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METHYL JASMONATE INDUCE ENHANCED PRODUCTION OF SOLUBLE BIOPHENOLS IN *PANAX GINSENG* ADVENTITIOUS ROOTS FROM COMMERCIAL SCALE BIOREACTORS

G. Sivakumar[1] and K. Y. Paek[2]

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The contents of soluble biophenols such as protocatechuic, gentisic, vanillic, caffeic, syringic, p-*coumaric, ferulic, salicylic, and cinnamic acids were screened and quantified from the adventitious roots of* Panax ginseng *by HPLC-MS. Control adventitious roots which showed the greatest accumulation of ferulic acid (0.09 mg/g DW) were observed in our experiments. An increase in the total soluble biophenol content of adventitious roots was observed 5 days after treatment with elicitor 200 μM/L methyl jasmonate (MeJa) i.e., 35 to 40 days of inoculation. Among the biophenols investigated, the salicylic acid content was higher (0.44 mg/g DW) in MeJa treated adventitious root culture in the bioreactor. MeJa might apparently increase soluble biophenols production in adventitious roots by enhancing the biosynthesis pathway from phenylalanine to salicylic acid and other simple biophenols.*

Key words: antioxidant, biophenols, bioreactors, ginseng, HPLC-MS.

Panax ginseng C. A. Meyer (Araliaceae) has been used as a natural traditional medicine for thousands of years in Asian countries for its reputed anti-stress, and anti-aging properties and enhances memory power as well as sperm production in men. The cultivation of ginseng root is a long and laborious process and, as a result, it is an expensive commodity in the international market. Biophenols are present in three different forms: soluble, esterified, and insoluble, bound to the cell wall, in Korean ginseng [1]. Ginseng soluble biophenols have recently gained great attention for their antioxidant, anticancer, antidiabetic, anticarcinogenic, antimutagenic, and immunomodulating activity [2]. Due to these factors, pharmaceutical and healthcare professionals are increasingly considering complementary as well as alternative approaches for the commercial production of ginseng bioactive biophenols from disease-free and pesticide-residue-free roots. Modern bioreactor culture systems provide a more advanced technology to produce higher *in vitro* natural biophenols from *P. ginseng* root tissues using artificial nutrients with MeJa in an aseptic environment. The objective of the present study was to screen the soluble biophenolic profiles from bioreactor-derived ginseng adventitious roots and to increase production of soluble biophenols using MeJa. This is the first report on screening and enhanced production of biophenols using MeJa from bioreactor-derived *P. ginseng* adventitious roots.

We previously reported the effects of various auxins and their combinations on callus induction and higher multiple adventitious root induction using the root explant of *P. ginseng* [3]. This paper presents an HPLC-MS screened soluble biophenol profile and enhanced soluble biophenol accumulation in *P. ginseng* adventitious roots using MeJa in bioreactor culture. By careful analysis of the chromatograms at different wavelengths in the scale of 200–800 nm, it was found that the chromatograms at 240 nm together with 280 nm could well represent the profile of the constituents. By comparing the chromatogram of control *P. ginseng* adventitious roots with those of its MeJa treatment extracts, the root derivation of each peak was confirmed in terms of the retention time and spectra achieved from the PDA detection. The identified soluble biophenol compounds are listed in Table 2. The presence of acetic acid in the mobile phases suppressed the dissociation of the biophenolic compounds and enhanced the selectivity of the elution system. Comparisons of chromatograms generated at different detection wavelengths support the use of 200–800 nm as a compromise for the detection of biophenols [4].

1) Biotech Genomics, ENEA, Casaccia, Rome, Italy, e-mail: sivakumar@libero.it; 2) Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 361-763, South Korea. Published in Khimiya Prirodnykh Soedinenii, No. 6, pp. 551-554, November-December, 2005. Original article submitted March 16, 2005.

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TABLE 1. Effects of Methyl Jasmonate on Adventitious Root Growth and Biophenol Production of *P. ginseng*

MeJa Con., μM/L	Biomass* fresh wt., g	Dry wt., g	% of dry wt.	Growth rate**	Total soluble biophenols*
Control	489.6 a	42.1 b	5.05 b	24.76	0.22 d
100	476.2 b	42.9 b	5.29 c	25.23	0.71 b
200	470.6 c	45.9 a	5.73 a	27.00	1.19 a
300	420.1 d	38.4 d	5.37 ab	22.58	0.45 c
400	332.0 e	34.6 c	6.10 d	20.35	0.39 c

Data was collected after 40 days of culture using a 5 L balloon type bioreactor having 4 L 3/4 MS medium.
*Mean followed by different letters within a column are significantly different at $P < 0.05$ by Duncan's multiple range test.
**The values are the quotients of the dry weight after cultivation and the dry weight of the inoculum. Similar experiments were done 3 times.

