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Seed mass affects the susceptibility of weed and crop species to phytotoxins extracted from red clover shoots

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Residues of legume crops used to increase soil fertility may also serve as sources of phytotoxins that can suppress the germination and early growth of weed and crop species. To test the hypothesis that weed and crop susceptibility to extracts of red clover shoots would be inversely proportional to seed mass, we (1) identified 18 weeds and 44 crops whose 100-seed masses ranged from 20 to 26,250 mg; (2) exposed their seeds in petri dishes and filter paper to a 2% aqueous extract of 'Marathon' red clover shoots or distilled water; and (3) measured germination percentage and radicle length of germinated seeds after incubation for 4 days. In a second experiment, we assessed germination and radicle growth of four crop and four weed species after exposure to 1% extracts of Marathon or 'Cherokee' red clover or distilled water. Germination inhibition by red clover extracts was greatest for lighter seeds and least for heavier seeds in Experiment 1 ($P = 0.0005$), but was unrelated to seed mass in Experiment 2. Radicle inhibition by red clover extracts was inversely proportional to seed mass in both Experiment 1 ($P < 0.0001$) and Experiment 2 ($P = 0.0047$), and, in Experiment 1, was greater for monocots than dicots ($P = 0.0002$). Our findings corroborate the general relationship between seed mass and stress tolerance observed by other investigators and indicate that small-seeded monocots are most likely to be susceptible to phytotoxins contained in red clover shoots.

Nomenclature: Red clover, *Trifolium pratense* L.

Key words: Allelopathy, legume green manure, phytotoxins, seed size, weed management.

Farmers have used legume residues for thousands of years to maintain or improve soil fertility in a practice called "green manuring" (Pieters 1927). The quantities of nitrogen mineralized from common legume green manures, such as alfalfa (*Medicago sativa* L.), hairy vetch (*Vicia villosa* Roth), and red clover, can be sufficient to satisfy much or all of the nitrogen demand of succeeding nonlegume crops, including corn (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.) Moench], and muskmelon (*Cucumis melo* L.) (Blevins et al. 1990; Bruulsema and Christie 1987; Fox and Piekielek 1988; Singogo et al. 1996). The use of legume green manures is complicated, however, by potential problems with phytoxicity of the residues for several weeks following incorporation into the soil. Fred (1916) and Pieters (1927, p. 115) recognized this phenomenon in the early part of the last century, and recent studies have confirmed that legumes used as green manures can serve as sources of chemicals that suppress plant germination and growth (Batish et al. 2001; Chung and Miller 1995; White et al. 1989).

Although allelopathy has been suggested as a potentially important component of new weed-management systems (Weston 1996), a well-defined strategy has yet to be developed for exploiting the phytotoxicity of legume green manures selectively, such that weed species are suppressed but crops are not. One possible approach derives from differences among species in stress tolerances that are related to differences in seed mass. Seedling size is positively correlated with seed mass (Leishman et al. 2000), and, as compared with seedlings of small-seeded species, seedlings of larger-seeded species are often more tolerant of stresses that include shade, nutrient deficits, drought, defoliation, burial by soil and plant litter, and resource competition from other plants (Westoby et al. 1996, 2002). Mohler (1996) noted that the seeds of many annual agronomic crops are one to three orders of magnitude larger than the seeds of the annual weeds that infest them, and suggested that phytotoxins released from green manure residues should have greater effects on small-seeded species, including weeds, than on large-seeded crops.

Variation among species in seed mass might contribute to differential susceptibility to phytotoxins for several reasons. First, as compared with large-seeded species, small-seeded species tend to have greater amounts of root length per unit of root mass (Seibert and Pearce 1993), and thus proportionally greater amounts of absorptive surface area through which phytotoxins might enter. Second, small-seeded species generally have fewer reserves with which to support seedling respiration during periods of stress-induced carbon deficit (Westoby et al. 2002), and thus might suffer disproportionate reductions in early season growth that limit their access to resources and competitive ability later. Finally, it is conceivable that differences in seed reserves might also contribute to variation among species in their ability to detoxify allelochemicals, though, to our knowledge, this hypothesis has not been tested.

