




Jasmonates promote enhanced production of bioactive caffeoylquinic acid derivative in *Eclipta prostrata* (L.) L. hairy roots

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Abstract

Eclipta prostrata (L.) L. is widely used in traditional medicine for treatment of hepatitis, poisoning from snake bites and viral infections. Pharmacological studies confirmed its antioxidant, anti-inflammatory and anticancer activities. The efficacy of *E. prostrata* (L.) L. extracts has been correlated to phenylpropanoids such as flavonoids, coumestans and caffeoylquinic acid derivatives. In this work, the production of wedelolactone, demethylwedelolactone and 3,5-di-O-caffeoylquinic acid (3,5-diCQA) in hairy root cultures of *E. prostrata* (L.) L. C19 clone was increased after addition of eliciting agents jasmonic acid (JA) or methyl jasmonate (MeJA) at multiple concentrations. Cultures elicited with 100 μ M of JA saw a 5.2 fold increase in wedelolactone (from 0.72 to 3.72 mg/g d.w.), a 1.6 fold increase in demethylwedelolactone (from 5.54 to 9.04 mg/g d.w.) and a 2.47 fold increase in 3,5-diCQA (from 18.08 to 44.71 mg/g d.w.). Obtained data validate the potential of *E. prostrata* (L.) L. hairy root cultures as a production system of wedelolactone, demethylwedelolactone and especially 3,5-diCQA, which has recently been reported to possess activity against coronavirus disease (Covid-19) by in silico computational studies.

Key message

The goal of this work was to evaluate the effect of jasmonic acid (JA) or methyl jasmonate (MeJA) on wedelolactone, demethylwedelolactone and 3,5-di-O-caffeoylquinic acid (anti-Covid-19 drug candidate) production from hairy root cultures of *Eclipta prostrata* (L.) L. C19 clone.

Keywords *Eclipta prostrata* (L.) L. · Hairy roots · Covid-19 · Coumestans · Jasmonates · Caffeoylquinic acid derivatives

Introduction

In vitro culture techniques are an alternative for rapid multiplication of rare plant genotypes, plant genome transformation, and production of plant bioactives (Espinosa-Leal et al. 2018). They provide unique opportunities for the use of biotechniques that may increase plant secondary metabolite production. Some methods can improve the technique such

as the use of plant transformation generation techniques. In response to *Rhizobium rhizogenes* genetic transformation, susceptible plants present a phenotype alteration known as hairy roots (Cardarelli et al. 1987; Spina et al. 1987; Schmülling et al. 1988). Applications of some elicitors and combinations of different stress stimuli can enhance the production of bioactive molecules by elicitor effects. Jasmonates are considered widespread elicitors since they affect several signaling pathways stimulating the production of different classes of secondary metabolites in plants and microorganisms (Lourenço et al. 2016). The more active derivative methyl jasmonate (MeJA) and the less active cis-jasmonate and dihydrojasmonic acid are the main plant stress compounds involved in the signaling of defense responses, primarily due to induction of genes involved in phytoalexin and phenolics biosynthesis (Sák et al. 2021).

Eclipta prostrata (L.) L., native to Brazil and other tropical and subtropical regions of the world, is an annual

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herbaceous species from the Asteraceae family (Souza et al. 2003). This species is widely used in traditional medicine against several diseases like hepatitis (Lu et al. 2016), poisoning by snake bites and treatment of viral infections (Manvar et al. 2012). Moreover, pharmacological studies have reported *E. prostrata* (L.) L. antioxidant, anti-inflammatory and anticancer activities (Chaudhary et al. 2011; Yuan et al. 2013; Ali et al. 2016). The efficacy of *E. prostrata* (L.) L. has been correlated to chemical constituents present in its extracts such as flavonoids (Malla et al. 2013), thiophenes (Wu et al. 2008; Han et al. 2013), coumarins (Zhang and Guo 2001), triterpenoid saponins (Yahara et al. 1994), steroids (Cheng and Hu 2010) and coumestans (Diogo et al. 2009). The coumestans wedelolactone (WL) and demethylwedelolactone (DWL) isolated from *E. prostrata* (L.) L. are the most explored compounds from the point-of-view of biological activity, displayed potency and selective inhibition activity of lipoxygenase subcategories 5-lipoxygenase (Wagner and Fessler 1986) and a trypsin-inhibiting activity that may be associated to the anti-inflammatory potential of the species (Syed et al. 2003). WL and DWL suppressed cancer cell motility, inhibiting the invasion and growth of breast cancer cells and they also exhibited an anti-invasive effect on human SK-HEP-1 hepatoma cells. Also, DWL suppressed lung cancer cells metastasis in mice (Lee et al. 2012). Moreover, the association of WL with the flavonoids apigenin and luteolin restrained both the in vitro and in vivo growth of prostate cancer cells (Lin et al. 2007; Tsai et al. 2009).

