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NATURAL PRODUCTS PHYTOTOXICITY
A Bioassay Suitable for Small Quantities of Slightly Water-Soluble Compounds[1]

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Abstract—A large variety of secondary metabolites that can inhibit germination and/or seedling growth are produced by plants in low quantities. The objective of this study was to develop a bioassay capable of reliably assessing reductions in germination percentage and seedling length of small-seeded plant species caused by exposure to minute quantities of these compounds. The germination and growth of alfalfa (*Medicago sativa*), annual ryegrass (*Lolium multiflorum*), and velvetleaf (*Abutilon theophrasti*) were evaluated against six known phytotoxins from five chemical classes; cinmethylin (a herbicidal cineole derivative) was selected as a comparison standard. Each phytotoxin, dissolved in a suitable organic solvent, was placed on water–agar in small tissue culture wells. After the solvent evaporated, imbibed seeds were placed on the agar; after three days, germination percentages and seedling lengths were measured. Compared to a commonly used filter paper procedure, this modified agar bioassay required smaller quantities of compound per seed for comparable bioassay results. This bioassay also readily permitted the measurement of seedling length, a more sensitive indicator of phytotoxicity than germination. Seedling length decreased sigmoidally as the toxin concentration increased logarithmically. Phytotoxicity was a function of both compound and plant species. Cinmethylin, a grass herbicide, reduced the length of annual ryegrass seedlings by 90–100%, whereas that of alfalfa and velvetleaf was inhibited slightly. The agar bioassay facilitated the rapid and reliable testing of slightly water-soluble compounds, requiring only minute quantities of each compound to give reproducible results.

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Key Words—Alfalfa, *Medicago sativa*, benzylisothiocyanate, cinmethylin, cinnamaldehyde, coumarin, germination, juglone, nigericin, plumbagin, ryegrass, *Lolium multiflorum*, seedling length, velvetleaf, *Abutilon theophrasti*.

INTRODUCTION

The phytotoxicity of various compounds, as demonstrated by reductions in germination and seedling growth, has been evaluated in Petri dishes with filter paper (Lehle and Putnam, 1982; Shilling and Yoshikawa, 1987; Tang and Young, 1982; Wolf, 1986), sand (El-Deek and Hess, 1986), and water agar (Pederson, 1986). Although the germination percentage (defined as the proportion of seeds with a protruded radicle) is typically measured to assess the phytotoxic response to a compound, root length and root fresh weight were found to be more sensitive growth parameters (Cope, 1982; Pederson, 1986; Shilling and Yoshikawa, 1987). Because bubbles form under filter paper when wetted, small seeds often received a nonuniform supply of moisture and gave inconsistent results. Pederson (1986) found that differences in white clover (*Trifolium repens*) root length were more readily detected when grown on agar treated with aqueous extracts from tall fescue (*Festuca arundinacea*) leaves than on germination paper.

The majority of phytotoxicity studies have been conducted with water-soluble compounds such as the phenolics. These materials are typically applied to a growth medium in aqueous solution (Leather and Einhellig, 1985; Lehle and Putnam, 1982; Tang and Young, 1982). Pederson (1986) combined liquid agar and aqueous leaf extracts and then allowed them to solidify prior to assay. Other phytotoxic compounds, such as nigericin, are poorly soluble in aqueous systems (Heisey and Putnam, 1986). These toxins are normally dissolved in organic solvents and applied to filter paper. After the solvent is evaporated, water is added to induce germination (Shilling and Yoshikawa, 1987; Wolf et al., 1984; Wolf, 1986). The application of slightly water-soluble compounds to an agar surface may facilitate the accurate measurement of their toxic effects on the seedling length of small-seeded plant species.

The application of phytotoxins to a relatively large surface area, such as 100-mm-diameter Petri dishes (Wolf et al., 1984; Pederson, 1986), requires substantial quantities of test materials. By reducing the area of the test surface, a greater proportion of the slightly water-soluble compound is in contact with the seedling. The quantity of material available for study is often limited in natural products research, precluding the accurate bioassay of toxic effects. A reduction in the dimensions of the germination container might reduce the quantity of compound required for replicated bioassay. The objective of this study was to develop a bioassay capable of reliably assessing effects of minimum

quantities of phytotoxin on germination and seedling growth of several small-seeded plant species. The toxicity of six phytotoxins from five chemical classes, and the grass herbicide cinmethylin, was evaluated on three plant species with an agar germination medium in small tissue culture wells.

