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Precursor Feeding Experiments with Elicitation in Suspension Cultures of *Eschscholtzia californica*

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The accumulation of benzophenanthridine alkaloids occurred in suspension cultures of *Eschscholtzia californica*. To increase alkaloid production, precursor feeding with tyrosine was studied under various culture conditions. Tyrosine feeding without any treatment resulted in no significant increase of alkaloid production. However, precursor feeding together with yeast extract elicitor, which induces metabolic enzyme activity, increased alkaloid production several fold. This enhancement was possible when tyrosine and elicitor were administered at the late exponential growth stage.

Introduction

Suspension cultures of *Eschscholtzia californica* accumulate the benzophenanthridine alkaloids sanguinarine, chelirubine, chelerythrine and macarpine, all of which are known to be constituents of the *Eschscholtzia* plant[1]. The benzophenanthridine alkaloids have recently been the subject of increasing interest because of their dental and medical uses[2]. The biosynthesis of benzophenanthridine alkaloids in *E. californica* has been well studied and some of the complex precursor relationships have been deduced[3,4]. Such knowledge might allow increased production of alkaloids through the addition of inexpensive precursors.

Several studies on the addition of biosynthetic precursors to plant cell cultures have been done to increase production of secondary metabolites. Sometimes the addition of precursor showed positive effect and product level was increased[5-7], but negative or no effects have also been reported[8,9]. In this study, tyrosine was used for precursor feeding experiments aiming at an increase of benzophenanthridine alkaloids in suspension cultures of *E. californica*. Also, elicitation with yeast extract elicitor was applied to increase alkaloid production *via* biosynthetic pathway induction. This was optimized by the investigation of quantitative response and production pattern of alkaloids to precursor and elicitor addition at different growth stages.

Materials and Methods

Cell Cultures: Cultures of *Eschscholtzia californica*, originally developed in 1984, were kindly provided by Dr. Henrik Pedersen (Rutgers Univ., NJ, USA). Suspension and callus cultures have been maintained on B5 basal medium supplemented with 2, 4-dichlorophenoxyacetic acid (5 μM), kinetin (0.5 μM) and 20 g/l of sucrose. The pH was adjusted to 5.8 with 1N KOH. For the maintenance of suspension cultures, 16 g of cells (fresh weight) were transferred into 200 ml medium

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in a 500 m*l* Erlenmeyer flask every 7 days. For all experimental shake flask cultures, 4 g of cells (fresh weight) were innoculated into 125 m*l* Erlenmeyer flasks containing 50 m*l* of growth medium on a gyratory shaker at 180 rpm at 26°C under 18 hr of Cool White fluorescent light (4 μEi/m^{2}s) per day.

Chemicals and analytical procedures were the same as described by Byun *et al.*[14].

Precursor feeding with and without elicitation: For tyrosine feeding experiments without addition of yeast elicitor in shake flasks, different levels of tyrosine per fresh cell weight (FCW) were administered at the 6th day from inoculation and duplicate samples were harvested after 36 hours. For feeding experiments with elicitation, different amounts of tyrosine (mg) and 60 μg of yeast elicitors per gram of fresh cell weight (g FCW) were added to shake flasks and the duplicate samples were harvested after 36 hours.

Results and Discussion

Tyrosine was reported as the precursor for the biosynthetic metabolism of benzophenanthridine alkaloids[10]. It is incorporated with dopamine into sanguinarine. The tyrosine feeding effects on cell growth and total alkaloid production are shown in **Fig. 1**. The cell growth was slightly reduced with increasing tyrosine concentration. Total alkaloid production, however, increased slightly with increasing tyrosine concentration and was highest around 5 mg tyrosine/g FCW. It is unclear whether this increase originated from the incorporation of precursor into the secondary metabolism or was induced by stress generated from tyrosine like an abiotic elicitor. If tyrosine was consumed in secondary metabolic pathways, it is necessary to find the optimum feeding time when enzymes involved in the metabolic pathways are fully translated and activated.

It was reported that treatment of *E. californica* cells with elicitor resulted in the massive induction of benzophenanthridine alkaloids[11,12]. Schumacher and Zenk[13] found that sanguinarine, chelirubine, chelerythrine, chelilutine and macarpine were specifically induced by cell wall components of *Penicillum* and *Saccharomyces* in suspension cultures of *E. californica*. One of the branch point enzymes, namely the berberine bridge enzyme, catalyzing the formation of (−)-scoulerine from (+)-reticuline, is strongly stimulated during the elicitation process. Their results clearly

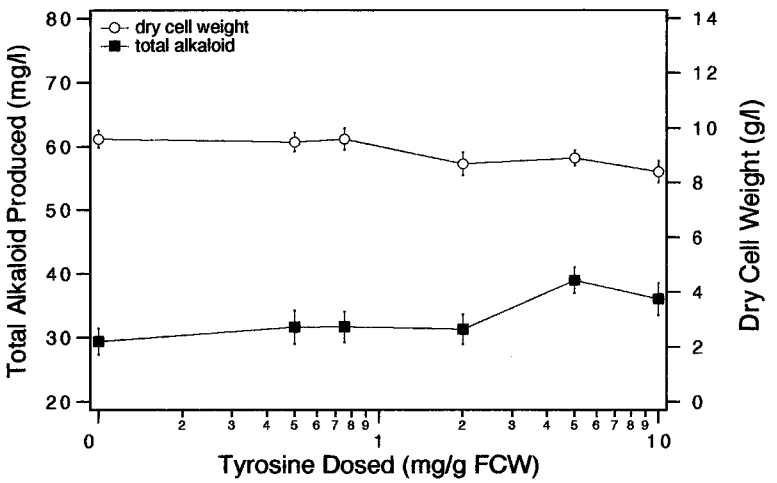

Fig. 1 Tyrosine feeding effects on cell growth and alkaloid production in suspension cultures of *E. californica*.
Different levels of tyrosine/g FCW were added at the 6th day from inoculation and duplicate samples were harvested after 36 hours.

