Search for a Standard Phytotoxic Bioassay for Allelochemicals. Selection of Standard Target Species†

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In the search for a standard bioassay of phytotoxicity for allelochemicals, 22 commercial varieties of eight plant species [four dicotyledons: lettuce (Compositae), carrot (Umbelliferae), cress (Cruciferae), tomato (Solanaceae); and four monocotyledons: onion (Liliaceae), barley, wheat, and corn (Gramineae)] proposed as models for the most common weed families have been tested at different pH and solution volumes per set conditions. Nine commercial varieties selected as standard target species (STS) were tested with standard commercial herbicides to ensure their sensitivity to phytotoxic compounds. Results are discussed to establish the proper growth requirements, and sensitivity of commercial seeds of STS and to find the most suitable commercial herbicides that allow comparison with an internal standard to validate the response of potential allelochemicals.

Keywords: *Allelopathy; bioassay; phytotoxicity; standard target species (STS); coefficient of variation (CV); commercial herbicides; pre- or postemergence treatments; Lactuca sativa L.; Lycopersicon esculentum L.; Daucus carota L.; Lepidium sativum L.; Allium cepa L.; Triticum aestivum; Hordeum vulgare L.; Zea mays L.*

INTRODUCTION

Herbicides help farmers to increase yields while reducing agricultural labor. They are a key component in most integrated weed management systems. Without herbicides, labor would be a major cost of crop production in developed countries. Nevertheless, the indiscriminate use of herbicides has provoked an increasing incidence of resistance in weeds, changes in weed population toward species more related to the crop, and potential environmental pollution and health hazards. New, more efficient, and specific herbicides are needed.

Allelopathy (Molisch, 1937; Rice, 1984) is an emerging branch of applied sciences which studies any process primarily involving secondary metabolites produced by plants, algae, bacteria, and fungi that influence the growth and development of biological and agricultural systems, including positive and negative effects (IAS, 1996).

Plants have defense mechanisms, and allelochemicals (biologically active natural products) are, in fact, natural herbicides. Allelochemicals may help in overcoming weed problems through the development of crop varieties having greater ability to smother weeds and the use of natural (from plants or microbes) or synthetic derivative phytotoxins as plant growth regulators.

In terms of application, one of the most important targets of allelopathic studies is the search for alternative herbicides. Progress in this area affords new possibilities for their development (Einhellig, 1988; Oloffdoter et al., 1994; Macías, 1995; Macías et al., 1996), apart from other chemical approaches (Davidonis, 1992; Schmidt, 1992; Streibig, 1988).

Searching for new herbicide templates, we have developed a research project, "Natural Product Models as Allelochemicals", in which we are evaluating different plant species for compounds with phytotoxic activity.

Bioassays are defined as the assessment of the potency of a compound via its application-induced response to the subject (Webster, 1980; Govindarajulu, 1988). In allelopathy (Rice, 1974), bioassays are necessary in each step of the isolation, purification, and identification processes of active compounds (Stowe and Kil, 1981; Macías et al., 1997). Since initial pioneering work in the 1940s and 1950s (Mitchell and Livingston, 1968), numerous inexpensive and easy-to-use bioassays have been used to check the activity of putative allelopathic compounds. The most widely used bioassay is seed germination, carried out in Petri dishes, with filter paper as the most common support (Leather and Einhellig, 1986).

Most allelopathic bioassays that are correlated to phytochemical studies evaluate the bioactivity by affecting germination and seedling growth (Steffens et al., 1986; Shilling and Yoshikawa, 1987). These parameters are accepted as indirect measures of other physiological processes affected by chemical interaction. In this way, a wide range of effects are covered, and such bioassays serve to select compounds that can be evaluated in greenhouse and field studies.

Usually, the most used species model for weeds is lettuce (*Lactuca sativa* L.). It has been used extensively as a test organism because of its fast germination and high sensitivity (Rasmussen and Einhellig, 1979; Leather, 1983; Yu and Matsui, 1994). It is used extensively and allows comparison of bioassay results for many different compounds. However, the concept of allelopathy includes both inhibition and stimulation (IAS, 1996), so it is necessary to consider models of some agronomically important species to gauge possible application of alle-

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lochemicals as plant growth regulators. On the other hand, living systems present a wide variability in their response to treatments, and one single agent may induce different effects in different species. This fact necessitates the evaluation of compounds by various weed and crop models.

Strictly speaking, the use of weed seeds as a bioassay implies the use of allelochemicals only as herbicides. However, the use of commercial crop seeds gives rise to a more general model, especially with respect to stimulatory effects. The latter avoids some of the more common problems implied by use of wild species. First, wild species are genetically more heterogeneous than commercial crops, and their growth under laboratory conditions is unpredictable. Viability of seeds depends on many conditions, and seeds have various degrees of sensitivity to similar treatments at different times. Second, many weed seeds do not germinate uniformly (King, 1966; Cutler and Cole, 1983), probably because of internal protection mechanisms. Moreover, germination and growth capacity often deteriorate with time. This requires a constant, periodic harvesting which is expensive and time-consuming. Commercial crop seeds have advantages of being more genetically homogeneous, germinating more uniformly, and being readily available.

Keeping the variability of wild seeds in mind, and combining both criteria of target species as models for weeds and crops, we propose using diverse standard target species (STS) such as dicotyledons: *L. sativa* L. (lettuce, Compositae), *Daucus carota* L. (carrot, Umbelliferae), *Lepidium sativum* L. (cress, Cruciferae), *Lycopersicon esculentum* L. (tomato, Solanaceae); and monocotyledons: *Allium cepa* L. (onion, Liliaceae), *Hordeum vulgare* L. (barley, Gramineae), *Triticum aestivum* L. (wheat, Gramineae), and *Zea mays* L. (corn, Gramineae). These families represent the most common weeds (Holm et al., 1979).

No articles have been published that establish standard procedures for phytotoxic bioassays, including growth conditions, or model species. The object of this work is to propose a standard bioassay of potential phytotoxicity for allelochemicals with wide application for this particular kind of allelopathic study. The work has been divided in two parts. The first part of this work includes the following: (i) the appropriate STS, (ii) the use of commercially available seeds, and (iii) the evaluation of the influence of pH and application volume on germination and growth.

Many reports have used different target species and conditions of growth (Leather and Einhellig, 1986) that we consider as valid as our proposed STS. However, many studies focused their assays on a low number of target species. This often limits the response of tested compounds and makes it difficult to establish a standard procedure for most phytotoxic bioassays. Thus, we propose to use nine target species as models of the most widespread weed families and for important crops. This allows us to achieve a wide response range, and not to limit assays to only a particular weed species.

