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The Effects of NO and $AgNO_3$ on Cell Growth and Salidroside Synthesis in Rhodiola Sachalinensis A.Bor. Cell Suspension Culture

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Abstract

The purpose of this work was to investigate the effect of abiotic elicitors on the production of salidroside in *Rhodiola sachalinensis* A.Bor. Different concentration of each elicitor was respectively added into the cell suspension culture in different periods of cell culture. The content of salidroside was determined by high performance liquid chromatography (HPLC). NO could enhance cell growth and the synthesis of salidroside, whereas $AgNO_3$ inhibited cell growth, and promoted the synthesis of salidroside. 50 μmol/L of SNP as the donator of NO and 60 μmol/L of $AgNO_3$ were added into cell suspension culture on the 12th day. And the contents of salidroside were significantly increased up to 2.2 fold and 2.0 fold respectively. Therefore the elicitation by NO and $AgNO_3$ can effectively promote accumulation of the secondary metabolite in plant cell culture.

Keywords: NO; $AgNO_3$; Elicitor; Salidroside; *Rhodiola sachalinensis* A.Bor

Abbreviations: $AgNO_3$: Silver Nitrate; DMSO: Dimethyl Sulfoxide; HPLC: High Performance Liquid Chromatography; MS: Murashige and Skoog (1962) medium; NAA: α-Naphthaleneacetic acid; NO: Nitric Oxide; PAL: Phenylalnine Ammonialyase; SNP: Sodium Nitroprusside; 6-BA: 6-Benzyladenine

Introduction

Rhodiola sachalinensis A.Bor., a perennial herb, was regarded as a rare and endangered traditional Chinese medicine plant. Modern pharmacological studies had shown that salidroside in *R.sachalinensis* has the bioactive effects of anti-anoxia, anti-cold, anti-fatigue and anti-radiation and anti-cancer (Zhang et al., 1989; Chen et al., 2002; Xu et al., 2004; Zheng et al., 2000; Wang et al., 1992). Natural resources of *Rhodiola* plants are on the edge of extinction because of the pollen abortion,extreme growing environment and man-made over-collection due to commercial demands (Wu,1988).

The biosynthesis of secondary metabolites in plant could be regulated by making use of biotechnology during development. The accumulation of these metabolites increases in response to stress and different process of growth under the various environments (Darvill and Albersheim, 1984). Many method were used to optimize the secondary metabolites in cell suspension culture,such as elicitation,immobilization,cell wall permeabilization and feeding of precursors (Brodelius et al., 1989; Godoy-Hemandez et al., 1997; Biondi et al., 2002; Shakirova et al., 2003) while exploiting abiotic elicitors was the very attractive strategy .

In this study abiotic elicitors were selected to induce salidroside in the suspension culture cells of *R.sachalinensis*, as well as to provide an effective approach in large-scale cultivation for the future.

Materials and Methods

Suspension cell culture of *rhodiola sachalinensis*

R.sachalinensis plants were collected from the Changbai Mountains in Jilin province of China. The callus was induced from the stem and leaf of *R.sachalinensis* using plant tissue and cell culture techniques by Laboratory of Traditional Chinese Medicines Biotechnology in Shenyang Pharmaceutical University. Suspension culture cells were selected from cell lines of fine dispersion, uniform characters, similar shapes and size, fast growing speed and more stable growing and production capacity. Suspension culture conditions: MS (Murashige and Skoog (1962) medium) + sucrose 25 g/L + NAA (α-Naphthaleneacetic acid) 2.0 mg/L +6-BA (6-Benzyladenine)1.0 mg/L, pH value was adjusted to 5.8 before the sterilization, rotation speed: 110 ± 5 rpm, culture temperature: 24±1°C, light time: 12 h/d, light intensity: 85 μmol/m^2•s, the concentration of vaccination: 30 g FW/L. Suspension culture was in 30 ml liquid medium of 100 ml flask.

Measurement of dry and fresh weight of cells

The cultured cells in shake flask were washed by deionized water, fresh weight were obtained after filtration in vacuo. The cells were dried in 60°C oven until constant weight, then the dry weight of the cells were measured.

