

## Metabolic gene expression and centelloside production in elicited *Centella asiatica* hairy root cultures

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### ABSTRACT

The most economically important centellosides, madecassoside and asiaticoside, are produced in very low amounts in *Centella asiatica* (L.) Urban roots. With the aim of overcoming this limitation, in this work we compared the effect of different elicitors on centelloside production in hairy root cultures established by *Agrobacterium rhizogenes* infection. Additionally, elicitor-induced changes in the expression of key biosynthetic genes were studied to shed light on the regulation of the triterpene metabolic pathway. The elicitors tested were coronatine and methyl jasmonate, added separately or together, and salicylic acid. The content of the four main centellosides (asiaticoside, madecassoside, asiatic acid and madecassic acid) was determined by HPLC/DAD, and the expression level of genes after elicitation was analyzed by real-time quantitative polymerase chain reaction. The greatest increase in the production of centellosides, especially madecassoside, was achieved with coronatine, applied alone or with methyl jasmonate. This treatment also enhanced the expression of the target genes, particularly at the start of elicitation. By far the most highly expressed were those involved in oxidations, indicating that the tested elicitors did not act specifically on key genes in the centelloside biosynthetic pathway. These results support that hairy roots are a promising biotechnological platform for improved centelloside production and that this approach warrants further research.

### 1. Introduction

*Centella asiatica* (L.) Urban is a herbaceous, perennial plant species of the Apiaceae (Umbelliferae) family. Requiring a humid climate, it grows up to 1800 m of altitude in tropical and subtropical regions such as southeastern Asia (Azerad, 2016). Reported to have neuroprotective, antioxidant, antidiabetic, antimicrobial, antitumor and antidepressant properties, among others, *C. asiatica* has been used in a wide range of medicinal and cosmetic applications.

The main bioactive compounds in *C. asiatica* are pentacyclic triterpenoid saponins called centellosides. The centelloside pathway is initiated via the mevalonate pathway to generate a sesquiterpene precursor, farnesyl diphosphate (FPP), for subsequent formation of an intermediate, squalene, by squalene synthase (James and Dubery, 2009). Squalene is then oxidized to 2,3-oxidosqualene, a branch point in the sterol and triterpenoid saponin biosynthesis, which cyclizes to a proto-steryl or dammarenyl cation. Cyclization through a dammarenyl cation

generates, among others, an oleanyl cation that, finally catalyzed by  $\alpha/\beta$ -amyrin synthase, will form  $\alpha$  or  $\beta$ -amyrin (Azerad, 2016). The last steps of centelloside pathway have not yet been fully elucidated. Following cyclization, additional diversity is conferred by modification of the products by oxidation, hydroxylation, glycosylation, and other substitutions mediated by cytochrome P450-dependent monooxygenases, glycosyltransferases, and other enzymes. Asiatic acid and madecassic acid are glucosylated by UDP-glycosyltransferases (UGTs) to obtain asiaticoside and madecassoside (O.T. Kim et al., 2017). These highly specialized secondary metabolites form part of the plant defense system against pathogens and herbivores (Ramirez-Estrada et al., 2016). The biological activities of *C. asiatica* are mainly attributed to the pentacyclic triterpenoid saponins madecassoside and asiaticoside, and their corresponding sapogenins, madecassic acid and asiatic acid. Madecassoside has anti-inflammatory properties and significantly increases collagen III secretion, while asiaticoside stimulates wound healing and is useful in the treatment of leprosy (Singh et al., 2015).

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In the last years, there has been growing interest in the therapeutic potential of *C. asiatica* extracts containing specific proportions of centellosides (Wen Su, 1995). Subsequent over-exploitation of wild-growing plants and excessive uprooting has brought *C. asiatica* to the brink of extinction (Mangas et al., 2008). As the chemical synthesis of centellosides is either extremely difficult or economically unviable, attention has turned to plant cell culture technology (plant biofactories) in the search for alternative systems of efficient secondary metabolite production (Verpoorte, 1999). In particular, hairy roots have been used as a biotechnological platform to produce plant bioactive compounds that are scarce in nature and whose complex structures make them difficult to synthesize. The advantages of hairy roots include genetic stability, hormonal independence, rapid growth and the capacity to produce the same metabolite spectrum as the roots of the whole plant (Gallego et al., 2014).

Endogenous levels of plant secondary metabolites are influenced by multiple environmental stresses and signals classified as biotic or abiotic (Gandhi et al., 2015). In plant *in vitro* cultures, it is possible to activate the secondary metabolism response to stress factors by the exogenous addition of biotic and abiotic elicitors (*i.e.*, phytohormones or plant pathogen particles) (Cusido et al., 2014), an approach commonly employed to boost metabolite production in plant-based production platforms (Thakur and Sohal, 2013). Furthermore, supplementing the media of a plant *in vitro* culture with elicitors under controlled conditions and observing the response in terms of gene expression can provide new insights into metabolite biosynthesis (Pauwels et al., 2009). Elicitors can be abiotic or biotic, the latter being the ones that have presented the best results to increase the production of secondary metabolites (Ramirez-Estrada et al., 2016). Among biotic elicitors, 100 mM methyl jasmonate (MeJa) has shown great results in achieving high terpene content in different cells and organ cultures *in vitro*. Thus, centelloside production in cell suspension cultures of *C. asiatica* was significantly

increased using 100  $\mu$ M MeJa (Bonfill et al., 2011). On the other hand, 100  $\mu$ M MeJa was the best elicitor to increase the production of ginsenosides in hairy roots of *Panax ginseng* (Kim et al., 2013) and that of the diterpene tanshinone in hairy roots of *Salvia miltiorrhiza* (Liang et al., 2012; Kai et al., 2012). Coronatine (COR) is a polyketide phytotoxin produced by microbes. In some cases, it has shown greater activity as elicitor than MeJa, such as in the production of sakuranetin and momilactone A in rice leaves (Tamogami and Kodama, 2000), or in cell suspension cultures of *Taxus media*, where 1  $\mu$ M COR had an activating effect on the production of taxanes (Onrubia et al., 2013). Vaccaro et al. (2017) obtained the best content of abietane diterpenes in *Salvia sclarea* hairy roots elicited with 100  $\mu$ M MeJa or with 1  $\mu$ M CORO. Salicylic acid (SA), a small molecule with a vital role in plant defense regulatory systems, has also shown interesting results, such as increased ginsenoside production after elicitation of *Panax ginseng* adventitious roots with 100  $\mu$ M SA (Tewari and Paek, 2011). On the other hand, in *Cichorium intybus* hairy root cultures, sesquiterpene lactones production increased after elicitation with 100  $\mu$ M SA (Malarz et al., 2007), similar results were observed on the diterpenoid andrographolide content in *Andrographis paniculate* hairy roots elicited with 100 mM SA (Sharmila and Subburathinam, 2013).

To improve centelloside production, more knowledge is required of their complex biosynthetic pathways (Fig. 1). Although many centelloside structures have been elucidated (Azerad, 2016), little is known about their metabolism, and only a few of the biosynthetic genes have been sequenced and cloned.

