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BIOLOGICALLY ACTIVE SECONDARY METABOLITES OF BARLEY. I. DEVELOPING TECHNIQUES AND ASSESSING ALLELOPATHY IN BARLEY

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Abstract—Allelopathic effects of barley (*Hordeum vulgare* L.) on white mustard (*Sinapis alba* L.) were assessed using modified bioassays that reduced other environmental influences. In a Petri dish bioassay, germination of white mustard was delayed and the radicle lengths were significantly inhibited at a density of 0.5 barley seed/cm^2. In a 'siphoning' bioassay apparatus, when the two species were sown together, radicle elongation of white mustard was not inhibited one day after sowing but became increasingly inhibited as bioassay time increased. Barley allelochemicals were released from the roots in a hydroponic system for at least 70 days after commencement of barley germination. Solutions removed from the hydroponic system of growing barley delayed germination and inhibited growth of white mustard. The allelopathic activity of barley was further confirmed at a density of 0.3 barley seed/cm^2 in a modified stairstep apparatus.

Key Words—Allelopathy, germination, bioassay, siphoning apparatus, hydroponics, stairstep assay, barley, *Hordeum vulgare*, *Sinapis alba*.

INTRODUCTION

Separation of allelopathy from other aspects of plant interference remains one of the most challenging tasks in studies of plant interference (Harper, 1977). Methods used in studying allelopathy have received more criticism than those

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for studying competition. This has resulted in some ecologists holding deep reservations concerning the significance of allelopathy.

Techniques applied to studies of allelopathy have frequently been crude, contributing to uncertainties about the significance of allelopathic phenomena. Leather and Einhellig (1986, 1988) reviewed the literature pertaining to the use of bioassays and discussed the general suitability of different assays for studying allelopathy, demonstrating that many reports of allelopathy are questionable because the bioassays were not suitable indicators. Soaking of plant parts in either water or organic solvents, for example, may lead to the release of chemicals that are not normally released into the environment (Lovett, 1982). Interpretation of "allelopathic" effects on plants or other organisms may be confounded in these circumstances. Allelopathy occurs only if the chemicals are not only produced by a plant but released into the vicinity of other plants and, ultimately, received under the influence of natural environmental conditions. Therefore, reliable investigations of allelopathy include tests of compounds released by intact living donor plants into the vicinity of receiver plants. This is a fundamental principle in investigations of allelopathy.

Barley (*Hordeum vulgare* L.) has been reported to be a smother crop, which can suppress the growth of weeds through competition for environmental resources (Overland, 1966). However, in the absence of competition, barley still inhibited germination of *Amaranthus hybridus* L. (slim amaranth) and *Chenopodium album* (Went et al., 1952), suggesting that phytotoxins might be involved (Overland, 1966). Overland (1966) further found that the inhibitory activity of barley was selective among broad-leaved plants, chickweed (*Stellaria media* L.) being more severely inhibited than shepherd's purse [*Capsella bursa-pastoris* (L.) Medic.].

The objectives of this research were to develop techniques for separating competitive influences from allelopathy and, through these techniques, to assess allelopathic activity of barley on white mustard (*Sinapis alba* L.).

METHODS AND MATERIALS

Petri Dish Bioassay. Allelopathic activity of germinating barley was bioassayed on filter paper in 9-cm Petri dishes in an incubator at 25°C in the dark using surface-sterilized seeds of white mustard (*S. alba*), used to simulate a broad-leaved weed of the same family as *C. bursa-pastoris*, but having synchronous germination. Barley (*H. vulgare*, cv. Triumph) seeds were evenly distributed on two Whatman No. 1 filter papers at rates of 0 (control), 0.13, 0.25, 0.5, 1.0, and 2.0 seeds/cm^2 with 10 white mustard seeds in each Petri dish for bioassay. The bioassay was designed with five replications.

Because germinating barley absorbs large amounts of water (Alabushev,

1977), an experiment was carried out to determine water uptake by germinating barley. Since radicle lengths of white mustard were greatest, and similar, at 5, 6, and 7 ml sterile distilled water under the bioassay conditions over the range of densities employed (Liu, 1991), 5, 6, and 7 ml sterile distilled water for controls (i.e., three controls without barley seeds) and 6.1, 6.2, 6.3, 6.6, and 7 ml for barley density of 0.13, 0.25, 0.5, 1.0, and 2.0 seeds/cm^2, respectively, were initially applied to the medium at the commencement of the experiment. Water uptake by barley was determined by weighing barley seeds after carefully removing free water from the barley seed surface. The compensating water for the uptake by barley was added 6, 18, 30, 45, and 60 hr after sowing. The criterion for determining the time and the amount of the compensating water was: 5 ml control $\leq$ water in all treatments, $\leq$7 ml control. At each observation, the water uptake by barley during the last period was added before placing in the incubator for continuation.

