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Inhibition of Ethylene Production in Fruit Slices by a Rhizobitoxine Analog and Free Radical Scavengers

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JAMES E. BAKER, MORRIS LIEBERMAN, AND JAMES D. ANDERSON
United States Department of Agriculture, ARS, AMRI, Postharvest Plant Physiology Laboratory, Beltsville, Maryland 20705

ABSTRACT

The rhizobitoxine analog, L-2-amino-4-(2-aminoethoxy)-*trans*-3-butenoic acid (Ro), which effectively inhibits ethylene production in apple (*Malus domestica Borkh.*) and other tissues at concentrations at about 68 micromolar, inhibited ethylene production by about 50 to 70% in green tomato (*Lycopersicon esculentum* Mill.) fruit slices but only by about 15% in pink and ripe tomato tissue slices. Ethylene production in climacteric-rise and postclimacteric avocado slices was likewise relatively insensitive to 68 micromolar Ro. At 340 micromolar Ro, inhibition of ethylene production increased up to 50% in pink tomato slices, whereas 680 micromolar Ro was required to inhibit ethylene production by 30% in avocado slices. Incorporation of ^{14}C from [^{14}C]methionine into ethylene in green and pink tomato tissues was inhibited by Ro to about the same extent as inhibition of total ethylene production. Results thus far are inconclusive as to the mechanism of Ro resistance in tomato and avocado tissues. At 1 millimolar, free radical scavengers such as benzoate, propyl gallate, nordihydroguaiaretic acid, and to a lesser extent, eugenol, inhibited ethylene production in both Ro-sensitive (green tomato and apple) tissues and Ro-resistant (pink tomato and avocado) tissues. Therefore, free radical steps are suggested in the ethylene-forming systems.

Since Lieberman *et al.* (6) showed that ethylene was derived from carbon atoms 3 and 4 of methionine in apple slices, evidence has accumulated that methionine is the natural precursor of ethylene in several plant tissues (2, 11). Despite this evidence, the exact reaction mechanism of the conversion of methionine to ethylene has not been established. With the discovery that rhizobitoxine inhibits ethylene production from methionine (11), a tool was provided for probing the pathway(s) of ethylene biosynthesis in plant tissues. Rhizobitoxine was found to inhibit effectively ethylene production of apple and banana slices, pea seedlings, tobacco leaves, sorghum seedlings (11), and flower tissues (5); the assumption was that methionine was the major precursor of ethylene in those tissues. Rhizobitoxine and certain of its analogs (Ro)[1] inhibit ethylene production irreversibly, presumably by binding to an enzyme involved in the first step of the methionine to ethylene pathway (7). One purpose of the present investigation was to extend study of the rhizobitoxine effect of other fruits and to determine whether sensitivity of the ethylene-producing system to rhizobitoxine varies with the different stages of maturation and senescence. A second purpose was to determine whether free radical scavengers inhibit ethylene production *in vivo,* as they have been shown to do in a model system which converts methional to ethylene following hydroxyl radical attack (4). A preliminary report of this work was published earlier (1).

MATERIALS AND METHODS

Plant Material and Procedure. Tomato (*Lycopersicon esculentum* Mill., cvs. Rutgers, Cal Ace, and Walter) and apple (*Malus domestica* Borkh., Red Delicious) fruit were grown at the Beltsville Agricultural Research Center, and unknown varieties of tomato were purchased from a local wholesale market. The responses of tomato fruit slices to inhibitors of ethylene production appeared to be independent of variety. Avocado (*Persea gratissima* Gaertn.) fruit were from a local market or provided by Calavo Growers of California. Tomato pericarp tissue was cut into about 0.5-cm cubes; avocado and apple tissues were cut into discs about 0.3 cm thick and 1 cm in diameter. Tissue samples (2 g) were incubated in 25-ml stoppered Erlenmeyer flasks containing 3 ml of 0.4 M sucrose in 0.027 M citrate-0.047 M Na-phosphate buffer (pH 4.6). The rhizobitoxine analog, sodium benzoate, gallic acid, and propyl gallate stock solutions were made up in that buffer. Nordihydroguaiaretic acid and eugenol solutions were prepared with ethanol, and the final concentration of ethanol in the incubation flasks was 1.5%. The flasks were incubated at 25 C, and gas samples were removed for ethylene analysis by the method of Meigh *et al.* (10).