In this work it has been determined the effect of different elicitors on the biotechnological production of the main centellosides in a selected fast-growing *C. asiatica* hairy root line and on the expression levels of genes encoding key enzymes in the triterpene biosynthetic pathway, analyzed by real-time quantitative polymerase chain reaction (RT-qPCR) assays. The aim was to identify which genes are essential for the

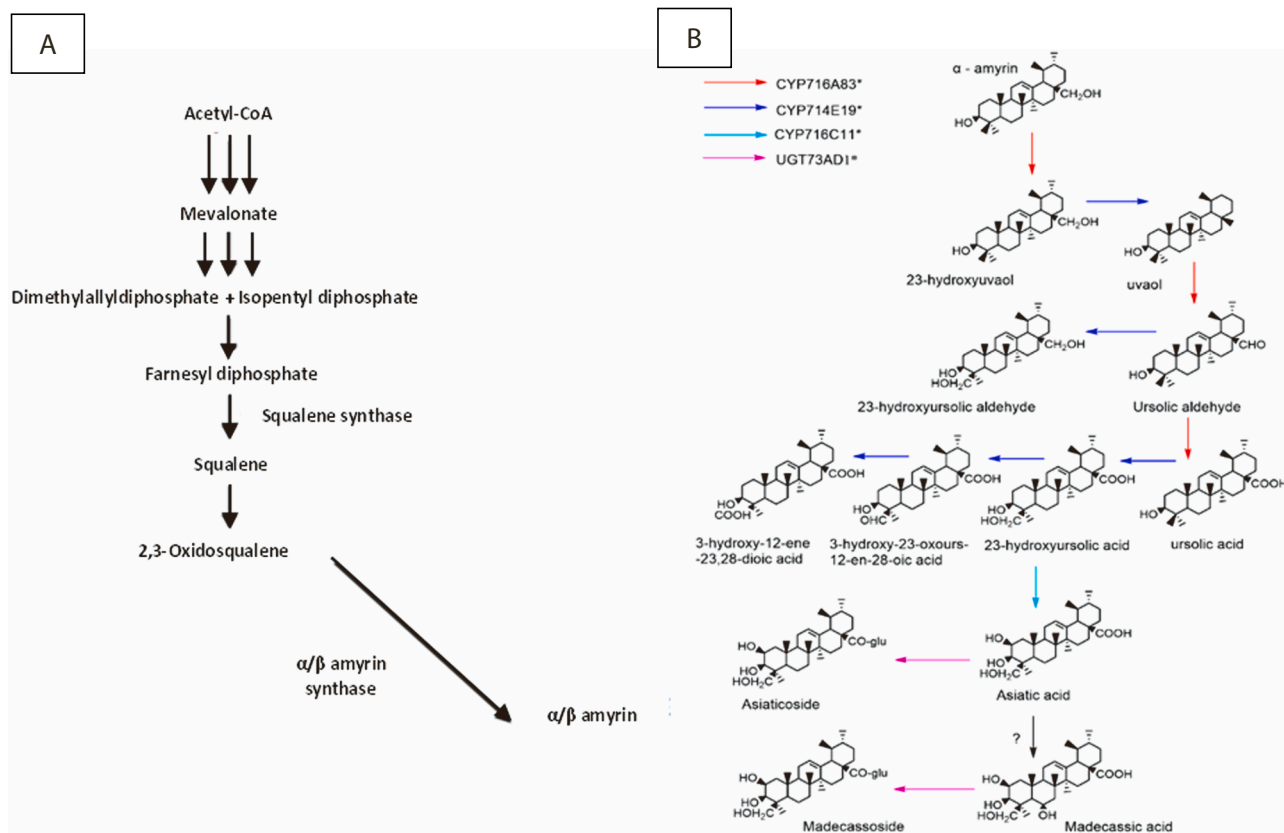


Fig. 1. A summarized scheme of centelloside biosynthesis. (A) Main steps in the biosynthesis of both  $\alpha$ - and  $\beta$ -amyrin. (B) Last steps of oxidations and glycosylations. Modified figure from Mangas et al. (2008) and Kim et al. (2018).

**Table 1**  
Primers used for confirmation of transformed roots.

Gene		Primer sequence (5'–3')	Temperature melting (°C)	Amplicon size (pb)	Accession number
virD	Forward	ATGTCGCAAGGCAGTAAGCCA	56	700	KX986281.1 ( <i>A. rhizogenes</i> A4)
	Reverse	GGAGTCTTTCAGCATGGAGCAA			
rolA	Forward	TGGAATTAGCCGACTAAAC	60	660	KX986281.1 ( <i>A. rhizogenes</i> A4)
	Reverse	GCGTACGTTGTAATGTGTTG			
rolB	Forward	AGTTCAAGTCGGCTTTAGGC	60	770	KX986281.1 ( <i>A. rhizogenes</i> A4)
	Reverse	TCCACGATTCAACCACTAG			
rolC	Forward	TAAATGGCTGAAGACGACC	60	534	KX986281.1 ( <i>A. rhizogenes</i> A4)
	Reverse	AAACTGCACTCGCCATGCC			

control of centelloside production and ascertain which elicitor most effectively increases the expression of triterpene biosynthetic genes.

## 2. Material and methods

### 2.1. Establishment of hairy root cultures

*In vitro* plants of *C. asiatica* were cultured on MS medium including vitamins (Murashige and Skoog, 1962) and supplemented with 30% (w/v) sucrose, and 2.7% (w/v) gelrite. They were maintained in a controlled growth chamber at 25 °C and with a long-day photoperiod (16 h light/8 h dark).

Leaves from *in vitro* cultured *C. asiatica* plantlets were used to obtain explants. They were separated in a sterile Petri dish and cut into sections of approximately 1.5–2 cm<sup>2</sup>. A colony of *Agrobacterium rhizogenes* A4 strain, grown at 25 °C on solid YEB medium for 48 h and subsequently stored at –70 °C, was detached with the tip of a scalpel and inoculated onto leaves from the underside. Leaves were placed on plates with solid MS medium, up to 4 leaf segments per plate, and incubated in the dark at 25 °C for 2 days. After 48 h, the explants were transferred to a new plate with solid MS medium + 500 mg/L cefotaxime and incubated at 25 °C for approximately 4 weeks.

After this time, the roots appearing on the leaf discs were separated and placed on new solid MS medium + 500 mg/L cefotaxime at 25 °C in darkness. This step was repeated every 2 weeks for approximately 2 months to remove any remaining *A. rhizogenes*. Transformed roots growing well were selected by cutting the top 5–7 cm of the root apex

**Table 2**  
Gradient used for HPLC separation of the centellosides.

Time (min)	Flow (mL/min)	Aqueous solvent (%)	Organic solvent (%)
0	1	80	20
15	1	62	38
30	1	30	70
35	1	30	70
37	1	80	20
45	1	80	20

**Table 3**  
Primers used for gene expression analysis.

Gene		Primer sequence (5'–3')	Melting temperature (°C)	Amplicon size (pb)	Reference – Accession number
Squalene synthase (SQS)	Forward	TGAGGAGAATTCGGTCAAGG	60	126	AY787628.1 ( <i>Centella asiatica</i> )
	Reverse	GCACAAAACCGAAGATAGC			
β-Amyrin synthase (β-AS)	Forward	TTACGTTTGCTGGGAGAAGG	60	134	AY520818.1 ( <i>Centella asiatica</i> )
	Reverse	TATACCCCAAGAACCGCAAG			
CYP716A83 (CYP83)	Forward	TAGCTCTGCATGCACCTTTCG-	60	105	KU878849.1 ( <i>Centella asiatica</i> )
	Reverse	CCAGGTCCCTTTGATTTGAC			
CYP714E19 (CYP19)	Forward	AACCACACACATCCTTTGG	60	116	KT004520.1 ( <i>Centella asiatica</i> )
	Reverse	TCCITTGATGAGCCCAAGAC			
CYP716C11 (CYP11)	Forward	CCCGATTCAACGACTCTTTC	60	104	KU878852.1 ( <i>Centella asiatica</i> )
	Reverse	CCGGTGTGTTGATCGACTTC			
UGT73AD1 (UGT)	Forward	TCTGGAAGCAGTTTGTGAGG	59	101	KP195716.1 ( <i>Centella asiatica</i> )
	Reverse	CGCCAATCTTCACTACATCG			
β-actin	Forward	TGACAATGGAACTGGAAATGG	58	80	Kim et al. (Kim et al., 2018) - KF699319.1 ( <i>Panax ginseng</i> )
	Reverse	CAACAATACTGGGGAACACT			

with its lateral branches and cultured on new solid MS medium at 25 °C in darkness. Each root line was designated a number. Transformed root lines were sub-cultured in the same conditions every 2 weeks.

