Effect of elicitation and precursor feeding on accumulation of 20-hydroxyecdysone in *Achyranthes aspera* Linn. cell suspension cultures

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Abstract 20-Hydroxyecdysone is one of the most common ecdysteroids in plants with potential therapeutic applications. In this study, cell suspension cultures of *Achyranthes aspera* were raised in shake flasks to investigate the production of 20-hydroxyecdysone. The quantification and characterization of 20-hydroxyecdysone in the cultures were done by High performance liquid chromatography (HPLC) and Liquid Chromatography-quadrupole time-of-flight mass spectrometry (LC-Q-TOF) analyses. For raising the suspension, calli initiated from in vitro grown leaf explants were cultured in liquid Murashige and Skoog (MS) medium augmented with combinations of 2, 4-dichlorophenoxyacetic acid (1 mg L^{-1}) and α -naphthaleneacetic acid (1 mg L^{-1}). Maximum growth index of the cell suspension was 9.9, which was achieved during 20th day of culture (final phase of exponential growth). At this stage, the biomass accumulated was $1.09 \pm 0.09 \text{ g dry weight (DW)}$ and the 20-hydroxyecdysone concentration was $0.24 \text{ mg g}^{-1} \text{ DW}$. Eliciting the cultures with 0.6 mM Methyl jasmonate for 6 days; enhanced the production of 20-hydroxyecdysone production to $0.35 \text{ mg g}^{-1} \text{ DW}$. By augmenting the cultures with the precursors namely cholesterol (10 mg L^{-1}) and 7-dehydrocholesterol (10 mg L^{-1}), production of 20-hydroxyecdysone was boosted to $0.31 \text{ mg g}^{-1} \text{ DW}$ and $0.28 \text{ mg g}^{-1} \text{ DW}$ respectively.

Keywords *Achyranthes aspera* · 20-Hydroxyecdysone · Methyl jasmonate · Cholesterol · 7-Dehydrocholesterol

Introduction

Phytoecdysteroids belong to a large group of biologically active plant steroids with structures resembling the insect-moulting hormones; called ecdysteroids (Lafont 1998). Ecdybase (<http://www.ecdybase.org>) delivers an up-to-date listing of the chemical and biological data regarding ecdysteroid analogues, their occurrence in plants, effects on various organisms and their applications (Lafont et al. 2002; Dinan et al. 2009). Literature suggest that they stimulate protein synthesis in animals and humans, and have adaptogenic, antimutagenic, hypocholesterolemic, immunostimulating, nutritive and tonic properties (Lafont and Dinan 2003; Dinan and Lafont 2006; Dinan 2009). The most common phytoecdysteroid is 20-hydroxyecdysone and it has potential therapeutic applications (Dinan 2001) and several agricultural applications. It enhances synchronous development of the larvae of *Bombyx mori*, and significantly increases the quantity of silk from the cocoon (Trivedy et al. 2003).

For several decades, plant cell cultures especially cell suspension cultures were used for the synthesis of various secondary metabolites of commercial interest (Vazquez et al. 2010). Recently, cell suspension cultures were established in numerous medicinal plant species, for producing secondary metabolites (Kolewe et al. 2008; Boonsongcheep et al. 2010; Bonfill et al. 2011; Silja et al. 2014; Deepthi and Satheeshkumar 2015). Besides, they can be scaled up and used for studying important biosynthetic pathways of secondary metabolites (Lim and Bowles 2012). Some of the important strategies for improving the

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throughput of these compounds include media optimization, screening and selection of high-producing cell lines, elicitation, addition of precursors, in situ product removal and immobilization (Verpoorte et al. 1999; Zhang and Furusaki 1999; Dias et al. 2009; Rhee et al. 2010).

In vitro plant cell cultures are deemed to be feasible routes for the production of phytoecdysteroids. They provide an excellent experimental system for elucidating ecdysteroid biosynthetic pathways and discovering their regulation mechanisms. Cell cultures also serve as possible means for the synthesis of specific ecdysteroids via bio-engineering techniques (Dinan et al. 2009). *Ajuga turkestanica* callus and suspension cultures synthesize 20-hydroxyecdysone two to six-folds higher than the level found in aerial parts and roots of the plant (Lev et al. 1990). Also, cell cultures of *Serratula tinctoria* (Corio-Costet et al. 1996) and *Vitex glabrata* (Thanonkeo et al. 2011) function as a source of ecdysteroids.

