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Influence of methyl jasmonate on ginsenoside biosynthesis in suspension cultures of *Panax quinquefolium* L.

SUMMARY

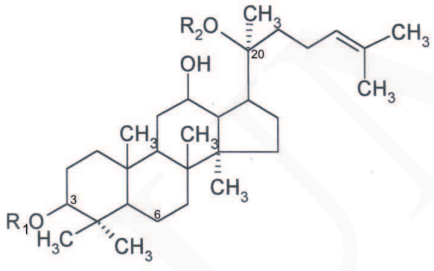
Panax quinquefolium L., belonging to the Araliaceae family, along with *P. ginseng* is one of the well-known species of ginseng. Multidirectional pharmacological action of this plant is attributed to triterpene saponins called ginsenosides. Pharmacopoeial raw material are roots obtained from the field crops which are time-consuming and require expensive agrotechnical procedures. Therefore, the new sources of ginseng biomass are sought such as *in vitro* suspension cultures. *P. quinquefolium* L. cell cultures, treated with the elicitation of methyl jasmonate (MJ) in concentration 50 and 250 μmol L^{-1}, synthesize more ginsenosides than control cultures. The highest increase (2.2-fold) of all examined compounds was noted using 250 μmol L^{-1} MJ. In this condition, the predominantly quantitative metabolite was Rb_1 ginsenoside belonging to protopanaxadiol derivatives.

Keywords: *Panax quinquefolium* suspension culture, methyl jasmonate, ginsenosides, elicitation

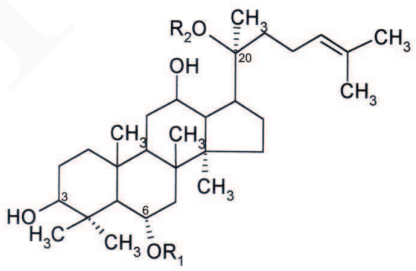
INTRODUCTION

American ginseng (*Panax quinquefolium* L.) is a herbaceous perennial plant in the *Araliaceae* family commonly used in traditional medicine. It is native to

eastern North America, though it is also cultivated in China (24). The pharmacological properties of ginseng are mainly attributed to ginseng saponins commonly named ginsenosides, their major and bioactive constituents. Ginsenosides are dammarane-type saponins and are classified into protopanaxadiol and protopanaxatriol groups which have a four-ring hydrophobic steroid-like structure with sugar moieties, but differ in the carbohydrate moieties at C3, C6 and C20 (Fig. 1). The metabolites such as Rb_1, Rb_2, Rc, Rd belong to protopanaxadiol derivatives. The protopanaxatriol derivatives are represented for example, by saponins Rg_1 and Re (Fig. 1) (25). Ginsenosides have been found to exhibit multiple pharmacological activities *via* different mechanisms and pathways *in vitro*, *in vivo* and clinical models (3, 4, 23). They are widely used as an antistress, anti-fatigue and anti-aging agents (9, 22). Moreover, ginsenosides have cardiovascular protection and neuroprotection activities (8, 10).



20(S) - protopanaxadiol	R_1	R_2
Rb_1	Glc-Glc-	Glc-Glc-
Rb_2	Glc-Glc-	Glc- Ara (p)
Rc	Glc-Glc-	Glc- Ara (f)
Rd	Glc-Glc-	Glc-



20(S) - protopanaxatriol	R_1	R_2
Re	H-	H-
Rg_1	Glc- Rha	Glc-
	Glc-	Glc-

Fig. 1. Structures of studied ginsenosides. Abbreviations mean: -Glc, D-glucopyranosyl; -Rha, L-rhamnopyranosyl; -Ara(f), L-arabinofuranosyl; -Ara(p), L-arabinopyranosyl

Pharmacopoeial raw material are the roots of ginseng that are mainly obtained from the field crops. However, the cultivation of ginseng is laborious and time-consuming; the seeds germinate slowly, the annual growth of the root is slight, and the plants are susceptible to infections with various fungal pathogens. Therefore, scientists have attempted to use plant cultures *in vitro* as an alternative and potentially more efficient source of ginseng biologically active compounds, especially since there were studies demonstrating that the biomass obtained by the biotechnological methods contained the same saponins as the roots originating from the field crops (27). Despite the many advantages of *in vitro* plant cultures, only few industrial cases have been implemented. Most often, these cultures pro-