In the bioassay experiment, the compensating water was added at the times and in the amounts as determined in the water uptake experiment. This design should eliminate the effect of water competition by germinating barley and ensure no effect of excess water on white mustard.

The germination of white mustard was observed at 3- to 6-hr intervals and after 72 hr the radicle length of white mustard was measured. The germination data were used to calculate the germination rate, which was defined as the time to reach the germination peak in the germination distribution curves (Hughes, 1977). Range tests were applied to determine statistically significant differences for the radicle length.

Siphoning Bioassay Apparatus. The apparatus consisted of a glass container (40 cm long, 40 cm wide, and 12 cm high), four pieces of glass (45 $\times$ 9 cm), sixteen 9-cm glass Petri dishes, and 32 pieces of Whatman No. 1 filter paper (25 $\times$ 3.5 cm). Four glass Petri dishes were set on each piece of glass. Two Whatman papers were placed, parallel, over the Petri dishes and the ends of the papers were suspended in the water container. The container was filled with distilled water. The apparatus was set in an incubator at 25°C in the dark. Figure 1 shows a siphoning apparatus similar to the one used in these experiments.

The experiment was designed to observe the effects of young barley seedlings on white mustard at different times. The treatments included white mustard seeds alone and white mustard seeds with barley seeds, harvesting at each day after sowing up to six days. There were four replications. Thus, the total number of Petri dishes was 48. Since the number of Petri dishes in the apparatus was 16, which allowed only two harvests, it was not possible to conduct the experiments at one time. Therefore, the experiment was split into three runs, in each of which the 16 Petri dishes were set up in 2 $\times$ 2 factorial designs with four replications. The harvests were day 1 and day 2 in the first run, day 3 and day

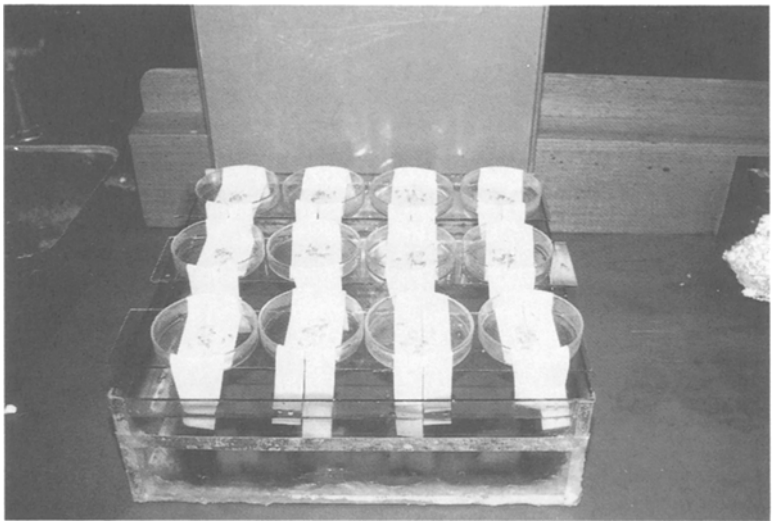

FIG. 1. Siphoning bioassay apparatus developed to eliminate competition for water while assessing the activity of allelochemicals released by germinating barley seeds.

4 in the second run, and day 5 and day 6 in the third run. Ten white mustard seeds in monoculture and 10 mustard plus 10 barley seeds in mixture were evenly sown in the Petri dishes, as appropriate. Four 6-mm filter paper disks were distributed in each dish and osmotic potentials were measured using a Wescor HR-33 dew point microvoltmeter on the harvest day.

The data for mean radicle length of white mustard collected at each harvest day were subjected to analysis of variance. The mean values of each four readings of osmotic potentials from each Petri dish at each harvest day were also subjected to analysis of variance.

