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Effect of Mixtures of Pesticides Used in the Direct Seeding Technique on Nontarget Plant Seeds

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Riparian communities are essential for maintaining the quality of the aquatic habitat in agro ecosystems. In particular, shoreline vegetation plays critical roles in soil stabilization, and therefore in reduction of erosion, and as a habitat to insects that contribute to the diet of fish (Blackburn and Boutin 2003). Plants of these communities next to agroecosystems are impacted by related activities, particularly the spray of herbicides. Soil seed banks are also important reservoirs of diversity. As a result, the assessment of the impacts of pesticides on seed viability is a relevant point to be considered in the environmental management of agroecosystems. There is a particular interest in studying riparian communities within the Pampa Ondulada, a large region of Argentina characterized by very rich soils used for grain production. The main crop of these agroecosystems in the last decade has been the transgenic RR variety of soybean. It is managed by a direct seeding technique, using glyphosate formulations as a weed killer and several insecticides such as cypermethrin, chlorpyrifos and endosulfan to control different pests (Pengue 2000; Jergentz et al. 2004; Marino and Ronco 2005). Depending on the stage of crop management, it is a common practice to spray either herbicide or insecticide formulations by themselves or in different mixtures.

Glyphosate is a non-selective herbicide known to inhibit aromatic amino acid synthesis, interfering in protein production and with other molecules such as auxins and polyphenols which require these amino acids as precursors (Salisbury and Ross 1994). Blackburn and Boutin (2003) have reviewed the literature on studies of the effects on the germination and growth of first generation seeds produced by plants sprayed with the herbicide glyphosate. The pyrethroid and the organophosphorate insecticides cypermethrin and chlorpyrifos are well known neurotoxicants with different modes of action for animal species (Ecobichon 1996). Because differing mixtures of pesticides are usually applied in agricultural practices, it is important to evaluate their impacts on the biota. They not only contain a mixture of the active ingredients, but also chemicals from coadjuvants of each formulation. The differential effects of active ingredients and formulations from herbicides indicate higher toxicity of formulations with respect to the first ones on non-target plants and animal species (Tsui and Chu 2003; *Sobrero, Rimoldi, Ronco 2005, unpublished*).

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Considering that the first days of seedling growth are often the most sensitive stage of plant development, adverse effects due to exposure to chemicals are most likely to take place during this phase (ASTM 1994). The following hypotheses were made: pesticides affect seed germination and root elongation to a different extent depending on plant species, and interactions in mixtures of pesticides determine variations in the toxicity of individual compounds on their formulations. The objective of the present study was to assess the effects on germination and root elongation of seeds exposed to the Roundup Max formulation of the glyphosate herbicide and the formulations Shooter and Sherpa of the insecticides chlorpyrifos and cypermethrin, separately and all of their possible mixtures in imbibition solutions. Effects of environmental samples from crop fields are also analyzed.

MATERIALS AND METHODS

The relative toxicity of Roundup®Max to different plant species was first assessed by means of a battery of tests using seeds from Compositae, Cruciferae, Amarilidaceae, Leguminoseae and Gramineae (OECD 1984; ASTM 1994; ISO 1995). A detailed assessment of all compounds was carried out using a standardized toxicity test assessing root elongation and germination inhibition of *Lactuca sativa* (USEPA 1989; Sobrero and Ronco 2004).

The species used in the battery of toxicity tests were *Lactuca sativa* L., *Brassica napus* L., *Allium cepa* L., *Medicago sativa* L., and *Lolium perenne* L. for the Compositae, Cruciferae, Amarilidaceae, Leguminoseae, and Gramineae families, respectively. These species have been found to have high sensitivity to 10 herbicides according to Boutin and Rogers (2000). Seeds were placed in 9.2 cm Petri dishes with two filter papers (Whatman 1) and 3.5 ml of the test solution, using 20 seeds per test chamber. Toxicity tests were performed in triplicate using a minimum of five concentrations. Range of concentration (mg/L) was for Roundup with *L. sativa*: 2.5–20 (root elongation) and 500–2500 (germination), *L. perenne* and *M. sativa*: 12.5–200, A. *cepa*: 100–500, and *B. napus*: 1000-1500. Also, for *L.* sativa, tested dilution of mixtures was 0.01–20 % v/v. Single product ranges (mg/L for each active principle) were for cypermethrin: 1-1000, chlorpyrifos: 250–500, and glyphosate: 1-20. Experimental conditions were as follows: 22±2 °C, in darkness (for *L. sativa* and *B. napus*) or 16 h day light (for all other species). Seeds were exposed for 120 h. Assessment end points were seed germination and seedling root elongation. Acceptability criterion of the tests was germination over 90% for negative controls. A positive control is routinely performed with the *L. sativa* test using Cr(VI) (Sobrero and Ronco 2004).