duce an unsatisfactory amount of desired secondary metabolites. Therefore, a lot of research hes are conducted how to increase the production of these metabolites for example, through selection of high-yielding cell lines, modifications of the nutrient composition, addition of appropriate precursors, cell immobilization and elicitation (21). Elicitation uses the fact that many plants produce secondary metabolites in response to stress factors which may be elicitors. The elicitors are chemical compounds of various origins or physical factors that trigger the physiological response of plant cells. They play a special role in inducing the synthesis of low-molecular substances with antimicrobial activity called phytoalexins, which are the part of the plant defense system (25). Elicitors are divided into abiotic and biotic (21). The first group includes inorganic salts of some metals (e.g. Cu, Cd, Hg), physical factors (change in pH, radiation) and mechanical damage to tissues. The second group consists of substances of biological origin (homogenates and filtrates of fungal and bacterial cultures). Considering the place of elicitor formation, they can be divided into endogenous (of plant origin) or exogenous (derived from a pathogen or originating outside the cell under the influence of endogenous elicitors) (26).

Methyl jasmonate (MJ) is commonly used as abiotic elicitor to increase the efficiency of the biosynthesis of active compounds in plant cell and tissue cultures. For instance, MJ enhances the production of paclitaxel (5), *trans*-resveratrol (1), centellosides (2) or rosmarinic acid (16) in suspension cultures of *Taxus* x *media* Rehder, *Vitis vinifera* L*, Centella asiatica* (L.) Urban and *Mentha x piperita* respectively.

Methyl jasmonate has also been used for the elicitation of *P. ginseng* C.A. Meyer cell culture. Lu et al. (18) treated this culture of MJ in concentrations: 50, 200 and 500 μmol L^{-1} on the day of inoculation and on the 10^{th} day of culture. The greatest content of ginseng saponins (almost 28 times higher than in the control) was obtained using 500 μmol L^{-1} MJ added to the suspension on the day of the culture inoculation. In the other research, the best results were achieved using 200 μmol L^{-1} MJ – the suspension culture of *P. ginseng* produced 1.4 and 3 times more ginsenosides of the Rg group and the Rb group respectively, comparing to control (28).

To our knowledge, there is no data on the effect of MJ on the accumulation of ginsenosides in suspension cultures of *P. quinquefolium* L. The aim of this paper was to study the effect of MJ on ginsenosides production in cultures cultivated in the shake flasks. Moreover, the optimal concentration of the elicitor was also determined.

MATERIALS AND METHODS

SUSPENSION CULTURE CONDITION

Suspension culture of *P. quinquefolium* L. was initiated from four week-old callus tissue which grew in dark on solid woody-plant (WP) (18) medium with 1 mg l^{-1} 2.4-dichlorophenoxy acetic acid (2.4-D), 1 mg l^{-1}(alpha)-naphthalene acetic acid (NAA), 0.5 mg l^{-1} 6-benzylamino purine (BAP). About 3 g fresh weight of callus from passage XXIII, was transferred into Erlenmeyer flasks (300 ml) containing 50 ml of liquid Murashige and Skoog (MS) (19) medium with 1 mg l^{-1} 2.4D, 0.1 mg l^{-1} kinetin (kin) and pH 5.6-5.8. The medium was sterilized in the temperature of 123ºC and 1 atm. pressure for 21 min. The flasks were placed on the rotary shaker (100 rpm), in 26±2ºC temperature and 90% humidity and in dark for 40 days. The average fresh biomass of inoculum was 3.05 g l^{-1}, and average dry weight was 0.24 g l^{-1}.

METHYL JASMONATE TREATMENT

The MJ (95% purity, Sigma Aldrich) was added to the MS medium in day 28 of culture. Then, the suspension culture of *P. quinquefolium* L. produced maximum total content of examined ginsenosides according to the previously determined dynamics of ginsenoside biosynthesis of the studied culture (data not shown). MJ was prepared as a stock solution in 96% ethanol (POCH, Poland), it was sterilized through a millipore filter (Merck Millipore Ltd. pore size 0.20 µm) and then it was added to the liquid media at the final concentrations of 50, 250 and 500 µmol L^{-1}. Ethanol was added to the control media in the same volume (50 µl/flask) as individual concentrations of jasmonate. The effect of elicitation on ginsenoside accumulation in suspension cultures of *P. qiunquefolium* L. was measured at day 3 after methyl jasmonate treatment. The biomass of cultures was separated through filtration (using vacuum pomp) and dried at the room temperature. This material was used for ginsenoside extraction.

