



# Glyphosate vulnerability explains changes in root-symbionts propagules viability in pampean grasslands



M. Druille <sup>a,\*</sup>, M.N. Cabello <sup>b,c</sup>, P.A. García Parisi <sup>a</sup>, R.A. Golluscio <sup>a</sup>, M. Omacini <sup>a</sup>

<sup>a</sup> IFEVA, Faculty of Agronomy, University of Buenos Aires, CONICET, Av. San Martín 4453, Buenos Aires C1417DSE, Argentina

<sup>b</sup> Instituto Spigazzini, Faculty of Natural Sciences and Museum, National University of La Plata, Av. 53 No 477, La Plata B1900AVJ, Argentina

<sup>c</sup> CICPBA, Argentina

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## ABSTRACT

Research into the impact of agricultural practices on plant symbionts is essential for understanding the factors that modulate plant community productivity and diversity. Although glyphosate is used worldwide as an herbicide, its effects on root symbionts under natural conditions have not been sufficiently studied. We performed a field experiment to evaluate the influence of glyphosate, used for promoting winter forage production, on the viability of arbuscular mycorrhizal fungi (AMF) and rhizobium propagules and other ecosystem traits in native grasslands. The number of viable propagules was strongly reduced with a single application at the recommended dose. Spore viability reduction was dependent on AMF species. Furthermore, changes in plant community composition and soil salinity were detected, which may eventually influence these symbionts in the future. Considering the low nutrient availability and high root-symbiont dependency of several species with forage value, repeated applications might lead to a loss in the grassland diversity and productivity, decreasing livestock production. Application of sublethal doses of this herbicide could avoid these damages, although success in increasing winter forage production would be less. Our results are relevant for understanding the effects of glyphosate on non-target species and designing sustainable land management systems.

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## 1. Introduction

There is great interest in understanding the factors that determine the persistence of plant symbionts because they are closely related to ecosystems productivity and stability (van der Heijden et al., 2006, 2008; Klironomos et al., 2011). In the past, ecologists have tried to explain the organization of plant communities focusing on the negative biotic interactions among plants (Tilman, 1994) and their enemies, such as herbivores (Olf and Ritchie, 1998; Bakker et al., 2006) and pathogens (van der Putten and Peters, 1997; Mills and Bever, 1998; van der Putten and van der Stoel, 1998; Maron et al., 2011), and on the impact of climate and soil abiotic properties (Milchunas et al., 1989; Robinson et al., 1998; Knapp et al., 2002). However, it is increasingly recognized that positive interactions between plants and microbial symbionts play a central role in determining plant community composition, diversity and productivity (van der Heijden et al., 1998a; Clay and Holah, 1999; van der Heijden

et al., 2006; Klironomos et al., 2011). Mechanisms involved may be diverse, including resource acquisition, protection against antagonists and habitat modification (Kothamasi et al., 2010; Omacini, 2014).

Among plant microbial symbionts, the most important are arbuscular mycorrhizal fungi (AMF) and nitrogen fixing bacteria, considering their benefits to host plants, and their worldwide distribution in different types of ecosystems (Kahindi et al., 1997; Zahran, 1999; Brundrett, 2009). Both types of soil-borne microbes form beneficial associations with their hosts. AMF colonize more than 80% of terrestrial plant species (Smith and Read, 2008) enhancing their growth and uptake of immobile mineral nutrients, particularly phosphorous (Smith et al., 2003), and improving water relations (Smith and Read, 2008). Rhizobia is a group of soil bacteria (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, or *Azorhizobium* spp.) that reduce atmospheric N<sub>2</sub> into NH<sub>4</sub><sup>+</sup> after becoming established in root nodules of legumes (Sprent, 2007) thus enhancing their host growth and, ultimately, N availability for other plants (Høgh-Jensen and Schjoerring, 2000). AMF and rhizobia spread among hosts through the soil; therefore, variations in the number of viable propagules have an impact on legumes and plants with high mycorrhizal dependency, altering

\* Corresponding author. Tel.: +549 114524 8000x4056.  
E-mail address: [druille@ifeva.edu.ar](mailto:druille@ifeva.edu.ar) (M. Druille).

plant composition and diversity (Denison, 2000; O'Connor et al., 2002).

