Bangladesh J. Bot. **47**(3): 479-485, 2018 (September)

EFFECTS OF SMOKE-WATER AND SMOKE-DERIVED BUTENOLIDE ON ACCUMULATION OF PHENOLIC ACIDS IN CULTURED HAIRY ROOTS OF *SALVIA MILTIORRHIZA* BUNG

JIE ZHOU, ZIXIN XU, ZHIFANG RAN[1], LEI FANG* AND LANPING GUO [2]

School of Biological Science and Technology, University of Jinan, Jinan 250022, PR China

Keywords: Salvia miltiorrhiza, Smoke-water, Butenolide, Phenolic acids, Hairy root culture

Abstract

Smoke-water (SW) and biologically active compound butenolide (KAR_1) isolated from smoke show stimulating effects on the plant growth of agricultural and horticultural crops, while little has been known on their effects on the secondary metabolism of medicinal plants. Hairy root culture of *Salvia miltiorrhiza*, an important medicinal plant, was treated with SW and butenolide. The results showed that the contents of salvianolic acid (SAB) and rosmarinic acid (RA) were enhanced with the treatments of SW and KAR_1. The expressions of genes (PAL, C4H, 4CL, TAT and HPPR) involved in phenolic acids biosynthesis were upregulated by treatments of SW and KAR_1. Responses of C4H and 4CL to SW and KAR_1 treatment were later than PAL, TAT and HPPR. It suggests that SW and KAR_1have the potential to be used as elicitor to increase the accumulation of secondary metabolites in *S. miltiorrhiza* hairy root.

Introduction

Salvia miltiorrhiza, called Danshen in Chinese, is one of the most important medicinal plants in traditional Chinese medicine (TCM) (Wang *et al*. 2016, Han *et al*. 2015, Su *et al*. 2015, Liu *et al*. 2011). It has been used for removing blood stasis, clearing away the heart-fire, relieving restlessness, cooling blood and eliminating carbuncle (Dowom *et al*. 2017). Bioactive compounds in *S. miltiorrhiza* mainly contain water-soluble phenolic acids and lipid-soluble tanshinones (Han *et al*. 2015). Phenolic acids have increasingly attracted the attention due to their marked pharmacological activities coupled with their traditional use from herbs steeped in boiling water in China (Zhang *et al*. 2014). Rosmarinic acid (RA) and salvianolic acid B (SAB), belonging to phenolic acids, are excellent antioxidants, used in the treatment of cardiovascular and cerebrovascular diseases, hepatic fibrosis and nephritis, and used as the index chemicals for quality control of *S. miltiorrhiza* (Song *et al*. 2015).

Hairy root culture obtained by infecting plant tissues with the natural occurring *Agrobacterium rhizogenes*, makes it convenient to improve the production of target metabolites by adding elicitors. So far hairy root culture of *S. miltiorrhiza* has been established and widely used for the mass production of bioactive components (Ming *et al*. 2013, Wu *et al*. 2016). In order to obtain a large number of phenolic acids, elicitors including yeast extracts, Ag^+ and methyl jasmonate have been widely studied to stimulate phenolic acids production in *S. miltiorrhiza* hairy root culture (Han *et al*. 2015). However, the content of phenolic acids in hairy root is often lower than that in mother plant, so various elicitations are being employed to increase the yield of bioactive components.

*Author for correspondence: < fleiv@163.com>. [1]School of Pharmaceutical Sciences, Shandong University of Traditional Chinese Medicine, Jinan 250355, PR China. [2]State Key Laboratory of Dao-di Herbs, National Resource Center for Chinese Medica, China Academy of Chinese Medical Sciences, Beijing 100700, PR China.

Plant-derived smoke-water (SW), which has been investigated to show stimulatory effects on seed germination and plant growth, has great potential for use in horticulture and agriculture. Butenolide (KAR_1), being considered as an active compound in SW, has been expected to be a new plant growth regulator (Martinez-Baniela *et al*. 2016, Morffy *et al*. 2016, Mojzes *et al*. 2015, Light *et al*. 2009). However, the effects of SW in promoting the accumulation of secondary metabolites in plants have not been well reported. The present research team has reported that the accumulation of indigo in *Isatis indigotica*, a Chinese medicinal plant could be improved with the treatment of SW (Zhou *et al*. 2011). Evaluation of SW and KAR_1 could be used as effective elicitors for improving the secondary metabolites yield in plants tissue culture. The effects and underlying mechanism of SW on phenolic acids production in *S. miltiorrhiza* are still not clear. In this study, effects of SW and KAR_1 on the accumulation of phenolic acids in *S. miltiorrhiza* hairy root were investigated. Expressions of key genes involved in the phenolic acids biosynthesis (PAL, C4H, 4CL, TAT and HPPR) were further detected to reveal the regulatory mechanism.

