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Inhibition of Ethylene Biosynthesis by Salicylic Acid

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ABSTRACT

Salicylic acid inhibited ethylene formation from ACC in self-buffered (pH 3.8) pear (*Pyrus communis*) cell suspension cultures with a K_i^{app} of about 10 micromolar after 1 to 3 hours incubation. Inhibition appeared noncompetitive. Among 22 related phenolic compounds tested, only acetylsalicylic acid showed similar levels of inhibition. Inhibition by salicylic acid was inversely dependent on the pH of the culture medium and did not require a continuous external supply of salicylate. When compared to known inhibitors of the ethylene forming enzyme, cobalt, *n*-propyl gallate, and dinitrophenol, inhibition by salicylic acid most closely resembled that by dinitrophenol but salicylic acid did not produce the same degree of respiratory stimulation. Results are discussed in terms of other known effects of salicylic acid on plants, pH-dependency, and the possible influence of salicylic acid on electron transport.

Applications of SA[2] and ASA to plants have been shown to influence a wide variety of biological processes including flower stimulation (12), vegetative bud formation (4), adventitious root initiation (13), disease resistance (25), stomate function (15), and heat production (24). Recently Leslie and Romani (16) further demonstrated that these compounds strongly reduce the conversion of ACC to ethylene in pear cell suspension cultures, suggesting they inhibit EFE, the putative terminal enzyme in ethylene biosynthesis. Rapid inhibition, proportional to the concentration of SA or ASA in the medium, maximized within 2 h and was followed by a slower reversal requiring a period of hours to days. This paper further characterizes this inhibition and compares SA activity to that of several previously demonstrated EFE inhibitors.

MATERIALS AND METHODS

These experiments employed a continuously cultured strain of pear cells established in 1972 by Pech and Fallot (19) from young fruit tissue of *Pyrus communis* cv Passe Crassane. The culture process was essentially that of Pech and Romani (20). Cell suspensions were grown at 25°C in 2 L flasks on a rotary shaker at 120 rpm and subcultured every 7 d using about 10% inoculum of a decanted cell suspension.

For experimental use, cultures were diluted with additional medium to a concentration of approximately 5×10^5 cells/mL and transferred in 50 mL aliquots to Morton capped, long-necked, 125 mL culture flasks, ACC (100 μM) added, and the flasks were placed on a rotary shaker at approximately 175 rpm under continuous light at room temperature. Experimental additions or manipulations were made following a minimum 1-h equilibration period. ACC was routinely added to increase ethylene production and amplify the effects of SA or other inhibitors. It has been shown, however, that SA inhibits both endogenous and ACC-stimulated ethylene production (16).

In experiments calling for pH adjustment all culture aliquots were supplemented with 40 mM phosphate buffer and pH was adjusted with HCl or KOH as needed. At this level the PO_4 did not itself affect ethylene production. For SA removal, the cultures were centrifuged (International model HN, swinging bucket rotor) at 1000*g* for 5 min, the supernatant discarded, and the cells gently resuspended in medium of appropriate pH without SA.

Ethylene measurements were a modification of the procedure used by Puschman and Romani (21). The 125 mL culture flasks were flushed with a vigorous air flow and capped for 30 min with rubber septa. Six mL head space samples were collected by syringe and ethylene concentrations measured by flame ionization gas chromatography using a Carle model 211 analytical gas chromatograph fitted with an alumina column held at 80°C and employing N_2 as a carrier gas.

Respiration readings employed an IR CO_2 analyzer (Horiba, model PIR 2000) to measure head space concentrations of CO_2. Cell vitality was determined by exclusion of Evan's blue dye and cell number was estimated by the packed cell volume (20).

IAA, ACC, $CoCl_2$, SA, ASA, benzoic acid, and chlorogenic acid were all purchased from Sigma Chemical Co. Benzoic acid derivatives were a gift from Dr. V. L. Singleton of the Viticulture Department, University of California, Davis. Stocks of SA, ASA, and other phenolics were prepared in 50% ethanol while $CoCl_2$, IAA, and ACC were dissolved in water. All stock solutions were kept refrigerated until use.

RESULTS AND DISCUSSION

Comparative Aspects. The ability of SA and ASA to inhibit ethylene biosynthesis by cultured pear cells was compared to that of similar phenolic compounds (Table I). The inhibitory nature of SA and ASA was unique among compounds examined.

Orthohydroxylation appeared to be a key element in activity. Monosubstituted benzoic acid derivatives hydroxylated at either the 3 or 4 position or chlorinated at the 2 position were not comparable to SA or ASA as ethylene inhibitors. Although several di- and trihydroxybenzoic acids showed fairly strong inhibition at higher doses, none showed the activity at low concentration that was demonstrated by SA and ASA.

