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short communication

Accumulation of madecassoside – a major component of centelloside – in centella (*Centella asiatica* (L.) Urban) cells elicited by salicylic acid

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Abstract

Background and Purpose: *Use of elicitors is one of the most important methods to increase the production of secondary metabolites in plants. Salicylic acid is a common chemical elicitor and also a signalling molecule in the signal transduction systems, stimulating biosynthesis of enzymes. However, its influence on the accumulation of madecassoside, a valuable bioactive compound, in centella cell cultures remains uncharacterized.*

Materials and Methods: *Callus of centella (*Centella asiatica *(L.) Urban) was used to establish cell suspension culture on the MS (Murashige and Skoog 1962) medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid and 3 mg/L thidiazuron. The cell biomass was harvested after 24 days of culture. Concentrations of salicylic acid from 50 to 200 µM were added to the medium at the beginning of cell culture. The timing of 100 µM salicylic acid addition on days 5, 10 or 15 after inoculation was also studied. Callus growth and biosynthesis of madecassoside in centella cells were characterized.*

Results and Conclusions: *The cell fresh (dry) biomass increased from the 3rd to 24th day to a maximum of 8.1±0.4 g/flask (0.95±0.05 g/flask). The highest madecassoside concentration of 27±0.7 mg/g dry weight was also obtained on the 24th day of culture. The cell growth was inhibited with salicylic acid additions at the beginning of culture (day 0), reaching the minimum at 200 µM salicylic acid. However, biosynthesis of madecassoside was significantly enhanced at all salicylic acid concentrations, with the maximum (82±3.5 mg/g dry weight) reached at 100 µM salicylic acid. The growth of cells treated with 100 µM salicylic acid was the lowest when salicylic acid was added at the beginning of the culture and the highest when the addition was postponed until day 15 (harvested on day 24). The madecassoside concentration reached as high as 114±3.1 mg/g dry weight in the treatment with 100 µM salicylic acid added after 10 days of culture (a 4-fold increase compared with the control without salicylic acid). In general, salicylic acid is effective in enhancing biosynthesis of madecassoside in centella cells.*

INTRODUCTION

Centella (*Centella asiatica* (L.) Urban) is a small and aromatic-smelling species of the Apiaceae family native to tropical and subtropical regions such as southeast Asia, India, Sri Lanka as well as South Africa and Madagascar *(1)*. Centella contains many valuable secondary compounds, especially centellosides (madecassoside, asiaticoside, madecassic acid and asiatic acid) belonging to triterpenoids *(2)*. The centellosides (also known as triterpene saponins) have a wide range of pharmaco-

logical applications, such as the antiplatelet, hypocholesterolemic, antitumoral, anti-HIV, immunoadjuvant, anti-inflammatory, antibacterial, insecticide, fungicide and anti-leishmanial agents *(3)*.

Application of elicitors is one of the most important methods to increase the production of secondary metabolites in plants *(4)*. There were some reports on *in vitro* cultures of centella treated by methyl jasmonate (MJ) to improve the biosynthesis of triterpene saponins: for example, stimulation of asiaticoside production in plantlets *(5)* and hairy-root cultures *(6)* by MJ, effect of MJ on triterpene metabolisms of planlets *(7)*, centelloside production in cells elicited by MJ *(8)*, and metabolomic analysis of MJ-induced triterpenoid production in cells *(9)*. However, there are no reports on the effects of salicylic acid (SA), a common chemical elicitor in plant *(10)*, on the accumulation of madecassoside in centella cell cultures. Meanwhile, many studies used SA to improve the biosynthesis of important metabolic compounds in cell or hairy-root cultures of different plant species, e.g. jaceosidin and siringin from *Saussurea medusa (11)*, flavonolignans and lipoxygenase from *Silybum marianum (12)*, phenolic metabolism in *Matricaria chamomilla (13)*, metabolic changes of *Catharanthus roseus* cell cultures *(14)*, podophyllotoxin from *Linum album (15)*, and taxol from *Taxus baccata (16)*. In a previous report, we showed a stimulatory effect of SA on the biosynthesis of asiaticoside in centella cells *(17)*. This study was aimed at investigating the role of SA in accumulation of another important component of centelloside, that is madecassoside.

MATERIALS AND METHODS

Plant materials

Callus of centella (*Centella asiatica* (L.) Urban) was used to establish cell suspension culture as described previously *(17)* with some modifications on component and concentration of plant growth regulators including 2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) and 3 mg/L TDZ (thidiazuron). Centella suspension cells were cultured in 250 mL Erlenmeyer flasks containing 50 mL of nutrient medium with an inoculum size 3 g, shaking speed 120 rpm, light intensity 360 lux and temperature 25±2°C for 18 days (Fig 1) to produce sufficient starting biomass.

