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Repeated annual glyphosate applications may impair beneficial soil microorganisms in temperate grassland

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ABSTRACT

Due to the worldwide use of the herbicide glyphosate, there is a growing interest in understanding its impact on beneficial soilmicroorganisms. However, most studies have been focused on evaluating the effects on these microorganisms of a single application in agricultural crops, despite the fact that repeated applications is a common scenario in different production systems. We evaluated the impact of four annual glyphosate applications on arbuscular mycorrhizal fungi (AMF), dark septate endophytes (DSE) and free-living diazotrophs in a temperate grassland. Sub-lethal (0.8 l ha^{-1}) and recommended field doses (3 l ha^{-1}) were analyzed. AMF viable spores and free-living diazotrophs densities were reduced by 56% and 82% respectively, after the fourth application even at sublethal dose. While total AMF root colonization in *Lolium arundinaceum* was not affected among treatments, arbuscules percentage was reduced in plants grown in plots treated with 3 l ha^{-1}. A similar response was detected in DSE root colonization. Considering the role they have in structuring plant communities, these deleterious effects on beneficial soil microorganisms might negatively impact on grassland productivity and diversity. It is necessary to investigate the resilience of the microbial community in order to develop a long-term strategic management of glyphosate applications that would achieve the desired objectives without irreversibly affecting soil biota.

1. Introduction

There is growing recognition of the role that reciprocal interactions between plants and beneficial soil microorganisms (BSM) play in determining plant communities structure and dynamics (Reynolds et al., 2003; van der Heijden et al., 2008). This group of microorganisms includes many species of fungi and bacteria, being arbuscular mycorrhizal fungi (AMF), dark septate endophytes (DSE) and free-living diazotrophs particularly highlighted due to their presence in most worldwide ecosystems (Jumpponen and Trappe, 1998; Postgate, 1998; Smith and Smith, 2011). They enhance plants performance through increases in nutrient and water availability and/or protection against pathogens (Dobbelaere et al., 2003; Smith and Read, 2008; Newsham, 2010). Therefore, the presence of BSM in ecosystems allows the coexistence of various plant species by reducing intra- and interspecific competition for soil resources (Reynolds et al., 2003) and avoiding loss of competitiveness in species more susceptible to pathogens (Dobson and Crawley, 1994). These benefits may result from additive effects of each microorganism, or from synergistic effects arising from the interaction among them (e.g. Awasthi et al., 2011; Ramasamy et al., 2011).

In recent years, there has been increasing interest in understanding how different agricultural practices affect BSM. Within agrochemicals, glyphosate (*N*-phosphonomethylglycine) is one of the most frequently used worldwide due to its effective weed control and low mammalian toxicity (Busse et al., 2001). It is a systemic non-selective herbicide, which inhibits 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, thereby interrupting synthesis of aromatic amino acids, lignin, flavonoids, phenolics and other secondary metabolites (Shah et al., 1986; Franz et al., 1997). Glyphosate is not exclusively used in agricultural crops, but also in forest production (Tanney and Hutchison, 2010) and natural grasslands for the eradication of exotic species or promotion of forage species (e.g. Barnes, 2007; Rodriguez and Jacobo, 2010).

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Thus, there is a growing interest in assessing the responses of non-target organisms, such as bacteria and fungi, to glyphosate application in different ecosystems (Araújo et al., 2003; Kremer et al., 2005; Ratcliff et al., 2006; Arango et al., 2014; Zaller et al., 2014; Cherni et al., 2015; Newman et al., 2016), since they also present the enzyme EPSP synthase (Padgette et al., 1995).

Glyphosate effects on BSM have been mostly evaluated in agricultural systems, with less focus on grassland ecosystems. In all cases, responses related to a single glyphosate application were evaluated. In agricultural crops, glyphosate application effects on AMF have been highly variable, depending on host plant and AMF species, glyphosate doses, application site and/or type of substrate used in each case (e.g. Morandi, 1989; Giovannetti et al., 2006; Ronco et al., 2008; Hart et al., 2009; Powell et al., 2009). In grassland communities, a reduction in AMF root colonization was detected under controlled conditions and a reduction in spore viability under controlled and field conditions (Druille et al., 2013a, b, 2015). It has also been demonstrated that there are different sensibilities to glyphosate application among AMF species under field conditions, both in agricultural crops (Sheng et al., 2012) and grasslands (Druille et al., 2015). There are very few studies that have evaluated glyphosate impact on free-living diazotrophs, none of which were conducted in grassland communities. There have been reports of negative effects of a single application at high doses (above the recommended field doses) and different sensibilities among species under controlled conditions (Mårtensson, 1993; Santos and Flores, 1995). Otherwise, Angelini et al. (2013) detected a decrease in free-living diazotroph population and alteration of the community structure in peanut-growing areas even at recommended field doses. Finally, no studies have reported the effect of glyphosate application on DSE in any system.

