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Short communication

Influences of polyunsaturated fatty acids (PUFAs) on growth and secondary metabolite accumulation in *Panax ginseng* C.A. Meyer adventitious roots cultured in air-lift bioreactors

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Abstract

The present study relates to different polyunsaturated fatty acids (PUFAs) which were used as elicitors to enhance biomass accumulation and ginsenoside production in *Panax ginseng*. Adventitious root cultures of ginseng were elicited with oleic and linolenic acid at 0, 1, 5, 10 or 50 μmol/l concentrations respectively. Elicitors were added to the medium of adventitious roots on the 40th day of culture and roots were harvested on day 47. Cultures supplemented with oleic acid decreased root biomass and ginsenoside accumulation. Cultures supplemented with 1 μmol/l linolenic acid enhanced ginsenoside accumulation, without the decrease of adventitious root biomass. Linolenic acid enhanced the biosynthesis of both protopanxatriols (2.95±0.048 mg/g DW) and protopanxadiols (5.66±0.043 mg/g DW) compared to that of control at (1.41±0.002 mg/g DW) and (1.58±0.006 mg/g DW) respectively. No changes in polysaccharides and phenolics content have been noticed upon elicitation with PUFAs. This is the first report on linolenic acid as an elicitor for ginsenoside accumulation in ginseng adventitious root cultures.

Keywords: Elicitation; Ginsenosides; Saponins; Triterpenes

1. Introduction

Panax ginseng C.A. Meyer (Araliaceae) is one of the most valuable oriental herbs. The medicinal value of ginseng has been of worldwide interest. Extensive effort is still being put into investigating its pharmacological effects and the identification of its bioactive components. The saponins, known as ginsenosides, are widely believed to be the major bioactive ingredients of ginseng. Ginsenosides are attributed to cardio-protective, immunostimulatory, anti-fatigue and hepato-protective physiological and pharmacological properties (Wu and Zhong, 1999). In addition, it has been reported that ginseng roots contain antioxidants, peptides, polysaccharides, fatty acids, alcohol and vitamins (Huang, 1993).

Recently, ginseng adventitious root cultures have been found to offer an alternative method for the production of ginsenosides, which, due to its fast growth and stable metabolite production, provides an efficient means for biomass production. A series of experiments was conducted in order to establish efficient ginseng adventitious root growth. Ginsenosides production in liquid media and a pilot scale culture system using bioreactors was also investigated (Choi et al., 2000; Paek et al., 2005; Sivakumar et al., 2005). Due to the low productivity of metabolite in ginseng tissue culture, various elicitation strategies have been developed to stimulate the biosynthesis of ginsenosides. Several reports indicate that elicitation with jasmonic acid (JA) and methyl jasmonate (MeJ), the most commonly known oxylipin compounds, has increased ginsenosides production in ginseng adventitious root cultures (Choi et al., 2000; Yu et al., 2000, 2005; Palazon et al., 2003; Paek et al., 2005; Ali et al.,

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2006; Jeong et al., 2006). However, the decrease of dry weight of the cultures following JA elicitation is the major problem.

Oxylipins are classes of biologically active compounds that are generated by oxidative catabolism of polyunsaturated fatty acids (PUFAs). The release of PUFAs, particularly 18:3 and 16:3, is believed to provide substrates for the synthesis of oxylipins during plant–pathogen and plant–insect interactions (Farmer et al., 2003; Li et al., 2003). The group of jasmonates, which are cyclopentanone derivatives, originate biosynthetically from linolenic acid (18:3) via an inducible octadecanoid pathway. Oleic acid (18:1) is converted into linolenic acid which is a substrate for JA biosynthesis (Kirsch et al., 1997) and can function as an elicitor for ginsenosides biosynthesis. In this study, we have tested the effects of oleic acid and linolenic acid as elicitors to enhance ginsenoside accumulation in ginseng adventitious roots cultured in bioreactors.

2. Materials and methods

2.1. Plant material

Adventitious root cultures of *P. ginseng* C.A. Meyer, viz., CBN-2, were obtained from an established root line collection, at Chungbuk National University, South Korea. These root cultures were generated from a 4-year-old mountain ginseng, through callus culture as described by Yu et al. (2000). For induction of adventitious roots from the callus, proliferated calli were inoculated on modified MS medium (Murashige and Skoog, 1962) supplemented with 3.0 mg/l indole-3-butyric acid (IBA) and 50 g/l sucrose (pH 6.0) and cultured at 22±1 °C in the dark for 1 month. Following 1 month of culture, adventitious roots were induced, which were proliferated further in 5 l air-lift balloon type bubble bioreactors (BTBB) on the same media by sub-culturing at 4-week intervals, and were used as explants in further experiments.

