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Strategies for the improvement of salidroside production in cell suspension cultures of *Rhodiola sachalinensis*

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Abstract Strategies of elicitation and precursor feeding were applied to improve salidroside production in cell suspension cultures of *Rhodiola sachalinensis.* Of the seven elicitors examined, that extracted from *Aspergillus niger* was the most effective, increasing the salidroside content by five-fold when added on the day of inoculation 40 mg carbohydrate is medium. Three possible precursors for salidroside synthesis, L-phenylalanine, L-tyrosol and L-tyrosine were added to the cultures. A high content of salidroside (1.440%) was attained with an initial L-tyrosol concentration of 0.5 mM in the medium. Combined application of the two strategies resulted in a significantly high salidroside content of 1.734%, corresponding to a salidroside yiel of 200 mg/l.

Key words *Rhodiola sachalinensis* · Salidroside · Elicitor · Precursor

Introduction

In traditional Chinese medicine salidroside (Fig. 1) has been accorded such medical properties as resisting anoxia, microwave radiation, and fatigue (Ming et al. 1986). It has also been suggested to extend human life (Saratikov 1968; Kurkin 1986). Salidroside can be synthesized chemically, but the yields are very low and the pharmacological activity is not comparable to that of the natural product (Troshchenko and Juodvirshis 1969; Ming et al. 1986). For this reason salidroside is usually extracted from plant sources, mainly the genus *Rhodiola* (Ming et al. 1988). Of the 90 species of *Rhodiola* found all over the world, *R. sachalinensis* is one of several species containing high level of salidroside (0.5–1.0%). However, the natural resource of *R. sachalinensis* is on the brink of extinction due to overgathering (Jiang et al. 1994), and studies on the artificial cultivation of *R. sachalinensis* have made little progress due to the temperature-sensitive nature of the plant and the frequent occurrence of root-rotting diseases (Meng et al. 1994). Therefore, production of salidroside by cell suspension cultures may be a possible alternative, but as yet the literature contains no reports on cell suspension cultures of *R. sachalinensis*.

Plant cell cultures usually encounter problems of low product yields and high cost, which discourage their commercialization (Dicosmo and Misawa 1985; Panda et al. 1989). In our previous work (Xu et al. 1996), the maximum salidroside content attained in the cultured cells was only 0.171%, very much lower than the content of intact plants (0.5–1.0%). The objective of the research reported here was to improve salidroside production in cell suspension cultures of *R. sachalinensis* through the application of two strategies. The first is elicitation (Chappell and Nable 1987; Furze et al. 1991) and second precursor feeding (Tabata et al. 1972; Robins et al. 1991). Both approaches have effectively enhanced the production of secondary metabolites in other plant cells. Seven kinds of fungal elicitors and three possible precursors of salidroside synthesis were employed in this research, and their effects on the salidroside content and biomass yield were investigated. The two strategies were also combined to obtain a high salidroside yield.

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Material and methods

Plant material

R. sachalinensis callus was initially induced from a stem explant in this laboratory. Cell suspension cultures were grown in Murashige

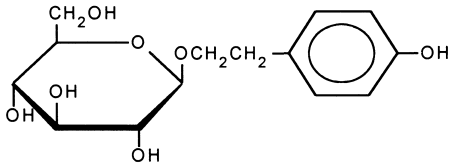

Fig. 1 Structural representation of salidroside

and Skoog medium containing 3% sucrose, 0.3 mg/L naphthaleneacetic acid and 3 mg/l 6-6-benzylaminopurine. The pH of the medium was adjusted to 5.8 before sterilization (121°C, 30 min). The stock cultures were maintained in 500-ml Erlenmeyer flasks containing 200 ml medium. Flasks were placed on a gyratory shaker at 120 rpm in the dark at 24°C. Subculture was carried out every 10 days with a 20% inoculum density. In all experiments, inoculum was kept identical (2.0 g/l) and the experiments were done in triplicate.

