INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 13, No. 4, p. 369-377, 2018

RESEARCH PAPER OPEN ACCESS

Effect of methyl jasmonate elicitation on biomass, gene expression and saponin accumulation in *Bacopa monnieri*

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Key words: Methyl jasmonate, *Bacopa monnieri*, Gene expression, Saponins

http://dx.doi.org/10.12692/ijb/13.4.369-377 Article published on October 30, 2018

Abstract

Bacopa monnieri (known as brahmi) is an important medicinal plant in Thailand containing numerous bacoside-saponin compounds and widely used for pharmacological activities. Biosynthesis is regulated by many enzymes and also affected by cellular and environmental factors. The aim of this study was to enhance the biomass (dry weight), key gene expression, and production of saponin in brahmi through the process of methyl jasmonate (MeJA) elicitation. Results showed that a low concentration (100μM) of MeJA treatment had minimal effect and slightly decreased the biomass (dry weight) of the plant (31.3±1.1mg/ plant), compared to untreated MeJA (38.1±2.7 mg/plant). By contrast, higher concentrations of MeJA treatment (200-800μM) resulted in a highly significant decrease in plant biomass. Gene expression of *BmAACT* was significantly up-regulated at 1-3 hours but started to down-regulate at 6-168 hours after 100µM MeJA-treated brahmi, eventually reaching the normal level of untreated control. *BmOSC* expression was significantly up-regulated at 6-12 hours but started to down-regulate at 48 hours after 100µM MeJA-treated brahmi, returning to a normal level of untreated control. Bacoside contents (A3, II, X, and C) were significantly increased by low and high concentrations of MeJA treatments after both 7 and 14 days, compared to untreated control. Bacoside accumulations at 14 days after individual MeJA treatment were significantly higher than at 7 days. Results suggested that low concentrations (100-200µM) of exogenous MeJA application could provide a potential protocol for the enhancement of bacoside contents in brahmi seedlings.

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Introduction

Bacopa monnieri, known as brahmi, is a member of the Scrophulariaceae family as a semi-aquatic annual medicinal plant widely distributed in the tropics and subtropics (Faisal *et al.*, 2018). This species is a highly valued medicinal plant, extensively used historically as a traditional Ayurvedic medicine in many countries (Singh and Dwivedi, 2018) and non-toxic for human consumption (Yadav *et al.*, 2012). Many previous publications have reported the high potential of brahmi to enhance memory through brain rejuvenation, promote longevity and improve intellectual functions by activating neurons (Gubbannavar *et al.,* 2012; Piyabhan and Wetchateng, 2014). Brahmi has also been used to treat Parkinson's disease (Jansen *et al.*, 2014), as an antidepressant (Rauf *et al.*, 2014), and to reduce anxiety (Pandareesh *et al.*, 2014). Triterpenoid saponins, as the main bioactive compounds of secondary metabolites, are present as complex entities in brahmi ((Majumdar *et al.*, 2011), forming the structures of bacoside A, bacopaside II, bacoside X, and bacoside C (Deepak *et al.*, 2005). Bacoside A is a core bioactive compound (Tripathi *et al.*, 2012; Singh *et al.*, 2014), and is considered responsible for memory enhancing effects and protection against morphine-induced cerebral toxicity (Hebert *et al.*, 2013). However, biosynthesis of these secondary metabolites is complicated and regulated by many key enzymes involving cellular and environmental factors related to mechanisms of biotic and abiotic condition (Mendosa *et al.*, 2018).

Several commercial products involving *B. monnieri* such as brahmi capsules are widely available in Thai markets (GPO, 2018). Most raw plant materials are collected from natural wild populations, and these are restricted to certain agroclimatic conditions in wetlands and muddy shores. Therefore, the quality and quantity of bacoside contents produced in field crops under natural conditions are extremely variable and depend on both biotic and abiotic conditions (Ncube *et al.*, 2012). These variables have affected and changed the growth rate of biomass and biosynthesis of secondary metabolites, leading to difficulties in controlling the quality and quantity of bioactive compounds in raw materials (Sangeetha and Ganesh, 2011). One approach, widely used as a strategy to increase biomass and saponin biosynthesis during field cultivation, involves elicitation application utilizing methyl jasmonate (MeJA) (Largia *et al.*, 2015; Khojasteh *et al.*, 2016; Thiem *et al.*, 2017).