Several studies have shown that human activities, such as tillage (Ferreira et al., 2000; Schalamuk and Cabello, 2010), fertilization (Beard and Hoover, 1971; Bradley et al., 2006) and use of pesticides (Johnson and Pflieger, 1992; Sugavanam et al., 1994; Singh and Wright, 2002) can alter the performance of AMF and rhizobia. Within pesticides, glyphosate (*N*-phosphonomethyl glycine) is one of the most studied because it is a broad-spectrum herbicide massively used worldwide for its effective weed control and low toxicity in mammals (Busse et al., 2001). Being rapidly degraded by microorganisms or adsorbed on soil particles, impacts of this herbicide on non-target microorganisms were assumed to be insignificant under field conditions (Anderson et al., 1993). Nevertheless, contradictory effects have been reported in agricultural crops, being positive, negative or neutral on AMF root colonization (Morandi, 1989; Mujica et al., 1999; Malty et al., 2006; Ronco et al., 2008; Powell et al., 2009; Savin et al., 2009) and neutral or negative on AMF spores (Giovannetti et al., 2006; Malty et al., 2006; Pasaribu et al., 2011). In relation to rhizobia, negative effects of glyphosate application on soybean nodulation and N<sub>2</sub> fixation have been reported (King et al., 2001; Reddy and Zablutowicz, 2003; Zablutowicz and Reddy, 2004).

The use of glyphosate is not limited to agricultural systems; it is also used in forest plantations, recreational areas and natural grasslands with the aim of eradicating exotic species or promoting winter forage species (Cole and Lunt, 2005; Barnes, 2007; Rodriguez and Jacobo, 2010; Helander et al., 2012). Glyphosate application and nitrogen fertilization are frequently used in native grasslands of the Flooding Pampa (Argentina), with the aim of increasing winter productivity (Rodriguez and Jacobo, 2010). This herbicide is sprayed in late summer to reduce competition of forbs and C<sub>4</sub> grasses, improving germination and establishment of cool-season annual C<sub>3</sub> grasses (Rodriguez and Jacobo, 2010). This practice increases winter forage production per hectare and allows improvement of stocking rate and meat production (Bilello and Zeberio, 2002). Greenhouse experiments have demonstrated that glyphosate application reduces AMF spore viability in soil from these grasslands and subsequently mycorrhizal colonization of plants (Druille et al., 2013a,b). A large reduction in spore viability was detected even when a dose that damaged but did not kill *Lolium multiflorum* plants (sub-lethal dose) was used, which demonstrates that ecosystem components may have different vulnerability to glyphosate. Additionally, it has been shown that total basal vegetation cover and plant species richness (mainly cool-season perennial grasses, warm-season tussock grasses, warm-season legumes) are negatively affected in grazed grasslands after several years of applying glyphosate and grazing (Rodriguez and Jacobo, 2010).

The objective of this study was to evaluate the effect of glyphosate application on soil availability of plant-symbiont propagules. The study was conducted in a Flooding Pampa grassland of Argentina, in which the abundance and viability of AMF and rhizobium propagules were evaluated after different doses of glyphosate application in late of summer. Although there are three types of AMF propagules (spores, external mycelium and infected root segments) we focused on spores, considering its importance as a source of propagules for AMF perpetuation and spread in the system and for optimal root colonization of plants (Smith and Read, 2008).

Additionally, changes in the plant community composition, basal cover of vegetation and litter, and soil salinity (measured as electrical conductivity of saturation extract) were analyzed, taking into account the close relationship between these variables and plant symbiont propagules (Singleton et al., 1982; Abbott and Robson, 1991; Craig et al., 1991). In these grasslands, a decrease in

vegetation cover may generate an increase in electrical conductivity, due to the ascent of salts from the B horizon toward the surface in periods of high temperature (Taboada et al., 2011). Our hypothesis is that under field conditions, glyphosate application, even at sublethal doses, reduces viable propagules of AMF and rhizobia, and increases soil salinity by reducing vegetation cover.