METHODS AND MATERIALS

Source of Materials. Alfalfa (*Medicago sativa* L., cv. Vernal, Kelly Seed Co., Peoria, Illinois), annual ryegrass (*Lolium multiflorum* L., Kelly Seed Co.), and velvetleaf (*Abutilon theophrasti* Medic., Azlin Weed Seed Service, Leland, Mississippi) seeds were obtained from cold storage (1°C) at the Northern Regional Research Center (NRRC), Peoria, Illinois). Cinmethylin {*exo*-1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)methoxy]-7-oxabicyclo[2.2.1] heptane} was supplied by E. I. du Pont de Nemours and Co., Inc., Wilmington, Delaware. *trans*-Cinnamaldehyde, coumarin, and nigericin were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin; juglone and plumbagin from Sigma Chemical Company, St. Louis, Missouri; and benzylisothiocyanate from ICN Pharmaceuticals, Inc., Plainview, New York. Cluster24 tissue culture dishes were obtained from Costar, Cambridge, Massachusetts. All solvents were reagent grade.

Solvent Optimization. Water agar was prepared by autoclaving 4.4 g Bactoagar (Difco Laboratory, Detroit, Michigan) in 400 ml distilled water for 20 min, and then diluted with an additional 400 ml of distilled water. One milliliter of agar was placed in each of the 24 3-ml wells, arranged in four rows of six columns each, in each disposable tissue culture dish. The volumes 0.0, 0.2, 0.4, 0.6, 0.8, or 1.0 ml of four solvent mixtures (100:0, 10:90, 1:99, or 0:100 of chloroform–hexane) were layered on solidified agar in each tissue culture well. The solvent was allowed to passively evaporate from the agar surface in a ventilated hood, typically requiring 4 hr, before seeds were placed on the agar.

Alfalfa and velvetleaf seeds were surface-sterilized in 10% Clorox for 15 m, thoroughly rinsed with distilled water, and imbibed for 4 hr on two layers of filter paper that were saturated with water. Five seeds of imbibed alfalfa and velvetleaf, and of unimbibed annual ryegrass, were placed on the agar in each well. The dishes were placed in a growth chamber at constant 25°C. After three days, seeds with a radicle protruding through the seed coat were counted as germinated. The seedling length (root plus shoot) of germinated seeds was measured after being frozen. Freezing over night softened the seedlings and facilitated the measurement of length. Seeds that did not germinate were not considered in seedling length measurements.

Agar Bioassay Method. A stock solution of each test compound was made

by dissolving the desired quantity in 1 ml chloroform and diluting to 100 ml with hexane. A dosage series of each compound was made by diluting the stock solution with 1:99 chloroform–hexane (v/v). One milliliter of each solution was carefully layered on the agar in each appropriate tissue culture well. Five imbibed alfalfa, annual ryegrass, or velvetleaf seeds were placed in each well. Germination percentages and seedling lengths were recorded according to the procedure described above.

Filter Paper Bioassay Method. Filter paper germination bioassays were conducted as described by Wolf et al. (1984). Two pieces of Whatman No. 1 filter paper were placed in each 9.0-cm-diameter glass Petri dish. Two milliliters of each compound, dissolved in 1:99 chloroform–hexane (v/v) as before, were pipetted on the filter paper. Approximately 1 hr after evaporating to dryness, the filter paper was rewetted with 4 ml distilled water and 20 imbibed seeds were spaced on the filter paper. The dishes were wrapped in aluminum foil and placed in a growth chamber maintained at 25°C. After three days, the number of germinated seeds was counted according to the procedure described previously.

