

Enhancement of polysaccharides accumulation in *Dendrobium officinale* by exogenously applied methyl jasmonate

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Abstract

The accumulation of polysaccharides, activities of sucrose metabolism enzymes, and the expression of sucrose biosynthetic genes in *Dendrobium officinale* were significantly affected by exogenous methyl jasmonate (MeJA). Application of MeJA increased the content of polysaccharides and the highest polysaccharide production occurred in the samples treated with 200 μ M MeJA. The MeJA application influenced polysaccharide biosynthesis rather than degradation because the activities of sucrose metabolism enzymes and the expressions of sucrose biosynthetic genes were upregulated by MeJA. Interestingly, low MeJA concentrations promoted accumulation of *Dendrobium* polysaccharides, while high MeJA amounts played an inhibitory role. The content of major constituent of polysaccharides, glucose and mannose, also increased after MeJA treatment.

Additional key words: gene expression, glucose, mannose, sucrose phosphate synthase, sucrose synthase.

Introduction

Dendrobium officinale, which belongs to the *Orchidaceae* family, is used in Chinese traditional medicine. The polysaccharides and alkaloids are main bioactive substances in *D. officinale* (Liu *et al.* 2005, Zhao *et al.* 2007), therefore, it is crucial to investigate regulatory mechanisms of their biosynthesis and possibility of its genetic manipulation (Yuan *et al.* 2014).

As an important endogenous signal molecule, methyl jasmonate (MeJA) is a major component in signal transduction pathways leading to biosynthesis of particular compounds under abiotic stresses, and it is also essential for the development of systemic acquired resistance under biotic stresses (Browse 2009). In the recent years, application of MeJA at non-toxic concentrations has been shown to be effective in the regulation of number of processes, such as plant growth (Pauwels *et al.* 2008), defence against oxidative stress (Schenk *et al.* 2000, Sasaki-Sekimoto *et al.* 2005), senescence and cell wall modification (Browse and Howe 2008), chlorophyll metabolism and photosynthesis

(Reymond *et al.* 2004, Jung *et al.* 2007). In addition, MeJA has been involved in inducing the formation of secondary metabolites, such as alkaloids and terpenoids (Okudera 2009, Vázquez-Flota *et al.* 2009).

Dendrobium polysaccharides can be hydrolyzed into monosaccharides glucose, mannose, xylose, and arabinose. These monosaccharides are produced by sucrose hydrolysis and derivatization. Sucrose phosphate synthase (SPS) is the key enzyme involved in the conversion of starch to sucrose in photosynthetic organs. SPS activity reflects the capacity of sucrose biosynthesis (Salvucci 1990). Sucrose accumulation in plants is due to the increase of SPS activity and decrease of invertase activity (Lingle 1999). However, the functions of sucrose synthase (SS) include degradation of sucrose, providing precursors for polysaccharide syntheses and regulating the assignment of sucrose into various metabolic pathways (Vaughn 2002). SPS and SS gene expressions are closely related with the accumulation of polysaccharides. Previous studies on polysaccharides

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Abbreviations: EDTA - ethylene diamine tetraacetic acid; HPLC - high performance liquid chromatography; MeJA - methyl jasmonate; MOPS - 3-(morpholino) propanesulfonic acid; MS - Murashige and Skoog; PDAD - photodiode array detector; PMP - 3-methyl-1-phenyl-2-pyrazolin-5-one; SPS - sucrose phosphate synthase; SS - sucrose synthase; UDPG - uridine diphosphate glucose.

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have focused mainly on the extraction and pharmacological analysis, while few studies have investigated their accumulation and biosynthesis. Herein, *D. officinale* seedlings were used to investigate the regulatory effects of exogenous methyl jasmonate on polysaccharide biosyntheses, enzyme activities, and the

expression of sucrose biosynthesis related genes. The results will provide a theoretical basis for efficient synthesis of polysaccharides in *D. officinale* as well as a further understanding of the regulatory mechanisms of plant secondary metabolism.

