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Inhibition of Ethylene Production in *Penicillium digitatum*[1]

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ABSTRACT

Production of ethylene by static cultures of *Penicillium digitatum*, which utilize glutamate and α-ketoglutarate as ethylene precursors, was inhibited by methionine, methionine sulfoxide, methionine sulfone, and methionine sulfoximine. Rhizobitoxine did not affect ethylene production but its ethoxy and methoxy analogues were effective inhibitors of ethylene production; its saturated methoxy analogue and kainic acid stimulated ethylene production. Tracer studies showed that the inhibitors blocked the conversion of [^{3}H]glutamate into [^{3}H]ethylene.

In shake cultures of this fungus, which utilize methionine as the ethylene precursor, rhizobitoxine and its unsaturated analogues all inhibited, while the saturated methoxy analogue stimulated ethylene production. In both types of cultures inhibition was irreversible and was diminished by increasing concentrations of the ethylene precursor. The inhibitory activity or lack of it by rhizobitoxine and its analogues appears to be a function of their structural resemblance to glutamate and methionine as well as of their size and stereoconfiguration. These data suggest similarities between the ethylene-forming system in the fungus and in higher plants despite differences in precursors under some cultural conditions.

The production of ethylene by *Penicillium digitatum* Sacc., the green mold fungus of citrus fruits, has been extensively studied (2, 6, 7, 17). Cultural conditions affecting ethylene production were described (5, 14, 15), and the biosynthetic pathway partially elucidated (4, 6, 7). When the fungus is grown in static culture, ethylene is derived from α-ketoglutarate or glutamate (4) but not from methionine, the only known precursor of ethylene in higher plants (8, 9, 16). Recently we reported (3) that under shake culture conditions ethylene was produced from methionine which also induced the ethylene-forming system in the fungal cells. Thus, in *P. digitatum*, ethylene can be produced from two different precursors depending on whether the fungus is cultured under static or shake conditions.

With the discovery that rhizobitoxine[3] and its analogues inhibit ethylene production in fruits and other plant tissues (10, 11) by blocking the conversion of methionine to ethylene, more detailed studies on ethylene biosynthesis became possible (8). In a recent paper (3) we reported that RO, the aminoethoxy analogue of rhizobitoxine, inhibited production of ethylene by both static and shake cultures of *P. digitatum*. This observation seemed to be in contrast with a previous finding (11) that rhizobitoxine did not inhibit ethylene production by the fungus in static culture. To resolve this apparent contradiction and to better understand the mode of ethylene production and its control in static and shake cultures of *P. digitatum*, we studied the effects of ethylene substrates, substrate analogues and inhibitors on the production of ethylene by this fungus. We report the results of this study and discuss their significance.

MATERIALS AND METHODS

P. digitatum (ATCC No. 10030) was grown on modified Pratt's liquid medium (15). Two types of cultures, static and shake, were used in this study. Static cultures were prepared by aseptically inoculating cotton-plugged, 50-ml, Erlenmeyer flasks containing 10 ml of medium. The fungus was grown statically for 4 days and the medium underneath the mycelial mat was then poured out of the flask and replaced with solutions of the chemicals tested at pH 4.5. Shake cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium. These flasks were incubated on a shaker as previously described (15). After 4 days of growth, the cultures were centrifuged, and portions (0.5 g) of the mycelial pellet were transferred into 25-ml Erlenmeyer flasks containing 5 ml of the test solutions at pH 4.5 and continued shaking. In tests lasting only several hr cotton plugs were removed, the flask was flushed with air and sealed with serum caps for 1 hr before a gas sample was withdrawn with a syringe for ethylene determination. In other experiments lasting several days, serum cups were fitted over the cotton plugs without flushing. In such experiments the medium was not replaced and all solutions introduced into the flasks were sterilized by filtration.

