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Polyamines Inhibit Biosynthesis of Ethylene in Higher Plant Tissue and Fruit Protoplasts[1]

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AKIVA APELBAUM[2], ALAN C. BURGOON, JAMES D. ANDERSON, AND MORRIS LIEBERMAN
Postharvest Physiology Laboratory, Beltsville Agricultural Research Center (W), Beltsville, Maryland 20705
RUTH BEN-ARIE AND AUTAR K. MATTOO[3]
Department of Fruits and Vegetables Storage, Agricultural Research Organization, The Volcani Center, P. O. Box 6, Bet Dagan, Israel

ABSTRACT

Ethylene production in apple fruit and protoplasts and in leaf tissue was inhibited by spermidine or spermine. These polyamines, as well as putrescine, inhibited auxin-induced ethylene production and the conversion of methionine and 1-aminocyclopropane-1-carboxylic acid to ethylene. Polyamines were more effective as inhibitors of ethylene synthesis at the early, rather than at the late, stages of fruit ripening. Ca^{2+} in the incubation medium reduced the inhibitory effect caused by the amines. A possible mode of action by which polyamines inhibit ethylene production is discussed.

Attention has been focused on the role of polyamines in plant metabolism following studies implicating them as essential growth factors (16). Many reports have since appeared showing that polyamines can delay senescence of plant tissue. For example, exogenous application of polyamines retards the aging of leaf protoplasts (5, 6) and preserves Chl in thylakoid membranes (15) during dark-induced senescence of detached leaves. Also, polyamines inhibit development of RNase and protease activity in barley leaf discs (15). The mechanism by which polyamines exert these effects is unknown. These effects, however, are the opposite of those caused by the plant growth regulator, ethylene, which promotes senescence of many plant tissues (8).

We investigated the effect of polyamines on ethylene biosynthesis, partly because polyamines and ethylene derive from the same precursor, *S*-adenosylmethionine (1, 2, 16), and because of the possibility that polyamines regulate plant metabolism by influencing the biosynthesis of ethylene.

MATERIALS AND METHODS

Chemicals. ACC[4] was purchased from Calbiochem; putrescine-HCl, spermidine-HCl, and spermine-HCl came from Sigma; Biofluor liquid scintillation fluid came from New England Nuclear; and [^{14}C-3,4]L-methionine came from Research Products International Corp.[5]

Plant Material. Apple fruits (*Malus sp.* cvs. Golden Delicious and Anna) were harvested at the preclimacteric stage and stored at 0 C until used or ripened at 20 C. Discs (1.0 cm in diameter and 2 mm thick) were cut from fruits at various stages of ripening. Six discs (1 g) were incubated in a 25-ml Erlenmeyer flask containing 3 ml incubation medium consisting of 600 mM sorbitol in 10 mM phsophate (pH 7). In some cases, Tris-HCl (pH 7) was used.

Protoplast Isolation. Protoplasts were prepared from Golden Delicious fruit tissue. A 3-g sample of apple discs was incubated overnight in 9 ml 800 mM sorbitol containing 100 mM $CaCl_2$, 10 mM Mes (pH 6.0), 10 μg/ml chloramphenicol, 5 μg/ml Fungizone, and 5% (w/v) Cellulysin (3). The macerate was filtered through glass wool; protoplasts were allowed to settle for 20 min and then transferred with a Pasteur pipette to fresh medium without Cellulysin. Aliquots (1-ml) of protoplasts were placed in 25-ml flasks and brought to a final volume of 2.1 ml with the appropriate solutions, according to the treatments described in Table I.

Pinto beans (*Phaseoleus vulgaris* L.) and tobacco (*Nicotiana tabacum* I.) plants were grown in a greenhouse under natural lighting at temperatures ranging between 25 and 30 C. Fully expanded leaves were harvested and washed in running tap water. Leaf discs (1 cm diameter) were cut with a corkborer and floated abaxially down under light in Petri dishes containing 0.1 mM IAA or 1 mM ACC in water. This procedure is referred to as preincubation. At the end of the preincubation, the discs were transferred to 25-ml Erlenmeyer flasks containing IAA or ACC and various polyamines to be tested as described for each experiment.