Experimental Design. Solvent and germination bioassays were conducted as split-plot completely randomized designs. Toxin or solvent mixture represented the main plots, and the dose represented the split plot. All experiments were replicated at least four times. The data were analyzed by general linear model and linear regression procedures of the Statistical Analysis System (SAS Innstitute Inc., Cary, North Carolina 27511-8000) and the curvilinear regression procedure of SigmaPlot (Jandel Scientific, Corte Madera, California 94925). Treatment effects were considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

Solvent Optimization. Many biologically active compounds isolated in natural products research are insoluble or slightly soluble in water, but are soluble in organic solvents. Because chloroform and hexane are inexpensive solvents that can readily dissolve a wide variety of poorly water-soluble compounds, an optimum solvent or solvent mixture for compound delivery was identified using these solvents. Alfalfa and velvetleaf germination and seedling length were equally affected by the solvent treatments (Figure 1). Only 0.2 ml chloroform reduced the germination percentage of alfalfa and velvetleaf by 80% (Figure 1). A volume of 0.4 ml chloroform or greater reduced the germination percentage by 100%. Seedling length was reduced 77% by 0.2 ml of chloroform, and 100% by 0.4 ml or more chloroform (Figure 1). Chloroform dissolved the surface of the polystyrene wells leaving a layer impervious to water movement between the seed and agar. Chloroform also penetrated the agar because its

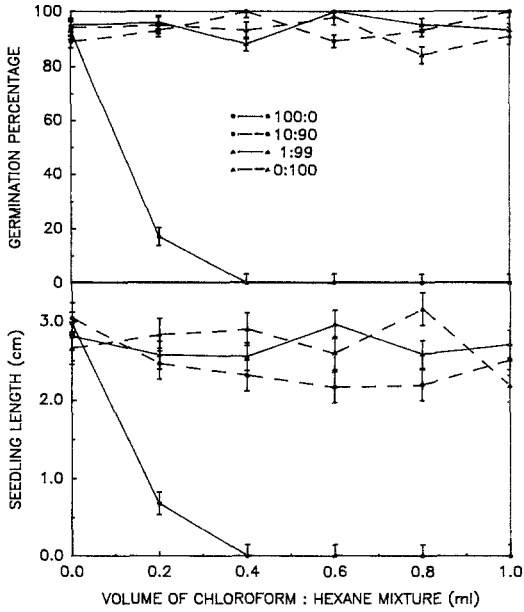

FIG. 1. The effect of chloroform, hexane, and representative mixtures of chloroform and hexane on alfalfa and velvetleaf seed germination and early seedling growth in polystyrene tissue culture wells ± 1 SE.

density is greater than that of water, preventing its complete evaporation from the agar.

Germination percentage was not affected by absolute hexane or either of the chloroform–hexane mixtures (Figure 1). Seedling length was reduced by 25% at all volumes of 10:90 chloroform–hexane, but was not reduced by hexane or 1:99 chloroform–hexane (Figure 1). Absolute hexane did not affect germination or seedling growth in the bioassay after evaporation but was incapable of dissolving several of the test compounds. A small proportion of chloroform (10:90, chloroform–hexane, v/v) proved nontoxic to germination, but reduced seedling length slightly. The optimum solvent was a 1:99 mixture of chloroform–hexane (v/v), which was nontoxic to both germination and seedling growth. When dissolved in a small volume of chloroform and diluted to the desired volume with hexane, all the test compounds, plus numerous others not reported here, could be solubilized and tested for phytotoxicity using the agar bioassay without an adverse solvent effect.

Agar versus Filter Paper Bioassay. All five compounds submitted to filter paper and agar bioassays reduced the germination of alfalfa, annual ryegrass, and velvetleaf to at least 20%, except for benzylisothiocyanate against vel-

vetleaf on filter paper (Tables 1–5). Both bioassay techniques indicated that juglone, plumbagin, and coumarin were more phytotoxic against germination than benzylisothiocyanate and cinnamaldehyde. A statistically significant curvilinear regression equation was fitted to the data for each plant–compound combination, indicating that the germination percentage was reduced linearly as the concentration of phytotoxin decreased. Using the agar bioassay, the LD_{50} (mg/replication) of juglone and plumbagin against velvetleaf was 20- and fourfold less, respectively, than the filter paper bioassay (Table 1). Similarly, between three- and sixfold less juglone or plumbagin was required to reduce the germination percentage of alfalfa or annual ryegrass with the agar bioassay (Table 2). With coumarin, the LD_{50} (mg/replication) values for alfalfa and annual ryegrass were six- and ninefold less with the agar bioassay (Table 3). Much lower doses of benzylisothiocyanate and cinnamaldehyde (26- to 187-fold) were required by the agar bioassay to reduce the germination percentages of alfalfa, annual ryegrass, and velvetleaf comparably to that of the filter paper bioassay (Tables 4 and 5). Without exception, the agar bioassay described herein

