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Increased accumulation of isoflavonoids in common bean (*Phaseolus vulgaris* L.) tissues treated with 1-oxo-indane-4-carboxylic acid derivatives

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ABSTRACT

Isoflavonoid phytoalexins (isoflavones: genistein, 2′-hydroxygenistein, and daidzein; isoflavanones: dalbergioidin and kievitone; coumestrol; pterocarpans: phaseollidin and phaseollin; and the isoflavan: phaseollinisoflavan) production in response to the application of eleven 1-oxo-indane-4-carboxylic acid derivatives (indanoyl esters and indanoyl amino acids conjugates), in cotyledons and hypocotyl/root of two common bean (*Phaseolus vulgaris* L.) cultivars was evaluated. The content of isoflavonoids depended on the cultivar, the treated tissue, the time after induction, the structure and concentration of the elicitor. The highest isoflavonoid contents were found when 1-oxo-indanoyl-amino acids conjugates were used as elicitors. Cotyledons and hypocotyl/root of the anthracnose-resistant cultivar produced significantly higher isoflavonoid contents as compared to the susceptible one. Maximum levels of phaseollin were obtained using 0.66 mM 1-oxo-indanoyl-L-isoleucyl methyl ester and between 72 and 96 h post-induction. So, 1-oxo-indane-4-carboxylic acid derivatives, may be used to enhance the amount of isoflavonoid phytoalexins in common bean and protect crops from phytopathogenic microorganisms.
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1. Introduction

Within the grain legumes, the common bean (*Phaseolus vulgaris* L.) is the most important for human consumption [1]. This leguminous is cultivated in 129 countries on five continents and consumed by almost 500 million people in the tropics [2]. Latin America is the area of greatest production and consumption; it is estimated that more than 45 % comes from this region where it is considered one of the basic products of the peasant economy [3]. In addition, the common bean provides a highly nutritious food, which contains protein, fiber, complex carbohydrates, vitamins and micronutrients [4]. Unfortunately, a fungal disease called anthracnose (causal agent *Colletotrichum lindemuthianum* [Sacc. & Magn.] Bri. and Cavi.) seriously affects this crop, causing losses that may exceed 90 % [5,6]. Anthracnose causes dark brown necrotic lesions and decreases the photosynthetic activity, which induces early senescence of the leaves and the death of the plant [7,8]. Traditionally, the disease has been successfully controlled by the application of fungicidal substances. Nevertheless, some of these compounds can cause adverse effects on human health and the environment, for their non-selective and biocide characteristics. In addition, pathogenic microorganisms have developed resistance to various fungicidal agents demanding the use of highest doses and most frequent applications in the crop. Therefore, new compounds and modes of action are required. Induced resistance appears as a new, effective and environmentally friendly alternative for the control of diseases in plants. In response to attack by pathogens, plants produce a wide array of secondary metabolites with antimicrobial properties. These compounds may act as constitutive (phytoanticipins) or induced (phytoalexins) chemical barriers [9]. In common bean, different isoflavonoid compounds have been recognized as phytoalexins (Fig. 1); their highest levels and fastest accumulation has been related with resistant cultivars to diseases [10,11].

These antimicrobial compounds can be biosynthesized and accumulated as a result of the infection or through the exogenous application of some compounds, called elicitors. Thus, elicitors can protect the plant against subsequent infections by pathogens. Additionally, elicitors have a non-biocidal character, which offers them ecological advantages over fungicide agents [12,13]. Moreover, elicitors can be used for selecting disease resistant

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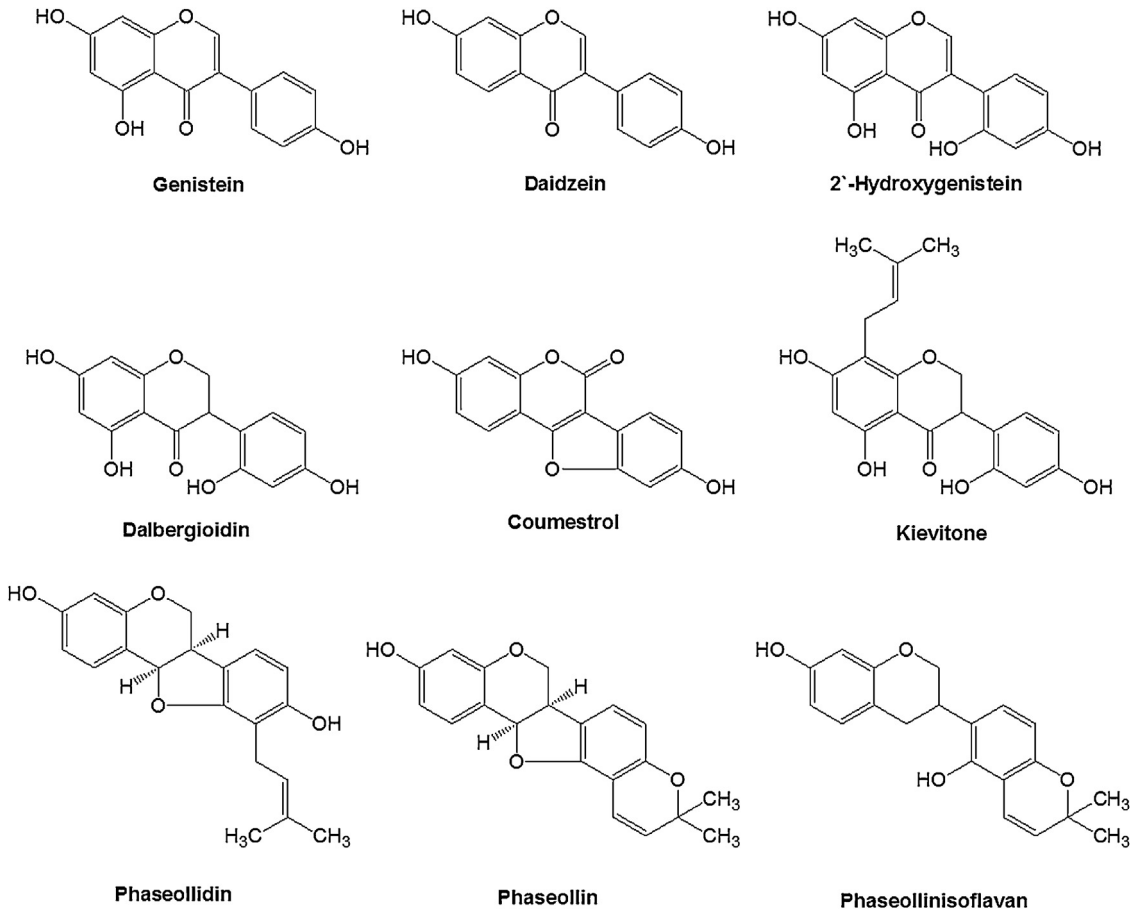

Fig. 1. Structure of phytoalexins from Common bean (*Phaseolus vulgaris* L.).

cultivars based on the phytoalexin production, and provide valuable information about how plants response to pathogens.

Jasmonic acid (**JA**) and methyl jasmonate (**MeJA**) are cyclopentanone compounds that act as plant hormones and elicitors. Both compounds are known to play an important role in many physiological processes in plant growth and development, and especially in plant responses to biotic and abiotic stresses. Exogenous applications of **JA** and **MeJA** have caused efficient reduction of diseases development [14]. Interestingly, a bacterial toxin named coronatine (Fig. 2) that structurally and functionally mimics **MeJA**, elicited a number of responses that are also induced by treatment with **JA**, including phytoalexins. In fact, coronatine was much more active than **JA** in eliciting glyceollins (pterocarpan-type phytoalexins) accumulation in soybean (*Glycine max* L.) cell cultures [15]. Coronatine stimulates the phytoalexin accumulation in rice leaves (*Oryza sativa* L.) [16], volatiles in Lima bean (*Phaseolus lunatus* L.) [17,18], and proteinase inhibitors [19], which are stress responses associated with herbivory. In addition, it has been shown that structurally-related indanoyl derivatives with coronatine, like as a 6-ethyl indanoyl-isoleucine conjugate (coronalon), elicit the accumulation of defense-related secondary metabolites in both cell cultures and plant tissues, and the stress-related genes expression [20,21]. In the present work, a series of indanoyl derivatives was prepared and its phytoalexins-elicitor effect in seedlings of two common bean cultivars grown in Colombia was evaluated.

2. Materials and methods

2.1. General methods

Reactions were monitored by Thin Layer Chromatography (TLC) on silica gel plates (60 F_{254}). As mobile phase, it were used mixtures

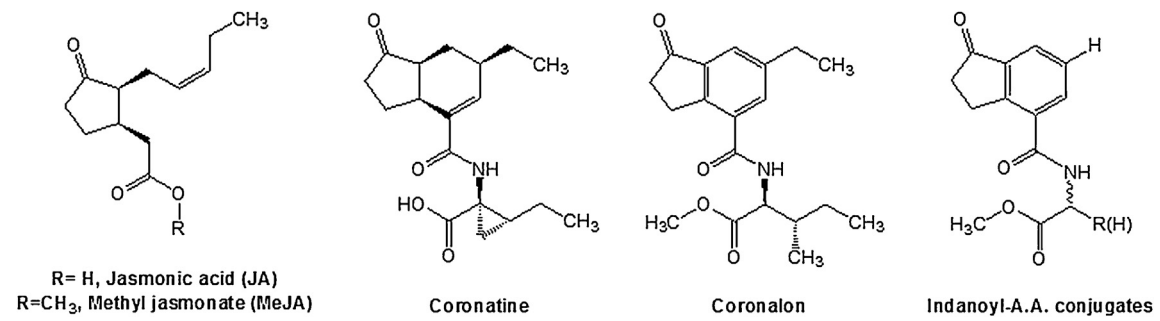

Fig. 2. Structure of jasmonic acid (**JA**), methyl jasmonate (**MeJA**), coronatine, coronalon and indanoyl-amino acid conjugates.

of organic solvents: *n*-hexane-ethyl acetate (EtOAc), 60:40; $CHCl_3$-acetone, 80:20; ethyl ether-*n*-hexane, 70:30 and dichloromethane (DCM)-methanol (MeOH), 98:2. The compounds were visualized under UV radiation (model lamp UVGL-58, Multiband UV-254/365 nm), and by spraying with a solution of acetic acid, sulfuric acid and water, 43:28:30, followed by a soft heating. ^{1}H NMR and ^{13}C NMR spectra were determined in $CDCl_3$ or methanol-d_4 solution by using a Bruker AMX 300 (300 MHz for ^{1}H and 75.42 MHz for ^{13}C) spectrometer. Chemical shifts are reported in δ (ppm) and coupling constants (J) in Hz. The determination of the carbon types present in the molecule was made by DEPT 135/JMOD experiments. Mass spectrometry using fast-atom bombardment (positive-ion FAB-MS) of low and high resolution was carried out on a JEOL JMS-700 spectrometer (Jeol, Tokyo, Japan). The matrix used was 3-nitro-benzylalcohol.