Achyranthes aspera Linn. (Fa. Amaranthaceae); is a medicinal herb found throughout the tropics. The ethanolic root extract of the plant is reported to have spermicidal activity in human sperm (Paul et al. 2006) and post-coital antifertility activity in female albino rats (Vasudeva and Sharma 2006). Reports also indicate that the ethyl acetate leaf extract of the plant showed mosquito larvicidal activity against early fourth instar larvae of *Aedes aegypti* and *Culex quinquefasciatus* (Bagavan et al. 2008). The content of 20-hydroxyecdysone in various parts of the plant is 0.25 mg/g (seeds), 0.09 mg/g (roots) and 0.04 mg/g (stem, leaves) (Banerjee and Chadha 1970; Banerji et al. 1971). Current knowledge about the biosynthesis and availability of 20-hydroxyecdysone in in vitro cell cultures of *A. aspera* is limited. The focus of this study is to establish cell suspension cultures of *A. aspera* and investigate the presence of 20-hydroxyecdysone. This study also analyses the influence of methyl jasmonate (elicitor), cholesterol and 7-dehydrocholesterol (precursors) on scaling up of 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*.

Materials and methods

Plant material

The plants of *A. aspera* L. were collected in October 2015 from Mahatma Gandhi University, Kottayam, Kerala, India and a voucher specimen was deposited at Regional Herbarium Kerala, India, with field no: 6302 and accession no: 7565. Seeds of the plant were surface sterilized in 0.1% mercuric chloride solution for 6 min and rinsed four to five times in sterile distilled water. The seeds were then inoculated on MS solid medium (Murashige and Skoog 1962).

The cultures were maintained in the culture room $25 \pm 2^\circ\text{C}$ with 70% relative humidity (RH) and under 16/8 h photoperiod at a photosynthetic photon flux density (PPFD) of $45\text{--}50\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ provided by cool white fluorescent lights (40 W, Philips, India).

Callus induction and establishment of cell suspension cultures

Young leaf explants excised from in vitro grown seedlings were placed on MS solid medium supplemented with varying concentrations and combinations of 2, 4-D ($0.5\text{--}4.0\ \text{mg L}^{-1}$) with NAA ($0.5\text{--}4.0\ \text{mg L}^{-1}$) and BAP ($0.5\text{--}4.0\ \text{mg L}^{-1}$; John et al. 2017). The calli induced were subcultured after every 30 days using fresh media.

For initiation of cell suspension cultures, friable calli obtained from in vitro grown leaf explants were used. The calli (0.5 g) were inoculated in 250 mL flasks containing 100 mL of MS liquid medium supplemented with of 2, 4-D ($1\ \text{mg L}^{-1}$) and NAA ($1\ \text{mg L}^{-1}$) and maintained on a rotary shaker at 100 rpm in complete darkness.

Growth kinetics of cell suspension culture

The cells were separated from suspension culture by filtration. The biomass was estimated by measuring the dry weight (DW). The dry weight (DW) was calculated after drying the cells at 60°C to a constant weight in a hot air oven. Cultures were harvested in triplicate and the biomass accumulation was examined periodically after every 5 days interval for a total period of 30 days. Growth curves of the cell suspension culture were plotted by taking both the dry weight of cells and growth index against the number of days of culture. The growth index of the cultures was calculated as described by Hernandez and Vazquez-Flota (2006).

$$\text{Growth index} = \frac{\text{Final dry weight} - \text{Initial dry weight}}{\text{Initial dry weight}}$$

Preparation and addition of elicitor and precursors

Stock solution (1 M) of elicitor methyl jasmonate (Sigma Aldrich, Saint Louis, USA) was prepared in dimethylsulfoxide (DMSO, Merck, India) and filter-sterilized through a sterile microfilter of $0.22\ \mu\text{m}$ (Himedia, India). Methyl jasmonate was then aseptically added to the 15-day old liquid culture medium to obtain a final concentration of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM.

Also, two different precursors namely cholesterol (Sigma Aldrich, Saint Louis, USA) and 7-dehydrocholesterol (Sigma Aldrich, Saint Louis, USA) were added aseptically at different concentrations (5, 10, 50, 100 mg

L^{-1}) to the 15-day old liquid culture medium. All the treated cultures were maintained on a rotary shaker at 100 rpm in complete darkness. The cells were harvested after, 3, 6, 9 and 12 days after addition of the elicitor and precursors. An appropriate control culture without elicitors and precursors was also maintained.

