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Differential accumulation of defense-related isoflavonoids in hypocotyls/roots of common bean (*Phaseolus vulgaris* L.) cultivars treated with salicylic acid and structurally related compounds

[Acumulación diferencial de isoflavonoides relacionados con la defensa en hipocótilos/raíces de variedades de poroto (*Phaseolus vulgaris* L.) tratados con ácido salicílico y compuestos estructuralmente relacionados]

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Abstract: Hypocotyls/roots of four (anthracnose-resistant: ICA Quimbaya and CORPOICA 106; anthracnose-susceptible: Cargamanto Rojo and Cargamanto Mocho) common bean cultivars treated with salicylic acid (SA) as elicitor, were analyzed to determine the capacity for synthesizing defense-related isoflavonoids. Time-course and dose-response studies indicated that the maximum levels of isoflavonoids, occurred at 1.45 mM SA and between 96 and 144 h post-induction. Overall, anthracnose-resistant cultivars produced the defense-related isoflavonoids to superior amounts than the susceptible ones. Additionally, crude isoflavonoid extracts from SA-treated tissues cvs. ICA Quimbaya and Cargamanto Rojo displayed higher inhibitory effect against *C. lindemuthianum* than those from water-treated tissues. A comparison of the isoflavonoid-eliciting activity of a series of structurally-related compounds to SA revealed that isoflavonoid production may be differentially controlled. Acetyl-salicylic acid showed the best isoflavonoid-inducing effect. Results might be useful for crop protection programs through the selecting of common bean cultivars with better prospects of disease resistance, and the development of better isoflavonoid-eliciting agents.

Keywords: elicitor, coumestrol, time-course experiments, dose-response profile, *C. lindemuthianum*, antifungal activity.

RESUMEN: Los hipocótilos/raíces de cuatro variedades de poroto (resistente a antracnosis: ICA Quimbaya y CORPOICA 106; susceptible a antracnosis: Cargamanto Rojo y Cargamanto Mocho) tratados con ácido salicílico (AS) como elicitor, se analizaron para determinar la capacidad para sintetizar isoflavonoides relacionados con la defensa. Los estudios en el curso del tiempo y dosis-respuesta indicaron que los niveles máximos de isoflavonoides, ocurrieron a una concentración de AS de 1.45 mM y entre 96 y 144 h post-inducción. En general, las variedades resistentes a la antracnosis produjeron los isoflavonoides relacionados con la defensa en cantidades superiores en comparación con las variedades susceptibles. Adicionalmente, los extractos de isoflavonoides crudos provenientes de tejidos tratados con AS var. ICA Quimbaya y Cargamanto Rojo desplegaron un efecto inhibitorio contra *C. lindemuthianum* mayor que aquellos resultantes de tejidos tratados con agua. Una comparación de la actividad inductora de isoflavonoides de una serie de compuestos estructuralmente relacionados con el AS reveló que la producción de isoflavonoides puede ser controlada diferencialmente. El ácido acetilsalicílico mostró el mejor efecto inductor de isoflavonoides. Los resultados pueden ser útiles para los programas de protección a cultivos a través de la selección de variedades con mejores perspectivas de resistencia a enfermedades, y el desarrollo de mejores agentes elicitores de isoflavonoides.

Palabras clave: Elicitor, coumestrol, efecto del tiempo y la dosis, *C. lindemuthianum*, actividad antifúngica

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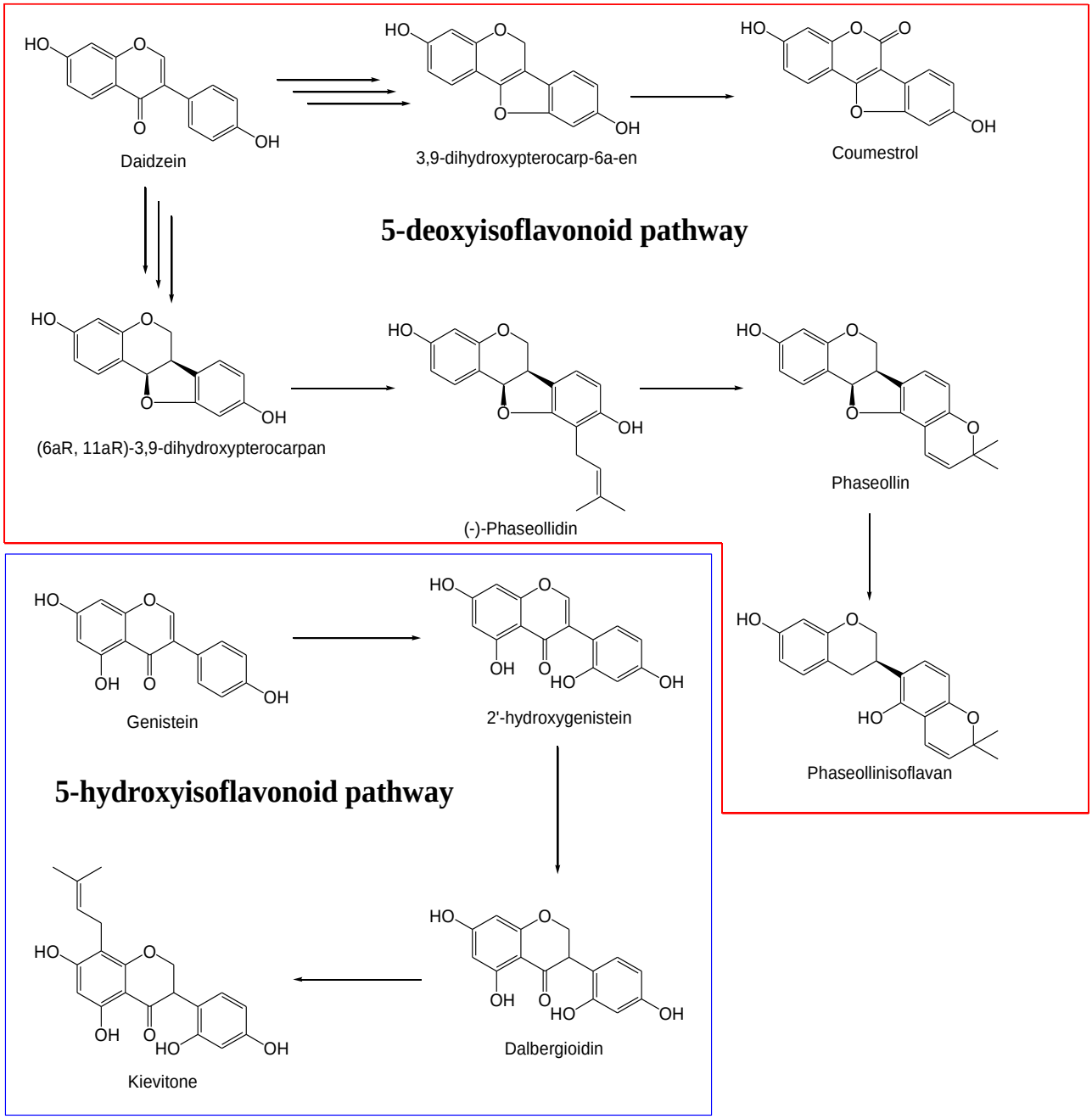
INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is the most important grain legume for direct human consumption (De La Fuente *et al.,* 2011). This crop represents a low cost and easy access protein source for over 500 million people in Latin America and parts of eastern and southern Africa (Broughton *et al.,* 2003). Also, this leguminous is an excellent source of starch, soluble and insoluble fiber, vitamins, and minerals. In Colombia, common bean is the major legume produced and consumed in all regions. Bean (comprising both dry and green beans) constitutes an important ingredient of numerous highly consumed typical dishes, and hence is well valuated within local cultures. Unfortunately, there are problems for bean production in Colombia which are mainly related with the high incidence of fungal and bacterial diseases, and insect pests.

Among the fungal diseases that affect the common bean crop, the anthracnose is the most predominant. The causal organism is *Colletotrichum lindemuthianum*, which mainly affects the quality of pods and grain, and consequently reduces its market value. Overall, anthracnose can lead to yield losses ranging between 38 and 95% depending on cultivar susceptibility (Guzman *et al.,* 1979; Garzón *et al.,* 2007). Traditionally, the disease has been acceptably controlled by the application of conventional synthetic fungicides such as the benzimidazoles, benomyl and carbendazim. However, some of these chemicals can present potential harmful effects on the environment and human health as a result of their low selectivity (Tripathi & Dubey, 2004). Also, its indiscriminate use has often resulted in development of fungicide resistance by pathogens, and increased production costs resulting of the need for apply higher doses and more frequents in the crop. On the other hand, the use of resistant varieties has also been considered an effective method for controlling the disease. Nevertheless, the emergence of new pathogenic races within relatively short periods of time, added to the difficulties in the evaluation of resistance in bean breeding programs through effective field screening (which are time-consuming and expensive), has been the major impediments (Miklas *et al.,* 2006). As a result of the above, researchers have actively been searching new biorational alternatives and compounds to control the disease, and new rapid screening methods for resistance to anthracnose (Buendia *et al.,* 2003; Lebeda & Svábová, 2010).

Nowadays, one of the alternatives that are being more intensely explored is based in the natural protective mechanisms of plants, induced by biotic (such as plant pathogenic fungi) and abiotic agents (such as salicylic acid and UV irradiation). Such inducible defense mechanisms include the accumulation of low molecular weight antimicrobial compounds synthesized from remote precursors (phytoalexins), the fortification of cell wall structure (through deposition of hydroxyl-proline rich glycoproteins, callose, lignin), the synthesis of antifungal hydrolytic enzymes (like chitinases, 1,3-β-glucanases) and other defense-related proteins, and the activation of hypersensitive reactions (Van Loon & Van Strien, 1999; Heil & Bostock, 2002). In legumes, however, studies have mainly been focused on phytoalexins and some biosynthetically related isoflavonoids; particularly for common bean, induction enhanced the amounts of the phytoalexins phaseollin, phaseollidin, phaseollinisoflavan, kievitone and coumestrol, and their biosynthetic precursors, genistein, daidzein, 2-hydroxygenistein, and dalbergioidin (Figure 1). Frequently, these isoflavonoids are absent or present in very low concentrations in healthy plants.

Figure 1
Biosynthetic pathways of isoflavonoid-type defensive metabolites on common bean (*Phaseolus vulgaris* L.)



The application of such biotic and abiotic agents (elicitors) to induce natural disease resistance could be part of an integrated pest management approach (Hammerschmidt, 1999; Edreva, 2004). Also, elicitors offer ecological advantages with respect to synthetic fungicides due to their non-biocidal character and mode of selective action. Moreover, induction experiments can provide useful information about the mechanism used by plants to defend themselves against pathogenic microorganisms, give a rapid indication of the varieties with better perspectives of resistance to diseases, and permit the selection of those elicitors that can be used in crop protection.