TABLE 1. RELATIVE PHYTOTOXICITY OF JUGLONE AND PLUMBAGIN AGAINST VELVETLEAF SEED GERMINATION IN AGAR AND FILTER PAPER BIOASSAY

	Filter paper bioassay[a]			Agar bioassay		
	mg/rep	Mole/seed (10^{-8})	Germination (%)	mg/rep	Mole/seed (10^{-8})	Germination (%)
Juglone						
	0.28	8	100	0	0	95
	0.56	16	74	0.017	2	80
	0.70	20	41	0.035	4	60
	2.09	60	6	0.053	6	35
				0.070	8	25
				0.088	10	10
LD_{50}	1.02	30		0.05	5	
$LSD_{0.05}$						14
Plumbagin						
	0.15	4	100	0	0	92
	0.38	10	66	0.031	3.3	92
	0.53	14	22	0.063	6.7	67
	0.60	16	12	0.094	10.0	50
				0.125	13.3	33
				0.157	16.7	17
LD_{50}	0.40	9		0.10	10	
$LSD_{0.05}$						22

[a] From Spencer et al. (1986).

TABLE 2. RELATIVE PHYTOTOXICITY OF 1,4-NAPHTHOQUINONES JUGLONE AND PLUMBAGIN AGAINST ALFALFA AND ANNUAL RYEGRASS SEED GERMINATION IN AGAR AND FILTER PAPER BIOASSAY

Filter paper bioassay				Agar bioassay			
		Germination (%)				Germination (%)	
mg/rep	Mole/seed (10^{-8})	Alfalfa	Ryegrass	mg/rep	Mole/seed (10^{-8})	Alfalfa	Ryegrass
Juglone							
0	0	95	97	0	0	95	90
0.003	0.1	92	95	0.017	2	90	95
0.035	1.0	87	97	0.052	6	80	55
0.348	10.0	58	80	0.087	10	70	45
0.523	15.0	46	33	0.131	15	20	0
0.697	20.0	20	11	0.174	20	7	0
LD_{50}							
mg/rep		0.43	0.43			0.10	0.07
Mole/seed		13.4	12.1			10.8	7.9
$LSD_{0.05}$		16	10			21	17
Plumbagin							
0	0	97	95	0	0	95	95
0.075	2.0	90	100	0.019	2	85	55
0.151	4.0	92	90	0.056	6	100	0
0.226	6.0	8	0	0.094	10	60	0
0.565	15.0	0	0	0.141	15	0	0
LD_{50}							
mg/rep		0.24	0.17			0.09	0.03
Mole/seed		4.2	3.9			9.0	2.9
$LSD_{0.05}$	9	9	4		18	18	10

TABLE 3. RELATIVE PHYTOTOXICITY OF COUMARIN IN AGAR AND FILTER PAPER BIOASSAY WITH ALFALFA, ANNUAL RYEGRASS, AND VELVETLEAF

Dose		Germination percentage		
mg/rep	Mole/seed (10^{-8})	Alfalfa	Annual ryegrass	Velvetleaf
Filter paper bioassay				
0	0	92	93	95
0.003	0.1	92	92	95
0.029	1.0	77	90	90
0.292	10.0	53	48	98
0.438	15.0	6	18	94
0.584	20.0	6	4	89
LD_{50}				
mg/rep		0.24	0.26	>0.58
Mole/seed		9.9	10.1	>20.0
$LSD_{0.05}$		18	34	8
Agar bioassay				
0	0	95	90	100
0.029	4	70	75	100
0.044	6	20	20	83
0.073	10	15	0	92
0.110	15	0	0	0
LD_{50}				
mg/rep		0.04	0.03	0.08
Mole/seed		6.6	4.9	9.6
$LSD_{0.05}$		16	13	16

required smaller quantities of the compounds tested to reduce the germination percentage comparably to that of the typical filter paper bioassay.

