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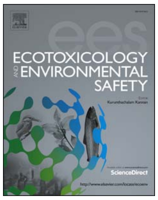

Effects of the herbicide glyphosate on non-target plant native species from Chaco forest (Argentina)

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ABSTRACT

Agriculture based on transgenic crops has expanded in Argentina into areas formerly occupied by Chaco forest. Even though glyphosate is the herbicide most widely used in the world, increasing evidence indicates severe ecotoxicological effects on non-target organisms as native plants. The aim of this work is to determine glyphosate effects on 23 native species present in the remaining Chaco forests immersed in agricultural matrices. This is a laboratory/greenhouse approach studying acute effects on seedlings after 21 days. A gradient of glyphosate rates (525, 1050, 2100, 4200, and 8400 g ai/Ha; recommended field application rate (RFAR) = 2100 g ai/Ha) was applied on four-week seedlings cultivated in a greenhouse and response variables (phytotoxicity, growth reduction, and sensitivity to the herbicide) were measured. This gradient of herbicide rates covers realistic rates of glyphosate applications in the crop field and also those that can reach vegetation of forest relicts by off-target drift and overspray. Testing was performed following guidelines for vegetative vigour (post-germination spray). All species showed lethal or sublethal effects after the application of the 25% of RFAR (50% of species showed severe phytotoxicity or death and 70% of species showed growth reduction). The results showed a gradient of sensitivity to glyphosate by which some of the studied species are very sensitive to glyphosate and seedlings died with 25% of RFAR while other species can be classified as herbicide-tolerant. Thus, the vegetation present in the forest relicts could be strongly affected by glyphosate application on crops. Lethal and sublethal effects of glyphosate on non-target plants could promote both the loss of biodiversity in native forest relicts immersed in the agroecosystems and the selection of new crop weeds considering that some biotypes are continuously exposed to low doses of glyphosate.

1. Introduction

Agriculture based on transgenic seeds (particularly soybean) has expanded into areas formerly occupied by native Chaco forests and glyphosate has become the main agrochemical used in Argentina (Cáceres, 2015). Biodiversity loss due to the transformation of natural ecosystems into lands destined to agribusiness is considered one of the main impacts that land-use changes have produced (Hails, 2002; Sanvido et al., 2007). In the non-Pampean regions of central Argentina, the process of capitalist expansion that is focused on the appropriation of nature through a non-diversified productive matrix that produces commodities for international markets (neoextractivism, according to Gudynas, 2009) was settled since the 1990s. This neoextractivist development model is producing the largest-ever transformation of natural capital into economic capital in the history of the region (Cáceres, 2015). For example, the Province of Córdoba (Argentina) has lost more than 95% of its original forest coverage (Hoyos et al., 2013; Piquer-Rodríguez et al., 2015).

Glyphosate (*N*-(phosphonomethyl) glycine) is a systemic non-selective herbicide, that has transformed agriculture and that is the most widely used in the world (Baylis, 2000). Even though glyphosate has been considered to be the less toxic alternative for weed control, however, its use is controversial as there is increasing evidence for possible profound ecotoxicological effects of this herbicide on the agroecosystem biodiversity (Bourguet and Guillemaud, 2016; Cuhra et al., 2016). For example, there have been recent reports of direct

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Table 1

Phytotoxicity in 23 non-crop plant native species sprayed with increasing rates of glyphosate (0, 525, 1050, 2100, 4200 and 8400 g ai/Ha). Data show the average percentage of observed damage in the seedlings treated with each of the glyphosate rates and its corresponding standard deviation. Damage percentage in the seedlings was determined according to the symptom manifestation of chlorosis, foliar damage, wilt and/or death in relation to the control sample (see Material and Methods section). In the seedlings used as control samples for each species there were not observed any of the phytotoxicity symptoms considered in this work. NOEC = No observed effect concentration. Observations were made 21 days after treatments. Species were ordered in decreasing value of phytotoxicity observed with the recommended field application rate (2100 g ai/Ha). Non-parametric analysis of variance was used to compare phytotoxicity among treatments of each species;different letters indicate significant differences; * = $p < 0.05$, ** = $p < 0.01$.

