Plant Omics Journal POJ

POJ 6(2):116-120 (2013) ISSN:1836-3644

Methyl jasmonate stimulates 20-hydroxyecdysone production in cell suspension cultures of *Achyranthes bidentata*

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Abstract

20-Hydroxyecdysone (20E), one of major phytoecdysteroids (i.e. analogues of insect steroid hormones) in plants, has important agrochemical, medicinal and pharmaceutical uses. In order to develop a sustainable source of 20E, cell suspension cultures were established from shoot cultures of *Achyranthes bidentata*. When cultivated in Murashige & Skoog medium supplemented with 1.5 mg/l 1-naphthlcetic acid (NAA) and 1.5 mg/l 6-benzyladenine (6-BA), *A. bidentata* cells in suspension culture grew rapidly, yielding 20E (5.4 mg/l) after 24 days. The increase of 20E was dependent on the growth stage of cell cultures as well as on the dose of methyl jasmonate (MeJA) applied. When cells of 18-day-old cultures were exposed to 0.6 mM MeJA for 6 days, it was found that total (intracellular and extracellular) 20E production reached the maximum yield 7.5 mg/l, a 2.6-fold increase over the control. This is the first report of 20E production in cell suspension cultures of *A. bidentata*.

Keywords: *Achyranthes bidentata*, cell cultures, methyl jasmonate, 20-hydroxyecdysone.
Abbreviation: ABPS_*Achyranthes bidentata* polysaccharides; 6-BA_6-benzylaminopurine; DMSO_dimethylsulfoxide; 20E_ 20-hydroxyecdysone; JA_Jasmonic acid; MeJA_methyl jasmonate; NAA_1-naphthlcetic acid.

Introduction

Achyranthes bidentata Blume (Amaranthaceae), an indigenous herb in hilly districts of China, Korea, Japan and India, is used in traditional Chinese medicine as a tonic and diuretic agent (Meng and Li, 2001). The majority of the bioactive constituents isolated from *A. bidentata* were of water-soluble polysaccharides with unit composition molecular weight of ~1400 D and fructose and glucose residues in the molar ratio of 8:1 (Chen et al., 2005). Previous study showed that *A. bidentata* polysaccharides (ABPS) could strengthen the immune system, restrain tumor metastasis, increase leucocytes number, activate thoracic cavity macrophages of human and enhance anti-malarial immunity (Deng et al., 2003; Jin et al., 2007; Chen et al., 2009; Zhu et al., 2012). The phytochemical investigations of *A. bidentata* have also reported the isolation of a rich array of bioactive phytoecdysteroids including 20-hydroxyecdysone (20E; also reported as β-ecdysone or ecdysterone), inokosterone, serfurosterone A and some new phytoecdysteroids containing a furan ring such as niuxixinsterone A, B, C (Meng et al., 2005; Li et al., 2007b; Wang et al., 2011; Zhang et al., 2012). Air-dried tissues from *A. bidentata* leaves, stem and roots have been reported to contain 20E 0.45, 0.15 and 0.24 mg/g, respectively (Li et al., 2007a).

20E, a major phytoecdysteroid present in plants, has various biological effects in animals such as hastening maturation of silkworm *Bombyx mori* (Trivedy et al., 2003), reducing the length of the moult cycle of shrimp *Alpheus heterochelis* (Mellon and Greer, 1987), stimulating carbohydrate metabolism and reducing hyperglycemic response in rats (Yoshida et al., 1971). 20E and its derivatives also displayed a wide array of health improvement (e.g. growth-promoting, wound-healing and stimulating protein synthesis) (Lafont and Dinan, 2003) and beneficial pharmacological effects (e.g. antidepression, antioxidation, antidiabete and neuron protection) (Sláma and Lafont, 1995; Najmutdinova and Saatov, 1999). The need of large amount 20E in many commercial anabolic preparations led to a boom for the exploration of 20E resources (Dinan and Lafront, 2006). Accumulation of 20E has been reported from various species in the main orders of higher plants Polypodiophyta, Gymnospermae, and Angiospermae, but 20E content in these species is generally low approximating only 0.1% or less of the dry weight (Dinan, 2001). Cell culture, as a viable route for biosynthesizing phytochemicals, provided a promising strategy for enhancing 20E production originally from plants. 20E was produced at a two- to six-fold higher level by cell and callus cultures of *Ajuga turkestanica* than the level found in root and aerial parts (Lev et al., 1990). The yield of 20E increased from 0.1% to 0.2% with the treatment of the mutagen *N*-nitroso-*N*-methylurea at 8 mM (Zakirova et al., 2000). With the medium optmization and precursor feeding, 20E production in cell suspension cultures of *Vitex glabrata* was stimulated (Thanonkeo et al., 2011). Although the polysaccharide content in callus and suspended cell cultures of *A. bidentata* has been described (Li et al., 2008), little information is available up to date about the pharmacologically significant 20E in the cultured *A. bidentata* cells. Since methyl jasmonate (MeJA), a plant signaling compound induced by insect damage, triggered an increase in phytoecdysteroid synthesis in spinach, *Spinacia oleracea* (Schmelz et al., 1999), the compound has potential as an elicitor to effectively enhance 20E production in cell cultures of *A. bidentata*. As a continuation of our interest in the *in vitro* production of medically important secondary metabolites from Chinese traditional medicinal herbs (Wang and Tan, 2002; Wang et

