United States Environmental Protection Agency
Environmental Research Laboratory Corvallis OR 97333
Research and Development EPA-600/D-82-274 August 1982

EPA ENVIRONMENTAL RESEARCH BRIEF

Development of a Test-Tube Stress-Ethylene Bioassay for Detecting Phytotoxic Air Pollutants

L. E. Craker
University of Massachusetts, Amherst, MA 01003

Abstract

The primary purpose of this study was to develop stress-ethylene production from plant seedlings as a simple, rapid and quantitative bioassay for detecting phytotoxic air pollutants. The developed procedure was to require only small quantities of gas for phytotoxic testing, have minimum cultural and space needs, be easily standardized for comparison of results from diverse testing laboratories, and provide an unbiased estimate of phytotoxic activity.

Wheat, *Triticum aestivum* L., and tomato, *Lycopersicon esculentum* L., seedlings growing on 3 ml of a defined agar nutrient media in test tubes were evaluated for their stress-ethylene production following exposure to phytotoxic air pollutants. The test tubes containing the seedlings served as both the pollutant exposure chamber and container for collection of stress-ethylene. Sensitivity of the seedling tissue to phytotoxic air pollutants and the presence of phytotoxicants in an air sample were indicated by increased ethylene evolution from seedlings exposed to pollutants as compared with control seedlings not exposed to pollutants.

Applicability of the bioassay was determined by testing five known phytotoxicants: sulfur dioxide, nitrogen dioxide, chlorine, hydrogen sulfide, and ozone. Except for ozone, seedling tissue was treated with the pollutant by sealing the top of the test tube with a rubber serum cap and injecting the pollutant through the serum cap with a hypodermic needle and syringe. Seedlings were exposed to ozone by placing the test tube (not sealed with serum cap) into a large ozone treatment chamber. Stress-ethylene production was measured by gas chromatography of a 2-ml gas sample removed from each tube.

Results indicated that stress-ethylene production from wheat or tomato seedlings could be used to indicate the presence of a phytotoxic pollutant. Since the test plants were grown on an agar medium in test tubes, the procedure could be replicated a large number of times in a matter of hours, requiring only a small amount of air pollutant and a minimum amount of space. The measurement of stress-ethylene provided an unbiased estimate of phytotoxic activity.

Introduction

In order to establish safe release guidelines, government agencies and industrial laboratories need a rapid, simple and accurate means of screening smokestack emissions, industrial compounds, and other air samples for phytotoxicants. Current air pollution screening bioassays generally involve large commitments of time and facilities because of relatively long-term growth periods required for test plants plus large quantities of pollutant and special fumigation chambers for exposing plants to pollutants. In addition, the injury patterns induced by phytotoxic substances can vary from species to species and from pollutant to pollutant, making visible assessment of air pollution subject to human bias.

The bioassay in this report is based on knowledge that injured or stressed plants produce ethylene, that the level of ethylene production by plants is generally related to the level of injury or stress, and that ethylene can be quantitatively analyzed with a gas chromatograph. Plant seedlings were chosen as the test organisms since they would have simple environmental and nutrient requirements and may be grown in large numbers in a small amount of space. Growth of the seedlings in test tubes allowed the use of the tubes as both the air pollutant treatment chamber and the container for stress-ethylene collection.

A defined air pollutant bioassay protocol was to be developed by testing the suitability of plant seedlings in a stress-ethylene bioassay and determining the optimal growth and treatment/response conditions for maximizing the sensitivity of the bioassay. Both phytotoxic and non-phytotoxic gases were to be tested to determine the accuracy of the

bioassay. Different concentrations of pollutants were to be tested to define the relationship between pollutant concentration and stress-ethylene production.

Experimental

Plant Material

Wheat, *Triticum aestivum* L., and tomato, *Lycopersicon esculentum* L., seedlings were selected for these studies because they could be easily grown in test tubes on a simple agar growth media and the plants have been shown to be susceptible to air pollutant injury. Experimental testing was done primarily with the wheat cultivar Olaf and the tomato cultivar Roma VF, although other varieties and cultivars were also tested. A total of 27 varieties of wheat were evaluated for ozone sensitivity by measuring stress-ethylene production.

