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Enhanced production of flavonoids by methyl jasmonate elicitation in cell suspension culture of *Hypericum perforatum*

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Abstract

Background: Flavonoids of *Hypericum perforatum* are important secondary metabolites which have been widely utilized in medicine for a range of purposes. The use of methyl jasmonate (MeJA) elicitation for the enhancement of flavonoid production in cell suspension culture of *H. perforatum* would be an efficient alternative method for the flavonoid production.

Results: MeJA influenced the cells growth and flavonoid production. The optimal elicitation strategy was treatment of the cell cultures with 100 μmol/L MeJA on day 15, which resulted in the highest flavonoid production (280 mg/L) and 2.7 times of control cultures. The activities of catalase (CAT) were inhibited after MeJA treatment in the cell cultures, while the activities of phenylalanine ammonia lyase (PAL) increased, which led to the enhancement of flavonoid production.

Conclusion: MeJA elicitation is a useful method for the enhancement of flavonoid production in cell suspension culture of *H. perforatum*.

Keywords: MeJA; *Hypericum perforatum*; Suspension culture; Flavonoids

Background

Hypericum perforatum, commonly known as St. John's wort, is a perennial herb native to Europe and also a traditional medicinal plant which has been utilized in Chinese folk medicine for a range of purposes [1,2]. The medicinal applications of *H. perforatum*, including skin wounds, eczema, burns, diseases of the alimentary tract, and psychological disorders, have been related to the phenolic composition of the plant, particularly to hypericins, hyperforins, and flavonoids [3,4]. The efficacy of medical constituents of *H. perforatum* is based on the whole secondary metabolites, rather than the presence of single chemical compound [5]. Flavonoids, specifically quercetin and its glycoside derivatives, comprise the major group of biologically active metabolites in *H. perforatum* and are important biochemical markers in authenticating the herbal plant materials [4,6].

The consumption of *H. perforatum*-derived products as pharmaceutical preparations or food additives has increased dramatically, and it is presently one of the most consumed medicinal plants in the world [4,7]. The extracts of *H. perforatum* currently used in foods and pharmaceutics, mostly composed of flavonoids, are mainly obtained from the top aerial parts collected in the flowering stage [6,8]. However, the quality of the flavonoid-rich extracts derived from field-grown plants may be affected by many environmental factors as well as biological processes [5]. Furthermore, field cultivation of *H. perforatum* requires a long growth period and plant management, which is a slow and laborious process [6]. Therefore, an alternative method for more efficient and controllable production of flavonoids from *H. perforatum* is urgently required.

Plant cell, tissue, and organ cultivation technology has been successfully applied to the production of pharmaceutically valuable compounds and other fine chemicals of commercial interest in recent years [9]. For instance, cell suspension cultures of *Panax ginseng* have been widely used for the production of saponin and other metabolites [10]. Plant-mediated green biomimetic synthesis of silver

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nanoparticle was considered a widely acceptable technology for rapid production of silver nanoparticles for successfully meeting the excessive need and current market demand and resulting in a reduction in the employment or generation of hazardous substances to human health and the environment [11]. Till now, a number of efforts have been made for the establishment of cell and/or adventitious root cultures of *H. perforatum* for the production of hypericins, phenolics, and xanthones [12-15]. The information on the factors affecting biomass and aforementioned metabolites of *H. perforatum* was also investigated through elicitation and culture optimization [1,16-18]. However, there are few reports on the production of flavonoids from cell suspension culture of *H. perforatum*, and little is known about the strategies for hyperproduction of biomass and flavonoids.

Many cell cultures have been established from plants, but they seldom produce sufficient amounts of the required secondary metabolites. Secondary metabolite biosynthesis in plants depends on environmental stresses; their accumulation can be stimulated by precursors and elicitors [19]. Precursors are intermediate compounds of the secondary metabolite biosynthesis cycles, which would be toxic to the culture if not used at an appropriate stage or concentration [20]. Elicitors are biological (components of microbial cells and poly- and oligosaccharides), chemical (heavy metals, pesticides, and the signaling compounds in plant defense responses), or physical (cold shock, UV, hyperosmotic stress, ultrasound, and pulsed electric field) factors that induce enzymatic activity against stress [19-21]. Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), have been proposed to be important signaling compounds in the process of elicitation leading to the hyperproduction of various secondary metabolites [14]. They have also been reported to play a key role in signal transduction processes that regulate defense responses in plants and shown effective to enhance the production of secondary metabolites in cell cultures [14,19].

In the present study, the effect of MeJA on cell growth and flavonoid biosynthesis in the *H. perforatum* cell suspension culture was investigated in small batches. Some parameters, such as elicitation time and MeJA concentration, on biomass and flavonoid production were studied in detail. The activities of key enzymes (catalase and phenylalanine ammonia lyase) related to plant stress responses and secondary metabolite biosynthesis were investigated as well. To the best of our knowledge, this is the first report on the induction of flavonoid production by MeJA in cell suspension culture of *H. perforatum*.

