Plant Physiol. (1982) 69, 1212–1216
0032-0889/82/69/1212/05/$00.50/0

Quantification of Allelopathic Potential of Sorghum Residues by Novel Indexing of Richards' Function Fitted to Cumulative Cress Seed Germination Curves[1]

Received for publication May 18, 1981 and in revised form January 13, 1982

FREDRIC R. LEHLE AND ALAN R. PUTNAM
Department of Horticulture, Pesticide Research Center, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

The inhibitory activity of aqueous extracts of field-grown sorghum (*Sorghum bicolor* cv. Bird-a-boo) herbage and roots was quantitatively indexed by three aspects of cumulative cress (*Lepidium sativum* cv. Curlycress) seed germination: the germination onset; weighted mean rate; and final germination percentage. Extract potency was greatest for herbage collected four weeks after planting but declined sharply thereafter as the plants matured. About 91% of the inhibitory activity obtained from four-week-old herbage was in a low molecular weight fraction. Differential effects of herbage and root extracts on cress seed germination suggest that the nature and/or proportion of biologically active substances extractable from these plant parts is dissimilar.

Mature foliage and roots of several members of the genus *Sorghum* contain water-soluble inhibitors of both seed germination and seedling growth (1, 7–9, 16, 18). Some of these have been isolated, but there has been little direct evidence that the quantities present were sufficient to account for the observed levels of inhibition (1, 8). Progress in this area has been impeded by the absence of systematic determinations of biological activity at increasingly sophisticated levels of chemical purification. This problem stems in part from the lack of standardized methods for quantifying bioassay responses.

Recently, considerable literature has accumulated which describes plant growth in terms of mathematical models (11). These reports describe applications of the 'functional' approach to plant growth analysis, in which cumulative growth data are fitted to either polynomial approximations of exponential functions or explicitly defined empirical models (25). With determinate growth data, statistically comparable descriptions can be achieved by either method, but practical considerations appear to favor the use of empirical models. Notable among these is the Richards' function, which defines a family of asymptotic curves, including as special cases the monomolecular, autocatalytic (logistic), and Gompertz functions (21).

The objective of the present study was preliminary characterization of water-soluble inhibitors in sorghum residue that might be useful for the biological control of weeds in agronomic crops. Plant growth analysis was used to quantify the simple bioassay response of cumulative seed germination. Despite its advantages, this functional approach to plant growth analysis has been applied to cumulative germination data in only a few instances (15, 23). The present study demonstrates how a novel indexing of the Richards' function fitted to cumulative seed germination curves can be utilized as a bioassay technique to quantify the inhibitory potential of plant materials suspected of containing allelopathic substances.

MATERIALS AND METHODS

Plant Material. Sorghum (*Sorghum bicolor* cv. Bird-a-boo; Taylor Evans Seed Co., Tulia, TX) was seeded July 16, 1979, in a Sprinks sandy loam at East Lansing, MI. Seed were drilled in a 4.3- × 31-m plot at 81 kg/ha in 17.7-cm rows. Starting 2 weeks after planting, plants from a 4-m^2 area were harvested weekly for 6 weeks. At each sampling time, plant height and leaf number were recorded; uprooted plants were divided into root and herbage subsamples. After drying at about 50°C for several d and grinding to pass a 40-mesh screen on a Wiley mill, the samples were stored frozen under N_2 until used.

Cress (*Lepidium sativum* cv. Curlycress) seeds (Burpee Seed Co., Warminster, PA) were dusted with 0.95 mg thiram and 0.95 mg captan/g seed, prior to use.

Aqueous Extract Preparation. A crude extract was prepared by stirring 10 g of ground sorghum material with 100 ml of H_2O overnight at 4°C. The mixture was then filtered through several layers of Miracloth and centrifuged at 12,000*g* for 30 min. The supernatant fraction was brought to 100 ml with H_2O and diluted as necessary prior to bioassay.

Alternately, 100 ml of H_2O were added to 10 g of ground sorghum material, and the mixture was dialyzed overnight against 2,300 ml of water at 4°C. The dialyzable portion was concentrated under reduced pressure at 50°C to a final volume of 100 ml. Prior to bioassay, this dialyzable component was diluted as necessary to give final specific conductance readings of 94, 188, 375, 750, and 1,500 μmhos/cm. The nondialyzable portion was purified further by the procedures described for crude extracts. H_2O used for all extracts was deionized and double-distilled.