TABLE 2. Soluble Biophenolic Profile of *P. ginseng*, Methyl Jasmonate (200 μM/L) Treated Adventitious Roots

Biophenol profile (acid)	Retention time	Control adventitious root biophenols, mg/g DW	MeJa treated adventitious root biophenols, mg/g DW
Gallic	3.15	Internal standard	Internal standard
Protocatechuic	5.34	0.01±0.01*	0.03±0.06*
Gentisic	8.90	0.03±0.01	0.15±0.01
Vanillic	10.21	0.01±0.01	0.11±0.05
Caffeic	11.16	0.01±0.01	0.04±0.02
Syringic	12.10	0.01±0.01	0.07±0.02
p-Coumaric	12.21	0.01±0.02	0.02±0.02
Ferulic	15.33	0.09±0.02	0.27±0.09
Salicylic	17.51	0.03±0.02	0.44±0.09
Cinnamic	23.74	0.02±0.01	0.09±0.05
Total soluble biophenols		0.190	1.21

Data was collected after 40 days of culture using a 5 L balloon type bioreactor having 4 L of 3/4 MS liquid medium.
*Mean ± standard error of 3 replicates.

A great advantage of mass spectrometry in terms of biophenols is that it provides prominent peaks for the molecular ions. The mass spectra of each soluble biophenol peak from *P. ginseng* adventitious roots show the presence of abundant $[M–H]^-$ ions at the appropriate *m/z* values, along with a unique retention time for each of the soluble biophenols. These chromatograms at *m/z* 170, 154, 154, 168, 180, 164, 194, 138, and 148 show the corresponding mass numbers of gallic acid (internal standard), protocatechuic, gentisic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, salicylic, and cinnamic acids, respectively. We carefully studied the mass spectra of these compounds and compared them with standards and reference data [5]. ESI overcomes the lack of analyte volatility by the direct formation or emission of ions from the surface of the condensed phase. Deprotonated molecular ions represented the base peak in the negative ion spectra of all ten biophenolic species.

Different concentrations of MeJa (100–400 μM/L) were added to adventitious root cultures of *P. ginseng* at culture day 35 after inoculation. Chaudry et al. [6] reported that after 48 h of jasmonate treatment the general decrease in protein synthesis is caused by the ribosome-inactivating protein JIP 60. These results were similar to our present report after treatment of MeJa resulted in inhibition of adventitious root growth but a higher accumulation of soluble biophenols (Table 1). In order to prevent a decreasing biomass of adventitious roots, the authors added MeJa 5 days before harvesting i.e., after 35 days of inoculation. The soluble biophenolic profile of MeJa-treated adventitious roots was dominated by salicylic, ferulic, and gentisic acids, with

lesser amounts of vanillic, cinnamic, syringic, caffeic and protocatechuic acids (Table 2). Most plant phenolic acids are derivatives of benzoic (C6–C1) or cinnamic (C6–C3) acids, and phenolic acids of low molecular weight occur widely in decomposing plant residues and in roots [7].