## 2. Materials and methods

### 2.1. Study site

The experiment was conducted in a humid mesophytic meadow, located near Ignacio Correas, in the northeast of the Flooding Pampa (35° 01' S, 57° 50' W) (Perelman et al., 2001). The average annual temperature in the region is 15.9 °C and annual precipitation is 885 mm year<sup>-1</sup> (Perelman et al., 2001). The soil is classified as a Typic Natraquoll/US Soil Taxonomy (Mollic Gleyic Solonetz/FAO Soil Taxonomy), characterized by an acidic, non-saline A1 horizon and a saline, highly alkaline B2 horizon (Lavado and Taboada, 1988), with 3.5% organic matter and 7 ppm P. Even though this type of grassland is commonly treated with glyphosate in late summer (Rodriguez and Jacobo, 2010), the study site had no history of herbicide treatment. The dominant species of the grassland are *Stenotaphrum secundatum* (Walt.) Kuntze, *Phyla canescens* (Kunth) Greene, *Lotus tenuis* Waldst. & Kit, *Eryngium ebracteatum* Lam., *L. multiflorum* Lam., *Paspalum dilatatum* Poir., *Bothriochloa laguroides* (DC) Herter, *Setaria geniculata* (Lam.) Beauv., *Chaetotropis elongata* (Kunt) Björkman, *Panicum gouinii* Fournier and *Paspalum vaginatum* Sw. Before glyphosate application, vegetation cover was measured in each experimental unit according the line intercept method (Canfield, 1941). The grassland presented a percentage of vegetation cover, litter and bare soil of 69%, 14% and 17%, respectively.

### 2.2. Experimental design and herbicide application

Experimental units were 30 plots of 2.25 m<sup>2</sup> randomly selected, with a similar floristic composition. The experiment had a completely randomized design with three glyphosate levels: 0, 384 and 1440 g acid equivalent ha<sup>-1</sup> (control, sublethal and recommended dose, respectively), with 10 replicates per treatment. Glacoxan<sup>®</sup> (48 g isopropylamine salt of glyphosate in 100 cm<sup>3</sup> of inerts and adjuvants) was applied in late summer (15th March), using a knapsack sprayer with a 20 L tank, operating at constant 3 bar pressure. In control plots, the sprayer was used to apply water in the same volume as in plots treated with glyphosate. During the course of the experiment, plots were kept surrounded with electric wire to prevent cattle grazing.

### 2.3. Measurements

#### 2.3.1. Spore separation and identification

Considering that AMF community is determined by host plant species (Bever, 2002), 300 g soil samples associated to roots of two species from different functional groups according to their life-form and ability to form symbiotic relationships with N<sub>2</sub>-fixing organisms (i.e. the perennial legume *L. tenuis* and the perennial grass *Paspalum dilatatum*) were collected 15 days after glyphosate application. Soil cores (8 cm diameter) were taken to a depth of 10 cm. Part of this rhizosphere soil was oven-dried (105 °C) to reach constant weight, in order to estimate soil moisture. With the remaining rhizosphere soil, two sievings were performed for spore extraction through wet sieving technique and decanting (Gerde-mann and Nicolson, 1963), followed by sucrose gradient centrifugation (Walker et al., 1982). One of the extractions was used for spore number estimation, and the other one for measuring spore

viability. To assess AMF identity, each morphotype was mounted on polyvinyl alcohol–glycerol–lactic acid (PVLG) and PVLG + Melzer reagent for identification. The spores were matched with species described by International Culture Collection of VA Mycorrhizal Fungi (INVAM, <http://www.invam.caf.wvu.edu>) and Błaszczkowski (2012).

### 2.3.2. Number and viability of AMF spores

To estimate spore number, only externally healthy morphotypes were counted, by direct observation under stereomicroscope. Total spore number in each sample was corrected considering its moisture content to express this value per gram of dry soil. The An and Hendrix (1988) method was used to determine viable spores, developing a red color with the tetrazolium bromide vital stain MTT [3-(4,5-dimethylthiazol-yl)-2,5-diphenyl-2H-tetrazolium bromide]. Spore suspensions were diluted 1:1 with a solution of 0.5 mg MTT ml<sup>-1</sup> and incubated for 40 h. This determination was made primarily in the entire community and subsequently in the four species that dominate AMF spore community.