Extraction and quantification of 20-hydroxyecdysone

For extracting 20-hydroxyecdysone, 0.3 g of dried powdered cells was extracted thrice with 20 mL methanol. The extracts were pooled and evaporated to dryness and dissolved in 1 mL methanol. This was then filtered through a nylon syringe filter of pore size 0.22 μm to remove any cellular debris and 20 μL of the solution was injected for analysis by High Performance Liquid Chromatography (HPLC). HPLC analysis of the extract was performed on Shimadzu Prominence LC 20AP (Japan) equipped with pump LC 20AP, UV–Vis detector SPD 20A/20AV and LC solution software. Enable RP C18 G250 column (250 mm $\times$ 4.6 mm $\times$ 5 μm , 120 $\text{\AA}$) was used throughout the study. HPLC was conducted using the procedure reported by Zimmer et al. (2006). 20-Hydroxyecdysone was identified with authentic standard 20-hydroxyecdysone (Sigma Aldrich, Saint Louis, USA). Quantitative estimation of 20-hydroxyecdysone in the cell suspension cultures was done by using a calibration graph of standard 20-hydroxyecdysone (10–200 $\mu g/ml$) (Sigma-Aldrich, Saint Louis, USA). The calibration plot was obtained by plotting the HPLC peak area of the 20-hydroxyecdysone against the concentration of the standard solutions (Fig. S1).

LC-Q-TOF analysis

For identification and characterization of 20-hydroxyecdysone, the cell suspension culture extract was subjected to Liquid Chromatography-quadrupole time-of-flight mass spectrometry (LC-Q-TOF). LC-Q-TOF analysis was performed using ACQUITY, BEH C18 column (50 mm $\times$ 2.1 mm $\times$ 1.7 μm) (Waters, Milford, MA, USA), ACQUITY Ultra Performance Liquid Chromatography system and Xevo G2 Quadrupole—Time-of-Flight mass spectrometer (Waters, Milford, MA, USA) operating in ESI- negative ionization modes. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The gradient system was as follows: 0–0.1 min, 10% B 90% A, 0.1–5.0 min 95% B 5% A, 5.5 min 95% B 5% A, 6.5 min 10% B 90% A and 1 min for stabilization. The sample injection volume was 10 μL . The mobile phase was delivered with a flow rate of 0.2 mL/min and the detection wave length was set as 242 nm. Mass spectra were recorded in negative ion mode and scan mode was set from 50 to

1000 m/z . The nebulization gas was set to 900 L/h at a temperature of 300 $^{\circ}C$, the cone gas was set to 50 L/h, and the source temperature was set to 135 $^{\circ}C$. Capillary voltage and cone voltage settings were 2.5 kV and 35 V, respectively.

Statistical analysis

All experiments were repeated thrice. The data shown represent the mean $\pm$ SE for three independent experiments. The mean comparison was done by One-way ANOVA analysis followed by Duncan's multiple range Test. The mean values which are not significantly different are depicted with the same letters. All statistical analyses were performed using SPSS Ver.18 (SPSS Inc. Chicago, IL, USA) statistical software package.

Results

In vitro seed germination and calli induction

On inoculating in MS solid medium, the seeds started germinating after 5 days (Fig. 1a). Calli were induced from leaf explants of in vitro grown seedlings. The leaf explants cultured on MS solid media, supplemented with combinations of 2, 4-D (1 $mg L^{-1}$) and NAA (1 $mg L^{-1}$), produced friable yellow calli (Fig. 1b). Green compact callus was produced in the medium augmented with 2 $mg L^{-1}$ of 2, 4-D and 0.5 $mg L^{-1}$ of BAP.

Initiation of cell suspension cultures of *A. aspera*

Cell suspension cultures of *A. aspera* were initiated, by transferring 20 day old yellow friable callus to liquid MS medium supplemented with combinations of 2, 4-D (1 $mg L^{-1}$) and NAA (1 $mg L^{-1}$). The callus was easily broken apart and dispersed into single cells and cell clumps. On agitation cell clumps rapidly disintegrated into single cells and small cell aggregates. The suspension cultures were regularly transferred to fresh MS media after every 3 weeks of culture. After 2 months of subculturing, actively dividing homogenous cell suspension culture was obtained.