For measurement of [^{14}C]ethylene derived from L-[3,4-^{14}C]methionine 3 g of tomato tissue and 5 ml of the sucrose-citrate-phosphate medium were incubated in 25-ml Erlenmeyer flasks stoppered with serum caps. Each flask contained 1.9 μCi of L-[3,4-^{14}C]methionine, 4.5 μCi/μmol. As much of the gas phase as possible was withdrawn into a 50-ml air-tight syringe twice at 2-hr intervals; thus, about 90% of the gas phase was withdrawn by this method as determined experimentally using ethylene standards. The gas samples were introduced into evacuated 50-ml Erlenmeyer flasks that contained 3 ml of 0.1 M mercuric acetate in glass-distilled methanol. Ethylene was absorbed in the mercuric acetate solutions; and after 1 hr these were transferred to liquid scintillation vials containing Aquasol[2] for counting. No detectable quantities of other hydrocarbon gases were produced from [3,4-^{14}C]methionine in the tomato system.

Chemicals. The analog of rhizobitoxine, L-2-amino-4-(2-aminoethoxy)-*trans*-3-butenoic acid, was a gift from Hoffman-La-Roche Inc. Sodium benzoate, propyl gallate, gallic acid, and eugenol were products of Eastman Kodak Co.; nordihydroguaiaretic acid was a product of Sigma Chemical Co.; and L-[3,4-^{14}C]-methionine was obtained from New England Nuclear. Methanol used as a solvent for mercuric acetate, was obtained from Burdick and Jackson Laboratories, Inc.

RESULTS AND DISCUSSION

Effects of Ro on Ethylene Production of Fruit Slices. In apple slices the ethoxy analog of rhizobitoxine is known to be a potent inhibitor of ethylene production from both enodgenous substrate

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[1] Abbreviations: Ro: ethoxy analog of rhizobitoxine; Inhib.: inhibition.

[2] The mention of trade names or manufacturers is made for the purpose of identification and does not imply endorsement by the U.S. Government.

and exogenous [^{14}C]methionine (11). This compound inhibits ethylene production of both preclimacteric and climacteric apple slices (unpublished data). Furthermore, ethylene derives from [^{14}C]methionine in apple tissues (6). Consequently, we conclude that methionine is the unequivocal precursor of ethylene in apples and that Ro inhibits ethylene production from methionine in this "classical" ethylene-forming system. In the present investigation Ro at 68 μM inhibited ethylene production in green tomato slices 50 to 69%, but had little effect (11-13% inhibition) in slices from climacteric (pink) and postclimacteric (red) fruit (Table I). These percentages are typical, but in one test out of dozens the inhibition by 68 μM Ro of ethylene production in pink tomato slices was somewhat higher and the inhibition in green tomato slices was a little lower (Fig. 1).

Ethylene production in avocado slices was likewise relatively insensitive to Ro. Our standard concentration of Ro (68 μM) did not significantly affect ethylene production over a period of 4 hr, and a concentration of 680 μM inhibited only 13 to 29% (Table II). Baur *et al.* (3) showed that climacteric-rise and climacteric avocado fruit tissue converted [^{14}C]methionine to [^{14}C]ethylene. If methionine is the precursor of ethylene in avocado fruit, then why did not Ro effectively inhibit ethylene production? The relative insensitivity of pink or red tomato slices and avocado slices to Ro could reflect an alternate pathway for ethylene production—one not involving methionine or one involving methionine but in a different pathway—or a change in enzyme sensitivity to Ro, among other possibilities.

Among other possibilities, Ro may be metabolized in pink tissue to a greater extent than in green tissue, or Ro may not be taken up as well by pink tissue as by green tissue. Pink tomato tissue was incubated in solutions containing Ro for 4 hr, and then removed and washed with Ro-free medium. The incubation medium strongly inhibited ethylene production by apple slices (data not presented). Also, extracts of Ro-treated tissue and the incubation medium yielded a compound that co-chromatographed with Ro, and which was not present in extracts of control tissue or control incubation medium (data not shown). The results indicate that Ro is taken up by pink tomato tissue, and at least a large portion of Ro remains in the incubation medium. Likewise, vacuum infiltration of pink tomato and avocado tissue with Ro solutions did not increase inhibition of ethylene production, indicating that Ro resistance of these tissues was not due to lack of Ro uptake.

