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Effects of Some Organic Solvents on Ethylene Evolution from Young Cotton Bolls

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ABSTRACT

The presence of promoter(s) of ethylene biosynthesis in young cotton (*Gossypium hirsutum* L.) fruits (bolls) was demonstrated by injection of an aqueous extract from bolls into other bolls and measurement of a 3-fold increase in rate of ethylene evolution. Injection of methionine did not affect rate of ethylene production, indicating that the promoter extracted from bolls was not methionine. Injection of the ethoxy analog of rhizobitoxine inhibited ethylene production, indicating that methionine is a precursor of ethylene in cotton bolls. Injection of organic solvents altered membrane permeability, as indicated by decreased resistance to electric current at 1,000 Hz, and stimulated ethylene evolution. The less polar solvents caused large increases in ethylene evolution, major loss of resistance, and visible evidence of membrane damage. The results support the hypothesis that membrane integrity affects rate of ethylene biosynthesis.

Ethylene production is increased by many stimuli, including insect injury, low temperature, water deficit, diseases, mechanical damage, and physical impedance (ref. 1 and papers cited therein). In addition, a decrease in net photosynthesis, detachment from the parent plant, and partial desiccation increased the rate of ethylene production in young cotton bolls (3, 4). Because of the diversity of these stimuli, a unifying mechanism of action for stimulation of ethylene biosynthesis is not readily apparent.

Membranes may play an important role in the regulation of ethylene synthesis. Osborne *et al.* (9) reported that an acidic, nonvolatile SF[2] diffused from senescent petioles and leaf blades, and that it could be extracted from healthy green leaves with ethanol. This SF promoted ethylene evolution and abscission when applied to healthy tissue. They postulated that SF is normally kept separate from sites of ethylene production by membrane compartmentation, and that wounding, senescence, or auxin could modify permeability of membranes and permit SF to leak out. They suggested that SF is ubiquitous in plants and that it functions *in vivo* as a regulator of ethylene production. Hanson and Kende (5), on the basis of a demonstrated enhancement by ethylene of ion and sucrose efflux, speculated that, "An ethylene-induced increase in tonoplast permeability could well allow mixing of the components of an ethylene-generating system." As they pointed out, such a mechanism could account for the autocatalytic properties of ethylene production.

Freezing disrupted membranes, caused leakage and mixing of cytoplasmic and vacuolar contents (14, 15), and stimulated ethylene production and abscission of citrus leaves (16). Chilling at 5 C was adequate to cause electrolyte leakage and increase ethylene production in bean leaves (13).

If a promoter of ethylene production is normally isolated from the site of ethylene synthesis by a membrane, then any condition which impairs membrane integrity should stimulate ethylene production. To test this hypothesis, I injected some lipid solvents of varying polarity into young cotton (*Gossypium hirsutum* L.) bolls and determined their effects on subsequent ethylene evolution. Electrical resistance of treated tissue was measured as an indication of membrane integrity.

MATERIALS AND METHODS

'Deltapine 16' cotton plants were cultured in a glasshouse in a 1:1:1 (v/v/v) mixture of peat, sand, and vermiculite. A complete nutrient solution (2) was added twice weekly, and deionized H_2O was added at other times as needed to prevent wilting. Bolls were harvested 5 days after anthesis and substances were injected in 25-μl portions with a syringe at the point of maximum boll diameter (13.9 mm average). A Teflon sleeve on the syringe needle limited penetration to 5 mm. The bolls were enclosed in polycarbonate centrifuge tubes and ethylene was collected and measured at intervals for up to 5.4 hr (3). The first measurement was made about 1 hr after injection and net changes were determined after this initial measurement. Electrical resistance was measured at 1,000 Hz (11) with a YSI model 31[3] conductivity bridge attached to an electrode made of two stainless steel needles set 5 mm apart in a block of plastic. Teflon sleeves limited penetration of the needles to 5 mm. Ethylene was not measured after electrical resistance measurement because of wounding caused by the electrode. In all tests in which substances were injected before ethylene measurements, 25-μl portions of water were added to control bolls to permit estimation of wound ethylene.

To test for the presence of a promoter of ethylene biosynthesis in cotton bolls, an extract from healthy bolls was injected into other bolls. The extract was obtained by slicing, freezing, thawing, and then homogenizing 5-day-old bolls in water at 25 C. Insoluble material was removed by centrifugation and the extract was concentrated to dryness *in vacuo*. The residue was dissolved in H_2O equivalent to half the fresh wt of bolls used. Half of the extract was heated for 5 min in a boiling water bath to test for heat stability. I then injected 25-μl portions of H_2O, boll extract, and heated boll extract and compared their effects on rate of ethylene production by injected bolls.

Organic solvents of various polarities were injected into bolls and their effects on ethylene production and electrical resistance

[1] In cooperation with the Arizona Agricultural Experiment Station.
[2] Abbreviation: SF:senescence factor.
[3] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

determined. In addition to organic solvents, some enzymes were also tested.