Eclipta prostrata (L.) L. produces phenolic acids such as chlorogenic acid and its derivative 3,5-di-O-caffeoylquinic acid (3,5-diCQA) (Lee et al. 2010). Chlorogenic acid and its derivatives have demonstrated several biological activities, such as antioxidant, antibiosis, anti-inflammatory, antiviral, and antitumor activities (Huang et al. 2014; Ali et al. 2017; Liu et al. 2018; Devrnja et al. 2020). More recently, studies by molecular docking using a serine-type protease (Mpro), a SARS-CoV-2 virus polyprotein, showed that the obtained binding energies of 3,5-diCQA were closed to remdesivir (antiviral compound), indicating stronger binding to Mpro protein and promising antiviral compounds that could treat Covid-19 (Shah et al. 2021). Additionally, the 3,5-diCQA was related as an emergency drug that can be used for targeting 2'-O-ribose methyltransferase of SARS-CoV-2 in silico (Sumon et al. 2021). In previous work, our group investigated the suitability of *E. prostrata* (L.) L. hairy root cultures as a system for the production of the coumestans wedelolactone (WL) and demethylwedelolactone (DWL) and a hairy root clone named C19 was selected considering its superior potential for producing coumestans (Diogo et al. 2009). Aiming to increase yields of bioactive compounds, we decided to evaluate the eliciting effect of JA and MeJA in *E. prostrata* (L.) L. hairy root C19 during the biosynthesis

of coumestans (WL and DWL) and especially 3,5-diCQA (Fig. 1), an promising anti-covid-19 compound.

Materials and methods

Chemicals

The chemical elicitors JA (jasmonic acid), MeJA (methyl jasmonate), and WL (wedelolactone) were purchased from Sigma-Aldrich® (St. Louis, MO).

Maintenance of *E. prostrata* (L.) L. C19 hairy root clone

Eclipta prostrata (L.) L. hairy root C19 clone was obtained by infecting *E. prostrata* (L.) L. micropropagated seedlings with *R. rhizogenes* LBA 9796 (Diogo et al. 2009). C19 clone was selected because its roots presented higher production of the coumestans WL and DWL. Monthly subculture of the C19 clone in MS liquid culture medium (Murashige and Skoog 1962) under agitation (100 rpm) at 24 °C in the dark in order to maintenance of clone. To determine the most appropriate period to add the eliciting agent to the culture, a growth curve was established inoculating hairy roots (2 g) into 100 ml of MS medium, in the same conditions as above. Hairy root samples were collected periodically at 3-day intervals, weighed and dried in a circulating air oven to determine fresh and dry weight of samples.

Determination of the kinetics of growth of *E. prostrata* (L.) L. hairy root

For determining the growth kinetics, the hairy roots (2.00 ± 0.01 g) were inoculated into 100 mL of MS liquid culture medium. Cultures were kept in the dark at 25 ± 1 °C under agitation (100 rpm). Two independent experiments were carried out in triplicate flasks. Samples were collected at 3-day intervals up to the 36th day of culture. After determining root fresh weight, the material was dried at 45 °C in a circulating air oven for 24 h to determine the root dry weight.

Elicitation of *E. prostrata* (L.) L. hairy roots with JA or MeJA

For the elicitation assays, hairy roots (1.0 g) were inoculated into 100 ml of MS liquid medium and maintained as above. On the 21st day, cultures were supplemented with two different concentrations (100 µM or 140 µM) of JA or MeJA (Wiktorowska et al. 2010) and kept under agitation as mentioned above. One, two and four days after addition of elicitor agent, samples were collected and dried in

a circulating air oven and then extracted to determine WL, DWL and 3,5-diCQA levels. Analysis of variance (ANOVA) was carried out to compare mean values and linear regression curves were constructed using SISVAR 5.3 software (Ferreira 2011). Mean values were compared by the Scott-Knott test ($p < 0.05$) (Scott and Knott 1974).