Regardless of the mechanisms involved, a better understanding of weed and crop responses to legume green manures is needed for the development of farming systems that are more reliant on ecological interactions and less dependent on purchased fertilizers and pesticides (Liebman and

Davis 2000). In the two experiments reported here, we conducted laboratory bioassays to determine how weed and crop taxa commonly encountered in temperate agroecosystems respond to aqueous extracts of red clover, a legume used as a green manure that can produce phytotoxic compounds (Chang et al. 1969; Inderjit 1996; Siquiera et al. 1991; Tamura et al. 1969).

Materials and Methods

Seeds

Weed and crop seeds spanning three orders of magnitude in mass were obtained from commercial seed dealers, other research groups, and our own field collections (Table 1). For some species, multiple cultivars or populations were obtained. Hereafter, we refer to each cultivar or population as an *accession*. The scientific nomenclature with which we have identified each accession follows that used by the U.S. Department of Agriculture (2005) and the Weed Science Society of America (2003). Weed or crop status of each accession was determined based on collection information provided by USDA (2005) and ecological information provided by Randall (2002). To determine the mean seed mass of each accession, three to five samples of 100 seeds were dried at 70 C in a forced-air oven for at least 1 d and then weighed. Seeds from the same sources that were not subjected to high-temperature drying were used in the subsequent experiments.

Experiment 1

Experiment 1 was conducted to determine (1) the influence of seed mass on weed and crop responses to an aqueous extract of red clover shoots, and (2) whether fundamental differences exist between monocots and dicots, and between weeds and crops, in the strength of their responses to red clover extract. Of the 62 accessions used for this experiment, 11 were monocot weeds, 7 were dicot weeds, 15 were monocot crops, and 29 were dicot crops (Table 1).

Shoots of Marathon red clover were collected from well-established plants growing at the Iowa State University Agronomy Farm, Boone, IA, on August 14, 1998. Plants were cut at ground level and then air dried at 28 C for 3 d, after which they were ground in a Wiley mill to pass through a 2-mm mesh screen and stored in a sealed plastic bag at 5 C until needed. Preliminary experiments indicated that a 2% solution of red clover shoots would provide marked, but not overwhelming, effects on a range of target species (data not shown). Consequently, in this experiment a 2% aqueous extract was produced by adding 4 g of the dried, ground red clover residue to 200 ml of distilled water in a 500-ml Erlenmeyer flask and placing the mixture on a rotary shaker set at 300 rpm for 16 h at 22 C in the dark. Plant material was removed from the mixture by vacuum filtration through filter paper,[1] followed by centrifugation at 4000 × *g* for 10 min at 22 C. The extract was stored in a freezer until needed. Just before the thawed extract was added to seeds, microorganisms were removed by filtration through a 0.45-μm nylon syringe filter.[2]

Weed and crop seeds were surface sterilized by soaking in 7% sodium hypochlorite solution for 10 min and then rinsed thoroughly with distilled water. Eighteen seeds of each accession were placed on two layers of filter paper[3] in sterile plastic petri dishes (100 mm diameter by 15 mm height). Two milliliters of red clover extract or 2 ml of distilled water (the control treatment) was pipetted into each petri dish. Each accession-by-extract treatment combination was replicated four times. The petri dishes were covered with their lids, placed in plastic boxes lined with a saturated paper towel to maintain high humidity, and germinated in alternating conditions of 30 C (16 h in light) and 20 C (8 h in dark). Locations of the replicate petri dishes were randomized within the germination chamber. After 4 d, 1.5 ml of 50% ethanol was added to each petri dish to stop germination and growth. Germination percentage and radicle length of each germinated seed were then determined. Radicle length was measured to the nearest millimeter.

Because of space limitations in the germination chamber, it was impossible to use the entire set of accessions at the same time. Instead, groups of 8 to 12 accessions were tested during each run of the experiment. The entire experiment was conducted twice between December 1998 and May 1999.

