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Methionine-induced Ethylene Production by *Penicillium digitatum*[1]

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ABSTRACT

Shake cultures, in contrast to static cultures of *Penicillium digitatum* grown in liquid medium, were induced by methionine to produce ethylene. The induction was concentration-dependent, and 7 mM was optimum for the methionine effect. In the presence of methionine, glucose (7 mM) enhanced ethylene production but did not itself induce ethylene production. The induction process lasted several hours, required the presence of viable mycelium, exhibited a lag period for ethylene production, and was effectively inhibited by cycloheximide and actinomycin D. Thus, the methionine-induced ethylene production appeared to involve induction of an enzyme system(s). Methionine not only induced ethylene production but was also utilized as a substrate since labeled ethylene was produced from [^{14}C]methionine.

Following induction by the fungus, filtrates of induced shake cultures also evolved ethylene in increasing amounts by both enzymic and nonenzymic reactions. Tracer experiments indicated that the ethylene released by the filtrate was derived from a fungal metabolite of methionine and not directly from methionine.

It is now well established that ethylene, a plant hormone, is produced not only by higher plants (11, 12) but also by a wide variety of microorganisms (7, 8, 20, 22, 25, 26). Because of its association with citrus fruits (3), ethylene production by *Penicillium digitatum* Sacc. has been studied extensively; cultural conditions have been defined (6, 24) and attempts made to elucidate its biosynthetic pathway (9, 10, 23). When the fungus is cultured on liquid media under static conditions (without shaking), ethylene production is high and appears to be related to the surface development of a mycelial mat (24). However, in shake culture the mycelium grows as submerged ball-like structures and produces little or no ethylene although growth is equivalent or exceeds that in static culture (6, 24). Ethylene produced by *P. digitatum* grown on liquid medium in static culture was shown (4, 9, 10) not to derive from methionine, the common precursor of ethylene in higher plants (11, 12, 25), but from 2-ketoglutarate and glutamate (4). Other, more recent studies of fungi (5, 18) and bacteria (20), however, have indicated that methionine may be associated with the production of ethylene in microorganisms.

In this study we show that shake cultures of *P. digitatum* could be made to evolve ethylene. We show that methionine induced the ethylene-forming system and also was the precursor of ethylene. Our report describes characteristics of this system.

MATERIALS AND METHODS

P. digitatum (American Type Culture Collection No. 10030) was grown on modified Pratt's medium (24). For preparation of stock cultures, potato dextrose agar slants were inoculated with the fungus, held at 22 C for several days, and stored at 4 C. A spore suspension (10^5 spores/ml) used as inoculum for a given test was made from a stock culture with sterile deionized H_2O. Test samples were prepared in triplicate or more, by aseptically pipetting 0.5 ml of the spore suspension into a cotton-plugged, 50-ml Erlenmeyer flask containing 10 ml sterile medium. In tests lasting only several hr, cotton plugs were removed, flasks were flushed with air and then sealed with rubber serum caps for 1 hr before a gas sample was withdrawn with a syringe for ethylene determination (24). In other experiments lasting several days, serum caps were fitted over the cotton plugs without flushing. The amount of ethylene initially present in the flasks was determined periodically and found to be 12 to 15% of the amount present in the flasks after being sealed for 1 hr. The initial amount of ethylene was found not to affect ethylene production, hence, it was readily corrected for in the calculation of the production rate of ethylene.

For tracer studies 0.95 μCi of L-[^{14}C]methionine, labeled in carbons 3 and 4 (4.5 μCi/μmol) was added to each 25-ml Erlenmeyer flask containing 5 ml unlabeled methionine (7 mM) and 0.5 g fungal cells from shake cultures. Labeled ethylene produced was trapped, at various times during a 24-hr incubation period, into 3 ml of freshly prepared, ice-cold, 0.1 M mercuric acetate in methanol. This was accomplished by connecting the 25-ml incubation flask to a serum capped, evacuated (650 mm mercury), 50-ml Erlenmeyer flask, containing the mercuric acetate solution, by means of Argyle extension tubing equipped with No. 22 Luer-slip needles on both ends. The flasks were kept connected for 15 min. Preliminary experiments showed that 57% of the ethylene in the incubation flask was transferred to the evacuated flask. The latter was then shaken and kept at 4 C for at least 2 hr for ethylene absorption in the mercuric acetate methanol solution, which was then transferred into counting vials containing Aquasol (New England Nuclear). Radioactivity was determined with a Packard Tri-Carb liquid scintillation counter.

