Differential Enhancement of Benzophenanthridine Alkaloid Content in Cell Suspension Cultures of Sanguinaria canadensis Under Conditions of Combined Hormonal Deprivation and Fungal Elicitation

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DIFFERENTIAL ENHANCEMENT OF BENZOPHENANTHRIDINE ALKALOID CONTENT IN CELL SUSPENSION CULTURES OF *SANGUINARIA CANADENSIS* UNDER CONDITIONS OF COMBINED HORMONAL DEPRIVATION AND FUNGAL ELICITATION[1]

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ABSTRACT.—An elicitation protocol, resulting in the accumulation of sanguinarine in suspension cultures of *Papaver bracteatum*, was assessed for induction of the same alkaloid in *Sanguinaria canadensis*. Although only a trace constituent of *P. bracteatum* plants, sanguinarine is a major alkaloid (1–3% dry wt) of *S. canadensis* rhizomes. By combining hormonal deprivation for various intervals and a 3-day fungal (*Verticillium dahliae*) elicitation, benzophenanthridine alkaloid accumulation was induced in *S. canadensis* cell suspensions. Chelirubine content increased (0.1–1.3% dry wt) consistently in elicited cell cultures while chelerythrine (0.01–0.10% dry wt) and sanguinarine (0–0.02% dry wt) levels were considerably less. Alkaloid accumulation always occurred upon removal of hormone but only at certain time intervals in the log phase upon fungal elicitation. Levels of dopamine, a precursor of the alkaloids, fluctuated over the incubation period, but displayed a 2- to 6-fold increase in cell suspensions grown without hormone. In some experiments dopamine accumulated to levels >20% dry wt, and these increases were enhanced by the addition of fungal elicitor. Although the same fungal elicitor induces benzophenanthridines in taxonomically related *S. canadensis* and *P. bracteatum*, it did not elicit the accumulation of the same alkaloid in the two different plant cultures.

Fungal elicitors have proven to be effective inducers of secondary metabolites, including specific alkaloids in tissue cultures of various plant genera (1–8). Augmentation of the content of a secondary metabolite under elicitation conditions is one criterion proposed for phytoalexins (9). Recently, the benzophenanthridine alkaloid sanguinarine has been shown to satisfy some of the criteria of a phytoalexin in *Papaver somniferum*, *Papaver bracteatum*, and *Eschscholtzia californica* (2,6,8). While sanguinarine is present only at trace levels in planta (10), we have observed a 50- to 500-fold increase (10% dry wt) in sanguinarine through a combination of fungal elicitation and hormone deprivation. Other constituents of these plants do not meet the same criteria. Thebaine and morphine, major alkaloids of *P. bracteatum* and *P. somniferum* are trace constituents of the plants in culture under control and elicited conditions (6,10,11 and references cited therein). Moreover, dopamine, a precursor of both benzophenanthridines and morphinans, normally occurs in millimolar concentrations in tissue cultures and in the intact plant (6,11,12).

Future exploitation of elicitation for the overproduction of certain metabolites necessitates understanding the factors involved in determining which metabolite is accumulated. Current evidence supports the notion that in cell culture the formation of normally abundant endogenous plant end-products is suppressed while metabolites considered trace constituents of the intact plant may become major products. In *Sanguinaria canadensis* L. (Papaveraceae) sanguinarine (average 3% dry wt) and chelerythrine

[1]Dedicated to the memory of Professor Edward Leete.

are considered major alkaloids present in rhizomes (13). Sanguinarine also occurs in *S. canadensis* tissue cultures (14). It was of interest to test the elicitation protocol previously used for *P. bracteatum* (6) for its ability to stimulate sanguinarine synthesis in cell cultures of *S. canadensis*. In addition, we measured levels of two related benzophenanthridine alkaloids (chelerythrine and chelirubine) and their precursor dopamine under conditions of hormonal deprivation and fungal elicitation. This would allow us to determine whether the same elicitor can induce the same alkaloid in related plants that differ in their alkaloidal metabolite profiles.

EXPERIMENTAL

PLANT CELL CULTURES.—*S. canadensis* cell cultures were maintained on Murashige and Skoog's revised tobacco medium (MSRT) containing 1 mg/liter 2,4-D as described (11).