To date there is no information about the impact of repeated annual glyphosate applications on BSM, even though this is a very common scenario in production systems worldwide (McCormack, 1994; Culpepper et al., 2006; Rodriguez and Jacobo, 2010). On the one hand, several applications of this herbicide might decrease glyphosate retention rate by soils due to the reduction of binding sites (Barrett and McBride, 2007; Shushkova et al., 2010). In addition, recurring applications may also prolong glyphosate persistence due to decreased rates of biodegradation (de Andréa et al., 2003), resulting in glyphosate accumulation in soils and increasing possible direct effects on BSM. On the other hand, indirect effects on these microorganisms caused by changes in host plant vigor (Druille et al., 2013b), root exudates (Bürgmann et al., 2005; Kremer et al., 2005), and plant community structure (Bever, 1999; Kowalchuk et al., 2002; Zak et al., 2003; Johnson et al., 2004) might increase with successive annual glyphosate applications. Therefore, field studies with appropriate spatial and temporal scale to recognize the outcome of reciprocal interaction of plant and BSM communities are needed.

The aim of the present study was to evaluate changes in BSM abundance after four annual glyphosate applications in a temperate grassland. The experiment was conducted in a humid mesophytic meadow (Flooding Pampa, Argentina), where glyphosate is frequently applied with the objective of increasing winter productivity, allowing a higher carrying capacity of the livestock system. Application of this herbicide in late summer reduces competition of forbs and C_4 grasses, improving germination and establishment of cool-season annual C_3 grasses (Rodriguez and Jacobo, 2010).This assay follows the study conducted by Druille et al. (2015) in which the effect of a single glyphosate application on root-symbionts propagules under field conditions was evaluated. Given that EPSP enzyme is also present in fungi and bacteria (Padgette et al., 1995), and considering their vulnerability to environmental changes (Abbott and Robson, 1991; Craig et al., 1991; Li et al., 2015; Yan et al., 2015), we hypothesize that in the medium-term, glyphosate has detrimental effects on AMF and DSE root colonization and on AMF spores and free-living diazotrophs density. Glyphosate applications were performed at recommended field dose ($3\,l\,ha^{-1}$) and at sub-lethal dose for plants ($0.8\,l\,ha^{-1}$). The latter allows, on the one hand, the detection of different sensitivities among organisms, and on the other hand, the knowledge of potential effects due to drift situations during herbicide application.

2. Materials and methods

2.1. Study site

A four-year field experiment was conducted in a humid mesophytic meadow of a commercial farm located in the northeast of the Flooding Pampa (35°01′S, 57°50′W) (Perelman et al., 2001). This region encompasses 90,000 km^2, mostly occupied by natural grasslands, where extensive livestock farming is developed. Annual average precipitation in the region is $885\,mm\,yr^{-1}$ and average annual temperature is 15.9 °C (Perelman et al., 2001). The soil is classified as a typical Natraquoll/US Soil Taxonomy (Mollic Gleyic Solonetz/FAO Soil Taxonomy), with 3.5% organic matter and $7\,mg\,kg^{-1}$ P. While late-summer applications of glyphosate are common in these grasslands, the experimental site had no history of herbicide treatment.