al., 2004; Guo and Wang, 2008), we tried for 20E production in cell cultures of *A. bidentata* with additional attention paid to the effect of MeJA in the cultures.

Results

Time course of cell growth and 20E production

The cell suspension cultures were established successfully from leaf explants of plantlets of *A. bidentata* (Fig. 1A). The callus was induced from the leaf and stem explants by 6-BA 0.5 mg/l and NAA 1.5 mg/l in the MS medium with 3% (w/v) sucrose and 0.8% (w/v) agar. On the medium, the yellowish callus was initiated from the leaf discs after two weeks (Fig. 1B). Then the callus was transferred to the MS medium with 6-BA 1.5 mg/l, NAA 1.5 mg/l and 3% (w/v) sucrose to establish cell suspension cultures (Fig. 1C). Data regarding the accumulation of biomass and 20E was presented in Fig. 2. After 24 days of cultivation, the biomass was about 17.7 g/l dw, about 4.2-fold of initial concentration (Fig. 2A). As shown in Fig. 2B, the intracellular contents of 20E firstly fluctuated between 0.17 mg/g and 0.25 mg/g with time, then decreased gradually after day 24. However, the pattern of 20E production in medium was almost close to that of biomass growth. The total 20E production increased linearly with time up to day 24 with the highest value of about 5.4 mg/l on day 24, and thereafter declined rapidly (Fig. 2C).

Effect of MeJA dosage

As shown in Fig. 3A, addition of MeJA at all six given concentrations did not result in any significant changes in biomass accumulation. However, the MeJA treatment stimulated the accumulation of 20E in cell cultures. Although the extracellular content of 20E was significantly stimulated by all concentrations of MeJA, the intracellular 20E was only influenced by MeJA at 0.6 and 0.8 mM (Fig. 3B). With 0.6 mM treatment 20E production reached its highest value (6.4 mg/l), 1.6-fold that of the control level (Fig. 3C). MeJA at 0.6 mM was then used for subsequent study.

Time course of elicitation

The time course of the effect of MeJA on 20E accumulation in 18-day-old cultures is shown in Fig. 4. After the treatment for 6 days, the intracellular content reached a maximum value (0.36 mg/g dw) as compared with that of the non-elicited control (0.12 mg/g dw). As a result, the maximum 20E production (7.5 mg/l) in the *A. bidentata* cultures was obtained when treated with 0.6 mM MeJA for 6 days, which was about 2.6-fold that of the control.

Discussion

While producing a large amount of polysaccharides in previous study (Li et al., 2008), the cell cultures of *A. bidentata* described here can synthesize as well the pharmacologically important 20E with the corresponding productivities (0.25 mg/g dw) comparable to those higher levels of the constituents (0.15-0.45 mg/g dw) in *A. bidentata* collected from different locations of China (Li et al., 2007a). The amount of 20E was found to be 0.33 mg/g dw in cell suspension cultures of *V. glabrata* (Sinlaparaya et al., 2007). The ultrahigh concentration (6.9 mg/g dw) of 20E was found in 10 to 15-month-old cell suspension cultures of *A. turkestanica* (Cheng et al., 2008). Owing to the conspicuous pharmacological activities of 20E, biotechnological production of 20E using *A. bidentata* cell

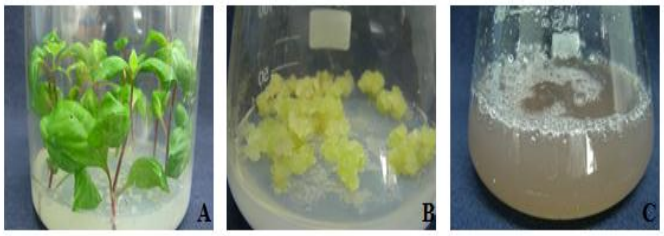

Fig 1. Establishment of *Achyranthes bidentata* cell suspension cultures. (A) 3-week-old plantlets. (B) Callus induced from leaves. (C) Cell suspension cultures.