Analytical procedures

Cells were harvested by vacuum filtration. The biomass was washed three times with deionized water and dried for 24 h at 80°C in an oven. The dry weight was then determined. The biomass yield is expressed as grams per liter. For the measurement of intracellular salidroside content, a Waters HPLC system was used. A 1.0-g mass of dry cells was extracted with 10 ml methanol and the suspension was sonicated at 125 W for 30 min. The extracts were filtered through 0.45-μm membrane filters and 10 μl of solution was injected. The HPLC system was equipped with a Waters Bondapak ODS column (3.9 mm×300 mm, 10 μm) and a Waters UV detector at 276 nm. A mobile-phase mixture of water (80%) and methanol (20%) and a flow rate of 1.0 ml/min were used. Salidroside was quantified by the external standard method with the peak height as the quantitative parameter. The retention time of salidroside was 9.8 min. The salidroside standard was provided by the Qinhai Plateau Medical Institute of China. The spectra of IR, EI-MS, ^{1}HNMR, and ^{13}CNMR of the salidroside standard were the same as those in the literature, mp 159–160°C.

Fungal cell cultures

Fungal cells were cultured in B_5 basal medium free of plant regulators. The pH of the medium was adjusted to 6.0 before sterilization. The suspension cultures were maintained on a gyratory shaker in the dark at 25°C and 140 rpm for 6 days.

Elicitor preparation

The cultured fungal mycelia were harvested by vacuum filtration. The mycelia were transferred to a homogenizer supplemented with a tenfold volume of deionized water, then homogenized at room temperature. The homogenized liquid was autoclaved for 20 min and filtered. The filtrate was used as elicitor (Ning et al. 1994). To determine the carbohydrate concentration in the fungal elicitor, the orcinol-sulfuric acid procedure was used (Francois et al. 1962). Glucose was the standard.

Additions of elicitors and precursors

Sterilized elicitors and precursors were added to the cell cultures at varying time. Cells were cultured for several days and harvested for analysis of salidroside content and biomass yield.

Results and discussion

Growth characteristics of *R. sachalinensis* cell suspension cultures

Figure 2 illustrates the profiles of biomass yield and salidroside accumulation as a function of culture time. The culture displayed a relatively slow growth curve with an almost growth-associated pattern for salidroside accumulation. A lag phase was not apparent from inoculation to the first sampling time at day 3. The maximum biomass attained was 14.01 g/l at the end of the exponential phase at day 21, which was also the peak for salidroside accumulation (0.171%). The stationary phase extended from days 21 to 24. The biomass then decreased steadily due to cell lysis. The specific growth rate was between 0.08 and 0.28 $days^{-1}$ at the exponential growth phase. This indicates that the cell doubling time was 2.5 days during the fastest growth phase.

Elicitation of salidroside production

Elicitors extracted from seven fungal species were tested for their effect on the induction of salidroside accumulation in cell suspension cultures of *R. sachalinensis*.Erlenmeyer flasks (250 ml) containing 50 ml medium were used for the experiments. The same concentrations of elicitors (50 mg carbohydrate/l medium) were added to the medium on the day of inoculation and cells were harvested after 20 days of culture. Table 1 shows that three of seven elicitors effectively stimulated salidroside formation. In particular, the elicitor extracted from *Aspergillus niger* brought about a fivefold increase in salidroside yield over that in the control condition. Thus further experiments concentrated on the use of *A. niger* elicitor.

Elicitor concentration is a factor that strongly affects the intensity of the response (Sang and Pedersen 1994).

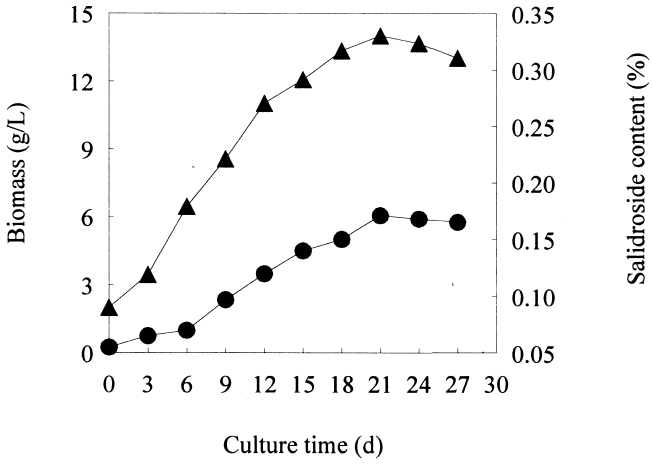

Fig. 2 Time course profiles of cell growth and salidroside production in *Rhodiola sachalinensis* cell suspension culture (▲ Biomass, ● salidroside content)