Materials and Methods

Smoke-water was prepared by continuously bubbling smoke from smouldering *Crataegus pinnatifida* and *Magnolia denudata* plant materials through 500 ml water for 45 min at a ratio of 6 kg of raw plant materials for 10 litre of distilled water (Light *et al*. 2009, Farley 2005, Flematti *et al*. 2004). Before burning, the materials were cut into small pieces and subjected to UV-B radiation with the dosages of 10 kJ/m^2 for 4 hrs at a distance of 30 cm above it and dried at 105°C for 24 to 48 hrs. Three SW dilutions were tested (v/v, 1 : 500, 1 : 1000 and 1: 2000). KAR_1 was isolated from SW by the method of Light *et al*. (2009) and 10^{-9} M was chosen for this experiment and the concentrations of SW and KAR_1 were tested in earlier experiments on *S. baicalensis* hair root culture.

S. miltiorrhiza hairy root culture was derived after infection of plantlets with *Agrobacterium rhizogenes* bacterium (ACCC10060). Successive transfer culture was conducted every 18 days. Experiments in this study were carried out in 250 ml shake flasks on an orbital shaker running at 110 rpm and 25°C kept in the dark. The SW and KAR_1 were applied to 18 day-old hairy root culture. The hairy root was harvested at 0, 1, 3, 5 and 9 days post-treatment, at which point the hairy root was harvested, respectively and fresh mass was recorded. Subsequently, an aliquot of the material was frozen in liquid nitrogen and stored at –80°C for analysis of expressions of genes. Another aliquot was oven-dried at 45℃ for determination of the content of phenolic acids. All experiments were performed in triplicate and the results were represented by their means ± standard deviation (Sd).

Extraction and qualitative analysis of phenolic acids in *S. miltiorrhiza* hairy root followed the methods described by Zhou *et al*. (2017) with minor modification. Briefly, the hairy root was dried until a constant weight was obtained, grounded into powder and sieved through a 0.45 mm screen. The sample powder (100 mg) was extracted with 20 ml of methanol: dichloromethane (3 : 1, v/v), sonicated for 1 hr and then kept at room temperature for 24 hrs. The supernatant was diluted with 70% methanol to 20 ml total volume, and the extract solution was filtered through a 0.45 µm organic membrane before injection into the HPLC system.

The qualitative analysis of phenolic acids was performed by an Agilent-1260 HPLC system with a ZORBAX SB-C18 chromatographic column (250 mm × 4.6 mm, 5 µm) at 30°C, a sample injection volume of 20 µl, flow rate of 0.8 ml/min, and a detection wavelength of 280 nm. A gradient elution of A (acetonitrile) and B (0.02% phosphoric acid solution) was used as followed: isocratic elution 95% B/5% A, 0 to 5 min; linear gradient from 95% B/5% A to 50% B/50% A, 5-55 min; isocratic elution 50% B/50% A, 55 to 65 min; linear gradient from 50% B/50% A to

95% B/5% A, 65 to 67 min. Standards of RA and SAB were purchased from the National Institute for the Control of Pharmaceutical and Biological Products.

Expressions of key enzyme genes PAL, C4H, 4CL, TAT and HPPR in the biosynthesis of phenolic acids were examined during the treatments. According to the manufacturer's scheme, total RNA was extracted from hairy root of *S. miltiorrhiza* using TRIzol reagent (Invitrogen). Primers were designed in order to detect transcript levels of PAL, C4H, 4CL, TAT and HPPR (Zhang *et al*. 2014). List of primer sequences was as follows: PAL forward primer 5´-ACCTACCTCGTCGCCCTATGC -3´; PAL reverse primer 5´- CCACGCGGATCAAGTCCTTCT -3´; C4H forward primer 5´- CCAGGAGTCCAAATAACAGAGCC -3´; 4CL reverse primer 5´-GAGCCACCAAGCGTTCACCAA-3´; 4CL forward primer 5´- ATTCGCATTCGCATTTCTCGG -3´; 4CL reverse primer 5´-GCGGCGTAGTGCTTCACCTTT -3´; TAT forward primer 5´- TTC AACGGCTACGCTCCAACT -3´; TAT reverse primer 5´- AAACGGACAATGCTATCTCAAT -3´; HPPR forward primer 5´- GACTCCAGAAACAACCCACATT -3´; HPPR reverse primer 5´-CCCAGACGACCCTCCACAAG -3´. As a control, part of the actin gene of *S. miltiorrhiza* (GenBank No. DQ243702) was amplified using the forward primer (5'-CTGCCGTGGAG AAGAACTAC-3') and reverse primer (5'-ATTCCAGCAGCTTCCATTCC-3'). PCR amplification was performed in a total volume of 50 µl. Real-time quantitative PCR was conducted in an thermocycler as follows: pre-denaturation for 4 min at 94°C for 1 cycle, denaturation for 15 s at 94°C and annealing for 60 sec at 60°C and extension72°C for 15 sec for 40 cycles, followed by 8 min at 72°C (Hao *et al*. 2016).

