J Plant Growth Regul (1989) 8:63–69

Journal of
Plant
Growth
Regulation

Salicylic Acid Inhibition of Ethylene Production by Apple Discs and Other Plant Tissues

Roger J. Romani, Betty M. Hess, and Charles A. Leslie

Department of Pomology, University of California, Davis, California 95616

Received January 14, 1988; accepted August 18, 1988.

Abstract. Ethylene production by apple discs is effectively inhibited by salicylic acid. Inhibition is pH dependent, being greatest from pH 3.5–4.5 and minimal at pH 6.5 and above. With 100 μM salicylic acid maximal inhibition, approximately 90%, is achieved in 3 h with an apparent K_i of 40 μM. At somewhat higher concentrations salicylic acid also inhibits the conversion of 1-aminocyclo-propane-1-carboxylic acid to ethylene by pear discs and mung bean hypocotyls. Salicylic acid interferes with action of the putative ethylene-forming enzyme and in this respect is somewhat more effective than cobalt ion. The inhibitory effects of salicylic acid and cobalt ion are not additive. Implications for the limits and locus of salicylic acid inhibition are discussed.

In addition to cobaltous ion (Yu and Yang 1979), several compounds including analogs of 1-aminocylopropane-1-carboxylic acid (ACC), protonophores, free radical scavengers (Apelbaum et al., 1981), and α(p-chlorophenoxy) isobutyric acid (Trebitsh and Riov, 1987) have been shown to inhibit the putative ethylene-forming enzyme (EFE) that converts ACC to ethylene. Although some of these inhibitors are appreciably less effective than others, each may provide some clue(s) to the locus and mode of action of EFE. In this regard, we reported that salicylic acid (SA) effectively inhibits EFE in suspension-cultured pear fruit cells (Leslie and Romani, 1986). The apparent K_i proved to be about 10 μM, inhibition was rapid, pH-dependent (best at pH 4–5), and reversible via a change in pH (Leslie and Romani, 1988). In view of its promise as an investigative tool it seemed important to know whether SA was an effective inhibitor in other plant tissues commonly used in studies of ethylene biosynthesis. We describe herein the effects of SA on ethylene production by apple discs, pear discs, and mung bean hypocotyls, and compare its action with that of cobalt ion, the more commonly used inhibitor of EFE.

Materials and Methods

Tissue discs (1 cm diameter and approximately 2 mm thick) were prepared from the flesh of apples (*Malus sylvestris*, cv. "Granny Smith") purchased at a local market. The discs were first suspended in cold (0–4°) 0.1 M mannitol from which randomly selected 3 g of discs were placed in 25 ml Erlenmeyer flasks containing 10 ml of 0.1 M mannitol and 40 mM potassium phosphate buffer at designated pH. The flasks were incubated at 30°C in a reciprocating water-bath shaker.

To assess rates of ethylene production, as described by Puschmann and Romani (1983), the flasks were first flushed for a few seconds with a stream of air and then closed with serum caps. After 30 min a 3 ml aliquot of the headspace gas was removed with a needle and syringe for injection in a gas chromatograph equipped with an alumina column and flame ionization detector. The flasks were flushed, capped, and sampled at stated intervals to assess changing rates of ethylene production. Times refer to the end of each 30 min incubation. Duplicate or triplicate samples of slices were used and each experiment was repeated at least once.

In comparative assays the efficacy of SA was tested with 3 g samples of tissue discs prepared from pear (*Pyrus communis*, cv. Bartlett) fruit and with 20 2 cm sections (approximately 2 g) of mung bean (*Vigna radiata*) hypocotyls cut from just below the hook after germination for 4 days in the dark at 25°C.

Results

In preliminary experiments with apple discs and either 0.25 M mannitol or sucrose as an osmoticum, there was little or no inhibition of ethylene production by SA. Penetration of the SA appeared to have been improved and good inhibition was observed when the concentration of mannitol was reduced to 0.1 M. As further aids to penetration, mild vacuum and/or 5% dimethylsulfoxide (DMSO) were used but neither appeared to improve inhibition by SA significantly.