RESULTS AND DISCUSSION

Injection of boll extract increased the rate of ethylene evolution 3-fold over that of the water-injected controls. Boiled extract also gave a 3-fold increase, indicating that the promoter(s) of ethylene production in cotton bolls is (are) heat-stable. Rates of ethylene production during a 2.5-hr period were 2.1 ± 0.2, 6.2 ± 0.5, and 6.7 ± 0.7 nl/g·hr for bolls injected with water, boll extract, and boiled boll extract, respectively. Slow penetration of the plasmalemma may have limited the effectiveness of boll extract in promoting ethylene evolution unless ethylene biosynthesis occurs outside the plasmalemma, which seems unlikely. Nothing was added to improve penetration because such a treatment would probably also affect permeability of any membranes that normally isolate an *in situ* promoter of ethylene biosynthesis.

If a promoter of ethylene production is normally isolated from the site of ethylene synthesis by a membrane, treatment with an organic solvent should alter membrane permeability and increase ethylene evolution. Organic solvents did stimulate ethylene production and increased the leakiness of membranes, as indicated by changes in electrical resistance (Table I) and appearance. Methanol and ethanol caused little visible change, but less polar solvents caused injection sites to become discolored, soft, and watersoaked. The reddish discoloration may have been caused by leakage of anthocyanin from vacuoles. Because of their diversity, it seems unlikely that these organic solvents served as substrates for ethylene production. Their effectiveness was more likely related to the membrane damage they caused. Effectiveness was not always directly related to polarity. For example, 1-propanol and 1-butanol were more effective than 2-propanol and 2-butanol in causing membrane damage and in stimulating ethylene production even though they are slightly more polar than their isomers.

Other workers have also reported a stimulation of ethylene production by substances that should affect membranes. Solomos and Laties (12) reported that bromine, which should add to double bonds of unsaturated fatty acids of phospholipids and thus alter the physical characteristics of cellular membranes, and chloroform, an effective lipid solvent, both caused a rapid onset of the climacteric, ethylene production, and ripening of avocados and bananas. Lossner (8) recently reported that aqueous solutions of acetone, methanol, ethanol, 1-propanol, and 2-propanol caused the synthesis of ethylene in etiolated pea seedlings. He noted that the ability of the alcohols tested to stimulate ethylene production increased with increasing boiling points. Iwata and Stowe (6) found that certain long chain lipids with low polarity, which they termed oleanimins, stimulated respiration, growth, and ethylene production when applied with auxin. Although they suggested that the site of action was a membrane, they did not mention changes in permeability.

Table I. Ethylene Evolution and Electrical Resistance of Bolls after Injection of Organic Solvents with Different Polarities

Polarities are indicated by dielectric constants at 25 C. Each solvent was injected at 25 µl per boll. Resistance at 1,000 Hz was determined, after ethylene measurements, with electrodes spaced 5 mm apart and inserted 5 mm into the boll on each side of the site of solvent injection. Data are averages of eight or more replications and standard errors of the means are shown.

Solvent Injected	Dielectric Constant	Ethylene Evolution	Resistance of bolls
	ε	nl/g·hr	Ohms
		First test	
None	...	0.4 ± 0.04	4308 ± 107
Water	78.5	1.6 ± 0.1	3986 ± 181
Methanol	32.6	10.8 ± 3.0	3143 ± 235
Ethanol	24.3	14.7 ± 3.6	2653 ± 263
2-Propanol	18.3	19.1 ± 2.0	2694 ± 211
2-Methyl-1-propanol	17.7	63.3 ± 4.2	1100 ± 129
2-Methyl-1-butanol	14.7	38.1 ± 3.6	825 ± 65
		Second test	
Water	78.5	1.5 ± 0.2	4250 ± 133
Ethanol	24.3	4.8 ± 0.4	2821 ± 149
Acetone	20.7	8.9 ± 1.0	2954 ± 73
1-Butanol	17.1	34.6 ± 2.0	1075 ± 41
		Third test	
Water	78.5	0.8 ± 0.05	4450 ± 206
1-Propanol	20.1	35.4 ± 3.2	2088 ± 229
2-Propanol	18.3	6.1 ± 0.6	2656 ± 240
1-Butanol	17.1	33.7 ± 1.9	1188 ± 96
2-Butanol	15.8	12.2 ± 1.7	1731 ± 153

