Plant Physiol. (1971) 48, 1–4

Inhibition of Ethylene Production by Rhizobitoxine

Received for publication December 14, 1970

L. D. Owens, M. Lieberman, and A. Kunishi
United States Soils Laboratory, Soil and Water Conservation Research Division, and Pioneering Research Laboratory, Market Quality Research Division, United States Department of Agriculture, Agriculture Research Service, Plant Industry Station, Beltsville, Maryland 20705

ABSTRACT

Rhizobitoxine, an inhibitor of methionine biosynthesis in *Salmonella typhimurium*, inhibited ethylene production about 75% in light-grown sorghum seedlings and in senescent apple tissue. Ethylene production stimulated by indoleacetic acid and kinetin in sorghum was similarly inhibited. With both apple and sorghum, the inhibition could only be partially relieved by additions of methionine. A methionine analogue, α-keto-γ-methylthiobutyric acid, which has been suggested as an intermediate between methionine and ethylene, had no effect on the inhibition.

Incorporation of ^{14}C from added methionine-^{14}C into ethylene was curtailed by rhizobitoxine to about the same extent as was ethylene production. These results suggest that rhizobitoxine interferes with ethylene biosynthesis by blocking the conversion of methionine to ethylene and not indirectly by inhibiting the biosynthesis of methionine. Ethylene production by *Penicillium digitatum*, a fungus which produces ethylene via pathways not utilizing methionine as a precursor, was not affected by rhizobitoxine.

Two model systems for the generation of ethylene in plant tissues have been described by Lieberman and co-workers, one utilizing methionine as a substrate (8), and the other utilizing linolenate (11). In addition, methionine can serve as a precursor of ethylene in plant tissues (2, 7). To help assess the physiological importance of methionine as an ethylene precursor, a specific inhibitor of methionine biosynthesis was sought. Rhizobitoxine appeared to offer that potential.

Rhizobitoxine is a phytotoxin produced by certain strains of the soybean root nodule bacterium *Rhizobium japonicum* (15). It inhibits greening of new leaf tissue of many plants and causes the main visual symptom of the disease in soybean known as rihizobial-induced chlorosis (14). The precise structure of rhizobitoxine remains to be elucidated; however, it is known to be a basic sulfur-containing amino acid which yields an ether derivative of homoserine upon desulfurization (13). Rhizobitoxine inhibits the growth of *Salmonella typhimurium* by inhibiting β-cystathionase, an enzyme in the methionine biosynthetic pathway (12). It also irreversibly inactivates β-cystathionase isolated from spinach leaves (4); however, the physiological effect of this lesion on the biosynthesis of methionine in spinach has yet to be assessed.

We report here that rhizobitoxine inhibits ethylene biosynthesis in sorghum seedlings and in senescent apple tissue by the unexpected mechanism of blocking the conversion of methionine to ethylene.

MATERIALS AND METHODS

Sorghum Experiments. Seeds of *Sorghum vulgare* var. Hegari were surface-sterilized by wetting with ethanol and then immersing in an aqueous solution of 0.2% $HgCl_2$ + 1% HCl for 2 min. After rinsing well, the seeds were germinated on moist filter paper in a Petri dish at 27 C in the dark. Two days after imbibition, the seedlings were transplanted to 50-ml Erlenmeyer flasks constructed with a side arm to collect CO_2. Six seedlings per flask (about 300 mg fresh wt) were supported on a nylon mesh screen held 1.0 cm above the flask bottom by three glass cylinders and 0.3 cm above the surface of the root solution (10 ml of water). The loosely capped flasks were placed in the dark at 27 C overnight, after which the treatments were added. One milliliter of 10% KOH was added to the side arm, and the flasks were closed with a rubber serum stopper and placed under a fluorescent lamp in the laboratory (about 250 ft-c) at about 25 C and 14-hr day length.

At daily intervals, O_2 was added to restore flasks to near atmospheric pressure with a gas-tight syringe. Three milliliters of gas were then withdrawn (replaced with air) for assaying ethylene. The total gas volume of the assembled flask system was 50 cc. Treatments were replicated three times and each experiment was performed at least twice except for the experiment with *P. digitatum*.