At the beginning of the experiment, grassland community was dominated by warm-season (C_4) grasses (*Stenotaphrum secundatum* Walt. Kuntze, *Paspalum dilatatum* Poir., *Bothriochloa laguroides* DC Herter, *Setaria geniculata* Lam. Beauv., *Chaetotropis elongata* Kunt Björkman, *Panicum gouinii* Fournier and *Paspalum vaginatum* Sw.), cool-season (C_3) grasses (*Lolium multiflorum* Lam., *Lolium arundinaceum* Schreb. Darbysh.), warm-season legumes (*Lotus tenuis* Waldst. & Kit) and forbs (*Phyla canescens* HBK Greene, *Eryngium ebracteatum* Lam.). Most of these species are native and typical of undegraded, humid mesophytic meadows (Perelman et al., 2001). As a result of late-summer glyphosate applications, vegetation cover changed over the four years. Prior to the fourth application, plots had, on average 79, 71 and 57% of total plant cover; 76, 63 and 40% of grasses; 0.8, 0.9 and 2.3% of legumes and 0.3, 4 and 12% of forbs in the treatments of 0, 0.8 and $3\,l\,ha^{-1}$ respectively.

2.2. Experimental design and herbicide applications

Experimental units were 21 randomly selected plots of 2.25 m^2, with a similar floristic composition at the beginning of the experiment (Druille et al., 2015). The assay had a completely randomized design with three glyphosate levels: control: $0\,l\,ha^{-1}$; sublethal dose: $0.8\,l\,ha^{-1}$ and field recommended dose: $3\,l\,ha^{-1}$, (0, 384 and 1440 g active ingredient ha^{-1}, respectively), with 7 replicates per treatment. Glacoxan® (48 g isopropylamine salt of glyphosate in 100 cm^3 of inerts and adjuvants) was applied in late summer 2012, 2013, 2014 and 2015, using a knapsack sprayer with a 20 l tank, operating at constant 3 bar pressure. The sprayer was used in control plots to apply water in the same volume as in plots treated with glyphosate. During the course of the experiment, plots were kept fenced with electric wire to prevent cattle grazing.

Fifteen days after the fourth glyphosate application, samples of *Lolium arundinaceum* roots and their associated soil were taken. This species and its associated soil (approximately 3–4 cm around the roots) were used to compare the effects of glyphosate application on BSM because of its presence in plots of all treatments. This allowed us to minimize the effect of changes in plant community structure generated by glyphosate application (Rodriguez and Jacobo, 2010; Druille et al., 2015) on soil microorganisms.

2.3. Measurements

2.3.1. AMF spore number and viability

Samples of soil associated to *L. arundinaceum* (8 cm diameter) were collected up to a depth of 10 cm. Part of this soil was oven-dried (105 °C) to reach constant weight, in order to estimate soil moisture. With the remaining soil, sievings were performed for spore extraction through wet sieving technique and decanting (Gerdemann and Nicolson, 1963), followed by sucrose gradient centrifugation (Walker et al., 1982).

Estimation of total spore number was made by direct observation under a stereomicroscope at up to 90× magnification, counting only healthy looking morphotypes. Total spore number was corrected considering the moisture content of each sample to express this value per gram of dry soil. After pesticide application, dead spores that appear normal may persist in soil for extended periods (McGraw and Hendrix, 1986). Thus, it is indispensable to measure spore viability in order to determine their potential as propagules (An et al., 1998). The An and Hendrix (1988) method was used to determine the percentage of spore viability 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^{-1} and incubated for 40 h. This determination was made in the entire community associated to *L. arundinaceum*. Viable spore number was obtained from multiplying total spores number of each treatment by the percentage of viability.

2.3.2. AMF and DSE root colonization

L. arundinaceum roots were washed in tap water and cleared with 10% KOH for 15 min at 90 °C, placed in 1% HCl for 10 min and then stained with 0.05% Trypan Blue for 5 min at 100 °C (Phillips and Hayman, 1970). Twenty root fragments (ca. 1 cm long) from plants of each treatment were mounted on slides in a polyvinyl alcohol–lacticacid–glycerol solution. Root colonization by AMF and DSE was measured with the method proposed by McGonigle et al. (1990) using a compound microscope at 200× magnification. In the case of AMF, total colonized roots and the fraction of root length containing arbuscules and vesicles were determined.