Experiment 2

Experiment 2 was conducted to determine (1) the influence of seed mass on weed and crop responses to a more dilute extract of red clover shoots than was used in Experiment 1, and (2) whether the effects of the extract were specific to a particular red clover cultivar. Seeds of four weed and four crop accessions (Table 1) and aqueous extracts of two red clover cultivars were used in the experiment.

Shoots of Marathon and Cherokee red clovers were collected from plants at the Iowa State University Agronomy Farm, Boone, IA, on September 2, 1999 and processed as in Experiment 1. A 1% aqueous extract of each red clover cultivar was prepared by mixing 2 g of red clover residue with 200 ml of distilled water. Surface-sterilized weed and crop seeds were placed in petri dishes and treated with 2 ml of the Marathon or Cherokee red clover extracts or distilled water. Each accession-by-extract treatment was replicated four times and the locations of the petri dishes within the germination chamber were randomized. The germination and seedling assessment protocols were the same as in Experiment 1. Groups of four accessions were tested during each run of the experiment. The entire experiment was conducted twice between December 1999 and February 2000.

Data Analysis

Petri dishes containing 18 seeds of an accession were treated as the experimental units, and mean values of germination percentage and radicle length were calculated for each replicate dish. These values were then averaged over all replicates used in both repetitions of each experiment and used to calculate the primary response variables of interest, which we called *germination inhibition* (GI) and *radicle inhibition* (RI). We defined germination inhibition as:

$$GI = ([GI_{control} - GI_{extract}]/GI_{control}) \times 100$$

where $GI_{control}$ was the mean germination percentage for an accession exposed to distilled water and $GI_{extract}$ was the mean germination percentage for the same accession ex-

TABLE 1. Seeds used in Experiments 1 and 2. Nomenclature follows that used by the Weed Science Society of America (2003) and the U.S. Department of Agriculture (2005). Cultivar names are provided when known.

