Chitosan activates defense responses and triterpenoid production in cell suspension cultures of *Betula platyphylla* Suk.

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Triterpenoid production and plant defense enzyme activity in suspension cultures of *Betula platyphylla* Suk. cells treated with chitosan elicitor were investigated. Chitosan, at the optimal concentration of 100 mgL\(^{-1}\) enhanced triterpenoid production by 2.6-fold, with a maximum yield of 202.75 versus 78.72 mgL\(^{-1}\) of the untreated control cells. Furthermore, the activities of superoxide dismutase, phenylalanine ammonia-lyase, endochitinases and exochitinases increased by 1.6 ~ 3.0 fold. These results indicate that triterpenoid accumulation in *B. platyphylla* Suk. cell are the consequence of a plant defense response to chitosan elicitation.

Key words: Chitosan, *Betula platyphylla* Suk., defense enzymes, triterpenoids.

**INTRODUCTION**

Triterpenoids, extracted from the bark of *Betula platyphylla* Suk., are an excellent drug for its antiviral, antibacterial, antitumor, anti-aids properties (Jing et al., 2005; Alakurtti et al., 2006; Wen et al., 2007). Due to the slow-growth of *B. platyphylla* Suk. trees in nature, *B. platyphylla* Suk. triterpenoids supply has to resort to alternative sources rather than the natural plants. In a previous study, we found that triterpenoids (betulin and oleanolic acid) may be produced in *B. platyphylla* Suk. cell culture, but their content was significantly lower than from the bark of *B. platyphylla* Suk. (Fan et al., 2009a). Therefore, enormous efforts have been made in the search and development of plant cell culture techniques for efficient production of triterpenoids (Fan et al., 2009b; Wang et al., 2008a; Wang et al., 2008b).

Chitosan (\(\beta\)-1,4-linked glucosamine) is a deacetylated derivative easily obtainable from various sources, particularly from the exoskeletons of crustaceans. It is also found in cuticles of insects as well as in the cell walls of fungi and some algae (Sanford and Hutchings, 1987). Being present in the wall of pathogenic microorganisms, it can be recognized as a microbe associated molecular pattern (MAMP) by the plant immune system, thus activating plant defense responses (Iriti and Faoro, 2009) and the related biosynthesis of secondary metabolites. Therefore, chitosan has been widely applied as a potent elicitor in plant cell suspension cultures to enhance secondary metabolite production, such as menthol (Chang et al., 1998), plumbagin (Nahálková et al., 1998), paclitaxel (Zhang et al., 2000), artemisinin (Putalun et al., 2007), phenylethanoid glycosides (Liu et al., 2008) and phenylpropanoid derivatives (Chakraborty et al., 2009).

However, the study of Sánchez-Sampedro and co-workers showed that different concentrations of chitosan (5 ~ 200 gL\(^{-1}\) culture medium) did not stimulate any increase in silymarin accumulation in *Silybum marianum* cells (Sánchez-Sampedro et al., 2005). Also, Eilert et al. (1984) found that addition of chitosan resulted in reduction in cell growth and artemisinin content in cultures of *Ruta graveolens*. From the above results it can be concluded that the function of chitosan as elicitor is species specific.

The objective of the current work was to verify the eliciting capacity of chitosan in improving plant growth...
and triterpenoid accumulation in *B. platyphylla* Suk. cell suspension cultures and to correlate this possible enhanced accumulation with the activation of plant general defense responses, that is, the activities of superoxide dismutase (SOD), phenylalanine ammonia-lyase (PAL), endochitinases (EDC) and exochitinases (EOC).

**MATERIALS AND METHODS**

**Plant materials**

The cell line used in the present study was developed from the auxiliary buds of 30-year-old *B. platyphylla* Suk. Suspension cultures were established and cultivated on optimized Nagata- Takebe (NT) medium supplemented with 0.1 mgL⁻¹ 6-benzyladenine (6-BA), 0.01 mgL⁻¹ thidiazuron (TDZ), and 20 gL⁻¹ sucrose, at an interval of 7 ~ 10 days. The medium pH was adjusted to 5.6 with 1 M NaOH before autoclaving. Although not shown in this report, previous work demonstrated a considerable variation in the amount of triterpenoids with subcultures. For this reason, experiments were done in stabilized cultures, that is to say when triterpenoid production was similar after four consecutive subcultures.