2.3.3. Free-living diazotrophs

The soil associated to *L. arundinaceum* was sampled 45 days after last glyphosate application (11th May, 2015) to determine the most probable number (MPN) of cultivable free diazotrophic bacteria capable of growth in a Nitrogen-free medium under aerobic conditions. The soil associated to six independent alive *L. arundinaceum* tillers was taken from each plot, up to 5 cm depth. The soil from each plot was mixed and stored at 4 °C. A subsample of 10 g was taken for determination of soil moisture. Six serial one in ten (1/10) soil dilutions with 3 repetitions each were prepared with 10 g of soil in 90 ml of sterile physiological solution for the first dilution step. For diazotrophic bacteria determinations, vials containing 4 ml of N-free semisolid malate medium (NFb, Döbereiner et al., 1976) were inoculated with a 0.2 ml aliquot of −4, −5 and −6 dilutions. Vials were incubated at 28 °C for 5 days and were scored for diazotrophic growth. Vials were considered "positive" when the characteristic white, dense, 1 mm fine pellicle was formed above the medium, and "negative" when it was not (Döbereiner et al., 1976).

2.4. Statistical analysis

Total spores number, percentage of spore viability, viable AMF spore number, and free-living diazotrophs number were analyzed using Analysis of Variance (ANOVA), where the fixed factor was glyphosate application. To analyze several variables at once (AMF total root colonization, percentages of arbuscules and vesicles and DSE root colonization) a multivariate analysis of variance (MANOVA) was carried out, using InfoStat software (Di Rienzo et al., 2011). Bonferroni correction was performed to correct the degrees of freedom to account for multiple pair-wise tests. Pillai's trace was used as the multivariate criterion. When MANOVA showed significant results, we used univariate ANOVA analysis to determine which of the response variables was most affected by glyphosate application (Scheiner, 2001). Percent data was arcsine square-root transformed ($y = \arcsin\sqrt{x}$) to meet the assumptions of ANOVA. Numerator and denominator degrees of freedom and factor and error degrees of freedom are shown in brackets when MANOVA and ANOVAs results are reported, respectively. Treatment means were compared using Tukey test when significant F values were found. The significance level was set at $\alpha = 0.05$.

3. Results

3.1. AMF spore number and viability

In plots without glyphosate application 1172 total spores/100 g of dry soil were detected, decreasing by more than 50% in plots treated with 0.8 and 3 l ha^{-1} (ANOVA, $F_{[2,18]} = 14.56$; $P = 0.0002$) (Fig. 1A). While a trend toward reduction was found in the percentage of spore viability in soils treated with this herbicide, those differences were not significant (ANOVA, $F_{[2,18]} = 2.94$; $P = 0.0787$) (Fig. 1B). Thus, the number of viable spores was reduced by more than 56% when glyphosate was applied at sub-lethal or recommended field dose (Fig. 1C).

3.2. AMF and DSE root colonization

A significant effect of four years of glyphosate application was found on fungal traits (MANOVA, $F_{[8,32]} = 5.48$; $P = 0.0002$). Average AMF total root colonization (Fig. 2A) and vesicles percentage in *L. arundinaceum* roots (Fig. 2C) were not affected by glyphosate application (ANOVA, $F_{[2,18]} = 1.70$; $P = 0.2113$ and $F_{[2,18]} = 1.39$; $P = 0.2750$, respectively). However, a significant effect of herbicide application on the percentage of arbuscules was detected, being 61% lower in plants growing in plots treated with the highest dose (ANOVA, $F_{[2,18]} = 21.23$; $P < 0.0001$) (Fig. 2B).

Reduction in root colonization by DSE was detected in plots treated with glyphosate at field recommended dose (ANOVA, $F_{[2,18]} = 15.82$; $P = 0.0001$). The plants of this treatment presented 68% less colonization by DSE than those of other treatments (0 and 0.8 l ha^{-1}) (Fig. 3).

3.3. Free-living diazotrophs

Density of free-living diazotrophs present in the soil associated to *L. arundinaceum* was significantly reduced after four years of glyphosate applications (ANOVA, $F_{[2,18]} = 10.40$; $P = 0.0036$) at both doses. Free-living diazotrophs density in soil treated with this herbicide was 82% lower compared to the untreated soil, regardless of the applied dose (Fig. 4).

4. Discussion

Our results support the hypothesis that repeated glyphosate applications have negative effects on different types of beneficial soil microorganisms (BSM). We demonstrated that four years of late-summer applications of this herbicide in a temperate grassland negatively affected AMF, DSE and free-living diazotrophs, with the latter being the most susceptible. As far as we know, this is the first time that the impact of repeated glyphosate applications on BSM has been evaluated in grassland ecosystems.

