RESEARCH ARTICLE

Elicitation with methyl jasmonate combined with cultivation in the Plantform™ temporary immersion bioreactor highly increases the accumulation of selected centellosides and phenolics in *Centella asiatica* (L.) Urban shoot culture

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Centella asiatica (L.) Urban is an important pharmacopoeial plant used not only in medicine but also in cosmetology. *C. asiatica* agitated shoot cultures were established to study the influence of ethephon, methyl jasmonate, L-phenylalanine (Eth 50 µM, MeJa 50 µM, L-Phe 2.4 g/L of medium, respectively; seven variants of the supplementation) on the accumulation of secondary metabolites: the main centellosides (asiaticoside and madecassoside) and selected phenolic acids, and flavonoids in the biomass. Microshoots were harvested two and six days after the supplementation. Secondary metabolites were analyzed in methanolic extracts by UPLC-MS/MS (centellosides) and by HPLC-DAD (phenolics). In comparison with the reference cultures, the concentrations of individual secondary metabolites increased as follows: centellosides up to 5.6-fold (asiaticoside), phenolic acids up to 122-fold (*p*-coumaric acid) and flavonoids up to 22.4-fold (kaempferol). The highest production increase of individual compounds was observed for different variants of supplementation. Variant C (50 µM MeJa), the most optimal for centellosides and flavonoid accumulation, was selected for the experiment with bioreactors. Bioreactor Plantform™, compared to RITA® system and agitated cultures, appeared to be the most advantageous for secondary metabolites production in *C. asiatica* shoot cultures. The phenolic acid, flavonoid, centelloside, and total secondary metabolite productivity in Plantform™ system is 1.8-fold, 1.7-fold, 2.8-fold, 2.1-fold, respectively, higher than in MeJa elicited agitated cultures, and 4.3-fold, 7.3-fold, 12.2-fold, 7.2-fold, respectively, higher than in control agitated cultures.

KEYWORDS

centellosides, flavonoids, phenolic acids, precursor and elicitor supplementation, temporary immersion bioreactor, UPLC-MS/MS

1 | INTRODUCTION

Centella asiatica (L.) Urban (Apiaceae), commonly known as Gotu Kola, is a perennial plant growing in tropical and subtropical swampy regions of Asia, Africa, South and North

Abbreviations: BAP, 6-benzylaminopurine; DW, dry weight; Eth, ethephon; GA₃, gibberellic acid; L-Phe, L-phenylalanine; MeJa, methyl jasmonate; MS, Murashige and Skoog medium; NAA, α-Naphthaleneacetic acid; PGRs, plant growth regulators; TIB, temporary immersion bioreactor.

America [1]. It has been widely used since ancient times in many traditional medicinal systems such as Ayurveda or traditional Chinese medicine. The plant is also appreciated by modern medicine due to its valuable therapeutic applications. *C. asiatica* herb is currently listed in the 9th Edition of European Pharmacopoeia [2]. It has been demonstrated in many studies to have anti-inflammatory, antioxidant, antibacterial, antiulcer, and anticancer activity. *C. asiatica* is well known for its neuroprotective and cognitive enhancing properties. It is also recommended in skin regeneration and wound healing treatment. *C. asiatica* extract influence collagen synthesis so it is used not only in medicine but also in cosmetology [1].

Centellosides (e.g. asiaticoside and madecassoside) – active metabolites of *C. asiatica* – belong to pentacyclic terpenoids [3,4]. Apart from it, *C. asiatica* is rich in antioxidant compounds such as flavonoids and phenolic acids [1].

Plant cell and tissue cultures are well known as an alternative possibility to produce secondary metabolites. Different methods are used to increase the accumulation of expected valuable chemical compounds, for instance culture supplementation with elicitors and precursors [5–10].

Bioreactors are considered as important biotechnological engineering systems that enable to scale-up the production of valuable secondary metabolites in plant in vitro cultures [11, 12]. The choice of the bioreactor type largely depends on the type of in vitro culture that is to be cultivated in. RITA[®] and PlantformTM are examples of temporary immersion bioreactors (TIBs). Such systems are widely used in plant biotechnology and provide the most natural environment for in vitro culture of shoots and seedlings [13].

There is a considerable research on the production of centellosides in suspension and hairy root cultures of *C. asiatica* but yields of the compounds have been generally low. The highest production was achieved in cultures treated with elicitors [14–17]. Only few studies concern centellosides production in shoot (or whole plant) cultures and none of them were conducted in temporary immersion bioreactors RITA[®] or PlantformTM [14,18,19].