EXTRACTION PROCEDURES

The samples of 1±0.1 g of dry raw material were placed in 250 ml flasks. They were extracted three times with 50 ml of 80% methanol for 30 min at solvent boiling temperature under a reflux condenser. The combined methanol (POCH, Poland) extracts were evaporated to dryness in a vacuum evaporator under lowered pressure at 60ºC. The flask with dried residues was placed in a desiccator filled with drying agent. The dried methanolic extract was weighed.

GINSENOSIDE ANALYSIS USING HPLC METHOD

The dried extracts were dissolved in 1 ml of methanol (HPLC-grade, J.T. Baker, Netherlands) and filtered through 0.2 µm pore diameter Millex®-FG Hydrophobic Fluoropore filters (PTFE) (Sigma-Aldrich). The volume of 20 µl

was introduced to the liquid chromatography system (column C18 LiChroART® 250-4 (Merck), Waters 600 Controlled pump, UV-Vis 996 detector combined with Pentium 60PC hardware equipped with Millennium software). The mixture of acetonitrile (J.T. Baker, Netherlands) with water (J.T. Baker, Netherlands) was used as eluent. For determination of saponins Rb_1, Rb_2, Rc and Rd (all purchased from C. Roth GmbH+Co Karlsruhe, Germany), acetonitrile to water in ratio 30:70 was used, analysis time was 45 min and flow rate was 2 ml min^{-1}. The eluent for determination of metabolites Rg_1 and Re (both purchased from C. Roth GmbH+Co Karlsruhe, Germany) was acetonitrile to water in ratio 18:82, flow rate was 3 ml min^{-1}, analysis time was 40 min. The detection of ginsenosde was made at 203 nm wavelength. Ginsenosides were quantified (mg g^{-1} d.w.) by comparing retention time and peak areas between standards and samples.

STATISTICAL ANALYSIS

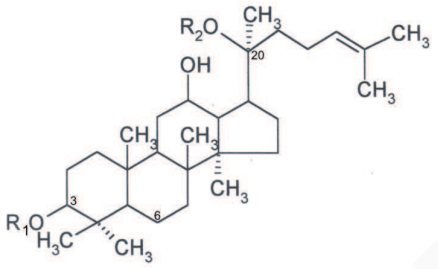
All the experiments were performed in triplicate. Data were analysed using the Kruskal-Wallis test with STATISTICA (StatSoft, Inc. 2011, version 10, www.statsoft.com) and $p<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

The study determined the effect of MJ on the level of six ginsenosides (Rb1, Rb2, Rc, Rd, Rg1, Re) in the suspension culture of *P. quinquefolium* L. in the shake flask cultures. The accumulation of all examined metabolites (calculated as a sum of ginsenosides of Rb and Rg group) was greater after 50 and 250 μmol L^{-1} MJ treatment compared to the control (untreated with MJ) cultures. The obtained results indicated that the level of total ginsenosides reached the maximum value at the use of 250 μmol L^{-1} MJ and it was 2.2-fold higher than in the control sample (Fig. 2). The similar results were obtained in the Wang et al. (29) study, in which 2.6 times higher content of ginsenosides was received, compared to the control in suspension culture *P. notoginseng* C. A. Meyer after elicitation of 200 μmol L^{-1} MJ (30). In addition, the amounts of these compounds were greater (18 mg g^{-1} d.w.) comparing to the level of ginseng saponin (4.2 mg g^{-1} d.w.) in *P. quinqefolium* L. described in this report. Furthermore, the studies on *P. ginseng* adventitious root cultures indicated that MJ at 100 μmol L^{-1}concentration may be optimal for ginsenoside accumulation (13).

On the other hand, using significantly lower concentration (for example 0.5 or 10 μmol L^{-1}) of methyl jasmonate also may enhance the production of secondary metabolites as it was described for suspension cultures of *Vaccinium phalae* (6) and *Catharanthus roseus* (7). Our findings also demonstrated that the increase of elicitor concentration to 500 μmol L^{-1} MJ caused the decrease of ginsenoside content (Fig. 2). Opposite to the results of the present investigation,

the addition of 600 μmol L^{-1} of MJ to the medium resulted in 2.6-fold increase of 20-hydroxyecdysone production over the control in cell suspension cultures of *Achyranthes bidentata* (29).