2.2. Adventitious root culture in bioreactor and PUFAs elicitation treatments

Bioreactor culture was performed in a 3 l air-lift BTBB containing 2 l modified MS medium supplemented with 3.0 mg/l IBA and 50 g/l sucrose. The inoculum was 10 g FW of excised adventitious roots. Different concentrations of oleic and linolenic acid (0, 1, 5, 10, 50 µmol/l) were added to the culture medium on day 40 of culture. Non-elicited bioreactors were considered as the control. The elicitors were diluted with ethanol to give the appropriate test range. The same amount of ethanol was added to the control culture. The airflow rate was adjusted at 0.1 vvm (200 ml/min) during cultivation. The air temperature was controlled at 25±1 °C and the experiment was run for 47 days (Fig. 1A).

2.3. Determination of root biomass

The roots were separated from the medium by passing through a stainless steel sieve. Fresh weight was recorded after the roots were rinsed with tap water and excess surface water was blotted away (Fig. 1B). Dry weight was recorded after the roots were dried to constant weight at 60 °C for two days. The growth ratio (GR) was determined as:

$$GR = \{harvested\ dry\ weight\ (\mathrm{g}) - inoculated\ dry\ weight\ (\mathrm{g})\} \div inoculated\ dry\ weight\ (\mathrm{g}).$$

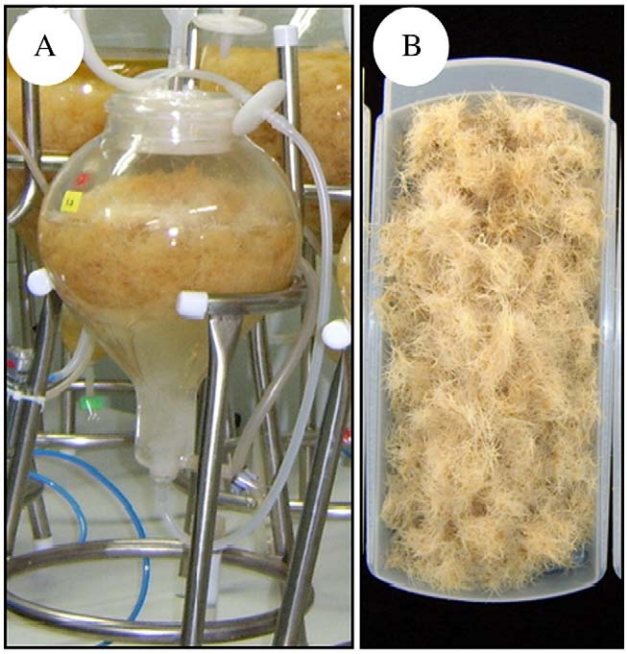

Fig. 1. Adventitious roots culture of *P. ginseng*: (A) roots cultured in a 3 l air-lift balloon type bubble bioreactor for 47 days; (B) harvested fresh roots.

2.4. Determination of ginsenosides content

Ginsenosides were extracted and determined according to Furuya and Yoshikawa (1987) and William et al. (1996) with modifications. One gram of ground-dried roots was extracted with 50 ml of 80% methanol at 80 °C for 1 h and filtered through Whatman filter paper. The residue was further extracted using the same method and a final volume of 100 ml was recovered. The extract was evaporated until dry and dissolved in 50 ml of HPLC grade water. Thereafter, the water-soluble fraction was washed twice with the same volume of ethyl ether. The water phase was extracted with 50 ml of water saturated n-butanol thrice and washed with 20 ml of water. The organic phase was evaporated until dry under vacuum at 30 °C. Following evaporation, the residue was dissolved in 5 ml of LC-MS CHROMASOLV grade methanol and filtered through 0.45 µm Millipore PTEF membrane filters (ADVANTEC MFS, Inc., USA) into vials. The ginsenoside fraction was analyzed using an HPLC system (Waters 2690 87 separation module; Waters 996 photodiode array detector; Waters millennium 2010 chromatography manager) on an Altec Platinum C18 column (φ1.5 m, 33 mm × 7 mm), with water and acetonitrile as the mobile phase. The ratios of water and acetonitrile for the first 10 and the last 25 min were 75:25 and 63:37, respectively. The

flow rate of the mobile phase was 1.2 ml/min and the ginsenoside was detected at 203 nm. The peak areas corresponding to ginsenosides from the samples, with the same retention time as authentic ginsenosides [Rg1, Re. Rb1, Rc (Wako, Osaka, Japan) and Rf, Rb2, Rd (Karl Roth, Germany)] were integrated by comparison with an external standard calibration curve. The total saponin content was calculated as the sum of these fractions.