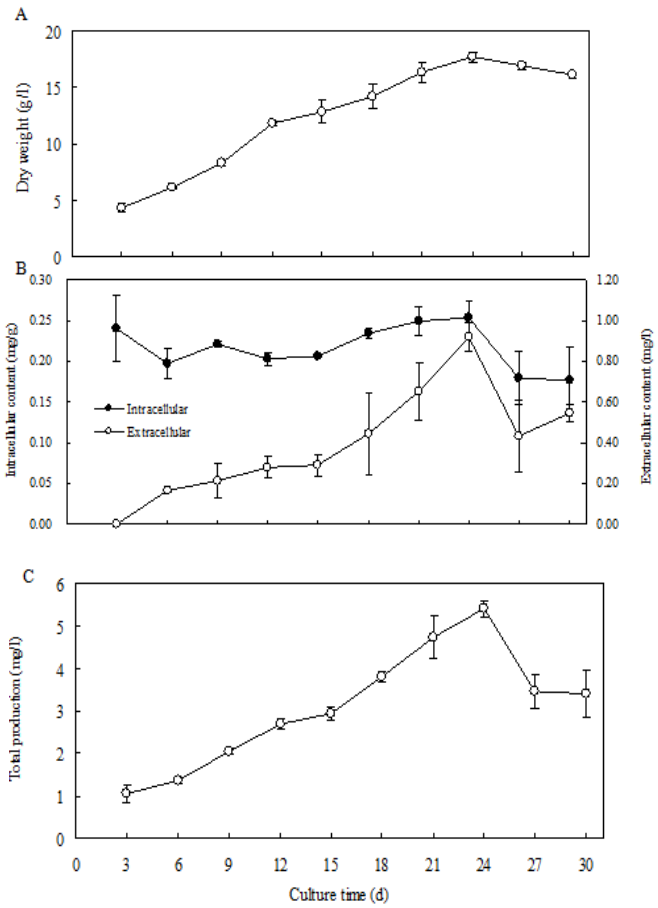

Fig 2. Time course for growth and 20-hydroxyecdysone (20E) accumulation in cell cultures of *Achyranthes bidentata*. (A) Biomass growth in cultures. (B) Intracellular and extracellular content of 20E in cultures. (C) 20E production in cultures. Production refers to sum of 20E recovered from cultured cells and liquid medium. Dates are expressed as mean ± standard deviation of three replicates.

cultures is of great practical value. This is the first report of 20E production in cell cultures of *A. bidentata.* The major barrier to commercial application of cell cultures in 20E production is mainly attributed to the low yields of the secondary metabolite. Therefore, various stimulation and process strategies including medium optimization, precursor feeding and elicitation have been exercised to improve 20E production in cell cultures (Sinlaparaya et al., 2007; Cheng et

