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ORIGINAL ARTICLE

Antioxidant responses under jasmonic acid elicitation comprise enhanced production of flavonoids and anthocyanins in *Jatropha curcas* leaves

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Abstract *Jatropha curcas* has significant potential for production of biodiesel and secondary metabolites with medical applications. The effect of jasmonic acid (JA) on flavonoid production and antioxidant responses in two Mexican *J. curcas* plants (accessions I-64 and I-52), growing under controlled environmental conditions, was studied. Foliar application of JA (0.25, 0.5, and 1.0 mM) increased flavonoid content by 3.3-fold (445.6 ± 62.2 µg QE g^{-1} DW) at 1.0 mM in the I-64 accession after 120 h. At this time, individual flavonoid levels of kaempferol, apigenin, and vitexin were 6.8 (106.8 ± 18.6 µg g^{-1} DW), 4.4 (15.5 ± 1.5 µg g^{-1} DW), and 8.1 (6.27 ± 1.3 µg g^{-1} DW) times higher than in the controls, while for the I-52 specimens, the highest increase of flavonoids occurred at 24 h. In treated I-52 plants, enhancement of 58% (74.7 ± 9.2 µg g^{-1} DW) in anthocyanins occurred after 120 h, while in I-64 plants, they remained similar to controls. Phenylalanine ammonia lyase showed a peak of activity after 120 h in treated I-64 plants, while the I-52 accession showed peaks at 24 and 120 h. Elicited I-64 plants presented H_2O_2 levels similar to controls with a 30.6% increase in catalase (CAT) and 3.3-fold in ascorbate peroxidase (APX) activities. In elicited I-52 plants, a 2.4-fold increase in H_2O_2 concentration was related to a 65.6% decrease of CAT and a 1.8-fold increase of APX activities. Therefore, under JA elicitation, *J. curcas* plants increased antioxidant responses including flavonoid and anthocyanin production to maintain cell redox balance.

Keywords Antioxidant responses · Flavonoids · *Jatropha curcas* · Jasmonic acid · Phenylalanine ammonia lyase

Introduction

Jatropha curcas L., commonly known as physic nut, is a perennial plant belonging to the Euphorbiaceae family, which is found mostly in tropical and subtropical regions. In the last decade, this species received much attention due to its high potential for biodiesel production (Kumar and Sharma 2008). Molecular analysis revealed wide genetic variations in *J. curcas* from Mesoamerica. This region has been suggested as the center of origin (Salvador-Figueroa et al. 2015) in contrast with accessions from Asia and Africa, where they have low genotypic diversity and a nearby clustering that indicate a common ancestor (Zhang et al. 2011). Most seeds of this plant are toxic due to their high content of diterpene phorbol esters. However, in Mexico, along with the toxic accessions, often used as living fences, there are those with low or null toxicity due to their low content of such esters (Zavala del Angel et al.

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2016). These plants are known as "piñon" or "piñoncillo" and are even used in cooking local dishes (Martinez-Herrera et al. 2010). Such situation occurs in those varieties found at similar altitude and climates in the state of Veracruz. Interestingly, in this region, a selective domestication process initiated by the ancient Totonacs, towards the non-toxic varieties, has been noted (Zavala del Angel et al. 2016).

J. curcas contains diverse phytochemical compounds, mainly alkaloids, triterpenes, and flavonoids, that exhibit a broad spectrum of biological activities (Prasad et al. 2012). Such is the case of the flavonoids that have been reported from leaves (Kumar and Sharma 2008), including apigenin, vitexin, isovitexin, and their glucosides, which possess pharmacological activity as antioxidant, anti-irritant, anti-inflammatory, and anti-cancer agents (Kumar and Pandey 2013).

Flavonoids are a group of polyphenolic secondary metabolites with several functions in plants (Winkel-Shirley 2002). Their biosynthesis involves the action of phenylalanine ammonia lyase (PAL), which forms cinnamic acid from phenylalanine. After four steps, naringenin is biosynthesized producing the primary C15 flavonoid skeleton. At this point, the route bifurcates to form flavones and dihydroflavonols which are oxidized to flavonols as kaempferol or reduced to leucoanthocyanidins, thus initiating the synthesis of anthocyanins (Falcone-Ferreyra et al. 2012). Flavonoids play a major role in plant responses to environmental signals, in particular during biotic and abiotic stresses due to their antioxidant function as scavengers of reactive oxygen species (ROS) (Agati et al. 2012). In addition, anthocyanins have the capacity to reduce the potential of oxidative damage by means of light attenuation and reducing ROS through scavenging and metal chelation (Franceschi and Grimes 1991).

It has been proposed that flavonoids form part of the antioxidant complex network that function in plants to regulate their growth and stress tolerance (Winkel-Shirley 2002). This network also involves other molecular signals such as jasmonates, ROS, and antioxidant enzymes. Jasmonic acid (JA) and its derivative methyl jasmonate (MeJA), both known as jasmonates, mediate plant defense responses to wound and environmental stresses (Wasternack and Hause 2013; Xia et al. 2015). JA elicits the hyperproduction of various secondary metabolites as alkaloids, phenylpropanoids, and isoprenoids by means of a complex transcriptional and metabolic reprogramming in a cell- and tissue-specific manner (De Geyter et al. 2012). Thus, JA induces production of triterpenes in relation with the stimulation of oxidative responses in in vitro dedifferentiated *J. curcas*-pale cell suspension cultures grown in darkness (Zaragoza-Martínez et al. 2016). It has been reported that in some species, jasmonates positively regulate the enzymes responsible for flavonoid biosynthesis, which have been shown to induce the activity of PAL (Gundlach et al. 1992), as well as to stimulate the accumulation of anthocyanins in light-grown soybean seedlings (Franceschi and Grimes 1991) and flavonoids such as kaempferol (Hendrawati et al. 2006). On the other hand, it has been demonstrated that jasmonates can induce H_2O_2 production and that this last molecule can act as a second messenger inducing the expression of a variety of defense-related genes (Orozco-Cardenas et al. 2001). Furthermore, the coordinated activation of antioxidant metabolic pathways mediated by jasmonates provides plant resistance to environmental stresses (Sasaki-Sekimoto et al. 2005).

