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Effects of Exogenous Salicylate on Basal and Stress-Induced Ethylene Formation in Soybean

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Abstract. Aqueous salicylate solutions stimulated ethylene formation only when injurious, or potentially injurious, concentrations were exogenously supplied to soybean cuttings. Stimulation occurred via the biochemical sequence involving ACC as an intermediate, and was attributable to stimulation of ACC synthesis but not of EFE activity. Similar results were obtained by testing wound-induced ethylene,whereas the production of virus-induced ethylene was not affected by salicylate. Prolonged salicylate treatments which did not produce evident injurious effects inhibited soybean growth and rooting, probably through the moderate antiauxinic property attributable to salicylates. These findings are discussed in relation to other results obtained from similar or different plant materials.

Exogenous tratment of plant materials with salicylate results in important biochemical and physiological events (for references, see Pennazio *et al.* 1987 and Leslie and Romani 1988).

Recently, Leslie and Romani (1986) found that salicylates could inhibit ethylene production in pear cell suspension cultures, and have also shown (1988) that salicylates were potent inhibitors of EFE activity when exogenously supplied to these cultures at low pHs. This finding was rather unexpected, because other results indicated that salicylates had no effect (van Loon and Antoniw 1982, Pennazio *et al.* 1985) or slightly stimulated ethylene biosynthesis (Pennazio *et al.* 1987, Meijer and Brown 1988).

The aim of the present work was to test the effect of exogenous salicylate on basal and stress-induced ethylene in soybean plants.

MATERIALS AND METHODS

Plants of soybean (*Glycine max* Merr., cv. Hodgson) were grown from seed in a glasshouse, in 10 cm pots containing a sterilized standard potting compost. On

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emergence, seedlings were transferred to a growth chamber and kept under a 12 h photoperiod, at 100 μmol $s^{-1}m^{-2}$ photosynthetically active radiations (Philips TLD 95 lamps), at 23 °C (± 1 °C) and 60 % R. H. When plantlets had reached the V_1 stage (Fehr *et al.* 1971) they were severed 3 cm below the primary leaves and the stems of these cuttings were immersed in aqueous salicylate solutions of different concentrations (Na salt, pH 5.8, Merck). Highly deionized water was used as controls. This procedure produced only a small cut surface (about 1.5 mm^2) and involved the release of minimal amounts of wound ethylene during the first hours, so allowing the direct determination of basal ethylene.

Estimates of basal ethylene were carried out by carefully introducing single salicylate-treated or control cuttings into vials, which were sealed and incubated for 1 h at 23 °C, in the dark. The ethylene released was determined by gas chromatography. Aqueous solutions of AVG (Fluka) and $CoCl_2$ (Merck) were used as inhibitors of ethylene biosynthesis (Yang and Hoffman 1984). EFE was tested by determining ethylene production from cuttings on stems immersed in 10 mM ACC

TABLE 1

Effects of salicylate on basal ethylene produced by soybean cuttings

Treatment		Ethylene production [nmol h^{-1} g^{-1} (f.m.)]
Concentration [mM]	Time [h]	
Water control	24 (12 h photoperiod)	0.05 ± 0.009
1		0.06 ± 0.01
2		0.15 ± 0.01*[a]
3		0.25 ± 0.10*[a]
Water control	3 (in the light)	0.01 ± 0.007
1		0.04 ± 0.007
2		0.12 ± 0.06*[b]
3		0.21 ± 0.05*[b]

Cuttings were treated as indicated. They were then incubated for 1 h in darkened sealed vials before determining ethylene by gas chromatography. Each value is the average of two repetitions (± s.d.), each including 6 replicates. The results statistically different from the corresponding water controls are asterisked (P = 1%).
(a) Visible symptoms of injury.
(b) Potentially injurious treatment.

Abbreviations used: ACC = 1-aminocyclopropane-1-carboxylic acid; AVG = Amino-ethoxyvinylglycine; EFE = ethylene-forming enzyme; TNV = tobacco mosaic virus.

dissolved in deionized water, for 3 h in the light. Free ACC was determined according to Pennazio and Roggero (1990).

Wound-induced ethylene was determined from discs of cuttings treated for 24 h with 1 mM salicylate or water (controls). After treatments, 5 mm diameter discs were punched from the primary leaves, halved and introduced in sealed vials which were incubated for 1 h in darkness before determing the released ethylene.

Virus-induced ethylene was determined during the hypersensitive reaction of soybean to TNV, since such a reaction is commonly accompanied by a burst of ethylene (van Loon 1984). The primary leaves of plantlets were inoculated with a suspension of TNV (of our Institute's collection), and 2 days later, when the ethylene release was maximal, cuttings were severed and incubated for 3 h with 3 mM salicylate or water (controls) in open vials, in the light. The vials were then sealed and incubated for 1 h in darkness before determining the ethylene released.