Methyl jasmonate is a key signaling compound in plant defense responses. Ethephon is converted by the plant into ethylene, a possible plant growth regulator (PGRs). Both compounds are used as elicitors in plant biotechnology to enhance the biosynthesis of secondary metabolites. L-phenylalanine (L-Phe) is an important intermediate of the shikimate pathway. This aromatic amino acid is considered as a precursor for a wide range of secondary metabolites such as phenolic acids, flavonoids, alkaloids, tannins etc.

The aim of the experiment was to examine the influence of the elicitors (methyl jasmonate and ethephon) and/or the precursor (L-phenylalanine) on the accumulation of selected secondary metabolites: the main centellosides, selected flavonoids, and phenolic acids in agitated shoot cultures of *C. asiatica* (L.) Urban. The best supplementation variant from

PRACTICAL APPLICATION

Centella asiatica (L.) Urban is an important pharmacopoeial plant widely used not only in medicine but also in cosmetology. *C. asiatica* shoot cultures, fast-growing in the liquid medium; seem to be an ideal object for temporary immersion systems. Bioreactor PlantformTM turned out to be the best system, compared to bioreactor RITA[®] and agitated flasks, in terms of productivity of selected secondary metabolites from the three chemical groups: centellosides, phenolic acids and flavonoids. PlantformTM system, with the possibility of simultaneous combining numerous single bioreactors, provides the way to scale-up the production of *C. asiatica* secondary metabolites by shoot cultures.

the experiment was used to compare the accumulation and productivity of the above-mentioned secondary metabolites in *C. asiatica* shoot cultures grown in agitated flasks and bioreactors (RITA[®] and PlantformTM).

To the best of our knowledge, this is the first study describing the production of three groups of secondary metabolites (centellosides, phenolic acids, and flavonoids) in *C. asiatica* shoots cultivated in agitated cultures and in two types of temporary immersion bioreactors: RITA[®] and PlantformTM. The experiment was carried out using the previously selected variant of supplementation with elicitor.

2 | MATERIALS AND METHODS

2.1 | Plant material

The plant material consisted of the aerial parts of *C. asiatica* (L.) Urban, which were obtained in 2014 from plants grown in the greenhouse of the Garden of Medicinal Plants, Jagiellonian University, Medical College, Kraków, Poland (Figure 1A). The identity of the plants was verified by Dr. Ewa Skrzypczak-Pietraszek based on the available literature, and voucher specimens were delivered to the Department of Pharmaceutical Botany. In vitro cultures were established using fresh young shoots of *C. asiatica*, whereas the leaves of the plant, having been dried at room temperature and stored in a refrigerator, were used for phytochemical analyses.

2.2 | Initiation of shoot cultures

The initial treatment of fresh young tops of *C. asiatica* shoots (about 5–6 cm long) involved sterilization with 70% v/v ethanol for 30 s followed by incubation in a 0.1% mercuric chloride (HgCl₂) aqueous solution for 10 min. Next, they

FIGURE 1 *Centella asiatica* (L.) Urban – (A) a greenhouse plant and (B–H) *in vitro* cultures. Description: (B) 4-week shoot culture on the agar medium; (C) agitated 2-week cultures; (D) temporary immersion bioreactor RITA®; (E) temporary immersion bioreactor Plantform®; (F) microshoots harvested from Plantform® bioreactor

were washed three times with sterile distilled water, cut into 4–5 cm lengths and transferred onto the Murashige and Skoog (MS) medium [20] that was supplemented with PGRs: α -naphthaleneacetic acid (NAA)–0.5 mg/L, 6-benzylaminopurine (BAP)–1.0 mg/L, gibberellic acid (GA_3)–0.25 mg/L, and solidified with agar (7.3 g/L). The shoot cultures were maintained in 300 mL Erlenmeyer flasks at $25 \pm 2^\circ\text{C}$ under constant artificial light ($16 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-2}$), and were passaged onto the same fresh medium every 5–6 weeks (Figure 1B).

2.3 | Selection of growth and production medium

The shoot cultures of *C. asiatica* were grown on four variants of MS medium (Table 1) (fresh shoots–3 g per flask; 300 mL Erlenmeyer flasks–100 mL of medium solidified with agar). The produced biomass was harvested in three replicates every seven days for five weeks, weighed, dried at 40°C (SPU 250 vacuum dryer, ZEAMiL Horyzont, Kraków, Poland) and weighed again. The growth index was then calculated as follows:

$$\text{GI} = \frac{(\text{final fresh weight} - \text{inoculum fresh weight})}{\text{inoculum fresh weight}} \quad (1)$$

The above experiment was repeated using a liquid medium without agar. Agitated shoot cultures were conducted on a

rotary shaker (Altel) at 140 rpm, with the temperature and light conditions being the same as stated previously (see Section 2.2). All the experiments were run in three independent series. The harvested biomass was subjected to analysis by HPLC-DAD (phenolics) and by UPLC-MS/MS (centellosides).