Khurana and Maheshwari (12) noted several SA derived compounds stimulated flowering and suggested activity was due to the salicyl moiety, but several of these compounds (salicyl alcohol, salicin) failed to produce ethylene inhibition in pear cell tests. Although *p*-amino salicylic acid and 3,5-dinitro salicylic acid proved relatively active among compounds tested, they were much less effective than SA itself. The ineffectiveness of alcohols and noncarboxylated phenols as ethylene inhibitors suggests the carboxyl group is also an important determinant of activity.

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[2] Abbreviations: SA, salicylic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ASA, acetylsalicylic acid; DNP, dinitrophenol; EFE, ethylene-forming enzyme.

Table I. *Inhibition of Ethylene Production by Benzoic Acid Derivatives and Related Phenolic Compounds*

Ethylene production was measured 1.5 h after addition of the inhibitor to cells pretreated with 100 μM ACC. Data are single samples from series of 4 different concentrations.

Inhibitor	Percent Inhibition	
	25	250
	μM	
Unsubstituted		
Benzoic acid	0	25
Monosubstituted		
2-Hydroxy benzoic acid (SA)	78	94
3-Hydroxy benzoic acid	−13	34
4-Hydroxy benzoic acid	1	4
2-Chloro benzoic acid	8	46
2-Acetoxy benzoic acid (acetylsalicylic acid)	79	92
Disubstituted		
2,3-Dihydroxy benzoic acid (*o*-pyrocatachuic acid)	−2	71
2,4-Dihydroxy benzoic acid (β-resorcylic acid)	−11	26
2,5-Dihydroxy benzoic acid (gentisic acid)	−15	37
2,6-Dihydroxy benzoic acid	−6	0
3,4 Dihydroxy benzoic acid (protocatechuic acid)	−6	2
3,5-Dihydroxy benzoic acid	−17	−20
3,5-Dimethyl benzoic acid	11	70
3-Methoxy, 4-hydroxy benzoic acid (vanillic acid)	19	65
3,4-Dimethoxy benzoic acid (veratric acid)	4	53
2,Hydroxy, 4-amino benzoic acid (*p*-amino SA)	20	82
Trisubstituted		
2,3,4-Trihydroxy benzoic acid	−11	24
2,4,6-Trihydroxy benzoic acid	4	−2
3,4,5-Trihydroxy (gallic acid)	0	4
2-Hydroxy, 3,5-dinitro benzoic acid (3,5-dinitro SA)	14	40
Alcohols and miscellaneous phenolics		
Salicyl alcohol (saligenin)	4	12
Salicin	9	2
Catechol	0	10
Phloroglucinol	2	−9
Pyrogallol	7	15
2,4-Dinitrophenol	79	100

Baker *et al.* (2) demonstrated inhibition of ethylene biosynthesis by benzoic acid at concentrations that were also active in this study (Table I), but benzoic acid was considerably less active in pear cells than SA or ASA. This disparity in ability to block ethylene biosynthesis contrasts sharply with observations of benzoic acid activity equal to or stronger than that of SA in flower induction (10, 26), vegetative growth (12), and disease resistance (27), suggesting different mechanisms may be involved.

SA can chelate metalic ions (26), and chelation has been suggested as a mechanism for biological activity (11). In pear cells neither EDTA nor EGTA showed any ability to inhibit ethylene production (data not shown), suggesting the chelating ability of SA is of little importance in ethylene inhibition.

Kinetics. Measurements of ethylene production from SA inhibited cells in self-buffered growth medium at pH 3.8 were taken during the period of maximum inhibition, approximately 2 h after SA application, and used to construct Dixon plots (Fig. 1). SA inhibition appeared to be noncompetitive with a mean K_i^{app} of 10.5 μM SA. It should be noted that convergence of these plots was never precise, ostensibly because measurements were taken from actively metabolizing cell cultures rather than isolated enzymes. In addition, the ability of SA to inhibit ethylene production varied among individual batches of cells in response to general cell quality. Those cultures with the highest vitality generally exhibited both the highest rate of ethylene production and the strongest SA inhibition. Accordingly, K_i^{app} values determined using different batches of cells ranged from 7.8 to 16.8 μM SA.

Effects of pH. The role of pH in SA activity was examined and inhibition of ethylene biosynthesis proved to be inversely dependent on the pH of the culture medium (Fig. 2). When cell cultures, which normally self-buffer their medium to a pH of approximately 3.8, were buffered to pH values ranging from 3.5 to 6.5, the ability of SA to inhibit ethylene production decreased sharply with increased pH.