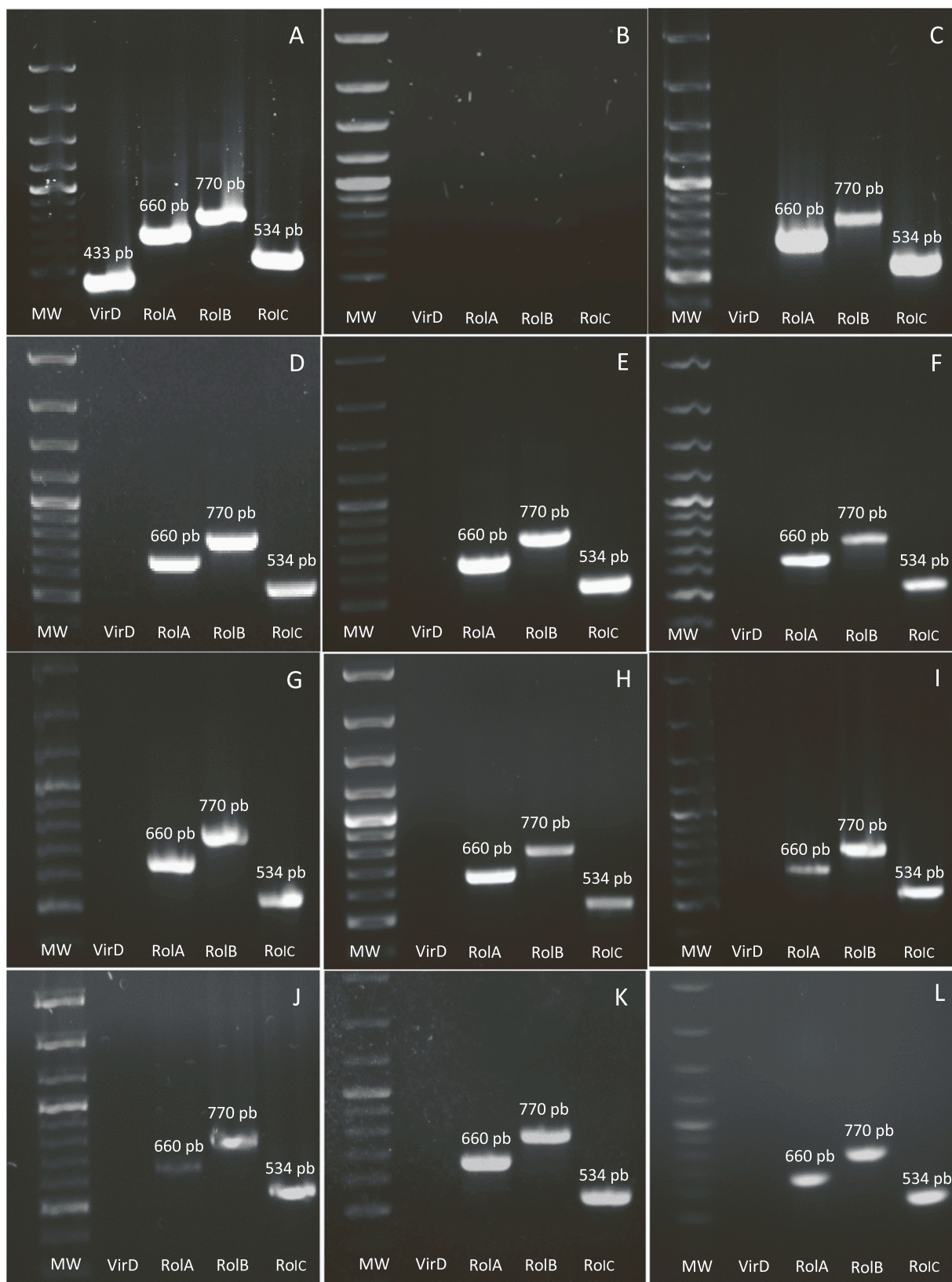
### 2.2. Confirmation of root transformation by PCR

Plant tissue (0.2 g) was pulverized in liquid nitrogen and placed in an Eppendorf tube, to which was added 0.75 mL of extraction buffer (50 mM EDTA Ph 8.0, 100 mM Tris pH 8.0 and 500 mM NaCl), 0.6 µl of β-mercaptoethanol and 50 µl of 20% sodium dodecyl sulphate (SDS). After incubation at 65 °C for 10 min, 250 µl of 5 M potassium acetate was added, followed by incubation on ice for 20 min and then centrifugation at 4 °C for 20 min at 9000 rpm. After retention of the supernatant, 1 mL of isopropanol was added, followed by incubation at –20 °C for 1 h. The pellet was centrifuged for 15 min at 8000 rpm, dried, and after the addition of 140 µl of T10E1 (Tris 10 mM EDTA 1 mM), centrifuged again for 10 min at 14,000 rpm. After retention of the supernatant, 15 µl of 3 M sodium acetate and 100 µl of isopropanol were added. The supernatant was retained, centrifuged for 10 min at 15,000 rpm, and the pellet was kept. The Eppendorf tube was dried at 37 °C for 10 min, and 30 µl of T10E1 buffer was added. Finally, 1 µl of RNase (10 mg/mL) was added and the purity of the DNA was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Amplification of *rol* (*rolA*, *rolB* and *rolC*) and *vir* (*virD*) genes was performed by PCR in 0.5 mL tubes. To each tube was added 12.5 µl of Green taq polymerase, 1 µl of forward primer, 1 µl of reverse primer, 2 µl of DNA and 8.5 µl of milliQ water. The specific primers used are described in Table 1. The PCR conditions for *rol* and *vir* genes were as follows: one cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min, and a final extension step of 5 min at 72 °C. The size of the PCR products was determined by agarose gel electrophoresis (1%).

### 2.3. Selection and culture of the different hairy root lines

To choose the best hairy root line for the elicitation studies, 1 g of each line was sub-cultured in a glass flask with 20 mL of liquid MS



**Fig. 2.** Gel electrophoresis of PCR products from *C. asiatica* transformed roots after *A. rhizogenes* infection. (A) *A. rhizogenes* (positive control), (B) non-transformed roots (negative control), (C–L) hairy root lines (L1, L2, L3, L4, L6, L7, L8, L10, L12 and L14). MW: 100 bp molecular weight marker.

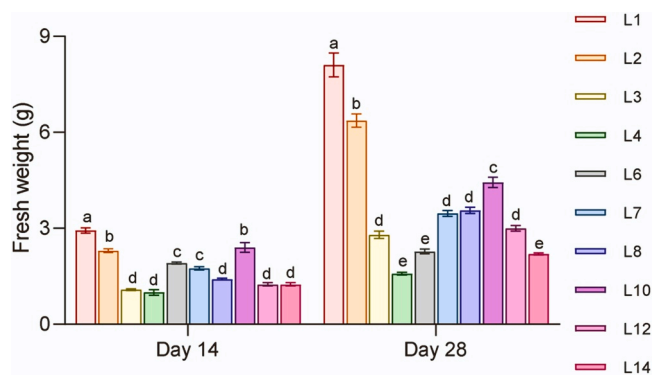


Fig. 3. Fresh weight of *C. asiatica* hairy roots. Data represent the mean  $\pm$  SD of three replicates. Different letters show significant differences ( $p < 0.05$ ) between hairy root lines at each time point.

medium for 4 weeks, maintained in a rotatory shaker at 115 rpm in darkness at 25 °C. The fresh weight (FW) was evaluated after 14 and 28 days. The growth of each hairy root line was established as the mean FW value of 3 replicates.