Ethylene Determination. All of the Erlenmeyer flasks were flushed with air between successive determinations, and gas samples were withdrawn with airtight hypodermic syringes. Ethylene was determined by GC.

[^{14}C]Methionine Feeding Experiment. Apple discs (1 g) were incubated for 5 h in 125-ml flasks with 5 ml of a solution containing 5 μCi[^{14}C-3,4]methionine (40 mCi/mmol), 600 mM sorbitol, and 10 mM phosphate buffer (pH 7.0). A gas sample was withdrawn from each flask for ethylene determination. A second 3-ml gas sample was then withdrawn and injected into a scintillation vial (sealed with a rubber serum cap) containing 3 ml 0.1 M mercuric acetate at 0 C in methanol. Next, 10 ml Biofluor scintillation fluid was added to the vial, and radioactivity was

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[2] On leave from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

[3] Present address: Postharvest Physiology Laboratory, Horticultural Science Institute, Beltsville Agricultural Research Center, Beltsville, MD 20705.

[4] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid.

[5] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be available.

Table I. *Effect of Spermine and Spermidine on Ethylene Production by Apple Protoplasts*

Protoplasts were isolated from midclimacteric fruit and handled and treated as described in "Materials and Methods."

	Ethylene Production					
	0 to 2 h		2 to 4 h		4 to 20 h	
	nl/g	%	*nl/g*	%	*nl/g*	%
Control	30	100	23	100	81	100
Spermidine, 1 mM	26	86	22	98	68	83
Spermine, 1 mM	25	75	18	78	39	48

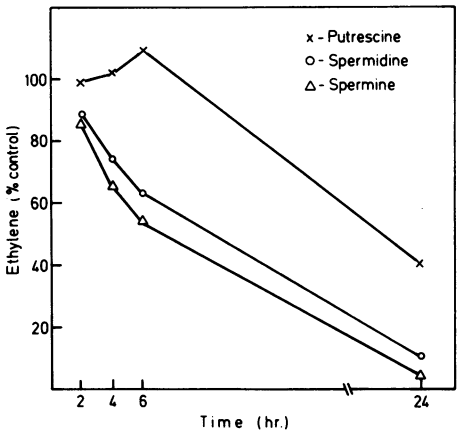

FIG. 1. Effect of diamine and polyamines on ethylene production by apple tissue. Discs from Golden Delicious apple fruits in midclimacteric were incubated in 25-ml flasks (6 discs weighing 1 g/flask) containing 600 mM sorbitol in 10 mM phosphate buffer (pH 7.0), 10 μg/ml D-chloramphenicol, and the amines (putrescine, spermidine, and spermine) at 1 mM. Rate of ethylene produced in control samples at 0 to 2, 2 to 4, 4 to 6, and 6 to 24 h were 45, 52, 55, and 37 nl/g·h, respectively.

assayed by scintillation spectrometry.

All experiments were repeated at least three times, with three replicates for each treatment. The data presented are the means of three replicates of a representative experiment.

RESULTS

Effect of Applied Diamine and Polyamines on Ethylene Production. The diamine and polyamines incorporated into the incubation medium markedly inhibited ethylene production in apple discs (Fig. 1) but did not affect respiration (data not shown). At least 4 h of incubation were needed for the polyamines to significantly inhibit ethylene production in the discs. The diamine, putrescine, was the least potent inhibitor (Figs. 1 and 2); at 0.1 mM, it had no effect, and, at 1 mM, it increased ethylene production slightly during the first 6 h before inhibiting production sharply after 24 h. In contrast, 1 or 10 mM of the polyamines spermidine and spermine inhibited ethylene production by 40 to 68% after 6 h and by 90 to 100% after 24 h.