Determination of salidroside

The cells were dried to constant weight in an oven at 55°C for24 h, powdered by triturator and sieved with 50 mesh. 0.1 g cells samples were extracted for 24 h with 10ml distilled water at room temperature. After extracting by ultrasonic for 30 min,

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the sample was centrifuged at the speed of 4000 rpm for 15 min. The supernatant was collected, and the remaining coarse debris was extracted once according to the mentioned step above. The two supernatant was pooled together. Salidroside was determinated by HPLC.

Quantification of Salidroside by HPLC

The supernatant was filtered with a 0.45 μm membrane.The amount of salidroside were measured by HPLC (Shimadzu Co., Kyoto, Japan) using a 4.6mm×250 mm RP-C18 column (DIAMONSILC-18) and an ultraviolet refractive index detector (SPD-10ATvp) at 275nm. The column temperature was controlled at 40°C. Water (85% (v/v)) and methanol (15% (v/v)) were used as the mobile phase with a flow rate of 1.0 ml/min.

Statistical analysis

All experiments were carried out at least in triplicate to ensure good reproducibility. All data were subject to average analysis and expressed as mean±SD.

Preparation of abiotic elicitor

As the donor of nitric oxide (NO), SNP (sodium nitroprusside) was dissolved in DMSO (dimethyl sulfoxide). $AgNO_3$ was diluted to the appropriate concentration by distilled water,and sterilized by 0.22 μm membrane filter respectively. They were respectively added into the sterilized nutrient medium directly according to various concentrations or during the different periods of the time in the processes of cell culture (Jian et al., 2006).

Methods of adding elicitor

The former two elicitors were added into the medium respectively, the concentrations were:

(1) SNP: 10,50,100,150 μmol / L; (2) $AgNO_3$: 30,60,120,200 μmol / L;

The elicitors were respectively added into the suspension culture system ,which had been pre-cultured for 12 days, and cultured under fermentation condition for 48h, then harvest. The fresh weight, dry weight and salidroside content were determined, respectively. The elicitors at optimal concentration was added into different growth period in order to determine the optimal time.

Results

Effect on the suspension cell growth and the content of salidroside accumulation of *Rhodiola sachalinensis* by NO.

Effect of NO concentration

The influence of suspension cell growth by different concentration of SNP was investigated. The results were shown in Figure 1.

When the concentration of SNP was between 10 ~ 100 μmol/L, dry weight of the cells were all higher than these without SNP. The cell dry weight was 9.49 g/L with the 50 μmol/LSNP. While the concentration of SNP was 150 μmol/L, dry weight of the cells was 5.80 g/L, 4.30 g/L less than the controlled one 10.10 g/L. It showed that low concentration of SNP could promote cell growth, while high concentration of SNP had some inhibitory effect on the cell growth.

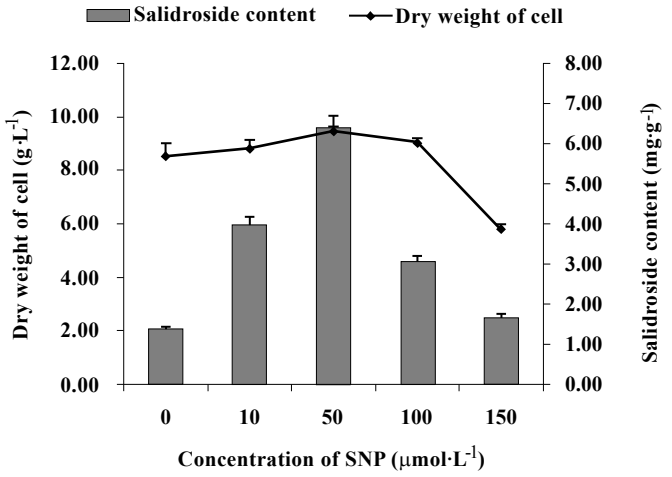

Figure 1: Effect on the dry weight and the salidroside content of different concentration of SNP.

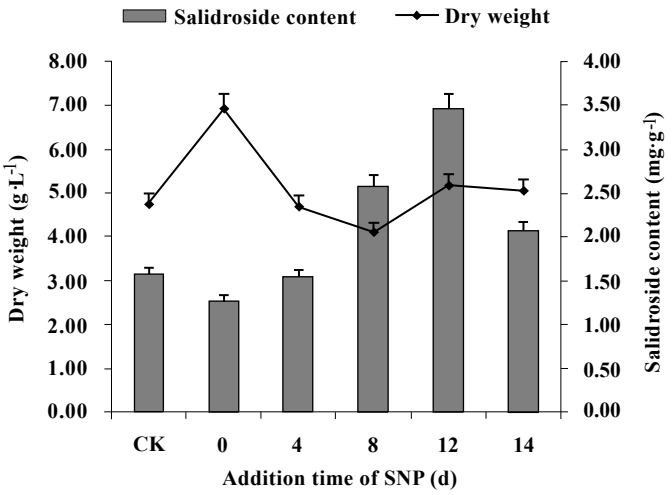

Figure 2: Effect on the dry cell weight and the salidroside content of different addition time of SNP(CK:blank control).