Nevertheless, commercial seeds are often treated with protectant fungicides or plant growth regulators to stimulate germination. These processes may affect their response to stimulatory or inhibitory activity of assayed compounds. Thus, it is necessary to assay them with different compounds of verified activity. To ensure sensitivity to phytotoxic compounds of commercial seeds of STS, nine herbicide formulations were bioassayed. This goal is covered in the second part of this report.

The second part of this work covers three objectives: (i) to test the sensitivity of proposed STS, (ii) to establish the activity profiles in a standard phytotoxic bioassay using commercial herbicides over nine STS (they have potential to be used as reference data in allelochemical assays to evaluate natural product herbicide templates), and (iii) to propose a particular formulation as an internal standard with reproducible activity to authenticate the phytotoxic responses of experimental allelochemicals. Thus, a novel system for standard phytotoxic bioassays is introduced. Seedling growth is high variable in different bioassays under identical conditions; this is exacerbated by the heterogeneous population of seeds and necessitates an internal standard.

EXPERIMENTAL METHODS

Materials. One-year-old seeds belonging to commercial dicotyledonous varieties were: seven *L. sativa* L. (lettuce) cvs. Batavia, Maravilla, Romana Rubia, Trocadero, Nigra, Grandes Lagos, and Roman; two *D. carota* L. (carrot) cvs. Berlikumer and Coral; three *L. sativum* L. (cress) cvs. Comun, Royalfleur, and Belplanto; two *L. esculentum* L. (tomato) cvs. Tres Cantos and Rio Grande, and four monocotyledonous species: two *A. cepa* L. (onion) cvs. Valenciana and Morada; two *H. vulgare* L. (barley) cvs. Flika and Wellam; two *T. aestivum* L. (wheat) cvs. Durabel and Cortex; and two *Z. mays* L. (corn) cvs. Duro and Oropesa.

pH. Aqueous solutions were buffered with 10 mM 2-[*N*-morpholino]ethanosulfonic acid (MES) and adjusted to different pH values with 1 N NaOH: pH was measured with a Crison micropH 2001.

Bioassays. Bioassays used Petri dishes in two sizes [90 and 140 mm diameter (diam)] with one sheet of Whatman No. 1 filter paper as support. Germination and growth were conducted in aqueous solutions at controlled pH. After adding seeds and aqueous solutions Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were incubated in the dark at 25 °C in a Memmert ICE 700 controlled environment growth chamber. Bioassays took 5 days for all species except for cress (3 days) and carrot (7 days). After growth, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process. This helped in the handling of plants, especially in accurately measuring root and shoot lengths.

Bioassay Design. Experimental design included two blocks of 100 seeds each. For dicotyledons and onion (seed diam <8 mm), 25 seeds were used. For big seeds (diam >8 mm) and the remaining monocotyledons (Gramineae), 10 and 50 seeds were used in 90- and 140-mm diam Petri dishes, respectively. Seeds were considered germinated only when a root length >2 mm was observed (see Leather and Einhellig, 1986). To make faster measurements, root and shoot length data were grouped in frequency intervals of 4 mm for dicotyledons and onion, and 8 mm for monocots (only the longest roots were measured). Mean values of germination level, and root and shoot lengths were calculated for all seeds from two blocks after statistical evaluation.

Statistical Analysis. Comparison between the two blocks assayed by applying Welch's test, a variant of Student's *t* test (Zar, 1984; Martin and Luna, 1990), calculating mean values for every parameter (germination average, and root and shoot elongation) and their population variance within a Petri dish.

Coefficient of variation (CV) was used to compare dispersion from different populations. This parameter is expressed as a percentage and represents the ratio between the standard deviation and the sample mean.

Influence of pH and Volume on Germination. Studies on the influence of pH used aqueous MES solutions buffered to 5, 6, and 7 and volumes of 10 mL. In assays evaluating volume influence, the pH was held at 6, but there were three

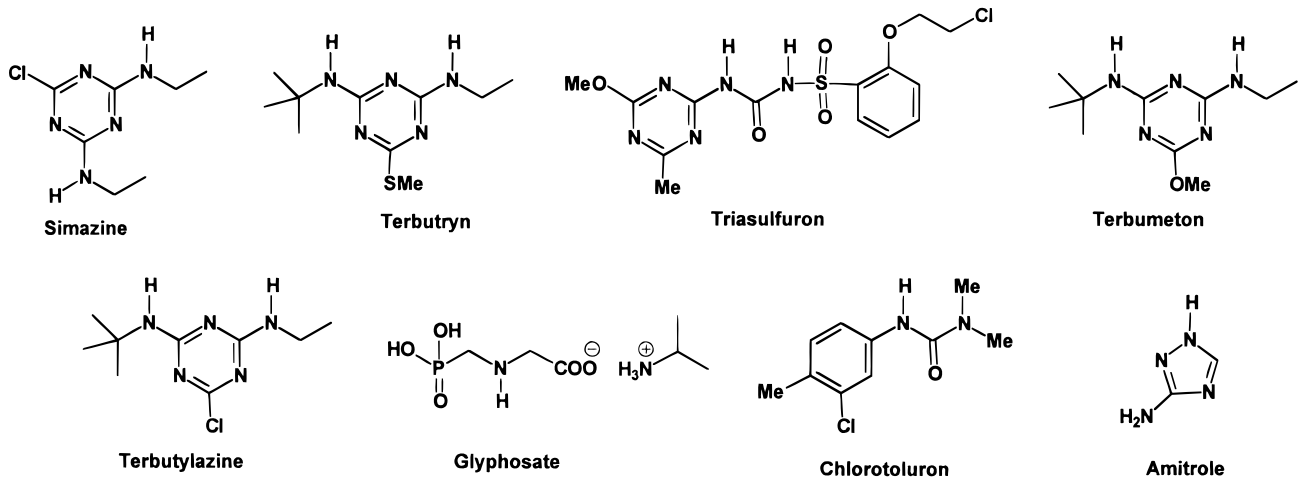

Figure 1. Structures of commercial herbicides used in standard phytotoxic bioassay.

different ratios between volume and seed number. Hence, 5, 10, or 15 mL were used for either 25 dicotyledons and onion seeds or 10 seeds for monocots. Bioassays using 140-mm-diameter Petri dishes employed 50 seeds with volumes of 25, 50, and 75 mL. In this way, ratios of 0.5, 1, and 1.5 mL per seed were obtained. For controls, 90-mm-diameter Petri dishes were used with the same ratios, using 5, 10, and 15 mL for 10 seeds per dish.

