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DYNAMICS OF BENZOPHENANTHRIDINE ALKALOID PRODUCTION IN SUSPENSION CULTURES OF *ESCHSCHOLTZIA CALIFORNICA* AFTER TREATMENT WITH A YEAST ELICITOR

MARGARET A COLLINGE and PETER E BRODELIUS*

Institute of Biotechnology ETH-Honggerberg CH-8093 Zurich, Switzerland

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Key Word Index— *Eschscholtzia californica*, Papaveraceae, yeast elicitor, suspension culture, benzophenanthridine alkaloids, tyrosine decarboxylase

Abstract—A number of benzophenanthridine alkaloids are induced in suspension cultures of *Eschscholtzia californica* after treatment with an elicitor prepared from yeast extract. The formation of the alkaloids sanguinarine, chelerythrine and macarpine has been studied in relation to, elicitor concentration, incubation time after elicitation, and culture age. A significant portion of these alkaloids is released into the medium. Sanguinarine and chelerythrine reach maximum levels a few hours after the time of elicitation Thereafter, their levels decline and the amount of macarpine increases. Viability of elicited cells, as determined by their subsequent growth, is not significantly reduced There is a good correlation between induced tyrosine decarboxylase activity and alkaloid formation

INTRODUCTION

Fungal elicitors have been widely used to induce secondary metabolism in plant cell suspension cultures. The formation of phytoalexins in response to elicitation has been most extensively studied [1, 2]. An increased production of alkaloids in various plant cell suspension cultures has also been observed after treatment with microbial elicitors [3–5]. In particular, it appears possible to induce isoquinoline alkaloids in cell suspension cultures [6–8].

A carbohydrate preparation from yeast extract induces increased berberine and benzophenanthridine alkaloid production in cell suspension cultures of *Thalictrum rugosum* [7] and *Eschscholtzia californica* [9], respectively. Recently other workers using a yeast elicitor were able to induce benzophenanthridine alkaloid formation in cell cultures of *Eschscholtzia* [10]. Furthermore, it has been shown that the induction of isoquinoline alkaloids in plant cell cultures is accompanied by an induction of the enzyme tyrosine decarboxylase (TDC) [11, 12] A possible role of this enzyme in isoquinoline biosynthesis has been suggested [11] This hypothesis is supported by the results reported in this communication We now report on the dynamics of benzophenanthridine alkaloid formation in suspension cultures of *E californica* after treatment with a yeast elicitor

RESULTS AND DISCUSSION

Cell suspension cultures of *E. californica* have been shown to produce various benzophenanthridine alkaloids [13]. The concentration of alkaloids in the culture was dependent on the culture conditions used

Treatment of suspension cultures of *E californica* with the yeast elicitor results in a rapid colour change of the culture We assumed that this change in colour was due to the formation of benzophenanthridine alkaloids. In order to test this assumption samples from elicited cells were analysed for alkaloid content by ion-pair reversed phase HPLC (Fig. 1). The non-elicited sample (Fig. 1A) contained very low amounts of the alkaloids sanguinarine, chelerythrine and macarpine. However, in the sample taken six hr after addition of elicitor (Fig. 1B) relatively large amounts of sanguinarine and chelerythrine were present, while a sample taken 48 hr after elicitation contained low amounts of these two alkaloids but significant amounts of the related alkaloid macarpine (Fig. 1C). Obviously, sanguinarine and chelerythrine are accumulated only transiently

Non-induced cells of *E californica* produced only trace amounts of the three alkaloids, sanguinarine, chelerythrine and macarpine over a 60 hr incubation period (Fig. 2) (at the beginning of which, the cultures were 7 days old). Furthermore, only low levels of tyrosine decarboxylase were found in cell free extracts of non-induced cells.

After elicitation, on the other hand, a rapid increase in TDC activity was observed (Fig. 2B) Maximum enzyme activity was seen three hr after addition of the elicitor. Subsequently, the cells produced significant amounts of the three benzophenanthridine alkaloids studied (Fig. 2B). The maximum levels of sanguinarine and chelerythrine were reached six and eight hr after elicitation, respectively. Significant amounts of sanguinarine were only present in the culture during a relatively short period of time Twelve hr after elicitation this alkaloid had been almost completely metabolized to other alkaloids or catabolized It may be pointed out that sanguinarine is a direct precursor of the end product macarpine ('5,12-dimethoxy-sanguinarine') as shown by Takao *et al.* [14]