Statistical analyses (one-way ANOVA) were conducted using SPSS 20.0 (SPSS Inc., Chicago, IL, USA) and the differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Smoke-water at the concentrations of 1 : 500, 1 : 1000 and 1 : 2000 (v/v) and KAR_1 at the concentrations of 10^{-9} M were separately added into the culture to investigate their effects on the accumulation of phenolic acids in *S. miltiorrhiza* hairy root. Effects of smoke-water and KAR_1 on the accumulation of SAB and RA in *S. miltiorrhiza* hairy root were shown in Fig. 1. In this experiment the treatments of SW and KAR_1 markedly increased the content of SAB ($p < 0.05$). With the treatment of SW at dilution of 1 : 500 (v/v), the accumulation of SAB in hairy root was dramatically stimulated on day 1, 3, 5 and 9 post-treatment, which increased by 32-, 17-, 4- and 3-folds of that in the control, respectively (Fig. 1A). The content of SAB was found to enhance significantly by 2833.08, 1134.85, 224.74 and 199.09% ($p < 0.05$), respectively with the treatment of KAR_1 at 10^{-9} M. The accumulation of RA in *S. miltiorrhiza* hairy root was dramatically increased on day 1 and day 3 post-treatment, while it was mildly increased on day 9 (Fig. 1B). The highest contents of SAB and RA were observed at SW at dilution of 1 : 500 treatment and increased to 1.12 to 10.26-folds (SAB) and 4.40 to 32.99-folds (RA) of the control. Time course of the treatment showed that the contents of SAB and RA were higher than the control levels during all the periods.

Elicitors, which could induce disease-resistance of plants, have been applied to stimulate the production of bioactive ingredients in plants (Han *et al*. 2015). Water-soluble phenolic acids have been suggested to be responsible for passive defense against microbial invasion in *S. miltiorrhiza*, and the content of RA could be increased by yeast elicitor (Chen and Chen 2000). In this study accumulation of phenolic acids in *S. miltiorrhiza* hairy root was found to be promoted by smoke-water and KAR_1 treatments. *Tulbaghia ludwigiana* treated with SW (1 : 500, v/v) had significantly higher contents of phenolic, flavonoid and condensed tannin comparing to the control. These results suggest the potentiality of smoke-water and KAR_1 used as an external elicitor enhance the

phytochemical content in medicinal plants tissue culture (Okem *et al*. 2015). Over all, smoke-water was more effective than KAR_1 and achieved the higher content of SAB and RA in *S. miltiorrhiza* hairy root.

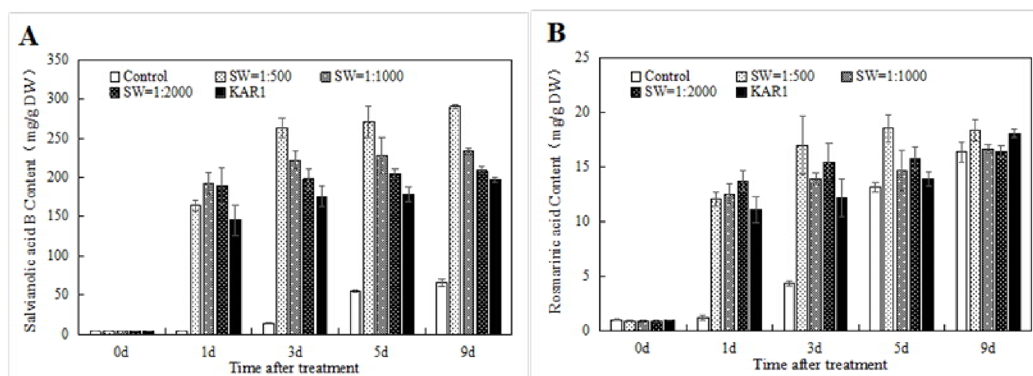

Fig.1. Effects of smoke-water and KAR_1 on the accumulation of salvianolic acid (A) and rosmarinic acid (B) in *S. miltiorrhiza* hairy root.

