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Elicitation and *In Situ* Recovery of Alkaloids in Suspension Cultures of California Poppy

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Large and rapid increases in benzophenanthridine alkaloid production occured in suspension cultures of *Eschscholtzia californica* cells treated with elicitors. Response to different biotic elicitors showed that elicitors prepared from yeast extract, *Collectotrichum lindemuthianum* and *Verticillium dahliae* induced alkaloid formation. Highest alkaloid accumulation was obtained with 60 μg of yeast extract elicitor per gram of fresh cell weight. In time course performance after elicitor addition, more than 40 hours were required to obtain saturated alkaloid accumulation. Compounded silicone fluid, an ideal accumulation phase for two-phase culture of *E. californica*, accumulated a large amount of alkaloids produced in a specific manner. Elicitation in two-phase culture clearly increased net alkaloid production as well as their concentrations in the accumulation phase.

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 (8). Sanguinarine has recently been the subject of increasing interest because of dental and medical uses (10, 19). Several studies have been reported to produce benzophenanthridine alkaloids in callus or suspension cultures of *E. californica*. Berlin *et al.* (8) found that suspension cultures of *E. californica* accumulate the dihydro forms of the benzophenanthridine alkaloids as well as oxidized forms. The specific yield of alkaloids varied from zero to 1.7% on a dry weight basis depending on the media conditions. They also developed an induction medium in which the alkaloid accumulation increased to 17 mg/g dry weight and 146 mg/*l*. It was reported that treatment of *E. californica* cells with elicitor resulted in the massive induction of benzophenanthridine alkaloids. Schumacher and Zenk (18) found that sanguinarine, chelirubine, chelerythrine, chelilutine and macarpine were specifically induced by cell wall components of *Penicillium* and *Saccharomyces* in suspension cultures of *E. californica*.

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Several approaches to increase productivity of secondary metabolites in plant cell cultures have been made. Special techniques including reactor application and downstream processing steps have been reported with remarkable results. Among them elicitation and two-phase culture are typical techniques of current interests. Application of these techniques to cell culture systems sometimes increased the productivity so highly that it has been a gateway to commercial success. However, any general rules or suggestions to apply these techniques have not been proposed because of the specificity of cell culture systems.

Elicitor induced accumulation of secondary metabolites in plant tissue culture has lately received increasing attention (11). This is due, in part, to the fact that the elicitation can improve the efficiency of secondary metabolite accumulation in systems where product formation appears near or after the late growth phase. Elicitation of secondary metabolite production is a function of the source material used for elicitor preparation. Some elicitors can cause induction and accumulation of secondary metabolites, whereas others may result in no response. In some cases, different elicitors may also result in unique responses within the same cell culture. For instance, a varied response to different elicitors was noted in cultu-

red cells of *Petroselinum hortense*. Irradiation with UV light induced formation of flavonoids while addition of fungal elicitors, in contrast, caused formation and accumulation of furanocoumarins (15). The elicitors are obviously representative of different types of stress and result in production of active compounds against UV light and pahtogens (20, 21).

The elicitor concentration is a factor that strongly affect the intensity of the response. The accumulation rate is highly affected by elicitor concentration at low elicitor concentration, but was virtually unaffected at high elicitor concentration. Inhibition by overdosed elicitor can reduce the accumulation of secondary metabolites as well as cell growth (12).

Secondary metabolites may be either stored within the cell or excreted. A low yield of secondary substances released into the medium may be attributable to several factors. In those cases where low yield is due to feedback inhibition of membrane transport, biosynthesis, gene activity, enzymatic or nonenzymatic degradation in the medium, or volatility of substances produced, it should be possible to increase the net production by the addition of an artifcial site for accumulation and/or conservation of secondary substances in the culture medium (9). Using 'two-phase culture' (6) the accumulation of a secondary substance inside the protoplast, in the culture medium, and in the accumulation/conservation phase should approach an equilibrium depending on the affinity, the capacity, and the amount of the second phase material. It is easily recognized that the appropriate second phase for the accumulation of a certain secondary metabolites has to be tailored to each substance. Up to the present only a very limited number of second phases have been tested experimentally. Only a few examples are known which show partitioning of cell products in a more or less nonspecific manner (7). No general rules about their influence on plant cells and about the prospect of their use can be formulated. However this technique can be a singificant breakthrough in secondary metabolite production in *E. californica*. Also, it is expected that the application of elicitation in two-phase culture can serve to eliminate bottlenecks to commercial application associated with poor yield.