Materials and methods

Plants and methyl jasmonate treatments: The capsules of *Dendrobium officinale* Kimura *et* Migo were procured from the Yandang Mountains, ZheJiang province, China. Healthy seeds of uniform size were selected and surface was sterilized with 5 % (m/v) solution of NaClO for 10 min followed by repeated washing with distilled water in ultra clean cabinet. Sterilized seeds were sown into culture flasks containing Murashige and Skoog (MS) basal medium with 30 g dm⁻³ sucrose. After pre-cultivation for 2 months, uniform size seedlings with completely expanded cotyledons were selected and transplanted into glass containers containing MS medium (60 seedlings per container). The experiment was carried out under a photosynthetic photon flux density of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a 12-h photoperiod, day/night temperatures of 25/25 °C, and a relative humidity of 65 - 67 %. After 60 d of culture, seedlings with the height of 3 - 5 cm were transferred to new glass containers with 30 seedlings per container containing MS medium supplemented with MeJa at final concentrations 0 (control), 50, 100, 200, 500, and 1000 μM . Each treatment had three replicates. Every 6 d, samples (5 g) of fresh seedlings from every treatment were stored in -70 °C for further use.

Polysaccharide extraction was performed according to Zhang (2011) with some modifications. The seedlings were ground to a powder after being dried in an oven (60 °C, 3 d). The crushed powder (40 meshes) was extracted with petroleum ether at 70 °C for 1 h thrice and filtered. The residue was hydrolyzed with 0.5 % (m/v) cellulase solution at 50 °C for 2 h, and then further extracted with 90 % (v/v) ethanol at 90 °C for 0.5 h. After each extraction, the solutions were separated from residues through filtration, and then extracted with double-distilled water at 25 °C for 2 h thrice. After concentrated using a rotary evaporator at 55 °C, the polysaccharide extracts were precipitated by adding 5-fold volume of ethanol at 4 °C for 24 h, followed by centrifugation at 1 500 g for 20 min. The precipitate was lyophilized to yield the crude extract. The polysaccharides was washed successively with ethyl acetate and acetone, and then dissolved in water, and then lyophilized to yield the purified polysaccharides.

The polysaccharide content was determined by the phenol-sulfuric acid method (Dubois *et al.* 1956). The

colour reaction was initiated by mixing 2 cm³ of polysaccharide solution with 1 cm³ of a 10 % (m/v) phenol solution and 5 cm³ of concentrated sulfuric acid. The reaction mixture was incubated in a boiling water bath for 15 min. After cooling to the room temperature, the absorbance (A) of the mixture was determined at 490 nm, and the total sugar content was calculated using D-glucose as a standard.

Determination of enzymes: Fresh samples of leaves were ground until obtaining a fine powder in the presence of liquid N₂, ice-cold 100 mM K-phosphate buffer (pH 7.0) containing 1 mM EDTA, and 1 mM phenyl methyl sulphonyl fluoride. After centrifugation at 14 000 g for 30 min, supernatant was collected and used as enzyme extract. The protein amount in the extract was determined by Bradford's method.

The activity of sucrose phosphate synthase (SPS) was assayed by the method of Lowell *et al.* (1989) with some modifications. SPS activity was measured by addition of 50 cm³ of dialysed enzyme preparation to 50 cm³ of a reaction mixture containing 100 mM 3-(morpholino) propanesulfonic acid (MOPS)-NaOH (pH 7.5), 20 mM glucose-6-phosphate, 4 mM fructose-6-phosphate, 3 mM uridine diphosphate glucose (UDPG), 5 mM MgCl₂, and 1 mM EDTA (reaction without UDPG served as a negative control).

The activity of sucrose synthase (SS) in sucrose synthesis direction was assayed by the method of Hubbard *et al.* (1989) with some modifications. Enzyme assay comprised of 50 cm³ of dialyzed enzyme preparation and 50 cm³ of a reaction mixture containing 50 mM MOPS-NaOH (pH 7.5), 15 mM MgCl₂, 25 mM fructose and 25 mM UDPG (reaction without UDPG served as a negative control).

Both the above mentioned reaction mixtures were incubated at 37 °C in a circulating water bath (60 min for SPS assay and 30 min for SS assay). The reactions were stopped by boiling in hot water bath for 3 min and allowed to cool to room temperature. Aliquots of 70 cm³ of the reaction mixture were reacted with equal volume of 30 % (m/v) KOH and boiled for 10 min and finally allowed to cool to room temperature. To this, 1 cm³ of freshly prepared anthrone reagent (containing 76 cm³ of H₂SO₄, 30 cm³ of distilled water and 150 mg of anthrone) was added and the reactions were further incubated at

37 °C for 20 min. Absorbance (A_{650}) was measured immediately in a microtiter plate using a plate reader (*Varioskan Flash*, *Thermo Fisher Scientific*, Waltham, USA). The amount of produced sucrose was used to estimate the enzyme activity.

