem* 48: 2167-2172.
- BOUÉ SM, SHIH FF, SHIH BY, DAIGLE KW, CARTER-WIENTJES CH AND CLEVELAND TE. 2008. Effect of biotic on enrichment of antioxidant properties and induced isoflavones in soybean. *J Food Sci* 73: 43-49.
- BUROW ME, BOUE SM, COLLINS-BUROW BM, MELNIK LI, DUONG BN, CARTER-WIENTJES CH, LI S, WIESE TE, CLEVELAND TE AND MCLACHLAN JA. 2001. Phytochemical glyceollins, isolated from soy, mediate antihormonal effects through estrogen receptor alpha and beta. *J Clin Endocrinol Metab* 86: 1750-1758.
- DANNE, DIERS B, BYRUM J AND HAMMERSCHMIDT R. 1998. Effect of treating soybean with 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) on seed yields and the level of disease caused by *Sclerotinia sclerotiorum* in field and greenhouse studies. *Eur J Plant Pathol* 104: 271-278.
- DAR BA, SAHU AK, PATIDAR P, PATIAL J, SHARMA P, SHARMA M AND SINGH B. 2012. An easy and efficient protocol for the synthesis of 2,3-dihydroquinazolinones using a low cost and reusable heterogeneous catalyst. *American J Chem* 2: 248-254.
- DOERGE DR, CHANG HC, CHURCHWELL MI AND HOLDER CL. 2000. Analysis of soy isoflavone conjugation *in vitro* and in human blood using liquid chromatography-mass spectrometry. *Drug Metab Dispos* 28: 298-307.
- DURANGO D, PULGARIN N, ECHEVERRI F, ESCOBAR G AND QUIÑONES W. 2013. Effect of salicylic acid and structurally related compounds in the accumulation of phytoalexins in cotyledons of common bean (*Phaseolus vulgaris* L.) cultivars. *Molecules* 18: 10609-10628.
- FETT WF AND OSMAN SF. 1982. Inhibition of bacteria by the soybean isoflavonoids glyceollin and coumestrol. *Phytopathology* 72: 755-760.
- GRAHAM TL AND GRAHAM MY. 1991. Glyceollin elicitors induce major but distinctly different shifts in isoflavonoid metabolism in proximal and distal soybean cell population. *Mol Plant Microbe Interact* 4: 60-68.
- GRAHAM T, KIM J AND GRAHAM M. 1990. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. *Mol Plant Microbe Interact* 3: 157-166.
- HAMMERSCHMIDT R. 1999. Induced disease resistance: how do induced plants stop pathogens? *Physiol Mol Plant Pathol* 55: 77-84.
- HATANO T, EDAMATSU R, HIRAMATSU M, MORI A, FUJITA Y, YASUHARA T, YOSHIDA T AND OKUDA T. 1989. Effects of the interaction of tannins with co-existing substances. VI: Effects of tannins and related polyphenols on superoxide anion radical and on 1,1-diphenyl-2-picrylhydrazyl radical. *Chem Pharm Bull* 37: 2016-2021.

- KAUSS H, THEISINGER-HINKEL E, MINDERMAN R AND CONRATH U. 1992. Dichloroisonicotinic and salicylic acid, inducers of systemic acquired resistance, enhance fungal elicitor responses in parsley cells. *Plant J* 2: 655-660.
- KEDARE SB AND SINGH RP. 2011. Genesis and development of DPPH method of antioxidant assay. *J Food Sci Technol* 48: 412-422.
- KIM HJ, SUH HJ, LEE CH, KIM JH, KANG SC, PARK S AND KIM JS. 2010. Antifungal activity of glyceollins isolated from soybean elicited with *Aspergillus sojae*. *J Agric Food Chem* 58: 9483-9487.
- LEE CH, YANG L, XU JZ, YEUNG SYV, HUANG Y AND CHEN ZY. 2005. Relative antioxidant activity of soybean isoflavones and their glycosides. *Food Chem* 90: 735-741.
- MCCUE P AND SHETTY K. 2004. Health benefits of soy isoflavonoids and strategies for enhancement: a review. *Crit Rev Food Sci* 44: 361-367.
- PIASECKA A, JEDRZECZAK-REYN AND BEDNAREK P. 2015. Secondary metabolites in plant innate immunity: conserved function of divergent chemicals. *New Phytol* 206: 948-964.
- QIAN ZG, ZHAO ZJ, XU Y, QIAN X AND ZHONG JJ. 2005. Novel synthetic 2,6-dichloroisonicotinate derivatives as effective elicitors for inducing the biosynthesis of plant secondary metabolites. *Appl Microbiol Biotechnol* 71: 164-167.
- QIAN ZG, ZHAO ZJ, XU Y, QIAN X AND ZHONG JJ. 2006. Novel chemically synthesized derivatives as an effective elicitor for inducing the biosynthesis of plant secondary metabolites. *Biotechnol Pro* 22: 331-333.
- RAJJOU L, BELGHAZI M, HUGUET R, ROBIN C, MOREAU A, JOB C AND JOB D. 2006. Proteomic investigation of the effect of salicylic acid on Arabidopsis seed germination and establishment of early defense mechanisms. *J Hort Sci Biotechnol* 81: 700-706.
- RIVERA-VARGAS LI, SCHMITTHENNER AF AND GRAHAM TL. 1993. Soybean flavonoid effects on and metabolism by *Phytophthora sojae*. *Phytochemistry* 32: 851-857.
- ROMANI A, VIGNOLINI P, GALARDI C, AROLDI C, VAZZANA C AND HEIMLER D. 2003. Polyphenolic content in different plant parts of soy cultivars grown under natural conditions. *J Agric Food Chem* 51: 5301-5306.
- ULANOWSKA K, TKACZYK A, KONOPA G AND WEGRZYN G. 2006. Differential antibacterial activity of genistein arising from global inhibition of DNA, RNA and protein synthesis in some bacterial strains. *Arch Microbiol* 184: 271-278.
- VANETTEN H, MANSFIELD J, BAILEY J AND FARMER E. 1994. Letter to the editor: two classes of plant antibiotics: phytoalexins versus phytoanticipins. *Plant Cell* 6: 1191-1192.
- VANETTEN H, TEMPORINI E AND WASMANN C. 2001. Phytoalexin (and phytoanticipin) tolerance as a virulence trait: Why is it not required by all pathogens? *Physiol Mol Plant Pathol* 59: 83-93.
- WANG Q, GE X, TIAN X, ZHANG Y, ZHANG J AND ZHANG P. 2013. Soy isoflavone: The multipurpose phytochemical (Review). *Biomed Rep* 1: 697-701.
- WAR AR, PAULRAJ MG, WAR MY AND IGNACIMUTHU S. 2011. Role of salicylic acid in induction of plant defense system in chickpea (*Cicer arietinum* L.). *Plant Signal Behav* 6: 1787-1792.
- ZHANG B, HETTIARACHCHY N, CHEN P, HORAX R, CORNELIOUS B AND ZHU D. 2006. Influence of the application of three different elicitors on soybean plants on the concentrations of several isoflavones in soybean seeds. *J Agric Food Chem* 54: 5548-5554.