CULTURE CONDITIONS.—Cell suspension cultures were prepared (40 g) from 14-day-old callus grown at 21–24°. Suspensions were grown in the dark at 24° on MSRT containing 1 mg/liter 2,4-D and maintained on a 14-day transfer schedule. At the third transfer, 70 g of cells filtered through Miracloth were divided equally into 300 ml MSRT either with or without hormonal supplement and allowed to incubate for an additional 18 h. Aliquots (5 ml) of the cell suspension (about 0.3 g fresh wt) were added to 50-ml flasks containing 10 ml of MSRT with or without hormone and grown on a rotary shaker at 21–24° in 12-h fluorescent light cycle prior to elicitation. In general, dry weights averaged 5–10% of the fresh wt of control and elicited cells.

ELICITOR PREPARATION AND ADDITION.—The elicitor consisted of an autoclaved conidial suspension (6×10^6/ml) of *Verticillium dahliae* prepared as described (6). The elicitor (1 ml) was added to the first set of 15-ml cell suspension cultures beginning on day 3 of the incubation period. Thereafter at days 7, 10, 14, 17, 21, or 27, additional sets of test flasks were elicited. Cells are harvested by filtration through Miracloth 72 h after elicitor addition, and levels of alkaloid and dopamine were determined. In preliminary studies 1 ml of either the fresh media or conidia-free spent media used for conidial preparation was added to 15-ml control flasks and their alkaloid content was found to be no different from that of untreated controls.

ALKALOID AND DOPAMINE ANALYSIS.— Total alkaloids were extracted from polytron-homogenized tissue with 0.1 N methanolic HCl. The cell homogenate was centrifuged ($100\times g$) and the alkaloid extract filtered prior to evaporation to dryness. The residue was reconstituted in MeOH, and the alkaloids were prefractionated on Extrelut columns, as previously described (10). Following evaporation of the alkaloid fraction, individual alkaloids were separated by preparative tlc (Si gel, Si250F). Using a $CHCl_3$-MeOH (97:3) mobile phase, chelirubine (R_f 0.63), sanguinarine (R_f 0.58), dihydrosanguinarine (R_f 0.71), and chelerythrine (R_f 0.33) were resolved. Alkaloids were identified by comparison with authentic standards kindly provided by Dr. L. Southard (Vipont Laboratories, Fort Collins, CO). Identification was based on comigration in different tlc [e.g., $CHCl_3$-MeOH (80:20, 97:3, 95:5); $CHCl_3$-hexane-MeOH (80:10:5); Me_2CO-toluene-EtOH-NH_4OH (40:40:12:4)] and hplc [hexane-MeOH-$CHCl_3$-diethylamine (290:28:10:0.1); $CHCl_3$-hexane-MeOH (80:10:5)] solvent systems as well as visible and fluorescence spectral properties (6, 10–12, and references cited therein).

Samples were eluted from the preparative plates with MeOH and quantified by uv-hplc on a (0.46×25 cm) 5 μm Si gel column (Beckman, San Ramon, CA) eluted with a mobile phase of hexane-MeOH-$CHCl_3$-diethylamine (290:28:10:0.1) with a flow rate of 1 ml/min and detection at 280 nm. In this system sanguinarine and chelerythrine comigrated (Rt 5.0 min), while chelirubine eluted more slowly (Rt 5.8 min) and dihydrosanguinarine more rapidly (Rt 3.5 min). Peak heights of the absorbance at 280 nm were compared to linear standard curves generated with authentic standards.

Dopamine content in cell extracts from 50-mg samples was prefractionated on alumina followed by quantification by hplc with electrochemical detection using a (0.46×15 cm) 7 μm octadecylsilica gel column (Spherisorb, Phase Separations, Norwalk, CT) eluted with EtOH/H_2O/HOAc containing paired ion reagents (flow rate 1 ml/min) as described (12).

STATISTICS.—Statistical analyses of the data were performed with the Student's *t*-test. In general, significant increases in alkaloid content were found upon removal of hormone. Significant differences detected upon elicitation in the presence or absence of hormone are shown in the figures.

RESULTS AND DISCUSSION

The presence of hormone had a significant effect upon growth (Figure 1). During the