Table 1 Biomass yield and salidroside accumulation in cell suspension cultures of *Rhodiola sachalinensis* after addition of seven kinds of fungal elicitors

Number	Fungal species	Biomass (g/l)	Salidroside content (%)	Salidroside yield (mg/l)
0	Control	14.12	0.171	24.14
1	*Ganoderma lucidum*	11.39	0.820	93.40
2	*Aspergillus niger*	11.55	0.992	114.6
3	*Fusarium oxysporum*	13.48	0.188	25.34
4	*Schizophyllum commune Fr.*	12.58	0.224	28.18
5	*Polystictus versicolor*	11.66	0.105	12.24
6	*Rhizopus delemar*	11.56	0.259	29.94
7	*Penicillium sp.*	12.34	0.783	96.62

Different concentrations of *A. niger* elicitor were added to the cultures on the day of inoculation. Figure 3 shows the quantitative accumulation of salidroside in response to different elicitor concentrations.

The accumulation pattern of salidroside versus elicitor concentration demonstrates a saturated phenomenon. Salidroside accumulation was strongly affected at low elicitor concentration but was virtually unaffected at high elicitor concentration. The maximum salidroside content (0.990%) was obtained at an elicitor concentration of 40 mg carbohydrate/l culture. Addition of elicitor changed the color of the cell culture but had only a slight effect on cell viability. A similar result was reported for elicitation of tropane alkaloid formation in *Datura stramonium* suspension culture (Ballica et al. 1993). Figure 3 also demonstrates that "overloading" of elicitor has adverse effects. Inhibition by overdosed elicitor reduced the both accumulation of salidroside and cell growth.

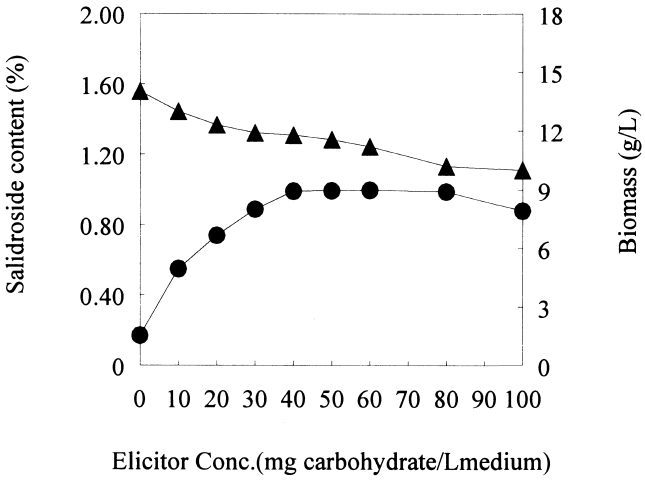

Fig. 3 Effects of elicitor concentration on cell growth (▲) and salidroside production (□)

The phase of plant cell growth when elicitation is applied also affects product accumulation. Elicitors (40 mg carbohydrate/l medium) were added to the cultures on the day of inoculation (day 0), at the pre-exponential phase (day 3), at the mid-exponential phase (day 12), and at the stationary phase (day 21). Figure 4a demonstrates that elicitation on the day of inoculation and at the pre-exponential phase produced the most significant effect, increasing the salidroside yield almost fivefold in both cases. Elicitation at the mid-exponential phase also enhanced product accumulation, but not as effectively. Adding elicitor at the stationary phase had no evident effect, but the biomass yield was the highest in this case (Fig. 4b). Ballica et al. (1993) reported that adding fungal elicitor at the stationary phase enhanced alkaloid yield fivefold in *D. stramonium* suspension cultures. In the report of Sang and Pedersen (1994), appropriate addition of yeast elicitor was at the exponential phase in cultures of *Escherichia californica* cells. However, Ning et al. (1994) suggested that for *Onosma paniculatum* cell cultures, adding fungal elicitor at the pre-exponential phase had the most significant effect. These differences demonstrate that the biochemical changes associated with the induction of secondary metabolism by adding precursors must be very complex; it is probable that different metabolic systems can be affected and that there is an enormous amount of biochemical alteration. The mechanism by which elicitors affect secondary metabolism still remains to be clearly defined. However, it has been widely accepted that elicitors facilitate secondary metabolite production by triggering a metabolic cascade. This function is somewhat similar to that of hormones, since a hormone also regulates the growth and product synthesis of plants by inhibiting or triggering metabolic cascades. In view of this we can suggest that elicitors may act as hormones regulating secondary metabolism in those plant cells which are able to receive, decode, and further modulate the molecular signal (Oba and Uritani 1979). However, different plant species with varying growth phases have shown different abilities to receive and further modulate the signal: the activity of elicitors varies with plant species and growth phase. In our research, the *A. niger* elicitor was active over the whole cell growth cycle (before the stationary phase). Therefore, it is appropriate to add it on the day of inoculation.