Salicylic acid (SA), an endogenous elicitor, plays a crucial role in the induction processes of systemic acquired resistance (SAR). SA is involved in

signal transduction systems, which stimulate particular enzymes that catalyze biosynthetic reactions to produce defense compounds (Van Loon & Antoniw, 1982). Interestingly, the exogenous application of SA can also result in the biosynthesis and accumulation of defense-related compounds and consequently, provides protection from attacks by a broad range of pathogens. Unfortunately, SA may display phytotoxicity at certain concentrations in some plant species, which is a critical limitation on the wide-spread use at field level (Schreiber & Desveaux, 2008). However, the key role of SA in modulating disease resistance along with its low cost and commercial availability made this molecule an interesting structural template for the development of new elicitors with improved properties. Thus, several structurally and functionally related compounds to SA have been identified in screening programs as capable of inducing resistance to disease. The 2,6-dichloroisonicotinic acid (INA) and acibenzolar-*S*-methyl (BTH), resemble SA by acting as exogenous chemical inducers of SAR and protecting the plants from fungal infections. In addition, BTH is considered a strong inducer of SAR with lower phytotoxicity than either SA or INA (Friedrich *et al.*, 1996).

Recently, we have reported the effect of SA in the isoflavonoid-phytoalexin accumulation in common bean cotyledons (Durango *et al.*, 2013). However, because it has been reported that the amounts of phytoalexins are depending on the plant organs (Goossens *et al.*, 1987), the aim of the present study was to examine the accumulation of the defense-related isoflavonoids on hypocotyls/roots of four common bean cultivars with different phytopathological behavior toward anthracnose, treated with SA and some structurally related compounds. It has been recognized that hypocotyls and roots are tissues constantly exposed to the pathogens as well as other potential elicitors (such as pesticides, fertilizers and other chemically synthesized products) present in the soil. Soil-borne pathogens like *Fusarium*, *Pythium*, or *Phytophthora* are infamous for being parasites that can devastate entire crops (Okubara & Paulitz, 2005). Also, hypocotyls and roots are essentials for the uptake and transport of nutrients and water, the environmental sensing, and the systemic defense system of the plant (Kaplan *et al.*, 2008). Recent studies show that plants can protect themselves from pathogens via systemically-induced defenses, and that defensive metabolites which are synthesized in roots may be transported aboveground to increase the resistance of rest of the plant (Erb *et al.*, 2009). Moreover, because roots are inaccessible for most pathogens, they play an important role as storage organs of precursors and defensive compounds. Both roots and hypocotyls can develop lesions when common bean seedlings emerge from anthracnose-infected seeds.

MATERIALS AND METHODS

Chemicals

Isoflavonoids genistein and daidzein were purchased from Sigma (St. Louis, USA). Dalbergioidin, 2'-hydroxygenistein, coumestrol, phaseollidin, phaseollin isoflavan, and phaseollin were obtained and identified from a previous work as described elsewhere (Durango *et al.*, 2002; Durango *et al.*, 2013). Salicylic acid (SA), acetyl salicylic acid (ASA), and benzoic acid (BA) were acquired from Merck (Darmstadt, GER), meanwhile thiosalicylic acid (TSA), 2-iodo benzoic acid (IBA), 4-amino salicylic acid (NAS), 2,5-dihydroxybenzoic acid (DHBA), salicylaldehyde (SAA), 2'-hydroxyacetophenone (HAP), 2',6'-dihydroxyacetophenone (DHAP), 2',5'-dihydroxyacetophenone (2-DHAP), benzo (1,2,3) thiadiazole-7-carbothioic acid S- methyl ester (BHT), acibenzolar acid (ABZ), 2,6-dichloropyridine-4-carboxylic acid (INA), and methyl 2-methyl benzoate (MBA) were from Sigma-Aldrich Co. (St. Louis, MO, USA). Methyl salicylate (MSA), ethyl salicylate (ESA), butyl salicylate (BSA), hexyl salicylate (HSA), phenyl salicylate (PSA), 2-phenethyl salicylate (PESA), 6-bromo hexyl salicylate (BHSA), 2-(2,2,2-trifluoroethoxy) benzoic acid (FBA), 2-hydroxyethyl salicylate (GSA), 3-phenylpropyl salicylate (PPSA), acetyl salicylic methyl ester (MASA) and 2,2,2-trifluoro ethyl salicylate (FSA) were prepared from ASA by conventional organic reactions (Smith & March, 2007).

Plant material

Seeds of two cultivars of bean (Cargamanto Mocho and Cargamanto Rojo) were obtained from Semillas & Semillas Ltda. (Medellín, Colombia). The bean cultivar ICA Quimbaya, developed through hybridization and successive selection in the Centro Internacional de Agricultura Tropical (CIAT, Palmira, Colombia) from the cross between Canadian Wonder and Perú 69 x Vermelho, was acquired from Semicol Ltda (Bogotá, Colombia). The resistant cultivar CORPOICA 106 was obtained for selection on local varieties (Cargamanto x

ICA Llanogrande), and provided by Dr. P. J. Tamayo (Corporación Colombiana de Investigación Agropecuaria, Antioquia, Colombia). Seeds from each Colombian bean (*Phaseolus vulgaris* L.) cultivar were surface-sterilized for 15 min in NaOCl (2.0%), washed with tap water, and sown in moist vermiculite. Germination was for 7 d in the darkness, to suppress chlorophyll production, at 25 °C. After this time, the testas were carefully removed from the seedlings.

Induction experiments

Dose-response profiles

Uprooted seedlings of each bean cultivar were placed vertically in sterile plastic trays and roots immersed for 4 hours in solutions of SA to various concentrations (0.36, 0.72, 1.45, 3.62, 7.25, and 14.50 mM). Before preparing all solutions, SA was dissolved in ethanol (0.2%). Then, SA solutions were discarded and the roots were covered with cellucotton soaked with distillated water. The trays were closed with plastic film and seedlings incubated for 96 h at 25 °C and in the darkness. Control experiments were carried out using ethanol (0.2%) instead SA and the same storage conditions. Next, cotyledons and hypocotyls/roots were carefully separated in order to preserve their integrity. Only hypocotyls/roots (10 g.) were considered in this study. Experiments were done at least three times.

Time-course experiments

Seedlings of each bean cultivar were placed vertically in sterile plastic trays under aseptic conditions and the roots immersed for 4 hours in solution of SA (1.45 mM). Solution was prepared in ethanol (0.2%). Then, SA solution was discarded and and the roots were covered with cellucotton soaked with distillated water. Polystyrene trays were covered with stretch film and plant materials were stored at 25 °C in the darkness during different times (24, 48, 72, 96, 120, and 144 h). Similarly, control experiments using seedlings treated with ethanol (0.2%) instead SA solution and stored during 24, 48, 72, 96, 120, and 144 h were performed. After that, cotyledons and hypocotyls/roots were carefully separated in order to preserve their integrity. Only hypocotyls/roots (10 g.) were considered in this study. Experiments were done at least three times.

Inducer effect of structurally related compounds to SA

Seedlings of bean cultivars Cargamanto Mocho and CORPOICA 106 were placed vertically in sterile plastic trays and the roots immersed for 4 h in solutions 1.45 mM of acetyl salicylic acid (ASA), thiosalicylic acid (TSA), benzoic acid (BA), 2-iodobenzoic acid (IBA), 4-amino salicylic acid (NSA), methyl 2-methyl benzoate (MBA), 2,5-dihydroxybenzoic acid (DHBA), methyl salicylate (MSA), ethyl salicylate (ESA), butyl salicylate (BSA), hexyl salicylate (HSA), phenyl salicylate (PSA), 2-phenethyl salicylate (PESA), 6-bromo hexyl salicylate (BHSA), 2-(2,2,2-trifluoroethoxy) benzoic acid (FBA), salicylaldehyde (SAA), 2-hydroxyethyl salicylate (GSA), 3-phenylpropyl salicylate (PPSA), acetyl salicylic methyl ester (MASA), 2,2,2-trifluoro ethyl salicylate (FSA), 2'-hydroxyacetophenone (HAP), 2',6'-dihydroxyacetophenone (DHAP), and 2',5'-dihydroxyacetophenone (2-DHAP). Treatments with benzo (1,2,3) thiadiazole-7-carbothioic acid (ABZ), benzo (1,2,3) thiadiazole-7-carbothioic acid S- methyl ester (BTH), and 2,6-dichloropyridine-4-carboxylic acid (INA) 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 distillated water. The trays were closed with stretch film and seedlings incubated for 96 h at 25 °C in the darkness. Similarly, seedlings immersed in ethanol (0.2%) were used as negative control. Next, cotyledons were discarded and hypocotyls/roots (10 g.) processed. Experiments were done at least three times.

Sample preparation

Hypocotyls/roots 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. The residue was redissolved in methanol (HPLC-grade MeOH, 5.0 mL), and 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 4° C until HPLC analysis.

HPLC Analysis

The analysis of the isoflavonoid phytoalexins was performed on a Gilson chromatograph equipped with a Gilson model 170 diode array detector, using a Phenomenex Security Guard cartridge C18 (4.0 x 3.0 mm) followed by a Phenomenex Luna 5µ C18 (2) reverse-phase column (150 mm x 4.6 mm i.d., 5µm) (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 µl. 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 200-800 nm to collect spectral data.

Identification and quantification of isoflavonoid phytoalexins

Isoflavonoid phytoalexins were characterized by comparing the retention times (Rt) of the standards with those of the extracts, and by co-elution. Additionally, retention times of these isoflavonoids, along with kievitone, were confirmed by liquid chromatography with mass spectrometry detection (LC-MSD) 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 positive ion mode, 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. Retention times of dalbergioidin, 2'-hydroxygenistein, daidzein, genistein, coumestrol, kievitone, phaseollidin, phaseollin isoflavan, and phaseollin were respectively 28.85, 30.50, 31.45, 34.48, 37.50, 40.89, 43.51, 44.15, and 45.78 min. Quantification of phytoalexins was carried out 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, dalbergioidin, 2'-hydroxygenistein, coumestrol, and phaseollin in 1, 10, 25, 50, and 100 mg/L concentrations. For phytoalexins without a pure standard phaseollinisoflavan and phaseollidin, and kievitone, concentrations were respectively estimated from the calibration curves for phaseollin, and dalbergioidin, and adjusted on the basis of differences in molecular weight. 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 ± standard deviation.

Antifungal assays

In order to investigate the toxicity of the crude isoflavonoid extracts proceeding from water- and SA-treated hypocotyls/roots against *C. lindemuthianum*, the poisoned food technique was used (Pineda *et al.*, 2012). The fungus was isolated from diseased *P. vulgaris* pods, and morphologically characterized. Resulting extracts from induction experiments obtained as described above (course-time experiments at 48, 96, and 144 h; dose-response assays at 0.72, 1.45, and 3.62 mM, and water-treated hypocotyls/roots) were dissolved in dimethylsulfoxide (DMSO, 70 µL) and diluted in Petri dishes (measuring 5 cm in diameter) with potato-dextrose agar (PDA, 20.0 mL) (concentrations aprox. of 200 to 400 µg/mL). Extracts from treatments and controls (water-treated hypocotyls/roots; incubated after 96 and 144 h) correspond to the same quantity of fresh material (30 g fresh weight). Petri dishes with and without DMSO were used as controls (solvent and absolute control, respectively). The Petri dishes were incubated at room temperature and the diameter of mycelial growth was measured each 24 hours for two days. The relative growth inhibition of the treatments (SA-treated and untreated hypocotyls-roots) compared to the controls was calculated as percentage, using the following formula: Inhibition (%) = [1 – radial growth of treatment (mm)/radial growth of control (mm)] x 100. The results are shown as mean values of three replications [± standard deviation (SD)].

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.