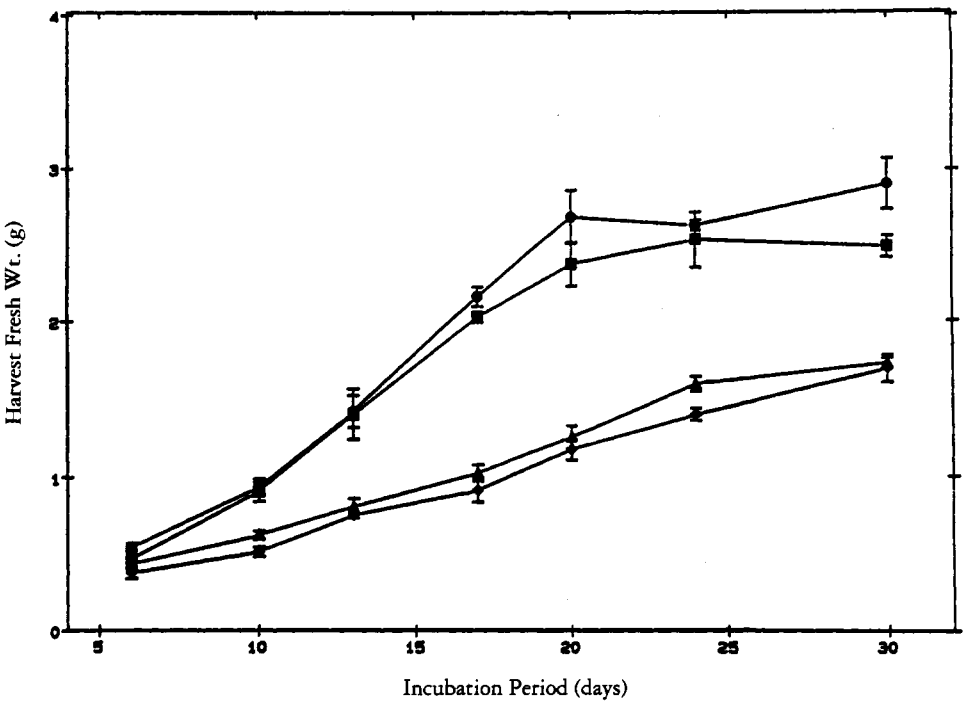

FIGURE 1. Effect of *Verticillium* elicitation on growth of *Sanguinaria canadensis* cell suspension cultures in the presence and absence of 2,4-D. *Verticillium* elicitor was added to cell suspension cultures at day 3, 7, 10, 14, 17, 21, or 27. Cells were harvested by filtration 72 h later and fresh wt determined. (●) 2,4-D, non-elicited; (■) 2,4-D, elicited; (▲) hormone-free, non-elicited; (◆)-hormone-free, elicited. In this and subsequent figures the number of days shown on the abscissa represents the total time of incubation including elicitation for each set of data above it. Each value represents the mean ±SD of 3 experiments.

log phase (10 to 17 days), suspension cultures supplemented with 2,4-D displayed a maximal doubling time of about 3 days. Overall growth was less rapid (6–7 day doubling time). In the absence of hormone, suspension cultures grew more slowly with a doubling time of about 8 to 9 days. Cell growth of cultures maintained in the presence of 2,4-D appeared to cease in the third week, which was considered to be the stationary growth phase. The absence of hormone promoted a slower but continous growth of the cultures through 30 days without an indication of reaching stationary growth phase in that time period. The presence of fungal elicitor in the last 3 days of each time interval did not appear to affect growth adversely beyond that of hormonal deprivation. In all experiments alkaloid content was estimated in two ways: total alkaloid (μg/flask) and concentration (μg/g fresh wt). This was done to determine whether alkaloid synthesis would parallel growth throughout the period studied.

Effects of hormonal deprivation alone produced the greatest change in total sanguinarine detected (Figure 2A). Total sanguinarine doubled after 6 days in hormone-free versus hormone-amended media, and the difference increased further through day 24. The maximum yield of sanguinarine represented only 0.02% of the dry wt. The presence of elicitor for a 3-day period resulted in a significantly higher accumulation of sanguinarine in the absence of hormone only for cells that were incubated for a total of 10 days.

