ALLELOPATHIC ACTIVITY IN WHEAT–CONVENTIONAL AND WHEAT–NO-TILL SOILS: DEVELOPMENT OF SOIL EXTRACT BIOASSAYS[1]

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Abstract—The primary objective of this research was to determine if soil extracts could be used directly in bioassays for the detection of allelopathic activity. Here we describe: (1) a way to estimate levels of allelopathic compounds in soil; (2) how pH, solute potential, and/or ion content of extracts may modify the action of allelopathic compounds on germination and radicle and hypocotyl length of crimson clover (*Trifolium incarnatum* L.) and ivy-leaved morning glory (*Ipomoea hederacea* L. Jacquin.); and (3) how biological activity of soil extracts may be determined. A water-autoclave extraction procedure was chosen over the immediate-water and 5-hr EDTA extraction procedures, because the autoclave procedure was effective in extracting solution and reversibly bound ferulic acid as well as phenolic acids from wheat debris. The resulting soil extracts were used directly in germination bioassays. A mixture of phenolic acids similar to that obtained from wheat–no-till soils did not affect germination of clover or morning glory and radicle and hypocotyl length of morning glory. The mixture did, however, reduce radicle and hypocotyl length of clover. Individual phenolic acids also did not inhibit germination, but did reduce radicle and hypocotyl length of both species. 6-MBOA (6-methoxy-2,3-benzoxazolinone), a conversion product of 2-*o*-glu-

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cosyl-7-methoxy-1,4-benzoxazin-3-one, a hydroxamic acid in living wheat plants, inhibited germination and radicle and hypocotyl length of clover and morning glory. 6-MBOA, however, was not detected in wheat debris, stubble, or soil extracts. Total phenolic acids (FC) in extracts were determined with Folin and Ciocalteu's phenol reagent. Levels of FC in wheat-conventional-till soil extracts were not related to germination or radicle and hypocotyl length of either species. Levels of FC in wheat-no-till soil extracts were also not related to germination of clover or morning glory, but were inversely related to radicle and hypocotyl length of clover and morning glory. FC values, solute potential, and acidity of wheat-no-till soil extracts appeared to be independent (additive) in action on clover radicle and hypocotyl length. Radicle and hypocotyl length of clover was inversely related to increasing FC and solute potential and directly related to decreasing acidity. Biological activity of extracts was determined best from slopes of radicle and hypocotyl length obtained from bioassays of extract dilutions. Thus, data derived from the water-autoclave extraction procedure, FC analysis, and slope analysis for extract activity in conjunction with data on extract pH and solute potential can be used to estimate allelopathic activity of wheat-no-till soils

Key Words—Cover crops, wheat, *Triticum aestivum*, soybean, *Glycine max*, soil extracts, germination bioassays, phenolic acids, hydroxamic acids, allelopathy, slope analysis, ivy-leaved morning glory, *Ipomoea hederacea*, crimson clover, *Trifolium incarnatum*.

INTRODUCTION

As growers in the United States implement the conservation requirements of the 1985 and 1990 Food Security Acts, many will turn to conservation or no-till agriculture (Worsham, 1990). Understanding how conservation tillage will impact crop production and sustainability of various crop management systems is therefore of considerable interest to U.S. farmers. Although many benefits are cited for no-tillage (i.e., control of soil erosion, soil water retention, reduced use of fossil fuel, etc.), reductions in crop yield and a perceived increased use of herbicides (potential for weed resistance, pollution, etc.) continue to be concerns. The development of effective management options using plant residues could, however, minimize the negative impacts on crop yield and lead to early weed control and a reduction in the use of preemergence herbicides (Worsham, 1991a,b).

Inhibition of weed germination and seedling growth by small grain mulches may be due to: (1) the physical barriers and shading associated with the residues (Worsham, 1989), (2) the immobilization of nitrogen (Broadbent and Tyler, 1962; Kimber, 1973), (3) a reduction in soil disturbance (Worsham, 1989), and/or (4) allelopathic compounds (Chou and Patrick, 1976; Liebl and Worsham, 1983; Shilling et al., 1985, 1986a,b; Barnes et al., 1986; Waller et al., 1987).

Potential allelopathic compounds that have been identified in living and decomposing tissue of small grains include phenolic acids (Chou and Patrick, 1976; Liebl and Worsham, 1983; Barnes et al., 1986; Blum et al., 1991), organic acids (Patrick, 1971; Chou and Patrick, 1976; Lynch, 1977; Tang and Waiss, 1978; Shilling et al., 1985), hydroxamic acids and related compounds (Willard and Penner, 1976; Niemeyer et al., 1989; Nair et al., 1990), and volatiles (Buttery et al., 1985; Bradow, 1991). The presence of such inhibitors in living and decomposing plant tissue does not, however, always translate into actual allelopathic activity, since the level of inhibition observed for a given weed or crop species depends upon: (1) the sensitivity of the species, (2) an adequate rate of release of inhibitors, (3) the activity and strengths of various "sinks" (i.e., clays, organic matter, microbes, seeds, roots) for inhibitors within the soil, and (4) environmental factors (i.e., temperature, soil nutrition, soil pH, etc.). These observations have led researchers to use soil extracts and various chromatographic techniques to identify and quantify potential inhibitors in soils. The identified potential inhibitors, usually in pure form, have then been tested for toxicity (Rice, 1984). Soil solutions, however, contain a complex mixture of inorganic and organic ions and/or molecules that affect the action of any given organic molecule in solution. The use of soil extracts to simulate the soil solution might thus be more meaningful in determining allelopathic potential. The primary objective of this research was, therefore, to determine if soil extracts could be used directly in bioassays for the detection of allelopathic activity.

We have chosen wheat (*Triticum aestivum* L. Coker 916) –soybean (*Glycine max* L. Merill Deltapine) no-till and conventional-till systems (Cecil and Appling gravelly sandy loam) for exploratory studies. The focus was primarily on phenolic acids and secondarily on 6-methoxy-2,3-benzoxazolinone, a conversion product of 2-*o*-glucosyl-7-methoxy-1,4-benzoxazin-3-one, a hydroxamic acid found in living wheat plants.

METHODS AND MATERIALS

Soil Extraction Procedures. Samples from A_1 and B_1 horizons (from the same pedon) of a Cecil (Typic Kanhaludults, clayey, kaolinitic, thermic) soil from the Piedmont of North Carolina were air-dried, sieved (0.25 mm), and characterized (Dalton et al., 1987). Air-dried soil was stored at room temperature in a laboratory until used. One gram of soil was placed in a 15-ml glass centrifuge tube, capped (Bacti-Capall, Sherwood Medical Industries), and autoclaved three times (15 min at 1.2 kg/cm^2 and 121°C) at three-day intervals. One milliliter of filter-sterilized (0.2-μm membrane filter) ferulic acid (1000 μg/ml), adjusted to pH 5 with NaOH, was added to each tube aseptically. The soil–ferulic acid mixtures were incubated at 24°C in a dark and high relative humidity environment. After 70 days, half the test tubes received 0.5 ml of filtered (Whatman No. 42) soil extract obtained from the appropriate Cecil soil material and 0.5 ml double-strength Hoagland's solution (Hoagland and Arnon, 1950) (pH 5). This reinfected the soil with microorganisms. The other half received 0.5 ml filter-sterilized soil extract and 0.5 ml filter-sterilized double-strength Hoagland's solution.

