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Phytohormone Effects with Elicitation on Cell Growth and Alkaloid Production in Suspension Cultures of *Eschscholtzia californica*

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In the suspension cultures of *Eschscholtzia californica*, phytohormone effects showed that alkaloid production was increased by IAA treatment without kinetin in both volumetric and specific way. Kinetin, however, suppressed alkaloid accumulation. Addition of ethephon inhibited cell growth. However, it enhanced the alkaloid production significantly in both volumetric and specific way. IAA promoted alkaloid production during elicitation. The highest alkaloid accumulation was observed at 5 μM of IAA. Ethephon also enhanced alkaloid production during elicitation. The highest alkaloid formation was observed at 460 mg/l of ethephon with elicitation. Elicitation with ethephon, however, altered cell growth and the pattern of benzophenanthridine alkaloids production.

It is generally necessary to add one or more growth substances, such as auxin, cytokinins, and gibberellins, for a well growth and a secondary metabolite formation in plant cell culture system. The requirement for these substances varies considerably with the tissue, and it is believed that it depends on their endogeneous level. In nature, auxins are involved with the elongation of stem and inter nodes, tropism, apical dominance, abscission, and rooting. In tissue cultures, auxins have been used for cell division and root differentiation. However, it is known that auxins prevent a good expression of secondary pathways in the cultured cells (21). According to Tabata (20) cytokinins, especially kinetin, promoted nicotine production in the absence of auxin, whereas auxin strongly inhibited nicotine formation even in the presence of kinetin.

Suspension cultures of *Eschscholtzia californica* accumulate benzophenanthridine alkaloids sanguinarine, chelirubine, chelerythrine and macarpine, all of which are known to be constituents of the *Eschscholtzia* plant (1). Sanguinarine has recently been the subject of increasing interest because of its application in dentistry and medicine (6, 19). Several studies on producing benzophenanthridine alkaloids in callus or suspension cultures of *E. californica* have been reported. Berlin *et al.* (1) found that the suspension cultures of *E. californica* accumulate dihydro forms of the benzophenanthridine alkaloids as well as oxidized forms. It was reported that a treatment of *E. californica* cells with an elicitor resulted in the massive induction of benzophenanthridine alkaloids (18). Byun *et al.* found that sanguinarine, chelirubine, chelerythrine, chelilutine and macarpine were specifically induced by the cell wall components of *Collectotrichum lindemuthianum, Verticillium dahliae* and the yeast extract in the suspension cultures of *E. californica* (4).

In this study, batch shake-flask culture experiments with various phytohormones were performed to investigate any changes in the cell growth and alkaloid production in the suspension cultures of *E. californica*. Also, the yeast extract elicitor was used with different levels of phytohormone concentration to increase the alkaloid productivity.

MATERIALS AND METHODS

Cell Cultures

Cultures of *Eschscholtzia californica* which had been developed in 1984 were kindly provided by Dr. Henrik Pedersen (Rutgers Univ., NJ, U.S.A.). Suspension cultures of *E. californica* have been known to produce benzo-

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phenanthridine alkaloids sanguinarine, chelirubine, chelerythrine and macarpine as well as their dihydro forms. Suspension and callus cultures have been maintained on the B5 medium (11) prepared from a B5 salt mixture (GIBCO Laboratories, Grand Island, NY) supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D, 5 μM), 6-furfurylamino purine (kinetin, 0.5 μM) and 20 g/l of sucrose as a carbon source. 0.5% (w/v) of agar was added to prepare a solid medium for callus maintenance. The pH was adjusted to 5.8 with 1N KOH for both of suspension and callus culture. For the maintenance of suspension cultures, 16 g of cells (fresh cell weight) was transferred into 200 ml of the medium in a 500 ml Erlenmeyer flask every 7 days. However, callus subculturing was carried out every 40 days by transferring a 'spoonful' of healthy callus on 50 ml of the solid medium. 125 ml Erlenmeyer flasks containing 50 ml of the growth medium were used for experimental batch cultures on a gyrotory shaker (Model G10, New Brunswick Scientific Co., Inc., Edison, NJ) at 180 rpm. The temperature of the culture room was 26℃ and the cultures were exposed to 18 hr of white fluorescent light per day.