Precursor feeding for salidroside production

Three possible precursors in the biosynthetic pathway of salidroside, L-phenylalanine, L-tyrosol and L-tyrosine (Fig. 5) were added to the cultures on the day of inocula-

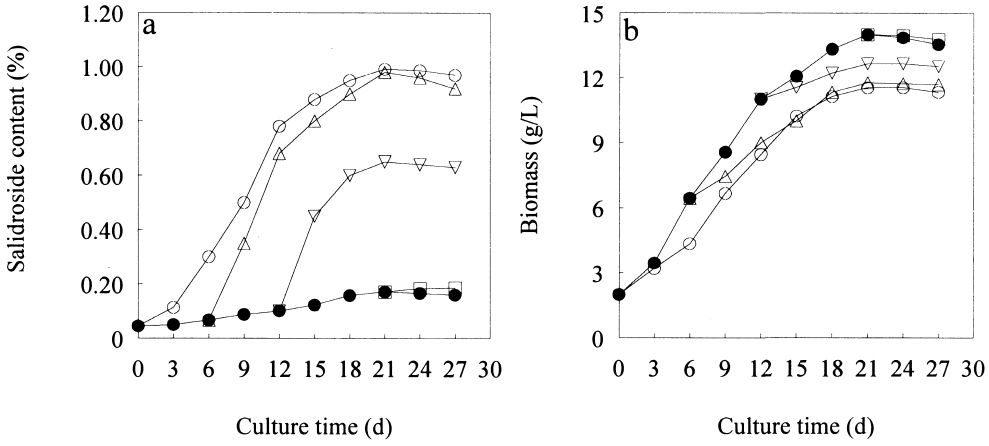

Fig. 4 The effect of elicitation time on salidroside accumulation (**a**) and biomass yield (**b**) (○ elicitation at day 0, △ at day 6, ▽ at day 12, □ at day 21, ● control)