Influence of Ro on Incorporation of ^{14}C from [^{14}C]Methionine into Ethylene by Tomato Tissue. We investigated the nature of the lower Ro sensitivity of pink tomato slices by comparing rates of [^{14}C]ethylene production of pink and green tomato slices incubated in [^{14}C]methionine. The rates of [^{14}C]ethylene production from [^{14}C]methionine, like total C_2H_4, were higher in pink tissue than in green tissue (Fig. 1, A and D). The rates of total and [^{14}C]ethylene production from green tissue increased through the incubation period with [^{14}C]ethylene increasing faster than total ethylene. The continuous increase in specific radioactivity of ethylene with time (Fig. 1G) suggests nonsaturation of the methionine pool used for ethylene synthesis in green tomato tissue. The smaller increase in specific radioactivity of ethylene from pink tissues, as well as the lower specific radioactivity of ethylene, suggest that the methionine pool for ethylene production is larger and easier to saturate in pink fruit than in green fruit.

Table I. Inhibition of Ethylene Production in Tomato Slices by the Ethoxy Analog (Ro) of Rhizobitoxine, Benzoate, Propyl Gallate, and Combinations of the Inhibitors

Treatment	Stage of Fruit					
	Mature Green		Pink		Red	
	C_2H_4 nl/g-hr[1]	% Inhib.	C_2H_4 nl/g-hr	% Inhib.	C_2H_4 nl/g-hr	% Inhib.
Control	6.9 ± 0.23		19.0 ± 0.63		10.2 ± 1.44	
1mM Benzoate	2.42 ± 0.46	64.9	5.92 ± 0.92	68.8	1.67 ± 0.23	83.6
68 μM Ro	2.13 ± 0.35	69.1	16.9 ± 1.15	11.1	8.86 ± 0.63	13.1
1mM Benzoate + 68 μM Ro	0.98 ± 0.06	85.8	4.08 ± 0.92	78.5	1.55 ± 0.12	84.8
1mM Propyl Gallate	1.84 ± 0.12	73.3			1.38 ± 0.23	86.5
1mM Propyl Gallate + 68 μM Ro	0.69 ± 0.06	90.0			1.27 ± 0.17	87.5

[1] Rates of ethylene production were measured after a 2-hr incubation period, and values represent averages of three samples with standard errors. Likewise, values given in subsequent tables and figures represent means of three samples.