2.2. Chemicals

Isoflavones, genistein and daidzein, were purchased from Sigma-Aldrich (St Louis, MO, USA). Dalbergioidin, 2′-hydroxygenistein, coumestrol, phaseollidin, phaseollinisoflavan, kievitone and phaseollin were obtained and identified from a previous work described by Durango et al. [22]. Methyl jasmonate (**MeJA**), palladium on carbon and oxalyl chloride was acquired from Sigma-Aldrich Co. (St Louis, MO, USA), whereas jasmonic acid (**JA**), anhydrous aluminum chloride ($AlCl_3$) and 2-carboxycinnamic acid were bought in Alfa Aesar Co. (Ward Hill, MA, USA). 1-oxo-4-indanoyl carboxylic acid was prepared by conventional organic reactions [23].

2.3. Plant material

Seeds of common bean, cultivars Cargamanto Blanco and ICA Quimbaya, were obtained from Semillas & Semillas Ltda. (Medellín, Colombia) and Semicol Ltda (Bogotá, Colombia), respectively. Cargamanto Blanco has high susceptibility to diseases, including anthracnose [24]. ICA Quimbaya is a cultivar resistant to the prevalent anthracnose races (*C. lindemuthianum* Sacc.), and tolerant to Rust (*Uromyces appendiculatus* (Pers.) Unger) and Angular Spot (*Phaeoisariopsis griseola* (Sacc.) Ferraris) [25]. Seeds of each cultivar were surface-sterilized for 10 min in sodium hypochlorite (NaClO, 5%), washed with distilled water and sown in quartz sand. After 6 days in darkness, germinated seedlings were removed from the sand bed to be used in induction experiments.

2.4. Preparation and characterization of indanoyl derivatives

Initially, 2-carboxycinnamic acid was reduced using hydrogen and palladium on carbon as catalyst (yield: 95 %). Then, the indanone skeleton was achieved via Friedel-Crafts intramolecular cyclization of 2-carboxyhydrocinnamic acid using anhydrous aluminum chloride ($AlCl_3$) (yield: 63 %). Next, the resulting 1-oxo-indane-4-carboxylic acid was put into a reaction with alkyl halides on presence of *N,N*-dimethylformamide (DMF). In addition, some esters were obtained by Fisher esterification refluxing 1-oxo-4-indane carboxylic acid with the appropriate alcohol and catalytic amounts of sulfuric acid. Also, indanoyl amino acid conjugates were synthesized using *N,N*'-dicyclohexylcarbodiimide (DCC) [23]. The prepared indanoyl derivatives corresponded to methyl 1-oxo-indane-4-carboxylate (**1**), ethyl 1-oxo-indane-4-carboxylate (**2**), isopropyl 1-oxo-indane-4-carboxylate (**3**), 2′,2′,2′-trifluoroethyl 1-oxo-indane-4-carboxylate (**4**), 2-isopentenyl 1-oxo-indane-4-carboxylate (**5**), butyl 1-oxo-indane-4-carboxylate (**6**), 2′-bromoethyl 1-oxo-indane-4-carboxylate (**7**), 4′-bromobutyl 1-oxo-indane-4-carboxylate (**8**), methyl 1-hydroxy-indane-4-carboxylate (**9**), 1-oxo-indanoyl-L-isoleucyl methyl ester (**C-1**), and 1-oxo-indanoyl-L-leucyl methyl ester (**C-2**).

2.4.1. Preparation of esters by Fischer esterification

A mixture of 1-oxo-indane-4 carboxylic acid with different alcohols (methanol, ethanol, 2-propanol, 1-butanol, 2,2,2-trifluoroethanol) and a few drops of sulfuric acid as catalyst was reacted under ambient temperature conditions and constant stirring. The resulting product was neutralized by sodium bicarbonate ($NaHCO_3$) and extracted with EtOAc. The unreacted alcohol was removed by reduced pressure distillation and the amorphous solid was subjected to purification by column chromatography. Spectroscopic properties of synthesize compounds are presented below.

*Compound (**1**).* Methyl 1-oxo-indane-4-carboxylate. Yield: 61 %. Yellow oil. IR (KBr, ū, cm^{-1}): 1705, 1595, 1260, 1120. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.78 (2H, t, J =6.0 Hz, H_2), 3.56 (2H, t, J =6.0 Hz, H_3), 4.01 (3H, s, $H_{1'}$), 7.54 (1H, t, J =7.8 Hz, H_6), 8.00 (1H, d, J =7.8 Hz, H_7), 8.34 (1H, d, J =7.8 Hz, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 27.2 (C_3), 36.1 (C_2), 52.2 ($C_{1'}$), 127.6 (C_6), 128.1 (C_7), 128.3 (C_{7a}), 136.4 (C_5), 138.4 (C_4), 156.7 (C_{3a}), 166.3 (−COO-), 206.6 (C_1). *Compound (**2**).* Ethyl 1-oxo-indane-4-carboxylate. Yield: 59 %. Yellow oil. IR (KBr, ū, cm^{-1}): 1705, 1590, 1250, 1150. ^{1}H NMR ($CDCl_3$, 300 MHz): 1.48 (3H, t, J =7.2 Hz, $H_{2'}$), 2.77 (2H, dd, J =6.0 Hz, 3.6 Hz, H_2), 3.55 (2H, t, J =6.0 Hz, H_3), 4.46 (2H, q, J =7.2 Hz, $H_{1'}$), 7.53 (1H, t, J =7.8 Hz, H_6), 8.00 (1H, d, J =7.8 Hz, H_7), 8.33 (1H, d, J =7.8 Hz, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 14.4 ($C_{2'}$), 27.3 (C_3), 36.2 (C_2), 61.2 ($C_{1'}$), 127.6 (C_6), 128.0 (C_7), 128.6 (C_{7a}), 136.4 (C_5), 138.4 (C_4), 154.2 (C_{3a}), 165.8 (−COO-), 206.5 (C_1). *Compound (**3**).* Isopropyl 1-oxo-indane-4-carboxylate. Yield: 41 %. Yellow oil. IR (KBr, ū, cm^{-1}): 1710, 1590, 1250, 1160, ^{1}H NMR ($CDCl_3$, 300 MHz): 1.45 (6H, d, J =6.3 Hz, 2xCH_3), 2.77 (2H, ddd, J =6.0 Hz, 5.7 Hz, 3.0 Hz, H_2), 3.55 (2H, t, J =6.0 Hz, H_3), 5.35 (1H, sept., J =6.3 Hz, $H_{1'}$), 7.53 (1H, t, J =7.8 Hz, H_6), 8.00 (1H, d, J =7.8 Hz, H_7), 8.33 (1H, d, J =7.8 Hz, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 22.1 (2xCH_3), 27.3 (C_3), 36.2 (C_2), 68.9 ($C_{1'}$), 127.6 (C_6), 127.9 (C_7), 129.0 (C_{7a}), 136.5 (C_5), 138.3 (C_4), 156.6 (C_{3a}), 165.4 (−COO-), 207.0 (C_1). *Compound (**4**).* 2′,2′,2′-trifluoroethyl 1-oxo-indane-4-carboxylate. Yield: 19 %. Yellow oil. IR (KBr, ū, cm^{-1}): 1705, 1590, 1250, 1150. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.80 (2H, dd, J =6.0 Hz, 3.3 Hz, H_2), 3.53 (2H, dd, J =6.3 Hz, 5.4 Hz, H_3), 4.80 (2H, q, J =8.1 Hz, $H_{1'}$), 7.58 (1H, t, J =7.8 Hz, H_6), 8.06 (1H, d, J =7.8 Hz, H_7), 8.38 (1H, d, J =7.8 Hz, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 27.2 (C_3), 36.1 (C_2), 60.7–61.1 ($C_{1'}$), 121.2–124.3 ($C_{2'}$), 126.6 (C_4), 127.0 (C_{7a}), 127.9 (C_6), 129.1 (C_7), 136.9 (C_5), 138.7 (C_4), 157.0 (C_{3a}), 164.0 (−COO-), 206.2 (C_1). *Compound (**5**).* Butyl 1-oxo-indane-4-carboxylate. Yield: 62 %. Yellow oil. IR (KBr, ū, cm^{-1}): 1705, 1590, 1260, 1140. ^{1}H NMR ($CDCl_3$, 300 MHz): 1.04 (3H, t, J =7.5 Hz, $H_{4'}$), 1.56 (2H, sext., J =7.5 Hz, $H_{3'}$), 1.78 (2H, quint., J =7.5 Hz, $H_{2'}$), 2.76 (2H, dd, J =6.0 Hz, 5.1 Hz, H_2), 3.53 (2H, dd, J =6.0 Hz, 5.1 Hz, H_3), 4.42 (2H, q, J =7.5 Hz, $H_{1'}$), 7.53 (1H, t, J =7.8 Hz, H_6), 8.01 (1H, d, J =7.8 Hz, H_7), 8.33 (1H, dd, J =7.8 Hz, 1.2 Hz, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 13.8 ($C_{4'}$), 19.4 ($C_{3'}$), 27.3 (C_3), 30.8 ($C_{2'}$), 36.2 (C_2), 65.1 ($C_{1'}$), 127.6 (C_6), 128.0 (C_7), 128.6 (C_{7a}), 136.5 (C_5), 138.4 (C_4), 156.6 (C_{3a}), 166.0 (−COO-), 206.7 (C_1). *Compound (**9**).* Methyl 1-hydroxy-indane-4-carboxylate. Yield: 55 %. IR (KBr, ū, cm^{-1}): 3300, 1240, 1160. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.05 (2H, m, −OH, H_2), 2.58 (1H, m, H_2), 3.18 (1H, m, H_3), 3.49 (1H, m, H_3), 3.94 (3H, s, CH_3), 5.30 (1H, t, J =5.4 Hz, H_1), 7.37 (1H, t, J =7.5 Hz, H_6), 7.65 (1H, d, J =7.5 Hz, H_7), 7.99 (1H, d, J =7.5 Hz, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 30.9 (C_3), 35.7 (C_2), 51.9 ($C_{1'}$), 76.0 (C_1), 126.8 (C_4), 127.0 (C_6), 128.7 (C_7), 130.3 (C_5), 145.7 (C_{7a}), 146.6 (C_{3a}), 167.3 (−COO-).

2.4.2. Preparation of esters by nucleophilic substitution

To 1-oxo-indane-4 carboxylic acid in anhydrous acetone was added potassium carbonate (K_2CO_3). After 30 min, a molar equivalent of an alkyl bromide (3,3-dimethylallyl bromide; 1,2-dibromoethane; or 1,4-dibromobutane) was added into the reaction. The reaction mixture was then poured into acidulate water and extracted with EtOAc. Evaporation of the solvent was done under reduced pressure to give an amorphous solid as final

product. Spectroscopic properties of synthesize compounds are presented below.