Growth kinetics of cell suspension culture

The growth kinetics of cell suspension culture based on dry weight and growth index was estimated. The cell suspension cultures showed a growth curve with an initial lag phase of 2–3 days. This was followed by an exponential phase of 5–20 days with a gradual increase in the cell biomass which reached a maximum value in day 20.

Fig. 1 Germination of seedlings and callus induction. **a** Germination of seedlings of *A. aspera*. **b** Callus formation from leaf explants on MS solid medium supplemented with 2, 4-D (1 mg L^{-1}) and NAA (1 mg L^{-1})

Maximum growth index of 9.9 was attained during 20th day of the culture, when the cells were in the final phase of the exponential growth (Fig. 2). Exponential phase terminated in a decline phase characterized by no increase in the cell biomass and growth index. The suspension culture was subcultured on fresh media at the end of the exponential phase.

Identification and quantification of 20-hydroxyecdysone

The identification of 20-hydroxyecdysone in *A. aspera* cell suspension culture was performed by HPLC with 20-hydroxyecdysone as standard. HPLC analysis of cell extract gave a peak with same retention time as that of authentic 20-hydroxyecdysone (Fig. S2).

On LC-Q-TOF analysis, mass spectrum of the cell extract was identical to that of the standard. The results revealed peaks at m/z 479 (M-H^-) and exhibited an adduct ion at m/z 525 ($\text{M} + \text{HCOO}^-$) (Fig. 3) in the negative ion

mode. This happened as a result of adding 0.1% formic acid in the mobile phase. In addition, fragment ions at m/z 319, 301, 283, 159 and 83 (Fig. 4) were also observed in the ESI-MS/MS spectrum. The HPLC and LC-Q-TOF analyses indicated the presence of 20-hydroxyecdysone in the cells suspension culture.

Production of 20-hydroxyecdysone in cell suspension culture of *A. aspera* is depicted in Fig. 5. An increase in 20-hydroxyecdysone accumulation was observed during the end of exponential growth phase of cell suspension culture. The initial inoculum of yellow friable calli multiplied and after 20 days of cultivation, the biomass accumulated was about $1.09 \pm 0.09 \text{ g DW}$ (gram/flask of 100 mL medium; Fig. 2). At this point, the growth index of the suspension was also the highest. After that a gradual decline in the cell dry weight and growth index was observed due to the depletion of nutrients the culture medium. Also, the concentration of 20-hydroxyecdysone increased progressively up to 20 days. The maximum concentration of 20-hydroxyecdysone i.e., 0.24 mg g^{-1} DW was reached on day 20, and thereafter, it declined rapidly (Fig. 5). The patterns of 20-hydroxyecdysone accumulation in cell suspension cultures of *A. aspera* were more or less similar to those of the biomass escalation and growth index.

Production enhancement of 20-hydroxyecdysone by elicitor (methyl jasmonate) and precursors (cholesterol and 7-dehydrocholesterol)

For enhancing the biosynthesis of 20-hydroxyecdysone in *A. aspera* cell suspensions, the cultures were elicited with different concentrations of methyl jasmonate (0.2–1.2 mM) for 3, 6 and 9 days and the content was estimated by HPLC. Methyl jasmonate enhanced the accumulation of 20-hydroxyecdysone in cell suspension cultures and 6 days after elicitation, maximum production was observed. Eliciting the culture with 0.6 mM of methyl jasmonate triggered the maximum quantitative enhancement of 20-hydroxyecdysone production (0.35 mg g^{-1} DW). This concentration was achieved 6 days after elicitation (Fig. 6) and was 1.5-fold higher than the concentration found in unelicited cells. Nevertheless, increasing the concentration of methyl jasmonate to 1 and 1.2 mM, prompted a noticeable reduction in the accumulation of 20-hydroxyecdysone to 0.2 and 0.16 mg g^{-1} DW respectively. Incidentally, these values were lower than the amounts of 20-hydroxyecdysone accumulated in unelicited control cultures.

The precursors (cholesterol and 7-dehydrocholesterol) at low (5 and 10 mg/L) and high concentrations (50 and 100 mg/L) were tested for their impact on 20-hydroxyecdysone production in *A. aspera* cell suspension cultures.