The production of soluble biophenols increased significantly compared to control (without MeJa), when up to 400 μM/L of MeJa was added. The optimum concentration of MeJa for the higher production of soluble biophenols was found to be 200 μM/L (Table 1), yielding a total soluble biophenol content of 1.21 mg/g DW. The maximum yield of salicylic acid was 0.44 mg/g DW when treated with 200 μM/L MeJa on the 40th day of inoculation (Table 2). The production of soluble biophenols did not change when MeJa was added at the zero day of inoculation but it inhibit the adventitious root biomass (data not shown). The time of addition of the MeJa had a significant effect on biophenol production compared to the control. These results also indicate that addition of MeJa at the early phase of *P. ginseng* adventitious root growth is not favored. Plata et al. [8] reported that the highest anthocyanin content was obtained after adding MeJa to sweet potato cell suspension cultures. Therefore it can be assumed that the presence of MeJa might be beneficial in triggering the expression of biophenol biosynthetic genes in the ginseng adventitious roots, which results in an increase in soluble biophenol production. Huang et al. [9] reported that ferulic acid was converted to vanillic acid and subsequently to protocatechuic acid by *R. rubra* under aerobic conditions. However, in the present study, salicylic acid accumulation was higher in MeJa-treated adventitious roots. Recent advances in root elicitor signalling pathway research have shown that roots are capable of differentially activating distinct defense pathways [10]. Depending on the type of elicitor encountered, the root appears to be capable of switching on the appropriate pathway or combination of pathways. The root signalling molecule salicylic acid plays an important role in this signalling network: blocking the response to either of these signals can render elicitor-treated roots more susceptible to stress [11]. We assume that higher accumulations of biophenols are associated with the elicitor of MeJa within ginseng adventitious root, which can enhance the activity of shikimic acid pathway enzymes. The authors observed the decreased biomass of ginseng adventitious roots when MeJa was added even after 35 days of inoculation. It may be, that MeJa can trigger the activation of proteinase inhibitor genes in *P. ginseng*. Kim et al. [12] identified a MeJa-responsive element in the promoter of a proteinase inhibitor II gene, indicating that this gene is a transcription ally regulated in response to MeJa.

In conclusion, although it has been recognized that MeJa is an excellent elicitor for increasing secondary metabolite production, the mechanism of MeJa-induced biophenols biosynthesis is still not well understood. The results of MeJa induction on soluble biophenol production may be useful for exploring new strategies to improve salicylic acid production. The main finding derived from this work is that *P. ginseng* adventitious root can supply bioactive biophenols for pharmaceuticals. In addition, its use in Western medicine has also been growing rapidly due to the growing interest in alternative remedies using natural *in vitro* herbal medicines. Furthermore, the model system presented here seems to be a good soluble biophenol screening method for *P. ginseng* adventitious roots.

EXPERIMENTAL

Induction of Callus. *Panax ginseng* C.A. Meyer was collected from the Mountain of KeumSan province in South Korea and the roots were selected as explants. They were surface-disinfected according to the Sivakumar and Krishnamurthy [13] method. Calluses were induced from root using Murashige and Skoog (MS) medium [14] supplemented with indolebutyric acid (IBA) in the range of 2.46–9.84 μM/L, 0.46–4.64 μM/L kinetin (Kin), and 3% sucrose, and solidified with 0.2% gelrite; subcultures were grown every 4 weeks. Adventitious roots were induced from 4-week-old well-grown, morphogenic callus cultures in 3/4 MS medium supplemented with 24.60 μM/L IBA and 0.2% gelrite under dark conditions. Adventitious roots were grown at a temperature of 20±2°C. The cultures were subcultured every 3 weeks.

Bioreactor Culture of Adventitious Roots. The pH of the medium was adjusted to 6.0 before autoclaving at 121°C and 1.2 $kg.cm^{-2}$ pressure for 40 min. The volume of input air in bioreactor was adjusted to 0.1 vvm (air volume/min). Bioreactors were maintained at 22±2°C in a dark room until harvest. 20 g fresh weight of adventitious roots was inoculated into 5 L balloon type bioreactors containing 4 L of the culture medium.

Elicitor Treatment. After 35 days culture, filter sterilized methyl jasmonate (MeJa), 100–400 μM/L, was added to the 3/4 MS medium. After 5 days, harvested adventitious roots were used for biomass and soluble biophenol analysis.

Determination of Root Biomass. Adventitious roots were separated from the medium by passing through a stainless steel sieve. Fresh weight was obtained after the roots were rinsed with tap water and the surface water blotted away. Dry weight was recorded after the roots were dried to constant weight at 50°C for two days.

The adventitious root growth rate was calculated as:

Growth rate = harvest dry weight (g)/inoculated dry weight (g).

Extraction and Characterization of Soluble Biophenol Fraction. Freeze-dried *P. ginseng* adventitious roots, each sample containing 20 g, were separately pitted by blending and homogenized in methanol–acetone (1:1. 80 ml) saturated with sodium metadisulfite at medium speed in an Ultraturrax homogenizer (Janke & Kunkel, IKA–Labortechnik, Germany) at 0°C for 3 min and centrifuged at 5000 g for 20 min at 4°C [15]. The supernatant was separated and the pellet resuspended four times in the same solvent until a colorless solution was obtained. The combined supernatants were used for soluble biophenol (SBP) analysis. The combined supernatants were evaporated under vacuum at 35°C. The dry residue was resuspended with pH 2 water solution and centrifuged to separate a cloudy precipitate. The clear supernatant was extracted five times with hexane at a hexane to water ratio of 1:1 to remove free fatty acids and other lipidic contaminants. The SBPs were then extracted with ether/ethyl acetate (1:1) six times at a solvent to water ratio of 1:1. The ether–ethyl acetate extracts were dehydrated with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum at 30°C. After evaporation to dryness, the residue was redissolved in LC-MS CHROMASOLV® grade methanol (2 mL) in vials containing an internal standard (IS) of known amount, i.e., gallic acid, and filtered through a 0.45 μm millipore filter.