When sanguinarine production was estimated on a concentration basis (Figure 2B),

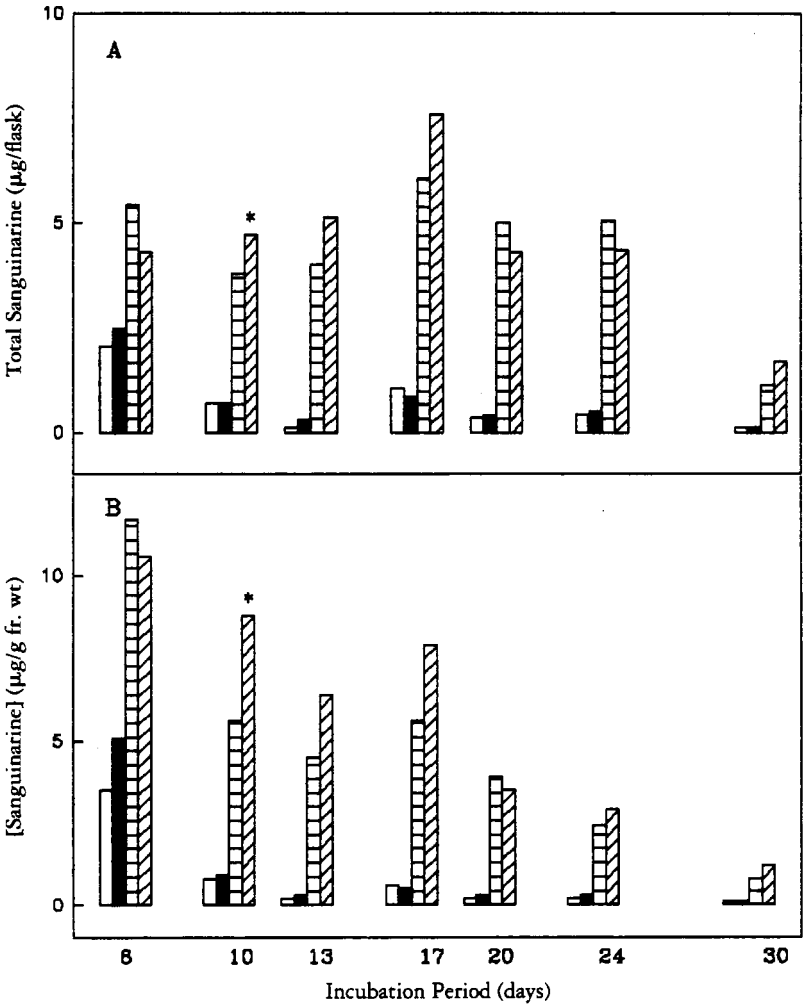

FIGURE 2. Sanguinarine, (A) total accumulation per flask and (B) concentration, in *Verticillium*-elicited cell suspension cultures of *Sanguinaria canadensis* grown in the presence of absence of 2,4-D. Conidial elicitor (1 ml) was added on day 3, 7, 10, 14, 17, 21, or 27 following transfer to test flasks. Cultures were incubated for an additional 72 h prior to harvest. Open and filled bars always represent hormone-supplemented cultures, whereas hatched bars are symbols for hormone-free cultures. Alkaloid concentrations were determined as described (see Experimental). (Open bars) 2,4-D, non-elicited; (filled bars) 2,4-D, elicited; (horizontal-hatched bars) hormone-free, non-elicited; (diagonal-hatched bars) hormone-free, elicited. Each value represents the mean of 3 experiments. Standard errors of the mean ranged from 4 to 28% except for values below 5 μg/g fresh wt where they were higher at times. *$P<0.05$ for elicited vs. non-elicited cultures in hormone-free experiments.

levels of the alkaloid were observed to be highest at the initial incubation period (6 days) and thereafter declined throughout the remaining 30 days of evaluation. In the presence of hormone, sanguinarine concentrations were virtually negligible after 6 days. In the absence of hormone, sanguinarine concentrations were significantly increased by elicitor in 10-day cultures.

Similar to the sanguinarine results, hormonal deprivation alone produced the greatest change in total chelerythrine (Figure 3A). In the presence of hormone, total chelerythrine accumulation remained low ($\leq$6 μg/flask) and elicitation was ineffective