Fig. 2 Growth curve of cell suspension cultures of *A. aspera* based on dry weight and growth index. Data represents mean $\pm$ SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan's multiple range test ($p \leq 0.05$)

Fig. 3 LC-Q-TOF analysis of 20-hydroxyecdysone from cell suspension cultures of *A. aspera* (m/z 479 [$M-H$] $^{-}$ and m/z 525 [$M + HCOO$] $^{-}$)

The addition of cholesterol and 7-dehydrocholesterol improved the production of 20-hydroxyecdysone. 20-hydroxyecdysone accumulation was the highest, 6 days after adding 10 mg L^{-1} of cholesterol (0.31 mg g^{-1} DW) (Fig. 7). On supplementing the medium with 10 mg L^{-1} of 7-dehydrocholesterol the amount of 20-hydroxyecdysone was enhanced to a maximum of 0.28 mg g^{-1} DW after 6 days (Fig. 8).

Discussion

In the present investigation, plant cell suspension cultures from *A. aspera* were initiated, maintained and analysed for the production of the phytoecdysteroid; 20-hydroxyecdysone. The ease of establishment of suspension culture from callus tissue was influenced by the friable nature of

the callus tissue. A fine suspension of cells in culture was obtained when yellow friable calli from leaf tissue were cultured and subcultured in MS medium supplemented with 2, 4-D (1 mg L^{-1}) and NAA (1 mg L^{-1}). According to previous reports, there was an increase in the degree of friability of the callus tissue when it was sustained on a semisolid medium for two to three passages (Bhojwani and Razdan 1996). In this study, cell suspension cultures of *A. aspera* were initiated in full strength MS liquid medium containing 2, 4-D (1 mg L^{-1}) and NAA (1 mg L^{-1}). Throughout the incubation period, a gradual increase in the biomass and growth index of suspension cultures was noted. Previously, it was reported that full-strength MS liquid medium had been appropriate for biomass accumulation and gymnemic acid production in *Gymnema sylvestre* cell suspension cultures (Nagella et al. 2011).

Fig. 4 LC-Q-TOF MS/MS (Negative mode) of the mass m/z 479($M-H$)[−], and its fragment

Fig. 5 Time course of 20-hydroxyecdysone production in cell suspension culture of *A. aspera*. Data represent mean $\pm$ SE of three replicates and the means with the same letter are not significantly different according to the Duncan's multiple range test ($p \leq 0.05$)

The secondary metabolite profile in suspension culture is usually presumed to be similar to that of the intact plant. However, these profiles may sometimes differ, with certain metabolites being absent and novel compounds which are not present in intact plants appearing in culture (Filippova et al. 2003). Our analysis of 20-hydroxyecdysone profile in cell suspension cultures of *A. aspera* ascertained the capability of the dedifferentiated cells to synthesize the phytoecdysteroid 20-hydroxyecdysone found in the intact plant. HPLC results and mass spectra obtained from LC-Q-TOF analyses of the cell suspension cultures of *A. aspera*, in comparison with the authentic standard confirmed the presence of 20-hydroxyecdysone. These results were supported by previous findings (Stevens et al. 2008; Felipe et al. 2014).

The cells cultured in MS liquid medium showed highest growth index and 20-hydroxyecdysone accumulation ($0.24 \text{ mg g}^{-1} \text{ DW}$) at the end of the exponential growth which was on the 20th day of culture. Depending on the plant species, the time of maximum accumulation of 20-hydroxyecdysone differs. There are reports of *Vitex glabrata* cultures showing accumulation of $0.33 \text{ mg g}^{-1} \text{ DW}$ of 20-hydroxyecdysone in cell suspension cultures (Thanonkeo et al. 2011). Also, Cheng et al. (2008) reported increased accumulation ($6.9 \text{ mg g}^{-1} \text{ DW}$) of 20-hydroxyecdysone in 10–15-month-old *Ajuga turkestanica* cell suspension cultures.