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, chelerythrine and macarpine as well as their dihydro forms. Suspension and callus cultures have been maintained on B5 medium (14) 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 a 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 cell weight) was transferred 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 on 50 ml solid medium. 125 ml Erlenmeyer flasks containing 50 ml of 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°C and the cultures were exposed to 18 h 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). Tetrabutylammonium phosphte for HPLC analysis and all the solvents used for HPLC such as acetonitrile, methanol and water were bought from Fisher Scientific (Rochester, NY). Compounded silicone fluid was supplied from Sigma Chemical Co. (St. Louis, MO). All other chemicals involved in this study were reagent grade.

Preparation of Alkaloid Standard

Macarpine was extracted from cultured cell mass because no commercial supply was available. Approximately 500 g of filtered cell mass of *E. californica* was dispersed in 1.5 liters of methanol. the mixture was stirred overnight room temperature and filtered. The filtered cells were then washed with 1 liter of methanol. The combined methanol extracts evaporated under reduced pressure. A 200 ml solution of acetic acid and water (50:50, v/v) was added to the residue and the mixture was again filtered. The filtrate was extracted with petroleum ether (200 ml) to remove colored materials and the aqueous layer was then made alkaline with 15% ammonium hydroxide and extracted with chloroform (400 ml). Evaporation of the organic solvent gave a crude mixture of *E. californica* alkaloids. The crude extract was dissolved in methanol and separated by FPLC (Pharmacia, Piscataway, NJ). Reversed phase silica C-18 (PepRPC™, Pharmacia) was used as a separation column. A programmed mixture of acetonitrile and water was used for the mobile phase where the water phase included 1 mM tetrabutylammonium phosphate with the pH adjusted to 2.0 using phosphoric acid. Macarpine

peaks were further isolated by solvent extraction with methylene chloride and positively identified with a mass spectrometer.

Preparation of Elicitors

Yeast elicitor was isolated from yeast extract (DIFCO) laboratories, Detroit, MI) by ethanol precipitation as described by Hahn and Albersheim (16). For fungal biotic elicitors three strains were used. The strains were *Phythophthora megasperma* f. sp. *glycinea* (ATCC 28001), *Colletotrichum lindemuthianum* (ATCC 11225), and *Verticillum dahliae* (ATCC 7611). Isolation procedures for fungal elicitors are described by Ayer *et al.* (2, 3).

Analytical Procedures

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 measurement of intracellular alkaloid concentration, 1.0 g of cells (FCW) were extracted with 10 m*l* of HPLC grade methanol and the suspension was sonicated at 125 W for 10 minutes. For the analysis of alkaloids formed in the accumulation phase of a two-phase culture, 1.0 m*l* of compounded silicone fluid was extracted with 20 m*l* of HPLC grade methanol and was stirred overnight. All extracts were filtered through 0.45 μm membrane filters and 10 μ*l* of the solution was injected. The HPLC system was also used with a Supelcosil™ LC-18-DB column and an UV detector at 280 nm. A mobile phase mixture of water (65%) and MeCN (35%) at a flow rate of 1.5 m*l*/min was used. The water phase 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* 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 the same concentration. Therefore, separate standard solutions were prepared in methanol for intracellular analysis and in water for extracellular analysis.

Carbohydrate concentration in various biotic elicitors: For the determination of carbohydrate concentration in fungal or yeast elicitors, the orcinol-sulphuric acid procedure (13) was used. Glucose was used as the standard.