Apple Tissue Experiments. Apple tissue samples were prepared from postclimacteric Stayman apples stored 6 to 9 months at 0 C or Summer Rambeau apples stored 2 weeks at 0 C and 1 week at 21 C. One apple was used for each experiment. Tissue plugs (1.0 cm in diameter, cut with a cork borer from 0.5-cm thick slices, and weighing approximately 1.5 g) were placed in 25-ml Erlenmeyer flasks containing 5 ml of solution and a small vial of KOH to absorb CO_2. The incubation solution contained 0.4 M sucrose, 0.1 M $NaHCO_3$, pH 8.5, and 0.05 mM EDTA. The flasks were sealed with serum stoppers and incubated in a shaker water bath at 30 C.

Tracer Experiments with Apple Tissue. L-Methionine-^{14}C was added to the experimental system described above. After incubation at 30 C for 5 hr, a 2-ml aliquot of the flask atmosphere was removed for ethylene analysis by gas chromatography. Then 86% of the remaining gas was removed with a gas-tight syringe, the ethylene was absorbed in cold mercuric perchlorate, and the radioactivity was determined by liquid scintillation counting as described (9).

The radiochemical purity of L-methionine-^{14}C(UL) (New England Nuclear Corp.) used in experiment 2 of Table II was determined by thin layer chromatography (cellulose) of a sample diluted with carrier L-methionine. Butanol-acetic acid-water (12:3:5 v/v) was used as the developing solvent, after which the cellulose was scraped off and the radioactivity was determined by placing directly in Bray's liquid scintillation solution. Only 10% of the radioactivity remained as methionine after storage in 50% ethanol for 6 months at −20 C.

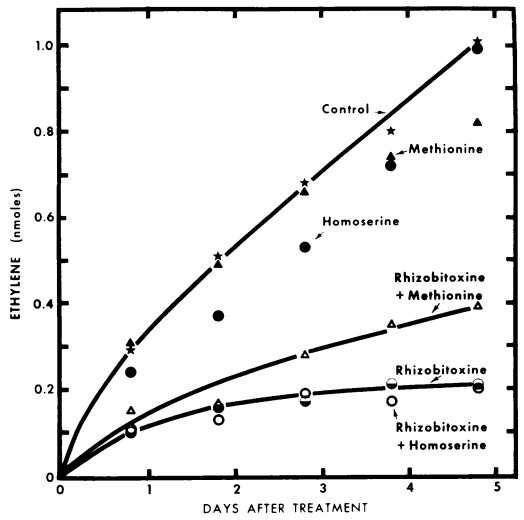

FIG. 1. Rhizobitoxine inhibition of ethylene production by sorghum seedlings. Rhizobitoxine concentration was 10 μM (41 μg). L-Methionine and L-homoserine treatments consisted of daily additions (with a syringe) of 10 μmoles of each compound to provide a minimum daily concentration of 1 mM. Root solution volume was 10 ml. Each value plotted is the average of three replicates.

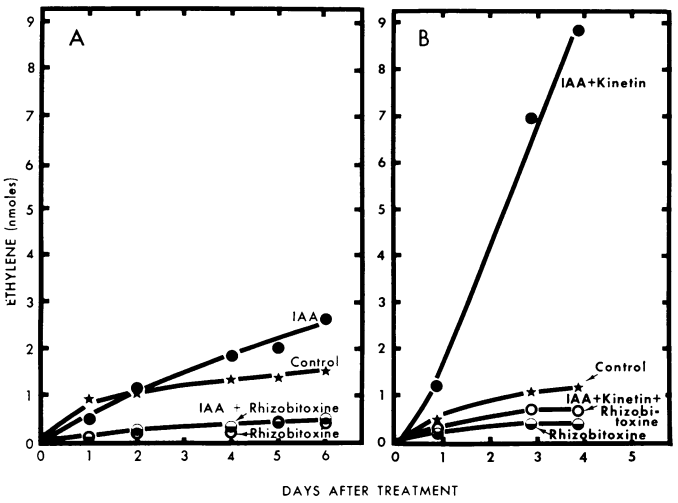

FIG. 2. Rhizobitoxine inhibition of IAA-enhanced (A) or IAA plus kinetin-enhanced (B) ethylene production of sorghum seedlings. Final concentration of additions were: rhizobitoxine, 2 μM (8 μg); IAA, 0.1 mM; and kinetin, 0.1 mM. All flasks were buffered at pH 6.9 with 1 mM potassium phosphate. Each value plotted is the average of three replicates.