Species	Phytotoxicity (%)							NOEC (g ai/Ha)	Statistical analysis
	Family		Glyphosate treatments (g ai/Ha)						
		0	525	1050	2100	4200	8400		
Solanum argentinum	Solanaceae	0 ± 0^{a}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	0	7.11^{**}
Passiflora morifolia	Passifloraceae	0 ± 0^{a}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	0	7.11^{**}
Bouteloua curtipendula	Poaceae	0 ± 0^{a}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	0	7.11^{**}
Setaria pampeana	Poaceae	0 ± 0^{a}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	0	7.11^{**}
Solanum palinacanthum	Solanaceae	0 ± 0^{a}	73.3 ± 5.8^{ab}	96.7 ± 2.9^{ab}	100.0 ± 0^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	1050	13.11^{**}
Bidens subalternans	Asteraceae	0 ± 0^{a}	91.7 ± 2.9^{ab}	98.3 ± 2.9^{b}	98.3 ± 2.9^{b}	100.0 ± 0^{b}	100.0 ± 0^{b}	525	11.82^{**}
Baccharis glutinosa	Asteraceae	0 ± 0^{a}	53.3 ± 5.8^{ab}	86.7 ± 12.6^{abc}	98.3 ± 2.9^{bc}	100.0 ± 0^{c}	100.0 ± 0^{c}	1050	13.16^{**}
Chromolaena hookeriana	Asteraceae	0 ± 0^{a}	85.0 ± 5.0^{ab}	96.7 ± 2.9^{abc}	96.7 ± 5.8^{bc}	100.0 ± 0^{c}	100.0 ± 0^{c}	1050	12.65^{*}
Jarava ichu	Poaceae	0 ± 0^{a}	73.3 ± 5.8^{ab}	95.0 ± 5.0^{abc}	96.7 ± 2.9^{bc}	100.0 ± 0^{c}	100.0 ± 0^{c}	1050	13.55^{*}
Fleishmannia prasiifolia	Asteraceae	0 ± 0^{a}	75.0 ± 5.0^{a}	96.7 ± 2.9^{ab}	96.7 ± 2.9^{ab}	100.0 ± 0^{b}	100.0 ± 0^{b}	2100	13.53^{*}
Rhynchosia edulis	Fabaceae	0 ± 0^{a}	25.0 ± 5.0^{ab}	88.3 ± 12.6^{abc}	96.7 ± 2.9^{bc}	100.0 ± 0^{c}	100.0 ± 0^{c}	1050	13.62^{*}
Solanum pseudocapsicum	Solanaceae	0 ± 0^{a}	28.3 ± 7.6^{ab}	85.0 ± 13.2^{abc}	95.0 ± 5.0^{bc}	100.0 ± 0^{c}	100.0 ± 0^{c}	1050	13.62^{*}
Amelichloa brachychaeta	Poaceae	0 ± 0^{a}	28.3 ± 7.6^{ab}	88.3 ± 10.4^{abc}	95.0 ± 5.0^{bc}	100.0 ± 0^{c}	100.0 ± 0^{c}	1050	13.62^{*}
Celtis ehrenbergiana	Celtidaceae	0 ± 0^{a}	53.3 ± 2.9^{ab}	78.3 ± 15.3^{abc}	93.3 ± 5.8^{bc}	98.3 ± 2.9^{c}	100.0 ± 0^{c}	1050	14.60^{*}
Ipomoea nil	Convolvulaceae	0 ± 0^{a}	68.3 ± 2.9^{ab}	81.7 ± 2.9^{abc}	93.3 ± 2.9^{bc}	100.0 ± 0^{e}	100.0 ± 0^{e}	1050	16.11^{**}
Piptochaetium sp.	Poaceae	0 ± 0^{a}	25.0 ± 5.0^{ab}	26.7 ± 2.9^{ab}	91.7 ± 2.9^{bc}	95.0 ± 5.0^{bc}	100.0 ± 0^{c}	1050	15.40^{**}
Acacia aroma	Fabaceae	0 ± 0^{a}	23.3 ± 2.9^{ab}	81.7 ± 16^{abc}	91.7 ± 7.6^{bc}	98.3 ± 2.9^{c}	100.0 ± 0^{c}	1050	13.44^{*}
Iresine diffusa	Amaranthaceae	0 ± 0^{a}	70.0 ± 10.0^{ab}	85.0 ± 5.0^{abc}	91.7 ± 2.9^{bc}	96.7 ± 2.9^{c}	100.0 ± 0^{c}	1050	15.77^{**}
Sida spinosa	Malvaceae	0 ± 0^{a}	11.7 ± 2.9^{ab}	73.3 ± 5.8^{abc}	90.0 ± 13.2^{bc}	98.3 ± 2.9^{c}	100.0 ± 0^{c}	1050	14.94^{**}
Ipomoea purpurea	Convolvulaceae	0 ± 0^{a}	71.7 ± 7.6^{ab}	80.0 ± 5.0^{abc}	90.0 ± 5.0^{bc}	98.3 ± 2.9^{c}	100.0 ± 0^{c}	1050	15.64^{**}
Rivina humilis	Phytolaccaceae	0 ± 0^{a}	23.3 ± 2.9^{a}	83.3 ± 5.8^{ab}	85.0 ± 5.0^{ab}	96.7 ± 2.9^{b}	100.0 ± 0^{b}	2100	15.87^{**}
Cardiospermum halicacabum	Sapindaceae	0 ± 0^{a}	15.0 ± 5.0^{ab}	16.7 ± 2.9^{ab}	78.3 ± 17.6^{bc}	93.3 ± 5.8^{bc}	100.0 ± 0^{c}	1050	15.47^{**}
Amphilophium carolinae	Bignoniaceae	0 ± 0^{a}	6.7 ± 2.9^{a}	58.3 ± 7.6^{ab}	60.0 ± 5.0^{ab}	68.3 ± 2.9^{b}	85.0 ± 8.6^{b}	2100	15.81^{**}

glyphosate effects on microorganisms and fungi (Druille et al., 2013), invertebrates (Casabé et al., 2007), amphibians (Relyea, 2005), and fishes (Soso et al., 2007). In wild mammals, domestic mammals and humans, recent evidence indicates that both the herbicide and its metabolite AMPA have teratogenic and genotoxic effects and show associations to diverse pathologies (López et al., 2012).

In vascular plants, it has been proved that glyphosate active ingredients are translocated either through the xylem or the phloem and are rapidly distributed through all the plant, inhibiting amino acid synthesis and interfering in other metabolic processes (Cuhra et al., 2016). Glyphosate is typically sprayed onto foliage and it is absorbed through the plant leaves. The negative effects on sensitive species can be observed in seedlings and adult plants through a reduction of growth, leaf chlorosis, tissue necrosis, wilt, reduction of seed production, among other indicators (Boutin et al., 2004, 2014; Gove et al., 2007).

Even though herbicides are used in the agricultural area to control certain plant species (target plants) that compete for the resources with crop plants, lethal and sublethal herbicide concentrations can reach the plants that live in the natural environments immersed in the agroecosystems. Non-target plants are those non-crop plants located outside of the crop treatment areas (European and Mediterranean Plant Protection Organization, 2003). Herbicides can reach these plants during or after the application, producing direct effects through toxicity, or indirect effects through, for example, competition among species, recruitment reduction, and differential pressure of herbivores or symbionts as mycorrhizae (Boutin et al., 2014).

The contact between non-target plants and herbicides can occur through off-target spray drift, overspray, among others (Marrs et al., 1989). Normally and under the recommended weather conditions of application, the drift is a little percentage of the total volume of the spraying solution. However, it has been proved that the off-target drift can cause mortality and the suppression of growth of sensitive plant species that are in the natural communities adjacent to the crop fields (Dixon et al., 2002; Gove et al., 2007; Marrs et al., 1993). In this sense, a study performed in greenhouse and field mesocosms of eight species showed an increase in mortality and biomass reduction, fecundity and survival of all the treated plant species through herbicide spray drift concentrations (Riemens et al., 2008). Low-level exposure to herbicide sublethal doses may result in long-term negative impacts on plant community structure and diversity (Boutin et al., 2004; De Snoo and Van der Poll, 1999; Kleijn and Snoeijing, 1997; Londo et al., 2014; Sullivan et al., 1996). These findings are of great concern in relation to biodiversity conservation since some wild species may become endangered in the near future (Schmitz et al., 2013) or currently endangered species may disappear (Schmitz et al., 2014).

At the same time, the continued use of glyphosate with agriculture intensification can produce changes in the weed flora associated with crops because extreme artificial negative environmental conditions are created for the growth and development of vegetation. The intense use of glyphosate has recently increased the abundance of wild species co-occurring with crops, which present tolerance or resistance to glyphosate (Vitta et al., 2004). For example, in Argentina, tolerance (or resistance) to glyphosate has been found in many species which are present near crops (for example: Powles, 2008; Puricelli et al., 2015; Vila-Aiub et al., 2008).

Although plants' differential sensitivity to glyphosate is yet not fully understood (Baucom, 2016; Norsworthy et al., 2001), some studies indicated the potential for low rates of herbicide to rapidly select high levels of resistance (Neve, 2007; Sammons and Gaines, 2014). In this way, glyphosate drift on non-crop plant species can exert a selection pressure, which sustained over time can promote the selection of new weeds (Baucom, 2016; Powles, 2008).