The effect of culture medium pH on SA activity was readily reversible (Table II). Not only did lowering the pH of the medium in the presence of SA produce a rapid drop in ethylene production, but cells strongly inhibited by SA at low pH showed rapid recovery of ethylene biosynthesis when the medium pH was raised. Similar dependence of SA biological activity on pH has been noted in other biological systems (6) and attributed to the lipid solubility and dissociation properties of SA (7, 26).

Experiments wherein SA was removed by gentle pelleting and resuspension of the cells at different pHs demonstrated that although inhibition of ethylene biosynthesis was proportional to the initial concentration of SA, it was not dependent on a continuous supply of free SA external to the cells (data not shown). Persistence of inhibition following SA removal from the culture medium suggests that consumption of external SA supply is not responsible for the slow reversal process observed (Fig. 3; Table III) following initial SA inhibition. Recovery of ethylene production is more likely due to glucose conjugation of internal SA (3, 5).

SA and Other EFE Inhibitors. Further indications of the nature of SA inhibition were sought by comparing SA activity

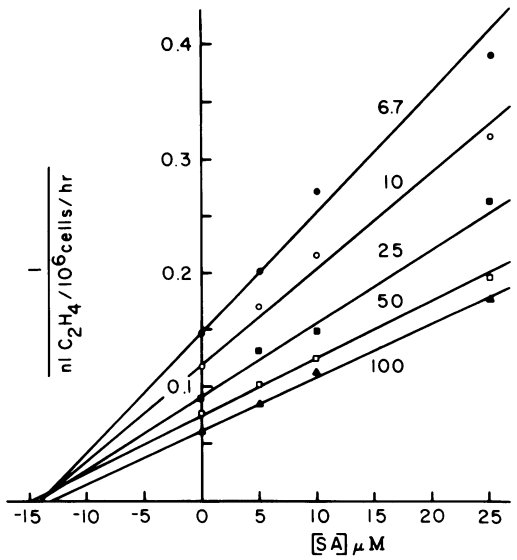

FIG. 1. Dixon plot for SA inhibition of ethylene production by pear fruit cells. Single flask readings taken 1.5 h after inhibitor addition to cells in growth medium. Numbers along the lines designate μM ACC. Data shown are from one of 5 experiments.

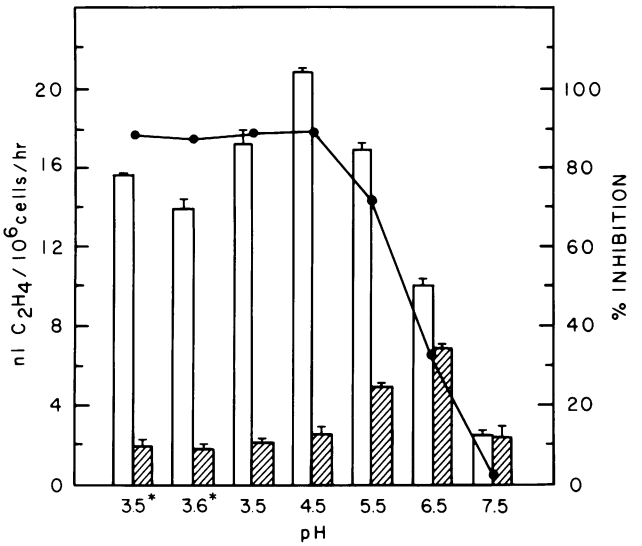

FIG. 2. Effects of media pH on ethylene production and its inhibition by salicylic acid. SA (50 μM) added to cells 1 h after the addition of 100 μM ACC; ethylene production measured 2 h thereafter. Control (open bars), +SA (cross-hatched bars), % inhibition (●), unbuffered (*), rest contained 40 mM PO_4. Rates represent the mean and error bars half the range of duplicate cultures.

Table II. *Effect of pH Shift on the Inhibition of Ethylene Production by 50 μM Salicylic Acid*

Ethylene production in the presence of 100 μM ACC was inhibited by addition of 50 μM SA and measured after 1.5 h. The pH of cell cultures was then readjusted (arrow) with HCl or KOH and inhibition measured after an additional 1.5 h.

pH Change	Ethylene Production in Presence of SA	
	nL/h/10⁶ cells	*% inhibition*
Experiment 1		
3.5 → 6.4	1.6 ± 0.01 → 6.9 ± 0.01[a]	88 → 50[b]
6.5 → 4.5	5.4 ± 0 → 1.1 ± 0.1	53 → 87
Experiment 2		
3.5 → 6.4	0.64 ± 0.07 → 3.26 ± 0.07	93 → 39
6.5 → 4.5	1.54 ± 0.3 → 0.48 ± 0.09	42 → 90
Experiment 3		
3.5 → 6.5	1.5 ± 0.1 → 5.1 ± 0.3	89 → 49
6.5 → 3.5	3.2 ± 0.1 → 0.5 ± 0.05	29 → 85

[a] Inhibited rates represent means and range of duplicate samples. [b] Percent inhibition is based on uninhibited rates at the same pH.

to that of several previously reported types of EFE inhibitors, including metalic ions, free radical scavengers, and uncouplers.