When SNP was between 10 ~ 50 μmol/L, the content of salidroside gradually increased with the SNP concentration increasing. The content of salidroside content reached the maximum 6.39 mg/g with 50 μmol/L of SNP, 4.65 fold higher than the control group. When the SNP concentration was higher than 100 μmol/L, salidroside content fell down. Salidroside content was only 1.67 mg/g with150 μmol/L of SNP. All these evidences indicated that low concentration of SNP played an important role in the promotion of salidroside induction, while high concentration was not conducive to the accumulation of salidroside. Therefore, the best concentration of SNP should be controlled at 50 μmol/L.

Effect of NO addition time

Based on the above experimental results, 50 μmol/L SNP were added on day 0, 4, 8, 12, 14 separately during the culture. It was harvested after culturing for 48 h. The influence of addition time on the cell growth and the salidroside accumulation were investigated. The results were shown in Figure 2. On day 0, SNP had more obvious promoting effect on cell growth than on day 4 or 8, which may indicate that cells accepted NO signal molecule at earlier time, then started to split rapidly.

When SNP was added on day 4 or 14, the dry weight of cells

and the salidroside content were all slightly improved. When the cells entering into log phase on the 8th and 12th day, the dry weight and the salidroside content were relatively enhanced. These results indicated it was the best time to add the elicitor on the 12th day during the culture. The content and production of salidroside were as high as 3.46 mg/g and 17.93 mg/L, which were 2.2 and 2.4 fold respectively. It illustrated that the elicitor induced during the advanced stage on the log phase of growth (the 12th day) showed better effect than induced during the earlier period.

Effect on the suspension cell growth and the salidroside accumulation in *Rhodiola* of $AgNO_3$.

Effect of $AgNO_3$ concentration

Similar with SNP, we selected the log phase the 12th day as the addition time of the elicitor for the first trial. In order to investigate the influence of the elicitor with different concentration on the cell growth and the salidroside metabolism, according to the concentrations: 30, 60, 120, 200 μmol/L , after culturing 12 days added the elicitor respectively, and cultured for 48h. The dry weight and salidroside content were measured. It was shown in Figure 3.

Our results suggested cell growth was suppressed with the concentration of $AgNO_3$ increased, the biomass significantly reduced. When $AgNO_3$ in the concentration of 30 and 60 μmol/L, salidroside content was increased. In the concentration of 60 μmol/L, the salidroside content reached the maximum with 3.52 mg/g. When the concentration of $AgNO_3$ exceeded 120 μmol/L, salidroside content would obviously decreased, and even lower than the control group. It may be caused by high toxic concentration of silver ions. In the process of culturing, a large number of death cells were observed. There were not enough energy and materials for the synthesis of salidroside. Consequently 60 μmol/L was chosen as the concentration of adding $AgNO_3$.

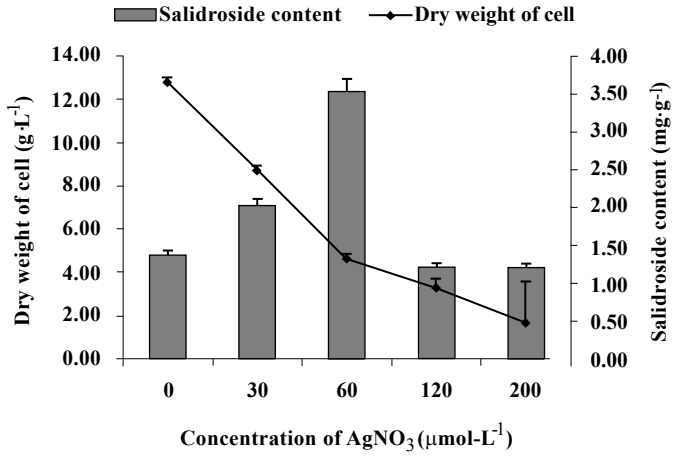

Figure 3: Effect on the cell dry weight and the content of salidroside of different concentration of $AgNO_3$.