Despite the vast interest on *J. curcas*, most of the research has focused on the seed oil, whereas by-products, as those found in the leaves that act as defensive metabolites, have not been studied in detail. Polyphenolic compounds as flavonoids are involved in *J. curcas* defense responses to abiotic stress such as that caused by salt (Zhang et al. 2014), while the expression of *JcPAL1* gene has been induced under different stresses (Gao et al. 2012). Considering that JA is a signaling molecule that induces secondary metabolite biosynthesis in coordination with antioxidant responses, the objective of this research was to investigate the relation among flavonoid and anthocyanin production, PAL activity, and antioxidant enzyme responses under foliar JA elicitation in toxic and non-toxic Mexican accessions of *J. curcas* plants.

Materials and methods

Plant material and growth conditions

Seeds of non-toxic (I-64) and toxic (I-52) *Jatropha curcas* accessions were obtained by a donation. Seeds were previously collected from different regions of the state of Veracruz, Mexico. The I-64 accession was collected from 19°11′N 96°20′W at 16 m a.s.l. and the I-52 accession, from 19°10′N 96°8′W at 10 m a.s.l. More details about the seed collections and their geographic localizations are found in Zavala del Angel et al. (2016). Representative sets of each seed are deposited in the Germplasm Bank of Colegio de Postgraduados, Campus Veracruz, Mexico.

Seeds were germinated on peat moss pellets in a Conviron chamber A1000 under controlled environmental conditions at 30 ± 2 °C and under long-day conditions (16 h of light/8 h of dark), with a maximum photosynthetic photon flux density (PPFD) of 200 µmol m^2 s^{-1} and 50–0% of relative humidity. After germination, seedlings of uniform size were transferred into plastic pots (1 L) filled with a mixture of peat moss-sand-expanded perlite (Agrolita®)

(2:2:1), watered with running water each 2 days, and kept under the above-controlled conditions.

Treatment with JA

All experiments were carried out in triplicate on 75-day-old *J. curcas* plants. Twelve specimens of the I-64 accession were sprayed on all leaves with JA (Sigma-Aldrich, St. Louis, MO) in 30% ethanol at concentrations of 0.0, 0.25, 0.50, or 1.0 mM JA (4 mL per plant) and harvested after 120 h of treatment. For the time-course experiments, 15 plants of each I-64 and I-52 accessions grown in plastic pots as described above were treated with 1.0 mM JA dissolved in 30% ethanol. In addition, a series of 15 control plants for each accession was treated with 30% ethanol. Both control plants and JA-treated specimens were sealed with transparent sun bags (Sigma-Aldrich) for 12 h, and then, the bags were removed (Supplementary Material Figure 1). After 0, 12, 24, 72, and 120 h of treatment, the leaves of each plant were harvested. For the metabolic analysis, the harvested leaves were immediately frozen under liquid nitrogen, ground to a fine powder, and freeze-dried; while for the biochemical analysis, the leaves were frozen and stored at −20 °C.

Extraction of flavonoids

Freeze-dried powdered leaves (200 mg) were extracted twice with MeOH (4 mL). The extracts were sonicated for 15 min and centrifuged at 3000 rpm for 10 min. The supernatants were combined, concentrated to dryness, and re-suspended in 1 mL of MeOH:90 mM phosphate buffer (1:1), pH 6.0. An aliquot of 500 µL was hydrolyzed as described by Flores-Sanchez and Verpoorte (2008) with slight modifications using butylhydroxytoluene (9 µM) as antioxidant. The hydrolyzed products were dissolved in MeOH for total flavonoid content determination and HPLC analyses.

Total flavonoids and anthocyanin content assay

Total flavonoid content was determined according to the method of Chang et al. (2002). The reaction mixture consisted of 0.5 mL of hydrolyzed extract and 1.5 mL of 95% ethanol, followed by 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm using a Genesys 10 V spectrophotometer (Thermo Scientific). Quercetin (0–20 µg) was used to make the calibration curve. The results were expressed as micrograms of quercetin equivalents per gram of dry weight (DW) (µg QE g^{-1} DW). Anthocyanin extraction was performed essentially as described by Onkokesung et al. (2014) with minor modifications. In brief, the frozen leaves were ground in 1 mL HCl 1% (v/v) in MeOH. Extracts were incubated at 4 °C for 1 h and 0.3 mL of distilled water and 0.5 mL of chloroform were added to remove chlorophylls through mixing. Anthocyanins contained in the aqueous phase were determined spectrophotometrically at 530 nm (A_{530}) and the anthocyanin content was expressed as µg per g of DW using the extinction coefficient for cyanidin-3-glucoside of 26 900 L cm^{-1} mol^{-1} using MW for cyanidin-3-glucoside of 449.2 g mol^{-1}.