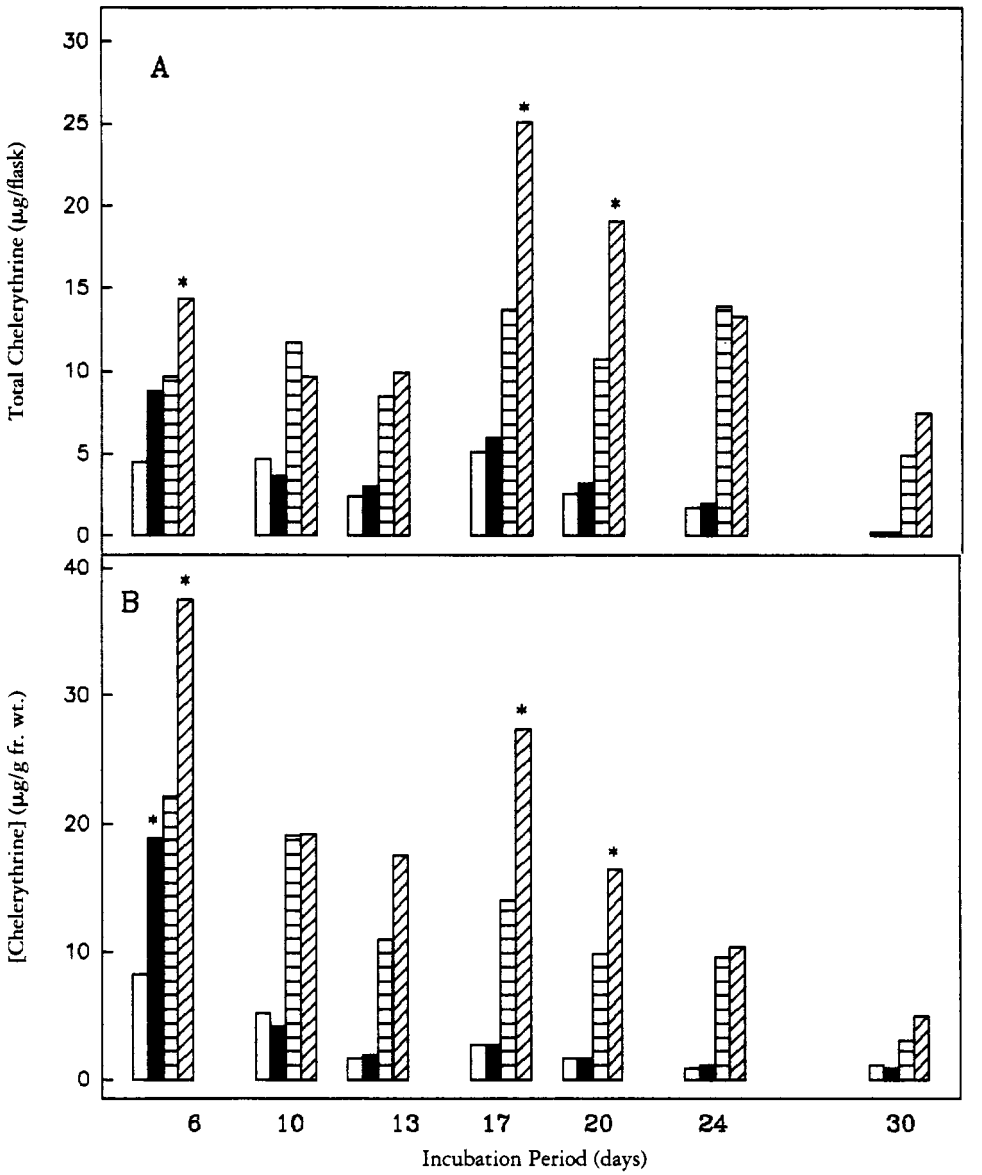

FIGURE 3. Chelerythrine, (A) total accumulation per flask and (B) concentration in *Verticillium*-elicited cell suspension cultures of *Sanguinaria canadensis* grown in the presence or absence of 2,4-D. See Figure 2 for experimental details. (Open bars) 2,4-D, non-elicited; (filled bars) 2,4-D, elicited; (horizontal-hatched bars) hormone-free, non-elicited; (diagonal-hatched bars) hormone-free, elicited. Each value represents the mean of 3 experiments. Standard errors of the mean were ≤20% except for values below 10 μg/g fresh wt where they were higher in some instances. *$P<0.05$ for elicited vs. non-elicited cultures.

in enhancing alkaloid yields. In the absence of hormone, elicitation increased total chelerythrine after 6, 17, and 20 day incubation periods. Peak production was observed in cultures grown for a total of 17 days (0.1% dry wt).

In the presence of hormone, the concentration of chelerythrine was only augmented by elicitation in 6-day cultures (Figure 3B). However, chelerythrine levels increased significantly in 6, 17, and 20 day elicited, hormone-free cultures over their non-elicited

counterparts. In general, chelerythrine concentrations were higher than those of sanguinarine.

Chelirubine was the major alkaloid produced in these experiments, with amounts at least an order of magnitude greater than the other two benzophenanthridines measured. Hormonal deprivation alone produced the greatest effect in chelirubine accumulation over the entire testing period (Figure 4A). Elicited hormone-free cultures