RESULTS AND DISCUSSION

Response to Different Biotic Elicitors

Plants accumulate toxins, often referred to as phytoalexins, in response to invading pathogens. The phytoalexin produced is thought to play an important role in disease resistance and occurs in response to a wide variety of microorganisms, both pathogenic and nonpathogenic. Molecules which elicit phytoalexin accumulation have been isolated from the mycelial walls (4, 5). Such molecules which stimulate phytoalexin accumulation are called elicitors. The term elicitor was proposed to apply to compounds of biological origin only, while the other treatments could be designated as abiotic stress.

The mycelial elicitors are glucans (2, 3). Ayer *et al.* (4, 5) have demonstrated that the glucans isolated from each of three reces of *Phytophthora megasperma* var. *sojae* have the same glucosyl linkage composition and possess identical abilities to stimulate the accumulation of phytoalexins in soybean tissue. They also found that the *Phytophthora megasperma* var. *sojae* elicitor cannot account for the varietal specificity exhibited by the *Phytophthora* races for their host, soybean. They suggested that the elicitor response was a part of a general mechanism of plant, a mechanism which might be activated by a wide variety of microorganisms. If this is indeed the case, then one should be able to find elicitors in the mycelial walls of other fungi.

Hahn and Albersheim (16) have demonstrated that an autolysate of 'brewers' yeast (*Saccharomyces cerevisiae*) possesses an elicitor very similar to the *Phytophthora* elicitor. The yeast glucan elicitor shares many characteristics with the glucan elicitor which had been isolated from *Phytophthora megasperma* var. *sojae*. These characeteristics included the fact that the yeast elicitor could be separated from other carbohydrate containing macromolecules using essentially the same techniques that were successful in isolating the *Phytophthora* elicitor. A comparison of the glycosyl linkage compositions showed the presence of the same linkages in both elicitors. There are some differneces between the yeast and *Phytophtora* elicitor glucans. The yeast glucan is rich in 6-linked glucosyl residues, whereas the *Phytophthora* glucan is rich in 3-linked glucosyl residues.

Four biotic elicitors were tested to study alkaloid production in suspension cultures of *E. californica*. Three different types of elicitors were prepared from each fungal elicitor (3). Therefore, 10 different elicitors (9 fungal elicitors and a yeast elicitor) were used in this experiment. 60 μg elicitor/g FCW was dosed at exponential growth phase and samples were harvested after 48 hours. Fig. 1 shows the responses to 10 different biotic elicitors. Elicitors propared from *Collectotichum lindemuthianum*, *Verticillium dahliae* and yeast extract induced the formation of benzophenanthridine alkaloids, whereas the elicitors from *Phytophthora* resulted in no response, The small extracellular elicitor from *Verticillium dahliae* showed no response either. The maximum increase in total alkaloid was 3.5-fold with yeast elicitor and *Verticillum dahliae* glucan elicitor. The differences in total alkaloid production may represent differences

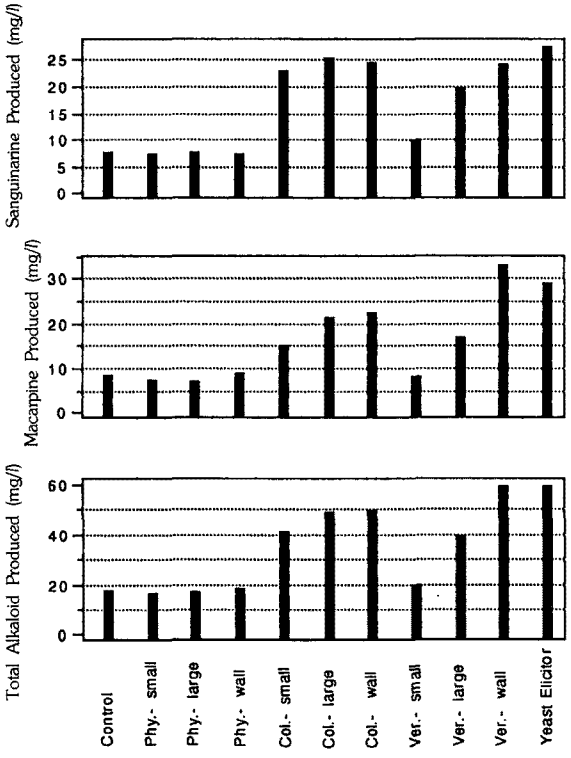

Fig. 1. Response to different elicitors in suspension cultured *E. californica*.
Phy.=*P. megasperma*, Col.=*C. lindemuthianum*, Ver.=*V. dahliae*, small=small extracellular elicitor, large=large extracellular elicitor, wall=wall-released elicitor (glucan).