Incubation procedures, culture weight determinations, and ethylene analyses were performed as previously described (24). Further details of techniques are described in legends of figures and tables.

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RESULTS

Effect of Glutamate and Methionine on Ethylene Production in Static and Shake Cultures. Comparison of the static cultures of *P. digitatum* showed that methionine in the growth medium inhibited production of ethylene by 30 to 65% (Fig. 1). In the absence of methionine, shake cultures produced virtually no ethylene. With methionine, however, they produced ethylene at a rate similar to that for static cultures without methionine. In both static and shake cultures, methionine (7 mM) inhibited growth by 30 to 35%, as indicated by culture weight (data not shown).

Ethylene was produced in shake culture in response to methionine at concentrations ranging from 0.07 to 70 mM with methionine most effective at 7 mM concentration (Fig. 2). However, we found that some exceptionally vigorous isolates of *P. digitatum* may produce ethylene in shake cultures without addition of methionine. These isolates also respond to methionine by producing even higher levels of ethylene.

The effects of glutamate and methionine on ethylene production differed in static and shake cultures. Table I shows ethylene production of shake and static cultures grown for the first 4 days in modified Pratt's medium and for 1 day on water (control), aqueous glutamate, or aqueous methionine. Glutamate increased the rate of ethylene production by about 3-fold in static cultures but had no effect on shake cultures. In contrast, methionine inhibited ethylene production in static cultures but markedly stimulated it in shake cultures. In the presence of RO,[3] the aminoethoxy analogue of rhizobitoxine, an inhibitor of ethylene production in higher plants (11), the stimulatory effect of glutamate on ethylene production and the basal level of ethylene in static culture were inhibited by 52 and 84%, respectively. In shake cultures, RO only slightly reduced the stimulatory effect of methionine.

Induction of Ethylene Production by Methionine in Shake Cultures. A time course study of ethylene production by shake cultures in response to methionine revealed a 2- to 3-hr lag period (Fig. 3). This suggests an induction phenomenon. Providing the fungal cells with 7 mM glucose in addition to methionine (7 mM) stimulated ethylene production by severalfold and shortened, but did not eliminate, the lag period. Glucose alone did not induce ethylene production. A lag period in ethylene production was evident also when the fungus, harvested after 4 days shake growth, was incubated in glucose for 10 to 20 hr before being treated with methionine (data not shown).

Induction of ethylene production by methionine required the presence of viable fungal cells since boiling cells for 10 min completely eliminated the production of ethylene. Cells that had been incubated in methionine solution for only 30 min did not produce ethylene. This suggests that the continued presence of methionine, at least beyond 30 min, is required for ethylene production.

The ethylene production of fungal cells transferred from culture medium to methionine was inhibited by the addition of cycloheximide, an inhibitor of protein synthesis. The degree of inhibition of the methionine-induced ethylene production depended on the time of application of cycloheximide (Fig. 4). Inhibition was greatest (95%) when the application of cycloheximide was at time 0 of transfer and decreased with later applications. When cycloheximide was added at 9 hr or later after the methionine-induced ethylene system was fully operative, inhibition was only 15% (Fig. 4). Similar results were obtained with actinomycin D, an inhibitor of DNA-dependent RNA synthesis. When applied at time 0, actinomycin D (10 μg/ml) inhibited ethylene production by 85%. D-Methionine, methionine sulfoximine, methionine sulfone, and methionine sulfoxide were completely ineffective in inducing ethylene production by shake culture cells; thus, a stereospecific requirement was suggested (2).