The extent to which the polyamines inhibited ethylene production depended largely on the ripeness of the fruit (Fig. 3). After 6 h of incubation, 10 mM spermine inhibited ethylene production in slices from early climacteric fruit by 85%, in those from midclimacteric fruit by 65%, and in those from climacteric-peak fruit by only 50% (Fig. 3). Addition of 10 mM Ca^{2+} to the medium did not significantly change the rate of ethylene production in the control during 6 h of incubation (data not shown). However, the presence of Ca^{2+} greatly relieved the inhibitory effects of 0.1 and 10 mM spermine (Fig. 4) and spermidine (data not shown). In the presence of Ca^{2+}, putrescine at 10 mM was totally ineffective as an inhibitor

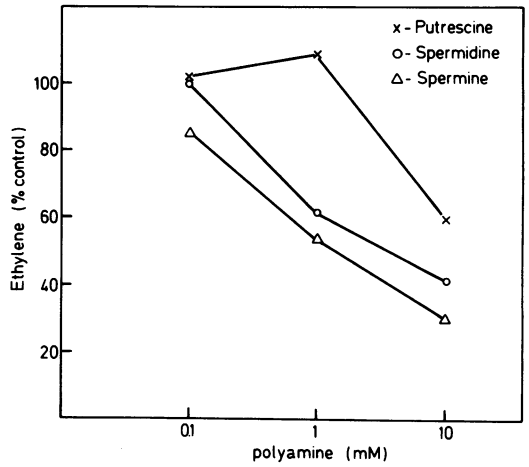

FIG. 2. Relation between concentration of putrescine, spermidine, and spermine and percentage inhibition of ethylene production in midclimacteric apple tissue. Methods and procedure were the same as described in legend to Figure 1. Rates of ethylene production were recorded after 6 h of incubation, when rate in control sample was 65 nl/g·h.

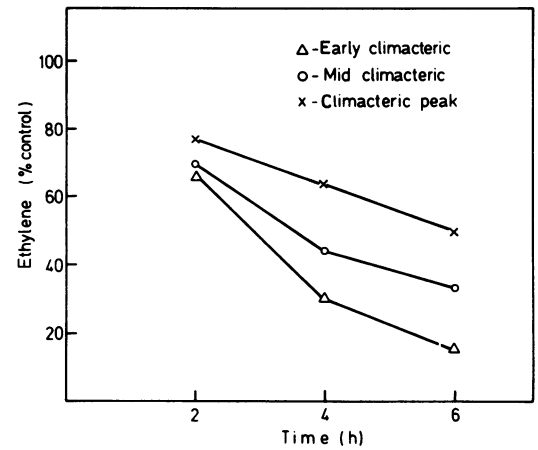

FIG. 3. Effect of spermine on ethylene production by apple fruits of different ripeness. Discs from preclimacteric, midclimacteric, or climacteric apple fruits (cv. Anna) were incubated in 25-ml flasks containing 600 mM sorbitol in 10 mM Tris-HCl (pH 7.0) in the absence and presence of 10 mM spermine. For those respective stages, ethylene production rates were 22, 155, and 242 μl/kg·h in whole apples and 15, 76, and 92 nl/g·h in control discs, as measured at 6 h.

(data not shown).

Spermine and spermidine also inhibited ethylene production in apple cortex protoplasts (Table 1). Between 4 and 20 h of incubation, spermine, the larger polyamine, was more effective in inhibiting ethylene production than was spermidine (52% *versus* 17%). The moderate inhibitory effect of polyamines on ethylene production by the protoplasts as compared to tissue slices was probably due to high Ca^{2+} (100 mM) content in the incubation medium; such high concentrations of Ca^{2+} are required for survival of apple protoplasts (3).

The conversion of [^{14}C-3,4]L-methionine to $^{14}C_2H_4$ in apple tissue was inhibited by 1 mM spermine or spermidine (Fig. 5). Inhibition was noticed within 2 h of incubation, and it increased gradually to about 60% during the next 4 h.