Hydrolysis of polysaccharides and derivatization of monosaccharides: The standard reference substance glucosamine hydrochloride, D-glucose, and D-mannose were dissolved in double-distilled water, and concentrations were 12 mg cm⁻³, 0.1 mg cm⁻³, and 0.1 mg cm⁻³, respectively. Polysaccharides of samples were dissolved in 10 cm³ of double-distilled water and were treated with 5 cm³ of 3.0 M HCl at 110 °C for 1 h. Samples were cooled to room temperature and pH was adjusted to 7.0 with 3.0 M NaOH.

According to Goubet *et al.* (2002), derivatization was carried out in tubes containing standards and polysaccharides. The hydrolyzed polysaccharide samples (0.4 cm³) were placed in the 2.0-cm³ centrifuge tubes, and then 0.4 cm³ of 0.5 M methanol solution of 3-methyl-1-phenyl-2-pyrazolin-5-one (PMP) and 0.4 cm³ of 0.3 M NaOH were added to each. The mixture was allowed to react in a water bath at 70 °C for 100 min, then cooled to room temperature, and neutralized with 0.2 cm³ of 0.3 M HCl. The resulting solution was separated by liquid-liquid extraction using a chloroform (three times, 2 cm³). After being shaken vigorously and centrifuged, the organic phase was carefully discarded to remove the excess reagents. Then, the aqueous layer was analyzed by high performance liquid chromatography (HPLC).

The analyses were performed with *Waters HPLC system 600* (Milford, MA, USA) equipped with photodiode array detector (PDAD) operating at 250 nm. The standard or supernatant was filtered and a 0.02 cm³ aliquot was injected onto the HPLC. Separations were carried out with symmetry column C18 (250 × 4.6 mm, 5 µm particle) and isocratic elution with a mobile phase of 20 µM ammonium acetate and acetonitrile in a ratio of 90:10 (v/v). The gradient elution mode separation was programmed, 0 - 20 min (B: 17 + 17 %), 20 - 30 min

(B: 17 + 37 %) with a column temperature of 30 °C and detection wavelength of 250 nm. The flow rate was set at 1.0 cm³ min⁻¹. The glucose and mannose content of polysaccharides were quantified by a calibration curve with glucosamine hydrochloride as internal standard.

Gene expression analysis: Real-time quantitative PCR was used to monitor the expressions of the *SPS* and *SS* genes at different times after treatments by different MeJA concentrations. Total RNA was extracted from leaves with the RNA prep pure kit (*Tiagen*, Beijing, China) and cDNAs used for PCR were synthesized with cDNA synthesis kit (*Fermentas*, Pittsburgh, USA). The *18S rRNA* gene was used as the internal reference gene. The PCR primers used were as follows: *SPS* forward, 5'-GTCTTGAAAGGGAACGAG-3', *SPS* reverse, 5'-GATAACATTTAGCCTGGTG-3', *SS* forward, 5'-GACATCGTCTTGAAAGGGAA-3', *SS* reverse, 5'-CACCACCTTTCTCCACGA-3', *18S rRNA* forward, 5'-CCAGGTCCAGACATAGTAAG-3', and *18S rRNA* reverse, 5'-GTACAAAGGGCAGGGACGTA-3'. For each primer/cDNA sample combination, PCRs were performed six-times. Relative quantitative real-time PCR was carried out on a *7500 Real-Time PCR System* (*Applied Biosystems*, Santa Clara, CA, USA) using a *SYBR®Green Real-Time PCR Master Mix* (*Toyobo*, Qsaka, Japan) following the manufacturer protocol. Cycling parameters were: 50 °C for 2 min, 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 1 min. Dissociation curves were analyzed at the end of each run to determine the purity of product and specificity of amplification.

Experimental design and statistical analysis: A completely randomized design was used for all treatments. All the experiments were repeated three times for each treatment. Data were statistically analyzed using analysis of variance (*ANOVA*). The mean separations were carried out using Duncan's multiple range tests and significance was determined at 0.5 % level.

Results and discussion

The polysaccharides were obtained from the seedlings of *D. officinale* by the method of water-extraction and ethanol-precipitation. The cellulase method was mild and effective in breaking cell wall, promoting the polysaccharide solubility and improving the yield. Purified polysaccharides were detected by the phenol-sulfuric acid method. Addition of MeJA significantly increased the content of polysaccharides in a concentration-dependent manner (Fig. 1). The maximum content of polysaccharides was obtained by adding 200 µM MeJA. Nevertheless, when the concentration of

MeJA was increased up to 500 and 1000 µmol dm⁻³, the polysaccharide production decreased. The polysaccharide production increased with the culture time. In addition, the low concentrations of MeJA promoted seedling growth and high concentrations of MeJA inhibited their growth (Fig. 2).

