BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, December 2015. Vol. **12**(3), 3061-3066

Influence of Acibenzolar-*S*-methylon on the Expression of Phenylpropanoid Biosynthetic Genes and the Accumulation of Phenylpropanoids in *Agastache rugosa*

Woo Tae Park[1], Sun Kyung Yeo[1], Thanislas BastinBaskar[1], Mariadhas Valan Arasu[2], Naif Abdullah Al-Dhabi[2], Jong Seok Park[3]* and Sang Un Park[1]*

[1]Department of Crop Science, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon, 34134, Korea
[2]Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia
[3]Department of Horticulture, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 34134, Korea.

DOI: http://dx.doi.org/10.13005/bbra/1989

(Received: 15 June 2015; accepted: 12 August 2015)

***Agastacherugosa*, (Korean mint), contains sesquiterpenes, essential oils, diterpenes, flavonoids, triterpenes, and carotenoids that are used for the treatment of cancer. Medicinal plants can activate defensive mechanisms upon exposure to pathogens, various chemicals, or physical stress. The present study aimed to determine the expression levels of phenylpropanoid pathway genes and accumulation of phenylpropanoids in *A.rugosa* plantlets in response to acibenzolar-*S*-methyl (ASM) treatment.ASM treatment stimulated the expression of phenylpropanoid biosynthetic genes such as *PAL*, *C4H*, *CHS*, *CHI*, *HPPR*, *TAT*, and *RAS* after 1, 3, 5, and 7 days of cultivation. The expression pattern of the upstream and downstream phenylpropanoid biosynthetic genes was directly proportional to the ASM exposure times. In particular, the expression level of the *RAS* gene was 1.59-, 2.88-, 1.36-, and 1.41-fold higher at 1, 3, 5, and 7 days after ASM treatment, when compared to respective controls. The levels of rosmarinic acid, tilianin, and acacetin accumulation were comparatively2.28-, 1.88-, and 1.61-fold higher than those of the control after 7 days of ASM treatment. Among the phenylpropanoids examined, rosmarinic acid was highest (5 mg/g dry weight) in the control and ASM-treated plantlets. Our results indicated that ASM enhances the expression of genes related to phenylpropanoids and accumulation of phenylpropanoids during the development of *A. rugosa* plantlets.**

Key words: *Agastacherugosa,* Acibenzolar-*S*-methyl, Phenylpropanoid genes, Rosmarinic acid, Tilianin.

Microbial pathogens such as bacteria, fungi, and viruses present in the soil and environments continuously attack plants irrespective of the plant family. Following a microbial attack, the defense mechanisms of plants, such as cell wall lignifications, papilla formation, and rapid changes in specific gene expression levels and subsequent synthesis of defensive proteins, are stimulated[1]. The proteins produced after infection have specific surface markers for systemic acquired resistance (SAR)[2],which is similar to the inborn immune system found in animals. This resistance is shown both at the site of pathogen attack and in uninfected parts

* To whom all correspondence should be addressed.
Phone: +82-42-821-5730; Fax: +82-42-822-2631;
E-mail: supark@cnu.ac.kr

of the plant. SAR is beneficial for plants not only in providing resistance to disease but also for recovery from disease once it has developed[3]. SAR can be caused by a wide range of pathogens and the resistance developed following the induction of SAR is effectual against a wide range of pathogens[4]. SAR has been reported in a wide range of flowering plants, including both dicotyledons and monocotyledons[5,6].

Various organic and inorganic compounds, such as 2,6-dichloroisonicotinic acid (INA) acibenzolar-*S*-methyl (ASM), and benzothiadiazole (BTH) have been employed as SAR inducers. Among these, acibenzolar-*S*-methyl (ASM) is a synthetic molecule manufactured by Novartis (Switzerland),and its role as a plant defense activator has been demonstrated in a number of annual plants, including *Arabidopsis*[7], tobacco[8], maize[9] , wheat[10], and cucumber[11]. It is commercially developed as a plant health enhancer of annual crops under the name of Actigard® (Syngenta, Switzerland), and it has been reported that pre-treatment of cucumber plants with ASM increases *PAL* expression in plants treated with 100ìM ASM or greater concentrations (500ìM)[12]. Therefore, the present study aimed to investigate the effect of ASM on the enhancement of rosmarinic acid in *Agastacherugosa*. The present study also aimed to determine whether supplementation with ASM plays a role in the expression of phenylpropanoid pathway genes in *A. rugosa*.