The agar bioassay provides a sensitive method for screening the phytotoxicity of both water- (Pederson, 1986) and organically soluble compounds that are available in limited quantity. One reason less compound was required for a similar effect is because of the smaller dimensions of the tissue culture wells in comparison to that of the Petri dishes. Poorly water-soluble compounds can be absorbed by seeds or seedlings through direct root contact or at dilute concentrations in the bulk flow of water to the root. A larger proportion of poorly water-soluble or insoluble molecules placed on the agar would be available for absorption by the seedlings because a greater proportion of the agar surface is in contact with a seedling, improving the efficiency of phytotoxin contact with, and uptake by, the seedlings.

TABLE 4. RELATIVE PHYTOTOXICITY OF BENZYLISOTHIOCYANATE IN AGAR AND FILTER PAPER BIOASSAY WITH ALFALFA, ANNUAL RYEGRASS, AND VELVETLEAF

Dose		Germination percentage		
mg/rep	Mole/seed (10^{-8})	Alfalfa	Annual ryegrass	Velvetleaf
Filter paper bioassay				
0	0	94	98	94
0.015	0.5	80	95	94
0.147	5.0	89	86	93
1.470	50.0	91	94	94
14.700	500.0	0	0	0
LD_{50}				
mg/rep		6.72	7.08	7.08
Mole/seed		252.7	251.8	251.8
$LSD_{0.05}$		10	8	6
Agar bioassay				
0	0	83	97	90
0.004	0.5	87	90	93
0.037	5.0	90	80	93
0.368	50.0	33	0	0
3.675	500.0	0	0	3
LD_{50}				
mg/rep		0.26	0.17	0.18
Mole/seed		40.3	24.9	25.2
$LSD_{0.05}$		30	12	16

A second reason for the requirement of less compound was because the efficacy of several compounds, namely benzylisothiocyanate and cinnamaldehyde, was enhanced in the agar bioassay. The LD_{50} values in mole per seed units for benzylisothiocyanate and cinnamaldehyde were approximately 10-fold less in the agar bioassay than the filter paper bioassay. The LD_{50} values (mole/seed) of juglone, plumbagin, and coumarin varied from sixfold less to no different in the agar bioassay than the filter paper bioassay. The interaction between compound and bioassay medium may be due to differences such as compound solubility and polarity.

Germination Percentage and Seedling Length. The agar bioassay can capably detect reductions in seedling length by phytotoxin doses lower than those required to detect reductions in the germination percentage, further minimizing the quantity of compound required for bioassay. After seedlings are killed and

TABLE 5. RELATIVE PHYTOTOXICITY OF *t*-CINNAMELDAHYDE IN AGAR AND FILTER PAPER BIOASSAY WITH ALFALFA, ANNUAL RYEGRASS, AND VELVETLEAF

Dose		Germination percentage		
mg/rep	Mole/seed (10^{-8})	Alfalfa	Annual ryegrass	Velvetleaf
Filter paper bioassay				
0	0	94	98	94
0.013	0.5	94	94	96
0.132	5.0	89	95	94
1.322	50.0	91	70	95
13.215	500.0	5	0	13
LD_{50}				
mg/rep		6.57	5.92	7.46
Mole/seed		265.2	246.9	290.8
$LSD_{0.05}$		17	26	26
Agar bioassay				
0	0	87	93	92
0.003	0.5	100	73	93
0.033	5.0	77	90	53
0.330	50.0	0	0	0
3.304	500.0	0	0	3
LD_{50}				
mg/rep		0.15	0.14	0.04
Mole/seed		24.9	25.2	24.4
$LSD_{0.05}$		17	26	26

softened by freezing, seedling length can be readily and reliably measured using the agar bioassay. The roots are restricted to the filter paper surface and can be difficult to remove from the paper without breaking, whereas they readily penetrate the agar and are easily removed. The germination percentage LD_{50} and the seedling length ID_{50} in the agar bioassay, when averaged across the plant species tested, were 8 and 6 $\times$ 10^{-8} mole/seed for juglone and plumbagin, and 7 and 2 $\times$ 10^{-8} mole/seed for coumarin (Tables 1–5, Figure 2), respectively. Others have also shown that many compounds can reduce seedling length (Cope, 1982; Pederson, 1986) and root length (Shilling and Yoshikawa, 1987) by a greater extent than germination at toxic concentrations.