Some lipases and proteases were injected to see if they would cause membrane damage and stimulate ethylene production. The proteases tested had little effect, but lipase (EC 3.1.1.3, Sigma type VII) always stimulated ethylene production. Lipase did not cause a measurable increase in membrane permeability, however. To determine whether stimulation of ethylene production by lipase was caused by the enzyme itself or by a heat-stable impurity, I heated a lipase solution in a boiling water bath for 30 min, cooled it, and injected 25-µl portions into 5-day-old cotton bolls. The heated lipase was just as effective as the same concentration of unheated lipase in stimulating ethylene production. Either the enzyme was very heat-stable or the stimulation was caused by a heat-stable impurity. A purer lipase (Sigma type VI) with much greater activity was then tested. Unlike the less pure type VII lipase, type VI lipase caused no increase in ethylene production. Perhaps lipase (EC 3.1.1.3) does not attack membrane lipids. A test was then conducted with phospholipase A_2 (EC 3.1.1.4) which should attack membrane lipids. Injection of phospholipase A_2 apparently caused a very slight increase in ethylene production, but had no effect on membrane permeability. Because none of the enzymes tested caused measurable changes in electrical resistance of boll tissue, I concluded that: (*a*) they were unable to penetrate to membrane sites; or (*b*) they were inactivated in cotton boll tissue; or (*c*) they were not effective against membrane lipids.

Methionine is known to be a precursor for ethylene biosynthesis (1) and could be the promoter of ethylene production extracted from cotton bolls and released by organic solvents injected into bolls. To test this possibility, I injected 0, 25, 50, and 100 µg of methionine/boll in water and in 50% acetone. Methionine caused no stimulation of ethylene production either with water or with acetone. Several possible explanations could be given for the failure of methionine to stimulate ethylene production: (*a*) Methionine did not penetrate to the site of ethylene biosynthesis. (*b*) The amount of methionine already present in bolls is not rate-limiting. (*c*) Methionine is not the precursor of ethylene in cotton boll tissue.

To test the possibility that methionine is not a precursor of ethylene in cotton bolls, I injected L-2-amino-4-(2'-aminoethoxy)-*trans*-3-butenoic acid hydrochloride, the ethoxy analog of rhizobitoxine, in water and in 79% ethanol at 4 µg/boll. Because rhizobitoxine and its analogs inhibit the conversion of methionine to ethylene (7, 10), rhizobitoxine should inhibit biosynthesis of ethylene only if methionine is a precursor. The ethoxy analog of rhizobitoxine did inhibit ethylene biosynthesis during the period from 1 to 3.4 hr after injection, but did not inhibit ethylene production during the following 2 hr (Table II). Therefore, some, if not all, of the ethylene produced during the first period must have been produced from methionine. Rhizobitoxine probably inhibited ethylene biosynthesis only in the small volume of tissue into which it penetrated; it inhibited production of wound ethylene in water-injected bolls, but did not inhibit ethylene production in the remainder of the boll. Therefore, as soon as wound-ethylene production ceased the apparent inhibition of ethylene production by the rhizobitoxine analog also

Table II. Ethylene Biosynthesis with and without Rhizobitoxine

Four µg per boll of L-2-amino-4-(2'-aminoethoxy)-trans-3-butenoic acid hydrochloride, the ethoxy analog of rhizobitoxine, were injected into 5-day-old cotton bolls in water and in 79% ethanol. Ethylene contents of sealed containers were determined 1, 3.4, and 5.4 hr after injection. Data are averages of 12 replications and standard errors of the means are shown.

Time after Injection	Water	Water plus Ro 1/	Ethanol	Ethanol plus Ro 1/
hr	nl/g·hr	nl/g·hr	nl/g·hr	nl/g·hr
1 to 3.4	2.4 ± 0.2	0.7 ± 0.1	10.4 ± 1.6	3.5 ± 0.6
3.4 to 5.4	0.7 ± 0.1	0.7 ± 0.1	7.0 ± 0.6	8.0 ± 0.8

1/ The ethoxy analog of rhizobitoxine

ceased because of the relatively large amount of tissue not permeated by the inhibitor. The apparent loss of inhibition of ethanol-stimulated ethylene production may have been caused by differential permeation of tissue by rhizobitoxine and ethanol. Ethanol probably moved more rapidly through the tissue and penetrated more cells than rhizobitoxine. If so, this would explain the results obtained. The results suggest that methionine is a precursor of ethylene in cotton bolls, but was not the promoter of ethylene production extracted from bolls.

Although the results obtained by injecting organic solvents into bolls generally support the hypothesis that membrane integrity affects the rate of ethylene production, they do not prove that a promoter of ethylene biosynthesis is normally isolated by membrane compartmentation. One or more enzymes involved in ethylene biosynthesis, rather than a promoter, could be isolated or imbedded in a membrane. This possibility could explain the lack of stimulation of ethylene production by injected methionine, but would not explain stimulation by boll extract; or, the synthesis of ethylene-producing enzymes may be induced by organic solvents, as suggested for long chain lipids by Iwata and Stowe (6). Unlike the results I obtained with organic solvents, however, they found that vitamins E and K_1 stimulated ethylene production only when they also added auxin.

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