Quantification of WL, DWL and 3,5-diCQA in *E. prostrata* (L.) L. hairy root C19 clone

For quantification of target secondary metabolites produced by *E. prostrata* (L.) L. hairy roots, dried roots (200 mg) were sonicated with 5.0 mL MeOH:H₂O (7: 3) for 30 min. The extracts were then filtered through a 0.45 μ m membrane and injected into a HPLC Shimadzu LC10AD vp (Shimadzu, Japan) coupled with a photodiode array detector. A Phenomenex Quinetex C-18 column (250 \times 4.0 mm, 5 μ m particles) was used for the analyses. The solvent system consisted of 0.1% acetic acid in water (A) and methanol (B). The analysis was performed on a linear gradient ranging from 10% B to 66% B in 32 min, returning to 10% B for up to 35 min, concluding the analysis in 40 min. The injection volume was 20 μ L and visualization at 330 and 350 nm. Quantification was performed by external standardization using standard curves from WL (at concentrations of 0.063 mg/mL to 0.50 mg/mL) and 3,5-diCQA (at concentrations of 0.063 mg/mL to 1.00 mg/mL).

Isolation and structural elucidation of 3,5-diCQA from *E. prostrata* (L.) L. hairy root C19 clone

C19 hairy roots cultured for 21 days in MS liquid medium were harvested and dried in a circulating air oven. The dried material (300 g) was macerated in an ethanol:water (7:3) solution (2 L) for 7 days and then with methanol (1 L) for 7 days. The methanolic extract was evaporated to dryness and the dry residue (60 g) was solubilized in methanol:water (1:9), partitioned with hexane and then with ethyl acetate (thrice each). The aqueous fraction obtained from the ethyl acetate partition was further fractionated by multi-step solid phase extraction (SPE-C18) and semi-preparative HPLC. The protocol used in C-18 solid phase extraction SPE (Supelco; 1 g) was as follows: the cartridge was conditioned with 6 mL of methanol and then with 6 mL of water. Then the crude extract (100 mg dissolved in 6 mL of water) was loaded onto the cartridge. The cartridge was washed with 6 mL of water, 6 mL of 8:2 methanol–water and 6 mL of ethyl acetate (wash residues). The water fraction (40 mg) was then submitted to semi-preparative HPLC using the solvent system: 0–32' (10:66; A:B), 32–35' (66:10; A:B), 3 mL.min⁻¹ flow rate, and detection at 330 and 350 nm during 40 min (A-water with 0,1% of acetic acid; B-MeOH); and an Agilent Zorbax Eclipse XDB-C18 column, 5 μ ,

250 \times 9.4 mm, to yield 3,5-diCQA (10 mg). Purified 3,5-diCQA was analyzed by one- and two-dimensional NMR in methanol-D₄ using a Bruker® (Billerica, MA) BioSpin Avance 400 MHz spectrometer. ESI-HR-MS was performed on a time-of-flight mass spectrometer (Jeol Accu TOF 4G, Tokyo, Japan).

Results and discussion

The *E. prostrata* (L.) L. hairy root C19 clone growth was evaluated for 21 days. In the first 3 days, the hairy roots were in the lag phase of growth, and the most intense cellular division occurred between the 6th and the 21st days of culture in which the fresh mass increased from 4.55 g/100 mL on the 6th day up to 31.7 g/100 mL on the 21st day, representing 1.3 fold increase at the beginning of the log phase and up to 14.9 fold increase at the end of the log phase (Supplementary data; Fig. S1). After 21 days, no mass increase was observed for the cultures indicating that the system reached the growth stationary phase and the death or decline phase was not observed in the course of experiments. The construction of growth curves allowed for the determination of the ideal time for addition of eliciting agents to cultures, which corresponds to the end of the logarithmic cell division phase. Obtained results indicated the 21st day of culture growth as the most effective day for starting elicitation. The growth profile of *E. prostrata* (L.) L. hairy roots resemble *Polygonum multiflorum* hairy roots where it was found that the ideal time for the induction of anthraquinones was between the 18th and 21st days of elicitation (Huang et al. 2014). Similarly, studies have reported the 21st day of elicitation as the end of the exponential growth phase of *Artemisia annua* hairy roots (Sivakumar et al. 2010). However, the log growth phase may vary depending on the plant species. Hairy roots of *Rhaponticum carthamoides*, also from the Asteraceae family, reported the period of 35 days for greatest mass production of fresh hairy roots (Skala et al. 2015).