HPLC analysis of flavonoids

The hydrolyzed extracts were dissolved in MeOH and injected (100 µL) into an HPLC system (Varian Chromatograph ProStar 333 with photodiode array detector, Varian Walnut Creek, CA) equipped with a reversed-phase C18 column (250 × 4.6 mm, 5 µm, Waters). The solvent system and the operational conditions were described by Liu et al. (2010) with slight modifications. The mobile phase consisted of MeOH (A) and 0.1% trifluoroacetic acid aqueous solution (B). The gradient was started with 33% A (0–10 min), followed by 33–65% A (10–30 min), 65–90% A (30–40 min), 90% A (40–50 min), and 90–33% A (50–60 min), using a flow rate of 1 mL min^{-1}. The detection wavelength of flavonoids was done at 330 nm. The flavonoid peaks were identified by comparing their retention times and UV spectra with those of the analytical standards (>95% purity, Sigma-Aldrich). The retention times for vitexin, isovitexin, kaempferol, and apigenin were 17.1, 23.7, 31.8, and 32.6 min, respectively. Injections in the HPLC equipment of mixtures of the standard compounds were particularly useful to verify the identity of kaempferol and apigenin, which eluted with close retention times. For quantification of flavones and flavonols, apigenin and kaempferol, respectively, were used as the reference compounds to construct the corresponding calibration curves. Results are expressed as mean values in µg g^{-1} DW.

Phenylalanine ammonia lyase activity

Fresh leaves (0.5 g) were frozen in liquid nitrogen and ground to a fine powder in a pre-chilled mortar. Proteins were extracted by homogenizing the powder with 1 mL of ice-cold 50 mM Tris–HCl buffer solution (pH 8.8), containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) polyvinylpyrrolidone (PVP) followed by centrifugation at 12,000 rpm for 15 min at 4 °C. The supernatant was assayed for enzyme activity according to Zimmerman and Hahlbrock (1975). Samples containing 100 µL of enzyme extract were incubated with 50 mM

Tris–HCl (pH 8.8) and 20 mM of L-phenylalanine in a 2 mL volume for 30 min at 30 °C. The PAL activity was expressed as units (1 µmol of *trans*-cinnamic acid produced per minute) per mg of protein (Hahlbrock and Ragg 1975).

Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) content in the leaves was measured by its reaction with potassium iodide according to Sergiev et al. (1997) and Velikova et al. (2000). Leaves (0.5 g FW) were frozen and finely ground with liquid nitrogen and then homogenized in 1 mL of trichloroacetic acid, 0.1% (w/v). The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. An aliquot of the supernatant (0.5 mL) was treated with 0.5 mL phosphate buffer 10 mM, pH 7, and 1 mL 1 M potassium iodide. The absorbance was measured at 390 nm and the H_2O_2 content was calculated from a standard curve, expressing the results as µmol g^{-1} DW.

Assays of antioxidant enzyme activities

Leaves (0.5 g FW) were used for protein extraction as described by Zaragoza-Martinez et al. (2016). Protein content was measured according to Peterson (1977) using bovine serum albumin as standard. The activity of catalase (CAT; EC: 1.11.1.6) was determined according to Aebi (1984). The reaction mixture contained 1.8 mL of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mL of 2% H_2O_2 (v/v), and 0.1 mL of protein extract. The reaction was monitored at 25 °C following the absorbance decrease at 240 nm every 30 s for 3 min. One unit of CAT was defined as the amount of enzyme required for the decomposition of 1 µmol of H_2O_2 per minute calculated from its extinction coefficient ($\varepsilon_{240} = 39.4\ mM^{-1}\ cm^{-1}$).

Ascorbate peroxidase (APX, EC: 1.11.1.1) activity was measured by monitoring the decrease in absorbance of ascorbic acid at 290 nm per 3 min according to Silva et al. (2010). The reaction solution consisted of 50 mM potassium phosphate pH 7.0, 0.5 mM ascorbic acid, 30 mM H_2O_2, and 0.1 mL of enzyme extract, in a total volume of 2 mL incubated at 25 °C. The enzyme activity was calculated using the molar extinction coefficient $\varepsilon_{290} = 2.8\ mM^{-1}\ cm^{-1}$ expressed as units of enzyme activity (oxidation of 1 µmol of ascorbate per minute). APX and CAT activities are reported as units of enzyme per milligram of protein (U mg^{-1} protein).

Statistical analyses

Results were analyzed by one-way ANOVA using the SPSS software version 21.0 (SPSS Inc., Chicago IL). Significant differences between the means of parameters were determined using the Tukey's test ($P < 0.05$).