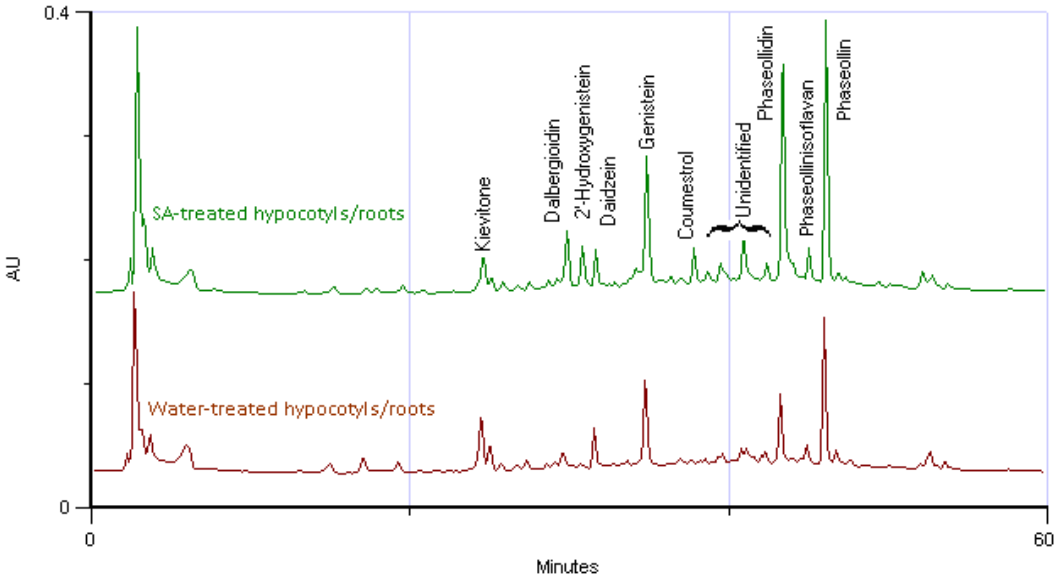
RESULTS AND DISCUSSION

Dose-response experiments

Due to the exogenous application of SA has resulted in phytotoxicity symptoms (Shettel & Balke, 1983; Schreiber & Desveaux, 2008), a dose-response

experiment was initially carried out. The effect of the concentration of SA in the synthesis of isoflavonoids on hypocotyls/roots was evaluated in the range 0.36-14.50 mM and after 96 h post-induction. Hypocotyls/roots treated with distilled water were used as controls. As can be seen from Figure 2, HPLC chromatograms of the isoflavonoids from SA- and distilled water-treated hypocotyls/roots showed generally the same pattern, except for a notorious enlargement of some peaks. Only the major isoflavonoids present in extracts were analyzed, although the total profile of metabolites was more complex. All the isoflavonoids, including those recognized as phytoalexins, were present at much lower concentrations (< 10 µg/g tissue) in tissues treated with distilled water alone. The presence of small quantities of isoflavonoids in common bean hypocotyls/roots treated only with distilled water might correspond to preformed isoflavonoids or some phytoalexins elicited due to micro-injuries and various environmental conditions (air pollutants, radiation). These mechanical micro-injuries may occur during the treatment (separation of cotyledons, and transfer of seedling from the vermiculite bed to plastic trays) and induce non-specific defense responses in tissues due to the release of endogenous elicitors. The injury and some environmental factors have been reported to trigger the accumulation of phytoalexins (Hargreaves & Bailey, 1978). Also, it has been described that bean cells contain a constitutive component capable of eliciting phytoalexin production (Hargreaves & Bailey, 1978). Although not shown, the HPLC profile of hypocotyls/roots induced by SA was much simpler as compared with cotyledons (Durango *et al.*, 2013).

Figure 2
HPLC profiles of induced metabolites in *P. vulgaris* (cv. CORPOICA 106) hypocotyls/roots treated with water (down) and 1.45 mM SA (up), and after 96 h post-induction. Chromatograms were analyzed by UV detection at 270 nm.



As shown in Figure 3, the application of SA induced the accumulation of several isoflavonoids, particularly the isoflavone kievitone, the coumestan coumestrol, and the pterocarpans phaseollidin and phaseollin. Results show that phaseollin and coumestrol were the major isoflavonoids for Cargamanto Mocho and ICA Quimbaya, whereas for Cargamanto Rojo and CORPOICA 106 were kievitone and phaseollidin, respectively. In general, levels of isoflavonoids in hypocotyls/roots increased steadily between 0.36 and 1.45 mM SA, in a dose-dependent manner. All cultivars showed a highly significant increase in coumestrol and phaseollin contents in the SA-treated hypocotyls/roots compared to the distilled water-treated tissues. In anthracnose-susceptible cultivars, coumestrol, phaseollin, and kievitone reached maximum levels at 1.45 mM SA, being about 12 to 15-fold higher than those in water-treated tissues (control).

Similarly, isoflavonoid accumulation in anthracnose-resistant cultivars demonstrated a positive dependence on the concentration of SA at 1.45 mM and below. Thus, coumestrol, phaseollin, and phaseollidin (particularly in CORPOICA 106) contents were progressively increased to reach their maximum concentrations at 1.45 mM SA, being respectively 4, 25, and 60-fold above that in the controls.

Interestingly, at 3.62 mM SA and above, the amount of isoflavonoids decreased rapidly for all cultivars. The reduction in the accumulation of isoflavonoids was accompanied by a loss of weight and seedling vigour along with a discoloration of the tissues, when is compared with the corresponding controls and treatments at 1.45 mM SA and below (*Data not shown*). These findings were in agreement with the fact that SA may cause phytotoxic effects (reduced weight, depigmentation, and wilting) (Hargreaves & Bailey, 1978; Shettel & Balke, 1983). For instance, the SA application prevented growth of seedling and reduced dry weight in corn (*Zea mays* L.), soybean (*Glycine max* L.), oat (*Avena sativa* L.), and three wild plants, which was reported by Shettel & Blake (1983). Likewise, barley plants grown in different SA concentration showed that root development was prevented, and chlorophyll amount decreased parallel to concentration increase, which was observed by Pancheva *et al*. (1996). Similar phytotoxic effects have also been reported with acetyl salicylic acid (Larque-Saavedra, 1978; Canakci & Munzuroglu, 2007). Moreover, the application of SA at high concentrations in plants has led to low phenolic contents (Rajjou *et al.,* 2006; Durango *et al.,* 2013). For instance, dose-response experiments using common bean cotyledons carried out by us have showed that a high amount of SA caused a notorious reduction in the defense-related isoflavonoid concentrations (Durango *et al.,* 2013). Due to the SA is a physiological inhibitor of some enzymes (catalase, ascorbate peroxidase, and a mitochondrial NADH:ubiquinone oxidoreductase), it seems possible to think that high concentrations of elicitor may inhibit important enzymes in the biosynthesis of isoflavonoids. Thus, it has been reported that plants of chickpea (*Cicer arietinum* L.) treated with 2 mM SA showed low activity of peroxidase and polyphenol oxidase (two important enzymes involved in plant defense against biotic and abiotic stresses), possibly due to its phytotoxicity (War *et al.,* 2011). Also, the inhibition of the enzyme catalase might promote the accumulation of H_2O_2, which is toxic for many plant cell functions. However, the molecular mechanism by which high concentrations of SA inhibited isoflavonoid formation is unclear. Since the application of SA at high concentration in common bean tissues [> 3.62 mM for cotyledons (Durango *et al.,* 2013) and > 1.45 mM for hypocotyls/roots] results in the decreased accumulation of isoflavonoid phytoalexins, it seems reasonable to suggest that SA at 1.45 mM or below could be safe to use as elicitor of defense-related isoflavonoids in common bean crops. The aforementioned coincides with the fact that SA can be utilized for the induction of defensive system of chickpea (another legume), being safe at concentrations of 1.5 mM and below, as reported by War *et al*. (2011). Nonetheless, further studies about the long-term physiological effects of SA in common bean seedlings are needed.

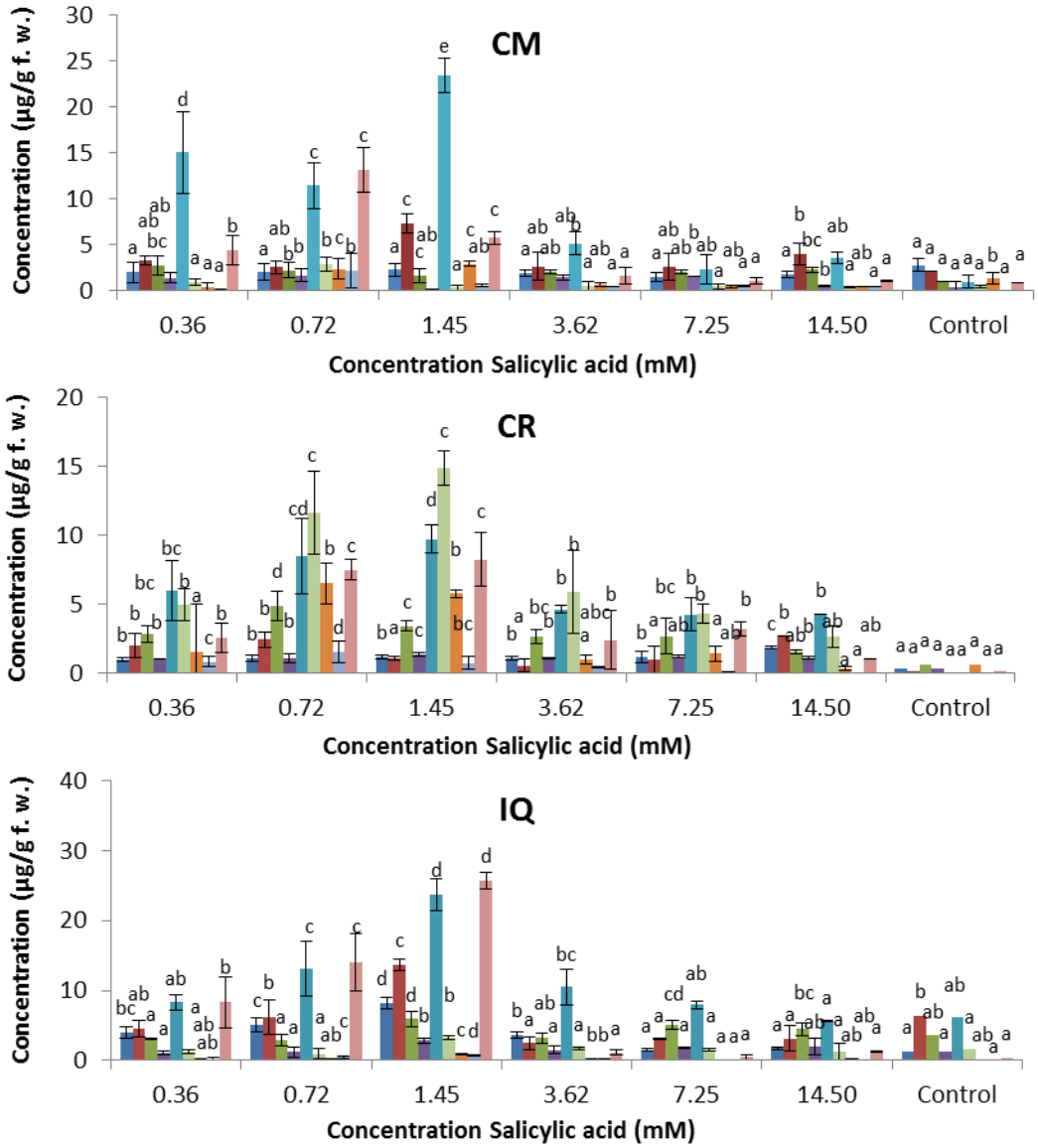
Overall, hypocotyls/roots from anthracnose-resistant cultivars (ICA Quimbaya and CORPOICA 106) accumulated significantly higher amount of phytoalexins as compared to the anthracnose-susceptible cultivars (Cargamanto Mocho and Rojo). For instance, phaseollin and coumestrol reached respectively, maximum levels of about 13 (at 0.72 mM SA) and 12 µg/µ fresh wt (at 1.45 mM SA) for Cargamanto Mocho, and about 23 and 26 µg/µ fresh wt (at 1.45 mM SA) for ICA Quimbaya; almost two times higher phytoalexin production in resistant cultivar in comparison with the susceptible one. Similarly, phaseollidin and phaseollin production in the anthracnose-susceptible cultivar Cargamanto Rojo was lower than that found in CORPOICA 106. It is noteworthy that hypocotyls/roots of cv. CORPOICA 106 accumulated high amounts of phaseollidin, being about 50-fold upper than in the other cultivars. This result is in agreement with an earlier study, in which the major phytoalexin in hypocotyls of the resistant bean cultivar Flor de Mayo after infection with *Colletotrichum lindemuthianum*, was phaseollidin (Soriano-Richards *et al.,* 1998).

