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Effect of Polysaccharide Elicitors on the Production of Decursinol Angelate in *Agelica gigas* Nakai Root Cultures

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Abstract Root cultures of *Angelica gigas* Nakai were found to be sensitive to elicitation by polysaccharide elicitors, such as methyl-β-cyclodextrin, glucan, carboxymethyl-β-chitin, chitosan, yeast extract and pectin. For the production of decursinol angelate, carboxymethyl-β-chitin and glucan were found to be the most efficient elicitors. The enhanced accumulation of decursinol angelate was proportional to the increase of the phenylalanine ammonia-lyase (PAL) activity after the treatment with most of the elicitors. However, carboxymethyl-β-chitin treatment did not stimulate the PAL activity, despite the 1.6-fold increase in the decursinol angelate production.

Keywords: *Angelica gigas*, decursinol angelate, elicitation, polysaccharide elicitor, root culture

INTRODUCTION

Utilization of phytochemicals, obtained from various kinds of plant, has increased all over the world. In East Asian countries, much research has been conducted to find useful products, such as anticancer agents, from traditional herd medicines. Compared to chemical syntheses, screening of natural plants has many advantages due to the long history of Oriental medical treatments.

A diverse range of important pharmaceuticals are produced by higher plants. There is still a continuing interest in the use of plant cell suspension, or organ, cultures as alternative sources for the field cultivation of plant-derived chemical productions [1]. This provides for the continuous, controllable and reliable production of industrially important phytochemicals originating from various higher plants in any location.

Angelica Radix, the root of *Angelica gigas* Nakai, a well-known crude drug, and traditional Korean herbal medicine, used in the treatment of gynecological diseases. Decursinol angelate and decursin are novel cancer chemotherapeutic candidates isolated from *A. gigas* Nakai [2]. Their cytotoxic activities against various human cancer cell lines are known to be very effective [3]. Decursin is a pyranocoumarin, and a major component in *A. gigas* Nakai roots. It has been used, not only to treat anemia, but also as a sedative, anodyne and as a traditional Korean tonic agent. Decursin has been shown to activate protein kinase C *in vitro* [4]. In addition to the presence of decursin and decursinol angelate, polysaccharide fractions, from the root of *A. gigas* Nakai, have been reported to have an immunostimulating activity related with T-lymphocyte activation. The immunostimulating polysaccharide was produced extracellularly in suspension cultures of *A. gigas* Nakai [5]. These cell suspension cultures could be preserved in liquid nitrogen with high viability [6].

Even though decursin and decursinol angelate were not produced by suspension cell cultures, it was found that decursinol angelate could be produced by root cultures of *Angelica gigas* Nakai [7]. There were differences in the specific yields between the primary and secondary roots cultivated in flasks. In addition, NAA (1-naphthaleneacetic acid) enhanced the production of decursinol angelate in root cultures. When a secondary metabolite is not produced in cell suspension cultures, differentiated organ cultures, such as root cultures, can be a substitute for the production.

In plant cell and organ cultures, the accumulation of secondary metabolites can be enhanced by the treatment of various kinds of elicitors, which can be either biotic or abiotic. Elicitation of root cultures has been reported for the production of several plant-derived chemicals [8,9].

In this study, the effects of various polysaccharide elicitors, on the production of decursinol angelate, in root cultures of *A. gigas* Nakai were investigated. The activity of phenylalanine ammonia-lyase (PAL) was monitored to examine the relationship between the production of decursinol angelate and the defense mechanisms of the plant.

The roots of the axenic plantlet of *A. gigas* Nakai were used for the initiation of root cultures in a liquid medium. Schenk and Hildebrandt (SH) medium, containing 30 g/L sucrose, 1 mg/L NAA and 0.6 g/L casein hydrolysate, was used for the initiation and maintenance of the root cultures. The pH of the medium was adjusted to 5.8

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using 1 M NaOH. Subcultures were performed every 15 days. The cultured roots were maintained in 250-mL Erlenmyer flasks, with 100 mL of fresh medium, on a gyratory shaking incubator operating at 120 rpm under dark conditions at 25°C.

Both the fresh and dry weights FW and DW, respectively) of the cultured roots were measured for the estimation of growth. The cultured roots were filtered through a Whatman No. 1 filter paper, under vacuum, and washed three times with distilled water to remove the residual sugar from the surface. The biomass was weighed as the fresh weight. The roots were then transferred to a pre-weighed aluminum tray, dried at 60°C for 2 days and the dry weight measured.