*Compound (**6**).* 2-Isopentenyl 1-oxo-indane-4-carboxylate. Yield: 31 %. IR (KBr, ū, cm^{-1}): 1750, 1600, 1250. ^{1}H NMR ($CDCl_3$, 300 MHz): 1.82 (6H, d, *J* =4.2 Hz, 2xCH_3), 2.75 (2H, m, H_2), 3.53 (2H, m, H_3), 4.88 (2H, d, *J* = 7.2, $H_{1'}$), 5.51 (1H, m, $H_{2'}$), 7.50 (1H, t, *J* = 7.5, H_6), 7.96 (1H, d, *J* = 7.5, H_7), 8.31 (1H, d, *J* = 7.5, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 18.2 (CH_3), 25.9 (CH_3), 27.3 (C_3), 36.2 (C_2), 62.0 ($C_{1'}$), 118.4 ($C_{2'}$), 127.5 (C_6), 128.0 (C_7), 128.6 (C_{7a}), 136.5 (C_5), 138.3 (C_4), 139.7 ($C_{3'}$), 156.7 (C_{3a}), 165.8 (−COO-), 207.0 (C_1). *Compound (**7**).* 2′-bromoethyl 1-oxo-indane-4-carboxylate. Yield: 29 %. IR (KBr, ū, cm^{-1}): 1750, 1590, 1250, 1150. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.79 (2H, ddd, *J* =6.0 Hz, 5.1 Hz, 3.3 Hz, H_2), 3.57 (2H, dd, *J* =6.0 Hz, 3.3 Hz, H_3), 3.72 (2H, t, *J* =5.7 Hz, $H_{2'}$), 4.73 (2H, t, *J* =5.7 Hz, $H_{1'}$), 7.55 (1H, t, *J* =7.8 Hz, H_6), 8.02 (1H, d, *J* =7.8 Hz, H_7), 8.37 (1H, dd, *J* =7.8 Hz, 1.2 Hz, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 27.4 (C_3), 28.9 ($C_{2'}$), 36.1 (C_2), 64.6 ($C_{1'}$), 127.7 (C_6), 127.8 (C_7), 128.5 (C_{7a}), 136.7 (C_5), 138.5 (C_4), 156.8 (C_{3a}), 165.3 (−COO-), 206.5 (C_1). *Compound (**8**).* 4′-bromobutyl 1-oxo-indane-4-carboxylate. Yield: 27 %. IR (KBr, ū, cm^{-1}): 1750, 1250, 1150. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.04 (4H, m, $H_{2'}$, $H_{3'}$), 2.75 (2H, ddd, *J* =6.0 Hz, 5.1 Hz, 3.3 Hz, H_2), 3.52 (4H, m, H3, $H_{4'}$), 4.42 (2H, t, *J* =6.3 Hz, $H_{1'}$), 7.51 (1H, t, *J* =7.8 Hz, H_6), 7.98 (1H, d, *J* =7.8 Hz, H_7), 8.30 (1H, d, *J* =7.8 Hz, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 27.3 (C_3), 27.4 ($C_{3'}$), 29.4 ($C_{2'}$), 33.1 ($C_{4'}$), 36.1 (C_2), 64.3 ($C_{1'}$), 127.6 (C_6), 128.1 (C_7), 128.3 (C_{7a}), 136.4 (C_5), 138.4 (C_4), 156.7 (C_{3a}), 165.7 (−COO-), 206.5 (C_1).

2.4.3. Amino acid coupling reaction

1-oxo-indane-4 carboxylic acid dissolved in dicloromethane was put to react with co-catalyst 1-hydroxybenzotriazole (HOBt) and methyl ester of some amino acids (L-isoleucine, L-leucine). The solution was cooled to 0 °C, and combined with the coupling agent DCC for 2 h. After this time, the solution was left at room temperature for two more hours, and the precipitate was filtered. Subsequently, neutralization was carried out with an aqueous solution of $NaHCO_3$, and extraction with EtOAc. The solvent was removed under reduced pressure, and an amorphous yellow solid was obtained. Spectroscopic data are in agreement with those reported by Krumm et al. [23].

*Compound (**C-1**).* 1-oxo-indanoyl-L-isoleucyl methyl ester. Yield: 35 %. ^{1}H NMR ($CDCl_3$, 300 MHz): 0.99 (6H, m, 2xCH_3), 1.28–1.60 (2H, m, $H_{3'}$), 2.09 (1H, m, $H_{2'}$), 2.77 (2H, m, H_2), 3.47 (2H, m, H_3), 3.84 (3H, s, −OCH_3), 4.88 (1H, dd, *J* = 8.1, 4.5, $H_{1'}$), 6.64 (1H, d, *J* = 8.1, −CONH-), 7.51 (1H, t, *J* = 7.5, H_6), 7.92 (2H, m, H_5, H_7). ^{13}C NMR ($CDCl_3$, 75 MHz): 11.7 (CH_3), 15.6 (CH_3), 25.4 ($C_{3'}$), 26.1 (C_3), 36.2 (C_2), 38.2 ($C_{2'}$), 52.4 (−OCH_3), 56.8 ($C_{1'}$), 126.6 (C_7), 127.8 (C_6), 132.7 (C_4), 133.0 (C_5), 138.3 (C_4), 154.1 (C_{3a}), 166.7 (−CON-), 172.6 (−COO-), 206.5 (C_1). *Compound (**C-2**).* 1-oxo-indanoyl-L-leucyl methyl ester. Yield: 33 %. ^{1}H NMR ($CDCl_3$, 300 MHz): 0.99 (6H, d, *J* = 5.1, 2xCH_3), 1.74 (3H, m, $H_{2'}$, $H_{3'}$), 2.71 (2H, m, H_2), 3.86 (2H, m, H_3), 3.77 (3H, s, −OCH_3), 4.76 (1H, t, *J* =5.7 Hz, $H_{1'}$), 7.48 (1H, t, *J* =7.5 Hz, H_6), 7.83 (1H, d, *J* =7.5 Hz, H_7), 7.90 (1H, d, *J* =7.5 Hz, H_5). ^{13}C NMR ($CDCl_3$, 75 MHz): 21.1 (CH_3), 22.5 (CH_3), 25.0 ($C_{3'}$), 25.6 (C_3), 36.0 (C_2), 40.1 ($C_{2'}$), 51.3 (−OCH_3), 52.0 ($C_{1'}$), 125.9 (C_7), 127.5 (C_6), 133.2 (C_4), 133.3 (C_5), 137.6 (C_4), 154.6 (C_{3a}), 168.4 (−CON-), 173.6 (−COO-), 208.0 (C_1).

2.5. Induction experiments

2.5.1. Inducer effect of indanoyl derivatives

Seedlings of Cargamanto Blanco and ICA Quimbaya common bean cultivars were immersed for 4 h in solutions 0.66 mM of the synthetized compounds: **1** to **9**, **C-1** and **C-2**. Each compound was first dissolved in a very small volume of ethanol and dispersed in appropriate volumes of water. By this way, all hydroalcoholic solutions were prepared in 0.05 % (v/v). Treatments with methyl jasmonate (**MeJA**) and jasmonic acid (**JA**) served as positive controls, whereas seedlings treated only with a hydroalcoholic solution (0.05 %) were used as negative control. Then, plant material was sown in wet cellu-cotton and placed in plastic containers in darkness at 25 °C during 96 h. After this time, cotyledons (5 g) and hypocotyls/roots (5 g) were carefully separated and analyzed independently.

2.5.2. Dose-response experiments

Seedlings of common bean cv. ICA Quimbaya were immersed for 4 h in 1-oxo-indanoyl-L-isoleucyl methyl ester solutions at different concentration (0.36, 0.66 and 4.45 mM). All solutions were prepared in ethanol (0.05 %). Then, plant material was sown in wet cellu-cotton and placed in plastic containers in darkness at 25 °C during 96 h. Control experiments were carried out only using distilled water and ethanol (0.05 %) at the same storage conditions described previously. After incubation time, hypocotyls/roots (5 g) were carefully separated and the isoflavonoid compounds extracted as it is described later. Cotyledons were discarded. All experiments were performed in triplicate.

2.5.3. Time-course experiments

Seedlings of common bean cultivars, Cargamanto Blanco and ICA Quimbaya were immersed for 4 h in solutions 0.66 mM of 1-oxo-indanoyl-L-leucyl methyl ester and 1-oxo-indanoyl-L-isoleucyl methyl ester. After induction, the vegetal materials were sown in cellu-cotton and put in plastic containers under dark conditions and room temperature. All assays were stored at different post-induction times (24, 48, 72, and 96 h) and each experiment was carried out by triplicate. As in the previous experiment, cotyledons (5 g) and hypocotyls/roots (5 g) were carefully separated and analyzed independently.

2.6. Extraction of isoflavonoid compounds

Cotyledons and hypocotyls/roots samples were macerated in 95 % ethanol (10 mL) and filtered through Whatman No. 1 filter paper. Subsequently, the extracts were concentrated at 40 °C under vacuum using a rotary evaporator (Buchi R-210). The resulting aqueous phase was extracted three times by liquid–liquid extraction using EtOAc. The organic phases were combined and brought to dryness under reduced pressure. For isoflavonoids analysis, the dry extracts were dissolved in 2.5 mL of methanol (HPLC grade) and the resulting samples were kept in amber glass vials and stored at 4 °C until HPLC analysis was carried out.

2.7. Detection and quantitative analysis of isoflavonoid compounds

Samples were analyzed using a Shimadzu Prominence LC 20AT chromatograph equipped with a diode array detector (DAD). Solvents used were 0.05 % acetic acid in water (solvent A), and methanol (solvent B). The elution gradient established was: from 10 to 70 % B in 40 min, then 70–90% B in 20 min, followed by 10 % B in 3 min., and re-equilibration of the column. The separation of isoflavonoid compounds was performed in a Luna C_{18} column (5 μm, 150 mm x 4.6 mm i.d.) operating at 30 °C and a flow rate of 0.7 mL/min. Injection volume was 20 μL. Detection was carried out in the DAD using 248, 259, 278, 286 and 343 nm as wavelengths.

The isoflavonoid compounds were characterized according to their UV, mass spectra and retention times, and comparison with authentic standards when available. The confirmation by liquid chromatography with mass spectrometry detection (LS-MSD) was carried out on an HP 1100 Series HPLC apparatus (Agilent Technologies Waldbronn, Germany) interfaced to an HP series 1100 mass selective detector with and 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, phaseollinisoflavan, phaseollidin and phaseollin were respectively: 34.0, 35.0, 36.0, 39.0, 43.0, 47.0, 49.0, 51.0 and 53.0 min. For quantitative analysis, calibration curves were prepared by injection of known concentrations of different standard compounds (1, 10, 25, 50, and 100 mg/L). The isoflavonoids that did not have pure standard, phaseollinisoflavan and phaseollidin, and kievitone were quantified respectively by estimation from the calibration curves of phaseollin and dalbergioidin, and were adjusted based on differences in molecular weight. Results were expressed as μg isoflavonoid/g fresh weight (g. f.w.)