Methyl jasmonate mediates plant responses to environmental stresses like wounding and insect/pathogen attack (Wasternack 2007). It also enhances the production of a

Fig. 6 Effect of methyl jasmonate on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*. Methyl jasmonate incubation period 3, 6 and 9 days are treated as different groups and data represents mean $\pm$ SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan's multiple range test ($p \leq 0.05$)

Fig. 7 Effect of cholesterol on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*. Cholesterol incubation period 3, 6, 9 and 12 days are treated as different groups and data represents mean $\pm$ SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan's multiple range test ($p \leq 0.05$)

variety of secondary metabolites (Bonfill et al. 2011; Hu et al. 2011; Qu et al. 2011). Methyl jasmonate and other jasmonic acid derivatives have been successfully used in many plant species for eliciting the enhanced synthesis of a particular secondary metabolite. The biosynthetic activity of cultured cells can be increased by these signaling molecules (Yoon et al. 2000; Suzuki et al. 2005). In the current study, by eliciting the culture with 0.6 mM methyl jasmonate, 1.5-fold increase in 20-hydroxyecdysone

content was observed in comparison to unelicited cultures. It concurs with a previous report in *Ajuga turkestanica* cell suspension cultures where the amount of 20-hydroxyecdysone was increased by adding 125 or 250 μ M methyl jasmonate (Cheng et al. 2008).

Supplementing exogenous precursor to culture media is a means to enhance the yield of the desired metabolites. Radiolabelled experiments established that mevalonic acid, cholesterol and acetyl-CoA are direct precursors of

Fig. 8 Effect of 7-dehydrocholesterol on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*. 7-dehydrocholesterol incubation period 3, 6, 9 and 12 days are treated as different groups and data represents mean $\pm$ SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan's multiple range test ($p \leq 0.05$)

phytoecdysteroid biosynthesis (Nagakari et al. 1994; Adler and Grebenok 1999). In *Ajuga lobata* D. Don suspension cultures, medium augmented with mevalonate, α -Pinene, and nitric oxide enhanced cell growth and 20-hydroxyecdysone accumulation (Qian et al. 2016). In the present study, addition of precursors namely, cholesterol (10 mg L⁻¹) and 7-dehydrocholesterol (10 mg L⁻¹) enhanced 20-hydroxyecdysone levels to 1.2–1.3 times the concentration found in control cultures. Thanonkeo et al. (2011) reported that, *Vitex glabrata* cell cultures supplemented with cholesterol (5 mg L⁻¹), yielded 1.11-fold accumulation of 20-hydroxyecdysone compared to the untreated control cultures. *A. aspera* cell suspension cultures synthesized 20-hydroxyecdysone to the levels of 0.24–0.35 mg g⁻¹ DW which was higher than the levels of 20-hydroxyecdysone reported in the field grown plants (Banerjee and Chadha 1970; Banerji et al. 1971).

Conclusion

Plant cell culture is a reliable substitute to whole plant cultivation for the synthesis of desired secondary metabolites. In the present study, suspension cultures of *A. aspera* were raised in MS liquid medium supplemented with combinations of two different auxins by inoculating the friable calli obtained from in vitro grown leaf explants. Biomass accumulation was monitored as dry weight and growth index. Suspension culture at the end of exponential growth yielded maximum 20-hydroxyecdysone content.

Addition of methyl jasmonate (elicitor), cholesterol and 7-dehydrocholesterol (precursors), enhanced the 20-hydroxyecdysone levels in cell suspension cultures in comparison to untreated controls and field grown plants. However, further search for superior biotic and abiotic elicitors with optimization of media and culture conditions are essential for enhancing the production of 20-hydroxyecdysone in cell suspension cultures of *A. aspera*.

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Compliance with ethical standards

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

References

- Adler JH, Grebenok RJ (1999) Occurrence, biosynthesis and putative role of ecdysteroids in plants. *Crit Rev Biochem Mol Biol* 34:253–264
- Bagavan A, Rahuman AA, Kamaraj C, Geetha K (2008) Larvicidal activity of saponin from *Achyranthes aspera* against *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae). *Parasitol Res* 103:223–229