For tracer studies 3.3 μCi of L-[3,4-^{3}H]glutamic acid (49 Ci/mmol) (New England Nuclear) were added to each 50-ml Erlenmeyer flask containing 3 ml of medium-replaced test solution. Labeled ethylene produced was trapped into 3 ml of freshly prepared, ice-cold, 0.1 M mercuric acetate in methanol. This was accomplished by connecting the incubation flask to a serum capped, evacuated (65 mm of mercury), 50-ml Erlenmeyer flask, by means of Argyle extension tubing equipped with No. 22 Luer-slip needles on both ends. Fifteen sec after the flasks were connected, another needle was introduced into the serum cap of the incubation flask allowing air to be bled into the flask. Preliminary experiments showed that by this method, 64% of the ethylene in the incubation flask was transferred to the evacuated flask.

All experiments were carried out in triplicate and repeated at least once.

Determination of radioactivity, inoculation and incubation procedures, culture weight determination, and ethylene analyses were performed as previously described (3, 15). Further details of techniques are described in the legends to the tables.

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[3] Abbreviations: rhizobitoxine: L-2-amino-4-(2'-amino-3'-hydroxypropoxy)-*trans*-3-butenoic acid; RO: L-2-amino-4-(2'-aminoethoxy)-*trans*-3-butenoic acid; MRO: L-2-amino-4-methoxy-*trans*-3-butenoic acid.

RESULTS

Inhibition of Ethylene in Static Cultures. The effects of known substrates of ethylene and their analogues on the production of ethylene by static cultures of *P. digitatum* are summarized in Table I. While ethylene production was stimulated approximately 3-fold by increasing concentrations of glutamate and α-ketoglutarate (from 10^{-4} to 10^{-2} M), it was inhibited some 50 to 66% by methionine and three of its analogues. Rhizobitoxine, as shown previously (11), had no effect on ethylene production at concentrations as high as 10^{-3} M; but its ethoxy analogue RO, and, to a much greater extent, its methoxy analogue, MRO, were effective inhibitors of ethylene production. The D-2-amino isomer of MRO, which contained 17% of the racemic D-L mixture, was less effective an inhibitor than was the L isomer. On the other hand, the saturated MRO caused a 2-fold increase in ethylene production even at the lowest concentration tested (10^{-5} M).

In a more detailed study of the inhibition of ethylene by RO in static cultures, the RO inhibition could not be reversed by addition of glutamate or α-ketoglutarate (10^{-6} to 10^{-3} M) to cultures that had been inhibited by RO for 3, 6, or 24 hr (data not shown). However, when RO and glutamate were added simultaneously, either inhibition or stimulation occurred depending on the concentrations used (Table I). At low concentrations (10^{-4} M) of glutamate the inhibition by RO was increased as RO concentrations were increased. However, at high glutamate concentrations (10^{-2}, 5×10^{-2} M), stimulation of ethylene production was reduced by RO.

Addition of 2×10^{-4} M RO to the growth medium of static cultures (Table II) during the initial 48 hr of growth almost completely inhibited ethylene production by the fungus and also reduced its growth by about 25 to 50%, as measured by the dry weight of the culture. Later applications of RO inhibited ethylene by about 70% and had little effect on growth. At 10^{-5} M, RO had no effect on either ethylene production or growth when added to the growth medium at any time during the initial 4 days of growth (data not shown).

Studies with ^{3}H-labeled Glutamate. The effects of rhizobitoxine, RO, and MRO on total ethylene production by static cultures of the fungus were further investigated in tests of the incorporation of [3,4-^{3}H]glutamate (49 Ci/mmol) into labeled ethylene (Tables III and IV). The results indicated that RO and, to a greater extent, MRO directly inhibited the conversion of glutamate into ethylene by the fungus. Methionine also inhibited, and kainic acid (Fig. 1) stimulated both total ethylene production and incorporation of glutamate into ethylene (Table IV). Saturated MRO stimulated total ethylene production but inhibited, by about 43%, [^{3}H]glutamate incorporation into [^{3}H]ethylene.