### 2.3.3. Viable propagules of rhizobia

Field soils were sampled in late spring (15th December) to determine the most probable number (MPN) of soil rhizobia capable of nodulating the selected host legume *L. tenuis*. Thirty 10 cm–diameter soil cores at a depth of 10 cm were taken, one from each experimental unit. Each soil core was mixed, subsampled for determination of moisture content, and stored at 4 °C overnight. Five serial ten-fold soil dilutions with 4 repetitions each were prepared as described by Somasegaran and Hoben (1985) with 10 g of soil on 90 ml of physiological solution for the first dilution step. Test plants growing under sterile conditions were inoculated with a 0.2 ml aliquot of the corresponding dilution. Plants were kept in sterile growing chambers supplied with an adequate volume of a sterile N-free nutrient solution. Plants were scored for nodulation 21 days after inoculation, considering as “positive” a plant with at least one functional nodule (determined by a pink color), and “negative” a plant without nodules. The MPN of rhizobia was determined considering the number of positive and negative plants in the serial dilutions (Somasegaran and Hoben, 1985). Four control plants were inoculated with the physiological solution used to prepare the dilutions. The four plants resulted negative for nodulation score.

### 2.3.4. Vegetation sampling and soil electrical conductivity

In late spring (15th December) measurements of vegetation and electrical conductivity were taken. Vegetation cover was measured according to the line intercept method proposed by Canfield (1941). As in previous studies conducted in these grasslands (Rusch and Oesterheld, 1997; Perelman et al., 2001; Rodriguez and Jacobo, 2010; Longo et al., 2013), plant species were classified into functional groups defined by life-form, phenology and the possibility to form symbiotic relationships with N<sub>2</sub>-fixing organisms: cool-season annual grasses (CSAG), cool-season perennial grasses (CSPG), warm-season grasses (WSG), cool-season legumes (CSL), warm-season legumes (WSL), sedges (S) and forbs (excluding legumes). Relative cover (%) of each functional group and bare soil plus litter (BS+L) were calculated from total basal cover. In estimating plant cover, both vegetation cover and litter were included.

Electrical conductivity (EC) is an easily measured and practical index of the total concentration of ionized solutes of a saturated soil (Rhoades et al., 1999). Therefore, to estimate soil salinity, three measurements of the EC of soil saturation extracts were performed in each plot, using a portable conductivity meter (HI93310,

HANNA®, Rhode Island, USA) (Rhoades, 1982). Soil moisture was close to field capacity at the moment of measurements.

## 2.4. Statistical analysis

One-way analysis of variance (ANOVA) was performed to determine principal effects of herbicide doses on the total number of spores, spore viability and total number of viable spores (a separate ANOVA for each plant species rhizosphere). Plant cover and EC data were also analyzed using one-way ANOVA. Treatment means were compared using Tukey test when significant *F* values were found. The significance level was set at  $\alpha=0.05$ . To obtain homogenous variances, percent data were arcsine square-root transformed before carrying out each analysis. Rhizobium number per gram of soil presented *Poisson* distribution and was analyzed using a generalized linear model (glm). Due to over dispersion of the data, “quasipoisson” family was specified to the model. The significance of the parameters estimated by the model was tested by applying Likelihood Ratio Test (LRT, R-cran Software). Floristic composition was a synthetic variable obtained by applying a Non-metric multidimensional scaling (NMS) ordination (Clarke, 1993) to plant species abundance (% cover) data. We performed one NMS per replicate of each treatment. With the location of each point on both axes a multivariate analyses of variance (MANOVA) was performed (Hotelling Test with Bonferroni correction). Statistically significant MANOVAs were followed up by simple ANOVAs and Tukey's post hoc tests to explore the trends observed for each individual parameter.