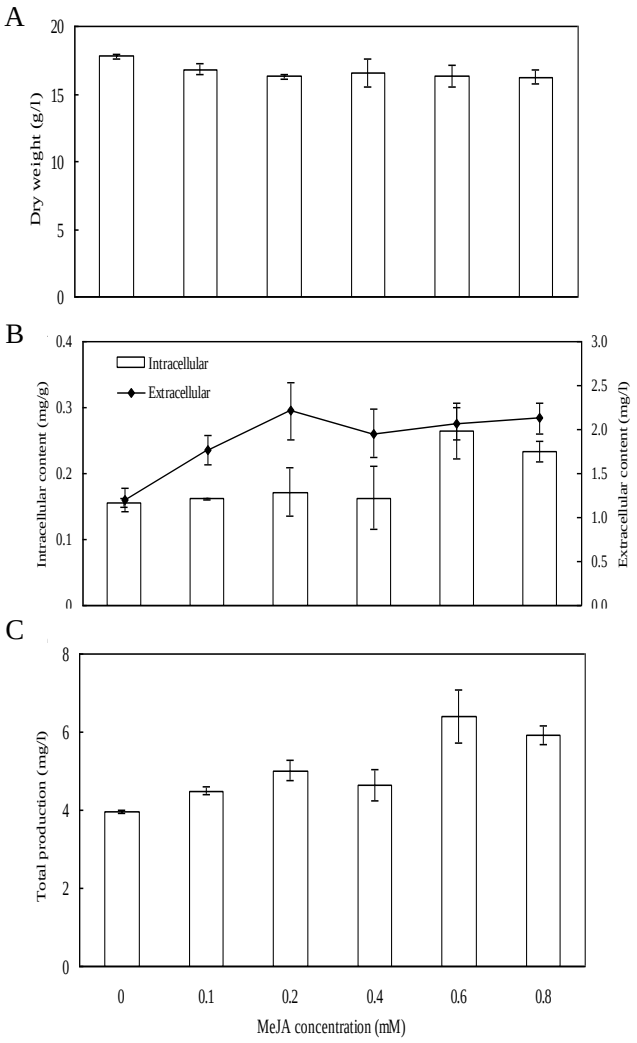

Fig 3. Effect of methy jasmonate (MeJA) at different concentrations on growth and 20-hydroxyecdysone (20E) accumulation in cell cultures *of Achyranthes bidentata*. MeJA at different concentrations were added to 18-day-old cell suspension cultures for 3 days treatment. (A) Effect on biomass growth in cultures. (B) Effect on Intracellular and extracellular content of 20E in cultures. (C) Effect on 20E production in cultures. Production refers to sum of 20E recovered from cultured cells and liquid medium. The control received 50 μl DMSO only. Dates are expressed as mean ± standard deviation of three replicates. *Values significantly different for $P \leqslant 0.05$ and **Values significantly different for $P \leqslant 0.01$.

al., 2008; Thanonkeo et al., 2011). 20E, a steroid hormone controlling arthropod development and reproduction, was demonstrated to have plant defensive functions towards phytophagous insects and soil nematodes (Kubo et al., 1983; Soriano et al., 2004). Our results showed that the accumulation of such an allelochemical can be stimulated by a plant signaling compound MeJA. It was reported that jasmonic acid (JA) and the analog MeJA could mediate the root damage-induced accumulation of 20E in spinach (Schmelz et al., 1999). In cell suspension cultures of *A. turkestanica*, 20E content increased 3- or 2-fold (23.6 or 14.7 μg/ mg dw, respectively) with the addition of 125 or 250 μM MeJA, compared to unelicited cultures (Cheng et al., 2008). Sinlaparaya et al. (2007) demonstrated that precursor feeding of cholesterol at 100 and 200 mg/l inhibited cell growth and 20-hydroxyecdysone production in *V. glabrata* suspension cultures. Feeding of cholesterol at lower concentration (5 mg/l), yielded 1.11-fold higher accumulation of 20E than that of the control cells (Thanonkeo et al., 2011). However, precursor feeding of cholesterol, sodium acetate or mevalonic acid at 50, 100 or 150 mg/l to medium did not increase 20E accumulation in cell cultures of *A. turkestanica* cell cultures (Cheng et al., 2008). In our present study, with the 6-day treatment of MeJA at 0.6 mM, 20E production reached its highest value (7.5 mg/l), 2.6-fold that of the control level. Furthermore, cell growth of *A. bidentata* could not be inhibited at this eliciting concentration (Fig. 3A). MeJA elicitation could be a promising strategy to increase 20E production in cell cultures of *A. bidentata*. It is important to note that MeJA could stimulate 20E both in cells and in medium. The cultured cells could exude 20E (0.92 mg/l) into the medium, which occupied about 17% of total production (Fig. 2B, 2C). When treated by MeJA, the extracellular 20E production increased to about 1.8-2.2 mg/l, which were about 30% that of total production (Fig. 3B), indicating that the enhancement of 20E release was mainly due to both the stimulated biosynthesis in cells, and the enhanced membrane permeability. Many other secondary metabolites can also be released and recovered in growth medium. In *Taxus baccata* cell suspension culture, 74.9% of total taxanes was released to growth medium (Kajani et al., 2010). Ye et al. (2004) found that up to 5.3% taxuyunnanine C and 25.1% phenolics were released into the liquid medium in *T. chinensis* cell culture (Ye et al., 2004). The extracellular paclitaxel levels in *T. canadensis* cell suspension cultures increased from 22.9 to 52.7 mg/l with 100 μM MeJA treatment (Roberts et al., 2003). Exudation offers a novel and efficient way of secondary metabolite production in cell cultures, allowing for stimulating cellular accumulation, extending culture time and reducing costs for product recovery (Cai et al., 2012). With precursor feeding and medium optimisation, the greatly enhanced production of 20E in cell suspension cultures of *A. bidentata* could be expected by using combined MeJA elicitation and exudation strategies including membrane permeabilization, cell immobilization and in situ product removal.