In Argentina, the constant increase in the use of glyphosate and the amounts applied can be related to the expansion of transgenic soybean cultivation to new deforested areas and the increasing weed tolerance

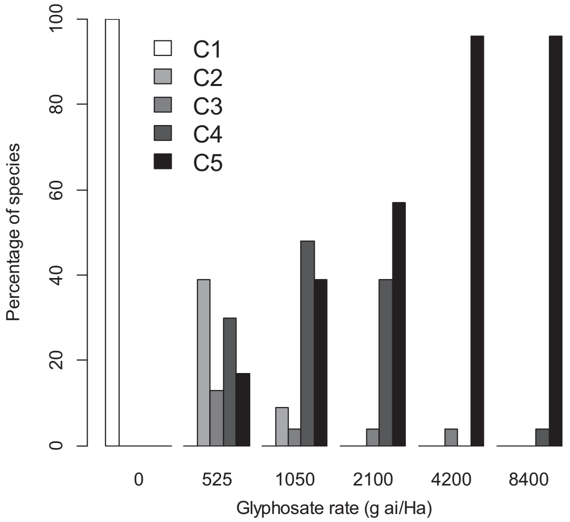

Fig. 1. Phytotoxicity in 23 non-crop plant native species sprayed with increasing rates of glyphosate (recommended field application rate 2100 g ai/Ha). The bars show the percentage of treated species for both each category of phytotoxicity and treatments with glyphosate (0, 525, 1050, 2100, 4200 and 8400 g ai/Ha). Phytotoxicity categories: C1) absence of phytotoxicity (0% damage), C2) slight phytotoxicity (1–30% damage), C3) medium phytotoxicity (31–69% damage), C4) severe phytotoxicity (70–99% damage) and C5) death (100% damage). Total of percentages from C1-5 for each treatment concentration is 100%. Seedlings were sprayed after four weeks post-emergence. Phytotoxicity symptoms were registered 21 days after the application of glyphosate on the seedlings (see Section 2.3.1).

and resistance to glyphosate (Cáceres, 2015; Christoffoleti et al., 2008). However, the consequences of glyphosate application on native plant species of the Chaco forest, which were repeatedly exposed to this herbicide during the last years, are still unknown.

The aim of this study was to determine the effects of different glyphosate rates (including drift and overspray situations) on 23 species present in native forest relicts immersed in agroecosystems of Argentina. The results may have agronomic and ecological implications since the effects of lethal and sublethal rates of glyphosate can, at the same time, promote the selection of new weed biotypes tolerant to this herbicide and produce a loss in biodiversity in native forest relicts.

2. Material and methods

2.1. Studied species

A total of 23 native plant species (Table 1) were selected to include a diversity of characteristics regarding: i) botanical families, ii) life forms (herbs, shrubs, vines, lianas, and trees), and iii) life cycles (annual and perennial). The Catálogo de las Plantas Vasculares del Cono Sur (2016) was used to determine the species status (i.e., native or non-native).

Seeds from 10 individuals of each species were collected in 20 Chaco forest relicts (Argentina, Province of Córdoba, Departments Santa María, Colón, and Capital) to perform the experiments. Chaco forests in central Argentina present high rates of landscape fragmentation due to land-use changes for agriculture and urbanizations. Forest fragments have an average isolation age of approximately 60–70 years and usually are less than 10–20 Ha (range < 1 to > 400 Ha) surrounded by crops as maize and soybean during spring and summer, and wheat during autumn and winter (Grilli et al., 2013). These forest relicts are immersed in agricultural matrices, in which glyphosate is applied regularly as the main herbicide.

Under greenhouse conditions, seeds were sowed into 4 × 4 × 6 cm plastic pots with a mixture of 1:2 of sterile sand and fertile soil. Pots were maintained with regular watering and under homogeneous conditions of light, temperature and humidity (16 h of light and 8 h of darkness, 25 ± 5 °C and 60 ± 15% of humidity). After emergence, the number of plants was thinned to one per pot. Experiments were conducted between August and November 2012.

2.2. Glyphosate application

When the seedlings had between 2 and 4 definitive leaves (approximately 4 weeks after being sowed), they were assigned, randomly, to six independent treatments, with increasing rates of glyphosate (Credit Full®, by Nufarm). This aqueous herbicidal comprises a mixture of glyphosate (540 g ai/L acid equivalent) with ammonium and potassium salts at a 70% w/v concentration. The herbicidal composition includes surfactants. In total, 414 seedlings were treated, 18 seedlings for each one of the 23 species, assigning 3 seedlings for each of the treatments within the gradient of glyphosate rates. The number of replications per treatment was related to a trade-off between the high number of species studied and the greenhouse space for the experiments. Testing was performed following the OECD (2006) guidelines for vegetative vigour (post-germination spray). The treatments applied were (i) the control sample (no herbicide was applied) and (ii-vi) 5 different rates of glyphosate (525, 1050, 2100, 4200 y 8400 g ai/Ha). Although the recommended field application rate (RFAR) for this herbicide is 2100 g ai/Ha, realistic rates applied at the studied sites ranges from 2100 to 8400 g ai/Ha, depending the crop level of weed infestation. This gradient of herbicide rates covers also those that can reach vegetation of forest relicts by off-target drift and overspray.

The seedlings were moved from the greenhouse to a plot outside for spraying. The herbicide was applied with a CO$_2$ pressurized precision backpack sprayer, coupled in a TeeJet 110.02 two-tip bar, positioned at 0.50 m from the target, with a spray relative consumption of 100 L/Ha.

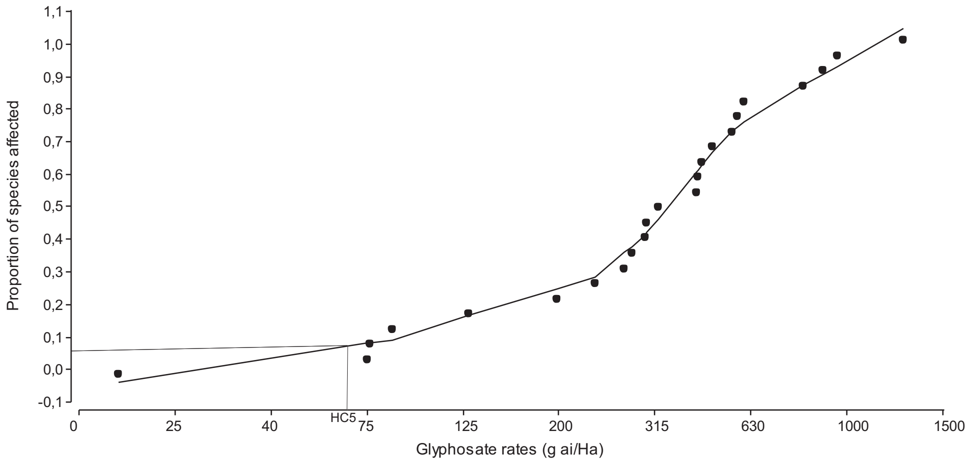

Fig. 2. Species sensitivity distribution (SSD) based on GR$_{50}$ values for 23 non-crop plant native species sprayed with increasing rates of glyphosate (0, 525, 1050, 2100, 4200, and 8400 g ai/Ha). Recommended field application rate = 2100 g ai/Ha. GR$_{50}$ = herbicide rate required to reduce growth by a 50% in relation to the growth of the untreated control samples. HC5 (Hazardous Concentration to 5% of the species) =72 g ai/Ha.