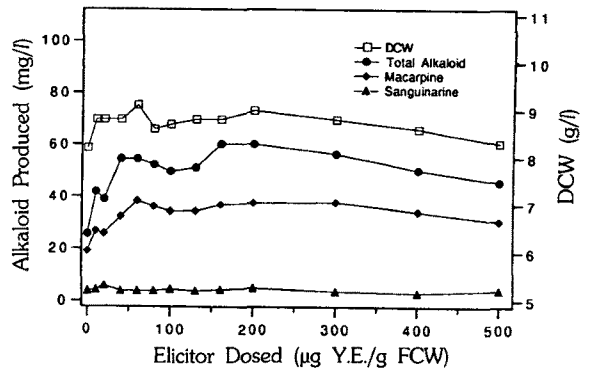

Fig. 2. Effects of elicitor concentration on cell growth and alkaloid accumulation.

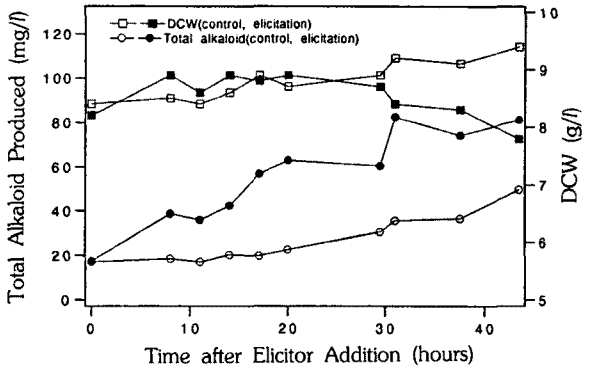

Fig. 3. Cell growth and alkaloid production after elicitor addition.

in optimum concentration for each elicitor. However, there is no genseral mechanism to explain that elicitors from *Phytophthora megasperma* var. *sojae* resulted in no response. There are some differences between the yeast and *Phytophthora* elicitor glucans. The yeast glucan is rich in 6-linked glucosyl residues, whereas the *Phytophthora* glucan is rich in 3-linked glucosyl residues. Another difference between them is the lower maximum level of phytoalexin production in soybean exhibited by the yeast elicitor (16). This phenomenon may result from different rates of degradation of two elicitors by gluconases present in the plant tissues (1, 17). The small extracellular elicitor of *Verticillium dahliae* showed no response. The only difference between small and large extracellular elicitor was the size of molecules. The small extracellular elicitor was composed of molecules dialyzed with the 8000 mol wt cutoff membrane.

The results of this experiment demonstrated that a wide variety of microorganisms could possibly be used as elicitors in suspension cultures of *E. californica*. These results also support the theory 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 extract elicitor.

Dependence of Alkaloid Production on Elicitor Concentration

The elicitor concentration is a factor which strongly affects the intensity of the response. Different levels of yeast elicitor were dosed at exponential growth phase and cells were harvested after 16 hours. Fig. 2 shows the quantitative accumulation of benzophenanthridine alkaloids in response to different elicitor concentrations. The accumulation pattern of alkaloids *versus* elicitor concentration demonstrates a saturated phenomenon. The accumulation rate was highly affected by elicitor concentration at low elicitor concentration, but was virtually unaffected at high elicitor concentration. The maximum accumulation of macarpine was observed at 60 μg of yeast elicitor per gram of FCW (μg Y.E/g FCW). The accumulation patterns of other secondary metabolites, sanguinarine and chelerythrine, were different. The maximum value of sanguinarine accumulation was obtained at 20 μg Y.E./g FCW while for chelerythrine, it was obtained at 40 μg Y.E./g FCW. Fig. 2 also demonstrates that

'overloading' of elicitor has adverse effects. Inhibition by overdosed elicitor reduced the accumulation of alkaloids as well as cell growth. The cell growth with elicitation was higher than that of control because it was measured after 16 hours from elicitor addition. After 25 hours from elicitation the cell mass started to decrease as shown in Fig. 3. Similar results with respect to cell growth have been reported for *Petroselinum hortense* (15).