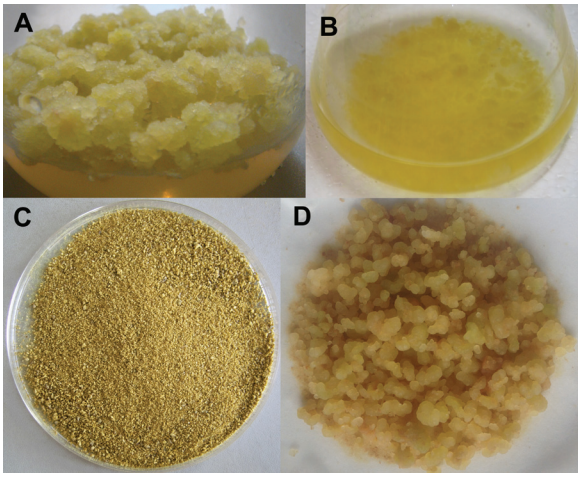

Figure 1. *A: Calli of centella. B: Centella suspension cells. C and D: Dry and fresh biomass of centella cells after 24 days of cuture.*

Salicylic acid elicitation

Elicitation effect of SA was studied by adding different concentrations (50 – 200 µM) to the medium at the beginning of 18-day-old cell culture (day 0) (experiments 1 and 2, Figs 2-3), and days 5, 10 or 15 after inoculation (experiment 3, Fig 4) with culture conditions similar to those described above for biomass production. The elicited cells were harvested after 30 days (Fig 2) or 24 days

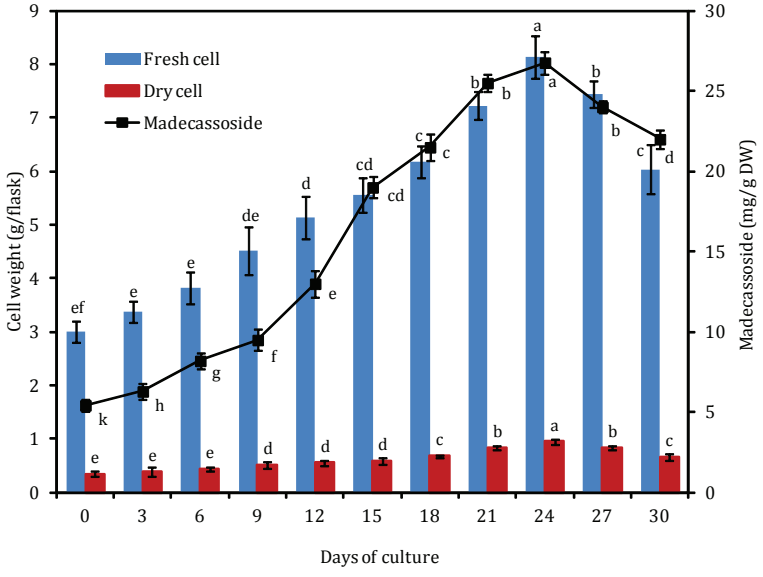

Figure 2. *Growth and madecassoside production by centella cells as influenced by duration of culture. For each mean, ± standard error bars are shown. Different letters indicate significantly different means (Duncan's test,* $p \leq 0.05$*).*

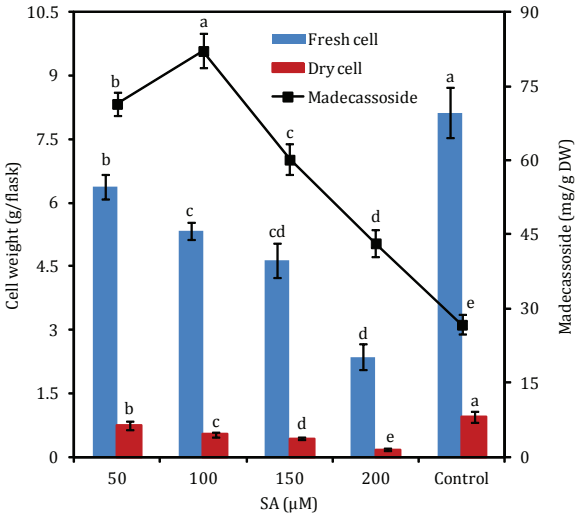

Figure 3. *Effect of SA concentration (50-200 µM) on growth and madecassoside production by centella cells after 24 days of culture. SA was added to the medium at the beginning of culture. Control: non-elicited cells. For each mean, ± standard error bars are shown. Different letters indicate significantly different means (Duncan's test,* p≤*0.05).*

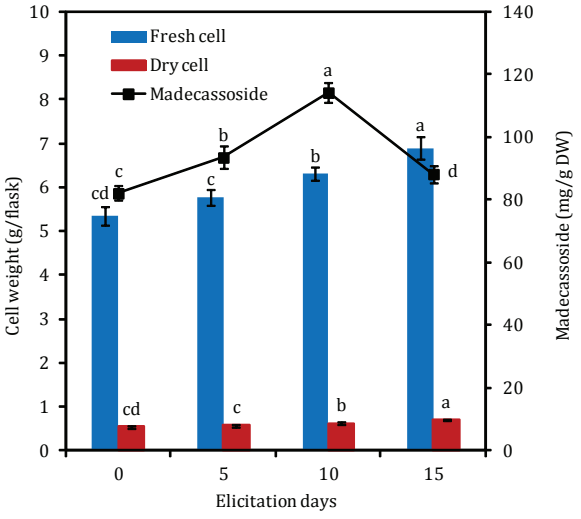

Figure 4. *Effect of timing of 100 µM SA addition on growth and madecassoside production by centella cells after 24 days of culture. 0: SA was added to the medium at the beginning of culture. 5, 10 or 15: SA was added to the medium after 5, 10 or 15 days of culture. For each mean, ± standard error bars are shown. Different letters indicate significantly different means (Duncan's test,* p≤*0.05).*

(Figs 3-4) by filtration; concentration of madecassoside was quantified by high performance liquid chromatography (HPLC).

