Original Papers Plant Tissue Culture Letters, **9**(3), 164~168(1992)

Elicitation of Alkaloid Production at Different Growth Stages in Cell Suspensions of *Eschscholtzia californica*

Sang Yo BYUN, Chul KIM* and Henrik PEDERSEN**

*Department of Biotechnology, *Department of Chemical Engineering, Ajou University,*
San5 Wonchon-dong, Kwonson-ku Suwon 441-749, Korea
**Department of Chemical and Biochemical Engineering,*
Rutgers, The State University of New Jersey, New Brunswick, NJ 08903, U. S. A.

(Received March 26, 1992)
(Accepted June 23, 1992)

Increases in benzophenanthridine alkaloids occurred in suspension cultures of *Eschscholtzia californica* cells treated with elicitor from yeast extract. Elicitation at different growth stages showed that the volumetric alkaloid production was highest when yeast extract elicitor was dosed at day 6 from inoculation which was the exponential growth stage. Elicitation at this stage increased alkaloid production when it was compared to the maximum alkaloid production without elicitation. Elicitation also decreased the time required to accumulate alkaloids to a certain level.

Introduction

The relationship between growth stage and formation of secondary metabolites in cultured plant cells is a topic of numerous publications. In normal plant cell culture systems, the majority of compounds are formed in the stationary stage. The elicitor response also proved to be dependent on the growth stage in most of the culture systems[1]. With a few exceptions, most of the cultures showed response to elicitation only during the growth stage. The growth stage of a culture may affect not only the quantitative response to elicitor treatment but also the production pattern. *Pythium* culture homogenate stimulated *N*-acetyltryptamine formation in 5-day-old *Catharanthus roseus* cultures; 10-day-old cells, in contrast, accumulated a whole spectrum of monoterpene indole alkaloids[2]. Elicitor treatment, when a culture has already started to accumulate the inducible compounds, does not enhance or accelerate accumulation[2,4] and can even suppress already activated biosynthesis[3]. Kombrink and Hahlbrock[4] speculate that the formation of furanocoumarins in parsley cultures in the stationary stage might be a result of autoelicitation on lysis of cells; endogenous elicitor might be released. The influence of the growth stage of cultured cells is important for purposes of secondary metabolite production by elicitation. In this study we have investigated the quantitative response and production pattern of alkaloids to elicitor treatment at different growth stages in suspension cultures of *Eschscholtzia. californica.*

Materials and Methods

Cell Cultures: Cultures of *Eschscholtzia californica* were kindly provided by Dr. Peter Brodelius (Lund Univ., Sweden) and were originally developed in 1984. Suspension cultures of *E. californica* have been known to produce the benzophenanthridine alkaloids sanguinarine, chelirubine, cheleryth-

rine and macarpine as well as their dihydro forms[5]. Suspension and callus cultures have been maintained on B5 medium prepared from 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 carbon source. 0. 5% (w/v) of agar was added to prepare solid medium for callus maintenance. The pH was adjusted to 5. 8 with 1 N KOH. For the maintenance of suspension cultures, 16 g of cells (fresh weight) were transaferred into 200 ml 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 onto 50 cc solid medium. For experimental shake flask cultures, 4 g of cells (fresh weight) were innoculated into 125 ml Erlenmeyer flasks containing 50 ml of growth medium on a gyrotory shaker (Model G10, New Brunswick Scientific Co., Inc., Edison, NJ) at 180 rpm at 26°C under 18 hr of Cool White fluorescent light (4 μEi/m^{2}s) per day.

Chemicals: Sanguinarine nitrate was supplied from Research Plus, Jnc. (Bayonne, NJ) and chelerythrine was from Atomergic Chemicals Corp. (Farmingdale, NY). Macarpine was extracted and purified from cultured cell mass because no commercial supply was available[1]. 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). Yeast elicitor was isolated from yeast extract (DIFCO Laboratories, Detroit, MI) by ethanol precipitation as described by Hahn *et al*[7]. All other chemicals involved in this study were reagent grade.

