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Linoleic and α-linolenic fatty acids affect biomass and secondary metabolite production and nutritive properties of *Panax ginseng* adventitious roots cultured in bioreactors

Chun Hua Wu [1], Elena V. Popova [2], Eun Joo Hahn, Kee Yoeup Paek*

Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 361-763, Republic of Korea

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ABSTRACT

The effect of elicitation with linoleic (C18:2) and α-linolenic (C18:3) fatty acids (LLA and α-LNA) was investigated in *Panax ginseng* C.A. Meyer adventitious roots cultured in 5 l balloon-type bioreactors. Fatty acids were added in culture medium at 0.0, 1.0, 2.5, 5.0, 10.0, and 20.0 μmol l^{-1} at day 40, at the end of exponential growth phase. Roots were harvested and assayed at day 47. Elicitation with both LLA and α-LNA enhanced accumulation of total polyphenolics and flavonoids in roots compared with control without elicitation. The highest accumulation of flavonoids was observed at 5.0 μmol l^{-1} for both elicitors. Total phenolics production reached its highest value of about 4.0 mg g^{-1} DW under the elicitation with 5.0 μmol l^{-1} LLA and 5.0–20.0 μmol l^{-1} α-LNA. Meanwhile, α-LNA was more effective than LLA for increasing biomass and ginsenoside production. The biomass of 11.1 g DW l^{-1} and maximal total ginsenoside content of 7.9 mg g^{-1} DW were achieved at 5 μmol l^{-1} α-linolenic acid. The essential polyunsaturated linoleic (C18:2) and α-linolenic (C18:3) fatty acids were accumulated in roots in response to elicitation while content of palmitic (C16:0) and oleic (C18:1) acids declined. The activities of SOD, G-POD and CAT were enhanced by two elicitors to similar extent while APX activity was preferably stimulated by α-LNA. Our results demonstrate that elicitation with α-linolenic acid stimulates production of biomass and secondary metabolites in bioreactor-cultured *P. ginseng* adventitious roots.

1. Introduction

Panax ginseng C.A. Meyer, a member of the Araliaceae family, is one of the most precious and important medicinal plants in East Asia. The principal active compounds in ginseng roots are ginsenosides (triterpenoid saponins) which possess a variety of pharmacological effects including immune system modulation, anti-stress, antioxidant, and anti-cancer activities [1]. Basing on structural differences, two main groups of ginsenosides exist as protopanaxadiol and protopanaxatriol classifications [1]. Polyphenolics and polysaccharides represent another class of bioactive components in ginseng roots [2]. Traditional field production of *P. ginseng* requires minimum 4 years of cultivation before root harvesting and cannot meet commercial needs. Recently, bioreactor culture of *P. ginseng* adventitious roots was developed as a reliable and competitive alternative to field-cultivated plants for the commercial production of specific ginseng metabolites [3]. This technology supposes elicitation with oxylipins, namely jasmonic acid (JA) and methyl jasmonate (MJ), as a key step to increase production of bioactive compounds [4,5]. However, the stimulation effect of jasmonate-based elicitors on biosynthesis was accompanied by significant biomass reduction [3,5]. Thus, it is essential to find other elicitors which allow increasing of secondary metabolite production without suppression of adventitious root growth.

The linoleic (C18:2) and α-linolenic (C18:3) polyunsaturated fatty acids (PUFAs) are known to possess biological activities in plant tissue cultures. For instance, exogenous PUFAs increased accumulation of secondary metabolites in the suspension cultures of *Lycopersicon esculentum*, *Tinospora cordifolia*, *Erythrina cristagalli* and *Eschscholzia californica* [6]. In addition, elicitation with α-linolenic acid enhanced activity of lipoxygenase, the key enzyme of oxilipin biosynthesis [7], in *Nicotiana tabacum* cell culture [8]. In the cell cultures of *Agrostis tenuis*, *Rauvolfia serpentine* and *N. tabacum*, addition of α-linolenic acid induced accumulation of JA and its bioactive precursor, 12-oxophytodienoic acid (12-oxo-PDA) [6]. These activities allowed to acknowledge the two acids as the potential elicitors. However, to our knowledge, they were not tested to increase metabolite production in cultured adventitious roots.

* Corresponding author. Tel.: +82 43 261 2529; fax: +82 43 272 5369.
E-mail address: paekky@chungbuk.ac.kr (K.Y. Paek).
[1] Current address: Dalian Agricultural Science Research Institute, Dalian 116036, China.
[2] Current address: National Agrobiodiversity Center, National Academy of Agricultural Science, RDA, Suwon 441-707, Republic of Korea.

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Here we investigated the effects of various concentrations of exogenous linoleic and α-linolenic fatty acids on biomass accumulation, production of saponins, polyphenolic and flavonoid compounds, fatty acid composition and activities of major antioxidant enzymes in *P. ginseng* adventitious roots cultured in balloon-type bioreactors.

2. Materials and methods

2.1. Adventitious root culture

Adventitious roots of *P. ginseng* C.A. Meyer viz. CBN-1 were induced and proliferated as described previously [5]. Roots were maintained in 5 l balloon-type airlift bioreactors containing 4 l of modified Murashige and Skoog (MS) liquid medium supplemented with 5 mg l^{-1} indole butyric acid (IBA) and 5% sucrose at 25 ± 1 °C in darkness [5]. Subcultures were performed every 40 days by transferring 5 g FW l^{-1} roots to the fresh medium.