Cress Germination Bioassay. Treatments consisted of 100 cress seeds imbibed and incubated in the dark at 25°C in 4 ml of either test solution or H_2O (controls) in a covered 9-cm Petri dish on a single sheet of Whatman No. 1 filter paper. The experimental design for the extract potency × harvest date experiment was a randomized complete block with a 2 × 6 factorial arrangement of treatments replicated five times. Replications consisted of Petri dishes randomly ordered on separate trays within a humid, clear plastic container. Percentage of germination in fluorescent light was recorded twice daily. Seed were scored as germinated, if the

[1] Supported by United States Department of Agriculture, Science and Education Administration, Competitive Grant 801-0410-07040 (to A. R. P.). Michigan Agricultural Experiment Station Journal Article No. 9536.

radicle exceeded the length of the longest seed coat dimension. Seed were removed from dishes upon reaching germination.

Curve Fitting and Germination Index Calculations. Richards' functions were fitted to cumulative cress seed germination curves for each replicate, separately using the nonlinear regression subprogram of SPSS,[2] which utilized Marquardt's method for sum of squares minimization (22). This program requires user specification of the function to be fitted as well as initial values for all parameters to be estimated. Using the nomenclature of Richards (21), the forms of the functions were defined as:

$$W = A(1 + be^{-kt})^{1/1-m} \quad \text{for} \quad m > 1 \qquad \text{eqn 1}$$

and

$$W = A(1 - be^{-kt})^{1/1-m} \quad \text{for} \quad m < 1 \qquad \text{eqn 2}$$

where W was cumulative cress seed germination, at time t, in d after the onset of imbibition. The parameters A, b, k, and m were empirically derived constants unique to each asymptotic curve. Of these, only the parameter A had any direct biological interpretation in that it represented the cumulative cress seed germination percentage as $t \rightarrow \infty$, *i.e.*, the final cumulative germination percentage. Since the shapes of many cumulative cress seed germination curves were similar to that of the Gompertz function (for which the Richards' function is undefined), poor fits were occasionally obtained using either eqn 1 or eqn 2. Such data were satisfactorily fitted to a close approximation of the Gompertz function by setting $m = 1.001$ in eqn 1, as suggested by Richards (21). The initial value and allowable ranges of parameters to be estimated were specified, as indicated in Table I.

Several biologically meaningful variables were calculated from the derived parameters for each replicate separately as follows. The weighted mean cumulative germination rate (R) was defined according to Richards as:

$$R = Ak(2m + 2)^{-1} \qquad \text{eqn 3}$$

Using the strategy of Lapp and Skoropad (15), the onset of germination ($t_{0.01A}$) was defined in terms of the time required to reach 1% of the final cumulative germination percentage (A) by substituting $W = 0.01A$ into either eqn 1 or eqn 2 and solving for t. The equations obtained were:

$$t_{0.01A} = ln\frac{(0.01)^{1-m}}{b}\frac{1}{-k} \quad \text{for} \quad m > 1 \qquad \text{eqn 4}$$

and

$$t_{0.01A} = ln\frac{1 - (0.01)^{1-m}}{b}\frac{1}{-k} \quad \text{for} \quad m < 1 \qquad \text{eqn 5}$$

where $t_{0.01A}$ was in terms of d after the onset of imbibition. To facilitate and simplify treatment comparisons, the onset of germination ($t_{0.01A}$), the weighted mean germination rate (R), and the final cumulative germination percentage (A) were incorporated into a single numerical index (I) defined as:

$$I = \frac{AR}{t_{0.01A}} \qquad \text{eqn 6}$$

Reductions of 50% in the index value (I_{50}) were estimated from linear calibrations of extract concentration *versus* index values for each replicate separately.