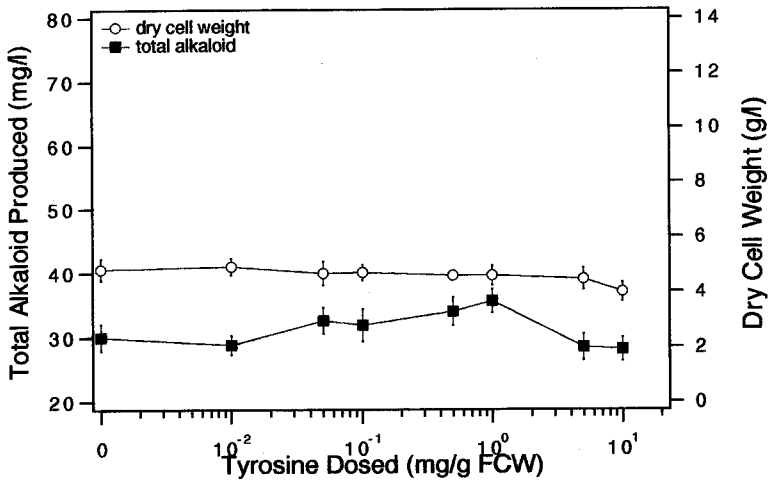

Fig. 2 Effects of tyrosine feeding with elicitation at early growth stage on cell growth and alkaloid production.
Sixty μg of yeast elicitor and different levels of tyrosine/g FCW were added at the 3rd day from inoculation and duplicate samples were harvested after 36 hours.

demonstrated the induction of the benzophenanthridine alkaloid biosynthetic pathway by microbial elicitors.

Experiments to prove tyrosine effect as a precursor were made with elicitation. Precursor feeding experiments with elicitation were carried out with tyrosine and yeast elicitor. The first experiment was done with suspension cultures which had been cultivated 3 days after inoculation. Yeast elicitor and different levels of tyrosine were dosed and the samples were harvested after 36 hours. **Fig. 2** is the result. Cell growth was suppressed as seen in the precursor feeding experiment without elicitation in **Fig. 1**. Alkaloid production was not much affected by increasing tyrosine concentration. The second precursor feeding experiment was done at day 7 from inoculation. The results is illustrated in **Fig. 3**. Cell growth was slightly reduced with increasing tyrosine concentration. Alkaloid production was increased with increasing tyrosine concentration and the

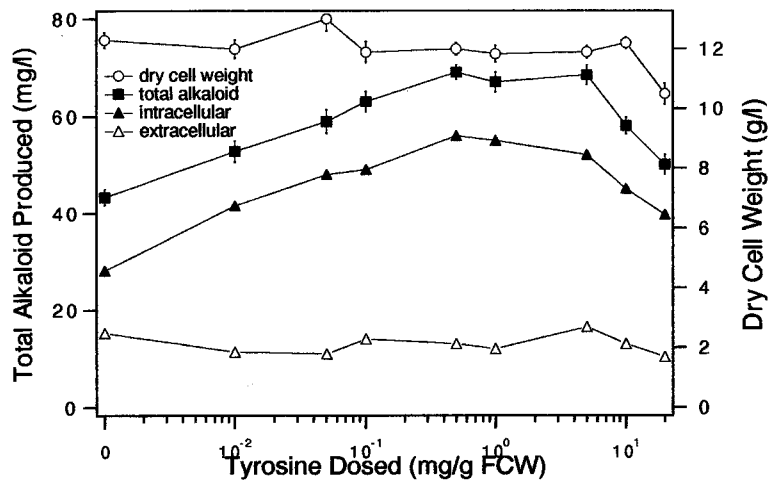

Fig. 3 Effects of tyrosine feeding with elicitation at late exponential growth stage on cell growth and alkaloid production.
Sixty μg of yeast elicitor and different levels of tyrosine/g FCW were added at the 7th day from inoculation and duplicate samples were harvested after 36 hours.

negative performance was observed at high tyrosine concentration. The addition of precursor with elicitor at day 7 from inoculation showed positive effect and the total alkaloid product level was increased, but negative or no effect was also observed when tyrosine was fed at the 3rd day from inoculation. The 7th day from inoculation was the late exponential growth stage and the 3rd day was the end of lag phase. **Fig. 3** also shows that this enhancement was mainly due to the increase in intracellular accumulation of alkaloids. Similar results were observed in elicitation without precursor feeding[14]. Elicitation at different growth stages showed that the highest alkaloid production was observed when the elicitor was administered at the late exponential growth phase.

Many studies for increased enzyme activity by elicitation have been reported. L-Tyrosine decarboxylase (TDC) activity was induced by yeast elicitor treatment of suspension cultured *E. californica*[15]. Highest TDC activity was observed 5 hours after elicitor addition. Increased tyrosine consumption is expected if TDC activity is induced. The source of tyrosine, however, is believed to be important for the feeding effect. In the first experiment, the tyrosine supplied was not the only source because primary metabolism of the cell could produce intracellular tyrosine from sugar left at the 3rd day from inoculation. If enough tyrosine could be supplied from intracellular metabolism, the uptake of tyrosine added would be reduced. In the second experiment, however, the uptake of tyrosine added was expected to be high because of lower amounts of tyrosine that could be supplied from intracellular metabolism, with no sugar left at the 7th day from inoculation.

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