Methyl jasmonate (MeJA), a natural volatile organic compound, has been identified as a signaling molecule in biotic and abiotic stresses. MeJA has been used to regulate gene expression associated with saponin biosynthesis including the acetyl-CoA C-acetyltransferase (*AACT*) gene in *B. monnieri* (Jeena *et al.*, 2017) and *Ginkgo biloba* (Chen *et al.*, 2017), and the *oxidosqualene cyclase* (*OSC*) gene in *Withania somnifera*)Dhar *et al.*, 2014) and *Betula platyphylla* (Yin *et al.*, 2014; Yin *et al.*, 2016). Furthermore, MeJA has been proven to increase the production of saponins in plants such as bacoside A in *B. Monneiri* (Largia *et al.*, 2015), phenolic contents in *Ajuga bracteosa* (Saeed *et al.*, 2017), and rosmarinic acid in *Satureja khuzistanica* (Khojasteh *et al.*, 2016) and *Thevetia peruviana* (Mendoza *et al.*, 2018). To meet market demand, an increase in bacoside quantity in brahmi is urgently required. Therefore, this paper evaluated the effect of MeJA-treated brahmi on biomass production, expression of both *BmAACT* and *BmOSCT* genes, and bacoside accumulation. Results demonstrate the potential ability to enhance bacoside contents of brahmi during field cultivation as raw material for drug production in Thailand.

Materials and methods

Plant material and MeJA treatment

Brahmi shoots (4-6 cm long) were cut and grown in pots containing clay soil and fertilizer tablets (N:P:K=15:15:15) in greenhouse condition at Naresuan University, Thailand. After one month of growth, the seedlings were subjected to MeJA treatments.

A stock solution of 100mm methyl jasmonate (MeJA) was prepared as a mixture of 200 µl MeJA (4590 mM, Sigma-Aldrich, USA) diluted in 10mL absolute ethanol.

The mixture was filter-sterilized through 0.22µM membrane filters (Millipore, USA), and stored at -20°C. Different MeJA concentrations (100, 200, 400, and 800 µM) of 100mL were applied by spraying one-month-old brahmi seedlings with sprayed water used as a control. After MeJA treatments, the pots were sealed with plastic bags, incubated for 2 days until MeJA was absorbed and then transferred to the greenhouse. Treated and untreated shoots were sampled at 7 and 14 days. Individual treatments were performed as three biological replicates. At least 10 plants per replicate were selected to measure various parameters such as shoot height, fresh weight, dried weight, leaf area (using an AM350 Portable Leaf Area Meter, ADC BioScientific Ltd., UK), stomatal size (under stereomicroscope, Olympus, Japan), and bacoside contents (using HPLC system, Shimadzu, Japan).

Total RNA extraction and first-strand cDNA synthesis

One-month-old brahmi seedlings were treated with 100µM MeJA. Aerial parts of the brahmi seedlings were individually collected at 1, 3, 6, 9, 12, 24, 48, 72, 120, and 168 hours after treatment, and then immediately immersed in liquid nitrogen and stored at -80°C for further total RNA extraction. Each treatment was performed with three biological replicates.

Total RNA was extracted using Total RNA Extraction Kit Maxi (RBC Real Genomics, Taiwan), according to the manufacturer's instructions. Quantity was measured with Synergy H1 Hybrid Multi-Model Microplate Reader (BioTek Instruments, USA).

For first-strand cDNA synthesis, a reaction mix was prepared using Tero cDNA Synthesis Kit (Bioline, USA) containing random hexamer (1µL), 10mM dNTP mix (1µL), 5X RT buffer (4µL), RiboSafe RNase Inhibitor (1µL), 200 U/µL

Tero Reverse Transcriptase (1µL), total RNA (1µg) and adjusted to a final volume of 20µL with nuclease-free water (Life Sciences, USA). The reaction was incubated at 25°C for 10 minutes, 45°C for 30 minutes, and 85°C for 5 minutes. The first-strand cDNA was immediately subjected to RT-PCR amplification and stored at -20°C for future use.

Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

The qRT-PCR reaction was performed using SennsiFAST™ SYBR No-ROX Kit (Life Sciences, USA) with *BmAACT* and *BmOSC* gene-specific primers, and the *Bm18s-rRNA* gene was used as internal control for normalization of all the reactions. Sequences of all the primers are shown in Table 1.

Table 1. Primer sequences used in this study

Primer Name	Primer Sequence 5'-3'	Reference
BmAACT-F	GACTACGGCATGGGAGTTTG	Vishwakarma *et al.* (2013a)
BmAACT-R	ATTCCACGCTCAAAACTTTGG	
BmOSC-F	GCATGTGGAATGCACTGCTTCTGT	Vishwakarma *et al.* (2013b)
BmOSC-R	TGCCTTCGCCACGGAGATTTCTAT	
Bm18S-rRNA-F	GCACGCGCGCTACACCGAAG	Vishwakarma *et al.* (2013a,b)
Bm18S-rRNA-R	GTCTGTACAAAGGGCAGGGACG	

The qRT-PCR analyses for the *BmAACT*, *BmOSC*, and *Bm18s-rRNA* genes were performed using total reaction containing Sennsi FAST™ SYBR No-ROX (10µL), 10µM of each forward and reverse primer (0.4µL), first-strand cDNA (1µL) and the final volume was adjusted to 20µL with nucleic acid free water. The qRT-PCR reaction was carried out under the following conditions: 1 cycle of 95°C for 2 minutes, followed by 45 cycles of 95°C for 5 seconds, 55.6 (*BmAACT*) or 60°C (*BmOSC* and *Bm18s-rRNA*) for 10 seconds, and 72°C for 20 seconds. Finally, a melting curve was realized by progressively heating the reaction from 75°C to 95°C using 1.0°C increments every 10 seconds to check the purity of the qRT-PCR product.

All reactions were run in triplicate and repeated twice. Data were normalized with *18sBmrRNA* as endogenous control. Relative expression of the target gene was analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) and performed as mean ± standard error. For statistical analysis, the baseline correction was automatically calculated to determine the cycle threshold (Ct) value in each reaction.

Data were normalized with *Bm18s-rRNA* as endogenous control. Relative expression of the target gene was analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

Quantification of bacoside contents

Bacoside contents were quantified using the modified method of Bansal *et al.* (2016). In brief, aerial parts of brahmi seedlings were individually dried at 45-50°C for 2 days and then ground to fine powder using a mortar and pestle. The fine powder (0.1g) was transferred into a 15mL amber centrifuge tube containing 3mL methanol, mixed thoroughly, and incubated at room temperature for 1 hour.

The mixture was sonicated in an ultrasonic water bath (S50R Elmasonic, Elma, Germany) for 15 minutes and then incubated in the dark at 4°C for 5 minutes. The final volume was made up to 10mL with methanol and filtrated through a 0.45µm nylon syringe filter (Tianjin Fuji Science & Technology Co., Ltd., China). The solution was kept in a 2mL screw neck ND9 vial (Amber, CeiExpert, Thailand) and stored at -80°C until required for injection into the chromatographic system.

A stock solution of the calibrating reference standard was prepared by weighing bacoside (Sigma-Aldrich, USA) 5mg, dissolved in methanol 1 mL, and further diluted with methanol to give a standard solution of various bacoside concentrations as 20, 40, 60, 80 and 100 ppm.

The standard solution (60 µL) was injected into the HPLC system (Shimadzu, Japan) equipped with a Purospher®STAR-RP-18 endcapped (5µm) LiChro CART® 250-4.6 HPLC cartridge (150 x 4.4mm) (Merck, Germany), an LC-10AD VP pump and a Rheodyne Injector (20µL loop) (Shimadzu, Japan).

Chromatographic conditions were used to quantify the bacoside content as follows: (i) the mobile phase consisted of phosphoric acid (0.2%) dissolved in a mixture of water and acetonitrile (65:35 v/v); the pH was adjusted to 3.0 with 5M NaOH, (ii) the flow rate was 1mL/min for saturation time of 30 minutes, and (iii) bacoside content was detected at 205nm using a SPD-10A VP UV-vis Detector (Shimadzu, Japan) and its content was calculated by comparing relative retention times with standard samples.

