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Research Article

An optimized biotechnological system for the production of centellosides based on elicitation and bioconversion of *Centella asiatica* cell cultures

Centella asiatica is a herbaceous plant of Asian traditional medicine. Besides wound healing, this plant is recommended for the treatment or care of various skin conditions such as dry skin, leprosy, varicose ulcers, eczema, and/or psoriasis. Triterpene saponins, known as centellosides, are the main metabolites associated with these beneficial effects. Considering the interest in these high value active compounds, there is a need to develop biosustainable and economically viable processes to produce them. Previous work using *C. asiatica* plant cell culture technology demonstrated the efficient conversion of amyrin derivatives into centellosides, opening a new way to access these biomolecules. The current study was aimed at increasing the production of centellosides in *C. asiatica* plant cell cultures. Herein, we report the application of a new elicitor, coronatine, combined with the addition of amyrin-enriched resins as potential sustainable precursors in the centelloside pathway, for a positive synergistic effect on centelloside production. Our results show that coronatine is a powerful elicitor for increasing centelloside production and that treatments with sustainable natural sources of amyrins enhance centelloside yields. This process can be scaled up to an orbitally shaken CellBag, thereby increasing the capacity of the system for producing biomass and centellosides.

Keywords: *Centella asiatica* / Copal resin / Coronatine / Manila elemi resin / Plant cell cultures

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1 Introduction

Advances in biotechnology, particularly plant cell culture technology, should provide innovative alternatives for the production of renewable sources of biomolecules. The major benefits of cell cultures include: (i) synthesis of bioactive secondary metabolites in a controlled environment, independently of climatic and soil conditions, (ii) elimination of negative biological influences that affect secondary metabolite production in nature (microorganisms and insects), (iii) possibility of improving the production of desired metabolites, and (iv) automatization of cell growth control and metabolic process regulation, which increases production and reduces the cost [1].

Thus, plant cell factories have emerged as a realistic technology for the production of bioactive components of medicines and cosmetics, including high added value products such as shikonin, arbutine, and taxol [2]. In recent years, a number of cosmetic companies have also begun to use plant cell cultures, referred to as "plant stem cells," directly as cosmetic ingredients, thus expanding the role of this technology. Plant stem cell-based products used for this purpose include PCT Malus Domestica and PCT Solar Vitis from Mibelle AG Biochemistry or Centella Stems GX and Echinacea Stems GX from Sederma [3]. Invariably, a biotechnological system based on plant cell cultures requires a previous optimization step, involving the selection of the most productive cell lines, and finding the optimum culture medium and conditions (e.g., temperature, mineral nutrients, carbon source, plant growth regulators, etc.). Moreover, in most cases, the highest yields of secondary metabolites in plant cell

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Abbreviations: CFW, cell fresh weight; **CORO**, coronatine; **GI**, growth index; **MeJA**, methyl jasmonate; **MER**, Manila elemi resin; **SA**, salicylic acid; **YE**, yeast extract

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cultures are achieved by harnessing the effect of biotic or abiotic elicitors [4].

Centella asiatica (L) Urban, traditionally named Gotu Kola, is a herbaceous plant belonging to the Apiacea family, widely used in health, food, and cosmetic industries. In traditional medicine, it is recommended as an antipyretic, diuretic, and antibacterial treatment [5]. The active metabolites of *C. asiatica*, triterpene saponins and their sapogenins, are known as centellosides, principally asiaticoside, madecassoside, asiatic acid, and madecassic acid, and several clinical studies have confirmed that they are responsible for the beneficial effects of *C. asiatica* extracts on various disorders [6]. Centellosides, like all triperpenes, are synthesized from the dimerization of farnesyl diphosphate supplied by the cytoplasmic mevalonate pathway, and although their biosynthesis is not well described, structural similarities with amyrins suggest the latter could potentially act as centelloside precursors [7].

Despite considerable research on the development of *C. asiatica* plant cell cultures, centelloside yields have been generally low (the in vitro production of centellosides has been reviewed in detail [7]). In most cases, the highest production was achieved in cell cultures treated with elicitors, principally methyl jasmonate (MeJA) [8, 9] and salicylic acid (SA) [10, 11], which were found to increase the expression of some of the known genes involved in centelloside biosynthesis. Additionally, the capacity of *C. asiatica* cell cultures to biotransform precursors such as amyrins and amyrin-rich resin extracts into centellosides has been reported [12, 13].