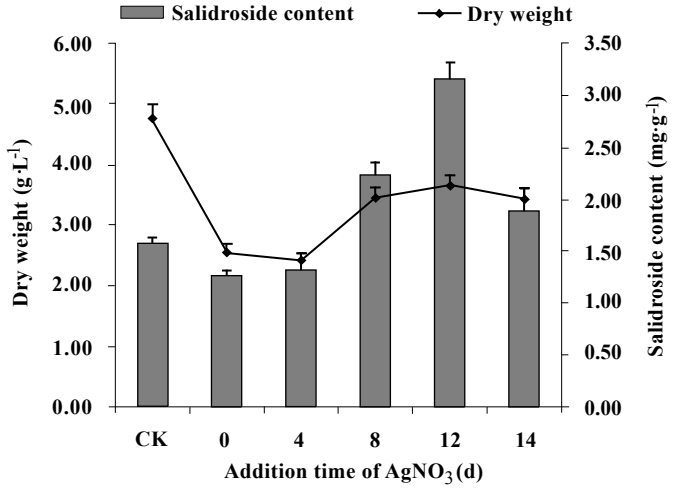

Figure 4: Effect on the dry cell weight and the salidroside content of different $AgNO_3$ addition time(CK:blank control).

Effect of $AgNO_3$ addition time

Based on the experimental results above, 60 μmol/L $AgNO_3$ was added on the 0th, 4th, 8th, 12th, 14th day. It was harvested after culturing for 48 h. Studied on the effect of the cell growth and accumulation of salidroside on addition time. The results were shown in Figure 4.

Compared with the control group, $AgNO_3$ inhibited the cell growth. $AgNO_3$ was added on day 0. It had a certain impact on the earlier cell growth and the later salidroside synthesis. With the proliferation of cells, the ability of resisting external additives was reinforced. The biomass increased with the $AgNO_3$ adding for a longer time. When $AgNO_3$ was added on the 12th day, the biomass reached the highest value 3.65 g/L. $AgNO_3$ had apparently better effects during on the late log phase (the 12th day) than on the early log phase. When induced on day 0, 4 and 14, the dry weight of the cells and the salidroside content had only a few improvement. In contrast, with the cells stepping into the log phase on the 8th day or 12th day, the dry weight and salidroside content had all been relatively enhanced after adding elicitor. The salidroside content and production reached the highest value 3.16 mg/g and 9.96 mg/L respectively, significantly increased up to 2.0 fold and 1.3 fold respectively, compared with the control group. The best time to add $AgNO_3$ was on the 12th day.

Discussion

This work clearly shows that abiotic elicitors such as NO and $AgNO_3$ can largely influenced the growth and production of salidroside in *Rhodiola sachalinensis* A.Bor.

In this research, the reaction to the elicitor of cells was closely related with the cell cycle. It not only affected the accumulation of secondary metabolites, but also played a significant role in the accumulation of its models.

NO played an important role in plant growth and development, seed germination and disease resistance response. It drew a great attention of researchers (Hu et al., 2003). The results in this study showed that NO enhanced cell growth and the salidroside synthesis, whereas $AgNO_3$ inhibited cell growth, but the salidroside synthesis was promoted.

Nitric oxide (NO) and active oxygen (ROS) are two common signal molecules in plant. They played a significant part in plant stress reactions, so as to enhance the content of secondary metabolites. H_2O_2 produced by oxidation may be the molecular signals of NO to provocate salidroside synthesis (Jian et al., 2005). Metal ions (Ag^+) can stimulate biosynthesis of many secondary metabolites in plant. And it can also be induced in the burst of

oxidation. It was reported that H_2O_2 could led many gene expression in defense and the biosynthesis of secondary metabolite, participation for sesquiterpene cyclase and PAL gene biosynthesis for instance (Mehdy, 1994). It was just the PAL that was the key enzyme in the biosynthetic pathway of salidroside. The action of the elicitor promoted the activity of PAL, so as to enhance the content of salidroside.

These results are useful for understanding effects of NO and $AgNO_3$ on salidroside and cell biosynthesis by cell cultures of *R. sachalinensis* A.Bor and important for exploring the possibility of scale-up of cell suspension cultures.

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