RESULTS

The potency of aqueous extracts prepared from sorghum herbage and roots was different relative to plant maturity, as shown by a significant F value (0.05 level) for the interaction of extract potency and harvest date (Fig. 1). Extracts prepared from herbage collected 2 and 4 weeks after planting were the most inhibitory. Extracts prepared from herbage samples collected more than 4 weeks after planting exhibited a pattern of decreasing inhibitory activity as the plants matured. Although biomass increased steadily throughout the 8-week period, toxicity actually decreased after 6 weeks. Hence, the maximum activity extractable from sorghum herbage grown under field conditions was reached at the mid-fifth to early sixth leaf stages (60–80 cm) of growth.

In contrast, the inhibitory activity of aqueous extracts prepared from sorghum root material declined with increased plant age (Fig. 1). The inhibitory activity of root material on a unit-mass basis was generally about one-half that measured for the herbage. The inhibitory activity of herbage and root extracts could not be explained on the basis of their acidity (pH range of 5.6–6.1), since cress seed germination in unbuffered water controls was similar

Table I. *Initial Values and Allowable Ranges of Richards' Function Parameters Specified in SPSS Nonlinear Subprogram*

Parameter	Initial Value	Allowable Range
	m > 1 and m = 1.001	
A	85–95	0–100
b	0.5–3.5	0–1,000
k	2.4	0–100
m	1.001	$1 < m < 10$[a]
	m < 1	
A	70–95	0–100
b	0.002–0.02	0–1,000
k	2.322–3.473	0–100
m	0.9999	$0 < m < 1$

[a] For $m > 1$ only.

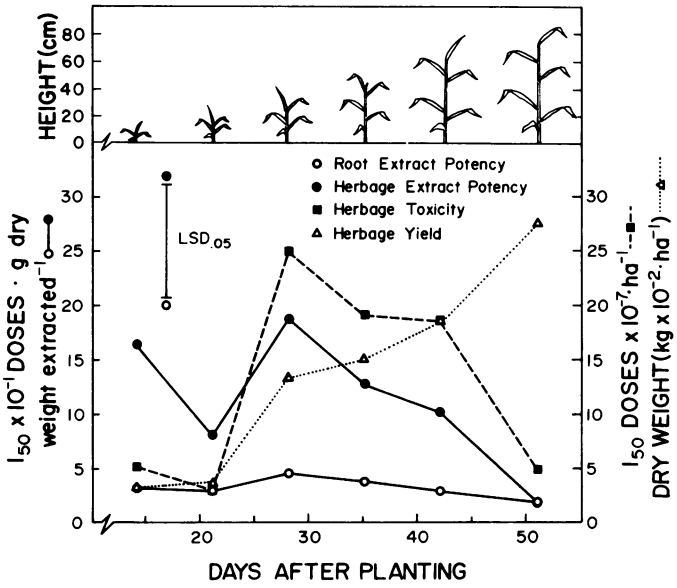

FIG. 1. Inhibitory activity of Bird-a-boo sorghum herbage and root material during early development under field conditions. Aqueous extracts (dialyzable component; 1:10, g dry weight extracted/ml H_2O) were diluted as necessary prior to cumulative cress seed germination bioassay, as described in "Materials and Methods." Extract potency (as measured by I_{50} doses/g dry weight extracted) was estimated from linear calibrations of germination index (eqn 6, "Materials and Methods") values *versus* extract concentrations. Herbage toxicity (as measured by I_{50} doses/ha) was extrapolated from herbage dry weight yields. Plant height and leaf stages for all harvests are illustrated in the top panel. $LSD_{0.05}$ for comparison of extract type at a given harvest date.

[2] Abbreviations: SPSS, Statistical Package for the Social Sciences; I_{50}, dose that results in a 50% reduction in the germination index value relative to control.