Total chlorophyll was determined as described elsewhere (Roggero and Pennazio 1988). Rooting was evaluated by counting the number of the root primordia developing at the base of stems.

Each experiment included several replicates and was repeated at least twice. Significant differences between salicylate-treated and control cuttings were detected by the Student *t*-test.

RESULTS

Effects of Salicylate on Basal Ethylene Formation

Prolonged (24 h or more) incubation of cuttings in 1 mM salicylate solution did not produce evident injury or alter ethylene production (Table 1). When cuttings were

TABLE 2

Effects of inhibitors of ethylene biosynthesis on salicylate-stimulated ethylene production by soybean

Treatment	Ethylene production [nmol h^{-1} g^{-1}]
Deionized water	0.05 ± 0.006
3 mM salicylate	0.25 ± 0.06
3 mM salicylate + 0.5 mM $CoCl_2$	0.07 ± 0.03
3 mM salicylate + 0.5 mM AVG	0.06 ± 0.02

Cutting were treated for 3 h with the solutions indicated, in the light. They were then incubated in sealed vials for 1 h in darkness before determining ethylene. Each value is the average of two repetitions, each including 6 replicates.

incubated in more concentrated solutions severe symptoms of toxicity appeared, together with increased ethylene production. Short (3 h) incubation of cuttings in the light produced early severe injury when the concentration was higher than 3 mM (Table 1). Short treatments with 2 or 3 mM salicylate stimulated ethylene production, which was inhibited by AVG or Co^{2+} (Table 2). Such concentrations did not produce early evident injury, but were potentially injurious since evident damage occurred some hours later.

Lowering the pH of the salicylate solution notably stimulated ethylene production by cuttings, but this stimulation was shown also with acidic buffer free of salicylate (Table 3).

Prolonged incubation (24 h) in 1 mM salicylate did not affect EFE activity nor accumulation of free ACC (Table 4). EFE was not stimulated by short pulse with 3 mM salicylate, but such pulse increased the free ACC content; this increase was abolished by AVG (Table 4).

Very prolonged incubation (up to 8 days) in 1 mM salicylate did not produce severe injury. However, growth and rooting of cuttings were partially inhibited, and the leaf chlorophyll content slightly decreased (Table 5). Such prolonged treatments did not alter ethylene production (Fig. 1).

Effects of Salicylate on Stress Ethylene

Salicylate had no effect on vound ethylene when cuttings were incubated for 24 h in 1 mM solution. However, statistically significant increase in wound ethylene was

TABLE 3

Effects of the pH of the salicylate solution on ethylene production by soybean cuttings

Treatment		Ethylene production [nmol h^{-1} g^{-1}]
Concentration [mM]	pH	
Water control	5.8	0.05 ± 0.004
1		0.05 ± 0.006
3		0.44 ± 0.05*
0.1 mM acetate buffer	3.8	0.24 ± 0.06
1		0.18 ± 0.03
3		0.22 ± 0.02

Cuttings were treated for 3 h with the solutions indicated, in the light. They were then incubated for 1 h in sealed vials, in darkness, before determining ethylene. Each value is the average from three repetitions, each including 6 replicates. Result statistically different from the water control is asterisked (P = 1%).

TABLE 4

Effects of salicylate on EFE activity and free ACC content in soybean cuttings

Treatment	EFE activity [nmol ethylene g^{-1}]	Free ACC content [nmol g^{-1}]
Water control for 24 h	13.7 ± 2.0	0.96 ± 0.31
1 mM salicylate for 24 h	13.3 ± 3.8	1.05 ± 0.38
Water control for 3 h	12.4 ± 0.6	0.60 ± 0.08
3 mM salicylate for 3 h	11.5 ± 1.4	1.17 ± 0.12*
3 mM salicylate + 0.5 mM AVG for 3 h	nd	0.43 ± 0.06

Cuttings were treated as indicated before determining EFE activity and free ACC content. Each value is the average of three repititions, each including 4 replicates. The value statistically different from the water control is asterisked (P = 1%).

found when cuttings were pre-incubated for 3 h in 3 mM salicylate (Table 6). No effect on TNV stress ethylene was encountered following 3 h incubation in 3 mM salicylate (Table 6).

