

CHAPTER 45

**Mechanism of Ethylene and Carbon Monoxide Production
by *Septoria musiva***

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S. musiva, a causative agent of premature defoliation of cottonwood trees, has been shown previously to produce ethylene and carbon monoxide (CO) on media containing glucose, methionine, and iron. Chemical analyses have shown that all three substances are present in the cottonwood leaves. Of seven carbohydrates tested, none supported the production of ethylene and only glucose supported CO production. From 24 amino acids employed as sole carbon sources only methionine and cysteine supported the production of ethylene and CO but four other amino acids supported ethylene production. Metabolic studies have demonstrated that the fungus transaminates methionine to 4-methylmercapto-2-oxobutyric acid, which presumably is acted upon by a peroxide in the presence of iron to form ethylene, formic acid, and dimethyldisulfide. Although the organism grew in a medium containing S-adenosylmethionine, glucose, and iron, no ethylene or CO was produced. Using cell-free preparations, dihydroxyacetone was implicated as a precursor of CO.

INTRODUCTION

S. musiva, a fungal plant pathogen of cottonwood trees (*Populus deltoides*), is known to produce ethylene and carbon monoxide (Brown-Skrobot et al. 1984) in amounts sufficient to cause premature defoliation. The purpose of this investigation was to shed some light on the pathway(s) by which these gases are produced by the pathogen.

MATERIALS AND METHODS

Cultures and media. The fungus, *Septoria musiva*, isolated from cottonwood (*Populus deltoides*) leaf spots, was received on potato dextrose agar (PDA) from Bernard Smyley of the USDA Forest Service, Stoneville, MS. Additional isolates were obtained in potato dextrose broth (PDB) in 6-oz prescription bottles fitted with serum stoppers. Cultures were maintained on tryptic soy agar (TSA), tryptic soy broth (TSB), and a glucose-peptone broth (G-P broth). Stock cultures were stored at 4 C. Identification of *S. musiva* was by the characteristic hyaline conidia (straight or curved with 1-4 septa) and the pink spore tendrils. All cultures were checked for purity by staining the mycelia and conidia with trypan blue to observe the hyaline conidia. Additionally, the culture was streaked on tryptic soy agar to determine whether bacterial contamination was present. Sections of

mycelia also were placed on either potato-dextrose agar (PDA) or rose-bengal agar (RBA) and observed for the presence of fungi other than *S. musiva*. Finally, the *S. musiva* culture was grown in G-P broth. After growth, the culture was examined microscopically for bacterial contamination and streak plates were prepared using nutrient agar (NA) and TSA.

All leaves and stems of cottonwood trees (*Populus deltoides*) were obtained from the Southern Hardwood Laboratories, Stoneville, MS, and were from clones 66 and 261. Both clones are susceptible to infection by *S. musiva*.

Mineral salts broth (MSB) was prepared as described by Brown et al. (1964), and consisted of 1.0 g KNO₃, 0.5 g K₂HPO₄·3H₂O, 0.2 g MgSO₄·7H₂O, 0.05 g FeCl₃·6H₂O per liter of distilled water. The pH was adjusted to 7.0 using 10% (v/v) hydrochloric acid. Mineral salts agar (MSA) was prepared by adding 1.5% (w/v) Bacto-agar to MSB.

Methionine-glucose broth (M-G broth) consisted of methionine (1.0% w/v), glucose (0.5% w/v), with K₂HPO₄·3H₂O (0.05% w/v), and MgSO₄·7H₂O (0.02% w/v) dissolved in distilled water. The medium was solidified by the addition of 1.5% (w/v) Bacto-agar (M-G agar).

All carbohydrates and amino acids employed as the sole carbon sources in growth studies were filter-sterilized (0.45 μm filter) and employed at a concentration of 1% (w/v). The remaining constituents of the media were either KNO₃ or NH₄Cl (0.5% w/v) as nitrogen source with K₂HPO₄·3H₂O (0.05% w/v), MgSO₄·7H₂O (0.02% w/v), and 1.5% (w/v) Bacto-agar with iron powder (100 mg/tube).

All media were either obtained from Difco Laboratories, Inc., or prepared from reagent grade chemicals.