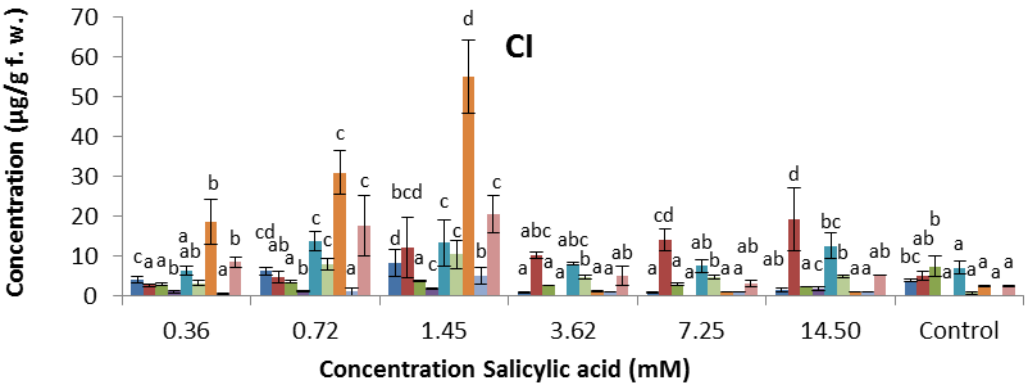
On the other hand, isoflavonoids found in water-treated tissues for both cultivars corresponded mainly to isoflavones daidzein, genistein, 2'-hydroxygenistein, and the isoflavanone dalbergioidin, which are the biosynthetic precursors of the phytoalexins phaseollidin, phaseollin, phaseollinisoflavan, and kievitone. These precursor isoflavonoids were also detected in slightly higher amounts in the water-treated hypocotyls/roots from anthracnose-resistant cultivars. The fact that pytoalexin-precursor isoflavonoids reaches upper basal levels in anthracnose-resistant cultivars compared to susceptible ones, led us to hypothesize that these metabolites could be acting as constitutive isoflavonoids, which could be rapidly

metabolized to the isoflavonoid phytoalexins. Moreover, preformed isoflavones could behave as a first barrier to the pathogenic microorganism, because they have been reported as antifungal compounds (Von Baer *et al.*, 2006). This trait allows to the anthracnose-resistant cultivars to have a first chemical barrier to infection more effective and also, a source of precursors at an upper level which may be rapidly metabolized to the isoflavonoid phytoalexins, like the pterocapans. However, the current study did not permit

Figure 3

Accumulation of defense-related isoflavonoids on hypocotyls/roots of common bean by SA at different concentrations. ■, genistein; ■, dalbergioidin; ■, phaseollinisoflavan; ■, phaseollidin; ■, daidzein; ■, 2'-hydroxygenistein; ■, kievitone; ■, coumestrol; ■, phaseollin. Bars represent the mean concentration of isoflavonoids ± standard deviation (n = 3). Cultivars: CM, Cargamanto Mocho; CR, Cargamanto Rojo; IQ, ICA Quimbaya; CI, CORPOICA 106. For each compound, bars with different letters are significantly different (p = 0.05; Fisher's LSD test).





to determine for sure if these phytoalexin-precursor isoflavones are present in constitutive form on water-treated tissues, or are a rapid response to the environmental or treatment conditions, being faster in anthracnose-resistant cultivars compared to susceptible ones.

Interestingly, under the experimental conditions used, kievitone was detected. This finding contrasts with our previous work in which this phytoalexin was not found in bean seedlings of these same cultivars but treated with $CuCl_2$ (Durango *et al.*, 2002). The above suggests that the kievitone accumulation in common bean might be dependent on the elicitor employed. Also, it has been reported that kievitone reaches higher amounts in bean cotyledons than in hypocotyls (Whitehead *et al.*, 1982). Nonetheless, the kievitone levels found in the present study were substantially lower (< 20 µg/g f.w.) compared with that reported from North American and European cultivars (Hargreaves, 1981; Hynes *et al.*, 1994). For instance, the accumulation of kievitone on wounded cotyledons (white bean cv. OAC Seaforth) following inoculation with *Fusarium solani* f. sp. *phaseoli* reached levels of 850 ± 251 µg/g f.w., which was reported by Hynes *et al.* (1994). Surprisingly, in the present study, the anthracnose-susceptible cultivar Cargamanto Rojo exhibited the highest levels of the isoflavanone phytoalexin kievitone.

In common bean cotyledons, coumestrol was the overwhelmingly predominant isoflavonoid in the anthracnose-resistant cultivars treated with SA under the same conditions (Durango *et al.*, 2002). This coumestan was also present in hypocotyls/roots, but at even lower levels. In addition, phaseollidin was detected in cotyledons cv. CORPOICA 106 as a comparatively minor component. These observations agree with the fact that relative amounts of defense-related isoflavonoids observed in elicited or infected tissue are influenced by the tissue type, as reported by Whitehead *et al*. (1982).

Time-course experiments

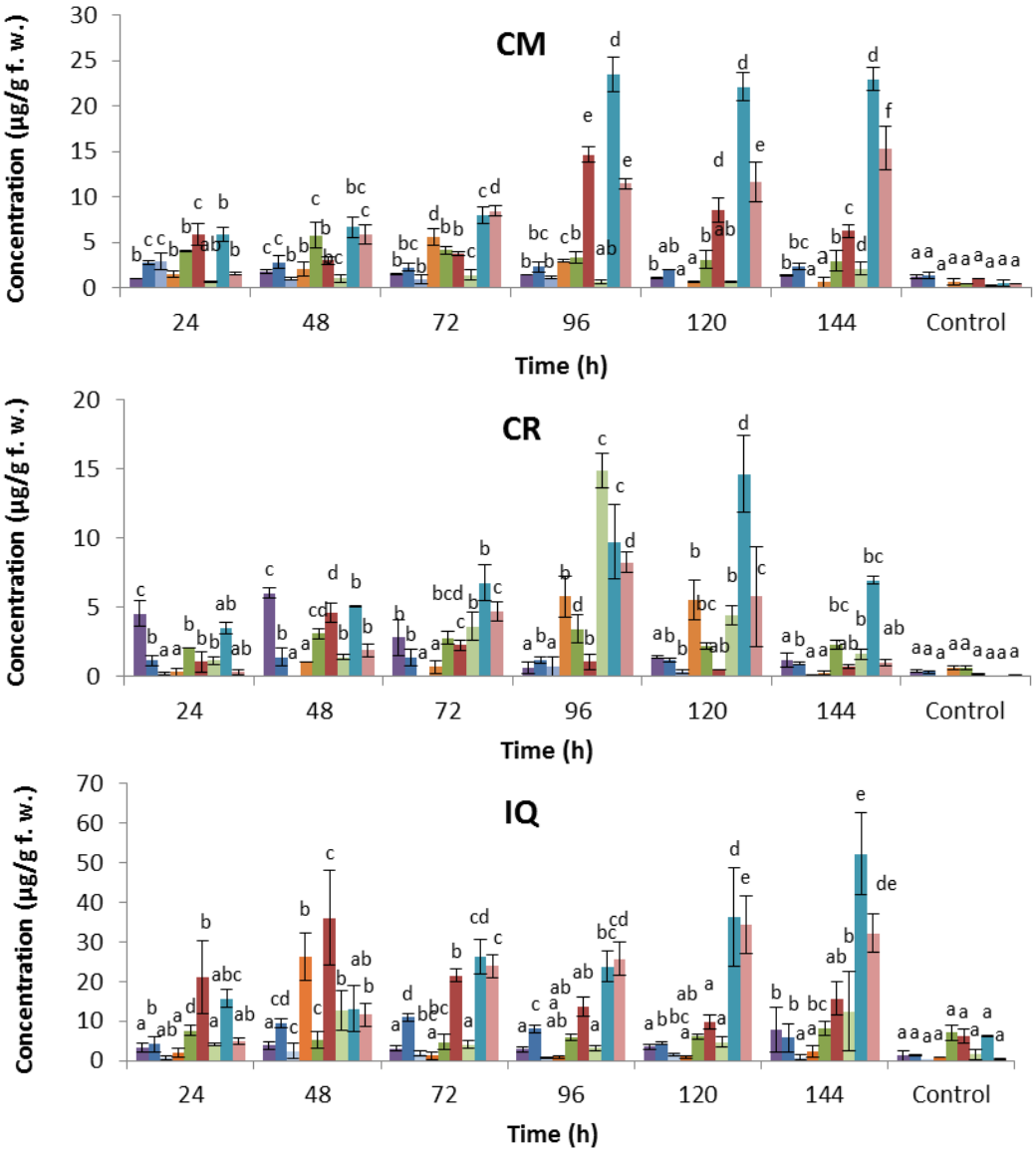
In order to study the accumulation of defense-related isoflavonoids in the time course, hypocotyls/roots of the different bean cultivars were immersed in a solution of 1.45 mM SA and incubated during 144 h. Each 24 h, isoflavonoid concentrations were quantitatively measured by HPLC analysis. The response of hypocotyls/roots of each cultivar to the elicitor is shown in Figure 4. Because distilled water-treated tissues for each day gave the same HPLC readings, these were averaged and referred to as control in Figure 4. Overall, the concentration of pterocarpans (phaseollin and phaseollidin), coumestrol, kievitone, 2'-hydroxigenistein and phaseollinisoflavan was significantly increased with the time. Optimal accumulations of the coumestan and pterocarpans were seen between 96 and 144 h after treatment with SA. Once more, anthracnose-resistant cultivars were able to accumulate isoflavonoids in response to SA at a greater rate than anthracnose-susceptible cultivars. In particular, SA induced the accumulation of coumestrol/phaseollin in cultivars Cargamanto Rojo and Cargamanto Mocho almost 12/8 and 14/14-fold