Material and methods

Plant material and callus induction

A. bidentata seeds, collected in June 2010 from the suburb of Nanjing, China with its voucher specimen (SCU-100812) identified by Prof. C.Y. Liu deposited in the Herbarium of Soochow University, were sterilized with 0.1% (w/v) $HgCl_2$ for 60 s and washed three times with sterile distilled water. Seeds were germinated on hormone-free Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium containing 2% sucrose (w/v) at pH 5.8. Germination started within 5 days and plantlets were used for callus induction. Young leaves of 3-week old plantlets were cut into small pieces and callus was induced on MS medium containing 0.5 mg/l 6-benzylaminopurine (6-BA), 1.5 mg/l 1-naphthlcetic acid (NAA) (medium A) in continuous darkness at 25 ± 1°C. Subcultures of the callus were carried out in MS medium containing 6-BA 1.5 mg/l, NAA 1.5 mg/l (medium B) in continuous darkness every 30 days.

Cell suspension cultures of A. bidentata

2.5 g fresh weight of calli were inoculated in 150 ml flasks containing 50 ml of medium B. Cell suspension cultures were maintained on a rotary shaker at 120 rpm in continuous darkness at 25 ± 1°C. During the 30 days culture period, cell

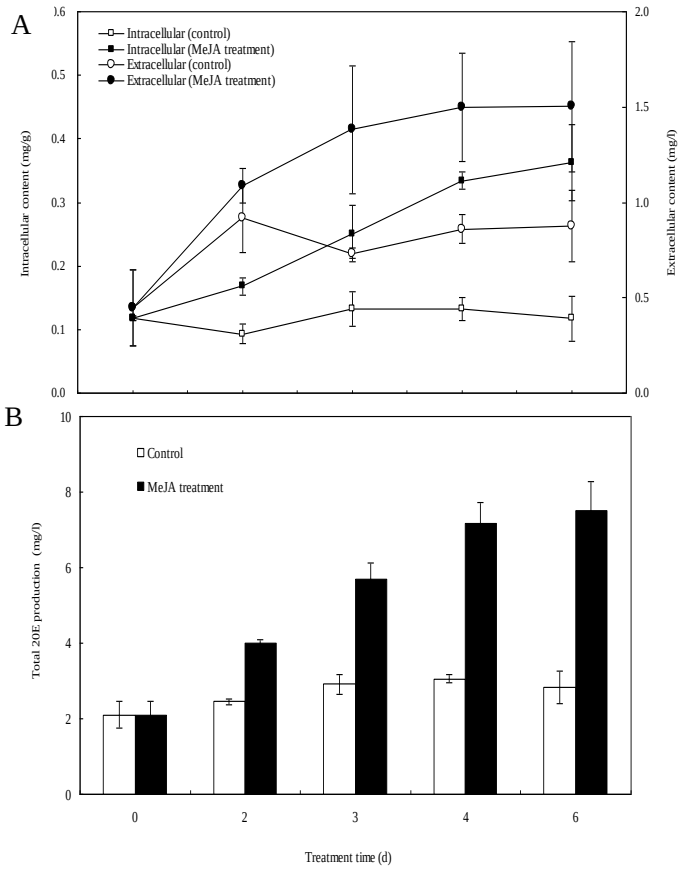

Fig 4. Effect of treatment time of methy jasmonate (MeJA) on 20-hydroxyecdysone (20E) accumulation in cell cultures of *Achyranthes bidentata*. MeJA at 0.6 mM was added to 18-day-old cell suspension cultures. (A) Effect on Intracellular and extracellular content of 20E in cultures. (B) Effect on 20E production in cultures. Production refers to sum of 20E recovered from cultured cells and liquid medium. The control received 50 μl DMSO only. Dates are expressed as mean ± standard deviation of three replicates. *Values significantly different for P ≤ 0.05 and **Values significantly different for P ≤ 0.01.