In previous work, we established the optimal conditions for *C. asiatica* callus induction [14] and cell suspension growth, and demonstrated the positive effect of 100 μM of MeJA on centelloside yield and on the expression of some centelloside biosynthetic genes [15]. The aim of the current study was to improve the biotechnological production of centellosides in *C. asiatica* cell cultures by testing a new elicitor, coronatine (CORO), which is a bacterial toxin and a jasmonate mimic produced by *Pseudomonas syringae* [16], together with resins rich in amyrins (monohydroxylated pentacyclic triterpene derivatives), potential centelloside precursors [12], before finally scaling up the optimized process to an orbitally shaken CellBag.

2 Material and methods

2.1 Plant cell cultures

Centella asiatica cell line UBCA17 was obtained as described by Mangas et al. [14] and cultured in Murashige and Skoog medium + 30 g sucrose/L + 2 mg of 2,4-dichlorophenoxiacetic acid (2,4-D)/L + 0.1 of benzil aminopurine mg/L, shaken at 100 rpm at 25°C in the dark. The *C. asiatica* cell line was routinely subcultured every 3 wk in fresh medium: 10 g of cell fresh weight (CFW) was inoculated in 250 mL flasks with 100 mL of culture medium. This cell line is characterized by a good growth capacity with an average doubling time of around 7 d and a stable centelloside production. In preliminary experiments, the growth curve (measured as CFW) showed an exponential growth phase until day 12 of culture and the onset of the stationary phase. At day 18, the growth of the cell line decreased significantly, when the growth index (GI: CFW harvested/CFW inoculum) of the system was approximately 3.8–4 (data not shown).

2.2 Elicitor treatments

We have previously reported that MeJA increased centelloside production in the *C. asiatica* cell line UBCA17 more than sevenfold compared to the control (untreated) cells [15], and recently Loc et al. [10, 11] reported that SA and yeast extract (YE) treatment of *C. asiatica* cell cultures enhanced asiaticoside production. In order to confirm these results, in 250 mL shake flasks, we tested the elicitors MeJA, SA, YE, and the new elicitor, CORO, which participates in the jasmonate signaling pathway and was recently found by our group to be more effective than MeJA in increasing taxane production in *Taxus* spp. cell cultures [16]. Thus, UBCA17 cell cultures were supplemented with MeJA (100 and 200 μM), SA (100 μM), YE (4 g/L), and CORO (1 and 2 μM) at day 12 (at the end of the exponential growth phase), and samples were taken in triplicate at days 3 and 6 after elicitation.

2.3 Bioconversion experiments

In the same shake flasks, in order to increase the centelloside production of the system, we tested the centelloside precursor α-amyrin at the concentration of 50 mg/L (120 μmol/L), as well as natural sources of amyrins: 250 mg/L copal resin (containing 20% of amyrins), 125 mg/L copal extract (Ext Copal) (containing 40% of amyrins) [12] and 25 and 50 mg/L Manila elemi resin (MER) (containing 39% of amyrins; quantified by GC analysis). In all the systems, 1% DMSO was added as a permeabilizing agent to enhance cell uptake of precursors [12], and as resins were dissolved in ethanol, 1% of ethanol was also added in mock conditions. Precursors were added after 12 days of growth, and samples were taken in triplicate after 3 and 6 days.

2.4 Combined treatment of elicitors and addition of potential precursors

Once the best elicitation conditions and the most efficient precursors were established, we carried out a combined treatment in the shake flask system in which 1 μM CORO was added to the culture medium at day 12 of growth, together with Ext Copal (125 mg/L) or MER (50 and 125 mg/L). In all the treatments, 1% of DMSO was added and samples were taken as in previous experiments at days 3 and 6 of the treatment. Results were compared with those of mock conditions in which only 1% of DMSO and 1% of ethanol were added to the cell cultures.