2.8. Antifungal assays

In order to investigate the fungitoxicity of the compounds synthetized against *C. lindemuthianum* and *Fusarium* spp., the poisoned food technique was used [26,27]. The compounds 1-oxo-indanoyl-L-isoleucyl methyl ester (**C-1**) and 1-oxo-indanoyl-L-leucyl methyl ester (**C-2**) were dissolved in ethanol (0.05 %), diluted at final concentration 1.0 mM using Potato-Dextrose Agar (PDA; potato dextrose agar DifcoTM 20 g and 1000 mL deionized water) for *Fusarium* spp., and oatmeal agar (oatmeal 60 g, agar 20 g, and 1000 mL deionized water) for C. *lindemuthianum*, and incorporated in Petri dishes (measuring 9 cm in diameter). Petri dishes with and without ethanol were used as solvent control and absolute control, respectively. Also, 1.0 mM **MeJA** was used as positive control. The mycelial growth was measured every 24 h during 7 days for *Fusarium* spp. and every 48 h during 16 days for *C. lindemuthianum* at room temperature, and the percentages of inhibition were calculated based on the growth of the control plates and using the following formula: (%) = [1- radial growth of treatment (mm)/ radial growth of control (mm)] x 100. The experiments were done by triplicate.

2.9. Statistical analysis

Data were expressed as mean ± standard deviation of three independent experiments. Data were subjected to one-way analysis of variance (ANOVA) and significant differences between samples were determined by the Fischer least significant differences (LSD) test at $p \leq 0.05$.

3. Results

3.1. Induction of phytoalexins using indanoyl esters

A series of indanoyl esters (**1** to **9**) was synthesized and evaluated as potential phytoalexins elicitors in two cultivars of common bean. Preparation of indanoyl esters was carried out using the route proposed by Nakamura et al. [28]. The synthesis started from 2-carboxy cinnamic acid, which was hydrogenated using H_2/C (Pd)(yield: 95 %). The desired indanone skeleton (1-oxo-indane-4-

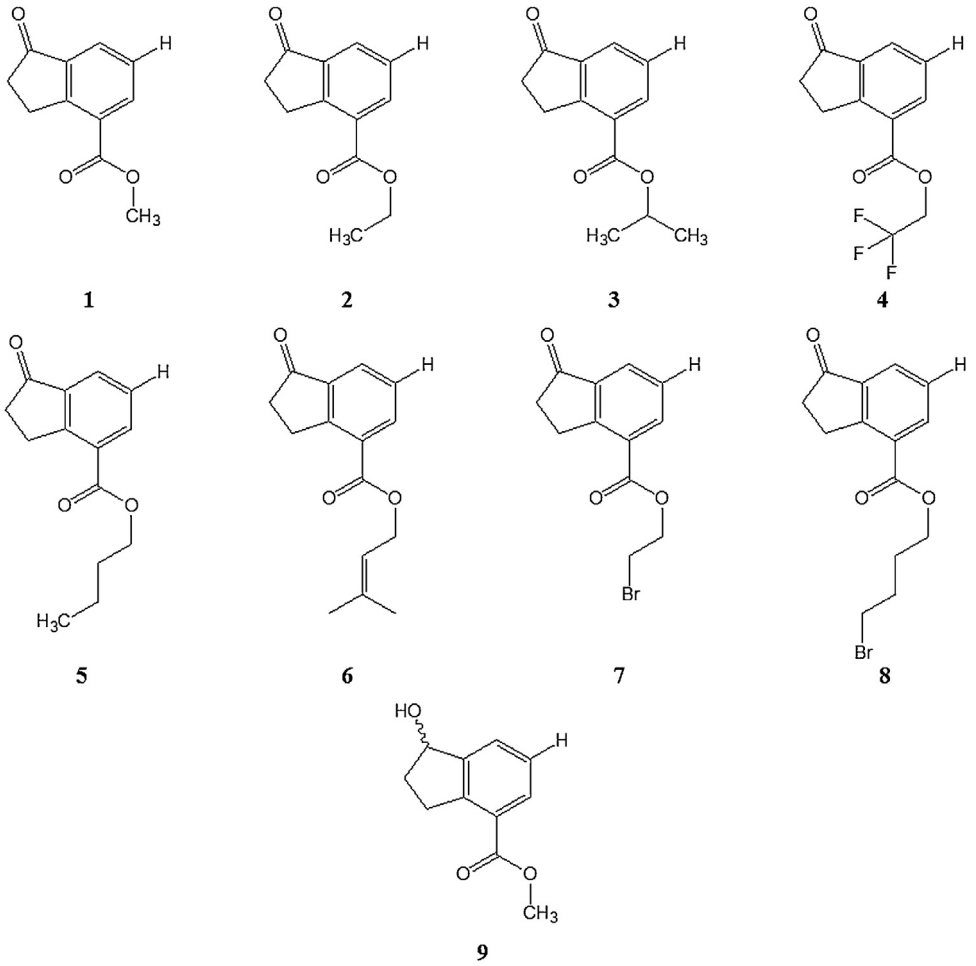

Fig. 3. Structure of indanoyl esters synthesized and evaluated as potential phytoalexins elicitors.

carboxylic acid) was obtained by intramolecular Friedel-Crafts acylation (yield: 63 %). Finally, esters were prepared by nucleophilic substitution (compounds **1** to **5**; overall yields between 19 and 62 %) or Fischer esterification (compounds **6** to **8**; overall yields between 42 and 48 %) using the respective alkyl halides or alcohols. Compound **9** was obtained by hydrogenation of **1**. Structure of compounds is shown in Fig. 3. The concentration of isoflavonoid phytoalexins in cotyledons and hypocotyl/root treated with the indanoyl esters solutions at 0.66 mM and after 96 h incubation is shown in Figs. 4 and 5, respectively.

As can be seen, chromatographic profile was dependent on the cultivar, tissue and elicitor evaluated. In general, the concentration of all isoflavonoids was significantly increased as result of treatments with the indanoyl esters compared to controls (water-treated tissues). Cotyledons displayed the highest level of genistein, 2′-hydroxygenistein, phaseollidin and kievitone whereas hypocotyls-roots exhibited the greatest levels of phaseollin and daidzein. This result agrees with those reported by Whitehead et al. [29], who found that after treatments with preparations heat-released from the cell walls of *C. lindemuthianum*, the major phytoalexin in cotyledons (kievitone) was different from that of hypocotyls (phaseollin). Likewise, Goossens and Vendrig [30] reported that the ratio of kievitone to phaseollin was highest in the cotyledons and decreased through the hypocotyl to the roots. In the cultivar Cargamanto Blanco, susceptible to anthracnosis, the higher accumulation of isoflavonoids was found when tissues were treated with the compound **2**, followed by **1**. For instance, the highest amount of phaseollin (38.4 μg/g) and phaseollidin (37.9 μg/g) was found in cotyledons (Fig. 4b) and hypocotyls-roots (Fig. 5b) of Cargamanto Blanco treated with the compound **2**, respectively. The increase in the concentration of phaseollin and phaseollidin corresponded to 5- and 30-fold that in the corresponding controls (water-treated tissues). Similarly, concentration of all phytoalexins in tissues of ICA Quimbaya, cultivar resistant to anthracnosis, was significantly increased as result of treatments with all indanoyl esters. Phaseollin reached maximum levels in cotyledons (50.4 μg/g, Fig. 4a) and hypocotyls-roots (78.5 μg/g, Fig. 5a) treated by **1**, being about 50 to 60-fold higher than those in water-treated tissues (controls). In addition, phaseollidin that was absent in controls, it was strongly augmented to 29.1, 49.6, 37.6 and 37.4 μg/g in cotyledons of ICA Quimbaya elicited by **1**, **2**, **3** and **6**, respectively. Interestingly, tissues of ICA Quimbaya treated with compounds having halogens (**4**, **7** and **8**) showed high levels of the isoflavones genistein, 2′-hydroxygenistein (cotyledons, Fig. 4a), and daidzein (hypocotyls-roots, Fig. 5a). This peculiar behavior could be due to some metabolic blockage caused by halogenated substituents.

In general, the amount of phytoalexins in the cultivar ICA Quimbaya was higher than that found in the susceptible one (cv. Cargamanto Blanco). This result is in agreement with earlier studies [11,22]. Interestingly, under the experimental conditions used, coumestrol presented a low and almost similar concentration between the treatments. This finding contrast with our previous works, which found that coumestrol was one of the major phytoalexin in common bean seedlings of the same cultivars but treated with salicylic acid derivatives [10,11].

So, indanoyl esters could be differentially inducing the metabolic pathways leading to the formation of coumestan and pterocarpans from the same precursor, daidzein. Figs. 4 and 5 show that after treatment with indanoyl elicitors, the concentrations of phaseollin and phaseollidin are strongly increased, while the concentration of coumestrol remains moderate. This result is

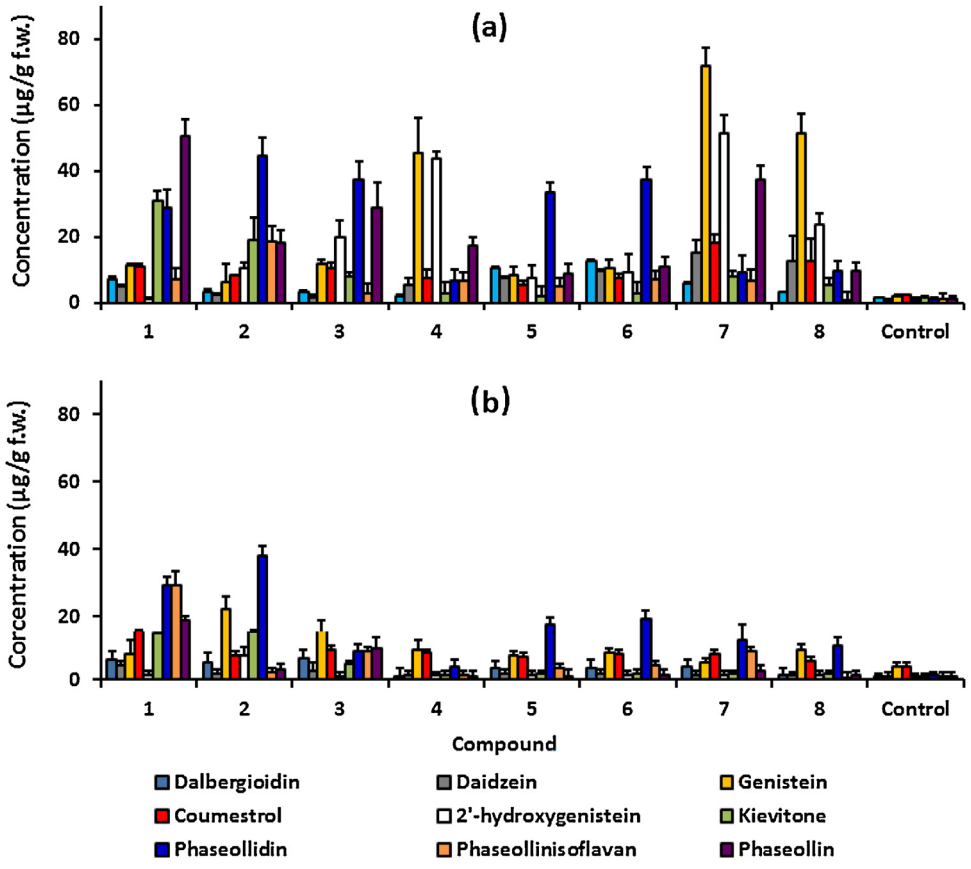

Fig. 4. Isoflavonoid accumulation on cotyledons of common bean, cultivars ICA Quimbaya (**a**) and Cargamanto Blanco (**b**). Bars represent the mean concentration of isoflavonoids ± standard deviation (n = 3). 96 h post-induction.