Materials and methods

Chemicals

MeJA was supplied by Drug Institute of East China University of Science and Technology (Shanghai, China). Salicylic acid (SA), ammonium metavanadate (NH_4VO_3), and nickel sulfate ($NiSO_4$) were purchased from Shanghai Zhong Lan Chemical Company (Shanghai, China). Acetonitrile of HPLC grade was purchased from TEDIA Company, USA. All other chemicals (analytical grade) were purchased from Shanghai Chemical Co., Ltd. (Shanghai, China).

Plant material and callus induction

H. perforatum plants were provided by Prof. Jie Qian (Tongji University, China) and were cultivated in the greenhouse. The fresh stem explants of *H. perforatum* were washed thoroughly with tap water, surface-sterilized with 75% ethanol for 60 s, and then soaked in 10% sodium hypochlorite solution for 30 s and rinsed seven times in sterile deionized water. The clean and sterilized stem explants were cut into 5-mm segments and inoculated on solid Murashige and Skoog (MS) [22] medium supplemented with 1.0 mg/L 2,4-D, 0.2 mg/L 6-benzyladenine (BA), and 25 g/L sucrose to induce callus formation. After 1 month, the successfully induced callus was separated from the explants and cultured separately until used for the establishment of cell suspensions.

Suspension cultures of *H. perforatum* cells

Cell suspension cultures were initiated from the friable callus and maintained in the liquid MS medium (pH 5.8) supplemented with 1.0 mg/L 2,4-D, 0.2 mg/L 6-benzyladenine (BA), and 25 g/L sucrose. A sterile standard sieve with aperture of 300 μm was used to filter the initial established suspension cells to obtain homogeneous cultures. The pre-weighed cells were cultured in a 250-mL Erlenmeyer flask containing a 50-mL medium; cultures were placed on a rotary shaker shaking at 120 rpm at 25 ± 2°C under continuous illumination. Every 20 days, cells were subcultured to fresh media with 5.0-g fresh weight (FW) in 250-mL flasks.

Elicitation of cell suspension culture

Four elicitors including MeJA, SA, NH_4VO_3, and $NiSO_4$ were used to study the elicitation effect on flavonoid production by the cell suspension cultures of *H. perforatum*. MeJA and SA were dissolved in ethanol, NH_4VO_3 and $NiSO_4$ were dissolved in water, and the four elicitors were filter-sterilized before adding into the suspension cultures. Based on the results of preliminary experiments, the feeding concentration of MeJA, SA, NH_4VO_3, and $NiSO_4$ was 50, 100, 50, and 15 μM, respectively. As a control, filter-sterilized ethanol was added to the cell suspension culture with a final concentration of 0.2%.

According to the results of the above experiments, MeJA was selected for further study. The MeJA induction time and feeding concentration, known to be crucial to plant cell culture, were investigated by single-factor

experimental design by varying a single factor at a time and keeping other factors at a constant value. All experiments were performed in triplicate, and data are expressed as the mean of three samples with standard deviation.

Extraction and HPLC analysis of secondary metabolites

H. perforatum extracts were prepared by soaking 0.5 g of the dried cells in 10 mL of methanol and treated with 1 h of ultrasonic extraction for two times. The extracts were then centrifuged at 10,000 rpm at 4°C. The supernatant was combined and used for HPLC analysis and flavonoid determination.

The HPLC system of Shimadzu LC-10Avp Plus with a PDA detector (SPD-M20A) and a C_{18} column (4.6 mm × 250 mm, 5 μm, Eclipse XDB C18) was used for the qualitative and quantitative analyses of flavonoids and other metabolites in the methanol extracts. The mobile phase was acetonitrile (A) and 0.1% trifluoroacetic acid (B). A gradient method was used for the separation of the extracted samples: 0-25 min, 15%-40% A; 25-40 min, 40%-100% A. The elution flow rate and detection wavelength were set at 1.0 mL/min and 254 nm, respectively. The identity of peaks separated by HPLC was confirmed by the injection of standard, and UV spectral analysis was carried out to confirm compound identification.

Determination of biomass and flavonoid content

The plant cell biomass is expressed as the gram fresh cell weight and/or dry cell weight per liter. Cell suspension cultures were filtered and washed by deionized water, then cells were collected and weighed to get the FW. The collected cells were dried in the vacuum drying oven at 45°C until constant weight (DW) was attained.