2.4. Elicitation studies

The elicitation experiment was performed using the transformed root line that produced the highest amount of biomass, applying the following elicitors at the indicated concentrations: 1  $\mu$ M coronatine (Coro), 100  $\mu$ M methyl jasmonate (Meja), 100  $\mu$ M salicylic acid (SA) and 1  $\mu$ M coronatine/100  $\mu$ M methyl jasmonate (Coro + Meja). These concentrations were the best concentrations studied up to now in different culture systems (Loc and An, 2010; Narayani and Srivastava, 2017; O.T. Kim et al., 2017; J.Y. Kim et al., 2017; Krishnan et al., 2019). As a pre-culture, inocula of 1 g FW of the selected hairy root line were placed in flasks containing 20 mL of liquid MS, and the cultures were maintained for 2 weeks in a rotatory shaker at 115 rpm in darkness at 25 °C.

After 2 weeks, the cultures were treated with the respective elicitors and maintained under the same conditions. After the treatment, samples were collected at 8 h and days 7 and 14 to determine the centelloside production, and at 0, 8, 12, 24, and 36 h and day 7 to study the expression of the targeted genes from the centelloside metabolic pathway. The samples were freeze-dried, and the dry weight (DW) was registered. The elicitation experiment was performed in triplicate.

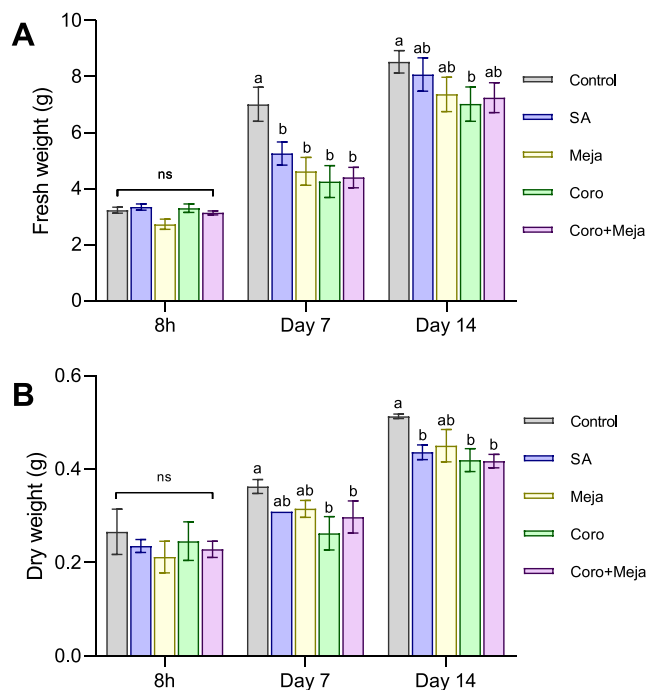


Fig. 5. Fresh (A) and dry (B) weight of elicited and non-elicited hairy roots. Data represents the mean  $\pm$  SD of three replicates. Different letters show significant differences ( $p < 0.05$ ) between elicited and non-elicited cultures at each time point.

2.5. Extraction and quantification of centellosides by HPLC/DAD

To determine the centelloside production, 0.5 g DW of elicited and non-elicited hairy roots were added to 10 mL of methanol: H<sub>2</sub>O (9:1), and this suspension was sonicated for 1 h at room temperature, followed by centrifugation at 20,000 rpm for 10 min. The supernatant was separated, and the previous step was repeated. The supernatants of the different samples were placed in porcelain mortars to be evaporated at 38 °C for approximately 24 h and finally redissolved in 1.5 mL of methanol. The methanolic extract was filtered through a 0.22  $\mu$ m filter for HPLC quantification of centellosides. The system consisted of a Waters 600 Controller Pump, a Waters 717 Autosampler Automatic Injector, a photodiode array (PDA) detector and Empower data analysis software version 1.5. Chromatographic analysis was performed by

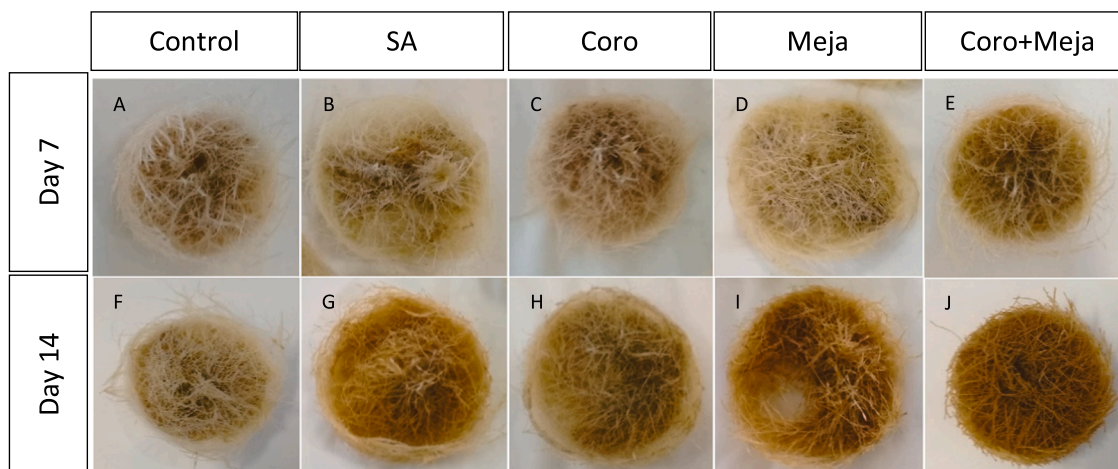
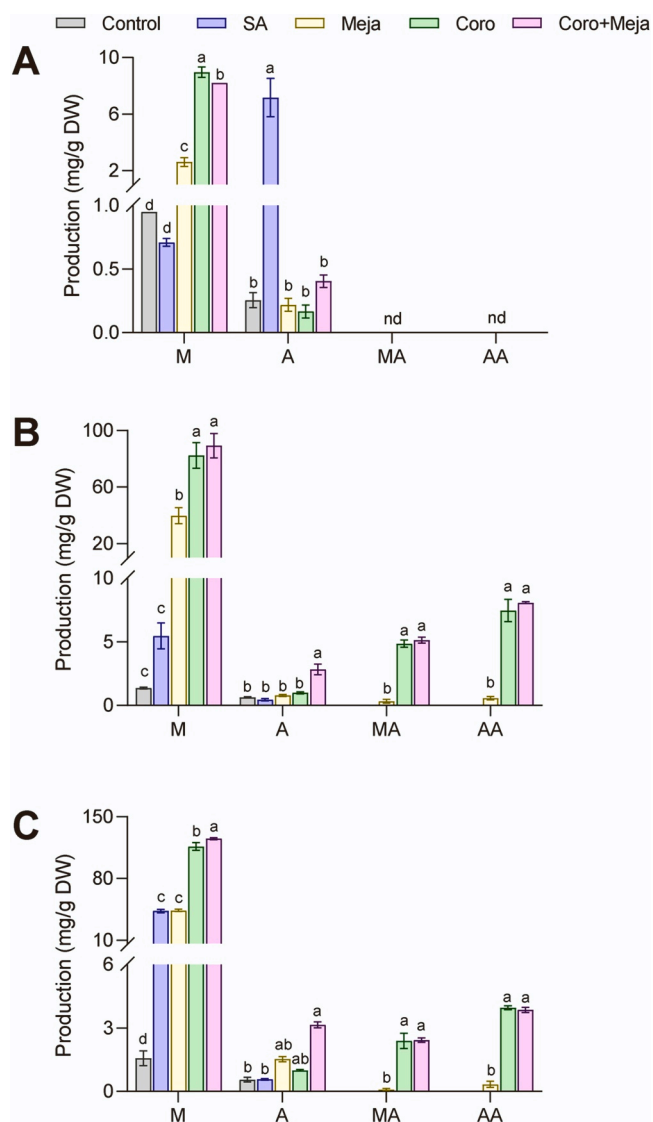