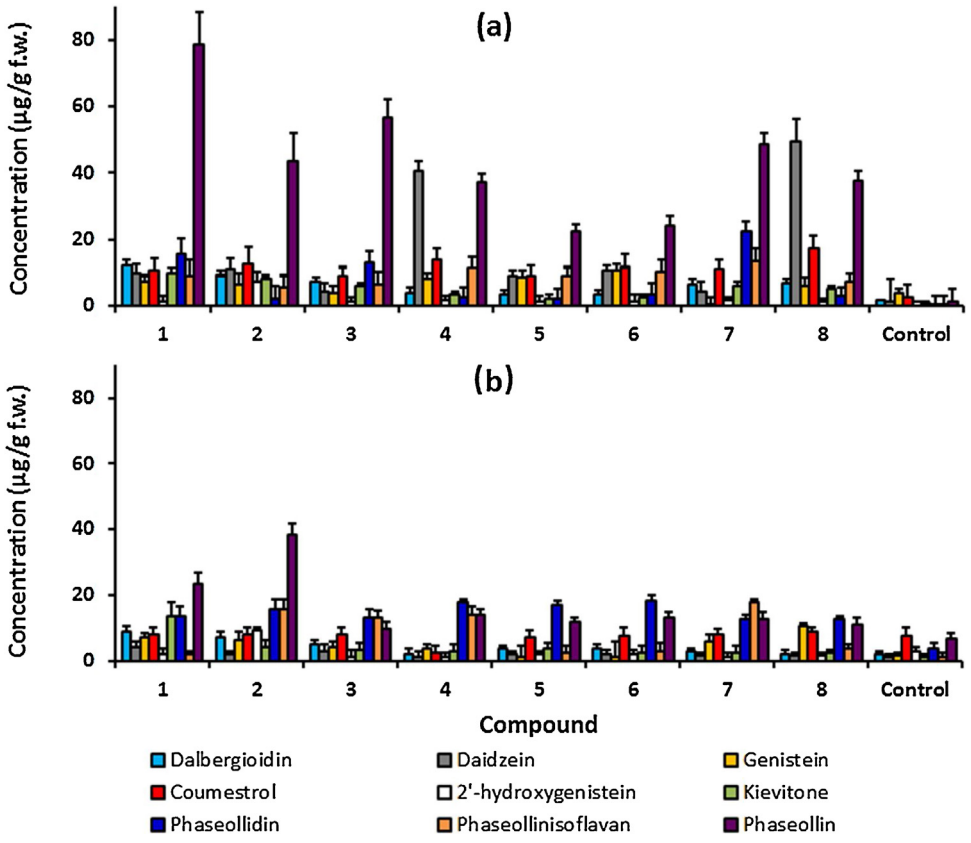

Fig. 5. Isoflavonoid accumulation on hypocotyls-roots of common bean, cultivars ICA Quimbaya (**a**) and Cargamanto Blanco (**b**). Bars represent the mean concentration of isoflavonoids ± standard deviation (n = 3). 96 h post-induction.

interesting considering that pterocarpans have been recognized for their powerful antifungal activities.

Variation of the substitution at C-4 also indicated that is possible modulate the biological activity. As the size of the carbon chain in the alkoxycarbonyl substituent increased, the eliciting effect of defense-related isoflavonoids decreased. This effect was most evident for phaseollin elicitation. Thus, the greatest increases in the concentration of isoflavonoids related to disease defense occurred when the tissues were treated with the methyl (**1**) and ethyl (**2**) indanoyl esters. In contrast, the long carbon chain indanoyl esters (such as *n*-butyl and 2-isopentenyl, **5** and **6** respectively) elicited the lowest levels of isoflavonoids. The above could be the result of the decrease in the polarity of the compound, which reduces its solubility in water. Additionally, small hydrocarbon chains in indanoyl esters may be essential for the ligand-receptor interaction.

3.2. Induction of phytoalexins using indanoyl-amino acid conjugates

Indanoyl-amino acid conjugates (**C-1** and **C-2**) were conveniently prepared by treatment of 1-oxo-indane-4-carboxylic acid with L-isoleucine methyl ester and L-leucine methyl ester using DCC/HOBt system as coupling reagent (yields: 35 and 33 %, respectively). The accumulation of defense-related isoflavonoids in cotyledons and hypocotyls-roots of common bean cultivars treated by **C-1**, **C-2**, along with the indanoyl esters **1** and **9**, and the well-known elicitors **MeJA** and **JA** is shown in Figs. 6 and 7. Compounds were evaluated at 0.66 mM and common bean seedlings were extracted after 96 h post-induction.

In general, concentration of defense related isoflavonoids in cotyledons and hypocotyls-roots treated with **MeJA**, **JA**, **C-1**, **C-2**, **1** and **9** was significantly increased in comparison to the respective controls (water-treated tissues). The major isoflavonoids detected in ICA Quimbaya cotyledons were phaseollinisoflavan, kievitone, phaseollidin, genistein, and phaseollin. The greatest increases occurred when compound **C-1** was used as elicitor; mainly, for the isoflavonoids phaseollinisoflavan (130.4 µg/g), kievitone (88.5 µg/g), and genistein (80.5 µg/g). The highest level of phaseollin (78.7 µg/g) and phaseollidin (78.7 µg/g) was reached when cotyledons were treated by **MeJA**. For the disease susceptible cultivar, Cargamanto Blanco, the composition of the extracts resulting from induction was more similar. The major phytoalexin was phaseollin, followed by kievitone (treatments with **MeJA** and **C-1**) or phaseollidin (treatments with **JA** and **C-2**). Phaseollin reached the highest amounts when **MeJA** (94.4 µg/g) and **JA** (90.6 µg/g) were used as elicitors, followed by **C-1** (67.0 µg/g) and **C-2** (61.7 µg/g) and finally, **1** (23.3 µg/g) and **9** (18.5 µg/g). It is noteworthy that the compound **C-1** increased the isoflavonoids concentrations in cultivar ICA Quimbaya to higher levels than **JA** and **MeJA**. Interestingly, the elicitor effect of **JA**, **MeJA**, **C-1** and **C-2** was even higher than that displayed by the indanoyl methyl ester **1**.

As can be seen in Fig. 7, exogenous elicitation of hypocotyls-roots with all compounds resulted in a strong increase in the phytoalexin amounts, mainly kievitone, phaseollin, and phaseollidin, in relation to the controls. Again, **JA**, **MeJA**, **C-1** and **C-2** displayed an isoflavonoids elicitor effect higher than the indanoyl methyl ester **1**. For the disease resistant cultivar, ICA Quimbaya, the highest levels were found for the pterocarpan phaseollin with all the elicitors used. Particularly, phaseollin reached a greatest concentration (153.2 µg/g) when the hypocotyls-roots were treated with **C-1**, being even higher than with the elicitors **JA** (59.8 µg/g) and **MeJA** (102.0 µg/g). The hypocotyls-roots of

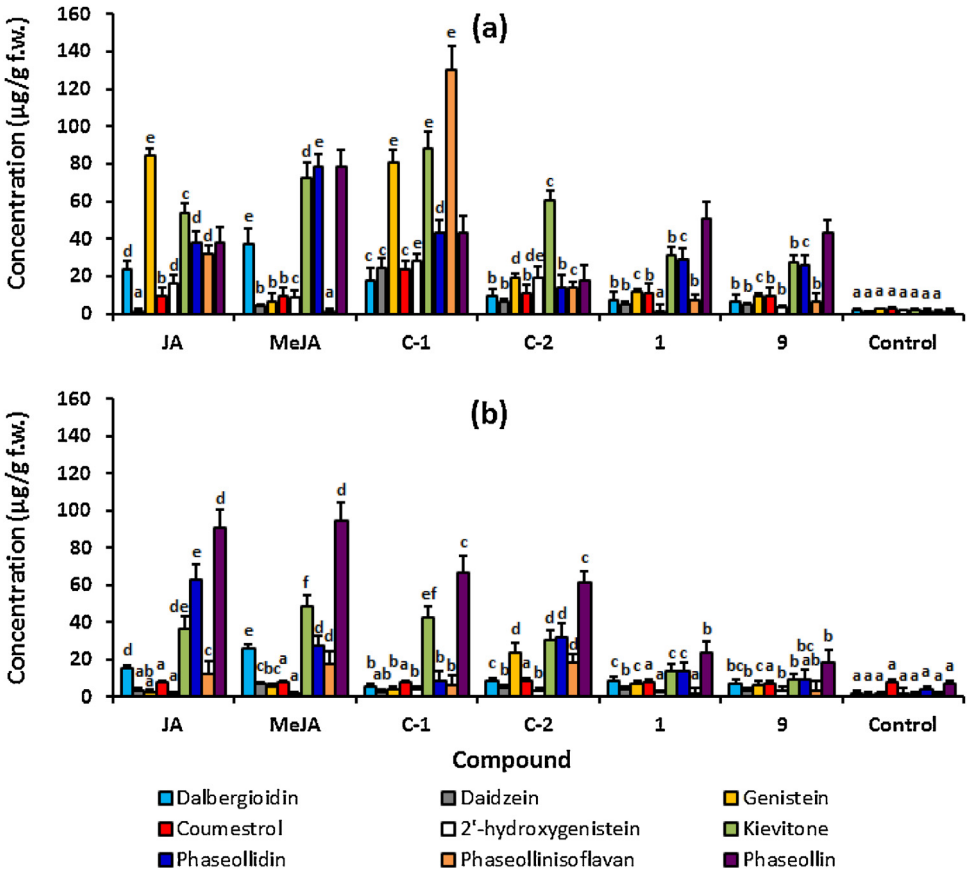

Fig. 6. Isoflavonoid accumulation on cotyledons of common bean, cultivars ICA Quimbaya (**a**) and Cargamanto Blanco (**b**). Bars represent the mean concentration of isoflavonoids ± standard deviation (n = 3). For each isoflavonoid, bars with different letters are significantly different (p = 0.05). 96 h post-induction.

cultivar Cargamanto Blanco presented similar levels for the major phytoalexins phaseollin, phaseollidin and kievitone when these were treated with **C-1** and **MeJA**.

Once again, the substituent at position 4 of the indanoyl system is a structural characteristic that influences eliciting activity. The presence of amino acids triggered the synthesis of isoflavonoids at higher levels than indanoyl esters. The methoxycarbonyl group in C-4 of the indanoyl system reduced the phytoalexins eliciting activity when is compared to the carbamoyl substituent. In fact, biological activity of **C-1** and **C-2** exceeds even that of **JA** and **MeJA** (Fig. 8).

