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Re-Elicitation with Methyl Jasmonate in *Eschscholtzia californica* Cell Suspension Cultures

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Abstract Elicited cells with methyl jasmonate continued to produce benzophenanthridine alkaloids throughout medium changes in suspension cultures of *Eschscholtzia californica*. Large increases in alkaloid production were observed by re-elicitations with medium changes. The total alkaloid production increased during the successive elicitation steps reaching a maximum level on the 4th elicitation. The highest total alkaloid produced was 250 mg/l, which was 2-fold higher than that of the single elicitation and 4-fold higher than that of the normal culture without elicitation. The large increases in alkaloid production in successive re-elicitations with medium changes are believed to be caused by the accumulation of the signal transduction compound, jasmonate.

Key words: Re-elicitation, memory effect, methyl jasmonate, *Eschscholtzia californica*

In plant cells, it has been reported that elicitor-induced mechanisms are specific, but very complex. It is now well accepted that the interaction of elicitors with their surface receptor is the initial step during the chain of events that lead to subsequent biological response. Therefore, the regulation of elicitors and surface receptors is crucial since it provides a point of control for these responses. Multiple effects can be elicited by an interaction of an elicitor with its receptor. One of multiple effector systems generates intracellular messengers or mediators, which bring about alterations of various intracellular metabolic pathways [6]. It is possible that some of the cytoplasmic signals can affect nuclear responses as well. Alternatively, the elicitor could interact directly with additional receptors on the nuclear membrane, or the elicitor-receptor complex on the plasma membrane, thus effecting directly on the nucleus by internalization.

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It has been suggested that jasmonic acid could be an integral part of a general signal transduction system regulating inducible defence genes in plants [4]. In search of the signal chain of reactions between the elicitor-receptor complex and the gene activation process, Gundlach *et al.* [5] have defined that using suspension cultures of plant species representing a wide taxonomic distribution, jasmonic acid and its established precursors were shown to have a position in this molecular cascade of events. Jasmonate is rapidly synthesized in response to treatment with an elicitor in suspension cultures of *Rauvolfia* and *Eschscholtzia*. Furthermore, exogenously applied methyl jasmonate induces the synthesis of specific low molecular weight compounds in the absence of elicitors, in all the plant cell suspension cultures tested. Moreover, exogenously applied methyl jasmonate induces *de novo* transcription of the gene needed for the key enzyme of the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL) [9], resulting in elevated levels of active enzyme in soybean (*Glycine max*) cell suspension cultures. The jasmonates are, therefore, key signal compounds in the elicitation process leading to *de novo* transcription and translation and, ultimately, to the biosynthesis of secondary metabolites in plant cell cultures [10, 12].

Suspension cultures of *Eschscholtzia californica* accumulate the benzophenanthridine alkaloids [1, 7]. They are biosynthetically derived of (+)-reticuline, the central intermediate in isoquinoline biosynthesis, which is transformed to (-)-scoulerine by action of the berberine bridge enzyme. This intermediate is then converted to benzophenanthridine alkaloids [3]. Several studies have been conducted to produce benzophenanthridine alkaloids in callus or suspension cultures of *E. californica* [2]. The enhanced productions of sanguinarine and macarpine, which are typical benzophenanthridine alkaloids produced in cell cultures of *E. californica*, have also been studied with elicitation [1, 11].

In some of the plant, cell and elicitor combinations such as *Catharanthus roseus*, *Phytium aphanidermatum*, and

Papaver somniferum. elicitor treatment continued to produce secondary metabolites in replenished media for some time [8]. It is known as the memory effect of elicitation with replenished media. By replenishing elicited cells with fresh medium. elicitor molecules remaining in the culture medium can be removed, and the number of elicitor-receptor complexes on the plasma membrane surface will decrease to zero by dissociation and/or deactivation. The only source left to cause re-elicitation is believed to be the internalized elicitor-receptor complexes or signal transduction compounds such as jasmonates. In this paper, memory effects of elicitation with methyl jasmonate in suspension cultures of *E. californica* are described. Furthermore, re-elicitation is applied to increase the production of benzophenanthridine alkaloids.