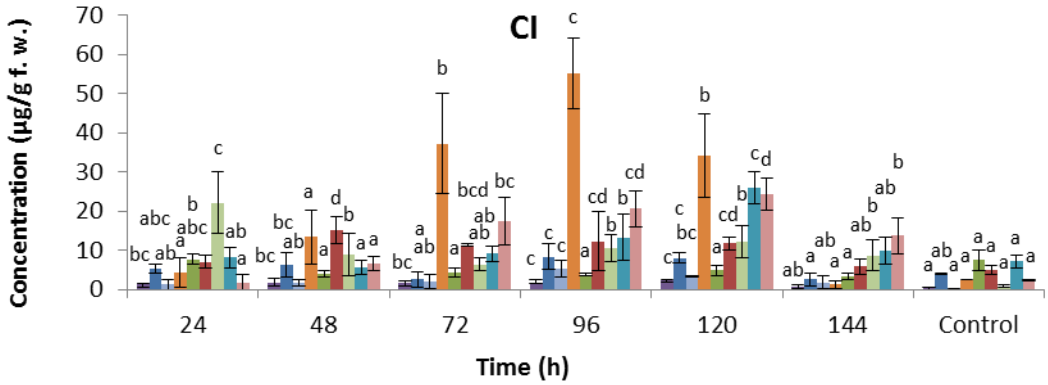
over control, whereas for ICA Quimbaya/CORPOICA 106 were respectively 50/35 and 25/25-fold above the corresponding control. The differences among the defense related-isoflavonoid levels between resistant and susceptible cultivars in response to SA, suggest that this feature could be determinant of the resistance capability in common bean. This fact is in agreement with data obtained from common bean (Durango *et al.*, 2002) and some other crops, e.g., rice (Dillon *et al.*, 1997), chickpea (Haware *et al.*, 1997), alfalfa (Vaziri *et al.*, 1981), sorghum (Nicholson *et al.*, 1987), carnation (Van Peer *et al.*, 1990), citrus (Sulistyowati *et al.*, 1990), among others. Meanwhile the concentration of the isoflavones genistein and daidzein, precursors of phaseollin and kievitone respectively, along with dalbergioidin remained almost constant over the whole period of the evaluation (144 h). Taking in account the 5-deoxy biosynthetic pathway, it seems to be a more efficient conversion from daidzein to coumestrol and phaseollin in the anthracnose-resistant cultivars. Otherwise, the ratio coumestrol/phaseollin was almost the same for all cultivars (between 1.0 and 1.5). So, the two branches of the biosynthetic route of the 5-deoxiisoflavonoides appear equally favored for all cultivars. Furthermore, although the amounts of 2´-hydroxygenistein and dalbergioidin were higher in anthracnose-resistant cultivars than susceptible ones, the maximum kievitone contents detected in the time-course experiments were almost similar in all cultivars (< 15 µg/g f. w.). Only the cultivar Cargamanto Rojo exhibited significantly higher levels of kievitone. Consistent with the 5-hydroxyisoflavonoid biosynthetic pathway (which is believed to be genistein → 2'-hydroxygenistein → dalbergioidin → kievitone), it seems to be certain delay in the biosynthetic step of prenylation in the anthracnose-resistant cultivars and the cv. Cargamanto Mocho. In this latter, the metabolic blocking appears to occur at earlier stages of the biosynthetic pathway (from genistein to 2´-hydroxygenistein), since the low levels of 2'-hydroxygenistein found. In contrast, Cargamanto Rojo show low levels of 2'-hydroxygenistein and dalbergioidin, but high amounts of kievitone in comparison with the other cultivars suggesting higher conversion efficiency.

Interestingly, although ICA Quimbaya and CORPOICA 106 are anthracnose-resistant varieties both exhibited a chromatographic profile quite different; coumestrol and phaseollin were the major phytoalexins for ICA Quimbaya while for CORPOICA 106 was found to be phaseollidin. So, it is possible to think that various isoflavonoid compositions may produce a comparable adverse environment for fungal growth. For cv. CORPOICA 106, there was a rapid increase in the phaseollidin contents within the first 96 h post-induction, reaching its maximum level of about 55.07 µg/g f. w.

Afterward, the phaseollidin level was slowly decreased until 120 h, and then, a strong reduction in the amount was observed. According to Soriano-Richards *et al*. (1998), the quick increase of the precursor phaseollidin sooner than phaseollin and at higher concentration suggests the presence of constitutive pools of phaseollidin in the tissue. They also confirmed the existence of preformed phaseollidin glycosides which may act as biosynthetic precursors. The pterocarpan conjugates could provide a rapid source of phaseollidin which could be mobilized under stress conditions if the availability of carbon sources for phenylpropanoid biosynthesis became rate-limiting (Santana *et al.*, 2004) or metabolized to more active compounds, like phaseollin or phaseollinisoflavan. In general, phaseollidin levels in hypocotyls/roots cv. CORPOICA 106 were considerably higher than that found by us in cotyledons (Durango *et al.*, 2013). On the other hand, the low accumulation of phaseollin accompanied by the high production of its biosynthetic precursor phaseollidin in cv. CORPOICA 106, may suggest failures or delays in biosynthetic step of cyclization and further dehydrogenation of the prenyl side chain. According to our previous study published in 2002, bean seedlings cv. CORPOICA 106 treated with $CuCl_2$ accumulated the highest amount of phaseollin compared to others nine cultivars evaluated (Durango *et al.*, 2002). The rapid conversion of daidzein to phaseollin seems to be lost in cv. CORPOICA 106 during the last 10 years. This finding is in agreement with a study which mentions that the resistant reaction of cv. CORPOICA 106 is not stable (Pedras & Ahiahonu, 2005).

Figure 4
Time-course accumulation of defense-related isoflavonoids in common bean hypocotyls/roots treated with SA. ■, genistein; ■, dalbergioidin; ■, phaseollinisoflavan; ■, phaseollidin; ■, daidzein; ■, 2'-hydroxygenistein; ■, kievitone; ■, coumestrol; ■, phaseollin. Bars represent the mean concentrations of the isoflavonoids ± standard deviation (n = 3). Cultivars: CM, Cargamanto Mocho; CR, Cargamanto Rojo; IQ, ICA Quimbaya; CI, CORPOICA 106. For each compound, bars with different letters are significantly different (p = 0.05; Fisher's LSD test).





The results from the present work and our previous study (Durango *et al.*, 2013) allow us say that common bean tissues (cotyledons and hypocotyls/roots) respond to treatment with SA by synthesis of defense-related isoflavonoids, being the accumulation dependent on the cultivar, the plant tissue, the time of treatment and the concentration of elicitor. Time-course and dose-response studies indicated that the maximum levels of isoflavonoids, particularly those described as phytoalexins, occurred at 1.45 mM SA and between 96 and 144 h post-induction. In general, anthracnose-resistant cultivars accumulated higher defense-related isoflavonoid contents as compared to the susceptible ones. Also, isoflavonoid profiles found in common bean hypocotyls/roots were rather different from that previously reported in cotyledons (Durango *et al.*, 2013). As mentioned above, phytoalexin production at different levels depending on the plant tissue under study has been reported by other authors (Bailey *et al.*, 1976: Goossens *et al.*, 1987; Tangey *et al.*, 2003). It is suggested that exits a relationship between the responses of plant organs related to the formation of phytoalexins and the resistance to organ-specific diseases (Bailey *et al.*, 1976). According to the results found by us, it is possible to use the accumulation of defense-related isoflavonoids in common bean treated by SA as an alternative to detect cultivars with better perspectives of resistance to fungal diseases.

Antifungal effect of extracts from Hypocotyls/Roots treated with SA

Isoflavonoid biosynthesis is believed to be an important defense mechanism of plants in response to pathogenic microorganisms. Therefore, the induction of defense-related isoflavonoids in common bean through the use of elicitors may be a valuable alternative to control some important fungal diseases, including the anthracnose. In order to evaluate if the treatment with the elicitor has effect in the antifungal activity against *C. lindemuthianum*, extracts from Cargamanto Rojo (anthracnose-susceptible) and ICA Quimbaya (anthracnose-resistant) hypocotyls/roots treated with water and SA (at different concentrations: 0.72, 1.45, and 3.62 mM and post-induction times: 48, 96, and 144 h) were analyzed using the poisoned food technique. Since common bean can produce an isoflavonoid mixture, it is possible to think that these compounds act synergistically to produce a more antifungal environment. For above, crude isoflavonoid extracts from tissues treated with water and SA, corresponding to the same quantity of fresh material (30 g fresh weight), were dissolved in dimethylsulfoxide, diluted in Petri dishes with potato-dextrose agar, and used as culture medium. Hypocotyls/roots treated with distilled water and incubated during 96 and 144 h were combined before analysis since they gave the same HPLC readings. Mycelial growth inhibitions were determined after 24 and 48 h. Results of antifungal activity, in terms of radial growth inhibition of *C. lindemuthianum* are summarized in Table 1.

Table 1
Time-course antifungal effect of crude isoflavonoid extracts from hypocotyls/roots of common bean cultivars treated with water or SA at different concentrations and post-induction times. Values are given as mean (± SD) of three experiments. For each column, means with different letters were significantly different according to the least significant difference test ($p = 0.05$).

Material	Concentration of SA (mM)	Time after elicitation (post-induction, h)	Inhibition (%) Evaluation time of fungal growth (incubation time, h)	
			24	48
Cargamanto Rojo	0.72	96	21.7 ± 0.0^{bc}	16.3 ± 3.5^{de}
	1.45	96	34.8 ± 13.0^{d}	16.3 ± 3.5^{de}
	3.62	96	17.4 ± 7.5^{abc}	4.1 ± 3.5^{a}
	1.45	48	17.4 ± 7.5^{abc}	14.3 ± 6.1^{cde}
	1.45	144	21.7 ± 0.0^{bc}	14.3 ± 6.1^{cde}
	Water-treated tissues	96 and 144	13.0 ± 7.5^{ab}	6.1 ± 3.5^{ab}
ICA Quimbaya	0.72	96	26.1 ± 7.5^{cd}	8.2 ± 6.1^{abc}
	1.45	96	34.8 ± 0.0^{d}	20.4 ± 6.1^{e}
	3.62	96	17.4 ± 7.5^{abc}	4.1 ± 3.5^{a}
	1.45	48	17.4 ± 7.5^{abc}	6.1 ± 3.5^{ab}
	1.45	144	26.1 ± 7.5^{cd}	16.3 ± 3.5^{de}
	Water-treated tissues	96 and 144	21.7 ± 0.0^{bc}	6.1 ± 3.5^{ab}
Solvent control			8.7 ± 0.0^{a}	4.1 ± 3.5^{a}

According to Table 1, all crude isoflavonoid extracts displayed a modest toxicity against the microorganism under the conditions used. It is noteworthy that extracts were evaluated without any additional purification and at a relatively low concentration (< 400 µg/mL). Overall, the highest inhibitions occurred during the first 24 h evaluation. Antifungal activity of isoflavonoid extracts from water- and SA-treatments, was comparable with that reported for crude extracts from cowpea, soybean and groundnut cotyledons (three genotypes) inoculated with fungi toward *Curvularia lunata* (percent inhibitions between 20 and 40%) (Tangey *et al.*, 2003). However, isoflavonoid extracts from hypocotyls/roots treated with SA showed a lesser antifungal activity against *C. lindemuthianum* than that exhibited by cotyledon extracts under the same conditions (Durango *et al.*, 2013). It can also be seen from Table 1 that extracts from tissues treated with water and SA exhibited inhibitions of *C. lindemuthianum* higher than that from solvent control (culture medium with solvent).