Gas Analysis. Ethylene produced in the flask atmosphere was determined by gas chromatography using an alumina column and a flame ionization detector (11).

Methionine Uptake. In one experiment the amount of L-methionine-^{14}C(UL) uptake by apple tissue was determined. At designated times the apple tissue was removed from duplicate flasks, separated from the incubation medium via filtration, washed on the filter with 5 ml of fresh medium, oven-dried, and oxidized by dry combustion. The CO_2 was collected in ethanolamine and counted by liquid scintillation (6).

***Pencillium* Culture.** Fifty-milliliter Erlenmeyer flasks containing 10 ml of modified Pratt's medium (16) were inoculated with 50,000 spores of *Pencillium digitatum* ATCC No. 10030. After incubation at 25 C for 91 hr with loose covers, the flasks were sealed with rubber serum stoppers. Filter-sterilized rhizobitoxine was added to some flasks at time of inoculation and to other flasks 24 hr after sealing (three replicates). Samples of gas were removed periodically for assaying ethylene by gas chromatography, and the mycelia were dried and weighed at the termination of the experiment.

Rhizobitoxine. The isolation and purification of rhizobitoxine from whole culture extracts of *Rhizobium japonicum* strain 94 (USDA *Rhizobium* culture collection, Beltsville, Md.) has been described elsewhere (4, 15). Reagent solutions of rhizobitoxine were prepared using an assumed molecular weight of 414 (Owens, L. D., J. F. Thompson, and K. Biemann, unpublished).

RESULTS

Rhizobitoxine inhibited ethylene production about 75% in both intact sorghum seedlings (Figs. 1 and 2) and senescent apple tissue slices (Tables I and II). This degree of inhibition was obtained with relatively low concentrations of rhizobitoxine: 2 μM in the case of sorghum seedlings (Fig. 2) and 12 μM with apple tissue (Table I). With sorghum seedlings, continuous presence of rhizobitoxine in the root solution was not required. A separate experiment showed that treatment with 10 μM rhizobitoxine for 1 day was sufficient to cause a 75% inhibition of ethylene production for the following 4 days.

Substantial additions of methionine succeeded in only partially relieving rhizobitoxine inhibition of ethylene production in both sorghum and apple tissue, the inhibitions being decreased from about 75% to about 60% (Fig. 1 and Table I). Additions of homoserine, a precursor of methionine, to sorghum had no effect on the inhibition. These results suggest that the inhibition of ethylene production by rhizobitoxine was mainly due to a block in the conversion of methionine to ethylene rather than in the biosynthesis of methionine

The *in vivo* conversion of added methionine to ethylene in apple tissue was followed by using ^{14}C-labeled methionine. In repeated experiments, the amount of label incorporated into ethylene was inhibited by rhizobitoxine to about the same extent as was the total amount of ethylene formed (Table II).

Rhizobitoxine had no effect upon the uptake of methionine-^{14}C by apple tissue (Table III). Uptake by both toxin-treated and control tissues was virtually linear during the 6-hr experimental period and amounted to about 40% of the added radioactivity by the end of the experiment (Table III).

Table I. *Rhizobitoxine Inhibition of Ethylene Production by Apple Tissue*

Each flask contained four plugs (about 1.4 g) taken from a Stayman apple stored 6 months at 0 C. Flask atmospheres were sampled after incubating 5 hr at 30 C. Each value represents the average of three replicates. The "no tissue" treatment consisted of filtered buffer solution that had been shaken briefly with the apple plugs.

Additions	Ethylene Production		Inhibition
	No rhizobitoxine	Rhizobitoxine, 12 μM	
	nmoles		%
None	25.7	6.2	75
L-Methionine, 1 mM	30.7	12.0	61
α-Keto-γ-methylthiobutyric acid, 1 mM	27.2	7.7	72
No tissue + 1 mM α-keto-γ-methylthiobutyric acid	0.3	...	...