In order to evaluate the regulation mechanism of smoke-water and KAR_1 the content of phenolic acids in *S. miltiorrhiza* hairy root, the expressions of key enzyme gene PAL, C4H, 4CL, TAT and HPPR in the biosynthesis of phenolic acids were determined. As shown in Fig. 2, transcripts of PAL, key enzyme genes in the phenylpropanoid pathway, was enhanced with a maximum value by treatment of SW (1 : 1000) reaching about 14.82% (on 1 d) higher than the control. The expression of C4H was significantly enhanced by 23.40% ($p < 0.05$) and 17.96% ($p < 0.05$) on 5d post-treatment with the treatment of SW at dilution of 1 : 500 and 1 : 1000. The expression of HPPR was increased on 5d, which was evaluated as 24.69% (SW 1 : 500), 40.36% (SW 1 : 1000), 29.42% (SW 1 : 2000) and 31.80% (KAR_1), respectively. The SW at dilution of 1 : 500 treatment resulted in a significant increase in the expressions of HPPR by 44.84% ($p < 0.05$) on 9d compared to the control (Fig. 2E). The highest expression levels of TAT were observed on 5d and 9d, and increased to 50.02% (SW 1 : 500) and 36.48% (SW 1 : 500) when compared to the control. For 4CL, the highest expression level was observed to be 23.44% which was higher than the control on 3d, and then it gradually decreased to the control level on day 5 post-treatment. In comparison, transcripts of 4CL, TAT and HPPR were more sensitive to the SW and KAR_1 elicitation, with maximum level reaching about 1.28-, 1.45- and 1.40-folds of control at 5d respectively.

It has been reported that SAB is derived from RA, which is considered as the core structure of most hydrophilic active compounds. RA is biosynthesized by two parallel pathways, namely the phenylpropanoid pathway and the tyrosine-derived pathway. The phenylpropanoid pathway consists of three successive steps, catalyzed by phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and hydroxycinnamate coenzyme A ligase (4CL). And the tyrosine-derived pathway consists of two steps, catalyzed by tyrosine aminotransferase (TAT) and 4-hydroxyphenylpyruvate reductase (HPPR) (Zhang *et al*. 2014). Enzymes in the phenylpropanoid pathway (PAL, C4H and 4CL) are ubiquitous in land plants and represent branch points in formation of many different secondary metabolites, such as flavonoids, chlorogenic acid and anthocyanin. Enzymes in the tyrosine-derived pathway are related to biosynthesis of much fewer metabolites compared to the enzymes in the phenylpropanoid pathway. These results demonstrate

that following the addition of SW and KAR_1 to *S. miltiorrhiza* hairy root culture medium, the phenylpropanoid pathway and the tyrosine-derived pathway were activated. The enzymes in the secondary metabolic pathway show different responses to the treatments of SW and KAR_1. In this study, it was observed that expressions of PAL, C4H, 4CL, TAT and HPPR were upregulated by treatments of SW and KAR_1. Responses of C4H and 4CL to SW and KAR_1 treatment were later than PAL, TAT and HPPR.

