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CHAPTER 70

Ethylene and Carbon Monoxide Production by *Septoria musiva*

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An investigation into the mechanism by which *Septoria musiva* causes the premature defoliation of cottonwood trees was undertaken. Gas-chromatographic analysis of the atmosphere overlying the original culture indicated that this fungus produced significant quantities of ethylene and carbon monoxide. Subcultures failed to produce either gas on a variety of commercially available laboratory media but did produce ethylene on media prepared from cottonwoood leaves. The addition of ashed leaves or metallic iron, zinc, or boron to a mineral-salts medium containing methionine and glucose resulted in the production of both carbon monoxide and ethylene. Inoculation of *S. musiva* onto cottonwood cuttings resulted in the same manifestations of disease as observed in nature. The organism was reisolated from these diseased plants. Additional isolates from diseased cottonwood leaves were all found to produce carbon monoxide and ethylene. *Sektoria musiva* produces ethylene and/or CO on plant tissues in amounts sufficient to cause premature defoliation of *Populus deltoides*.

INTRODUCTION

Septoria musiva produces cankers (Filer et al. 1971; Zalasky et al. 1968) and leaf spots (Thompson 1941) on cottonwood trees (*Populus deltoides*) and results in premature defoliation of the trees, followed by dieback or growth loss, and eventual death of the trees. The disease caused by *S. musiva* poses a serious problem to the pulpwood industry in that the paper produced from diseased pulp has lower tensile and bursting strength, burst factor, and breaking length (M. E. Ostry and H. S. McWebb, in press) than uninfected pulp.

Since ethylene is known to cause defoliation (Beyer 1972; Lieberman 1979; Jackson et al. 1973; Baur et al. 1971) and carbon monoxide (CO) is known to mimic the physiological effects of ethylene (Kader et al. 1978), the defoliation may be caused by the production of either and/or both of these gases by the fungus. Therefore, the objective of this investigation is to demonstrate that *S. musiva* is the cause of disease in cottonwood and can cause premature defoliation by the production of ethylene and/or carbon monoxide.

MATERIALS AND METHODS

Materials

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. These cultures were transferred routinely and 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*. Additionally, the *S. musiva* culture was grown in G-P broth. After growth, *S. musiva* was examined microscopically for bacterial contamination and streak plates prepared using nutrient agar (NA) and TSA.

All of the following media were obtained from Difco Laboratories, Inc.: NA, PDA, PDB, RBA, TSA, and TSB.

Reagent grade chemicals were employed for the preparation of all media.

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

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 while agitating on a New Brunswick Rotary Shaker at 110 rpm/min. The fungus was harvested by filtering through on a 0.45 nm Millipore filter and washed with 25 m*M* phosphate buffer, collected 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).

Synthetic Media

Mineral salts broth (MSB) was prepared as described by Brown et al. (1964), and consisted of 1.0 g KNO_3, 0.5 g $K_2HPO_4 \cdot 3H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $FeCl_3 \cdot 6H_2O$ in 1 liter of distilled water. The pH was adjusted to 7.0 using 10% (v/v) HCl.

The G-P broth consisted of 10.0 g of glucose and 5.0 g of peptone per liter of distilled water. The broth was dispensed in 50-ml quantities in 6-oz prescription bottles and sterilized at 121 C for 15 min.

Methionine-glucose agar (M-G agar) consisted of methionine (1.0% w/v), glucose (0.5% w/v), Bacto-agar (1.5% w/v) with potassium phosphate (0.05% w/v), and magnesium sulfate (0.02% w/v) dissolved in distilled water. The agar was dispensed in 5-ml quantities in 16 × 150 mm test tubes and sterilized at 121 C for 15 min.

Preparation of Natural Media

Branches of cottonwood were obtained from the Southern Forest Experiment Station, Stoneville, MS, and divided into leaves, young stems, and hard bark. Each material was mascerated in MSB or water, using a Waring Blender and employed in concen-

trations of 1.0% (w/v) and 10.0% (w/v). The mixtures were employed as solid media using 2.0% (w/v) agar.

Surface-sterilization of plant material was accomplished by washing with a 2.6% (v/v) hypochlorite solution and rinsing with distilled water.