Table II. *Rhizobitoxine Inhibition of ^{14}C Incorporation from Labeled Methionine into Ethylene by Apple Tissue*

Experimental conditions were the same as in Table I, except that rhizobitoxine concentration was 20 μM and that the tissue used in experiment 2 was from a Summer Rambeau apple stored for 2 weeks at 0 C and 1 week at 21 C. In experiment 1 each flask received 0.89 μc of radioisotope. However, an undetermined portion of this probably represented degradation products of methionine-3,4-^{14}C. The ratios of specific radioactivities of methionine and ethylene were calculated on the basis of 2 precursor ^{14}C atoms per mole of methionine.

Treatment	Ethylene					Specific Radioactivity Ethylene/Specific Radioactivity Methionine
	nmoles	*% inhibition*	*nc*	*% inhibition*	*μc/mmole*	
Experiment 1						
1 mM L-methionine-3,4-^{14}C	37.9		1.03		0.027	
1 mM L-methionine-3,4-^{14}C + 0.02 mM rhizobitoxine	10.8	72	0.22	78	0.020	
No tissue + 1 mM L-methionine-3,4-^{14}C	.1					
None	26.3					
20 μM rhizobitoxine	5.4	80				
Experiment 2						
1 mM L-methionine-^{14}C(UL), 28.2 nc (0.0056 μc/μmole)	73.1		0.20		0.0027	1.2
1 mM L-methionine-^{14}C(UL), 28.2 nc + 20 μM rhizobitoxine	17.4	76	0.03	85	0.0017	0.8
None	62.2					

Virtually all of the ethylene produced by apple tissue treated with methionine came from the added methionine, since the specific radioactivity of ethylene-^{14}C recovered (Table II) was similar to that of the added methionine-^{14}C (specific radioactivity of the ethylene-precursor carbon atoms 3 and 4 of methionine was calculated to be 0.0022 μc/μmole). Apparently the large amounts of methionine-^{14}C taken up by the tissue (Table III) were in great excess of the endogenous methionine supply.

Addition of the α-keto-analogue of methionine, α-keto-γ-methylthiobutyrate, to apple tissue caused virtually no stimulation of ethylene production in the absence of rhizobitoxine and provided virtually no relief of rhizobitoxine inhibition of ethylene production (Table I). In both ways it was less effective than its analogue methionine. Similar experiments could not be performed with sorghum seedlings because peroxidase, exuded from roots or from sloughed off cells, caused large amounts of ethylene to be formed from α-keto-γ-methylthiobutyrate in the root solution. No such problem was encountered with apple tissue.

Ethylene production by sorghum seedlings was stimulated about 2-fold by 0.1 mM IAA alone (Fig. 2A) and 8-fold by addition of IAA and kinetin to the root solution (Fig. 2B). Inhibition of ethylene production by rhizobitoxine was virtually as severe in flasks receiving IAA or IAA plus kinetin as in unsupplemented flasks.

Table III. *Uptake of L-Methionine-^{14}C by Apple Tissue in Presence and Absence of Rhizobitoxine*

Uptake was determined by total combustion of the apple tissue used in experiment 2 of Table II and of additional tissue samples prepared from the sample apple and incubated in a like manner. The $^{14}CO_2$ formed was collected and counted by liquid scintillation. Rhizobitoxine concentration was 20 μM and L-methionine-^{14}C(UL), 1 mM (5 μmoles total). Values listed are averages of two replicates for the 1- and 2-hr and three replicates for the 6-hr treatment.

Treatment Time	Uptake	
	No rhizobitoxine	Rhizobitoxine, 20 μM
hr	*μmoles*	
1	0.53	0.54
2	1.02	1.25
6	2.21	2.30

Table IV. *Ethylene Production by P. digitatum*

Cultures were sealed from the atmosphere 91 hr after inoculation and assayed for ethylene and harvested 139 hr after inoculation. Values represent the average of three replicates.

Treatment	Mycelium	Ethylene	
	mg dry wt	*nmoles*	*nmoles/mg*
None	2.4	22.8	9.5
Rhizobitoxine, 10 μM	3.5	36.4	10.1
Rhizobitoxine, 10 μM, added at t = 115 hr	2.5	26.9	10.8

The effect of rhizobitoxine on *Pencillium digitatum*, a high ethylene producer, was tested. Rhizobitoxine slightly stimulated the growth of this fungus but had no effect on ethylene production per unit weight of mycelium (Table IV).