Kinetics: Time Course Performance after Elicitor Addition

The kinetics of alkaloid accumulation in response to treatment with elicitor is not a simple mechanism. Elicitor signal transmission, mRNA transcription and enzyme translation are involved prior to product formation. This is the reason that a lag period commonly appears before product accumulation in tissue culture systems. In suspension cultures of *E. califiornica*, yeast elicitor was added during the exponential growth phase and the time dependent performance was observed. Adverse effect on cell growth was monitored as shown in Fig. 3. From 25 hours after elicitor addition the DCW started to decrease. The alkaloid accumulation pattern *versus* elapsed time demonstrated a saturated phenomenon. The alkaloid accumulation reached the maximum value around 30 hours after elicitor dosing. After this point a slow decrease in alkaloid accumulation was expected by degradation with exoenzymes excreted through cell lysis. Fig. 4 clearly shows the accumulation pattern of benzophenanthridine alkaloids. Sanguinarine, an intermediate of the macarpine synthesis pathway,was aaccumulated in the substrate-regulated system and was then metabolized to a product further down the biosynthetic pathway. The time required for activation of the biosynthetic pathway is plant specific and the time courses of accumulation of secondary metabolites should be considered when investigating a new system.

In Situ Recovery of Alkaloids in Two-phase Culture

A suitable accumulation phase for *E. californica* cultures was discovered (9). Compounded silicone fluid has the properties reuqired of the ideal accumulation phase for suspension cultures of *E. californica*. It binds benzophenanthridine alkaloids specifically and releases them by simple treatment. It is stable under conditions of repeated autoclaving and does not change the medium composition because it binds only end products. It was also observed that the benzophenanthridine alkaloids absorbed were stable in it. It also offers large accumulation capacity. It is insoluble in the culture medium and it is clearly separated from cellular or medium phases.

In spite of the fact that silicone is generally known to be nontoxic for cell cultures, we tested the toxicity of compounded silicone fluid against suspension cultures of *E. californica*. Different amounts of compounded silicone fluid were added into suspension cultures which had been maintained 3 days from inoculation. Samples were harvested after 36 hours. Cell growth was not affected by the addition of the accumulation phase (Fig. 5). Cellular sanguinarine accumulation and total alkaloid production were not influenced either. No evidence has been shown that compounded silicone fluid caused any negative effect on suspension cultures of *E. californica* so far.

The biggest advantage of compounded silicone fluid in suspension cultures of *E. californica* is that it causes an enormous increase in the production of benzophenanthridine alkaloids. The large accumulation capacity made it possible to store a large amount of alkaloids in it and lessened feedback inhibition on alkaloid production. It is better to add accumulation phase as much as possible to enhance alkaloid production and recovery as far as the accumulation phase does not inhibit cell growth or alkaloid production. The driving force between

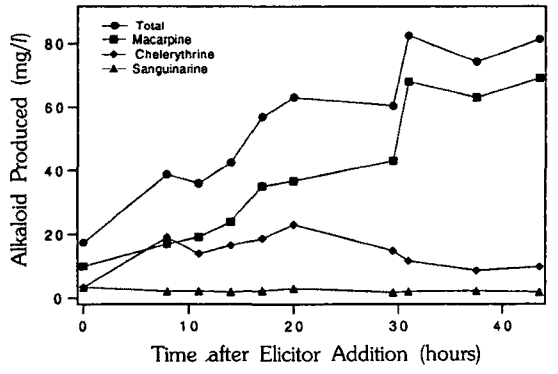

Fig. 4. Accumulation pattern of akaloids after elicitor addition.

