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Project Summary

Development of a Protocol for Testing Effects of Toxic Substances on Plants

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The purpose of this study was to devise a rapid, simple, and reproducible bioassay procedure to determine the effects of toxic substances on vegetation and to provide a standardized procedure for evaluating and comparing the effects of diverse compounds.

Eight different plant species were evaluated for rapid production of leaf tissue, uniformity within the particular cultivar, structural characteristics, and potential for high ethylene production when exposed to mild stress.

The plants were grown in a growth chamber in small plastic pots in a commercial potting mix, beans for 9 to 10 days and cucumbers for 14 days prior to spraying. A photoperiod of 12 hours produced plants which evolved the most stress ethylene. The test compounds were applied with a modified pendulum sprayer equipped to spray a single plant placed beneath the center of its arc of swing. Prior to spraying, the plants were exposed to light for two hours. Thirty minutes after spraying, the plants were encapsulated under half gallon glass jars with a water seal, and were incubated for 24 hours in a dark chamber at °C. Five dosages of test compounds were used with 8 replicates for the final evaluation.

Ethylene samples were removed from the jars with a syringe having a bent needle. Concentrations of ethylene were determined with a calibrated Aerograph 1520 gas chromatograph. A computer was used to plot the stress-ethylene evolved from plants versus the amount of compound applied from the equation: $\log_e$ (ethylene concentration) = $\log_e A + B$ (concentration of the toxicant).

Seven compounds were tested: two organic herbicides (Paraquat and Endothall), three inorganic plant toxicants (Phytar, sodium fluoride and sodium chlorate), and two insecticides (Orthene and Diazinon). The statistical parameters, slope, intercept and correlation coefficients were recorded.

Reproducibility of the method was tested with two successive runs with Endothall. The slopes were 143.6 and 136.6 with correlation coefficients of 0.91 and 0.96, respectively. Analysis of covariance showed there was no significant difference between these slopes at the 95% confidence interval.

This Project Summary was developed by EPA's Environmental Research Laboratory, Corvallis, OR, to announce key findings of the research project which is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

A rapid, simple, inexpensive and reproducible bioassay procedure or protocol was needed to determine the deleterious effects of toxic substances in the environment on vegetation, and provide a standardized procedure where-

by laboratories could test these diverse substances under standard conditions.

To develop this protocol, a decision was made to use ethylene evolution and visible injury symptom as indices of toxicity. Stress-induced ethylene production by plants is an indication of an injury occurring after very mild trauma or unfavorable growing conditions. The injury was first observed after very mild adverse chemical treatment, and was later caused by insect injury, temperature extremes, drought, irradiation, disease, and by wounding, pressure, or abrasion.

Air pollutants such as ozone and Cl_2, which have induced stress ethylene production in several plants, have been suggested as a measure of ozone injury on plants. Some investigators have advocated the use of ethylene and ethane production to measure SO_2 injury.

A spectrum of plant species from diverse families were to be tested under standardized conditions with precise levels of toxic substances and measurement of the amount of stress ethylene produced. Correlations of dosage and level of ethylene produced were to be made using statistical procedures.

Experimental

Two growth chambers which control temperature, light and humidity were used for growing the plants.

A gas-chromatograph equipped with a 2-ml sample loop, and a column of Poropak N, gave good separation of ethylene and precise, reproducible determinations.

Toxicants were applied with pendulum sprayer; an average deposit of liquid from 5 applications was 2.69 mg/cm² with a standard deviation of .06 mg/cm² (Table 1).

Several different methods were employed for enclosing treated plants and sampling the amount of ethylene produced. The best method consists of covering the treated plant with a two-quart widemouth glass jar; a small aluminum weighing dish is placed in the bottom of a six-inch saucer. The pot containing the treated plant is then placed in the aluminum dish and covered by the inverted jar. The plant saucer is filled with water to seal the opening. A gastight syringe, equipped with a bent hyprodermic needle, is inserted beneath the jar to withdraw ethylene samples.

Because incubation temperature affected ethylene production, causing higher ethylene production at increased temperatures, the plants were held in a darkened incubation box and warm air from the growth chamber held the temperature at about 24°C for the 24 hours of incubation.