DISCUSSION

This study was undertaken to test the ability of rhizobitoxine to act as an *in vivo* inhibitor of ethylene biosynthesis from methionine. The rationale for this test was based upon the knowledge that rhizobitoxine blocks methionine biosynthesis in *Salmonella* (12), although some question remains as to whether its toxicity to higher plants can be explained solely by this same mechanism (4).

Low concentrations of rhizobitoxine were indeed found to greatly inhibit ethylene production by sorghum seedlings and apple tissue. Contrary to our expectations, however, additions of methionine to either system failed to overcome the inhibition completely. To explain these results we might consider three possible ways by which rhizobitoxine could inhibit ethylene production. Rhizobitoxine may (*a*) block the biosynthesis of methionine, (*b*) block the conversion of methionine

to ethylene, or (*c*) do both. If only the biosynthesis of methionine were inhibited, then addition of methionine should have relieved the inhibition. It did not, to any substantial degree. The failure of methionine to relieve the inhibition in apples cannot be attributed to poor entry, since about 40% or some 2000 nmoles of methionine-^{14}C were apparently taken up by the tissue during the 5 hr experiment, both in the presence and absence of rhizobitoxine (Table III).

The second possibility, a block in the conversion of methionine to ethylene, should be relieved by addition of an intermediate which occurs between methionine and ethylene and which follows the step at which the inhibitor acts. The fact that the putative intermediate, the α-keto analogue of methionine, did not relieve the inhibition means that either the block is between the α-keto analogue and ethylene, or that the analogue is not an intermediate. In any case the results point to a block in some step of the conversion of methionine to ethylene.

This conclusion is further substantiated by the methionine-^{14}C feeding experiments (Table II). Rhizobitoxine curtailed the conversion of added methionine-^{14}C to ethylene-^{14}C to approximately the same extent (about 70–85%) as it did total ethylene production. Since the uptake of methionine-^{14}C was not affected by rhizibitoxine, these results directly demonstrate that rhizobitoxine blocks the conversion of methionine to ethylene.

Pencillium digitatum is known to produce ethylene via pathways not utilizing methionine as a precursor (5). The failure of rhizobitoxine to inhibit ethylene production by this fungus is evidence that rhizobitoxine inhibits ethylene biosynthesis only where methionine serves as the precursor.

As mentioned above, the α-keto analogue of methionine, α-keto-γ-methylthiobutyrate, has recently been considered as a likely intermediate in the conversion of methionine to ethylene (10) but has been questioned on the basis of its slow rate of conversion to ethylene when compared with methionine (1). The inability of α-keto-γ-methylthiobutyrate to stimulate ethylene production appreciably in the apple tissue experiments reported here or to relieve rhizobitoxine inhibition of ethylene production casts additional doubt upon its possible role as an intermediate in the conversion of methionine to ethylene. However, our results do not rule out the possibility that the α-keto analogue is an intermediate and that rhizobitoxine blocks its conversion to ethylene.

Auxin has long been known to stimulate ethylene production in seedlings of higher plants, and recently kinetin has been found to enhance this auxin effect greatly (3). These findings raised the question as to whether the ethylene-forming system induced by auxin and kinetin is the same as the endogenous system. Our observations that both systems in sorghum seedlings exhibit similar sensitivities toward rhizobitoxine suggest that the endogenous and the induced ethylene-forming systems are, in fact, identical.

The main finding reported here, that rhizobitoxine inhibits the conversion of methionine to ethylene, was unexpected since rhizobitoxine is known to act as an antimetabolite of cystathionine in both spinach (4) and *Salmonella* (12). Apparently rhizobitoxine has at least two sites of action: one which interferes with methionine biosynthesis and ultimately prevents the greening of young leaf tissue, and the other which blocks the conversion of methionine to ethylene. The inhibition of ethylene production apparently does not contribute to chlorosis induction, since the symptom develops normally in rhizobitoxine-treated sorghum seedlings growing in an atmosphere containing relatively high levels of ethylene (>1 μl/liter).