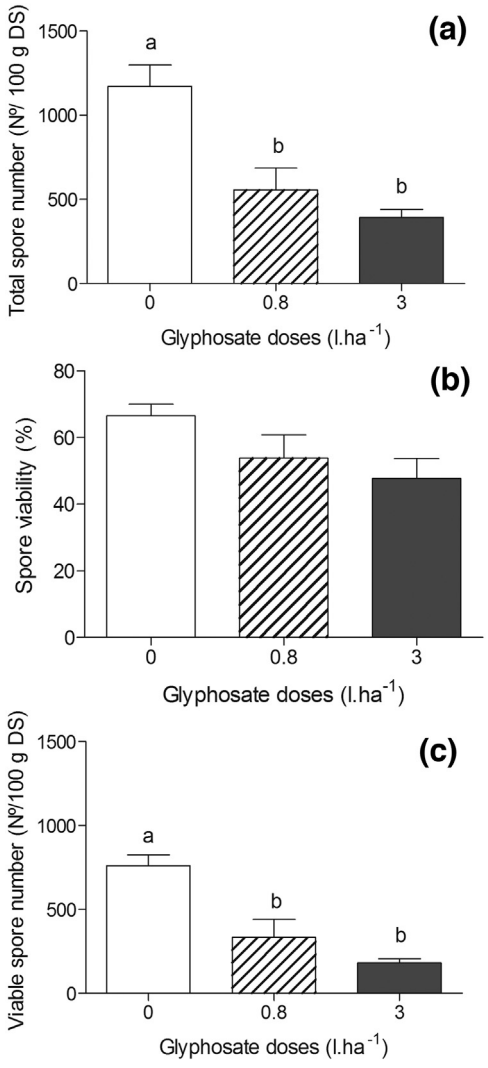

Fig. 1. Total AMF spore number (a), percentage of spore viability (b) and viable spore number (c) present in the soil associated to *Lolium arundinaceum* 15 days after the fourth annual glyphosate application. Error bars indicate standard error estimates. The same letter above bars indicates that the values did not differ significantly between treatments according to ANOVA and Tukey test ($P \leq 0.05$).