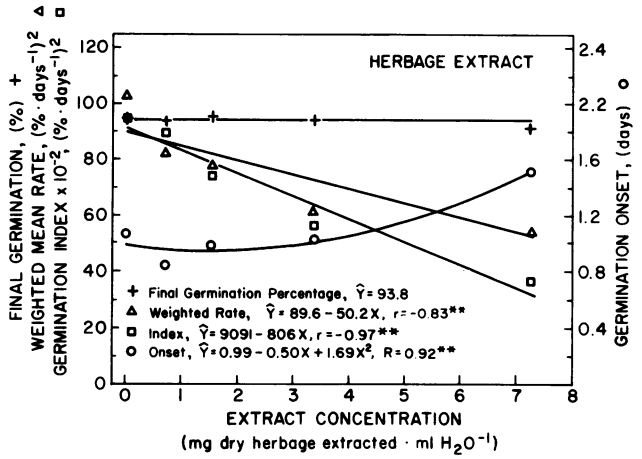

FIG. 2. Effect of aqueous extracts (dialyzable component) of 4-week-old Bird-a-boo herbage on several aspects of cress seed germination. Bioassay conditions are described in "Materials and Methods." All points are mean values of five replications. **, F value from trend analysis was significant at $p < 1\%$ level of significance.

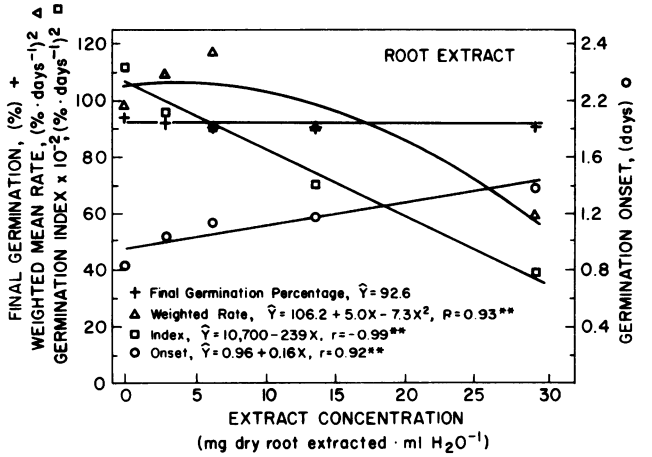

FIG. 3. Effect of aqueous extracts (dialyzable component) of 4-week-old Bird-a-boo root material on several aspects of cress seed germination. Bioassay conditions are described in "Materials and Methods." All points are mean values of five replications. **, F value from trend analysis was significant at $p < 1\%$ level of significance.

Table II. *Aqueous Extract Potency of Four-Week-Old Bird-a-boo Sorghum Herbage*

Extracts (1:10, g dry weight extracted/ml H_2O) were diluted as necessary prior to cumulative cress seed germination bioassay, as described in "Materials and Methods." Extract potency was determined, as described in Figure 1.

Extract	Extract Potency
	I_{50} *doses/g dry wt extracted* ± SD
Crude	217 ± 11
Dialyzable component	192 ± 33
Nondialyzpble component	20 ± 3

over an initial solution pH range of 5.3 to 6.7 (data not presented).

The effects of herbage and root extracts on cress seed germination were qualitatively different for the majority of the harvest dates (Figs. 2 and 3). The most striking differences between extracts prepared from herbage and those prepared from root material were in the rate and onset of cress seed germination. At low extract concentrations, herbage extracts were generally inhibitory only to the weighted mean germination rate, whereas extracts

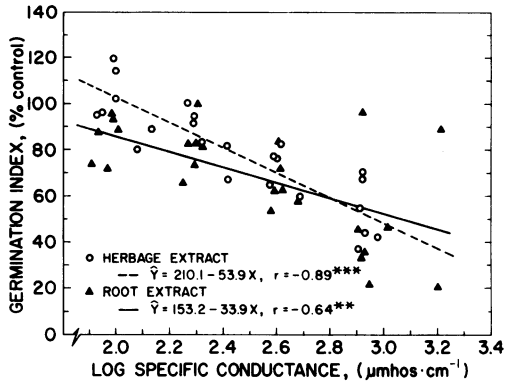

FIG. 4. Relationship between specific conductance of aqueous extracts (dialyzable component) of Bird-a-boo herbage and root material and germination index values obtained with cumulative cress seed germination bioassay. Index values were obtained from data used in Figure 1. **, ***, Significant at $p < 1\%$ and $p < 0.1\%$ level, respectively.

prepared from root material were generally inhibitory only to the germination onset. At higher extract concentrations, however, both herbage and root extracts significantly inhibited both the weighted mean rate and onset of cress seed germination. In the range of extract concentrations tested, neither herbage nor root extracts had a significant effect on the final germination percentage. Significant reductions in the final germination percentage were achieved only with extract concentrations several orders of magnitude higher. Fractionation of crude extracts of 4-week Bird-a-boo herbage into dialyzable and nondialyzable components indicated that about 91% of the recoverable activity was present in the dialyzable component (Table II).