Most experiments were conducted in 16 × 150 mm serum-stoppered test tubes.

Preparation of inoculum. Generally the *S. musiva* isolate was grown in 6-oz bottles containing 50 ml of G-P broth for 14 d, with incubation at room temperature using agitation at 110 rpm/min on a New Brunswick Rotary Shaker. The fungus was collected on a 0.45 μm Millipore filter and washed with 25 mM phosphate buffer, removed from the filter, and resuspended in the same solution. The cells were homogenized in a sterile Waring blender for 1 min. The suspension was diluted with buffer to the point where a 1:10 dilution of fungal cells gave a reading of 50% on a Bausch & Lomb Spectronic 20 Spectrophotometer (590 nm).

Resting cell techniques. Cell suspensions were prepared by washing *S. musiva* cells grown in M-G broth and iron with 25 mM phosphate buffer (pH 7.0), removing the iron with a magnet, blending in a Waring blender, and resuspending the cells in the phosphate buffer. The fungal cell suspension was then disrupted in an American Instrument Company French Press stainless steel cylinder (3/8 in) (20,000 psi). Tests were performed in serum-stoppered 5-ml serum vials containing 0.5 ml of substrate solution (DHA 100 μmol).

Analytical techniques. All gas-chromatographic (GC) analyses were carried out using a Precision Pressure-Lok syringe and a Fisher Model 1200 Gas Partitioner, using helium as the carrier gas at a flow rate of 30 ml/min, a column temperature of 75 C, and a bridge current of 200 mA. The first column (6.5 ft long by 1/8 inch diameter aluminum) was packed with 800-100 mesh Columpack™ PQ. Column

two (11 ft long by 3/16 in diameter aluminum) was packed with 60–80 mesh molecular sieve 13X.

The GC-MS analysis was conducted by the Mississippi State Chemical Laboratory using a Finnigan Model 45–10 GC-MS with either a capillary column (DV-5 liquid phase) for detection of ethylene or a molecular sieve 5A (Linde 6 ft column) with 120–140 mesh for detection of CO.

Hydrolysis of the cottonwood leaves was achieved using sulfuric acid as specified by Browning (1967). Derivatization was achieved using the methodology of Chen and McGinnis (1981). The gas-liquid chromatographic analyses were conducted using a Perkin-Elmer 3920 gas-liquid chromatograph. Nitrogen was employed as the carrier gas with a nickel alloy column packed with 1.0% stabilized diethyl-glycoadipate on 100–120 mesh chromosorb WHP.

The free amino acids were separated from the protein bound amino acids using a solvent extraction system described by Bielecki and Turner (1966). Hydrolysis of the protein bound amino acids was conducted according to Penke et al. (1974). Analysis of cottonwood leaves for free and protein-bound methionine was conducted using thin-layer chromatography according to the method of Pataki (1968). Cottonwood leaves were analyzed for the presence of iron using an Instrumental Laboratories Model 353 Atomic Absorption Spectrophotometer by the Mississippi State Chemical Laboratory.

Analysis for the presence of formic acid was performed using two methods described by Feigl (1958).

RESULTS AND DISCUSSION

A previous investigation into the mechanism by which *S. musiva* causes premature defoliation of cottonwood trees revealed that this pathogen produces copious quantities of both ethylene (0.22–0.26 $\mu\text{mol/h/g}$ dry wt mycelium) and carbon monoxide (0.13 $\mu\text{mol/h/g}$ dry wt mycelium) when grown on media containing methionine, glucose, and iron (Brown-Skrobot et al. 1984). Further, the quantities of ethylene and CO produced were sufficient to cause premature leaf fall of *Populus deltoides* (Brown-Skrobot et al. 1984).

All three of the key ingredients of this medium would be expected in cottonwood leaves and chemical analyses proved their presence: methionine (determined by TLC), iron (determined by atomic absorption), and glucose (determined by GLC). In order to identify other carbohydrates that could serve as a substrate for the growth of *S. musiva*, leaves were subjected to acid hydrolysis and gas-liquid chromatographic analysis for aldoses. As expected, glucose was the predominant aldose in both frozen and refrigerated leaves. Other aldoses present in both frozen and refrigerated leaves were D-arabinose, D-mannose, D-rhamnose, D-ribose, and D-xylose. The above analyses were conducted employing leaves of Clone 261, but when refrigerated leaves of Clone 66 were analyzed, galactose was found in addition to the other aldoses present in Clone 261.