To initiate uniform germination and growth, the wheat seeds were cold stratified for five days at 5 ° and the tomato seeds were imbibed in distilled water at 25 ° for 24 hours before planting. Seeds were subsequently surface sterilized in a 1% sodium hypochlorite solution for 10 minutes, rinsed with distilled water and individually planted in 23 ml (1.5 × 15 cm) test tubes containing 3 ml of sterilized agar growth medium [1% agar and half-strength Hoagland's solution (Hoagland and Arnon, 1950) (Table 1)]. Following planting, test tubes containing seeds were stoppered with cotton plugs to prevent airborne pathogenic contamination and placed into controlled environment chambers for germination and growth [16 hr day/8 hr night cycle; 65 $\mu E\ m^{-2}\ s^{-1}$ (400-700 nm); with a constant temperature of 18 ° for wheat, 27 ° for tomato]. To provide for water loss and to maintain stomatal openings, 0.5 ml of half-strength Hoagland's solution was supplied to wheat plants every five days and to tomato plants the day before exposure to pollutants.

Air Pollutant Treatments

Sulfur dioxide, chlorine, hydrogen sulfide, nitrogen dioxide, and ozone were selected for studying stress-ethylene production by wheat and tomato seedlings because of their known phytotoxicity to plants. Concentrations of pollutant tested were selected by determining the minimum amount of pollutant that would initiate stress-ethylene production by the seedlings during the short exposure period.

Exposure of plant tissue to all air pollutants except ozone was accomplished by replacing the cotton plugs of each test tube with a rubber serum cap and injecting pollutant into the sealed tube with a syringe and needle to produce a known concentration. Each test tube was then returned to the same controlled environment chamber used to grow the seedlings for an exposure/collection period during which the pollutant interacted with the seedling and the stress-ethylene produced by the seedlings in response to the pollutant treatment was trapped in the sealed test tube.

Seedlings treated with ozone had the cotton plugs removed and were then placed in a Plexiglas treatment chamber (102 cm × 58 cm × 36 cm). Ozone, generated by ultraviolet light, was pumped into the treatment chamber as necessary to maintain a fixed level of ozone for treatment of the seedlings. At the end of the treatment period, the test tubes containing the treated seedlings were sealed with rubber serum caps and replaced in a controlled environment chamber for collection of stress-ethylene.

Table 1. Agar Growth Medium for Growing Test Plants

Ingredient	*Concentration (g/l)*
Agar	10.0
$Ca(NO_3)_2 \cdot 4H_2O$	.590
KNO_3	.253
$MgSO_4 \cdot 7H_2O$	.247
KH_2PO_4	.068
H_3BO_3	1.43×10^{-3}
$MnCl_2 \cdot 4H_2O$	9.05×10^{-4}
$ZnSO_4 \cdot 7H_2O$	1.1×10^{-4}
$CuSO_4 \cdot 5H_2O$	4.0×10^{-5}
$H_2MoO_4 \cdot H_2O$	1.0×10^{-5}
NaFe EDTA	3.94×10^{-3}

Stress-Ethylene Measurement

At the end of the exposure/collection period or collection period (ozone), a 2-ml gas sample was removed from each test tube and quantitatively analyzed for ethylene using a gas chromatograph (sensitive to five ppb ethylene). Following ethylene analysis, fresh weight of the top growth from each plant was determined. Control seedlings were grown the same as pollutant-treated seedlings, except there was no pollutant treatment. Differences in ethylene production (expressed on a per unit fresh weight of leaf tissue basis) between pollutant-treated plants and control plants indicated the stress-ethylene resulting from pollutant exposure.

The optimum age at which the seedlings could be effectively used to test for phytotoxicity was determined by exposing different age seedlings to pollutants for two hours. The most effective length of the exposure/collection period was determined by monitoring ethylene production from seedlings exposed to pollutants for different lengths of time.

All tests were replicated a minimum of three times with five samples per treatment per replicate. Differences in amounts of ethylene produced between treated and control seedlings, differences among age of seedlings and differences among exposure and collection periods were tested for significance by analysis of variance. Ethylene production by wheat and tomato seedlings in response to various concentrations of phytotoxicants was examined by regression analysis.

Results and Discussion

Measurable increases in ethylene production were observed in both wheat (Table 2) and tomato (Table 3) seedlings exposed to phytotoxic air pollutants. The amount of ethylene

Table 2. Stress-Ethylene Production by Olaf Wheat Seedlings Exposed to Air Pollutants. The wheat seedlings were grown on an agar medium in test tubes for six days and then exposed to indicated concentrations of pollutants for 2 hr. Mean ± S.E.

Pollutant	Ethylene Production nl/g fresh wt · 2 hr	% above control
Cl_2 @ 100 µl/l	4.12 ± 0.25	182
O_3 @ 0.35 µl/l	2.78 ± 0.32	90
SO_2 @ 100 µl/l	2.15 ± 0.18	47
NO_2 @ 100 µl/l	2.05 ± 0.14	40
H_2S @ 100 µl/l	1.56 ± 0.31	7
CO @ 200 µl/l	1.38 ± 0.12	0
Control	1.46 ± 0.16	0

produced varied with both the pollutant and the plant species. Wheat plants treated with 200 μl/liter of carbon monoxide (a non-phytotoxic gas except at very high concentrations) did not stimulate ethylene production above that of control plants.