2.5 CellBag cultures

In order to scale up the process, a 2 L CellBag (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with a working volume of 1 L, shaken in a Khuner orbital shaker in the dark at 25°C and 35–38 rpm, with a shaking diameter of 50 mm, was used. We initiated the culture at 35 rpm and gradually the shaking was increased up to 38 rpm to obtain a good distribution of cell biomass

and oxygen transference without foaming. The sterile airflow was 0.2 L/min and the inoculum size was 10% w/v. In previous experiments, these conditions were, determined as optimal for the growth of the cell line and to avoid out-of-phase phenomena (data not shown). After 10 days of culture (end of the exponential phase in these conditions), MER (125 mg/L), 1 μM CORO, and 1% of DMSO were added. Samples were taken daily in triplicate from day 10 to day 16, in order to measure the biomass and centelloside production. The experiment was run in triplicate.

2.6 Growth measurement

In order to measure the growth capacity of the systems, samples were filtered through 35 mm porous nylon filters, and cell biomass was weighed before (CFW) and after freeze-drying (cell dry weight, CDW).

2.7 Centelloside extraction and quantification

To quantify the centellosides (madecassoside, asiaticoside, madecassic acid, and asiatic acid), freeze-dried samples (1 g) and culture media were extracted as previously reported [17]. HPLC-UV analyses were carried out following the method of Inamdar et al. [18] with some modifications [19]. The analyses were performed at room temperature with a Spherisorb 51 ODS2 (250 $\times$ 4 mm) column (Waters Milford, MA, USA) using a mobile phase of ACN and water with 10 mM ammonium dihydrogen phosphate $NH_4H_2PO_4$ (pH 2.5 with orthophosphoric acid) in gradient conditions (ACN: 0–35 min, 20–70%); the flow rate was 1 mL/min and the detector was set at 214 nm. Standard purity was determined by MALDI-TOF MS. Analyses were carried out in triplicate.

2.8 Statistics

Statistical analysis was performed with Excel software. All data are the average of three determinations $\pm$ SD. A multifactorial ANOVA analysis followed by Tukey's multiple comparison tests were used for statistical comparisons. A p-value of <0.05 was assumed for significant differences.

3 Results

3.1 Elicitation experiments

In previous growth kinetic studies of the *C. asiatica* cell line UBCA17, we demonstrated that the end of the exponential growth phase and the beginning of the stationary phase is the best moment to perform the elicitor treatment [15]. Thus, the elicitors (see Section 2) were added to the culture at this point, and samples were taken 3 and 6 days later. Figure 1A shows the effect of the different elicitor treatments on the growth capacity measured as the GI (CFW harvested/CFW inoculum). In control conditions and after a culture period of 18 days, the cell biomass of the system increased more than fourfold, which represents an average doubling time of 9 days. Elicitor treatments

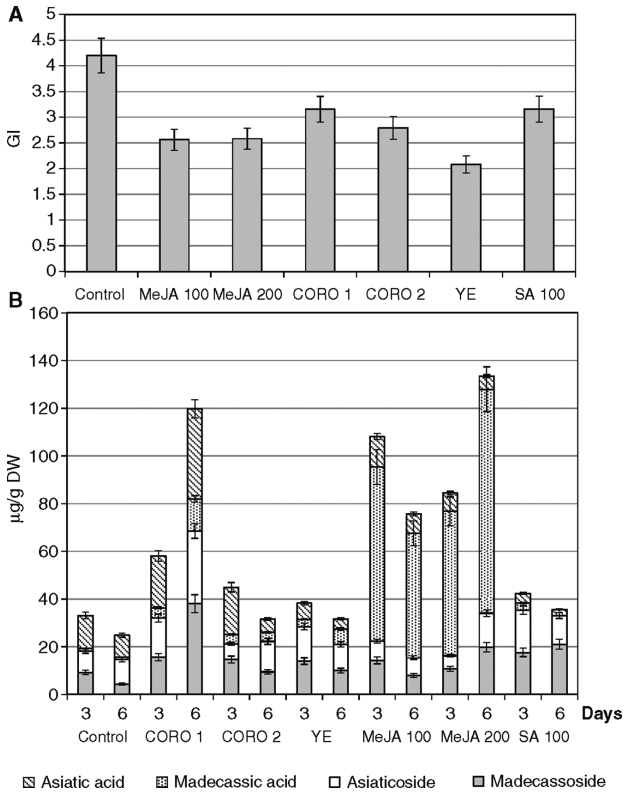

Figure 1. (A) GI (CFW sample/CFW inoculum) of the cell cultures after 18 days of culture. (B) Centelloside production expressed as microgram per gram DW measured 3 or 6 days after the elicitation treatment. CORO 1: coronatine 1 μM; CORO 2: coronatine 2 μM; MeJA 100: methyl jasmonate 100 μM; MeJA 200: methyl jasmonate 200 μM. All the values are the average of three replicates $\pm$ SD.

decreased the growth capacity of the cell line, especially MeJA and YE (Fig. 1A).