Growth on Carbohydrate Media

All aldoses found in the leaves were tested for their ability to serve as substrates for the production of ethylene and/or carbon monoxide. For these experiments,

the carbohydrate sources were filter-sterilized into test tubes containing MSB with either KNO_3 or NH_4Cl as the nitrogen source along with iron powder. After inoculation, the tubes were serum-stoppered and incubated at room temperature. After 30 d, gas chromatographic analyses using 1.0-ml samples of the atmospheres from duplicate tubes revealed that none of the carbohydrate sources stimulated the production of ethylene, but carbon monoxide ($0.07 \mu\text{mol}$) was produced by *S. musiva* growing on glucose with either KNO_3 or NH_4Cl as a nitrogen source. The remaining carbohydrate sources did support growth as indicated by oxygen consumption and carbon dioxide production.

Growth on Amino Acids

Methionine was shown to be a substrate for ethylene production by *S. musiva*. To test the ability of other amino acids to serve as substrates for the production of ethylene and carbon monoxide, growth studies were conducted employing several amino acids as carbon sources. Gas chromatographic analyses of the overlying atmosphere from duplicate samples after 30 d incubation revealed that L-cysteine stimulated the production of an average of $0.09 \mu\text{mol}$ of ethylene and $0.98 \mu\text{mol}$ of carbon monoxide, and L-methionine stimulated the production of $0.18 \mu\text{mol}$ of ethylene and $0.29 \mu\text{mol}$ of carbon monoxide.

In contrast, L-aspartic acid, DL-ethionine, L-glutamic acid, and DL-homocysteine stimulated the production of ethylene ($0.09 \mu\text{mol}$) but not carbon monoxide. Amino acids supporting growth but not stimulating the production of either ethylene or carbon monoxide within 30 d were L-alanine, L-arginine, L-asparagine, L-cystine, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, DL-norleucine, DL-norvaline, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine.

Pathway of Ethylene Production by S. Musiva

Methionine has been shown to be a substrate for the production of ethylene by *S. musiva*. Two possible pathways for the production of ethylene from methionine have been reported in the literature. One pathway proceeds through the formation of S-adenosylmethionine (Fig. 1) while the second pathway proceeds by way of transamination to the alpha-keto acid (Fig. 2). To determine if *S. musiva* could utilize S-adenosylmethionine as a substrate for the production of ethylene, S-adenosylmethionine was substituted for methionine in M-G broth. The organism grew well in this medium but failed to produce either ethylene or CO . Next, *S. musiva* was grown in M-G broth in the presence of isonicotinic acid hydrazide, a known transaminase inhibitor. The lack of ethylene production in the presence of the inhibitor suggests that transamination of methionine was occurring since ethylene was produced in the same system in the absence of the inhibitor. In the transamination pathway, the alpha-keto acid (Fig. 2) formed from the methionine is converted to ethylene via reactions involving peroxides. Since the alpha-keto acid was unavailable and it is known that methional is converted to ethylene via reaction with hydroxyl radicals, an experiment was conducted using disrupted *S. musiva* cells with methional as the substrate. It was found that ethylene was produced from the methional with and without the disrupted cells but the amount of ethylene produced in the presence of disrupted cells was 25% greater than that obtained in the absence of the disrupted cells indicating that