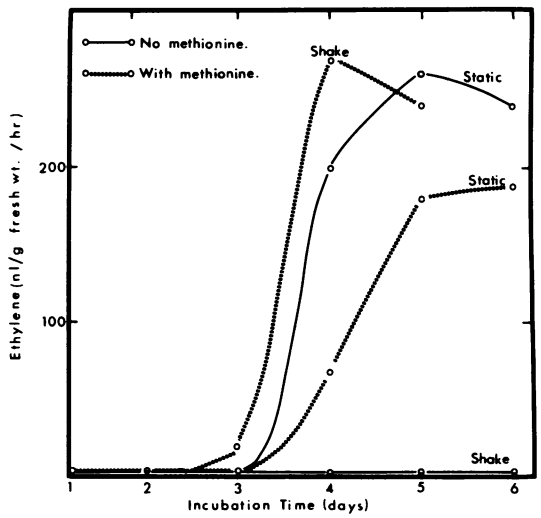

FIG. 1. Ethylene production by static and shake cultures of *P. digitatum* in the absence and presence of methionine (7 mM) in the medium. Cultures were incubated in cotton-plugged, 50-ml Erlenmeyer flasks containing 10 ml modified Pratt's medium. The same flasks were analyzed daily for ethylene production and were sealed, without removal of the cotton plugs, for 1 hr prior to sampling. Daily, three other flasks were used for weight determination of the fungal growth.

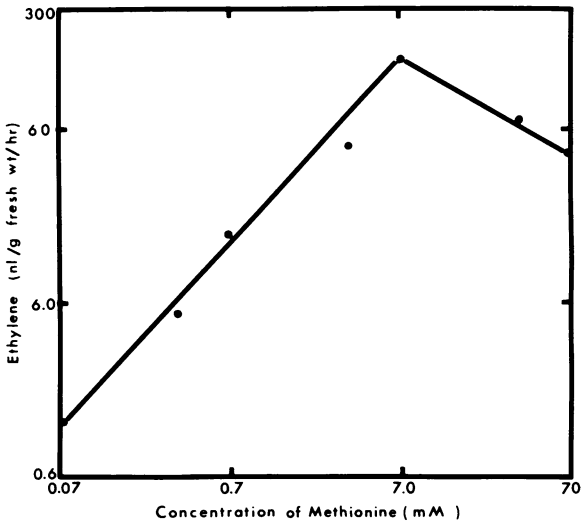

FIG. 2. Effect of methionine concentrations on ethylene production by shake cultures of *P. digitatum*. Cultures were grown for 4 days on modified Pratt's medium in 50- or 250-ml Erlenmeyer flasks under shake conditions, then centrifuged at 18,000 rpm for 10 min. Portions (0.5 g) of mycelium pellet were transferred into 25-ml Erlenmeyer flasks containing 5-ml solutions of methionine. Ethylene was measured 24 hr later.

Conversion of C-3, 4-[^{14}C]Methionine to Ethylene. In a test to verify the assumption that ethylene produced in methionine-induced shake cultures was derived from methionine, [^{14}C]methionine labeled in carbons 3 and 4 was added to the incubation solution. Over a 24-hr incubation period, [^{14}C]ethylene was produced at about the same rate as that of the total ethylene production induced by the unlabeled methionine, which was present at a concentration more than 100 times that of the [^{14}C]methionine (Fig. 5). These data indicate that methionine was converted to ethylene by the shake culture cells of the fungus.

Evolution of Ethylene from Medium by Nonenzymic Reaction. Further studies of the production of ethylene by shake

[3] Abbreviations: RO: L-2-amino-4-(2-aminoethoxy) *trans*-3-butenoic acid; SMKB: 4-S-methyl-2-ketobutyric acid.

Table I. Effects of glutamate, methionine and L-2-amino-4-(2(aminoethoxy) trans-3-butenoic acid on ethylene production by P. digitatum in static and shake cultures

The fungus was grown under static and shake conditions for 4 days on modified Pratt's medium. The medium was then replaced with 10 ml water, aqueous glutamate (70 mM) or methionine (70 mM) ± RO (0.5 mM) or aqueous RO. Ethylene was measured 24 hr later. Values in parentheses are S.E.