Hydroponics. An 18-cm-diameter plastic saucer covered the top of a glass container (Figure 2). The volume of the glass container was periodically increased from a minimum of 0.15 liters to a maximum of 2.5-liter capacity. Hoagland's solution, varying in strength to match plant growth, was used to fill the glass containers. A glass Petri dish, 9 cm diameter and 2 cm in depth, was set on the top of the plastic saucer. There was a 3-cm hole through the glass Petri dish and plastic saucer. Two pieces of 2.5 × 4.0-cm glass were set parallel over the hole of the glass Petri dish, and roots of plant seedlings were placed through the gap between the two pieces of glass into the glass container. The epicotyls of the seedlings were surrounded by a gravel and sand mixture in the glass Petri dish. A RENA 301 air pump was used to pump air into the containers. The container was surrounded by sand in a pot, which had free drainage. Water was added to the sand around the container to avoid over heating.

FIG. 2. Hydroponic system developed to study the release of allelochemicals by intact plants over time. The tube was connected to a RENA 301 air pump, which is not shown in the picture.

The hydroponic system was set up in a glasshouse with natural light and temperature range of 16–27°C. We (Liu and Lovett, 1990) have described the experimental design and reported the response in radicle length of white mustard to growing barley in a hydroponic system. The second part of the experiment is reported here.

Stairstep Apparatus. A stairstep apparatus with four recycling nutrient units (Figure 3) was set up in a glasshouse with a mean temperature of 18°C (range 16–20°C) with natural light supply. The apparatus was developed from an earlier model (Lovett and Jokinen, 1984). Each unit comprised six steps. The nutrient solution from the top reservoir flowed, by gravity, to an incubation tray (57 × 35 × 18 cm) through three lines of pots (15 cm diameter, 14 cm height), with

FIG. 3. A modified stairstep apparatus for assessment of allelopathic effects by barley.

four replications within each set of steps, to a collector tank (50-liter capacity, on the first and sixth steps) from which it was automatically recycled by an electric pump (FP Model, Onga Pump).

Between steps 2 and 3, 3 and 4, and 4 and 5, there was an opaque tube (55 mm diameter, 800 mm long), to collect solutions from the preceding step. The topmost tube was joined to the incubation tray by a 10-mm-diameter black pipe. Connections from this tube to the next level and between lower levels were by 3-mm-diameter black tubes. A bent 3-mm-diameter black tube with a 150° angle was inserted into the opaque tube over each pot for individual solution supply. This bent tube was designed for a fine adjustment of the flow rate of solution by turning the tube clockwise or counterclockwise. The opaque tube was mounted on two steel semicircular supporters; thus, a coarse adjustment of flow rate could be obtained. In this system the flow rate of solution could be varied from 1 to 20 liters/hr.

Three centimeters of gravel (5–10 mm particle size) was sandwiched by two nylon mesh (0.8 mm) liners on the base of the incubation tray. Twelve centimeters of sand (0.7–1.5 mm particle size) covered the nylon mesh. Individual pots were filled in similar fashion and contained 500 g gravel and 2500 g sand. On day 1 the collection tank was filled with 50 liters of half-strength Hoagland's solution. Flow rates were gradually increased from 1 to 6 liters/hr during the experiment and the solution was cycled for 2 hr twice a day. Before each recycling, the electrical conductivity (EC) and pH of the solution were

monitored. A 10% Hoagland's solution was added to each unit collecting tank at day 12 and 12% Hoagland's solution was further added at day 17, in order to maintain the EC range of 1.0–1.5 mS/cm^2. The value of pH did not vary from 6.8 during the period of the experiment.

The treatments consisted of: (a) without barley (control); (b) barley at a density of 0.3 seed/cm^2 sown at the same day as white mustard; (c) barley at a density of 0.3 seed/cm^2 growing for 28 days before introduction of white mustard; and (d) 100 g oven dried plant materials of (c).

Eight seeds of white mustard were sown directly into each test pot and thinned to four seedlings in uniform positions at day 3 after sowing. The white mustard was harvested after growing for 21 days. The plants were separated into leaf, stem, and roots. The leaf area was determined using an electronic planimeter (Paton, Stepney Australia). The fresh and oven dry weights of each component were measured.

RESULTS

Petri Dish Bioassay. Total water uptake during the bioassay was up to 5.5 ml/dish (Table 1). After competition for water uptake, radicle length of white mustard was still significantly inhibited by germinating barley at a density of 0.5 seed/cm^2 or more. The critical density of barley seeds, where such inhibition may begin, was about 0.3 seed/cm^2 (determined by regression curves) under our experimental conditions. The final germination of white mustard with barley seeds was not significantly different from that without barley in general (data not shown), but the time to peak germination (germination rate) of white mustard was delayed by germinating barley. The delayed germination rate was significant, as indicated by the trends of regression equation (Figure 4).