Effect of pH

As with cultured pear cells, SA inhibition of ethylene production by apple discs was strongly pH dependent (Fig. 1). Acid pHs (3.5–5) resulted in roughly 90% inhibition after a 3 h exposure to 100 μM SA. At pH 6.5 or higher SA was no longer an effective inhibitor. Three hours after the start of an experiment utilizing 20 μM phosphate, the pH of the medium, as shown in parentheses along the abscissa (Fig. 1), was altered by acidic exudates from the discs. More effective pH control and similar inhibition patterns were obtained with 40 and 100 μM phosphate. However, as observed by Chalutz et al. (1980), the higher phosphate concentration itself interfered with ethylene production (data not shown). Accordingly, all subsequent experiments were conducted with 20 or 40 μM phosphate, as indicated.

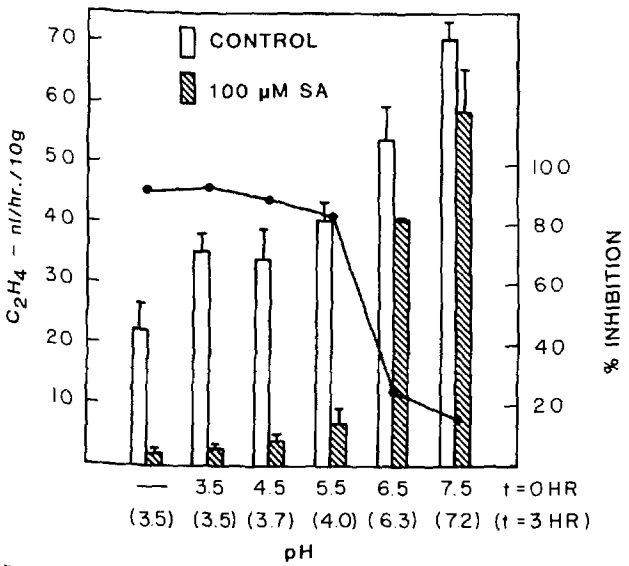

Fig. 1. Effects of medium pH on ethylene production by apple discs (*open bars*) and its inhibition by 100 μM salicylic acid (*cross-hatched bars*). Percentage inhibition (●———●). The first 2 bars subtended by (———) represent ethylene production rates in the absence of phosphate buffer. All others represent rates in the presence of 20 μM potassium phosphate at the indicated initial (t = 0) pH with the final (t = 3 h) pH shown in parentheses. Error lines represent the range from the mean of duplicate batches of discs.

The overall increase in ethylene production at higher pHs is unexplained, and is opposite the trend observed with cultured pear cells (Leslie and Romani 1988).

SA Concentration

Kinetic studies with tissue discs are seriously compromised by the postcutting decline in ethylene production, by barriers to penetration of the inhibitor, and by unknown interactions between the inhibitor and cytoplasmic components other than those pertaining to the reaction under investigation. Nonetheless it is obvious from data such as those in Fig. 2 that inhibition of ethylene production by apple discs is affected by both time and SA concentration. Inhibition by 20 μM SA is minimal and transient, 100 μM SA results in effective inhibition lasting at least 5 h, and 500 μM SA results in more rapid but not greater final inhibition. Data from a comparable experiment presented in more conventional kinetic form (Fig. 3) are helpful in estimating half-maximal inhibition at about 100 μM and 40 μM for a 1 h and a 3 h exposure, respectively. While such apparent K_is are useful for comparative purposes, they are likely, for reasons noted above, to bear little relevance to inhibition mechanisms at the enzymic level.