- Banerjee A, Chadha MS (1970) Insect moulting hormone from *Achyranthes aspera*. *Phytochemistry* 9:1671
- Banerji A, Chintalwar GJ, Joshi NK, Chadha MS (1971) Isolation of ecdysterone from Indian plants. *Phytochemistry* 10:2225–2226
- Bhojwani SS, Razdan MK (1996) Plant tissue culture: theory and practice, a revised edition. Elsevier, Amsterdam, p 68
- Bonfill M, Mangas S, Moyano E, Cusido RM, Palazon J (2011) Production of centellosides and phytosterols in cell suspension cultures of *Centella asiatica*. *Plant Cell Tissue Organ Cult* 104:61–67
- Boonsongcheep P, Korsangruang S, Soonthornchareonnon N, Chintapakorn Y, Saralamp P, Prathanturug S (2010) Growth and isoflavonoid accumulation of *Pueraria candollei* var. *candollei* and *P. candollei* var. *mirifica* cell suspension cultures. *Plant Cell Tissue Organ Cult* 101:119–126
- Cheng DM, Yousef GG, Grace MH, Rogers RB, Gorelick-Feldman J, Raskin I, Lila MA (2008) In vitro production of metabolism-enhancing phytoecdysteroids from *Ajuga turkestanica*. *Plant Cell Tissue Organ Cult* 93:73–83
- Corio-Costet MF, Chapuis L, Delbecq JP (1996) *Serratula tinctoria* L. (Dyer's Savory): in vitro culture and the production of ecdysteroids and other secondary metabolites. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*. Vol 37, medicinal and aromatic plants IX, vol 37. Springer, Berlin, pp 384–401
- Deepthi S, Satheeshkumar K (2015) Enhanced camptothecin production induced by elicitors in the cell suspension cultures of *Ophiorrhiza mungos* Linn. *Plant Cell Tissue Organ Cult* 124:483–493
- Dias LLC, Santa-Catarina C, Ribeiro DM, Barros RS, Floh EIS, Otoni WC (2009) Ethylene and polyamine production patterns during in vitro shoot organogenesis of two passion fruit species as affected by polyamines and their inhibitor. *Plant Cell Tissue Organ Cult* 99:199–208
- Dinan L (2001) Phytoecdysteroids: biological aspects. *Phytochemistry* 57:325–339
- Dinan L (2009) The Karlson lecture. Phytoecdysteroids: what use are they? *Arch Insect Biochem Physiol* 72:126–141
- Dinan L, Lafont R (2006) Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. *J Endocrinol* 191:1–8
- Dinan L, Harmatha J, Volodin V, Lafont R (2009) Phytoecdysteroids: diversity, biosynthesis and distribution. In: Smagghe G (ed) *Ecdysone: structures and functions*. Springer, Amsterdam, pp 3–45
- Felipe DF, Brambilla LZS, Porto C, Pilau EJ, Cortez DAG (2014) Phytochemical analysis of *Pfaffia glomerata* inflorescences by LC-ESI-MS/MS. *Molecules* 19:15720–15734
- Filippova VN, Zorinyants SE, Volodina SO, Smolenskaya IN (2003) Cell cultures of ecdysteroid-containing *Ajuga reptans* and *Serratula coronata* plants. *Russ J Plant Physiol* 50:501–508
- Hernandez GG, Vazquez-Flota FA (2006) Growth measurements: estimation of cell division and cell expansion. In: Loyola-Vargas VM, Vazquez-Flota F (eds) *Plant cell culture protocols, methods in molecular biology*, vol 318. Humana Press, Totowa, pp 51–58
- Hu YH, Yu YT, Piao CH, Liu JM, Yu HS (2011) Methyl jasmonate and salicylic acid-induced D-chiro-inositol production in suspension cultures of buckwheat (*Fagopyrum esculentum*). *Plant Cell Tissue Organ Cult* 106:419–424
- John R, Shajitha PP, Devassy A, Mathew L (2017) Multiple Shoot induction and production of insect moulting hormone 20-hydroxyecdysone from the callus cultures of medicinal plant *Achyranthes aspera* L. In: Jisha MS, Rajan MM, Ashok AK (eds) *Plant science technology for sustainable development*. SKJ Publishers, Jaipur, pp 85–99
- Kolewe ME, Gaurav V, Roberts SC (2008) Pharmaceutically active natural product synthesis and supply via plant cell culture technology. *Mol Pharm* 5:243–256
- Lafont R (1998) Phytoecdysteroids in world flora: diversity, distribution, biosynthesis and evolution. *Russ J Plant Physiol* 45:276–295
- Lafont R, Dinan L (2003) Practical uses for ecdysteroids in mammals including humans: an update. *J Insect Sci* 3:7