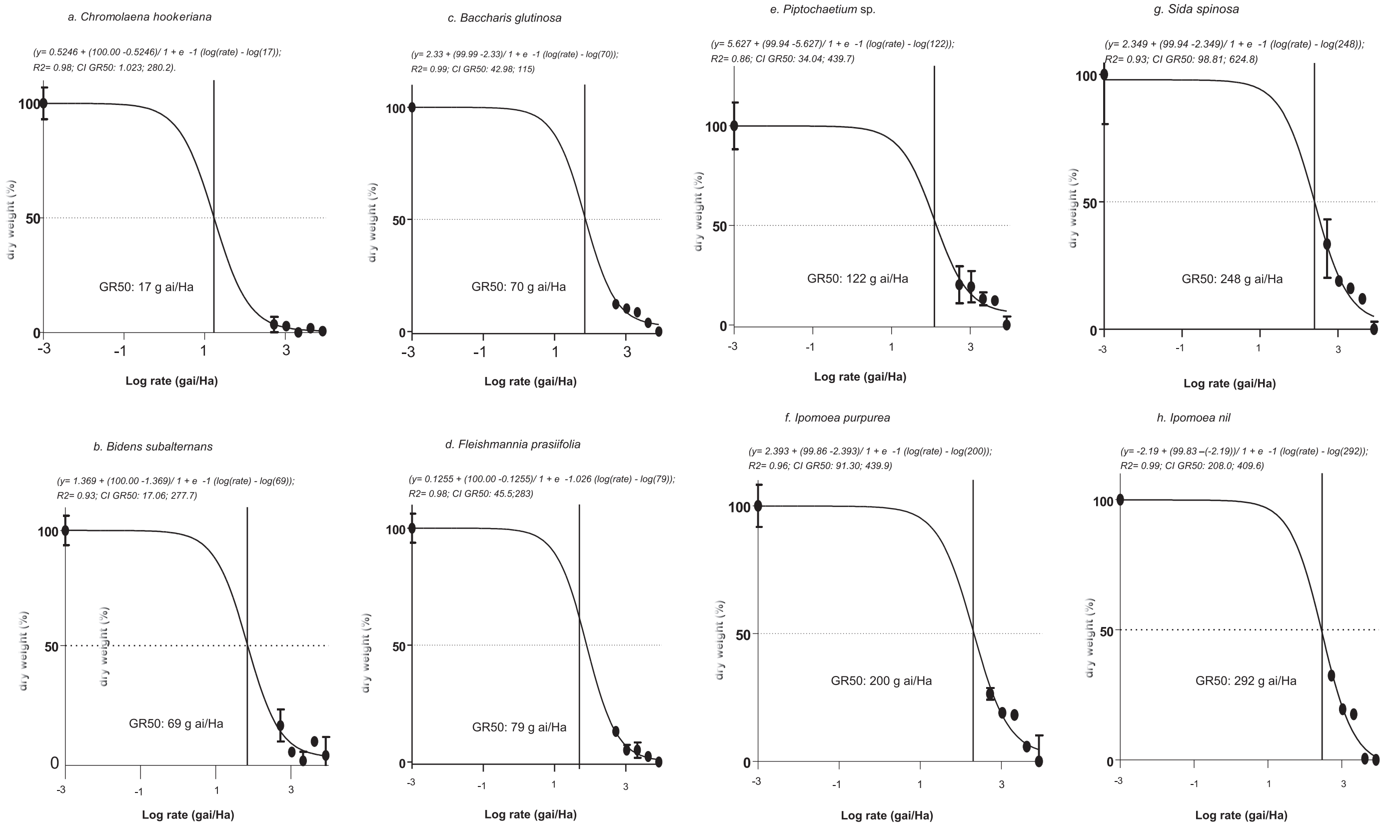

Fig. 3. Rate-response curves using the log-logistic model: a rate-response curve was calculated for each of the treated 23 non-crop plant native species, in which the aboveground dry weight reduction compared to control per rate was calculated and analyzed using nonlinear regression with a logistic growth curve: $y = c + (d - c)/ 1 + e^{-b\ (\log(rate)\ -\ \log(e))}$ (Seefeldt et al., 1995) with four parameters b, c, d, and e. The lower limit (c), the upper limit (d), the slope (b) and the GR_{50} (e) were estimated. Data: dry weight percentages with respect to control against glyphosate rates (g ai/Ha). Line is the response curve predicted from nonlinear regression. The R^2 value and GR50 confidence intervals (CIs) for each species are also included. Glyphosate rates on the *x*-axis were log transformed. Observations were made 21 days after treatment. Species were ordered according sensitivity to glyphosate from most sensitive to least sensitive (using its GR_{50}).

This pressure was regulated to simulate, as close as possible, the usual spraying made on the crops. Seedlings were transferred back to the greenhouse, 24 h after the treatments with herbicide.

2.3. Assessment of glyphosate effects

2.3.1. Phytotoxicity

Twenty one days after herbicide application, visual observations were made to determine the extent of the condition according to the manifestation of phytotoxicity symptoms: chlorosis, foliar damage and/or wilt (Riemens et al., 2008). In accordance with the manifestation of phytotoxicity symptoms for each of the glyphosate rates applied, the treated species were classified in five categories. The categories used were: 1) absence of phytotoxicity (0% damage), 2) slight phytotoxicity (1–30% damage, slight chlorosis: yellow spots or leaftips), 3) medium phytotoxicity (31–69% damage, severe chlorosis, slight to moderate necrosis: yellow spots or leaftips and wilting of the plant), 4) severe phytotoxicity (70–99% damage, severe chlorosis and severe necrosis, wilt: brown coloration), and 5) death (100% damage). For each plant species the no-observed-effect concentration (NOEC) was determined. The NOEC value is the highest substance concentration (herbicide in this case) used in an experimental bioassay that has no significant statistical effect comparing control with treated plants.

2.3.2. Quantitative assessment

The species response to increasing rates of herbicide was assessed by comparing growth reduction (seedlings' dry weight) between control (glyphosate-free seedlings) and treated plants (seedlings with each of the glyphosate rates). The aerial parts of the treated seedlings were harvested 21 days after glyphosate application and then dried at 40 °C, for a period of 72 h. Each individual was weighed in a precision balance of 0.0001 g.