Before elicitation and after 21 days of culture of the *E. prostrata* (L.) L. hairy root C19 clone, the ethanolic extracts from hairy root C19 clone was prepared and submitted to chromatographic procedures and semi-preparative HPLC. 3,5-diCQA was obtained as a white powder and characterized by MS and one- and two-dimensional NMR. NMR data showed three important signals from the aromatic ring at δ 7.05 (s, H-2'/2''), δ 6.79; 6.76 (H-5'/5''), and δ 6.5 (H-6'/6''), also the ¹H-NMR spectrum showed two sets of double bonds H-7' and H-8' at δ 7.61/7.54 and δ 6.33/ 6.21, respectively, related to caffeoyl moieties. Signals of the quinic acid subunit were confirmed with the presence of methylene groups (C-2 and C-6) at δ 34.3 and δ 36.2 (Supplementary data; Table S1 and Fig. S2–S5). HRMS data confirm the proposed molecular formula (Supplementary data; Fig.

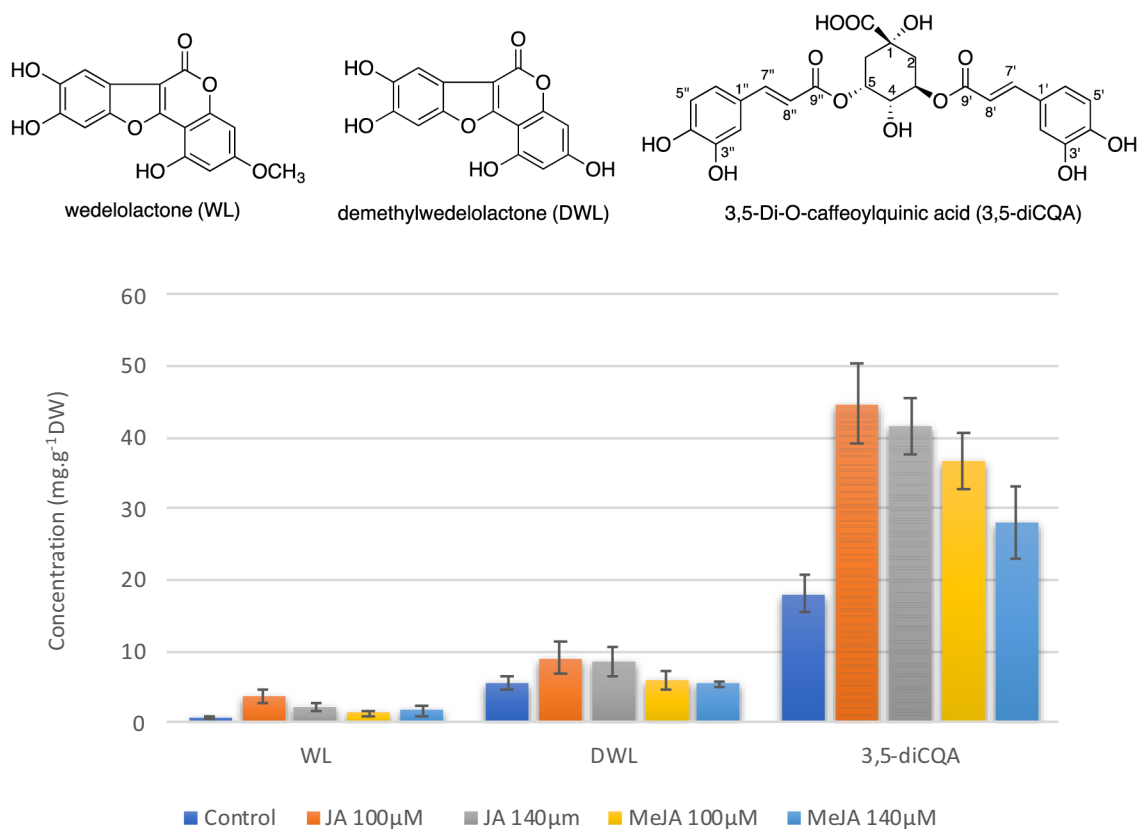


Fig. 1 Production of WL, DWL and 3,5-diCQA in *E. prostrata* (L.) L. hairy roots on 4th day of cultivation after elicitation with JA and MeJA

S6–S7). All NMR data allowed us to establish the structure of 3,5-diCQA in comparison with literature data (Wu et al. 2007; Wan et al. 2017).

Elicitation with JA and MeJA indicated that all variables analyzed (exposure period, type and concentration of the elicitor) affected the production of the target compounds WL, DWL and 3,5-diCQA, as shown in Fig. 1 (Supplementary data; Table S2). When examining WL production in hairy roots elicited with JA or MeJA, regarding exposure time, samples of all treatments collected 2 or 4 days after addition of elicitors showed an increased production of WL compared to control. Hairy roots elicited for 2 days presented an increase on WL production in the range of 1.30 mg/g d.w. and 1.20 mg/g d.w. for elicitation with 100 μM JA and 100 μM MeJA and of 0.65 mg/g d.w. and 1.05 mg/g d.w. for 