Results

Effect of JA concentration on the content and profile of flavonoids

To investigate the effects of JA on the flavonoid content, plants of *J. curcas* (I-64) were elicited with several concentrations of JA for 120 h. The total flavonoid content was markedly enhanced ($P < 0.05$) when increasing JA concentration. The increase with 0.5 mM JA was 2.5-fold (305.8 ± 27.8 µg QE g^{-1} DW) compared to control plants (136.0 ± 11.7 µg QE g^{-1} DW), while with 1 mM JA reached a maximum up to 3.3-fold (445.6 ± 62.2 µg QE g^{-1} DW) (Fig. 1). The individual flavonoid content changed widely depending on the applied JA concentrations (Fig. 2). It was found that a dose of JA 0.5 mM sprayed on the leaves enhanced mainly vitexin and isovitexin content by 44- and 10-fold, respectively (0.6 ± 0.1 to 26.6 ± 5.4 and 1.6 ± 0.5 to 16.3 ± 1.0 µg g^{-1} DW). However, the highest induction of all evaluated flavonoids occurred at 1 mM JA. The increase in individual flavonoids was quantified for kaempferol 4.7-fold (57.4 ± 0.4 in elicited and 12.1 ± 2.1 µg g^{-1} DW in control), apigenin 2.5-fold (15.5 ± 4.4 in elicited and 6.3 ± 3.5 µg g^{-1} DW in control), vitexin 7.1-fold (6.4 ± 2.3 in elicited and 0.6 ± 0.1 µg g^{-1}

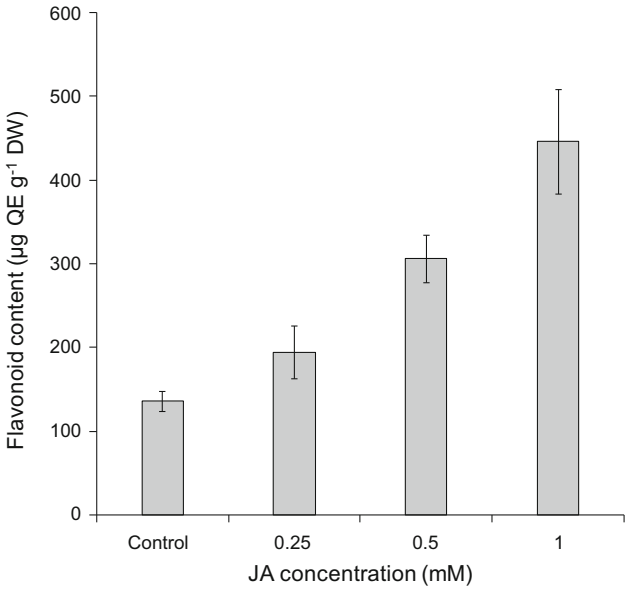

Fig. 1 Flavonoid content in leaves of *Jatropha curcas* (I-64 accession) at 120 h after JA treatment using three different concentrations. Control plants were treated with 30% ethanol and elicited plants with JA in 30% ethanol. Values represent mean ± DE ($n = 3$)

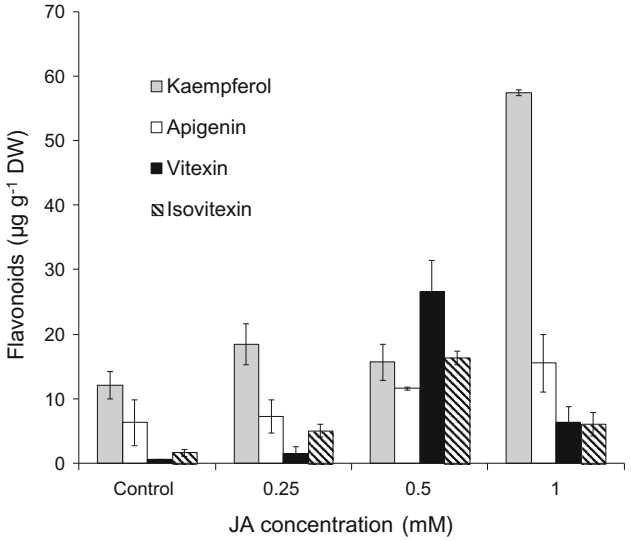

Fig. 2 Flavonoid content profile in leaves of *Jatropha curcas* (I-64 accession) at 120 h after JA treatment using three different concentrations. Control plants were treated with 30% ethanol and elicited plants with JA in 30% ethanol. Values represent mean ± DE ($n = 3$)

DW in control), and isovitexin 3.8-fold (6.1 ± 1.8 in elicited and 1.6 ± 0.5 µg g^{-1} DW in control) (Fig. 2).

Changes in the flavonoid profile after JA elicitation in *J. curcas*

To investigate changes in concentration of kaempferol, apigenin, vitexin, and isovitexin in each accession of *J. curcas* after foliar application of 1 mM JA, flavonoid accumulation was examined through the time (Fig. 3). In the I-64 plants treated with JA, kaempferol levels were 6.8 times higher (106.8 ± 18.6 µg g^{-1} DW) than control plants at 120 h (Fig. 3a). For JA-elicited *J. curcas* I-52, the levels of kaempferol increased up to 3.9 times at 24 h (50.1 ± 13.8 µg g^{-1} DW) compared to the control, and then, it began to decrease (Fig. 3a).

In I-64 specimens, a high increase of both apigenin and vitexin was found at 120 h after elicitation reaching 4.4-fold (15.5 ± 1.5 µg g^{-1} DW) and 8.1-fold (6.27 ± 1.3 µg g^{-1} DW), respectively, in relation with control levels (Fig. 3b, c), while in the I-52 plants, a biphasic response was observed. An increase of 6.0- and 2.5-fold in apigenin and of 3.4- and 1.8-fold in vitexin concentrations occurred at 24 and 120 h, respectively (Fig. 3b, c). Isovitexin concentrations showed a rise at 120 h after JA elicitation in both plants, in I-64 by 3.6-fold (6.1 ± 0.9 µg g^{-1} DW) and in I-52 by 2.3-fold (2.7 ± 0.3 µg g^{-1} DW) (Fig. 3d).