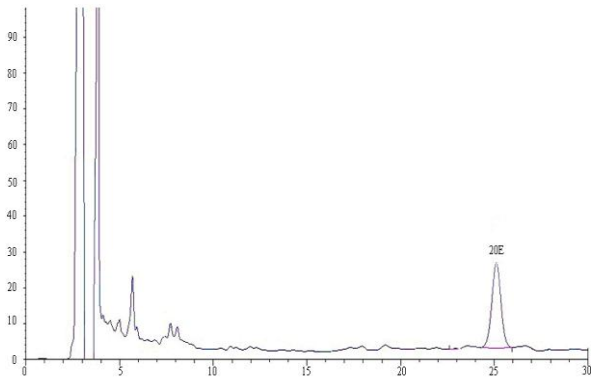

Fig 5. HPLC analysis of 20-hydroxyecdysone (20E) isolated from cultured cells of *Achyranthes bidentata*. The isolated 20E was analyzed by HPLC employing Aglient HPLC system consisting of a diode array detector and HC-C_{18} column (250 mm × 4.6mm) by UV detection at 242 nm. 20 μl samples were eluted with a mobile phase consisting of 14.9: 85.1 (v/v) acetonitrile / water-acetic acid (41:1, by vol.) at a flow rate 1 ml/min.

growth was evaluated by determining biomass (dry weight, dw) every three days. Measurements were performed until there was no longer an increase in cell growth (stationary phase). Each experiment was done in triplicate.

MeJA elicitor preparation and treatment

MeJA (Sigma, USA) was dissolved in dimethylsulfoxide (DMSO) at 1.0 M followed by filter sterilized. To determine the optimal elicitor concentrations, MeJA was added aseptically to the individual culture medium at the end of the exponential growth phase (day 18), to give final concentrations of 0.1, 0.2, 0.4, 0.6, and 0.8 mM. The treated samples were harvested for analysis after 3 days. To determine the most effective treatment time of MeJA, we added optimum concentration (0.6 mM) of MeJA to cultures on 18th day after inoculation. The cells were harvested 2, 3, 4 and 6 days after addition of MeJA. For the control, 50 μl DMSO was added to each flask.

20E extraction and analysis

For extraction of intracellular 20E, about 0.1 g dry cells were ground into powder and extracted with 30 ml methanol under sonication for 90 min. The extract was then evaporated to dryness and dissolved in 1 ml methanol. For the extracellular 20E, the liquid medium were collected and filtered through a nylon syringe filter of pore size 0.40 μm to remove any cellular debris, and concentrated by freeze-drying (Labconco) to remove water. The final concentrate was dissolved in 1 ml methanol. Quantification of 20E content was carried out by the modified method of Chen et al. (2008). HPLC analysis conditions: Aglient 1280 HPLC system equipped with 250 mm × 4.6 mm Agilent HC-C_{18} column; samples were eluted with 14.9: 85.1 (v/v) acetonitrile / water-acetic acid (41:1, by vol.) at a flow rate 1 ml/min and monitored at 242 nm. 20E was quantified with genuine standards (Sigma). Fig. 5 presented a typical chromatogram of 20E in cells and its retention time (25.1 min) acquired under the condition. 20E production refers to sum of 20E recovered from the cells and the medium.

Statistical analysis

The experimental date was expressed as means ± standard deviations. One-way analysis of variance (ANOVA) was carried out to determine significant differences ($p < 0.05$) between the means by SPSS (version 11.0, SPSS Inc.).

Conclusion

In conclusion, results of present study demonstrate cell suspension culture as an optimum culture for 20E production in *A. bidentata*. The MS medium containing 1.5 mg/l 6-BA, 1.5 mg/l NAA and 3% (w/v) sucrose was found suitable for the production and secretion of 20E in *A. bidentata* cell cultures. The total (intracellular and extracellular) 20E production (5.4 mg/l) was obtained after 24 days of culture. The production of the secondary metabolite in cultures can be maximized by employing MeJA as both an elicitor to stimulate 20E biosynthesis and an inducer for 20E release into the extracellular medium. With the 6-day treatment of MeJA at 0.6 mM, 20E production reached its highest value (7.5 mg/l), 2.6-fold that of the control level, while cell growth of *A. bidentata* could not be inhibited. MeJA elicitation could be a promising strategy to increase 20E production in cell cultures of *A. bidentata*.

Acknowledgements

The authors are grateful to the fifth Jiangsu Leading Discipline Program (No. BU 132802), the Graduate Program of Higher Education in Jiangsu Province (No. CXLX12-0851), Soochow Scholar Program (No. 14317363) and the projects sponsored by the NNSF (No. 81273487), PAPD and SRF for ROCS (No. K513201011) for financial support of this work.

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