TABLE 1. TOTAL WATER LOSSES, TOTAL AMOUNT OF COMPENSATING WATER ADDED TO SYSTEM, AND EFFECT OF GERMINATING BARLEY ON RADICLE LENGTH OF WHITE MUSTARD IN BIOASSAY

	Density (seed/cm^2)					
	0.00	0.13	0.25	0.50	1.00	2.00
H_2O losses (ml)	0.5	0.8	1.1	1.7	3.0	5.5
Compensation (ml)	0.0	0.2	0.5	1.0	2.9	6.0
Radicle length (mm)[a]	38.5 a	39.7 a	36.4 a	28.8 b	21.8 c	8.9 d

[a] Means of radicle length of white mustard that share a common letter are not significantly different at 5% level, by Student's range test.

Siphoning Bioassay Apparatus. When barley seeds and white mustard seeds were sown together, there were no significant differences between radicle length of white mustard with or without barley at day 1. However, the radicle length of white mustard was significantly ($P < 0.01$) inhibited at days 2, 3, 4, 5, and 6 (Figure 5). Although it was not possible to analyze the variance of the radicle length of white mustard between the different harvests, there was a decrease in radicle length from day 5 to day 6. Some of the mustard roots on day 5 and day 6 were decayed, possibly due to infection by microorganisms.

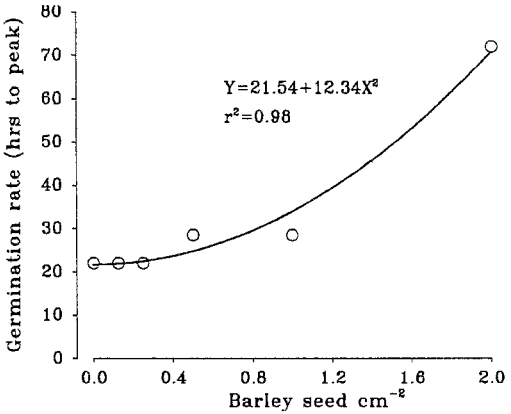

FIG. 4. Germination rates (hours to peak germination) of white mustard as affected by germinating barley seeds on filter paper at increasing densities.

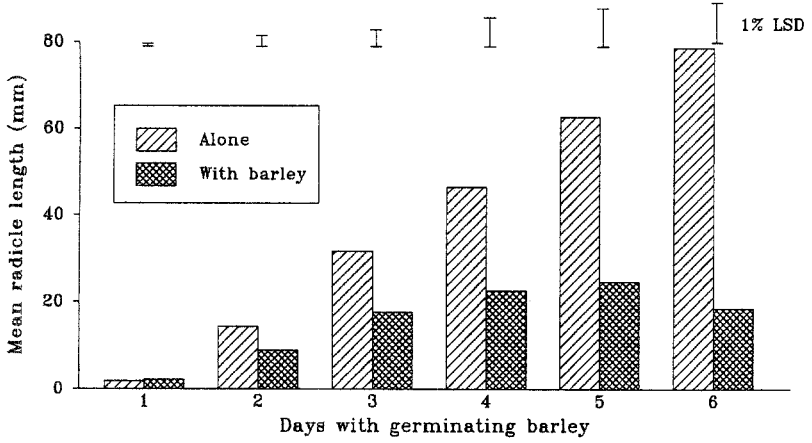

FIG. 5. Radicle lengths of white mustard as affected by germinating barley over time, where competition for water was not a factor.

There were significantly ($P < 0.01$) higher osmotic potentials on the filter paper with barley than on those without barley at all harvests (Table 2). The ranges of osmotic potentials were from −0.33 bars on day 1 to −0.95 bars on day 6, and from −0.83 bars on day 1 to −1.51 bars on day 6 for white mustard alone and white mustard with germinating barley, respectively.