Statistical analysis

The data (plant height, fresh weight, dry weight, gene expression, and bacoside content) were statistically assessed using one-way analysis of variance (ANOVA). Mean comparisons between multiple treatments were assessed by Tukey's test at *p*-value less than 0.01 statistical significance using Statistical Product and Service Solution version 17.0 software (SPSS Inc., Chicago, USA). All values were expressed as mean ± standard error (SE).

Results and discussion

Effect of MeJA elicitation on growth of brahmi

To evaluate the effect of MeJA-treated brahmi on growth parameters, seedlings (4-6 cm long) were grown in soil for one month and then subjected to various concentrations of MeJA (0, 100, 200, 400 and 800µM). After treatments for 7 and 14 days, seedling growth was measured. Results revealed that all MeJA treatments decreased growth rate such as plant height, number of axillary shoots, fresh weight, dry weight, and leaf area (at 1st, 2nd, or 3rd leaf), with high significance at *p*-value ≤ 0.01 compared to the untreated MeJA control (Table 2). By contrast, untreated and all treated MeJA samples showed no significant differences in stomata size (at the 1st, 2nd, or 3rd leaf) at *p*-value ≤ 0.01 (Table 2).

A significant decrease in plant biomass (dry weight) was also observed at higher MeJA concentrations of 200, 400 and 800μM at *p*-value ≤ 0.01, compared to both untreated and treated 100μM MeJA (Table 2).

However, low treated MeJA concentration (100μM) had minimal effect, with a slight decrease in plant biomass (dry weight) at 31.3±1.1mg/ plant compared to untreated MeJA at 38.1±2.7mg/ plant. This result clearly indicated that MeJA treatment at different concentrations had significantly different impacts on brahmi growth development. Exogenous MeJA had a

significant inhibitory effect on growth reduction of plants at higher concentrations by decreasing growth in plant cells (Sukito and Tachibana, 2016). This finding concurred with Sharma *et al.* (2013) who reported that MeJA treated shoots of *B. monnieri* showed decreased growth compared to the control. Largia *et al.* (2015) also reported that higher concentrations of MeJA significantly inhibited biomass production in shoot cultures of *B. monnieri.*

Effect of MeJA elicitation on expression patterns of BmOSC and BmAACT genes

The previous section showed that MeJA treatment at low concentration (100 μM) slightly affected growth reduction of brahmi biomass. Expression patterns of *BmAACT* and *BmOSC* genes involved in early and later phases of triterpenoid saponin biosynthesis, respectively in brahmi were further investigated at 11 time points within a 7 day period (168 hours) after treatment by 100μM MeJA on one-month-old brahmi seedlings, compared to a mock solution used as a control. At all the time points, the whole plant was sampled with three biological replicates, and 100mg of individual plants was randomly sampled for total RNA extraction. Results indicated that the *BmAACT* gene was significantly up-regulated for 1 and 3 hours after MeJA-treated brahmi but down-regulated after 6 hours until 168 hours after MeJA treatment (Fig. 1). Thus, expression level of the *BmAACT* gene strongly increased at early time points (1 and 3 hours) for MeJA treated brahmi, and then decreased to the normal level at later time points.

This occurred because acetyl-CoA C-acetyltransferase (AACT), transcribed from the *AACT* gene, is the first core enzyme which catalyzes and converts three acetyl-CoA molecules into isopentenyl diphosphate (IPP) in the cytosolic mevalonic acid (MVA) pathway (Cui *et al.* 2012). This IPP precursor has been used in the initial step of triterpenoid biosynthesis (Chen *et al.*, 2017). Jeena *et al.* (2017) reported that the *AACT* gene involved in the first phase of triterpenoid biosynthesis was strongly up-regulated after 3 hours of MeJA treatment in bacopa. Similarly, the *GbAACT* gene showed a positive increase in expression level after 1mM MeJA treatment of *Ginkgo biloba* at a time point of 8 hours (Chen *et al.*, 2017).