There was an effect of seedling age on the synthesis of ethylene by the plant tissue (Figure 1). Significant differences in ethylene production between seedlings exposed to phytotoxic air pollutants and controls were observed in wheat at all seedling ages tested and in tomato at all seedling ages tested except for five days.

Table 3. Stress-Ethylene Production by Roma VF Tomato Seedlings Exposed to Air Pollutants. The tomato seedlings were grown on an agar medium in test tubes for six days and then exposed to indicated concentrations of pollutants for 2 hours. Mean ± S.E.

Pollutant	Ethylene Production nl/g fresh wt · 2 hr	% above control
Cl_2 @ 100 μl/l	4.49 ± 0.07	193
SO_2 @ 100 μl/l	2.55 ± 0.47	67
NO_2 @ 400 μl/l	2.43 ± 0.17	59
H_2S @ 400 μl/l	2.81 ± 0.50	84
Control	1.53 ± 0.21	0

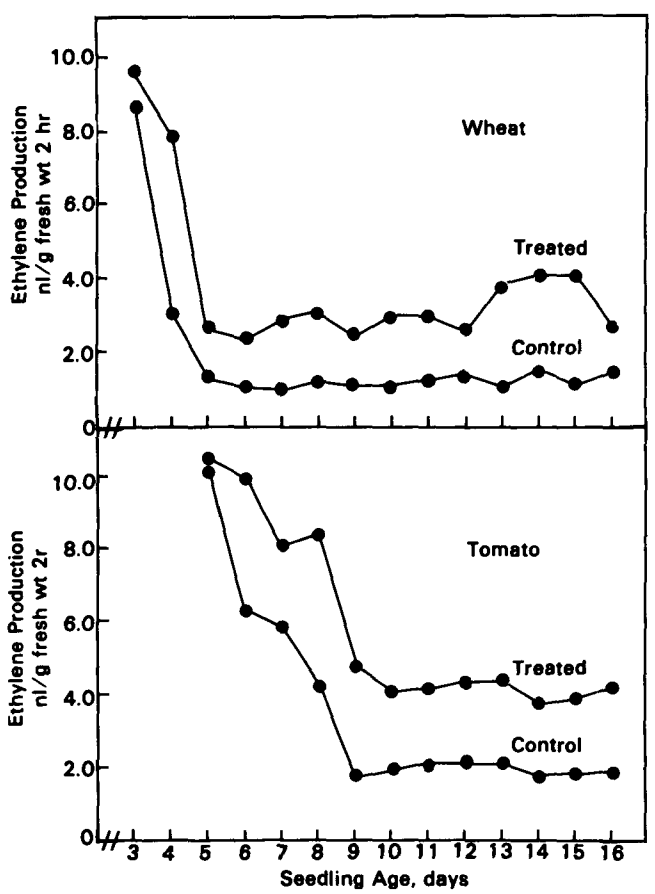

Figure 1. Seedling age and stress-ethylene production. Wheat and tomato seedlings, grown on an agar medium in test-tubes for indicated days, were exposed to 0.35 μl/l O_3 or 100 μl/l Cl_2, respectively, for 2 hours (Treated) or not treated with a pollutant (Control).

The constancy of ethylene production by wheat seedlings ranging in age from 5 to 16 days from seeding and by tomato seedlings ranging in age from 9 to 16 days from seeding, suggested that any age seedling within these ranges could be used in a stress-ethylene test for phytotoxicants. However, there were limitations created by the size of the test tube. After 8 days in wheat and 12 days in tomato, the seedlings had grown so large that there was a physical restriction on continued leaf expansion in tomato due to the sides of the tube and in wheat due to the cotton stopper at the top of the tube. In the case of wheat over 8 days old, the tissue was easily injured when the rubber serum cap was placed in the top of the test tube. To avoid problems of increased ethylene production due to growing and mechanical stresses, wheat plants less than 8 days old and tomato plants less than 12 days old should be used in the test procedure.

The relatively large amounts of ethylene produced in both the pollutant-treated and control seedlings at early ages (3 to 4 days in wheat; 5 to 8 days in tomato) were associated with the early development of the plant tissue. To insure that all seedlings had reached the stage of development where ethylene was produced at a constant level, seedlings older than 5 days in wheat and 9 days in tomato should be used. We recommend using 6 day old wheat and 11 day old tomato seedlings.