The relationship between the specific conductance of herbage and root extracts and cress germination index values is shown in Figure 4. For both extracts, specific conductance provided a rapid method for estimating extract potency prior to actual bioassay, greatly facilitating the preparation of appropriate extract concentrations.

DISCUSSION

In this study, a technique for indexing three aspects of cumulative seed germination curves has been described which permits the use of seed germination as a bioassay to quantify the toxicity of plant extracts. This technique was developed to overcome problems associated with comparing germination curves which have detracted from the practical advantages of speed and convenience with which a seed germination bioassay can be performed.

Existing procedures for comparing cumulative germination curves consist essentially of two approaches: creating a curve index based on an area under a cumulative germination curve (17, 20, 24); or comparing fitted, cumulative germination curves graphically (6, 12, 15). While curve indexes based on area offer considerable convenience in terms of treatment comparison, their interpretation is difficult, since they are derived from measures which have little or no biological meaning. This problem of interpretation is particularly acute when identical index values are obtained for seed populations of widely different germination patterns (6, 10). On the other hand, while graphic comparison of fitted germination curves offers biological clarity, this approach is cumbersome when a large number of treatments are to be evaluated and is not as amenable to statistical analysis as a numerical index. Our approach in overcoming these problems has been to develop a numerical index based entirely on biologically meaningful measures; specifically, only those which are sensitive to the manner in which cumulative germination curves can potentially differ.

Unlike previous indexes based on area, selection of germination aspects for inclusion in the proposed index was based on the observation that potential differences of cumulative cress germination curves are of three types: differences in the maximum germination percentage achieved; differences in the cumulative germination rate; and differences in the position of the cumulative germination curve along the horizontal, or time, axis. From a biological standpoint, this latter difference was more meaningful when interpreted as a difference in the cumulative germination onset. These three aspects of cumulative seed germination have been previously used to characterize the germination behavior of the desert seeds *Atriplex dimorphostegia* and *Artemisia monosperma* (13, 14). Figure 5 illustrates that the extremes of germination pattern differences can be defined in terms of these three germination aspects. In this illustration, hypothetical germination patterns are described by four different curves. Regardless of the curve chosen for comparison, the remaining three curves differ from it in only one of the three germination aspects discussed above. Thus, valid assessment of these curve differences can only be made by examining all three of these germination aspects. While this illustration demonstrates the usefulness of these germination parameters for describing the extremes of pattern differences, curve dissimilarities involving differences in two or more aspects are also readily discerned.

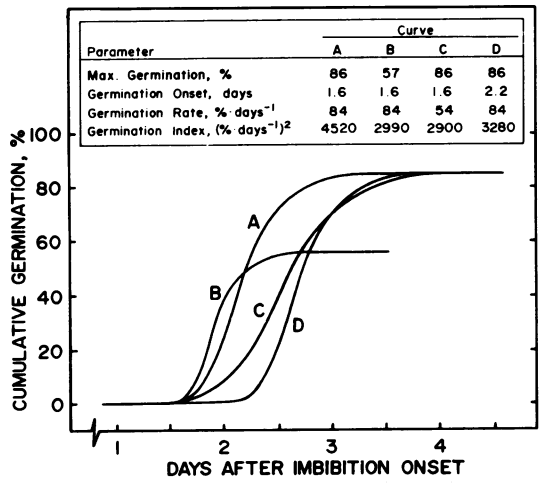

FIG. 5. Hypothetical germination curves generated from Richards' function illustrating that the extremes of germination pattern differences can be defined in terms of the germination aspects of maximum germination percentage achieved, germination rate, and germination onset. Germination aspects and index values were derived from Richards' function parameters, as described in "Materials and Methods."