140 μM JA and 140 μM MeJA, respectively, while the control produced 0.73 mg/g d.w. Moreover, hairy roots elicited for 4 days presented enhanced production of WL in all tested conditions, though it was observed that the best eliciting agent was JA in the concentration of 100 μM, which produced 3.72 mg/g d.w., thereby an increase of 5.2 fold in WL production if compared to the control which produced 0.72 mg/g d.w. Linear regression curves constructed for evaluating WL production after elicitation

for 2 and 4 days showed that on the 2nd day there was no significant difference between elicitation with JA or MeJA agents; however, there was a correlation between dose and effect of elicitor once 100 μM of either JA or MeJA induced higher stimulating effect than 140 μM. Regression analysis data showed that higher production of WL could be obtained in hairy roots elicited with 53 μM of JA (Fig. 2A). Prolonged period of co-culture with JA resulted in more pronounced effect on the production of WL, 3.72 mg/g d.w., by roots exposed to 100 μM JA, maximum yield at 78 μM of JA, than those exposed to 140 μM JA (Fig. 2B).

In general, WL production in *E. prostrata* (L.) L. hairy roots was superior in cultures elicited by JA than in cultures elicited with MeJA. The best JA concentration on 2nd day should be 53 μM whilst the best JA concentration on 4th day should be 78 μM, in follows that one could reduce elicitor dosage and save money. Hairy roots exposed to JA for 4 days showed enhanced DWL production compared to the control. Cultures elicited with 100 μM JA produced 9.04 mg/g d.w. of DWL while those co-cultured with 140 μM JA produced 8.63 mg/g d.w. of DWL. Obtained yields represent a 1.6 fold increase on DWL contents compared to the control.

Besides coumestans, *E. prostrata* (L.) L. hairy roots produced significant amounts of 3,5-diCQA, and the elicitation