Ashed cottonwood leaves (100 mg per tube) were prepared in an oven at 600 C for 15 min and added to M-G agar in 16 × 150 mm test tubes. The *S. musiva* isolate was inoculated onto the media and the test tubes closed with serum stoppers. The inoculated tubes were incubated at room temperature.

Preparation of Metal-Containing Media

The metallic compounds (100 mg per tube) added to media were: heated iron powder, zinc, zinc chloride, nickel chloride, cupric sulfate, manganese chloride, boric acid (pH adjusted), and molybdenum. The compounds were placed in 16 × 150 mm test tubes and sterilized. Sterilized M-G agar was added to the tubes and the medium allowed to harden at a slant.

Preparation of Growing Cottonwood Cuttings

Growth studies using cottonwood cuttings were conducted using Ball® Mason jars containing 200 g of sterile potting mix. The cottonwood stem-cuttings (2-3 in in length depending upon the position of the node) were surface-sterilized with 2.6% (v/v) sodium hypochlorite solution and planted in the sterile potting mix. After 14 d, the cuttings began leafing out and were inoculated with a 1.0% (w/v) *S. musiva* mycelial and spore suspension in 25 m*M* phosphate buffer (pH 7.0) in quantities of 1 ml per cutting.

Gas-Chromatographic Analyses

A dual-column Fisher Model 1200 Gas Partitioner gas chromatograph was employed to analyze the atmospheres of the serum-stoppered containers. Helium was employed as the carrier gas with a flow rate of 30 ml/min, column temperature set at 75 C, bridge current set at 200 mA, and the injector temperature set at 100 C. The first column (6.5 ft long by 1/8 in diameter aluminum) was packed with 80-100 mesh Columpak^{AM}PQ. Column two (11 ft long by 3/16 in diameter aluminum) was packed with 60/80 mesh molecular Sieve 13X. The sample size was 250 μl.

Confirmation of ethylene was achieved using another set of gas-chromatographic columns for analysis. Helium was employed as the carrier gas, with a flow rate of 30 ml/min. Column 1 was a 20 ft × 1/8 in aluminum column packed with 37.5% DC-200/500 on 80/100 mesh chromosorb P-AW. Column 2 was a 6 ft × 3/16 in aluminum column, 80/100 mesh chromosorb P-AW and a 12 ft × 3/16 in aluminum column, 60/80 mesh molecular sieve, 13X. The sample size was 250 μl.

Results and Discussion

Preliminary Results

Upon receipt of the *S. musiva* isolate, it was transferred to stoppered 6-oz prescription bottles containing PDB. After 14 d of growth, analyses showed that the isolate had produced an average of 164 μmol of CO_2, 38 μmol of CO, 4 μmol of ethylene, 51

μmol of H_2, and 42 μmol of CH_4 for duplicate samples. The identification of ethylene and CO was confirmed using a second set of columns (hydrocarbon columns) in the gas chromatograph.

Upon subculture of the *S. musiva* isolate in fresh PDB, only CO_2 was produced. The isolate then was inoculated onto branches of cottonwood and after growth was observed, the branches were placed in stoppered 6-oz prescription bottles. After incubation, gas-chromatographic analysis of the atmospheres of the bottles containing the infected branches indicated the production of CO_2 and H_2, while the uninfected branches of cottonwood yielded only CO_2. A portion of the infected cottonwood tissue was removed using a razor blade and placed onto the surface of a petri plate containing RBA. After growth, a section of agar containing *S. musiva* was inoculated onto a piece of uninfected cottonwood tissue resting on solidified Bacto-agar in a stoppered test tube. Controls consisted of *S. musiva* on Bacto-agar (without cottonwood tissue), and cottonwood tissue on Bacto-agar (without *S. musiva*). After 48 h of incubation, an average of 16 μmol of CO_2, 0.6 μmol of CO, 4 μmol of H_2, and 2.6 μmol of ethylene was present in quadruplicate tubes containing the inoculated cottonwood tissue, while only CO_2 was produced in the control tubes. This procedure was repeated several times with the same results.

Growth on Prepared Laboratory Media

Subculturing of the cottonwood pathogen onto a variety of laboratory media including NA, PDA, PDB, RBA, TSA, TSB, and G-P broth resulted in excellent growth of the isolate. The CO_2 was produced and O_2 was utilized but neither ethylene nor CO was produced. The lack of ethylene and CO production could be the lack of nutrients or cofactors required by the isolate for the production of these gases.