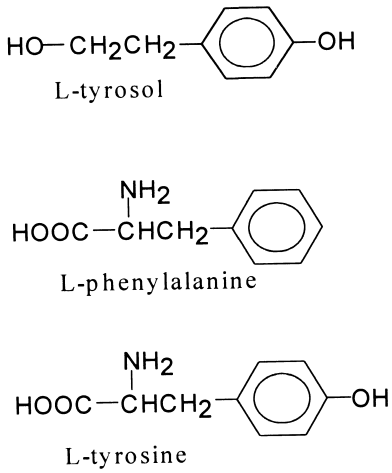

Fig. 5 Structural representation of L-tyrosol, L-phenylalanine, and L-tyrosine

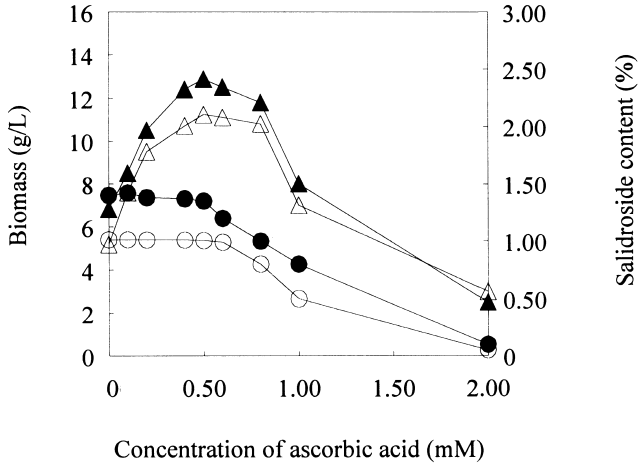

Fig. 6 The effects of ascorbic acid addition on biomass yield and salidroside production in *R. sachalinensis* cell suspension cultures supplemented with 0.5 mM L-tyrosol or L-tyrosine (△ biomass for addition of L-tyrosol, ▲ biomass for addition of L-tyrosine, ● salidroside content for addition of L-tyrosol, ○ salidroside content for addition of L-tyrosine)

Table 2 Effect of precursor addition on salidroside production and biomass concentration in *R. sachalinensis* cell suspension cultures

Precursor added	Precursor concentration (mM)	Salidroside content (%)	Biomass (g/l)	Salidroside yield (mg/l)
Control		0.171	14.12	24.14
L-Phenylalanine	0.05	0.188	12.40	23.31
	0.1	0.180	11.26	20.27
	0.5	0.173	10.23	17.70
	1	0.166	9.91	16.45
L-Tyrosol	0.05	0.880	10.86	95.57
	0.1	1.132	8.11	91.80
	0.5	1.440	6.82	98.20
	1	1.443	4.50	64.94
L-Tyrosine	0.05	0.681	10.23	69.56
	0.1	0.850	7.34	62.39
	0.5	1.012	5.17	52.32
	1	1.015	4.98	50.55

tion to enhance the salidroside synthesis. The results are shown in Table 2. Both L-tyrosol and L-tyrosine addition improved the salidroside content significantly. L-tyrosol had the strongest effect. The salidroside content of cells was nearly nine times higher in the culture supplemented with 0.5–1.0 mM L-tyrosol than that in the control culture. The final reaction step in salidroside biosynthesis is believed to be the combination of L-tyrosol with a glucose through a glycosidic bond (Ming 1986). The fact that L-tyrosol addition significantly enhances salidroside synthesis supports this suggestion. However, the conversion of L-tyrosol from L-tyrosine in cultured cells has not been confirmed experimentally, but the rise in salidroside content following addition of L-tyrosine seems to be related to this conversion step.

On the other hand, addition of precursors also caused a decrease in biomass yield. Adding L-tyrosine and L-tyro-

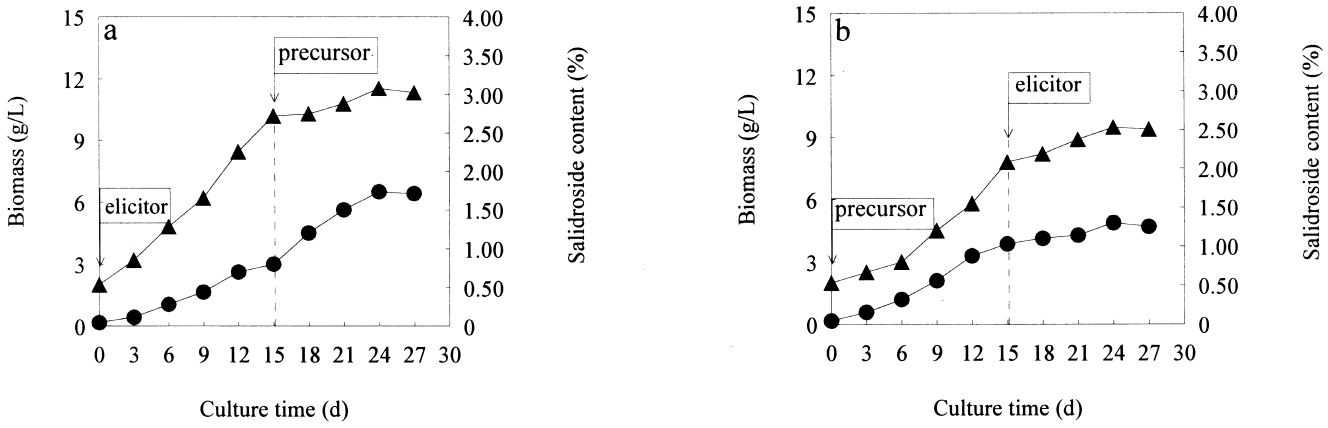

Fig. 7 a, b Time course of salidroside accumulation (●) and biomass yield (▲) when the elicitor and L-tyrosol were added at different growth phases of plant cells

