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Effect of ethylene on sanguinarine production from *Papaver somniferum* cell cultures

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ABSTRACT

A Papaver somniferum cell line capable of producing sanguinarine equivalent to 3% of cell dry weight was used to determine if ethylene was involved in signalling the biosynthesis of this alkaloid. A 3.3-fold increase in ethylene emanation from these cell suspension cultures was observed 7 h after elicitation with a Botrytis fungal homogenate. The rate of ethylene release then decreased to near zero after 48 h, suggesting that a pulse of ethylene production may be involved in sanguinarine production. However, sanguinarine biosynthesis was not promoted when either the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), or the ethylene releasing agent, 2-chloroethylphosphonic acid (ethephon), was added to the culture. These results strongly suggest that ethylene is not intimately involved in the production of sanguinarine from Papaver somniferum cell cultures or in the transduction of the elicitation event.

ABBREVIATIONS

ACC 1-aminocyclopropane-1-carboxylic acid.

INTRODUCTION

The promise that plant cell cultures hold for use as a tool in studying pharmaceutical alkaloid biosynthesis has increased through enhanced production levels induced by fungal (Chappell et al., 1987; Eilert et al., 1985; Eilert and Constabel, 1986) and metal ion (Grill et al., 1985; Grill et al., 1987; Grill et al., 1988) elicitation. The mechanism by which either elicitation process occurs is not currently known. Fungal elicitation may stimulate the activity of the enzymes involved in secondary product biosynthesis (Collinge and Brodelius, 1989) whereas metal ions may induce the synthesis of phytochelatins (Grill et al., 1987).

In an attempt to better understand its physiological mechanism, there have been recent reports investigating the role of ethylene associated with elicitation in plant cell cultures. Schumacher et al. (1987) elicited Eschscholtzia californica cell cultures with either Penicillium or Saccharomyces culture homogenate and did not see any change in the rate of ethylene emanation, although there was a slight increase in the content of 1-aminocyclopropane-carboxylic acid (ACC) and malonyl-ACC. On the other hand, Cho et al. (1988) reported that an ethephon-mediated ethylene release promoted alkaloid production in Coffea arabica and Thalictrum rugosum cell suspension cultures. Ongoing research in our laboratory is directed toward understanding the physiology associated with sanguinarine (a benzophenanthridine alkaloid) biosynthesis from Papaver somniferum cell cultures. Therefore, this report describes the effect of ethylene on the elicitation of these cells and its role in sanguinarine production.

MATERIALS AND METHODS

Cell Culture and Elicitation

Papaver somniferum L. cell cultures were initiated from hypocotyl explants cultured in B5 medium (Gamborg et al., 1968) containing 1 mg/L 2,4-D and 1 g/L casein hydrolysate (hereafter referred to as 1B5C). These callus cultures were initiated by Tam et al. (1980) and were maintained by biweekly subcultures to fresh agar-solidified 1B5C or were used as inoculum for suspension cultures (approximately 4 g callus inoculum into 75 ml 1B5C) which were subcultured weekly. Cell suspension cultures were elicited by adding 0.2 ml Botrytis homogenate to 7-day old cultures (the elicitor was prepared by inoculating a 1 cm^3 mass of Botrytis mycelium into 100 ml of nutrient medium and culture at 25°C for 1 week (200 rpm) followed by autoclaving and aseptic homogenization with a Polytron). Cells were harvested and extracted for sanguinarine (as described later) after an additional 2 days of culture. All suspension cultures were incubated in the dark at 25°C with the suspensions agitated on a gyrotary shaker at 150 rpm.

Ethylene Assessment

Seven-day old suspension cultures were elicited with the Botrytis homogenate and ethylene emanation was measured at time intervals over a 2 day period. Ethylene was measured by transferring 10 ml of medium containing approximately 2 g of elicited cells to a sterile 125 ml Erlenmeyer flask which was aseptically sealed with a septum and incubated at 22°C on a shaker (150 rpm). Following a 1 h emanation period, a 1 ml volume of the atmosphere within each flask was collected and injected into a gas chromatograph (with Porapak R column and flame ionization detector) for ethylene quantification.

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Ethylene emanation from callus was assessed by growing cultures in 130 ml jars with screw-cap lids containing 30 ml medium per jar. The screw-cap lids were fitted with a septum for headspace sampling with a syringe.

In a related experiment, 0, 0.1 or 1.0 mM 1-aminocyclopropane-1-carboxylic acid (ACC) was filter sterilized into 1B5C. For liquid cultures, approximately 4 g of established suspension cells were inoculated into each flask. Ethylene was monitored during a 7 day culture period, after which the cultures were elicited and sanguinarine was assessed by placing 3 g of vacuum-aspirated suspension culture cells into 30 ml absolute methanol with 0.5% concentrated HCl. The cells were then disrupted with a Polytron and centrifuged prior to HPLC analysis (as described by Tyler et al., 1988). Ethylene measurement from callus was performed as described above after inoculating approximately 1 g of cells per culture jar.