Growth on Natural Media

The failure to obtain ethylene or CO production by *S. musiva* using conventional laboratory media led to the testing of a variety of media prepared from cottonwood materials. Cottonwood-leaf agar, cottonwood-stem agar, and cottonwood-bark agar were employed, but even after 6 wk of incubation, no ethylene was produced although a trace of CO was produced from the cottonwood-leaf agar. The lack of significant quantitites of gas production suggests that the compound(s) necessary for production of ethylene and/or CO were either absent from the media or they were heat-labile since these media were sterilized in the autoclave.

The next series of tests were conducted using sections of cottonwood leaves and stems which were surface-sterilized and placed on Bacto-agar slants in serum-stoppered test tubes. The *S. musiva* was harvested from G-P broth, washed with buffer, and resuspended in buffer. The cells were homogenized using a Waring Blender, diluted to the standard inoculum concentration, and quadruplicate tubes of surface-sterilized leaves were inoculated with 0.5 ml of the fungus. After 7 d, *S. musiva* produced an average of 0.12 μmol of ethylene but not CO.

Additional experiments were conducted employing surface-sterilized leaves infected with *S. musiva*. Petri dishes containing inoculated leaves were placed in a lighted incubator at 22 C for 10 d. The isolate was transferred from the leaves onto new cottonwood leaves within quadruplicate, stoppered test tubes containing Bacto-agar.

The tubes were incubated at room temperature and in 7 d both ethylene (0.09 μmol avg) and CO (0.56 μmol avg) were produced. It is interesting to note that both ethylene and CO were produced by the isolate after being subcultured on natural material, whereas only ethylene was produced on the same medium when the inoculum was derived from G-P broth. Whole cottonwood leaves, cottonwood-leaf filtrate, and mascerated leaves and filtrate in serum-stoppered test tubes were inoculated from a G-P broth-grown culture. After 10 d of growth, ethylene was produced from the whole cottonwood leaves and the mascerated leaves with filtrate, but not from the leaf filtrate. Once again, transfer of the isolate to natural media directly from G-P broth resulted in the production of ethylene only.

Growth on Chemically Defined Media

Methionine is reported to be the substrate from which some microorganisms and plants produce ethylene, and glucose with methionine has been shown to stimulate ethylene production (Lynch and Harper 1974). Therefore, *S. musiva* was grown on M-G agar, but once again neither ethylene nor CO were produced. Since trace elements may be required for the production of the ethylene and CO, quadruplicate tubes of M-G agar were supplemented with ashed leaves and after 30 d of growth of *S. musiva*, ethylene (trace amount) and CO (0.49 μmol avg) were detected in the atmosphere of the culture vessels.

In an attempt to determine if metallic compounds in the ashed leaves were responsible for ethylene and CO production, heated iron powder was substituted for the ashed leaves. Previous work had shown that traces of ethylene and CO are absorbed on the iron powder. Therefore, to alleviate this problem, the iron powder was heated to drive off the absorbed gases. After 30 d of growth on this medium, *S. musiva* produced both ethylene and CO (Table 1).

A variety of other metallic compounds were substituted for the iron and/or ashed leaves in the M-G agar. The results in Table 1 clearly show that zinc, boron, and iron powder stimulated the production of both ethylene and CO, while cupric sulfate stimulated the production of only ethylene.

TABLE 1. *Growth of* S. musiva *on methionine-glucose agar supplemented with metallic compounds*

Metal Source	Ethylene Produced (μmol of gas/tube and standard deviation)	Carbon Monoxide (μmol of gas/tube and standard deviation)
	μmol	μmol
Iron powder	0.09 ± 0.01	0.25 ± 0.07
Zinc	0.06 ± 0.00	0.48 ± 0.05
Zinc chloride	0.00 ± 0.00	0.00 ± 0.00
Nickel chloride	0.00 ± 0.00	0.00 ± 0.00
Boric acid	Trace	0.49 ± 0.00
Cupric sulfate	0.09 ± 0.01	0.00 ± 0.00
Manganese chloride	0.00 ± 0.00	0.00 ± 0.00
Molybdenum	0.00 ± 0.00	0.00 ± 0.00

Conditions of Test: The autoclaved M-G agar supplemented with the respective metallic compound was solidified using 1.5% (w/v) Bacto-agar in serum-stoppered test tubes. Incubation was at room temperature for 30 d.