Standard Target Species (STS) Selection. The most suitable species were selected based on the lower CV in root and shoot length parameters. Selection was made from specific varieties of carrot, tomato, onion, barley, wheat, and corn and selected varieties of lettuce (Grandes Lagos, Nigra, and Roman) and cress (Comun and Royalfleur). Similarly, two blocks of 100 seeds were bioassayed at specific pH and ratio volumes, and the number of seeds chosen from previous assays (dicotyledons and onion, 25 seeds/5 mL using 4 dishes; monocots, 10 seeds/5 mL using 10 dishes at pH 6).

Bioassay of Commercial Herbicides and Statistical Treatment. Seeds were obtained from the Spanish company, Fitó S.A. The bioassay consisted of germinating 25 seeds for 5 days (3 days for germination and 2 days for root and shoot growth) for *L. esculentum* L. (tomato), *L. sativa* L. cvs. Nigra and Roman (lettuces), and *A. cepa* L. (onion); 25 seeds for 3 days for *L. sativum* L. (cress); 25 seeds for 7 days (4 for germination) for *D. carota* L. (carrot); 10 seeds for 5 days for *H. vulgare* L. (barley), *T. aestivum* L. (wheat), and *Z. mays* L. (corn) in the dark at 25 °C into a growth chamber, in 9-cm plastic Petri dishes containing a 9-cm sheet of Whatman No. 1 filter paper and 5 mL of a test or control solution, except for corn (15 mL).

Selected commercial herbicides with different metabolic targets, provided by Novartis (Figure 1), were bioassayed alone or in mixtures: simazine (Gesatop 90 WP), terbutryn + triasulfuron (Logran Extra), terbutryn + triasulfuron + chlorotoluron (Tricuran 64), terbutryn + chlorotoluron (Dicuran Extra), terbutryn (Igran Liquid), terbumeton + terbuthylazine (Caragard), terbuthylazine + glyphosate (Folar), simazine + amitrole (Saminol 1089) and terbumeton + terbuthylazine + amitrole (Vinagard). Commercial formulations were used alone or in mixtures, pre- or postemergence (Table 1) using dicotyledon or monocotyledon annual, or perennial weed seeds. All of them were applied in an equivalent concentration of active compounds (a.c.) of 10^{-2} M. Test solutions (10^{-2} M) were prepared as initial stock solutions. Test solutions (10^{-3}–10^{-9} M) were obtained by diluting the stock solution. Parallel controls consisted of deionized water. Three replicates (total 100 seeds), except for barley, wheat, and corn (19 replicates, total 100 seeds), for each treatment, and parallel controls were prepared. All pH values were adjusted to 6.0 before the bioassay.

Significant differences in germination and root and shoot lengths of treated and controlled tests were determined by

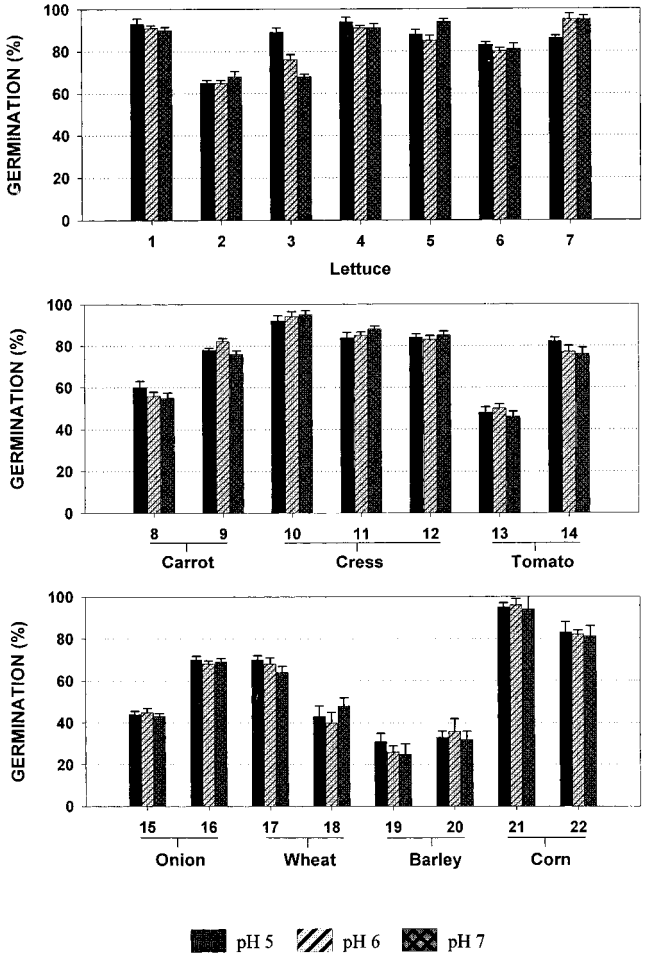

Figure 2. Germination levels of different varieties of *L. sativa* L. (1–7), *D. carota* L. (8, 9), *L. sativum* L. (10–12), *L. esculentum* L. (13, 14), *A. cepa* L. (15, 16), *T. aestivum* L. (17, 18), *H. vulgare* L. (19, 20), and *Z. mays* L. (21, 22) at three pH values.

Welch's test (Zar, 1984), being significant with $p \leq 0.01$. Results are presented in figures, where units are expressed as percentage of control. Thus, zero values indicate treatment equal to control, positive values indicate stimulation, and negative values demonstrate inhibition.