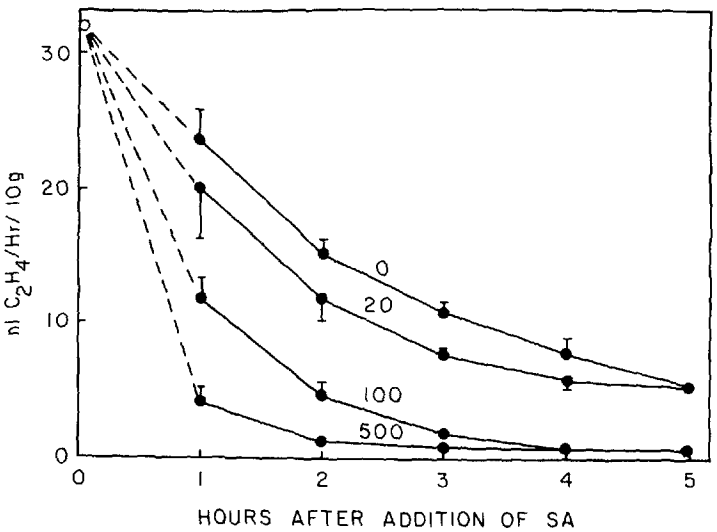

Fig. 2. Effects of time and concentration on the salicylic acid inhibition of ethylene production by apple fruit tissue discs. Ethylene production rate at time 0 is an arbitrary projection from control rates at 1 and 2 h. Bars represent the SD from 3 replicates. Numbers indicate the μM concentration of SA.

SA vs. Cobalt Ion

In Fig. 4 SA inhibition of ethylene production by apple slices is compared with that by equimolar cobalt, a commonly used inhibitor of EFE (Yu and Yang 1979). Over a period of 3 h SA is both faster acting and somewhat more inhibitory than cobalt, a difference that is even more pronounced in cultured pear cells (Leslie and Romani 1988). There is no additive effect of the 2 inhibitors. Over more extended periods SA inhibition is gradually reversed, whereas the effect of cobalt persists (data not shown).

Inhibition in Other Tissues

Pear tissue discs were found to produce little ethylene unless the fruit was first stored at 0°C. This is in keeping with the observations of Blankenship and Richardson (1985) with d'Anjou pear discs. Exogenous ACC further enhanced ethylene production of the d'Anjou pear discs, as it did with discs from Bartlett pears (Table 1). Under such conditions and after 3 h incubation, 100 and 200 μM SA resulted in roughly 50 and 70% inhibition of ethylene production, respectively (Table 1).

With mung bean hypocotyl segments, whose ethylene production was stimulated by the addition of 100 μM ACC, 200 μM SA resulted in about 50% inhibition (Table 2). At 600 μM SA, the highest concentration tested, inhibition increased to near 80%. Rather severe vacuum infiltration (exp. 2), which itself

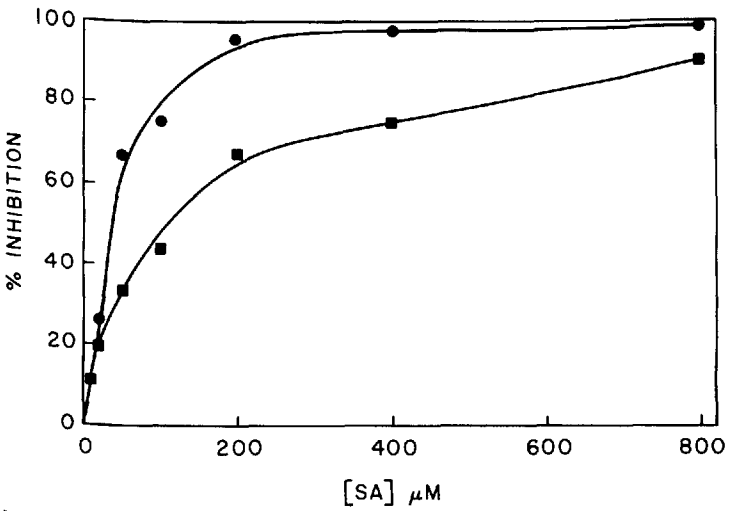

Fig. 3. Inhibition of apple disc ethylene production by increasing levels of salicylic acid. Ethylene production measured 1 h (■———■) and 3 h (●———●) after addition of the inhibitor. Uninhibited rates of ethylene production were 15.3 ± 1.7 and 7.1 ± 0.3 nl/h/10g units at 1 and 3 h, respectively.

diminished ethylene production, resulted in only a modest increment of inhibition of SA.