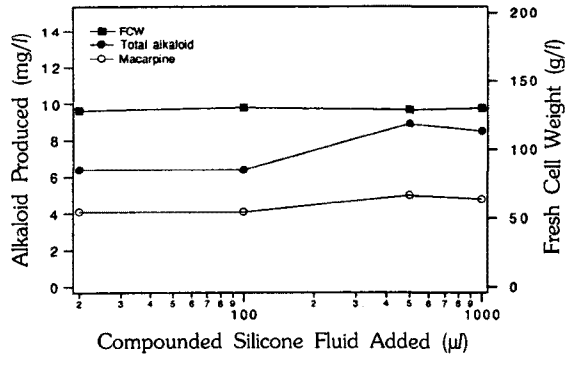

Fig. 5. Effect of compounded silicone fluid on cell growth and alkaloid accumulation.

the cellular and accumulation phase can be increased by addition of more accumulation phase which dilutes the alkaloid concentration in the accumulation phase. Another advantage of increasing the accumulation phase in a culture system is that it can offer a larger contact area between accumulation and culture phase. Increased driving force and contact area in two phase culture can enhance transfer rates of secondary metabolites.

The net alkaloids production was highest at 23% of accumulation phase in 50 ml flasks and it decreased at higher concentrations which was mainly due to lack of oxygen supply. This phenomenon was mainly due to a decrease in cell growth because the specific net total alkaloid productions were same for both 23% and higher accumulation phase concentrations (9).

We found that pH is an important factor for two-phase culture of *E. californica*. pH elevation led to an increase in alkaloid accumulation in the compounded silicone fluid. Altering the pH changes the net charge of the alkaloid. Among the benzophenanthridine alkaloids produced in *E. californica*, sanguinarine was found to be mostly dependent on pH (9).

Elicitation of Alkaloids Production in Two-phase Culture

To find out elicitor effects on the time course behavior, 60 μg Y.E./g FCW was injected at the same time the compounded silicone fluid was added. With all culture conditions the same, samples were harvested at the same time that elicitor free samples were taken and the results were compared. No difference in cell growth was observed until 68 hours from the staring point. After that point, elicitation inhibited cell growth. The total alkaloid production per 50 ml flask was increased 2.5~10.5 fold by elicitation (9). Elicitation also reduced the time requried to reach high alkaloid concentration.

In Fig. 6 the total alkaloid concentration in the accumulation phase was compared to that in the cellular phase. A major part of the alkaloid produced was observed to be transferred from the cellular phase to the accumulation phase. Therefore the alkaloid produced was not accumulated in the cellular phase, which eliminated feedback inhibition with alkaloid accumulation. pH dependence of alkaloid accumulation in two-phase culture was also observed during elicitation. Fig. 6 shows the relationship between pH and total alkaloid produced by elicitation.

Elicitation in two-phase culture clearly increased net alkaloid production as well as the concentration found in the accumulation phase. Although it is a combination of elicitation and two-phase culture, it has several advantages that can not be acquired by the individual methods. Typical advantages of elicitation in two-phase culture over the two individual methods are: it eliminates feedback inhibition by secondary metabolites produced.; it induces metabolic activities like transcriptional mRNA synthesis and corresponding translation that increases secondary metabolite formation, whereas the single two-phase culture method doesn't. In conclusion, elicitation in two-phase culture significantly increased benzophenanthridine alkaloid production in *E. californica*. It is expected that the application of elicitation in two-phase culture can climinate some major problems associated with the poor yield of secondary metabolite production in plant cell culture.

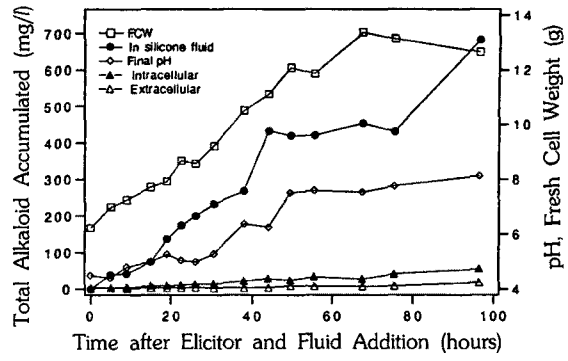

Fig. 6. Distribution of alkaloids produced in two-phase culture with elicitation.

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