2.5. Determination of total phenolic contents

Total phenolic content in plant methanolic extracts was determined by a colorimetric method according to Folin and Ciocalteu (1972) with modifications. One hundred microlitres of methanolic extracts was mixed with 2.5 ml deionized water, followed by addition of 0.1 ml (2 N) Folin-Ciocalteu reagent. They were mixed well and allowed to stand for 6 min before 0.5 ml of a 20% sodium carbonate solution was added. The color was developed after 30 min at room temperature and the absorbance was measured at 760 nm using a UV visible spectrophotometer (UV-1650PC, Shimadzu, Japan). The measurement was compared to a standard curve of prepared gallic acid solution and expressed as means (±S.E.) mg of gallic acid equivalent per gram of plant material for the triplicate extracts.

2.6. Determination of total flavonoid contents

Total flavonoid content was determined by a colorimetric method according to Zhishen et al. (1999), Dewanto et al. (2002) and Sakanaka et al. (2005). Briefly, 0.25 ml of the methanolic plant extract or (+)-catechin standard solution was mixed with 1.25 ml of distilled water, followed by the addition of 75 µl of a 5% sodium nitrite solution. After 6 min, 150 µl of a 10% aluminum chloride solution was added. The mixture was allowed to stand for a further 5 min, at room temperature, before 0.5 ml of 1 M sodium hydroxide solution was added. The absorbance was measured immediately at 510 nm using a spectrophotometer (UV-1650PC, Shimadzu, Japan). The results were expressed as means (±S.E.) mg of (+)-catechin equivalents per gram of plant material, in triplicate.

2.7. Determination of polysaccharides content

After ginsenoside extraction (2 g) with 80% methanol, the sediment was collected and desiccated in an oven at 60 °C. The sediment (0.2 g) was re-suspended in 5 ml 5% (v/v) sulphuric acid and placed in boiling water for 2 h. After acidic hydrolysis, the liquid–solid mixture was diluted to 50 ml with distilled water. The supernatant was separated by sedimentation, and the polysaccharide in the supernatant was assayed according to the carbazole reaction method as follows. A sample of 0.2 ml was taken from the above supernatant, mixed with 5 ml concentrated sulphuric acid, held in a boiling water bath for 20 min and cooled. Then, 0.2 ml carbazole-absolute ethanol (0–15%, v/v) was added and the contents mixed vigorously. After a reaction time of 2 h in darkness at room temperature, a purplish red color developed and absorbance was measured at 530 nm. D-Galacturonic acid was used as a standard.

2.8. Experimental design

Experiments were set up in a completely randomized design and data were subjected to Duncan's multiple range test using SAS software (Version 6.12, SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

Ginseng root biomass, ginsenosides, phenolics, flavonoids and polysaccharides accumulation were influenced by oleic and linolenic acid at different concentrations. Except that of 1 µmol/l linolenic acid, all PUFAs concentrations significantly decreased biomass production in terms of fresh weight, dry weight, % of dry weight and growth ratio (Table 1). The adventitious roots did not display any visible morphological changes in all the PUFAs treatments. Fig. 2 presents the accumulation of ginsenosides groups as influenced by PUFAs elicitation. The principal active components of ginseng are triterpenoid saponins known as ginsenosides. The ginsenosides Rb_1, Rb_2, Rc and Rd belong to the Rb group (protopanxadiols), while Re, Rg_1 and Rf belong to the Rg group (protopanxatriols). Cultures supplemented with 1 µmol/l linolenic acid enhanced both the Rg ginsenoside group (2.95±0.048 mg/g DW) and Rb ginsenoside group (5.66±0.043 mg/g DW) compared to that of control at (1.41±0.002 mg/g DW) and (1.58±0.006 mg/g DW) respectively. Also, the total ginsenosides was increased nearly three times than that of the control. Oxylipins such as JA and MeJA are generated by oxidative catabolism of PUFAs (adding oxygen to the 9 or 13 position of the C18 chain of linoleic and linolenic acids) by the coordinated action of lipases, lipoxygenase, and a group of cytochromes *P450* (CYP74 family),

Table 1
Effect of PUFAs elicitation on biomass production of *P. ginseng* roots.