Up to 400 ppm of the ethylene releasing compound, 2-chloroethylphosphonic acid (ethephon), was filter sterilized into 7-day old suspension cultures grown in 1B5C. These cultures were allowed to incubate for an additional 2 days during which ethylene was measured periodically. The sanguinarine content was determined upon termination of the experiment.

RESULTS AND DISCUSSION

Ethylene emanation was initially measured in cell suspensions following addition of Botrytis elicitor to determine if ethylene is present during elicitation (Table 1). In cells prior to elicitation, the ethylene emanation rate was approximately 60 nL g^{-1} h^{-1}. This rate increased to nearly 250 nL g^{-1} h^{-1} 5 to 7 h after elicitation and decreased thereafter to less than 10 nL g^{-1} h^{-1} after 48 h.

Table 1. Effect of fungal elicitation on ethylene emanation from Papaver somniferum cell suspension culture

Hours after Elicitation	Ethylene Emanation nL g^{-1} h^{-1}
Control (0)	45.9 ± 6.1[a]
1	68.4 ± 4.0
3	118.9 ± 9.5
5	126.6 ± 13.8
7	196.0 ± 40.4
24	18.0 ± 7.9
48	7.1 ± 3.1

[a] Values represent averages from 4 replications ± S.E. from 2 independent experiments.

To determine if this ethylene release was involved directly in elicitation, suspension cultures were grown in the presence of ACC, the immediate precursor of ethylene (Adams and Yang, 1980). In the control cultures without any ACC, the rate of ethylene emanation was about 60 nL g^{-1} h^{-1} at the start of this experiment and reached a maximum rate of nearly 160 nL g^{-1} h^{-1} after 2 days of culture (Table 2). The 0.1 and 1.0 mM ACC treatments showed the same pattern for ethylene emanation with nearly the same rate as the control at the beginning of the experiment (60 nL g^{-1} h^{-1}) and maximum values of approximately 275 and 178 (after 3 days of culture) for each respective ACC level (Table 2). Since a greater rate of ethylene emanation was expected in these ACC treatments, this experiment was repeated using ACC from a different supplier. However, similar results were obtained (data not shown). This suggests that these suspension cultures are not efficient at converting exogenous ACC to ethylene. These ACC-treated cell cultures were elicited after 7 days of growth. The ethylene release rate 24 h after elicitation was 3.6-fold greater in the cells exposed to 1 mM ACC as compared to the control (Table 2), but that which was 48 h after elicitation declined to 2 nL g^{-1} h^{-1}. This suggests that the cells utilized the ACC during the early stages of elicitation.

Table 2. Effect of 1-aminocyclopropane-1-carboxylic acid (ACC) on ethylene emanation (nL g^{-1} h^{-1}) and sanguinarine production from Papaver somniferum cell suspension cultures

Time in Culture (h)	ACC (mM)		
	0	0.1	1.0
0	58.0±2.8	65.4±4.9	63.8±4.3[a]
24	92.4±9.8	67.4±9.3	88.8±9.6
48	157.5±18.2	184.1±41.7	161.4±7.9
72	145.0±15.0	273.5±5.0	178.4±23.0
168	96.3±12.7	182.1±40.3	106.1±23.0
24 h after elicitation	14.3±5.3	ND[b]	66.1±26.6
48 h after elicitation	6.6±0.5	ND	2.3±1.8
Sanguinarine (mg/g dry wt)	7.0±0.4	ND	7.1±0.6

[a] Values represent averages from 4 replications ± SE.
[b] ND = Not Determined

Ethylene release was then measured in Papaver somniferum callus cultures to assess if a solid culture environment could promote the conversion of ACC to ethylene. The initial ethylene emanation rate was 9.5 nL g^{-1} h^{-1} for the control callus and those used for the ACC treatments. The rate of emanation increased in the control to a maximum of 14 nL g^{-1} h^{-1} after 21 days of culture. In the ACC treatments, the ethylene release peaked at approximately 23 and 35 nL g^{-1} h^{-1} for the 0.1 and 1.0 mM ACC treatments, respectively, after 9 days of culture (Table 3). This indicates that callus cultures are similar to suspension cultures in the ability to convert ACC to ethylene.

Table 3. Effect of 1-aminocyclopropane-1-carboxylic acid (ACC) on ethylene emanation (nL g^{-1} h^{-1}) from Papaver somniferum callus cultures

Time in Culture	ACC (mM)		
	0	0.1	1.0
0	9.5±1.8[a]	--	--
1 d	6.6±0.2	10.8±1.5	10.7±1.2
2 d	5.6±0.1	7.8±0.8	8.1±0.7
3 d	6.3±1.8	10.9±1.3	10.2±1.3
5 d	13.5±0.8	19.8±1.7	18.7±3.2
9 d	13.3±1.8	22.8±3.0	35.3±11.4
21 d	14.1±1.8	16.3±2.4	17.1±2.9

[a] Average from 5 replications ± SE.