The activities of *SPS* and *SS* in *D. officinale* were significantly enhanced after the seedlings were treated with 50, 100, and 200 µM MeJA. But the *SPS* activity decreased after treatment with 500 and 1000 µM MeJA. The *SS* activity decreased after treatment with 1 000 µM

MeJA. Thus, the maximum SPS and SS activities were obtained by adding 200 μ M MeJA (Fig. 3). The results were consistent with polysaccharide content determination. As the polysaccharides affect pharmacological values of *Dendrobium*, their purification and structural elucidation are still needed. MeJA increased the

polysaccharide content of *Dendrobium*, but further research is needed to find its specific effects on polysaccharides components. MeJA regulated SPD and SS activities but as concern other enzymes involved in sugar metabolism (Zhang *et al.* 2003) further studies are needed.

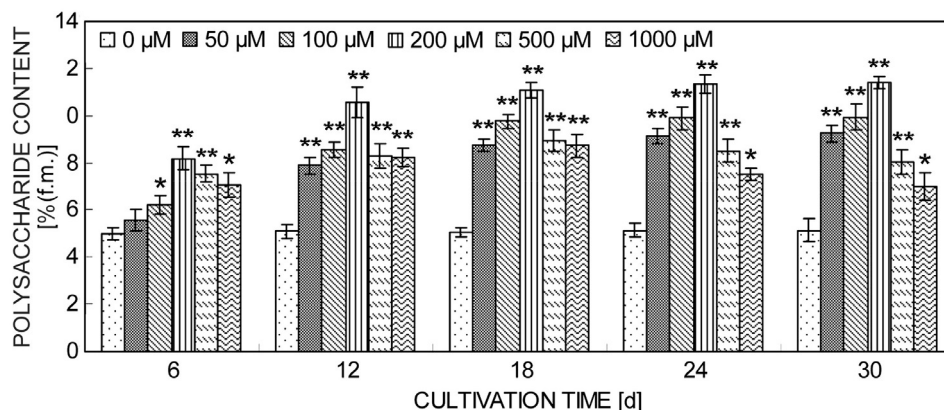


Fig. 1. Effect of different concentrations of methyl jasmonate on polysaccharide content in *Dendrobium officinale* seedlings. Means \pm SEs of three independent experiments. *,** - indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, according to Duncan's multiple range test.



Fig. 2. Effect of methyl jasmonate on seedlings of *D. officinale*: A - control, B - 50 μ M, C - 100 μ M, D - 200 μ M, E - 500 μ M, and F - 1 000 μ M MeJA.

HPLC was often employed to determine the content of polysaccharides. The derivatization and chromatographic conditions were optimized to obtain chromatograms with a good resolution of adjacent peaks within a short analysis time. The monosaccharide composition of the polysaccharides is mainly mannose and glucose (Xia *et al.* 2012). The concentration of derivatizing reagent PMP should be adequate enough to guarantee the sufficient reaction of the analytes. In this study, the introduction of PMP into the molecules of monosaccharides increased the hydrophobicity of the analytes and made them elute at increased retention times. Thus, the interaction between the analytes and the column was greatly increased. A large excess of PMP was used for derivatization reaction, therefore, removal of residuary reagent by chloroform extraction before HPLC

analysis was necessary. The effects of derivatization reaction time on peak area of derivative products in HPLC were essential. PMP derivatization reaction time was usually selected to be 100 min and the peak area of derivative of each sugar exhibited the maximum (Dai *et al.* 2010). This method was validated to be sensitive, precise and accurate, and has been successfully applied to the quantitative determination of monosaccharide components in polysaccharide samples. The content of monosaccharides was calculated according to the peak area. There was a considerable variation in the yield and ratio of monosaccharides depending upon the MeJA concentration (Fig 4). The content of glucose and mannose significantly increased with increased MeJA concentration up to 200 μ M and also with the culture time.