DISCUSSION

Aqueous salicylate solution exogenously supplied to soybean cuttings stimulated basal ethylene only when injurious, or potentially injurious, concentrations were administered. Stimulation occurred via the biochemical sequence involving ACC as an intermediate and was attributable to a stimulation of ACC synthesis but not of EFE activity. Similar results were obtained by testing wound-induced ethylene

TABLE 5

Effects of 8-d salicylate treatment on growth, rooting and leaf chlorophyll content in soybean cuttings

Treatment	Growth [mg]	Rooting (N° of root primordia)	Chlorophyll (A_{665nm})
Water control	94 ± 21	3.7 ± 1.2	607 ± 42
1 mM salicylate	52 ± 19	0.6 ± 0.03*	535 ± 31*

Each value is the average of 12 replicates. Values statistically different from water control are asterisked (P = 1%)

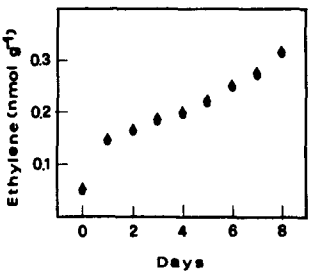

Fig. 1. Effect of salicylate treatment on basal ethylene production in soybean cuttings. Cuttings were treated for the indicated time with deionized water (controls; •), or 1 mM salicylate (▲). They were then incubated in sealed vials for 1 h, in the dark, before determining the released ethylene by gas chromatography. Each value is the average from 12 repetitions. Standard errors are within the limit of the symbols.

production which increased only if cuttings were incubated in potentially injurious concentrations. Such concentrations did not affect the production of TNV-induced ethylene, but this is not surprising considering that the TNV hypersensitive reaction incited a conspicuous production of ethylene, of about an order of magnitude higher than the putative increase due to salicylate.

TABLE 6

Effects of salicylate on production of stress ethylene

Stress	Treatment		Ethylene production [nmol h^{-1} g^{-1}]
	Concentration [mM]	Time [h]	
Wounding[a]	Water control	24	0.18 ± 0.05
	1		0.19 ± 0.04
	Water control	3	0.16 ± 0.02
	3		0.54 ± 0.08*
TNV[b]	Water control	24	0.04 ± 1.08
	1		4.17 ± 1.18
	3		4.38 ± 1.27

(a) Discs of 5 mm diameter were punched from cuttings treated as indicated. The discs were halved and incubated for 1 h sealed vials, in the dark, before determining ethylene. Each value is the average of 12 replicated. The result statistically different from the control is asterisked (P = 1%).
(b) The primary leaves of cuttings were TNV-inoculated. After 48 h, cuttings whose primary leaves had an equivalent number of necrotic local lesions (about 60 lesions per leaf) were incubated in the indicated solutions in open vials. The vials were then sealed and incubated for 1 h in the dark before determining ethylene. Each value is the average of 12 replicates.

These findings are in close agreement with previous results obtained for detached tobacco leaves (Antoniw and van Loon 1982), intact tobacco plants (Pennazio *et al.* 1985) and asparagus bean cuttings (Pennazio *et al.* 1987), and support the results of Meijer and Brown (1988) who worked with different plant materials. Salicylate did not in any instance inhibit ethylene biosynthesis.

In contrast, Leslie and Romani (1986) found that salicylates inhibited ethylene production by pear cell suspension cultures via an inhibitory effect on EFE activity. More recently, they (1988) concluded that inhibition was due to the effect of salicylates on some membrane-associated function. Salicylates can affect cell membranes by depolarizing the membrane potential (Glass and Dunlop 1974, Macri *et al.* 1986) but John *et al.* (1985) concluded that EFE activity was not dependent on membrane potential. Thus, the origin of the discrepancy between the results of Leslie and Romani (1986, 1988) and the above results remains difficult to explain. Leslie and Romani (1988) did not observe inhibition of ethylene production when incubating at neutral pHs, the condition adopted in our experiments. However, incubation of soybean cuttings in salicylate solutions at low pH had no additional effect on ethylene production, that was increased due to the acidic buffer alone.

Prolonged salicylate treatment inhibited the growth of soybean cuttings and prevented rooting. This was not unexpected because the allelopathic properties of salicylate on plant growth and development are well known (Harborne 1980, Rice 1984). In particular, the observed effect on rooting may be due to the slight capacity of the salicylates to inactivate indol-3-ylacetic acid (Lee and Skoog 1965) probably via a moderate stimulation of auxin oxidase activity (Lee *et al.* 1982). Although the antiauxinic activity of salicylates is rather low, prolonged treatments like these we have tested may result in the observed inhibition of rooting. It is interesting to note that Conti *et al.* (1988) have recently obtained similar results for *Datura* plants.

Since prolonged treatment inhibiting growth and rooting of the soybean cuttings did not affect ethylene biosynthesis, a direct involvement of this plant regulator in the observed events appears unlikely.

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