Response to the application of glyphosate of beneficial soil fungi associated with Lotus tenuis

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ABSTRACT

In recent years, the Lotus tenuis promotion through glyphosate application has been adopted in Flooding Pampa grasslands, due to the important contribution in forage quantity and quality of this species. However, several studies have shown that the use of glyphosate can cause undesirable effects on beneficial soil microorganisms. The objective of this study was to evaluate the effect of glyphosate application on the arbuscular mycorrhizal fungi and dark septate endophytes associated to L. tenuis. Two treatments were applied in plots located in a L. tenuis pasture: promoted Lotus tenuis and established Lotus tenuis. In the first case, plots were sprayed in winter (late August) with 3.5 l/ha of glyphosate, while in the second treatment plots did not receive herbicide application. Our results demonstrated that the number of viable spores of arbuscular mycorrhizal fungi was 52% lower, and the percentage of arbuscules in L. tenuis plants was 40% lower in the Promoted Lotus tenuis treatment in relation to Established Lotus tenuis treatment. Root colonization by dark septate endophytes was not affected by glyphosate application. The loss of functionality of the mycorrhizal symbiosis might in the medium-term affect not only L. tenuis biomass production, but also the diversity and productivity of the complete plant community. The information generated in this study will be useful to redesign management practices that allow food production with a sustainable use of resources.

Keywords: Arbuscular mycorrhizal fungi, Dark septate endophytes, Herbicide, Lotus tenuis promotion.

INTRODUCTION

The importance of Lotus species as forager is based on several reasons. On the one hand, they have similar or higher nutritional characteristics when compared to alfalfa and white clover (Escaray et al., 2012), which leads to a high animal response (Acosta et al., 2012; Wen et al., 2002). On the other hand, they have a positive impact on the system due to the nitrogen contribution from the biological fixation. This not only may increase N availability in the soil, but also to facilitate the growth of grasses that coexist in the soil cover (Castro et al., 2009; Quinos et al., 1998).

L. tenuis has naturalized in pastures of the Flooding Pampa. Its importance also lies in its tolerance to waterlogging, drought and saline-sodic environments (Garcia and Mendoza, 2014; Teakle et al., 2010). The presence of this species guarantees quality summer forage in environments where other summer legumes could not thrive.

Due to the aforementioned characteristics, in recent years the promotion of L. tenuis through the application of glyphosate in late winter (Bailleres and Sarena, 2011) has been promoted. This practice reduces the competition of other plant species at the time of implantation and allow to increase its dominance. However, several studies have shown that the use of this herbicide can affect beneficial soil microorganisms (Angelini et al., 2013; Druille et al., 2016; Reddy et al., 2001; Santos and Flores, 1995).
This group of microorganisms includes arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE), which colonize the plant roots and benefitting plants through different pathways. AMF increase the availability of poorly mobile nutrients such as phosphorus (P), improve water conditions in plants, and provide protection against pathogens (Smith and Read, 2008). On the other hand, the DSE benefit plants through the synthesis of phytohormones, increases in the mineralization of organic compounds and protection against pathogens (Newsham, 2010). Considering the marginal environments destined to the production of L. tenuis (nutrient-poor soils, alternating excesses and water deficits), both the AMF and DSE would play a key role in its growth.

Glyphosate can direct and indirectly alter these fungi (Druille et al., 2013). In the first case, fungal structures in the soil (spores and external hypha) could be affected when in contact with the herbicide, since the EPSPS enzyme (inhibited by glyphosate) is not only found in plants, but also in fungi (Padgette et al., 1995). Because the external spores and hyphae are propagation structures, a reduction in their number could alter the subsequent root colonization. The indirect route would occur when glyphosate leads to a lower flow of carbohydrates towards the roots, which in turn damage the fungi that are colonizing the plant. Regardless of the pathways involved, a reduction in the functionality of the AMF and DSE in the system could lead to a reduction in the production of L. tenuis, as it is highly dependent on these microorganisms (mainly AMF) in marginal environments (Mendoza and Pagani, 1997; Wilson and Hartnett, 1998).