20(S) - protopanaxadiol	R_1	R_2
Rb_1	Glc-Glc-	Glc-Glc-
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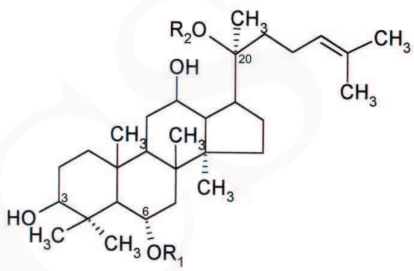

20(S) - protopanaxatriol	R_1	R_2
Re	Glc- Rha	Glc-
Rg_1	Glc-	Glc-

Fig. 2. The effect of MJ elicitation on ginsenoside accumulation in suspension cultures of *P. quinquefolium* L. cultivated in shake flasks. "C" means control sample without MJ

The content of individual ginsenosides also depended on the concentration of MJ (Fig. 3). For example, the level of Rc and Rb_1 ginsenosides achieved maximum with addition of 250 μmol L^{-1} MJ in the medium and was respectively 7 and 2.6 times greater than in the control culture. The research on *P. notoginseng* suspension cell cultures showed a stronger, approximately 9-fold increase of Rb1 concentration after MJ treatment (30). Similar observations were noted in hairy and adventitious root cultures of *P. quinquefolium* and *P. ginseng* growing in the shake flasks (12, 13, 14). In our study Rc and Rb_1 metabolites were not detected at 500 μmol L^{-1} concentration of MJ.

We also noticed that another metabolite – ginsenoside Rd – was not found in the untreated with MJ samples and at 50 μmol L^{-1} of MJ. It appeared after addition of 250 μmol L^{-1} MJ to the medium. Greater amount of elicitor reduced Rd content. In *P. notoginseng* cell culture this saponin was also absent in untreated samples, however the use of MJ in the concentration of 5–500 μmol L^{-1} stimulated its synthesis. Then, the optimal concentration of elicitor for Rd production was 200 μmol L^{-1} of methyl jasmonate above which Rd content was reducing (31). The other observation of our study concerned metabolite Rb2. This saponin was present in the control samples and it achieved the maximum yield at 50 μmol L^{-1} MJ. Similar to compound Rd, greater amount of elicitor reduced its content. Our findings are in agreement with the results demonstrated by Gaines 2004 (7). The increase of the

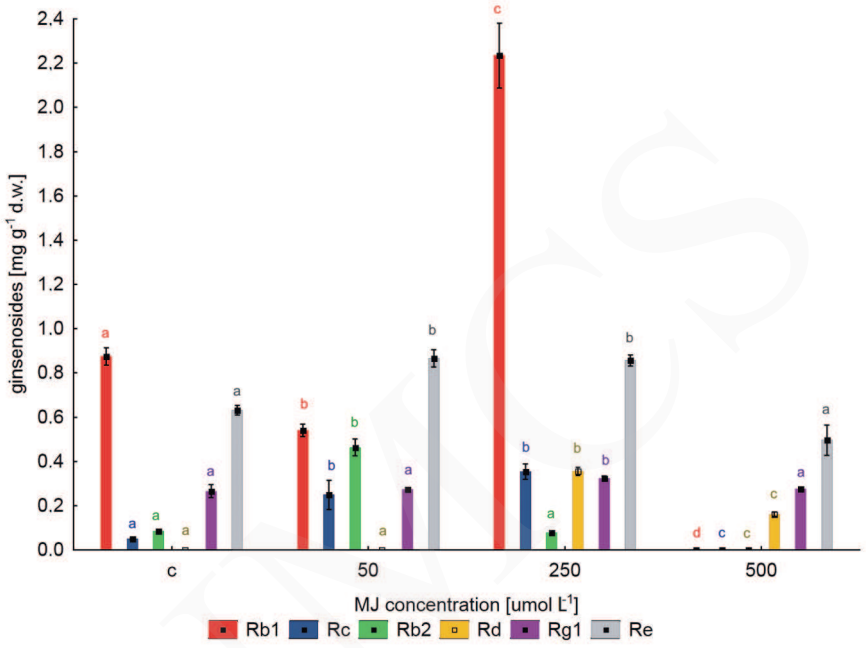

Fig. 3. The content of individual ginsenosides in suspension cultures of *P. quinquefolium* L. treated with MJ. "C" means control sample. Each value represents the mean of three replicates ± SE. The various letters for the same parameter means statistically significant differences at $p \leq 0.05$ (Kruskal-Wallis test)

concentration of methyl jasmonate above the optimal level for serpentine accumulation caused the absence of its detection in the suspension culture of *C. roseus*.

