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# Glyphosate reduces spore viability and root colonization of arbuscular mycorrhizal fungi

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ABSTRACT

Glyphosate is the most widely used herbicide in the world, but its effects on non-target organisms, such as arbuscular mycorrhizal fungi (AMF), are unclear. No studies have been found that made reference to effects of glyphosate on AMF spore viability despite its importance as a source of propagules for the perpetuation and spread of AMF in the system. The objective of this study was to evaluate the effect of glyphosate application on AMF spore viability, and their ability to colonize roots. Soil samples were collected from a grassland area located in the Flooding Pampa region (Argentina). We evaluated three herbicide rates: 0, 0.26 and 1× recommended field rate, 10 and 30 days after application. Part of the soil from each tray was used to estimate the spore viability, and the remainder was used as substrate for growing *Lolium multiflorum* Lam. One month after sowing, total root colonization and percentage of arbuscules and vesicles were determined. The spore viability in herbicide untreated soils was between 5.8- and 7.7-fold higher than in treated soils. This reduction was detected even when the lower rate was applied. Root colonization was significantly lower in plants grown in glyphosate treated soil than in untreated ones. A decrease in arbuscular colonization (but not in vesicles) was found in plants grown in soils treated with the highest herbicide rate. That would indicate that symbiosis functionality was affected, given that arbuscules are the main site for host–fungus nutrient exchange. The results indicate that soil residence time of glyphosate and/or its degradation products was enough to reduce AMF spore viability and their ability to colonize roots. This decrease in propagules viability may affect plant diversity, taking into account the different degrees of mycorrhizal dependency between plant species that may coexist in grassland communities.



## 1. Introduction

Arbuscular mycorrhizal fungi (AMF), common to all world ecosystems (Brundrett, 2009), are obligate biotrophs horizontally transmitted by three kinds of propagules from the soil: spores, external mycelium and infected root segments (Smith and Read, 2008). They obtain photosynthates from host plants in exchange for an improvement in their access to water and low-mobility soil minerals, drought tolerance and resistance against pathogenic infections (Augé, 2001; Smith and Read, 2008). The plant–AMF relationship has species-specific traits. On the one hand, different plant species have different degrees of mycorrhizal dependency (Habte and Manjunath, 1991). On the other hand, growth response of colonized plants varies according to colonizing AMF specific identity (Bever, 1999), leading to changes in their competitive abilities, and thus affecting plant community structure. As a consequence, AMF may play a key role in the definition of plant diversity, ecosystem variability and productivity (van der Heijden et al., 1998).

AMF community function and its interactions with plant community, are severely affected by human induced disturbances, such as cattle rangeland use or pesticide use. For instance, livestock can cause increases, decreases or null effects on the percentage of root colonization, depending on host plant type and type of plant tissue removed (Barto and Rillig, 2010). Furthermore, AMF spores and extra-radical hyphae were not affected or enhanced by defoliation of perennial grasses or mixes of perennial grasses and forbs (Barto and Rillig, 2010). Effect of pesticides can be even more variable, depending on the pesticide formulation, dosage, soil type, environmental conditions and specific identity of both host plants and AMF (Jansa et al., 2006).

Although glyphosate (N-phosphonomethylglycine) is the most widely used herbicide in the world due to low production costs and high efficiency (Baylis, 2000), its effects on non-target microorganisms are still unclear. Regarding symbiotic microorganisms,

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negative effects were observed on $N_2$ fixing bacteria (Reddy et al., 2000), and recent studies have shown conflicting results about the effect of glyphosate on AMF root colonization, ranging from increases to decreases or neutral (Morandi, 1989; Malty et al., 2006; Ronco et al., 2008). Besides, no studies have been found in the relevant literature that made reference to effects of glyphosate on AMF spore viability. Understanding possible AMF alterations caused by glyphosate is essential, given the widespread use of this herbicide, not only for weed control in agricultural crops, but also to eradicate exotic species or promote cool-season ($C_3$) annual grasses in grasslands (Barnes, 2007; Rodriguez and Jacobo, 2010).