There is evidence of negative effects on beneficial soil microorganisms of glyphosate application when it is used, for example, to promote Lolium multiflorum in pastures of the Flooding Pampas (Druille et al., 2015; Druille et al., 2016). However, this information could not be extrapolated to what happens during the promotion of L. tenuis, since in both cases the time of application of the herbicide is different (summer vs. winter), and impact on different stages of the life cycle of microorganisms.

Therefore, if we desire to make a sustainable use of the forage resource it is necessary to study the particular impact on these microorganisms of L. tenuis promotion. Until now, only one study analyzed the effect of this management practice on the bacterial community (Nieva et al., 2016), but there is a lack if information on the impact on the soil fungal community. The aim of this work was to evaluate the effect of glyphosate application on arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE) associated with Lotus tenuis.

**MATERIALS AND METHODS**

**Study site and experimental design**

The trial was conducted in the experimental field of the Faculty of Agronomy of the University of Buenos Aires (34° 37’ S, 58° 50’ W). The soil was classified as typical Argiudol, with 3.1% of OM, 3 ppm of P, pH: 5.5 and EC: 0.3dS/m. The rainfall and the average annual temperature were 932 mm/year and 17.8 °C, respectively.

Two treatments were conducted on a L. tenuis pasture implanted in April 2013: promoted Lotus tenuis (PL) and established Lotus tenuis (EL). In the first case, the pasture was sprayed with 3.5 l/ha of glyphosate at the end of August 2014. The seedlings were re-established from seeds present in the soil bank. In the LE treatment, no herbicide was applied (control), and a cleaning cut was performed at 8 cm from the soil. The experimental units were plots (Area: 1.05 m x 2 m), with four repetitions per treatment. Sixty days after the application of glyphosate, plants of L. tenuis and their associated soil were collected.

**Determinations**

- **Separation of AMF spores and viability estimation**
  The spores were extracted from 50 g of sub-samples of air-dried soil for each sample. We used the wet sieving and decantation method (Gerdemann and Nicolson, 1963) followed by centrifugation in sucrose gradient (Walker et al., 1982). The obtained spores were immersed in a solution of Tetrazolium Bromide (MTT) and incubated for 40 hours (An and Hendrix, 1988).
  The viability estimation was performed under a stereoscopic microscope, counting as viable those spores that presented rosy-reddish tones, and as non-viable to those that maintained the spore’s natural color or turned black during the incubation in MTT.

- **Root colonization by AMF and DSE**
  The roots of L. tenuis were clarified in 10% potassium hydroxide (KOH) for 15-20 minutes at 90 °C. Subsequently, they were acidified in 1% hydrochloric acid (HCl) for 10 minutes at room temperature and then stained with 0.05% trypan blue (Phillips and Hayman, 1970). Finally, the roots were washed and mounted in polyvinyl alcohol for observation with optical microscope (200x). The percentage of total mycorrhizal colonization (hyphae + vesicles + arbuscules), colonization by vesicles and colonization by arbuscules separately, and the percentage of dark septate endophytes, according to the methodology proposed by Mc Gonigle et al. (1990).

**Statistical analysis**

We performed an analysis of the variance (ANOVA) to determine the effect of glyphosate on the total number of spores, viability percentage and number of viable AMF spores. The attributes of the colonization (percentage of total root colonization by AMF, percentages of arbuscules and of vesicles, and percentage of colonization by DSE) were analyzed by a three-way analysis of multivariate variance (MANOVA).

When significant results were obtained with MANOVA, we used the ANOVA univariate analysis to determine...
which of the response variables were the most affected by the treatment (Scheiner, 2001). For the data expressed in percentage, an angular transformation was performed (\(y = \arcsen\sqrt{x}\)) before conducting each statistical analysis in order to obtain homogeneous variances. The level of significance was set at \( \alpha = 0.05 \).

**RESULTS**

**Number of spores of arbuscular mycorrhizal fungi (AMF)**

Glyphosate application did not modify the number of total AMF spores (\(p = 0.9159\)). The average was 160 spores/100 g of dry soil (Figure 1A). However, the viability percentage varied significantly (\(p = 0.0093\)), that is, 50% lower in the spores in the PL treatment when compared to the spores in the EL treatment (Figure 1B). As a consequence, the number of viable spores (arising from the multiplication of the two previous variables) was also modified between treatments (\(p = 0.0346\)) (Figure 1C).