RESULTS AND DISCUSSION

I. Standard Target Species Selection. pH and volume requirements (which strongly influence growth)

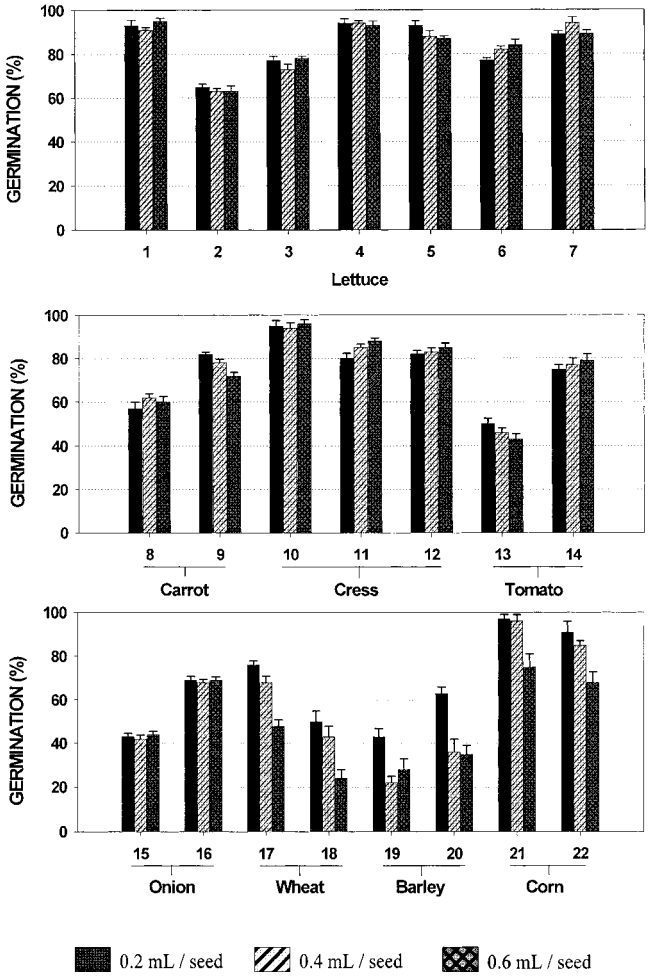

Figure 3. Germination levels of different varieties of *L. sativa* L. (1−7), *D. carota* L. (8, 9), *L. sativum* L. (10−12), *L. esculentum* L. (13, 14), *A. cepa* L. (15, 16), *T. aestivum* L. (17, 18), *H. vulgare* L. (19, 20), and *Z. mays* L. (21, 22) at three ratios of volume and number of seeds.

for both dicotyledons and monocotyledons were evaluated in the search for varieties with germination levels between 60 and 80% which were sensitive to possible stimulatory effects.

Dicotyledons. Among the different lettuce varieties assayed we found two groups with dissimilar germination levels which were independent of pH (Figure 2). Thus, with Batavia (**1**), Maravilla (**4**), Romana Rubia (**5**), and Trocadero (**7**) germination was >85%, whereas with Nigra (**2**), Grandes Lagos (**3**), and Roman (**6**) it was near 70%. The same was observed with two varieties of carrot and tomato, Berlikumer (**8**) and Rio Grande (**13**), which were near 60% each, and with Coral (**9**) and Tres Cantos (**14**), which were 80% at any pH tested. Homogeneous germination levels for all varieties were found in cress, Belplanto (**10**), Comun (**11**), and Royalfleur (**12**), which were consistently >80% at any pH.

Because almost all species had similar germination levels at the three pH values assayed, pH was not a determining factor in promoting germination. Although pH 6 is generally the optimal pH for plant growth (Castellano, 1997), pH could be modified according to stability of tested allelochemicals, without creating significant differences on germination.

For assaying wild lettuce seeds, 0.4 mL/seed has been recommended (Leather and Einhellig, 1988). To establish growth conditions for commercial seeds, two additional ratios were assayed for dicotyledons at pH 6. Varieties of lettuce (Figure 3) showed the same germination levels at the three ratios assayed and were similar to those obtained in pH assays. Similarly, the differences observed in germination levels of varieties of carrot, cress, and tomato could be explained by an inherent seed variability, as opposed to a volume effect. Consequently, it was concluded that the optimum conditions for commercial seeds are pH 6, and 5 mL per 25 seeds per Petri dish (0.2 mL/seed).

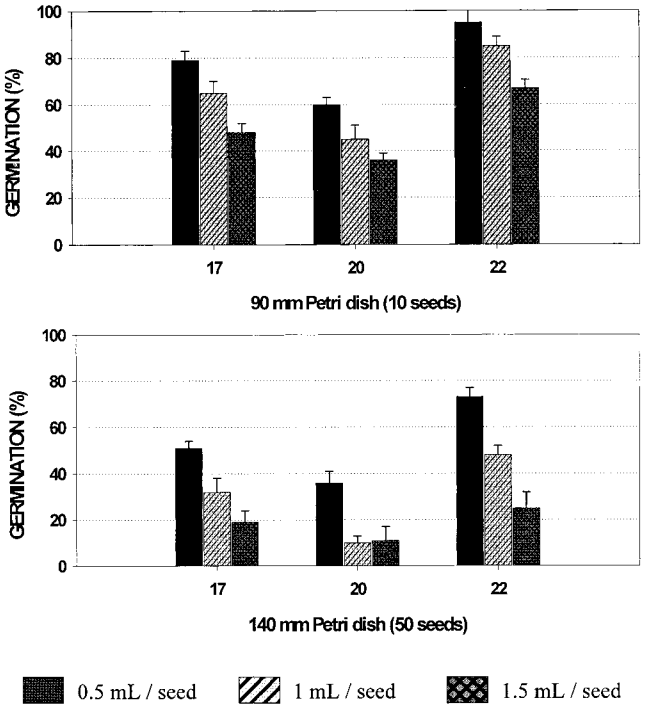

Figure 4. Germination levels of varieties of *T. aestivum* L. (17), *H. vulgare* L. (20), and *Z. mays* L. (22) with three ratios of volume and number of seeds in Petri dishes with different diameter.

Monocotyledons. As with dicotyledons, there were no differences among germination levels of monocotyledons at the three pH values assayed (Figure 2). There were variations within varieties, but not in the varying conditions. Onion and wheat varieties showed different germination. Morada (**15**) and Durabel (**18**) had 50% germination and Valenciana (**16**) and Cortex (**17**) had 70%. Barley varieties, such as Flika (**19**) and Wellam (**20**), showed an extremely low germination level, whereas the corn varieties, Duro (**21**) and Oropesa (**22**), were greater than 80%.

To determine the effects of volume on seeds and to establish the possibility of assaying onion under the same conditions as dicotyledons, three ratios of volume and seed number were assayed at pH 6 (Figure 3). Onion varieties did not show any variation at different ratios, so we concluded that optimum conditions for onion germination were pH 6, with 5 mL/25 seeds in each Petri dish (0.2 mL/seed). However, germination percentages of monocots increased when volumes decreased. So, wheat varieties decreased germination by 40% for **18** and 30% for **17** when a ratio of 1 mL/seed was used instead of 0.5 mL/seed. Similarly, barley varieties decreased their germination by 75% for **20**, and 95% for **19**.