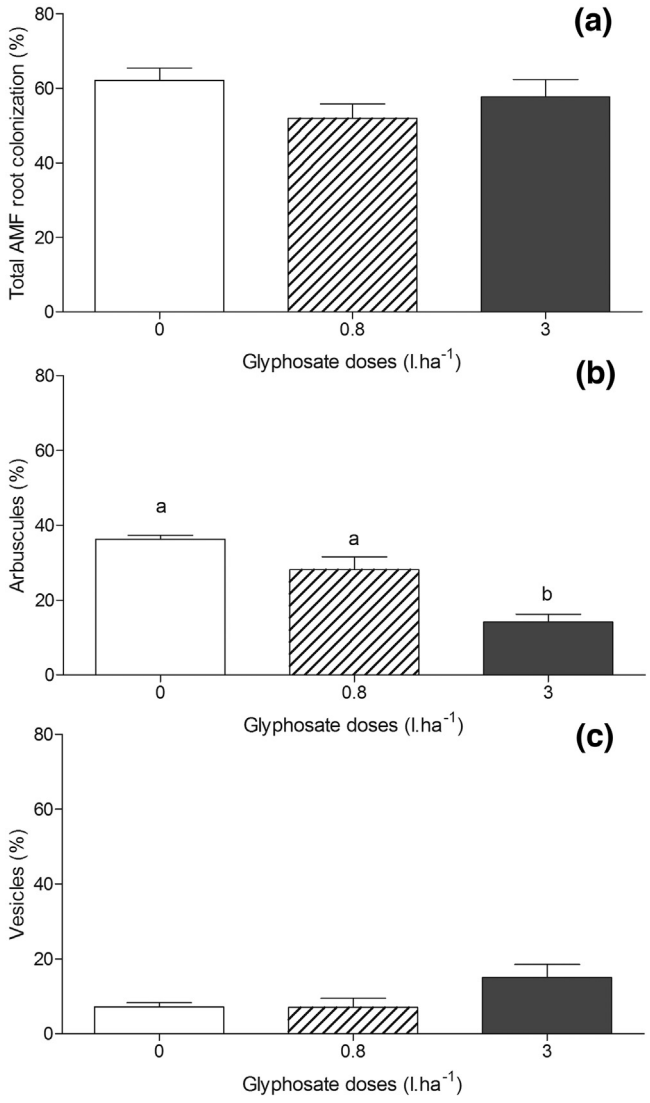

Fig. 2. Total root colonization (a), arbuscules (b) and vesicles (c) in plants of *Lolium arundinaceum* 15 days after the fourth glyphosate application. Error bars indicate standard error estimates. The same letter above bars indicates that the values did not differ significantly between treatments according to ANOVA and Tukey test ($P \leq 0.05$).

Our approach was adequate to detect that, even at sublethal doses, this herbicide has a substantial influence on the contribution of different BSM on total soil community composition. The responses found could be due to the salt of glyphosate itself, coadjutants and/or degradation products (e.g. AMPA). Regardless of the mechanisms involved, it can be expected that these deleterious effects on BSM generated by current agro-ecosystems management might negatively affect productivity and diversity of plant communities (van der Heijden et al., 1998, 2008; Dobbelaere et al., 2003; Newsham, 2010).

The number of AMF viable spores was reduced by four years of annual glyphosate application, even when a dose much lower than the recommended one was applied. This response was also detected with a single application, although only at recommended field dose (Druille et al., 2015). The decrease in viable spore number with a single application was due to a reduction in the percentage of spore viability, the total number being unaffected. Conversely, after four late-summer applications, there was a reduction in total number of spores, with no differences in the percentage of spore viability between treatments. The repeated use of glyphosate may have created a selection pressure on the

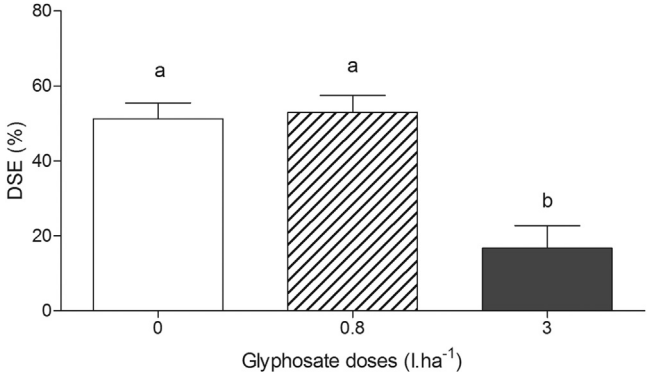

Fig. 3. Percentage of dark septate endophytes (DSE) colonization in roots of *Lolium arundinaceum* 15 days after the fourth glyphosate application. Error bars indicate standard error estimates. The same letter above bars indicates that the values did not differ significantly between treatments according to ANOVA and Tukey test ($P \leq 0.05$).

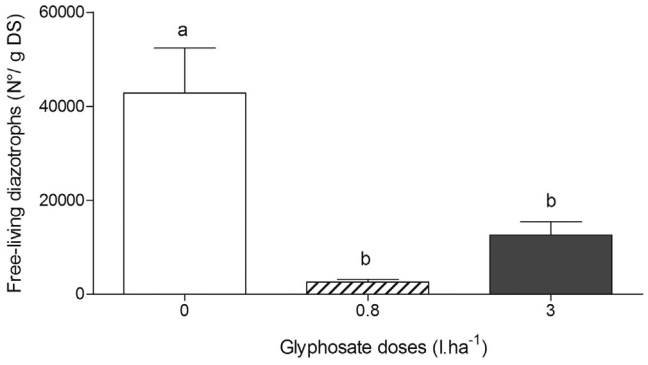

Fig. 4. Free-living diazotrophs in the soil associated to *Lolium arundinaceum* 15 days after the fourth glyphosate application. Error bars indicate standard error estimates. The same letter above bars indicates that the values did not differ significantly between treatments according to ANOVA and Tukey test ($P \leq 0.05$).

AMF community, since there is proof of different sensibilities to this herbicide among spores of different species (Sheng et al., 2012; Druille et al., 2015). The smaller number of viable spores may reduced the ability of these fungi to disperse spatially and to resist adverse conditions (Allen, 1987; Warner et al., 1987; Abbott and Robson, 1991).