Measuring the total centellosides as the sum of madecassoside + asiaticoside + madecassic acid + asiatic acid, two treatments dramatically increased the production capacity of the cultures (Fig. 1B). The addition of 200 μM MeJA gave a centelloside production of 134 μg/g DW, which was 5.8-fold higher ($p < 0.05$) than in control conditions on the same day (day 18), although this treatment significantly changed the centelloside pattern, producing a strong increase in madecassic acid. In contrast, treatment with 1 μM CORO increased the centelloside content 5.2-fold ($p < 0.05$) compared with the control but without changing the centelloside profile of the cell line. For this reason, this elicitor was selected for further experiments.

3.2 Bioconversion experiments

The capacity of the *C. asiatica* cell cultures to bioconvert the potential precursor α-amyrin into centellosides, thereby significantly increasing the yield of these bioactive compounds, has been demonstrated [13]. The high cost of pure amyrins impedes their use in industrial cell culture processes, so we decided

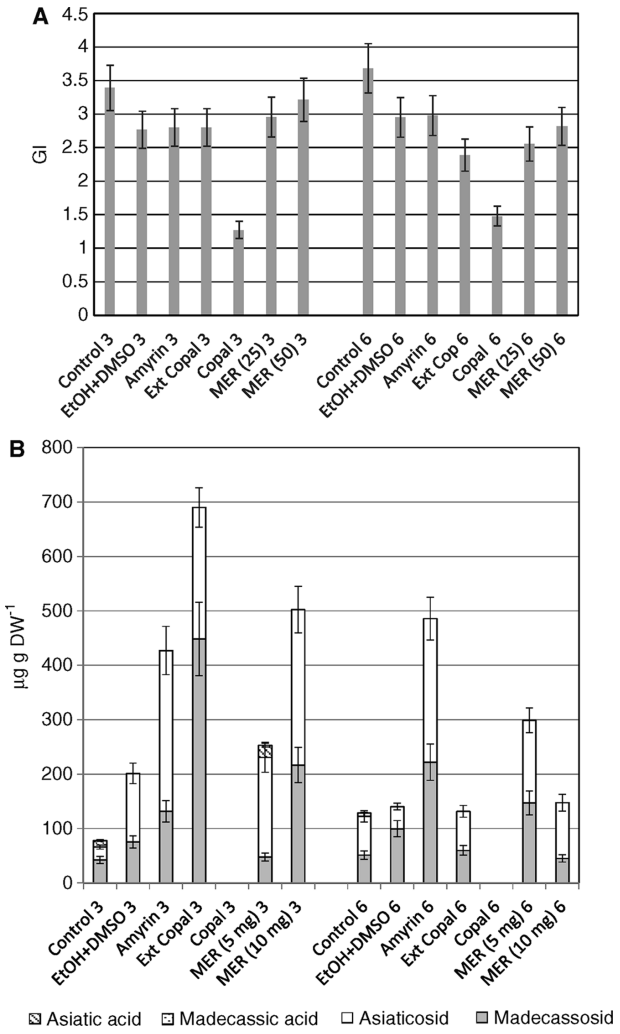

Figure 2. (A) GI (CFW sample/CFW inoculum) of the cell cultures after 18 days of culture. (B) Centelloside production expressed as μg/g DW measured 3 or 6 days after the treatment. CE: copal extract; CR: copal resin; MER: MER at the concentration of 25 or 50 mg/L. All the values are the average of three replicates ± SD.

to test copal and MER, natural and sustainable sources rich in these precursors [12]. Thus, based on our previous results, we supplemented the *C. asiatica* cell cultures at day 12 (end of the exponential growth phase) with 50 mg/L α-amyrin, 250 mg/L copal resin, 125 mg/L Ext Copal, and 25 and 50 mg/L MER. Amyrin and resins were dissolved in ethanol. In order to increase cell permeability and facilitate amyrin uptake by the cells, 1% DMSO was also added.