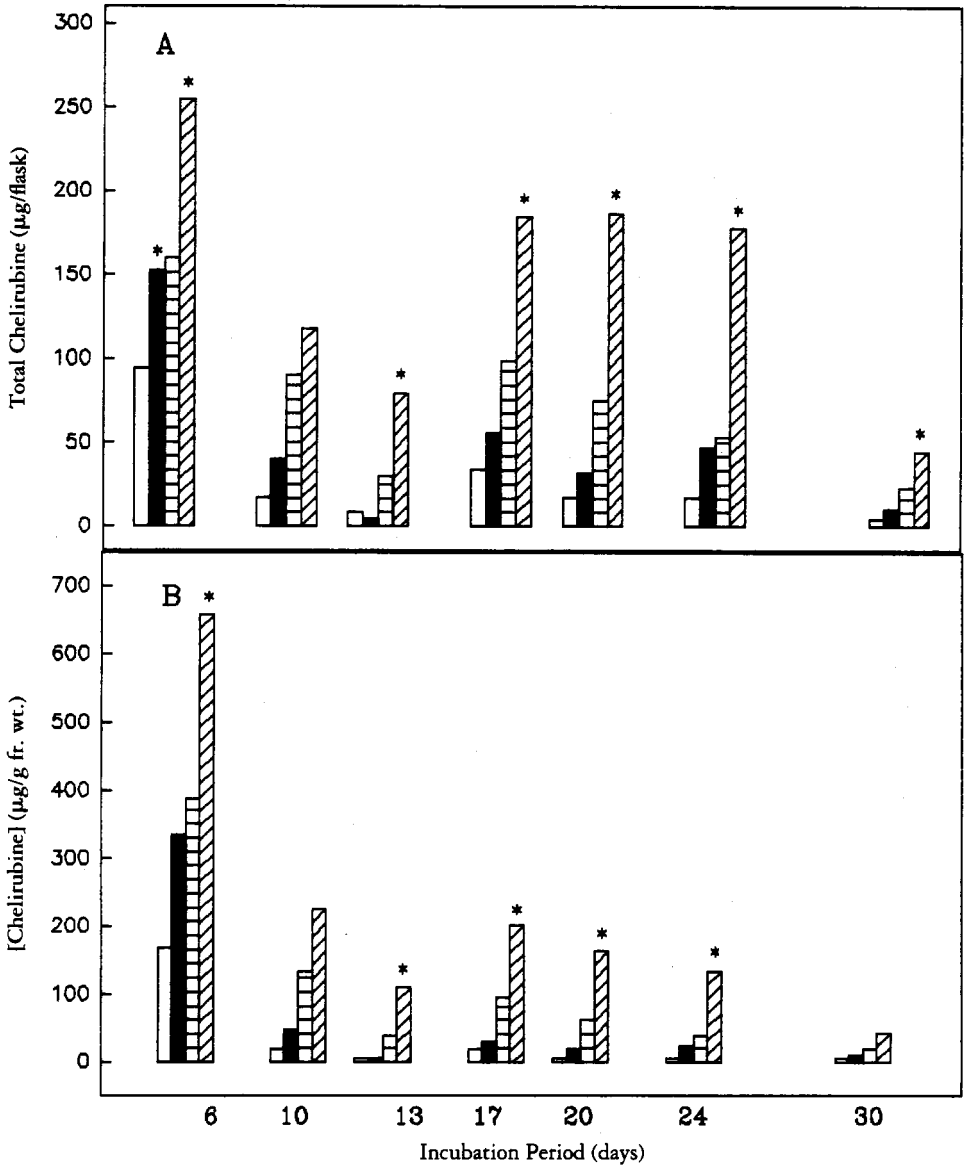

FIGURE 4. Chelirubine, (A) total accumulation per flask and (B) concentration in *Verticillium*-elicited cell suspension cultures of *Sanguinaria canadensis* grown in the presence or absence of 2,4-D. See Figure 2 for experimental details. (Open bars) 2,4-D, non-elicited; (filled bars) 2,4-D, elicited; (horizontal-hatched bars) hormone-free, non-elicited; (diagonal-hatched bars) hormone-free, elicited. Each value represents the mean of 3 experiments. Standard errors of the mean were ≤19% except for values below 50 µg/g fresh wt where they were higher in some instances. *$P<0.05$ for elicited vs. non-elicited cultures.

accumulated the largest amount of alkaloid (13.3 mg/g dry wt), which was considerably greater than non-elicited hormone-supplemented controls. Chelirubine elicitation was only observed for hormone-supplemented 6-day cultures but occurred at most time intervals for hormone-free cultures. Total chelirubine decreased in cultures grown longer than 6 days under all conditions tested.

In 6-day cultures chelirubine concentration were over an order of magnitude higher than those of sanguinarine or chelerythrine, and elicitation was optimal in the absence of hormone (Figure 4B). Again elicitation of chelirubine levels was prevalent at most time intervals in hormone-free cultures. After 6 days of incubation, levels declined regardless of treatment.

Total dopamine increased with the length of incubation in hormone-amended 6, 10, 13, and 17 day cultures and in hormone-free 6, 10, and 13 day cultures, leveling off after that time period (Figure 5A). The highest level of accumulation (12.2 mg/flask) occurred in elicited cultures grown without hormone for a total incubation period of 13 days. This corresponded to a cellular concentration in excess of 20% dry wt. In some experiments elicitation of total dopamine was observed.