Hydroponics. The inhibition of radicle length of white mustard in bioassay by the hydroponic solution in which barley roots were grown from 7 to 75 days after transplanting was previously reported (Liu and Lovett, 1990). The first-day germination was greatly reduced by hydroponic solution with barley growing in it (Figure 6). Thus, the peak germination of white mustard was delayed. The

TABLE 2. OSMOTIC POTENTIAL ON FILTER PAPER WHERE WHITE MUSTARD GERMINATED WITH OR WITHOUT GERMINATING BARLEY

	Mean osmotic potential (bars)					
	Run 1		Run 2		Run 3	
Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Without barley	−0.33	−0.44	−0.73	−0.87	−0.93	−0.95
With barley	−0.83	−1.07	−1.33	−1.41	−1.49	−1.51
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
$P < 0.05$ LSD	−0.06	−0.08	−0.10	−0.05	−0.05	−0.07
$P < 0.01$ LSD	−0.09	−0.12	−0.15	−0.08	−0.08	−0.11

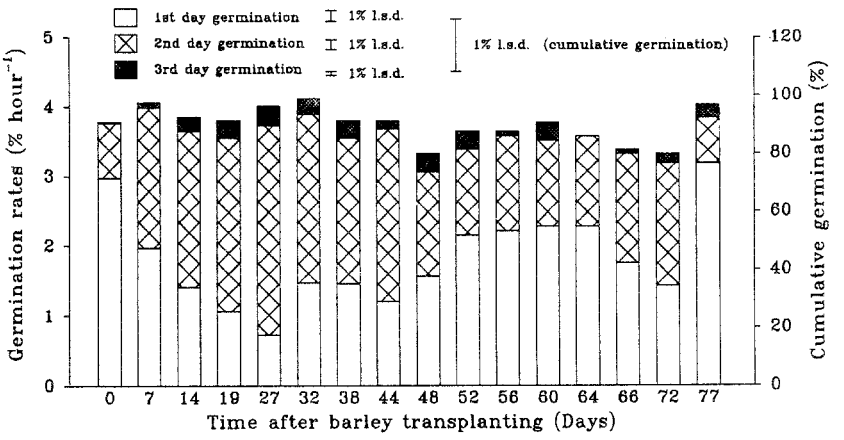

FIG. 6. Germination speed (% hour^{-1}) and cumulative germination (%) of white mustard as affected by allelochemicals from barley growing in a hydroponic system.

effect of germination, in terms of germination per hour at the first day, was not attributable to electrical conductivity, but can be attributed to the effects of allelochemicals released from barley roots (Figure 7).

Stairstep Apparatus. The barley sown simultaneously, sown 28 days before the start of the experiment, and the oven dried barley resulted in reductions of 13%, 45%, and 20% in leaf area, in reductions of 10%, 42%, and 22% in total fresh weight, and in reductions of 10%, 38%, and 24% in total dry weight, respectively (Figure 8). It was clear that barley sown 28 days before the start of the experiment exhibited the greatest inhibition of the growth of white mustard among the treatments.

The ratio of shoot to root dry weight was not significantly different ($P > 0.05$) between control and that associated with barley sown simultaneously (Figure 9). The ratio from the treatment associated with barley sown 28 days before the start of the experiment was lower, and the ratio from that associated with dead barley was higher. This result indicated that the inhibition by barley sown 28 days before introduction of white mustard inhibited shoots more than roots