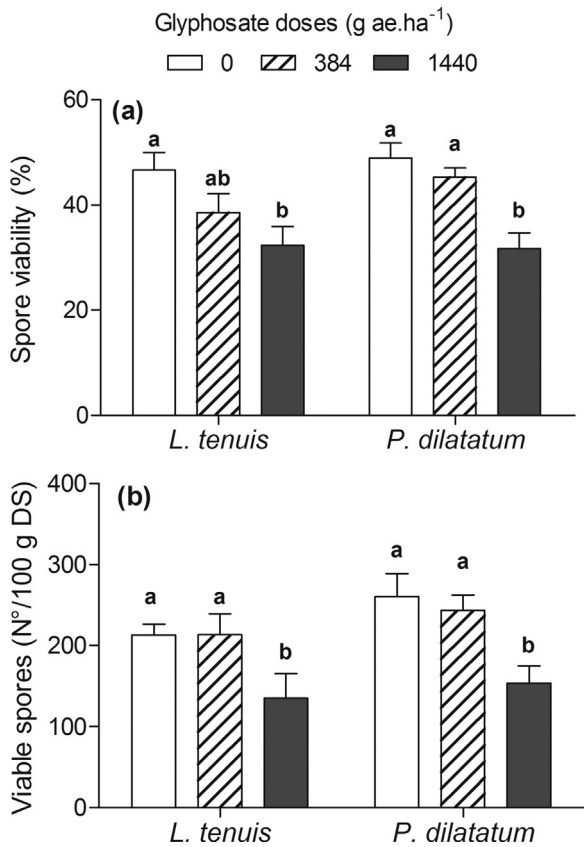
## 3. Results

### 3.1. Viable propagules of AMF and rhizobia

Total number of AMF spores present in the communities associated to *L. tenuis* and to *P. dilatatum* rhizosphere varied between 419 and 561 spores/100 g dry soil, respectively, and did not differ between the treatments ( $F_{2,27}=1.69$ ,  $P=0.202$  and  $F_{2,27}=0.22$ ,  $P=0.807$ , respectively). A total of 19 AMF morphospecies were identified in soil from all treatments (Supporting Information, Table 1). Four species accounted for 74%–89% from the total AMF spore community in all treatments: *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler, *Claroideoglossum etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler, *Septoglossum constrictum* (Trappe) Sieverd., G. A. Silva & Oehl and *Funneliformis caledonium* (T.H. Nicolson & Gerd.) C. Walker & Schüßler.

Spore viability was affected by glyphosate application in the AMF community associated to *L. tenuis* ( $F_{2,27}=4.21$ ;  $P=0.025$ ) and *P. dilatatum* ( $F_{2,27}=11.69$ ,  $P<0.001$ ). There was a significant reduction in viability of 31% and 35% in the AMF community associated to *L. tenuis* and *P. dilatatum*, respectively, when the highest dose of the herbicide (1440 g ae ha<sup>-1</sup>) was applied. In the case of the AMF community associated with *L. tenuis*, a tendency to decrease was demonstrated when applying 384 ae ha<sup>-1</sup> (see Tukey's results in Fig. 1a). Therefore, the number of viable spores (resulting from multiplying the number of spores of each treatment by the percentage of viability) was reduced by 36% and 41% when the higher dose of glyphosate was applied (Fig. 1b) in both communities ( $F_{2,27}=3.44$ ,  $P=0.046$  and  $F_{2,27}=5.80$ ,  $P=0.008$  in *L. tenuis* and *P. dilatatum*, respectively).

When focusing the analysis on the four species dominating the community (Supporting Information, Table 1), a significant interaction between species and glyphosate was observed ( $F_{6,115}=2.25$ ,  $P=0.043$ ). Spore viability was significantly reduced by 52% and 72% in *F. caledonium* and *S. constrictum*, respectively, but not in the other two species: *F. mosseae*, *C. etunicatum* (Fig. 2).

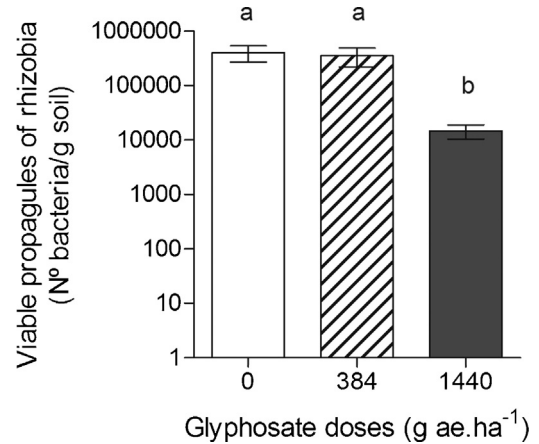


**Fig. 1.** Percent of spore viability (a) and number of viable spores (b) associated to *L. tenuis* and *P. dilatatum* soils, subjected to different glyphosate doses. Values represent means  $\pm$  S.E. of ten repetitions. The same letter above bars indicates that the values did not differ significantly between treatments according to ANOVA and Tukey test ( $P \leq 0.05$ ).

Glyphosate application significantly reduced the viable propagules of rhizobia ( $\chi^2_{2} = 10.9$ ,  $P = 0.004$ ). In soils treated with the recommended dose, viable propagules of rhizobia was more than ten-fold lower than in untreated soils (Fig. 3).