MATERIALAND METHODS

Seed sterilization and germination

Seeds of *A. rugosa* were purchased from Aram Seed Company (Seoul, Korea). The collected seeds were washed with sterile distilled water and then surface-sterilized with 70% (v/v) ethanol for 1min,followed by a 4% (v/v) sodium hypochlorite solution containing a few drops of Tween 20 for 10min,and finally rinsed three times with sterilized distilled water. Single seeds were planted in plastic pots containing nursery box soil and germinated at 25°C under standard cool white fluorescent tubes using a 16-h photoperiod. After 1 month of germination, plantlets showing ordinary growth rate were used for ASM treatment.

ASM treatment

For ASM treatment, Actigard® (Syngenta, Switzerland), which stimulates SAR in a number of annual plants, was dissolved in distilled water (200mg/l).Immediately after preparation, ASM solutions were sprayed on the leaves of 1-month-old*A. rugosa*. To examine time effects, ASM-treated leaves were randomly collected at 0, 1, 3, 5, and 7 days after the treatment. The collected leaves were immediately frozen in liquid nitrogen, and stored at -80°C until used.

Total RNA extraction and cDNA preparation

Total RNA was extracted from collected samples using a modified Trizol method. Harvested plantlet samples were finely ground in liquid nitrogen using a mortar and pestle. One hundred grams of the ground sample was dissolved in 1ml of Trizol, to which 200ìl of chloroform was added for phase separation. The aqueous phase was collected and centrifuged at13,000 rpm using a micro-high-speed centrifuge (Micro 17TR, Hanil Science Medical, Korea) for 15min at 4°C to pellet RNA. The supernatant containing RNA was carefully collected and washed with 70% ethanol and the pellet was resuspended in DEPC water. The integrity of the RNA was determined using a NanoVue™ Plus Spectrophotometer (GE Healthcare, UK) and electrophoresis with formaldehyde RNA agarose gels.

Quantitative real-time PCR for gene expression analysis

First-strand DNA of the fragment was synthesized using 1ìg of total RNA by following kit instructions(ReverTra ace, Toyobo, Japan). The reverse-transcribed cDNA products were used as templates for gene expression analysis using gene-specific primers (Table 1). The expression level of each gene was presented as a relative expression, i.e., the Ct value of each gene was compared to that of a housekeeping gene (*Actin*). Quantitative real-time PCR (qRT-PCR) was performed using a CFX96 real-time system (BIO-RAD Laboratories, USA) with the 2× Real-Time PCR Smart mix (Bio FACT, Korea) under the following conditions: 95°C for 15min, followed by 40cycles of 95°C for 15 s, annealing for 15s at 55°C, and elongation for 20s at 72°C. Transcript levels were normalized relative to the *Actin* housekeeping gene. Three replications of each sample were used for real-time PCR analysis and significant differences between treatments were evaluated by standard deviation.

Analysis of phenylpropanoids by HPLC

Phenylpropanoids were quantified using high-performance liquid chromatography (HPLC).For the analysis, the samples were freeze-dried in a vacuum dryer for at least 48 h, ground into a fine powder using mortar and pestle, and then 100-mg samples were vortexes with 5ml of 100% methanol for 1 hat 60°C. Phenylpropanoids, namely rosmarinic acid and tilianin, were extracted using methanol. After centrifugation, the supernatant was filtered through a 0.45-µm PVDF filter (Whatman, GE Healthcare, UK) and the extracts were analyzed using an HPLC system (NS-4000, Futecs, Korea)and monitored using a UV detector at 340 nm and reverse-phase column (C18, 250 mm × 4.6 mm, 5ìm) (Prontosil, Bischoff, Germany) at 30°C. The mobile phase was a gradient mixture of absolute methanol and 0.1% (v/v) acetic acid in water. The flow rate was maintained at 1.0 ml/min and the sample injection volume was 20 ìl. The concentration of phenylpropanoids in samples was calculated using a standard curve. Standard compounds were purchased from Sigma-Aldrich Corporation (USA). Mean values were obtained from three independent replicates.