Fig. 4. Morphology and coloring of *C. asiatica* hairy roots (Line 1) at day 7 (photographs in the top row) and day 14 (photographs in the bottom row): non-elicited (A and F); elicited with salicylic acid (B and G); elicited with coronatine (C and H); elicited with methyl jasmonate (D and I); elicited with coronatine and methyl jasmonate (E and J).

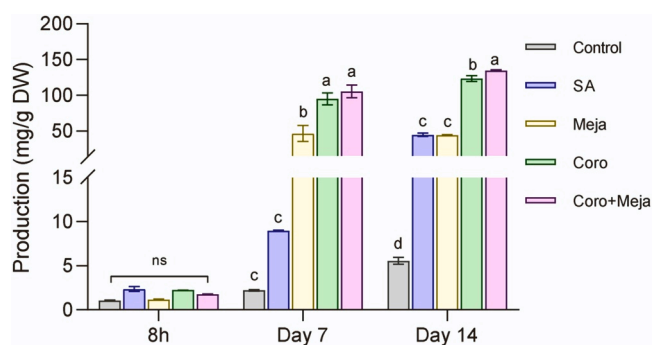


**Fig. 6.** Centelloside production (mg/g DW) in *C. asiatica* transformed roots. Madecassoside (M), asiaticoside (A), madecassic acid (MA) and asiatic acid (AA) production with different elicitation conditions: salicylic acid (SA), methyl jasmonate (Meja), coronatine (Coro), Coro + Meja, and control (no elicitation). (A) 8 h, (B) 7 days, and (C) 14 days after elicitation treatment. Data represent the mean  $\pm$  SD of three replicates. Different letters show significant differences ( $p < 0.05$ ) between elicited and non-elicited cultures for each centelloside. nd = not detected.

gradient (Table 2) at room temperature using a Lichrospher 100 RP18 5  $\mu$ m column (250  $\times$  0.4 mm). The mobile phase consisted of acetonitrile and ammonium phosphate (10 mM) acidified to pH 2.5 with orthophosphoric acid. The acidification improved the definition of the compound peaks. The flow rate was 1 mL/min, and the injection volume was 10  $\mu$ l. The detector wavelength was set at 214 nm, 1.00 au/v, and the run time was 45 min. To quantify the centellosides (asiatic acid, madecassic acid, asiaticoside and madecassoside), standards of these four compounds were used at concentrations of 10, 25, 50, 100, 250 and 500 ppm to prepare the respective calibration curves.

## 2.6. Expression of centelloside pathway genes analyzed by RT-qPCR

The studied genes are representative of the centelloside biosynthetic pathway: squalene synthase (SQS),  $\beta$ -amyrin synthase ( $\beta$ -AS), CYP716A83 (CYP83), CYP714E19 (CYP19), CYP716C11 (CYP11) and



**Fig. 7.** Total centelloside production (mg/g DW) with elicitation treatments over time. Data represent the mean  $\pm$  SD of three replicates. Different letters show significant differences ( $p < 0.05$ ) between elicited and non-elicited cultures at each time point.

## UGT73AD1 (UGT).

Total RNA extractions were obtained using elicited and non-elicited *C. asiatica* transformed roots. Elicited and control samples of approximately 5 g FW collected at each time point were frozen in liquid nitrogen and stored at  $-70$   $^{\circ}$ C. The PureLink RNA Mini Kit (Invitrogen) was used according to the manufacturer's instructions to extract the RNA. The amount and quality of each RNA sample was quantified using the NanoDrop 2000 Spectrophotometer (Thermo Scientific). The integrity of RNA was also assessed by agarose gel electrophoresis.

DNase treatment was performed using the total RNA with a fixed concentration as a template (1.5  $\mu$ g of RNA). For this purpose, the required sample volume was calculated considering the volume of DNase I and buffer needed and made up to a final volume of 10  $\mu$ l per sample with sterile H<sub>2</sub>O. After addition of the DNase mix, the samples were heated at 37  $^{\circ}$ C for 30 min; then 1  $\mu$ l of 50 mM EDTA was added per sample to inactivate DNase by incubation at 65  $^{\circ}$ C for 10 min. First-strand cDNA was synthesized using the SuperScript™ IV First-Strand Synthesis System (Invitrogen) and 2  $\mu$ l of RNA according to the manufacturer's instructions.

After designing primers with Primer3Plus software (Table 3), the RT-qPCR assays were performed using the LightCycler 480 System (Roche Diagnostics). Amplifications were carried out in 7.5  $\mu$ l reaction solutions containing 2.5  $\mu$ l first-strand cDNA (diluted 1:5), 1.7  $\mu$ l of sterile H<sub>2</sub>O milliQ, 2.5  $\mu$ l of iTaq™ Universal SYBR® Green Supermix (BIO-RAD) and 0.4  $\mu$ l of each specific primer, at a concentration of 10  $\mu$ M. PCR conditions were 95  $^{\circ}$ C for 30 s followed by 45 cycles of 95  $^{\circ}$ C for 15 s and 60  $^{\circ}$ C for 30 s. The specificity of each pair of primers was checked by melting curve analysis (95  $^{\circ}$ C for 15 s, 65–95  $^{\circ}$ C, with an increment of 0.25  $^{\circ}$ C/s ramp rate followed by 95  $^{\circ}$ C for 15 s). To check reproducibility, each assay was performed with technical triplicates for each of the three biological samples. The  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001) was used to analyze the data obtained, using the constitutively expressed  $\beta$ -actin as the reference gene.