### 3.2. Floristic composition

A total of 38 plant species were identified in plots from all treatments (Supporting Information, Table 2). Plant functional

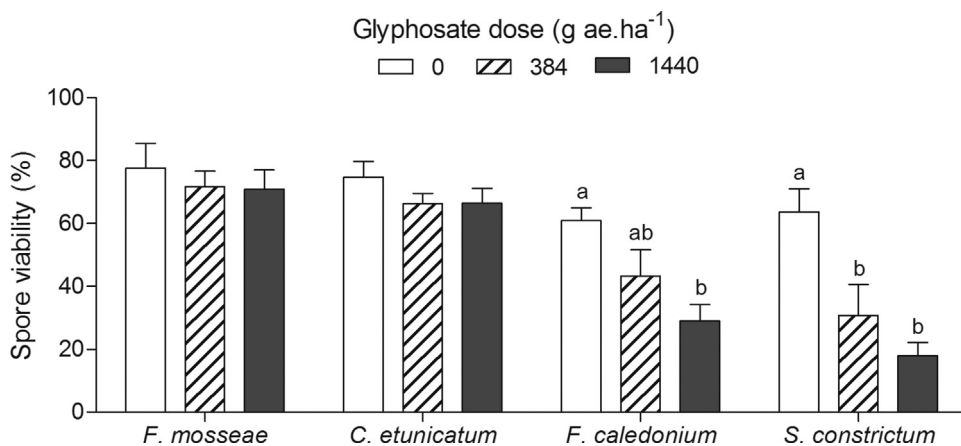


**Fig. 3.** Rhizobial bacteria density in soils treated with different doses of glyphosate according to most probable number estimation after ten-fold successive dilutions of the soil solution. Values represent means  $\pm$  S.E. of ten repetitions. The same letter above bars indicates that the values did not differ significantly between treatments according to ANOVA and Tukey test ( $P \leq 0.05$ ).

group composition diverged among the three doses as demonstrated by the non-metric multidimensional scaling and MANOVA analyses ( $P < 0.001$ ). The best solution for the NMS ordination was made up by two significant axes (Monte Carlo test,  $P = 0.039$ ) with a cumulative  $r^2$  of 0.91. The final stress for 2-dimensional solution was 14. Axis 1 of the ordination diagram of the multivariate trait analysis (NMS) was closely and negatively correlated with cool-season annual grasses (CSAG,  $r = 0.73$ ), bare soil and litter (BS+L,  $r = 0.91$ ), and positively correlated with cool-season perennial grasses (CSPG,  $r = 0.89$ ), and axis 2 was closely and positively correlated with warm-season grasses (WSG,  $r = 0.73$ ) and warm-season legumes (WSL,  $r = 0.53$ ) (Fig. 4).

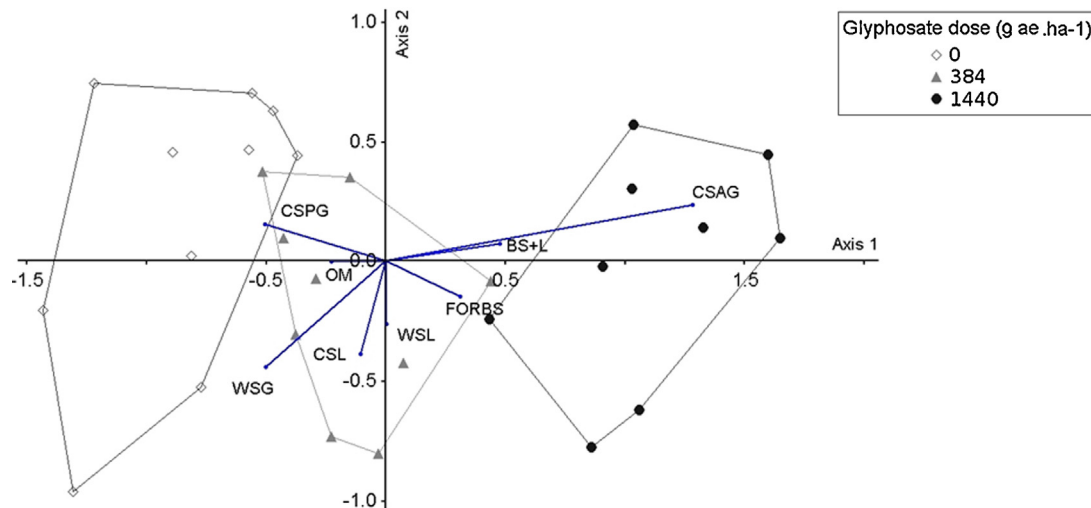
### 3.3. Vegetation cover and electrical conductivity

Vegetation cover was reduced by herbicide application ( $F_{2,27} = 9.69$ ,  $P < 0.001$ ), obtaining a similar reduction when doses of 384 and 1440 ae.ha<sup>-1</sup> were applied. In contrast, electrical conductivity was increased by herbicide application ( $F_{2,27} = 8.64$ ,  $P = 0.003$ ) being significantly higher in plots treated with the highest dose of glyphosate, surpassing the electrical conductivity of the control plots by 50% (Fig. 5).