Analytical Procedures: Cells were harvested by vacuum filtration and the filtrates were collected for the analysis of extracellular benzophenanthridine alkaloids in the medium. For the measurement of intracellular alkaloid concentration, 1. 0 g of cells (fresh weight) were extracted with 10 ml of HPLC grade methanol and the suspension was sonicated at 125 W for 10 min. All extracts were filtered through 0. 45 μm membrane filters and 10 μl of the solution was injected. The HPLC system was equipped with a Supelcosil™ LC-18-DB (15 cm×4. 6 mm, Supelco Inc., Bellefonte, PA) column and a UV detector at 280 nm. A mobile phase [H_2O-MeCN (13 : 7) at a flow rate of 1. 5 ml/min was used. The water phase contained 1 mM tetrabutylammonium phosphate and was adjusted to pH 2. 0 with phosphoric acid. The retention time of macarpine peak was 4. 9 min. Sanguinarine and chelerythrine were eluted faster than macarpine. 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 alkaloids in water in spite of being the same concentration. Therefore, separate standard solutions were prepared in methanol for intracellular analysis and in water for extracellular analysis. The total alkaloid is the summation of sanguinarine, chelerythrine chelirubine and macarpine analyzed. However, the chelirubine peak detected was negligible. The HPLC system was also used for the simultaneous analysis of sucrose and its hydrolyzed products, glucose and fructose. K Supelcosil™ LC-NH_2 (25 cm×4. 6 mm, Supelco Inc., Bellefonte, PA) was used with RI detector. The mobile phase was MeCN-H_2O (15 : 5) and the flow rate was 2 ml/min. For the determination of carbohydrate concentration in yeast elicitors, the orcinol-sulphuric acid procedure[8] was used. Glucose was the standard.

Elicitation at different growth stages: To study alkaloid production by elicitation at different growth stages in suspension cultures of *E. californica*, 60 μg of yeast elicitors per g of cell fresh weight were added to shake flasks at different growth stages. Cells in duplicate flasks were harvested after 2 days from elicitor addition.

Results and Discussion

Suspension cultures of *E. californica* showed typical kinetic behaviors of cell growth and product formation. The total benzophenanthridine alkaloid production was partly growth associated as shown in **Fig. 1**. The total alkaloid is the summation of sanguinarine, chelerythrine and macarpine. The macarpine formation pattern was different from that of total alkaloid because it is the final product. It was non-growth associated. The increase in macarpine formation after the stationary stage was probably due to conversion of other alkaloids into macarpine. Large and rapid increases in alkaloids production occured in suspension cultures treated with elicitor from yeast extract. It was well described by Byun *et al*[9].

This study showed that the influence of growth stages of cultured cells was obvious in product accumulation with elicitation. The volumetric production of macarpine was highest when yeast. elicitor was dosed at day 6 after inoculation as shown in **Fig. 2**. This is also true for the volumetric production of total alkaloid as shown in **Fig. 3**. Similar performances for sanguinarine and chelerythrine were represented in **Fig. 4** and **5**. These results disprove the negative opinion that elicitation could not increase production yield but just decrease the time required to accumulate product to a

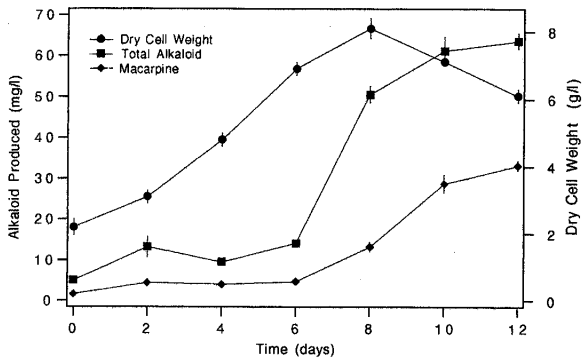

Fig. 1 Time course changes of cell growth and alkaloid production in suspension cultures of *E. californica*.

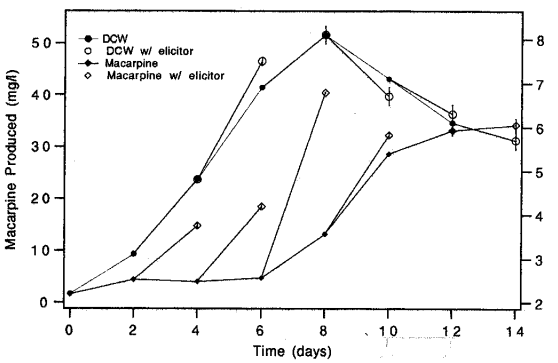

Fig. 2 Elicitation of macarpine production at different growth stages.
Cells in duplicate flasks were harvested 2 days from elicitation at different growth stages. Changes by elicitation are represented with straight lines from closed to open marks.

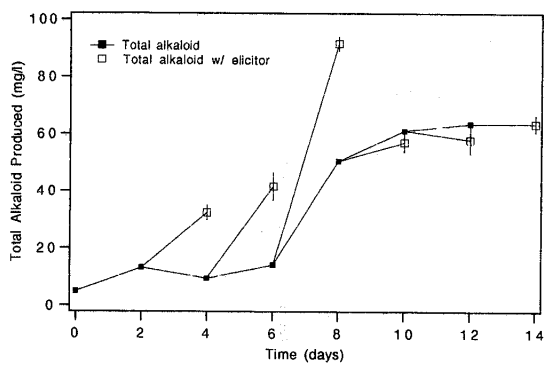

Fig. 3 Elicitation of total alkaloid production at different growth stages with the same experimental conditions as **Fig. 2**.
Changes by elicitation are represented with straight lines from closed to open marks.