Soil extracts (300 ml of water per 150 g soil material) for reinfestation were obtained from soil–sand mixtures (1 : 2 soil–sand by weight) that had been treated with 5 ml of a phenolic acid mixture (1230 μg/ml; composition of the seven phenolic acid mixtures is described subsequently) and 30 ml of double-strength Hoagland's solution and incubated in the dark at 30°C for three days (Blum and Shafer, 1988). Thirty days after reinfestation, soil samples were extracted. Three extraction procedures were used: (1) immediate water extraction, (2) water-autoclave extraction (45 min) (Blum et al., 1991), and (3) EDTA extraction (0.5 M EDTA adjusted to pH 8, 5 hr extraction time) (Dalton et al., 1987). The final solution volume in each tube was approximately 4 ml. Actual water content was determined gravimetrically on a subset of test tubes (105°C). Tubes were centrifuged for 10 min at 12,100*g*. The resulting supernatants were filter-sterilized (0.2 μm membrane filters) and injected into an HPLC to quantify extracted ferulic acid (Blum et al., 1991).

Field Soil Samples and Extracts. Soil samples (0–2.5 cm depth) were obtained from soybean plots of a low-input sustainable agriculture (LISA) study located 5 km south of North Carolina State University campus. Soybean plots selected included plots with wheat (*Triticum aestivum* L. Coker 916) stubble (no-till), plots in which the wheat stubble had been tilled into the soil, and plots without wheat. Detailed information on the field plots used has been described by Blum et al. (1991). Soil [Cecil and Appling gravelly sandy loam (Typic Kanhaludults)] samples covering a range of treatments, harvests, and soil depths were chosen from 371 samples collected during July–October, 1989, and 72 samples collected during June–November, 1990. Upon collection, soil samples were sieved (3-mm sieve), placed into plastic bags, and stored at −20°C. Soil samples to be extracted were thawed overnight at 10°C. Water content was determined gravimetrically on 10–15 g of soil (105°C).

To obtain extracts, 50–75 g of soil, depending on the experiment, was placed in a 500-ml Erlenmeyer flask with 100 ml deionized water. Changes in the amount of soil extracted per milliliter of water were made to adjust the baseline concentrations of the extracts. Loosely capped flasks were autoclaved for 45 min, removed from the autoclave, and cooled to room temperature over 20–30 min. The slurry in the flask was centrifuged for 10 min at 27,200*g*, and the resulting supernatant was filtered (Whatman No. 42). Except for the final bioassay experiment, filtered supernatants were adjusted with NaOH or HCl to pH 5 (extract 1) or adjusted to pH 2 with HCl, centrifuged for 10 min at 27,200*g* to remove humic acid, and then adjusted to pH 5 with NaOH (extract 2).

Determination of phenolic acid content in extracts is described below. Freezing point depression of extracts was determined with a Osmette Precision Osmometer (Precision Systems Inc., Natick, Massachusetts). No-till soils for final soil extract bioassay were also analyzed for nitrate, ammonium (Cataldo et al., 1974), phosphorus (Taussky and Shorr, 1953), and potassium (atomic absorption spectroscopy).

Solute Potentials of Extracts. Data from Lang (1967) for sodium chloride (20°C) and a model from Steuter (1981) for polyethylene glycol (PEG) 6000 in conjunction with freezing point depression data were used to estimate the solute potential of salt and PEG solutions. This was done only to identify the range of solute potentials of extracts. Regressions for bioassays, to determine the relationship between soil extract solute potential and seed response, were based on freezing point depression values (milliosmoles, mosmol).

Phenolic Acid Determinations. Five milliliters of ferulic acid (standard phenolic acid) solutions (pH 5) or extracts 1 or 2 (0.5 ml) plus 4.5 ml deionized water were mixed with 0.75 ml of 1.9 M Na_2CO_3 and 0.25 ml Folin and Ciocalteu's phenol reagent. This mixture was incubated in the dark at room temperature for at least 1 hr before absorption at 750 nm was measured (Box, 1983; Blum et al., 1991). This procedure will be referred to subsequently as the FC method. Units of total phenolic acid are in micrograms ferulic acid equivalents per gram of soil or milliliter of soil extract. Individual phenolic acids were determined by HPLC analysis (Blum et al., 1991). Folin and Ciocalteu's phenol reagent and phenolic acids, with the exception of sinapic acid, were purchased from Sigma Chemical Company (St. Louis, Missouri). Sinapic acid was purchased from Aldrich Chemical Company (Milwaukee, Wisconsin).

6-MBOA Determinations. Folin and Ciocalteu's phenol reagent also reacted with 6-methoxy-2,3-benzoxazolinone (6-MBOA). 6-MBOA was purchased from Sigma Chemical Company. The amount of 6-MBOA dissolved in water (pH 5) could be estimated by the FC method and the following equation: μg 6-MBOA $= -1.427 + (98.341 * \text{ABS})$, $R^2 = 0.998$, $P \leq 0.0001$, where ABS = the optical density at 750 nm. The HPLC procedure used to detect and quantify phenolic acids was also used for 6-MBOA. 6-MBOA was detected and quantified, however, at 290 nm instead of 254 nm. 6-MBOA concentrations before and after autoclaving procedures were essentially identical. Since 6-MBOA and *p*-coumaric acid have very similar HPLC retention times, thin-layer chromatography of extracts was also used to confirm the absence or presence of 6-MBOA.

Germination Studies. Twenty crimson clover seeds (*Trifolium incarnatum* L. Jacquin. Tibbee; Funk Seeds International, Bloomington, Illinois) inoculated with *Rhizobium* (Nitragin Co., Milwaukee, Wisconsin) or 10 ivy-leaved morning glory seeds (*Ipomoea hederacea* L.; F&J Seed Service, Woodstock, Illinois) were placed in 9-cm-diameter petri dishes containing Whatman No. 1 filter paper and 5 ml of a solution to be tested. Petri dishes were incubated at 30°C for 48 hr before germination, and the total length of the radicle and hypocotyl were determined.