The effects of hormone deprivation and elicitation on dopamine concentration are shown in Figure 5B. Cell suspensions grown without hormone, possessed from 2- to 6-fold higher concentrations of dopamine than paired hormone-treated controls. Elicitation of dopamine levels was seen both in the presence and absence of hormone.

In summary, the experiments described above demonstrate that hormone deprivation was a major influence on alkaloid production in *S. canadensis*. Effects of elicitation on cells grown with or without 2,4-D were seen more consistently for chelirubine and dopamine. Levels of chelerythrine and, to a lesser extent, sanguinarine were occasionally enhanced.

When sanguinarine is a minor constituent of plant tissue, as is the case for *P. bracteatum*, its levels were strongly influenced by elicitor (2,6). Although we have used the same *Verticillium* preparation that successfully induced sanguinarine accumulation in *Papaver* cell cultures, it did not influence sanguinarine production in *Sanguinaria* under the same conditions. Apparently, the elicitor induces benzophenanthridine synthesis, but does not determine the production of a specific alkaloid in the two plants, despite the fact they are both members of the Papaveraceae. These results emphasize the need to measure the flux of a pathway as well as levels of individual intermediates.

The low levels of sanguinarine in *S. canadensis* cell cultures are reminiscent of the morphinans, thebaine and morphine. They represent major alkaloids of the *P. bracteatum* and *P. somniferum* plants, respectively (10), but are minor products of cell cultures, only observed under conditions of hormonal deprivation (11,15). Accumulation of chelirubine as a major alkaloid of cell cultures contrasts with its trace amounts in the intact plant (13,16). In this case, *S. canadensis* cell cultures produce chelirubine in response to hormone deprivation and elicitation similar to *P. bracteatum* and *P. somniferum* accumulation of sanguinarine. Accordingly, it is a phytoalexin candidate in this plant. One can also speculate that the plant will not overproduce an alkaloid that represents a major constituent, but will increase the synthesis of a trace metabolite to give itself a wider array of compounds to be used in the defense mechanism.

Of the 17 enzymes implicated in the biosynthesis of sanguinarine from two molecules of tyrosine, nine have been shown to require molecular oxygen (17–19). Dihydrobenzophenanthridine oxidase is an oxygen-dependent enzyme that catalyzes the conversion of dihydrosanguinarine to sanguinarine (18–20). The activity of this oxidase from *S. canadensis* cell cultures was elevated 2–3-fold under the same elicitation conditions described here in the presence and absence of hormone (19). Chelirubine