Chemicals tested	C_2H_4 Production	
	Static cultures	Shake cultures
	nl/g fresh wt·hr	
H_2O	290 (± 22)	4.2 (± 0.8)
Glutamate	1020 (± 102)	5.0 (± 0.6)
Glutamate + RO	350 (± 60)	4.1 (± 0.7)
Methionine	202 (± 41)	260 (± 31)
Methionine + RO	153 (± 23)	190 (± 29)
RO	46 (± 26)	3.2 (± 0.6)

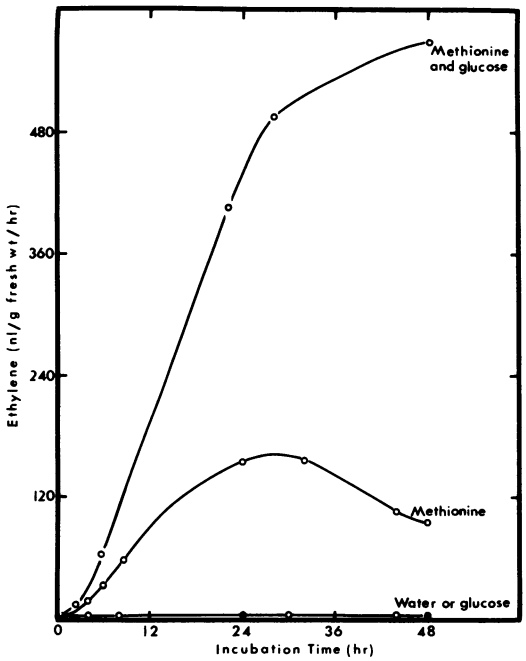

FIG. 3. Ethylene production by shake cultures of *P. digitatum* transferred to a solution containing methionine or glucose or both. Procedure was similar to that described in Figure 2. One-hr accumulations of ethylene were determined at various intervals during the following 48 hr.

cultures of *P. digitatum* revealed that part of the ethylene was evolved from the incubation solution. After 24 hr of contact with the fungus, the filtrate of the incubation solution continued to evolve 75 to 85% as much ethylene as the amount produced by an unfiltered culture. Tracer studies indicated that labeled ethylene was released by the filtrates only when [^{14}C]methionine had been added to the solution when the fungus was still present. Radioactive ethylene was not produced when labeled methionine was directly added to the solution after filtration. The evolution of ethylene by the filtrate was only partially inhibited (Table II) by boiling of the filtrate or by the addition of pronase, a protein-degrading enzyme. These inhibitions (40 and 50%) indicate that much of the ethylene was produced nonenzymically. The addition of chelating agents or of compounds shown to inhibit ethylene production (15) only partially inhibited the evolution of ethylene by the filtrate (Table II). Addition of SMKB (10^{-4} M), previously indicated as a substrate for ethylene (13), to both the nonboiled and boiled filtrates resulted in a markedly increased ethylene evolution, which was further stimulated by the addition of horseradish peroxidase (Table III). Similarly, filtrates from the 24-hr shake-incubated fungal cells in water released ethylene when SMKB was added. SMKB is known (13) to be readily converted to ethylene nonenzymically especially at low pH, such as that of the filtrate and incubation solution (Table II). However, we were unable to detect SMKB, by chromatography or by the ethylene-peroxidase assay (13) in the methionine-containing solutions, either during 24 hr of incubation with the fungus or subsequently. Nor could we detect activity of peroxidase, glucose oxidase, L-amino-acid oxidase or xanthine oxidase, enzymes which can degrade SMKB to ethylene (13), in filtrates of the induced and noninduced cultures. The addition of these enzymes to the filtrates without exogenous substrates did not effect the production of ethylene.

Comparison of Ethylene Production by Fungus and by Culture Filtrate. Methionine-induced ethylene production in shake cultures was initiated in the fungal cells and was virtually absent in the filtrate during the early hours of ethylene production. As ethylene production increased with time, increasing amounts of ethylene were produced by the culture filtrate. Between 12 and 24 hr after induction of ethylene production most of the ethylene