Ginsenosides Rg_1 and Re were found in all the examined samples. The level of Rg_1 was constant, meanwhile Re content increased about 37% in relation to control at concentration 50 and 250 µmol L^{-1} of MJ. Previous studies indicated that the concentration of protopanaxatriol derivatives typically increases 2- to 3-fold after MJ treatment (12, 31).

Stimulating effect of methyl jasmonate on ginseng saponin production may be explained on the genetic level. MJ treatment greatly enhances the expression of the key genes involved in the ginsenoside biosynthesis pathway and increases the quantity of major intermediates such as: squalene, 2,3-oxidosqualene, and dammarenediol II that are precursors for biosynthesis of ginsenosides (14, 17).

The results presented in this paper and research of other authors indicates that the effect of methyl jasmonate elicitation can depend on the species of the plant, the growth condition of cell lines and the concentration of the elicitor. Although the using of MJ as an elicitor usually leads to enhance the production of

different type of secondary metabolites, the level of the concentration of methyl jasmonate should be set individually for a tested culture to achieve a successful elicitation process.

CONCLUSIONS

1. Methyl jasmonate influenced on the ginsenoside production in suspension cultures of *P. quinquefolium* L.
2. The most preferred concentration of MJ for ginsenoside biosynthesis was 250 μmol L^{-1}. The greater content of the elicitor- 500 μmol L^{-1}- in the medium inhibited biosynthesis.

REFERENCES

1. Belchí-Navarro S., Almagro L., Lijavetzky D., Bru R., Pedreño M.A. 2012. Enhanced extracellular production of trans-resveratrol in *Vitis vinifera* suspension cultured cells by using cyclodextrins and methyljasmonate. Plant Cell Rep. 31(1):81–89.
2. Bonfill M., Mangas S., Moyano E., Cusido R.M., Palazon J. 2011. Production of centellosides and phytosterols in cell suspension cultures of *Centella asiatica*. Plant Cell Tissue Organ. Cult. 104(1):61–67.
3. Choi K.T. 2008. Botanical characteristics, pharmacological effects and medicinal components of Korean *Panax ginseng* C. A. Meyer. Acta Pharmacol. Sin. 29(9): 1109–1118.
4. Ernst E. 2010. *Panax ginseng*: an overview of the clinical evidence. J. Ginseng Res. 34(4): 259–263.
5. Exposito O., Syklowska-Baranek K., Moyano E., Onrubia M., Bonfill M., Palazon J., Cusido R.M. 2010. Metabolic responses of *Taxus media* transformed cell cultures to the addition of methyl jasmonate. Biotechnol. Prog. 26(4):1145–1153.
6. Fang Y., Smith M.A.L., M.F. Pépin. (1999). Effects of exogenous methyl jasmonate in elicited anthocyanin-producing cell cultures of ohelo (*Vaccinium phalae*). *In vitro* Cell Dev. Biol. Plant 35(1):106-113.
7. Gaines J.L. 2004. Increasing alkaloid production from *Catharanthus roseus* suspensions through methyl jasmonate elicitation. Pharmaceutical Engineering. 24(4):1–6.
8. Gómez-Serranillos M.P. 2015. Potential neuroprotective activity of Ginseng in Parkinson's disease: a review. J. Neuroimmune Pharmacol. 10(1):14–29. doi: 10.1007/s11481-014-9569-6.
9. Kim E.H., Kim I.H., Ha J.A., Choi K.T., Pyo S., Rhee D.K. 2013. Antistress effect of red ginseng in brain cells is mediated by TACE repression via PADI4. J. Ginseg Res. 37(3): 315–323.
10. Kim J.H. 2017. Pharmacological and medical applications of *Panax ginseng* and ginsenosides: a review for use in cardiovascular diseases. J. Ginseng Res. 1–6 article in press. https://doi.org/10.1016/j.jgr.2017.10.004.
11. Kim O.T., Bang K.H., Shin Y.S., Lee M.J., Jung S.J., Hyun D.Y., Kim Y.C., Seong N.S., Cha S.W., Hwang B. 2007. Enhanced production of asiaticoside from hairy root cultures of *Centella asiatica* (L.) Urban elicited by methyl jasmonate. Plant Cell Rep. 26(11):1941–1949.
12. Kim Y.S., Han J.Y., Lim S., Choi Y.E.2009. Ginseng metabolic engineering: regulation of genes related to ginsenoside biosynthesis. J. Med. Plants Res. 3(13),1270–1276.
13. KimY.S., Hahn E.J., Murthy H.N., Paek,K.Y. 2004. Adventitous root growth and ginsenoside accumulation in *Panax ginseng* cultures as affected by methyl jasmonate. Biotechnol. Lett. 26(21);1789–1792.