The following solutions were tested for inhibitory effects: (1) soil extracts adjusted to pH 5, unless otherwise stated; (2) PEG 6000 solutions (pH 5) ranging from 0 (distilled water) to 10%; (3) Hoagland's solution (pH 5) ranging from 0 strength (distilled water) to double-strength; (4) a phenolic acid mixture (12, 11, 17, 12, 33, 10, and 5% of the *p*-hydroxybenzoic, caffeic, vanillic, syringic, *p*-coumaric, ferulic, and sinapic acids, respectively; at total concentrations of 0–394 μg/ml; initial solution pH was 5) with various concentrations of PEG or strengths of Hoagland's solution; (5) individual phenolic acids (caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, vanillic, sinapic, or syringic) or 6-MBOA solutions ranging from 0 to 2 mM (pH 5); or (6) 6-MBOA (0–1 mM) with various concentrations of PEG or various strengths of Hoagland's solution. Molecular weights of phenolic acids and 6-MBOA ranged from 138.1 for *p*-hydroxybenzoic acid to 224.2 for sinapic acid. The proportions of the phenolic acids in the mixture were based on the average values for soil extracts (extract 2) from wheat–no-till plots (Blum et al., 1991). Hoagland's solution of various strengths and PEG solutions of various concentrations were used to generate different solute potentials.

General Data Analyses. Data were analyzed by analysis of variance, regressions (PROC GLM), and response surface (PROC RSREG) analysis (SAS Institute Inc., 1988). An alpha value of 0.05 was used in all instances.

Analysis for Final Soil Extract Bioassay. Experimental details for preliminary bioassays, which varied somewhat from the final soil extract bioassay, are given in the results section. Each of the 33 soil extracts from no-till plots was tested at full-, half-, quarter-, and zero-strength using three subsamples of each extract. All extracts showed a trend towards a greater inhibition at higher concentrations. For the data from each extract, the simple linear regression of clover germination (and, separately, of radicle and hypocotyl lengths) on extract strength (0, 0.25×, 0.5×, 1×) was obtained using a common intercept. The resulting slope for an extract served as a measure of its biological activity (e.g., ability to inhibit germination or radicle and hypocotyl length); the steeper the slope, the higher the activity. For completeness, an overall test for quadratic effects was run and found to be not significant. Thus, the use of simple linear slopes was deemed to be adequate. The slopes were used as input (dependent variable) to a further analysis.

Other characteristics of the extracts were also obtained including pH, FC value, freezing point depression, NO_3^-, NH_4^+, K, and P. Model-building tools such as stepwise regressions (SAS PROC LEAPS) and response surface analysis (SAS PROC RSREG) were used to determine which combinations of these characteristics best accounted for the variability in slopes. The resulting regression model related the extract slopes to the measured characteristics of the extracts.

RESULTS

Soil Extraction Procedures. One hundred days after the addition of 1000 μg/g ferulic acid; water, water-autoclave, and EDTA procedures recovered 28, 32, and 37%, respectively, from sterile Cecil A_1, and 17, 30, 52% from sterile Cecil B_1 soil materials, respectively (Table 1). For soils reinfested with microorganisms, recovery was reduced to less than 2%.

Seventy-two and 83% of the ferulic acid added to sterile Cecil A_1 and B_1 soil materials, respectively, could not be recovered by the water-extraction procedure after 100 days (Table 2). Ferulic acid has been shown to be both reversibly and nonreversibly fixed in Cecil soils (Dalton et al., 1987). Six and 16% of the sorbed ferulic acid was recovered from Cecil A_1 and B_1 soil materials, respectively, by the water-autoclave procedure and 13 and 43% by the EDTA procedure. More than 96% of the sorbed ferulic acid recovered from sterile soil by the EDTA procedure was absent in soil extracts from reinfested soils. This was also true for the sorbed ferulic acid recovered from Cecil B_1 soil material by the water-autoclave procedure. Only 55%, however, of the sorbed ferulic acid recovered from the Cecil A_1 soil material by the water-autoclave procedure was accessible to microbes. The Cecil A_1 and B_1 soil material contained 37 and 2 g organic matter/kg, respectively (Dalton et al., 1987). This suggested that at least part of the sorbed ferulic acid recovered by the water-autoclave procedure came from the organic matter in the soil.

The EDTA procedure recovered 2–2.6 times more sorbed ferulic acid than the water-autoclave procedure. EDTA soil extracts, however, could not be used directly for germination bioassays, because clover and morning glory seeds (our bioassay species) would not germinate in the presence of these extracts. In

TABLE 1. RECOVERY OF ADDED FERULIC ACID FROM CECIL SOIL MATERIAL BY THREE EXTRACTION PROCEDURES

Soil and horizon	Microorganisms[a]	Solution pH just before extraction[b]	Recovery (μg/g ± standard error) Water	Autoclave	EDTA
Cecil A_1	Absent	5.0	277 ± 3	322 ± 10	373 ± 19
	Present	7.4	0 ± 0	20 ± 4	4 ± 0.4
Cecil B_1	Absent	4.6	167 ± 12	304 ± 9	524 ± 6
	Present	5.2	0 ± 0	4 ± 0.4	0.4 ± 0.2

[a] Soil materials were reinfested with microbes 70 days after addition of ferulic acid; extracts of soil were made 30 days after reinfestation; three replicates.

[b] Because of the small volume, solutions from five test tubes were combined for pH measurements. Initial pH values of soils were 5.5 and 5.0 for Cecil A_1 and B_1, respectively.

TABLE 2. ESTIMATES OF FERULIC ACID ADDED TO CECIL SOIL MATERIAL SORBED IN SOIL AFTER 100 DAYS RECOVERED BY AUTOCLAVE AND EDTA PROCEDURES, AND AVAILABLE TO SOIL MICROBES

	Ferulic acid (μg/g)				
		Autoclave procedure		EDTA procedure	
Soil and horizon	Sorbed in soil[a]	Recovered[b]	Utilized by microbes[c]	Recovered[b]	Utilized by microbes[c]
Cecil A_1	723 (72%)[d]	45 (6%)[e]	25 (55%)[f]	96 (13%)[e]	92 (96%)[f]
Cecil B_1	833 (83%)	137 (16%)	133 (97%)	357 (43%)	357 (100%)

[a] Ferulic acid, 1000 μg, was added to 1 g of soil material. Sorbed in soil = amount not recovered by water extraction from sterile soil materials.
[b] For sterile soils. The difference in recovery between the water and autoclave or EDTA procedures.
[c] Difference between sorbed recovered by autoclave or EDTA procedures from sterile soils and soils reinfested with microbes.
[d] Percentage of 1000 μg initially added.
[e] Percentage of sorbed amount that was recovered.
[f] Percentage of recovered amount that was utilized by microbes.

addition, Folin and Ciocalteu's phenol reagent was reduced by EDTA immediately upon contact, thus EDTA soil extracts could not be used to estimate total phenolic acid equivalents in soil extracts. The EDTA extraction procedure also was very ineffective in extracting phenolic acids from plant debris. The EDTA extraction procedure extracted no detectable phenolic acids (HPLC analysis) from ground (60 mesh) wheat stubble (500 mg/100 ml), whereas extracts from the water-autoclave procedure contained 11 distinct peaks (HPLC analysis). Concentrations for ferulic, vanillic, *p*-coumaric, and *p*-hydroxybenzoic were 33, 22, 1034, and 47 μg/g dry weight, respectively. The water-autoclave procedure was, therefore, used in all subsequent studies.