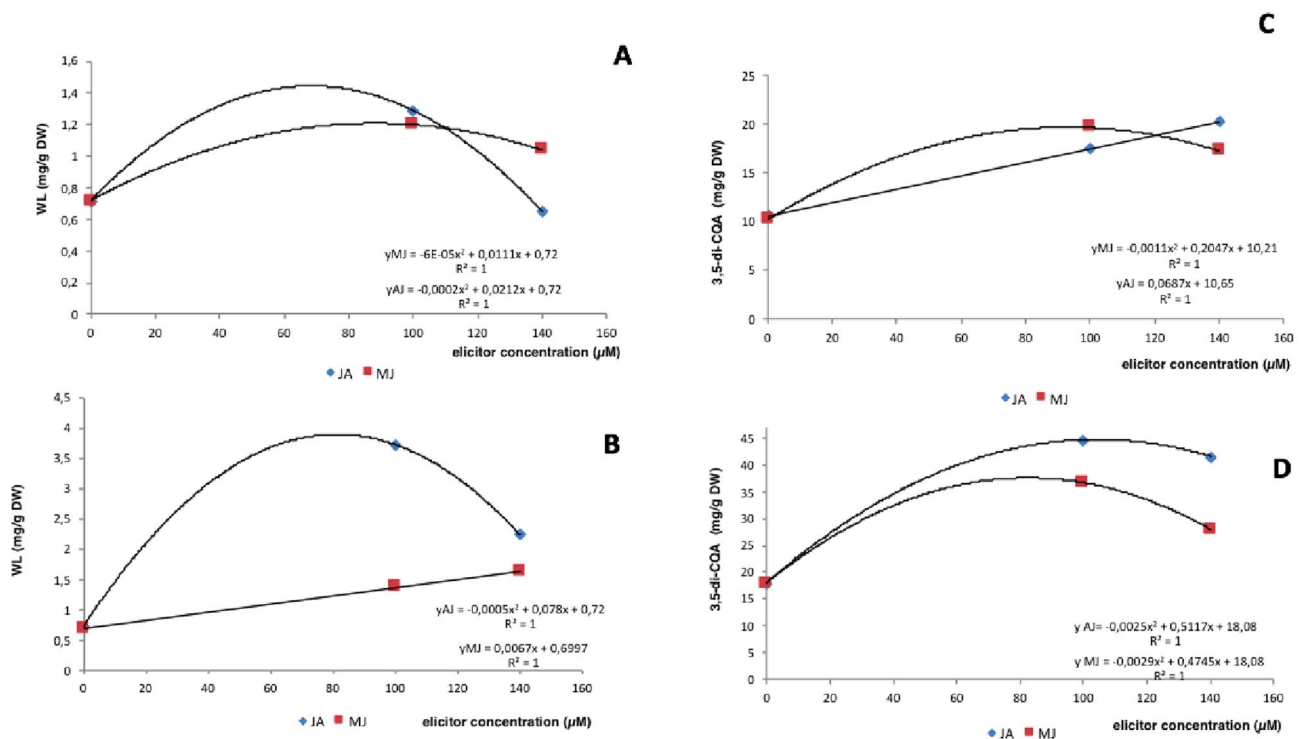


Fig. 2 Linear regression curves of WL and 3,5-diCQA produced by *E. prostrata* (L.) L. hairy roots as a function of period of elicitation and concentration of JA or MeJA elicitors. **A** WL after 2-day elicit-

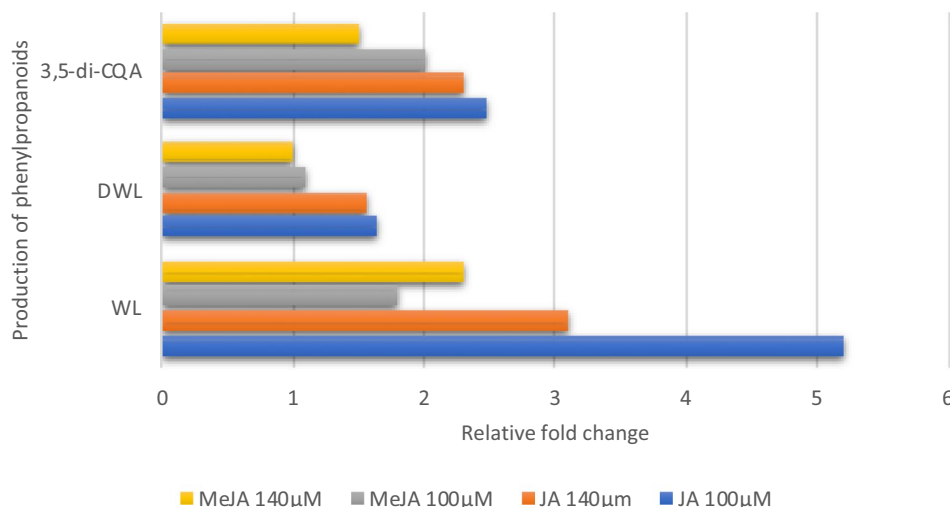
ation; **B** WL after 4-day elicitation; **C** 3,5-diCQA after 2-day elicitation; **D** 3,5-diCQA after 4-day elicitation