Growth on Cottonwood Cuttings

Having isolated *S. musiva* from infected trees and having demonstrated in the laboratory that the isolate could produce ethylene and CO which are known to cause the manifestations of the disease, it seemed appropriate to carry out Koch's postulates using this organism. To accomplish this, cottonwood cuttings were inoculated with *S. musiva*. The cottonwood plant response to the *S. musiva* infection was flexing, then leaf spots, with eventual leaf dropping, the same phenomena observed in cases of the disease in nature.

Fifteen leaves from the infected plants were surface-sterilized and placed on PDA in petri dishes and incubated at 22 C. After 10 d, mycelia were removed, stained with trypan blue, and identified by microscopic examination as *S. musiva*.

Ethylene and Carbon Monoxide Production by Other S. musiva *Isolates*

Experiments were conducted to determine if ethylene and CO production was unique for the *S. musiva* isolate employed in this study or whether it is a general characteristic of *S. musiva*. Accordingly, 24 additional isolates of *S. musiva* were obtained and inoculated onto duplicate M-G agar slants containing heated iron powder. After 10 d of incubation, all of the isolates had produced ethylene (1.10 ± 0.14 μmol) and CO (0.1 ± 0.05 μmol). Since Pratt and Goeschl (1969) and Lieberman (1979) reported that 0.06 ppm ethylene damaged the plant and Agrios (1978) stated that 0.05 ppm ethylene was toxic, the concentrations of ethylene [1,408 ppm (v/v)] produced by the *S. musiva* isolates was more than enough to cause defoliation.

Conclusions

It is concluded that *S. musiva* infects cottonwood leaves and produces ethylene and CO in plant tissues in sufficient quantities to cause premature leaf fall of *Populus deltoides*.

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Literature Cited

Agrios, G. N. 1978. *Plant Pathology*. Academic Press, New York p. 62 and 163.

Baur, A. H., S. F. Yang, H. K. Pratt, and J. B. Biale. 1971. Ethylene biosynthesis in fruit tissues. *Plant Physiol.* 47:696–699.

Beyer, E. M. 1972. Mechanism of ethylene action. *Plant Physiol.* 49:672–674.

Brown, L. R., R. J. Strawinski, and C. S. McCleskey. 1964. The isolation and characterization of *Methanomonas methanooxidans*k (Brown and Strawinski). *Can. J. Microbiol.* 10:792–799.

Filer, T. H., F. I. McCracken, C. A. Mohn, and W. K. Randall. 1971. *Septoria* canker on nursery stock of *Populus deltoides*. *Plant Dis. Rep.* 55:460–463.

Jackson, M. B., C. B. Hartley, and D. J. Osborne. 1973. Timing abscission in *Phaseolus vulgaris* L. by controlling ethylene production and sensitivity to ethylene. *New Phytol.* 72:1251–1260.

Kader, A. A., G. A. Chastagener, L. L. Morris, and J. M. Ogawa. 1978. Effect of carbon monoxide on decay, physiological responses, ripening, and composition of tomato fruits. *Am. Soc. Hort. Sci.* 103:665–670.

Lieberman, M. 1979. Biosynthesis and action of ethylene. *Annu. Rev. Plant Physiol.* 30:533–591.

Lynch, J. M., and S. H. T. Harper. 1974. Formation of ethylene by a soil fungus. *J. Gen. Microbiol.* 80:187–195.

Pratt, H. K., and J. D. Goeschl. 1969. Physiological roles of ethylene in plants. *Annu. Rev. Plant Physiol.* 20:541–584.

Thompson, G. E. 1941. Leaf-spot diseases of poplars caused by *Septoria musiva* and *S. populicola*. *Phytopathology* 31:241–254.

Zalasky, H., O. K. Fenn, and C. H. Lindquist. 1968. Reactions of poplars to infection by *Septoria musiva* and *Diplodia tumefaciens* and to injury by frost in Manitoba and Saskatchewan. *Plant Dis. Rep.* 52:829–833.