Discussion

SA is an effective inhibitor of ethylene biosynthesis in apple discs and somewhat less so in pear discs and mung bean hypocotyls. Time and concentration effects imply that penetration is a limiting factor, although diffusion barriers overcome by vacuum infiltration or DMSO do not appear to be involved. SA is faster acting than Co and slightly more effective over the first few hours.

SA's effectiveness as an inhibitor of ethylene biosynthesis is clearly pH dependent. An interesting and close analogy exists between the concentration, time, and pH dependency of SA's inhibition of ethylene biosynthesis and its inhibition of K^+ absorption by root segments from oak seedlings (Harper and Balke 1981). Because the pK_a of SA is about 3, Harper and Balke (1981) reasoned that at acid pHs the more prevalent undissociated form (SA°) penetrates the plasmalemma and then accumulates in the cytoplasm as the active, dissociated form (SA^-). This mode of distribution, and hence its effectiveness as an inhibitor, was also apparent from pH shift and "wash" experiments conducted with cultured pear fruit cells.

Given its moderate lipid solubility, SA may accumulate in hydrophobic regions of cell membranes (Harper and Balke 1981, and references cited therein). Such an event would not be out of keeping with SA's uncoupler-like action in

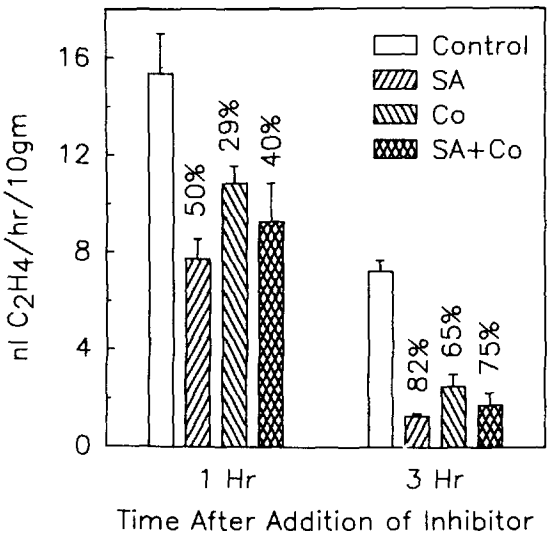

Fig. 4. Ethylene production by apple discs as affected by separate or combined presence of 100 μM salicylic acid and 100 μM cobalt ion. Data represent the mean of triplicate 3 g samples of discs with the SD as indicated. Numbers above the bars indicate percentage inhibition.

Table 1. Salicylic acid inhibition of ACC-stimulated ethylene production by pear fruit discs

	nl C_2H_4/10 g/h		% Inhibition	
Treatment	1 h	3 h	1 h	3 h
Control	44 (±8)	23 (±2)		
+ACC	89 (±9)	105 (±8)		
+ACC + 100 μM SA	44 (±6)	51 (±18)	51	52
+ACC + 200 μM SA	33 (±5)	28 (±3)	63	73

Pear discs were prepared from fruit stored at 0°C for 30 days. Assay procedure as described for apple discs except that 100 μM ACC was added to the incubation medium where indicated. Data represent the mean and SD of triplicate samples.

inhibiting ethylene biosynthesis (Leslie and Romani 1988) and its exacerbation of heat production in *Arum* spadicies (Raskin et al. 1987), ostensibly via activation of the alternative electron transport pathway. Membrane-localized action of SA is also consistent with the presumed location of EFE (McKeon and Yang, 1987). In short, SA should not only serve as an effective inhibitor of EFE in tissues tolerant of low pH but may also provide some clues to the localization and membrane-dependency of the final step in ethylene biosynthesis.