3 | SUPPLEMENTATION EXPERIMENT

For the supplementation experiment, the agitated shoot cultures were initiated by inoculating 3 g of shoot fresh mass in 300 mL Erlenmeyer flasks containing 100 mL of liquid MS medium and the same PGRs as for the best growth and production medium selected previously: 1 mg/L BAP, 0.5 mg/L NAA, 0.25 mg/L GA_3 (see Section 2.3). The flasks were agitated on a rotary shaker (Altel) at 140 rpm, under the same temperature and light conditions as previously (see Section 2.2). Two weeks after inoculation (Figure 1C), 100 mL of the fresh liquid medium (with the same composition as before) was added. After another week, the cultures were supplemented with 50 μM methyl jasmonate (MeJa; Sigma-Aldrich), 50 μM ethephon (Eth; Sigma-Aldrich) and 2.4 g/L L-phenylalanine (L-Phe; Merck) according to a predetermined scheme (Figure 2).

The experiment was preceded by the preparation of stock solutions of MeJa, Eth and L-Phe. Before being administered to the medium, MeJa was dissolved in 96% v/v ethanol, Eth, and L-Phe were separately dissolved in water, and

TABLE 1 *Centella asiatica* agitated shoot cultures—selection of the best growth and production medium

Variant of the medium—PGRs content [mg/L]			Agitated cultures—growth index ^a					Metabolite content in 3-week culture [mg/100 g DW]		
BAP	NAA	GA ₃	1 week ^b	2 weeks ^b	3 weeks ^b	4 weeks ^b	5 weeks ^b	Chlorogenic		
								Asiaticoside	acid	Rutin
1	0.5	—	0.5 ± 0.1	3.8 ± 0.4	7.1 ± 0.5	6.9 ± 0.6	6.7 ± 0.6	95 ± 9	37 ± 8	171 ± 9
2	1	—	0.4 ± 0.1	3.2 ± 0.4	6.8 ± 0.6	6.7 ± 0.5	6.6 ± 0.5	77 ± 6	45 ± 5	180 ± 10
1	0.5	0.25	0.6 ± 0.1	5.9 ± 0.4	12 ± 0.6	11.6 ± 0.5	11.4 ± 0.6	124 ± 9	59 ± 11	214 ± 34
2	1	0.25	0.5 ± 0.1	4.1 ± 0.4	8.6 ± 0.5	8.5 ± 0.6	8.4 ± 0.5	89 ± 7	41 ± 5	162 ± 13

All experiments were conducted in triplicates. Data are means ± SE.

^aGrowth index of fresh microshoots GI = (final fresh weight – inoculum fresh weight)/inoculum fresh weight.

^bCultures harvested after 1–5 weeks.

FIGURE 2 Elicitation and feeding experiment in agitated shoot cultures of *Centella asiatica*. Variants of supplementation: B, C, D, E, F, G, H. Control culture: variant A

all three solutions were filter-sterilized (0.22 μm, Millex® Millipore). The control was represented by variant A. Variants without MeJa, Eth or L-Phe were supplemented with sterile ethanol or water, respectively. The final concentration of ethanol in the culture medium was 0.1% v/v. The biomass and the medium (100 ml per flask) were harvested after two and six days (on 23rd and 27th day of cultivation, respectively). The biomass was dried at 40°C (see Section 2.3) and the medium was frozen and lyophilized. They were subsequently used to prepare extracts to be analyzed by HPLC-DAD and UPLC-MS/MS. The experiments were performed with three independent samples for biological replicates.

4 | SHOOT CULTURES IN BIOREACTORS

4.1 | Bioreactor setup

Two types of commercially available TIBs were used: RITA® (VITROPIC, Saint-Mathieu-de-Treviers, France) and Plantform™ (Plant Form AB, Sweden). The details of both TIBs are available on manufacturer websites. Bioreactors were steam sterilized before use (1 bar, 120°C for 20 min). The details of the experimental parameters are described in Table 2.

TABLE 2 Experimental parameters of the temporary immersion systems

Parameter	Temporary immersion system	
	RITA®	Plantform™
Periodic aeration*	1 min/90 min	—
	5 min/90 min	5 min/90 min
Working volume	200 mL	500 mL

*Periodic aeration was applied at 1 vvm (vvm—a volume of the air per the unit of a medium and the unit of a time)

4.2 | Experiment

Based on the earlier results (see Section 2.3), MS medium with 1 mg/L BAP, 0.5 mg/L NAA and 0.25 mg/L GA₃ (the best growth and production medium) was used in the study. Both TIBs were inoculated at 2:100 (w/V) ratio (4 g of shoots/200 mL of the medium per RITA® bioreactor and 10 g of shoots/500 mL of the medium per Plantform™ bioreactor).