After the application of drastic treatments, seemingly alive spores, which are actually dead, may persist in soil for extended periods (McGraw and Hendrix, 1986). For this reason, it is essential to measure spore viability in order to determine their potential as propagules (An et al., 1998). The objectives of this paper were to assess the effect of glyphosate application on AMF spore viability and their ability to colonize roots. We tested the hypothesis that this herbicide decreases spore viability and, therefore, root colonization. Results of this study can improve the knowledge of deleterious effects of glyphosate on the function of non-target soil microorganisms, which is necessary for the development of sustainable management practices.

## 2. Materials and methods

### 2.1. Study site and sample collection

Soil samples were collected in spring 2010 from a grazed grassland located near Azul, in the center of the Flooding Pampa region (36°40′S, 59°32′W, 80 m above sea level). The average annual temperature in the region is 14.6 °C, and annual precipitation is 895 mm $yr^{-1}$ (Perelman et al., 2001). The soil was classified as a typical Natraquoll/US Soil Taxonomy (Mollic Gleyic Solonetz/FAO Soil Taxonomy), with 3.5% organic matter and 5.6 ppm P. The grassland was dominated by a humid mesophytic meadow community (Perelman et al., 2001), and dominant species include *L. multiflorum* Lam., *Paspalum dilatatum* Poir., *Bothriochloa laguroides* (D.C.) Herter, *Sporobolus indicus* (L.) R. Br., *Panicum milioides* Nees ex Trin., *Nassella neesiana* (Trin. & Rupr.) Barkworth, *Briza subaristata* Lam., *Piptochaetium montevidensis* (Spreng.) Parodi, and *Danthonia montevidensis* Hack. & Arechav. In this type of grasslands, glyphosate is applied in late summer to promote the growth of winter annuals (Rodriguez and Jacobo, 2010), but this field had no history of herbicide treatment. Composite soil samples from the top 10 cm were taken, packed, labeled and transported to the laboratory. Samples were mixed and sieved using a 2 mm mesh to remove plant tissues, and the equivalent of 160 g dry soil was placed in 21 × 16 cm trays, 2 cm deep.

### 2.2. Experimental design

We used a completely randomized design with a 3 × 2 factorial array of treatments and five replications. Factors were herbicide rate and sampling date. Single trays were the experimental units. Three levels of glyphosate rate were used: 0, 0.8 and 3 l $ha^{-1}$ (0, 0.26 and 1× recommended field rate, respectively). Sampling date had two levels: 10 and 30 days after application.

In addition to determining effects of glyphosate on spore viability we also assessed plant mortality for each herbicide rate. To do this we set up 15 additional trays in which we grew *L. multiflorum* (15 plants per tray) for 1 month prior to treatment. We included five of these trays at each herbicide rate during glyphosate application. Seven days later, we evaluated the survival rate (as the percentage, out of 15, of plants alive within a tray) and chlorophyll content in leaves using a Minolta chlorophyll meter SPAD 502. The latter was measured to assess the vigor of the plants after herbicide application. This information is presented in Table 1.

**Table 1**
Survival rate and chlorophyll content in plants of *L. multiflorum* subjected to different rates of glyphosate[a].

| Rate (l $ha^{-1}$) | Survival rate (%) | Chlorophyll content (SPAD units) |
|---|---|---|
| 0 | 94.8[a] ± 3.18 | 24.51[a] ± 0.97 |
| 0.8 | 66.6[b] ± 6.96 | 12.89[b] ± 1.55 |
| 3 | 32.2[c] ± 4.91 | 9.24[b] ± 0.67 |

[a] Values are mean ± S.E. of 5 replications. Similar lower-case letters indicate similar means among treatments according to Tukey's test ($P \leq 0.05$).