14. Kochan E., Balcerczak E., Lipert A., Szymańska G., Szymczyk P. 2018. Methyl jasmonate as a control factor of the *synthase squalene* gene promoter and ginsenoside production in American ginseng hairy root cultured in shake flasks and a nutrient sprinkle bioreactor. Industrial Crops and Products 115: 182–193.
15. Krzyżanowska J., Czubacka A., Pecio, L., Przybys M., Doroszewska T., Stochmal A., Oleszek W. 2011. The effects of jasmonic acid and methyl jasmonate on rosmarinic acid production in *Mentha* × *piperita* cell suspension cultures. Plant Cell Tissue Organ. Cult. 108(1):73–81.
16. Lee Y.S., Park H.S., Lee D.K., Jayakodi M., Kim N.H., Koo H.J., Lee S.C., Kim Y.J., Kwon S.W., Yang T.J. 2017. Integrated transcriptomic and metabolomic analysis of five *Panax ginseng* cultivars reveals the dynamics of ginsenoside biosynthesis. Front Plant Sci. 19, 8:1048. doi: 10.3389/fpls.2017.01048.
17. Lloyd G., McCown B. 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Int. Plant Prop. Soc. Proc. 30:421 427.
18. Lu M.B., Wong H., Teng W. 2001. Effects of elicitation on the production of saponin in cell culture of *Panax ginseng*. Plant Cell Rep. 20(7):674–677.
19. Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473–497.
20. Namdeo A.G. 2007. Plant cell elicitation for production of secondary metabolites: a review. Pharmacogn. Rev. 1(1):69–79.
21. Oliynyk S., Oh S. 2013. Actoprotective effect of ginseng: improving mental and physical performance. J. Ginseng Res. 37(2):144–166.
22. Park J.D., Rhee D.K., Lee Y.H. 2005. Biological activities and chemistry of saponins from *Panax ginseng* C. A. Meyer. Phytochem. Rev. 4(2-3):159–175.
23. Pengelly, A., Bennett, K. 2011. Appalachian plant monographs: *Panax quinquefolius* L., American ginseng. http://www.frostburg.edu/aces/appalachianplants.
24. Qi L-W., Wang Ch-Z., Yuan Ch-S (2011). Ginsenosides from American ginseng: Chemical and pharmacological diversity. Phytochemistry72(8):689–699. 10.1016/j.phytochem.2011.02.012.
25. Ramirez-Estrada K., Vidal-Limon H., Hidalgo D., Moyano E., Golenioswki M., Cusidó R.M., Palazon J. 2016. Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. Molecules. 21(2):182. doi: 10.3390/molecules21020182.
26. Szymańska G., Kochan E., Szymczyk P. 2013. Field cultivation and *in vitro* cultures, root-forming callus cultures and adventitious root cultures, of *Panax quinquefolium* as a source of ginsenosides. Z. Naturforsch. C. 68:(11–12):482–488.
27. Thanh N.T., Murthy H.N., Yu K.W., Hahn E.J., Paek K.Y. 2005. Methyl jasmonate elicitation enhanced synthesis of ginsenoside by cell suspension cultures of *Panax ginseng* in 5-l balloon type bubble bioreactors. Appl. Microbiol. Biotechnol. 67(2):197–201.
28. Wang Q.J., Zheng L.P., Sima Y.H., Yuan H.Y., Wang W.J. (2013) Methyl jasmonate stimulates 20-hydroxyecdysone production in cell suspension cultures of *Achyranthes bidentata*. POJ 6(2):116–120.
29. Wang W., Zhang Z.Y., Zhong J.J. 2005. Enhancement of ginsenoside biosynthesis in high-density cultivation of *Panax notoginseng* cells by various strategies of methyl jasmonate elicitation. Appl. Microbiol. Biotechnol. 67(6):752–758.
30. Wang W., Zhong J.J. 2002. Manipulation of ginsenoside heterogeneity in cell cultures of *Panax notoginseng* by addition of jasmonates. J. Biosci. Bioeng. 93(1):48–53.
31. Zhang C.H., Mei X.G., Liu L., Yu L.J. 2000. Enhanced paclitaxel production induced by the combination of elicitors in cell suspension of *Taxus chinensis*. Biotechnol Lett. 22(19):1561–564.