It can be also seen that the amino acid L-isoleucine displayed a greater eliciting effect of isoflavonoids than the amino acid L-leucine. This finding agrees with that reported by Krumm et al. [23] and Mithöfer et al. [31], who found after systematic permutation with different amino acids (L- and D-amino acids) in C-4, that the 1-oxo-indanoyl conjugate with L-isoleucine was the most active eliciting the biosynthesis of volatiles in *P. lunatus*. According to Mithöfer et al. [31], only aliphatic amino acids resembling the size and polarity of L-isoleucine result in active conjugates. Reduction of the carbonyl group (compound **9**) in the indanoyl ester **1** also decreased the eliciting effect of isoflavonoids.

3.3. Dose-response and time-course experiments

In order to better understand the eliciting effect of 1-oxo-indanoyl L-isoleucine conjugate, a dose-response and time-course study was performed. For the dose-response experiments, common bean seedlings cv. ICA Quimbaya were treated with solutions of **C-1** at different concentration (0.36, 0.66 and 4.45 mM) and the synthesis of phytoalexins evaluated after 96 h. Dose-response results in hypocotyls-roots are summarized in Table 1. Concentration of defense-related isoflavonoids was significant increases after treatment with **C-1** in relation to the control. In addition, levels of isoflavonoids increased steadily in a dose-dependent manner.

On the other hand, time-course experiments over common bean seedlings, cultivars Cargamanto Blanco and ICA Quimbaya, were carried out. The seedlings were treated using solutions of C-1 and C-2 at 0.66 mM for 4 h. Every 24 h and during four consecutive days, seedlings were extracted and its composition analyzed. The chromatographic profiles of common bean hypocotyls-roots elicited by the 1-oxo-indanoyl L-isoleucine conjugate (**C-1**) in the course of time is shown in Fig. 9. The treatment with **C-1** results after 24 h in a massive accumulation of defense related isoflavonoids. For this time, the peaks that prevail are those with retention times between 25 and 40 min, corresponding to the more polar isoflavones, dalbergioidin, 2′-hydroxygenistein, daidzein, and genistein. Other peaks that were not identified are also present in this range. Subsequently, a decrease in the concentration of isoflavones was observed in line with an increase in the intensity of the peaks with retention times between 40 and 55 min. Within this range are kievitone, coumestrol, phaseollidin, phaseollin, and phaseollinisoflavan. The intensity of the peaks corresponding to these compounds continues to increase until the end of the evaluation. Longer times were not considered as a consequence of the evident deterioration presented by the seedlings. Concentration of defense-related isoflavonoids in the course time is shown in Figs. 10 and 11.

As can be seen, exogenous elicitation of hypocotyls-roots and cotyledons with **C-1** resulted in a dramatic increase in the defense-related isoflavonoids amount, mainly genistein, 2′-

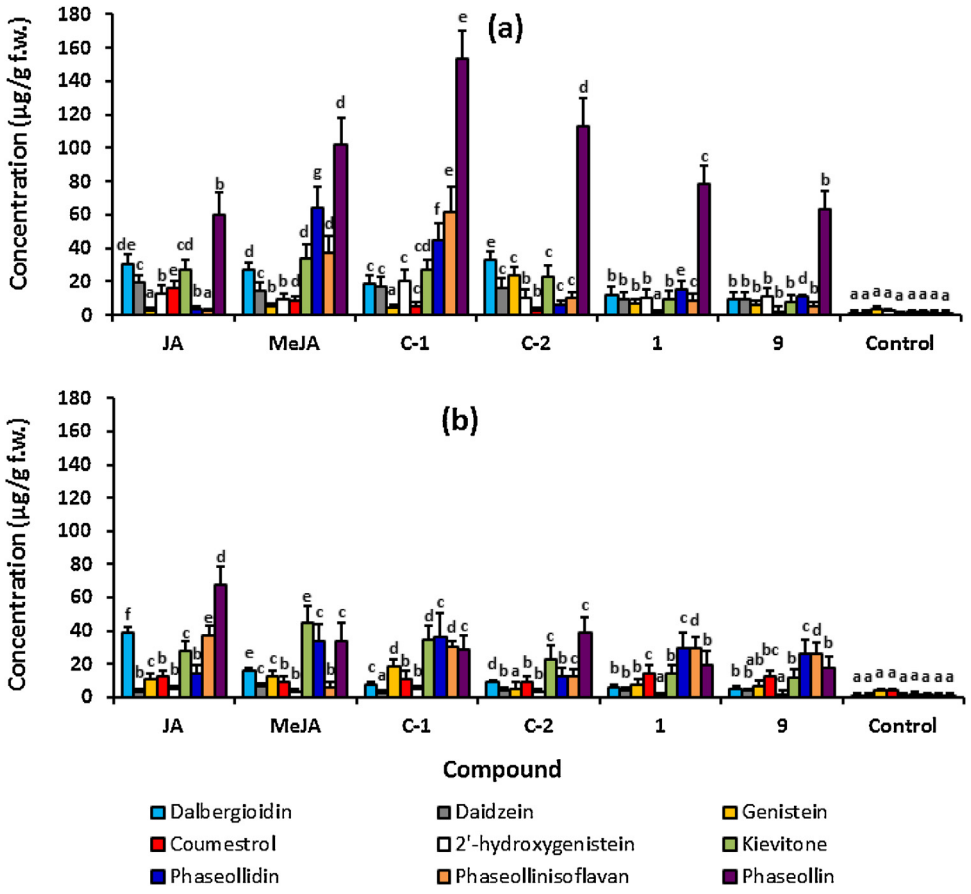

Fig. 7. Isoflavonoid accumulation on hypocotyls-roots of common bean, cultivars ICA Quimbaya (**a**) and Cargamanto Blanco (**b**). Bars represent the mean concentration of isoflavonoids ± standard deviation (n = 3). For each isoflavonoid, bars with different letters are significantly different (p = 0.05). 96 h post-induction.

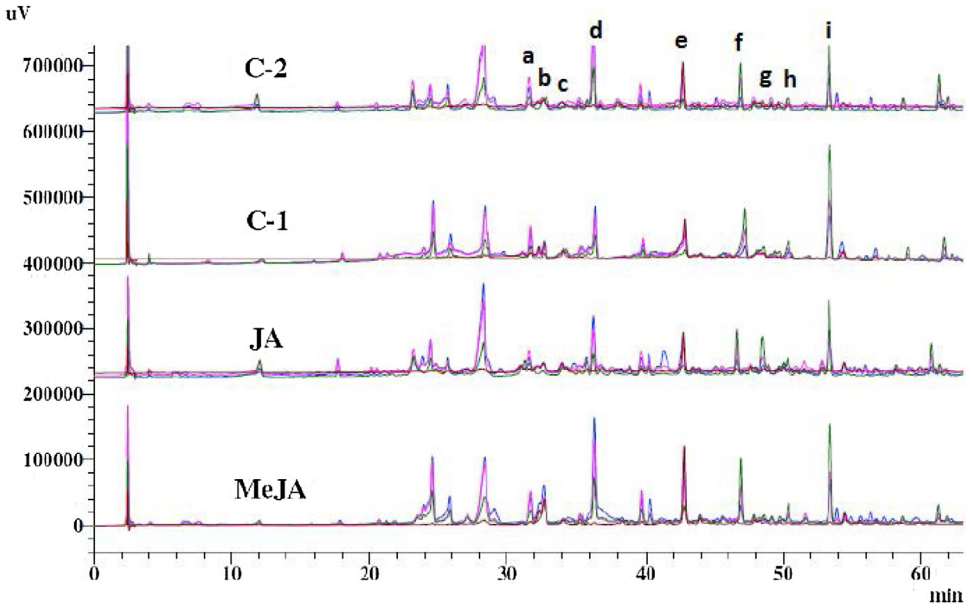

Fig. 8. Chromatographic profiles of common bean seedlings (cv. Cargamanto Blanco) elicited by 1-oxo-indanoyl-L-isoleucyl methyl ester. **a**, dalbergioidin; **b**, 2′-hydroxygenistein; **c**, daidzein; **d**: genistein; **e**, coumestrol; **f**, kievitone; **g**, phaseollidin; **h**, phaseollinisoflavan; **i**, phaseollin. λ analysis: 248 (blue), 259 (green), 278 (green) and 343 (red) nm.

Table 1
Effect of different concentrations of 1-oxo-indanoyl-L-isoleucyl methyl ester (C-1) on defense-related isoflavonoids contents of common bean hypocotyls-roots (cv. ICA Quimbaya).

Treatment	Dalb.	2′-OHG	Daid.	Gen.	Coum.	Kiev.	PLD	PIS	PHA
0.36 mM	15.2 ± 3.8	3.9 ± 1.7	18.3 ± 5.1	9.9 ± 2.6	17.8 ± 5.3	19.7 ± 4.8	37.9 ± 9.5	55.6 ± 10.7	80.3 ± 15.8
0.66 mM	18.6 ± 4.9	5.1 ± 2.7	17.0 ± 4.6	4.1 ± 1.7	20.2 ± 6.9	27.0 ± 6.3	44.6 ± 10.1	61.4 ± 15.6	153.2 ± 16.8
4.45 mM	17.9 ± 5.6	4.4 ± 2.9	15.7 ± 3.9	7.5 ± 3.3	22.7 ± 5.7	33.5 ± 5.6	55.8 ± 9.7	70.8 ± 20.8	135.6 ± 25.6
Control	1.5 ± 1.7	1.2 ± 1.1	1.3 ± 1.2	3.8 ± 1.1	2.4 ± 1.2	1.9 ± 1.1	1.5 ± 1.1	1.3 ± 1.0	1.3 ± 1.1

Dalb., dalbergioidin; 2'**-OHG**, 2'-hydroxygenistein; **Daid.**, daidzein; **Gen.**, genistein; **Coum.**, coumestrol; **Kiev.**, kievitone; **PLD**, phaseollidin; **PIS**, phaseollinisoflavan; **PHA**, phaseollin. Data are expressed as mean concentration of isoflavonoids ± standard deviation (n = 3).