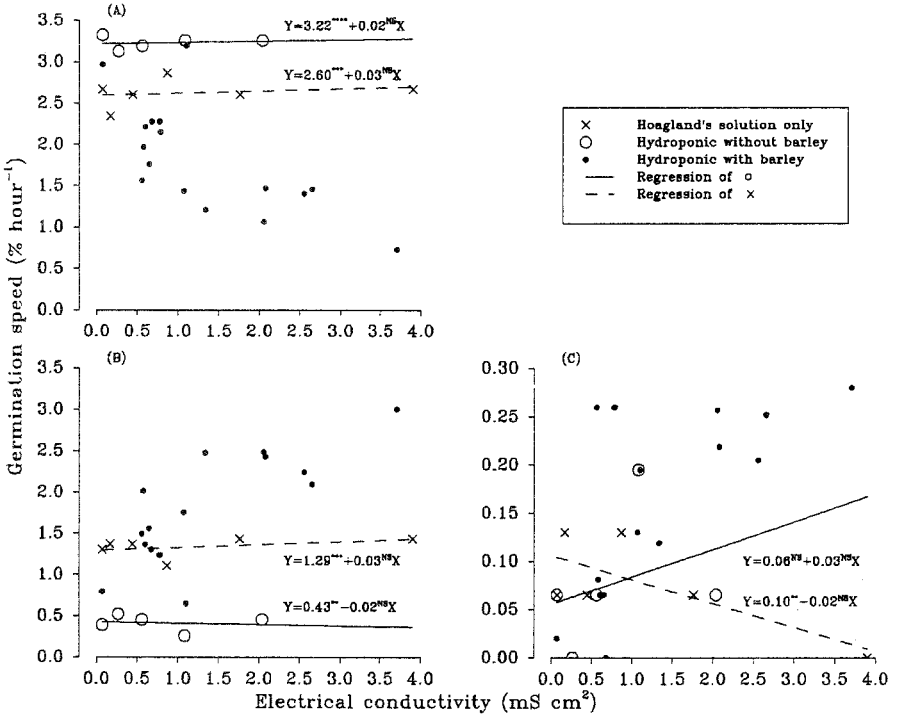

FIG. 7. Relationship between electrical conductivity and germination speed of white mustard seeds at the first day (A), the second day (B) and the third day (C).

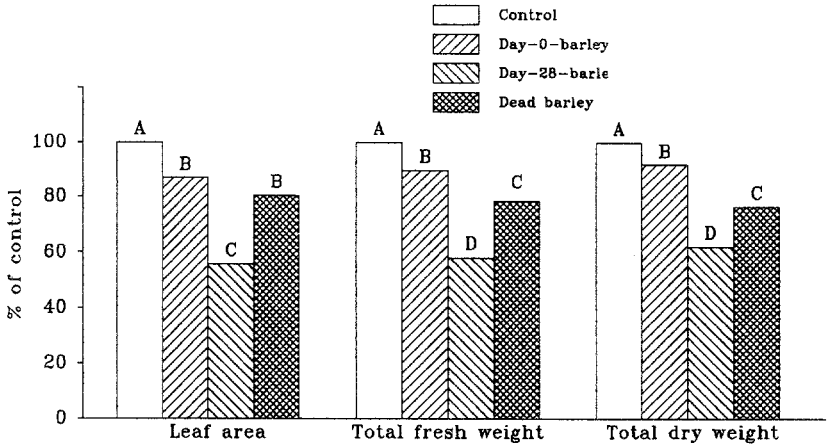

FIG. 8. Allelopathic effects of barley on leaf area, total fresh weight, and dry weight of white mustard after three weeks in a modified stairstep apparatus. The data for control are presented as 100% and the data for the treatments are presented as percent of control after analysis of variance. Any two bars within the same parameter not marked by the same letter are significantly different from each other at the 1% level, by Duncan's multiple-range test.

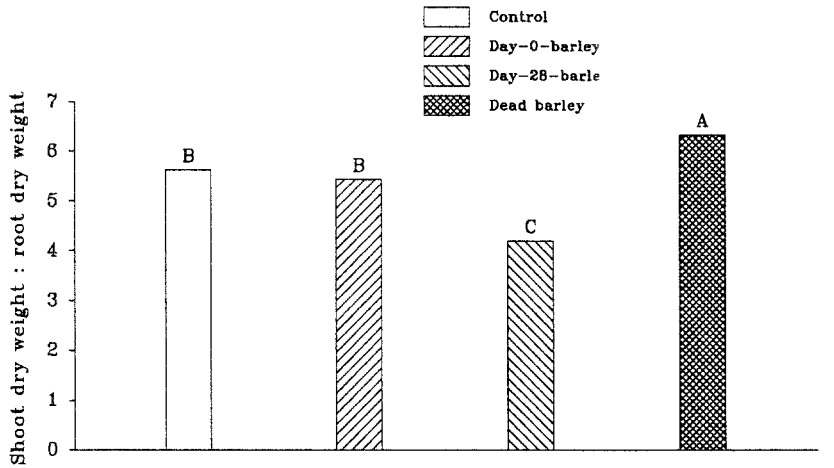

FIG. 9. Allelopathic effects of barley on the ratio of shoot dry weight to root dry weight of white mustard after three weeks in a modified stairstep apparatus. Any two bars not marked by the same letter are significantly different from each other at 1% level, by Duncan's multiple-range test.

of white mustard, while the inhibition by oven dried barley inhibited roots more than shoots. In addition, the seedlings of white mustard associated with the oven dry barley became chlorotic one week after sowing, but this symptom gradually declined two weeks after sowing and disappeared by the harvest.