### 2.3. Herbicide application and growing conditions

The herbicide was applied using a stationary spray, with a flat fan nozzle (Bertolotti 110SF04), delivering a volume of 120 L $ha^{-1}$ at a speed of 4.6 km $h^{-1}$ and a pressure of 3 bar. Control trays only received water. After application, the trays were irrigated with 200 $cm^3$ of water to allow the incorporation of the herbicide in depth.

Part of the soil of each tray was used to estimate the spore viability, and the remainder was used as substrate for growing *L. multiflorum*. Both the assessment of spore viability and the sowing of *L. multiflorum* were carried out in the two sampling dates proposed (10 and 30 days after application). This species was used as a host plant to assess AMF spores ability to colonize roots. Following glyphosate application, the trays were kept in a greenhouse at 25–35 °C until harvest of *L. multiflorum*. These were distributed following a completely random design and rotated weekly. Soil was kept at field capacity during all the experiment.

### 2.4. Measurements

#### 2.4.1. AMF spore isolation and viability assessment

Spores were extracted from 50 g sub-samples of air-dried soil for each sample. They were wet-sieved and decanted (Gerdemann and Nicolson, 1963) and the supernatant was centrifuged in a sucrose gradient (Walker et al., 1982). An and Hendrix (1988) procedure 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 $ml^{-1}$ and incubated for 40 h.

#### 2.4.2. Percentage of root colonization

Plants were harvested 33 days after sowing. 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% lactic–glycerol–Trypan Blue for 5 min at 100 °C (Phillips and Hayman, 1970). A total of 20 root fragments (ca. 1 cm long) from each plant were mounted on slides in a polyvinyl alcohol–lactic acid–glycerol solution and examined under microscope at ×200 magnification. Root colonization was assessed with the method of McGonigle et al. (1990); the total colonized roots, and the fraction of root length containing arbuscules and vesicles were determined.

### 2.5. Statistical analysis

Analysis of variance (ANOVA) was performed to determine principal effects of herbicide rate on survival rate and chlorophyll content in leaves (Table 1), and spore viability. Fungal traits (total root colonization, and percentages of arbuscules and vesicles) were

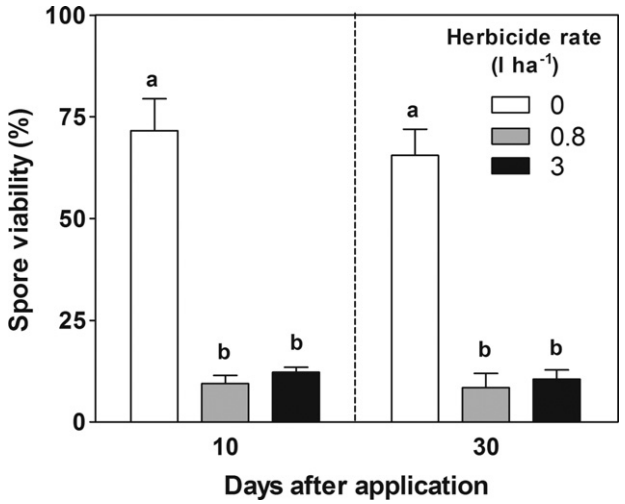


**Fig. 1.** AMF spore viability (%) under different rates of glyphosate, 10 and 30 days after application. Values are means ±SE for 5 replicates. The same letter above bars indicates that values do not significantly differ among the six treatments.

analyzed in a three-way MANOVA. When MANOVA showed significant results, we used univariate ANOVA analysis to determine which of the response variables were most affected by treatments (Scheiner, 2001). Percent data was arcsine square-root transformed ($y$ = arcsine $\sqrt{x}$) before carrying out each analysis to obtain homogenous variances. The significance level was set at $\alpha = 0.05$. Treatment means were compared using Tukey test when significant $F$ values were found.