Data in Table 1 show that antifungal activity was higher in crude isoflavonoids extracted from tissues treated with SA at 1.45 mM, followed by those treated at 0.62 and 3.72 mM for Cargamanto Rojo and ICA Quimbaya. Interestingly, the former also exhibited the highest levels of defense-related isoflavonoids for both cultivars according to the dose-response experiments, being the major constituents found coumestrol and phaseollin (for ICA Quimbaya), and kievitone, phaseollin, coumestrol, and phaseollidin (for Cargamanto Rojo). Thus, it is not possible to ascribe the antifungal activity exclusively to one individual compound. Also, all these compounds have been defined as phytoalexins for common bean and their antimicrobial activity have been well documented (Fett & Osman, 1982). Particularly at 24 h incubation, isoflavonoid extracts from Cargamanto Rojo and ICA Quimbaya treated with 1.45 mM SA, significantly inhibited the radial growth of *C. lindemuthianum* (almost 35%) compared to solvent control and distilled water-treated control. As mentioned above, a significantly increased content of isoflavonoids was

found in 1.45 mM SA-treated tissues as compared to water-treated control hypocotyls/roots. These extracts also showed the highest inhibitory effects after 48 h of incubation (~20% inhibition). Thus, our data indicate that a correlation between the isoflavonoid contents and the inhibitory effect against *C. lindemuthianum* was present.

Comparing the time-course treatments with SA, the higher antifungal activity was exhibited by the isoflavonoid extracts obtained at 96 h post-induction followed by those at 144 and 48 h. Again, inhibitory effects were significantly higher in 1.45 mM SA-treated hypocotyls/roots at 96 h post-induction in comparison to those at 48 and 144 h post-incubation and the controls (water-treated tissues and solvent control) for both cultivars. The above data suggest that the enhanced antifungal activity found to crude extracts from hypocotyls/roots treated with SA at 1.45 mM and 96 h post-induction for both cultivars was in agreement with the highest levels of defense-related isoflavonoids detected.

For Cargamanto Rojo, time-course experiments show that isoflavonoid levels were increased during the first 96 h and then, these were slightly decreased from 96 to 144 h. This is consistent with the variable behavior of inhibitory activity observed for extracts in the time course. Furthermore, crude isoflavonoid mixture extracted from 1.45 mM SA-treated tissues of common bean cv. Cargamanto Rojo at 144 h post-induction exhibited a slightly higher inhibitory activity than water-treated hypocotyls/roots. These extracts are mainly differentiated by the content of coumestrol. Strangely, the crude extracts obtained from cv. ICA Quimbaya treated under the same experimental conditions (1.45 mM SA and 144 h post-incubation) exhibited a slightly lower antifungal activity than that at 96 h post-incubation, despite having very similar chemical profiles. Again, the major difference between isoflavonoid profiles of both treatments appears to be in the accumulation of coumestrol, being greater with the advancement of time. The high concentration of coumestrol in the tissues of common bean cv. ICA Quimbaya treated with 1.45 mM SA and 144 h post-incubation did not seem to contribute appreciably to fungistatic activity. In fact, this coumestan might be exhibiting a subtractive effect in the antifungal activity against *C. lindemuthianum* of crude isoflavonoid extract. This finding contrasts with our previous work, in which extracts from common bean cotyledons cv. ICA Quimbaya having coumestrol as the overwhelmingly predominant component, showed the highest antifungal activity against *C. lindemuthianum* (Durango *et al.*, 2013). The controversial antifungal activity of coumestrol is in agreement with previous studies (Fett & Osman, 1982) and appears contrary to the role of phytoalexins as a defense mechanism against pathogens. Nevertheless, phytoalexins are not only antifungal compounds, but rather they can modulate other events related to plant and other plant-microorganism relationships (Lee *et al.*, 2012). For instance, coumestrol shows antibacterial activity and is able to enhance the nodulation (Lee *et al.*, 2012). On the other hand, extracts from hypocotyls/roots treated with SA at 3.62 mM and 96 h post-induction or 1.45 mM and 48 h post-induction exhibited lower antifungal activity than the other treatments with SA. These extracts also displayed a low amount of isoflavonoids. Therefore, our results from *in vitro* antifungal assay suggest that the SA may enhance the defense-related isoflavonoid levels and so, the defensive capacity of common bean against fungal disease.

Otherwise, crude isoflavonoids from water-treated tissues cv. ICA Quimbaya (anthracnose-resistant) were a little more active against *C. lindemuthianum* than those from cv. Cargamanto Rojo (anthracnose-susceptible). In general, only relatively minor differences (mainly in phytoalexin precursor isoflavones daidzein, 2'.hydroxygenistein, and genistein) are seen between the isoflavonoid profiles of the distilled water-treated hypocotyls/roots for both cultivars, being higher in the anthracnose-resistant cultivar. These compounds could be acting as a first defensive barrier to inhibit the advance of fungi until plants biosynthesize the isoflavonoid phytoalexins. It is not clear, however, whether they are preformed or are induced isoflavonoids for environmental or treatment stress.

Despite the differences observed in phytoalexin levels (time-course and dose-response experiments), our results show that no highly significant differences were found in the antifungal activity against *C. lindemuthianum* between both common bean cultivars treated with SA. However, a little trend toward a higher antifungal activity for the isoflavonoid extracts of ICA Quimbaya was observed. In general, it is not possible to assign the fungistatic activity of bean extracts solely to one or two individual components. It is possible that additive effect between the major constituents, different for both cultivars in this case, may produce a similar antifungal environment. Also, it may indicate that minor components also play a role in the fungistatic effect of the extracts. Likewise, it seems to be that the individual isoflavonoid levels may not be as determinants in the activity against *C. lindemuthianum*,

as is its diversity. For ICA Quimbaya, 1.45 mM SA-treated tissues at 96 and 144 h post-induction show that two metabolites were the overwhelmingly predominant isoflavonoids (coumestrol and phaseollin), thus reducing the chemical diversity. Instead, Cargamanto Rojo under the same conditions shows four isoflavonoids (coumestrol, phaseollin, kievitone, and phaseollin) at almost the same amounts. The biosynthesis of a wide group of isoflavonoids can be beneficial for the plant because it increases the chance that the pathogen is confronted with at least one toxic compound. Similar conclusions were reached by Lindig-Cisneros *et al*. (2002) when isoflavonoid phytoalexins and resistance traits were studied in two systems of wild and cultivated *Phaseolus* spp. seedlings. As they suggested, the effectiveness of the phytoalexin natural mixture depends more on its diversity than its total levels in seedlings. Alternatively, as mentioned above, the accumulation of the same defense-related isoflavonoids but in different proportions could produce a similar fungistatic environment. ICA Quimbaya tissues accumulated higher levels of coumestrol, phaseollin, and 2'-hydroxygenistein compared to Cargamanto Rojo. In contrast, Cargamanto Rojo showed upper amounts of kievitone and phaseollidin (along with lesser levels of coumestrol and phaseollin) with respect ICA Quimbaya. Although levels of kievitone and phaseollidin in Cargamanto Rojo are not as high as those of phaseollin and coumestrol in ICA Quimbaya, the first could be having synergy or additive effects among components, and consequently causing the antifungal activity of both cultivars is nearly equal.

From the results given in Table 1, it can also be seen that inhibitory effects of extracts (from dose-response and course-time experiments) rapidly decreased with the incubation time, a fact that suggests that *C. lindemuthianum* has a rapid adaptation to the medium composition. Indeed, all extracts only inhibited fungal growth by no more than 20% after 48 h. This behavior is in agreement with the recognized ability of plant pathogenic fungi to avoid some of the plant chemical defenses through metabolism and detoxification (Van den Heuvel & Vollaard, 1976). It has been previously reported that different races of *C. lindemuthianum* were capable of metabolizing phaseollin *in vitro* (Burden *et al.,* 1974; Van den Heuvel & Vollaard, 1976). It is important to mention that in plant tissues resisting a fungal attack, the microorganism is exposed to increasing amounts of fungitoxic isoflavonoids, whereas in our *in vitro* bioassay only a specific concentration is employed at the beginning of assay. So, although it is unlikely that the conditions at the infection site can be completely reproduced by the *in vitro* assay, it is possible to think that the conditions during first hours of incubation are more similar than those found *in planta* by the pathogens. Our findings support the fact that before the elicitors can be tested effectively in plant, it is necessary to understand the relationship between the metabolites that they induce and the effects of these metabolites have on fungi.

SA is a well-known natural elicitor involved in the disease resistance of plants. It may enhance the plant defensive capacity against a broad array of pathogens after appropriated treatment. For example, the exogenous application of SA has the capacity to induce resistance to *Colletotrichum lagenarium* in cucumber (Mills & Wood, 1984) and *Erysiphe graminis* f. sp. *tritici* in barley (Walters *et al.,* 1993). Our results from mycelial growth bioassay using the fungus *C. lindemuthianum* show that, extracts from hypocotyls/roots treated with 1.45 mM SA and 96 h post-induction for both cultivars, have a significant higher percent inhibition than extracts from distilled water-treated hypocotyls/roots. These observations suggest that the application of elicitor may enhance the fungitoxic isoflavonoid levels in bean tissues and thus, provides protection from attacks by the microorganism. Furthermore, elicitor concentration and post-induction time were important parameters for the fungitoxic effect observed in the SA-treated bean hypocotyls/roots extracts.

In the current study, hypocotyls/roots of common bean cultivars showed significant increases in the defense-related isoflavonoid contents and the antifungal activity against *C. lindemuthianum* after treatment with SA, in relation to the distilled water-treated control. However, at the concentrations required to induce resistance, SA could exhibit phytotoxicity symptoms in some plant species. So, there is an increasing interest in the search of new functionally and structurally related compounds to SA for controlling important plant diseases. Such compounds should have enhanced properties such as higher water solubility or lower phytotoxicity than SA. Thus, screening programs have identified compounds that mimicked SA function, like 2,6-dichloroisonicotinic acid (INA) and benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester (BTH). Indeed, BTH induced resistance of bean to the rust fungus *Uromyces appendiculatus* (Maffi *et al.,* 2011). In order to determine the most effective compound inducing defense-related isoflavonoids in common bean of a

series of structurally related compounds to SA, a study of structure-activity relationship was carried out. Therefore, the isoflavonoid accumulation on common bean hypocotyls/roots treated with 28 different compounds was evaluated.