DISCUSSION

Many of the problems associated with the separation of allelopathy from plant interference can be attributed to a lack of convincing methodology (Fuerst and Putnam, 1983). The common methods used in such studies were reviewed by Liu (1991). The key criteria for developing convincing methods must be that the investigations are on intact plants and that experiments should reasonably simulate the real situation of plant interference. Since numerous compounds are already present within plants (Putnam, 1988), results from, for example, testing the effects of extracted plant materials using solvents or from maceration and grinding of plant materials are certainly unreliable due to the liberation of chemicals that might not be released in nature (Lovett, 1982).

Germinating seeds are able to take up water to 88% of their dry weight for cereal crops and up to 113% of their dry weight for legumes (Alabushev, 1977). Liu (1991) showed that the average water uptake by germinating barley was 83% and 91% of their initial dry weight on filter paper and sand media, respectively. The amount of water absorbed by germinating barley at barley densities of 0.5 seeds/cm^2 or more (Table 1) could certainly alter the water available for other species. Therefore, addition of water to compensate for water depletion by barley is essential in a bioassay when an attempt is made to reduce the physical effects (competition), especially in a limited test medium such as Petri dishes or in a bioassay where the number or size of germinating seeds is large. In the siphoning bioassay apparatus (Figure 1), water was continuously supplied to the system so that the amount of water in each should, theoretically, be the same and any water uptake or evaporation should be automatically compensated. Therefore, this technique enabled conclusive separation of competition for water from allelopathic interference. In addition, since there were no physical barriers limiting the early growth of germination seeds in the open system, a bioassay could be conducted much longer than is permitted by conventional Petri dish techniques.

Allelopathic interference by germinating barley, as previously reported (Overland, 1966; Prutenskaya, 1972), was further confirmed by the modified bioassay technique. Such effect was not attributable to osmotic potentials (Table 2) as these osmotic potentials were higher than could result in an inhibition (Liu, 1991), nor to competition for water as the feature of the system (Figure 1), but

was attributable to allelochemical release by the early seedlings of barley (Liu and Lovett, 1990).

In the field situation, germinating seeds of plants are generally subject to infection by fungi, bacteria, and virus diseases. The results of allelopathic activity of crop species that delay germination (Figures 4, 6, and 7) or reduce radicle elongation of weed seedlings (Figure 5) could complement these stresses, to the advantage of establishing crop seedlings. Since the delayed germination and inhibition of root growth of weeds is a secondary expression of primary disturbance of metabolic activity by germinating seeds (Winter, 1961), the ability of seeds to provide microbial inhibitors may be important (Einhellig, 1987). Decaying roots of white mustard were noted using the siphoning bioassay apparatus suggesting that the germinating seeds were less resistant to disease, including those caused by microorganisms. Therefore, crop plants may exhibit allelopathy as indirect self-defense through interaction with other environmental stresses, and the combined effects of allelopathy and pathogenicity may help to eliminate some weed seedlings.

The aerated hydroponic system (Figure 2) provided a technique to further assess phytotoxic activity. In this system plant roots and nutrients were in the dark. Plant stands were supported by the gravel and sand mixture, thus simulating field conditions despite the roots being in the solution. Since hydroponic solution without barley did not interfere with seedling growth of white mustard, the inhibition of white mustard was attributable to the presence of barley roots. Interference by competition for plant nutrients was eliminated in this system but inhibition of white mustard was observed up to 75 days (Liu and Lovett, 1990). The results strongly suggest that inhibition of white mustard occurs as a result of the release of allelochemicals by barley and that such release is not confined to the seedling stage.

The stairstep apparatus (Figure 3) was built with opaque materials, thus keeping it free from algal growth, as observed by Lovett and Jokinen (1984). The potential for adjustment provided uniform delivery of solution over a range of flow rates. Thus, flow rate could be adapted to different experimental needs and to the changing water requirements of plants at different stages of growth. Further evidence for allelopathic effects of living barley was found using the modified stairstep apparatus. Barley, already growing for 28 days, exhibited more inhibition of the seedling growth of white mustard than barley sown simultaneously with white mustard (Figures 8 and 9).

Overall, separation between allelopathy and competition is the most difficult task in the studies of plant interference. In our studies, attempts have been made to develop convincing techniques for such separation. These techniques, at least in part, simulate plant growth in nature and have significant advantages over conventional methods.

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