## 3. Results

### 3.1. Spore viability

Glyphosate application significantly reduced AMF spore viability ($F_{[2,24]} = 85.60$; $P < 0.0001$) (Fig. 1). The spore viability in herbicide untreated soils was between 5.8- and 7.7-fold higher than in treated soils. Although the impact of glyphosate application on survival rate of *L. multiflorum* was statistically different among the three rates tested (Table 1), the AMF spore viability was not significantly different between rates of 0.8 and 3 l ha$^{-1}$. Neither sampling date ($F_{[1,24]} = 1.23$; $P = 0.2801$), nor the interaction between glyphosate rate and sampling date ($F_{[2,24]} = 0.07$; $P = 0.9312$) were significant, indicating that the magnitude of this reduction was similar after 10 and 30 days of herbicide application.

### 3.2. Root colonization

A significant effect of sampling date and glyphosate rate was found (but not of the interaction between them) on fungal traits (MANOVA, Table 2). Univariate ANOVA showed that the percentage of root colonization was significantly lower in plants grown in soil treated with glyphosate than in untreated soils ($F_{[2,24]} = 21.14$; $P < 0.0001$) (Fig. 2A). An effect of sampling date, with a higher percentage of root colonization 30 days post-application was also found ($F_{[1,24]} = 61.82$; $P < 0.0001$). No interaction occurred between glyphosate rate and sampling date for mycorrhizal root colonization ($F_{[2,24]} = 1.25$; $P = 0.3071$).

Analyzing the different structures of the fungus within the root, a decrease in percentage of arbuscules was found in plants grown in treated soils ($F_{[2,24]} = 14.36$; $P < 0.0001$). This reduction was significant only between untreated soils and highest level of glyphosate treatment (Fig. 2B). In this case, we also found an