2.2. Determination of residual sugars in the media

Media samples (20 ml) were taken every 5 days during the course of 80-days bioreactor culture. After filtering through 0.2 μm membrane filter (Advantec, Japan), the medium was diluted 10-fold and 1 ml probe was used for sugar analysis. Content of sucrose, glucose and fructose in the media were determined by an HPLC system (Waters 600S controller, Waters 626 pump, Waters Co., Mildford, USA) with high performance carbohydrate column (300 mm × 7.8 mm, Waters Co., Mildford, USA) coupled to a reflex Differential Refractometer (Waters Co., Mildford, USA). A mobile phase consisting of 75% acetonitrile was used at flow rate of 1.0 ml min^{-1}. Glucose, fructose and sucrose peaks were identified and quantified using Sigma standards.

2.3. Elicitation procedure and harvesting

For elicitation experiments, 5 g FW l^{-1} roots were inoculated into 5 l bioreactors containing 4 l of modified MS liquid medium supplemented with 5 mg l^{-1} IBA and 5% sucrose. The airflow rate during cultivation was adjusted to 0.1 vvm and air temperature was controlled to 25 ± 1 °C. Solutions of linoleic (LLA) and α-linolenic (α-LNA) fatty acids were purchased from Chromadex (Santa Ana, CA, USA), diluted with ethanol and filtered through 0.45 μm filters (Advantec, Japan). Fatty acid solutions were aseptically added to culture medium at day 40 after the inoculation in amounts required to achieve final concentrations of 0.0, 1.0, 2.5, 5.0, 10.0, and 20.0 μmol l^{-1}. Roots were harvested 7 days after the elicitation and washed with running tap water for 5–7 min followed by double rinse with distilled water. Samples were blotted dry and fresh weight was measured. Dry weight (DW) was recorded for roots dried at 60 °C for 3 days. The growth ratio was calculated as (final DW − inoculum DW)/inoculum DW.

2.4. Determination of ginsenosides content

The extraction and analysis of ginsenosides were performed by the method of Yu et al. [4]. The ginsenoside fraction was analyzed using an HPLC system with an Altec Platinum C18 column (particle size 1.5 μm, 33 mm × 7 mm) by eluting water/acetonitrile at 3:1 (v/v) for 10 min then at 63:37 (v/v) for 25 min with a flow rate of 1.2 ml min^{-1}. Ginsenosides were detected at 203 nm. Ginsenoside standards were purchased from Chromadex. The total ginsenoside content was calculated as the sum of the ginsenoside fractions.

2.5. Determination of total phenolics and flavonoids content

For total phenolics and flavonoids assay 1 g of dry root material was extracted with 100 ml of 80% methanol at 60 °C for 2 h and filtered through filter paper (Advantec, Japan).

The total phenolics content was determined by Folin-Ciocalteu colorimetric method [9] using gallic acid as a standard. Hundred microliters of root extract were mixed with 2.5 ml deionized water followed by addition of 0.1 ml (2N) Folin-Ciocalteu reagent. The mixture was vortexed 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 in a spectrophotometer (UV-1650PC, Shimadzu, Japan). The measurement was compared to a standard curve of prepared gallic acid solution and the results were expressed as means mg of gallic acid equivalent per gram of root material.

The total flavonoids content in roots was determined by colorimetric method [10]. Briefly, 0.25 ml of methanolic root extract or (+)-catechin standard solution was mixed with 1.25 ml of distilled water followed by addition of 0.75 ml of a 5% sodium nitrite solution. After 6 min, 0.15 ml of a 10% aluminum chloride solution was added. The mixture was brought to 2.5 ml with distilled water and shaken well. The absorbance was measured immediately at 510 nm using a spectrophotometer (UV-1650PC, Shimadzu). The results were expressed as mg of (+)-catechin equivalents per gram of root material.

2.6. Determination of fatty acid composition

The contents of palmitic (C16:0), oleic (C18:1), linoleic (C18:2) and α-linolenic (C18:3) fatty acids were measured in roots 7 days after the elicitation as described by Wang et al. [11]. Briefly, fatty acids after extraction and methylation were separated in a gas chromatograph (GC, HP6890, Hewlett Packard Co.) equipped with fused silica capillary column (100 m × 0.25 mm, i.d. ×0.20 μm thickness, Supelco, SP™-2560, USA). Helium was used as a carrier gas. The initial oven temperature was controlled to 170 °C for 5 min then increased by 4 °C min^{-1} to 200 °C (held for 40 min). The injector and detector temperatures were controlled to 250 and 260 °C, respectively. The fatty acids were identified and quantified by comparing with Sigma standards.

2.7. Extraction and assay for enzyme activities

Extraction and assay for superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) activities were performed as described previously [12].

2.8. Statistical analysis

In elicitation experiments, three bioreactors were harvested for control and for each concentration of linoleic and α-linolenic fatty acids. Analysis of residual sugars in the medium as well as analysis of ginsenosides, total phenolics and flavonoids, and fatty acids in adventitious roots was performed for triplicate extracts. Data were analyzed by ANOVA and Duncan's multiple range tests (DMRT) following arcsine transformation, using the SAS 8.1 software (SAS Institute Inc., Cary, NC, USA). Tables and figures present mean values with their SE.

3. Results

3.1. Growth of adventitious roots and medium characteristics

P. ginseng adventitious roots cultured in balloon-type bioreactors exhibited the standard S-type growth curve including the