As shown in Fig. 2A, the precursors had very different effects on the GI of the cell cultures. Whereas cell growth was not affected by α-amyrin and MER, with the biomass at the end of the culture being similar to that in mock conditions (supplemented with 1% DMSO and 1% ethanol), it was largely inhibited ($p < 0.05$) by copal resin. In further experiments, increasing the MER concentration up to 125 mg/L did not negatively affect the growth capacity of the cell line (data not shown). The organic solvents ethanol and DMSO had negative effects on the cell growth and GI, which were lower in mock conditions than in control (untreated cells).

Centelloside production (Fig. 2B) was significantly enhanced ($p < 0.05$) by both α-amyrin and Ext Copal, in some cases more than 70-fold. In the case of Ext Copal, the centelloside content was higher after 3 days of treatment than at day 6, whereas the effects of α-amyrin were most prominent at day 6. In contrast, copal resin was found to cause cell death, probably due to components that are eliminated in the copal extract by washing with petroleum ether [12]. MER treatment also increased the total centelloside content of the culture, being more effective when added at 50 mg/L, and achieving a yield at day 3 comparable to α-amyrin and lower than Ext Copal (Fig. 2B).

3.3 Combined effect of elicitation and addition of potential precursors

In a further approach, we tested the effects of a treatment combining elicitors and precursors. CORO (1 μM) was selected as the best elicitor (Fig. 1) and Ext Copal (125 mg/L) and MER (50 and 125 mg/L) were used as potential precursors, since they enhanced centelloside production without significantly affecting cell growth ($p < 0.05$) (Fig. 2). Neither the CORO treatment nor the addition of resins affected the growth capacity of the cell line (Fig. 3A). The highest centelloside yields were obtained at day 3 by treating the cells with Ext Copal (125 mg/L) and MER (125 mg/L), and were 10.9- and 8.7-fold higher ($p < 0.05$), respectively, compared to control conditions (untreated cells). In both cases, the centelloside production peaked at day 3 of the treatment, decreasing thereafter until day 6 (Fig. 3B).

3.4 Scaling up the process

To scale up the process, a 2 L CellBag with a working volume of 1 L was used, shaken at 35 to 38 rpm in an orbital shaker. The system was working in batch mode. After 10 days (at the end of the stationary growth phase in these conditions), the cell culture was treated with 1 μM CORO, 125 mg/L MER and 1% DMSO. Samples were taken daily. After 10 days of culture, the GI peaked at 4.8, then the cell density decreased at day 11 (after the treatment) but remained stable until the end of the experiment (Fig. 4A). Despite the negative effects of the treatment on cell growth, the combined treatment of elicitor and resin greatly improved the production of total centellosides, with a progressive increase until a maximum yield (7.3 mg g DW^{-1}) at day 15 (Fig. 4B). As in the shake-flask system, the predominant centelloside produced in the bioreactor was madecassoside. The high biomass and centelloside production of the system shows the suitability of the disposable orbitally shaken bioreactor for the culture of *C. asiatica* cell suspensions.

4 Discussion

Centella asiatica has been used in Ayurvedic (Indian) medicine to treat conditions such as skin illnesses, nervous disorders, and

