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ELICITATION OF DIHYDROBENZOPHENANTHRIDINE OXIDASE IN *SANGUINARIA CANADENSIS* CELL CULTURES

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Key Word Index—*Sanguinaria canadensis*; Papaveraceae; blood root; elicitation; benzo[c]-phenanthridine alkaloids; dihydrobenzophenanthridine oxidase; methyl jasmonate; salicylic acid.

Abstract—Dihydrobenzophenanthridine (DHBP) oxidase catalyses the last step in the biogenesis of the benzo[c]phenanthridine alkaloid sanguinarine. Addition of autoclaved fungal preparations or putative plant defence signalling intermediates (jasmonic acid (JA), methyl jasmonate (MeJ), acetylsalicylic acid (ASA)) to *Sanguinaria canadensis* cell suspension cultures elicited an increase in the activity of DHBP oxidase. MeJ and ASA were better inducers of oxidase activity than were the fungal elicitor and JA. Enzyme-specific activity could be induced in a dose- and time-dependent manner up to 4- to 14-fold, respectively, when cells were treated with MeJ or with ASA. A change in total enzyme activity in cultured cells was observed only at the highest concentration of MeJ and not at any level of ASA tested. The results suggest that MeJ and ASA may play a role in the *S. canadensis* defence against pathogens by eliciting the enzymes involved in the synthesis of the phytoalexin benzophenanthridine alkaloids. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Benzophenanthridine alkaloids accumulate in cell cultures of members of the Papaveraceae, Rutaceae, Fumariaceae, Caprifoliaceae and Sapindaceae families [1]. Although this family of isoquinoline alkaloids occurs constitutively in some of these plants, they have been shown to be inducible in many others and represent phytoalexin components of their defence responses. For example, the benzophenanthridine alkaloid sanguinarine accumulates in large amounts following elicitation with fungal elicitors in cell cultures of *Sanguinaria canadensis*, *Papaver bracteatum*, *Papaver somniferum* and *Escholtzia californica* [2–9]. Elicitation of alkaloids, as for many other phytoalexins, entails a complex signalling cascade that includes the participation of a family of octadecanoic acids [8–12]. Jasmonic acid (JA), its methyl ester (MeJ), and their metabolic precursor, α-linolenic acid, accumulate following pathogenic or environmental stress. In addition, pathogen-induced plant defence responses can be simulated upon exogenous administration of JA or MeJ. These results suggest that the octadecanoic acids are components of an intracellular signalling system between the pathogen activated receptor and the defence responses, which include increased accumulation of alkaloids, other phytoalexins and activation of a family of wound-inducible proteinase inhibitor genes.

A ubiquitous putative plant defence signal that has been implicated in systemic acquired resistance to pathogens is salicylic acid (SA) [13–18]. SA accumulates after wounding or pathogenic attack during a process known as the pathogenesis reaction which triggers the expression of several low molecular weight pathogenesis-related (PR) genes. The exogenous application of acetyl salicyclic acid (ASA) is known to parallel the effect of pathogen-induced defence responses with regard to the activation of PR-1 and PR-2 genes. SA is also an inducer of the genes activated after tobacco mosaic virus (TMV) infection [18] and nopaline synthase [19]. In contrast, both SA and ASA were found to be potent inhibitors of systemin-induced and JA-induced synthesis of proteinase inhibitor mRNA and protein (Doares *et al.* [20] and references cited therein). Thus, SA and ASA can act either as inducers or inhibitors. Recently, evidence has been gained indicating that, although an essential transducer for systemic acquired resistance, SA may not be the translocated signal [21]. Moreover, SA binds non-specifically to iron containing enzymes including catalase, suggesting that it could simply be a phytoalexin [22].

Although JA, MeJ and other intermediates in the octadecanoate signalling pathway have been shown to elicit benzophenanthridine alkaloids [8, 23, 24], little is

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known about the effect of salicylates on the benzophenanthridine phytoalexins and their enzymes of synthesis. In this study we compared the time and dose response of *S. canadensis* cell suspension cultures to elicitation by MeJ and ASA by measuring dihydrobenzophenanthridine (DHBP) oxidase activity [6, 25, 26], the last enzyme in the biosynthesis of sanguinarine.