The flavonoid content was assayed using UV colorimetric method as reported elsewhere [5,13]. Briefly, 0.5 mL of the methanolic plant cell extract was mixed with 2 mL of distilled water and subsequently with 0.15 mL of a 5% $NaNO_2$ solution. After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and the mixture was allowed to stand for a further 6 min before 2 mL of 4% NaOH solution was added. Absorbance of the mixed solution was measured at 510 nm using a UV-Vis spectrophotometer (UV-1650PC, Shimadzu, Japan). Rutin was used as standard compound for the quantification of total flavonoid. Results were expressed as milligram of rutin equivalents per gram of dry cells. Data were expressed as means ± SD for three replications.

Enzyme activity analysis

Suspension culture cells were harvested for evaluation of the catalase (CAT) activity level as described by Georgiew et al [23]. The decomposition of H_2O_2 was followed by measuring the decrease in absorbance at 240 nm. One unit of CAT is the amount that decomposes 1 μmol of H_2O_2 for 1 min at pH 7.0 and 25°C. Protein was estimated by the procedure of Lowry et al. [24], using crystalline bovine albumin as standard. Phenylalanine ammonia lyase (PAL) was extracted from fresh *H. perforatum* cells with borate buffer (pH 8.8). The cells were ground in the buffer (0.15 g/mL) for 2 min with a pestle and mortar on ice and then centrifuged at 10,000 rpm and 4°C for 20 min to obtain a solid-free extract. The PAL activity was determined based on the conversion of L-phenylalanine to cinnamic acid as described by Wu and Lin [21].

Results and discussion

Callus induction and cell suspension culture establishment

In the preliminary work, calyx, leaf, petal, and stem segments of *H. perforatum* were used as explants for callus induction. It revealed that callus induction was significantly affected by the type of explants and the stem was the best explant for the initiation of a friable callus (Figure 1A,B,C). The stem-derived callus was incubated at 25 ± 2°C under continuous illumination on a solid MS medium and subcultured every 4 weeks. Cell suspension culture was initiated once the stable cell lines were obtained on the solid MS medium. Given the potential for deriving useful secondary metabolites from plant resource, cell suspension cultures of *H. perforatum* were established to produce hypericin, naphtodianthrones, and phenylpropanoids [12,14,18]. In addition, some efforts have been made for the establishment of adventitious root cultures of *H. perforatum* for the production of flavonoids, phenolics, and xanthones [5,15-17]. In the present study, cell suspension culture was successfully established (Figure 1D) from the stem-derived calli of *H. perforatum* for further investigation of flavonoids' production performance.

To understand the growth and flavonoid accumulation in *H. perforatum* cells for determining the optimal cultivation time, cultures were analyzed throughout a 30-day period (Figure 2A). The cell growth exhibited a lag phase or slow growth period in the first 10 days; subsequently, the cells entered their exponential growth phase with the maximum DW of 8.2 g/L reached on day 20. As shown in Figure 2B, the flavonoid production is related to cell growth and its content reached maximum with a value of 16 mg/g DW during stationary or declining phase between day 20–25. The content of total flavonoids in cell suspension culture of *H. perforatum* (16 mg/g DW) was lower than that of the adventitious root cultures (42.7 mg/g DW) as reported earlier [5]. However, the culture period for the suspension cells of *H. perforatum* (3 weeks) was much shorter than that of the adventitious root cultures (6 weeks). In addition, the flavonoid production in cell suspension culture of *H. perforatum* can be further improved by the supplementation of effective elicitors

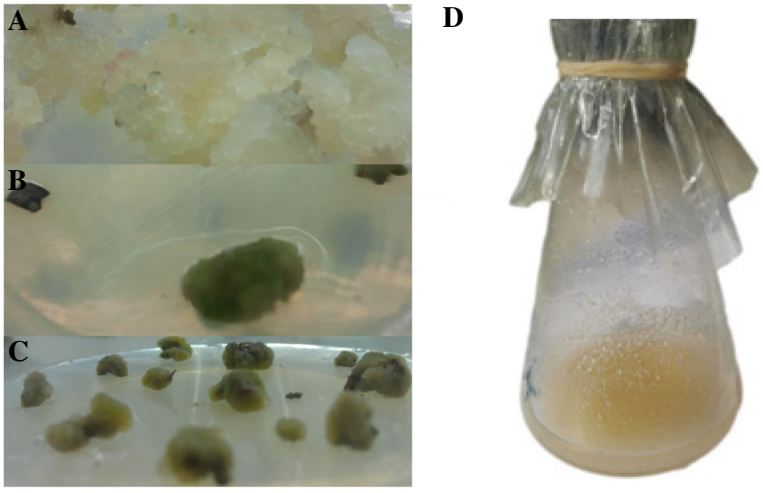

Figure 1 Callus induced from different explants of *H. perforatum* and established cell suspension cultures. (A) Callus induced from stem explants; **(B)** callus induced from leaf explants; **(C)** callus induced from calyx explants; **(D)** cell suspension culture.