This observation points to two advantages of using Gramineae in phytotoxic bioassays: first, it is possible to increase germination levels by using only 5 mL/10

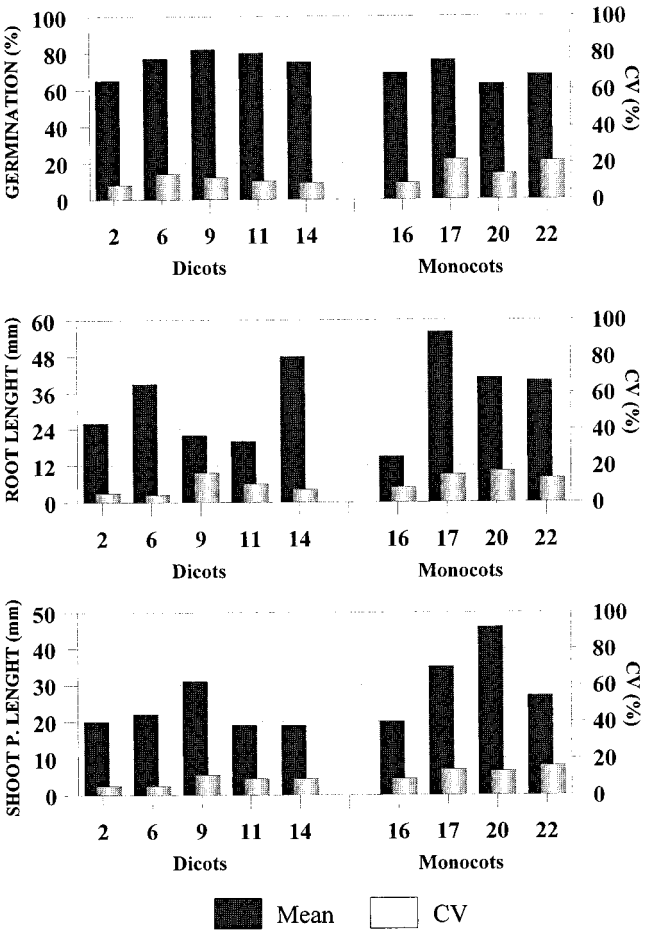

Figure 5. Mean and CV values for germination and root and shoot length of different dicotyledons (2, 6, 9, 11, and 14) and monocotyledons (16, 17, 20, and 22).

Table 1. Weed Characteristics and Treatments with Commercial Herbicide Formulations[a]

	treatment		weeds			
herbicide	pre-	post-	annual	perennial	dicot.	monocot.
Gesatop 90 WP	+		+		+	+
Logran Extra	+	+	+			+
Tricuran 64	+	+	+			+
Dicuran Extra	+	+	+			+
Igran Liquid	+	+	+		+	+
Caragard	+	+	+	+	+	+
Folar		+	+		—[b]	—[b]
Saminol 1089	+	+	+	—[b]	—[b]	
Vinagard		+	+	+	—[b]	—[b]

[a] Commercialized by Novartis. [b] Without specification.

seeds per Petri dish, and second, the larger seeds do not require an increased amount of compound, which is often limited.

The higher germination level with lower liquid volume, can be explained by the amount of oxygen available for each seed (Leather and Einhellig, 1986.). As the ratio between the volume and seed number becomes greater, the volume of air in the chamber becomes less in each sealed Petri dish and the germination level is lower. To test this hypothesis, we used larger Petri dishes (140 mm diameter).

Petri dishes with 140 mm diam have three times greater volume than the small (90 mm) Petri dishes and allow the numbers of seeds to be increased five times, while maintaining the same ratios of 0.5, 1, and 1.5 mL/seed. Under these conditions, larger dishes (with 25, 50, or 75 mL for 50 seeds) had a lower internal volume than smaller dishes (5, 10, or 15 mL for 10 seeds). This implies a smaller amount of oxygen per seed and, subsequently, a reduction in germination (Figure 4). This was observed in both sizes of Petri dishes. Hence, seeds appear to be competing for oxygen and not water.

The most suitable germination conditions for Gramineae are pH 6 and a ratio of 0.5 mL/seed in small Petri dishes, except for corn which has a high germination level (95%). In this case, the germination level is reduced by using 1.5 mL/seed (65%). Other conditions such as 0.5 mL/seed (75%) in larger Petri dishes would not be acceptable because of the lack of growth of corn (Figure 4). We emphasize that the use of commercial seeds under these conditions allows for a reduced amount of test compound, thus making it is possible to increase the number and range of target species.

Proposed Varieties. After establishing the most suitable conditions for achieving germination levels between 60 and 80%, the second step was to propose those dicotyledonous and monocotyledonous varieties that present the highest and most homogeneous growth. Parameters of root and shoot length were evaluated. The coefficient of variation (CV) for every variable was used, in addition to mean values. The coefficient of variation is used in this article as the standard deviation/mean value ratios. Among varieties of lettuce (Table 2), **6** presented the highest mean value with the lowest CV. However, **2** has the lowest germination level, so it should not be discarded as a possible STS because it is useful for observing stimulatory effects. Thus, both varieties of lettuce were chosen, that is, **2** for specific germination tests and **6** for evaluating effects on plants growth. Both evaluations together allow for different responses.

Following the same criteria of highest mean values with lowest CV for most of the considered variables, it is possible to establish as more suitable STS: variety **9** for carrot, **11** for cress, and **14** for tomato (Table 2). Those varieties of monocotyledons assayed under optimal conditions (Table 2) allowed us to establish as more suitable experimental species: variety **16** for onion, **17** for wheat, **20** for barley, and **22** for corn.

Conclusions. From comparison of three measured variables for all different proposed STS (Figure 5), we conclude:

1. All selected varieties show germination levels in the desirable range of 60−80%, which allows evaluation of both stimulatory and inhibitory effects on germination. Variability (highest CV) is larger for the Gramineae (size seeds >8 mm), because they are more sensitive to the conditions described, not only with respect to oxygen competition, but also because of their own autotoxic character (Waller et al., 1987), which may affect germination.

2. Root length mean values are high enough for evaluating activity clearly, all of them with a low standard deviation for small seeds (except carrot).

3. Shoot length mean values show that CV is similar in all cases, which makes this variable more suitable for comparing the effects of a given compound on different species.

4. The most suitable dicotyledon is *L. sativa* L. cv. Roman, which shows the highest homogeneous growth. This fact improves assay reproducibility and increases the statistical level of acceptability. Its germination level is near 80%, however, so it is necessary also to use *L.*

Table 2. Mean and CV Values for Root and Shoot Length of Different Varieties of Lettuce (2, 3, and 6), Carrot (8, 9), Cress (11, 12) and Tomato (13, 14), Onion (15, 16), Wheat (17, 18), Barley (19, 20) and Corn (21, 22), in Water at pH 6 and 0.2 mL/seed

	lettuce			carrot		cress		tomato		onion		wheat		barley		corn	
	2	**3**	**6**	**8**	**9**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
root length																	
$\bar{x}$ (mm)	26	12	39	12	22	20	19	43	48	14	15	56	46	45	41	35	40
CV (%)	5	15	4	13	16	10	20	11	7	10	8	15	20	17	17	18	13
shoot length																	
$\bar{x}$ (mm)	20	15	22	13	31	19	20	21	31	18	20	35	22	43	46	20	27
CV (%)	5	13	5	8	11	9	19	10	5	9	9	14	21	7	13	14	16

sativa L. cv. Nigra to evaluate stimulatory effects. Combined use of both varieties allows a reliable measure of the three variables.