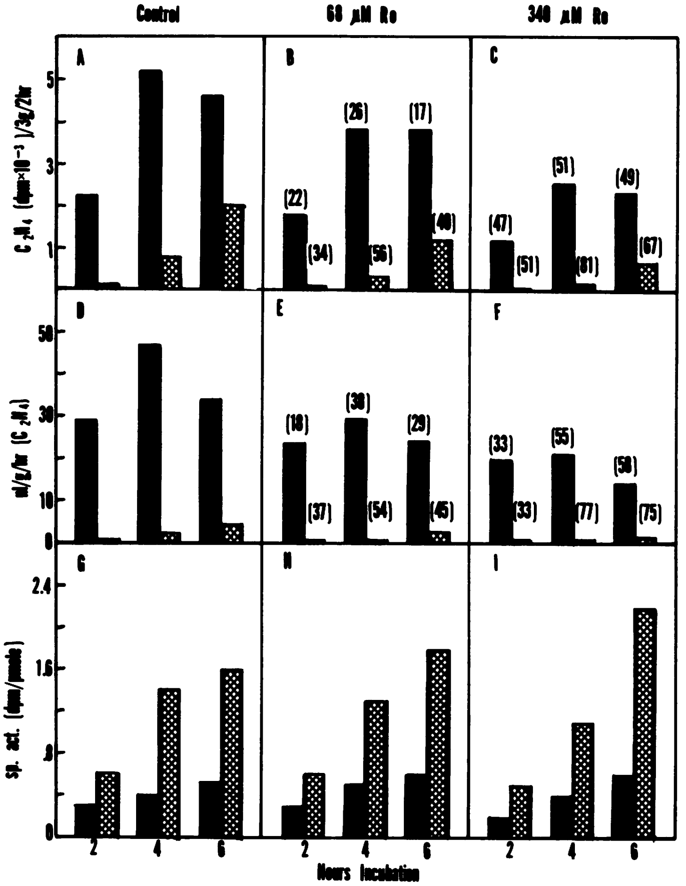

FIG. 1. Influence of Ro on ethylene production and ^{14}C incorporation from L-[3,4-^{14}C]methionine into ethylene in tomato tissue. (■): values for pink tissue; (▨): values for green tissue. Figures in parentheses over the bars indicate per cent inhibition. Radioactivity in ethylene from controls (A), 68 μM Ro-treated tissues (B), and 340 μM Ro-treated tissues (C). Total ethylene production by control tissues (D), 68 μM Ro-treated tissues (E), and 340 μM Ro-treated tissues (F). Specific radioactivity of ethylene from controls (G), 68 μM Ro-treated tissues (H), and 340 μM Ro-treated tissues (1).

Table II. Influence of Ro and Free Radical Scavengers on C_2H_4 Production in Avocado Slices

Treatment	Time (hr)			
	2		4	
Climacteric-rise tissue	C_2H_4 nl/g-hr	% Inhib.	C_2H_4 nl/g-hr	% Inhib.
Control	1.6 ± 0.1		4.8 ± 0.3	
68 μM Ro	1.7 ± 0.04	(6.2)	4.8 ± 0.3	
680 μM Ro	1.6 ± 0.05		4.2 ± 0.2	12.6
Control	2.5 ± 0.3			
1mM benzoate	0.5 ± 0.2	80.0		
1mM propyl gallate	0.3 ± 0.1	88.0		
Postclimacteric tissue				
Control	0.7 ± 0.06		2.4 ± 0.3	
68 μM Ro	0.8 ± 0.05	(1.4)	2.5 ± 0.2	(4.2)
680 μM Ro	0.7 ± 0.05		1.7 ± 0.1	29

[1] Percentages in parentheses indicate values higher than that of the control. The data for benzoate and propyl gallate effects were from a different experiment than that for Ro effects.

Ro reduced incorporation of ^{14}C from methionine into ethylene, and total ethylene production (Fig. 1, B, C, E and F) about equally in the respective tissues, but the over-all patterns of ethylene production with respect to time were similar to those of the controls (Fig. 1, A and D). However, Ro was considerably more effective in inhibiting both total ethylene production and ^{14}C incorporation into ethylene in the green than in the pink tissue. Possibly the difference in sensitivity of green and pink fruit to Ro is simply explained by competitive action of a larger methionine pool, as suggested above, for ethylene production in the pink fruit. However, one cannot rule out at this time the formation during ripening of a second pathway utilizing methionine that differs in sensitivity to Ro, a change in enzyme sensitivity, or other factor such as altered uptake or metabolism of Ro.

Free Radical Scavengers and Ethylene Production. The free radical scavengers, benzoate and propyl gallate, at 1 mM, effectively inhibited ethylene production in tomato, avocado, and apple slices; and in tomato slices these compounds, unlike Ro, did not lose effectiveness with increasing ripeness of the tissue (Table I). Benzoate was effective only at acid pH in avocado and apple slices, perhaps reflecting better entry of the undissociated acid than the anion into those tissues. Although the magnitude of inhibition of ethylene production by these compounds varied from experiment to experiment, 1 mM benzoate and propyl gallate inhibited ethylene production in tomato and avocado by as much as 80 to 88% during a 2-hr incubation (Tables I and II). Benzoate and propyl gallate inhibited ethylene production by apple tissue about 50 and 62%, respectively, during a 4-hr incubation (data not shown). Propyl gallate or benzoate in combination with Ro produced a stronger inhibition of ethylene production in green or pink tomato tissue than did Ro or the free radical scavengers alone (Table I).