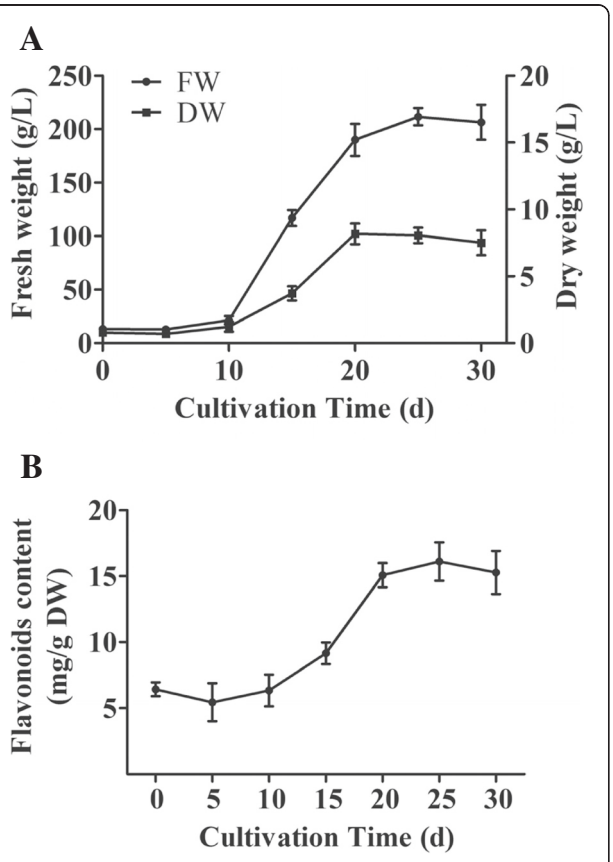

Figure 2 Determination of optimal culture time in *H. perforatum* cells. Kinetic profiles of cell growth **(A)** and content of flavonoids **(B)** in *H. perforatum* cell suspension cultures.

[18,19]. Therefore, it is crucial to screen potential elicitors for the hyperproduction of flavonoids in a shortened culture period by cell suspension culture of *H. perforatum*.

HPLC was used to recognize the flavonoids biosynthesized in the cell cultures on the basis of the retention times and UV spectras with those of reference standards. As shown in Figure 3A,B,C,D, hyperin and quercetin were the main flavonoids obtained from the established cell suspension cultures, which was in accordance with a previous study [6]. In addition, the medium was examined, and no flavonoids were detected.

Selection of MeJA as the elicitor in cell suspension culture of *H. perforatum*

Some known abiotic elicitors for plant secondary metabolites such as signaling molecules (MeJA and SA) and inorganic salts (NH_4VO_3 and $NiSO_4$) were tested for their effect on cell growth and flavonoid production by the *H. perforatum* cell suspension culture. Four treatments, MeJA (50 μM), salicylic acid (100 μM), NH_4VO_3 (50 μM), and $NiSO_4$ (15 μM) were applied to the cell cultures on day 15 based on our previous lab results. As shown in Figure 4, the growth of cell cultures was inhibited by the addition of the four elicitors as compared to the control cultures. Similar findings were also observed by Dong and Zhong [25], in which the application of elicitation in suspension cell culture severely inhibited the growth of *Taxus chinensis* cells. However, elicitation treatments may have varied effects on different cell lines. Some researchers have reported that the growth of cell cultures was not affected by elicitors [26]. The effects of the elicitors on flavonoid production are also given in Figure 4. Flavonoid content was promoted by MeJA and