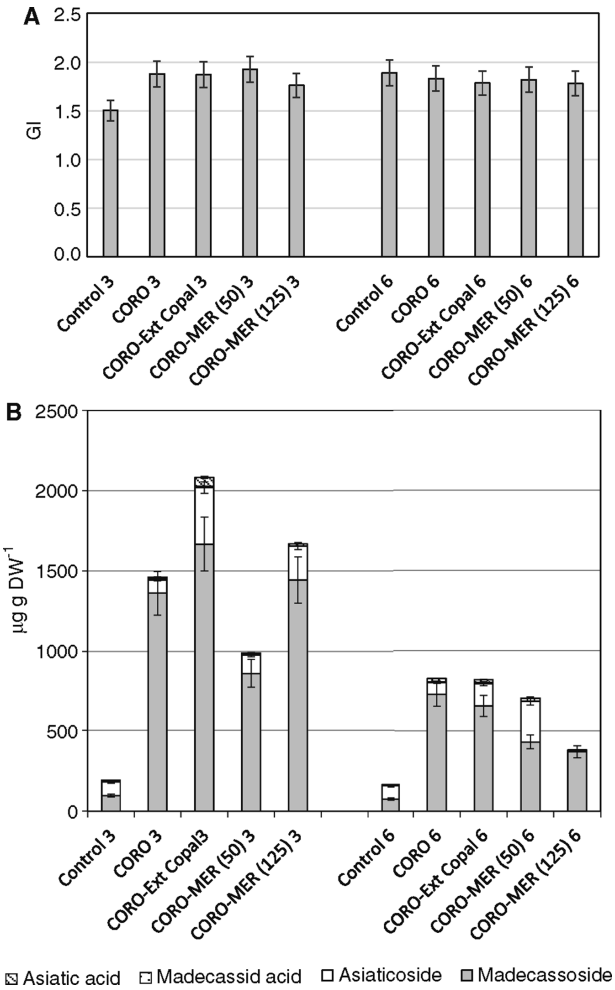

Figure 3. (A) GI (CFW sample/CFW inoculum) of the cell cultures after 18 days of culture. (B) Centelloside production expressed as microgram per gram DW measured 3 or 6 days after the treatment. CE: copal extract; MER: Manila elemi resin at the concentration of 50 or 125 mg/L. All the values are the average of three replicates ± SD.

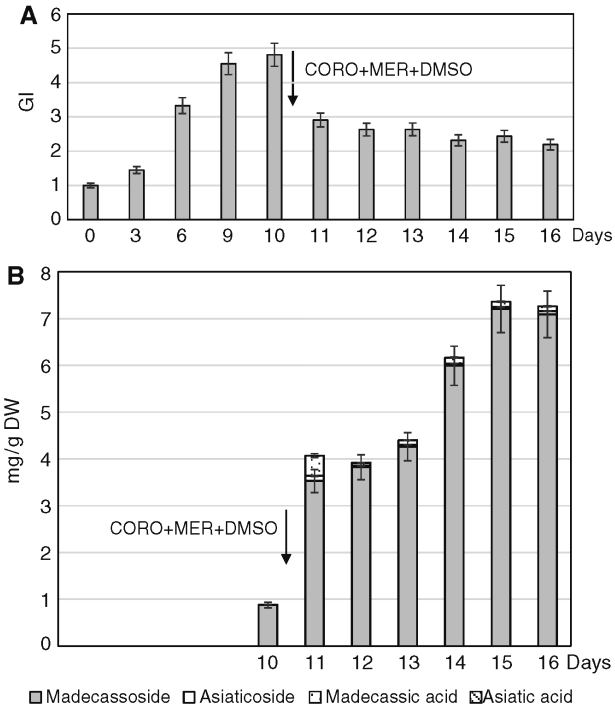

Figure 4. (A) Changes in the GI (CFW sample/CFW inoculum) during the culture period studied. (B) Time course of the centelloside production from the moment of elicitation to the end of the culture period. All the values are the average of three replicates ±SD. Black arrows indicate the time point of the combined elicitor/precursor treatment.

venous insufficiency, and its potent antioxidant, antimicrobial and antifungal, anti-inflammatory, and antirheumatoid activities, among others, are supported by scientific evidence [20–23]. These properties, together with recent applications of *C. asiatica* stem cells as cosmetic ingredients [3], have generated growing interest in *C. asiatica* cell cultures as a new source of centellosides, the triterpenoid saponins responsible for the biological activity of the plant and cell extracts. *Centella asiatica* cell line UBCA17 is characterized by a high capacity for biomass and centelloside production under elicitation [15].

The positive effects of treatments with elicitors such as MeJA, SA, copper ions, and YE on centelloside production have been previously demonstrated in both *C. asiatica* plant cell and hairy root cultures [7]. In this work, we corroborated the positive effect of MeJA, SA, and YE on centelloside biosynthesis, and also for the first time demonstrated the effectiveness of the new elicitor CORO in increasing centelloside production (more than fivefold), even at concentrations 100-fold lower than MeJA, without affecting the growth capacity of the cell cultures (Fig. 1). Additionally, unlike MeJA, which principally enhanced yields of madecassic acid, CORO-treated cultures produced the same centelloside pattern as the control (untreated cells), with madecassoside and asiaticoside predominating (Fig. 1). CORO has been previously used as an elicitor to increase the biotechnological production of other bioactive compounds, such as taxanes in *Taxus* spp. plant cell cultures, achieving a higher activation of taxane biosynthetic gene expression than MeJA and consequently an increased production of taxanes [14].