At all the time points of MeJA-treated brahmi, the *BmOSC* gene exhibited up-regulation expression at 3 hours, showing highest expression from 9 to 12 hours with highly significant difference at p-value ≤ 0.01, and approximately 5-fold higher than untreated control. After 48 hours of MeJA treatment, the *BmOSC* gene showed down-regulation expression level (Fig. 1). This indicated that MeJA treatment had a positive effect on promoting the expression level of the *BmOSC* gene at 9-12 hours after MeJA treatment. These results concurred with previous reports that the expression of the *OSC* gene in forms of lupeol synthase (*BpW*) and beta-amyrin synthase (*BpY*) in the triterpenoid synthesis pathway in *Betula platyphylla* was up-regulated at 12 hours after 1 mM MeJA treatment in birch but decreased at 7 days and 14 days after MeJA treatment)Yin *et al.*, 2014; Yin *et al.*, 2016). Similarly, three *OSC* gene, beta-amyrin synthase (*WsOSC/BS*), was up-regulated after 24 hours of 100μM MeJA-treated *Withania somnifera*)Dhar *et al.*, 2014). The expression profile of the *BmOSC* gene tended to up-regulate later that the *BmAACT* gene after MeJA treatment. This occurred because the *BmOSC* gene played an important role in construction of the basic triterpenoid skeletons, and was catalyzed and cyclized by the oxidosqualene cyclase (OSC) enzyme involved in the penultimate step of triterpenoid biosynthesis (Zhan *et al.*, 2016; Chen *et al.*, 2017).

Effect of MeJA elicitation on bacoside accumulation

One-month-old brahmi seedlings were MeJA-treated at different concentrations (0, 100, 200, 400 and 800 μM). After treatments for 7 and 14 days, total bacoside contents (bacoside-A3, bacoside-II, bacoside-X and bacosidesaponin-C) of the seedlings were quantified using HPLC assay as shown in Table 3. Overall, all bacoside contents (A3, II, X, and C) significantly increased using both low and high concentrations of MeJA treatment after 7 and 14 days compared to untreated MeJA control.

Bacoside A3 and bacoside II contents were significantly increased by low MeJA concentrations (200 and 100μM, respectively) after treatment for 7 days, while their contents were significantly decreased by high MeJA concentrations (400-800μM) at 7 days after treatment compared to the control)Table 3(. Moreover, bacoside X and bacoside C contents showed more significant increases at higher MeJA concentrations at both 7 and 14 days after treatment compared to the control) Table 3(. These results indicated that accumulation of the four bacoside contents was significantly enhanced at 7 and 14 days after MeJA treatment, compared to control. However these accumulations at 14 days after MeJA treatment were significantly higher than at 7 days. Methyl jasmonate (MeJA) has been proposed as an important compound for signal transduction that acts to up-regulate key genes involved in metabolic biosynthesis and enhances production of many secondary metabolites in plants (Zhao *et al.*, 2010). For this reason, MeJA-elicitation has been widely used to enhance metabolite production in both *in vitro* and *in vivo* conditions. Previous reports suggested that production of the bioactive compound bacoside A was enhanced using 50μM MeJA as an elicitor for *in vitro* shoot culture of *B. monneiri* (Largia *et al.*, 2015). Total phenolic contents were enhanced after 14 days of 0.6 mg/L MeJA-elicitated root culture of *Ajuga bracteosa* (Saeed *et al.*, 2017), and production of rosmarinic acid was enhanced after 16 days of 100μM MeJA-treated cell culture of *Satureja khuzistanica* (Khojasteh *et al.*, 2016). Phenolic compounds in cell suspension increased at 96 hours after 0.3μM MeJA-treated *Thevetia peruviana* (Mendoza *et al.*, 2018). For the *in vivo* condition, terpenoid accumulation was promoted after 64-96 hours of 1mM MeJA-treated seedlings of *Gingko biloba* (Chen *et al.*, 2017). Similarly, lycopene and total carotenoid contents were significantly enhanced at 8 and 11 days after MeJA treatment of mature green fruit of *Solanum lycopersicum* (Liu *et al.*, 2018).