Cobalt. Cobalt is one of several metallic ions that strongly inhibit EFE function, perhaps by complexing with protein sulfhydryl groups (29). When SA and cobalt were compared, the pattern of slow expression and absence of reversal exhibited by cobalt contrasted sharply with the rapid and reversible inhibition demonstrated by SA (Fig. 3). Time of exposure was important in comparing effectiveness of these inhibitors. Over short time intervals of several hours, SA was clearly the more effective inhibitor, but over longer intervals SA inhibition, particularly at low doses, began to reverse while cobalt effectiveness continued to increase and cobalt became the more effective inhibitor. The very different inhibition patterns exhibited by these two compounds suggest SA and cobalt act by separate mechanisms to block EFE function.

Free Radical Scavengers. Rainsford (22) has suggested some SA activities may be attributable to an ability to scavenge free radicals. Others (1, 2) have noted that approximately 1 mM doses

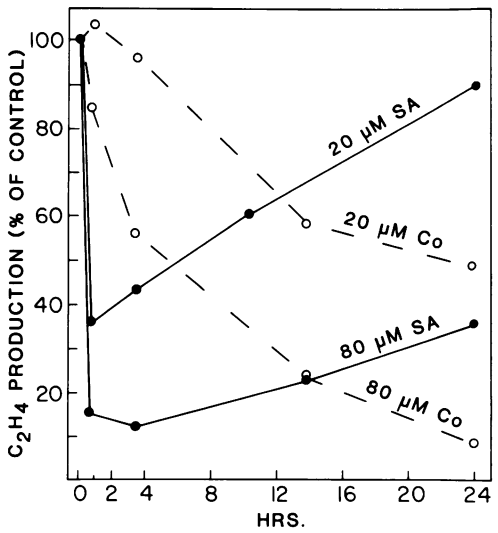

FIG. 3. Time course: inhibition of ethylene production by cobalt and salicylic acid. Inhibitors were added 1 h after addition of 100 μM ACC. Symbols represent consecutive readings from the same culture flask. (●——●), SA: (O——O), Co. Control rates: 0 h, 21.35 nL C_2H_4/10^6 cells/h; 24 h, 13.95 nL C_2H_4/10^6 cells/h. One of three corroborative experiments.

Table III. *Effects of Time on the Inhibition of Ethylene Biosynthesis by n-Propyl Gallate and Salicylic Acid*

Inhibitors added immediately before ACC and ethylene production rates determined after 1, 3, and 24 h.

Inhibitor	Percent Inhibition[a]		
	1 h	3 h	24 h
μM			
n-Propyl gallate			
100	72	45	13
200	94	70	17
400	99	79	44
Salicylic acid			
100	82	89	45
200	88	92	76
400	95	96	81

[a] Uninhibited rates of ethylene production were 6.5, 10.3, and 7.2 nL/10^6 cells/h at 1, 3, and 24 h, respectively. One of two corroborative experiments.

of several free radical scavengers, including *n*-propyl gallate, inhibit ethylene production.

In pear cells, ethylene inhibition by *n*-propyl gallate showed concentration effects and a pattern of rapid onset very similar to those of SA, but the two compounds differed noticeably in the persistence of their inhibition (Table III). Doses of 200 μM *n*-propyl gallate showed significant loss of inhibitory effect within 3 to 4 h and even the effects of a 400 μM dose substantially reversed within 24 h, while SA doses of 100 μM SA remained effective for 24 to 48 h.

Inhibition by *n*-propyl gallate was also accompanied by noticeable darkening of the pear cell cultures which then recovered their normal color as the effects of the inhibitor reversed. Similar darkening of cells was not observed with SA doses as high as 2.5 mM. Differences in reversal times and cell color change suggest that SA does not inhibit EFE by acting as a free radical scavenger, although one cannot discount the fact that these differences could be explained by a relatively much more rapid cellular detoxification of SA.

Uncouplers. The ability of uncouplers to inhibit ethylene pro-

duction was demonstrated by Yu *et al.* (28) although the mechanism remains to be clarified. Suggestions have included EFE dependence on membrane potential (8) or on a membrane bound electron transport system (17).