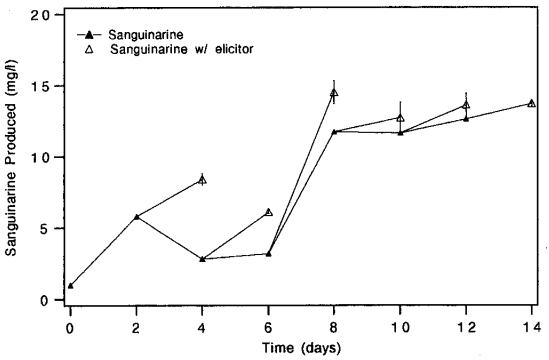

Fig. 4 Elicitation of sanguinarine production at different growth stage with the same experimental conditions as **Fig. 2.** Changes by elicitation are represented with straight lines from closed to open marks.

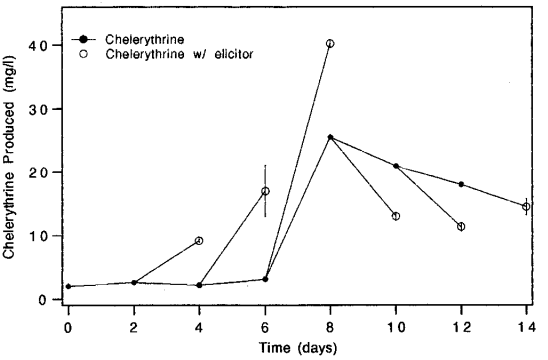

Fig. 5 Elicitation of chelerythrine production at different growth stage with the same experimental conditions as **Fig. 2.** Changes by elicitation are represented with straight lines from closed to open marks.

Table 1. Changes in alkaloid production rate and consumption rate by elicitation at day 4 from inoculation.

	Control	Elicatation
Sanguinarine production rate	0.20 mg/*l* day	1.65 mg/*l* day
Macarpine prodution rate	0.35 〃	7.25 〃
Total alkaloid production rate	1.00 〃	16.30 〃
Fructose consumption rate	1.43 g/*l* day	2.28 g/*l* day
Glucose consumption rate	1.15 〃	2.26 〃

Samples were harvested after 2 days of treatment.

certain level. When elicitor was dosed at an appropriate growth stage, alkaloid producsion increased in suspention cultures of *E. californica.*

Production rate increase was observed when elicitor was dosed at day 4 from inoculation and samples were harvested after 2 days of treatment. In this period, sugar consumption rate also increased as summarized in **Table 1**. More sugar was consumed for the expressjon of secondary metabolism because elicitor treatment at this growth stage didn't increase cell growth significantly. The sugar consumption pattern was different from that without elicitation. The glucose consumption rate was the same as that of fructose, which is quite different from normal culture where fructose was preferred to glucose.

The accumulation pattern of macarpine without elicitor showed a typical nongrowth associated result. However, elicitation changed that pattern into growth associated as shown in **Fig. 2.** Elicitation decreased the time required to accumulate alkaloids to a level which was possible at the stationary stage without elicitation. As a result, this experiment clearly showed that elicitation at an appropriate growth stage increased alkaloid production to values that were higher than that of the stationary stage without elicitation. This means that elicitation ultimately increased alkaloid production. The influence of the growth stage of cultured cells is obvious and of importance for the purpose of secondary metabolite production.

Acknowledgements

This work was supported in part by a research grant from the Research Center for New Bio-Materials in Agriculture, Korea Science and Engineering Foundation.

References

1) Eilert, U., 1987. In "Cell Culture and Somatic Cell Genetics of Plants Vol. 4" (ed. by Constabel, F., U. Eilert), p. 174-176, Academic Press, San Diego.
2) Eilert, U., F. Constabel, W. G. W. Kurz, 1986. J. Plant Physiol., **126**: 11-12.
3) Oba, K., I. Uritani, 1979. Plant Cell Physiol., **20**: 819-826.
4) Kombrink, E., K. Hahlbrock, 1985. Plant Cell Reports, **4**: 277-280.
5) Berlin, J., E. Forche, V. Wray, J. Hammer, W. Hösel, 1983. Z. Naturforsch, **38c**: 346-352.
6) Byun, S. Y., H. Pedersen, C.-K. Chin, 1990. Phytochemistry, **29**: 3135-3139.
7) Hahn, M. G., P.Albersheim, 1978. Plant Physiol., **62**: 107-111.
8) François, C., R. D. Marshall, A. Neuberger, 1962. Biochem. J., **83**: 335-341.
9) Byun, S. Y., H. Pedersen, 1991. J. Microbiol. Biotechnol., **1**: 220-226.