Table 2. Effect of MeJA-treated brahmi on growth development 7 and 14 days after spraying.

Trait	Duration after MeJA treatment at various concentrations									
	0 μM		100 μM		200 μM		400 μM		800 μM	
	7 days	14 days	7 days	14 days	7 days	14 days	7 days	14 days	7 days	14 days
PH(cm)*	9.2±0.33^{a}	11.8±0.37^{a}	7.0±0.55^{b}	7.9±0.34^{b}	6.4±0.2^{bc}	6.3±0.33^{c}	5.8±0.19^{bc}	5.6±0.21^{c}	5.1±0.45^{c}	5.5±0.30^{c}
Number of nodes	6.3±0.21^{a}	6.4±0.17^{b}	6.0±0.26^{a}	5.7±0.16^{a}	4.7±0.33^{a}	4.8±0.17^{c}	4.3±0.21^{a}	4.1±0.14^{c}	4.3±0.21^{a}	4.2±0.13^{c}
FW (mg)	419.1±37.0^{a}	526.0±10.6	328.7±16.2^{a}	393.1±19.0^{b}	266.4±19.4^{b}	317.5±15.5^{c}	265.1±17.5^{b}	297.7±4.65^{c}	264.0±14.1^{b}	288.7±14.2
DW (mg)	38.1±2.7^{a}	45.3±2.2^{a}	31.3±1.1^{b}	37.7±1.8^{b}	23.3±3.2^{c}	30.5±1.5^{b}	23.5±0.8^{c}	23.8±1.2^{c}	24.1±0.6^{c}	22.2±0.7^{c}
LA-N1 (mm²)	46.0±2.6^{a}	76.7±1.9^{a}	24.3±4.4^{b}	64.0±4.5^{a}	29.0±2.6^{ab}	42.3±2.7^{b}	41.0±5.0^{ab}	47±1.0^{b}	33.0±4.6^{ab}	35.7±3.7^{b}
LA-N2 (mm²)	54.0±2.0^{a}	93.3±1.9^{a}	39.0±3.0^{b}	80.3±2.8^{b}	42.0±0.6^{b}	63.3±2.3^{c}	54.7±0.9^{a}	56.0±3.5^{c}	40.0±1.0^{b}	54.3±1.5^{c}
LA-N3 (mm²)	77.7±6.9^{a}	125.7±3.9^{a}	65.3±3.0^{ab}	92.7±10.2^{ab}	54.7±4.0^{ab}	101.0±6.^{a}	74.7±1.2^{ab}	82.7±5.8^{ab}	55.7±4.8^{b}	72.3±11.4^{b}
SL-N1 (μm)	27.8±1.13^{ab}	30.4±1.09^{ns}	30.8±1.50^{ab}	32.3±1.94^{ns}	30.5±0.74^{ab}	31.0±1.66^{ns}	26.7±1.0^{b}	28.5±0.90^{ns}	32.2±1.48^{a}	29.8±0.71^{ns}
SL-N2 (μm)	29.7±2.44^{ns}	27.9±1.33^{b}	30.6±1.00^{ns}	35.5±1.88^{a}	32.4±1.47^{ns}	29.6±1.18^{b}	28.2±1.68^{ns}	29.6±0.91^{b}	31.1±1.47^{ns}	28.4±0.74^{b}
SL-N3 (μm)	30.7±1.08^{b}	33.7±1.11^{a}	32.3±0.84^{ab}	32.9±0.45^{ab}	36.7±1.36^{ab}	36.0±1.16^{a}	31.5±1.12^{b}	32.6±0.67^{ab}	32.2±1.39^{ab}	30.0±0.57^{b}
SW-N1 (μm)	16.4±0.33^{ns}	16.3±0.45^{ns}	16.4±0.66^{ns}	17.3±1.05^{ns}	15.7±0.60^{ns}	16.9±0.79^{ns}	16.6±0.62^{ns}	17.4±0.48^{ns}	17.5±0.73^{ns}	18.0±1.62^{ns}
SW-N2 (μm)	17.06±0.20^{b}	18.5±0.62^{ab}	17.65±0.46^{b}	21.9±1.03^{a}	16.64±0.69^{b}	17.3±0.72^{b}	17.4±0.42^{b}	19.7±1.02^{ab}	20.0±0.57^{a}	18.4±1.67^{ab}
SW-N3 (μm)	18.3±1.27^{ns}	19.4±0.58^{ns}	18.6±0.70^{ns}	19.9±0.85^{ns}	17.5±0.43^{ns}	22.6±1.26^{ns}	18.45±0.88^{n}	20.9±1.20^{ns}	19.3±0.79^{ns}	19.3±0.70^{ns}