Chemicals

Sanguinarine nitrate was supplied from Research Plus, Inc. (Bayonne, NJ) and chelerythrine was from Atomergic Chemicals Corp. (Farmingdale, NY). Macarpine was extracted from a cultured cell mass because no commercial supply was available (3). Tetrabutylammonium phosphate for HPLC analysis and all the solvents used for HPLC such as acetonitrile, methanol and water were bought from Fisher Scientific (Rochester, NY). All other chemicals involved in this study were reagent grade.

Preparation of Elicitor

Yeast elicitor was isolated from a yeast extract (DIFCO laboratories, Detroit, MI) by ethanol precipitation as described by Hahn and Albersheim (14). To determine the concentration of carbohydrate in the yeast elicitor, the orcinol-sulphuric acid procedure (9) was used. Glucose was used as the standard.

Alkaloid Analysis

Cells were harvested by vacuum filtration and the filtrates were collected for the analysis of extracellular benzophenanthridine alkaloids in the medium. For the measure the concentration of intracellular alkaloid, 1.0 g of cells (FCW) was extracted with 10 ml of HPLC grade methanol, and the suspension was sonicated at 125 W for 10 minutes. All extracts were filtered through 0.45 μ membrane filters and 10 μl of the solution was injected into the HPLC system. The HPLC system was used with a Supelcosil™ LC-18-DB column and UV detector at 280 nm. A mobile phase mixture of water (65%) and MeCN (35%) at a flow rate of 1.5 ml/min was used. The water contained 1 mM tetrabutylammonium phosphate and was adjusted to pH 2.0 with phosphoric acid. Using the conditions described above, linear standard curves were obtained up to 100 mg/l of sanguinarine and 80 mg/l of macarpine. However, the alkaloids dissolved in methanol gave a different peak shape, retention time, and integrated area from those of the alkaloids in water in spite of the same concentration. Therefore, standard solutions were prepared both in methanol for an intracellular analysis and in water for an extracellular analysis.

RESULTS AND DISCUSSION

Response to Different Phytohormones

Berlin *et al.* (1) tested the effect of 2, 4 D on the cell growth and the alkaloid production in *E. californica*. When the cells were grown on a B5-medium free of 2, 4 D for the first time, there was only a slight reduction in FCW and no changes in DCW. When these cells were transferred to another 2, 4 D-free medium, their growth decreased and sporadic morphological structures were detected. After 4 passages without 2, 4 D, a great number of root like structures were found. However, after the first transfer to the 2, 4 D-free medium, alkaloid productions increased on average 4-fold while those of phenolics increased 2-fold higher in comparison to the growth medium.

The effects of three different auxins (2, 4 D, NAA, and IAA) were examined with kinetin or kinetin-free medium. Without kinetin, all of the auxins tested gave a positive effect on the cell growth as shown in Fig. 1. However, the alkaloid production with 2, 4 D and NAA was decreased with the concentration increase. Total alkaloid is the summation of benzophenanthridine alkaloids sanguinarine, chelirubine, chelerythrine and macarpine. IAA had a positive effect on the cell growth and the

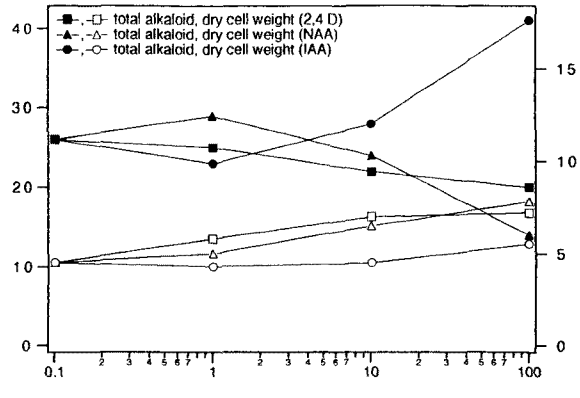

Fig. 1. Phytohormone effect on cell growth and alkaloid production in suspension cultures of *E. californica*. No kinetin was added.