Inhibition of Ethylene by *P. digitatum* Grown in Shake Cultures. Rhizobitoxine, RO, and MRO inhibited the methionine-induced ethylene production (3) in shake cultures of *P. digitatum* (Table V). In this system, rhizobitoxine was a more effective inhibitor than its ethoxy analogue, RO, but, as in the static cultures, MRO was the most effective inhibitor. Saturated MRO stimulated ethylene production. All three inhibitors were more effective at low than at high methionine concentrations. They were equally or more effective when applied to shake cultures which had already been induced to produce ethylene than when they were applied with methionine at time zero of the induction period (data not shown).

Table I. Effect of ethylene substrates and their analogues on the production of ethylene by static cultures of P. digitatum.

Chemicals	Concentration of chemical applied					
	10^{-5}M	10^{-4}M	5×10^{-4}M	10^{-3}M	10^{-2}M	5×10^{-2}M
	% of control[1]					
Glutamate	101	103	98	106	276	338
α-Ketoglutarate	106	109	107	117	298	
Methionine	102	75	72	73	58	50
Methionine sulfoxide	88	71	60			
Methionine sulfoximine	75	60	62			
Methionine sulfone	80	72	44			
Rhizobitoxine	101	103	102	96		
RO	96	60	28			
MRO	28	10	5			
MRO D isomer[2]	109	56	14			
MRO saturated	315	302	310	299		
RO + Glutamate (5×10^{-2}M)	335	333	168			
RO + Glutamate (10^{-2}M)	250	156	110			
RO + Glutamate (10^{-4}M)	97	82	42			

[1] Absolute values of water controls ranged from 2.6 to 4.6 μl ethylene/g dry weight/hr, in the different experiments summarized in the table.

[2] Contains approximately 17% of the racemic D-L mixture.

The fungus was grown for 4 days on modified Pratt's medium. The medium was then replaced with 3 ml water (control) or aqueous solutions of the chemicals tested, at pH 4.5. Ethylene was measured 22 hr later.

Table II. Effect of time of addition of RO during the growth of static cultures on ethylene production and on growth of P. digitatum.

Time of addition of RO (2×10^{-4}M)	Time of assay	Ethylene production	Dry weight of culture
hr	hr	% of control[1]	
0	72	2	48
24	72	2	65
48	72	8	76
72	96	25	87
96	120	30	96

[1] Absolute values of controls at 72, 96 and 120 hr of growth were 0.95, 2.5 and 30 μl ethylene/g dry weight/hr, and 0.07, 0.10 and 0.11 g dry weight of culture, respectively.

The fungus was grown in 50 ml Erlenmeyer flasks containing 10 ml modified Pratt's medium. RO was added (2×10^{-4}M) aseptically to the medium at time 0 of growth or at various times thereafter, as indicated. Ethylene production started 48 to 72 hr after inoculation.

Table III. Effects of inhibitors on production of ethylene and on incorporation of [^{3}H] glutamate into [^{3}H]ethylene by static cultures of P. digitatum.

Treatment	Total ethylene production	[^{3}H]ethylene production
	μl/g dry weight/hr	cpm/g dry wt. $\times 10^{-4}$
Water control	3.2	9.5
Rhizobitoxine	3.3	9.9
RO	2.6	6.5
MRO	1.0	1.6

The fungus was grown for 4 days in 50 ml Erlenmeyer flasks containing modified Pratt's medium. The medium was then replaced with 3 ml water or aqueous rhizobitoxine, RO or MRO (5×10^{-4}M) at pH 4.5. After a 2 hr incubation period 3.3uci of labelled [3,4-^{3}H] glutamate specific activity 49 Ci/mm (29,084 cpm/pmole)] were added to each flask. One hour after addition of labelled glutamate the flasks were sealed for 1 hr and ethylene concentrations and radioactivity in ethylene accumulated in the flask atmosphere was determined. Values were corrected for counting efficiency by internal standardization. Each flask contained 0.1g dry weight of fungal cells.