Cultures of *Eschscholtzia californica* which were originally developed in 1984 were kindly provided by Dr. Henrik Pedersen (Rutgers University, New Jersey). Suspension cultures of *E. californica* have been known to produce benzophenanthridine alkaloids sanguinarine. chelirubine, chelerythrine, and macarpine. Suspension and callus cultures were maintained on B5 medium supplemented with 2.4-dichlorophenoxyacetic acid (5 μM), kinetin (0.5 μM). and 20 g/l of sucrose as carbon source. The pH level was adjusted to 5.8 with 1 N KOH. For the maintenance of suspension cultures, 16 g of cells (fresh cell weight) were transferred into 200 ml of medium in a 500-ml Erlenmeyer flask every 7 days. Erlenmeyer flasks (125 ml) containing 50 ml of growth medium was used for experimental batch cultures on a gyratory shaker at 180 rpm. The temperature of the culture room was maintained at 26°C and exposed to 18 h of fluorescent light (4 $\mu E/m^2s$) per day. Sanguinarine was supplied from Sigma Chemical Co. (St. Louis, U.S.A.) and chelerythrine was from Atomergic Chemical Corp. (Farmingdale, U.S.A.). Macarpine and chelirubine were extracted and purified from cultured cells, because commercial supplies were not available [1]. Methyl jasmonate. which is the precursor of jasmonic acid and the signal transducer, was supplied from Aldrich Chemical Co. (Milwaukee, U.S.A.). All other chemicals used in this study were of reagent grade.

For the cell growth measurement, suspension cells were filtered and washed with distilled water. The washed cells on a preweighed aluminium tray were dried in a 60°C oven to constant weight. Dry cell weight (DCW) was expressed as grams per liter. For the alkaloid analysis. cells were harvested by vacuum filtration, and the filtrates were collected to analyze the extracellular alkaloids in the medium. To measure the intracellular alkaloid concentration, 1.0 g of cells (FCW) were extracted with 10 ml of HPLC-grade methanol and the suspension was sonicated at 124 W for 10 min. Extracts were filtered through 0.45 μm membrane filters and 10 μl of the solution was injected. The HPLC system was equipped with a reversed phase C-18 column and a UV detector at 280 nm. A mobile phase mixture was consisted of water (65%) and MeCN (35%), and the flow rate was 1.5 ml/min. The water phase contained 1 mM of tetrabutylammonium phosphate and was adjusted to pH 2.0 with phosphoric acid. Linear standard curves were obtained up to 100 mg/l of sanguinarine and 80 mg/l of macarpine.

By replenishing suspended cells treated with methyl jasmonate with fresh medium, jasmonate molecules remaining in the culture medium can be removed. The only source left to elicit secondary metabolite production is believed to be the internalized jasmonate. The number of internalized jasmonate molecules, however. decreased with an increasing number of replenishments, i.e. subcultures with fresh medium. To study memory effects on cell growth and alkaloid production in suspension cultures of *E. californica*, 50 μM of methyl jasmonate was injected in batch cultures and elicited cells were replenished with fresh medium. Every 4 days, elicited cells were replenished with fresh medium and were inoculated into flasks containing 50 ml of fresh medium. Figure 1 shows the memory effects on cell growth and alkaloid formation. Elicited cells were recovered throughout medium changes which caused the increase of cell growth. The gradual decrease of alkaloid production indicates memory effect that is believed to be caused by internalized jasmonate. The degradation rate of this possible internalized compound appeared low and, therefore. more medium replenishments are required to reach a normal cell culture and alkaloid production which are 11 g/l and 61 mg/l. respectively. The internalized jasmonate is believed to continue to induce secondary metabolites as far as it is remained and the degradation of it will end the memory effect.

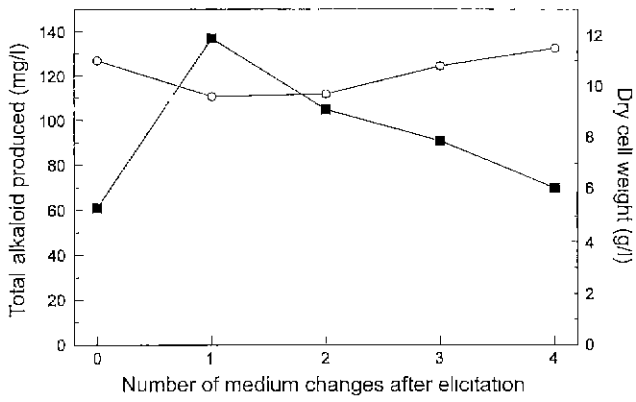

Fig. 1. Memory effects after elicitation on cell growth and alkaloid production in suspension cultures of *E. californica*.
The number 0 of medium change after elicitation means the normal culture without elicitation. Symbols: ○, dry cell weight; ■, total alkaloid produced.