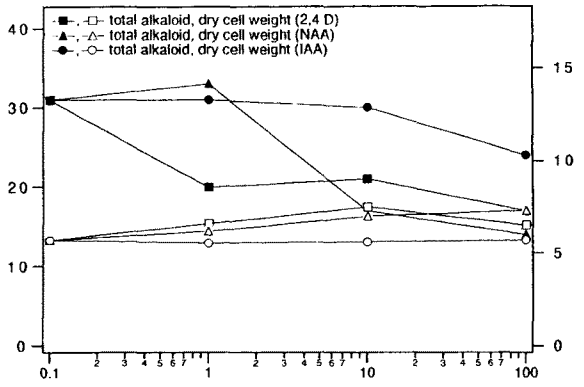

Fig. 2. Phytohormone effect on cell growth and alkaloid production.
0.5 μM of kinetin was added.

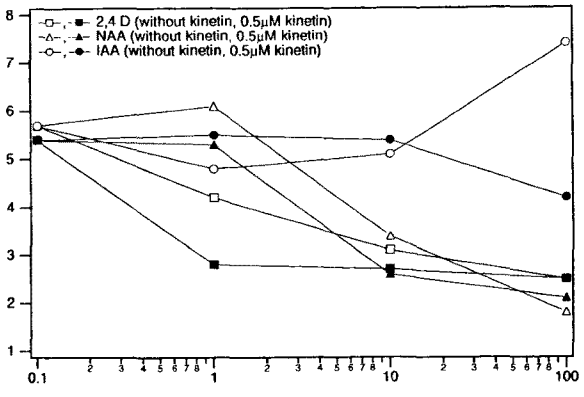

Fig. 3. Phytohormone effect on specific alkaloid production.

alkaloid accumulation at the concentration of 100 μM. The effect of kinetin with three different auxins was tested by using 0.5 μM of kinetin. All of the auxins tested showed a negative effect on the alkaloid formation with increasing auxin concentration as shown in Fig. 2. However the cell growth was not much affected.

When the alkaloid formation was calculated per unit cell mass (specific alkaloid production), it could be used to prepare a production medium. Fig. 3 shows that the alkaloid level in suspension cultures with IAA is fairly high in spite of the low cell growth. This is also true for the phytohormone-free medium. In conclusion, hormone-free or appropriate IAA concentration could be used for the preparation of production medium. The production medium has been used in the second stage (production stage) of a two-stage culture where cell growth was not important.

Ethephon Effects as an Ethylene Precursor

Ethylene is known to be a natural regulating substance of the plant metabolism, which acts and interacts with other recognized plant hormones in trace amounts. It is also known to have significant effects on the cell growth and the secondary metabolite formation. A reduction of cell growth by 20 and 30% for rose and *Ruta* cultures, respectively, was reported when 560 ppm of ethylene was supplied to the medium (16). However, an application of 25 ppm ethylene enhanced the growth of tobacco-suspension cells three-fold (10). Kim (15) reported that the ethephon as an ethylene precursor inhibited the cell growth of *Thalictrum rugosum* suspension culture and made the growth rate slower. The cell morphology also appeared to be changed by the ethephon treatment resulting in compact cells. In addition ethephon was found to enhance the berberine production in suspension cultures.

To investigate how ethephon effects the cell growth and the alkaloid production in suspension cultures of *E. californica*, different amounts of ethephon were injected into the cultures which had been maintained for 3 days from inoculation. Samples were harvested 36 hours after the time of ethephon injection. As shown in Fig. 4, the growth was inhibited by the ethephon treatment. However, the treatment enhanced the alkaloid production significantly in both volumetric and specific ways. In volumetric yield, alkaloid production was increased 84%, whereas specific productivity was increased 63%. Interestingly enough, ethephon also enhanced the intracellular alkaloid accumulation as in Fig. 5. This result possibly suggests that ethylene may be involved in cell membrane permeability.