Table IV. Effects of saturated MRO, kainic acid and methionine on production of ethylene and on incorporation of [^{3}H]glutamate into [^{3}H] ethylene by static cultures on P. digitatum.

Treatment	Total ethylene production	[^{3}H] ethylene production
	% of control[1]	cpm/flask $\times 10^{-3}$
Water control	100	9.3
MRO-saturated	128	5.3
Kainic acid	130	11.4
Methionine	82	7.0

[1] Absolute value of water control was 3.4 μl ethylene/g dry weight/hr.

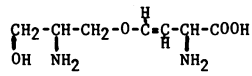
Procedure was similar to that described in Table III. Concentration of chemicals was 5×10^{-5}M, pH 4.5. Labelled glutamate was added at 28.2 nmoles/ml, and its specific activity was 16,049 cpm/pmole.

DISCUSSION

Studies on the inhibition of ethylene production in higher plants by rhizobitoxine (11) and by its ethoxy and methoxy analogues (10) have indicated that these inhibitors block the

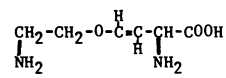
(1) Rhizobitoxine:

L-2-amino-4-(2'-amino-3'hydroxypropoxy)-trans-3-butenoic acid.



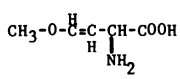
(2) RO:

L-2-amino-4-(2-aminoethoxy) trans-3-butenoic acid.

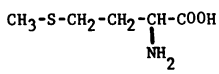


(3) MRO:

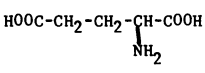
L-2-amino-4-methoxy-trans-3-butenoic acid.



(4) Methionine:



(5) Glutamate:



(6) Kainic acid:

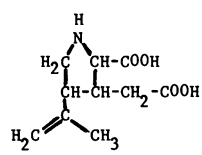


FIG. 1. Inhibitors and substrates of ethylene production.

conversion of methionine to ethylene. The failure of rhizobitoxine to inhibit production of ethylene by static cultures of *P. digitatum*, in which glutamate, but not methionine, is the ethylene precursor (4), was considered (11) as evidence that rhizobitoxine inhibits ethylene production only where methionine serves as the ethylene precursor. Results which support this view are reported in this study. Rhizobitoxine did not inhibit ethylene production in static cultures of the fungus (Table I) but did inhibit ethylene in shake cultures (Table V) which produce ethylene from methionine (3). Our results also showed that the ethoxy and methoxy analogues of rhizobitoxine were effective inhibitors of ethylene synthesis in static cultures (Tables I and II), blocking the conversion of glutamate to ethylene (Table III). Thus, while the effects of rhizobitoxine and its analogues on ethylene production are similar in higher plants (1, 10, 11) and in shake cultures of the fungus (Table V), the action of rhizobitoxine is different from that of its analogues in static cultures (Tables I and III).

Stimulation of ethylene production by kainic acid, which inhibits glutamate metabolism (13), was probably caused by the increased availability of glutamate for the ethylene reaction pathways as a result of the inhibition, by kainic acid, of alternate uses of this amino acid. This is substantiated by the findings that kainic acid stimulated the incorporation of [^{3}H]glutamate into [^{3}H]ethylene (Table IV) and that high concentrations of glutamate stimulated ethylene production (Table I).

It has been suggested (10) that the nature of the inhibition of rhizobitoxine and its analogues is related to the structural resemblance of their molecules to the methionine molecule. Our results indicate that in addition to structural resemblances to methionine and to glutamate, the effectiveness of each inhibitor of ethylene production, as shown by static cultures of the fungus, may be also related to molecular size (Fig. 1). The smaller the molecule of the inhibitor, the greater is its effectiveness as an ethylene inhibitor (Tables I and III). In addition to the size of the molecule, its stereospecificity seems critical since the D-2-amino isomer of MRO was a much less effective inhibitor than was the L isomer (Table I). Likely, the inhibition observed with the D isomer was due, at least in part, to the 17% content of the racemic D-L mixture in the D isomer preparation used.