Table 1. Reproducibility of Sprayer Delivery

Date	8/22/79	1/24/79	11/29/78	12/27/78
Nozzle #	4001	4001	6501	6501
Distance from Sprayer Tip (cm)	30	30	45	45
Delivery (Mg/cm^2)	2.06	2.63	1.18	1.77
	2.07	2.67	1.26	1.77
	2.14	2.70	1.31	1.79
	2.09	2.66	1.26	1.78
		2.79	1.31	1.76
			1.28	1.77
			1.31	1.79
			1.22	1.68
			1.33	1.75
			1.31	1.77
Mean	2.09	2.69	1.28	1.76
Standard Deviation	.04	.06	.05	.03

Eight species of plants were tested. The criteria considered in choosing the test plants were: fast growth; e.g., rapid production of leaf tissue, uniformity, plant habitus; e.g., structural characteristics that make it suitable for this particular application, and the potential for high ethylene production when exposed to mild stress.

Banana Squash (UCR selection), Corn (Early Sunglow), Cucumber (Pickling SMR-58), Bush Bean (Blue Lake), and Kidney Bean (Pink), Radish (Scarlet Globe), Spinach (Thickleaved Nobel), and Sunflower (Mammoth 307) were grown in growth chambers.

Phytar and Endothall, both weed killers known to cause ethylene production, were applied as sprays at low concentrations. The ethylene accumulated during the 24-hours after this application was measured (Table 2). Cucumbers and kidney beans were selected due to their fast growth and high ethylene production.

Environmental conditions for growth and development of the test plants with kidney beans (pink) and cucumbers (pickling SMR-58) were to grow them in six-ounce styrofoam cups (180 ml) with drainage holes in a commercial product.*

*Jiffy Products of America
250 Town Road
Chicago, IL 60185

The seeds were planted two-centimeters in cups deep and placed in trays filled with tap water. When germinated, the plants were irrigated with one-half strength NCSU phytotron nutrient solution. Light intensity in growth chambers was 322 Einsteins/m^{-2}/sec^{-1}. A 12-hour photoperiod was compared with a 16-hour photoperiod. Beans grown under the shorter photoperiod produced considerable more ethylene after spraying with Endothall and Phytar (Table 3), but slightly less when treated with sodium fluoride. The day and night temperatures and relative humidity established for beans were 27°C, 21°C, and 65%, respectively, and for cucumbers 30°C, 26°C, and 80%. Kidney beans (pink) were ready for testing 9 to 10 days after planting while cucumbers required 14 days.

A series of tests showed that the optimum light period following darkness and prior to spray application for stress ethylene is between 1.5 hours and 2.5 hours (Table 4). Incubation, in a lighted growth chamber following spraying and encapsulation, caused a 11°C temperature rise which caused ethylene evolution in controls; thereafter, the plants were encapsulated as soon as dry and incubated in the dark.

To establish some definite period during which the treated plants were allowed to evolve ethylene during the dark incubation, beans were treated with Endothall and cucumbers with Phytar. Rates of evolution were recorded for 24 hours or longer. Both plants had sigmoid rate curves. The bean plants had plateaued, or were regressing, at 24 hours. The cucumbers reached a plateau

between 30 and 46 hours with 70% of the total having been evolved in 24 hours. Because the beans had plateaued, and 70% of maximum ethylene had been produced by the cucumbers, 24 hours was selected as fixed period for sampling.

Preparation of suitable dilutions of test compounds which limit water solubility can present problems. Oil-soluble materials can often be dissolved in acetone and/or a non-toxic oil, such as olive oil; and emulsifier added, and a stable oil-in-water emulsion prepared. Odorless kerosene can also serve as a primary solvent. These emulsions can often be diluted to obtain suitable concentrations. Less soluble compounds can often be dissolved in acetone which is then dispersed in water with violent agitation. Water solutions require a non-toxic wetting agent to obtain uniform leaf coverage. In these tests, X-77* was used.

These procedures were used for formulation of the compounds tested as follows:

1. Paraquat - water solution + X-77
2. Phytar - water solution + X-77
3. Endothall - water solution + X-77
4. Sodium Fluoride - water solution + X-77
5. Sodium Chlorate - water solution + X-77
6. Orthene - water solution + X-77
7. Diazinon - dissolve in acetone-dispersed in water + X-77

Stress ethylene produced by plants when exposed to a toxicant increases proportionately with the toxicant concentration and up to a limit the increases in the stress-ethylene production can be modeled using the following equation: Log_e (ethylene concentration) = Log_e A + B (concentration of the toxicant). In this equation, A is an estimate of the ethylene production of nontreated plants and B is the slope parameter which is a measure of the increase in stress-induced ethylene production in relation to the stress concentration. Our studies showed that this slope parameter can be used to express the relative toxicity of aqueous solution or suspensions of toxicants on vegetation. All plots and slope parameters are based upon the values on the linear portion of the curve plus the control values.