It is not clearly known how the ethephon affects the cell metabolism. Endogenous ethylene evolution was increased by the treatment of ethephon in relation to abscission of various olive organs (7) and peach leaves (17). The decomposition of ethephon after application to the plant tissue or cell may also induce it to synthesize fur-

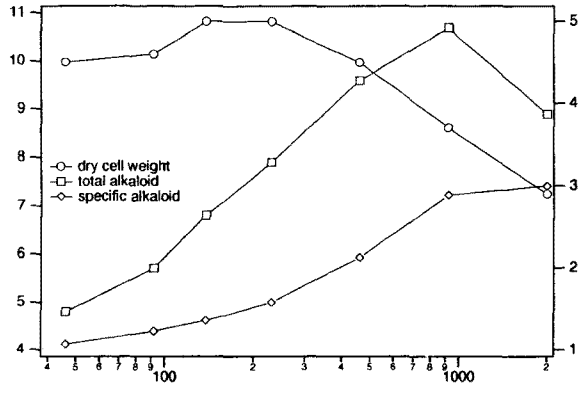

Fig. 4. Ethephon effect on cell growth and alkaloid production.

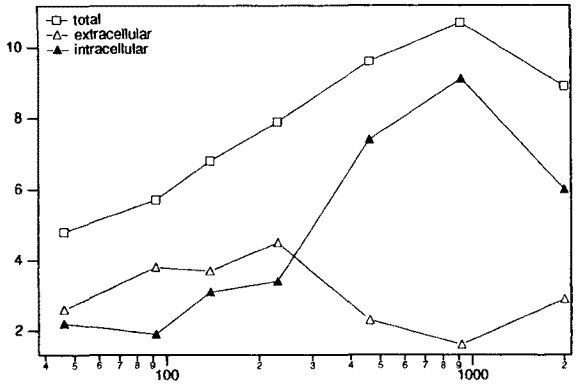

Fig. 5. Ethephon effect on accumulation pattern of alkaloid produced.

ther the enhanced levels of endogenous ethylene. When ethephon was applied at the final stage of culture, no effect was observed.

Since the pH of cultured medium was in the range of 4.5 to 5.6, ethephon is expected to decompose to ethylene. The decomposition of ethephon has been expressed as a function of pH and temperature (2, 17). Perry claimed that ethylene evolution rate was increased when the pH was increased to between 5.0 and 8.0. Cho (5) demonstrated similar enhancement of the alkaloid production in *Coffea arabica* cell suspension culture with an ethephon treatment.

Further experiments for the optimization of dosage, injection time and exposure time are necessary so as to use ethephon efficiently. In conclusion, ethylene which is known to be a natural plant growth regulator should draw more attention as an enhancer of secondary metabolite production.

Elicitation of Alkaloids Production

Several biotic elicitors were tested to study the alkaloid accumulation in the suspension cultures of *E. californica* (4). The results demonstrated that a wide variety of microorganisms could be used as elicitors in the suspension cultures of *E. californica*. These results also support the idea that the elicitor-stimulated accumulation of phytoalexins represents a widespread method by which plants defend themselves. Further experiments in this study to find application of elicitation have been done only with yeast elicitor.

The elicitor concentration is a factor which strongly affects the intensity of the response. Erlenmeyer flasks (125 m*l*) containing 50 m*l* of the growth medium and suspended cells were used for an experimental shake-flask culture. Different levels of yeast elicitor were dosed at exponential growth phase and cells were harvested after 16 hours. The accumulation pattern of alkaloids versus elicitor concentration demonstrated a saturated phenomenon (data not shown). The accumulation rate was highly affected by the elicitor at low level of elicitor concentration, but was virtually unaffected at high elicitor concentration. The maximum accumulation of total alkaloid was observed at 60 μg of yeast elicitor per gram of fresh cell weight (μg Y.E./g F.C.W). Overdosed elicitor reduced the accumulation of alkaloids as well as cell growth. Similar results in regard to the cell growth have been reported for *Petroselinum hortense* (13).