PUFAs conc. (µmol l^{-1}) [a]		Biomass production (g/3 l bioreactor)			
		Fresh weight	Dry weight	% of dry weight	Growth ratio
Control	0	369.39 a [b]	26.95 a	7.30 a	29.28 a
Oleic acid	1	357.90 b	23.61 b	6.60 b	25.53 b
	5	360.91 b	23.95 b	6.64 b	25.91 b
	10	355.29 b	23.31 b	6.56 b	25.19 b
	50	311.62 c	19.87 d	6.38 c	21.33 d
Linolenic acid	1	370.52 a	27.71 a	7.48 a	30.13 a
	5	305.45 c	22.41 c	7.34 a	24.18 c
	10	298.59 d	22.00 c	7.37 a	23.72 c
	50	361.75 b	24.08 b	6.66 b	26.06 b
Significance [c]					
Elicitor type (ET)		***	***	***	***
Elicitor conc. (EC)		***	***	***	***
ET × EC		***	***	***	***

[a] Elicitors were added on the 40th day of culture and roots were harvested on day 47.
[b] Mean separation within columns by Duncan's multiple range test at 5% level.
[c] ***Significant at $P \leq 0.001$.

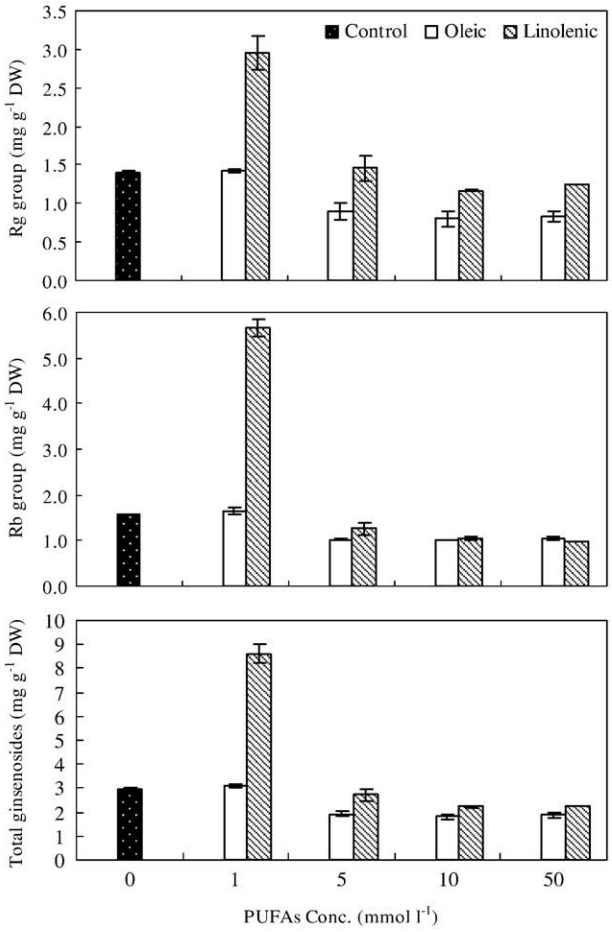

Fig. 2. Ginsenosides accumulation (Rg group, Rb group, total ginsenosides) in *P. ginseng* roots as affected by PUFAs elicitation (elicitors were added on the 40th day of culture and roots were harvested on day 47).