HPLC. The soluble biophenol fraction was analyzed using a HPLC system (Waters 2690 separation module with autoinjector; Waters 996 photodiode array detector; Waters millennium 2010 chromatography manager, USA) on a C_{18} (25 cm × 4.6 mm i.d.) column; the eluates detected range from 200 to 800 nm. The extracted BP fractions (20 μL) were injected into HPLC column. Elution was run at a 0.2 ml/min flow rate using the following mobile phases: methanol (solvent A) and water/acetic acid, pH 3.3 (0.1%) (solvent B). The selected gradient started with 10% A 90% B; then solvent A was raised to 90% in 60 min and acquisition was stopped. The column was washed 10 min with 100% A and then kept at the initial conditions another 10 min. HPLC eluate was introduced into the ESI-MS interface.

MS-ESI. MS detection was carried out using a Waters Micromass ZQ, USA. The system was monitored by a Compaq P720 Power PC computer equipped with MASSLYNX^{TM} 4.0 software for instrument control and data acquisition, data reprocessing, and solute quantification. Nitrogen was employed as the nebulizing gas at a pressure of 60 p.s.i. The temperatures of the electrospray source and desolvation gas were 100 and 200°C, respectively. ESI mass spectra were acquired in the negative mode by scanning over the *m/z* range 100–1000 with unit mass resolution. Ions generated in the ion source were sampled into the mass analyzer by passing through a 25 μm i.d. orifice (voltage from 0 to 30 V) at the rear end of the atmospheric chamber. To prevent solvent vapors and contaminants from entering the vacuum chamber, we used a flow rate of 0.2 mL/min during all experiments, and 0.1 ml/min when the instrument was set in overnight standby.

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REFERENCES

1. M. Y. Jung, B. S. Jeon, and J. Y Bock, *Food Chem.*, **79**, 105 (2003).
2. D. D. Kitts, A. N. Wijewickreme, and C. Hu, *Mol. Cell. Biochem.*, **203**, 1 (2000).
3. G. Sivakumar, K. W. Yu, and K. Y. Paek, *Eng. Life. Sci.*, **5** (4), 333 (2005).
4. S. Tian, K. Nakamura, T. Cui, and H. Kayahara, *J. Chromatog. A*, **1063**, 121 (2005).
5. J. Zhang, M. Cui, Y. He, H. Yu, and D. Guo, *J. Pharm. Biomed. Anal.*, **36**, 1029 (2005).

6. B. Chaudry, F. Muller-Uri, V. Cameron-Mills, S. Gough, D. Simpson, K. Skriver, and J. Mundy, *Plant J.*, **6**, 815 (1994).
7. U. Blum, S. R. Shafer, and M. E. Lehman, *Crit. Rev. Plant Sci.*, **18**, 673 (1999).
8. N. Plata, I. Konczak-Islam, S. Jayram, K. McClelland, T. Woolford, and P. Franks, *Biochem. Eng. J.*, **14**, 171 (2003).
9. Z. Haung, L. Dostal, and J. P. N. Rosazza, *Appl. Environ. Microbiol*., **59**, 2244 (1993).
10. M. J. C. Pieterse and L. C. van Loon, *Trends Plant Sci.*, **4**, 52 (1999).
11. T. Gaffney, L. Friedrich, B. Vernooij, D. Negrotto, G. Nye, S. Uknes, E. Ward, H. Kessmann, and J. Ryals, *Science*, **261**, 754 (1993).
12. S. R. Kim, J. L. Choi, M. A. Costa, and G. An, *Plant Physiol*., **99**, 627 (1992).
13. G. Sivakumar and K. V. Krishnamuthry, *Russ. J. Plant. Physiol.*, **51**, 713 (2004).
14. T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 23 (1962).
15. K. Krygier, F. Sosulski, and L. Hogge, *J. Agr. Food Chem.*, **30**, 330 (1982).