In addition to improving the sensitivity of the bioassay, the measurement of seedling length permits identification of certain compounds as being toxic that would be overlooked if considering germination percentage alone. Rela-

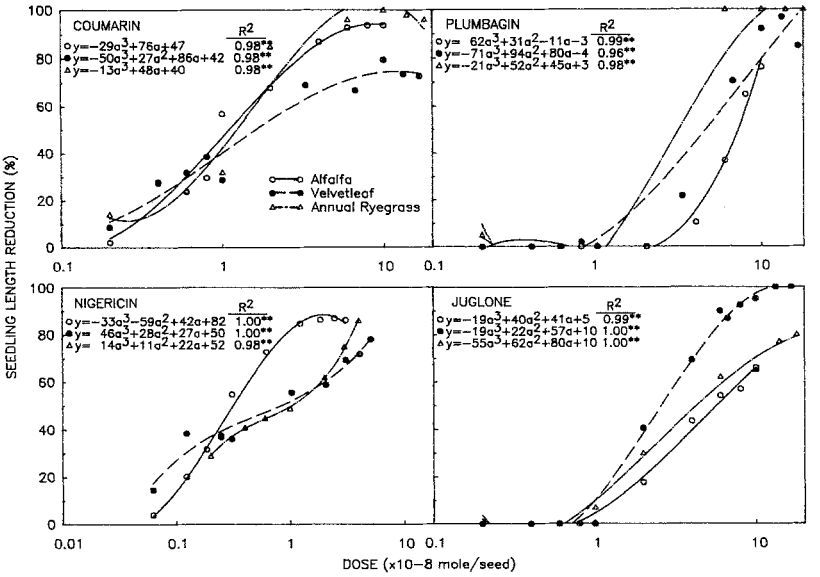

FIG. 2. Reductions in the seedling growth of alfalfa, annual ryegrass, and velvetleaf resulting from increased dosages of several phytotoxins delivered using the modified bioassay (** indicates significance at the 1% level).

tively few compounds directly inhibit the germination process. Many compounds, however, inhibit one or more aspects of seedling growth and metabolism. Nigericin did not inhibit the germination of alfalfa, annual ryegrass, or velvetleaf at the highest doses (data not shown), but was substantially more inhibitory to seedling growth than any of the other compounds tested (Figure 2). Greater potential for identifying inhibitory compounds may be realized by measuring a general parameter of seedling growth such as length.

A sigmoidal reduction in alfalfa, annual ryegrass, and velvetleaf seedling length was caused by coumarin, juglone, nigericin, and plumbagin in the agar bioassay (Figure 2). Low and high doses of each compound caused little change in seedling length. The curvilinear regression equations were significant and described the dose response for each compound and plant species. Dose-response characteristics differed among phytotoxins. The ID_{50} values for nigericin, coumarin, juglone, and plumbagin were, respectively, 0.5, 2.0, 6.6, and 7.2×10^{-8} mole/seed for alfalfa; 1.2, 1.2, 7.4, and 4.0×10^{-8} mole/seed for annual ryegrass; and 1.7, 3.9, 3.6, and 5.6×10^{-8} mole/seed for velvetleaf. Nigericin exhibited the greatest phytotoxicity against seedling length, followed by coumarin, juglone, and plumbagin. Heisey and Putnam (1986) reported that

nigericin strongly inhibited radicle elongation of garden cress (*Lepidium sativum* L.). The agar bioassay is useful for determining the dose–response characteristics of a large variety of compounds.