IAA Effect with Elicitation

The concentration of growth regulators in the nutrient medium can affect expression of secondary metabolism in cultured cells quite dramatically. Only a few studies have dealt with the influence of this factor on the cell's response to elicitation. Haberlach *et al.* (12) examined the effect of cytokinin and auxin concentration on resistance of *Nicotiana tabacum* callus cultures against *Phytophthora parasitica.* Susceptibility or resistance was a function of the phytohormone balance. A high cytokinin ratio favored infestation and suppressed hypersensitive responses. In the suspension cultures of bean, the ratio of kinetin to auxin as well as the type of auxin used markedly affected *Botrytis cineria* homogenate induced phaseollin accumulation (8). Cultures grown in the presence of 2,4 D accumulated lower levels than those grown with NAA and auxin.

The production of benzophenanthridine alkaloids by *E. californica* was promoted by the increase of IAA level with elicitation. Different levels of IAA were injected into the suspension cultures elicited by an identical amount of elicitor. The cultures used were 3 days old from inoculation and the samples were harvested 30 hours after injection. The accumulation of benzophenanthridine alkaloids was enhanced by IAA without elicitation as shown in Fig. 1. IAA also promoted the alkaloid production during elicitation. The highest alkaloid accumulation was observed at 5 μM of IAA as shown in Fig. 6. As from the experiment without elicitation, IAA increased the cell growth at low concentration, whereas it decreased the dry cell weight at high concentration

Ethephon Effect with Elicitation

To find out how ethephon affects the cell growth and the alkaloid formation during elicitation, ethephon was injected into the suspension cultures of *E. californica.* 60 μg Y.E./g F.C.W was also injected at the same time. Samples were harvested after 32 hours and analyzed to illustrate cell growth and alkaloid content. During elicitation, ethephon enhanced total alkaloid production as shown in Fig. 7. The highest total alkaloid production was observed at 460 mg/*l* of ethephon. Unlike the result seen for total alkaloid production, no significant increase of macarpine formation was observed. The increase in total alkaloid production was mainly due to enhanced

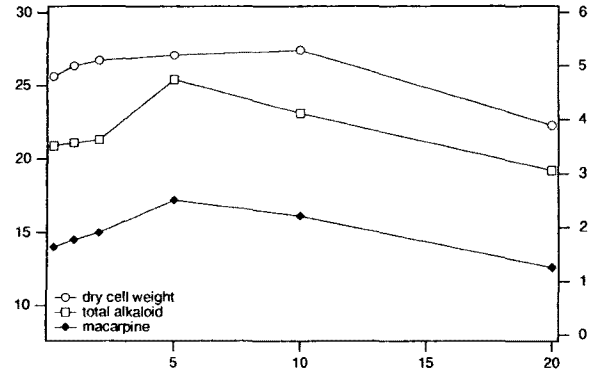

Fig. 6. IAA effect with elicitation on cell growth and alkaloid production.

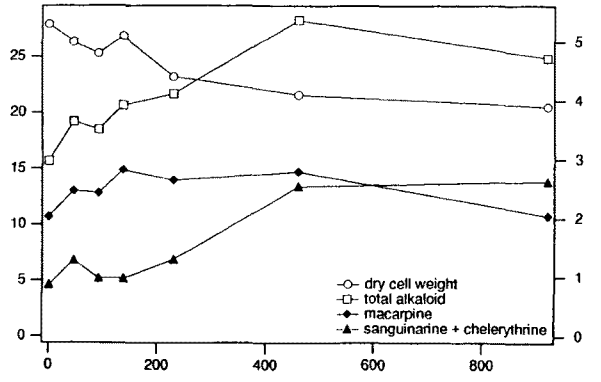

Fig. 7. Ethephon effect on cell growth and alkaloid production with elicitation.

sanguinarine and chelerythrine formations. Another different result from that without elicitation was observed with the cell growth. The addition of ethephon during elicitation suppressed cell growth, whereas without elicitation the cell growth was inhibited only at a high ethephon concentration.

It is not clearly known how ethephon affects the cell metabolism. From this experiment it could be expected that ethephon has specific enzymes which become active during elicitation. It didn't activate the enzymes that convert sanguinarine and chelerythrine into macarpine. This is a different result from that without elicitation.

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