In static cultures of *P. digitatum*, the saturated MRO was completely ineffective in ethylene inhibition. Rather, it strongly stimulated ethylene production at low concentrations (Table I), as it did in higher plants and in the copper-ascorbate model system (10). Our results also showed (Table IV) that saturated MRO reduced the conversion of [^{3}H]glutamate into [^{3}H]ethylene. These findings suggest that saturated MRO may serve as a substrate for ethylene production, and that the inhibition by rhizobitoxine, RO, and MRO of ethylene production by the fungus is related to the unsaturated double bond that all three inhibitors have in common (Fig. 1). It is this common part of the molecules that probably attaches to the ethylene-forming enzyme thereby blocking the attachment site for the ethylene precursor.

The mode of action of rhizobitoxine and its analogues in the inhibition of ethylene production is not definitively known. The results on ethylene inhibition obtained in this study, with both the static and shake cultures of the fungus, in which ethylene is being produced from different precursors (3, 4), show similarities to those obtained with higher plants (10, 11). They support the hypothesis that these compounds inhibit ethylene production by irreversibly attaching to the ethylene-forming enzyme. When the inhibitors were added to the fungus simultaneously with the ethylene precursor, inhibition was less at high glutamate (Table I) or at high methionine (Table V) concentrations than it was at low concentrations of these substrates. The relation between extent of inhibition and substrate concentration indicates that the inhibitors probably compete with the substrate by binding to the same site on the ethylene-forming enzyme. Once inhibition took place, however, it could not be reversed by addition of glutamate or α-ketoglutarate to RO-inhibited static culture. These results also indicate that in the static system, RO, which

Table V. Effect of ethylene inhibitors on methionine-induced ethylene production by shake cultures of P. digitatum.

Chemicals	Concentrations of chemical tested		
	10^{-5}M	10^{-4}M	2 x 10^{-4}M
	% of control[1]		
Rhizobitoxine			
in 7 mM methionine	71	66	46
in 0.7 mM methionine	61	38	30
RO			
in 7 mM methionine	97	80	75
in 0.7 mM methionine	95	71	54
MRO			
in 7 mM methionine	60	19	13
in 0.7 mM methionine	38	8	3
MRO saturated			
in 7 mM methionine	125	133	127
in 0.7 mM methionine	140	152	142

[1]Absolute values of methionine controls were 179 and 43 nl ethylene/g fresh weight/hr for 7 mM and 0.7 mM of methionine, respectively.

The cultures were grown for 4 days on modified Pratt's medium in 250-ml Erlenmeyer flasks under shake conditions, then centrifuged at 18,000 rpm for 10 min. Portions (0.5g) of the mycelium pellet were transferred into 25-ml Erlenmeyer flasks containing 5 ml of water, aqueous methionine or aqueous methionine + inhibitor solutions at pH 4.5, and returned to shake conditions. Ethylene was measured 22 hr later.

has been implicated as an inhibitor of transaminase (12), did not inhibit the transaminase which converts glutamate to α-ketoglutarate. The inhibitory effect of methionine and its analogues on ethylene production by static cultures (Table I) may, thus, also be explained on the basis of similarities of their molecular structures to glutamate. The part of the molecule of methionine or its analogues common to that of glutamate may become attached to the ethylene-forming enzyme; but, in static cultures of the fungus, these compounds appear not to be converted to ethylene. Consequently, incorporation of labeled glutamate into labeled ethylene is reduced in the presence of methionine (Table IV).

In this study some similarities are shown between the effects of the inhibitors on ethylene production by static cultures of *P. digitatum*, and their effects on ethylene production by shake cultures or by higher plants (10). These findings suggest that the ethylene-forming system in the fungus and in higher plants have similarities despite the two different precursors used for the production of ethylene. It also appears that the substrate-binding site in the ethylene-forming systems of the fungus and higher plants may differ in dimension or steric configuration.

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