Preliminary Bioassays of Soil Extracts. In an experiment using 1989 soil samples, the phytotoxicity of various concentrations of soil extract (0, 10, 20, 40, 80%) was determined. Data for germination (radicle emergence), mean radicle and hypocotyl length based on all seeds in a Petri dish (RAD), and mean radicle and hypocotyl length based on germinated seeds in a Petri dish (ARAD) of crimson clover were regressed against dilutions of extracts used (see "Analysis for Final Soil Extract Bioassay" in Methods and Materials section for details). Significant slopes for all individual extracts were linear. Six, 50, and 62% of the slopes for extract 1 (crude extract) and 38, 94, and 100% of the slopes for extract 2 (humic acid removed) were significantly greater than zero for germination, RAD, and ARAD, respectively. Thus the removal of humic acid by acidification and subsequent adjustment to pH 5 with NaOH apparently

changed the toxicity of the organic compounds in extract 2 and/or the solute potential of the solution. We suspect it was primarily the solute potential, since the milliosmole values (determined by freezing point depression) of extracts 1 and 2 were 1.25 $\pm$ 0.58 and 38.5 $\pm$ 2.40, respectively, and the removal of humic acid resulted in only minor changes in the free phenolic acid concentrations in the extract (Blum et al., 1991).

When slopes representing the activity of each extract for germination, RAD or ARAD, were regressed against the FC (total phenolic acid expressed as ferulic acid equivalents) and the milliosmole values of the original extract, only the extract 2 slopes were significantly greater than zero. An analysis based directly on germination, RAD, and ARAD values (not slopes) and FC and milliosmole values of the original extract (no extract dilutions were included in the analysis) resulted in F values that were 50% smaller than the F values from the slope (activity) analysis.

The pellet of humic acid produced by acidification of extract 1 was redissolved in the original volume from which it was recovered by adjusting the water solution to pH 5 with NaOH. The resulting solutions were tested against clover seeds. No significant effects on germination or radicle and hypocotyl length were observed.

For the experiments just discussed, each 0.42 $\pm$ 0.005 g of soil was extracted with 1 ml of water (2.35 ml/g). For the following experiment, 0.67 $\pm$ 0.005 g (wheat–conventional till) and 0.65 $\pm$ 0.006 g (wheat–no till) of soil were extracted with 1 ml of water (1.49 and 1.65 ml/g). These changes were made to adjust the baseline concentrations of the extracts. Extract 1 solutions of 1990 soils from wheat–no-till plots (extract dilutions were not included in this experiment) inhibited RAD and ARAD of clover and ARAD of morning glory (Table 3). The importance of FC and/or milliosmole values in determining RAD and ARAD responses depended on which of these variables was first placed in the response surface analyses (Table 3). This suggested that a colinearity existed between FC values and solute potential of the extract solutions. Extract 1 solutions of 1990 soils from conventional wheat–tilled plots did not affect germination or radicle and hypocotyl length of clover or morning glory.

Solute Potentials of Extracts. Modeling of NaCl solution freezing point depression values with solute potentials from Lang (1967) resulted in the following equation: J/kg of NaCl solution $= -2.133 \times 10^{-5} - (2.0924 \times 10^{-6} \times \text{mosmol}) - [1.3706 \times 10^{-9} \times (\text{mosmol})^2]$; $P \leq 0.0001$; $R^2 = 0.99$. Modeling of freezing point depression values for PEG 6000 with solute potential values generated from the PEG 6000 model of Steuter (1981) resulted in the following equation: J/kg of PEG $= 3.843 \times 10^{-6} - (1.426 \times 10^{-6} \times \text{mosmol})$; $P \leq 0.0001$; $R^2 = 0.99$. J/kg and mosmol in both equations represents joules per kilogram and milliosmoles, respectively.

Applying these equations, we found that NaCl (representing Hoagland's

TABLE 3. SEQUENTIAL MEAN SQUARE VALUES FROM GENERAL LINEAR MODELS PROCEDURE FOR RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN SOIL EXTRACTS FROM WHEAT/NO-TILL PLOTS[a]

Source	*df*	Clover RAD[b]	Clover ARAD	Morning glory ARAD
FC[c]	1	26.5244 (0.035)[d]	97.7233 (0.001)	622.3217 (0.004)
Milliosmoles	1	4.3415	2.6400	7.9543
$(FC)^2$	1	45.1372 (0.007)	18.1270	320.9215 (0.034)
$(\text{Milliosmoles})^2$	1	0.3406	0.0022	2.0604
FC × Milliosmoles	1	0.0761	2.6921	1.5955
Error	34	5.5056	7.7218	66.1608
Milliosmoles	1	23.4226 (0.047)	55.2458 (0.011)	315.2734 (0.036)
FC	1	7.4433	45.1175 (0.021)	315.0026 (0.036)
$(\text{Milliosmoles})^2$	1	14.9904	7.6883	159.1491
$(FC)^2$	1	30.4874 (0.024)	10.4408	163.8328
FC × Milliosmoles	1	0.0761	2.6921	1.5955
Error	34	5.5056	7.7218	66.1608

[a] Analysis run twice using different order of sources.
[b] RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.
[c] FC values (estimated phenolic acid values) ranged from 49 to 174 μg/ml ferulic acid equivalents. Milliosmole values ranged from 8 to 24. These values do not describe the factor space because they are not entirely independent.
[d] Values in parentheses are *P* values; if no *P* values are given then $P > 0.05$.

solution) and PEG solutions of 20 mosmol, for example, would equal -6.4×10^{-5} J/kg (-0.064 MPa) and -2.6×10^{-5} J/kg (-0.026 MPa), respectively. The solute potential of a 20 mosmol NaCl solution was thus 2.46 more negative in value than a 20 mosmol PEG solution. The J/kg values for 80 mosmol NaCl and PEG solutions were -2.0×10^{-4} (-0.20 MPa) and -1.1×10^{-4} (-0.11 MPa), respectively. In this case the NaCl solution was 1.82 times more negative than the PEG solution.