The GR_{50} value represents the herbicide rate required to reduce growth by a 50% in relation to the growth of the untreated control samples (Diez De Ulzurrun and Leaden, 2012). The GR_{50} values are used to compare species and allow us to have a reference on the sensitivity of this set of native species. The ratio between the GR_{50} of a given species with an unknown sensitivity and the GR_{50} of the most sensitive reference species is called Sensitivity Index (SI; Diez De Ulzurrun and Leaden, 2012). In this study, the most sensitive species to glyphosate was *Chromolaena hookeriana* so its GR_{50} was used as reference to assess the SI of each of the remaining 22 species.

The sensitivity for a group of species can be evaluated through the species-sensitivity distribution (SSD). According to Van den Brink et al. (2006), this concept can be used "to reduce the uncertainty relating to differences in the sensitivity of standard test species and those expected to be exposed in nature and uses interspecific variation in sensitivity to toxicants to predict effects at the community level". The SSD is defined as a cumulative distribution function of the toxicity of a single compound or mixture to a set of species that constitutes an assemblage or community (Van den Brink et al., 2006). The SSD approach is promising but available data are sparse and mostly based on the lethal responses of a small group of testing laboratory species (Baird and Van den Brink, 2007). A small value in the left tail of the SSD can be selected to estimate a concentration below which the fraction of species exposed above their NOEC is considered acceptable. Usually a cut-off value of 5% is chosen and their corresponding concentration is named HC5 (Hazardous Concentration to 5% of the species; Van den Brink et al.,

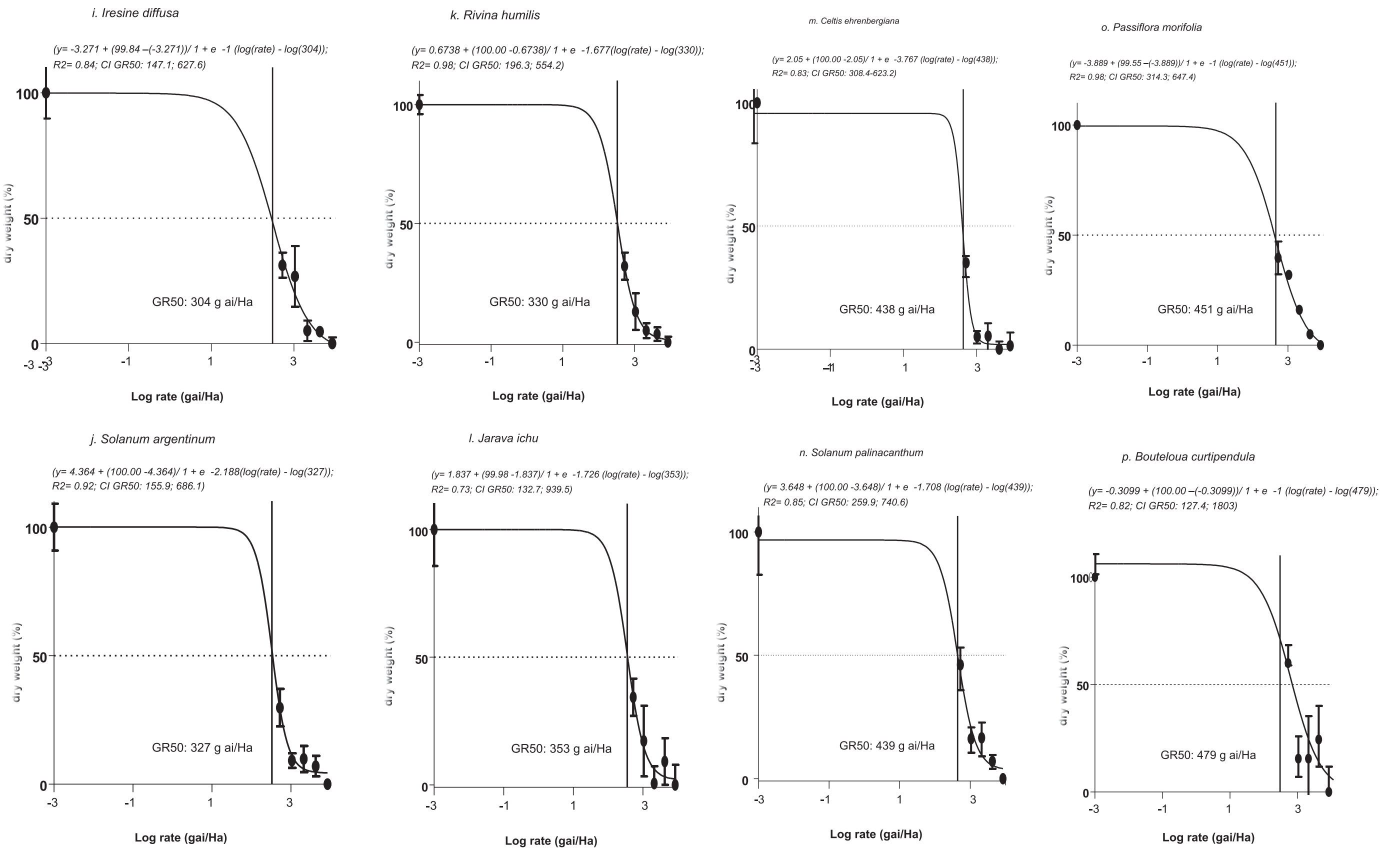

Fig. 3. (*continued*)

2006). The SSD was constructed by ranking the GR_{50} for all species in ascending order, using Infostat Software (2016). In addition, hazardous concentration (i.e., HC5) was estimated.

2.4. Data analysis

Parametric and non-parametric analyses of variance were used to compare the response variables (phytotoxicity and growth reduction) in the seedlings of each species treated with different rates of glyphosate, running the *car* package on R (R Core Development Team, 2014**)**.

The aboveground dry weight reduction compared to control per rate was calculated for each species and analyzed using nonlinear regression analysis with a logistic growth curve: $y = c + (d - c)/ 1 + e^{-b\ (\log(dose) - \log(e))}$ (Seefeldt et al., 1995) with four parameters b, c, d, and e. The lower limit (c), the upper limit (d), the slope (b) and the GR_{50} (e) were estimated. Regressions were performed using the statistical program SigmaPlot V13 (2016). Seedlings' dry weight was log transformed to perform the statistical analysis. Glyphosate rates on the x-axis were log transformed to perform the rate response curves. Nonlinear regressions were run using the *car* package on R (R Core Development Team, 2014**)**.

3. Results

3.1. Phytotoxicity

Different rates of glyphosate caused symptoms of phytotoxicity for all the treated species (Fig. 1). These symptoms were clearly observed five days after of the herbicide application. The symptoms increased during the duration of the experiment because the systemic effects of this herbicide can be produced up to 20 days after its application. After the application of lower glyphosate rates, the most evident symptom was chlorosis (yellow spots on the leaves). In the treatments with higher rates, tissue necrosis, wilt and death occurred. In general, phytotoxicity symptoms were greater as the applied glyphosate rates were increased (Table 1). Fifty percent of species showed severe phytotoxicity or death with 25% of the RFAR (Table 1). All species presented moderate to severe phytotoxicity or death with the RFAR (2100 g ai/Ha, Fig. 1).