Germination percentage was often positively correlated with reduced seedling length. Correlation coefficients between germination percentage and seedling length were 0.80 ($P = 0.01$), 0.91, and 0.61 for coumarin, juglone, and plumbagin with alfalfa; and 0.49, 0.94, and 0.89 with velvetleaf. In contrast, nigericin reduced alfalfa and velvetleaf seedling length but did not affect germination. Lower germination percentages were associated with reduced seedling length in the agar bioassay, even though only germinated seeds were considered in seedling length measurements. Phytotoxins that caused the largest reductions in germination percentage did not necessarily cause the largest reductions in seedling length.

Selectivity. The agar bioassay was capable of detecting differences in phytotoxicity among compounds and plant species. Alfalfa and velvetleaf germination were unaffected by cinmethylin. Cinmethylin (0.6×10^{-8} mole/seed) reduced the germination percentage of annual ryegrass by 70%. The germination of velvetleaf was reduced by 89% and 82% by 10×10^{-8} mole/seed of juglone and plumbagin, respectively (Table 1), whereas that of alfalfa was reduced by 26% and 39%, and annual ryegrass by 50% and 100% (Table 2). In contrast, 10×10^{-8} mole/seed coumarin reduced the germination of alfalfa and annual ryegrass by 84% and 100%, respectively, but did not reduce that of velvetleaf. Both naphthoquinones inhibited the germination of alfalfa and annual ryegrass more than that of velvetleaf, whereas coumarin did not reduce that of velvetleaf.

Cinmethylin reduced alfalfa and velvetleaf seedling length maximally by 30% and 18%, respectively, but that of annual ryegrass was reduced 97% by 1×10^{-9} mole/seed cinmethylin (Figure 3). Velvetleaf seedling length was inhibited more by plumbagin and juglone than was alfalfa (Figure 2). Juglone reduced alfalfa and velvetleaf seedling length by 66% and 95% at 1×10^{-7} mole/seed. Plumbagin reduced alfalfa and velvetleaf seedling length by 76% and 85% at 1×10^{-7} mole/seed. In contrast, the highest test doses of nigericin and coumarin reduced alfalfa seedling length by 86% and 94%, and velvetleaf by 78% and 73%.

Summary. The use of sensitive organisms such as garden cress (*Lepidium sativum* L.) in bioassaay is suggested as a way to identify toxic compounds. While useful in some applications, they may be too sensitive in others. The agar bioassay represents an alternative method able to test the effects of compounds on small-seeded agronomic, horticultural, or weed species, thereby emphasizing agricultural relevance.

An agar bioassay method is described that requires smaller quantities of plant growth inhibitors than the frequently used filter-paper bioassay to dem-

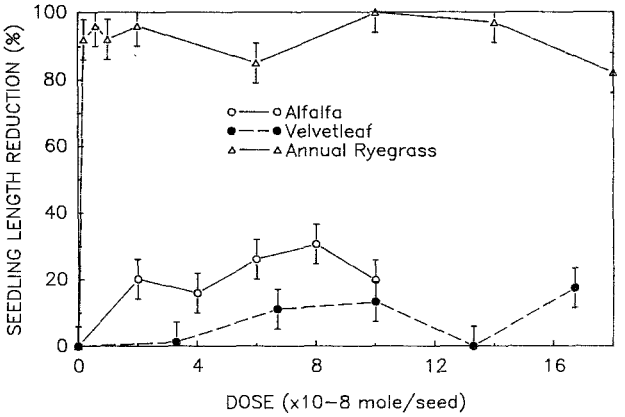

FIG. 3. The dose-response of cinmethylin on early seedling growth of alfalfa, annual ryegrass, and velvetleaf using the agar ± 1 SE.

onstrate comparable reductions in germination percentage. The use of agar as a growth medium in small tissue culture wells permits the accurate, reliable, and fast measurement of both the germination percentage and seedling length. The agar bioassay indicated that seedling length was more responsive than germination to compound phytotoxicity and was sensitive to selectivity among species. Dosage response dynamics of various compounds and plant species can be evaluated rapidly and analyzed statistically with the agar bioassay, permitting the rapid evaluation of poorly water-soluble organic compounds available in limited quantity.

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