Statistical analysis

For qRT-PCR and HPLC statistical analysis, the data were analyzed using the statistical analysis software (SAS version 9.3, SAS Institute Inc., USA). All data are given as the average (mean) and standard deviation of triplicate experiments. The experimental data were subjected to an analysis of variance (ANOVA), and significant differences among the means were determined by Duncan's multiple-range test.

RESULTS AND DISCUSSION

Effect of ASM on phenylpropanoid biosynthetic pathway genes

The effect of ASM on *A. rugosa* plantlet phenylpropanoid biosynthetic pathway gene expression was determined by harvesting at 0, 1, 3, 5, 7 days after treatment. The expressions of genes (*PAL*, *C4H*, *CHS*, *CHI*, *HPPR*, *TAT*, and *RAS*) were determined in each treatment by using qRT-PCR (Fig. 1). Among the genes, *CHI* and *RAS* showed higher levels of expression in all the sampling periods. The results indicated that the transcript levels of *CHI* increased markedly with increasing time of exposure to ASM up to 3 days and then started to decline: the expression level was 1.62-, 6.44-, 2.38-, and 1.19-fold higher at 1, 3, 5, and 7 days after treatment. The expression level of *RAS* and *TAT* was 2.81, 4.42, and 2.64 times higher and directly proportional to the exposure time to ASM for 1, 3, and 5 days and then showed a moderate decline. The higher level of expression compared to the control was maintained up to 5 days after ASM treatment, but thereafter the expression level was lower than that of respective controls. Although the level of *C4H*expression was lower at 1 and 3 days, it then increased very sharply up to 7 days after treatment. The level of *C4H* expression was 2.9 and 5.3 times higher at 5 and 7 days after ASM treatment, respectively, compared to the respective controls. The level of *CHS* expression was the highest at 1 day after ASM treatment and then started to decline. The higher expression level of *CHS* was maintained up to 3 days after ASM treatment, and then the expression level became considerably lower than that of the respective control. The expression level of CHS was 4.74 and 2.04 times higher at 1 and 3 days after ASM treatment. For HPPR, the level of expression increased from 3 days after ASM treatment, continued to increase up to 5 days after treatment, and then declined sharply. The level of expression of HPPR was 2.03 and 2.72 times higher at 3 and 5 days after ASM treatment, respectively, compared to the respective controls.

Quantification of phenylpropanoids

The contents of phenylpropanoids, rosmarinic acid, tilianin, and acacetin present in the plantlets of *A. rugosa* treated with ASM were determined by harvesting after 0, 1, 3, 5, and 7 days of incubation (Figs. 2, 3, and 4). The levels of rosmarinic acid, tilianin, and acacetin increased both in plantlets treated with ASM and in the controls. However, the increasing rate was higher in cases of ASM treatment. The level of rosmarinic acid was 1.8-, 2.15-, 2.23-, and 2.28-fold higher at 1, 3, 5, and 7 days after ASM treatment. This increasing tendency was still apparent beyond 7 days after ASM. Similar to rosmarinic acid, the amount of tilianin accumulation was 1.62, 1.87, 1.82, and 1.88 times higher at 1, 3, 5, and 7 days after ASM treatment. The increasing levels of acacetin were slightly lower than those of rosmarinic acid and tilianin. This result revealed that the increasing tendency of acacetin production was observed for

up to 3 days after treatment, but then the accumulation tended to decline compared to the respective controls.

The results of this investigation showed that the production of rosmarinic acid, tilianin, and acacetin in *A. rugosa* plantlets was stimulated by ASM treatments. The quantified phenylpropanoids were present at maximum concentrations after 3 to 5 days of ASM treatment, whereas control plantlets produced the highest levels nearly 2 days later than those in the ASM treatment, indicating that the ASM treatment activated SAR, thereby stimulating accumulations of phenylpropanoids such as rosmarinic acid, tilianin, and acacetin.