Our results indicated an exposure/collection period of two hours was adequate for both inducing the plant tissue to produce ethylene and for collection of sufficient ethylene for measurement by gas chromatography (Figure 2). Although differences in ethylene production between pollutant-treated and control plants could be measured at exposure/collection

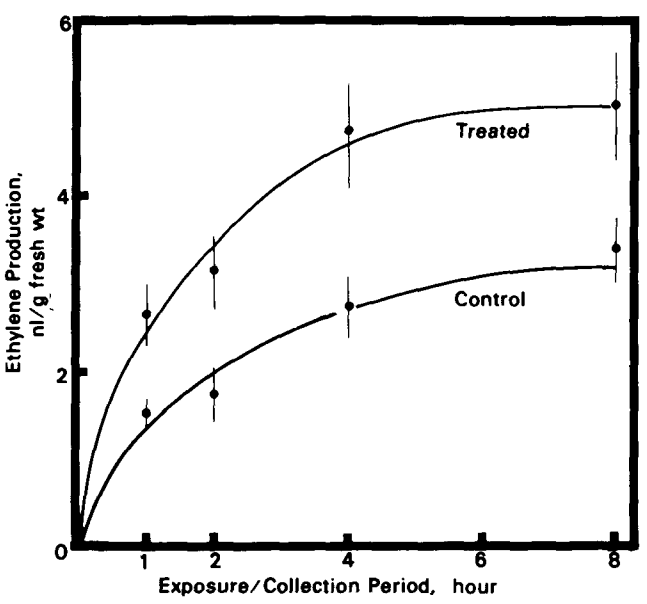

Figure 2. Time course of stress-ethylene production. Tomato seedlings, grown on an agar medium in test-tubes for 11 days, were either exposed to 40 μl/l Cl_2 for indicated times (Treated) or not treated with a pollutant (Control). Vertical lines indicate S.E.

Table 4. Relationship of Ethylene Production to Pollutant Concentration

Plant Species	Pollutant and Range Tested (μl/l)	Linear Response Range (μl/l)	Regression Equation for Linear Response[a]	r^{2}[b]
Wheat	Cl_2, 0-100[c]	0-80	Y = 2.00 + 0.044X	0.90
	SO_2, 0-100[c]	0-100	Y = 1.09 + 0.013X	0.90
	NO_2, 0-400[d]	0-400	Y = 1.41 + 0.004X	0.90
	H_2S, 0-400[d]	0-400	Y = 1.41 + 0.004X	0.90
Tomato	Cl_2, 0-100[c]	0-40	Y = 1.86 + 0.045X	0.90
	SO_2, 0-100[c]	20-100	Y = 0.68 + 0.022X	0.94
	NO_2, 0-400[d]	0-400	Y = 1.21 + 0.002X	0.98
	H_2S, 0-400[d]	0-300	Y = 1.73 + 0.004X	0.79

[a]All regression coefficients were significant at $P \leq 0.001$.
[b]Coefficient of determination.
[c]Concentrations of pollutant increased in units of 20 μl/l.
[d]Concentrations of pollutant increased in units of 100 μl/l.

periods less than two hours, low concentrations of phytotoxic gases did not induce the production of enough stress-ethylene for distinguishing between control and pollutant-treated seedlings. Any collection period with a duration of 2 to 8 hours (longest exposure/collection period studied) would be satisfactory, since the magnitude of difference in ethylene production between treated and control seedlings was maintained. However, the two-hour exposure/collection period was sufficient and allowed for completion of the phytotoxicity test within one working day.

The amount of ethylene produced by pollutant-treated wheat and tomato seedlings was related to the concentration of the pollutant to which the plants were exposed. Based on this relationship, it appears that the magnitude of pollutant concentration within an air sample can be evaluated by the amount of stress-ethylene produced (Table 4, Figure 3).

For all the phytotoxic gases tested, there was a range of pollutant concentrations where an increase in concentration initiated an increase in ethylene production. Concentrations above this range gave only small or no further increases in production of stress-ethylene by the plant tissue, depending upon the pollutant.

Significant differences in stress-ethylene production following exposures to ozone were observed in wheat cultivars (Table 5). These differences suggest a test-tube stress-ethylene bioassay may be capable of distinguishing plant sensitivity to air pollutants.