- Lafont R, Harmatha J, Marion-Poll F, Dinan L, Wilson ID (2002) Ecdybase, a free ecdysteroid database. <http://ecdibase.org>. Accessed 12 Mar 2015
- Lev SV, Zakirova RP, Saatov Z, Gorovits MB, Abubakirov NK (1990) Ecdysteroids from tissue and cell cultures of *Ajuga turkestanica*. *Chem Nat Compd* 1:51–52
- Lim E, Bowles D (2012) Plant production systems for bioactive small molecules. *Curr Opin Biotechnol* 23:271–277
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays for tobacco tissue cultures. *Physiol Plant* 15:473–497
- Nagakari M, Kushiro T, Matsumoto T, Tanaka N, Kakinuma K, Fujimoto Y (1994) Incorporation of acetate and cholesterol into 20-hydroxyecdysone by hairy root clone of *Ajuga reptans* var. *atropurpurea*. *Phytochemistry* 36:907–910
- Nagella P, Chung IM, Murthy HN (2011) In vitro production of gymmemic acid from cell suspension cultures of *Gymnema sylvestre* R. Br. *Eng Life Sci* 11:537–540
- Paul D, Bera S, Jana D, Maiti R, Ghosh D (2006) In vitro determination of the contraceptive spermicidal activity of a composite extract of *Achyranthes aspera* and *Stephania hernandifolia* on human semen. *Contraception* 73:284–288
- Qian J, Yang Y, Li X, Chi D (2016) 20-Hydroxyecdysone accumulation and regulation in *Ajuga lobata* D. Don suspension culture. *Biosci Biotechnol Biochem* 80:591–599
- Qu JG, Zhang W, Yu XJ (2011) A combination of elicitation and precursor feeding leads to increased anthocyanin synthesis in cell suspension cultures of *Vitis vinifera*. *Plant Cell Tissue Organ Cult* 107:261–269
- Rhee HS, Cho HY, Son SY, Yoon SYH, Park JM (2010) Enhanced accumulation of decursin and decursinol angelate in root cultures and intact roots of *Angelica gigas* Nakai following elicitation. *Plant Cell Tissue Organ Cult* 101:295–302
- Silja PK, Gisha GP, Satheeshkumar K (2014) Enhanced plumbagin accumulation in embryogenic cell suspension cultures of *Plumbago rosea* L. following elicitation. *Plant Cell Tissue Organ Cult* 119:469–477
- Stevens JF, Reed RL, Morré JT (2008) Characterization of phytoecdysteroid glycosides in meadowfoam (*Limnanthes alba*) seed meal by positive and negative ion LC-MS/MS. *J Agric Food Chem* 56:3945–3952
- Suzuki H, Srinivasa-Reddy MS, Naoumkina M, Aziz N, May GD, Huhman DV, Sumner LW, Blount JW, Mendes P, Dixon RA (2005) Methyl jasmonate and yeast elicitor induce differential transcriptional and metabolic re-programming in cell suspension cultures of the model legume *Medicago truncatula*. *Planta* 220:696–707
- Thanonkeo S, Chamnipa N, Thanonkeo P (2011) Induced accumulation of 20-hydroxyecdysone in cell suspension cultures of *Vitex glabrata* R.Br. *Afr J Biotechnol* 10:10612–10617
- Trivedy K, Nair KS, Ramesh M, Gopal N, Kumar SN (2003) Effect of phytoecdysteroid on maturation of silkworm, *Bombyx mori* L. *Indian J Seric* 42:75–77
- Vasudeva N, Sharma SK (2006) Post-coital antifertility activity of *Achyranthes aspera* L. roots. *J Ethnopharmacol* 107:179–181
- Vazquez LH, Mangas S, Palazon J, Navarroca A (2010) Valuable medicinal plants and resins: commercial phytochemicals with bioactive properties. *Ind Crops Prod* 31:476–480
- Verpoorte R, Van der Heijden R, ten Hoopen HJG, Memelink J (1999) Metabolic engineering of plant secondary metabolite pathways for the production of fine chemicals. *Biotechnol Lett* 21:467–479

- Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann Bot* 100:681–697
- Yoon HJ, Kim HK, Ma CJ, Huh H (2000) Induced accumulation of triterpenoids in *Scutellaria baicalensis* suspension cultures using a yeast elicitor. *Biotechnol Lett* 22:1071–1075
- Zhang W, Furusaki S (1999) Production of anthocyanins by plant cell cultures. *Biotechnol Bioprocess Eng* 4:231–252
- Zimmer AR, Bruxel F, Bassani VL, Gosmann G (2006) HPLC method for the determination of ecdysterone in extractive solution from *Pfaffia glomerata*. *J Pharm Biomed Anal* 40:450–453