RESULTS

In previous studies DHBP oxidase activity was found to be induced by fungal elicitor in cell cultures from three members of the Papaveraceae, *S. canadensis*, *P. bracteatum*, *P. somniferum* and *E. californica* [6, 8]. Given the greater growth rate and relatively high levels of activity seen for *S. canadensis*, it appeared to be a good cell line in which to examine the elicitation of this enzyme. Induction of DHBP oxidase activity was estimated under conditions of fungal elicitation or in the presence of octadecanoates, ASA or its positional isomer, 4-acetoxybenzoic acid (Fig. 1). In all elicitation experiments the culture media turned brownish-orange, which is indicative of benzophenanthridine alkaloid accumulation in these cells [4,7]. Of the substances tested, ASA clearly displayed the highest induction of oxidase specific activity.

Treatment of cells with MeJ resulted in a dose-dependent increase in oxidase specific activity compared to untreated controls (Fig. 2A). At 1 mM MeJ total cellular DHBP oxidase activity was also enhanced (Fig. 2B). Augmentation of oxidase specific activity was first detected after 8 hr of MeJ treatment, and by 24 hr it was three-to-four-fold higher than untreated cells (Fig. 3).

In parallel experiments, the effect of JA on oxidase activity was also assessed. Treatment of *S. canadensis* cell cultures with the same concentration of JA produced a less pronounced increase of DHBP oxidase activity (approximately two-fold). Maximum induction of oxidase occurred with 0.25 mM JA (308 pkat mg^{-1} protein; Fig. 1) and decreased at higher levels of this octadecanoid. No change in total oxidase activity was observed in the presence of this range of concentrations of JA (data not shown).

ASA dose-dependency studies on *S. canadensis* cell

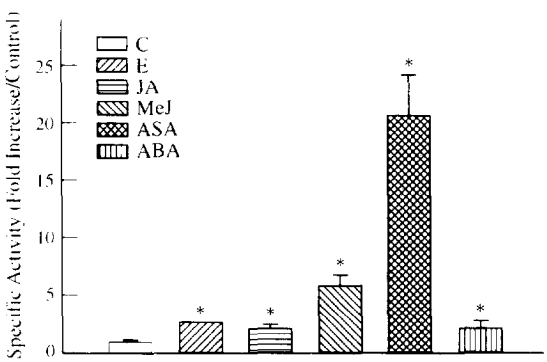

Fig. 1. Induction of dihydrobenzophenanthridine oxidase specific activity in *S. canadensis*. Cells were grown as described in the Experimental and treated for 24 hr with either 6×10^5 ml^{-1} conidia of *Verticillium dahliae* fungal elicitor (E), 0.25 mM JA, 0.5 mM MeJ, 10 mM ASA or 10 mM 4-acetoxybenzoic acid (ABA). Specific activity is expressed as *x*-fold increase over control (C) values (mean = 140 pkat mg^{-1} protein) which were set at 1. $^*P < 0.05$ vs controls.

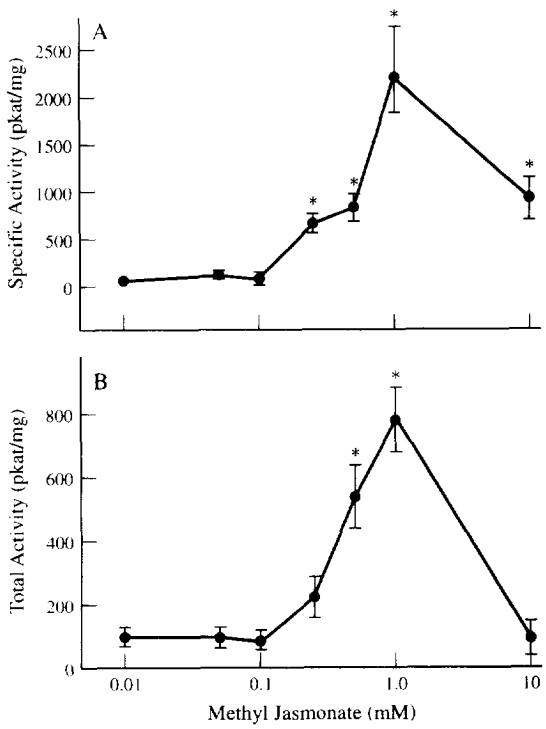

Fig. 2. Dose-dependant enhancement of DHBP oxidase specific (A) and total (B) activity upon addition of MeJ to *S. canadensis* cell cultures. Cell suspension cultures were treated for 24 hr with the indicated concentrations of MeJ. Specific activity is expressed as pkat mg^{-1} protein and total activity as pkat g^{-1} cells. $^*P < 0.05$ vs controls; $n = 3$–8.