A single stock culture grown in a 1000 ml Erlenmeyer flask was used as inoculum for the experimental flasks. All experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of the corresponding liquid media with 20 gL⁻¹ sucrose and inoculated with 4.0 g fresh weight of 8-day-old cell suspension cultures. The Erlenmeyer flasks were incubated on a rotary shake (110 rpm) at 25°C. Illumination was regulated so as to give 14 h of light (photon flux density 1400 - 2000 μmol·m⁻²·s⁻¹) provided by fluorescent tubes (mixing Osram fluora and Osram daylight types) with a photon flux density (400 - 700 nm).

**Chitosan**

Stock solutions at 50 mgml⁻¹ of water-soluble chitosan (minimum 85% deacetylation, average molecular weight 5 KDa, Shandong, Aokang Bio-Technology Co., Ltd.) were prepared by dissolving them in distilled water and filter sterilized.

**Dry cell weight and triterpenoid determination**

For dry cell weight determination, cells were harvested and collected by centrifugation at 3000 rpm for 15 min and washed with distilled water. The fresh cells were dried at 60 ± 2°C to a constant dry weight. Extraction of triterpenoids was done with 95% methanol (Fan et al., 2008a). Extract was thereafter analyzed by ultraviolet spectrophotometer under 510 nm wavelength.

**Enzyme extraction and activity assay**

Fresh cells were frozen and homogenized in an ice bath with extraction buffer which consists of 0.05 M Na phosphate buffer (pH 7.0), 2% polyvinylpyrrolidone, 0.25 M sucrose, 2 mM EDTA, 5 mM dithiothreitol and 5 mM MgCl₂. The homogenate was filtrated through a 4-layer of nylon cloth and the filtrate was centrifuged at 13000 rpm for 15 min at 4°C. The supernatant was used for enzyme assays. Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) activity was based on the PAL conversion of L-phenylalanine to cinnamic acid using a modified method of Zhang (2002). Superoxide dismutase (EC 1.15.1.1) activity was assessed by monitoring the inhibition of photochemical reduction by nitro blue tetrazolium reaction (NBT), according to the method of Beyer and Fridovich (1987). Endochitinases (EC 3.2.1.14) and exochitinases (EC 3.2.1.52) were determined following the method of Jeuniaux (1966), using colloidal chitin as substrate. Colloidal chitin was prepared by the method as described by Sandhya (2004).

**Statistical analysis**

All experiments were repeated three times. The data obtained were statistically analysed by SPSS (8.0) Means and standard error were calculated from three replicates.

**RESULTS**

**Elicitor dose**

In preliminary experiments, chitosan elicitor was screened at various doses (0 ~ 500 mgL⁻¹) to optimize the concentrations to obtain maximum triterpenoid accumulation (Figure 1). Addition of chitosan elicitor to *B. platyphylla* Suk. cells enhanced cell dry weight and triterpenoid production with an increase in the dose up to 100 mgL⁻¹. Above this level, decreased cell growth and triterpenoid accumulation were observed. The maximum triterpenoid production of the treated cell cultures was 202.75 mgL⁻¹ under 100 mgL⁻¹ elicitor dose, which is 2.6-fold higher than that of the control without elicitation (78.72 mgL⁻¹). On the basis of the results the concentration of 100 mgL⁻¹ in the medium was chosen for further experiments.

**Culture age**

The response to elicitation is dependent on growth phase of the culture, which not only affects the quantitative response but also the product pattern in general (Komaraiah et al., 2002). Therefore, *B. platyphylla* Suk. cell cultures at various ages (3, 8 and 13-day-old) were
treated by the selected chitosan elicitor of 100 mgL⁻¹, and then dry weight and triterpenoid accumulation were analyzed after a five-day induction (Figure 2). It was observed that the maximum dry weight (9.90 gL⁻¹) and triterpenoid production (230.01 mgL⁻¹) were obtained by eliciting 13-day-old cells. However, the triterpenoid production under chitosan treatment at 8 days (31.57), 13 days (24.24) and 3 days (18.49) were different. So, the optimum age of the culture for elicitation was on the 8th day.