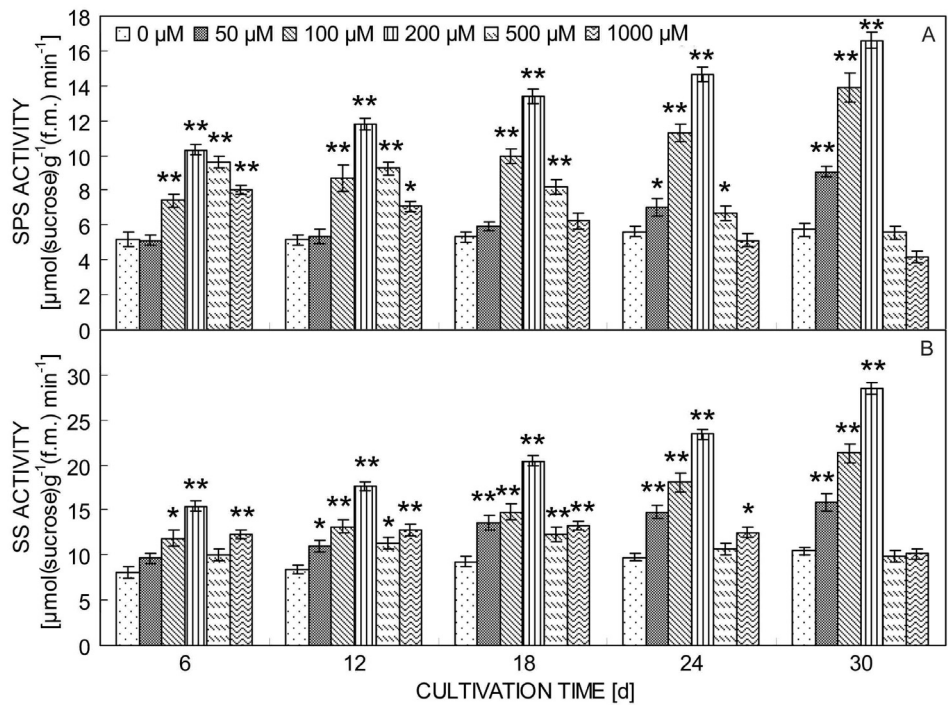


Fig. 3. Effect of methyl jasmonate on SPS (A) and SS (B) activities in *D. officinale* seedlings. Means \pm SEs of three independent experiments. *, ** - indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, according to Duncan's multiple range test.

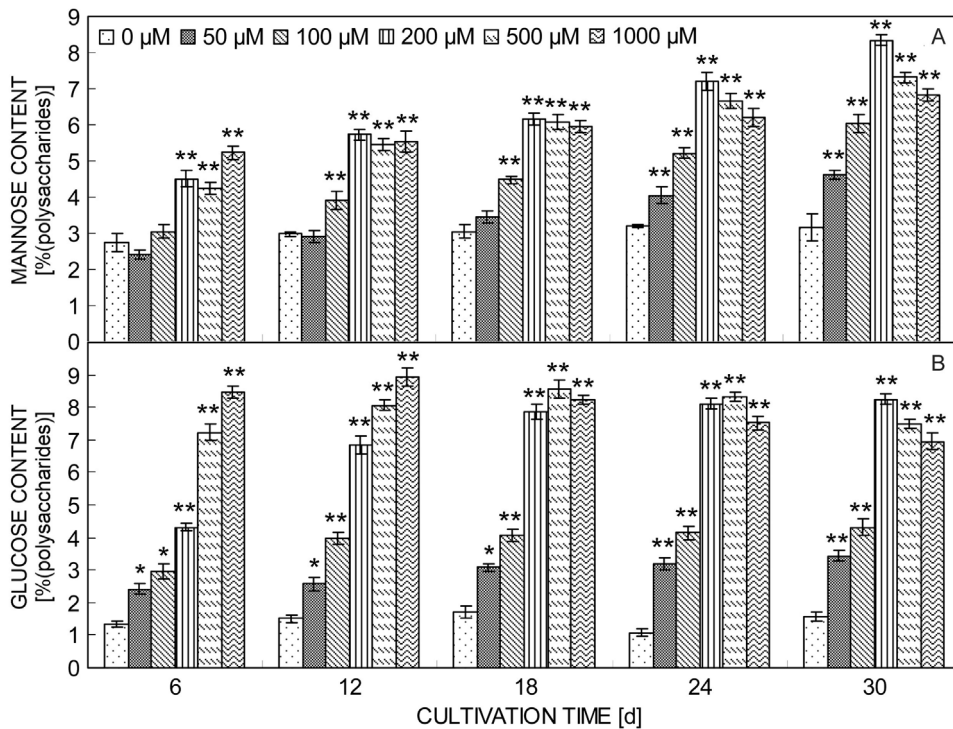


Fig. 4. Effect of different concentrations of methyl jasmonate on mannose (A) and glucose (B) content in *D. officinale* polysaccharides determined by HPLC. Means \pm SEs of three independent experiments. *, ** - indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, according to Duncan's multiple range test.