Botanical name	Weed (W) or crop (C)	Dicot (D) or monocot (M)	100-seed weight (mg)	Used in Expt. 1	Used in Expt. 2
Abutilon theophrasti Medik.	W	D	1037	+	+
Amaranthus caudatus L. cv. Love Lies Bleeding	C	D	54	+	
Amaranthus cruentus L.	C	D	54	+	
Amaranthus cruentus L.	C	D	69	+	
Amaranthus cruentus L. cv. K343	C	D	72	+	
Amaranthus hybrid (*A. caudatus* L. × *A. quitensis* Kunth)	C	D	36	+	
Amaranthus hypochondriacus L.	C	D	91	+	
Amaranthus retroflexus L.	W	D	35	+	
Amaranthus rudis Sauer	W	D	20	+	+
Amaranthus tricolor L.	C	D	123	+	
Avena fatua L.	W	M	1690	+	
Avena sativa L. cv. Premier	C	M	2515	+	
Beta vulgaris L. subsp. *vulgaris* cv. Beta 1996 (sugarbeet)	C	D	801	+	
Brassica hirta Moench	C	D	392	+	
Brassica hirta Moench cv. Tilney	C	D	657	+	
Brassica juncea (L.) Czern. & Coss.	C	D	199	+	
Brassica juncea (L.) Czern. & Coss.	C	D	496	+	
Brassica kaber (DC.) L.C. Wheeler	W	D	183	+	+
Brassica napus L. var. *napus*	C	D	350	+	
Brassica nigra (L.) W.J.D. Koch	C	D	48	+	
Brassica nigra (L.) W.J.D. Koch	C	D	118	+	
Brassica nigra (L.) W.J.D. Koch	C	D	260	+	
Bromus inermis Leyss. cv. Bounty	C	M	303	+	
Carthamus tinctorius L. cv. Finch	C	D	3170	+	
Dactylis glomerata L. cv. Duke	C	M	84	+	
Eriochloa villosa (Thunb.) Kunth	W	M	697	+	
Fagopyrum esculentum Moench	C	D	2439	+	
Glycine max (L.) Merr. cv. Stine 2870	C	D	15890	+	
Hordeum vulgare L. subsp. *vulgare*	C	M	2440	+	
Ipomoea hederacea (L.) Jacq.	W	D	1967	+	
Kochia scoparia (L.) Schrad.	W	D	115	+	
Kummerowia stipulacea (Maxim.) Makino	C	D	193	+	
Lactuca sativa L. cv. Black Seeded Simpson	C	D	106		+
Lolium perenne L.	C	M	298	+	
Lens culinaris Medik. subsp. *culinaris* cv. Pardina	C	D	3870	+	
Lens culinaris Medik. subsp. *culinaris* cv. Brewer	C	D	5430	+	
Lotus corniculatus L. cv. Norsen	C	D	135	+	
Medicago minima (L.) Bartal.	C	D	154	+	
Medicago sativa L. subsp. *sativa* cv. Pioneer 5454	C	D	215	+	
Panicum miliaceum L.	W	M	522	+	
Panicum virgatum L. cv. Alamo	C	M	98	+	
Panicum virgatum L. cv. Cave in Rock	C	M	185		+
Phalaris arundinacea L. cv. Venture	C	M	78	+	
Raphanus sativus L.	C	D	1236	+	
Secale cereale L. subsp. *cereale*	C	M	1818	+	
Setaria faberi Herrm.	W	M	170	+	+
Setaria glauca (L.) Beauv.	W	M	290	+	
Setaria italica (L.) Beauv.	C	M	177	+	
Setaria italica (L.) Beauv.	C	M	242	+	
Setaria italica (L.) Beauv.	C	M	260	+	
Setaria italica (L.) Beauv.	C	M	315	+	
Setaria sphacelata (Schumach.) Stapf & C. E. Hubb.	W	M	89	+	
Setaria sphacelata (Schumach.) Stapf & C. E. Hubb.	W	M	284	+	
Setaria viridis (L.) Beauv.	W	M	81	+	
Setaria viridis (L.) Beauv.	W	M	250	+	
Solanum ptycanthum Dun.	W	D	44	+	
Sorghum bicolor (L.) Moench	W	M	1437	+	
Sorghum bicolor (L.) Moench cv. CSCO 700 × 50	C	M	3150	+	
Sorghum halepense (L.) Pers.	W	M	470	+	
Trifolium alexandrinum L. cv. Multicut	C	D	339	+	
Trifolium pratense L. cv. Marathon	C	D	170	+	
Trifolium repens L.	C	D	63	+	
Triticum aestivum subsp. *aestivum* cv. Arapahoe	C	M	3109		+
Triticum aestivum subsp. *aestivum* cv. Jagger	C	M	3050	+	
Zea mays L. subsp. *mays* cv. B73 × MO17	C	M	26250	+	+

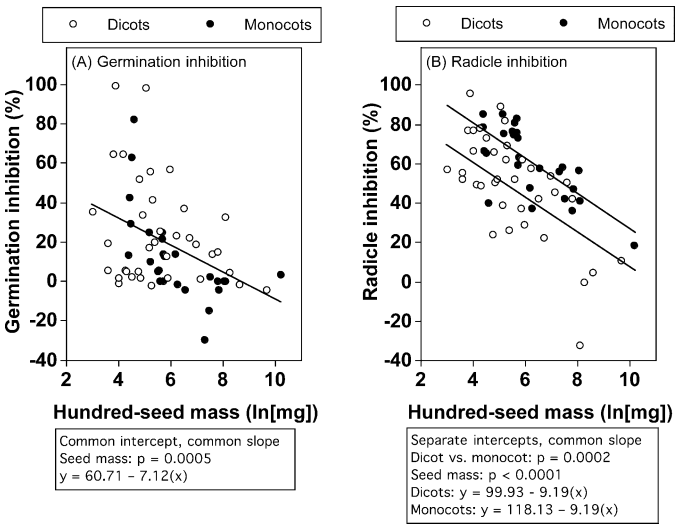

FIGURE 1. Germination inhibition (A) and radicle inhibition (B) of seeds in Experiment 1 as a function of seed mass and taxonomic class (monocot vs. dicot). Eighteen weed and 44 crop accessions were exposed to a 2% aqueous extract of Marathon red clover or distilled water. See text for methods of calculating germination inhibition and radicle inhibition.