Although AMF total root colonization in *L. arundinaceum* did not vary between treatments, percentage of roots colonized by arbuscules was significantly lower in plants grown in treated plots than in those untreated. On the one hand, this reduction could be generated directly through glyphosate that ended up on the soil surface and then reached fungi structures. This decrease in AMF viable spores and a reduction in external hyphae might then lead to a lower arbuscules formation. On the other hand, this reduction could be generated indirectly, by the reduction of carbohydrate flux from host plant to internal fungal structures (Druille et al., 2013b). Arbuscule percentage diminution implies that the symbiosis functionality is being affected by the herbicide, as these structures are the main site of nutrients exchange between host plant and fungus (Smith and Gianinazzi-Pearson, 1988). DSE root colonization was also reduced in plants grown in plots treated at recommended field dose, this study being the first report of negative effects of glyphosate application on these beneficial soil fungi. Changes generated by this herbicide in other soil organisms, which in turn might influence the functioning of AMF and DSE (e.g. Garbaye, 1994; Klironomos and Moutoglis, 1999; Gormsen et al., 2004) should not be discarded. A lower functionality of AMF and DSE could lead to reductions in plants productivity and diversity due to reduced nutrient availability and inferior resistance to drought and pathogens (Newsham et al., 1995; Augé, 2001; Addy et al., 2005; Smith and Read, 2008; Newsham, 2010).

Free-living diazotroph number decreased by over 70% when sublethal or field recommended doses of glyphosate were applied. This response is consistent with those reported by Angelini et al. (2013) who detected a decrease in the size of nitrogen-fixing bacterial population after a single application of 3.5 l ha^{-1} of this herbicide under greenhouse conditions. Santos and Flores (1995) reported reductions in viable cells number and size of *Azotobacter chroococcum* and *A. inelandii*at high doses in culture medium. The inhibition of EPSP synthase and glyphosate divalent metal cation chelation properties are proposed by these authors as possible mechanisms. Free-living diazotrophs convert N_2 into ammonia, which can be used by plants (Dobbelaere et al., 2003). Additionally, phytohormones production by these microorganisms and increased mineral uptake by plants (Dobbelaere et al., 2003) have been reported. Given that bacterial symbiotic diazotrophs are also susceptible to glyphosate (Hernandez et al., 1999; Reddy et al., 2001; Cherni et al., 2015; Druille et al., 2015), reductions in biological N fixation and plant mineral uptake (Dobbelaere et al., 2003) could be expected in this grassland.

In summary, this study demonstrates deleterious consequences of four years of late-summer glyphosate applications on BSM in grassland communities. AMF spores and free-living diazotrophs, with higher exposition to direct contact with the herbicide, were adversely affected even at sublethal doses (the latter being more susceptible than AMF). Fungal structures located within the root (arbuscules and DSE) were also impaired, but only at the field recommended dose. Taking into account the low nutrient availability and late spring to summer droughts typical of these environments (Soriano et al., 1991), the loss of the benefits provided by these microorganisms to plants, may in the long term, generate a reduction in the grassland productivity (van der Heijden et al., 2008).This would also adversely affect the profitability of livestock enterprises, due to decreases in meat production or increases in the amount of inputs needed to maintain production levels. These results should be complemented by future research to elucidate the mechanisms involved in the responses found. In addition, the effects of alternative management practices that could achieve the same objective (e.g. late-summer intensive grazing, mechanical defoliations or the application of less harmful herbicides) on BSM should be evaluated.

Future studies should consider direct effects due to glyphosate contact with soil and indirect effects generated by alterations in the plant physiology and plant community. Changes in soil biotic (*e.g.* predators, fungivorous, pathogens) and abiotic (*e.g.* pH, salinity) characteristics not considered in this study should also be taken into account. Furthermore, various groups of soil microorganisms should be included, since the responses may not be additive because of synergism among them (Garbaye, 1994; Tsimilli-Michael et al., 2000). Finally, it is necessary to investigate the resilience of the microbial community, to develop a long-term strategic management that would achieve the desired objective of controlling undesirable plant species without irreversibly affecting soil biota.

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