Previously reported that addition of yeast elicitor to plant cells enhanced the production of rosmarinic acid, where as benzothiadiazole treatment stimulated the activation of SAR in plants. Benzothiadiazole can be used as a tool for

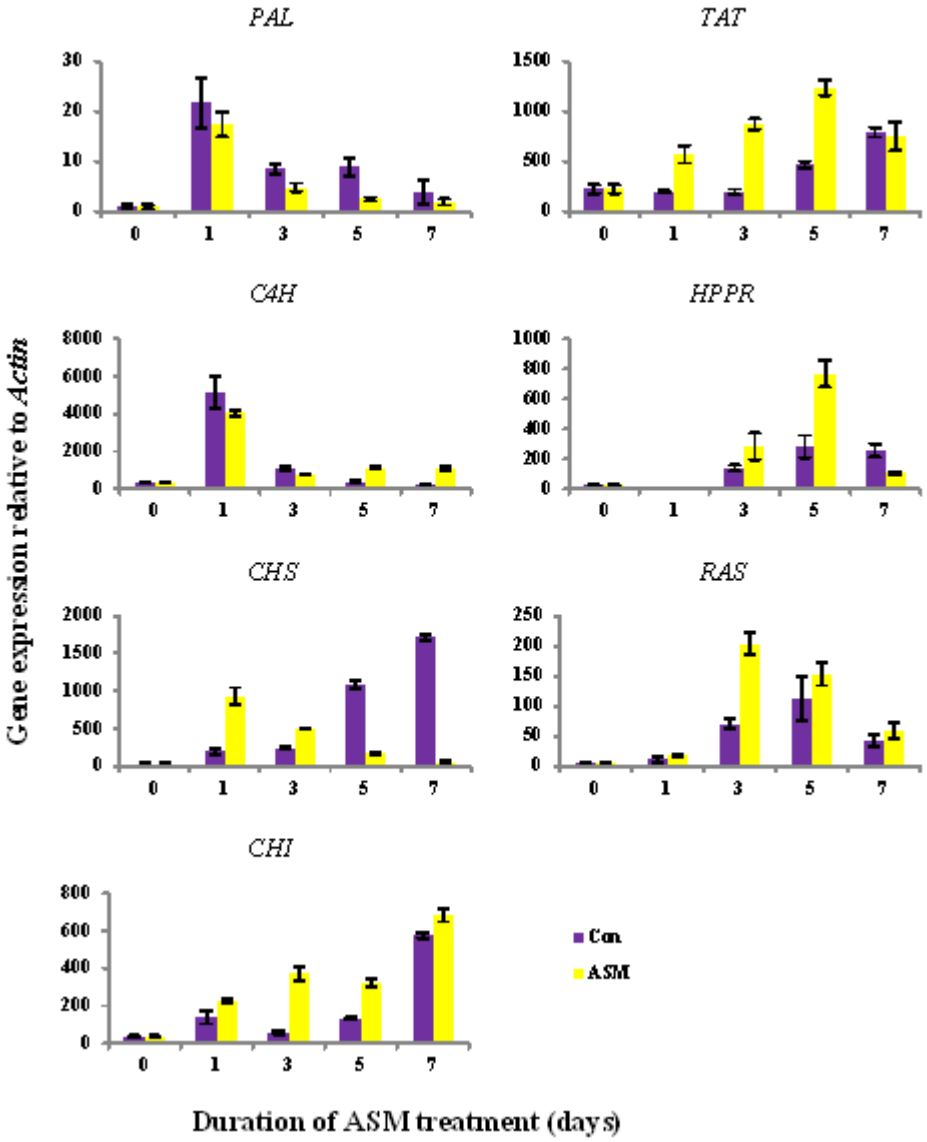

Fig. 1. Expression levels of phenylpropanoid biosynthetic genes in ASM-treated *Agastacherugosa* plantlets. Transcript levels from each of the three experimental groups were analyzed relative to that of actin. Error bars show standard deviation values

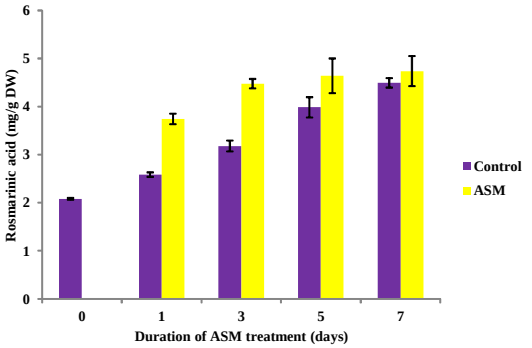

Fig. 2. Content of rosmarinic acid in ASM-treated *Agastacherugosa* plantlets. The level of accumulated rosmarinic acid in each of the three experimental groups was analyzed by HPLC. Error bars show standard deviation values. DW, dry weight.