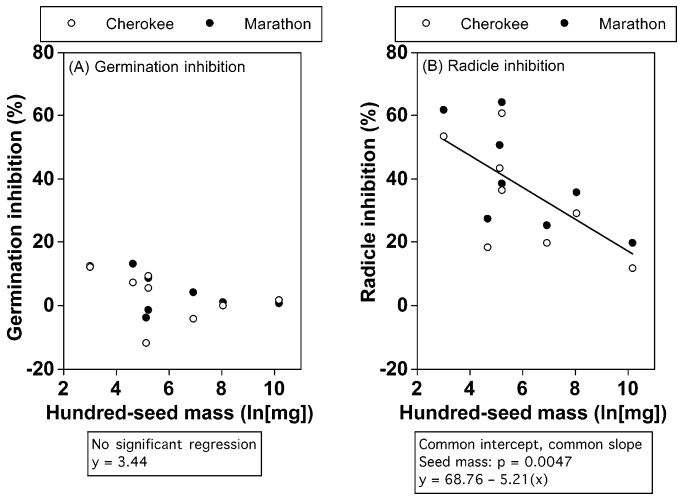

FIGURE 2. Germination inhibition (A) and radicle inhibition (B) of seeds in Experiment 2 as a function of seed mass. Four weed and four crop accessions were exposed to 1% aqueous extracts of Cherokee or Marathon red clover, or distilled water. See text for methods of calculating germination inhibition and radicle inhibition.

posed to an aqueous extract of red clover. We defined radicle inhibition as:

$$RI = ([RL_{control} - RL_{extract}]/RL_{control}) \times 100$$

where $RL_{control}$ was the mean radicle length for an accession after exposure to distilled water and $RL_{extract}$ was the mean radicle length for the same accession exposed to red clover extracts. Calculations of RI excluded those seeds that never germinated.

Both GI and RI are relative indices of responses to extract treatments, with values close to 0 indicating little effect on germination and radicle growth, and values close to 100 indicating strong inhibition. Use of relative indices is desirable because it avoids confounding differences in germination percentage and radicle length that are due to inherent, accession-specific differences in seed biology and mass with differences in germination and radicle growth that are due to exposure to red clover extracts.

For each experiment, mean GI and RI values of the accessions were subjected to analysis of covariance, using the $\log_e$-transformed mean 100-seed mass (in milligrams) of each accession as a quantitative explanatory factor, and class (monocot or dicot) and status (crop or weed) (Experiment 1), or red clover cultivar (Experiment 2) as qualitative explanatory factors. Analyses were conducted using the MGLH module of SYSTAT 5.2.1 (Wilkinson et al. 1992). Correlations between GI and RL were examined using the CORR module of SYSTAT 5.2.1 (Wilkinson et al. 1992).

Results and Discussion

GI and RI by red clover extracts in Experiment 1 were greatest for lighter seeds and least for heavier seeds (GI: $P = 0.0005$; RI: $P < 0.0001$) (Figures 1A and 1B). Additionally, for a given seed mass, RI was greater for monocotyledonous than dicotyledonous species ($P = 0.0002$) (Figure 1B). Further analyses of covariance indicated that neither the interaction between status (weed or crop) and seed mass nor the main effect of status influenced GI or RI in Experiment 1. No correlation was detected in Experiment 1 between RI and GI ($r = 0.2348$, $n = 62$, $P = 0.0663$), indicating that target plants differed in their susceptibilities to red clover extracts: those accessions that exhibited reduced radicle growth were not necessarily those that exhibited reduced germination, and vice versa.