Based on the supplementation studies results (see Section 2.4), variant C (50 μM MeJa) was used in the bioreactor cultures and variant A (without any supplements) for the control cultures. The produced biomass was harvested on 23rd day of the experiment (two days after supplementation), dried at 40°C (see Section 2.3) and subjected to analysis by HPLC-DAD (phenolics) and by UPLC-MS/MS (centellosides).

The bioreactor cultures were conducted in the same temperature and light conditions as stated previously (see Section 2.2).

4.2.1 | Extraction

The dried biomass from in vitro cultures and aerial parts of dried greenhouse plants were pulverized in a mortar. Samples of 1 g each were subjected to extraction with boiling methanol (2 × 100 mL, 2 h). The extracts were combined (200 mL) and concentrated to dryness (40°C, Büchi rotary vacuum evaporator). The residues were dissolved in 10 mL of methanol (HPLC grade, Merck). Likewise, residues of the lyophilized media were also dissolved in 10 mL of methanol (HPLC grade, Merck). All the methanolic solution samples

were filtered (0.22 μm Millipore syringe filters, Millex-GP), after which they were analyzed by HPLC-DAD (phenolics) and UPLC-MS/MS (centellosesides).

4.3 | HPLC analyses of secondary metabolites

Phenolic acids and flavonoids were analyzed by HPLC-DAD method. The method and the used HPLC apparatus was earlier described in details [21]. Centellosesides were analyzed by UPLC-MS/MS method.

4.3.1 | Reagents for UPLC-MS/MS

Acetonitrile, methanol, and formic acid (all – LC/MS-grade) were obtained from Sigma-Aldrich. HPLC-grade water was obtained from HLP 5 (HYDROLAB Poland) apparatus and was filtered through 0.2 μm filter before use. Chloramphenicol ($\geq 98\%$, TLC) and analytical standards, asiaticoside, and madecassoside ($\geq 98.5\%$, HPLC and $\geq 95\%$, HPLC, respectively) were obtained from Sigma-Aldrich.

4.3.2 | Preparation of centellosesides standard solutions

Chloramphenicol was used as internal standard in centellosesides standard solutions. The final concentrations of asiaticoside and madecassoside stock solutions were 100 $\mu\text{g/mL}$ and 1000 $\mu\text{g/mL}$, respectively. Details of the solution preparations are included in the Supporting Information.

Centellosesides stock solutions were stored at -10°C and used to make dilutions for calibration curves.

4.3.3 | Preparation of calibration samples

Asiaticoside and madecassoside stock solutions were diluted to obtain centelloseside concentrations within the range of 1–10 $\mu\text{g/mL}$ and 0.039–5.0 $\mu\text{g/mL}$, respectively. Details of the dilution are included in the Supporting Information.

4.3.4 | Preparation of samples

From each sample, 100 μL of solution was taken, then 100 μL of 10 $\mu\text{g/mL}$ chloramphenicol internal standard solution was added and brought to 1 mL with water making dilutions of the stock solutions of each sample. Each dilution was analyzed in triplicate with UPLC-MS/MS.

4.3.5 | Quantitative analysis

In order to determine asiaticoside and madecassoside in the samples, the quantitative UPLC-MS/MS method was developed.

The UPLC-MS/MS system consisted of the Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to the Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Chromatographic separations were carried out using the Acquity UPLC

BEH (bridged ethyl hybrid) C_{18} column, $2.1 \times 100 \text{ mm}$, and 1.7 μm particle size. The column was maintained at 40°C , and eluted under the following conditions:

- asiaticoside–linear gradient elution from 95 to 0% of eluent A over 10 min, afterwards 100% of eluent B over 1 min, at a flow rate of 0.3 mL/min.
- madecassoside–linear gradient elution from 100 to 90% of eluent A over 2 min, afterwards linear gradient elution from 90 to 0% of eluent A over 3 min and 100% of eluent B over 1 minute, at a flow rate of 0.3 mL/min.

Eluent A: water/formic acid (0.1%, v/v); eluent B: acetonitrile/formic acid (0.1%, v/v). 1 μL of each sample was injected in triplicate.

The Waters TQD mass spectrometer was calibrated for the quantitative analysis using asiaticoside or madecassoside solution with a concentration of 10 $\mu\text{g/mL}$ at a flow rate of 20 $\mu\text{L/min}$ and the mixture of eluent A and B 1:1 v/v at a flow rate of 0.28 mL/min. Optimized settings were the following: source temperature of 150°C , desolvation temperature of 350°C , desolvation gas flow rate of 600 L/h, capillary potential of 3 kV, collision gas flow of 0.1 mL/min. The cone potential and collision energy were individually optimized for each transition using asiaticoside or madecassoside solution. Nitrogen was used as nebulizer and as drying gas. Argon was used as collision gas. Traces of analyzed compounds were analyzed using MRM (Multiple Reaction Monitoring) method.