5. The remaining dicotyledons grow with suitable root and shoot length mean values after only 3 (cress), 5 (tomato), and 7 days (carrot).

6. The Gramineae are suitable for bioassay, with barley exhibiting the lowest variability. The required liquid volume ratio for corn often limits use of the species because of the generally low amount of allelochemical available.

Considering all these factors and keeping in mind that a difficult step in phytotoxic bioassays may be the availability of compounds, it is essential to establish a STS hierarchy. The proposition is based on particular biological characteristics, phytotoxic responses, and statistical analyses and it is recommended as follows: lettuce (**2** and **6**), onion (**16**), cress (**11**), tomato (**14**), barley (**20**), carrot (**9**), wheat (**17**), and corn (**22**).

II. Sensitivity of Standard Target Species. The main objective of this part is to establish that commercial seeds of STS are suitable for phytotoxic assays. Seed treatments that prevent fungal or bacterial attack and those that stimulate germination may interfere with a bioassay. To establish sensitivity of STS, the selected nine target species have been assayed with nine formulated herbicides.

Bioassay results for nine target species with six selected formulated herbicides (in a.c. 10^{-2} M concentration) are presented in Figure 6 and represent six different possible treatments (Table 1). Those formulations not presented showed similar activity profiles. We want to point out that these bioassays evaluated mixtures (of different active compounds in several weight/volume ratios) of commercial herbicides. These will be used as references to select allelopathic agents as new potential herbicide templates. The use of mixtures is in fact not important, because our purpose is obtaining firm data of sensitivity of commercial seeds.

All proposed STS have a strong sensitivity to phytotoxic agents and include both stimulatory and inhibitory effects. However, as expected, different responses on measured parameters were obtained depending on the respective formulations.

Effects on germination of dicotyledonous species are generally lower than effects expressed in monocotyledons; but, in general, all species exhibit inhibition. Terbumeton + terbuthylazine (C, pre- and postemergence, Figures 6−8) exhibited the greatest inhibition. It is also important to note the low influence of simazine (G, preemergence) on germination, except for lettuce, maybe because this compound only affects the photosynthetic pathway and all bioassays were performed in dark conditions.

With respect to growth, and except for simazine, all herbicide formulations induced powerful inhibitory effects over all the species. However, in these cases there are no significant differences between dicotyledons and monocotyledons. Moreover, no differences are shown among monocotyledonous species of different seed size (onion vs wheat, barley, or corn). On the other hand, it is not possible to establish significant differences among herbicides in postemergence and mixtures, although it is true that those mixtures have larger inhibitory effects on root and shoot length. Finally, simazine (G, preemergence) did not show a consistent activity profile for all species, being stimulatory for some, but inhibitory, or without effects, for others.

The data allow us to conclude that the proposed species are suitable STS for estimating the activity of inhibitory or stimulatory compounds (Figures 7 and 8). Selective response, especially in germination, makes it more evident that a wide range of species is necessary.

Activity Profiles of Pre- or Postemergence Herbicides in a Standard Phytotoxic Bioassay. Apart from testing the sensitivity of proposed STS using commercial herbicide formulations, this work also attempted to determine the activity profiles of these herbicides. The goal was to obtain reference data to compare responses of potential new herbicide templates. In this way, we have developed in our laboratory a research project named "Natural Product Models as Allelochemicals" in which we evaluate plant species for compounds with phytotoxic activity. Several bioassays are being undertaken with these agents in comparison with commercial herbicides to evaluate the potential of allelopathic agents as new herbicides.

Using these formulations, with field application rates (10^{-2} M) and the range of activity shown by the most common allelopathic agents (10^{-4}−10^{-9} M), Figures 7 and 8 show results of selected dicotyledonous and monocotyledonous STS, respectively. To facilitate presentation, results from 10^{-6} and 10^{-8} M have been omitted, because the most important point is to offer an activity profile rather than an exhaustive collection of particular results.

In general, for dicotyledonous and monocotyledonous species used in this standard phytotoxic bioassay, herbicides show strong inhibitory activities only at concentrations between 10^{-2} and 10^{-3} M; at lower concentrations this effect disappears or becomes stimulatory (10^{-5}−10^{-9} M).

This lack of activity at lower concentrations did not hold for the preemergence herbicide simazine (G), which shows greater inhibitory activity on germination at intermediate concentrations of 10^{-4}−10^{-5} M, especially for lettuce (both varieties), than at 10^{-2} M. These herbicides also show slight growth stimulation. With allelochemicals, the greatest activity may not always occur at the highest concentrations. Conversely, it is not possible to establish response differences between the rest of the assayed herbicides (postemergence and