3.2. Quantitative assessment

Species sensitivity distribution (SSD) was constructed by ranking the GR_{50} values for the glyphosate experiments in ascending order (Fig. 2) based on the nonlinear regression curves for all species (Fig. 3). The estimated HC5 was 72 g ai/Ha (Fig. 2). A gradient of sensitivity to glyphosate was observed for this set of 23 species (Table 2**)**. The different values obtained for the Sensitivity Index (SI) showed that some of the studied species are very sensitive to glyphosate while other species can be considered as herbicide-tolerant (Table 2). Results showed that glyphosate caused a significant growth reduction (GR) on all the treated species (Fig. 3). In general, the GR was greater as the glyphosate rates are increased (Table 2; Fig. 3).

According to the SSD, 70% and 92% of the treated species are affected by 25% and 50% RFAR (525 and 1050 g ai/Ha, respectively; Fig. 2) showing lethal or sublethal effects. The most sensitive species to glyphosate was *Chromolaena hookeriana* (Table 2), having the lowest GR_{50} value (17 g **ai/Ha;** Fig. 3a). This value means that a rate of 17 g ai/Ha of the herbicide is necessary to diminish 50% GR in the seedling biomass.

ANOVAs comparing aboveground dry weight in the seedlings of each species treated with different herbicide rates, showed that glyphosate caused a significant dry weight reduction on most species (Table 2). In only one species (*Bouteloua curtipendula*) significant differences were not found (Table 2). It is interesting to note that *Bouteloua curtipendula, Amphilophium carolinae*, *Amelichloa brachychaeta*, and *Solanum palinacanthum* showed estimated NOEC values equivalent o

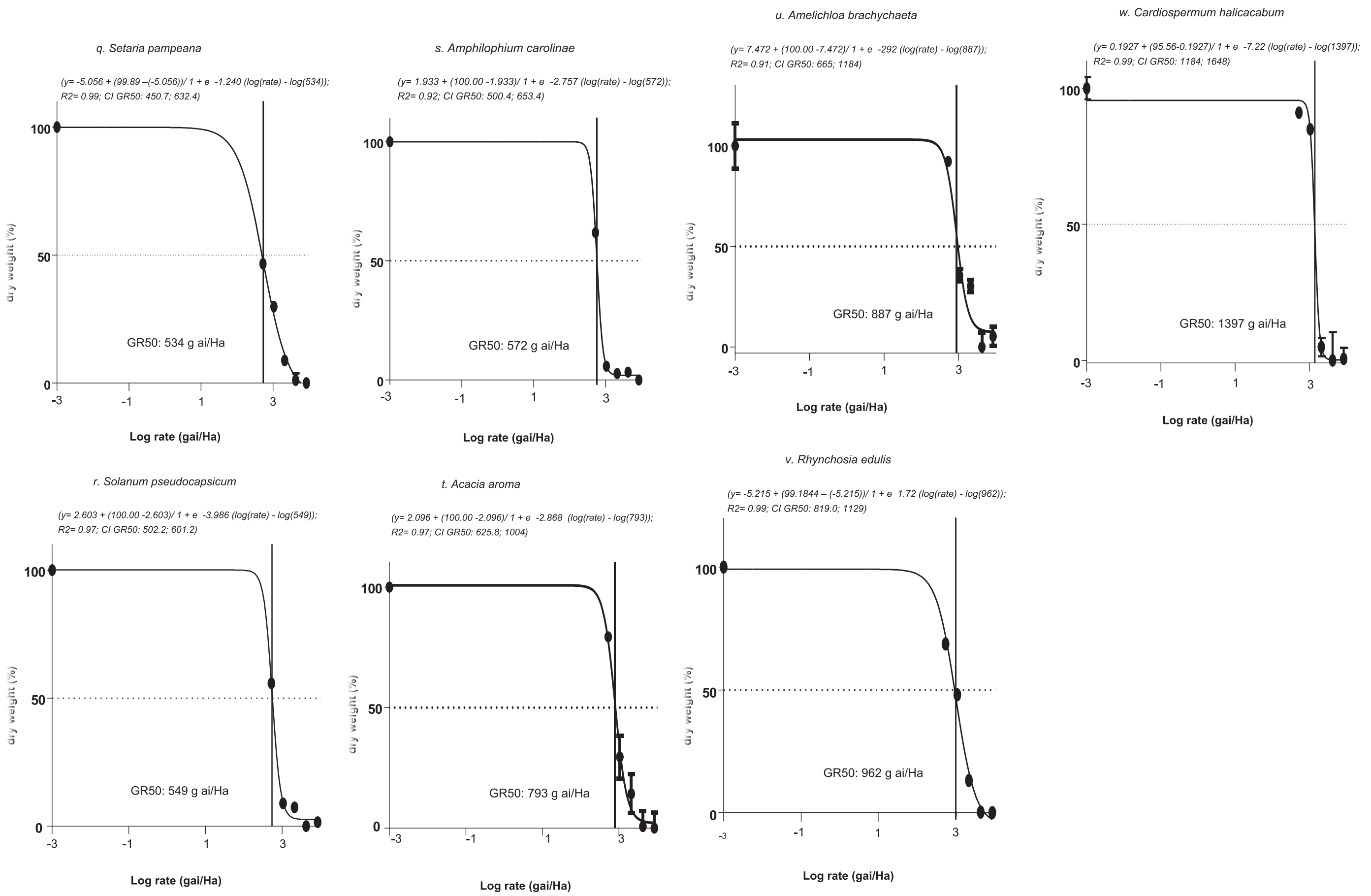

Fig. 3. (*continued*)

higher than RFAR (i.e., ≥ 2100 g ai/Ha; Table 2).

4. Discussion

4.1. Glyphosate effects on the survival of non-target plant native species

Lethal or sublethal effects (phytotoxicity and growth reduction) were observed for this set of native species, even when 25% of the RFAR was applied. These effects would be even greater in the margins of forest relicts where vegetation is exposed to the rate that is usually applied in the field (i.e., RFAR) and reaches the forest margins directly through overspray. Under ideal spraying conditions and appropriate application methods, total spray drift (i.e. the portion of applied spray outside the treated area) must be very low (1–2% of the volume applied) but strong winds and pour application procedures may result in total spray drift as high as 37% (Nordby and Skuterud, 1975). Schmitz et al. (2014) reported that the applied herbicide rates (30% of the RFAR) were consistent with their average input rates (drift + overspray) in the first meter of a field margin directly adjacent to the field. In general, our results suggest that the vegetation present in the native forest relicts would be strongly affected by glyphosate application on the adjacent agricultural lands. Boutin et al. (2014) have demonstrated that non-crop plants were affected during herbicide spray. Based on EC50 values, they reported many species at early vegetative stages affected by glyphosate, including some Poaceae, Asteraceae and Solanaceae (Boutin et al., 2004, 2014), as we found here.