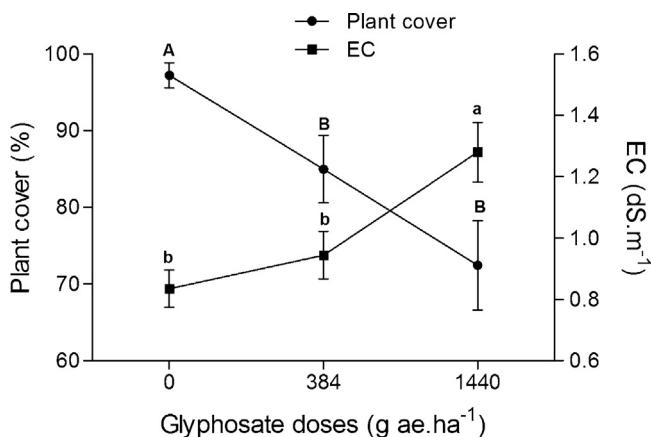


**Fig. 2.** Spore viability of the four dominant species of AMF in soils treated with different doses of glyphosate. Values represent means  $\pm$  S.E. of five repetitions. The same letter above bars indicates that the values did not differ significantly between treatments according to ANOVA and Tukey test ( $P \leq 0.05$ ).





**Fig. 4.** Ordination plot of vegetation data based on a non-metric multidimensional scaling (NMS). Plots are grouped according to bare soil and litter (BS+L), and plants functional groups: cool-season annual grasses (CSAG), cool-season perennial grasses (CSPG), warm-season grasses (WSG), cool-season legumes (CSL), warm-season legumes (WSL), sedges (S) and forbs. Length and direction of the vectors shown represent the correlations between functional groups and each axis.



**Fig. 5.** Plant cover (%) and electrical conductivity in plots treated with different glyphosate doses (0, 384 and 1440 g ae ha<sup>-1</sup>). Values represent means  $\pm$  S.E. of ten repetitions. Same letters indicate that values are not significantly different between treatments according to ANOVA and Tukey test ( $\alpha < 0.05$ ).

#### 4. Discussion

To our knowledge, this is the first time that the influence of glyphosate application on root-symbionts in native grasslands is studied under field conditions. Our results indicate that this herbicide negatively affects AMF and rhizobia propagule viability, when the recommended dose is applied. Given the benefits that these symbionts confer to their host plant, it is likely that nutrient availability and protection against pathogens would be reduced if propagules viability decreased, and external inputs such as pesticides and fertilizers would be required to maintain productivity and quality of these grasslands (Tikhonovich and Provorov, 2007; Gianinazzi et al., 2010; Andrews et al., 2011). This intensification of forage production systems may unfavorably impact on economic results and on the environment (Tilman et al., 2001).

AMF spore viability was reduced in the entire community and in two of the four dominant species. This result suggests that the lack of response found in the abundance of spores may be explained by the fact that after the application of drastic treatments, seemingly alive spores, which are actually dead, may persist in soil for extended periods (McGraw and Hendrix, 1986). Therefore, it could

be argued that the AMF propagule availability was reduced due to one glyphosate application, since the percentage of viability decreased. Furthermore, AMF community structure may also be affected, as the reduction in viability was not similar between the four species that dominated spore community. A decrease in the relative abundance of *S. constrictum* and *F. caledonum* and an increase in *C. etunicatum* and *F. mosseae* might be expected as direct effects of this herbicide. These results agree with those reported by Sheng et al. (2012), who detected changes in the structure of the AMF community with glyphosate application in agricultural crops, with *F. mosseae* being less sensitive to this herbicide. These changes in AMF relative abundance and identity may eventually alter plant community composition and diversity, for example through the modification of plant competition interactions (van der Heijden et al., 1998a,b,b; Scheublin et al., 2007).