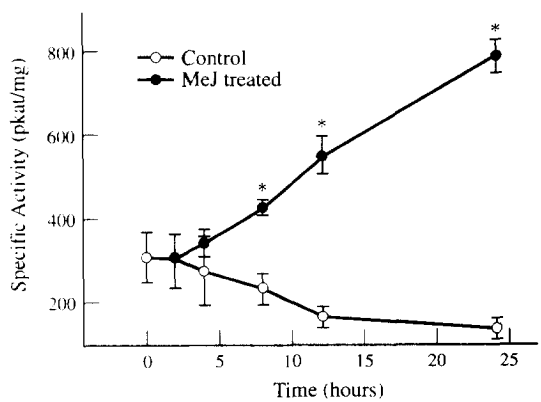

Fig. 3. Time course of DHBP oxidase specific activity upon MeJ addition to *S. canadensis* cell cultures. Cells were treated with 0.5 mM MeJ and maintained for the indicated time periods. $^*P < 0.05$ vs controls; $n = 3$–5 for controls, $n = 6$ for treated.

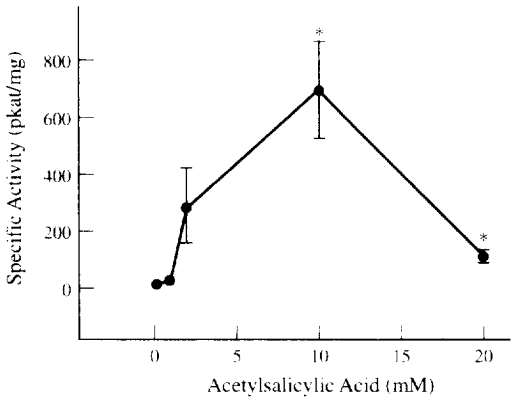

Fig. 4. Dose–response curve of DHBP oxidase specific activity of ASA to *S. canadensis* cultures. Cell suspensions were treated with the indicated concentrations of ASA and maintained for an additional 48 hr. $^*P < 0.05$ vs controls; $n = 3$ to 4.

suspension cultures revealed a maximum induction of DHBP oxidase specific activity at 10 mM (Fig. 4). Total activity did not differ significantly from controls (55±5 pkat g^{-1} treated cells versus 52±5 pkat g^{-1} control cells). ASA induction of oxidase specific activity reached a maximum of 14-fold in 24 hr. The specific activity then declined slowly (Fig. 5).

DISCUSSION

Two transducers of biotic stress (MeJ and JA), ASA and autoclaved fungi were compared for elicitation of DHBP oxidase activity in *S. canadensis*. Of the above, MeJ proved to be the most potent inducer of oxidase activity. Since there is evidence that the octadecanoids are intracellular components of the signal transduction cascade initiated by fungal pathogens [8, 24, 27, 28], their relative potency in comparison to autoclaved fungi is a function of a number of variables. These include the stability and rate of uptake of octadecanoids by the cell, the levels of the fungal elicitor in autoclaved fungi and its signal amplification efficacy. Uptake factors

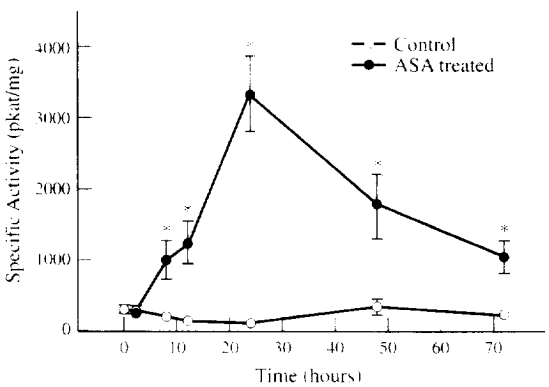

Fig. 5. Time course of ASA induction of DHBP oxidase specific activity. *Sanguinaria canadensis* cell suspensions were treated with 10 mM ASA and maintained for the indicated time periods. $^*P < 0.05$ vs controls; $n = 3$–8.

may also explain the differences in potency between the volatile MeJ and JA. These same uptake factors as well as cellular and subcellular compartmentation of endogenous jasmonates make it difficult to correlate their media levels with those *in planta* (< 1 μM for *E. californica*) [23].