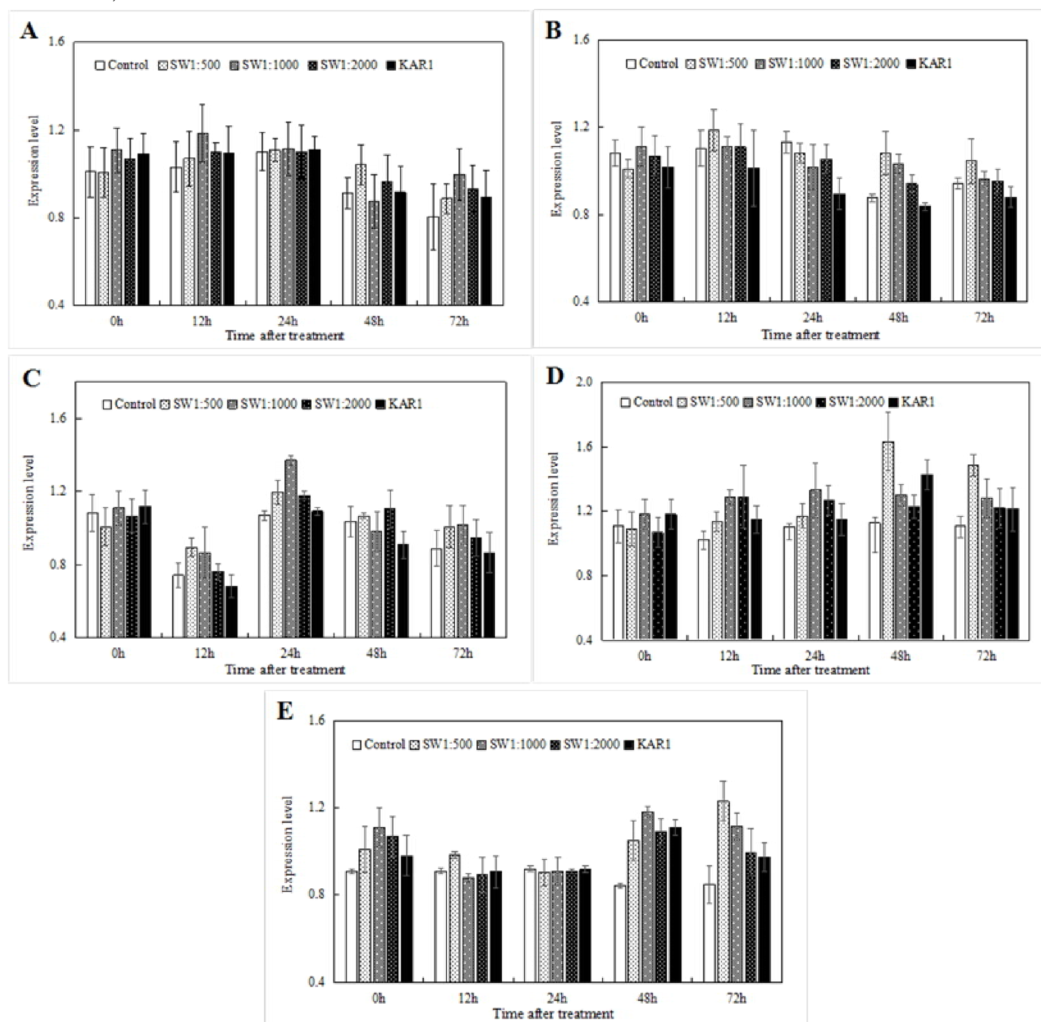

Fig.2. Effects of smoke-water and KAR_1 on the expression of key enzyme genes of PAL (A), C4H (B), 4CL (C), TAT (D) and HPPR (E) in the pathways of phenolic acids biosynthesis.

This work suggests that smoke-water and KAR_1 have the potential to be used as an elicitor to increase the accumulation of secondary metabolites in *S. miltiorrhiza* hairy root. This work provides insights into the mechanisms of biosynthesis of water-soluble phenolic acids in *S. miltiorrhiza*, and will encourage further research on the effects of smoke-water on the accumulation of secondary metabolite of herbal medicines.

Acknowledgements

This work was financially supported by Natural Science Foundation of China (81673527), Shandong provincial key research project (2017GSF19115, 2017CXGC1303), National key research and development project (2017YFC1702702, 2017YFC1700705) and the Construction Project for Sustainable Utilization of Valuable Traditional Chinese Medicine Resources (2060302).

References

Chen H and Chen F 2000. Effects of yeast elicitor on the growth and secondary metabolism of a high-tanshinone-producing line of the Ti transformed *Salvia miltiorrhiza* cells in suspension culture. Process Biochem. **35**: 837-840.

Dowom SA, Abrishamchi P, Radjabian T and Salami SA 2017. Enhanced phenolic acids production in regenerated shoot cultures of *Salvia virgata* Jacq. after elicitation with Ag^+ ions, methyl jasmonate and yeast extract. Ind. Crop. Prod. **103**: 81-88.

Farley GJ 2005. The implications of a reproducible method for making smoke water on seed dormancy studies. International Workshop on Seeds-germinating New Ideas. International Society for Seed Science 130-130.

Flematti GR, Ghisalberti EL, Dixon KW and Trengove RD 2004. A Compound from Smoke that Promotes Seed Germination. Science **305**: 977-997.

