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6. Production of Ethylene and Carbon Monoxide by Microorganisms

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Abstract. Various quantities of ethylene and carbon monoxide were produced on PDA by *Fusicladium effusum, Pestilotia nucicola, Alternaria tenuis,* and *Fusarium oxysporum* subcultured from diseased pecan shucks. Repeated subculturing of these fungi on potato dextrose broth supplemented with iron powder produced ethylene. The production of ethylene by these microorganisms in vitro shows that ethylene should be considered important in the etiology of the disease.

Introduction. Ethylene causes epinasty, leaf abscission, fruit and flower maturation, and other physiological effects associated with aging in plants (1,3,5,7). The production of ethylene has been attributed not only to plants but also to various microorganisms (2,5). The present investigation was initiated to determine if the ethylene produced by fungal and/or bacterial infections causes premature abscission of pecan fruits. The symptom associated with the disease to be studied is similar to pecan stem-end blight and shuck disease as described by Schaller and Ken Knight (8).

Materials and Methods. Diseased blackened shucks with necrotic tissue were collected at Leland, MS, in August 1981, from 16-year-old pecan trees of varieties "Success" and "Desireable." To obtain fungal isolates, diseased shucks were surface-sterilized with 2.6% sodium hypochlorite for 3 min and placed on acidified potato dextrose agar (PDA) in petri plates. The hyphal tips of microorganisms isolated were transferred to PDA slants in screw cap test tubes. The overlying atmospheres were analyzed for ethylene 7 days after the tubes were inoculated with the isolates.

Ethylene production by the microorganisms was tested by gas chromatographic analyses. The pecan isolates were grown in serum-stoppered test tubes containing 10 ml of the following commercially obtained media: nutrient agar (NA), rose bengal agar (RBA), potato dextrose agar (PDA), tomato juice agar (TJA), tryptic soy agar (TSA), and potato dextrose broth (PDB). They were also tested for ethylene production on other media prepared in the laboratory, including: pecan bark agar (PBA), pecan leaf agar (PLA), pecan bark broth (PBB), pecan leaf broth (PLB), pecan shucks (PS), and potato yeast extract mannitol agar (PYEMA). All the above media were sterilized at 121 C for 15 min.

To determine the importance of iron in synthetic media for the production of ethylene, electrolytic iron powder (60 mesh) was added to PDB in various concentrations (0.1-10% w/v). Impurities from the iron were removed by heating to 600 C for 1 hr. Gas chromatographic analysis was used to verify the absence of ethylene and carbon monoxide in the iron after heating.

Two separate gas chromatographic analyses were used to determine the amount of ethylene and carbon monoxide. The first gas analysis was carried out by injecting 100 µl samples of the overlying atmosphere from cultures (room temperature) with a Precision® Pressure-Lok gas syringe into a dual aluminum column Fisher Gas Partitioner Model 1200 gas chromatograph. Helium was employed as the carrier gas with a flow rate of 30 ml/min; column temperature was 75 C, and bridge current was set at 200 mA. In the first dual column, Column 1 (1.98 m long by 0.32 cm in diameter, aluminum) was packed with 80-100 mesh Columpak ™PQ. Column 2 (3.35 m by 0.48 cm in diameter, aluminum) was packed with 60-80 mesh molecular sieve 13X. Attenutation setting was x1.

To replicate the procedure, a second gas chromatographic analysis was performed by injecting 100 µl of the overlying atmosphere from cultures to confirm that the gases produced by the microorganisms were ethylene and carbon monoxide. Helium was employed as the carrier gas with a flow rate of 30 ml/min; column temperature was 50 C, and bridge current was set at 200 mA. Column 1 was 6.1 m by 0.32 cm (Aluminum), packed with 37.5 percent DC-200/500 on 80-100 mesh chromosorb P-AW. Column 2 was 1.82 meters by 0.48 cm (aluminum), 60-80 mesh molecular sieve 13x. Attenuation setting was x1.

Results and Discussion. The following microorganisms were isolated from diseased pecan shucks: *Fusicladium effusum, Pestilotia nucicola, Alternaria tenuis, Fusarium* sp., *F. roseum, F. oxysporum, and a bacterial sp.*

The results of the gas chromatographic analyses of the overlying atmosphere above the microorganisms from the sealed screw cap test tubes are given in Table I. Detectable quantities of ethylene and carbon monoxide were produced by isolates of *F. effusum, P. nucicola, A. tenuis,* and *F. oxysporum,* while the bacterial isolate produced only ethylene. The presence of ethylene and carbon monoxide was confirmed using the second gas chromatographic system. The unidentified species of *Fusarium* and *F. Roseum* did not produce ethylene or carbon monoxide.