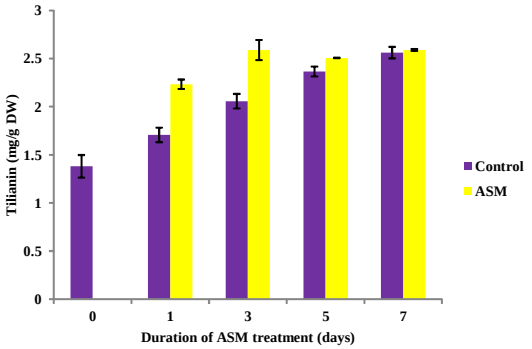

Fig. 3.Content of tilianin in ASM-treated *Agastacherugosa* plantlets. The level of accumulated tilianin in each of the three individual experimental groups was analyzed by HPLC. Error bars show standard deviation values. DW, dry weight

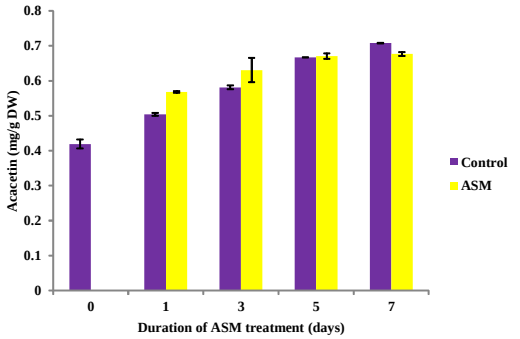

Fig. 4.Content of acacetin in ASM-treated *Agastacherugosa* plantlets. The level of accumulated acacetin in each of the three individual experimental groups was analyzed by HPLC. Error bars show standard deviation values. DW, dry weight

improving secondary metabolite accumulation in cell cultures, particularly that of phenylpropanoids[13]. Addition of methyl jasmonate (MeJa) also increases rosmarinic acid accumulation in suspension cell cultures of *Coleus blumei*[14] and *Lithospermumerythrorhizon*[15]. MeJa treatment might also be an efficient natural strategy to protect grapevine berries in vineyards[16]. Recently,[17] claimed that MeJa induced the production anthocyanin and glucosinolates in radish.

Exogenous salicylic acid treatment not only protects plants against stress but also enhances their growth and productivity[18]. Salicylic acid treatment leads to increased activities of PAL and soluble peroxidases, whereas cell wall-bound peroxidases were down-regulated in a concentration-dependent manner by salicylic acid[18]. These results provide evidence that the differences in phenylpropanoid metabolism induced by salicylic acid could provide as a defense system contributing to a reduction in oxidative cellular damage, as suggested by the high anti-lipid oxidation activity in *Cistus* extracts[18].

The high transcription levels of *PAL*, *C4H*, *CHS*, and *CHI* in ASM-treated *A. rugosa* may explain the high levels of acacetin and its derivative tilianin observed in the present study. Similarly, the low expression levels of *ArPAL*, *ArC4H*, and *Ar4CL* may be correlated with the trace amounts of tilianin and acacetin detected in roots[19]. The expression of *PAL*, *C4H*, and *4CL* has previously been correlated with flavonoid contents in various plants[20,21].

CONCLUSION

In conclusion, the application of ASM enhances the expression levels of the upstream and downstream genes of phenylpropanoid biosynthetic pathways. The quantification of the individual compounds confirmed that ASM treatment stimulated greater production of these compounds during the development of *A. rugosa* plantlets.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of trade, industry, and energy, Republic of Korea (Project No. 2015-0484-01).