The results we report here are consistent with the view that rhizobitoxine directly affects an enzymatic step in the conversion of methionine to ethylene, but they do not rule out the possibility that the effect is a secondary one. However, if we assume that the conversion of methionine to ethylene resembles a number of other amino acid transformations, then we can see two possible similarities between this transformation and the one that rhizobitoxine is known to inhibit, namely the β-cleavage of cystathionine. First, both substrates have some structural similarities; rhizobitoxine may be an antimetabolite of methionine as well as cystathionine. Second, both transformations result in the elimination of part of the amino acid molecule. β-Cystathionase and other enzymes catalyzing amino acid cleavage reactions require pyridoxal phosphate as a cofactor. We know that pyridoxal phosphate is intimately involved in enzyme inactivation by rhizobitoxine (4). It is therefore possible that an enzyme involved in converting methionine to ethylene may also require pyridoxal phosphate as a cofactor and be susceptible to inactivation by rhizobitoxine.

Whether rhizobitoxine acts directly or indirectly upon the conversion of methionine to ethylene can finally be answered only when the complete pathway is known and the appropriate enzyme(s) have been isolated. Nevertheless, rhizobitoxine should prove useful as a tool in studying the role of ethylene in the regulation of plant growth and development.

Lastly, these results showing the marked inhibition by rhizobitoxine of both the production of ethylene and the conversion of methionine to ethylene suggest that methionine is the major, if not sole, metabolic precursor of ethylene.

Acknowledgments—The authors thank Mr. Gerald Jones and Mr. Leroy Beall, Crops Research Division, Agricultural Research Service, United States Department of Agriculture, for use of their combustion apparatus.

LITERATURE CITED

1. Baur, A. H. and S. F. Yang. 1969. Precursors of ethylene. Plant Physiol. 44: 1347–1349.
2. Burg, S. P. and C. O. Claggett. 1967. Conversion of methionine to ethylene in tissue and fruits. Biochem. Biophys. Res. Commun. 27: 125–130.
3. Fuchs, Y. and M. Lieberman. 1968. Effects of kinetin, IAA, and gibberillin on ethylene production, and their interactions in growth of seedlings. Plant Physiol. 43: 2029–2036.
4. Giovanelli, J., L. D. Owens, and S. H. Mudd. 1971. Mechanism of inhibition of spinach β-cystathionase by rhizobitoxine. Biochim. Biophys. Acta. 227: 671–684.
5. Jacobsen, D. W. and C. H. Wang. 1968. The biogenesis of ethylene in *Penicillium digitatum*. Plant Physiol. 43: 1959–1966.
6. Jeffay, H. and J. Alvarez. 1961. Liquid scintillation counting of carbon-14. Use of ethanolamine-ethylene glycol monomethyl ether-toluene. Anal. Chem. 33: 612–615.
7. Lieberman, M., A. Kunishi, L. W. Mapson, and D. A. Wardale. 1966. Stimulation of ethylene production in apple tissue slices by methionine. Plant Physiol. 41: 376–382.
8. Lieberman, M. and L. W. Mapson. 1964. Genesis and biogenesis of ethylene. Nature 204: 343–345.
9. Lieberman, M., L. W. Mapson, A. T. Kunishi, and D. A. Wardale. 1965. Ethylene production from methionine. Biochem. J. 97: 449–459.
10. Mapson, L. W., J. F. March, and D. A. Wardale. 1969. Biosynthesis of ethylene. 4-Methyl-mercapto-2-oxobutyric acid: An intermediate in the formation of methionine. Biochem. J. 115: 653–661.
11. Meigh, D. F., K. H. Norris, C. C. Craft, and M. Lieberman. 1960. Ethylene production by tomato and apple fruit. Nature 186: 902–903.
12. Owens, L. D., S. Guggenheim, and J. Hilton. 1968. Rhizobium-synthesized phytotoxin: An inhibitor of β-cystathionase in *Salmonella typhimurium*. Biochim. Biophys. Acta 158: 219–225.
13. Owens, L. D. and J. F. Thompson. 1967. Characterization of the chlorosis-inducing phytotoxin synthesized by *Rhizobium japonicum*. Bacteriol. Proc. p. 19.
14. Owens, L. D. and D. A. Wright. 1965. Rhizobial-induced chlorosis in soybeans: Isolation, production in nodules, and varietal specificity of the toxin. Plant Physiol. 40: 927–930.
15. Owens, L. D. and D. A. Wright. 1965. Production of the soybean-chlorosis toxin by *Rhizobium japonicum* in pure culture. Plant Physiol. 40: 931–933.
16. Spalding, D. H. and M. Lieberman. 1965. Factors affecting the production of ethylene by *Penicillium digitatum*. Plant Physiol. 40: 945–948.