Of the polysaccharide elicitors, the methyl-β-cyclodextrin, pectin and chitosan were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The glucan and carboxymethyl-β-chitin were provided by Pacific Chemical Co. (Suwon, Korea). Each elicitor was added to the cultures on the 14th day, to make 100 mg/L. The yeast elicitor was prepared from yeast extract, obtained from Difco Laboratories (Detroit, MI, USA), using ethanol precipitation. The total carbohydrate content of the yeast elicitor was measured using the phenol-sulfuric acid method [10]. Yeast extract elicitor was added at a concentration of 160 mg glucose/L. Chitosan, originating from crab shells, was used after purification. One gram of chitosan was dissolved in 90 mL of 0.1 M acetic acid and centrifuged at 8,000 rpm for 30 min to remove the precipitate. The pH of the supernatant was adjusted to 8.0 using 5 M NaOH and the newly formed precipitate collected, washed with distilled water and freeze-dried for use as an elicitor.

The extraction of the PAL and the measurement of its activity were performed using the methods described by Ahn *et al.* [11], with slight modification. The activity of PAL was measured at 290 nm after the reaction with L-phenylalanine as a substrate. A fraction of the cultured root (0.1 g) was homogenized with liquid nitrogen and extracted with lysis buffer. The supernatant was used for the assay, with D-phenylalanine used as the negative control. One unit of PAL activity was defined as the production of 1 nM *trans*-cinnamic acid in 1 min under assay conditions.

Ethanol was used to extract the decursinol angelate from the dried roots. An HPLC system, consisting of a Model 910 pump (Young-In Scientific Co., Seoul, Korea), UV detector and Rexchrome S5-100-ODS column (4.6 × 250 mm), was used to analyze the decursinol angelate. The mobile phase was a mixture of water and acetonitrile at the ratio of 50:50 (v/v), and was used under isocratic conditions at a flow rate of 1 mL/min, and the decursinol angelate detected at 330 nm.

In order to enhance the decursinol angelate production from the *A. gigas* Nakai root cultures, various polysaccharide elicitors were used as treatments. The polysaccharide elicitors used in this study were glucan, methyl-β-cyclodextrin, carboxymethyl-β-chitin, chitosan, yeast elicitor and pectin, which mostly originated from the cell walls of fungi or plants. The effects of the various

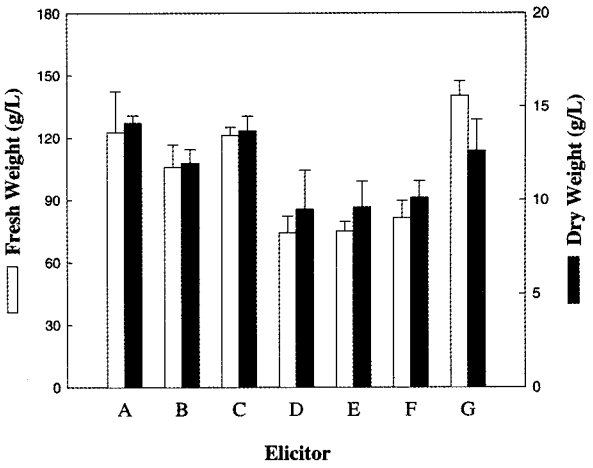

Fig. 1. Effects of various elicitors on the growth of *Angelica gigas* Nakai roots. Elicitors were added into the media at day 15, and the root growth measured 3 days after the treatment. All experiments were performed in triplicate. (A) control; (B) methyl-β-cyclodextrin; (C) glucan; (D) carboxymethyl-β-chitin; (E) chitosan; (F) yeast extract; (G) pectin.