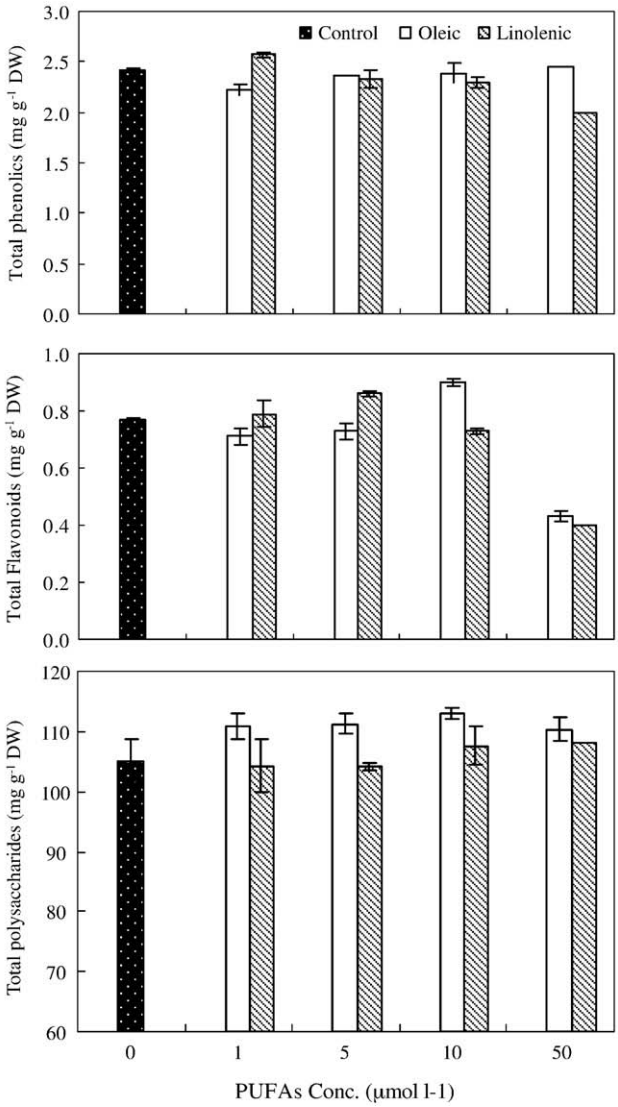

Fig. 3. Total phenolics, flavonoids and polysaccharides in *P. ginseng* roots as affected by PUFAs elicitation (elicitors were added on the 40th day of culture and roots were harvested on day 47).

including allene oxide synthase, and hydroperoxide lyase (Zhao et al., 2005). The first step in oxylipins biosynthesis is the release of fatty acid precursors from membrane lipids. Oleic acid (18:1) is converted into linolenic acid (18:3) (Kirsch et al., 1997) which is a substrate for JA biosynthesis. In the present study, linolenic acid may stimulate endogenous JA biosynthesis in ginseng roots leading to accumulation of ginsenosides. However, a non-enzymatic oxidation of 9- or 13-linoleic and linolenic acids also leads to the production of another group of cyclopentenone oxylipins, which also show biological activities, such as induction of defense gene expression and phytoalexin biosynthesis. Peroxidation products of linolenic acid were isolated from tobacco leaves and cell cultures; these molecules proved able to stimulate defense response genes and phytoalexin accumulation in tobacco and bloodroot cell cultures (Thoma et al., 2003).

Fig. 3 presents the effects of PUFAs elicitation on accumulation of total phenolics, flavonoids and polysaccharides in ginseng roots. No changes in polysaccharides and phenolics content have been noticed upon elicitation with PUFAs. It has been reported that exogenously applied JA induces *de novo* transcription of PAL, the key enzyme of the phenylpropanoid pathway (Gundlach et al., 1992). However, in our study, high concentrations of oleic acid and linolenic acid resulted in a decrease in flavonoids contents. The improvement in accumulation of Rb as well as Rg ginsenosides suggests that linolenic acid stimulates the metabolic pathway leading to ginsenosides biosynthesis and this is the most significant finding of this study (Fig. 4). MeJA elicitation has been applied to enhance the accumulation of ginsenosides in adventitious roots of ginseng; however, a considerable reduction in the biomass was the major problem (Yu et al., 2000, 2005; Jeong et al., 2006). In the present study, linolenic acid has not only promoted the biosynthesis of ginsenosides but also does not affect the biomass production as compared to the control. The promotion of ginsenosides accumulation is probably due to stimulation of endogenous JA biosynthesis upon elicitation with linolenic acid in ginseng roots.

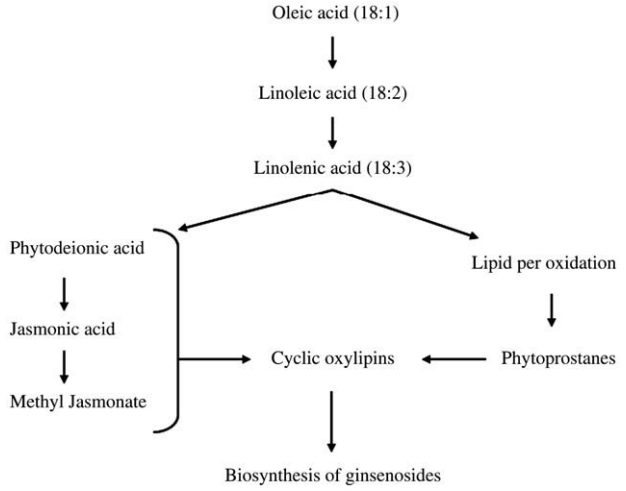

Fig. 4. Metabolic route of oleic acid and linolenic acid leading to ginsenosides biosynthesis.

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