PEG and PEG–Phenolic Acid Mixtures. As the milliosmoles of the PEG solutions increased, germination, RAD, and ARAD declined (Table 4; Figure 1). A solution of 20 mosmol, for example, reduced clover germination, RAD and ARAD by 0, 13, and 5%, respectively. Morning glory germination, RAD, and ARAD were reduced 28, 44, and 36%, respectively.

As the milliosmoles of the PEG solutions increased, the effects of the phenolic acid mixture declined for RAD and ARAD of clover (Table 5; Figure 2). RAD and ARAD of morning glory were not affected by the concentrations

TABLE 4. PARTIAL REGRESSION COEFFICIENTS AND R^2 VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN POLYETHYLENE GLYCOL SOLUTIONS[a]

Variable[b]	Intercept	Linear	Quadratic	Cubic	R^2
Clover					
GERM	0.7992	0.0018	-7.48×10^{-5}		0.57[c]
RAD	26.8512	−0.1799			0.46
ARAD	30.9000	−0.0848			0.15
Morning glory					
GERM	0.7871	−0.0124	7.44×10^{-5}		0.47
RAD	27.4575	−0.7162	0.0053		0.52
ARAD	36.8790	−1.0335	0.0213	-1.4×10^{-4}	0.24

[a] Milliosmole values ranged from 0 (distilled water) to 84 for clover and from 0 to 90 for morning glory; 10 replicates.
[b] GERM = germination; RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.
[c] $P \leq 0.0001$ for all regressions.

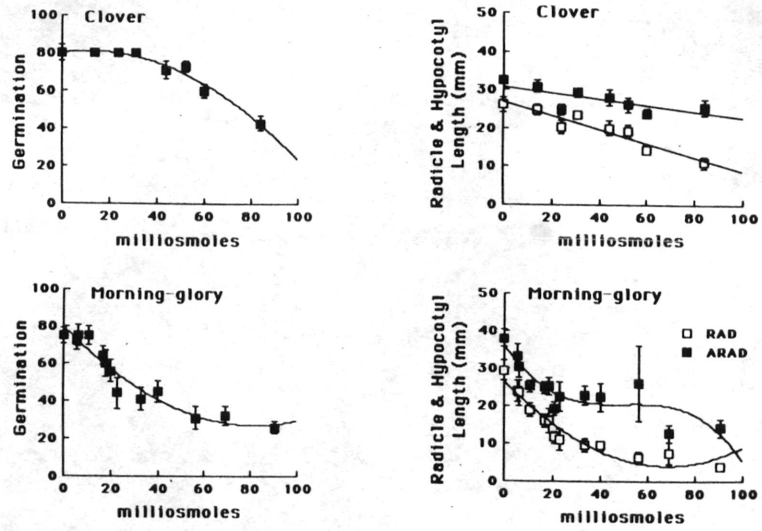

FIG. 1. Effects of PEG 6000 solutions with different freezing point depressions (milliosmoles) on germination and radicle and hypocotyl length (RAD, ARAD) of clover and morning glory. Means ± standard errors.

TABLE 5. PARTIAL REGRESSION COEFFICIENTS AND R^2 AND P VALUES FOR RADICLE AND HYPOCOTYL LENGTH OF CLOVER SEEDS GERMINATED IN VARIOUS CONCENTRATIONS OF PHENOLIC ACID MIXTURE AND POLYETHYLENE GLYCOL SOLUTION[a]

	RAD[b]	ARAD
Intercept	26.0402	31.8511
Phenolic acid mixture (PM)	-2.5703×10^{-2}	-3.433×10^{-2}
Milliosmoles	-0.1194	-0.1253
$(PM)^2$	-4.2733×10^{-7}	9.8475×10^{-6}
$(Milliosmoles)^2$	-5.6096×10^{-4}	-1.2369×10^{-4}
PM × milliosmoles	2.8964×10^{-4}	3.3072×10^{-4}
R^2	0.61	0.61
P	0.0001	0.0001

[a] Milliosmole values for PEG solutions ranged from 0 (distilled water) to 90. Phenolic acid mixtures ranged from 0 to 393.6 μg/ml of solution. Mixtures were composed of 15% *p*-hydroxybenzoic, 10% caffeic, 17% vanillic, 10% syringic, 35% *p*-coumaric, 9% ferulic and 4% sinapic; 5 replicates.

[b] RAD = sum of radicles and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (20 seeds); ARAD = sum of radicles and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.

of the phenolic acid mixture used. The phenolic acid mixture consisted of seven phenolic acids proportioned to that observed for soils from no-till wheat–soybean plots (see Table 5 for composition) (Blum et al., 1991). Germination of both species was not altered by the concentrations of the phenolic acid mixture used. The increasing milliosmoles of the PEG solution did, however, reduce germination of both species as noted earlier (Table 4; Figure 1).

Hoagland's, Hoagland's–Phenolic Acid Mixtures. Clover germination, RAD, and ARAD were reduced with increasing strength of Hoagland's solution (Table 6; Figure 3). A solution of 20 mosmol reduced clover germination, RAD, and ARAD by 64, 71, and 35%, respectively. Morning glory germination was not affected by the zero to double-strength Hoagland's solutions. RAD and ARAD values of morning glory increased and then decreased (Table 6; Figure 3) as the strength of Hoagland's solution increased.

Since morning glory germination and radicle and hypocotyl length were not affected by the concentrations of the phenolic acid mixtures used, only clover seeds were treated with concentrations of the phenolic acid mixture in various strength of Hoagland's solution. Germination of clover was not affected significantly by the concentrations of the phenolic acid mixture. The effects of zero (distilled water) to double-strength Hoagland's solution on RAD and ARAD

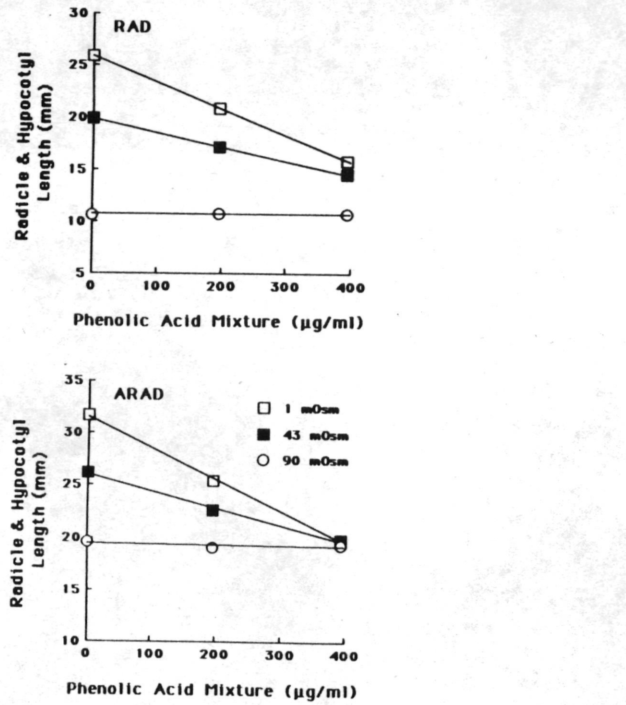

FIG. 2. Modification of the effects of a phenolic acid mixture on radicle and hypocotyl length (RAD, ARAD) of clover by PEG 6000 solutions with different freezing point depressions (milliosmoles). Lines derived from models in Table 5.

were independent of the effects of the phenolic acid mixture, i.e., the effects were additive (Table 7; Figure 4).