*Author to whom correspondence should be addressed

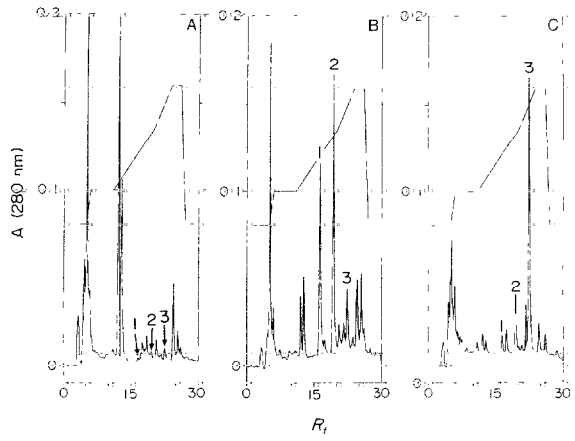

Fig 1 Reversed phase HPLC chromatograms of medium extracts from suspension cultures of *E californica* A Un-treated culture, B and C Treated cultures taken 6 and 48 hr after addition of yeast elicitor (50 μg carbohydrate/g fr wt of cells), respectively 1 sanguinarine (16 3 min), 2 chelerythrine (19 3 min), 3 macarpine (22 6 min)

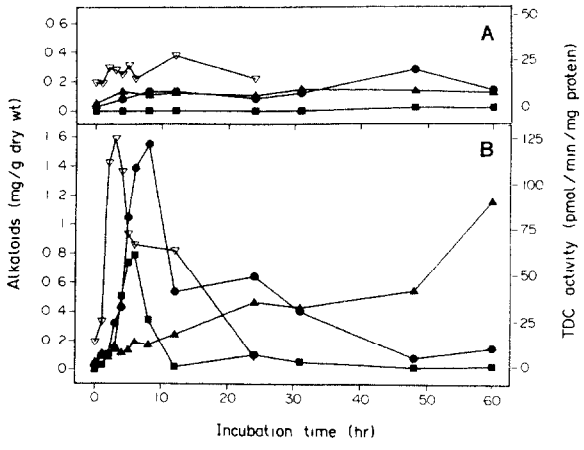

Fig 2 Time course of changes in TDC activity and alkaloid content in suspension cultures of *E californica* A Non-induced cells; B Elicitor treated cells (50 μg carbohydrate/g fr wt of cells) ▽—▽, TDC activity; ■—■, sanguinarine; ●—●, chelerythrine, ▲—▲, macarpine

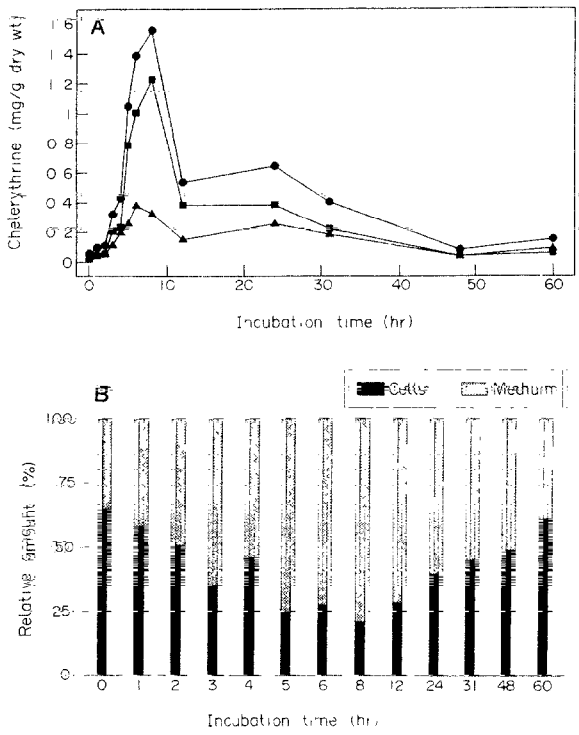

Fig 3 Time course of chelerythrine formation in elicitor-treated cells (50 μg/carbohydrate/g fr wt of cells) of *E californica* A Total extractable chelerythrine (●), amount of chelerythrine found in medium (■) and amount of chelerythrine found in cells (▲) B Relative distribution of chelerythrine in cells and medium

Chelirubine ('12-methoxy-sanguinarine') is the intermediate between these two alkaloids and therefore it may be assumed that one of the unidentified peaks in Figs 1B and 1C represents chelirubine

Chelerythrine was also metabolized or broken down by the cell cultures, although more slowly, and after 48 hr little chelerythrine was left in the cultures. Chelilutine ('12-methoxy-chelerythrine') could be a product of this possible conversion The presence of chelirubine and chelilutine in *Eschscholtzia* cultures after treatment with a yeast elicitor has been reported [10]

It is interesting to note that a major part of the alkaloids produced is excreted into the medium as shown for chelerythrine in Fig 3A. Its relative distribution between cells and medium is depicted in Fig. 3B.