**Incubation time**

The effect of treatment time with elicitors is presented in the Figure 3. Effective induction of triterpenoid accumulation in culture cells varied from 1 to 7 days. A maximum triterpenoid yield of 239.77 mgL⁻¹ DW was obtained after 3 days, whereas triterpenoid production declined during an extended time of incubation. This reveals that the duration of cultivation in the elicitor was rather important with respect to triterpenoid production.

**Effect of chitosan on defense enzyme elicitation**

Activation of defense responses of *B. platyphylla* Suk. cells to chitosan elicitation was assessed by determining phenylalanine ammonia-lyase (PAL), superoxide dismutase (SOD), and chitinase activities at different time intervals from 0.5 to 72 h after treatment (Figure 4). PAL activity slowly increased with the extension of elicited time in *B. platyphylla* Suk. cells, and the maximum was reached 24 h after elicitation, at which time it was 2.0-fold higher than in non-elicited cells. SOD activity was transiently increased in elicited cells at 4 h, being 2.1-fold higher than control. Chitinase activities was also transiently increased after treatment, with a peak at 6 h, though the enhancement of endochitinase (EDC) was different from that of exochitinase (EOC), being 1.6 and 3.0 fold higher than control, respectively.

**DISCUSSION**

Our results demonstrate that chitosan is effective in enhancing triterpenoid biosynthesis in *B. platyphylla* Suk. suspension cell cultures. Triterpenoid production of treated cell cultures reached 202.75 mgL⁻¹, which is 2.6-fold higher than that of control (78.72 mgL⁻¹). The results also show that elicitor dose, cell culture age and incubation time significantly influence biomass growth and triterpenoid production in *B. platyphylla* Suk. cells. This is in agreement with that reported for plumbagin production in suspension cultures of *Plumbago rosea* L. and phenylethanol glucosides biosynthesis in *Cistanche deserticola* cell suspension cultures elicited with chitosan (Komaraiah et al., 2002; Cheng et al., 2006), confirming that optimization of induction conditions is rather important with respect to secondary metabolite production.

Defense reactions that result from elicitors are usually composed of a multitude of biochemical events including Oxidative burst, accumulation of wall-bound phenolic compounds, induction of enzymes for lignin and phytoalexin synthesis, synthesis of hydrolytic enzymes like chitinase and β-1,3-glucanase, and accumulation of secondary metabolite (Qi et al., 2008). Among elicited enzyme activities, phenylalanine ammonia-lyase (PAL) is
the first to be activated, catalyzing the initial step of the phenylpropanoid pathway, that regulates the production of precursors for lignin biosynthesis and other phenolic defensive compounds in plant cells (Liu and Cheng, 2008). Superoxide dismutase (SOD) is instead activated to scavenge the overproduction of reactive oxygen species (ROS) produced during the initial oxidative burst following elicitation (Iriti and Faoro, 2007). Thus, the increased activities of SOD and PAL we found suggest that a typical defense reaction has been activated by chitosan elicitation (Chakrabortya et al., 2009), and this is possibly responsible for enhanced triterpenoid biosynthesis.

Plant chitinases, which are induced by pathogen infection and elicitor treatments, are pathogenesis-related (PR) proteins that play a role in the defense system in addition of being useful markers for host defense responses (Shinya et al., 2007). Besides defense responses, plant chitinases are involved in tolerance, to abiotic stresses, symbiosis, and plant development (Wiweiger et al., 2003). Because individual plant species have many different chitinases (Brunner et al., 1998; Truong et al., 2003), it is important to understand the regulation and functions of different chitinase genes. In this work, we found that chitosan differentially affected chitinases activity, with endochitinase (EDC) and exochitinase (EOC) raised 1.6 and 3.0 fold, respectively, in comparison with non-elicited control. The significance of this differential raising is not known and needs further investigation. Nevertheless, the fact that chitosan raised chitinases activity in B. platypylla Suk. cell suspension cultures confirms its capability in eliciting defense mechanisms also in this plant species. In turn, this suggests that the observed enhanced triterpenoid biosynthesis may be part of these mechanisms.

In conclusion, the possibility of enhancing significantly the biosynthesis of important triterpenoids such as those of B. platypylla Suk. in cell cultures by elicitation with chitosan, a cheap natural polysaccharide, deserves particular attention in view of a possible large scale practical application. In this regard, studies are underway to demonstrate the possibility of further increase in the production of triterpenoids with repeated elicitation cycles, in both shake flasks and bioreactors.
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