Pesticide test mixtures were prepared in distilled water with formulations at the concentrations and in the proportions used in the direct seeding technique. Table 1 shows the details of proportion and the types of pesticide formulations. Solutions used for toxicity testing were prepared by scaling down the amounts in order to obtain 0.5 L stocks. The insecticide formulations Shooter (480 mg/L of chlorpyrifos) and Sherpa (250 mg/L of cypermethrin mix of isomers), and the herbicide active ingredient and Roundup® Max (74.4% glyphosate) were also tested individually. Dilutions of pesticide sample stocks (mixtures or individual formulations) were prepared in distilled water and immediately used in preliminary or definitive toxicity tests. Concentration of pesticides in test mixtures were analyzed at day 0, 3 and 6 on the free liquid and paper bound solution (only insecticides) for two tested dilutions. Insecticides were analyzed by high-performance liquid chromatography, reverse phase separation, with acetonitrile: water in a 25 cm × 4.6 ID C18 column, at 1 ml/min, using a UV detector (230 nm) (Marino and Ronco 2005). Free liquid solution sample was directly analyzed and paperbound previous sample extraction using dichloromethane as solvent. Extracted samples were roto-evaporated and the solvent was changed to acetonitrile. Glyphosate concentration was tested by liquid chromatography according AOAC standardized methods (1990). J.T Baker solvents used were for pesticide analysis. Standards of glyphosate, cypermethrin and chlorpyrifos used for chemical analysis (95, 97 and 94 % w/w, respectively) were from SENASA (Argentinean National Service for Sanitary and Quality of Agriculture and Food).

Table 1. Pesticide formulation mixtures used in toxicity tests with *Lactuca sativa* seeds according to proportions used in the field at a rate of 100L/ha. Concentration of active ingredient in each formulation is also given.

	Glyphosate (Roundup®Max) 7440 mg a.i./L	Cypermethrin (Sherpa) 250 mg a.i./L	Chlorpyrifos (Shooter) 3840 mg a.i./L
Mixture 1	X	X	X
Mixture 2	X	X	
Mixture 3		X	X
Mixture 4	X		X

Environmental samples consisted of surface waters and sediments obtained simultaneously from a stream (Arrecifes river first order tributary, Province of Buenos Aires, Argentina) next to crop fields. Further details of the sampling place and dates of sampling, related agricultural activities (application of glyphosate, cypermetrin or chlorpyrifos), and rain events can be seen in Peruzzo et al. (2003) and Marino and Ronco (2005). Water samples were tested on the direct sample and sample dilutions were prepared with distilled water. Sediment samples were extracted with distilled water at a sediment:water ratio of 1:4. The aqueous extract was filtered through qualitative filter paper Negative controls were carried out with distilled water.

Results of tests, given as IC50 (referring to initial pesticide concentration), were estimated by the TOXSTAT program version 3.5 or graphical methods (USEPA 1993). Regression analysis of logarithm transformation of root elongation data for different plant seeds at several concentrations of Roundup, followed by slope comparisons was performed to assess differences in sensitivity among species (Zar 1999). A similar comparison was performed to evaluate the sensitivity of lettuce seeds to different treatments (exposure to formulations and mixtures of formulations).