When a high concentration of chelerythrine was present in the culture (5–8 hr after elicitation) at least 75% of the alkaloid was found in the medium A similar pattern was observed for sanguinarine (data not shown) At the end of the incubation (60 hr after elicitation) more than 50% of the end product macarpine was found in the medium

After separation of cells and medium at any time during the incubation, the concentration of alkaloids in the medium remained constant, indicating that there are no catabolizing enzymes (e g peroxidases) present in the medium The fact that macarpine accumulates in the medium also indicates that no breakdown occurs extracellularly The disappearance of sanguinarine and chelerythrine from the medium may therefore be due to uptake by the cells This possibility is now being investigated by feeding experiments with non-induced and elicitor treated cultures of *E californica*

The effect of elicitor concentration on macarpine formation is shown in Fig 4 Maximum induction is obtained at a carbohydrate concentration of 30 μg/g fr. wt of cells. Maximum TDC activity is also observed at this elicitor concentration. The results shown in Fig 4 were obtained 48 hr after elicitation, when only macarpine was present in significant amounts Other experiments, where alkaloid content was measured six hr after elicitation, showed that the optimum elicitor concentration for sanguinarine formation is also 30 μg carbohydrate/g fr. wt of cells At this point it may be pointed out that the time course of disappearance of sanguinarine and chelerythrine varies somewhat between experiments c f Figs 2 and

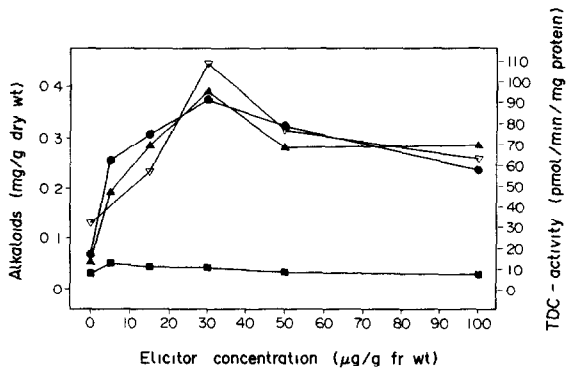

Fig 4. TDC activity and alkaloid formation as functions of elicitor concentration The samples for TDC activity and alkaloid determinations were taken 3 and 48 hr after elicitation, respectively Elicitor concentration is expressed as μg carbohydrate/g fr wt of cells ▽—▽, TDC-activity, ■—■, sanguinarine, ●—●, chelerythrine, ▲—▲, macarpine

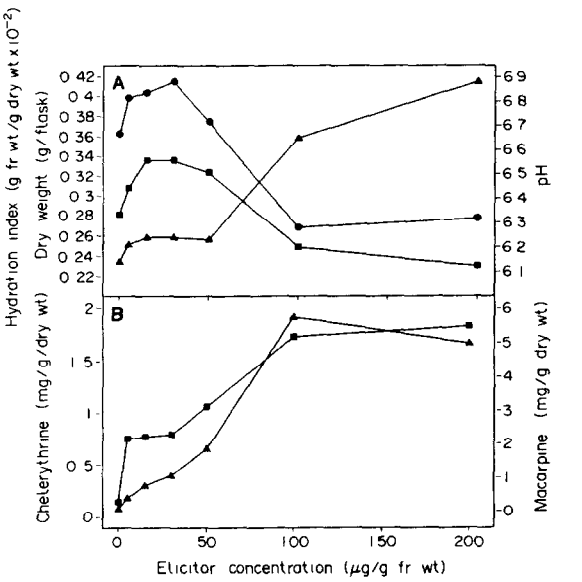

Fig 6. Cell growth and alkaloid yield as functions of elicitor concentration Fresh medium was added to the cells 24 hr after elicitation Dry wt and alkaloid content were determined 7 days after addition of fresh medium A. ■—■, dry wt, ●—●, hydration index, ▲—▲, pH B ■—■, chelerythrine; ▲—▲, macarpine