using JA and MeJA improved the production in all treatments as compared to control (Fig. 1). Effects of a longer elicitation period were correlated with the elicitor concentration, and more enhanced production was observed in the roots harvested after a 4-day elicitation period. JA showed more effective results on the production of 3,5-diCQA producing 44.71 mg/g d.w. and 41.62 mg/g d.w. in cultures elicited for 4 days with 100 μM and 140 μM JA, respectively (the control produced 18.08 mg/g d.w.). Although a stimulating effect of MeJA was inferior when compared to JA, 3,5-diCQA yields ranged from 36.71 mg/g d.w. to 28.02 mg/g d.w. in hairy roots elicited with 100 μM and 140 μM MeJA, respectively (Fig. 1). When comparing 3,5-diCQA production in the control culture it was possible to infer a production 2.5 and 2.3 fold superior in cultures elicited with 100 μM and 140 μM JA and 2.0 and 1.5 fold in cultures elicited with 100 μM and 140 μM MeJA, respectively (Fig. 2). The best JA concentration on the 2nd day should be 112.16 μM whilst the best JA concentration on the 4th day should be 102.34 μM . It is important to highlight that the JA concentration above 102.34 μM reduced 3,5-diCQA production. Linear regression analysis (Fig. 2) indicated that secondary metabolite production in cultures elicited with JA was dependent on elicitor concentration, that is, the lower the elicitor concentration the superior the caffeoylquinic acid

derivative production. Furthermore, cultures elicited with 140 μM MeJA showed toxic effects and subsequent reduction in the production of target bioactives. Obtained results indicate that both elicitor agents added to *E. prostrata* (L.) L. hairy root cultures were able to improve production of the target compounds, but JA was more effective than MeJA.

The most elicited compound is the WL, followed by 3,5-diCQA and DWL respectively, suggesting that these chemical agents predominantly activate the enzymes of the phenylpropanoids via synthesis. WL achieved was 5.2 fold, while the yields of 3,5-diCQA and DWL were 2.5 and 1.6 fold higher, respectively (Fig. 3). WL is biosynthesized by shikimate and acetate pathways, has been a high commercial value, and considerable amounts were produced after elicitation experiments. The 3,5-diCQA is biosynthesized by the shikimate pathway and is generally involved in plant disease-resistance responses to biotic or abiotic stress (Wan et al. 2017). Recently, 3,5-diCQA has been shown as a high value biological compound because it displays *in silico* anti-covid-19 activity [30–31, 39–40] (Joshi et al. 2020; Shah et al. 2021; Sumon et al. 2021; Kadioglu et al. 2021) and *E. prostrata* (L.) L. hairy root cultures proved to be an exceptional biological source for high 3,5-diCQA biotechnological production.

Fig. 3 Relative fold change of phenylpropanoids in *E. prostrata* (L.) L. hairy roots after elicitation with JA and MeJA on the 4th day



Conclusions

The biosynthesis of wedelolactone, demethylwedelolactone and 3,5-diCQA were positively influenced by elicitor concentration and elicitor exposure time of both chemical elicitors tested. The optimum period of co-cultivation with each elicitor was 4 days. Compared to controls, the highest yield of WL achieved was a 5.2 fold increase, while the yields of 3,5-diCQA and DWL were 2.5 and 1.6 fold higher, respectively. Under these conditions, *E. prostrata* (L.) L. hairy roots cultures showed productivity of 0.15 mg/g/day of WL, 0.36 mg/g/day of DWL and 1.19 mg/g/day of 3,5-diCQA. Obtained data validate the potential of *E. prostrata* (L.) L. elicited hairy root cultures as an efficient system for the production of bioactive phenylpropanoids, specially the 3,5-diCQA, a potential anti-Covid-19 therapeutic agent as determined by computational evidence.

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Author contributions MVL and AAL conceived the study design; GM carried out the experiments; SCF, MVL, AAL and BWB analyzed and interpreted data; CLC carried out the identification of the compounds, MVL prepared the draft manuscript; SCF, AAL made a critical revision of the manuscript; and all authors approved the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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