All analytical data were processed using the MassLynx V4.1 software (Waters Corporation, Milford, MA, USA).

4.3.6 | Validation of UPLC-MS/MS method

The described method was validated for the determination of asiaticoside and madecassoside by UPLC-MS/MS method according to ICH guidelines [22]. Details of the validation are included in the Supporting Information.

4.4 | Statistical methods

The experiment was prepared according to the well-known rules of the designed experiment [23]. Four factors controlled at two levels were selected: a harvesting time (2nd day vs. 6th day), a specific concentration of L-Phe (0 vs. 2.4 g/L), MeJa (0 vs. 50 μM) and Eth (0 vs. 50 μM). Three quantitative outputs were selected: total phenolic acids, total flavonoids and total centellosesides.

The two-level full factorial 2^4 experimental design [23] with 16 different treatments was selected as a scheme for the experiment. The design was planned as a balanced one with 3 measurement replications in each treatment. In the further analysis, the original design was split into two controlled factors: two-level harvest day (2nd day vs. 6th day) and eight-level the supplements (A, B, C, D, E, F, G, H; Figure 2).

The initial model was a fixed effects model with interactions up to and including the second order. The analysis of effects, combined with the estimation of a pure error on the basis of replications, allowed to determine statistically significant terms of the model. In the next step, insignificant model components were removed and diagnostic tests of the model were used: the normality test of residuals and a lack-of-fit test. The reduced model served as a base for the ANOVA analysis to estimate the individual impact of significant model terms.

Tukey-Kramer HSD test [24] was used to identify the homogeneous groups inside treatments and the group related to the maximum of the respective outcome was selected. Finally, the interpretation of the results was developed. All the computations were performed using Statistica software.

5 | RESULTS AND DISCUSSION

5.1 | Selection of growth and production medium

C. asiatica shoot cultures were initiated from tops of young shoots of the greenhouse plants. MS medium with 1 mg/L BAP, 0.5 mg/L NAA, 0.25 mg/L GA₃ was chosen based on our previous experience with plant shoot cultures [21,25,26]. After 1–2 weeks, 3–4 additional buds appeared at the base of the shoot tops and after 2–3 weeks formed young shoots (Figure 1B). The growth of the shoots was very slow so media with three other variants of PGRs were tested (Table 1). Regardless of the added PGRs, the growth of the shoot cultures was still very slow on all agar-solidified media. The values of the growth index after 3–4–5 weeks did not exceed 1.5–2 and for individual medium variants did not differ statistically (data not shown). Next, agitated shoot cultures were established and quick, considerable growth of them was observed (Table 1).

C. asiatica is a wetland plant, which is probably the cause why the liquid medium provides more favorable conditions for growth. In addition, the availability of nutrients and PGRs in shaken liquid cultures is also favorable in comparison with solid cultivation where diffusion is the only possibility for motion of medium constituents. After three weeks, the growth index reached the highest value in the range of 6.8–12.0 (Table 1). MS medium supplemented with 1 mg/L BAP, 0.5 mg/L NAA and 0.25 mg/L GA₃ was the best growth medium and, at the same time, it proved to be the best production medium (Table 1–selected results for metabolite content).

Plant growth regulators (PGRs) are a group of crucial signal molecules. They do not only regulate all aspects of plant growth, development and cell, and tissue differentiation, but also are involved in plant secondary metabolism [27]. Many PGRs have been used as efficient elicitors to stimulate biosynthesis of secondary metabolites. Gibberelic acids (GA₃) significantly increased tanshinone [28] and phenolic acid [29]

production in *Salvia miltiorrhiza* hairy root cultures. It was possible that GA₃ had the crucial impact on the secondary metabolite production in our experiment.

5.2 | Supplementation experiment

Ten selected secondary metabolites from three chemical groups were quantitatively analyzed in the harvested biomass: centellosides (asiaticoside, madecassoside), phenolic acids (chlorogenic, *p*-coumaric, rosmarinic, and vanillic acids) and, flavonoids (apigenin, kaempferol, rutin, and quercitrin). The aforementioned secondary metabolites were previously reported and identified as chemical constituents of *C. asiatica*.