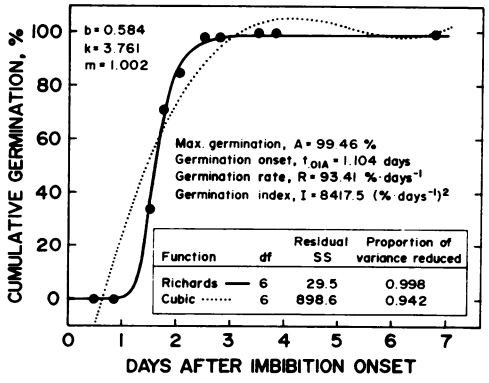

FIG. 6. Comparison of curve fitting by Richards' function and cubic polynomial for cumulative germination of 100 cress seeds imbibed in H_2O at 25°C in the dark. Germination aspects were derived from indicated Richards' function parameters, as described in "Materials and Methods."

Several factors favored quantifying the bioassay response of cumulative seed germination in terms of a numerical index derived from these three germination parameters, rather than in terms of the individual aspects themselves. In the present study, the measure chosen for quantification of biological toxicity was the dose of plant extract required for a 50% reduction in bioassay response relative to controls, *i.e.* the I_{50} dose. This value was estimated from a dose-response curve, in which bioassay response *versus* extract concentration was plotted. Determination of I_{50} values for all three germination aspects individually would have required preparation of three separate dose-response curves of differing concentration ranges appropriate to the sensitivity of each germination aspect. Such a procedure was considered impractical in the present study due to the large number of anticipated treatment comparisons. However, quantification by I_{50} values was manageable, if all three germination aspects were assessed simultaneously by the proposed numerical index, since a single dose-response curve sufficed for this purpose. Apart from this convenience, estimation of extract concentrations corresponding to I_{50} values from index-based dose response curves was also simplified, due to the linear relationship between the proposed index and plant extract concentration (Figs. 2 and 3).

A second advantage to quantifying the bioassay response of cumulative seed germination using a numerical index was an increase in bioassay sensitivity over that expressed by any of the three germination aspects separately. This resulted because germination pattern differences of cress in response to increasing concentrations of crude plant extracts typically involved parallel changes in two or more germination aspects simultaneously (Figs. 2 and 3). The arrangement of included germination aspects in the proposed index was such that stimulatory responses increased the index value while inhibitory responses decreased its value. Thus, simultaneous responses of similar direction in two or more germination aspects were proportionally magnified by definition. By convention, stimulatory responses were defined according to the index as increases in germination rate and final germination percentage, as well as an earlier onset of germination; opposite trends were interpreted as inhibitory responses. The disadvantage of this arrangement of germination aspects in the proposed index was that changes in each aspect were reflected equally in the index value. Such an arrangement permitted the possibility of obtaining similar index values for widely different germination patterns, when two or more of the germination aspects describing such patterns responded in nullifying directions (Fig. 5). In the present study, this problem did not occur, since all germination parameters were increasingly inhibited, as defined with increasing concentrations of plant extracts. The only exceptions were a few extracts that delayed the onset of germination relative to controls but, once growth commenced, had germination rates actually faster than that of controls (data not presented).

The three germination aspects—maximum germination percentage achieved, weighted mean germination rate, and germination onset—were estimated from an empirical growth model fitted to the raw cumulative cress germination data. A number of curve-fitting strategies were considered for this purpose, but, inasmuch as no theoretical basis was intended, final selection of the appropriate model was based on three criteria: the model should fit the raw observations closely; the form of the model should be biologically reasonable; and the model should not be restrained by assumptions concerning the shape of cumulative germination curves. Based on these criteria, the Richards' function (21) proved superior to other curve-fitting approaches considered. This extension of von Bertalantffy's model of animal growth has been used to describe a variety of seedling and mature plant-growth responses (5, 21, 25). In comparison to a polynomial exponential approach, the Richards' function produced better fits of more

reasonable biological form than did a polynomial equation of equal parameter number (Fig. 6). Although the shapes of several cumulative cress seed germination curves were close to that of the Gompertz function, this three-parameter model, which represents a special case of the Richards' function, was rejected due to an inflexible assumption concerning curve shape which held true for cress only occasionally. By definition, fitting the Gompertz equation exclusively to cumulative germination data makes the unrealistic assumption that the curve inflection point will always occur at a constant ratio relative to the final germination percentage achieved (21). Curve-fitting approaches, such as the logistic function (23) and the normal distribution function (12), were similarly rejected primarily for their restrictive *a priori* assumptions concerning the shape of cumulative germination curves (19).