Han MY, Guo WL, Liang ZS , Yang DF, Yan XJ, Zhu YH and Liu Y 2015. Effects of cerous nitrate on growth and tanshinone production in *Salvia miltiorrhiza* hairy roots. J. Rare Earth. **33**: 1228-1235.

Hao GP, Jiang XY, Feng L, Tao R, Li YY and Huang LQ 2016. Cloning, molecular characterization and functional analysis of a putative R2R3-MYB transcription factor of the phenolic acid biosynthetic pathway in *S. miltiorrhiza* Bge. f. alba. Plant Cell Tiss. Org. Cult. **124**: 151-168.

Light ME, Daws MI and Staden JV 2009. Smoke-derived butenolide: Towards understanding its biological effects. S. Afr. J. Bot. **75**: 1-7.

Liu HY, Wang XD, Wang DH, Zou ZR and Liang ZS 2011. Effect of drought stress on growth and accumulation of active constituents in *Salvia miltiorrhiza* Bunge. Ind. Crop. Prod. **33**: 84-88.

Martinez-Baniela M, Carlon L, Diaz TE, Bueno A and Fernandez-Pascual E 2016. Plant-derived smoke and temperature effects on seed germination of five *Helianthemum* (Cistaceae). Flora **223**: 56-61.

Ming QL, Su CY, Zheng CJ, Jia M, Zhang QY, Zhang H, Khalid R, Han T and Qin LP 2013. Elicitors from the endophytic fungus *Trichoderma atroviride* promote *Salvia miltiorrhiza* hairy root growth and tanshinone biosynthesis. J. Exp. Bot. **64**: 5687-5694.

Mojzes A, Csontos P and Kalapos T 2015. Is the positive response of seed germination to plant-derived smoke associated with plant traits?. Acta Oecol **65-66**: 24-31.

Morffy N, Faure L and Nelson DC 2016. Smoke and hormone mirrors: Action and evolution of karrikin and strigolactone signaling. Trends Genet **32**: 176-188.

Okem A, Kulkarni MG and Staden JV 2015. Enhancing phytoremediation potential of *Pennisetum clandestinum* Hochst in cadmium-contaminated soil using smoke-water and smoke-isolated karrikinolide. Int. J. Phytorem. **17**: 1046-1052.

Song ZQ and Li XF 2015. Expression profiles of rosmarinic acid biosynthesis genes in two *Salvia miltiorrhiza* lines with differing water-soluble phenolic contents. Ind. Crops. Prod. **71**: 24-30.

Su CY, Ming QL, Khalid R, Han T and Qin LP 2015. *Salvia miltiorrhiza*: Traditional medicinal uses, chemistry, and pharmacology. Chin J. Nat. Med. **13**: 0163-0182.

Wang YJ, Ye S, Zhuo S, Le Z, Ning DL, Chao J, Rong Z and Huang LQ 2016. Comparative proteomic analysis of the response to silver ions and yeast extract in *Salvia miltiorrhiza* hairy root cultures. Plant Physiol. Biochem. **107**: 364-373.

Wu CF, Bohnert S, Thines E and Efferth T 2016. Cytotoxicity of *Salvia miltiorrhiza* Against Multidrug-Resistant Cancer Cells. Amer. J. Chin. Med. **44**: 871-894.

Zhang SC, Yan Y, Wang BQ, Liang ZS, Liu Y and Liu FH 2014. Selective responses of enzymes in the two parallel pathways of rosmarinic acid biosynthetic pathway to elicitors in *Salvia miltiorrhiza* hairy root cultures. J. Biosci. Bioeng. **117**: 645-651.

Zhang Y, Yan YP, Wu YC, Hua WP, Chen C, Ge Q and Wang ZZ 2014. Pathway engineering for phenolic acid accumulations in *Salvia miltiorrhiza* by combinational genetic manipulation. Metab. Eng. **21**: 71-80.

Zhou J, Staden JV, Guo LP and Huang LQ 2011. Smoke-water improves shoot growth and indigo accumulation in shoots of *Isatis indigotica* seedlings. S. Afr. J. Bot. **77**: 787-789.

Zhou W, Huang Q, Wu X, Zhou ZW, Ding MQ, Shi M, Huang FF, Li S, Wang Y and Kai GY 2017. Comprehensive transcriptome profiling of *Salvia miltiorrhiza* for discovery of genes associated with the biosynthesis of tanshinones and phenolic acids. Sci. Rep. **7**: 10554.

(Manuscript received on 26 March, 2018; revised on 24 April, 2018)