As mentioned above, I-52 plants treated with JA for 120 h showed a reduction (35%) of kaempferol levels in relation with the control (Fig. 3a), which can be associated with the enhancement by 58% in anthocyanin accumulation (74.7 ± 9.2 µg g^{-1} DW) (Fig. 4). In contrast, anthocyanin content in the elicited I-64 plants remained similar to the control (Fig. 4).

Activity of PAL after JA elicitation in *J. curcas*

In JA-treated *J. curcas* I-64, PAL activity had a significant peak after 120 h (Fig. 5), showing three times more activity (63.23 ± 2.9 U mg^{-1} protein) than the control plants (20.62 ± 3.5 U mg^{-1} protein). On the other hand, in JA-elicited I-52 plants, PAL activity reached a 1.9-fold peak after 24 h of elicitation compared to control (19.19 ± 3.7 U mg^{-1} protein), followed by a minor peak until the end of the experiment (Fig. 5).

Hydrogen peroxide and antioxidant responses to JA elicitation in *J. curcas* accessions

The concentration of H_2O_2, as well as CAT and APX enzyme activities in leaves of I-64 and I-52 plants during 120 h of elicitation with JA is shown in Table 1. *J. curcas* I-64 plants presented H_2O_2 levels similar to those of controls after 120 h of elicitation, but with enhancement in CAT activity by 30.6% (Table 1). However, at 12, 24, and 72 h after JA treatment, the H_2O_2 levels increased 12.3, 55.6, and 31.6%, respectively, while CAT activity decreased 31.1% at 24 h and increased 23.3% at 72 h after elicitation. In the I-52 specimens, a significant increase in H_2O_2 concentration (2.4-fold) and a decrease in CAT activity (65.6%) took place in relation with control plants at 120 h. It was found that JA treatment increased the APX activity between 1.4- and 3.3-fold in the I-64 specimens and between 1.1- and 2.2-fold in the I-52 plants in relation with controls (Table 1).

Discussion

The differential increments obtained in production of flavones (vitexin and isovitexin) with respect to flavonols (kaempferol) according to the treatments with 0.5 or 1 mM JA were also found in other species in which the biosynthesis of secondary metabolites is stimulated in specific steps of the metabolic pathway (Gundlach et al. 1992; Ali et al. 2007). In view of the fact that the highest induction of all evaluated flavonoids occurred at 1 mM JA, we used such concentration for the subsequent experiments. Our results showed that JA treatment had an important effect on accumulation of individual flavonoids, since a high increase in kaempferol occurred in both elicited *J. curcas* accessions, mainly after 24 h for I-52 and 120 h for I-64. It