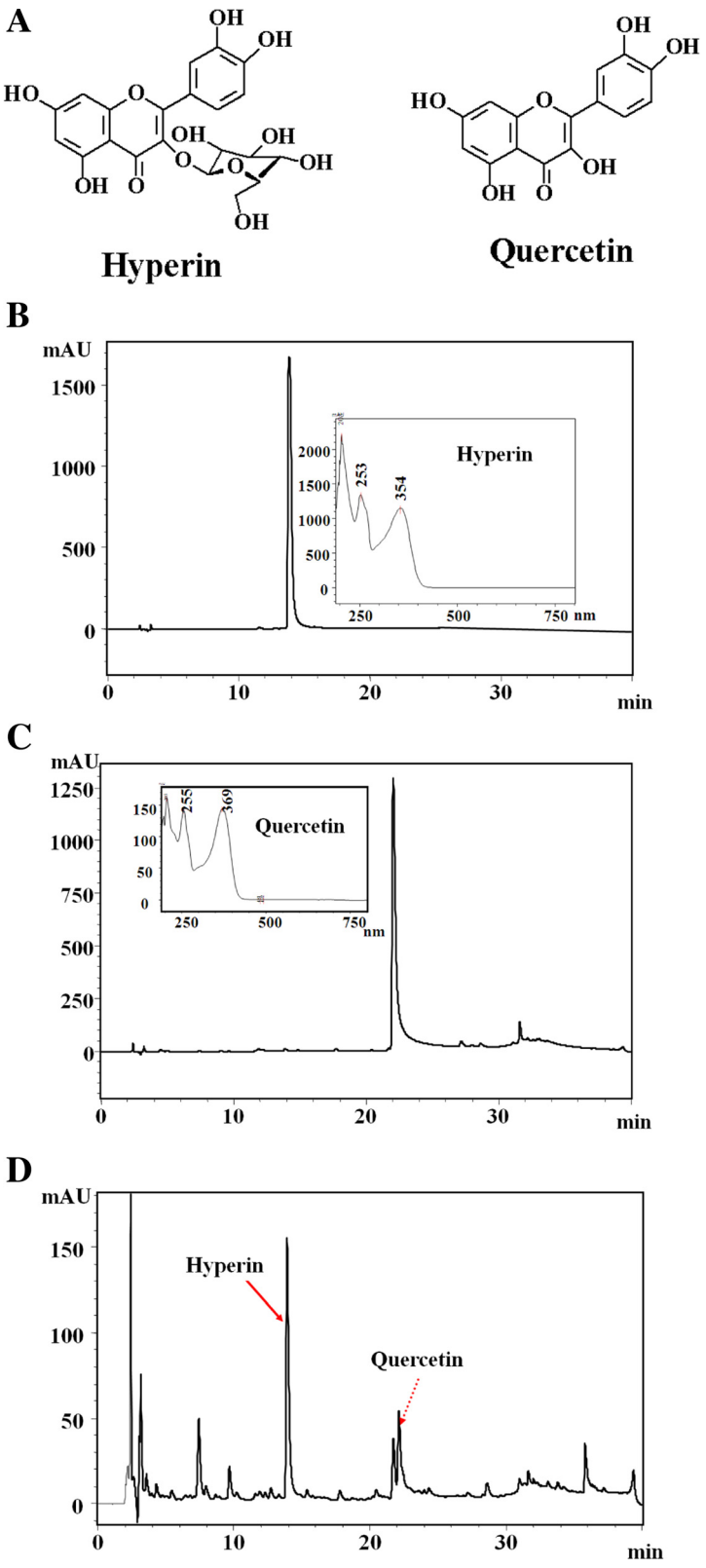

Figure 3 Hyperin and quercetin obtained from the established cell suspension cultures. Chemical structures of hyperin and quercetin **(A)** and chromatographic profile of hyperin (B), quercetin (C) and the suspension cell extracts **(D)**. The two insets are the UV spectrums of standard hyperin **(B)** and quercetin **(C)**.

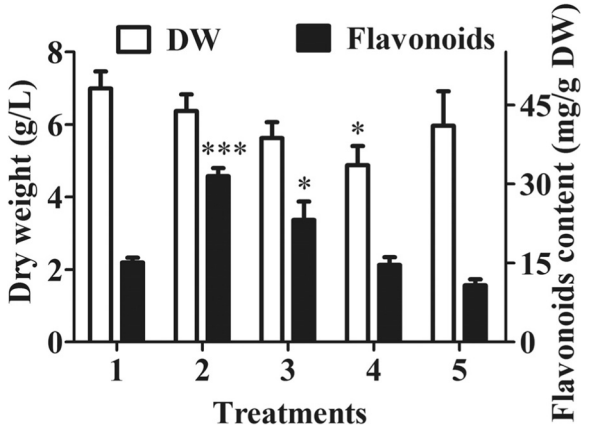

Figure 4 The effects of four abiotic elicitors on *H. perforatum* cell growth and flavonoid accumulation. 1 Control; 2 MeJA; 3 SA; 4 $NH4VO_3$; 5 $NiSO_4$ (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate statistical significance compared to the control).

SA induction, which were 2.1 and 1.5 times higher in comparison to control cultures (15.36 mg/g DW), respectively. Several studies on other plant cell cultures have shown that MeJA and SA elicitation can enhance the production of secondary metabolites. For instance, MeJA was recognized as an effective elicitor that could increase the production of paclitaxel in *Taxus candensis* and *T. cuspidate* [27], anthocyanin in *Tulipa gesneriana* [28], and gymnemic acid in *Gymnema sylvestre* [29]. In SA-elicited cell suspension culture of *H. perforatum*, the production of both hypericin and pseudohypericin has doubled as compared to control cell suspension cultures [18]. As shown in Figure 4, MeJA and SA both induced flavonoid accumulation in *H. perforatum* cell suspension cultures, but SA was less efficient than MeJA in promoting flavonoid accumulation under the tested feeding concentration.