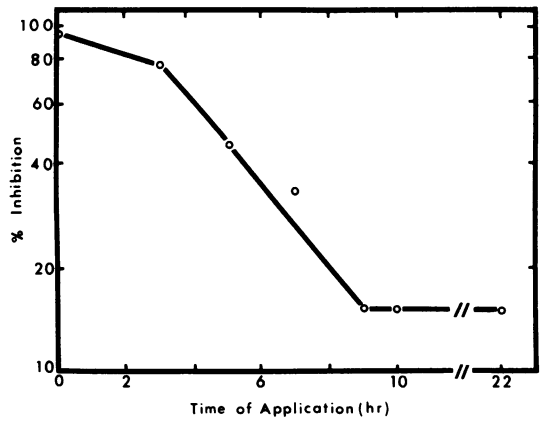

FIG. 4. Effect of time of application of cycloheximide on inhibition of ethylene production by shake cultures of *P. digitatum*. Fungus was grown for 4 days on modified Pratt's medium and then transferred to aqueous methionine solution (7 mM). Cycloheximide was added with methionine at time of transfer and at various intervals as indicated in the figure. Inhibitor was applied in a methanolic solution (6 μl/ml), and final concentration was 50 μM. Absolute value of methanol control, 24 hr after transfer, was 148 nl ethylene/g fresh wt·hr. Other conditions were similar to those described in Figure 2.

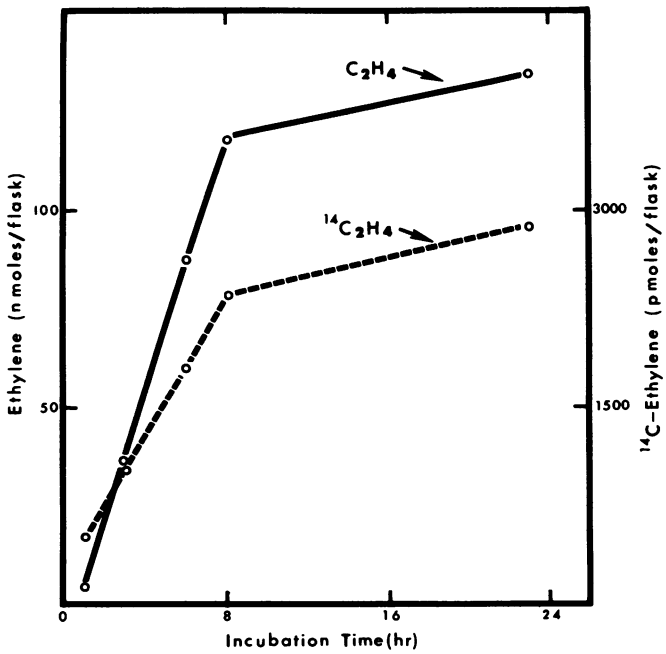

FIG. 5. Comparison between rate of labeled and total ethylene production by shake cultures of *P. digitatum*. Four-day-old shake cultures were incubated with cold (O——O) or labeled (O- - -O) methionine. Specific radioactivity was 76.8 cpm/nmol methionine. Other conditions were as described in Figure 2 and in the text.

Table II. Effects of various treatments on the rate of ethylene evolution from filtrates of methionine-induced shake cultures of P. digitatum

Procedure for ethylene induction was similar to that described in Fig. 2: after 24 hr contact with mycelium, the methionine (7mM) incubating solution was removed and the ethylene evolved was determined. Absolute control value was 15 nl C_2H_4/ml·hr. The pH of the solution was 3.

Treatment	Concentration	Relative Rate of C_2H_4 Evolution % of control
None		100
Boiled 10 min		60
Pronase	10 µg/ml	50
RO	5×10^{-4} M	75
Propyl gallate	10^{-3}M	50
Sodium benzoate	10^{-3}M	50
EDTA	5×10^{-4}M	82
DIECA	5×10^{-4}M	66
Bathocuproine	10^{-4}M	103
Catalase	5 µg/ml	50
Ascorbate	10^{-3}M	82
$CuSO_4$	10^{-4}M	92

Table III. Effect of SMKB and horseradish peroxidase on evolution of ethylene from filtrate of methionine-induced shake cultures of P. digitatum

Procedure used was similar to that described in Table II.

Additions	C_2H_4 Evolution Control	Boiled 10 min
	nl/ml·hr	
None	16	10
Horseradish peroxidase (2µg/ml)	14	10
SMKB (10^{-4}M)	25	34
Horseradish peroxidase and SMKB	43	33

was produced by the filtrate. Therefore, there appear to be three systems for ethylene production which develop to shake cultures as a result of induction by methionine. The first system is located in the fungal cells and uses methionine for ethylene production. The second and third systems occur in the filtrates and do not use methionine directly to produce ethylene. One of these filtrate systems is nonenzymic. Furthermore, both filtrate systems depend initially on leakage from the fungal cells.