Transfer of the subcultures onto new PDA slants resulted in good growth, but neither ethylene nor carbon monoxide were produced by any of the isolates. The absence of ethylene and carbon monoxide was attributed to the lack of some essential component in the synthetic media for gas production. When the isolates were placed on pecan shucks in screw cap tubes on slants for 3 days, the atmospheres overlying the pathogens were analyzed, and it was found that trace amounts of ethylene were produced by the isolates of *F. effusum*, *P. nucicola*, and the bacterial isolate, while neither ethylene nor carbon monoxide were produced by any of the remaining isolates.

Table I. Gas production by microbial isolates on potato dextrose agar

Organism [a/]	Ethylene [μ moles of gas/tube]	Carbon monoxide [μ moles of gas/tube]
Alternaria tenuis	0.43	0.80
Bacterium	18.37	-
Fusicladium effusum	0.43	0.40
Fusarium sp.	-	-
Fusarium oxysporum	0.43	0.40
Fusarium roseum	-	-
Pestalotia nucicola	0.43	0.40

[a/] Microbial isolates were transferred directly from diseased pecan shucks to PDA, and subsequently transferred to test tubes of PDA, sealed, and incubated for 168 hrs.

Subculturing of the isolates onto the same media failed to produce ethylene or carbon monoxide, although there was sufficient mycelium growth of the microorganism. Since substrates appear to dictate the metabolic by-products obtained from fungi, a variety of media were employed, including NA, RBA, PBA, TJA, TSA, PDB, and PYEMA. While mycelial growth was observed for all of the isolates employed, no ethylene was produced. However, when the isolates were cultured on autoclaved pecan shucks, trace amounts of ethylene were observed. It was reasoned that media prepared from other parts of pecan trees also might result in ethylene production; therefore, PBA, PLA, PBB, and PLB were tested, but no ethylene was detected.

In further attempts to develop a laboratory medium from which the fungi would produce ethylene, it was found that growth on PDB supplemented with iron powder did yield ethylene but no carbon monoxide was produced. Uninoculated media prepared in this fashion failed to yield any carbon monoxide or ethylene on slants in serum-stoppered test tubes. Repeated subculturing of the isolates on the same medium resulted in the production of ethylene on slants in the serum-stoppered test tubes (Table II). No ethylene was produced by subcultures growing on media without iron. The role of iron is not known at the present time, but ethylene production in PDB-Iron powder

supplement could have been the result of the presence of the iron itself or the fact that iron acts as an oxidation/reduction poising agent. Also, the varying amounts of ethylene produced by the cultures may reflect the variance from optimum conditions experienced by each isolate.

Lipe and Morgan (6) reported that the normal level of production of ethylene in pecan fruits is below 0.04 μm/kg fresh wt/hr and the level of production is raised to 0.67-0.80 μm/kg fresh wt/hr immediately prior to dehiscence. The *Fusarium* sp. cultures in our studies produced ethylene and carbon monoxide in vitro in excess of the level shown to cause dehiscence. Since both carbon monoxide and ethylene have the same physiological effect (4), the amounts produced by the pathogens (Table I) are equal to the levels of ethylene found in pecan fruits immediately prior to dehiscence. Isotope studies are underway to determine if ethylene produced by the microorganism triggers the host plant to increase the levels of ethylene.

The fact that the microorganisms produced ethylene and carbon monoxide in quantities, similar to amount of ethylene produced by the pecan tree during normal shuck dehiscence (6) suggests that the isolates should be considered important in the etiology of premature abscission, pecan shuck and stem-end blight disease.

Table II. Gas production by microbial isolates on PDA media with heated iron powder (1.0 mg/tube) after 72 hours

Organism	No. of replications	Ethylene produced μ moles of gas/tube ± standard deviation
Alternaria tenuis	2	0.12 ± 0.00
Bacterial sp.	7	0.11 ± 0.05
Fusicladium effusum	3	0.00 ± 0.00
Fusarium sp.	3	0.20 ± 0.07
Fusarium oxysporum	3	0.20 ± 0.07
Fusarium roseum	9	0.25 ± 0.06
Pestalotia nucicola	3	0.00 ± 0.00

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