Some literatures reported the enhancement of metabolite production by NH_4VO_3 and $NiSO_4$ elicitation [30]. However, the two elicitors revealed negative effects on biomass and flavonoid accumulation in *H. perforatum* cell cultures (Figure 4). It was probably due to the significant biological toxicity caused by the inorganic salts [30]. Some other elicitors such as fluoro- and hydroxyl-containing derivatives of methyl jasmonate, which were proven more potent than methyl jasmonate in suspension culture of *Taxus* [31,32], may also serve as possible options for the enhancement of flavonoid biosynthesis in *H. perforatum*. In addition, some new elicitors have been reported to have significantly promoted secondary metabolite biosynthesis by plant cell cultures [33]. However, elicitation treatments may have varied effects among different cell lines as mentioned above. Therefore, efforts devoted into the enhancement of flavonoid production by *H. perforatum* cell suspension culture are still required considering its further application. In the present investigation, the addition of MeJA (50 μM) resulted in maximum flavonoid production, which indicated the potential of this abiotic elicitor for the enhancement of flavonoid biosynthesis in cell suspension culture of *H. perforatum* and was selected for further study.

Effects of MeJA feeding time on cell growth and flavonoid production

The elicitor induction time is one of the key factors that affect the cell growth and product yield for plant cell suspension culture [33]. The effects of MeJA addition time on cell growth and flavonoid accumulation was evaluated by the addition of 50 μM MeJA into the cell cultures on lag phase (day 5 and day 10) and exponential phase (day 15 and day 20). Figure 5A,B,C showed the biomass and flavonoid production of *H. perforatum* cell cultures after MeJA addition. As shown in Figure 5A,B, C, the cell growth and production of flavonoids were suppressed deleteriously when MeJA was added on the lag phase. MeJA treatment on the exponential phase (day 15 and day 20) led to a slow decrease in DW (19.0% and 9.7%, respectively); however, the flavonoid content and production were significantly increased as compared to control cultures (Figure 5A,B,C). A similar phenomenon was also observed by Huang and Zhong [30], in which elicitation treatment on the log phase of *P. ginseng* reduced the DW and enhanced ginsenoside accumulation. Notably, MeJA treatment on day 15 resulted in the highest value of flavonoid content (38.26 mg/g DW) or production (229.79 mg/L). Therefore, MeJA treatment on day 15 was taken as the optimal addition time for flavonoid production.

Effects of MeJA dosage on cell growth and flavonoid production

A suitable elicitor concentration is important for the cell growth and product yield in plant cell suspension culture process [34]. To study the effects of MeJA addition concentrations, cell suspension cultures of *H. perforatum* were treated with different levels (50-200 μM) of MeJA after 15 days of cultivation. The DW and flavonoid content on day 20 were shown in Figure 6A. It is clear that all the tested concentrations of MeJA had an inhibitory impact on the cell growth; similar results were also obtained in the SA-induced cell suspension culture of *H. perforatum* [18]. Induction with 50 or 100 μM of MeJA decreased DW by 11.9%-23.1%, while higher concentration of MeJA (150 μM) severely decreased the DW from 7.0 ± 0.5 g/L (control cultures) to 5.0 ± 0.5 g/L. Addition of low dosage (<150 μM) of MeJA elicitor to the cultured cells of *H. perforatum* increased the flavonoid accumulation. As shown in Figure 6A, the flavonoid content was increased about 1.1-fold with a dosage of 50 μM and