REFERENCES

1. Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., Ryals, J. Acquired resistance in Arabidopsis. *Plant Cell.,* 1992; **4**: 645-656.
2. Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., Hunt, M.D. Systemic acquired resistance. *The Plant Cell.*, 1996; **8(10)**: 1809.
3. Braun-Kiewnick, A., Kiewnick, S., Jacobsen, B., Sands, D. Induction of systemic resistance by antagonistic *Bacillus* sp. and the chemical inducer benzothiadiazole controls *Cercospora* leaf spot of sugar beet. *Phytopathology*., 1998; **88**: S10.
4. Cohen, Y.R. β-aminobutyric acid-induced resistance against plant pathogens. *Plant disease*., 2002; **86(5)**: 448-457
5. Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science*., 1995; **270(5243)**: 1804-1806.
6. Gómez-Gómez, L. Boller, T. FLS2: An LRR receptor–like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular cell*., 2000; **5(6)**: 1003-1011.
7. Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., Ryals, J. Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *The Plant Journal*., 1996; **10(1)**: 71-82.
8. Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M.G., Meier, B., Dincher, S., Staub, T., Uknes, S. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *The Plant Journa.*, 1996; **10(1)**: 61-70.
9. Morris, S.W., Vernooij, B., Titatarn, S., Starrett, M., Thomas, S., Wiltse, C.C., Frederiksen,R.A., Bhandhufalck, A., Hulbert, S., Uknes, S. Induced resistance responses in maize. *Molecular plant-microbe interactions.*, 1998; **11(7)**: 643-658.
10. Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *The Plant Cell Online*., 1996; **8(4)**: 629-643.
11. Narusaka, Y., Narusaka, M., Horio, T., Ishii, H. Comparison of local and systemic induction of acquired disease resistance in cucumber plants treated with benzothiadiazoles or salicylic acid. *Plant and cell physiology.*, 1999; **40(4)**: 388-395.
12. Cools, H.J., and Ishii, H. Pre-treatment of cucumber plants with acibenzolar-S-methyl systemically primes a phenylalanine ammonia lyase gene (PAL1) for enhanced expression upon attack with a pathogenic fungus. *Physiological and Molecular Plant Pathology.,* (2002); **61**: 273-280.
13. Kim, H.K., Oh, S.R., Lee, H.K., Huh, H. Benzothiadiazole enhances the elicitation of rosmarinic acid production in a suspension culture of Agastacherugosa O. Kuntze. *Biotechnology letters.*, 2001; **23(1)**: 55-60.
14. Szabo, E., Thelen, A., Petersen, M. Fungal elicitor preparations and methyl jasmonate enhance rosmarinic acid accumulation in suspension cultures of Coleus blumei. *Plant cell reports*., 1999; **18(6)**: 485-489.
15. Mizukami, H., Ogawa, T., Ohashi, H., Ellis, B.E. Induction of rosmarinic acid biosynthesis in Lithospermum erythrorhizon cell suspension cultures by yeast extract. *Plant cell reports*., 1992; **11(9)**: 480-483.
16. Belhadj, A., Telef, N., Saigne, C., Cluzet, S., Barrieu, F., Hamdi, S., Mérillon, J.M. Effect of methyl jasmonate in combination with carbohydrates on gene expression of PR proteins, stilbene and anthocyanin accumulation in grapevine cell cultures. *Plant Physiology and Biochemistry.*, 2008; **46(4)**: 493-499.
17. Al-Dhabi, N.A., Arasu, M.V., Kim, S. J., Uddin, Md.R., Park, W.T., Lee S. Y., Park, S.U. Methyl jasmonate and light induced glucosinolate and anthocyanin biosynthesis in radish seedlings. *Natural Product Communications*., 2015; **10(7)**: 1211-1214.
18. López-Orenes, A., Martínez-Moreno, J.M., Calderón, A.A., Ferrer, M.A. Changes in phenolic metabolism in salicylic acid-treated shoots of Cistus heterophyllus. *Plant Cell, Tissue and Organ Culture (PCTOC).*, 2013; **113(3)**: 417-427.
19. Tuan, P.A., Park, W.T., Xu, H., Park, N.I., Park, S.U. Accumulation of tilianin and rosmarinic acid and expression of phenylpropanoid biosynthetic genes in Agastache rugosa. *J Agri Food Chem*., 2012; **60**: 5945–5951
20. Xu, F., Cai, R., Du, H., Wang, Y., Cheng, S. Molecular cloning, characterization and expression of phenylalanine ammonia-lyase gene from Ginkgo biloba. *Afr J Biotechnol*., 2008a; **7**: 721-729.
21. Singh, B.N., Singh, B.R., Singh, R.L. Polyphenolics from various extracts/fractions of red onion (Allium cepa) peel with potent anti-oxidant and antimutagenic activities. *Food and Chemical Toxicology*., 2009a; **47**: 1161-67.