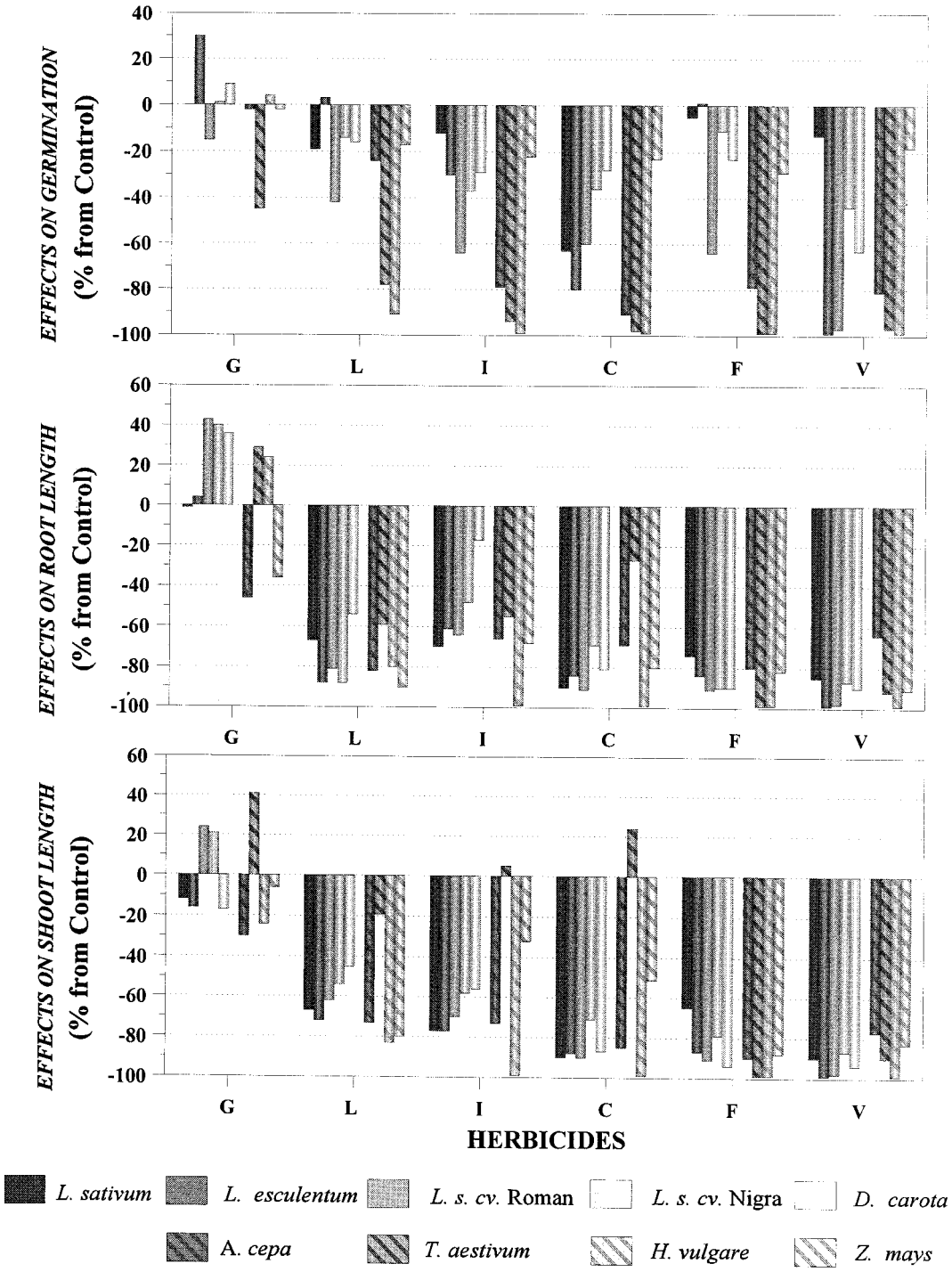

Figure 6. Effects of six selected commercial herbicide formulations (in 10^{-2} M): simazine (G, preemergence), terbutryn + triasulfuron (L, mix.), terbutryn (I, mix.), terbumeton + terbuthylazine (C, mix.), terbuthylazine + glyphosate (F, postemergence), and terbumeton + terbuthylazine + amitrole (V, postemergence), on germination and growth of dicotyledonous target species *L. sativum* cv. Comun, *L. esculentum* cv. Tres Cantos, *L. sativa* cvs. *Nigra and Roman* and *D. carota* cv. Coral, and monocotyledons *A. cepa* cv. Valenciana, *T. aestivum* cv. Cortex, *H. vulgare* cv. Wellam, and *Z. Mays* cv. Oropesa.

mixtures), because different formulations produce similar effects in all STS.

With respect to the different sensitivities and selectivities shown by species to the same formulations, it is interesting to notice the following:

1. A significant difference exists between effects on germination of two varieties of the same species (*L. sativa* L.), for example, var. Roman is much more sensitive. This fact highly supports the need for using a wide variety of species (when quantities of compounds allow it) so that particular effects, will not be missed.

2. In other cases, the combined use of two different species allows one to confirm those effects that are not easily explained, for example, effects of terbutryn (I, mix.) on root length in dicotyledonous species. Specifically, although all the formulations induced powerful inhibitory effects, this herbicide had no inhibitory effect at all and, additionally, it showed great stimulation at low concentrations.

3. Selectivity with allelochemicals that have potential use as agrochemicals is an important point, because it would allow use in agriculture with the desirable effect