The effective concentration range for benzoate and propyl gallate to inhibit ethylene production in tomato tissue was 1 mM and above (Table III). We used 1 mM routinely in this study because higher concentrations substantially inhibited respiration. At 1 mM, benzoate and propyl gallate inhibited respiration 30% or less during 2 hr of incubation (data not shown). At 0.5 mM and below, the inhibitory effects of these compounds on ethylene production dropped markedly. At 0.1 mM, benzoate and propyl gallate slightly, but consistently, stimulated ethylene production (Table III).

In some experiments tomato slices were incubated with benzoate and propyl gallate (1 mM) for 2 hr and then washed and incubated for 4 more hr in fresh medium without the inhibitors. The benzoate-treated tissue continued to exhibit low rates of ethylene production, whereas the propyl gallate-treated tissue showed a 14.6% stimulation of ethylene production (Table III). This suggests that benzoate or a metabolite of benzoate cannot be washed out of the tissue, is not metabolized to an inactive form, or is bound very tightly to the ethylene-forming system. On the other hand, propyl gallate either diffuses out of the tissue or is metabolized so that its effectiveness is approximately that of a 0.1 mM concentration level (Table III). Two other free radical scavengers, *i.e.* nordihydroguaiaretic acid, and to a lesser extent, eugenol, inhibited ethylene production in apple and tomato slices (data not shown). Gallic acid did not inhibit ethylene production in apple and tomato tissues.

Table III. Effects of Various Concentrations of Benzoate and Propyl Gallate on Ethylene Production of Pink Tomato Slices

Treatment	Time (hr) of Incubation 2 C_2H_4 nl/g-hr	2 % Inhib.	6 C_2H_4 nl/g-hr	6 % Inhib.
Control	13.0 ± 0.3		13.7 ± 1.2	
Benzoate, 2mM	3.8 ± 0.4	70.8	1.3 ± 0.3	90.5
Benzoate, 1mM	7.2 ± 0.5	44.6	4.9 ± 0.5	64.2
Benzoate, 0.5mM	12.9 ± 0.2	0.8	11.6 ± 0.9	15.3
Benzoate, 0.1mM	14.0 ± 0.5	(7.7)	15.7 ± 0.7	(14.6)
Propyl gallate, 2mM	3.3 ± 0.05	74.6	2.6 ± 0.2	81.0
Propyl gallate, 1mM	5.3 ± 0.7	59.2	11.3 ± 1.0	17.5
Propyl gallate, 0.5mM	9.5 ± 0.4	26.9	16.2 ± 0.5	(18.2)
Propyl gallate, 0.1mM	14.3 ± 0.4	(10.0)	16.2 ± 0.7	(18.2)
Benzoate, (1mM) removed after 2 hr[1]	7.6 ± 0.7	41.5	6.8 ± 0.5	50.4
Propyl gallate, (1mM) removed after 2 hr[1]	5.1 ± 0.4	60.8	15.7 ± 1.05	(14.6)

[1] Media containing benzoate or propyl gallate were removed from the tissue, the tissue was washed with 10 ml fresh medium, and then incubated in 3 ml of the latter for measurement of ethylene production 4 hr later. Percentages in parentheses indicate values higher than that of the control.

Our results on the effect of free radical scavengers suggest that *in vivo* ethylene production by both Ro-sensitive and Ro-insensitive tissues involves free radicals. Free radical mechanisms have been proposed for ethylene and ethane production in various model systems (4, 8, 9, 12, 13). Benzoate inhibited ethylene production from methional in a model system apparently by scavenging hydroxyl radicals (4).

We have shown that tomato (pink and ripe) and avocado tissues do not behave like apple, certain flower, and other tissues that produce ethylene from methionine via Ro-sensitive pathways. Whether or not there are substrates other than methionine, or different pathways for ethylene production from methionine, or other factors involved in Ro resistance in these tissues is not known. While the ethylene-producing systems of ripe tomato and avocado differ from that of apple in regard to Ro sensitivity, they are similar to that of apple in their responses to free radical scavengers. This suggests that free radical steps are universally involved in ethylene-producing systems.

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