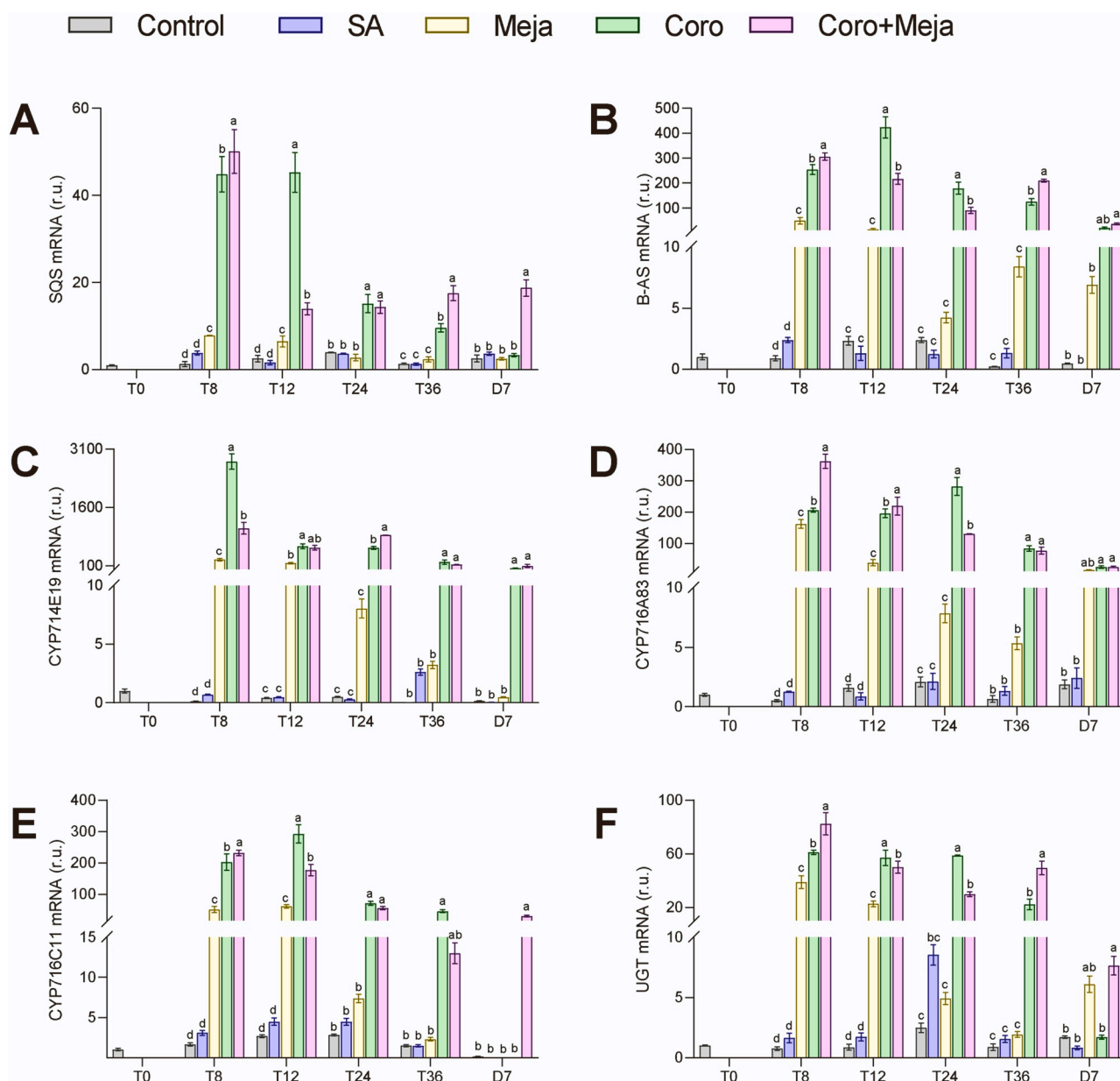
## 2.7. Statistical analysis

The statistical analysis was carried out using GraphPad PRISM software. Data are presented as the mean  $\pm$  standard deviation. Multifactorial ANOVA analysis was performed, followed by Tukey's multiple comparison test for statistical comparison. A  $p$ -value of  $< 0.05$  was assumed as a significant difference for growth parameters and centelloside production, and  $< 0.01$  for gene expression analysis.

## 3. Results and discussion

### 3.1. Culture of hairy roots and confirmation of their transformed nature

The integration of *A. rhizogenes* plasmid T-DNA in the *C. asiatica*



**Fig. 8.** RT-qPCR analysis of different genes from the centelloside biosynthetic pathway. (A) SQS, (B) β-AS, (C) CYP714E19, (D) CYP716A83, (E) CYP716C11 and (F) UGT gene expression in *C. asiatica* hairy roots after different elicitation treatments: salicylic acid (SA), coronatine (Coro), methyl jasmonate (Meja), Coro + Meja and control (no elicitation) at 0 (T0), 8 (T8), 12 (T12), 24 (T24), and 36 h (T36) and 7 days (D7). Data represent the mean ± SD of three replicates. Different letters show significant differences ( $p < 0.01$ ) between elicited and non-elicited cultures at each time point.

**Table 4**

Correlation matrix between genes from the centelloside biosynthetic pathway. The asterisk indicates a significant difference ( $p < 0.001$ ).

Gene expression	SQS	β-AS	CYP19	CYP83	CYP11	UGT
SQS	1					
β-AS	0.792*	1				
CYP19	0.669*	0.681*	1			
CYP83	0.706*	0.844*	0.729*	1		
CYP11	0.855*	0.890*	0.639*	0.730*	1	
UGT	0.656*	0.837*	0.712*	0.921*	0.702*	1

genome was used to confirm the transformed nature of the root lines. For this purpose, three *rol* genes (*rolA*, *rolB* and *rolC*) involved in hairy root formation (Ruslan et al., 2012) were analyzed by PCR. Different DNAs (from the hairy root lines, *A. rhizogenes* and untransformed *C. asiatica*

roots) were used as a template. Also, the *virD* gene was used as a negative control to check for residual *A. rhizogenes* in the transformed tissues; this gene is present in *A. rhizogenes* but not inside the T-DNA region, so does not integrate in plant cells (Sevón and Oksman-Caldentey, 2002). *Rol* genes (*rolA*, *rolB* and *rolC*) (660 pb, 770 pb and 534 pb, respectively) were found in *A. rhizogenes* (positive control) (Fig. 2A) and transformed *C. asiatica* root lines (Fig. 2C-L) but not in untransformed roots (Fig. 2B). The *virD* gene was only found in *A. rhizogenes* (Fig. 2A) and not in transformed and untransformed *C. asiatica* roots. The next step was to select the transformed root line with the highest biomass production measured as fresh weight (FW) after 3–4 weeks of growth.

### 3.2. Selection of the transformed root line with the highest biomass production

The growth course of the different transformed root lines is shown in

**Table 5**Correlation matrix between gene expression and centelloside production. One to three asterisks indicate significant differences ( $p < 0.05$ ,  $0.01$ ,  $0.001$  respectively).

Gene expression																
UGT					CYP11					CYP83					CYP19	
D7	T36	T24	T16	T8	D7	T36	T24	T16	T8	D7	T36	T24	T16	T8	D7	
0.067	0.574 *	0.740**	0.611*	0.551*	0.366	0.516*	0.715**	0.595*	0.671 **	0.531*	0.694**	0.685**	0.659**	0.521*	0.599*	
0.526 *	0.813***	0.836***	0.947***	0.978***	0.548*	0.592 *	0.916***	0.802***	0.979***	0.908***	0.931***	0.856***	0.925***	0.930***	0.828***	
0.440	0.811***	0.872***	0.898***	0.911***	0.578*	0.571*	0.913***	0.760**	0.958***	0.866***	0.923***	0.856***	0.913***	0.889***	0.836***	

**Fig. 3.** As the growth capacity of *line 1* (*L1*) was significantly higher from the second week of culture, it was selected for the subsequent elicitation studies.

### 3.3. Effect of different elicitors on growth and centelloside production

The morphology and changes in color of hairy roots (*L1*) was observed at days 7 and 14 after elicitation (days 21 and 28 of the experiment). In general, elicitation caused hairy roots to darken with respect to the control, an effect that intensified over time (Fig. 4).

The growth course of the transformed roots (*L1*) under control or elicited conditions during a culture period of 14 days is shown in Fig. 5. The results indicate that elicitors generally inhibited the growth of the transformed roots, measured as FW and DW, compared to the non-elicited cultures.

The transformed root line producing the most biomass was used to analyze the production of the main centellosides in *C. asiatica*, madecassoside and asiaticoside, and their respective sapogenins, madecassic acid and asiatic acid. The yields were compared between elicited and non-elicited cultures and expressed in mg/g DW.