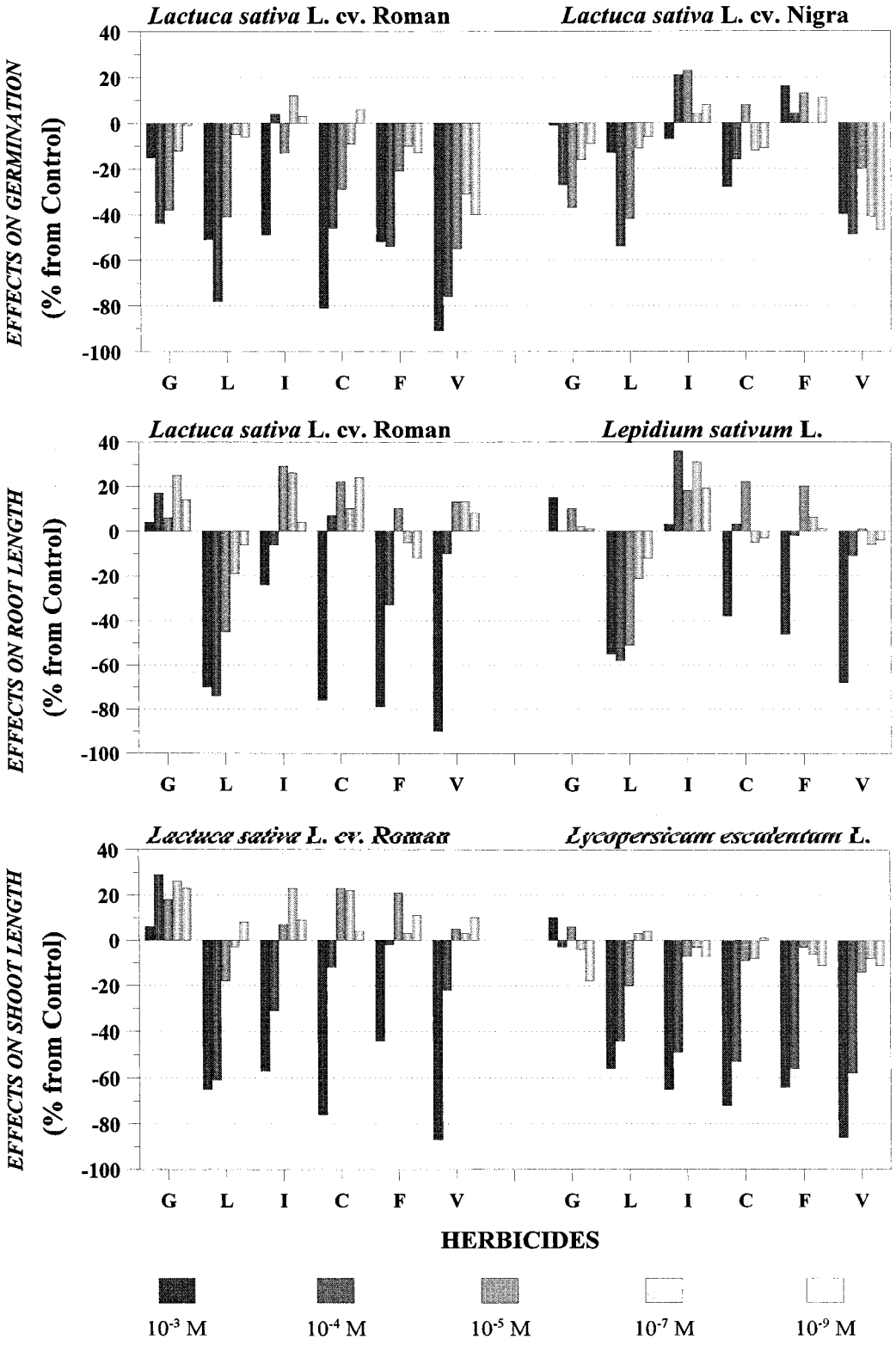

Figure 7. Effects of six selected commercial herbicide formulations: simazine (G, preemergence), terbutryn + triasulfuron (L, mix.), terbutryn (I, mix.), terbumeton + terbuthylazine (C, mix.), terbuthylazine + glyphosate (F, postemergence) and terbumeton + terbuthylazine + amitrole (V, postemergence) on germination and growth of selected dicotyledonous target species *L. sativa* cvs. Roman and Nigra, *L. sativum* cv. Comun and *L. esculentum* cv. Tres Cantos.

of suppressing weeds and stimulating crops, simultaneously. Using weeds and crop models in the same bioassay as target species would allow testing the same formulation at the same concentration to exhibit inhibition for monocotyledonous weeds models and stimulation for monocotyledonous crops. Results from Figure 8 suggest that a particular formulation can be a herbicide when applied to weeds (high concentrations) and after partial degradation of them in the soil (low concentration) they could cause stimulation to economically important crops such as barley, wheat, or corn (data not presented in this paper).

Selection of an Internal Standard. We propose the use of an internal standard of known phytotoxic activity which validates responses of the test chemicals.

It is important to note that most allelochemicals are

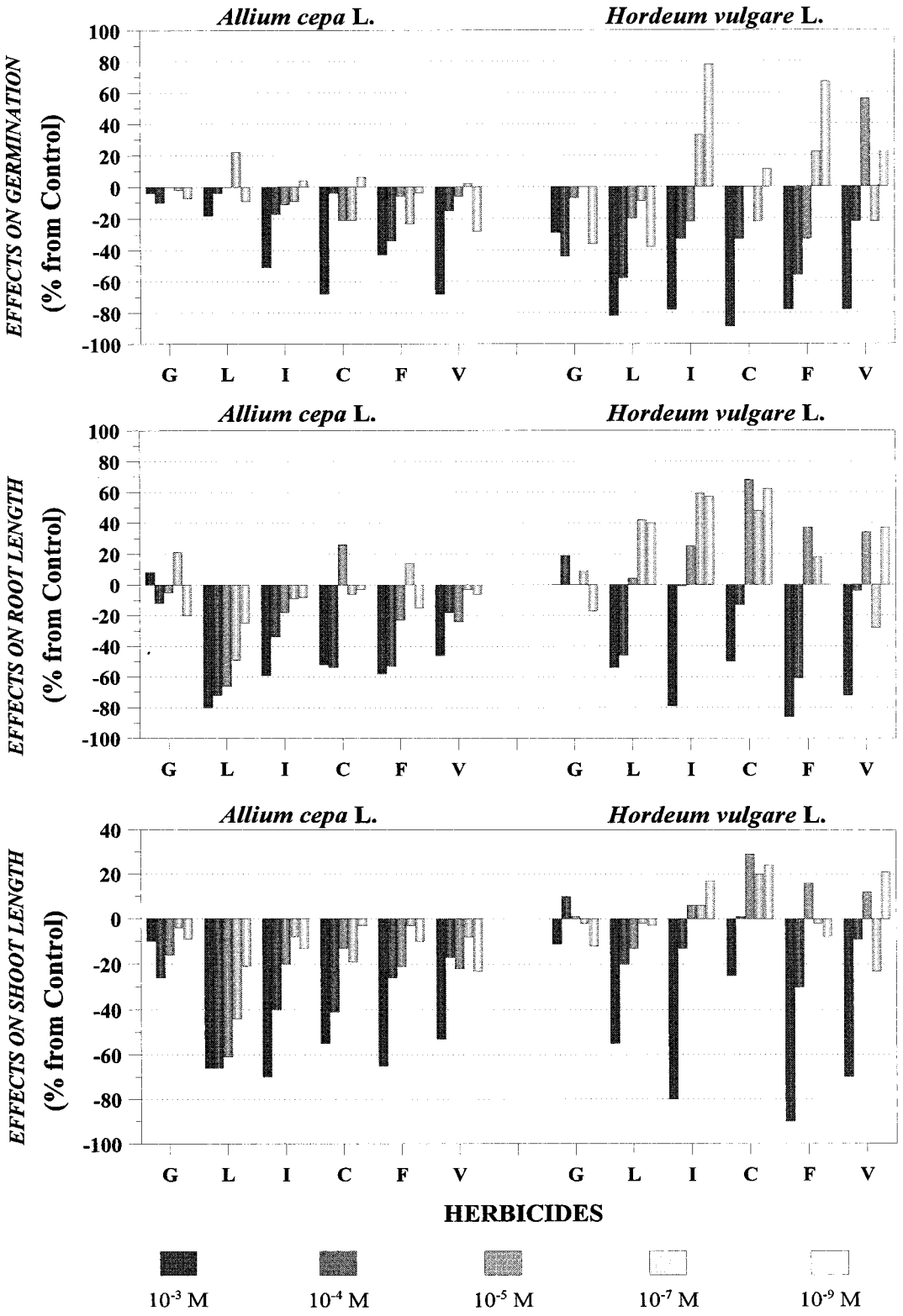

Figure 8. Effects of six selected commercial herbicide formulations: simazine (G, preemergence), terbutryn + triasulfuron (L, mix.), terbutryn (I, mix.), terbumeton + terbuthylazine (C, mix.), terbuthylazine + glyphosate (F, postemergence) and terbumeton + terbuthylazine + amitrole (V, postemergence) on germination and growth of monocotyledonous target species *A. cepa* cv. Valenciana and *H. vulgare* cv. Wellam.

often isolated in very low quantities which only allow for their bioassay at concentrations near 10^{-4} M and lower. This forces us to select the formulation that maintains a certain activity at a concentration under 10^{-3} M. Thus, we ensure that all compounds with activity profiles equal or higher than those offered for the internal standard are excellent candidates to be considered as potential new herbicides templates.

Based on the most consistent profile of activity of the nine test formulations, which represent eight herbicides at different rates (Figures 7 and 8), the combination of terbutryn + triasulfuron (L, mix.) was selected. This combination maintains an inhibitory effect on growth of *L. sativa* L. and *L. sativum* L. at 10^{-5} M and a significant inhibitory effect, at low concentrations, on growth of *A. cepa* L.

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