4. The chelerythrine concentration 48 hr after elicitation is around 0.1 and 0.4 mg/g dry wt of cells, respectively, in these two experiments

The effect of elicitor concentration on some other parameters has been investigated as summarized in Figs 5 and 6. A decreased conductivity of the medium was observed after addition of elicitor (Fig. 5) indicating that no extensive cell lysis occurs upon elicitation. Consequently, the release of alkaloids into the medium, to the extent observed (Fig 3), cannot be accounted for by cell lysis and must be due to release from intact cells It is clear from Fig. 5 that an increase in medium pH and cell respiration is observed after elicitation An increased respiration was also observed in cells of *Thalictrum rugosum* producing berberine after addition of the yeast elicitor [7].

Moderate concentrations of elicitor (less than 100 μg carbohydrate/g fr. wt of cells) resulted in a somewhat increased biomass formation over a 7 day incubation period (Fig 6A). This effect is only observed when the elicited cells are transferred to fresh medium within a short time period (24 hr) after elicitation If the cells are left in a 'spent' medium after addition of elicitor a slight decrease in biomass formation is seen after 48–60 hr (data not shown). A similar effect was observed for *T rugosum* cells after elicitation [7].

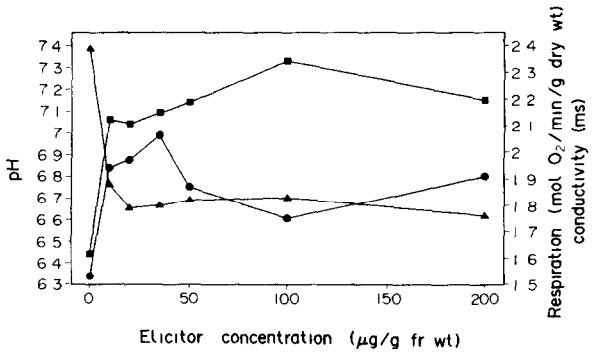

Fig. 5 Conductivity and pH of medium and cell respiration as functions of elicitor concentration Measurements were taken 24 hr after elicitation ▲—▲ conductivity, ■—■, pH, ●—●, respiration

On the other hand, at higher concentrations of elicitor (100 and 200 μg carbohydrate/g fr. wt of cells), decreases in biomass and hydration index are observed after a seven day incubation period (Fig. 6A). The change in cell size may be explained by the fact that the morphology of the culture is altered At high elicitor concentrations the cultures are highly aggregated. The aggregation of cells in combination with the availability of an energy source (sucrose) may be responsible for the significantly higher alkaloid concentrations observed at these elicitor concentrations (cf. Figs 4 and 6B). In conclusion, the yield of alkaloids may be considerably improved by supplying the elicited cells with fresh medium (energy source).

Finally, TDC and alkaloid formation is maximally induced at late exponential or early stationary growth phase (data not shown). A similar pattern was observed for the induction of TDC [11] and berberine synthesis in cultures of *T. rugosum* [7].

EXPERIMENTAL

Chemicals Sanguinarine and chelerythrine were supplied by Roth (FRG), Gamborg's B5 medium by Flow Laboratories (Scotland)

Cultivation of cells. Stock suspension cultures of *E californica* were cultivated on a rotary shaker (120 rpm), in the dark at 26°, in Gamborg's B5 medium supplemented with 1 0 mg/l 2,4-D, 0.1 mg/l kinetin, and 2% w/v sucrose. Cells were transferred to fresh medium every seven days at an inoculum density of 7.5 g fr wt per 100 ml culture medium.

Preparation of elicitor Elicitor was isolated from yeast extract (Gistex-X-11, Roba AG, Basel) as described by Hahn and Albersheim [15] The concn of carbohydrate (as determined by

the orcinol–sulphuric acid method [16] with glucose as reference) in the concd extract formed after 60% EtOH pptn (the 'elicitor') was 6 0 mg/ml Before it was added to cell cultures, the elicitor preparation was filter sterilized

Induction with elicitor Unless otherwise stated, the elicitor was added to cultures when they were 7-days-old at a concn of 50 μg carbohydrate/g fr wt of cells