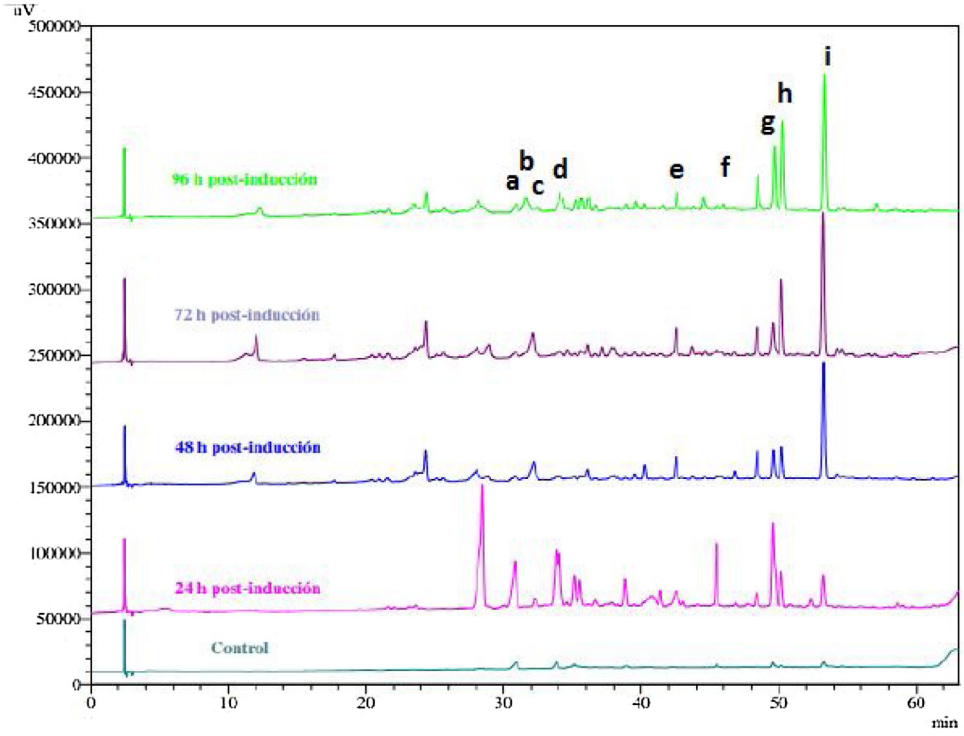

Fig. 9. Chromatographic profiles of common bean hypocotyls-roots (cv. Cargamanto Blanco) treated with 0.66 mM 1-oxo-indanoyl-L-isoleucyl methyl ester (**C-1**) and after 24, 48, 72 and 96 h post-induction, and the control treatment (water-treated seedlings and after 96 h). **a**, dalbergioidin; **b**, 2′-hydroxygenistein; **c**, daidzein; **d**: genistein; **e**, coumestrol; **f**, kievitone; **g**, phaseollidin; **h**, phaseollinisoflavan; **i**, phaseollin. λ analysis: 287 nm.

hydroxygenistein, phaseollidin, phaseollinisoflavan and phaseollin in relation to the controls. During the first 48 h, concentration of genistein was quickly increased in the anthracnose-resistant cultivar, ICA Quimbaya, reaching a maximum level after 48 h (95.1 μg/g for hypocotyls-roots, and 175.2 μg/g for cotyledons). Then, the amount of genistein decreased while the levels of kievitone, phaseollidin, phaseollinisoflavan and phaseollin were progressively increased until reaching the highest concentration after 72–96 hours. In hypocotyls-roots of ICA Quimbaya, kievitone exhibited the highest level after 72 h (33.8 μg/g) whereas for phaseollidin, phaseollinisoflavan and phaseollin it was reached after 96 h (43.9, 61.6, and 153.2 μg/g, respectively).

Likewise, the anthracnose-susceptible cultivar, Cargamanto Blanco, exhibited a maximum concentration of genistein (65.2 μg/g) and 2′-hydroxygenistein (51.1 μg/g) in hypocotyls-roots, and of dalbergioidin (66.1 μg/g) and daidzein (43.5 μg/g) in cotyledons after 24 h, which then gradually decreased. The highest level of kievitone, phaseollidin, phaseollinisoflavan and phaseollin was also found between 72 and 96 h. In general, amounts of coumestrol remained almost constant over the whole period of the evaluation.

Once again, the amount of defense-related isoflavonoids in the resistant cultivar ICA Quimbaya was higher than that found in hypocotyls-roots and cotyledons of the susceptible one.

3.4. Mycelial growth inhibition of Fusarium *spp. and* Colletotrichum lindemuthianum

Mycelial growth inhibition displayed by the compounds **C-1** and **C-2** was determined using the poisoned food technique. **MeJA** was also incorporated in the assay. All compounds were tested at 1.0 mM. The inhibitory effects of **MeJA**, **C-1** and **C-2** against *Fusarium* spp. and *C. lindemuthianum* are shown in Fig. 12.

As can be seen, the three compounds showed significant inhibitions on mycelial growth of both fungi when compared to controls. Compound **C-1** reduced the mycelial growth of *Fusarium* spp. and *C. lindemuthianum* about 17 and 34 mm after 168 and 384 h, respectively. Meanwhile, **C-2** caused inhibitions of mycelial growth for *Fusarium* spp. ranging from 100 (after 24 h) to 44 % (after 168 h). Likewise, the inhibitory effects of **MeJA** and **C-1** diminished with increasing time. Antifungal activity of **MeJA**

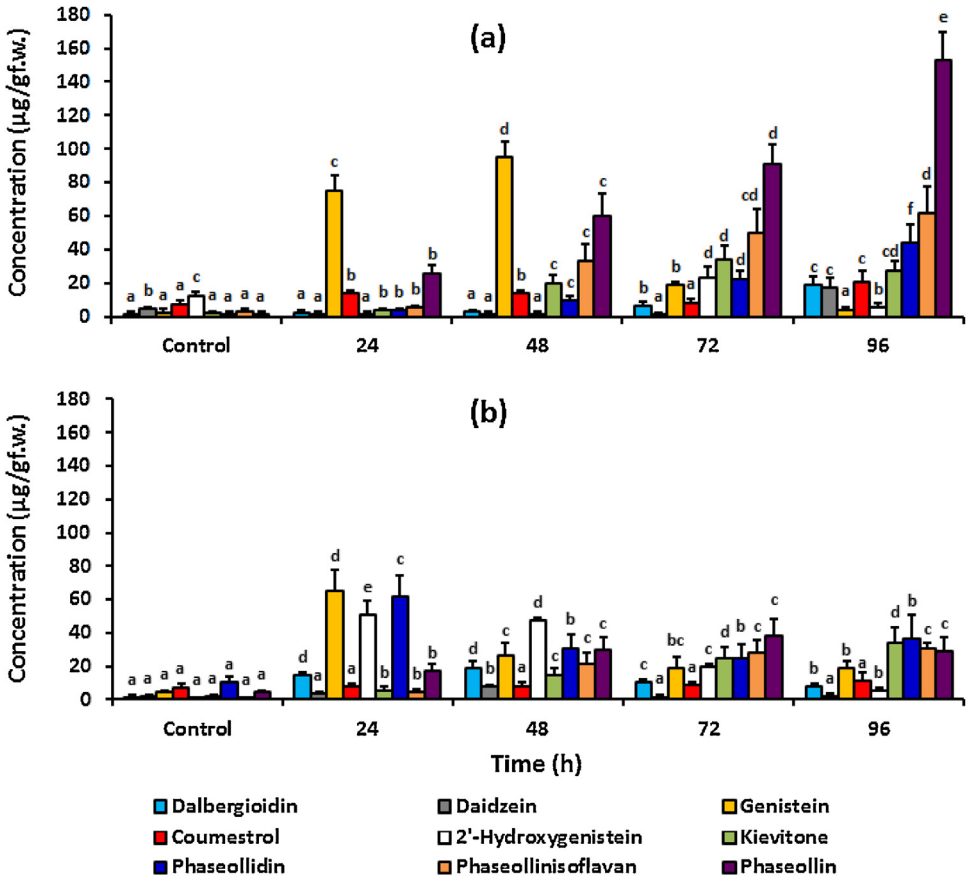

Fig. 10. Time-course accumulation of phytoalexins in common bean hypocotyls-roots, cultivars ICA Quimbaya (**a**) and Cargamanto Blanco (**b**), treated with 0.66 mM 1-oxo-indanoyl-L-isoleucyl methyl ester (**C-1**). Control treatments correspond to water-treated seedlings and after 96 h. For each time point, the bars headed by the same letter do not differ at p = 0.05 (Fisher's LSD test).

against both fungi was slightly lower than **C-1** and **C-2**. No significant differences were found for **C-1** and **C-2** against *C. lindemuthianum*. In general, a moderated to weak antifungal activity was displayed for the three compounds.

4. Discussion

Currently, there is an interest in the search for new elicitors that can be used for controlling important plant diseases, like anthracnose in common bean, by increasing the amounts of defense-related compounds in the tissues. Here, the elicitor effect of isoflavonoid phytoalexins in common bean (cv. ICA Quimbaya and Cargamanto Blanco) tissues of eleven 1-oxo-indane-4-carboxylic acid derivatives (esters and conjugates with amino acids), along with **JA** and **MeJA** was evaluated. The 1-oxo-indane-4-carboxylic acid derivatives are structurally related compounds with the coronatine (a highly active bacterial elicitor) and the coronalon (a synthetic mimic of coronatine). **JA**, **MeJA**, coronatine and coronalon (a 6-ethyl indanoyl-isoleucine conjugate) are known to trigger the defense or stress responses in many species of plants. In the present paper, the 1-oxo-indane-4-carboxylic acid was synthesized from the 2-carboxycinnamic acid by hydrogenation (yield: 95 %) and Friedel-Crafts intramolecular acylation (63 %) according to the methodology described by Krumm et al. [23], with some modifications. Then, esters were conveniently prepared by nucleophilic substitution (overall yields between 19 and 62 %) or Fischer esterification (overall yields between 42 and 48 %) using the respective alkyl halides or alcohols. Otherwise, 1-oxoindanoyl amino acid conjugates were obtained using DCC and HOBt (yield: 33–35 %). In general, the synthetic route represented an approach rapid and efficient enough for accessing to large amounts of the 1-oxo-indanoyl derivatives.