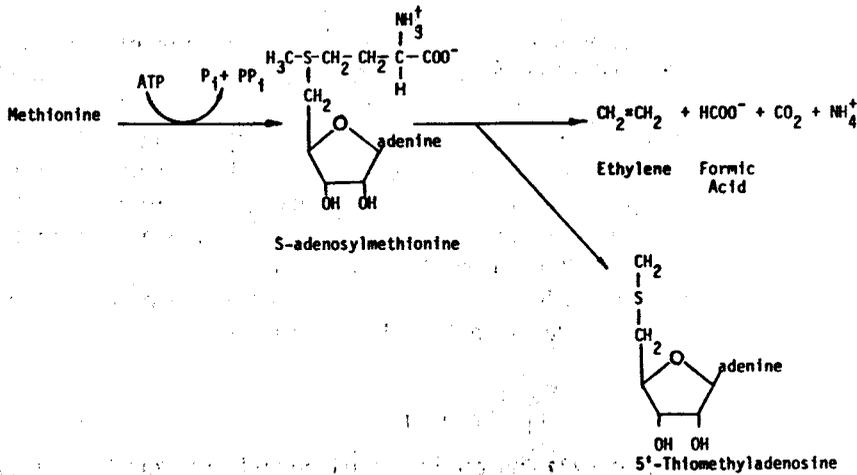


FIG. 1. Pathway for the conversion of methionine to ethylene via S-adenosylmethionine (Metzler 1977).

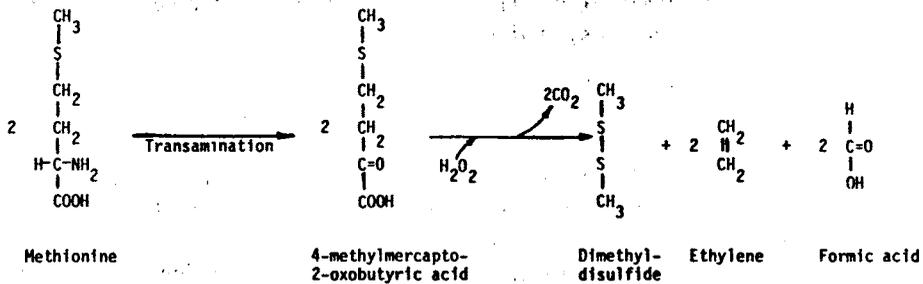


FIG. 2. Pathway for the conversion of methionine to ethylene via transamination. Drawn data are from Metzler (1977) and Hislop et al. (1973).

they stimulated ethylene production, possibly through the formation of hydroxyl radicals. This finding does not prove that the alpha-keto acid is converted to ethylene through the action of peroxide, but it does support the contention that methionine is converted to ethylene via the transamination pathway.

It also may be observed (Fig. 2) that in the transamination pathway, dimethyl-disulfide and formic acid are produced along with ethylene. Therefore, the atmospheres from *S. musiva* grown on leaf material and *S. musiva* grown on artificial media were subjected to GC-MS analysis. These analyses showed that dimethyl-disulfide was present in the atmospheres of both samples. Establishing that dimethyl-sulfide was present in the atmosphere overlying the *S. musiva* cultures adds further evidence that the production of ethylene proceeds via transamination of methionine.

When the medium (M-G broth) from a growing *S. musiva* culture producing ethylene was tested for the presence of formic acid by two separate colorimetric tests, both tests were positive. While none of these experiments disprove the existence of the S-adenosylmethionine pathway for the production of ethylene, they

do show that the transamination pathway for the production of ethylene is operative in *S. musiva*. Additionally, it should be pointed out that there is no absolute proof that all the ethylene produced by *S. musiva* is by way of methionine.

Other investigations were undertaken using cell-free preparations to determine intermediates in the pathway of carbon monoxide production by *S. musiva*. Disrupted cells of *S. musiva* were offered dihydroxyacetone and produced carbon monoxide (0.18 μmol) and a trace of ethylene. This result was not unexpected since Brown and Brown (1981) showed that carbon monoxide was produced by *Rhizoctonia solani* using dihydroxyacetone; thus, *S. musiva* may produce carbon monoxide by a pathway similar to that employed by *R. solani*.

CONCLUSIONS

It is concluded that *S. musiva* can produce ethylene and CO from the glucose, methionine, and iron available within cottonwood trees. Further, metabolic studies have demonstrated that ethylene is probably produced via transamination of methionine to 4-methyl mercapto-2-oxobutyric acid, which reacts with peroxide in the presence of iron to form dimethyldisulfide, ethylene, and formic acid while the CO is produced from DHA. The organism was unable to produce ethylene or CO from S-adenosylmethionine.

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