RESULTS AND DISCUSSION

Results obtained with herbicides showed that sensitivity to Roundup is very variable among the families. The IC50 values, expressed as mg/L of a.i. for root elongation inhibition as an assessment end point, are shown in Table 2. Values show differences of two orders of magnitude between the most and least sensitive species. With respect to this end point, the sensitivity rank is as follows: *L. sativa*> *L. perenne* > *M. sativa* > *A. cepa* > *B. napus*. The analysis of the slopes from dose response curves for each species indicates no significant differences among *L. perenne*, *M. sativa*, and *B. napus*. Seeds from *L. sativa* and *A. cepa* differ from one another and with the rest (Table 3). The lettuce seeds are the most sensitive to the herbicide and also exhibit greater effects to small variation in the concentrations (higher slope).

No effect on seed germination were observed with any of the testing species at the highest concentrations tested (2500, 1400, 400, 100, and 100 mg Roundup/L for *L. sativa*, *B. napus*, *A. cepa*, *M. sativa*, and *L. perenne*, respectively).

Table 2. Results of toxicity tests with Roundup® Max with seeds from five species. IC50 values (as mg/L of a.i.) correspond to root elongation inhibition.

	IC50 Analysis		Regression Analysis				
Species	IC50	CI	r^2	b	A	SE	n
L. sativa	9.89	8.73-12.64	0.81	-1.940*	3.14	0.21	27
L. perenne	15.31	12.79-19.38	0.55	-0.008*	2.82	0.30	10
M. sativa	56.21	45.13-76.69	0.75	-0.008*	3.07	0.16	18
A. cepa	131.80	-	0.51	-0.166*	2.31	0.23	18
B. napus	1164.31	1124.26-1195.78	0.77	-0.006*	2.93	0.19	10

CI= confidence interval; r^2= determination coefficient; b=slope (regression coefficient); A= elevation; SE= standard error; n= number of pair of *x* and *y* values. (*) Significantly different from zero slope. (-) Graphical interpolation CI not estimated.

The detailed study performed with *L. sativa* shows that the sensitivity to the formulation Roundup® Max (IC50= 7.3 [6.5-9.4] expressed as mg/L of a.i.), in comparison with the glyphosate active principle (IC50= 9.8 [8.2-11.8] mg/L), is very close. The formulation was shown to be slightly more toxic. Significant differences were detected according to the corresponding confidence intervals from the data. It is interesting to note that the effects on root elongation by exposure to the herbicide remain constant between 1200 mg/L and 2500 mg/L (maximum concentration tested) with an 88% mean inhibition.

The study of the effects of mix and unmixed pesticides (see Table 1) on *L. sativa* inhibition of root elongation indicates higher toxicity of mixtures (according to the IC50 value expressed as mg a.i./L) containing the herbicide with cypermethrin or both insecticides when compared to the unmixed herbicide (Table 4). Results indicate an increment up to seven times when mixed with the insecticide cypermethrin (a quotient IC50-gly: IC50-gly in Mix 2 of 7.68). It is important to note that cypermethrin formulation does not exhibit effects on root elongation even at concentrations as high as 500 mg a.i./L (at this level there is a high impact on germination, the IC50 value for inhibition of germination is 460.3 mg a.i./L, but there is no inhibition of elongation on germinated seeds), but there is an effect on elongation at a concentration 20 times lower in the mixture of both pesticides. To the contrary, a significantly lower toxicity was observed when glyphosate was mixed with chlorpyrifos formulation (a quotient IC50-gly: IC50-gly in Mix 4 of 0.54). Chlorpyrifos formulation exhibits very low toxicity by itself. However, the level of effects of the mixture with the three pesticides is closer to the herbicide formulation (a quotient IC50-gly: IC50-gly in Mix 1 of 1.24).

Table 3. Slope comparisons of linear regressions from dose response plots. Taken from toxicity tests with Roundup with seeds from five species.