The diamine and polyamines also inhibited IAA-induced ethylene production in pinto bean leaf discs (Fig. 6) much the same as they did in apple tissue, without affecting CO_2 evolution. At 5 mM, both spermidine and spermine reduced ethylene production noticeably (about 20%) within 2 h of incubation and maximally

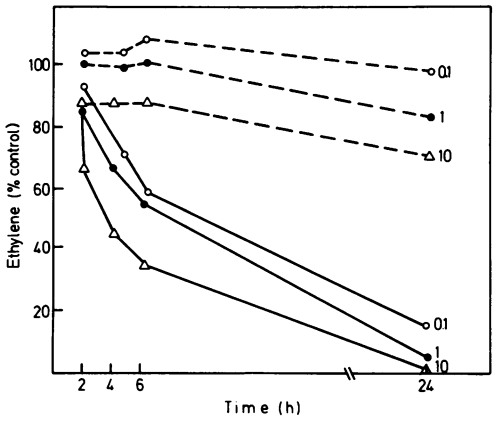

FIG. 4. Effect of Ca^{2+} on the inhibition of ethylene production in apple tissue by spermine. Spermine concentrations were 0.1 mM, (○); 1 mM, (●); or 10 mM, (▲); with (- - -) or without (——) 10 mM $CaCl_2$. Rate of ethylene produced in control samples between 0 to 2, 2 to 4, 4 to 6, and 6 to 24 h was 50, 55, 64, and 42 nl/g·h, respectively.

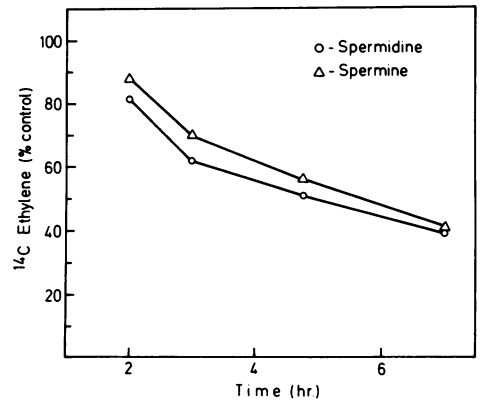

FIG. 5. Effect of spermidine and spermine on the conversion of [^{14}C-3,4]methionine to labeled ethylene in apple tissue. Golden Delicious apple discs were incubated in 25-ml flasks (6 discs weighing 1 g/flask) containing 600 mM sorbitol in 10 mM phosphate buffer (pH 7), 5 μCi [^{14}C-3,4]L-methionine (49 mCi/mmol), and 1 mM spermidine or spermine.

after 6 h (about 80%). Putrescine was much less inhibitory, as previously noted. In most cases, prolonging the incubation to 24 h only slightly increased the inhibitory effect of the polyamines and partially reversed that of putrescine.

The conversion of ACC to ethylene in tobacco leaf discs was stimulated between 10 and 30% by putrescine, spermidine, and spermine, when applied at 0.1 mM (data not shown). However, increasing the concentration of spermine or spermidine to 1 mM resulted in a 20 to 30% inhibition in 4 h and 50 to 60% inhibition in 24 h (Fig. 7). At 10 mM, both of these polyamines severely inhibited ethylene production by about 90% in 24 h (Fig. 7). Putrescine was inhibitory only at 10 mM.

DISCUSSION

This study demonstrates that polyamines inhibit ethylene biosynthesis in apple fruit slices and protoplasts and IAA-induced ethylene production in pinto bean leaf discs. One of the reasons for this inhibition could be charge neutralization of the plant membrane, since high concentrations of Ca^{2+} prevented or partly reversed the polyamine effect. However, calcium by itself had no effect on ethylene production in apple tissue during a 6-h incubation period, by which time polyamine inhibition was already evident.