Individual Phenolic Acids. None of the seven phenolic acids (0–2 mM; *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, or sinapic) tested affected germination of clover or morning glory (data not shown). Clover RAD and ARAD were reduced by the cinnamic acid derivatives ferulic acid and *p*-coumaric acid and the benzoic acid derivatives *p*-hydroxybenzoic acid and vanillic acid (Table 8; Figure 5). RAD of morning glory was reduced only by *p*-hydroxybenzoic acid and ARAD of morning glory was reduced only by *p*-coumaric acid (Table 8; Figure 6). ARAD of clover, for example, was reduced 26, 11, 10, and 5% by 0.5 mM *p*-coumaric, ferulic, vanillic, and *p*-hydroxybenzoic acids, respectively. ARAD of morning glory was reduced 7% by 0.5 mM *p*-coumaric acid.

6-MBOA, PEG– or Hoagland's–6-MBOA Mixtures. Germination, RAD, and ARAD of both species were reduced by 6-MBOA (Table 9; Figure 7). For

TABLE 6. PARTIAL REGRESSION COEFFICIENTS, R^2, AND P VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN VARIOUS STRENGTHS OF HOAGLAND'S SOLUTION[a]

Variable[b]	Intercept	Linear	Quadratic	R^2	P
Clover					
GERM	0.5999	−0.0231	0.0002	0.69	0.0001
RAD	10.8237	−0.4946	0.0054	0.72	0.0001
ARAD	19.0369	−0.4040	0.0034	0.55	0.0001
Morning-glory					
RAD	32.4311	1.1830	−0.0163	0.23	0.031
ARAD	44.3235	1.2762	−0.0189	0.33	0.005

[a]Milliosmoles of the various strengths of Hoagland's solution ranged from 0 to 61; 5 replicates.
[b]GERM = germination; RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.

example, a 0.5 mM 6-MBOA solution reduced clover germination, RAD, and ARAD values by 24–28%. Morning glory germination, RAD, and ARAD were reduced 14, 23, and 22%, respectively.

As the milliosmoles of the PEG solutions increased, the effects of 6-MBOA on clover germination and clover and morning glory RAD values declined (Table 10; Figures 8 and 9). The effects of mixtures of PEG and 6-MBOA on clover and morning glory ARAD were independent, i.e., the effects were additive.

As the milliosmoles of the various Hoagland's solutions increased, the effects of 6-MBOA on germination and RAD values of clover declined (Table 11). The various strengths of Hoagland's solution did not alter clover ARAD or morning glory RAD and ARAD values. Germination of morning glory was also not affected by 0–1 mM 6-MBOA.

Final Bioassay of Extracts. A subset of 1990 wheat–no-till soil samples was extracted by the water-autoclave procedure. The 33 soil samples chosen represented a cross section of the soil samples collected. Soil extract (extract 1) characteristics were as follows: pH 5.7 ± 0.058, freezing point depression 11.35 ± 0.757 mosmol, FC 121.94 ± 4.26 μg/ml ferulic acid equivalents, NO_3^- 6.016 ± 0.878 μg/ml, NH_4^+ 5.27 ± 0.1267 μg/ml, K 109.72 ± 5.496 μg/ml, and P 17.81 ± 0.951 μg/ml (number of extracts = 33). Since each milliliter of extract represented 0.60 ± 0.01 g soil, 1.68 ml of extract represented 1 g of soil. Five milliliters of extract or diluted extract were used in each Petri dish, thus the extract in each dish represented ≤3 g of soil or ≤0.15 g per crimson

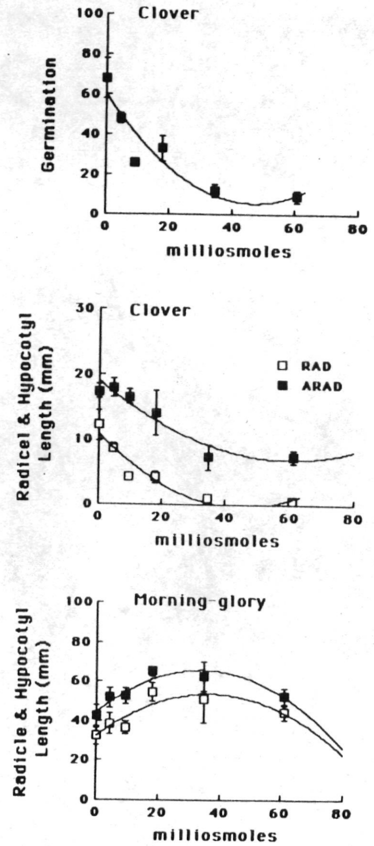

FIG. 3. Effects of Hoagland's solutions with different freezing point depressions (milliosmoles) on germination and radicle and hypocotyl length (RAD, ARAD) of clover and/or morning glory. Means ± standard errors.

clover seed (20 seeds per dish). See "Analysis for Final Soil Extract Bioassay" in Methods and Materials section for additional details.

The phytotoxicity of various dilutions of extract (0, 0.25×, 0.5×, and 1× strength) was determined on clover seeds. Sixty-three, 97, and 100% of the slopes were significantly greater than zero for clover germination, RAD and ARAD, respectively. Slopes were regressed against FC (μg/ml ferulic acid equivalents); milliosmoles; concentrations (μg/ml) of K, P, NO_3^- and NH_4^+, and pH of original soil extracts. PROC LEAPS, PROC RSREG, and PROC GLM procedures of SAS were used to identify the significant independent variables and to generate models (Tables 12 and 13; Figure 10). Partial regres-

TABLE 7. PARTIAL REGRESSION COEFFICIENTS, R^2, AND P VALUES FOR RADICLE AND HYPOCOTYL LENGTH OF CLOVER SEED GERMINATED IN VARIOUS CONCENTRATIONS OF PHENOLIC ACID MIXTURE AND VARIOUS STRENGTHS OF HOAGLAND'S SOLUTION[a]

	RAD[b]	ARAD
Intercept	8.5955	16.7421
Phenolic acid mixture	−0.0062	−0.0139
Milliosmoles	−0.3125	−0.0754
(Milliosmoles)^2	0.0038	
R^2	0.37	0.19
P	0.0001	0.0001

[a] Milliosmole values for the various strengths of Hoagland's solution ranged from 0 (distilled water) to 61. Phenolic acid mixture ranged from 0 to 393.6 μg/ml of solution. See Table 3 for composition of the phenolic acid mixture; 5 replicates.
[b] RAD = sum of radicles and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (20 seeds); ARAD = sum of radicles and hypocotyl lengths in a Petri dish divided by the number of germinated seed in the dish.

sion coefficients for FC values, milliosmoles, and pH were significant for clover RAD slopes, but only the partial regression coefficient for FC values was significant for clover ARAD slopes.