These results follow the same tendency observed in previous works done in other biogeographical regions, in which immediate lethal and sublethal effects of herbicides in non-target plants are described (for example, Boutin and Jobin, 1998; Boutin et al., 2004, 2014; Gove et al., 2007; Schmitz et al., 2013, 2014). Moreover, these effects can accumulate and become stronger over time considering that the natural areas included within intensive agricultural management are subjected to periodic application of low doses of herbicide drifts (Marshall, 2001). It has been reported that, in certain species, a gradually reduction of population size can be observed after the third year of treatments (Schmitz et al., 2014). This population reduction of the most sensitive species could be even faster and greater when other stress factors are added (for example: herbivores, extreme weather conditions), affecting the recovery and reproduction of the species regionally (Carpenter and Boutin, 2010). In this way, the differences among the responses of the species to herbicide drift can modify the composition of the plant community through changes in biological interactions, fecundity or seed production (Gove et al., 2007; Marrs et al., 1991).

On this basis, it is reasonable to assume negative effects of glyphosate on native forest plant species, determining changes in the structure and composition of natural communities of Chaco forests. In particular, most sensitive species could be undergoing local extinctions, as noted in plant community field experiments (for example, Schmitz et al., 2014). Failure to adequately assess and properly regulate herbicide effects can have important ecological consequences to plant communities within agroecosystems (Boutin et al., 2014).

4.2. Selection of new glyphosate-tolerant biotypes

A gradient of sensitivity to glyphosate was observed (using growth reduction and phytotoxicity symptoms) by which some species die with 25% of RFAR, while others either survive with moderate effects or can be considered tolerant species to the glyphosate RFAR. The continuous and intensive use of glyphosate to control weeds in transgenic soybean crops in Argentina exerts a high pressure of weed selection with negative consequences. For example, the density of glyphosate-tolerant

Table 2
Glyphosate effects according to quantitative assessment (measuring aboveground dry weight) in 23 non-crop plant native species sprayed with increasing rates of glyphosate (0, 525, 1050, 2100, 4200 and 8400 g ai/Ha; recommended field application rate = 2100 g ai/Ha). Data show mean and standard deviation for dry weight of seedlings for the different treatments of each species. Observations were made 21 days after treatments. SI (Sensitivity Index) = the ratio between the GR_{50} of a given species with an unknown sensitivity and the GR_{50} of the most sensitive reference species to glyphosate (see Section 2.3.2). Species were ordered according sensitivity to glyphosate from most sensitive to least sensitive. NOEC = No observed effect concentration. Parametric analysis of variance was used to compare plant dry weight (log transformed) among treatments of each species; different letters indicate significant differences; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Aboveground plant dry weight (mg)							**NOEC (g ai/Ha)**	**Statistical analysis**	**SI**
Species	**Glyphosate treatments (g ai/Ha)**								
	0	**525**	**1050**	**2100**	**4200**	**8400**			
Chromolaena hookeriana	13.43 ± 7.29^{a}	1.13 ± 0.15^{bc}	1.67 ± 0.57^{bc}	0.80 ± 0.56^{c}	1.50 ± 0.56^{bc}	0.77 ± 1.73^{ab}	0	8.63^{**}	1.00
Bidens subalternans	111.53 ± 31.21^{a}	24.50 ± 2.89^{b}	21.20 ± 10.88^{ab}	21.18 ± 20.61^{b}	19.55 ± 27.73^{b}	18.15 ± 7.22^{ab}	0	7.05^{**}	4.06
Baccharis glutinosa	21.25 ± 0.35^{a}	3.25 ± 0.07^{b}	2.85 ± 0.07^{bc}	2.51 ± 0.01^{c}	1.55 ± 0.07^{d}	0.75 ± 0.07^{e}	0	120.33^{***}	4.12
Fleischmannia prasiifolia	6.63 ± 3.38^{a}	3.38 ± 1.84^{ab}	1.93 ± 0.10^{b}	1.68 ± 0.51^{b}	2.23 ± 0.26^{ab}	1.83 ± 1.05^{b}	525	3.84^{*}	4.65
Piptochaetium sp.	8.83 ± 3.34^{a}	2.70 ± 1.44^{b}	2.63 ± 1.22^{b}	2.15 ± 0.51^{b}	2.08 ± 0.17^{b}	1.13 ± 0.67^{b}	0	11.03^{**}	7.18
Ipomoea purpurea	488.00 ± 50.49^{a}	64.55 ± 67.53^{b}	73.00 ± 52.33^{b}	135.85 ± 4.31^{ab}	80.95 ± 7.85^{ab}	133.85 ± 37.31^{b}	0	3.58^{*}	11.76
Sida spinosa	21.55 ± 25.39^{a}	9.35 ± 8.67^{b}	9.25 ± 0.49^{b}	8.35 ± 0.21^{b}	7.05 ± 0.35^{b}	3.30 ± 1.41^{b}	0	6.53^{*}	14.59
Ipomoea nil	684.70 ± 0.28^{a}	235.55 ± 0.07^{b}	149.65 ± 0.21^{c}	136.75 ± 0.07^{d}	23.45 ± 0.21^{e}	20.03 ± 0.03^{f}	0	25.07^{***}	17.18
Iresine diffusa	5.80 ± 1.26^{a}	2.06 ± 0.61^{ab}	1.82 ± 1.47^{bc}	0.64 ± 0.49^{cd}	0.62 ± 0.16^{cd}	0.36 ± 0.30^{d}	525	17.49^{***}	17.88
Solanum argentinum	72.83 ± 63.08^{a}	4.98 ± 1.85^{b}	2.38 ± 0.71^{b}	2.45 ± 1.29^{b}	2.10 ± 1.00^{b}	1.21 ± 0.16^{b}	0	18.88^{***}	19.23
Rivina humilis	107.20 ± 25.87^{a}	15.40 ± 13.29^{ab}	5.15 ± 4.03^{b}	2.25 ± 0.78^{b}	5.65 ± 2.47^{b}	2.00 ± 2.12^{b}	525	3.23^{*}	19.41
Jarava ichu	1.16 ± 0.62^{a}	0.52 ± 0.28^{b}	0.32 ± 0.22^{b}	0.20 ± 0.11^{b}	0.37 ± 0.07^{b}	0.28 ± 0.22^{b}	0	3.81^{*}	20.76
Celtis ehrenbergiana	20.90 ± 5.34^{a}	10.57 ± 1.88^{a}	5.81 ± 1.11^{b}	5.86 ± 2.39^{b}	5.00 ± 1.39^{b}	5.23 ± 2.38^{b}	525	15.32^{***}	25.76
Solanum palinacanthum	66.53 ± 41.55^{a}	23.45 ± 8.01^{ab}	8.08 ± 2.66^{ab}	14.19 ± 12.84^{ab}	5.98 ± 2.58^{b}	11.50 ± 7.60^{b}	2100	3.47^{*}	25.82
Passiflora morifolia	11.35 ± 0.07^{a}	2.89 ± 0.02^{b}	2.48 ± 0.04^{b}	2.88 ± 0.04^{b}	1.75 ± 0.07^{c}	3.43 ± 0.04^{d}	0	27.85^{***}	26.53
Bouteloua curtipendula	4.00 ± 0.14^{a}	3.81 ± 1.07^{a}	2.10 ± 0.28^{a}	2.10 ± 0.57^{a}	2.30 ± 0.42^{a}	2.05 ± 0.78^{a}	> 8400	1.69	28.18
Setaria pampeana	14.15 ± 0.07^{a}	7.80 ± 0.14^{b}	5.80 ± 0.14^{c}	3.30 ± 0.14^{c}	6.80 ± 0.14^{bc}	6.23 ± 0.04^{bc}	0	819.92^{***}	31.41
Solanum pseudocapsicum	18.38 ± 1.26^{a}	11.06 ± 5.82^{ab}	3.80 ± 0.83^{bc}	3.55 ± 0.84^{bc}	2.38 ± 1.56^{c}	2.63 ± 1.16^{c}	525	9.69^{**}	32.29
Amphilophium carolinae	24.16 ± 9.97^{a}	18.78 ± 6.64^{ab}	10.86 ± 3.87^{ab}	10.42 ± 4.04^{ab}	11.82 ± 3.88^{ab}	10.08 ± 4.49^{b}	4200	3.37^{*}	33.65
Acacia aroma	54.20 ± 0.28^{a}	47.75 ± 0.35^{b}	25.50 ± 0.71^{c}	29.75 ± 0.35^{d}	21.10 ± 0.14^{e}	25.20 ± 0.14^{c}	0	162.30^{***}	46.65
Amelichloa brachychaeta	1.97 ± 1.42^{a}	1.96 ± 0.00^{a}	0.90 ± 0.10^{ab}	0.80 ± 0.10^{ab}	0.23 ± 0.23^{b}	0.33 ± 0.15^{bc}	2100	10.47^{**}	52.18
Rhynchosia edulis	90.20 ± 0.28^{a}	63.65 ± 0.07^{b}	46.05 ± 0.07^{c}	16.50 ± 0.14^{d}	5.55 ± 0.07^{e}	5.35 ± 0.07^{f}	0	432.47^{***}	56.59
Cardiospermum halicacabum	18.50 ± 0.42^{a}	17.58 ± 0.04^{a}	16.95 ± 0.07^{a}	8.75 ± 0.35^{b}	9.50 ± 0.71^{b}	8.58 ± 0.04^{b}	1050	219.28^{***}	82.17