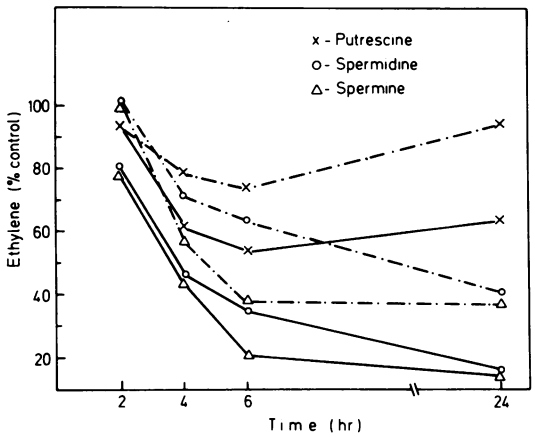

FIG. 6. Effect of a diamine and polyamines on auxin-induced ethylene production. Pinto bean leaf discs were preincubated for 1 h in light with 0.1 mM IAA, and then the indicated amines were added at concentrations of 1 mM (-·-·-) and 5 mM (——). Further incubation was carried out in the dark. Gas samples were withdrawn periodically, and ethylene content was determined by GC. Control values minus IAA were negligible (1 nl/g·h); with IAA, control values were 40, 34, and 20 nl/g·h at 2 to 4, 4 to 6, and 6 to 24 h, respectively.

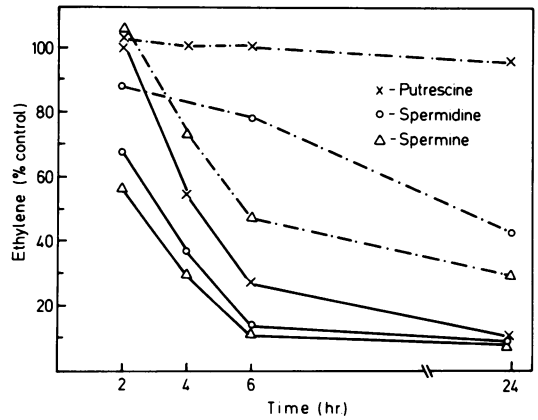

FIG. 7. Effect of a diamine and polyamines on ACC-induced ethylene production in tobacco leaves. Tobacco leaf discs (6/flask) were preincubated in light in 25-ml flasks for 1 h with 1 mM ACC, and then the indicated amines were introduced to the media at concentrations of 1 (-·-·-) or 10 mM (——). The flasks were sealed with serum caps and incubated in the dark, and gas samples were withdrawn periodically. Rate of ethylene produced in control samples with 1 mM ACC was 57, 51, 59, and 177 nl/g·h at 0 to 2, 2 to 4, 4 to 6, and 6 to 24 h, respectively.

The polyamine effect may be directed at the step for converting ACC to ethylene (1). This step appears to be associated with a particulate fraction of the cell (7, 11). The fact that ethylene production *in situ* (particularly the ACC-to-ethylene step) is susceptible to osmotic shock (4, 12, 14), cold shock (4, 14), lipophilic membrane perturbants (4, 9–11, 14), and ionic and nonionic detergents (4, 1, 12, 14) indicates involvement of a membrane function in ethylene biosynthesis. Therefore, polyamines may interact with the plant membranes in a way that inhibits ethylene evolution by a physical mechanism. Polyamines may bind ionically to membrane targets, causing conformational changes that impair the functionality of the ethylene-synthesizing system. This hypothesis appears to be incompatible with a report (6) that polyamines stabilize the integrity of membranes and, thus, may enable them to retain their functions, including ethylene production. However, this contradiction may not be real if a specific membrane microenvironment is required for ethylene biosyn-

thesis, as suggested earlier (4, 9–12, 14), and if polyamines alter that microenvironment.

On the other hand, although ethylene biosynthesis seems to require membrane stability and integrity, ripening (climacteric) fruits, whose membranes are presumably undergoing dynamic changes (13, 17), sharply increase their ethylene production. Moreover, the inhibiting effect of polyamines on ethylene production is more pronounced at the early stages of ripening (Fig. 3), *i.e.* before ethylene production has entered its presumed autocatalytic stage. Therefore, changes in, or degradation of, certain membrane components or compartments specific for polyamines may lead to less effectiveness of polyamines during progressive senescence as inhibitors of ethylene production.

The reduced ethylene production by polyamine-treated tissues seen in our study may have been (a) due to a general nonspecific effect of polyamines on cellular membranes; (b) directly responsible for retarding senescence; or (c) a consequence of retarded senescence.

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