Results of Experiment 2 indicated that GI was unaffected by red clover extracts, regardless of seed mass (Figure 2A). In contrast, inhibition by clover extracts of radicle growth in Experiment 2 was inversely proportional to seed mass ($P = 0.0047$) (Figure 2B), as in Experiment 1. Although there was a trend for Marathon clover to have a stronger suppressive effect on radicle growth than Cherokee clover, red clover genotype did not significantly affect RI, either when considered as part of an interaction with seed mass ($P = 0.9908$) or as a main effect in a reduced model ($P = 0.3476$). No evidence was detected in Experiment 2 for significant effects of monocot vs. dicot classification, or weed vs. crop status. The latter results are not surprising, given the small numbers of accessions per group. As in Experiment 1, no correlation was detected in Experiment 2 between GI and RI ($r = 0.2972$, $n = 16$, $P = 0.2636$).

The strength of radicle inhibition differed between Experiments 1 and 2. For example, for an accession whose 100-seed mass was 400 mg, the predicted value for RI in Experiment 1 was 63% for monocots and 45% for dicots (Figure 1B), whereas in Experiment 2 the predicted value was 38% (Figure 2B). This difference was consistent with the weaker strength of the extracts used in Experiment 2 compared with Experiment 1, and with the results of Ohno et al. (2000), who demonstrated in laboratory bioassays that inhibition of wild mustard [*Brassica kaber* (DC.) L.C. Wheeler, syn. *Sinapis arvensis* L.] seedling growth by aqueous extracts of red clover shoots and roots was linearly proportional to clover concentration; a 0.25% extract reduced wild mustard radicle length by about 15%, whereas a 1.25% extract reduced radicle length by about 65%, relative to a deionized water control.

Results of this study are consistent with reports from other investigators working with nonlegume species and chem-

icals with allelopathic potential, and target species representing a range of seed masses. In field experiments examining the effect of cover crop residues on weed and crop germination and seedling growth, Putnam and DeFrank (1983) observed that residues of barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), rye (*Secale cereale* L.), wheat (*Triticum aestivum* L.), and sorghum (*Sorghum bicolor* L.) suppressed the germination and growth of smaller-seeded vegetables (e.g., lettuce, *Lactuca sativa* L.) and weeds (e.g., smooth crabgrass, *Digitaria ischaemum* Schreb. ex Muhl.), whereas the residues had no effect on or stimulated the growth of larger-seeded vegetables (e.g., snap bean, *Phaseolus vulgaris* L.). Burgos and Talbert (2000) applied aqueous extracts of rye or distilled water to seeds of six crop and nine weed species in filter paper and petri dishes, and found that inhibition of root and shoot growth was greater for small-seeded species than for larger-seeded species. Similarly, Petersen et al. (2001) placed seeds of five weed species and wheat in filter paper and petri dishes, exposed them to methanol–water solutions containing isothiocynates, which are present in residues of turnip-rape [*Brassica rapa* (L.) var. *rapa* ssp. *oleifera* (DC.) Metzg.], and observed a negative correlation between seed mass and germination percentage: smaller-seeded species were most inhibited and larger-seeded species were most tolerant. To our knowledge, the present study is the first to quantify relationships between seed size and susceptibility to phytotoxins derived from a legume green manure for a large group of weed and crop species. We believe it is also the first study to distinguish quantitatively between the responses of monocot and dicot species when seed size was held constant statistically.

Although the two red clover cultivars tested in the present study were found to have similar phytotoxic effects (Figure 2), heritable variation in allelochemical concentrations might exist in this species, as it does for a number of cereal crops (Olofsdotter and Andersen 2004). Protocols have been developed for selecting and breeding rice for allelopathic weed suppression (Olofsdotter 2001a, 2001b), and similar approaches could be considered for red clover and other legume green manures.

No attempt was made in the present study to isolate the chemical agents present in red clover shoots that led to the observed suppression of germination and seedling growth, though previous investigations lead us to believe that the responsible agents were likely to have been isoflavonoid and phenolic acid compounds (Chang et al. 1969; Ohno et al. 2000; Tamura et al. 1969). It is possible, however, that the effects reported here were caused by osmotic factors, because solute concentrations were not equalized between the red clover extracts and distilled water control. Based on the results of experiments conducted by Chon et al. (2004), that hypothesis appears unlikely to be true. In a comparison of the effects of alfalfa leaf extracts with those of agar media adjusted with polyethylene glycol (PEG) to various osmotic potentials, Chon et al. (2004) found that root elongation rate was unaffected or stimulated by PEG, whereas it was strongly reduced by alfalfa extracts. PEG slightly delayed germination, whereas alfalfa extracts strongly delayed germination. If an analogous situation exists for red clover, the suppressive effects observed in the present study can be attributed mainly or entirely to phytotoxins derived from red clover shoots.