Table 4. Effect of ethephon on ethylene emanation (nL g^{-1} h^{-1}) and sanguinarine production in Papaver somniferum cell suspension cultures

Ethephon (ppm)	Ethylene Emanation[a] 24h	48h	Sanguinarine (mg/g dry wt)
0	63.1±10.4	45.6±3.4[b]	0.48±0.02
50	634±91	629±27	--
100	1219±87	1268±89	0.28±0.12
200	2466±611	3773±296	0.44±0.17
400	3078	4935	0.38

[a] Ethylene was assessed 24 or 48 h after addition of ethephon (pH 6.2). The initial ethylene emanation rate from the cell cultures prior to addition of the ethephon was approximately 60 nL g^{-1} h^{-1}.

[b] Values represent averages from 2 replications ± S.E. Values without S.E. were not replicated but show the trend associated with the other treatments in this experiment.

The ethylene releasing ability of 2-chloroethylphosphonic acid (ethephon) was then utilized to determine if an exogenous ethylene source would be important in the elicitation of these suspension cultures. Previous work in our lab showed that the pH of our cultures after 7 days of culture was approximately 6.2. Therefore, the pH of the ethephon was adjusted to 6.2 before its addition to the 7-day old cultures (ethephon spontaneously releases ethylene when the pH is above 3.5). Immediately before the addition of ethephon, the cultures were releasing ethylene at a rate of 70 nL g^{-1} h^{-1}. Twenty-four hours after the aseptic addition of 7.5 ml of fresh 1B5C medium containing 0, 50, 100, 200, or 400 ppm ethephon, the cultures markedly increased their emanation rate by 9.0, 18.3, 38.1, and 47.8-fold (over the 0 ppm ethephon control), respectively (Table 4). After 48 h, the ethylene release rates from the 400 ppm ethephon treatment was over 100-fold greater than the 0 ethephon control (Table 4). However, the ethephon-mediated ethylene burst did not signal the production of sanguinarine in our cultures (Table 4). This is contrary to what Cho et al. (1988) observed regarding alkaloid production in Coffea arabica and Thalictrum rugosum cell suspension cultures.

These experiments show that ethylene has little if any role in fungal-mediated sanguinarine production in Papaver somniferum. The increase in ethylene emanation in the first few hours after adding the fungal homogenate is likely associated with the stress conditions that the cell experiences during elicitation. Our findings agree with that of Grosskopf et al. (1989) where a rapid increase in ethylene production from elicited tomato cells was not related to a simultaneous increase in phenylalanine ammonia lyase activity.

The limited ability of ACC to enhance the ethylene emanation rate in our cell cultures is similar to that reported in sunflower suspension cultures where a 2.5-fold increase in ethylene emanation was observed (Sauerbrey et al., 1988). However, ACC has been reported to promote the rate of ethylene emanation by 22-fold in maize callus cultures (Songstad et al., 1988) and 6-fold in orchardgrass leaf cultures (Songstad et al., 1989). Robinson et al. (1987) reported that the ability of sunflower shoot tip cultures to convert ACC to ethylene varied with regard to genotype. Perhaps other genotypes of Papaver somniferum may more efficiently produce ethylene from ACC.

Although both the callus and suspension cultures are of the same genotype and arose from the same primary culture, we observed higher ethylene emanation rates from the suspensions compared to the callus cultures. The suspension culture growth rate is faster than that of the callus (2.1- and 6.0-day doubling times for suspension and callus, respectively) and may be partly the reason for the higher ethylene levels. However, it may be the liquid culture environment (ie. combination of less oxygen available to the cells and damage associated with agitating the cultures at 150 rpm) which results in stress conditions and increased ethylene production.

The addition of ethephon to our cultures resulted in ethylene emanation rates up to 107-fold greater than the control. The exogenous supply of ethylene did not appear to alter the pigmentation of the cells and it did not promote the release of any unusual effluvium (associated with volatile release during stress). Furthermore, these ethephon treatments had little effect on the growth rate (15.6 and 13.8 g growth for the 0 and 400 ppm ethephon treatments, respectively). These results agree with Cho et al. (1988) where ethephon did not influence growth of Coffea arabica or Thalictrum rugosum cell cultures.

The Papaver somniferum cell suspension culture used in this research has experienced considerable variation in chromosome number to result in aneuploidy. Ordinarily this species is a diploid with

22 chromosomes (Hegi, 1958) but now contains approximately 60 to 70. From the present study, this cell line also shows little sensitivity to ethylene (ethephon) and is relatively poor at converting ACC to ethylene. Perhaps this culture-induced variation has resulted in the ethylene insensitivity of these cells as well as the poor ability to convert ACC to ethylene. Other Papaver somniferum genotypes may then be more sensitive to ethylene-mediated elicitation in the approach described by Cho et al. (1988).

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