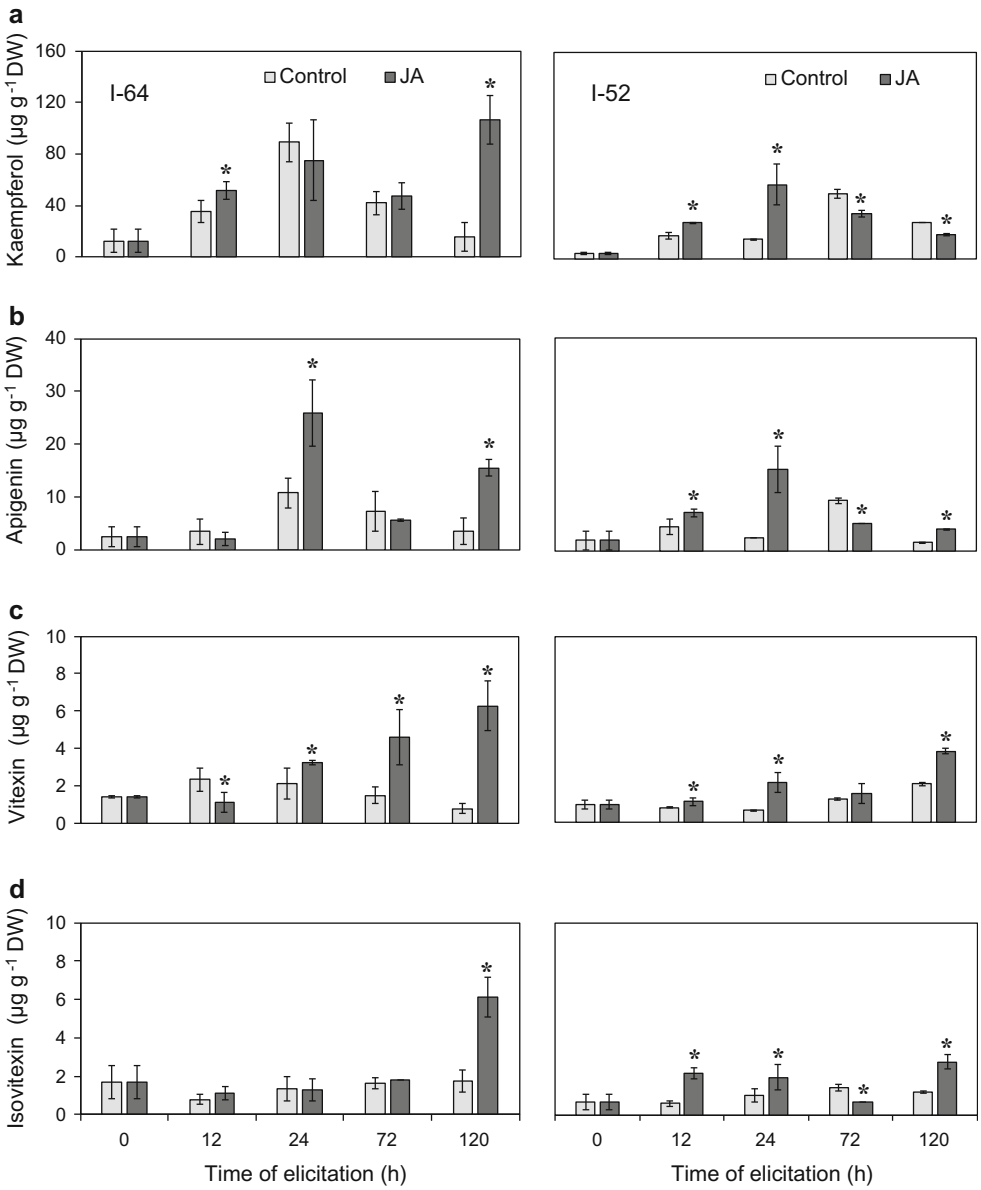

Fig. 3 Time course of (**a**) kaempferol (**b**) apigenin (**c**) vitexin, and (**d**) isovitexin content in leaves of I-64 and I-52 *Jatropha curcas* accessions after JA treatment. Control plants were treated with 30% ethanol and elicited plants with 1 mM JA in 30% ethanol. Values represent mean ± DE ($n = 3$). Significantly different values at $P < 0.05$ are represented by *

has been reported that in other species such as *Arabidopsis thaliana*, treatment with MeJA 5 mM applied to leaves rapidly enhanced accumulation of kaempferol glycosides (Hendrawati et al. 2006). In addition, in red raspberries, MeJA treatments increased the content of kaempferol itself (de la Peña et al. 2010). The observed kaempferol accumulation in JA-elicited *J. curcas* suggests that the increase of flavonol concentrations could be associated with the enhancement of gene expression of flavonol 3-hydroxylase (*F3′H*) and flavonol synthase as described for *Arabidopsis* (Winkel-Shirley 2002).

The high increase, over fourfold in relation with control, of apigenin, vitexin, ad isovitexin at 120 h after elicitation was found in the I-64 accession, while in the I-52 specimens, a biphasic response was observed for the flavones with increments at 24 and 120 h. The different responses of both accessions are in close relation with a high PAL activity at 120 h in I-64, leading the flow of precursors for biosynthesis of flavones and flavonols, while in the I-52 accession, the high PAL activity at 24 h may be related to the increase of these flavonoids at such time. The minor peak of PAL activity at 120 after JA elicitation of I-52 may correspond principally to the moderate increments in the flavone contents. In *Fagsopyrum esculentum* seedlings, flavones as apigenin were not affected by MeJA (Horbowicz et al. 2011), but an enhancement in levels of apigenin, vitexin, and isovitexin was observed in *J. curcas* callus cultures after treatment with high doses of ultraviolet-B light (Alvero-Bascos and Ungson 2012). The role of flavonoids in plant defense is well known; thus, apigenin

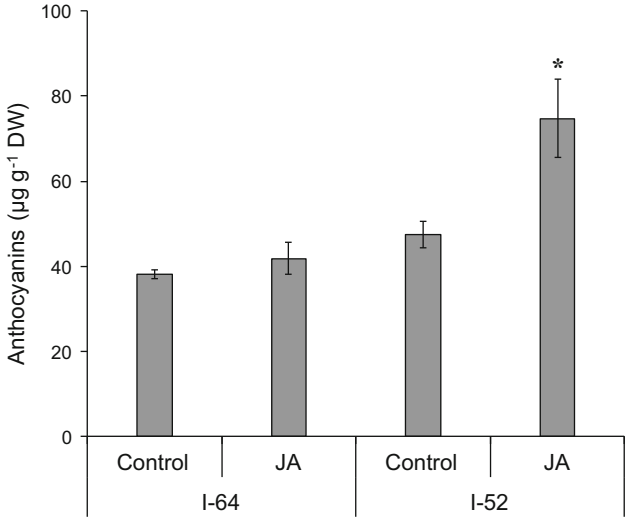

Fig. 4 Anthocyanin levels in leaves of I-64 and I-52 *Jatropha curcas* accessions at 120 h after JA treatment. Control plants were treated with 30% ethanol and elicited plants with 1 mM JA in 30% ethanol. Values represent mean ± DE ($n = 3$). Significantly different values at $P < 0.05$ are represented by *