Only the highest levels of MeJ used in this study altered total oxidase activity. These results are consistent with previous findings on the effect of MeJ on DHBP oxidase in *E. californica*, wherein lower levels of this octadecanoid (0.1 mM) were used and less than two-fold enzyme induction was reported [8]. In contrast, a 6.5- to 16-fold increase in the five membrane-associated enzymes in the sanguinarine pathway was elicited by 0.1 mM MeJ. Thus, the DHBP oxidase may be a rate-limiting step of sanguinarine synthesis.

ASA has been implicated as a systemic response inducer leading to acquired resistance in plants. Thus, it may represent a signalling element of a different defence pathway than the jasmonates. The role of ASA in phytoalexin induction has not been delineated. Here the induction of DHBP oxidase specific activity by this salicylate was greater than that seen with MeJ. Although higher levels of ASA were required to achieve this effect, the magnitude of the induction was unexpected. The same concentration of 4-acetoxybenzoic acid, a positional isomer of ASA, was not as effective an inducer of oxidase specific activity. These results argue against a non-specific effect caused by stress on the cultures. Since, unlike MeJ, ASA did not change DHBP oxidase total activity, only the jasmonates may be capable of activating expression of the oxidase gene. Rather, ASA may be acting to reduce protein synthesis other than that involved in benzophenanthridine alkaloids. Thus, ASA-treated cells may prove to be an advantageous starting material for the purification of the oxidase.

EXPERIMENTAL

Materials. Benzophenanthridine alkaloids were provided by Atrix Labs (Fort Collins, CO). Polyclar AT (polyvinylpyrrolidone) was a gift from GAF (New York). JA was a gift from T. Hasegawa USA Inc. (Cerritos, CA) and ASA was obtained from Merck & Co. (Rahway, NJ). MeJ, sodium metabisulphite, and most of the other chemicals were purchased from either Sigma-Aldrich Corp. (St Louis, MO) or Fisher Scientific (St Louis, MO).

Sanguinaria canadensis *growth conditions and elicitation. Sanguinaria canadensis* callus (0.8–2.0 g) grown for 24 days on agar containing Gamborg B_5 media supplemented with 1 ppm 2,4-D was used to inoculate 50 ml on the same nutrient in 250 ml flasks. The cell suspension was grown for 17 days on a gyrotory shaker (120±5 rpm) at 21–24°C under constant light. The cell suspensions were then treated for time period(s) indicated with self-sterilizing ethanol solutions (pH 5.5–5.8) of ASA or MeJ. For each measurement, cells from 1–4 flasks were harvested,

pooled and frozen at −70° until assayed. Fungal elicitation was achieved by treating cells with an autoclaved conidial suspension of *Verticillium dahliae* as described previously [4, 6].

Preparation of crude extracts for enzyme assay. Approximately 1 g frozen cells were ground to a fine powder under liquid nitrogen in a chilled mortar and extracted with 5 ml of buffer A (0.1 M potassium phosphate pH 7.0, 10 mM sodium metabisulphite, 10% glycerol (v/v)), containing 0.5 g polyclar AT. The homogenate was centrifuged at 20 000*g* for 30 min at 4°. The supernatant was filtered through four layers (prewetted in buffer A) of Miracloth and stored at 4°. The enzyme assay was performed on the same day as the preparation of crude extract.

DHBP oxidase assay. The reaction was initiated by adding dihydrosanguinarine (20 μM final concentration) to crude extracts in 0.1 M potassium phosphate (pH 7.0) [26]. It was allowed to proceed for 5, 10, 15 and 20 min at 37°. For each of the five time points, a 500 μl aliquot was removed and the reaction terminated by addition of 1 ml aqueous ethanol (79%). The formation of the reaction product, sanguinarine, was measured spectrofluorimetrically (Aminco-Bowman) at 324 nm excitation and 408 nm emission wavelengths [29]. Protein level was measured using the micro-Bradford assay at 595 nm (Bio-Rad, Hercules, CA).

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