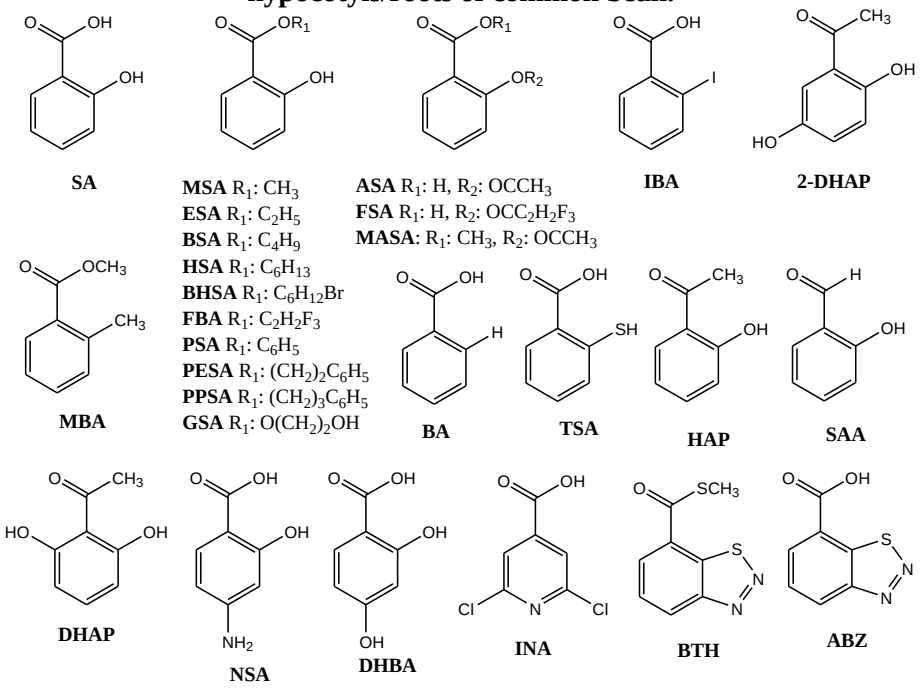
Inducer effect of structurally related compounds to SA

In order to evaluate the accumulation of defense-related isoflavonoids in response to different inducers structurally related to SA (Figure 5), hypocotyls/roots of Cargamanto Mocho and CORPOICA 106 were immersed in solutions at 1.45 mM and 96 h post-induction. Esters were prepared by acylation of corresponding alcohols with SA chloride in anhydrous dichlorometane in the presence of triethylamine at room temperature. SA chlorides were obtained from ASA and oxalyl chloride under an N_2 atmosphere. Induction results are shown in Figure 6 and Table 2. Isoflavones were grouped into three groups according to their structural similarities; isoflavo-nes/isoflavanones, which includes genistein, daidzein, 2'-hydroxygenistein, dalbergioidin, and kievitone; the coumestan, coumestrol, and the prenylated pterocarpans/isoflavan, which includes phaseollin, phaseollidin, and phaseollinisoflavan. The induction of the isoflavonoid phytoalexins, phaseollin, phaseollidin, phaseollinisoflavan, kievitone and coumestrol is highly desirable, since they are the final products of the biosynthetic pathways. In addition, a high amount of isoflavones and isoflavanones involve a source of biosynthetic precursors and an increase in diversity, and consequently in the antifungal activity. As can be seen, chemical profiles of phytoalexins in extracts from hypocotyls/roots of common bean were quantitatively different depending on the cultivar and the elicitor evaluated. Particularly for the cultivar CORPOICA 106, the application of SA and structurally related compounds substantially increased the levels of phaseollidin and phaseollidinisoflavan. For cv. CORPOICA 106, the relation coumestrol/pterocarpans-isoflavan was almost 0.50. However, some elicitors induced lesser relations, i.e. SA (0.16), ASA (0.18), 3,4-DHBA (0.29). This fact shows that the treatment with these elicitors favors the biosynthetic branch of pterocarpans/isoflavan compared with that of coumestrol. The decrease in the relation coumestrol/pterocarpans-isoflavan could enhance the antifungal activity of common bean extracts and consequently the defensive capacity of the plant, as a result of the channeling effect of precursor toward the most active pterocarpanic phytoalexins.

The differential induction using structurally related compounds to SA varied with the substituents on the benzene ring of the BA and SA, the modifications on the chains bearing the carboxylic acid function, and the substitution on C2 (*ortho*-carboxylic group) and of the 2-hydroxyl group. Also, the replacement of the carboxylic acid group for the aldehyde or ketone functions affected the isoflavonoid contents. The higher content of isoflavones/isoflavanones in cultivars CORPOICA 106 and Cargamanto Mocho was obtained by the treatments with SA and TSA respectively. Coumestrol, reached the maximum level when hypocotyls/roots of cultivar CORPOICA 106 were treated with ASA. In contrast, the application of ASA in Cargamanto Mocho resulted in a strong decrease in coumestrol amount, possibly due to phytotoxic effects. On the other hand, ASA exhibited a remarkably inducer effect of prenylated pterocarpans/isoflavan in cv. CORPOICA 106. Furthermore, the highest accumulation of pterocarpans/isoflavan in hypocotyls/roots of Cargamanto Mocho was obtained by response to INA, followed by SAA, DHBA, and SA.

In general, the replacement of the carboxylic acid function by methyl ketones or aldehydes (HAP or SAA) affected the inducer effect. In comparison with SA, these compounds showed similar levels of isoflavones/isoflavanones but slightly lower of coumestrol (for SAA) and pterocarpans/isoflavan (for HAP), when they were applied to bean hypocotyls/roots cv. Cargamanto Mocho.

Figure 5
Structurally related compounds to SA evaluated as potential isoflavonoid-eliciting agents in hypocotyls/roots of common bean.



Also, the isoflavonoid-eliciting activity of SAA in this cultivar was higher than that observed for BTH and ABZ. In addition, although the application of SAA on hypocotyls/roots of cv. Cargamanto Mocho resulted in a weak increase of pterocarpans/isoflavan content as compared to SA, it was found to be much less efficient for inducing these phytoalexins in cv. CORPOICA 106. Moreover, the incorporation of two hydroxyl groups in the benzene ring of the acetophenone (DHAP, 2-DHAP) nearly suppressed the biosynthesis of coumestrol and pterocarpans.

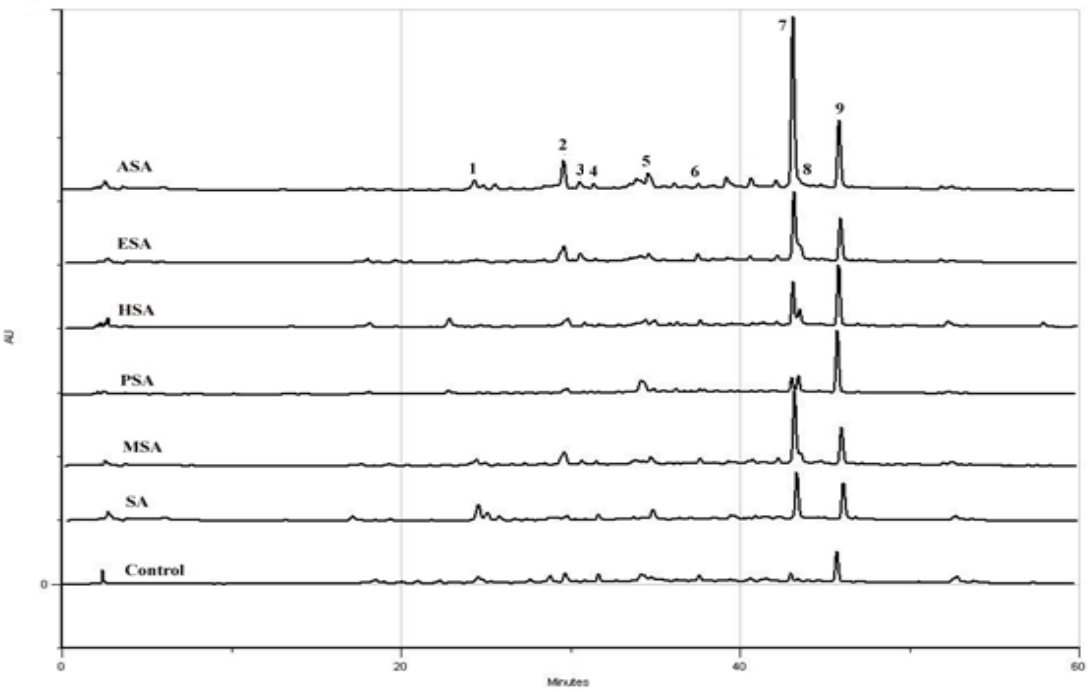
In addition, salicylates (esters MSA, ESA, BSA, HAS, BHSA, FBA, PSA, PESA, PPSA, and GSA) were found to have lower elicitor activity compared with SA. The aliphatic chain bearing the carboxylic acid function reduced the isoflavones/isoflavanones and pterocarpans/isoflavan levels. Nonetheless, coumestrol content in hypocotyls/roots treated with salicylates was similar (for cv. Cargamanto Mocho) or slightly higher (for cv. CORPOICA 106) than that reached in response to SA. Also, the elongation of the carbon chain attached to the carbonyl group decreased the elicitor effect. For instance, HAS (a compound with a carbon chain having six atoms) showed a lesser isoflavonoid-eliciting effect. Nonetheless, some of the compounds evaluated were elicitors comparable or more potent than the wide recognized inducers, BTH or INA. Unfortunately, the length of the alkyl side-chain affects the water solubility of these compounds, which in some cases limits their use as elicitors. In contrast, methyl salicylate which is implicated in the systemically acquired resistance (SAR), induced high contents of coumestrol and pterocarpans/isoflavan; this compound would have the additional advantage of acting on neighboring plants. The incorporation of a bromide group in the carbon chain of HAS (like BHSA) resulted in weak increases of coumestrol and pterocarpans/isoflavan content.

Table 2
Elicitor effect of related structurally compounds to SA (at 1.45 mM and after 96 h post-induction) in hypocotyls/roots of CORPOICA 106 and Cargamanto Mocho