If elicitation is a useful method in inducing biosynthetic processes in plant cell cultures, re-elicitation could be a great opportunity to increase the secondary metabolite production along with industrial application. Some prerequisites, however, are necessary for elicitors being re-injected again to assure effectiveness in inducing biosynthetic pathways. These conditions could be explained under the basis of elicitor-receptor dynamic behavior. Firstly, no elicitors previously injected should be left in the medium. Secondly, the minimum number of free receptors required for elicitation should be synthesized or recovered. Finally, metabolic activities in the cell should be maintained without any lack of nutritional requirements. These prerequisites, however, should be changed when signal transduction compounds such as jasmonates are used as elicitors. Minimum numbers of free receptors are not required because signal transduction compounds such as jasmonates are directly internalized and elicit secondary metabolite production. In order to study re-elicitation effects on cell growth and alkaloid production in suspension cultures of *E. californica*, 50 μM of methyl jasmonate was injected in batch cultures. After 4 days, elicited cells were washed with fresh medium and they were inoculated into flasks containing 50 ml of fresh medium. After 2 days of inoculation, 50 μM of methyl jasmonate was re-injected in the flask containing washed cells with fresh medium. Re-elicitations with medium exchanges were continued until the cells could maintain viability.

Figure 2 shows the re-elicitation effects on cell growth and alkaloid production. The total alkaloid production increased during the successive elicitation steps for reaching a maximum on the 4th elicitation. The total maximum alkaloid produced was 250 mg/l, which was 2-fold higher than that of the simple elicitation. When the maximum value was compared with that of normal culture without elicitation, it was found to be 4-fold higher. However, the cell growth was lower compared to the normal culture and it dropped after the 3rd elicitation. Large increases in alkaloid production in successive re-elicitations with medium changes are believed to be caused by the accumulation of a large amount of signal transduction compounds or second messengers. By the re-elicitation with methyl jasmonate, the signal transduction compound, jasmonate, is believed to be accumulated in the cell by internalization. The accumulated signal transduction compounds possibly induced the transcription, translation, and product formation, which enabled the large increase in alkaloid production to take place. The re-elicitation effects can be explained by a different point of view. The storage of secondary metabolites in the vacuole can be increased by the elicitation. More secondary metabolites can be stored in the vacuole by the repeated elicitations with medium changes. However, this is not always possible, because secondary metabolites production may interfere by acting as product inhibitors. In spite of the fact that the mechanism for re-elicitation-induced production of secondary metabolites is not well understood, re-elicitation can improve the efficiency of secondary metabolite production in plant cell culture systems where elicitation has received much attention to increase productivity. Application of this technique can be one of the gateways to achieve commercial success of secondary metabolites production that has been limited by poor yield.

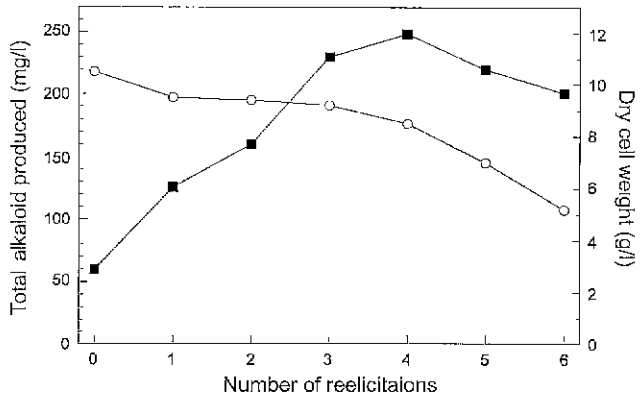

Fig. 2. Re-elicitation effects with medium changes on cell growth and alkaloid production in suspension cultures of *E. californica*.

The number 0 of re-elicitation means the control culture without elicitation. Symbols: ○, dry cell weight. ■, total alkaloid produced.

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