The expression of sucrose metabolism related genes, such as *SPS* and *SS*, significantly increased after the addition of MeJA (Fig. 5). This suggests that MeJA enhanced sucrose accumulation in *D. officinale* was

caused by regulating the expressions of sucrose biosynthetic genes. Nevertheless, when the concentration

of MeJA was increased up to 500 and 1000 $\mu\text{mol dm}^{-3}$, the expression of sucrose synthetic genes was inhibited.

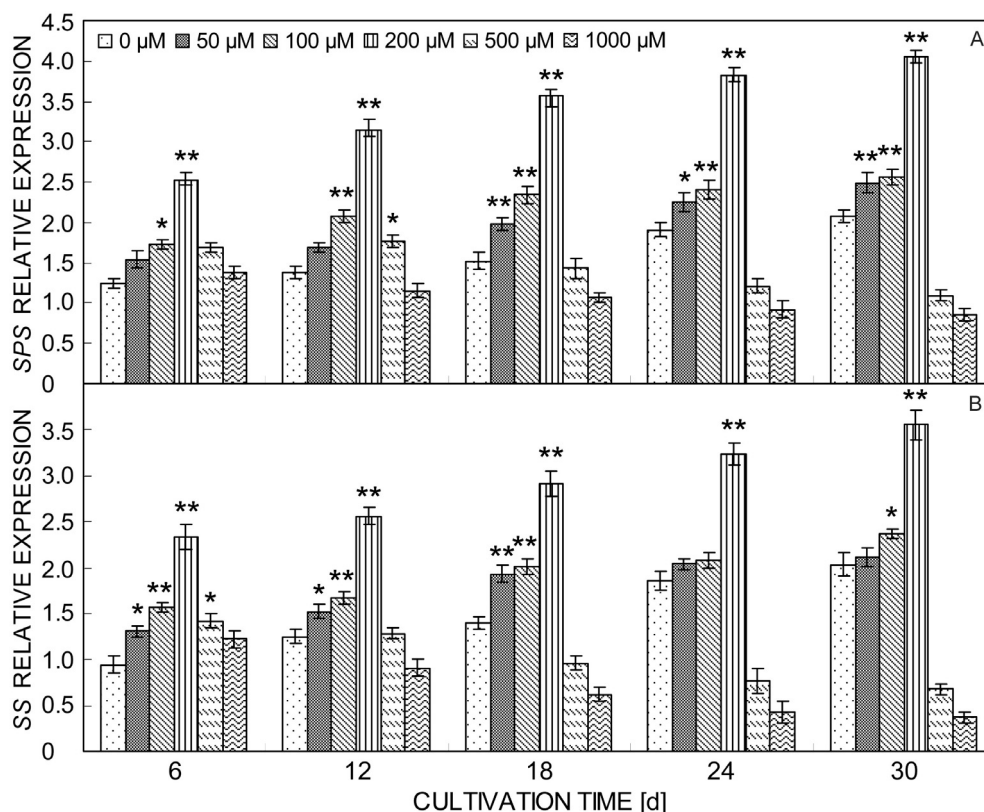


Fig. 5. Effect of methyl jasmonate on genes expression of *SPS* (A) and *SS* (B) in *D. officinale*. Means \pm SEs of three independent experiments. *, ** - indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, according to Duncan's multiple range test.

The polysaccharides in this species were composed mainly from mannose, glucose, galactose, fructose, and xylose. These monosaccharides were produced by sucrose hydrolysis. Thus, sucrose content was directly correlated with polysaccharide content. We found that MeJA influenced the content of polysaccharides, the activities of SPS and SS and *SPS* and *SS* genes expression. To further understand the molecular mechanisms for the polysaccharide syntheses, the

promoter regions and tissue-specific expressions of *SPS* and *SS* will be analysed in the future. In addition, there is evidence that more than one *SPS* gene and *SS* gene family exist in flowering plants (Okamura *et al.* 2011, An *et al.* 2014). So the number and expression profiles of *SPS* gene and *SS* gene in *D. officinale* will be analyzed in the future. These studies should provide information for a better understanding of polysaccharide synthesis mechanisms.

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