Quantification of madecassoside

Fresh cell biomass was dried at 50°C to a constant weight, then ground into a fine powder. One gram of the powder was soaked in 10 mL of 90% v/v ethanol for 48 h. The extract was then filtered and concentrated at 70°C using a vacuum rotary concentrator (Heidolph, Germany). The concentrate was dissolved in 10 mL of 100% ethanol, filtered through Minisart 0.25 µm membranes (Sartorius, Germany), and diluted 5-fold. An aliquot of 20 microliters of diluted extract was subjected to HPLC equipped with a LiChrospher 18e column (5 µm, 4 mm×250 mm), flow rate of 1 mL/min, run time of 10 min, detector wavelength of 254 nm, stationary phase of silica gel (reverse phase) and mobile phase of ethanol:water (6:4 ratio). A standard curve of madecassoside (purchased from Sigma, USA) was used for determination of madecassoside concentration in the extract. HPLC analysis was performed on a LC-20A Prominence system (Shimadzu, Japan). All solvents and standard chemicals were of analytical grade and were purchased from Sigma (USA) and Chromadex (USA).

Statistical analysis

All experiments were repeated at least three times, and the representative data were shown. The means of samples were compared using one-way ANOVA followed by Duncan's test at $p \leq 0.05$.

RESULTS

Effect of SA concentration

The cell fresh (dry) biomass increased from the 3rd to 24th day to a maximum of 8.1±0.4 g/flask (0.95±0.05 g/flask) (Fig 2), the values 1.35- (1.67-) fold higher compared with those achieved on the MS medium supplemented with 1 mg/L BAP (benzylaminopurine) and 1 mg/L NAA (naphthaleneacetic acid) in our previous study *(17)*. The dynamics of madecassoside accumulation was similar to that of the cell growth, with the highest concentration of 27±0.7 mg/g dry weight (DW) obtained on the 24th day of culture.

Different concentrations of SA were added to the medium at the beginning of culture. The callus growth was significantly inhibited with SA addition, reaching the minimum at 200 µM SA (Fig 3). Biosynthesis of madecassoside was enhanced significantly at all SA concentrations, with the maximum reached at 100 µM SA (almost 3-fold higher than in the non-elicited cells, Fig 3). This SA concentration was used in further experiments to study the effect of timing of SA addition.

Effect of elicitation day

An addition of 100 µM SA at days 0, 5 or 10 days resulted in decreased cell growth compared with the addition on day 15 (Fig 4).

The optimum SA addition for elicitation of madecassoside production was the 10th day of culture. The dynamics of madecassoside accumulation in the elicited and non-elicited cells was similar (data not shown).

DISCUSSION

According to Dučaiová et al *(18)*, SA is considered a potent phytohormone because of its key regulatory roles in plant metabolism. It is also a signalling molecule in the signal transduction systems, stimulating the biosynthesis of special enzymes for reactions forming defense compounds such as terpenoids (including triterpene saponins), phenols, alkaloids or pathogenesis-related proteins *(19, 20)*.

The elicitor role of exogenous SA was studied in plant cell cultures regarding accumulation of secondary metabolites. According to Li et al *(21)*, the SA treatment also slightly inhibited the growth of *Salvia miltiorrhiza* cell cultures. Bulgakov et al *(22)* found earlier that increasing the SA concentrations in the media strongly suppressed the growth of *Rubia cordifolia* callus cultures.

The results of the present study are consistent with previous studies on the stimulatory effect of SA on asiaticoside biosynthesis *(17)* and the expression of genes (*CaSQS*, *CabAS* and *CaCYS*) associated with the isoprenoid pathway in centella cells *(23)*. *CabAS* gene coding for β-amyrin synthase, a key enzyme in biosynthesis of triterpene saponin (madecassoside is a member of this secondary compound group), was more strongly expressed in the cells elicited with 100 µM SA on day 10 after inoculation than in the non-elicited control *(23)*. Study of Wen et al *(19)* in grape berry (*Vitis vinifera* L. cv. Cabernet Sauvignon) also showed that SA could induce the accumulation of *PAL* mRNA (phenylalanine ammonia-lyase (PAL) is a key enzyme in the phenylpropanoid pathway).

CONCLUSION

We found that concentration of 100 µM SA significantly decreased growth of centella cells when added on day 10 after inoculation. However, the madecassoside concentration was 4-fold higher than in the non-elicited control.

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