5.2.1 | Centellosides

MeJa had the strongest impact on the production of centellosides in *C. asiatica* agitated shoot cultures (the variant C). Two days after supplementation, asiaticoside, madecassoside, and the combined concentration of the two centellosides increased up to 445 ± 7 mg/100 g dry weight (DW) (3.6-fold), 240 ± 19 mg/100 g DW (6.2-fold), and 685 ± 22 mg/100 g DW (4.2-fold), respectively (Figure 4A–C; variant C versus variant A – reference culture). After a further four days, the highest concentrations of the compounds reached 546 ± 12 mg/100 g DW, 288 ± 32 mg/100 g DW, and 834 ± 26 mg/100 g DW, respectively. These values were 3.5, 5.6, and 4.1-fold higher, respectively, than those for the reference culture. Supplementation with MeJa together with Eth (variant G) was not as advantageous as option C. Eth, used separately (variant D), exhibited a very weak eliciting activity. Asiaticoside and madecassoside contents increased only to 203 ± 6 mg/100 g DW and 90 ± 9 mg/100 g DW, and to 212 ± 13 mg/100 g DW and 94 ± 11 mg/100 g DW, two and six days after supplementation, respectively.

5.2.2 | Phenolic acids

The effects of supplementation on the concentrations of four phenolic acids (chlorogenic, vanillic, rosmarinic, and *p*-coumaric acid) were investigated. Rosmarinic (Figure 3C) and chlorogenic (Figure 3A) acids were the main *C. asiatica* phenolic acids – 231–270 mg/100 g DW and 59–81 mg/100 g DW, respectively (variant A – reference culture). The concentrations of chlorogenic (Figure 3A), vanillic (Figure 3B) and *p*-coumaric (Figure 3D) acids significantly increased in *C. asiatica* shoot cultures after supplementation of the medium with *L*-Phe (variants B, E, F, H). The highest content values were observed for the variant H (*L*-Phe, MeJa, Eth) reaching up to 451 ± 7 mg/100 g DW, 10.0 ± 0.5 mg/100 g DW, and 377 ± 23 mg/100 g DW, respectively, on the 27th day of the culture. These values are 5.6, 4.5, and 122-fold higher than those of the reference cultures. Different supplementation variants had the strongest influence on rosmarinic acid

FIGURE 3 Supplementation experiment – phenolic acid (A–D) and flavonoid (E–H) content in *Centella asiatica* agitated shoot cultures supplemented with MeJa, Eth and L-PHE according to the scheme Figure 2. Variant A – control culture. The biomass was harvested after 2 or 6 days after supplementation

accumulation. The highest increases in the concentration of that compound were observed for variants C (MeJa) and G (MeJa+Eth): up to 566 ± 58 mg/100 g DW (2.1-fold) and up to 577 ± 45 mg/100 g DW (2.1-fold), respectively. Total

phenolic acid content (Figure 4E)–the sum of four analyzed phenolic acids–was the highest for variant H (27-day culture) reaching up to 1141 ± 15 mg/100 g DW. It was 3.2-fold higher comparing to reference culture.

FIGURE 4 Supplementation experiment – content of (A) asiaticoside, (B) madecassoside, (C) centellosides (asiaticoside+madecassoside), (E) phenolic acids (the sum of four compounds), (F) flavonoids (the sum of four compounds), (G) secondary metabolites (phenolic acids+flavonoids+centellosides) in *Centella asiatica* agitated shoot cultures, (H) growth index of cultures [GI = (final fresh weight – inoculum fresh weight)/inoculum fresh weight], (D) content of L-phenylalanine in culture medium. Cultures were supplemented with MeJa, Eth and L-PHE according to the scheme Figure 2. Variant A – control culture. The biomass was harvested after 2 or 6 days after supplementation

5.2.3 | Flavonoids

Four flavonoids were analyzed: apigenin, kaempferol, rutin, and quercitrin. Rutin (Figure 3F) was the main flavonoid in *C. asiatica* shoots – 212–214 mg/100 g DW (variant A – reference culture). All variants of supplementation caused an increase in rutin accumulation, however to a different degree. The highest increase in its concentration was observed for variant C (MeJa) – up to 910 ± 121 mg/100 g DW, 4.3-fold (23-day culture). Both elicitors, MeJa and Eth, used together had a weaker influence on the production of rutin. Its concentration grew only up to 723 ± 112 mg/100 g DW (variant G). Eth used separately caused an increase in rutin content, but only up to 590 ± 54 mg/100 g DW (2.8-fold, variant D). The influence of the elicitors on the accumulation of rutin decreased in the following days, resulting in, e.g. for variant C, a rutin content of just 725 ± 108 mg/100 g DW (27-day culture).

The other analyzed flavonoids (apigenin, Figure 3G; kaempferol, Figure 3E; quercitrin, Figure 3H) were found in much smaller quantities in the reference cultures than rutin, amounting to 1.1–2.3 mg/100 g DW, 2.1–4.2 mg/100 g DW, 3.0–5.2 mg/100 g DW, respectively.