When SA was compared to the uncoupler DNP the ethylene inhibition curves for the two compounds proved nearly identical but their effects on respiration were very different (Fig. 4). Low DNP concentrations produced a dramatic rise in respiratory rate, generally thought to be the result of uncoupling, and levels exceeding 25 μM DNP began to show inhibitory effects on respiration. In contrast, even moderately high concentrations of SA generally produced a respiratory rise of only 20 to 30%. Only at concentrations exceeding 500 μM did SA affect respiration by being inhibitory (data not shown).

Although SA can act as a protonophore, this may not be its mode of action in inhibiting EFE. The capacity of SA as a protonophore is generally orders of magnitude less than that of the classical protonophore DNP (7, 18), a difference reflected here in the respiration data. Moreover, SA inhibition of EFE in pear cells persisted when SA was removed from the external medium, whereas uncoupling by SA of pea mitochondria ceased under these conditions (18). Finally, Marci *et al.* (18) found no uncoupling by ASA, yet this compound is as strong an EFE inhibitor as SA (Table I).

This absence of correlation between uncoupling behavior and ability to inhibit EFE was also observed by John *et al.* (9) and led them to conclude that EFE activity was not dependent on membrane potential. Our observations lend support to suggestions EFE is dependent instead on some other membrane associated function, perhaps electron transport *per se* hypothesized by McKeon and Yang (17). This possibility is enhanced by the recent finding that SA also induces heat production in Arum lilies (24), ostensibly via induction of the alternative electron transport system.

Concluding Remarks. Further testing of salicylate related drugs for EFE inhibition could be of interest, particularly 5-chlorosalicylic acid which is both a highly effective flower inducer in *Lemna* (26) and potent anti-inflammatory compound (22) and diflunisal, a highly therapeutic biphenyl derivative of salicylate (22). The use of newly developed pro-drugs whose effects depend on their metabolism *in vivo* to form SA or ASA could also aid understanding of how salicylate affects ethylene production.

SA or SA derivatives have been widely isolated from many plants at levels (14, 23) comparable to those employed here to inhibit ethylene production and at least one instance of physiological activity by endogenous SA has been demonstrated (24). The possibility that endogenous SA also plays a role in ethylene regulation in plants or that some effects of exogenous SA application are mediated by altered ethylene metabolism remains to be explored.

In view of its diverse biological effects and apparent role in membrane related physiology, further exploration of the effects of SA in plants seems warranted. Regardless of any endogenous role as an inhibitor of EFE activity, SA should prove useful in studying ethylene biosynthesis.

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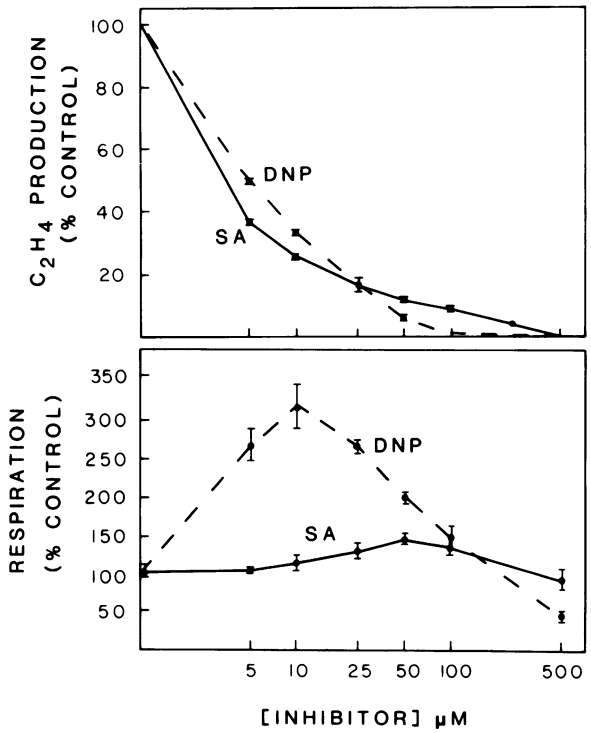

FIG. 4. Inhibition of cellular ethylene production and respiration by equimolar concentrations of dinitrophenol and salicylic acid. Inhibitors added to cells in growth medium 1 h after the addition of 100 μM ACC. Respiration readings were taken 1.5 h later and ethylene production rates were determined 2 h after inhibitor application. Symbols represent the mean rates and range from duplicate culture flasks. Control ethylene production rate: 10.5 nL $C_2H_4/10^6$ cells/h. One of two corroborative experiments.

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