Madecassoside was the main centelloside detected in all cultures and at all time points (8 h, days 7 and 14). At 8 h of elicitation with Meja, Coro, or Coro + Meja, its production was significantly higher compared with the untreated samples, whereas SA did not have a significant effect. Conversely, SA induced a significant increase in asiaticoside, whose levels were not changed by the other treatments. Asiatic acid and madecassic acid were not detected at 8 h (Fig. 6 A). After 7 days of elicitation, madecassoside production was significantly higher in all the elicited samples compared with the control; the yields of most of the targeted centellosides were increased by elicitation with Coro or Coro + Meja (Fig. 6B).

Madecassoside production continued to increase significantly, in all treated cultures, at 14 days of elicitation. Coronatine or Coro + Meja significantly enhanced the production of all the centellosides, whereas SA and Meja did not affect madecassic and asiatic acid yields. The maximum production of madecassoside was achieved with Coro or Coro + Meja (116 and 125 mg/g DW, respectively) after 14 days of elicitation (Fig. 6 C).

The total centelloside production (measured as the sum of madecassoside, asiaticoside, madecassic acid and asiatic acid) was higher in *C. asiatica* transformed roots treated with different elicitors (SA, Coro, Meja and Coro+ Meja) than in the untreated samples (control) and their levels increased throughout the elicitation period. The treatments resulting in the highest increase were Coro and Coro + Meja (Fig. 7).

### 3.4. Gene expression analysis of control and elicited *C. asiatica* hairy root cultures

To understand how the elicitation treatments were affecting centelloside biosynthesis, the expression levels of six genes involved in different steps of the biosynthetic pathway were analyzed by RT-qPCR in elicited *C. asiatica* transformed roots sampled at 0, 8, 12, 24, and 36 h and 7 days after treatment (Table 3). The results are expressed in relative units, using  $\beta$ -actin as a reference gene, and non-elicitation as an internal

control. For each treatment, expression levels were compared with the control sample (transformed but not elicited).

Expression levels of all targeted genes were significantly higher in samples treated with Coro or Coro + Meja compared with the control samples, at 8, 12, and 24 h post-treatment (Fig. 8). Both treatments had significantly upregulated all the genes at 36 h, except CYP716C11 in the case of Coro + Meja (Fig. 8E). Only elicitation with Coro + Meja, at day 7, had a significant enhancing effect on the expression levels of all the genes, except CYP714E19 (Fig. 8 C). Treatment with Meja induced a significant upregulation of all the genes at 8 and 12 h, but at subsequent time points (24 h, 36 h and 7 days) no significant differences with control samples were found (Fig. 8). After SA elicitation, the expression levels of all the genes remained quite constant in all tested samples, the only significant increase being observed for UGT at 24 h (Fig. 8 F).

The different elicitors had variable effects on the gene expression patterns during the study period (from 8 h to day 7). Levels of SQS reached a maximum at 8 h with Coro + Meja and at 8 and 12 h with Coro, subsequently decreasing, although remaining significantly higher compared to the control (Fig. 8A). The expression of  $\beta$ -AS, CYP714E19, and CYP716A83 was consistently higher than control levels in samples treated with Coro or Coro + Meja, reaching peaks at 12 h with Coro, at 8 h with Coro, and at 8 h with Coro + Meja, respectively (Fig. 8B, C and D). CYP716C11 and UGT were also upregulated throughout the study period by Coro + Meja, but their expression level had fallen at day 7 in the Coro-treated samples (Fig. 8E and F). Finally, Meja increased the expression levels of SQS, CYP714E19 and CYP716C11 at 8 and 12 h, which decreased thereafter (Fig. 8A, C and E). The same effect was observed on  $\beta$ -AS, CYP716A83 and UGT, except their levels had increased again at day 7 (Fig. 8B, D and F).

A high correlation was observed between the expression levels of all the genes, especially between  $\beta$ -AS and the others, while the highest correlation value was found between UGT and CYP716A83 (Table 4).

A high level of correlation was also found between centelloside production and the expression levels of most genes, above all  $\beta$ -AS and CYP716A83, and mainly at 7 days post-elicitation. However, the expression of UGT at day 7 was not correlated with the production values at the earliest and last time points analyzed (Table 5).

### 3.5. Discussion

In this work it has been studied how different elicitors act at the transcriptomic level to increase centelloside production, with the aim of determining any correlation between improved yields and the upregulation of key biosynthetic genes. Hairy root cultures were used due to their numerous advantages as a biotechnological production platform for bioactive plant secondary compounds (Gutierrez-Valdes et al., 2020). These include: a rapid growth capacity, hormone-independent growth, genetic stability, and a capacity to biosynthesize the same type of compounds as the roots of the mother plant. The transformed *C. asiatica* roots had a high growth rate (Figs. 5 and 6) and were able to biosynthesize centellosides (Figs. 7 and 8).

Although the underlying mechanism has not been fully clarified, hairy roots can act as a self-sufficient production system of secondary metabolites without any special induction signal or metabolic



Gene expression															
CYP19				β-AS				SQS				Time			
T36	T24	T16	T8	D7	T36	T24	T16	T8	D7	T36	T24	T16	T8		
0.636*	0.694**	0.640*	0.634*	0.577*	0.685**	0.679**	0.667**	0.669**	0.412	0.574*	0.746**	0.566*	0.709**	8 h	Centelloside production (mg/g DW) after elicitation
0.756**	0.924***	0.956***	0.811***	0.870***	0.906***	0.840***	0.832***	0.961***	0.573*	0.842***	0.844***	0.724**	0.968***	Day 7	
0.767***	0.930***	0.915***	0.793***	0.878***	0.917***	0.839***	0.823***	0.951***	0.615***	0.801***	0.896***	0.703**	0.961***	Day 14	

engineering strategy (Hayat et al., 2010). However, this self-induction has not been observed in *C. asiatica*, and therefore strategies such as elicitation are required for centelloside production (Hidalgo et al., 2017). Elicitation is routinely used to manipulate secondary metabolite yields, imitating stress-induced production in nature. Considerable research has been dedicated to enhancing centelloside biosynthesis in *C. asiatica* by the application of biotic and abiotic elicitors, with different results achieved depending on the elicitor and/or type of culture used (Gallego et al., 2014). Hidalgo et al. (2017) and Mangas et al. (2006) found that all tested elicitors inhibited the growth of *C. asiatica* cell and plant cultures, measured as FW and DW.

One of the elicitors used in the present work is salicylic acid, a well-known inducer of systemic acquired resistance to many plant pathogens of secondary metabolites productions (Pieterse and Van Loon, 1999; Walker et al., 2002). It was observed that the application of SA improved the production of asiaticoside at 8 h post-elicitation and madecassoside at days 7 and 14. Demonstrating the effects of SA on the yields of a specific centelloside, Loc and Giang (2012) reported a production of 45.35 mg of asiaticoside/g of DW in non-elicited *C. asiatica* cell cultures, which they later improved five-fold by applying 100 μM of SA, reaching a value of 229.83 mg/g of DW (Loc and An, 2010).