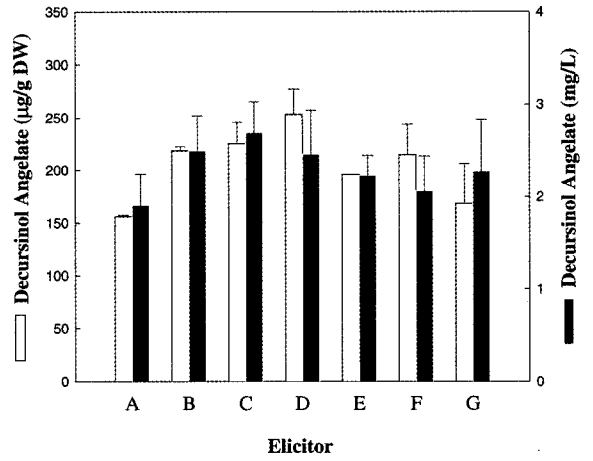

Fig. 2. Effects of various elicitors on the production of decursinol angelate in 18-day-old roots of *A. gigas* Nakai. (A) control; (B) methyl-β-cyclodextrin; (C) glucan; (D) carboxymethyl-β-chitin; (E) chitosan; (F) yeast extract; (G) pectin.

elicitors on the growth of the cultured roots is shown in Fig. 1. On the addition of 100 mg/L glucan to the root cultures, the root growth measured from the dry weight was almost the same as that of the control culture. Methyl-β-cyclodextrin and pectin inhibited the growth to some extent. Severe growth inhibition was caused by the addition of carboxymethyl-β-chitin, yeast elicitor and chitosan.

The effects of the various polysaccharide elicitors on the production of decursinol angelate are shown in Fig. 2 The results are totally different from those of the root growth. The addition of any of the polysaccharide elicitor

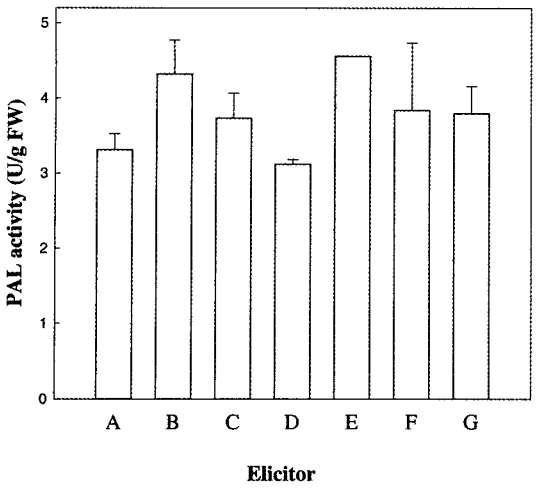

Fig. 3. Effects of various elicitors on the PAL activity in root cultures. (A) control; (B) methyl-β-cyclodextrin; (C) glucan; (D) carboxymethyl-β-chitin; (E) chitosan; (F) yeast extract; (G) pectin.

enhanced the production of decursinol angelate, even though the levels of enhancement were slightly different. In terms of the volumetric levels of decursinol angelate, glucan enhanced the production the most, up to 2.5 mg/L, which was a 1.3-fold increase compared to that of the control culture. With respect to the specific levels of decursinol angelate in the root biomass, the addition of carboxymethyl-β-chitin gave the best result, with a 1.6-fold increase. This high specific level was due to the low cell mass as a result of the severe growth inhibition caused by the carboxymethyl-β-chitin. Despite this severe growth inhibition, the biosynthesis of decursinol angelate was significantly stimulated by the addition of carboxymethyl-β-chitin.

Chitin and pectin were used as elicitors to enhance the production of plumbagin, and its release into the medium, in *Drosophyllum inusitanicum* Link suspension cultures [12]. Chitosan has been reported to act as an elicitor for the improved production of menthol in *Mentha piperita* cultures [13]. In hairy root cultures of *Brugmansia candida*, tropane alkaloid production was increased up to 3-fold by the addition of yeast extract as an elicitor [14].

In order to clarify the involvement of the general eliciting pathway related to the plant defense mechanism, that is the phenylpropanoid pathway, the PAL activity was measured during the treatment with the various polysaccharide elicitors. The results are shown in Fig. 3. With the exception of the carboxymethyl-β-chitin treatment, the PAL activity in elicitor-treated cultures was higher than that in the control culture. In the case of chitosan, a 1.4-fold increase in the PAL activity was obtained. From this result, the mechanism of the enhanced production of decursinol angelate in carboxymethyl-β-chitin-treated root culture was assumed to be different from those of the other elicitors. Elicitors are usually known to activate PAL [15]. Therefore, the carboxymethyl-β-chitin probably enhanced the production of decursinol angelate by activating a pathway other than the phenylpropanoid pathway. All the other elicitors were thought to be related to the PAL.

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