Compound	CORPOICA 106			CARGAMANTO MOCHO		
	Isoflavones/ Isoflavanones*	Coumestan	Prenylated pterocarpans/Isoflavan†	Isoflavones/ Isoflavanones*	Coumestan	Prenylated pterocarpans/ Isoflavan†
AS	36.90 ± 14.83^{a,c,d}	13.36 ± 5.80^{c}	84.22 ± 15.83^{a,c,d}	21.89 ± 2.21^{a,b,c,d}	23.48 ± 1.90^{a,b,c,c}	15.62 ± 0.80^{a,b,d}
ASA	16.23 ± 5.82^{c}	16.62 ± 4.45^{a}	94.81 ± 34.07^{a,c,d}	13.82 ± 3.02^{a,c}	3.28 ± 0.50^{b,c,d}	10.79 ± 0.36^{a,b}
MSA	13.84 ± 3.21^{c}	25.41 ± 2.13^{a,d}	51.28 ± 7.65^{a,c,d}	8.35 ± 2.21^{b}	20.17 ± 3.45^{a,c,d}	6.35 ± 1.17^{a,b,c}
ESA	25.18 ± 4.32^{c,d}	26.83 ± 5.68^{a,d}	59.70 ± 9.67^{a,c,d}	7.94 ± 1.75^{b}	19.28 ± 1.25^{a,c}	7.39 ± 1.61^{a,b,c}
BSA	14.31 ± 3.54^{c}	21.69 ± 4.14^{a,d}	47.52 ± 7.33^{a,c,d}	n.d.	n.d.	n.d.
HSA	8.61 ± 2.33	3.25 ± 1.23^{c,d}	16.40 ± 4.14^{a}	12.85 ± 0.87^{a,c}	5.29 ± 3.12^{a,b,d}	3.31 ± 1.30^{b,c,d}
BHSA	9.48 ± 3.14	10.11 ± 3.21^{c}	25.57 ± 5.98^{a,d}	17.42 ± 5.60^{a,c,d}	10.08 ± 2.37^{a,b,d}	4.22 ± 1.99^{b,c,d}
FBA	n.d.	n.d.	n.d.	20.78 ± 3.29^{a,b,c,d}	42.32 ± 9.45^{a,b,c,d}	7.66 ± 1.30^{a,b,c}
GSA	n.d.	n.d.	n.d.	20.91 ± 3.02^{a,b,c,d}	4.21 ± 2.33^{b,d}	1.43 ± 0.61^{b,c,d}
PSA	8.49 ± 2.45	14.19 ± 3.45^{c}	36.53 ± 6.78^{a,c,d}	n.d.	n.d.	n.d.
PPSA	16.42 ± 4.44^{c}	30.18 ± 4.78^{a,c,d}	22.65 ± 5.25^{a,d}	18.05 ± 3.36^{a,c,d}	53.05 ± 23.84^{a,b,c,d}	4.37 ± 1.23^{b,c,d}
PESA	n.d.	n.d.	n.d.	13.83 ± 4.97^{a,c,d}	3.96 ± 1.85^{b,c,d}	1.09 ± 0.97^{b,c,d}
MASA	11.16 ± 1.30^{a}	4.59 ± 2.09^{c,d}	2.98 ± 0.36^{c,d}	n.d.	n.d.	n.d.
MBA	8.84 ± 2.12^{a,d}	1.18 ± 0.26^{a,c,d}	5.49 ± 5.95^{c,d}	11.98 ± 1.31^{a,c}	2.93 ± 0.02^{b,c,d}	3.68 ± 2.07^{b,c}
BA	15.33 ± 4.12^{c}	6.53 ± 1.23^{c,d}	14.67 ± 2.87^{a,c}	11.16 ± 2.35^{a,b,c}	9.28 ± 1.91^{a,b}	12.89 ± 4.69^{a,b}
TSA	18.49 ± 3.17^{c}	8.49 ± 0.87^{c}	17.73 ± 5.36^{a}	24.70 ± 9.22^{a,b,c,d}	12.54 ± 1.56^{a}	9.81 ± 2.93^{a,b}
IBA	22.03 ± 3.82^{c,d}	11.70 ± 0.07^{a,c}	30.20 ± 10.03^{a,d}	17.33 ± 5.48^{a,c}	5.70 ± 1.39^{b,c,d}	4.43 ± 2.29^{b,c}
FSA	8.29 ± 1.54^{a,d}	3.00 ± 2.09^{a,c,d}	0.97 ± 0.28^{c,d}	10.74 ± 6.14^{a}	10.52 ± 7.04^{a}	1.25 ± 1.38^{b,c,d}
2,5-DHBA	15.06 ± 2.39^{c}	8.81 ± 1.77^{c}	24.78 ± 5.06^{a,d}	20.79 ± 1.15^{a,c,d}	36.99 ± 3.16^{a,b,c,d}	17.71 ± 3.81^{a,d}
3,4-DHBA	18.20 ± 3.72^{c}	11.13 ± 2.47^{c}	8.46 ± 13.27^{a,c,d}	n.d.	n.d.	n.d.
NSA	13.08 ± 1.07^{c}	5.51 ± 1.24^{c,d}	24.15 ± 0.23^{a,d}	12.63 ± 3.24^{a,c}	11.09 ± 6.82^{a}	3.89 ± 2.81^{b,c,d}
HAP	n.d.	n.d.	n.d.	18.47 ± 4.59^{a,c}	19.02 ± 12.89^{a,c}	10.14 ± 2.25^{a,b}
DHAP	n.d.	n.d.	n.d.	24.58 ± 10.59^{a,b,c,d}	2.54 ± 0.59^{b,c,d}	4.24 ± 0.69^{b,c}
2-DHAP	n.d.	n.d.	n.d.	13.85 ± 8.01^{a,c}	15.46 ± 5.33^{a,c}	7.47 ± 1.82^{a,b,c}
SAA	16.62 ± 3.71^{c}	8.13 ± 2.13^{c}	14.80 ± 1.12^{a,c}	17.43 ± 4.45^{a,c}	14.45 ± 1.13^{a,c}	17.95 ± 2.94^{a,d}
INA	n.d.	n.d.	n.d.	14.64 ± 2.53^{a,c}	18.58 ± 5.14^{a,c}	22.23 ± 7.04^{a,c,d}
ABZ	14.16 ± 1.69^{c}	12.23 ± 4.20^{a,c}	12.98 ± 5.54^{a,c}	10.58 ± 1.58^{a}	15.80 ± 0.52^{a,c}	9.32 ± 0.41^{a,b,c}
BTH	7.77 ± 0.54^{a,d}	21.82 ± 5.65^{a,d}	22.24 ± 1.37^{a,d}	6.73 ± 1.35	8.46 ± 1.84^{a,b,d}	14.03 ± 2.91^{a,b,d}
Control	17.53 ± 4.37^{c}	7.21 ± 1.69^{c,d}	3.19 ± 0.28^{c,d}	4.48 ± 0.67	0.52 ± 0.37^{b,c,d}	1.15 ± 0.32^{b,c,d}

Data correspond to the mean concentrations of the grouped isoflavonoids ± standard deviation (n = 3). * Dalbergioidin, 2'-hydroxygenistein, daidzein, genistein, kievitone. † Phaseollidin, phaseollinisoflavan, phaseollin. n.d. not determined. Significant difference (p = 0.05) between treatment and control (^{a}), INA (^{b}), BHT (^{c}), and ABZ (^{d}).

Figure 6
HPLC profiles of the induced isoflavonoids after treatment of common bean (cv. CORPOICA 106) hypocotyls/roots with structurally related compounds to SA (1.45 mM and 96 h post-induction). Control: distilled water-treated hypocotyls/roots; SA: salicylic acid; MSA: methyl salicylic acid; PSA: phenyl salicylic acid; HAS: hexyl salicylic acid; ESA: ethyl salicylic acid; ASA: acetyl salicylic acid. 1: kievitone; 2: dalbergioidin; 3: 2'-hydroxygenistein; 4: daidzein; 5: genistein; 6: coumestrol; 7: phaseollidin; 8: phaseollin isoflavan; 9: phaseollin.



The data also show that the addition of a hydroxyl or phenyl group to the ethyl chain bearing the carboxylic acid function (ESA vs. GSA or PESA) abolished the production of coumestrol and pterocarpans/isoflavan. In contrast, the substitution of hydrogen atoms in the methyl group by flourine on the ethyl chain (ESA vs. FBA) was found to improve the isoflavonoid content in hypocotyls/roots of Cargamanto Mocho. Otherwise, the presence of a benzene ring attached in the carbon chain showed unpredictable behaviors; whereas PPSA induced higher coumestrol content than SA, the compounds PSA and PESA were much less effective.

On the other hand, a comparison in the inducing effect between compounds which have different substituents in the *ortho*-position to carboxylic group of BA (SA, TSA, IBA, ASA, FSA) showed that ASA was the best elicitor of pterocarpans/isoflavan in cv. CORPOICA 106, followed by SA. Nonetheless, the accumulation of isoflavones/isoflavanones, coumestrol, and pterocarpans/isoflavan in response to AS was slightly higher than that for ASA in cv. Cargamanto Mocho. The replacement of hydroxyl group in C2 on SA by H- (BA), HS- (TSA), and I- (IBA), resulted in a reduction of phytoalexin-eliciting effect, especially of coumestrol and pterocarpans/isoflavan, compared to SA. Hypocotyls/roots of cultivar CORPOICA 106 treated with compounds having different substituents in C2 accumulated higher contents of pterocarpans/isoflavan and coumestrol using IBA, followed by TSA, BA, and FSA. Meanwhile, the accumulation of pterocarpans/isoflavan in bean hypocotyls/roots cv. Cargamanto Mocho was higher for BA, followed by TSA, IBA, and FSA. In fact, the presence of the substituent trichloroethoxy (FSA) or iodine (IBA) in

ortho-position almost abolished the elicitor effect in cv. Cargamanto Mocho. Also, the insertion of acetyl group along with the methyl chain attached to the carboxylic acid function (MASA) extinguished the isoflavonoid-eliciting effect. A similar effect was observed with MBA. Overall, the isoflavonoid accumulation in response to compounds having different substituents in C2 was contradictory.

Results also show that the attachment of an electron-donor substituent to the aromatic system of SA, affects the elicitor activity. Thus, the addition of an amino group in C4 (NSA) or a hydroxyl group in C5 (2,5-DHBA) decreased the isoflavonoid-inducing effect as compared to AS. However, pterocarpans/isoflavan levels were found to be similar to that on bean hypocotyls/roots cv. CORPOICA 106 treated with BTH. Interestingly, the 3,4-dihydroxy substitution pattern (3,4-DHBA) was also effective to induce pterocarpans/isoflavan in a comparable level to that elicited by BTH. Our results suggest that isoflavonoid production induced by SA and structurally-related compounds maybe differentially controlled.

CONCLUSIONS

According to dose-response experiments, the application of SA at 1.45 mM and below increased the defense-related isoflavonoid production in a dose-dependent manner. However, the major isoflavonoids were different depending on the variety evaluated. Thus, the phytoalexins coumestrol and phaseollin were the major isoflavonoids for Cargamanto Mocho and ICA Quimbaya whereas for Cargamanto Rojo and CORPOICA 106 were kievitone and phaseollidin, respectively. These compounds reached their maximum levels at concentrations between 0.62 and 1.45 mM SA. From these results, it may be suggested that SA at 1.45 mM or below could be used for the induction of defense-related isoflavonoids in common bean, and consequently, to enhance the resistance to pathogens. In relation to time-course experiments, there was steady increase in phytoalexin contents (particularly coumestrol and phaseollin) in all varieties. Depending on the variety, the maximum levels of phytoalexins were reached between 96 and 144 h. However, biosynthesis of isoflavonoid phytoalexins in anthracnose-resistant cultivars was faster that in susceptible ones. Even so, the present study indicates that the activity of extracts from hypocotyls/roots of both cultivars treated with SA against *C. lindemuthianum* was only slightly enhanced. The data show that the most active extracts against *C. lindemuthianum*, were those that accumulated the highest levels of isoflavonoid phytoalexins. However, the inhibitory effect against the pathogenic microorganism decreased in the time-course suggesting a possible detoxification mechanism. Additionally, the structure-activity relationship analysis showed that phytoalexins content varied with the substituents on the benzene ring of the BA and SA, the modifications on the chains bearing the carboxylic acid function, and the substitution on C2 (*ortho*-carboxylic group) and of the 2-hydroxyl group. Also, the replacement of the carboxylic acid group for the aldehyde or ketone functions affected the isoflavonoid contents. Thus, isoflavonoid-eliciting effect was decreased in comparison to SA by: the addition of a hydroxyl group in C5 or amino in C4, the esterification of the carboxylic acid function, the replacement of the 2-hydroxyl substituent by SH-, H-, I-, CH_3O-, CF_3CH_2O-, among others. Esters of SA were found to have lower isoflavonoid-inducing activity compared to the SA; the inducer effect was found to be depending on the length of the carbon chain attached to the carbonyl group. Nonetheless, some of the compounds evaluated were elicitors similar or more potent than the wide recognized inducers, like BTH or INA. The alkylation of the hydroxyl group along with the esterification of carboxylic acid group almost abolished the inducer effect. Acetylsalicylic acid showed a strong isoflavonoid-eliciting effect in common bean cv. CORPOICA 106.

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