**Root colonization by AMF and DSE**

Total root colonization of AMF in L. tenuis plants did not significantly different between treatments (\(p = 0.3246\)) (Figure 2). As for the percentage of arbuscules, a reduction of 40% was detected in the case of plants promoted with the herbicide when compared to the percentage found in established plants (\(p = 0.0394\)). Finally, the percentage of vesicles was similar between treatments (\(p = 0.5708\)).

No significant differences were detected in the colonized root percentage by DSE between the established L. tenuis

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Figure 1. Number of total spores (A), viability percentage (B) and number of viable spores (C) of AMF in the soil associated to promoted Lotus tenuis (PL) and established Lotus tenuis (EL). The bars correspond to the standard error. Asterisks indicate significant differences between treatments.

Figure elaborated for this edition.

Figure 2. Percentage of total root colonization, arbuscules and AMF vesicles in promoted Lotus tenuis (LP) and established Lotus tenuis (LE) plants. The bars correspond to the standard error. Asterisks indicate significant differences between treatments.

Figure elaborated for this edition.

Figure 3. Percentage of root colonization of DSE in promoted Lotus tenuis plants (PL) and established Lotus tenuis (EL). The bars correspond to the standard error.

Figure elaborated for this edition.
plants and those promoted with glyphosate (p= 0.4633), that is, an average of 48% (Figure 3).

DISCUSSION

The number of AMF viable spores was 52% lower in the PL treatment when compared to the EL treatment. The effect of glyphosate on the spore viability apparently would not depend on the time of application of the herbicide since a similar effect was reported with an application at the end of the summer (Druille et al., 2015). This suggests that the aforementioned glyphosate action occurs at different stages of the life cycle of these microorganisms.

The response reported is due to the direct effect of the herbicide on the spores in the soil. This would imply that although glyphosate is rapidly inactivated by microbial degradation and adsorption to edaphic particles (Giesy et al., 2000), the time that it remains in the soil is enough to generate toxic effects in non-target organisms.

One would expect that this reduction in the number of viable spores will lead to a reduced root colonization, taking into account the importance these structures have as a source of propagule (Smith and Read, 2008).

Although total root colonization by AMF did not vary between treatments, the percentage of arbuscules was lower in the PL treatment. This response coincides with previous reports, conducted both under greenhouse conditions and in the field (Druille et al., 2016; Druille et al., 2013; Ronco et al., 2008).

The results obtained with this experimental design do not allow to determine the causes of the observed effect. Future studies are required to determine if this reduction is due to a direct or indirect effect of the herbicide and/or to the contribution of the age difference of the plants between treatments (60 days plants in the PL treatment vs. adult plants in the EL treatment).

Probably, this reduction implies a functionality loss of the symbiosis, considering that the arbuscules are the structures where the exchange of nutrients between the plant and the fungus takes place (Smith and Gianinazzi-Pearson, 1988).

However, this management practice would not be compromising the temporary storage of AMF reserves since the percentage of vesicles in L. tenuis roots was not affected by the glyphosate application.

In our study, the percentage of root colonization by DSE in L. tenuis plants did not show significant differences between treatments. This response differs from that reported by other authors after repeated applications of glyphosate for the promotion of Lolium multiflorum (Druille et al., 2016), which indicates that the effect of glyphosate on root colonization by DSE depends on multiple factors. Among them, the identity of the host plant, the time of application of the herbicide and the number of glyphosate applications may be mentioned.

The annual repetition of this management practice could compromise the production and future quality of L. tenuis, considering the important role of mycorrhizal symbiosis in the establishment of seedlings and subsequent growth of legumes (Van der Heijden, 2004; Wilson and Hartnett, 1997).

Future studies should be conducted to further understand the mechanisms involved, since the reported responses could be due to the active principle of the herbicide, to the products of its degradation (eg AMPA) or to changes in the biological and chemical properties of the soil.

The information generated in this work contributes to the redefinition of management practices that allow the production of food with a sustainable use of resources.

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