Three lines of evidence lead us to believe that the suppressive effects of red clover extracts we observed in our soilless laboratory bioassays are relevant to weed and crop performance under field conditions. First, though soil particles can adsorb phenolic compounds and reduce their concentrations in the soil solution (Dalton et al. 1989; Makino et al. 1996), aqueous extracts of soil from field plots amended with red clover were found to have higher concentrations of phenolic compounds than unamended soil, and suppression of wild mustard radicle growth by soil:water extracts was found to be proportional to their phenolic C concentrations (Doolan 1997; Ohno et al. 2000). Thus, soil can reduce but will not necessarily eliminate the phytotoxicity of red clover residues. Second, Ohno et al. (2000) found that suppression of wild mustard seedling growth in agar was similar when agar contained an aqueous extract of red clover shoots and roots or an aqueous extract of field soil into which an equivalent amount of red clover had been incorporated 8 d before soil collection. That is, soil-free and soil-derived red clover extracts produced similar results when soil samples were collected shortly after tillage. Finally, the seed-mass–related patterns of GI and RI we observed under laboratory conditions were similar to responses elicited by clover green manure in field experiments examining growth and yield of several small-seeded weeds and large-seeded crops. Dyck and Liebman (1994) and Dyck et al. (1995) reported that incorporation of crimson clover (*Trifolium incarnatum* L.) into soil reduced the emergence rate, density, biomass, and competitive ability of the small-seeded weed common lambsquarters (*Chenopodium album* L.), but had negligible effects on the emergence and growth of the large-seeded crop sweet corn. Similarly, field experiments with wild mustard, sweet corn, and bean exposed to red clover residue showed that growth of the two crops was unaffected or promoted by clover green manure, whereas the weed species was suppressed (Davis and Liebman 2001; Liebman and Gallandt 2002). Though there is an important need for additional research on more weed and crop species grown under a wider range of environmental conditions, the foregoing results and those of the present study lead us to conclude that suppressive effects of red clover green manure on weed and crop species growing in the field will tend to be inversely proportional to seed mass.

Despite the potential to exploit allelopathic legume green manures as agents for weed suppression, their use poses several challenges. Moles and Westoby (2004) reviewed relationships between seed mass, tolerance of various forms of stress (drought, burial, nutrient deficits, defoliation, shading, and resource competition from other plants), and fecundity, and concluded that the stress-tolerance disadvantage of smaller-seeded species might be offset by their ability to produce more seeds. Thus, small-seeded weeds such as common waterhemp (*Amaranthus rudis* Sauer) and eastern black nightshade (*Solanum ptycanthum* Dun.), which were highly susceptible in the present study to red clover extracts, but which can produce thousands to millions of seeds per plant (Bassett and Munro 1985; Hartzler et al. 2004), might persist abundantly in a cropping system that included red clover unless their survival and fecundity were greatly diminished. Additionally, because clover green manure had less effect on large-seeded dicots than on small-seeded monocots, clover-based cropping systems might select over time for a greater

proportion of large-seeded dicot weeds. Consequently, red clover residue should be viewed as one possible component of weed-management strategies that comprise multiple tactics.

Sources of Materials

[1] Filter paper (No. 1), Whatman International Ltd., St. Leonard's Road, Maidstone, Kent, United Kingdom, ME16 OLS.

[2] Nylon syringe filter (0.45-μm mesh), Millipore Corp., 290 Concord Rd., Billerica, MA 01821.

[3] Filter paper (P5), Fisher Scientific, 2000 Park Lane Dr., Pittsburgh, PA 15275.

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