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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 ($0.8\,\mathrm{l\,ha^{-1}}$) and recommended field doses ($3\,\mathrm{l\,ha^{-1}}$) 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 $3\,\mathrm{l\,ha^{-1}}$. 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.