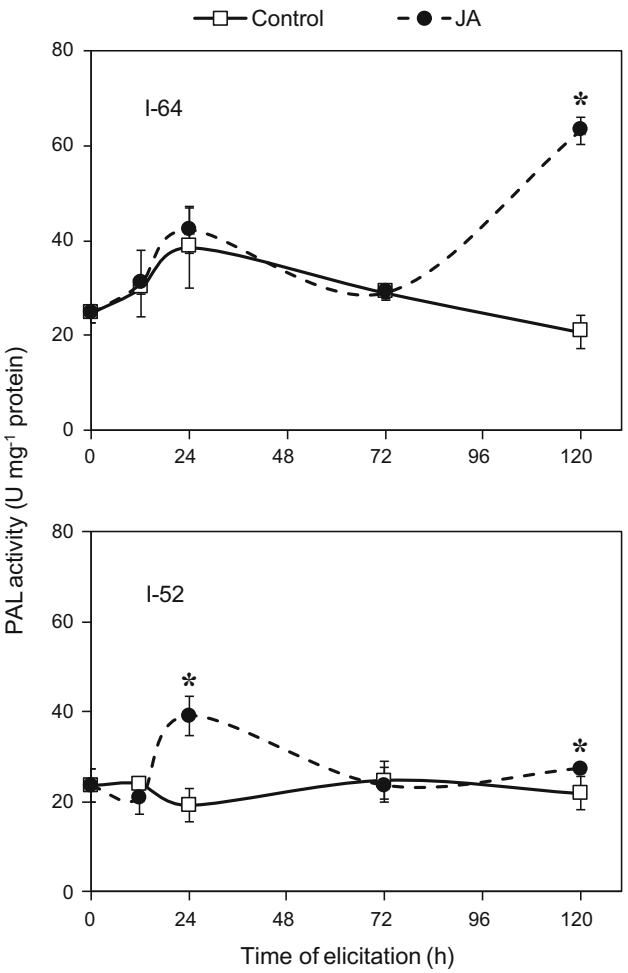

Fig. 5 Time course of PAL activity in leaves of I-64 and I-52 *Jatropha curcas* accessions after JA treatment. Control plants were treated with 30% ethanol and elicited plants with 1 mM JA in 30% ethanol. Values represent mean ± DE ($n = 3$). Significantly different values at $P < 0.05$ are represented by *

derivatives can act as feeding deterrents to *Lepidoptera* larvae (Erhard et al. 2007), while vitexin and isovitexin show a possible defensive role in plants infected by pathogens (Mierziak et al. 2014) and virus (Krcatović et al. 2008). These roles should not be excluded that they may occur in the elicited *J. curcas* leaves.

As mentioned above, the toxic I-52 *J. curcas* plants treated with JA for 120 h showed a reduction (35%) of kaempferol levels in relation with the control, which can be associated with the enhancement by 58% in anthocyanin accumulation together with a two-fold increase of H_2O_2. In contrast, the anthocyanin and H_2O_2 contents in the elicited non-toxic I-64 plants remained similar to the control. In this accession, the increase in the activity of antioxidant enzymes and the flavonoid concentrations, in particular for kaempferol, may be decreasing the H_2O_2 contents to values similar to the control. Under these conditions, production of anthocyanins to reduce the potential for oxidative damage would not be stimulated. It has been reported that the metabolic flow in the flavonoid biosynthetic pathway is controlled by substrate competition between flavonol synthase and dihydroflavonol 4-reductase (Falcone-Ferreyra et al. 2012). Moreover, an antagonistic relationship between the accumulation of flavonols, primarily kaempferol derivatives, and anthocyanins in *Arabidopsis* has been proposed (Gou et al. 2011). Research in diverse plant species has yielded evidence for both the photoprotective and antioxidant hypotheses of anthocyanins (Franceschi and Grimes 1991). The difference in the anthocyanin content in the two *J. curcas*-treated accessions might be related to different levels of resistance to environmental stresses and also by the selective domestication processes towards the non-toxic accessions (Zavala del Angel et al. 2016). In several plant species, it has been demonstrated that jasmonate signaling stimulates anthocyanin accumulation (Shan et al. 2009) and their biosynthesis is controlled by numerous regulatory factors at the transcriptional level. Overexpressed *Arabidopsis* plants with the MYB75 transcription factor showed an enhancement in anthocyanin accumulation as a result of re-channeling of the flavonols quercetin or kaempferol. In consequence, this metabolic decline affected the plant defense mechanisms against a specialist caterpillar (Onkokesung et al. 2014).

Herein, it was found that the PAL activity measured over the time in the I-52 and I-64 plants shows a biphasic trend that can be correlated with the flavonoid contents after elicitation with 1 mM JA. In different taxa, the PAL plays a key regulatory role in controlling the biosynthesis

Table 1 Time course of H_2O_2 levels, and CAT and APX activities in *Jatropha curcas* leaves (I-64 and I-52 accessions) elicited with JA 1 mM

Time (h)	Treatment	I-64 accession			I-52 accession		
		H_2O_2 (μmol g^{-1} DW)	CAT (U mg^{-1} protein)	APX (U mg^{-1} protein)	H_2O_2 (μmol g^{-1} DW)	CAT (U mg^{-1} protein)	APX (U mg^{-1} protein)
0	Control	370.4 ± 11.1	6.3 ± 0.4	25.6 ± 0.8	495.5 ± 77.2	3.5 ± 0.2	75.2 ± 0.9
12	Control	402.7 ± 9.2	6.1 ± 0.1	25.1 ± 0.1	589.4 ± 35.5	4.1 ± 0.4	78.2 ± 4.2
12	JA	497.8 ± 22.5*	6.4 ± 0.4	24.0 ± 7.8	957.8 ± 142.4*	2.1 ± 0.8*	87.2 ± 9.0*
24	Control	390.3 ± 19.9	6.8 ± 0.6	35.4 ± 4.0	447.1 ± 49.0	4.8 ± 0.9	68.4 ± 13.3
24	JA	615.6 ± 30.9*	4.7 ± 0.7*	51.4 ± 2.7*	652.2 ± 42.6*	3.7 ± 0.3	96.1 ± 1.3*
72	Control	392.0 ± 28.6	6.0 ± 0.3	34.2 ± 4.1	609.9 ± 22.9	6.0 ± 0.3	79.1 ± 4.5
72	JA	516.5 ± 33.7*	7.4 ± 0.3*	85.2 ± 4.8*	924.4 ± 82.6*	2.8 ± 0.2*	175.0 ± 17.0*
120	Control	444.0 ± 14.8	5.2 ± 0.2	25.1 ± 0.1	434.6 ± 107.3	5.2 ± 0.2	80.6 ± 2.6
120	JA	453.7 ± 28.8	6.8 ± 0.4*	82.6 ± 2.1*	1049.3 ± 179.7*	1.8 ± 0.1*	144.0 ± 2.1*