Like microorganisms, plant cell cultures are able to carry out regio- and stereoselective hydroxylations, hydrogenation, glycosidation, etc. of exogenous substrates, biotransforming them into other compounds with improved pharmacological actions [24]. Thus, biosynthetic precursors have been frequently used to improve the biotechnological production of bioactive compounds in plant cell cultures [25], although this strategy is only suitable for industrial purposes when the precursors accumulate in natural sources in high amounts or can be chemically synthesized. The capacity of *C. asiatica* cell cultures to bioconvert the potential precursor α-amyrin into centellosides with an efficacy of 84% has been reported [13], but the high market price of this compound impedes the scale up of the process

to an industrial level. However, resins such as Mexican copal or Manila elemi are known to be rich natural and sustainable sources of amyrins [13], so they were selected to feed the *C. asiatica* cell cultures in the present work. Copal resin was found to be highly toxic, unlike Ext Copal, which suggests that cleaning copal resins with petroleum ether removes toxic compounds. Both Ext Copal and MER significantly enhanced the centelloside accumulation in the cell cultures, demonstrating that the system effectively transformed the exogenous amyrins into centellosides.

Empirical approaches to improving secondary metabolite production in plant cell cultures can involve the combination of several strategies. In this study, we tested the effect of resin addition to CORO-elicited *C. asiatica* cells after corroborating the effectiveness of this elicitor. This type of combined treatment has previously achieved good results in, for example, improving taxane production in *Taxus* spp. cell cultures [26]. We found that the two treatments had a synergistic effect, as centelloside production in cells treated with both Ext Copal and CORO increased more than 16-fold compared with the 2.8-fold improvement achieved with the Ext Copal alone.

Rather than in reusable bioreactors, plant cell cultures are increasingly being grown in disposable bioreactors with volumes of up to 200 L for the production of high added value compounds such as proteins and secondary metabolites [27]. These bioreactors have several advantages, as they require less sterilization work, are time saving and cost saving, and produce less waste and environmental contamination [28, 29], but until now they have not been tested for the production of centellosides. In this work, we used a 2 L CellBag and tested one of the treatments producing the best results in the shake-flasks, i.e. elicitation with CORO (1 μM) and feeding with MER (125 mg/L). MER was chosen as the precursor instead of Ext Copal, as it does not require any preparation before adding to the plant cell culture, which is an advantage for industrial application. Under these conditions, the *C. asiatica* cell line grew very well, achieving a GI of 4.8 after 10 days of culture with a final centelloside production of 7.3 mg g DW^{-1}, which was 4.2-fold higher than in shake flasks under the same conditions, thus demonstrating the suitability of this system for scaling up centelloside production.

In summary, our results demonstrate that *C. asiatica* plant cell cultures are a potential sustainable technology for the production of centellosides. In this context, we demonstrated that a combined treatment in which cell cultures are supplemented with the new elicitor CORO and fed with natural amyrin-rich products, such as Ext Copal or MER, significantly enhanced the centelloside production of the system. Moreover, scaling up to a 2 L CellBag was easy and improved the growth and centelloside production capacity of the *C. asiatica* cell suspension. Although this system is limited to 10 L bags, the results obtained open the possibility of scaling up the process further to obtain these valuable compounds in commercial biosustainable plant cell biofactories. New orbital shaken bioreactors with disposable bags with a working volume of up to 50–200 L are available on the market to perform this type of approach.

Practical application

Centella asiatica extracts are widely used in pharmaceutical and cosmetic industries due to the properties of their bioactive components known as centellosides. Currently, with the utilization of plant stem cells as cosmetic ingredients, there is a need to develop biotechnological systems based on *C. asiatica* plant cell cultures producing high amounts of centellosides. This study shows that a treatment combining elicitors with culture feeding with natural sources of amyrins (centelloside precursors) such as copal and Manila elemi resins can improve centelloside production in *C. asiatica* cell suspensions. The successful scale up of the system to a disposable bench-top bioreactor shows it can be applied for the development of *Centella* plant cell cultures with increased yields of centellosides.

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The authors have declared no conflicts of interest

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