biotypes of different species increases and the evolution of glyphosate-resistant plant populations could be promoted (Neve et al., 2014; Pedersen et al., 2007; Powles, 2008).

The evolution of glyphosate-resistant biotypes is becoming a serious problem in agroecosystems all over the world (Duke and Powles, 2008). Selection of tolerant or resistant biotypes requires some years of particular and repeated practices. For example, *Ipomoea purpurea* has been mentioned as tolerant species in the current soybean production systems in Argentina (Vitta et al., 2004). However, we observed relatively low GR_{50} values for this species indicating sensitivity differences between populations of the same plant species. A plausible explanation for these results would be that the seedling were treated here during initial developmental stages (2–4 leaves) that could be more sensitive and/or that seedling come from seeds collected in more sensitive populations located within forests relicts. Generally herbicide sensitivity decreases as plant developmental stages progress (Boutin et al., 2014**;** Marrs et al., 1991; Shrestha et al., 2007).

Numerous studies in other regions confirm the potential of very low doses of glyphosate to select highly resistant phenotypes in many species (Neve and Powles, 2005; Neve et al., 2014). Our results suggest the development of tolerant biotypes in many species of Chaco forests. Some of these native species may potentially become crop weeds considering that some biotypes are continuously exposed to low doses of glyphosate. This repeated strategy can cause a great pressure to select biotypes and a subsequent resistance to the herbicide (Christoffoleti et al., 2008).

4.3. *Synthesis and applications*

Considering our results, weed management system linked to glyphosate-resistant soybean could lead, on the one hand, to the reduction of the landscape complexity through the gradual loss of biodiversity in the agroecosystems. On the other hand, glyphosate-tolerant biotypes **could be** selected turning the agroecosystem dynamics into a very problematic circle to the current model of industrial agricultural production. Therefore, it is necessary and urgent to discuss the current use of glyphosate and the magnitude of its effects on non-target organisms.

Although good agricultural practices are regulated, better controls are needed for acceptable weed management in the crops, preventing the appearance of new tolerant biotypes, and maintaining biodiversity integrity in the adjacent natural environments. Herbicide spraying techniques and equipment, weather conditions during the application, operator's abilities, buffer zones, etc. have a crucial importance on the drift level and herbicide overspray in non-target areas (Nuyttens et al., 2006a, 2006b). Drift measurements in the forest margins have shown that up to 5% of the applied dose can be deposited as far as 2 m in the forest interior, and up to 1% as far as 10 m (Gove et al., 2004). Most serious impacts of herbicide drift on non-target organisms are generally confined in less than 10 m from the application point (Felsot et al., 1996; Marrs et al., 1989), but sublethal effects on the plants have been observed much further than that distance (Marrs et al., 1993). Hence, it can be on the basis of the results of this work and the previous ones, concluded the need and urgency for the implementation of no-spray buffer zones large enough to protect the biodiversity of the forest relicts which are immersed in agricultural matrices and, at the same time, avoid the selection of tolerant or resistant biotypes.

The adequate understanding and evaluation of the consequences of herbicide drifts in non-target areas result even more urgent and necessary if the deforestation crisis of the Chaco forests is taken into consideration. In the Province of Córdoba (Central Argentina), land-use changes have reduced the native forest to fragments of different sizes immersed in matrices of cultural vegetation. The Gran Chaco is a large, dry forest region covering about 1,080,000 km^2 in Argentina (Piquer-Rodríguez et al., 2015) and in the province of Córdoba more than 1000,000 Ha of seasonally dry forests of the semiarid Chaco were lost from 1970 to 2000 (Hoyos et al., 2013).

In this context, it is crucial to guarantee the conservation of all the native forest remnants because they play an important role in the agricultural landscape, since they provide many environmental, ecosystem and social services as pollinator sources and pollination, soil formation and fertility, among other services (IPBES, 2016). In addition, native forest relicts sustain biocultural diversity and they are the only remaining habitat for numerous threatened species. Consequently, it would be reasonable to apply the Precautionary Principle regarding the actual use of glyphosate in agroecosystems and the expansion of agribusiness over Chaco forests. Scientific uncertainty about the effects of glyphosate drift on non-target organisms justifies the adoption by farmers of adequate practices to guarantee biodiversity integrity in agroecosystems.

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