Control plants were treated with 30% ethanol and elicited plants with JA in 30% ethanol. Values represent mean ± DE ($n = 3$)

Significant differences are indicated by * at $P < 0.05$

of phenylpropanoids as the entry-point enzyme of this pathway that was transiently increased after jasmonates treatment (Gundlach et al. 1992; Ali et al. 2007). Likewise, PAL activity regulated the formation of naringenin from L-phenylalanine, resulting in accumulation of flavonoids, particularly kaempferol in red raspberries treated with 250 μM MeJA (de la Peña et al. 2010). It is worth to mention that anthocyanin biosynthesis also include transcription factors which predominantly regulate the expression of the "late" over the "early" anthocyanin biosynthetic genes such as *PAL* (Shan et al. 2009). In plants, PAL is encoded by a multi-gene family presenting multiple isoforms which differentially respond to various abiotic and biotic stimuli. Each PAL gene may have distinct metabolic functions as *PAL*1 and *PAL*2 which could have functional specialization in triggered flavonoid biosynthesis (Zhang and Liu 2015). In rice cell suspension cultures, a dose-dependent biphasic profile of PAL activity induction might rely upon the presence of different elicitors in fungal hydrolysates. The early and late elevations in PAL activity were based on the sequential induction of two different isoenzymes (Giberti et al. 2012). These findings could suggest a plausible explanation for the biphasic trend of PAL activity in JA-elicited *J. curcas* accessions which could involve responses towards molecular signals as JA and H_2O_2.

The CAT and APX enzymes are responsible for detoxification of elevated levels of ROS, whereas CAT activity appears to be critical for maintaining the redox balance during oxidative stress (Mhamdi et al. 2010). In JA-elicited I-64 plants, the CAT activity decreased only within the first 24 h, followed by a recovery of the antioxidant enzyme activities which maintained the H_2O_2 levels similar to the controls at 120 h after elicitation. These results show that there was a mitigation of oxidative stress due to the CAT increase, while in I-52 plants, a significant increase in H_2O_2 concentration was closely related to a sustained decrease in CAT activity. The APX activity in both I-52 and I-64 plants was increased after the JA treatment. In *Panax ginseng* roots treated with 200 μM MeJA showed an increased in H_2O_2 content associated with a decrease of CAT activity and an increment of APX activity after the addition of the elicitor (Ali et al. 2007). In plant cells, APX plays a central role in detoxification of low concentrations of H_2O_2; therefore, their activity may have a critical function controlling the H_2O_2 levels (Shigeoka et al. 2002). Flavonoids in the vacuole of plant mesophyll cells are capable of removing H_2O_2 freely diffusing out of the chloroplast (or the peroxisomes) when the activity of APX or CAT is strongly depressed (Agati et al. 2012). Consequently, the increase of antioxidant enzyme activities, CAT and APX, along with levels of other non-enzymatic antioxidants such as flavonoids could maintain a redox balance with low H_2O_2 concentrations that favor essential regulatory mechanisms in the cell after JA treatment.

A significant difference between the toxic I-52 and non-toxic I-64 accessions is that the I-52 plants contain phorbol esters (Zavala del Angel et al. 2016) which could be an important factor for their differential responses. Their presence might also be triggering responses in the production of anthocyanins and H_2O_2 concentration in JA-elicited I-52 specimens. Phorbol esters have been suggested to be involved in biotic and abiotic stress responses, either directly through the activation of plant protein kinases or via protein analogous (Baudouin et al. 1999; 2002), which are key components of JA signaling (Wasternack and Hause 2013). It has been proposed that this could be the mechanism for induction of secondary

metabolite accumulation in carrot and *Sanguinaria canadensis* elicited cultures treated with phorbol esters (Kurosaki et al. 1987; Mahady et al. 1998).

Conclusions

Our results suggest that different doses of JA stimulate the flavonoid metabolism in *J. curcas*. Application of JA 1 mM to the leaves of I-64 and I-52 plants induces an increment in the PAL activity, as well as in accumulation of total flavonoids and consequently in the individual flavonoids including kaempferol, apigenin, vitexin, and isovitexin. In elicited I-64 plants, the presence of H_2O_2 levels similar to the control could be the result of enhanced CAT and APX activities as well as an increment in the total flavonoid content. In contrast, the I-52 plants showed an increase in the levels of H_2O_2, which should be associated with the decrement of CAT activity and related to the anthocyanin increase. The leaves of *J. curcas* stimulated with JA can be a rich source of valuable bioactive products such as flavonoids and anthocyanins.

Author contribution statement All authors contributed extensively to the work presented in this paper and approved the manuscript in its final form. GLC and FZM contributed for carrying out the experiments, the data collection, and statistical analysis. ACRV conceived and designed the experiments, and TPN, CMCGR, GTT, and FEG analyzed and discussed the results and implications commented on the manuscript at all stages.

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