DISCUSSION

Results of this study indicate that *P. digitatum* produces ethylene by two different pathways, depending on whether the fungus is cultured under static or shake conditions (Fig. 1, Table I). Glutamate is the precursor of ethylene production in static cultures (4); whereas methionine, previously believed not to be utilized by the fungus as an ethylene precursor (4), is here shown to be the precursor (and inducer) in shake cultures.

Our finding that RO, the rhizobitoxine analogue, considerably reduced the stimulatory effect of glutamate on ethylene production in static cultures contrasts with a previous finding that rhizobitoxine did not inhibit ethylene production by *P. digitatum* static cultures (19). The difference between these results may be due to a 20-fold difference in concentration of the inhibitors used. Although the effects of rhizobitoxine and its analogue on ethylene production by higher plants are similar (1, 16, 19), their effects may be different in the fungus and may be related to inhibition of transaminase, which converts α-ketoglutarate to glutamate (21). In shake cultures RO only slightly inhibited the production of ethylene stimulated by methionine (Table I). This would be expected because methionine can compete with RO (16), and the ratio of methionine to RO in the incubating solutions was high. In fact, when ethylene production was induced in shake culture by much lower concentrations of methionine, a greater inhibition by RO—of more than 50%—was demonstrated.

The production of ethylene by shake culture in response to methionine seems to involve induction of an ethylene-synthesizing enzyme system(s) in the fungal cells. The lack of ethylene production in the absence of methionine, the requirement of viable fungal cells, the existence of a lag period, the effective inhibition by cycloheximide (Fig. 4) or actinomycin D, the lowered inhibition by cycloheximide when added after 3 to 9 hr, and the lack of induction by D-methionine and other methionine analogues, all support this view. The induction of ethylene required several hr; and during induction two periods could be distinguished, as shown by the two slopes in Figure 4. During the first period, cycloheximide inhibited very effectively, presumably because little or no ethylene-forming enzyme had been synthesized. During the second period (after 3 hr), increasing amounts of the ethylene-forming enzyme had been synthesized before addition of cycloheximide, and, consequently, its effectiveness gradually decreased with time. Induction of ethylene production by methionine in *P. digitatum* resembles similar phenomena reported for *Escherichia coli* (20) and for *Mucor hiemalis* (18).

About half of the ethylene evolved by filtrates of the methionine-induced cultures was produced nonenzymically (Table II). Propyl gallate, which quenches free radicals and thus inhibits ethylene production in higher plants (1) and in the horseradish peroxidase model system (25), was as effective an inhibitor of ethylene evolution as boiling. Chelators which have been shown to inhibit ethylene production from methionine in both the model systems (14, 17) and in apple slices (15) also somewhat inhibited ethylene production by the incubating solution. Their inhibitory effects were not greater probably because of the low pH of the filtrate. Results with the chelators indicate that a heavy metal ion, possibly an oxidized Cu, may be involved in the reactions; and the inhibition by catalase suggests the involvement of H_2O_2. SMKB stimulation of ethylene production in filtrates from both the methionine-induced and the water control shake cultures (Table III) suggests that constituents of a model system may leak out of the fungal cells into the incubation solution, as they have been shown to do from higher plants (13).

The data from tracer experiments indicate that methionine is a precursor of ethylene produced by the fungal cells but not by the filtrates. Filtrates of cultures evolved labeled ethylene only when radioactive methionine had been added to the incubating solution when they still contained the fungal cells. These results indicate that the fungus took up methionine from the solution and, presumably, released a metabolite of methionine into the medium, which, in turn, was converted to ethylene. Ethylene-forming systems of cell-free filtrates of other microorganisms (18, 20) and of *P. digitatum* may, in fact, be similar and require further investigations.

Because *P. digitatum* was shown to synthesize ethylene from glutamate, its ethylene-forming system was thought to be entirely different from that of higher plants (4). Our study showed, however, that under slightly altered cultural conditions, this fungus produced ethylene from methionine. Thus, ethylene could be produced in *P. digitatum*, and perhaps in other organisms, from more than one precursor.

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