On the other hand, two separate biosynthetic pathways are believed to exist for the formation of the isoflavonoid phytoalexins in common bean (*P. vulgaris* L.); the 5-hydroxy- and the 5-deoxyisoflavonoid pathway [29]. The first route involved the sequence genistein → 2′-hydroxygenistein → dalbergioidin → kievitone. The 5-deoxyisoflavonoid pathway uses daidzein as a precursor, and then branches into two routes: one that passes through phaseollidin to phaseollin and phaseollinisoflavan, and the other that produces coumestrol. The composition of infected or elicited tissues is usually much more complex, and other minor metabolites may be present. In the present paper, the composition of defense-related isoflavonoids in common bean tissues was influenced by:

i.) the cultivar; in general, the anthracnose-resistant cultivar (ICA Quimbaya) accumulated defense-related isoflavonoids in higher amounts than the susceptible one (cv. Cargamanto Blanco). With some exceptions, levels of the precursor isoflavones (daidzein, genistein, 2′-hydroxygenistein, and dalbergioidin) were slightly similar in both cultivars. However, the amounts of phaseollidin, phaseollin, phaseollinisoflavan and kievitone were significantly highest in the cultivar ICA Quimbaya. This finding is consistent with previous reports [22]. The above suggests that metabolic delays may occur in the diseases-susceptible cultivars in the final stages of the biosynthesis of pterocarpans,

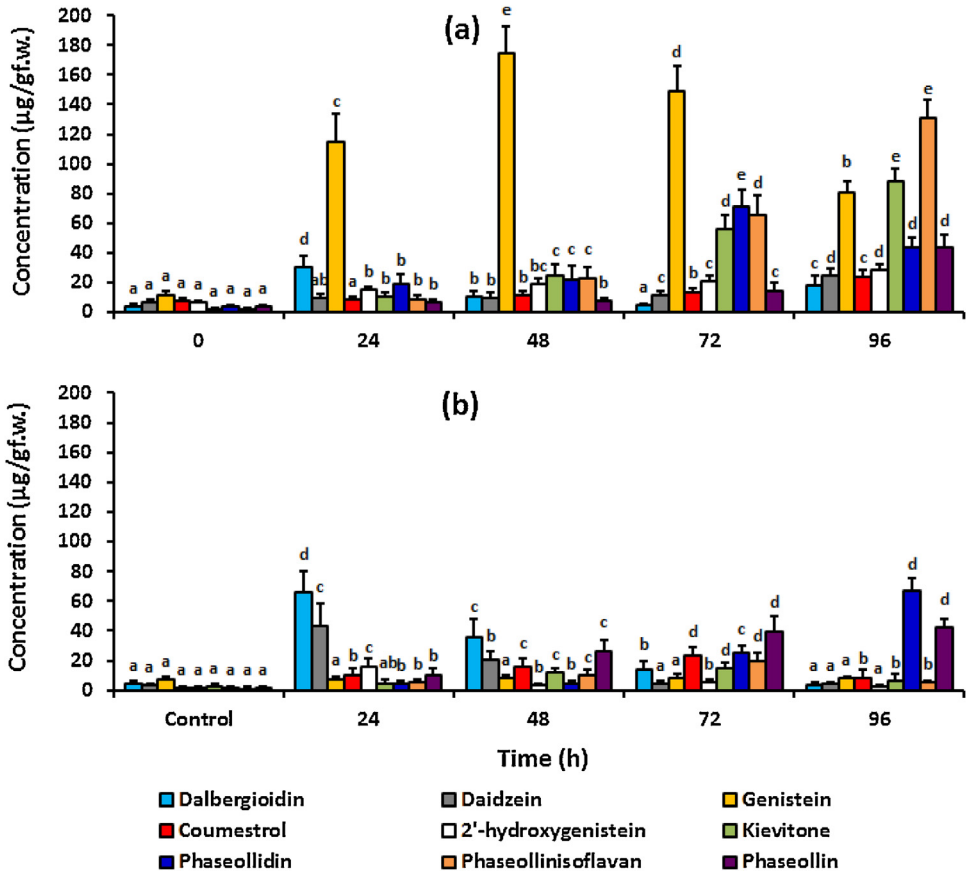

Fig. 11. Time-course accumulation of phytoalexins in common bean cotyledons, cultivars ICA Quimbaya (**a**) and Cargamanto Blanco (**b**), treated with 0.66 mM 1-oxo-indanoyl-L-isoleucyl methyl ester (**C-1**). Control treatments correspond to water-treated seedlings and after 96 h. For each time point, the bars headed by the same letter do not differ at p = 0.05 (Fisher's LSD test).

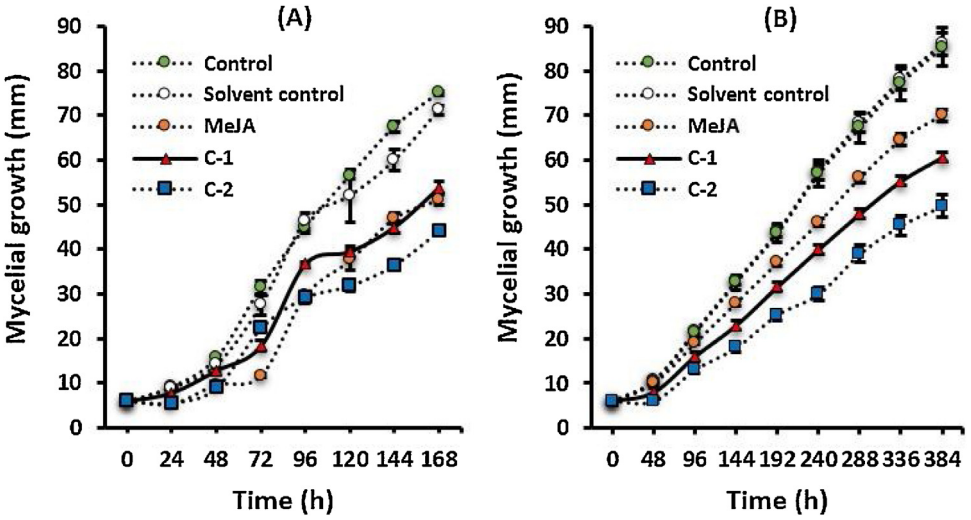

Fig. 12. Effect of methyl jasmonate (**MeJA**), 1-oxo-indanoyl-L-isoleucyl methyl ester (**C-1**), and 1-oxo-indanoyl-L-leucyl methyl ester (**C-2**) on mycelial growth of *Fusarium* spp. (**A**) and *C. lindemuthianum* (**B**).

phaseollinisoflavan and kievitone. It is possible to hypothesize that these delays occur in the prenylation stage of both the pterocarpanoid skeleton and the isoflavanone dalbergioidin to produce kievitone. A cDNA for pterocarpan 4-dimethylallyltransferase catalyzing the dimethylallylation in the biosynthetic pathway of the soybean pterocarpanoid phytoalexin, glyceollin, has been cloned and characterized [32]. In addition, prenylated pterocarpans has been seen to exhibit an increased toxicity against phytopathogenic fungi [33]. So, the identification and expression of the enzymes involved in the prenylation of phaseollin and kievitone would provide information on the molecular mechanisms involved in the resistance of common bean to pathogens and would enable rapid detection of those cultivars with better prospects for diseases resistance.

ii.) the tissue type; it has been reported that tissues composition of *P. vulgaris* treated with the same elicitor is very different. Thus, kievitone was the major phytoalexin accumulated in cotyledons treated with fungal cell wall preparation from *C. lindemuthianum*, whereas phaseollin was the major in hypocotyls [29]. Similarly, the elicitation of cotyledons and hypocotyls-roots of common bean with salicylic acid and derivatives have shown differences in the isoflavonoids composition depending on the treated tissue [10,11]. In the present study, genistein and kievitone were the major phytoalexins accumulated in cotyledons, especially for the anthracnose-resistant cultivar ICA Quimbaya, whereas hypocotyls-roots accumulated more phaseollin. The results are in according to the fact that the 5−OH and 5-deoxy pathways of isoflavonoid phytoalexins may be under separated control [34]. In addition, it is clear that the 5−OH pathway leading to kievitone formation prevails in cotyledons, while the 5-deoxy pathway leading to phaseollin overcomes in hypocotyls-roots.

iii.) the elicitor structure; taking into account that pterocarpans (phaseollidin and phaseollin), phaseollinisoflavan, coumestrol and kievitone are formed in the last stages of the biosynthesis of the 5-deoxy and 5-hydroxy isoflavonoids, it is possible to compare the eliciting activity of indanoyl derivatives by means of a correlation with the production of these phytoalexins. Bioassays with indanoyl esters of increasing size of the alkoxy substituent revealed a reduction in pterocarpans content as chain size increases. If the alkylic chain in the indanoyl ester is longer than three carbons, the contents of phaseollin, phaseollinisoflavan and kievitone found in the tissues are lower than those elicited by **1** and **2**. An additional advantage of indanoyl methyl and ethyl esters is their greater solubility in water. The reduction of the carbonyl group at C-1 to form a hydroxyl group also caused a decrease in the accumulation of phytoalexins. On the other hand, the permutation of the alkoxy substituents by the amino acids L-isoleucine (**C-1**) and L-leucine (**C-2**) demonstrated that these conjugates are much more actives. Compounds **C-1** and **C-2** also triggered the emission of volatiles in Lima bean (*P. lunatus*) [23] and pterocarpane-type phytoalexins in soybean cell cultures [15]. In fact, common bean tissues (cultivars ICA Quimbaya and Cargamanto Blanco) treated with **C-1** (1-oxo-indanoyl-L-isoleucyl methyl ester) and **C-2** (1-oxo-indanoyl-L-leucyl methyl ester) exhibit similar or even higher amounts of kievitone, phaseollin and phaseollidin than those elicited by **JA** or **MeJA**. It has been reported that only the indanoyl conjugates with aliphatic amino acids resembling the size and polarity of L-isoleucine, are active compounds [23]. Conjugates **C-1** and **C-2** more closely resemble coronatine and the endogenous elicitors jasmonoyl-L-isoleucine and (+)-7-iso-jasmonoyl-L-isoleucine than the indanoyl esters. Interestingly, coumestrol that was one of the major phytoalexins found in tissues of common bean elicited by salicylic acid and structurally-related compounds [11], presented low levels in cotyledons and hypocotyls-roots treated with indanoyl derivatives. Therefore, it is possible to think that the biosynthetic pathway of 5-deoxyisoflavonoids is being channeled towards the production of pterocarpans instead of coumestan. Given that the antifungal activity of pterocarpane-type phytoalexins has been recognized, the application of indanoyl elicitors could be improving the protection of the plant against pathogenic fungi by allowing the direct conversion of the precursor isoflavones into pterocapans. On the other hand, **C-1** and **C-2** exhibited moderate to weak inhibitory effects against *C. lindemuthianum* and *Fusarium* spp. which would suggest a dual mode of action for controlling fungal diseases; increasing host resistance and reducing pathogen inoculum.

iv.) *the post-induction time*; phytoalexin accumulation increased progressively according to the post-induction time; the highest amounts of phytoalexins were accumulated between 72 and 96 h. During the first 48 h, the dominant compounds were the isoflavones genistein, daidzein and 2′-hydroxygenistein and the isoflavanone dalbergioidin, which is consistent with the two biosynthetic pathways. Then, the concentration of these precursors was decreased or keep almost constant, while the levels of kievitone, coumestrol, phaseollin, phaseollidin, and phaseollinisoflavone were increased.

5. Conclusions

A series of derivatives of the 1-oxo-indane-4-carboxylic acid (esters and conjugates with some L-amino acids) was prepared. Then, the phytoalexins-elicitor effect was evaluated on cotyledons and hypocotyls-root of common bean (*P. vulgaris* L. cvs. ICA Quimbaya and Cargamanto Blanco). Results showed that the accumulation of isoflavonoid phytoalexins was dependent on the cultivar, the tissue type, the elicitor structure and the post-induction time. The indanoyl derivatives, especially indanoyl amino acid conjugates, exhibited an isoflavonoid-elicitor effects in common bean tissues. These compounds could be used for enhancing the amount of phytoalexins in common bean tissues and protect crops against fungal pathogens.

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CRediT authorship contribution statement

Leidy Botero: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Samuel Vizcaíno:** Conceptualization, Methodology, Investigation. **Winston Quiñones:** Resources, Writing - review & editing, Supervision. **Fernando Echeverri:** Resources, Investigation. **Jesús Gil:** Resources, Writing - review & editing, Supervision. **Diego Durango:** Resources, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2021.e00601.

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