sol, the cultured cells and broth turned brown and the biomass yield dropped sharply as the amount of these precursors added increased. As a result, the salidroside yield was low although the cultured cells had a high salidroside content. In contrast, when adding L-phenylalanine, the cells did not turn brown and the biomass yield was relatively higher. Since both L-tyrosol and L-tyrosine have the phenol hydroxide group, the browning of the cultured cells and broth may be caused by the oxidization of the phenol hydroxide group by polyphenol oxidase. This enzyme is known to occur in natural plant tissue (Luh and Phithalcpal 1972). To inhibit the activity of the polyphenol oxidase in the cultures when adding L-tyrosol and L-tyrosine, we used ascorbic acid, an anti-oxidization chemical. Different concentrations of ascorbic acid were added to the cultures together with 0.5 mM of L-tyrosol and L-tyrosine on the day of inoculation. Figure 6 shows that adding ascorbic acid up to 0.5 mM can increase the biomass yield up to 12.88 g/l (with L-tyrosol) and 11.23 g/l (with L-tyrosine), values close to the productivity in cultures without adding precursors. Salidroside contents were hardly affected and the salidroside yields were high: up to 173.9 mg/l for L-tyrosol addition and 112.9 mg/l for L-tyrosine addition, representing a maximum salidroside yield sevenfold higher than that in the control condition. The cultured cells and medium did not turn brown during the culture period, indicating that the activity of the polyphenol oxidase had been inhibited by the ascorbic acid, and the L-tyrosol remained unoxidized. However, excess ascorbic acid (>1 mM) had an adverse effect on cell growth: at 2 mM ascorbic acid, almost all the inoculated cells turned white and cell growth virtually ceased.

Addition of precursors at the early stationary phase of growth has been reported to enhance secondary metabolite yield (Ballica et al. 1993). However, in our research, adding 0.5 mM L-tyrosol at the early stationary phase (day 18) and growing for another 10 days had no significant effect. This difference may be caused by the growth-associated feature of salidroside synthesis in *R. sachalinensis* cell suspension cultures, since at the stationary phase, salidroside synthesis in cultured cells virtually stopped, corresponding to the cessation of cell growth. It is possible that addition of precursor at this time did not enhance the salidroside synthesis.

Combined application of elicitation and precursor feeding for enhancing salidroside production

Both elicitation and precursor feeding were able to significantly increase the salidroside content in cultured cells. In the following experiments, a culture medium supplemented with *A. niger* elicitor at 40 mg carbohydrate/l medium, 0.5 mM L-tyrosol, and 0.5 mM ascorbic acid was prepared for the culture of *R. sachalinensis* cells. Unexpectedly, a relatively low salidroside content of 0.64% was obtained after 20 days of culture and the biomass yield was only 8.50 g/l. This suggests that the mechanisms by which elicitation and precursor feeding promote secondary metabolite synthesis are quite different, and the metabolic pathway in the cultured cells is disturbed when the two strategies are applied together. However, if the elicitor and precursor were to be added at different phases of cell growth, the results may be different. Figure 7 shows the time course of salidroside accumulation and biomass yield when 40 mg carbohydrate/l medium of elicitor and 0.5 mM L-tyrosol were added at the day of inoculation (day 0) and at mid-exponential phase (day 15) alternatively. Ascorbic acid (0.5 mM) was added with the L-tyrosol. Two different patterns of salidroside accumulation were observed. In case one, that is elicitation first followed by precursor feeding at day 15, a high salidroside content of 1.73% was obtained (Fig. 7a). In contrast, in case two, the salidroside content was relatively low (1.28%) with the reverse order for elicitor and precursor addition (Fig. 7b). The mechanism for enhancing salidroside accumulation in these two cases remains to be elucidated. However, a high salidroside yield of 200 mg/l was attained in our research, almost eightfold higher than that in the original experiments. This salidroside productivity is expected to be economically acceptable.

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