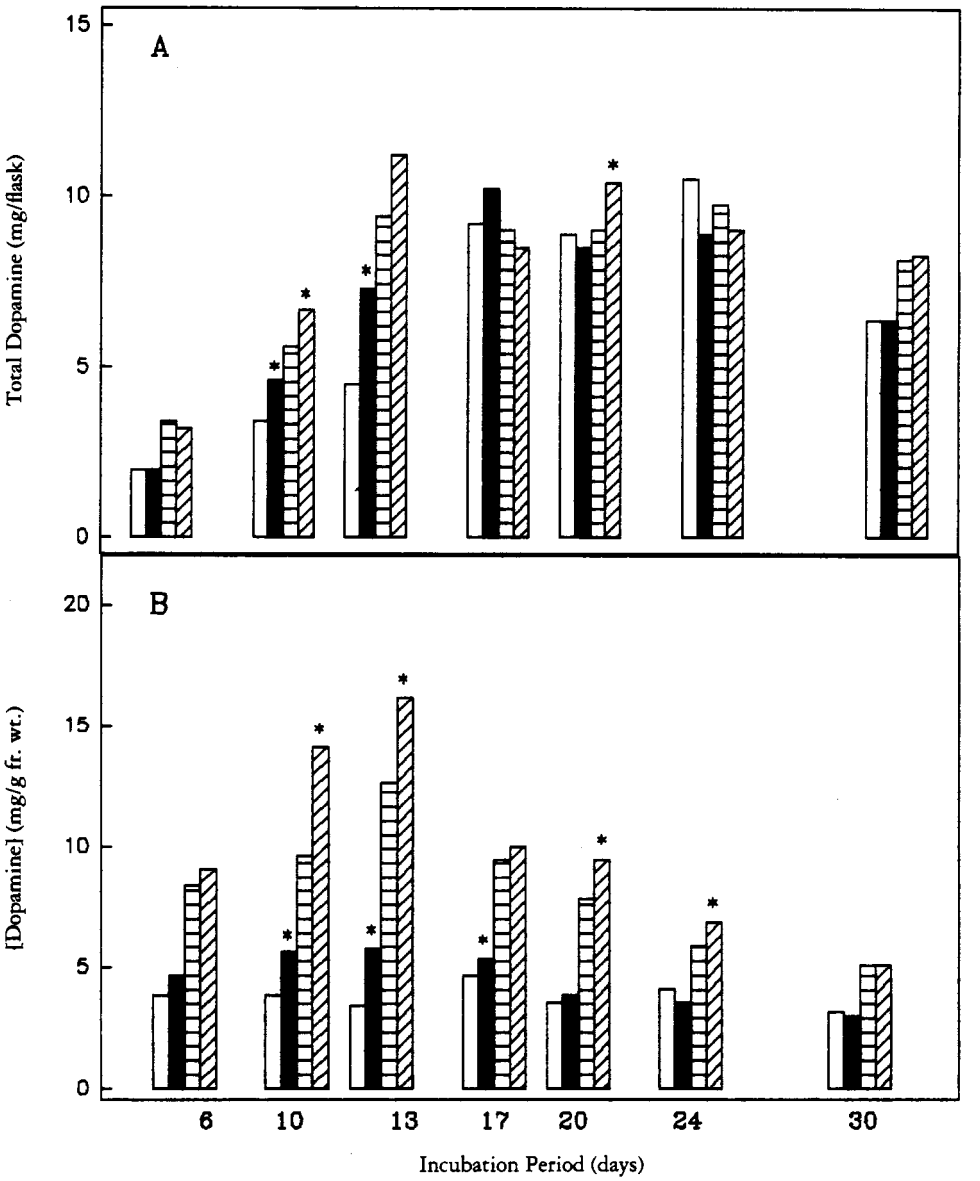

FIGURE 5. Effect of *Verticillium* elicitation and hormonal deprivation on dopamine (A) total accumulation per flask and (B) cell concentration in *Sanguinaria canadensis* cell suspension cultures. See Figure 2 for experimental details. (Open bars) 2,4-D, non-elicited; (filled bars) 2,4-D, elicited; (horizontal-hatched bars) hormone-free, non-elicited; (diagonal-hatched bars) hormone-free, elicited. Each value represents the mean of 3 experiments. Standard errors of the mean were ≤10% *$p<0.05$ for elicited vs. non-elicited cultures.

appears to be formed from dihydrosanguinarine by a sequence that includes a monooxygenase-catalyzed hydroxylation followed by methylation (21,22). Therefore, of the array of oxygenases and oxidases that hydroxylate aromatic and alkyl carbon atoms involved in the benzophenanthridine alkaloid synthesis, it is possible that many are induced under these elicitation conditions. The 50-fold differences in sanguinarine and chelirubine content seen here are consistent with this notion.

Further support for the hypothesis that alkaloids of elicited plant tissue cultures preferentially undergo biological oxidation is seen in studies of *E. californica*. Both the intact plant and tissue cultures contain primarily dihydroderivatives of sanguinarine, chelirubine, macarpine, and chelerythrine that upon fungal elicitation are converted to their corresponding oxidized quaternary alkaloids, suggesting induction of the activity of dihydrobenzophenanthridine oxidase(s) (8,23). Furthermore, it was demonstrated that activity of the berberine-bridge-forming enzyme which utilizes molecular oxygen to convert (*S*)-reticuline to (*S*)-scoulerine is also induced. This oxidase catalyzes a key step in the biosynthesis of benzophenanthridine alkaloids.

This propensity for biological oxidation is not restricted to *Papaver* elicitation. In *Catharanthus roseus* the same *Verticillium* preparation utilized here initially elicited the accumulation of the indole alkaloid, ajmalicine, but ultimately serpentine (19). Serpentine is not on the main pathway of indole alkaloid biosynthesis in this plant, but it represents an oxidized side product. A review of the phytoalexin literature reveals that many other elicited secondary metabolites are highly oxidized (9). In addition, oxygenases are required for the production of lignin and hydroxyproline-rich glycoproteins and for other response mechanisms to wounding and infection. Also included among oxidative processes is the synthesis of jasmonic acid, which has been implicated as an intracellular component of the cell signalling pathway involved in alkaloid elicitation (24). Viewed collectively, the data support the notion that preferential induction of oxygen-utilizing enzymes may accompany the elicitation process, raising the interesting question of genetic regulation of such oxidative processes in plant cell adaptation.

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