Enhanced paclitaxel productivity and release capacity of *Taxus chinensis* cell suspension cultures adapted to chitosan

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Abstract

This work characterized the growth and paclitaxel production and extracellular release of *Taxus chinensis* cell suspension cultures adapted to chitosan stress by comparing with the unadapted cells. Without elicitor treatment, the paclitaxel yield of the chitosan-adapted cells was two-fold that of the unadapted cells. When both cell cultures were elicited by Ag^+ and methyl jasmonate (MJ), the paclitaxel yield of the chitosan-adapted cells was 4.6- and 3.2-fold that of the unadapted cells, respectively; the paclitaxel release ratio of the chitosan-adapted cells was 2.8- and 3.2-fold that of the unadapted cells, respectively. Furthermore, upon elicitor treatment, the activation of phenylalanine ammonia-lyase (PAL) activity, a common and important response of plant cells to biotic and abiotic stresses, as well as the cell viability and permeability of the chitosan-adapted cells was significantly higher, compared with the unadapted cell cultures, which corresponded well to the superior paclitaxel yield and release. These results suggest that adaptation to chitosan is an effective strategy for improving paclitaxel yield and release of *T. chinensis* cells.
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1. Introduction

Paclitaxel is a novel anticancer drug, which has been approved by the United States Food and Drug Administration to treat breast, ovarian and lung cancers as well as AIDS-related Kaposi's sarcoma. Since the supply of paclitaxel from the *Taxus* tree is in great shortage, large-scale cell suspension cultures of *Taxus* spp. are viewed as a promising production process. Various strategies have been examined to enhance the productivity of paclitaxel in *Taxus* cell suspension cultures, mainly including precursor [1] and nutrient (sugar) feeding [2], in vitro extraction (two phase culture) [3], and treatment with elicitors, such as, chitosan [poly (1,4-β-ᴅ-glucopyranosamine)], silver ion (Ag^+), benzoic acid, fungal elicitors, methyl jasmonate (MJ) and arachidonic acid [4]. Among them, elicitation is the most effective strategy. However, elicitors also severely reduce cell viability and growth at the optimal

concentration [3]. So we have interest in improving cell tolerance against elicitor stress in *Taxus* cell cultures.

Chitosan is the deacetylated form of chitin, which is the main component of the cell walls of some fungal species and of the exoskeletons of insects and crustaceans, being the second most abundant natural polysaccharide on the earth just next to cellulose. As a natural, biocompatible, cationic biopolymer, chitosan mimics the effects of some pathogenic microorganisms to stimulate plants to biosynthesize secondary metabolites. Therefore, chitosan has been widely applied as a potent elicitor in plant cell suspension cultures to enhance secondary metabolite production [5,6]. In addition, chitosan promotes plant secondary metabolite release from the cells as well [7] and has been shown to trigger paclitaxel production alone or in combination with MJ and Ag^+ in *T. chinensis* cell cultures [4]. Also it is shown that chitosan stimulates the immunity of plants, protects plants against microorganisms [8,9] and promotes plant growth and development [10].

Sub-lethal biotic or abiotic stress enhances the tolerance or resistance of plants against the same or similar stresses, although the molecular mechanism remains largely unclear

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[11,12]. Plants have the ability to acquire an enhanced level of resistance to pathogen attack after being exposed to specific stimuli, such as necrotizing attackers, nonpathogenic root-colonizing pseudomonads and some natural and synthesized inducers including SA and MJ [13,14]. The induced resistance makes the plant cells to react more quickly and efficiently to subsequent elicitor treatment or pathogen attack, a process called 'priming', fostering secondary metabolism [13,14].

Taxus chinensis cell cultures adapted to chitosan were established by sub-cultured in medium containing chitosan. The objective of this work was to characterize the growth and paclitaxel production and extracellular release of the chitosan-adapted cells by comparing with the unadapted cells. In addition, the activation of phenylalanine ammonia-lyase (PAL), a common and important response of plant cells to biotic and abiotic stresses, and extracellular protein content, an indicator of cell permeability, were also investigated.