Fitting of the Richards' function to raw cumulative germination data is computationally complex and is best handled by general-purpose, statistical computer programs such as the nonlinear regression program of SPSS used in this study (22). While this particular program performed adequately, several limitations prevent its unqualified endorsement. First, this program required specification of initial estimates of Richards' function parameters for each fitting, which was tedious when a large number of data sets were to be fitted. Occasionally, when initial estimates were poor, convergence of the residual sum of squares was not smooth and reached a minimum only after forcing one parameter estimate to its allowed limit. While the regressions obtained in such instances were statistically comparable to those where convergence was rapid, the parameter estimates were not. Thus, comparison of curve differences based on the Richards' function parameters themselves was statistically meaningless as a result of this parameter estimate irregularity. An algorithm for estimating initial estimates of Richards' function parameters from the raw data prior to actual curve fitting has been proposed by Causton (3) but was not evaluated in the present study. A second limitation of the SPSS nonlinear program concerns its inability to evaluate more than one form of the Richards' function per program run. In the present study, an initial attempt was made to fit the $m > 1$ form of the Richards' function (eqn 1) to all raw, cumulative germination data sets. While this approach was successful for the majority of cases, a few data sets could not be fitted with this equation, due to sudden instabilities encountered during the minimization process, which prevented further residual sum of squares convergence. Specification of the SPSS implementation of the Gauss-Newton method for sum of squares minimization instead of Marquardt's method did not alleviate this problem. In such instances, satisfactory fits were obtained by considering either the $m < 1$ form (eqn 2) or the restricted form of eqn 1 ($m = 1.001$), but this necessitated the inconvenience of repeated program runs. Unlike SPSS, other general-purpose, statistical computer programs such as the Statistical Analysis System overcome this inconvenience by permitting consideration of several functions per program run (2).

The observation that the inhibitory level of Bird-a-boo sorghum was maximal after only 4 weeks of growth was surprising, considering the high levels of inhibition obtained with extracts of mature residues by others (1, 7, 9). These results were, nevertheless, encouraging from a practical agronomic point of view, since they were consistent with preliminary field trials at Michigan State University. These trials indicated that, using reduced tillage culture, residues of immature Bird-a-boo enhanced weak control significantly when used in conjunction with conventional herbicides (4). In contrast to previous results, in which extracts prepared from mature root or rhizome material were about equally as inhibitory as those prepared from mature herbage (1, 7, 9), extracts prepared from Bird-a-boo herbage collected 2 and 4 weeks after planting were significantly more inhibitory than were those prepared from the roots.

Qualitative differences in the effect of herbage and root extracts on the rate and onset of cress germination strongly suggest that the biologically active components from these sources either were present in different proportions and/or were chemically dissimilar. Dialysis fractionation of extracts prepared from herbage collected 4 weeks after planting indicated that about 91% of the total inhibition could be accounted for by relatively low mol wt compounds. The negative correlation between specific conductance of herbage and root extracts and cress germination index values suggests that the biologically active compounds present in this low mol wt fraction were ionizable (Fig. 4). Such a hypothesis is consistent with previous reports attributing the phytotoxicity of mature foliage and roots of several members of the genus *Sorghum* primarily to phenolic acids (1, 8). Removal of soil particles from root samples was not exhaustive, and, presumably, this explains the greater variation in specific conductance measurements obtained with these extracts. It is unclear whether the relationship observed between specific conductance and cress germination index values holds for other tissues or bioassay systems.

Aknowledgments—The authors wish to thank Joseph DeFrank of the Department of Horticulture, Michigan State University, for his valuable ideas concerning this research, and Satoru Miyazaki of the Pesticide Research Center, Michigan State University, for reviewing the manuscript. Thanks are also extended to Sylvia Denome and Kathy Braun for their expert technical assistance in conducting the bioassays. We are also grateful to David Zeitler of the Michigan State University Computer Laboratory for his programming assistance.

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