Test For	Null Hypotesis	Statistic Test	Critical Value	Accept Reject
Differences among slope	b1=b2=b3=b4=b5	F=46.27	F=2.53	R
Multiple comparisons among slopes	*Medicago* = *Allium*	q=4.05	Q=4.16	A
	Medicago = *Lactuca*	q=12.09	Q=4.16	R
	Medicago = *Lolium*	q=0.00	Q=4.16	A
	Medicago = *Brassica*	q=0.89	Q=4.16	A
	Lactuca = *Allium*	q=14.45	Q=4.16	R
	Lactuca = *Lolium*	q=15.03	Q=4.16	R
	Lactuca = *Brassica*	q=17.61	Q=4.16	R
	Lolium = *Brassica*	q=0.00	Q=4.16	A
	Lolium = *Allium*	q=4.70	Q=4.16	R
	Brassica = *Allium*	q=5.32	Q=4.16	R

The comparison of slopes from the dose response curves (Table 5) of unmixed Roundup with all mixtures containing the herbicide shows no significant difference. On the contrary, unmixed Roundup differs significantly with the insecticides separately or their mixture. The highest slopes correspond to mixtures containing the herbicide or to the unmixed Roundup (Table 4). Formulated insecticides differ between them and with their mixture. These comparisons, together with the IC50 values, allow for the observation that although glyphosate determines the variation of the effect with the concentration of toxicants, the presence of the insecticide formulations provides an additional effect on root elongation.

Pesticide concentrations in environmental samples are given in Table 6. Results of toxicity tests performed with the stream water samples and sediment eluriate (1:4, sediment: water) only showed significant effects on root elongation inhibition of *L. sativa* seeds for a sediment eluriate corresponding to the application event where the mixture of the herbicide plus the two insecticides (13.03 % inhibition at the direct sample eluriate) is in agreement with the maximum load of pesticides in the sediment. A field study with caged experiments performed simultaneously in the

Table 4. Results of toxicity tests of pesticide mixtures with *Lactuca sativa*. IC50 values correspond to root elongation inhibition expressed as the % dilution of mixture and as concentration of a.i. for each toxicant.

Tested Toxicants	Application Mixture IC50 % v/v and CI	Application Mixture or single formulation IC50 mg a.i./L and CI	r^2	b	A	SE	n
Mixture 1 = (Gly + Chl + Cyp)	0.08 [0.07-0.09]	(Gly) 5.95 [5.43-6.91] (Chl) 3.07 [2.80-3.57] (Cyp) 0.20 [0.18-0.23]	0.79	-2.389*	3.31	0.22	18
Mixture 2 = (Gly + Cyp)	0.010 [0.012-0.013]	(Gly) 0.95[0.93-1.11] (Cyp) 0.032[0.031-0.039]	0.50	-1.305*	3.01	0.37	18
Mixture 3 = (Chl + Cyp)	8.06 [7.43-8.60]	(Chl) 309.50 [285.31-330.24] (Cyp) 20.15 [18.57-21.50]	0.84	-0.091*	3.43	0.18	11
Mixture 4 = (Gly + Chl)	0.18 [0.17-0.19]	(Gly) 13.39 [12.65-14.14] (Chl) 6.91 [6.53-7.30]	0.65	-1.547*	3.06	0.32	24
Roundup	-	(Gly) 7.35 [6.49-9.44]	0.81	-1.941*	3.14	0.21	27
Sherpa	-	(Cyp) 460.28# [357.30-500.59]	0.71	-0.014*	3.59	0.16	12
Shooter	-	(Chl) 489.75 [451.09-498.43]	0.68	-0.036*	3.59	0.10	12

Dose response curves correspond to % dilution and elongation in mm. r^2= determination coefficient; b=slope (regression coefficient); A= elevation; SE= standard error; n= number of pair of x and y values. (*) Significantly different from zero slope. (#) Data corresponds to germination inhibition. Abbreviations: Gly=glyphosate; Chl=chlorpyrifos; Cyp=cypermethrin, CI= confidence interval. (-) No data, correspond to unmixed pesticide.

same area and published elsewhere (Martin et al., 2003) showed an inhibition of chlorophyll content and total biomass in exposed Lemanaceae (75% and 28%). This indicates that laboratory tests with environmental samples were capable of detecting toxic effects from exposure to a sediment eluriate sampled at a time and in a place where field tests with aquatic plants were also affected, showing an impact of the agricultural activity in a surface water body adjacent to a crop field.