Similarly as described above for AMF, the number of viable propagules of rhizobia was also decreased in plots treated with 1440 g ae ha<sup>-1</sup> of glyphosate. Previous studies reported negative effects of this herbicide on nodulation (Reddy et al., 2001; dos Santos et al., 2005) and bacteroid nitrogenase activity (Hernandez et al., 1999) under controlled conditions, and nodule biomass in soybean under field conditions (Reddy and Zablotowicz, 2003). Moreover, it has been shown that glyphosate sensitivity depends on the strain of rhizobia (Zabaloy and Gómez, 2005), which could lead to changes in the community of these symbionts with repeated applications of this herbicide. The reduction in rhizobium propagule availability in natural grasslands could lead to a loss of basal legume cover, thus affecting soil N pools, productivity and forage quality (Graham and Vance, 2003).

Indirect effects of glyphosate application on root-symbiont propagules could be expected, mediated by the changes detected in plant community structure. On the one hand, the shift in the floristic composition in plots treated with glyphosate once, mainly explained by a reduction in C<sub>4</sub> grasses and legumes and an increase in forbs basal cover, may eventually affect AMF community (Bever et al., 1996; Johnson et al., 2004). In this study, the detected decrease in AMF spore viability only reflects a direct effect of glyphosate, since it was estimated 15 days after application, when no changes in floristic composition were still evident. The reduction in legume cover may, in turn, compromise rhizobium community, since their fitness depends on the reproductive success during the symbiotic stage (Denison, 2000). On the other

hand, plots treated with 1440 ae ha<sup>-1</sup> presented higher soil salinity, probably due to the reduction in vegetation cover (Taboada et al., 2011). Previous studies have demonstrated that soil salinity delays germination and limits growth of hyphae from propagules of AMF (McMillen et al., 1998; Juniper and Abbott, 2006), and decreases the number of nodules per plant and the amount of nitrogen fixed per unit weight of nodules (Manchanda and Garg, 2008). Since rhizobium response was measured when the plant community structure was already modified due to glyphosate application (late spring), the observed reduction in their number of viable propagules could be due to direct and/or indirect effects of this herbicide.

More empirical research is required to evaluate the physiological or molecular mechanisms underlying root-symbionts response in our field study. Glyphosate causes the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase activity, leading to the inhibition of aromatic amino acid (phenylalanine, tyrosine, and tryptophan) biosynthesis, the reduction of protein production and the prevention of secondary product formation (Franz et al., 1997). Since the ESPS enzyme is present not only in plants but also in bacteria and fungi (Padgett et al., 1995), it can be speculated that alterations in the ESPS enzyme might explained, at least in part, the obtained results. Further studies should also evaluate possible toxic effects generated by adjuvants accompanying the active ingredient, or degradation products of the herbicide, as AMPA (aminomethylphosphonic acid).

A single glyphosate application at recommended dose was enough to reduce the number of viable propagules of AMF and rhizobia. The increase in the percentage of bare soil generated by glyphosate suggests that subsequent applications may magnify the detected damages since a larger amount of active product could contact the root-symbiont propagules. It has been demonstrated under greenhouse conditions that this herbicide reduces AMF spore viability when applied on bare soil, while spores remain intact when glyphosate is applied to plant foliage (Druille et al., 2013b). Considering the low nutrient availability and high root-symbiont dependency of several species with high forage value (van der Heijden et al., 2008), repeated applications might lead to a loss in the grasslands quality and productivity, decreasing livestock production. The use of sublethal doses of glyphosate could avoid the damage generated on the root-symbionts, although success in the establishment of winter forage species would be less. Additionally, other tools that would achieve the same objective as glyphosate, such as intensive grazing or mechanical cutting (Deregibus et al., 1994; Arzadun and Mestelan, 2009), should be considered.

To conclude, we found that glyphosate can play a role in determining root-symbiotic relationships in grassland communities. In the short term, glyphosate application directly reduces the abundance of viable root-symbiont propagules. In the long term, this herbicide could indirectly affect AMF and rhizobium communities by modifying plant community and increasing soil salinity levels. Based on these results, we suggest that this management practice will affect the productivity and stability of these systems, where nutrient availability is low, decreasing livestock production. It is therefore necessary to continue studying the impact that this highly popular herbicide generates on non-target species in order to design sustainable land management systems.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.agee.2014.12.017>.

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