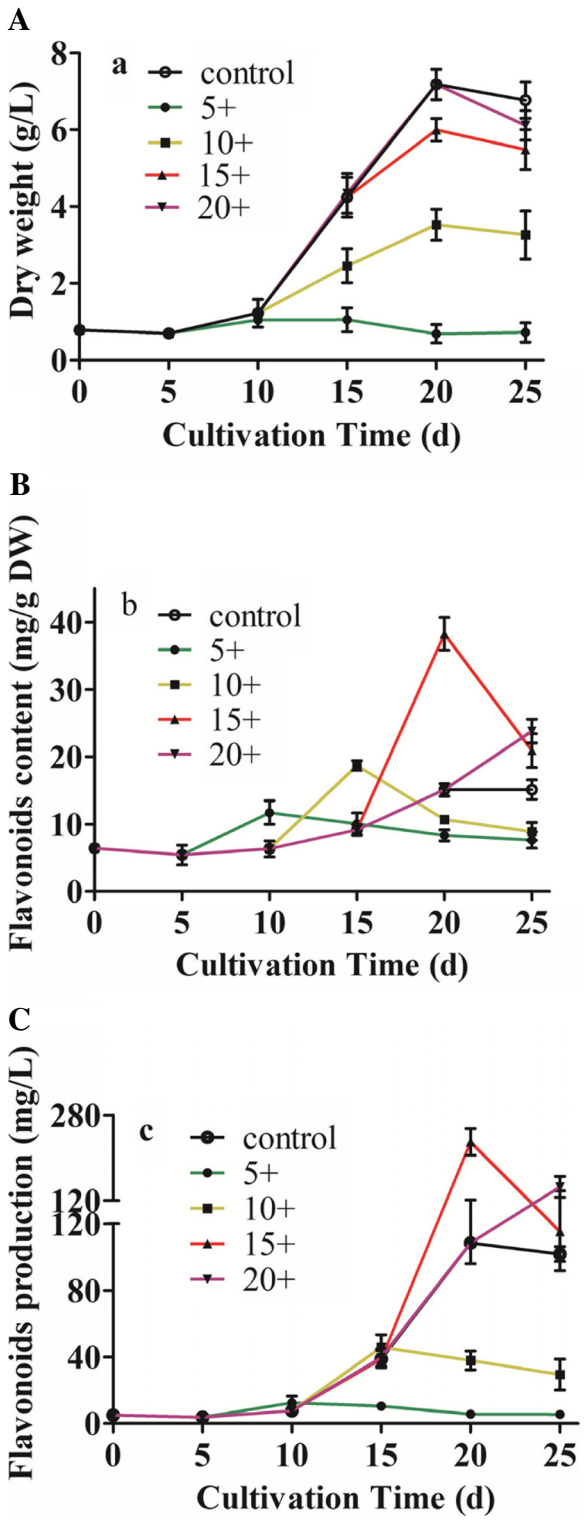

Figure 5 Effects of MeJA (50 μM) feeding time on cell growth (A), flavonoid content (B), and flavonoid production (C). 5+, 10+, 15+, and 20+ indicate MeJA feeding on day 5, 10, 15, and 20.

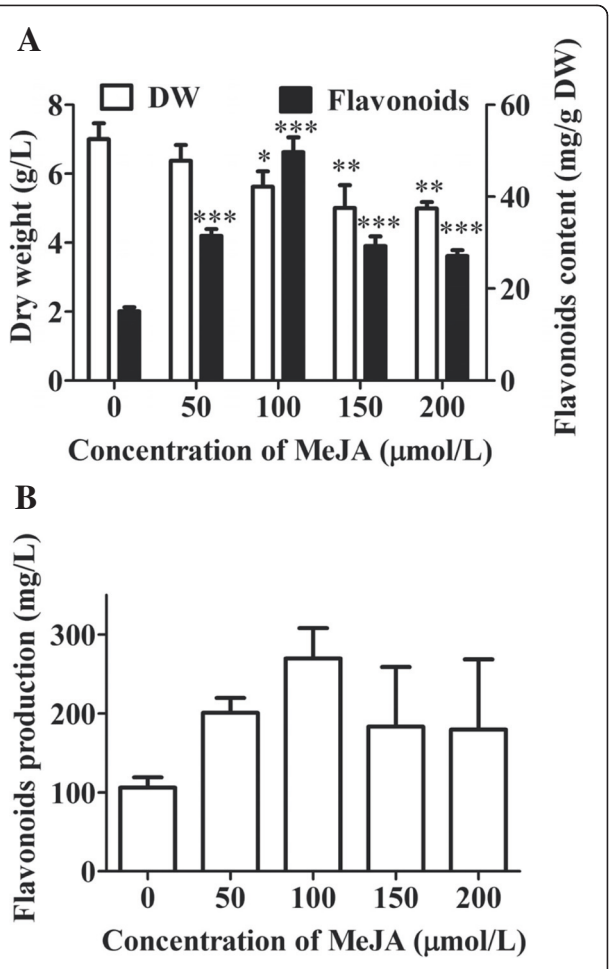

Figure 6 Effects of MeJA dosage on cell growth and flavonoid content (A) and flavonoid production (B). *$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$ indicate statistical significance compared to the control.

about 2.3-fold with a dosage of 100 μM as compared to the control cultures. The flavonoid content reached maximum (52.8 mg/g DW) at the elicitation dose of 100 μM, which was relatively higher than the previously reported values in the adventitious root culture (i.e., 42.7 and 48.6 mg/g DW) [5,17]. However, further dosage increase (150-200 μM) decreased the flavonoid biosynthesis compared to moderate MeJA levels (50-100 μM). Sensitivity of suspension cell cultures to elicitor concentration differs with plant species [19,29]. The negative effect of MeJA at higher concentrations (200 μM) on cell growth and metabolite production was also reported in *Gymnema sylvestre* cell suspension cultures [29]. The best dose of the elicitor with maximum production (279.5 mg/L) of flavonoids was 100 μM as shown in Figure 6B, which was about 2.7-fold the value of control cultures. The above-mentioned results indicate that the optimal MeJA elicitation strategy was treatment on day 15 with a dosage of 100 μM.