*Colloidal Products Corp.
P.O Box 666
Sausalito, CA 94965

Table 2. *Ethylene[1] produced by Five Different Plant Species After Spraying with Phytar or Endothall*

g/l	Banana squash	Corn	Cucumber	Bush beans	Kidney beans
		PHYTAR			
0.0	48	10	63	37	45
0.095		2.8	81		
0.19		5.3	144		
0.38	58	11	443		232
0.76	86	11	1722	146	517
1.82	146		2673		744
3.04				669	
Age, days	21	80	14	9	9
Leaf area, cm^2	190	—	67	130	165
		ENDOTHALL			
0.0	47	0.5	60	16	24
0.01	74		84		
0.02				810	938
0.03			151		
0.04	161			1223	2835
0.08	667				
0.125					
0.250		1.2			
0.375					
0.500		1.6			
Age, days	17	8	14	9	9
Leaf area, cm^2	115	—	72	130	165

[1]*Parts per billion.*
[2]*6-8 plants per concentration.*

The reproducibility of the method was determined by two tests with Endothall. The slopes were, respectively, 143.6 and 136.6; the correlation coefficients were 0.91 and 0.96. Analysis of covariance showed that there is no significant difference between these slopes; the 95% confidence interval of the mean is 140 ± 8.0. This interval was determined for n=40; namely, five concentrations at eight plants each. For n=30, the interval would be 140 ± 10 and for n=20, 140 ± 12. These results suggest that a smaller number of plants per concentration would not affect the results dramatically.

The relative toxicity of test compounds was determined as above, and the slope, intercept and correlation coefficients, are shown in Table 5.

Evaluation of the seven test compounds (Table 5) shows that correlation coefficients on some earlier runs showed little significance. However, as the relative importance of key experimental factors was recognized and better controlled, better results were obtained and reproducibility from run-to-run was good.

Table 3. *Ethylene Evolution of Kidney Beans and Cucumbers Grown with 2 Photoperiods*

		Endothall (Beans)		
98	12	19	604	3195
98	16	11	196	774
97	12	6	609	1833
97	16	5	133	77
99	12	16	1363	3229
99	16	17	326	1437
		Phytar (Beans)		
		0	.30 g/l	.60 g/l
101	12	12	171	318
101	16	10	16	256
Run 3	Light hrs	Control ppb	.020 g/l ppb	.040 g/l ppb
		NaF (Cucumbers)		
			2.62 g/l	5.25 g/l
100	12	19	62	139
100	16	33	51	296

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Table 4. *Effect of Light Period before Spray Application on Ethylene Evolution (ppb)*

Light Period	Beans, Endothall .02 g/l						Cucumbers, Sodium Fluoride 5.25 g/l					
(hrs.)	Run 58		Run 69		Run 70		Run 51		Run 63		Run 72	
0	44.97	6.4†							0.42	0†	80.14	19.7†
0.5			374.54	5.6†	786.63	8 6†					77.22	9.6‡
1									20.20	.6†		
1.5	272.26*	4.4†	509.81	27.8†	814.96*	1.1†	158.40*	20.2†			68.43	12.1†
2									86.45*	0†		
2.5			609.71*	10 7†	523.70	0†					93.19*	12.8†
3									27.69	1.7†		
3 5	242.46	2.4†	489.99	14.5†	419 53	0†	21.00	8.4†			55.51	8†
5.5	155.16	0†					21.58	1.2†				
7.5	15 73	0†					3.63	0†				

*= maximum response.
† nontreated controls.

Table 5. *Slope Parameter and Correlation Coefficient with Kidney Beans and Cucumbers Treated with Seven Plant Toxicants*

Plant Species	Phytotoxicant	Slope	Correlation Coefficient
Beans	Endothall	143.6	0.91
Beans	Endothall	136.6	0.96
Cucumbers	Endothall	19.1	0.47
Beans	Phytar	8.4	0.93
Cucumbers	Phytar	5.8	0.93
Beans	Paraquat	2.0	0.70
Cucumbers	Paraquat	0.14	0.14
Beans	NaF	0.04	0.80
Cucumbers	NaF	0.25	0.62
Beans	$NaClO_3$	0.23	0.69
Cucumbers	$NaClO_3$	0.05	0.24
Beans	Orthene	0.04	0 38
Cucumbers	Orthene	0.001	0.06
Cucumbers	Diazinon	0 106	0.74

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***David T. Tingey** is the EPA Project Officer (see below).*
The complete report, entitled "Development of a Protocol for Testing Effects of Toxic Substances on Plants," (Order No. PB 81-157 901; Cost: $6.50, subject to change) will be available only from:
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