Time course of TDC-activity and alkaloid production after elicitation An appropriate number of suspension cultures in 250 ml flasks, containing 70 ml medium, were set up from pooled stock cultures At time 0, two flasks were taken and determinations were made of the fr and dry wt of the cells, and the conductivity and pH of the medium Cells and medium were then frozen in liquid N_2 for TDC-activity and alkaloid analysis later Some of the remaining flasks were treated with elicitor (30 μg carbohydrate/g fr wt of cells) At appropriate times non-induced and induced flasks were harvested and treated as the time 0 samples

TDC-activity and alkaloid formation in relation to elicitor concentration Suspension cultures were set up from pooled stock cultures as described above Three flasks were taken to determine the mean fr wt, and the remaining flasks were elicited at different carbohydrate concentrations (four replicates per concn) After 3 hr half of the cultures were harvested for determination of TDC activity and after 48 hr, the remaining cultures were harvested and determinations were made of fr wt, dry wt, and alkaloid content of cells and medium

Effect of elicitor concentration on medium pH and conductivity and on cell respiration Measurements were carried out 24 hr after elicitation in cultures prepared as above

Effect of elicitor concentration on subsequent growth and alkaloid production At time 0 6-day-old stock cultures were pooled and divided into aliquots of 10 ml in 25 ml flasks These were elicited at various carbohydrate concentrations (3 replicates per concentration) After 24 hr, the cells were transferred to 100 ml flasks containing 30 ml fresh medium, 7 days later, determinations were made of medium pH and fr and dry wt of cells, and alkaloid content of cells and medium

Effect of culture age on response to elicitation On day 0, cultures were set up from pooled stock cultures in the normal way On this and the following 8 days, one flask was elicited After 3 hr, a sample was removed from this and a non-elicited flask for TDC activity measurement, and 48 hr later, the alkaloid content of the cells and medium in both flasks was determined

Analytical procedures Fr wt Cells were collected by filtration under reduced pressure onto a nylon net (50 μm) Dry wt Samples of known fr wt were dried at 50° on preweighed Al foil until constant wt Respiration Cells (200 mg fr wt) were suspended in H_2O (5 0 ml) The mixture was saturated with air and respiration measured with an O_2 electrode TDC activity was determined in crude, desalted, enzyme extracts (without ammonium sulphate fractionation), as described [12] Conductivity Determined immediately after removal of cells with a conductivity meter (Orion Research, U.S A). Alkaloid extraction Cells (1–2 g fr wt) were extracted with MeOH (30–50 ml) by refluxing The filtered MeOH extract was evapd to dryness at 40° under red pres The residue was dissolved in MeOH (1 0 or 2 0 ml) and its alkaloid content analysed by HPLC The total cell culture medium was extracted with EtOAc (3 ×) after adjustment of the pH to 9 0 (with 1 0 M NaOH) The combined EtOAc extracts were dried with dry Na_2SO_4 and evapd to dryness at 50°, under red pres The residue was dissolved in MeOH (1 0 or 2 0 ml) and analysed by HPLC Alkaloid identification Sanguinarine and chelerythrine isolated from culture extracts were identified by their UV spectra and co-chromatography with standard samples in all of the TLC and HPLC systems described below Macarpine, extracted from cell cultures, was identified by its UV and ^{1}H NMR spectra TLC In both systems silica gel plates (Kieselgel-60, Merck, F R G) were used, and detection was by fluorescence after excitation at 366 nm System A toluene–MeOH (5 1), system B toluene–MeOH (50 1) HPLC System A this was the method used for determination of the alkaloid content of culture extracts Column Nucleosil C8 7 μm 250 × 4 6 mm Precolumn Nucleosil C8 5 μm 20 × 4 6 mm The mobile phase consisted of A Aq 5 mM 1-pentanesulphonic acid sodium salt, and 10 mM 1-octanesulphonic acid sodium salt adjusted to pH 2 8 with ortho-phosphoric acid, and B, MeCN Gradient system 0 to 5 min 40% B, 5 to 6 min 40 to 50% B, 6 to 11 min 50% B, 11 to 20 min 50 to 67% B, 20 to 24 min 67 to 80% B, 24 to 26 min 80% B, 26 to 27 min 80 to 40% B, 27 to 30 min 40% B Flow rate 1 ml/min. Detection UV at 280 nm. System B Column Hypersil Silica 5 μm 250 × 4.6 mm Precolumn Hypersil Silica 5 μm 20 × 4.6 mm Mobile phase hexane–toluene–MeOH (93 6 1). Flow rate 1 ml/min. Detection UV at 280 nm.

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