Clover RAD slopes, representing extract activity, became more negative with increasing FC values (linear), milliosmole values (concave), and pH values (linear). The more negative the slope, the more inhibitory the factor. The relationship of FC, milliosmoles, and acidity on clover RAD were independent, i.e., the effects were additive.

A separate analysis based on actual clover germination, RAD, and ARAD values (not slopes) and undiluted extracts confirmed the earlier observation (based on F values) that slope analysis was a more powerful tool in detecting the significant extract characteristics and their effects on germination and radicle and hypocotyl length.

DISCUSSION

An effective protocol for the identification of a functional allelopathic cover crop should provide: (1) an estimate, preferably a single integrated value, of the levels of active compounds in the soil; (2) an estimate of the biological activity of the observed action fraction; and (3) an estimate of how physical and chemical soil factors (e.g., pH, water potential, fertility, etc.) may moderate the effects of this active fraction. In addition, the relationship between the cover crop and crop performance must also ultimately be determined.

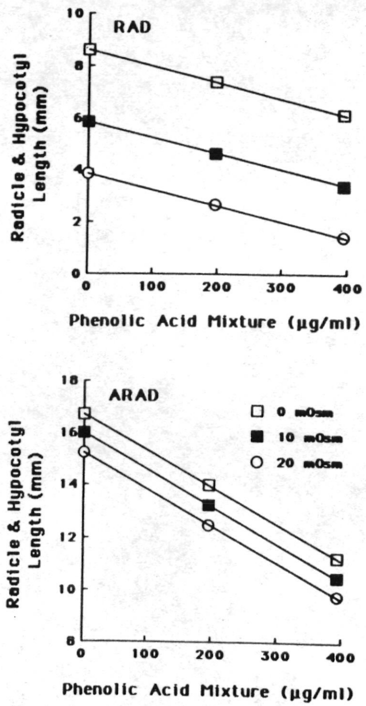

FIG. 4. Modification of the effects of a phenolic acid mixture on radicle and hypocotyl length (RAD, ARAD) of clover by Hoagland's solutions with different freezing point depressions (milliosmoles). Lines derived from models in Table 7.

Estimating Levels of Active Compounds in Soil. All productive agricultural soils contain a large number of organic substances, many of which can affect germination and growth of weeds and crops. Experience and the literature, however, suggest that certain types of compounds are more likely than others to inhibit germination and growth. In wheat systems, these include phenolic acids (Chou and Patrick, 1976; Liebl and Worsham, 1983; Barnes et al., 1986; Blum et al., 1991), organic acids (Patrick, 1971; Chou and Patrick, 1976; Lynch, 1977; Tang and Waiss, 1978; Shilling et al., 1985), hydroxamic acids and related compounds (Willard and Penner, 1976; Niemeyer et al., 1989; Nair et al., 1990), and volatiles (Buttery et al., 1985; Bradow, 1991).

Based on the recovery of ferulic acid, a phenolic acid, from sterile and nonsterile Cecil soils (Tables 1 and 2), water (immediate) and EDTA (0.5 M; pH 8; 5-hr extraction) extractions will provide a reasonable and adequate estimate of the active fraction of free or readily exchangeable phenolic acids in soils, but not phenolic acids in plant debris and/or soil organic matter. The

TABLE 8. PARTIAL REGRESSION COEFFICIENTS, R^2 AND P VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN PHENOLIC ACID SOLUTIONS[a]

Phenolic acid	Variable[b]	Intercept	Linear	Quadratic	R^2	P
Clover						
FER[c]	RAD	29.1632	−5.6626		0.47	0.0046
	ARAD	37.4753	−7.9734		0.73	0.0001
PCO	RAD	39.1294	−28.3452	7.9082	0.84	0.0001
	ARAD	43.5217	−26.2946	6.7138	0.88	0.0001
POH	RAD	27.9264	−3.2470		0.29	0.0374
	ARAD	36.9121	−3.7198		0.29	0.0392
VAN	RAD	25.1801	−4.6527		0.28	0.0445
	ARAD	31.1549	−6.0399		0.53	0.0020
Morning glory						
POH	RAD	55.0781	−7.4888		0.23	0.0415
PCO	ARAD	61.4919	−8.1800		0.47	0.0018

[a] Phenolic acid concentrations ranged from 0 to 2 mM; 3 replicates.
[b] RAD = sum of radicles and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in a dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicles and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.
[c] FER = ferulic; PCO = *p*-coumaric; POH = *p*-hydroxybenzoic; VAN = vanillic.

immediate water extraction will provide an estimate of phenolic acid concentrations in soil solution, and the difference in the recovery of phenolic acid concentrations by the water and EDTA extraction will provide an estimate of that which is immediately available to go into soil solution. EDTA extracts of soil, which contain soil solution and reversibly bound phenolic acids, however, could not be used directly in germination bioassays, because 0.5 M, pH 8 EDTA solutions completely inhibited clover and morning glory germination. Longer-term soil extractions with water, to recover at least some of the reversibly bound phenolic acids in soils, could not be used because microbes utilize phenolic acids as a carbon source during the extraction period (Blum and Shafer, 1988). We therefore chose to use the water-autoclave procedure. This procedure is rapid, eliminates microbial activity in the extract, and changes extract acidity within acceptable limits (soil pH 6.5 ± 0.06, extract pH 5.6 ± 0.05, $N = 72$). Adjustments of extracts to a common pH were not desirable due to the variations this generated in solute potential for soil extracts. Soil extracts produced by the water-autoclave procedure were thus used directly in germination bioassays.