The development of this bioassay has minimized the quantity of test gas necessary for a phytotoxicity test and reduced the need for greenhouse and fumigation facilities. The recommended procedure for a test-tube stress-ethylene bioassay (TTSEB) is presented in Table 6. Positive phytotoxic responses to SO_2 have been observed in tests conducted over 18 months. The bioassay developed in this proposal should provide a relatively inexpensive method of rapidly screening large numbers of air pollutants for phytotoxicity activity by both government agencies and private industry.

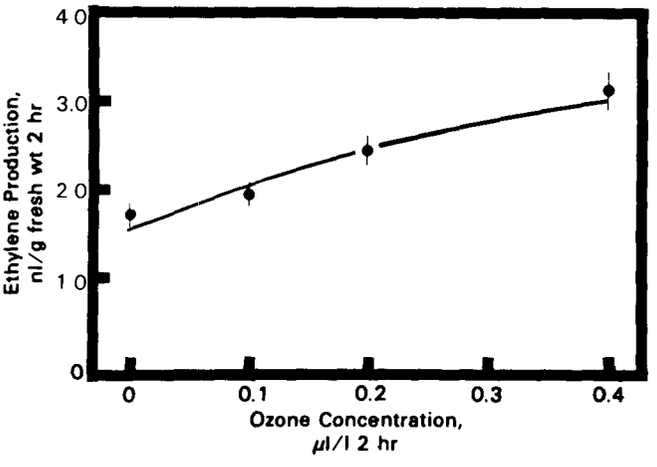

Figure 3. The effect of ozone concentration on ethylene production by wheat seedlings. Olaf wheat seedlings were grown on an agar medium in test-tubes for 6 days and then exposed to indicated concentration of ozone for 2 hours. Each point represents the mean ± S.E. of 4 replicates (Grant, 1980).

Literature Cited

Grant, L. 1980. The effectiveness of stress-ethylene as a screening technique for a varietal susceptibility to air pollution. M.S. Thesis, Dept. of Plant and Soil Science, University of Massachusetts, Amherst, MA 01003.

Hoagland, D.R. and D.I. Arnon. 1950. The water culture method for growing plants without soil. Circular 347. University of California Experiment Station Bulletin.

Table 5. Sensitivity of Wheat Cultivars to Ozone as Indicated by Stress-Ethylene Production (Grant, 1980)

Cultivar	Stress-Ethylene Production[1] (nl/g Fwt · 2 hr)
Waldron*[2]	0.37 a[3]
Coteau*	0.43 a
Coteau*	0.56 bc
Olaf*	0.64 cd
Butte*	0.70 d
Olaf*	0.73 d
Prodax*	0.73 d
Olaf*	0.74 d
Fortuna*	0.82 ef
Newana*	0.86 fg
Ellar	0.89 fgh
Newana*	0.90 gh
Eureka	0.95 hi
Butte*	0.99 ij
WS 25	1.04 jk
Chris	1.05 jk
Waldron*	1.11 kl
Solar	1.13 l
Parker 76	1.16 l
Triumph	1.16 l
Kitt	1.25 m
Prodax*	1.25 m
Protor	1.29 mn
Profit 75	1.36 no
Bounty 309	1.37 o
Eagle	1.39 o
Centurk	1.41 o
Era*	1.51 p
Fortuna*	1.55 pq
Newton	1.56 pq
WS 1809	1.63 qr
Angus	1.69 r
Lew	1.88 st
Era*	1.92 t
James	2.08 u
Funk W444	2.84 v

[1] Difference between control and ozone-treated seedlings. Each value represents the mean of 4 replicates.

[2] Asterisks indicate that more than one seed source of this cultivar was tested.

[3] Mean separation within cultivars by Duncan's new multiple range test. Values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 6. Procedures Used In Test-Tube Stress-Ethylene Bioassay (TTSEB)

1. Prepare sterile growth medium in 23 ml test tubes (3 ml/tube).
2. Plant individual seeds of wheat in each test tube containing growth media and stopper tubes with sterile cotton plugs.
3. Allow seeds to germinate and seedlings to develop.
4. At 5 days from seeding, add 0.5 ml of water to each tube to prevent water stress.
5. At 6 days from seeding, seal test tubes containing wheat seedlings with rubber vaccine caps and add air sample containing suspected phytotoxicant(s) through vaccine cap with hypodermic syringe and needle.
6. Allow 2 hours for exposure of plant to phytotoxicant(s) and collection of stress-ethylene in sealed tube.
7. Remove gas sample from each tube with hypodermic syringe and needle and quantitatively analyze for ethylene by gas chromatography.
8. Determine vegetative fresh weight of each seedling.
9. Express stress-ethylene production for each seedling as nl/g FWT · 2 hr and compare with ethylene production from wheat seedlings not exposed to phytotoxicant.