Effects of MeJA addition on CAT and PAL activities

The enzyme activity changes were often related to the secondary metabolite accumulation [35]. For a better understanding of the elicitation effect of MeJA, the activities of two important enzymes (CAT and PAL) under MeJA treatment were determined. Reactive oxygen species (ROS) are toxic intermediates resulting from successive steps in the reduction of molecular O_2 in plant cells. The ROS exerts various effects on plant defense responses, including cell wall reinforcement, hypersensitive cell death, defensive gene activation, as well as defensive compound induction [36]. CAT is a well-known intracellular enzyme which protects against the ROS generated within cells [37]. As shown in Figure 7A, the enzyme activity increased with the cultivation time and reached maximum on day 19, thereafter decreased. MeJA down-regulated the CAT activity after its addition to the cell cultures on day 15 and kept at a relatively lower level as compared to the control (Figure 7A), which led to a higher accumulation of ROS and resulted in a promotion of secondary metabolites production. Similar results were observed on phytoalexin production in the suspension cell culture of *Glycine max*, which indicated that the ROS mediated elicitor-induced accumulation of secondary metabolites [38].

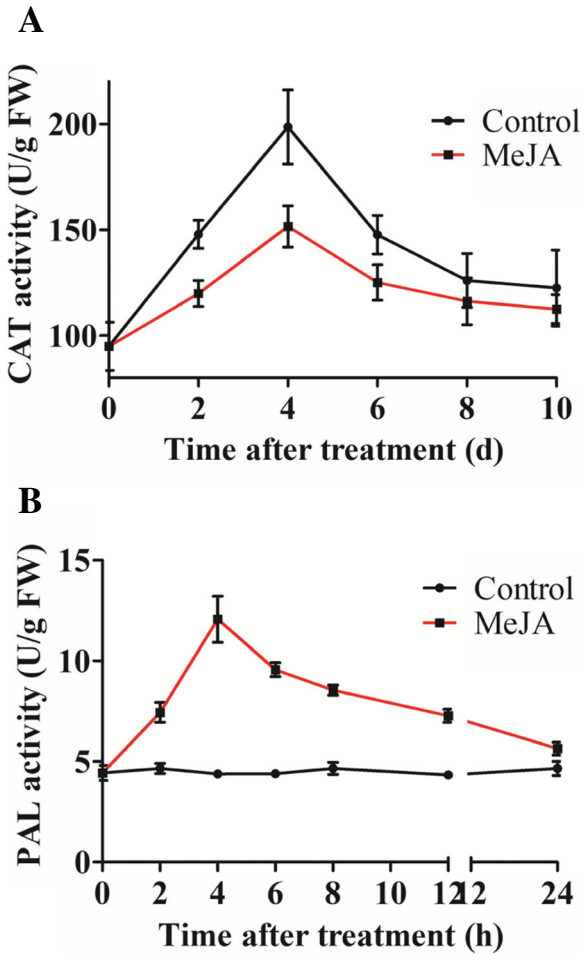

Figure 7 Dynamic changes of CAT (A) and PAL (B) activity with 100 μM of MeJA added on day 15 in cell suspension culture of *H. perforatum*.

PAL is the first enzyme of phenylpropanoid biosynthesis in plants and plays an important role in the biosynthesis of flavonoids, lignins, and many other compounds [36]. An increase in PAL activity after elicitor treatment often results in enhanced secondary metabolism in plant cells [19]. Figure 7B shows the changes of intracellular PAL activity in the suspension cultures after the treatment by MeJA (100 μM). As shown in Figure 7B, MeJA induced the increase of PAL activities and reached maximum (12.1 U/g FW) 4 days after treatment, thereafter decreased. However, cell cultures without MeJA treatment (control) revealed little change of PAL activities. In the presence of MeJA, PAL activity was much higher than that of control (Figure 7B), the elicitor-induced up-regulation of the PAL activity resulted in an improved flavonoid production in the suspension cultures (Figure 6B). Zhao et al. [19] have observed similar results in *Salvia miltiorrhiza* cell cultures, which indicated that both biotic and abiotic elicitation treatment resulted in the up-regulation of PAL activity and improved tanshinone accumulation as compared to the control cultures. These results demonstrated that MeJA is an efficient elicitor for the induction of flavonoid production in the suspension cell culture of *H. perforatum*.

Conclusions

Plant cell suspension culture is an efficient alternative method for the production of useful biochemicals. In this work, MeJA-elicited cell suspension cultures of *H. perforatum* enhanced the production of flavonoids through the down-regulation of the CAT activity and up-regulation of the PAL activity. The flavonoid production reached 280 mg/L after the optimal elicitation conditions, which was 2.7-fold of the control cultures. These results would be useful for the hyperproduction of flavonoids from *H. perforatum* and would be useful for the production of valuable compounds from other plant cell cultures.

Abbreviations

CAT: Catalase; DW: Dry cell weight; FW: Fresh cell weight; MeJA: Methyl jasmonate; PAL: Phenylalanine ammonia lyase; SA: Salicylic acid.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

J W was in charge of the experiments and paper writing. LY Y participated in the experiments and paper writing. J Q kindly offered experimental plant and guided the experiments. YH L directed the study as the tutor. All authors read and approved the final manuscript.

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