Note: Values are expressed as means ± standard error, different superscript letters (in same row) indicate significant differences between treatments by Tukey's test at $p \leq 0.01$.

* Before staring the experiment, all explant heights were approximately 5 cm.

PH represents plant height; FW represents fresh weight; DW represents dry weight; LA-1, 2 or 3 represents leaf area at 1st, 2nd, or 3rd node, respectively; SL-1, 2 or 3 represents stomata length at 1st, 2nd, or 3rd leaf, respectively; and SW-1, 2 or 3 represents stomata width at 1st, 2nd, or 3rd leaf, respectively.

Table 3. Bacoside contents accumulation after MeJA treatment of *B. monnieri* for 7 and 14 days.

MeJA con. (µM)	Content (% dry weight)							
	Bacoside A_3		Bacopaside II		Bacopaside X		Bacopasaponin C	
	7 days	14 days	7 days	14 days	7 days	14 days	7 days	14 days
0	2.73±0.009 c	2.38±0.008 d	3.51±0.003 d	3.08±0.013 d	2.26±0.001 c	1.89±0.007 d	3.4±0.005 c	3.01±0.029 e
100	2.85±0.124 b	5.45±0.012 c	4.04±0.004 a	7.12±0.017 b	2.27±0.007 c	4.23±0.008 c	3.54±0.010 c	6.52±0.027 d
200	2.96±0.031 a	6.13±0.008 b	3.79±0.010 b	7.16±0.016 b	2.34±0.008 b	5.36±0.039 a	3.70±0.014 b	8.50±0.025 b
400	2.72±0.001 c	5.46±0.005 c	3.60±0.003 c	6.76±0.120 c	2.29±0.001 b	5.22±0.014 b	3.62±0.003 b	8.40±0.004 c
800	2.71±0.040 c	6.53±0.003 a	3.59±0.024 c	8.87±0.017 a	2.47±0.019 a	5.18±0.014 b	4.44±0.020 a	9.25±0.011 a

Note: Values of individual bacoside content are expressed as means ± standard error of triplicate experiments. Mean values with different letters (in same the gene) are significantly different at $p < 0.01$, analyzed by one-way ANOVA with Tukey's HSD (honestly significant difference) test using SPSS version 17.0 software program.

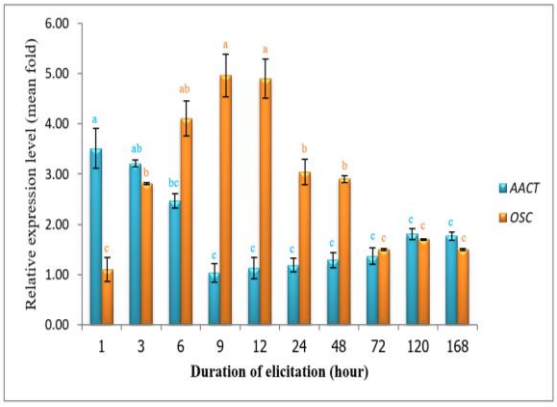

Fig. 1. Expression patterns of BmOSC and BmAACT genes after MeJA-treated brahmi at different time periods

Mean values with different letters (in same the gene) are significantly different at $p < 0.01$, analyzed by one-way ANOVA with Tukey's HSD (honestly significant difference) test using SPSS version 17.0 software program.

Gene expression levels of *BmAACT* and *BmOSC* in mock solution treatment were set to 1, and their *BmAACT* and *BmOSC* at different time points were calculated accordingly and presented as the relative fold changes, respectively. All reactions were normalized using the *Bm18s-rRNA* gene.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

This study was financially supported by Naresuan University, Thailand (Project number R2559B015).

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