Supplementation increased the accumulation of these compounds, but in a variety of ways. L-Phe had the strong impact on apigenin accumulation. Two days after supplementation, its content reached 9.8 mg/100 g DW (9-fold) for variant B. The addition of the elicitors together with L-Phe was not so favorable. The concentration of apigenin amounted to 5.1, 6.3, 7.5 mg/100 g DW for variant E, F, H, respectively. After six days of the supplementation, a significant influence of the elicitors, added in combination with L-Phe, could be noticed. The highest concentration of apigenin was observed for variant F (L-Phe+Eth), 27 ± 3.5 mg/100 g DW (13.5-fold increase), in comparison to variant B (L-PHE), 10.1 ± 2.4 mg/100 g DW (5.1-fold increase), and variant E (L-PHE+MeJa), 17 ± 0.7 mg/100 g DW (8.5-fold increase).

The strong impact of L-PHE (variant B) and L-PHE with both elicitors (variant H) on quercitrin accumulation was observed six days after supplementation. The flavonoid content reached up to 27.2 ± 4.7 mg/100 g DW (9-fold increase) and up to 62 ± 6 mg/100 g DW (20.7-fold increase), respectively.

The highest kaempferol accumulation was observed for variant D (Eth) and G (MeJa+Eth) reaching up to 45 ± 6 mg/100 g DW (22.4-fold increase) and 48 ± 7 mg/100 g DW (24.1-fold increase), respectively, two days after supplementation.

Total flavonoid content (Figure 4F) – the sum of four analyzed flavonoids – was higher for variant C (23-day culture) reaching up to 927 ± 119 mg/100 g DW. It was 4.2-fold higher comparing to reference culture.

All analyzed secondary metabolites (centellosides, phenolic acids and flavonoids) were summed up (Figure 4G). The highest content of compounds was observed for variant C (23-day culture) amounting up to 2295 ± 55 mg/100 g DW (3.4-fold increase). It was the reason the variant C was used in the next step of the experiment.

5.3 | Shoot cultures in bioreactors

Accumulation of selected secondary metabolites in *C. asiatica* shoot cultures grown in bioreactors RITA® (R-1 and R-5) and Plantform™ (P-5) was compared with results for agitated cultures (AC) and the greenhouse plants (GP) (Figure 5). The productivity of the systems was expressed as mg of compounds per liter of the medium per day of the culture. The biomass was harvested on the 23th day of culture. Two days earlier, cultures were supplemented with 50 μ M MeJa (variant C). Variant A (without supplementation) was prepared as a reference. Phenolic acid, flavonoid and centelloside content was the sum of four analyzed phenolic acids, four flavonoids and two centellosides, respectively.

C. asiatica shoot cultures had enough space in the bioreactors and could grow freely (Figure 1D–F), which was not ensured by conical flasks used for agitated cultures.

Total secondary metabolite content in greenhouse grown plants was compared with shoots grown as reference cultures – variant A (Figure 5D). Culture supplementation with MeJa caused a significant increase in metabolite accumulation – 3.6-fold for P-5 and about 3-fold for AC, R-1, R-5 in comparison with greenhouse-grown plants. The greenhouse-grown plants accumulated more centellosides (Figure 5C), less phenolic acids (Figure 5A) and similar amounts of flavonoids (Figure 5B) comparing to reference shoot cultures (variant A).

The metabolite content in *C. asiatica* shoot cultures elicited by 50 μ M MeJa is highest for Plantform™ system (Figure 5A–D). Bioreactor RITA® in comparison with agitated cultures (AC) gets higher production of compounds only in the case of centellosides (Figure 5C), with more advantageous 5-minute aeration (R-5) than 1-min one (R-1). The comparison of the system productivity (Figure 5E–H) gives more explicit results as follows: $AC < R-1 < R-5 < P-5$. The phenolic acid (Figure 5E), flavonoid (Figure 5F), centelloside (Figure 5G) and total secondary metabolite (Figure 5H) productivity in Plantform™ system is 1.8-fold, 1.7-fold, 2.8-fold, 2.1-fold, respectively, higher than in MeJa elicited agitated cultures, and 4.3-fold, 7.3-fold, 12.2-fold, 7.2-fold, respectively, higher than in control agitated cultures.