The elicitor traditionally considered most effective in *C. asiatica* is Meja, which together with jasmonic acid, is produced widely in plants as a “stress hormone”, in response to insect attacks (Kim et al., 2018). When exogenously applied to plant cell cultures of a variety of species, Meja (100–200 μM) enhances the workflow of secondary biosynthetic pathways, leading to an increased production of diverse compounds, including terpenoids, flavonoids, alkaloids and phenylpropanoids (Ramirez-Estrada et al., 2016). Recent studies in whole plant cultures demonstrated that the transcript levels of *C. asiatica* squalene synthase (CaSQS) and *C. asiatica* dammarenediol synthase (CaDDS), both genes associated with the triterpenoid pathway, are increased by the application of 100 μM Meja (Kim et al., 2018). In the present work, Meja elicitation mainly improved the production of madecassoside, which was higher than in the control at all time points (8 h, and days 7 and 14) (Fig. 6). Different studies have improved centelloside production using 100 μM Meja. Kim et al. (2007) obtained 7.12 mg/g DW of asiaticoside in transformed *C. asiatica* roots after 3 weeks of elicitation, a 5-fold increase compared to the control, and Ruslan et al. (2012) reported a very similar 4.9-fold increase in asiaticoside in cell suspension cultures. Yoo et al. (2011) described an enhanced production of both asiaticoside and madecassoside, but a reduction in madecassic acid levels, an effect we also observed, the amount decreasing from 0.32 mg/g to 0.09 mg/g of DW (Fig. 6).

The strongest elicitation effect on centelloside production reported to date was recently achieved by the exogenous application of the bacterial toxin coronatine (Coro) in cell suspension cultures (Onrubia et al., 2013). Due to a similar chemical structure, Coro mimics the function of the isoleucine-conjugated form of jasmonic acid and has a comparable mode of action to Meja. As an elicitor, Coro is of particular interest, as it enhances metabolite production more effectively than Meja and at lower concentrations (Ramirez-Estrada et al., 2016). This was confirmed in the present study, where the production of all centellosides was improved by Coro treatment, especially at day 14 after elicitation (Fig. 7). Similar results have been described by Hidalgo et al. (2017) in cell suspensions

of *C. asiatica*, and Onrubia et al. (2013) in cell cultures of *Taxus media*, in which Coro induced a higher taxane production than Meja. This superior performance could be due to the greater stability of Coro (Gallego et al., 2014), which would allow a more constant response in the plant tissue throughout the treatment period. The highest total centelloside production in *C. asiatica* hairy roots was achieved at day 14, the end of the experiment, when applying Coro, either alone or in combination with Meja, and the centelloside with the highest yield was madecassoside (Figs. 7 and 8). Possible explanations are that madecassoside (and asiaticoside) is more stable in intracellular spaces than its acid form, and, that the elicitors favored the glycosidation of these compounds.

Apart from improving metabolite production, elicitation of plant cultures provides a biological platform in which the stress response can be studied under controlled conditions (Lee et al., 2004). Changes in gene expression and proteins can be correlated with the accumulation of metabolites and messenger RNAs (mRNAs). Transcriptomes are highly dynamic, and mRNA levels provide information about gene expression within the signal response period. Therefore, to better understand the observed changes in centelloside production, key biosynthetic genes were analyzed by qRT-PCR, and the transcriptomic and metabolomic data derived from each elicitor treatment were integrated.

Among the targeted genes, SQS is involved in the formation of the universal precursor of triterpenoid saponins and phytosterols (Azerad, 2016). The elicitation treatments enhanced its expression, above all in cultures treated with Coro or Coro + MeJa at the beginning of the experiment (8 and 12 h, Fig. 8A). The subsequent decrease in expression is likely because SQS activity is not limited to centelloside biosynthesis. As SQS plays a key role in the upregulation of triterpene production (Lee et al., 2004), modulating its expression could avoid high accumulations of saponins and phytosterols.

β-amyrin synthase is a versatile gene involved in multiple steps in the centelloside metabolic pathway (Bonfill et al., 2011), catalyzing the formation of α and β-amyrin, the initial precursors of centellosides (Kim et al., 2007). The overexpression of β-AS reached a peak (an increase of up to 400-fold) at 12 h after elicitation with Coro, and remained consistently high throughout the study period in hairy roots treated with Coro or Coro + Meja, decreasingly slightly by day 7 (Fig. 8B). As CYP450s (CYP716A83, CYP714E19 and CYP716C11) are involved in the biosynthesis of different saponins derived from α and β-amyrin, an expression pattern similar to that of β-AS could be expected. Thus, maximum upregulation of CYP716A83 and CYP716C11 was observed at 8 h with Coro + Meja and at 12 h with Coro treatments, respectively (Fig. 8D and E). The expression of CYP714E19 underwent a highly significant increase, being almost 3000-fold higher compared to the control at 8 h in Coro-treated roots (Fig. 8C); this result could be explained by its involvement in more metabolic steps than the other CYP450s.

At the end of the biosynthetic pathway, UGT glycosylates saponogenins (acids) to yield the corresponding saponins (Kim et al., 2018). The expression of UGT increased in roots treated with Coro or Coro + Meja throughout the elicitation period, with maximum levels observed at 8 h of Coro + Meja treatment (Fig. 8F), like other targeted genes. The low yields (mg/g DW) of madecassic acid compared with madecassoside in these two treatments could be related with the elicitor-induced overexpression of UGT, which, as mentioned, is involved in the production of madecassoside from madecassic acid. Additionally, the complete

conversion of madecassic acid may have stimulated further production of this saponin from asiatic acid. The overexpression of UGT could also explain why madecassoside was the most produced centelloside in the other elicitor treatments. At day 7, the expression levels had mostly decreased, although they were maintained in Coro- and Coro + Meja-elicited cultures, probably due to transcript accumulation (Fig. 8).

Although the relative expression levels of genes varied throughout the period of elicitation (8–36 h), the most noticeable differences were observed in Coro and Coro + Meja treatments at 8 and 12 h (Fig. 8). Meja and SA also increased gene expression levels but not enough to have a clear impact on centelloside production, especially in the case of SA. A high degree of correlation was observed between the expression of all the studied genes, especially between  $\beta$ -AS and the others, which indicates that  $\beta$ -AS plays a major role in the regulation of the centelloside biosynthetic pathway (Kim et al., 2007).

The results demonstrate that the upregulation of biosynthetic genes can result in a higher centelloside production, especially long-term (7 days after elicitation), as shown by the values in Table 5. The key genes in the regulation of centelloside biosynthesis were found to be  $\beta$ -AS and CYP716A83 (Table 5); the former is responsible for modifying  $\beta$ -amyrin into oleanane-type saponins and the CYP gene family acts in multiple steps toward the end of the metabolic pathway (Kim et al., 2018). However, the most highly expressed genes after elicitation were mainly those involved in oxidations (CYP19), indicating that the tested elicitors did not act only on the key centelloside biosynthetic genes ( $\beta$ -AS, CYP83 and UGT).

Although gene expression was measured at specific hours and centelloside production on different days, any correlation between the two events is not necessarily evident immediately and, they can be separated by many intermediate steps. The lag between gene expression and secondary metabolite production is a common feature in *in vitro* cultures, as demonstrated in other studies. Ramirez-Estrada et al. (2015) observed that although taxane production was related with the expression of biosynthetic genes, other factors may also be involved, such as post-transcriptional and post-translational regulation.

#### 4. Conclusions

New insights into metabolism and its regulation were obtained by studying centelloside production in elicited *C. asiatica* hairy roots together with the expression of key biosynthetic genes. Coro, especially in combination with Meja, proved to be the most effective elicitor in terms of enhanced gene expression and centelloside production, above all that of madecassoside. These findings may have useful application for increasing centelloside production in biotechnological platforms based on metabolically engineered *C. asiatica* hairy roots.

#### CRediT authorship contribution statement

Mercedes Bonfill supervised the project and wrote the draft manuscript, Javier Palazon designed and lead the Project, Elisabeth Moyano reviewed the manuscript, Rosa Maria Cusido analyzed all the data, Miguel Angel Alcalde carried out the experiments.

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#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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