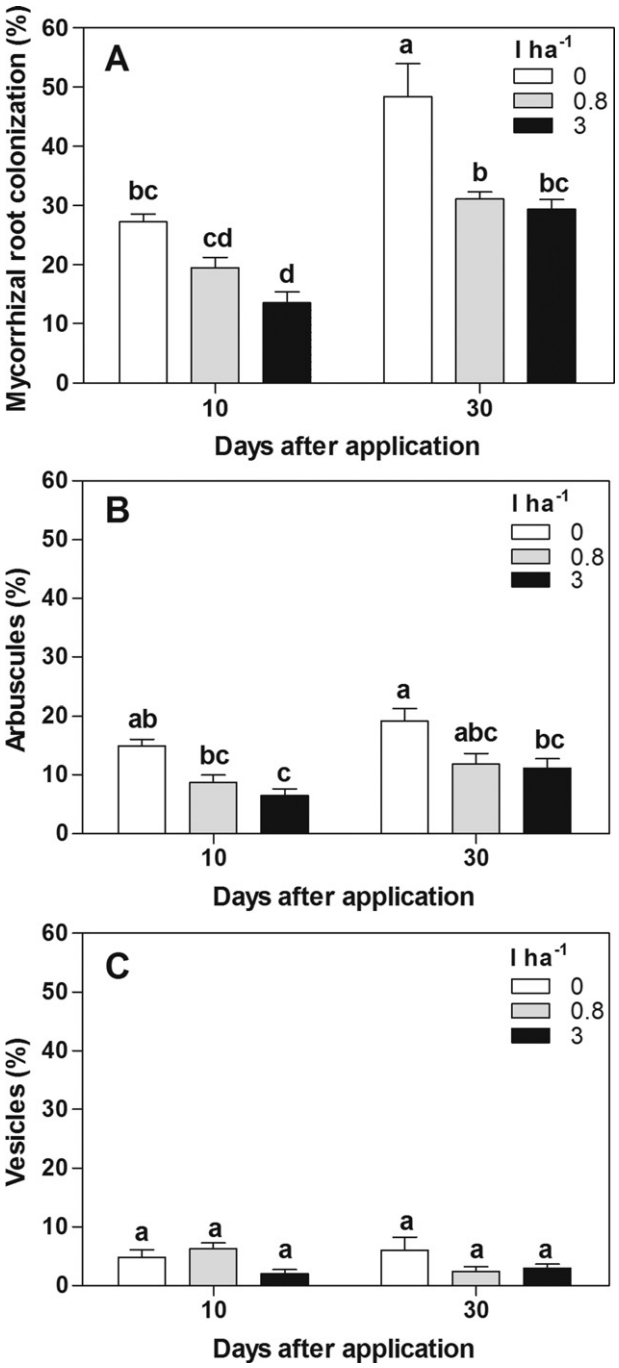


**Fig. 2.** Percentage of root colonization by AMF (A), arbuscules (B) and vesicles (C) under three different rates of glyphosate, 10 and 30 days after application. Values are means ±SE for 5 replicates. The same letter above bars indicates that values do not significantly differ among the six treatments according to ANOVA and Tukey's test ($P \leq 0.05$).

**Table 2**
Results of multivariate analysis of variance (MANOVA) for the effects of sampling date and glyphosate rate on fungal traits (total root colonization, percentage of arbuscules and percentage of vesicles).

| Effect | Pillai's trace | d.f. (numerator, denominator) | $F$ | $P$-level |
|---|---|---|---|---|
| Sampling date (S) | 0.74 | 3.22 | 21.30 | <0.0001 |
| Glyphosate rate (GLY) | 0.74 | 6.46 | 4.53 | 0.0011 |
| S × GLY | 0.29 | 6.46 | 1.27 | 0.2878 |

Pillai's trace was used as the multivariate criterion. For means and SE of the original variates, see Fig. 2.

effect of sampling date, with a higher percentage of arbuscules 30 days post-application ($F_{[1,24]} = 8.85$; $P = 0.0066$). No effect was found in the interaction between glyphosate rate and sampling date ($F_{[2,24]} = 0.20$; $P = 0.8161$).

Glyphosate application determined a marginally significant reduction in percentage of vesicles ($F_{[2,24]} = 3.39$; $P = 0.0503$) (Fig. 2C). This reduction is evidenced by the intermediate and high herbicide rate only 30 days after application, showing a marginally significant effect on the interaction between herbicide rate and sampling date ($F_{[2,24]} = 3.37$; $P = 0.0513$).

## 4. Discussion

The results of this study supported our hypothesis that application of glyphosate reduces AMF spore viability and root colonization in plants growing in soils previously exposed. Risks of glyphosate toxicity to non-target organisms in soils are generally considered as marginal, since glyphosate in the soil solution is prone to rapid microbial degradation or almost immediate inactivation by sorption to the soil matrix (Giesy et al., 2000). However, the results of this study indicate that the residence time of glyphosate and/or its toxic metabolite AMPA (aminomethylphosphonic acid) in the soil was enough to reduce AMF spore viability and their ability to colonize roots. A decrease in spore viability may lead to changes in AMF diversity, AMF population dynamics and functionality of the symbiosis, considering the importance of the spores as a source of propagules for AMF perpetuation and spread in the system and for optimal root colonization of plants (Smith and Read, 2008).

The reduction in spore viability was detected even when the lower herbicide rate (0.8 l $ha^{-1}$) was applied. At this dosage, glyphosate application reduced the survival rate of *L. multiflorum* 29.7% and chlorophyll content 47.4%, while the decrease in AMF spore viability was 87%. This suggests that there are different sensitivities to glyphosate among an ecosystem components and in this case it was higher in organisms for which the agrochemical was not designed (non-target organism). There is evidence from other non-target organisms that are affected by glyphosate. For example, Casabé et al. (2007) evaluated the effect of this herbicide on earthworms, and found a decrease in cocoon viability using 1.440 g acid equivalent $ha^{-1}$. Another study has observed negative effects of glyphosate on nodule biomass and leghemoglobin content in soybean (*Glycine max* (L.) Merril) (Reddy et al., 2000).