SA has a myriad other effects on plant development including shoot formation, root initiation and vegetative reproduction (references in Leslie and Romani 1988). These effects occur at levels of SA that could conceivably moderate intracellular ethylene concentration. Further study of the physiological and metabolic effects of low levels of SA seems warranted.

Table 2. Salicylic acid inhibition of ACC-stimulated ethylene production by mung bean hypocotyl sections

Treatment[a]	nl C_2H_4/h/20 section[b]		% Inhibition	
	1 h	3 h	1 h	3 h
Exp 1				
No SA control	15.7 (±0.9)	9.1 (±0.6)	—	—
+200 μM SA	7.0 (±0.2)	5.5 (±0.4)	55	40
+600 μM SA	3.7 (±0.1)	1.9 (±0.1)	76	79
Exp 2				
No SA control	12.9 (±1.1)	19.1 (±2.4)		
+200 μM SA	6.9 (±1.1)	10.3 (±1.0)	46	46
no SA + Vac	10.3 (±1.1)	9.4 (±1.2)		
+200 μM SA + Vac	4.0 (±0.4)	3.2 (±0.3)	52	66

[a] Assay conditions: Hypocotyl sections were placed in 40 mM PO_4, 100 mM mannitol, 10 μM ACC. Exp. 1, initial pH = 3.5, after 3 h = 4.8–5.1. Exp. 2, initial pH = 4.5, after 3 h = 5.1. Vacuum, approx. 65 mmHg for 5 min.

[b] Rates: Exp. 1, means ± half the range for duplicate samples. Exp. 2, means ± SD for triplicate samples except for "no SA + Vac," which are means ± range of duplicate samples.

References

Apelbaum A, Wang SY, Burgoon AC, Baker JE, Lieberman M (1981) Inhibition of the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene by structural analogs, inhibitors of electron transfer, uncouplers of oxidative phosphorylation, and free radical scavengers. Plant Physiol 67:74–79

Blankenship SM, Richardson DG (1985) Development of ethylene biosynthesis and ethylene-induced ripening in d'Anjou pears during the cold storage requirement for ripening. J Am Soc Hort Sci 110:520–523

Chalutz E, Mattoo A, Fuchs Y (1980) Biosynthesis of ethylene: the effect of phosphate. Plant Cell Environ 3:349–356

Harper JR, Balke NE (1981) Characterization of the inhibition of K^+ absorption in oat roots by salicylic acid. Plant Physiol 68:1349–1353

Leslie CA, Romani RJ (1986) Salicylic acid: a new inhibitor of ethylene biosynthesis. Plant Cell Rep 5:144–146

Leslie CA, Romani RJ (1988) Inhibition of ethylene production by salicylic acid: kinetic and comparative aspects. Plant Physiol 88:833–837

McKeon TA, Yang SF (1987) Biosynthesis and metabolism of ethylene. In: Davies PJ (ed) Plant hormones and their role in plant growth and development. Martinus Nijhoff, Dordrecht, pp 94–112

Puschmann R, Romani R (1983) Ethylene production by auxin-deprived, suspension-cultured pear fruit cells in response to auxins, stress, or precursor. Plant Physiol 73:1013–1019

Raskin I, Ehmann A, Melander WR, Meeuse BJD (1987) Salicylic acid—a natural inducer of heat production in *arum* lilies. Science 237:1601–1602

Trebitsh T, Riov J (1987) Inhibition of the ethylene-forming enzyme by α(p-chlorophenoxy) isobutyric acid. J Plant Regul 5:207–215

Yu Y-B, Yang SF (1979) Auxin-induced ethylene production and its inhibition by aminoethoxyvinylglycine and cobalt ion. Plant Phyiol 64:1074–1077