2. Materials and methods

2.1. Cell cultures

The *T. chinensis* cell culture, adapted to grow in medium containing chitosan (from now on referred as chitosan-adapted cell culture), and the unadapted cell culture, E2, were used in this work. The chitosan-adapted cell culture was derived from E2, which was derived from zygotic embryos and maintained in MS medium with the half concentration of iron ions as previously described [4,15]. In brief, after the removal of the hard thick episperm, the seeds were washed under running tap water for 9 days. Then the embryos were isolated from seeds, and inoculated onto the solid B5 medium [16] with 0.5 g/l lactalbumin hydrolyzate (LH) + 0.1 g/l Vitamin C + 4 g/l activated carbon (AC) + 0.5 mg/l 6-benzylaminopurine (6-BA) + 0.1 mg/l dichlorophenoxyacetic acid (2,4-D) + 30 g/l sucrose + 8 g/l agar, and incubated at $25 \pm 1\,^{\circ}$C under 11 h light (1000 lx)/13 h dark regime for germination. The embryos germinated and grew to about 10 mm long green plantlets but without roots after about 2 weeks incubation from about 2 mm in length at the time when isolated from the seeds. If the plantlets either continuously incubated in the medium or transferred to the callus induction medium (B5 supplemented with 0.25 mg/l 2,4-D, and 0.01 mg/l 6-BA), they would form calli; if they were very carefully transferred to the above mentioned medium but excluding any plant regulator avoiding any wounding, they would root and form normal plantlets. The callus from the embryo was selected to initiate cell suspension cultures. E2 was screened from the formed callus lines.

The adaptation of cells to chitosan was carried out by sequentially sub-culturing the cells in the modified MS medium containing increasing concentration of chitosan, i.e., 10, 25 and 50 mg/l, for two sub-culture cycles at each concentration, and then maintaining in the medium containing 75 mg/l chitosan at a sub-culture interval of 20 days. Chitosan (Sigma, min. 85% deacetylated) was added to the medium at the desired concentration before autoclave. At the beginning of the experiments, the cells were sub-cultivated in medium containing 75 mg/l chitosan for 10 growth cycles and exhibited stable growth profiles.

2.2. Experimental protocol

The suspension cultures at the late exponential growth phase of the growth curve (days 18–20 post-inoculation) were filtered and the cells were transferred to the production medium for paclitaxel production as follows: 5 g (fresh weight) of cells were inoculated into a 125 ml Erlenmeyer flask containing 25 ml of the production medium. The production medium was Gamborg's B5 medium [16] supplemented with 2.5 mg/l α-naphthalene acetic acid (NAA) and 50 g/l sucrose. Cell suspensions were maintained in a rotary shaker at 120–130 rpm in the dark.

To determine the effect of elicitors on paclitaxel production, an abiotic elicitor, silver ions (Ag^+) and a biotic elicitor, MJ, were added to the chitosan-adapted and unadapted cell cultures on the 12th day of growth. Ag^+ was used in its stable form, silver thiosulfate ($Ag_2S_2O_3$) solution, prepared by adding the stock solution of $AgNO_3$ (Sigma) drop by drop to the stock solution of $Na_2S_2O_3$ (Sigma) with vigor stirring until the final molar ratio of $AgNO_3$ to $Na_2S_2O_3$ at 1:4 [17]. MJ was obtained from Serva (Texas, USA). MJ stock solution was prepared with dimethyl sulphoxide (DMSO). The maximal final DMSO concentration in the culture medium along with the addition of MJ was less than 0.05% (v/v). Control was treated with DMSO at 0.05% (v/v) without MJ.

2.3. Analysis

The biomass was measured on dry weight (DW) basis. The cells were separated from the culture medium by filtration through a Whatman filter paper (No. 3) under vacuum. The cell mass on the filter was rinsed twice with double distilled water and then dried in an oven at 50 °C until constant weight, to determine the DW [18]. Paclitaxel content within the cells and in the culture medium was measured by HPLC as previously described [4]. The release ratio of paclitaxel was calculated by dividing the paclitaxel content in the medium by the total paclitaxel content (paclitaxel in the medium + paclitaxel within the cells).

The measurement of 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 Bolwell (1985) and Heide et al. (1989) as reported previously [19].

Protein in the culture medium was determined by Coomassie brilliant blue dye using calf albumin as the standard [20].

Cell viability was determined by staining with fluorescein diacetate (FDA) following the method of Widholm [21]. A fluorescent microscopy was utilized to distinguish viable (fluorescent) from nonviable (nonfluorescent) cells as described previously [19].