TIBs are widely used for in vitro organ cultures [11,12,30]. As the systems providing huge amounts of biomass, they can be used in secondary metabolites production in in vitro conditions. TIBs are mostly used for adventitious hairy root cultures. There are numerous examples of plant micropropagation in TIBs, but there are also more and more

FIGURE 5 Comparison of the metabolite content (A–D) in *Centella asiatica* greenhouse plant (GP) with agitated shoot cultures (AC) and bioreactor cultures (R-1, R-5 – bioreactor RITA® with 1 min and 5 min aeration, P-5 – bioreactor Plantform™ with 5 min aeration). Phenolic acids = the sum of four compounds. Flavonoids = the sum of four compounds. Centellosides = the sum two compounds. Total secondary metabolites = phenolic acids + flavonoids + centellosides. (E–H) productivity of the systems expressed as mg of compounds per liter of the medium per day of culture. Variant A – control culture, variant C supplemented with 50 μ M MeJa. The duration of the cultures 23 days

TABLE 3 Effect of the elicitors on centelloside production in different in vitro systems of *Centella asiatica*

Type of culture	Elicitor	Production [mg/g DW]		References
		Asiaticoside	Madecassoside	
Shoot cultures (agitated flasks)	MeJa (50 μ M)	4.45	2.40	Our results
Shoot cultures (Plantform TM 5 min/90 min)	MeJa (50 μ M)	7.91	3.81	Our results
Whole plant	MeJa (100 μ M)	1.40 (in leaves)	0.60 (in leaves)	[34]
Whole plant	CdCl (5 mM)	3.98	–	[14]
	CuCl ₂	4.20	–	
	yeast extract (0.1 g/L)	5.91	–	
	MeJa (10 μ M)	6.74	–	
Cell suspensions	MeJa (100 μ M)	1.10	Sum of both centellosides	[15]
Cell suspensions	copal extract (125 mg/L)	0.45	0.25	[17]
Hairy roots	MeJa (100 μ M)	7.12	–	[35]
Hairy roots (Pg FP5 gene)	MeJa (100 μ M)	3.80	1.70	[36]

information about secondary metabolites production in shoot cultures [30–33].

Our results with PlantformTM system, in comparison with earlier research of other authors on different types of *C. asiatica* cultures (e.g [14,15,17,34–36]), seem to be very interesting and promising (Table 3).

5.4 | Validation of UPLC-MS/MS method

The developed UPLC method was specific to the examined compounds and guaranteed that well shaped peaks were obtained. No interference that could have an influence on the obtained results was possible because the MRM method was applied. Details of the validation are included in the Supporting Information.

5.5 | Statistical analysis

Statistical analysis confirms previously mentioned observations.

Phenolic acids. ANOVA analysis revealed a statistical significance (p less than 0.05) of the harvest day and the supplement variant as well as their interaction. The marginal means plot showed that supplements differentially impose phenolic acids outcome for all variants while the harvest day strongly imposes the H variant. The Tukey's HSD test detected five homogeneous groups i.e. those treatments for which the total production of phenolic acids is not statistically different. Noteworthy is the combination of day six and variant H, which has the highest production value and is a separate homogeneous group.

Flavonoids. ANOVA analysis revealed a statistical significance (p less than 0.05) of the harvest day and the supplements while their interaction is not significant ($p = 0.89$).

The marginal means plot showed that supplement treatments differentially impose the flavonoids outcome for all variants. The Tukey's HSD test detected four homogeneous groups i.e. those treatments for which the total production of flavonoids is not statistically different. Unfortunately, the group with the highest productivity is quite fuzzy and includes five combinations: 2nd day with variants C, D, G and 6th day with variants C and G.

Centellosides. ANOVA analysis revealed statistical significance (p less than 0.05) of the supplement variant and its interaction with the harvest day while the direct influence (the linear effect) of the harvest day is statistically insignificant. The marginal means plot showed that supplement treatments differentially impose the centellosides outcome for all variants while the harvest day strongly imposes the C variant. The Tukey's HSD test detected 8 homogeneous groups i.e. those treatments for which the total production of centellosides is not statistically different. Noteworthy is the combination of day six and variant C, which has the highest production value and is a separate homogeneous group.

Details are shown in the Supporting Information.

6 | CONCLUDING REMARKS

The highest production increase of individual compounds was observed for different variants of supplementation. Variant C (50 μ M MeJa), as the most optimal for centellosides and flavonoid accumulation, was selected for the experiment with bioreactors.

Bioreactor PlantformTM turned out to be the best system, compared to bioreactor RITA[®] and agitated flasks, in terms of productivity of selected secondary metabolites from the three chemical groups: centellosides, phenolic acids and flavonoids. Their productivity in PlantformTM system is

2.8-fold, 1.8-fold, 1.7-fold, respectively, higher than in MeJa elicited agitated cultures, and 12.2-fold, 4.3-fold, 7.3-fold, respectively, higher than in control agitated cultures.

PlantformTM system, with the possibility of simultaneous combining numerous single bioreactors, provides the way to scale-up the production of *C. asiatica* secondary metabolites by shoot cultures.

Obtained results can be helpful for biotechnological processes of other plant species cultures. Elicitation significantly increases the production of secondary metabolites. The experimental selection of the optimal elicitation variant (type of elicitor/elicitors, variant of concentration etc.) for special type of secondary metabolite is crucial. PlantformTM system is very convenient for fast growing shoot cultures.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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