Table 5. Slope comparisons of linear regressions from dose response plots taken from toxicity tests with pesticide mixtures and single compounds using the *Lactuca sativa* test system.

Test for	Null Hypotesis	Statistical Test	Critical Value	Accept Reject
Differences among slope	b1=b2=b3=b4=b5=b6=b7	F=104.59	F=2.19	R
	Roundup = Mix1	q= 1.79	q= 4.24	A
	Roundup = Mix2	q= 2.87	q= 4.24	A
	Roundup = Mix3	q= 14.62	q= 4.24	R
	Roundup = Mix4	q= 1.75	q= 4.24	A
	Roundup = Shooter	q= 16.42	q= 4.24	R
	Roundup = Sherpa	q= 15.77	q= 4.24	R
	Mix 1 = Mix 2	q= 3.30	q= 4.24	A
	Mix 1 = Mix3	q= 11.38	q= 4.24	R
	Mix 1 = Mix4	q= 2.49	q= 4.24	A
	Mix 1 = Sherpa	q= 12.28	q= 4.24	R
	Mix 1 = Shooter	q= 12.15	q= 4.24	R
	Mix 2 = Mix 3	q= 7.81	q= 4.24	R
	Mix 2 = Mix 4	q= 0.97	q= 4.24	A
	Mix 2 = Shooter	q= 8.67	q= 4.24	R
	Mix 2 = Sherpa	q= 8.59	q= 4.24	R
	Mix 3 = Mix 4	q= 9.58	q= 4.24	R
	Mix 3 = Shooter	q= 7.13	q= 4.24	R
	Mix 3 = Sherpa	q= 8.58	q= 4.24	R
	Mix 4 = Shooter	q= 10.40	q= 4.24	R
	Mix 4 = Sherpa	q= 10.38	q= 4.24	R
	Sherpa = Shooter	q= 3.02	q= 4.24	A

With regard to the stability of the pesticides in the testing conditions, results of chemical analysis (given as the mean reduction detected at two tested concentrations respect to initial concentration) during exposure time indicate that glyphosate concentration does not differ between the first and third day of exposure, and there is a 25% reduction in concentration at the end of exposure. For the case of cypermethrin and chlorpyrifos, a 65% reduction was detected in the content of the first and only a 5% reduction of the second insecticide (considering the content in both, supernant liquid and paper bound). Recovery of analytical methods was over 98%.

We conclude that no effect of the herbicides was observed on seed germination for any of the tested seeds. Effects on root elongation vary considerably between species. The high sensitivity of lettuce seeds within the test battery favors its selection among the others for the type of applications analyzed here. The relevance of the assessment of mixtures of pesticides on lettuce seeds reveals higher impacts in root elongation for some specific mixtures, according to toxicity test results with both application cocktails and environmental samples. Cypermethrin formulation only affects seed germination when tested separately. Although, a high effect on root elongation is observed when mixed with the herbicide.

Table 6. Pesticide content in environmental samples and related sprays and rain events (from Peruzzo et al. 2003 and Marino and Ronco 2005)

	Glyphosate		Cypermethrin		Chlorpyrifos	
Event related To Sampling	Water (mg/L)	Sediment (mg/Kg)	Water (µg/L)	Sediments (µg/Kg)	Water (µg/L)	Sediments (µg/Kg)
Gly+Cyp spray	0.4	3.1	0.5	100		
After first rain	0.3	<0.5	<0.2	7.5		
Gly+Cyp+Chl spray	0.5	3.9	2.0	399	5.0	15.0
After first rain	0.6	1.2	3.5	4.6	10.8	3.3

Abbreviations: Gly+Cyp= glyphosate plus cypermethrin; Gly+Cyp+Chl= glyphosate plus cypermethrin plus chlorpyrifos.

It is important to point to the relevance of using a battery of seeds to screen the relative impact of pesticides. Although the use of commercial seeds from vascular plants as surrogate organisms is recommended, the potential use of seeds from non-target plants of regional significance, in accordance with previous published suggestions (Boutin and Rogers 2000), should always be considered.

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