The percentage of root colonization was significantly lower in plants grown in soil treated with glyphosate than in untreated soils. There was an effect of sowing date on root colonization, being higher in plants which seeds were sowed later (30 days after herbicide application). This result is related to the effects of a slightly higher growing temperature in the glasshouse which resulted in larger plants (data not shown). However, the response in the percentage of root colonization under glyphosate presence was similar in both sampling dates. These results are similar to those reported by Ronco et al. (2008), who found a reduction in the percentage of root colonization of 18% and 33% in pepper (*Capsicum annuum* L.) growing in soil treated with 0.4 and 4 l $ha^{-1}$, respectively. However, these results do not agree with those reported by Malty et al. (2006), who found no changes in the percentage of root colonization by AMF in soybean growing in soils exposed to glyphosate at a rate equivalent to 10 l $ha^{-1}$. Possibly, these differences are due to host plant and AMF species used in each paper and soil *P* levels, as the dose and soil texture were similar. Our results also showed that the percentage of arbuscules was significantly reduced in plants growing in soil treated with the recommended field dose, indicating that the functionality of the symbiosis was affected, given that these structures are the main site for host–fungus nutrient exchange (Smith and Gianinazzi-Pearson, 1988).

Although this study did not examine the mechanisms by which glyphosate application affects AM spore viability, it is reasonable to assume that inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase is partly responsible for this effect. Glyphosate inhibits this enzyme in the shikimic acid pathway (Amrhein et al., 1980), and this leads to the inhibition of the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan), the arrest of protein production and the prevention of secondary product formation (Grossbard and Atkinson, 1985; Franz et al., 1997). The EPSP synthase is found not only in plants but also in bacteria and fungi (Padgette et al., 1995). Therefore, the inhibition of this enzyme may explain, at least partially, the decreased AMF spore viability when exposed to glyphosate. In addition, possible toxic effects of AMPA on AMF should be considered, given that inhibitory effects of this metabolite have been reported on germination of winter wheat seeds (Barry, 2009; Bott et al., 2011).

Data shown in this paper result from applying glyphosate in soil contained in trays (2 cm deep), with a post-application irrigation, in order to ensure contact between the herbicide and spores. Under field conditions, the chances that AMF spores are in contact with glyphosate, and the magnitude of the effect of this herbicide on them, are not exclusively linked to the dosage and time of application. For the same herbicide rate, the magnitude of the effect will be greater at lower plant cover, and shorter time between application and a rainfall event, since both factors may increase the amount and mobility of glyphosate in soil. Management practices, such as phosphate fertilization, generate a remobilization of glyphosate residues in soils because P competes for adsorption sites (Bott et al., 2011), and therefore it should be considered as an additional potential pathway for glyphosate toxicity to AMF spores. In addition, glyphosate can reach spores through root exudations to the rhizosphere of plants treated with this herbicide (Neumann et al., 2006). This process can have a major impact on the number of propagules of AMF due to the greater number of spores in rhizosphere soils compared to nonrhizosphere soils (Hindumathi and Reddy, 2011). In this case, the magnitude of the effect will be greater at higher plant cover, compared to random leaching of glyphosate by events of rainfall.

In conclusion, our work allows us to affirm the existence of a direct effect of glyphosate application on AMF spores, and their ability to colonize roots. This decrease in the number of propagules could be added to a possible indirect effect of this herbicide on AMF due to alterations in the flow of carbohydrates to the fungus as a result of stress in the host plant. The combination of direct and indirect effects of glyphosate application on AMF may affect plant diversity, taking into account the different degrees of mycorrhizal dependency between plant species that may coexist in grassland communities. Further studies are needed to better understand the magnitude of these effects under field conditions. Additionally, it should be assessed whether glyphosate affects certain AM fungal species, *i.e.* eradicate some species while not affecting others, changing AMF diversity and therefore impacting on the plant community.

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