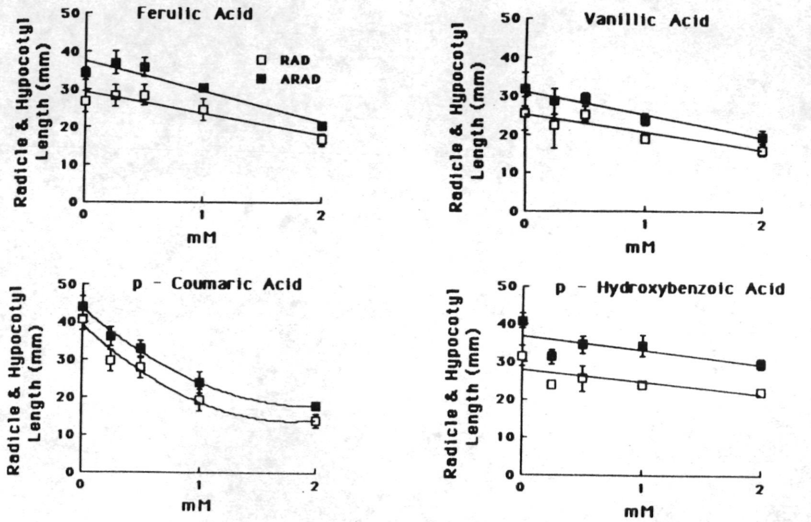

FIG. 5. Effects of individual phenolic acids on radicle and hypocotyl length (RAD, ARAD) of clover. Means ± standard errors.

Identification and quantification of individual phenolic acids in soil extracts by HPLC analysis were very time-consuming (approximately 1 hr per sample); so a rapid means of estimating phenolic acid content was required. An estimate of total phenolic acid, expressed as ferulic acid equivalents, in soil extracts was obtained by the use of Folin and Ciocalteu's phenol reagent (FC method). The resulting values were correlated ($r \geq 0.95$ for 1989 data and $r \geq 0.85$ for 1990 data) with the sum of seven phenolic acid concentrations in water-autoclave extracts as determined by HPLC analysis (Blum et al., 1991). Average values estimated by the FC procedure were 16.75 (1989) and 18 (1990) times greater than those determined by HPLC analysis. The phenol reagent used in the FC procedure was immediately reduced by EDTA, and thus EDTA extracts of soil could not be used to estimate total phenolic acid as ferulic acid equivalents. The phenol reagent is also reduced by a number of other organic and inorganic substances (Box, 1983) including 6-MBOA. 6-MBOA, however, was not detected by HPLC analysis or thin-layer chromatography in any of the wheat debris and soil extracts. This does not, however, preclude the presence of other hydroxamic acids or related products. Hydroxamic acids are constitutive secondary metabolites in several grasses (Zuniga et al., 1983), including wheat, maize, and rye (Willard and Penner, 1976). Wheat contains primarily 2-*o*-glucosyl-7-methoxy-1,4-benzoxazin-3-one (DIMBOA glucoside) (Willard and Penner, 1976; Nair et al., 1990). Upon cell injury, this glucoside is hydrolyzed and spontaneously converted to 6-MBOA. Since DIMBOA does not appear

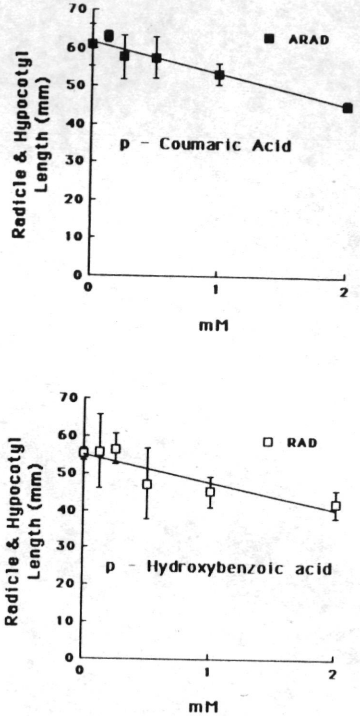

FIG. 6. Effects of individual phenolic acids on radicle and hypocotyl length (RAD, ARAD) of morning glory. Means ± standard errors.

to be released by living wheat plants (Argandona et al., 1987; Perez and Ormeno-Nunez, 1991), we assumed that injury of tissue with associated conversion to 6-MBOA was required. 6-MBOA was, however, not detected in soil or wheat debris extracts.

Potential Soil Extract Characteristics and Germination Bioassays. Germination and radicle and hypocotyl growth of weed and crop seeds are affected by many physical and chemical characteristics of soil extracts, such as pH, solute potential, nutrient content, and organic matter content. Effects of soil extracts on seed germination and radicle and hypocotyl length thus are not the exclusive property of allelopathic agents present in the extract, but are a product of all such factors acting independently or in concert with each other.

Crimson clover and ivy-leaved morning glory were used as bioassay species to characterize the role of some of these factors and their interactions. Crimson clover was chosen because of frequently poor early fall stands of clover seeded into our wheat-no-till soybean plots. Ivy-leaved morning glory was chosen

TABLE 9. PARTIAL REGRESSION COEFFICIENTS AND R^2 VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN 6-MBOA[a]

Variable[b]	Intercept	Linear	R^2	P
Clover				
GERM	0.5347	−0.2774	0.66	0.0001
RAD	9.1971	−5.1132	0.58	0.0002
ARAD	18.3101	−9.4975	0.75	0.0001
Morning glory				
GERM	0.8732	−0.2502	0.47	0.0016
RAD	44.6090	−20.7662	0.65	0.0001
ARAD	54.0485	−23.7037	0.75	0.0001

[a] 6-Methoxy-2-benzoxazolinone (6-MBOA) concentrations ranged from 0 to 2 mM; 3 replicates.
[b] GERM = germination; RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.

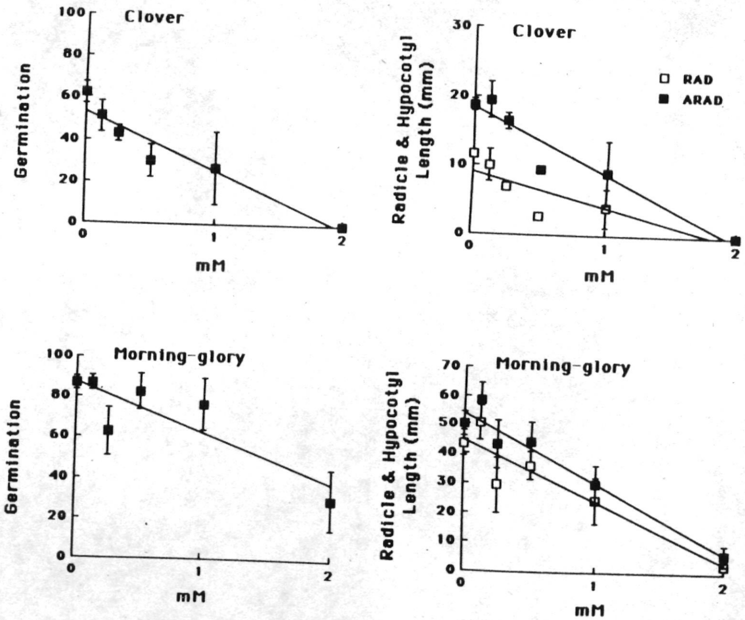

FIG. 7. Effects of 6-MBOA on germination and radicle and hypocotyl length (RAD, ARAD) of clover and morning glory. Means ± standard errors.